NON-TARGETED RADIATION EFFECTS IN ESOPHAGEAL ADENOCARCINOMAS

NON-TARGETED RADIATION EFFECTS IN ESOPHAGEAL ADENOCARCINOMAS

BY

CHRISTINE HANU, BMRSc

A THESIS

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

OF MCMASTER UNIVERSITY

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Doctor of Philosophy (2016) McMaster University

(Medical Physics & Applied Radiation Sciences) Hamilton, Ontario, Canada

TITLE:	Non-targeted radiation effects in esophageal adenocarcinomas
AUTHOR:	Christine Hanu BMRSc
SUPERVISOR:	Dr. Carmel E. Mothersill
NUMBER OF PAGES:	xvi, 168

I dedicate this dissertation to my parents and my husband Andrei Hanu.

First and foremost, to my father, Augusto Pinho, your love and strength knew no limitations when it came to your family. You are my role model. You've showed me an amazing example of hard-work and determination. Had it not been for you, I would not be at this point in my life.

Last but not least, I am dedicating this thesis to my very best friend, Andrei Hanu, who has been my rock and support throughout this whole journey. From you, I truly learnt to stay focus and strong no matter the circumstances.

Abstract

The primary aim of this thesis is to investigate non-targeted radiation effects (NTE) in cancer patients undergoing radiotherapy. One of the main NTE being studied was radiation-induced bystander effects (RIBE). A pre-existing research project was being conducted on esophageal adenocarcinoma cancer (EAC) patients at our local Juravinski Cancer Centre (JCC). High dose-rate (HDR) brachytherapy is a specific type of radiotherapy used to treat advanced stages of esophageal cancer. One study followed 15 esophageal cancer patients throughout their entire fractionated brachytherapy by using a number of sample based colony-forming assays. Another study investigated 60 esophageal cancer patients' responses to a single exposure of brachytherapy by using a blood serum based colony-forming assay. The mechanisms underlying RIBE have remained elusive to date. Serotonin dependent mechanisms have been shown to have a role in radiation-induced bystander signaling and response pathways for human keratinocytes and breast cancer cells, but there are no previous studies investigating bystander effects in esophageal cancer. Overall thesis findings are summarized below:

Summary of research questions and findings

Research Questions	Finding	Publication
Q1:Are bystander effects produced in brachytherapy patients?	Yes, they are produced in non-smokers	[1]
Q2:Are they detectable in non-tumour samples such as blood and urine?	Yes, best in blood samples	[2]
Q3:Is serotonin involved in the mechanism in patient samples and EAC cells ?	Serotonin was important in some patient samples but not in the two cell lines	[3] (manuscript submitted)
Q4:Do they persist during fractionated treatments?	Yes, an adaptive response initiated in bystander cells	[2]
Q5:Are available esophageal cell lines useful for studying bystander effects?	Yes, EAC cells produce bystander signals	(manuscript accepted subject to revision)

This thesis may contribute to the knowledge on bystander or even abscopal effects in radiotherapy. The investigations presented in this thesis motivates other research to identify the propagated soluble factors that promote radiation-induced signaling pathways in esophageal cancer, to develop risk based radiation exposure guidelines, and to develop clear bioassays and biomarkers for bystander effects.

Acknowledgements

I began my graduate research journey roughly 5 years, 1 month, and 28 days ago. During this time many people were instrumental in shaping my academic career, and here lies a small tribute to those people. First of all, I wish to thank my supervisor, Dr. Carmel Mothersill, for her never-ending encouragement, guidance, and support throughout my thesis while allowing me the room to grow in my own way. I would also like to thank my committee members, Dr. Joe Hayward and Dr. Riamond Wong, for their constant guidance and input over the years during meetings and manuscript submissions.

A special thanks to Dr. Colin Seymour for his constant guidance and support over the years. I will never forget your helpful suggestions on data analysis and graph plotting throughout the last few years. I would also like to acknowledge Dr. Ranjan Sur for his help, early on during my MSc, while acquiring eligible cancer patients for the clinical studies discussed in this thesis. Also for his continuous support during the later part of my PhD. I am also indebted to Emilia Timotin for all of her help with patient consent, sample collection, and blood sera extraction. Your friendly and outgoing personality truly kept me grounded throughout graduate school. I would like to thank the Hamilton Health Sciences Juravinski Cancer Centre (JCC) brachytherapy staff for assisting in sample collection. I would also acknowledge and thank all the patients who participated in the pilot and phase II clinical studies.

I am forever grateful to have been blessed with friendly, helpful, and often times a very resourceful group of ladies working in the Medical Physics office during my thesis. Thank you for all of your help! During my daily lab work, I had been fortunate to work alongside an absolutely amazing group of people over the years: Michelle Le, Hedi Mohseni, Jenn Fazzari, Cris Fernadez-Palomo, Hayley Furlong, and Nicole Pachal. Hedi and Cris, you both were one of my first friends in graduate school! It was a pleasure to see you both grow and succeed over these years. Michelle, it has been a pleasure being your desk neighbour and becoming very good friends! Hopefully you will find a new neighbour to help you stock up the snack cupboard for those long days in the office. Not only did I gain an absolutely exceptional education, but also I met some amazing friends along the way that will last a lifetime. Thanks everyone for making my graduate experience so memorable and always for your encouragement throughout the years.

A special thanks to the Medical Physics and Applied Radiation Sciences department and Dr. Diana Moscu. I feel so very fortunate to have been given the opportunity to become a Sessional Lecturer for a third year undergraduate course on Research Methods for Medical

Radiation Sciences. I had received such an amazing experience that helped me to develop new skills and a confidence in myself that I know will help me to succeed in the future. The feeling of genuinely helping future professionals is truly gratifying; I hope my career path in life brings me back to teaching one day. I would like to extend a very special thanks to Dr. Carmel Mothersill who helped made it possible for me to gain permission from graduate studies to accept such an amazing opportunity.

I would like to thank my parents for their continuous love and support. Thank you for raising me to be the person I am today and always being there for me. To my second family, Marin and Mihaela Hanu, thank you for welcoming me into your home during my graduate studies and for always keeping me motivated to succeed. Thank you to Krista-Marie Salem, Felicia Beck, Jurgen Beck, and Alex Crisan for always being such great friends and family. To my brother and his beautiful family, and also my sisters, for always being there for me. I feel so very blessed to be surrounded with such wonderful people in my life. To my husband,very best friend and biggest supporter, Andrei, you gave me the courage to continue along this crazy journey. No matter how hard things got, you always gave me the strength to conquer any obstacle that came my way. I am truly lucky to have shared a substantial portion of my graduate studies alongside with you. To my very special golden feeder, Ollie. I know you cannot read this thesis, but I know others who love you as dearly as I do will appreciate this! Thanks for always being there to greet me at the door at 3:30 am during those very hard last 4 months of experiments. After a long day in the lab, you always had those ever so squinty eyes, piercing back at me, and that full body wiggle which always made me laugh.

THANK YOU!

Notation and abbreviations

CCCM	control cell conditioned medium
CDK	cyclin dependent kinase
COX-2	cyclooxygenase-2
CT	computed tomography
CTV	clinical target volume
DNA	deoxyribonucleic acid
EAC	esophageal adenocarcioma
EBRT	external beam radiation therapy
EGFR	epidermal growth factor receptor
Erk	extracellular-signal-regulated kinase
ESCC	esophageal squamous cell carcinoma
EUS	endoscopic ultrasound
FBS	foetal bovine serum
GEJ	gastroesophageal junction
GERD	gastroesophageal reflux disease
GJIC	gap-junction intercellular communication
GPCRs	G protein-coupled receptors
GTV	gross tumour volume
HDR	high dose rate
HRS	hyper-radiosensitivity
IAEA	International Atomic Energy Agency
IARC	International Agency for Research in Cancer
iNOS	inducible nitric oxide synthase
ICCM	irradiated cell conditioned medium
^{192}**Ir**	Iridium-192
IRR	induced radioresistance
JCC	Juravinski Cancer Centre
JNK	c-Jun N-terminal kinase
LET	linear energy transfer
LGICs	ligand-gated ion channels
LOH	loss of heterozygosity
MAPK	mitogen activated protein kinase
Mdm2	mouse double minute 2 homolog
miRNA	micro RNA

MN	micronuclei
MRI	magnetic resonance imaging
nAChRs	nicotinic acetylcholine receptors
NO	nitric oxide
NPO	nil per os
NTE	non-targeted radiation effect
PET	position emission tomography
PI3K	phosphatidylinositol 3-phosphate kinase
PTV	planning target volume
RIBE	radiation-induced bystander effects
RIGI	radiation-induced genomic instability
ROS	reactive oxygen species
TNM	tumour node metastasis
TP53	protein p53

Contents

List of Figures

Chapter 1

Background

This thesis investigates the presence and implications of radiation-induced bystander effects (RIBE) in cancer patients undergoing radiotherapy. Generally, RIBE describe a response by non-irradiated "bystander cells", which themselves have not been directly traversed by a radiation track, but respond as if they have [1–8]. A resultant stress response is observed in the bystander cells due to signals received from directly irradiated cells.

The most common effects observed in bystander cells are a death response [2, 5, 9, 10] and influxes of intracellular calcium following exposure to irradiated cell conditioned medium (ICCM) [1, 11]. Numerous studies point toward a serotonin driven mechanism in human keratinocyte cultures [12–16]. The elusive "bystander pathway" has proven difficult to unravel, as the main signaling factors have yet to be discovered. Much of the existing data on RIBE come from cultured cells [1, 3, 4, 17] rather than from using complex human tissues [18]. Consequently, the presence, relevance, and implications of bystander effects in radiotherapy are not fully understood. This thesis explores whether bystander signals can be detected in human samples following radiotherapy. The results give further insight into biologically relevant effects that may need to be considered during treatment planning or as a preventive approach for limiting adverse effects in healthy tissues.

This thesis is divided into two major parts. Part I encompasses work performed to investigate RIBE in samples taken from esophageal cancer patients who were undergoing

fractionated [19] and single [20] exposures to high dose rate (HDR) brachytherapy procedures. HDR brachytherapy is an internal radiotherapy technique by which high doses of radiation are precisely delivered to the tumor without irradiating the immediate adjacent normal tissue. This type of radiotherapy is used for treating advanced stages of esophageal cancer to improve dysphagia symptoms in patients [21, 22]. Part II presents data from studies using two human esophageal adenocarcioma (EAC) cell lines to investigate the generation of bystander signals and response to bystander signals in esophageal cancer.

Part I presents data from two clinical studies undertaken at the Juravinski Cancer Centre (JCC). The reason for selecting esophageal cancer was due to a pre-existing research project being conducted on esophageal cancer patients at our local Juravinski Cancer Centre (JCC), which is affiliated with McMaster University. HDR brachytherapy is currently a standard practice at JCC for esophageal cancer. The project ethical approval also permits tumor and normal tissue sampling by endoscopy as part of the treatment procedure. This portion of the thesis includes three published research papers detailing work performed to investigate the existence of bystander signals in patients' samples following radiotherapy. Part I starts with chapters 2 and 3 introducing a small pilot study performed on 15 esophageal cancer patients who were being followed throughout their entire fractionated treatments of HDR brachytherapy. During the pilot study, blood and urine samples were collected at the start and end of each fraction of HDR brachytherapy. Ethics approval allowed for biopsy samples to be collected proximal to the tumour from the mucosa layer of the esophagus. Tissue samples were collected prior to the first fraction of brachytherapy and immediately after the final fraction of HDR brachytherapy. Initially this clinical trial started off as a one-year pilot study that continued on as an additional phase II study. In chapter 4, the results of a follow-up study are presented which employed a larger sample size of 60 esophageal cancer patients and cancer-free control subjects. During the phase II study, blood samples alone were collected at the start and end of a single fraction of HDR brachytherapy. Between

the two studies, a total of 75 Canadian adults, aged 49 to 90 years, with clinically diagnosed esophageal cancer were studied. An additional group consisting of 15 cancer-free control subjects were also assessed. A variety of human samples were tested to find and develop a fast, sample-based assay, which could be used to detect the production of radiation-induced bystander signals.

Part II consists of work performed on human EAC cell lines testing whether bystander signals are produced and if these cells can respond to these signals, see chapter 5 (manuscript accepted subject to revision). The two EAC cell lines selected were OE33 and OE19. Both cells lines were derived from primary tumours which had similar cancer type and staging characteristics found in the patients enrolled in our clinical studies, making them a suitable choice. Table 1.1 highlights the key differences and characteristics for both EAC cell lines used in this thesis. Parts of the research also focused on a series of *in vitro* bystander experiments with EAC cell lines in the presence and absence of serotonin, see chapter 6. Concluding remarks along with a brief discussion and suggestions for future work are presented in chapter 7.

Table 1.1. EAC cell line characteristics

Characteristics	OE33	OE19
Patient		
Gender	Female	Male
Age	73	72
Ethnicity	white	white
Tumour		
Barrett tumour	Yes	No
Tumour location	distal EAC	cardia AC
Nodal Involvement	No	Yes
Metastases	Unknown	Unknown
Clinical cancer staging	Stage IIA	Stage III
Cell line		
Year of establishment	1993	1993
Site of origin	primary tumour	primary tumour
TP53 status	mutated (p.Cys135 Tyr)	mutated

Table was taken from Boonstra et al. (2010) [23] and adapted.

RIBE have not been fully explored in human samples [24], specifically samples irradiated *in vivo*. The existence and relevance of bystander signal production in cancer patients undergoing single and fractionated radiotherapy is still very much unclear. Furthermore, the role of certain patient characteristics such as gender, environmental modifiers and cancer staging needs to be considered to see whether these variables influence bystander signal production. Serotonin

driven mechanisms have a role in radiation-induced bystander signal production and also in bystander response in human keratinocytes [12, 13, 16] and the breast cancer line MCF7 [15], but other cell lines require further investigation. The goal of this thesis is to explore whether bystander signals can be detected in a cohort of humans exposed to radiotherapy and in EAC cell lines, using both clinical and in vitro samples, by combining clonogenic assays and ratiometric calcium measurement techniques.

Based on evidence from the literature [1, 11–13, 16, 25, 26], it was hypothesized that directly irradiated tumour cells undergoing *in vivo* or *in vitro* irradiation will generate bystander signals, which will elicit a death response in non-irradiated cells. Table 1.2 summarizes the most important literature motivating this PhD research. Past work by our research group has shown that tissue samples harvested from female non-smokers are able to produce bystander signals, whereas male smokers are not [25]. Therefore, it was hypothesized that certain patient characteristics such as gender and smoking status will influence the bystander signal production in human samples harvested from cancer patients, refer to Hypothesis Summary Table 1.3. Lastly, it was hypothesized that serotonin driven mechanisms will have a role in bystander signal generation and bystander responses [12, 13, 16] but that p53 status will have a role in the bystander response pathway alone [27], refer to Hypothesis Summary Table 1.3. It was further hypothesized that bystander effects will lead to a significant decrease in the reporter cell colony-forming assay as measured by the clonogenic assay technique for both *in vivo* and *in vitro* irradiation. Fractionated ICCM exposure using bystander cells has been shown to result in either an induced radioresistant response [28] or further cell killing after the second dose of ICCM [29]. As evident above there are contradictory findings concerning fractionation and bystander effects, however, we predict that the fractionated treatments of HDR brachytherapy will cause less cell killing in reporter cells during later fractions of radiation as was found in earlier work on fractionating

ICCM exposure [28]. This work will contribute to the current knowledge concerning non-targeted radiation effect (NTE) and will further our understanding of the effects of low doses of radiation and the potential risks involved in humans.

Table 1.2. Summary of articles motivating this PhD dissertation research

Experimental System	Effect	Endpoint	Mediator of bystander effect ‡	Publication
Human keratinocyte HPV-G cell line	Reduced clonogenic survival	Clonogenic assay		[1]
γ-ray	Rapid calcium fluxes (within 30 s*)	Ratiometric calcium measurements	Influx of calcium ions	[1, 11, 26]
	Increase reactive oxygen species (ROS) production (6 hrs *)	2,7- dichlorofluorescein diacetate	ROS	[1, 11, 26]
	loss of mitochondrial membrane potential	Fluorescent dye (Rhodamine 123)		[1, 11, 26]
	Apoptosis	Morphology characteristics		[1, 26]
	Activation of MAPK pathway	Immunofluorescence	Activation of ERK and JNK pathways (abolish effect with inhibitor)	[26]
	Reduced clonogenic survival; influx of calcium ions	Clonogenic assay; ratiometric calcium measurements	Serotonin driven mechanism	[12, 13, 16]
Fractionated ICCM	Enhance cell killing; reduced sparing effect	Clonogenic assay	-	[29]
Normal human lung fibroblasts (HFL-1) cell line	Fractionated ICCM	Clonogenic assay		[28]
α-irradiation	induced radioresistance			
Human keratinocyte HaCaT cell line	Reduced clonogenic survival	Clonogenic assay	Release of soluble factors (PMA did not change effect*)[9]	[9, 30]
γ-ray	Reduced clonogenic survival	Clonogenic assay	Serotonin has no role	[31]
	Calcium fluxes	Ratiometric calcium measurements	Influx of calcium ions	[32]
	Increase ROS production	2,7- dichlorofluorescein diacetate	ROS (catalase reduced effect)	[32]
Human colon tumour cell line (HCT 116)				
γ-ray				
p53 wildtype	Reduced clonogenic survival	Clonogenic assay	p53 pathway contributory role in bystander response	[27]
p53 null	Bystander signals detected in p53 functional reporters	Clonogenic assay	P53 pathway has no role in bystander signal pathway	[27]
p53 wildtype	insignificant reduction in micronuclei (MN)	Cytokinesis-block micronucleus assay	Supplemented serotonin (100 ng/ml)	[15]
Human urothelium tissue samples				
ex vivo γ-ray irradiation	Reduced clonogenic survival;			
	Female non-smokers produce signals	Tissue based clonogenic assay	-	[25]

*PMA-phorbol myristate is a gap junction inhibitor; first study reporting the release of soluble factors
‡ The soluble factors eliciting the bystander effect are unknown
⋆ Amount of time that has elapse after ICCM exposure

Table 1.3. Hypothesis Summary Table

Publications	Hypothesis
Fractionated ICCM Enhance cell killing [29]; Induced radioresistance [28]	**H1:** Fractionation will induce less cell death in reporter cells following each fraction of brachytherapy
Female non-smokers produce strong bystander signals [25]	**H2:** Gender and smoking status will influence bystander effects
Bystander effects are typically reported as a death response [1, 9, 30, 31]	**H3:** Bystander effects will lead to a significant decrease in reporter cell survival
Serotonin mechanisms have a role in bystander effects [12–14, 16, 33]	**H4:** Serotonin will modulate bystander effects in esophageal cancer
p53 pathway has a contributory role in bystander response [27]	**H5:** Cell lines possessing mutated p53 will not respond to bystander signals

1.1 Radiation-induced bystander effects

Non-targeted phenomena include radiation-induced genomic instability (RIGI) [34, 35], RIBE [1, 3, 17, 26, 36], abscopal effects [37], radiation-induced clastogenic effects [38–42], and hyper-radiosensitivity (HRS)/induced radioresistance (IRR) [43] responses. Prior to the early 1990s, a deoxyribonucleic acid (DNA) centric perspective was widely held where direct damage to DNA was either repaired and the reproductive integrity of cells was maintained, or the cell underwent death as a result of misrepaired radiation damage accumulated to the point that the cell could no longer function [44]. However evidence of NTE date back to 1915, when Murphy and Norton reported the first results of direct x-ray exposure leading to non-irradiated effects in distant organs of mice [37]. From the late 1960s and the mid 1980s, other studies observed the induction of chromosomal aberrations in unirradiated cultures following their exposure to irradiated human and animal blood samples [39, 40, 45].

In 1986, Seymour and others found a high yield of lethal mutations in the descendants of irradiated cells. Similar findings were found shortly after by Weissenborn [35] and Kadhim [46]. These findings ultimately suggested that the target for radiation damage must be much larger than the cell itself. Even after such vital clues had surfaced regarding the existence of NTE, the mindset remained unchanged until Nagasawa and Little (1992) and Mothersill and Seymour (1997) presented very compelling evidence that non-irradiated cells are able to produce damage without the nucleus of a cell being traversed by radiation. Nagasawa and Little named this phenomenon "radiation-induced bystander effects" and, since 1992 and a number of researchers have since used this terminology [3, 5–7, 17]. In their study, Nagasawa and Little quantified a 30% increase in sister chromatid exchanges within unirradiated cells, yet 1% or less of the cells were traversed by low doses of α particles. Shortly afterwards, a paradigm shift within the radiobiology field occurred where genetics and environmental factors were seen to play an integral role in direct and indirect radiation exposure responses

[25, 47–49].

Over the past two decades, bystander effects have been reported as increased apoptosis [1, 3, 17, 26, 36], delayed cell death [5, 6, 29], clonogenic survival reductions [9, 30], sister chromatid exchanges [8], influx of calcium ions [12, 13, 16], and MN formation [42]. Furthermore, the effects have been observed across a number of cell lines including human HaCaT keratinocytes [17, 31], human HPV-G keratinocytes [1, 11, 14, 26, 50], human T98G glioblastoma [51], human SW48 colorectal adenocarcinoma [17, 30], human MCF-7 breast adenocarcinoma [15], and human HCT 116 p53 wildtype colorectal cancer cells [27, 52]. Irradiation of tissue explants can also lead to a production of soluble factors visualized using reporter cell models [9, 30, 53, 54]. Most of the evidence of RIBE *in vivo* comes from blood-borne factors often referred to as clastogenic factors following radiation exposure [38–42] and from using animal models [6, 47, 55].

Bystander effects can be induced in at least two ways including the gap-junction intercellular communication (GJIC) [56] and by transferring extracellular soluble factors [17]. In the first mode of communication bystander signals are transferred through gap-junctions, whereas the medium transfer method requires no cell contact to transmit the signal. Recently it has been shown that the emission of ultraviolet radiation from directly irradiated cells may be an additional mode of communication [57, 58]. Since there are different ways that bystander signals are communicated between cells, this thesis focuses on the indirect mode of communication with bystander signals transmitted via medium transfer. Below is a description of medium-transfer bystander effects including potential signaling candidates, mechanisms, and bystander responses found in *in vitro* and *in vivo* experiments.

1.1.1 *In vitro* Studies

In 1997, Mothersill and Seymour published data showing medium-transfer of bystander signals [17]. In this technique sterilized culture media from directly irradiated cells, called

donors, is transfered onto non-irradiated "bystander" cells. Increased clonogenic cell death compared with controls was observed in directly irradiated cells as well as in non-irradiated cells. The possibility of radiation producing a toxic product in the culture media was eliminated because culture medium containing no cells was irradiated and had no observable effect on bystander cell clonogenic survival. Other bystander effect experiments which involve shared medium use a Transwell insert co-culture system. This technique requires the use of inserts containing irradiated cells placed in petri dishes containing bystander cells so that only the medium is shared and there is no contact between the two groups of cells [59].

Bystander effects have been characterized as a low dose phenomenon that appears at a threshold dose of 2 mGy with the effect saturating at 0.5 Gy in human keratinocytes. This means a significant bystander response becomes apparent above a dose of 2 mGy [60] and the magnitude of the bystander response increases as the dose is increased up to 0.5 Gy. Above a dose of 0.5 Gy, the bystander response remains significant but does not increase any further in magnitude above that elicited by the 0.5 Gy dose. In this respect, significant bystander responses have been observable at least up to doses of 5 Gy in human keratinocytes and probably at higher doses as well. At 5Gy the contribution of bystander cell death to total cell death is approximately 50% [4, 30]. Further investigations found that bystander cells require exposure to ICCM for a minimum of 30 minutes in order to observe a significant reduction in survival [17]. Even 60 hours after irradiation, bystander signals are still present in ICCM. Furthermore, the effect cannot be reversed in bystander cells even after the ICCM has been replaced with fresh medium.

Bystander signal production and response are very much cell line specific, as not all cells are capable of generating these cytotoxic factors [25, 30, 61, 62], nor are they able to respond to these signals [27, 61, 62]. The nature of the signal is still unclear, but it has been shown to survive freezing-thawing and is abolished at high temperatures of 70°C [9]. Recently, the role of exosomes has been shown to be important during radiation-induced

bystander signaling in human breast cancer MCF7 cells [63] and HaCaT human keratinocytes [64]. Once the exosomes had been extracted from ICCM, bystander effects were no longer observed in the human keratinocyte reporter cells. Exosomes have been implicated in eliciting immune responses [65] and also tumour growth [66] in other areas of research since these small vesicles are involved in releasing signals to nearby cells. These small vesicles are only 30 to 100 nanometers in size [64]. They often contain micro RNA (miRNA) and proteins that can be delivered to nearby cells [65]. Xu et al. (2014) found the role of small non-coding RNAs mediating bystander effects, in particular, an up-regulation of miRNA-21 in bystander human fetal lung MRC-5 fibroblast cells [67].

1.1.2 *In vivo* Studies

Most of the NTE reported in humans are due to clastogenic factors. Clastogenics factors are chromosome damaging substances present in the blood of patients exposed to cytotoxic stressors such as ionizing radiation [68]. Early work by Goh and Sumner (1968) led to the detection of a blood-borne factor following irradiation. This was referred to as radiation-induced clastogenic effect [45]. In this work, leukocytes in culture were exposed to blood plasma taken from patients receiving total body irradiation. The authors observed a significant increase in chromosomal aberrations within the cultivated leukocytes. Clastogenic factors also been observed up to two decades after radiation exposure by a number of other researchers [38–42]. Nearly a decade after radiation exposure, Goh (1975) was still able to detect a substantially higher amount of chromosomal aberrations in the peripheral blood of these patients compared to healthy controls. More than two decades after Chernobyl, Marozik and colleagues observed elevated MN formation and enhanced cell death in human keratinocytes exposed to blood serum samples taken from Chernobyl liquidators [42]. In 2006, Seymour and Mothersill developed a simple blood based colony-forming assay to predict overall radiation response of patients immediately following their first treatment of radiation,

midway during treatment and six weeks after radiation therapy [69]. This same technique was adopted for the blood serum based colony-forming assays for the first part of this thesis.

Other NTE being reported in humans are commonly referred to as abscopal effects. These are often termed "distant" bystander effects, as this phenomenon commonly occurs in remote sites from the initial radiation insult [70]. For instance, remote tumour regression was observed in patients with hepatocellular carcinoma [71], melanoma[72], papillary adenocarcinoma[73], and malignant lymphoma[74]. There is also considerable evidence of *in vivo* radiation-induced signaling and responses using animal models [6, 47, 55]. Mice partially exposed to radiation showed epigentic changes such as DNA methylation in non-irradiated tissues [75]. Other work by the same research group exposed the cranial region of mice to 20 Gy x-rays and measured epigenetic factors in distant non-irradiated spleen tissues [76]. A year later, this research group reported that radiation-induced bystander signaling is sex-specific in mice [47]. These authors reported that male non-irradiated spleen tissues exhibited higher levels of miRNA deregulation than females. Both environmental and genetic factors have been shown to influence radiation-induce bystander signal production in human and mouse tissue samples [25, 48, 49].

1.1.3 Does the presence of a tumour affect radiation-induced bystander signals and responses?

The relevance of bystander signals and responses in radiotherapy has often been questioned [10, 18, 24], yet the answers remain elusive. There have been many bystander effect experiments performed in cultured cells [1, 3, 4, 17], but very few performed on humans. Not only do normal cells generate bystander signals, but irradiated tumour cells can as well. Some human tumour cell lines will express bystander effects while others will not. For example, the human colon cancer cell lines HCT 116 p53 +/+ [27] and SW48 [17, 30] produce, and respond to, bystander signals whereas human colon HT29 [30] and prostate PC3 cell

lines cannot [77]. In p53 functional and non-functional studies on human colon cancer cells [27], bystander signals were shown to be undetectable when the expression of functional p53 protein is completely eliminated in the bystander cells. The human SW48 colon cancer cell line has functional p53 whereas the human PC3 prostate cancer cells possess mutant p53. Perhaps having a mutation that renders the p53 pathway inactivate may prevent cells from responding to cell signals in bystander cells. However HPV-G keratinocytes are very useful reporter cells for bystander effects. These cells are transfected with the E6 protein of the HPV virus and express only 30% of the normal amount of wild type p53 protein. It is suggested that a reporter cell with partial function of its wild type p53 protein[78] still can facilitate the transduction of the signal [79]. A few studies have suggested that the presence of a tumour in humans [25] and animals [47, 80] weakens the bystander signal to the point where it either blocks the signal entirely, or causes a significant change in normal expression of RIBE. Similar effects have been observed in normal samples being treated with a carcinogen, (nitrosamine) [81], or when tissue samples were harvested from individuals who were heavy smokers [25]. It is obvious that this area of research requires further investigation, since very few studies have investigated radiation-induced signaling and responses in tumour-bearing individuals and animals.

1.1.4 Possible mechanisms of bystander effects

The mechanisms of bystander effects still are not fully understood. However, the induction of a RIBE may be a result of different cellular pathways, where some pathways produces the bystander signals and the others orchestrates responses to the signals. During the last decade, the involvement of serotonin in bystander effects has surfaced as one of the mechanisms involved in bystander signal production [12–16]. Poon et al. (2007) studied the role of signaling candidates that may initiate bystander responses in human keratinocytes HPV-G cultures. In this report, micromolar levels of serotonin were depleted from the medium

during cell irradiation by attaching to the 5-HT_3 receptors. Experiments by Mothersill and others found a correlation between the level of serotonin in foetal bovine serum (FBS), supplemented in culture medium, and the ability of cells to produce bystander signals [14]. From this work, a threshold of 25 ng/ml of serotonin was suggested for human keratinocytes where below this concentration the production of bystander signals did not occur. A study using antagonist serotonin receptors, such as Ketanserin (5-HT $_2$) and Granisetron (5-HT $_3$), found bystander signals were abolished in HPV-G reporters by these receptor inhibitors [16]. These studies suggested that serotonin was a key signaling molecule in generating bystander signals within human keratinocytes cultures. In an inter-laboratory study, serotonin was shown to have a mechanistic role in radiation-induced bystander signaling in some but not all systems used [33]. Other potential signaling candidates which have been reported in the literature are ROS production [32, 82, 83], nitric oxide (NO) [84], transforming growth-factor-β1 (TGF-β1) and tumour necrosis factor-α (TNF-α)[83, 85] and secondary release of UV photons from direct radiation exposure [57, 58].

While there have been several studies showing serotonin driven mechanisms to be part of bystander signaling pathways, other work has suggested that a p53-dependent apoptotic pathway has an important role in bystander response pathways [86, 87]. The protein p53 (TP53) or the "guardian of the genome" is a tumour suppressor gene that has a key role in DNA repair processes through regulation of the cell cycle and of apoptotic pathways. These functions lead to suppression of the development of cancers [88]. In 2005, Mansfield et al. irradiated cytochrome c +/+ normal and -/- null mouse embryonic fibroblast cells, which were co-cultured in a mix/match protocol. Cytochrome c +/+ normal donors co-cultured with autologous reporters had a significant increase in MN formation whereas cytochrome c -/- null reporters had diminished MN formation. Other work with hepatoma cells possessing functional p53 showed they have the ability to induce bystander effects which have been linked to the release of cytochrome c from mitochondria into the cytoplasm [87]. In 2011,

Mothersill and colleagues studied the role of p53 pathway in the transduction of bystander effects. This study found that bystander signals were generated with the HCT 116 p53+/+ and -/- cells, but functional p53 is necessary for cells to respond to the signals in the human colorectal cancer cell line [27]. The HPV-G human keratinocytes have roughly 30% expression of wild-type p53 [78, 79]. It is apparent that the role of p53 is a complicated one, which is cell line specific, because this role does not seem to be universal in all bystander responding cell lines. For example, in HaCaT human keratinocytes bystander effects are seen but these cells possess a point mutation that does not affect the DNA binding site for p53 and these cells can undergo an alternative pathway for apoptosis [89, 90].

The elusive “bystander pathway" has not been elucidated, but the extracellular signaling candidates, with the exception of UV photons, are presumed to interact with plasma or nuclear membrane receptors to elicit a response in nearby non-irradiated cells. The driving force behind such bystander responses has primarily been reported to be a cell death effect, which is likely to be attributed to apoptosis. In fact one paper reports high apoptosis levels in bystander cell populations once the mitogen activated protein kinase (MAPK)/extracellular-signal-regulated kinase (Erk) pathway has been inhibited [26], and in another paper the bystander effect can be abolished all together with apoptotic inhibitors [36]. Table 1.4 outlines a list of studies reporting different modes of cell death in the bystander response pathway. To date, apoptosis seems to one of the most frequent modes of cell death in bystander cells. According to Mattson and Chan (2003), apoptosis is triggered by an increase in extracellular calcium at the cell membrane possibly explaining the role of serotonin which binds to ligand-gated ion channels (LGICs) allowing calcium to enter the cell. Increases in the cellular calcium concentrations can result in openings in the outer membrane of the mitochondria leading to a leakage of cytochrome c. Diffusion of cytochrome c into the endoplasmic reticulum can initiate further release of calcium that is uptake into the mitochondrial matrix [92]. Subsequently the mitochondria undergo depolarization and increase production of ROS [92].

These distinct cellular responses, mitochondrial depolarization [1, 11, 93], intracellular calcium fluxes, cyclooxygenase-2 (COX-2) signaling cascade [94], and elevated ROS production have been found in bystander cells following exposure to ICCM [1, 11]. Other researchers have found that the MAPK pathway, including the Erk and c-Jun N-terminal kinase (JNK) pathways, are activated in cells following direct radiation exposure and also in bystander cells exposed to ICCM [26]. Other modes of cell death that have also been reported recently are senescence [52] and mitotic catastrophe [95].

Bystander cells can die by a number of different mechanisms, including apoptosis [1, 3, 17, 26, 36], mitotic catastrophe [95] and senescence [52, 96] as summarized in Table 1.4. As these modes of cell death are all essential for both direct and indirect radiation effects, each subsubsection will touch upon a brief overview of each type of cell death.

Table 1.4. Mode of cell deaths in the bystander response

Experimental System	Mode of cell death	Publication
Human fibroblast cells (AGO15)	Increase apoptosis	[3]
Human keratinocyte cells (HaCaT)	Apoptosis at 48 hr*	[17]
Human keratinocyte cells (HPV-G)	increase apoptosis at 48 hr*	[1]
Human keratinocyte cells (HPV-G)	Inhibition of Erk pathway increases apoptosis	[26]
Human keratinocyte cells (HPV-G)	Apoptosis inhibitors abolish bystander effect	[36]
Human colon cancer cells (SW48)	Apoptosis at 48 hr*	[17]
Human colorectal carcinoma cells (HCT116 p53 +/+)	Larger number of senescent cells	[52]
Human breast cancer cell lines (MDA-MB-231-2A)	Link between senescence and a bystander effect in response to radiation	[96]
Human breast cancer cells (MDA-MB-231)	Cells die by late apoptosis due to mitotic catastrophe	[95]

* Time elapsed after exposure to ICCM

1.1.4.1 Apoptosis

Apoptosis is a regulated form of cell death that occurs once cells have reached the end of their lifespan or have sustained cellular damage from radiation or deleterious agents [97]. During this programmed suicide, there is no dependence on inflammation since the release of cell damaging proteins are contained within membrane-bound apoptotic bodies that are removed by phagocytes [98]. As dying cells are removed, this process helps to maintain tissue homeostasis and physiological processes including embryonic development [99]. There are excellent comprehensive reviews on apoptosis published by Elmore (2007) and Adams and

Cory (2007). In brief, we discuss below the intrinsic and extrinsic apoptotic pathways.

There are two distinct pathways responsible for apoptosis within a cell [99], refer to Figure 1.1. As cells sustain radiation damage, the intrinsic or mitochondrial pathway triggers antiapoptotic Bcl-2 that in turn initiates pro-apoptotic effectors, such as Bax [99]. Antiapoptotic BH1234 proteins (Bcl-xL and Bcl-2) are inhibitors for apoptosis as they bind to two proapoptotic BH123 proteins (Bax and Bak) [100]. Both Bax and Bak are stopped from forming pores in the mitochondrial outer membrane when Bcl-xL and Bcl-2 are present. This subsequently prevents the release of cytochrome c resulting in no apoptosis.

The BH3-only proapoptotic proteins (Bim and Puma) inhibit the apoptotic inhibitors (Bcl-2 and Bcl-xL) by forming complexes and leading to both Bax and Bak proapoptotic proteins to be free to form holes in the mitochondrial outer membrane and release cytochrome c. The release of cytochrome c in turn leads to a cascade of caspases eventually resulting in cell death. The other apoptotic pathway is known as the extrinsic or the death receptor pathway. This pathway is initiated by an extracellular ligand that binds to the death cell surface receptor, such as tumour necrosis factor receptor, on the plasma membrane [97, 102]. Once the ligand is bound to the receptor, this initiates adaptor proteins that in turn activate caspase 8 proteins followed by downstream executioner caspase proteins. Both the intrinsic and extrinsic pathways merge into one pathway, after the activation of the executioner caspase 3,6, and 7 proteins, that will breakdown cellular proteins ultimately destroying the cell.

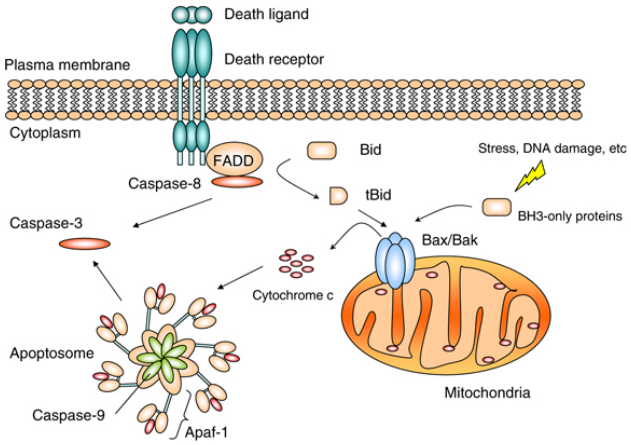


Figure 1.1. This schematic illustrates the two apoptotic pathways: intrinsic or mitochondrial and extrinsic or death receptor pathway. This image has been reprinted from a Nature Review by Li and Yuan (2008) [101].

1.1.4.2 Mitotic catastrophe

Mitotic cell death is associated with aberrant mitosis where the cells have attempted to divide but have been unsuccessful [103]. The replicative potential is technically lost, however, these cells may undergo a few more cell divisions before colony death occurs [103, 104]. Chromosome aberrations and MN formations are two of the morphological characteristics that can be observed under a microscope [99]. Defects in the G2 cell-cycle checkpoint result in premature mitosis that ultimately leads to cells dying from mitotic catastrophe.

1.1.4.3 Senescence

Senescence is an irreversible cell growth arrest characterized morphologically and metabolically by the flattening of cytoplasm and by an increased β-galactosidase expression, respectively. This process can also be caused by radiation-induced DNA damage [105] and telomere dysfunction [106]. Once this mode of cell death has been activated by radiation damage the cells enter a permanent cell-cycle arrest. In contrast to other modes of cell death, these cells remain metabolically active and can release cytokines amongst other factors involved in senescence-associated secretory phenotype (SASP), which have the ability to promote tumour progression [107].

1.2 Esophageal cancer

Since the focus of my research has been to follow esophageal cancer patients undergoing HDR brachytherapy, this section will provide a more thorough description of this type of cancer and this particular radiotherapy modality.

HDR brachytherapy is a specific type of radiotherapy used to treat advanced stages of esophageal cancer [21]. The Greek word *Brachy* literally means 'short distance.' In the case of esophageal cancer, this treatment involves the placement of a radionuclide source

inside the esophagus and is often referred to as intraluminal brachytherapy. An important advantage of this type of cancer treatment is that the radiation can target the tumour quite effectively without major surrounding organs or tissues being damaged [108]. Other benefits of using HDR brachytherapy in comparison to external beam radiation therapy (EBRT) are that it has a substantially shorter fractionation period and higher patient throughput [109].

The esophagus is a long muscular tube beginning at the oral pharynx descending distally towards the entry of the stomach [110]. It is a part of the gastrointestinal tract and is the carrier of food from the pharynx to the stomach. The esophagus is normally about twenty-five centimeters in length and consists of three main parts, including the cervical esophagus, thoracic esophagus and the lower esophagus [110]. At any of these locations, the normal squamous epithelial cells residing in the esophagus may change abnormally into a premalignant state, which can eventually progress into cancer.

Esophageal cancer is ranked sixth in cancer-related deaths [111]. It mainly affects adult males, chronic alcohol users, heavy tobacco users, and individuals with a high dietary intake of red meats [112]. Even though esophageal cancer is quite rare in comparison to other epithelia tumours [113], it has a very poor prognosis with roughly 90% of cases resulting in death amongst the US and Canadian adults [113, 114]. Such poor outcomes are mainly attributed to the disease being detected at advanced stages where curative treatments are no longer an option.

The two most common forms of esophageal cancer are squamous cell carcinoma and adenocarcinoma, which have been named according to the type of cells becoming malignant [115]. For instance, esophageal squamous cell carcinoma (ESCC) arises from the flat, thin, squamous cells, which line the esophagus whereas EAC arises from glandular metaplastic cells in the esophageal mucosa. ESCC is the most common type of esophageal cancer worldwide

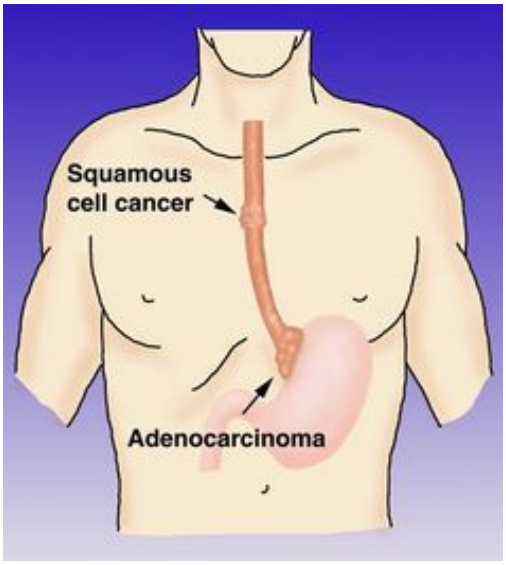


Figure 1.2. This diagram illustrates the location of the two types of esophageal cancer: esophageal squamous cell carcinoma and esophageal adenocarcinoma [116].

and is located at the upper two thirds of the esophagus whereas EAC is typically located at the lower third of the esophagus or gastroesophageal junction (GEJ) [115], as illustrated in Figure 1.2. The type of esophageal cancer varies considerably worldwide. Adenocarcinomas are much more prevalent in the United States [117], United Kingdom [118], and Canada [119], whereas squamous cell carcinoma are more common in Asia, Africa, and parts of Europe [120]. Andenocarcinoma of the esophagus is more predominant in Caucasians versus African Americans at a ratio of 4:1 whereas squamous cell carcinoma is more prevalent in African Americans than Caucasians at a ratio of 6:1 [121]. Our research is focused on NTE in patients with adenocarcinoma of the esophagus instead of squamous cell carcinoma since it has a higher incidence in this region of the world.

Both cigarette smoking and chronic alcohol use have been shown to enhance the risk of developing ESCC and EAC by tenfold and threefold, respectively [122]. One of the most important differences between ESCC and EAC is adenocarcinomas of the esophagus develops from a precancerous condition known as gastroesophageal reflux disease (GERD) which occurs from persistent reflux of gastric acid [112]. In Sweden, one of the largest population based case-control studies found individuals living with long-term gastric acid reflux for 20 plus years had an adjusted odds ratio of 43.5 for EAC compared to the control group [123]. The authors also found no association between gastric acid reflux and squamous cell carcinoma. In this same study, patients with adenocarcinoma of the esophagus were eight times more likely to have acid reflux symptoms weekly compared to ESCC patients. Within developed

countries, obesity seems to be another important contributing factor to the sudden increase in EAC, since these regions of the world have a higher incidence of obese individuals who also have GERD [124]. Gastric reflux also has a strong association for a condition called Barrett's esophagus [125], which was named in 1950 by Norman Barrett [126]. To combat the constant acidic environment,the cells in this condition adapt by undergoing intestinal metaplasia which replaces the normal squamous epithelium cells in the esophagus with columnar epithelium from the stomach [125]. Barrett's esophagus is a condition, which occurs in both males and females, although it does seem to, statistically, be more common in males [112, 127]. One very important complication for this condition is it may eventually progress into adenocarcinoma of the esophagus. Studies have shown this condition can increase the risk for developing EAC by 30x to 125x more than those in the general population [128, 129].

1.2.1 Progression of Barrett's esophagus to adenocarcinoma

Cancer develops from a number of spontaneous mutations, as it is a multistep process. Usually these alterations can result in uncontrollable cell division and further tumour development and proliferation [130]. The complete loss of heterozygosity (LOH) of 17p (p53) [131, 132] and p16 [132–134] are common alterations while cells transition into adenocarcinoma of the esophagus. Even though every type of cancer seems to be different, all cancers seem to share six hallmarks of carcinogenesis. In 2011, a review published by Hanahan and Weinberg made it clear that many cancerous cells provide a competitive advantage over normal cells due to their unlimited supply of growth signals that drive the indefinite proliferation of cells, avoidance of growth-inhibitory signals, loss of apoptotic sensitivity, angiogenesis formation, tumour invasion and proliferation within other neighbouring tissues [130, 135]. Below is a brief summary of the six hallmarks of carcinogenesis described in regards to cancer along with a few alterations that commonly aid in the progression of Barrett's esophagus into adenocarcinoma of the esophagus.

Cancer cells have the ability to free themselves from exogenous growth factors and produce their own growth signals to stimulate cell division in their favour. Both Barrett's esophagus and esophageal cancers have an overexpression of cyclin D and cyclin E, which allows the cells to progress uncontrollably through the G1 checkpoint [136]. Furthermore, esophageal carcinomas have been observed to have an increased abnormality with the tyrosine-kinase growth factor receptors and their associated ligands epidermal growth factors [137, 138].

Apart from cancer cells reproducing without receiving growth signals, they can also continue to grow even in the presence of anti-growth signals. The inactivation of tumour suppressor genes by deletion or mutation is one of the driving forces behind this hallmark of carcinogenesis. In esophageal cancers, the p16 tumour suppressor gene has been found to be involved in blocking cellular proliferation and preventing entry into S-phase of the cell cycle. Studies have reported a significant loss of the p16 expression by LOH and as a result of promoter hypermethylation in esophageal carcinomas [133, 139].

Cancer cells also resist cell death when their DNA has been damaged. Cancers evolve to limit the sensitivity of apoptosis by the loss of TP53 tumour suppressor function. Normal cells undergo apoptosis in a p53 dependent manner when irreparable DNA damage presents itself. The TP53 expression is degraded when bound to its negative regulator, mouse double minute 2 homolog (Mdm2). When both TP53 and Mdm2 become phosphorylated following some kind of cytotoxic stressor, the two proteins can no longer interact. As a result, this leads to an accumulation of p53 in damaged cells that can activate growth arrest and apoptotic effector pathways independently by upregulating cyclin dependent kinase (CDK) inhibitor p21 and pro-apoptotic proteins (Bax and Puma), respectively [88]. The loss of TP53 tumour suppressor function is very common in a number of tumours including esophageal cancer [99, 130, 131], in fact, 52% to 93% of EACs have complete loss of p53 function [131, 140]. Since the majority of cancers have complete loss of p53 proteins and apoptosis is highly regulated

by p53, tumours tend to eliminate this critical DNA damage response pathway resulting in resistance to cell death [141]. However, not all mutant p53 tumours result in loss of function but may still allow the p53 protein to respond to stress. 'Hot spot' amino acid residues have been reported which allow p53 to interact with its DNA binding sites to upregulate key genes needed for the DNA damage response [142]. For instance, the p53 mutant human keratinocyte HaCaT has been discussed above to have a gain of function mutation allowing an alternative apoptotic pathway following exposure to a cellular stressor.

Like normal tissues, tumours can develop new blood vessels to supply the cancer cells with nutrients. The process of angiogenesis becomes active and remains switched on in cancer cells. This ensures that a constant supply of nutrients is available which aids in the growth of tumours [143]. In the absence of a blood supply, it has been reported that the maximum size a tumour would reach is roughly 23 mm in size since nutrients would be limited by diffusion [144]. Cancer cells typically secrete vascular endothelial growth factors (VEGF) to aid in cell proliferation and migration of cancer cells into neighbouring tissues [145]. Both highly dysplastic Barrett's esophagus and EAC been shown to have remarkable increases in VEGF receptors [146].

Unlike normal cells, cancer cells have an unlimited replicative potential that drives cancer cells to proliferate indefinitely. One possible explanation for this can be attributed to the restabilization of telomeres [130, 135]. After each cell cycle, the telomeres known as the repeating non-coding regions of the chromosome are lost. Once the telomere reaches a certain length where the coded regions of the chromosome are no longer protected, the cell undergoes a form of cell death known as senescence. Almost all human cancers, roughly 85 to 95%, have an overexpression of an enyzme called telomerase that prevents the shortening of the telomeres [130, 147]. High expressions of telomerases have been reported in high grade dyplastic cancers and EAC [148, 149].

Finally, cancer cells can break loose and evade distant tissues, which is known as

metastasis. Loss of cell-to-cell adhesion molecules known as E-cadherin is one process that aids in tissue invasion and metastasis [135, 150–152]. Cancer cells invade nearby tissues by escaping into nearby blood and lymphatic vessels [135]. Unlike normal cells that have high expressions of E-cadherin, these act as an antagonist for metastasis, the expression of the molecules are normally much lower in cancer cells [150–152].

1.2.2 Patient diagnostic workup and treatments

When a patient presents with symptoms of esophageal cancer, this usually means difficulty while swallowing known as dysphagia [21]. There are varying degrees of dysphagia that can be quantified using the scale developed by Mellow and Pinkas in 1985, as shown in Table 1.5. Dysphagia is scored from 0 to 4, with 0 indicating the patient having the ability to eat a normal diet whereas a score of 4 indicates the patient is unable to swallow liquids including their own saliva. The need to mitigate such symptoms is an area of active research for esophageal cancer in order to improve patients' quality of life by being dysphagia-free [21, 22].

Table 1.5. Dysphagia Scores Description

Scores	Description
0	No dysphagia: able to eat normal diet
1	Moderate passage: able to eat some solid foods
2	Poor passage: able to eat semi-solid foods
3	Very poor passage: able to swallow liquids only
4	No passage: unable to swallow anything

A barium swallow is one of the first tests used to visualize the contours of the esophagus for aberrations or obstructions [154]. Any irregularities seen on the radiographs that may be suggestive of cancer require further evaluation with an endoscope to visualize abnormal masses [155]. Also, several diagnostic imaging modalities can be used for confirmation and cancer staging including magnetic resonance imaging (MRI), computed tomography (CT), position emission tomography (PET) and endoscopic ultrasound (EUS). However, EUS is predominantly used for tumour node metastasis (TNM) cancer staging for patients presenting with advanced stages of EAC [156]. From a meta-analysis consisting of 49 studies, EUS has been shown to have excellent sensitivity 92.4% (95% CI: 89.2-95.0) and specificity of 97.4%

(95% CI: 96.6-98.0) in accurately staging advanced cases of esophageal cancer [156]. It also shows remarkable delineation of the individual histological layers of the esophagus [157] with a much higher sensitivity in detecting lymph node involvement in comparison to CT and PET [158]. Both CT and MRI cannot accurately image the different esophageal mucosa layers [156], and, therefore, have difficulty in cancer staging with an accuracy of roughly 40% of the time [159, 160].

Esophageal carcinomas are staged based upon the TNM classification system. Table 1.6 summarizes the staging groups used for esophageal tumours, and the primary tumour (T), regional lymph nodes (N), and distant metastasis (M). Stage 0 tumours have an *in situ* cancerous mass that lie within the inner lining of the esophageal mucosa. Once the cancerous mass has penetrated into the submucosa of the esophagus, this tumor becomes invasive and is classified as a stage I cancer. The classification of stage II cancers comprises of two stages called IIA and IIB.

Table 1.6. Cancer Staging

Stage Group	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T2	N0	M0
	T3	N0	M0
Stage IIB	T1	N1	M0
	T2	N1	M0
Stage III	T3	N1	M0
	Any T	Any N	M0
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

Table was adapted from [161] .

In stage IIA, the cancerous cells have become more invasive and have invaded into the muscular layers of the esophagus. Stage IIB carcinomas have invaded the lymphatic systems or nearby lymph nodes. Once the cancer has transitioned to stage III, the malignancy has invaded the lymph nodes and spread to nearby tissues or organs. Stage IV cancer has metastasized to 'distance' tissues or organs [161]. Figure 1.3 illustrates the various stages of esophageal cancers according to the TNM classification system [162].

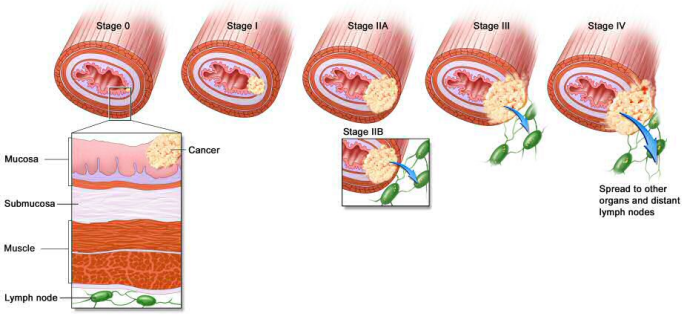


Figure 1.3. This figure shows an illustration of esophageal carcinoma staging [162].

Treatment selection is greatly dependent on the stage of cancer found at time of diagnosis. This type of cancer is typically detected at advanced stages where curative treatments are no longer an option. The aggressive nature of this cancer combined with late detection at advanced stages often leads to a very poor prognosis [21, 22, 112]. Consequently, those that do survive have only a 14% chance of surviving for 5 years [113]. The higher death rate amongst Canadian adult males is not unique to this country, rather this seems to be a common characteristic of the disease that has been observed throughout the United States, Australia, and Asian adult populations [112]. Symptoms often develop only after the tumor has grown fairly large in size or has spread to distant organs. Surgery, radiation, and chemotherapy are commonly used for esophageal cancer treatments. However, only 10% to 20% of esophageal cancer cases are suitable for surgical removal [163]. Even in those patients that do qualify for surgery, the surgical intervention tends to be palliative [164]. This can be considerably difficult to justify considering the possibility of postoperative complications and even the chance of mortality [165].

There is some debate as to the optimal order in which to deliver HDR brachytherapy, EBRT, and chemotherapy to the esophagus [166–170]. Different institutions may vary the order in which they use these three modalities in an attempt to achieve a faster and more effective dysphagia relief in advanced stages of esophageal cancer. Many anti-cancer therapies focused on advanced stages of esophageal cancer treatments can be used alone or together as a boost to HDR brachytherapy. Both EBRT and chemotherapy have been documented in the literature to be used either prior to or after brachytherapy [109, 170–173]. Both radiation therapy and chemotherapy are used to enhance tumour cell killing. Radiation

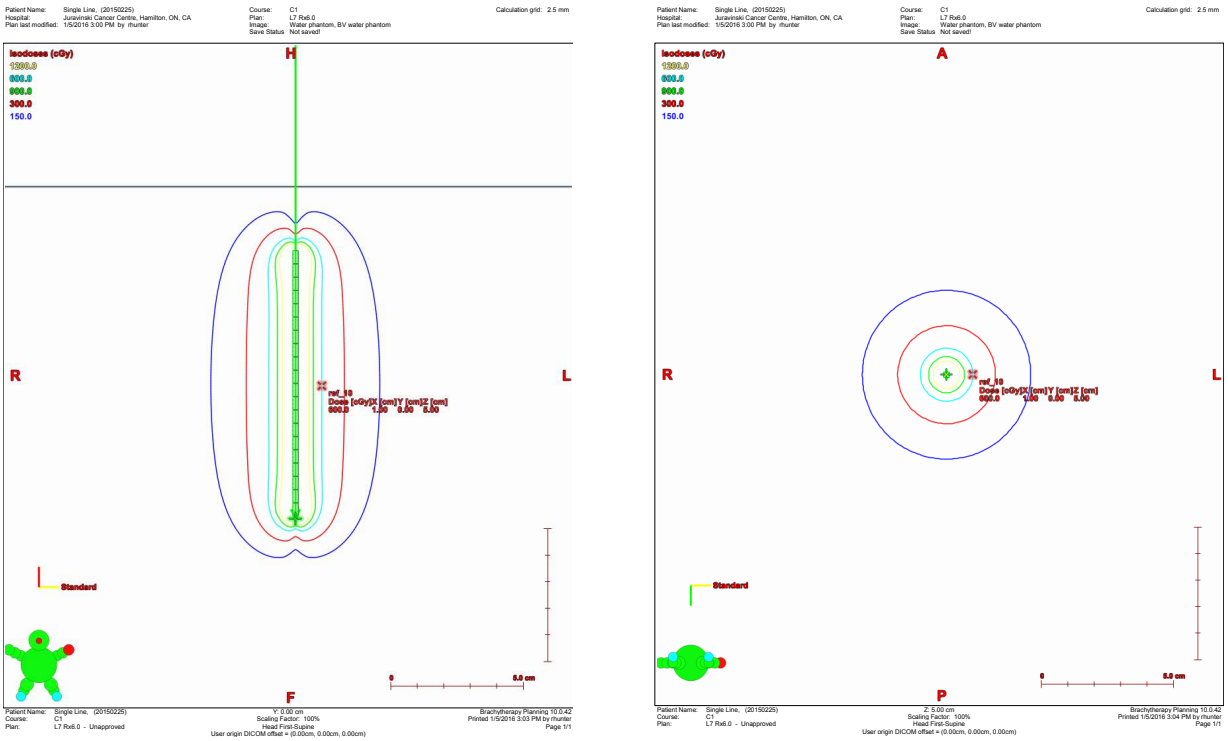
treatments target the DNA of esophageal tumour cells by using either high energy x-rays (EBRT) or gamma-irradiation (brachytherapy) [174], whereas chemotherapy targets various cellular mechanisms of rapidly dividing cells [109, 170, 175]. Chemotherapy cannot selectively target cancer cells since these are not the only cells dividing in the body. For example, hair follicles and the digestive tract have normal cells undergoing mitosis and are often affected in chemotherapy [175]. In some cases, the patients often experience hair loss and emesis [175, 176]. Unlike cancer cells, normal cells are better at repairing damage to their DNA, and radiotherapy uses these facts by trying to damage the DNA in the cancerous cells. Thus, for the purpose of our clinical studies, none of the patients received any EBRT or chemotherapy prior to and during the HDR brachytherapy treatments. However, EBRT is often given as a boost to improve the effectiveness of brachytherapy, but this typically follows HDR brachytherapy regimens [170].

1.2.3 HDR brachytherapy for esophageal cancer

Since HDR brachytherapy is an effective treatment to improve dysphagia-free survival, this treatment is often preferred to any other palliative radiotherapy. Between the years of 1980 to 2013, studies demonstrated the potential use of HDR brachytherapy as a palliative treatment for advanced cases of esophageal cancer [109, 169, 170, 177, 178]. In 1995, a preliminary study found low dose rate (LDR) brachytherapy was highly effective at achieving dysphagia relief since 90% of the patients were able to eat semi-solid foods until death [168]. Another research group found HDR brachytherapy improves dysphagia in most patients, and also significantly increased the median survival time in patients with smaller size tumours (12.1 months) compared to larger size ones (6.4 months). A retrospective study was undertaken from January 2006 to 2010, the authors reported that 56% of the patients had a significant improvement in their dysphagia symptoms following HDR brachytherapy [169]. An International Atomic Energy Agency (IAEA) randomized study was performed on

219 esophageal cancer patients [170]. The investigators assigned patients either to receive two fractions of HDR brachytherapy alone or two fractions of HDR brachytherapy followed by 10 fractions of EBRT. These clinical trials involved multiple hospitals across six different countries (Brazil, China, Croatia, India, South Africa and Sudan). The authors found a global dysphagia score of 1.23 and 0.79 for HDR brachytherapy and HDR brachytherapy plus EBRT, respectively. This resulted in a 0.44 drop in dysphagia scores for HDR brachytherapy plus EBRT procedures. Therefore, two fractions of HDR brachytherapy followed by 10 fractions of EBRT provide better dysphagia-relief in esophageal cancer patients. Even with inter-hospital and inter-patient variability, it is apparent that many clinicians have observed significant improvements in their patients' dysphagia-free survival. Overall, these studies suggest HDR brachytherapy to be a very promising palliative radiation treatment option for patients with advanced stages of esophageal cancer.

Iridium-192 (^{192}Ir) is considered to be a suitable radionuclide when treating esophageal cancer with HDR brachytherapy [179]. ^{192}Ir has a short half-life and high specific activity allowing the temporary placement of a small source in the esophageal lumen. These treatments typically last for only a couple of minutes. The ^{192}Ir radioactive source has an initial activity of 10 Curie (Ci) when supplied by the manufacturer [180, 181]. This source is typically replaced after one half-life to eliminate prolonging treatment times [182]. This equates to the source being changed roughly every 3 months because it has a half-life of 73.83 days [183]. Outline below is a typical example of isodose lines for an esophageal cancer case shown with two views, a coronal (a) and transverse (b) view.



(a) (b)

Figure 1.4. The VariSource^{TM} iX HDR Brachytherapy afterloader and treatment planning BrachyVison software are used for esophageal cancer patients' treatments at the JCC. These images show an example of a coronal (a) and transverse (b) view of isodose lines for an esophageal cancer case. A dose of 600 cGy is prescribed 1 cm from the source axis.

Afterloading is a method used to deliver the radioactive source to an area in close proximity to the tumour by using an applicator that is connected to the remote afterloader [181]. This esophageal applicator is positioned by use of a flexible guide wire inserted into the esophagus under endoscopy guidance. Following the removal of the endoscope, an esophageal applicator is passed along the guide wire through the esophagus to the tumour. A dummy catheter containing radiopaque markers is then threaded through the application to allow definition of treatment margins. In general, a 2 cm treatment margin is added proximal and distal to the tumour to ensure the entire tumour volume has been irradiated, as shown in Figure 1.5.

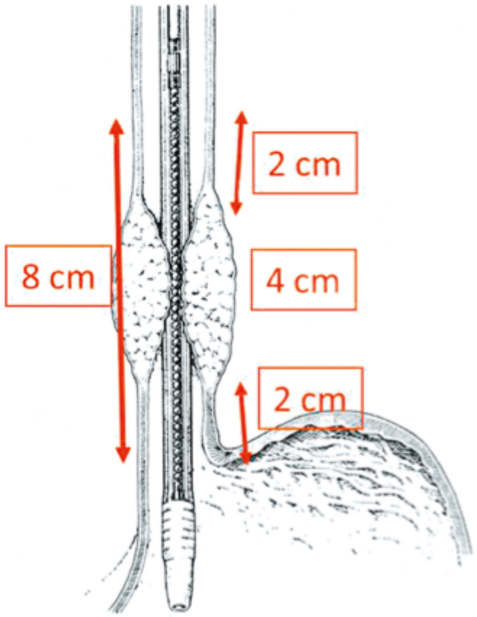


Figure 1.5. An illustration of an esophageal applicator, containing a catheter with radiopaque markers, placed in close proximity to a 4 cm tumour [184]. The treatment volume extends an additional 2 cm proximal and distal to the tumour in order to ensure the entire tumour has been treated.

This 2 cm treatment margin may change from patient to patient depending on their endoscopy results. Fluoroscopy is used to confirm the applicator has been placed properly prior to treatment and to establish the treatment length. The dummy catheter is then removed and is replaced by an active catheter that will allow the passing of the active source. The radiation is administered to the patient safely from a remote location to minimize occupational exposure. The afterloader moves a single ^{192}Ir source in specified steps, known as dwell positions [181]. At each dwell position, the source stops for a certain length of time referred to as dwell time. The source moves through a series of dwell positions without the need to use multiple sources since one ^{192}Ir source can simulate a series of sources simply by altering the dwell time [181, 185]. The total dose of each treatment fraction was typically 600 cGy and was completed within two weeks. Furthermore, the time interval between fraction 1 and 2 was 6 days whereas the time interval between fraction 2 and 3 was 24 hours.

1.2.4 Relevance of radiation-induced bystander effects

The vast majority of published data in the bystander and NTE field concern low dose risk (as reviewed in [186, 187]). However, bystander effects have also been shown to occur at high doses and can contribute significantly to total cell death at the doses used in radiotherapy [18, 188]. As they are linked to induction of genomic instability [189], they may also play

a role in promoting carcinogenesis or the recurrence of cancers [24, 190, 191]. One of the only studies to show the carcinogenic potential of bystander effects *in vivo* was by Mancuso and colleagues, in 2008 [192]. These authors used a radiosensitive Patched-1 heterozygous mouse model to investigate bystander effects. The development of medulloblastoma was observed in the cerebellum following x-ray exposure of the remainder of the body. This work has been suggestive to be a type of 'distant' bystander effect [186]. Other work has suggested bystander effects to be beneficial where more cell death could mean less pre-cancer mutations available to increase carcinogenesis risk, but on the other hand, higher damage in bystander cells could mean this phenomenon is a detrimental effect [193, 194]. Several reviews on NTE have emphasized a link to carcinogenesis [18, 79, 186], which may signify the bystander non-targeted phenomenon heading into a more clinical setting to seek out the risk involved in radiotherapy. It may also have wider applicability in other human exposure situations including environmental exposures, accident situations, and planned low dose medical procedures, since bystander responses are influenced by both environmental and genetic factors [25, 48].

In summary, the aim of this thesis is to examine the expression of bystander effects in brachytherapy patients as a subset of radiotherapy patients. The reason for choosing brachytherapy was related to existing funding available to support this work. This thesis focused on the following questions:

1. **Are bystander effects produced in brachytherapy patients?**
   Cell culture work suggests that bystander effects may have relevance in radiotherapy, but very few studies were performed on a cohort of patients undergoing radiotherapy (as reviewed in [10, 18, 24]).

2. **Are they detectable in non-tumour samples such as blood and urine?**
   Both blood and urine samples have been found to carry signals post-irradiation and elicit a response in non-irradiated mice [47, 55, 195], but this is not typically shown with human models.

3. **Do they persist during fractionated treatments?**

There are conflicting findings from *in vitro* work investigating fractionated ICCM versus one single ICCM exposure. One study demonstrated a higher level of cytotoxic bystander factors being produced [29] while another study showed an adaptation to previously exposed soluble bystander factors during fractionation [28]. Such results are one of the main motivations behind the pilot study following cancer patients throughout their entire fractionated HDR brachytherapy treatments.

4. Are RIBE in any way related to outcome, stage of the cancer, gender, lifestyle factors such as smoking status?

Earlier work shows that bystander signal production is influenced by gender [25, 47]. Samples collected from females yield significant reductions in cloning efficiency in healthy patients [25], but animal mouse models show conflicting findings that suggest males produce larger bystander effects [47]. In addition, smoking status and the presence of a malignancy have been suggested to influence bystander signal generation [25, 77]. The role of bystander effects in modulating patient outcome has not been investigated to date, but they have been proposed to have importance in radiotherapy [24]. Based upon past work, I have hypothesized that certain patient characteristics such as gender and smoking status will influence the bystander signal production in this cohort of cancer patients.

5. Are available esophageal cell lines useful for studying bystander effects and their underlying mechanisms such as the role of serotonin?

There are many studies showing the involvement of serotonin in bystander signal production and responses [12–16], but there is no work on bystander effects in esophageal cancer cells. Therefore, I hypothesized that if bystander effects are induced in esophageal cancer cell lines serotonin driven mechanisms will play a part in bystander signal generation and bystander responses [12, 13, 16]. There is also a chance that the p53 status of the esophageal cancer cell lines may have a role to play in determining whether these cells can respond to bystander signals as shown in past work [27].

1.3 References

[1] F. Lyng, C. Seymour, and C Mothersill. "Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis". In: *British journal of cancer* 83.9 (2000), p. 1223.

[2] C Mothersill and C Seymour. "Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells." In: *Int. J. Radiat. Biol* 71.4 (1997), pp. 421–427.

[3] K. Prise. "Studies of bystander effects in human fibroblasts using a charged particle microbeam". In: *International journal of radiation biology* 74.6 (1998), pp. 793–798.

[4] C. B. Seymour and C. Mothersill. "Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve". In: *Radiation research* 153.5 (2000), pp. 508–511.

[5] C. Seymour and C Mothersill. "Delayed expression of lethal mutations and genomic instability in the progeny of human epithelial cells that survived in a bystander-killing environment". In: *Radiation oncology investigations* 5.3 (1997), pp. 106–110.

[6] W. F. Morgan. "Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects in vivo, clastogenic factors and transgenerational effects". In: *Radiation research* 159.5 (2003), pp. 581–596.

[7] T. K. Hei et al. "Mechanism of radiation-induced bystander effects: a unifying model". In: *The Journal of pharmacy and pharmacology* 60.8 (2008), p. 943.

[8] H. Nagasawa and J. B. Little. "Induction of sister chromatid exchanges by extremely low doses of α-particles". In: *Cancer research* 52.22 (1992), pp. 6394–6396.

[9] C. Mothersill and C. Seymour. "Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for

release during irradiation of a signal controlling survival into the medium". In: *Radiation research* 149.3 (1998), pp. 256–262.

[10] C. B. Seymour and C. Mothersill. "Radiation-induced bystander effects-implications for cancer". In: *Nature Reviews Cancer* 4.2 (2004), pp. 158–164.

[11] F. Lyng, C. Semour, and C Mothersill. "Early events in the apoptotic cascade initiated in cells treated with medium from the progeny of irradiated cells". In: *Radiation protection dosimetry* 99.1-4 (2002), pp. 169–172.

[12] R. Poon et al. "Bystander effects of ionizing radiation can be modulated by signaling amines". In: *Environmental research* 105.2 (2007), pp. 200–211.

[13] R. Saroya et al. "Injection of resperpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: confirmation in vivo of a role for serotonin in the mechanism". In: *Dose-Response* 8.3 (2010), dose–response.

[14] C. Mothersill et al. "Serum serotonin levels determine the magnitude and type of bystander effects in medium transfer experiments". In: *Radiation research* 174.1 (2010), pp. 119–123.

[15] E. Kalanxhi and J. Dahle. "The role of serotonin and p53 status in the radiation-induced bystander effect". In: *International journal of radiation biology* 88.10 (2012), pp. 773–776.

[16] J. Fazzari et al. "Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect". In: *International journal of radiation biology* 88.10 (2012), pp. 786–790.

[17] C Mothersill and C Seymour. "Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells." In: *International journal of radiation biology* 71.4 (1997), pp. 421–427.

[18] K. M. Prise and J. M. O'Sullivan. "Radiation-induced bystander signalling in cancer therapy". In: *Nature Reviews Cancer* 9.5 (2009), pp. 351–360.

[19] C. Pinho et al. "Assessing patient characteristics and radiation-induced non-targeted effects in vivo for high dose-rate (HDR) brachytherapy". In: *Int. J. Radiat. Biol* (2015), pp. 1–9. DOI: 10.3109/09553002.2015.1068458.

[20] C Hanu et al. "The influence of smoking on radiation-induced bystander signal production in esophageal cancer patients". In: *Environmental Research* (2016).

[21] R. K. Sur et al. "Fractionated high dose rate intraluminal brachytherapy in palliation of advanced esophageal cancer". In: *International Journal of Radiation Oncology* Biology* Physics* 40.2 (1998), pp. 447–453.

[22] R. K. Sur et al. "Prospective randomized trial of HDR brachytherapy as a sole modality in palliation of advanced esophageal carcinoma-an International Atomic Energy Agency study". In: *International Journal of Radiation Oncology* Biology* Physics* 53.1 (2002), pp. 127–133.

[23] J. J. Boonstra et al. "Verification and unmasking of widely used human esophageal adenocarcinoma cell lines". In: *Journal of the National Cancer Institute* 102.4 (2010), pp. 271–274.

[24] K. Butterworth et al. "Bystander signalling: exploring clinical relevance through new approaches and new models". In: *Clinical Oncology* 25.10 (2013), pp. 586–592.

[25] C. Mothersill et al. "Individual variation in the production of abystander signal'following irradiation of primary cultures of normal human urothelium". In: *Carcinogenesis* 22.9 (2001), pp. 1465–1471.

[26] F. M. Lyng et al. "The involvement of calcium and MAP kinase signaling pathways in the production of radiation-induced bystander effects". In: *Radiation Research* 165.4 (2009), pp. 400–409.

[27] C. Mothersill et al. "A role for p53 in the response of bystander cells to receipt of medium borne signals from irradiated cells". In: *International journal of radiation biology* 87.11 (2011), pp. 1120–1125.

[28] R. Iyer and B. E. Lehnert. "Alpha-particle-induced increases in the radioresistance of normal human bystander cells". In: *Radiation research* 157.1 (2002), pp. 3–7.

[29] C. Mothersill and C. Seymour. "Bystander and delayed effects after fractionated radiation exposure". In: *Radiation research* 158.5 (2009), pp. 626–633.

[30] L. A. Ryan et al. "Dilution of irradiated cell conditioned medium and the bystander effect". eng. In: *Radiation research* 169.2 (Feb. 2008), pp. 188–196. ISSN: 0033-7587. DOI: 10.1667/RR1141.1.

[31] F. M. Lyng et al. "The importance of serum serotonin levels in the measurement of radiation-induced bystander cell death in HaCaT cells". In: *International journal of radiation biology* 88.10 (2012), pp. 770–772.

[32] F. M. Lyng, O. L. Howe, and B. McClean. "Reactive oxygen species-induced release of signalling factors in irradiated cells triggers membrane signalling and calcium influx in bystander cells". In: *International journal of radiation biology* 87.7 (2011), pp. 683–695.

[33] C. Mothersill et al. "A laboratory inter-comparison of the importance of serum serotonin levels in the measurement of a range of radiation-induced bystander effects: overview of study and results presentation". In: *International journal of radiation biology* 88.10 (2012), pp. 763–769.

[34] C. Seymour, C. Mothersill, and T. Alper. "High yields of lethal mutations in somatic mammalian cells that survive ionizing radiation". In: *International Journal of Radiation Biology* 50.1 (1986), pp. 167–179.

[35] U Weissenborn and C Streffer. "Analysis of structural and numerical chromosomal aberrations at the first and second mitosis after X irradiation of two-cell mouse embryos". In: *Radiation research* 117.2 (1989), pp. 214–220.

[36] C Mothersill et al. "Involvement of energy metabolism in the production of 'bystander effects' by radiation". In: *British journal of cancer* 82.10 (2000), p. 1740.

[37] J. B. Murphy and J. J. Norton. "The effect of x-ray on the resistance to cancer in mice". In: *Science* (1915), pp. 842–843.

[38] K.-o. Goh. "Total-Body Irradiation and Human Chromosomes: IV. Cytogenetic Follow-Up Studies 8 and 10 1/2 Years after Total-Body Irradiation". In: *Radiation research* 62.2 (1975), pp. 364–373.

[39] G. S. Pant and N Kamada. "Chromosome aberrations in normal leukocytes induced by the plasma of exposed individuals". In: *Hiroshima Journal of Medical Sciences* 26.2-3 (Sept. 1977), pp. 149–154. ISSN: 0018-2052. URL: `http://www.ncbi.nlm.nih.gov/pubmed/591380` (visited on 04/04/2012).

[40] G. B. Faguet, S. M. Reichard, and D. A. Welter. "Radiation-induced clastogenic plasma factors". In: *Cancer genetics and cytogenetics* 12.1 (1984), pp. 73–83.

[41] I Emerit et al. "Transferable clastogenic activity in plasma from persons exposed as salvage personnel of the Chernobyl reactor". In: *Journal of cancer research and clinical oncology* 120.9 (1994), pp. 558–561.

[42] P. Marozik et al. "Bystander effects induced by serum from survivors of the Chernobyl accident". In: *Experimental hematology* 35.4 (2007), pp. 55–63.

[43] P Lambin et al. "Hypersensitivity of a human tumour cell line to very low radiation doses". In: *International journal of radiation biology* 63.5 (1993), pp. 639–650.

[44] C. Mothersill and C. Seymour. "Changing paradigms in radiobiology". In: *Mutation Research/Reviews in Mutation Research* 750.2 (2012), pp. 85–95.

[45] K Goh and H Sumner. "Breaks in normal human chromosomes: are they induced by a transferable substance in the plasma of persons exposed to total-body irradiation?" eng. In: *Radiation research* 35.1 (July 1968), pp. 171–181. ISSN: 0033-7587.

[46] M. Kadhim et al. "Transmission of chromosomal instability after plutonium α-particle irradiation". In: *Nature* 355.6362 (1992), pp. 738–740.

[47] I. Koturbash et al. "Radiation-induced bystander effects in vivo are sex specific". In: *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 642.1 (2008), pp. 28–36.

[48] C. Mothersill et al. "Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo". In: *Radiat. Res.* 163.4 (2005), pp. 391–399.

[49] S. A. Lorimore et al. "The influence of p53 functions on radiation-induced inflammatory bystander-type signaling in murine bone marrow". In: *Radiat. Res.* 179.4 (2013), pp. 406–415.

[50] F. M. Lyng, C. Seymour, and C Mothersill. "Initiation of apoptosis in cells exposed to medium from the progeny of irradiated cells: a possible mechanism for bystander-induced genomic instability?" In: *Radiation research* 157.4 (2002), pp. 365–370.

[51] S. C. B. Fernandez-Palomo C and C. Mothersill. "Inter-relationship between low-dose hyper-radiosensitivity and radiation-induced bystander effects in the human T98G glioma and the epithelial HaCaT cell line". In: *Accepted. Rad Res.* (2016).

[52] J. Poleszczuk et al. "Connecting Radiation-Induced Bystander Effects and Senescence to Improve Radiation Response Prediction". In: *Radiation research* 183.5 (2015), pp. 571–577.

[53] H. Furlong et al. "Apoptosis is signalled early by low doses of ionising radiation in a radiation-induced bystander effect". In: *Mutat Res Fund Mol Mech Mut* 741 (2013), pp. 35–43. DOI: 10.1016/j.mrfmmm.2013.02.001.

[54] H. Furlong et al. "Identification of Key Proteins in Human Epithelial Cells Responding to Bystander Signals From Irradiated Trout Skin". In: *Dose-Response* 13.3 (2015), p. 1559325815597669.

[55] Y. Chai and T. K. Hei. "Radiation induced bystander effect in vivo". In: *Acta Medica Nagasakiensia* 53 (2008), S65.

[56] E. I. Azzam et al. "Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles". In: *Radiation research* 150.5 (1998), pp. 497–504.

[57] M. Le et al. "An Observed Effect of Ultraviolet Radiation Emitted from Beta-Irradiated HaCaT Cells upon Non-Beta-Irradiated Bystander Cells". In: *Radiation research* 183.3 (2015), pp. 279–290.

[58] M Le et al. "Factors affecting ultraviolet-A photon emission from β-irradiated human keratinocyte cells". In: *Physics in medicine and biology* 60.16 (2015), p. 6371.

[59] H. Yang, V. Anzenberg, and K. D. Held. "The time dependence of bystander responses induced by iron-ion radiation in normal human skin fibroblasts". In: *Radiation research* 168.3 (2007), pp. 292–298.

[60] Z. Liu et al. "A dose threshold for a medium transfer bystander effect for a human skin cell line". In: *Radiation research* 166.1 (2006), pp. 19–23.

[61] M. V. Sokolov and R. D. Neumann. "Radiation-induced bystander effects in cultured human stem cells". In: *PLoS One* 5.12 (2010), pp. 141–195.

[62] U. Kaźmierczak et al. "Investigation of the bystander effect in CHO-K1 cells". In: *Reports of Practical Oncology & Radiotherapy* 19 (2014), S37–S41.

[63] A. H. Al-Mayah et al. "Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation". In: *Radiation research* 177.5 (2012), pp. 539–545.

[64] K Kumar Jella et al. "Exosomes are involved in mediating radiation induced bystander signaling in human keratinocyte cells". In: *Radiation research* 181.2 (2014), pp. 138–145.

[65] C. Théry, M. Ostrowski, and E. Segura. "Membrane vesicles as conveyors of immune responses". In: *Nature Reviews Immunology* 9.8 (2009), pp. 581–593.

[66] J. Skog et al. "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers". In: *Nature cell biology* 10.12 (2008), pp. 1470–1476.

[67] S. Xu et al. "MiR-21 is involved in radiation-induced bystander effects". In: *RNA biology* 11.9 (2014), pp. 1161–1170.

[68] I Emerit et al. "Clastogenic factors in the plasma of Chernobyl accident recovery workers: anticlastogenic effect of Ginkgo biloba extract". In: *Radiation research* 144.2 (1995), pp. 198–205.

[69] C. Seymour and C. Mothersill. "Development of an in vivo assay for detection of non-targeted radiation effects". In: *Dose-Response* 4.4 (2006), dose–response.

[70] J. M. Kaminski et al. "The controversial abscopal effect". In: *Cancer treatment reviews* 31.3 (2005), pp. 159–172.

[71] K Ohba et al. "Abscopal regression of hepatocellular carcinoma after radiotherapy for bone metastasis". In: *Gut* 43.4 (1998), pp. 575–577.

[72] S. M. Hiniker, D. S. Chen, and S. J. Knox. "Abscopal effect in a patient with melanoma". In: *N Engl J Med* 366.21 (2012), p. 2035.

[73] G. Ehlers and M. Fridman. "Abscopal effect of radiation in papillary adenocarcinoma". In: *The British journal of radiology* 46.543 (1973), pp. 220–222.

[74] M. P. Nobler. "The Abscopal Effect in Malignant Lymphoma and Its Relationship to Lymphocyte Circulation 1". In: *Radiology* 93.2 (1969), pp. 410–412.

[75] I Koturbash et al. "Irradiation induces DNA damage and modulates epigenetic effectors in distant bystander tissue in vivo". In: *Oncogene* 25.31 (2006), pp. 4267–4275.

[76] I. Koturbash et al. "Role of epigenetic effectors in maintenance of the long-term persistent bystander effect in spleen in vivo". In: *Carcinogenesis* 28.8 (2007), pp. 1831–1838.

[77] C. Mothersill, C. Seymour, and M. Joiner. "Relationship between radiation-induced low-dose hypersensitivity and the bystander effect". In: *Radiation research* 157.5 (2002), pp. 526–532.

[78] L. Pirisi et al. "Continuous cell lines with altered growth and differentiation properties originate after transfection of human keratinocytes with human papillomavirus type 16 DNA". In: *Carcinogenesis* 9.9 (1988), pp. 1573–1579.

[79] C. Mothersill and C. Seymour. "Radiation-induced bystander effects: past history and future directions". In: *Radiat. Res.* 155.6 (2001), pp. 759–767.

[80] C. Fernandez-Palomo et al. "Use of synchrotron medical microbeam irradiation to investigate radiation-induced bystander and abscopal effects in vivo". In: *Physica Medica* (2015).

[81] F. M. Lyng et al. "Effect of a tobacco-related nitrosamine on intercellular communication in human urothelial cells: a possible factor in smoking-related bladder carcinogenesis." In: *Oncology research* 8.9 (1995), pp. 371–378.

[82] G. Kashino et al. "Effective suppression of bystander effects by DMSO treatment of irradiated CHO cells". In: *Journal of radiation research* 48.4 (2007), pp. 327–333.

[83] B. J. Blyth and P. J. Sykes. "Radiation-induced bystander effects: what are they, and how relevant are they to human radiation exposures?" In: *Radiation research* 176.2 (2011), pp. 139–157.

[84] C. Shao et al. "Nitric oxide-mediated bystander effect induced by heavy-ions in human salivary gland tumour cells". In: *International journal of radiation biology* 78.9 (2002), pp. 837–844.

[85] S. L. Irons et al. "The effect of genetic background and dose on non-targeted effects of radiation". In: *International journal of radiation biology* 88.10 (2012), pp. 735–742.

[86] K. D. Mansfield et al. "Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-α activation". In: *Cell metabolism* 1.6 (2005), pp. 393–399.

[87] M He et al. "Radiation-induced intercellular signaling mediated by cytochrome-c via a p53-dependent pathway in hepatoma cells". In: *Oncogene* 30.16 (2011), pp. 1947–1955.

[88] B. G. Wouters and A. C. Begg. "Irradiation-induced damage and the DNA damage response". In: *Basic clinical radiobiology* (2009), pp. 11–26.

[89] T. A. Lehman et al. "p53 mutations in human immortalized epithelial cell lines". In: *Carcinogenesis* 14.5 (1993), pp. 833–839.

[90] M. He et al. "A mutant P53 can activate apoptosis through a mechanism distinct from those induced by wild type P53". In: *FEBS letters* 517.1 (2002), pp. 151–154.

[91] M. P. Mattson and S. L. Chan. "Neuronal and glial calcium signaling in Alzheimer's disease". In: *Cell calcium* 34.4 (2003), pp. 385–397.

[92] K. Zhang and R. J. Kaufman. "From endoplasmic-reticulum stress to the inflammatory response". In: *Nature* 454.7203 (2008), pp. 455–462.

[93] D. R. Green and J. C. Reed. "Mitochondria and apoptosis". In: *Science* 281.5381 (1998), p. 1309.

[94] H. Zhou et al. "Mechanism of radiation-induced bystander effect: role of the cyclooxygenase-2 signaling pathway". In: *Proceedings of the National Academy of Sciences of the United States of America* 102.41 (2005), pp. 14641–14646.

[95] A. Luce et al. "Death receptor pathways mediate targeted and non-targeted effects of ionizing radiations in breast cancer cells". In: *Carcinogenesis* 30.3 (2009), pp. 432–439.

[96] E. Liao et al. "Radiation induces senescence and a bystander effect through metabolic alterations". In: *Cell death & disease* 5.5 (2014), e1255.

[97] R. C. Taylor, S. P. Cullen, and S. J. Martin. "Apoptosis: controlled demolition at the cellular level". In: *Nature reviews Molecular cell biology* 9.3 (2008), pp. 231–241.

[98] S. Elmore. "Apoptosis: a review of programmed cell death". In: *Toxicologic pathology* 35.4 (2007), pp. 495–516.

[99] B. G. Wouters. "Cell death after irradiation: how, when and why cells die". In: *Basic Clinical Radiobiology. 4th ed. London: Hodder Education* (2009), pp. 27–40.

[100] J. Adams and S Cory. "The Bcl-2 apoptotic switch in cancer development and therapy". In: *Oncogene* 26.9 (2007), pp. 1324–1337.

[101] J Li and J Yuan. "Caspases in apoptosis and beyond". In: *Oncogene* 27.48 (2008), pp. 6194–6206.

[102] Z. Jin and W. S. El-Deiry. "Overview of cell death signaling pathways". In: *Cancer biology & therapy* 4.2 (2005), pp. 147–171.

[103] E. J. Hall and A. J. Giaccia. *Radiobiology for the Radiologist*. Lippincott Williams & Wilkins, 2006.

[104] H. B. Forrester et al. "Using computerized video time lapse for quantifying cell death of X-irradiated rat embryo cells transfected with c-myc or c-Ha-ras". In: *Cancer research* 59.4 (1999), pp. 931–939.

[105] J. Campisi and F. d. di Fagagna. "Cellular senescence: when bad things happen to good cells". In: *Nature reviews Molecular cell biology* 8.9 (2007), pp. 729–740.

[106] Y. Deng, S. S. Chan, and S. Chang. "Telomere dysfunction and tumour suppression: the senescence connection". In: *Nature Reviews Cancer* 8.6 (2008), pp. 450–458.

[107] J.-P. Coppé et al. "The senescence-associated secretory phenotype: the dark side of tumor suppression". In: *Annual review of pathology* 5 (2010), p. 99.

[108] P. M. Devlin. *Brachytherapy: applications and techniques*. Lippincott Williams & Wilkins, 2007.

[109] R. Sur et al. "Randomized prospective study comparing high-dose-rate intraluminal brachytherapy (HDRILBT) alone with HDRILBT and external beam radiotherapy in

the palliation of advanced esophageal cancer". In: *Brachytherapy* 3.4 (2004), pp. 191–195.

[110] H. B. Gelberg. "Comparative anatomy, physiology, and mechanisms of disease production of the esophagus, stomach, and small intestine". In: *Toxicologic pathology* 42.1 (2014), pp. 54–66.

[111] Y. Zhang. "Epidemiology of esophageal cancer". In: *World journal of gastroenterology: WJG* 19.34 (2013), p. 5598.

[112] B. J. Reid et al. "Barrett's oesophagus and oesophageal adenocarcinoma: time for a new synthesis". In: *Nature Reviews Cancer* 10.2 (2010), pp. 87–101.

[113] Canadian Cancer Statistics AdvisoryCommittee. *Canadian Cancer Statistics 2015*. Toronto, ON, 2015.

[114] American Cancer Society. *Cancer Facts & Figures 2015*. Atlanta, 2015.

[115] D. Hendricks and M. I. Parker. "Oesophageal cancer in Africa". In: *IUBMB life* 53.4-5 (2002), pp. 263–268.

[116] Lin J, Reddy R. *The patient guide to heart, lung, esophageal surgery*. 2015.

[117] C. Hur et al. "Trends in esophageal adenocarcinoma incidence and mortality". In: *Cancer* 119.6 (2013), pp. 1149–1158.

[118] C. Lepage et al. "Continuing rapid increase in esophageal adenocarcinoma in England and Wales". In: *The American journal of gastroenterology* 103.11 (2008), pp. 2694–2699.

[119] J. R. Parfitt, Z. Miladinovic, and D. K. Driman. "Increasing incidence of adenocarcinoma of the gastroesophageal junction and distal stomach in Canada–An epidemiological study from 1964 to 2002". In: *Canadian journal of gastroenterology* 20.4 (2006), p. 271.

[120] L. Leichman and C. R. Thomas Jr. "Squamous cell cancer of the esophagus: the forgotten one". In: *Gastrointestinal cancer research: GCR* 4.1 (2011), p. 22.

[121] W. J. Blot and J. F. Fraumeni Jr. "Trends in esophageal cancer mortality among US blacks and whites." In: *American journal of public health* 77.3 (1987), pp. 296–298.

[122] M. Koshy et al. "Multiple management modalities in esophageal cancer: epidemiology, presentation and progression, work-up, and surgical approaches". In: *The oncologist* 9.2 (2004), pp. 137–146.

[123] J. Lagergren et al. "Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma". In: *New England journal of medicine* 340.11 (1999), pp. 825–831.

[124] J. Jeon, E. G. Luebeck, and S. H. Moolgavkar. "Age effects and temporal trends in adenocarcinoma of the esophagus and gastric cardia (United States)". In: *Cancer Causes & Control* 17.7 (2006), pp. 971–981.

[125] M. T. Suchorolski et al. "Warburg and Crabtree effects in premalignant BarrettâĂŹs esophagus cell lines with active mitochondria". In: *PloS one* 8.2 (2013), e56884.

[126] N. Barrett. "Chronic peptic ulcerz of the œophagus and âĂŸœsophagitisâĂŹ". In: *British Journal of Surgery* 38.150 (1950), pp. 175–182.

[127] B. J. Kendall et al. "Leptin and the risk of BarrettâĂŹs oesophagus". In: *Gut* 57.4 (2008), pp. 448–454.

[128] R. E. Sampliner, P. P. C. of the American College of Gastroenterology, et al. "Updated guidelines for the diagnosis, surveillance, and therapy of BarrettâĂŹs esophagus". In: *The American journal of gastroenterology* 97.8 (2002), pp. 1888–1895.

[129] M. Conio et al. "Long-term endoscopic surveillance of patients with Barrett's esophagus. Incidence of dysplasia and adenocarcinoma: a prospective study". In: *The American journal of gastroenterology* 98.9 (2003), pp. 1931–1939.

[130] a. S. S. Morales C Souza R. "Hallmarks of cancer progression in Barrett's oesophagus". In: *The Lancet* 360.4 (2002), pp. 1587–1589.

[131] B. J. Reid et al. "Predictors of progression in Barrett's esophagus II: baseline 17p (p53) loss of heterozygosity identifies a patient subset at increased risk for neoplastic progression". In: *The American journal of gastroenterology* 96.10 (2001), pp. 2839–2848.

[132] P. C. Galipeau et al. "Clonal expansion and loss of heterozygosity at chromosomes 9p and 17p in premalignant esophageal (Barrett's) tissue". In: *Journal of the National Cancer Institute* 91.24 (1999), pp. 2087–2095.

[133] H. Suzuki et al. "Intragenic mutations of CDKN2B and CDKN2A in primary human esophageal cancers". In: *Human molecular genetics* 4.10 (1995), pp. 1883–1887.

[134] G. Jenkins et al. "Genetic pathways involved in the progression of Barrett's metaplasia to adenocarcinoma". In: *British journal of surgery* 89.7 (2002), pp. 824–837.

[135] D. Hanahan and R. A. Weinberg. "Hallmarks of cancer: the next generation". In: *cell* 144.5 (2011), pp. 646–674.

[136] D Coppola et al. "Cyclin D1 expression correlates with degrees of dysplasia in Barrett's esophagus". In: *Laboratory Investigation*. Vol. 76. 1. Williams & Wilkins, Baltimore, MD. 1997, pp. 298–298.

[137] M Brito et al. "Transforming growth factor α expression in gastro-oesophageal tumorigenesis may reflect altered processing of the precusor peptide". In: *Int J Cancer* 60 (1995), pp. 27–32.

[138] J Jankowski et al. "Increased expression of epidermal growth factor receptors in Barrett's esophagus associated with alkaline reflux: a putative model for carcinogenesis." In: *The American journal of gastroenterology* 88.3 (1993), pp. 402–408.

[139] D. J. Wong et al. "p16INK4a promoter is hypermethylated at a high frequency in esophageal adenocarcinomas". In: *Cancer Research* 57.13 (1997), pp. 2619–2622.

[140] M. Younes et al. "p53 protein accumulation in Barrett's metaplasia, dysplasia, and carcinoma: a follow-up study". In: *Gastroenterology* 105.6 (1993), pp. 1637–1642.

[141] C. Morgan et al. "In vitro acid exposure has a differential effect on apoptotic and proliferative pathways in a Barrett's adenocarcinoma cell line". In: *The American journal of gastroenterology* 99.2 (2004), pp. 218–224.

[142] D. Bergamaschi et al. "p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis". In: *Cancer cell* 3.4 (2003), pp. 387–402.

[143] D. Hanahan and J. Folkman. "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis". In: *cell* 86.3 (1996), pp. 353–364.

[144] T. A. Martin et al. "Cancer Invasion and Metastasis: Molecular and Cellular Perspective". In: (2000).

[145] M. G. Achen et al. "Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4)". In: *Proceedings of the National Academy of Sciences* 95.2 (1998), pp. 548–553.

[146] M. I. Auvinen et al. "Incipient angiogenesis in Barrett's epithelium and lymphangiogenesis in Barrett's adenocarcinoma". In: *Journal of clinical oncology* 20.13 (2002), pp. 2971–2979.

[147] J. Shay and S Bacchetti. "A survey of telomerase activity in human cancer". In: *European journal of cancer* 33.5 (1997), pp. 787–791.

[148] C. P. Morales, E. L. Lee, and J. W. Shay. "In situ hybridization for the detection of telomerase RNA in the progression from Barrett's esophagus to esophageal adenocarcinoma". In: *Cancer* 83.4 (1998), pp. 652–659.

[149] R. V. Lord et al. "Telomerase reverse transcriptase expression is increased early in the Barrett's metaplasia, dysplasia, adenocarcinoma sequence". In: *Journal of Gastrointestinal Surgery* 4.2 (2000), pp. 135–142.

[150] S. Swami, S. Kumble, and G. Triadafilopoulos. "E-cadherin expression in gastroesophageal reflux disease, Barrett's esophagus, and esophageal adenocarcinoma: an

immunohistochemical and immunoblot study." In: *The American journal of gastroenterology* 90.10 (1995), pp. 1808–1813.

[151] U. Cavallaro and G. Christofori. "Cell adhesion and signalling by cadherins and Ig-CAMs in cancer". In: *Nature Reviews Cancer* 4.2 (2004), pp. 118–132.

[152] W Zschiesche et al. "Expression of E-cadherin and catenins in invasive mammary carcinomas." In: *Anticancer research* 17.1B (1996), pp. 561–567.

[153] M. H. Mellow and H. Pinkas. "Endoscopic laser therapy for malignancies affecting the esophagus and gastroesophageal junction: analysis of technical and functional efficacy". In: *Archives of internal medicine* 145.8 (1985), pp. 1443–1446.

[154] B. Kumbasar. "Carcinoma of esophagus: radiologic diagnosis and staging". In: *European journal of radiology* 42.3 (2002), pp. 170–180.

[155] J. W. van Sandick et al. "Impact of endoscopic biopsy surveillance of BarrettâĂŹs oesophagus on pathological stage and clinical outcome of BarrettâĂŹs carcinoma". In: *Gut* 43.2 (1998), pp. 216–222.

[156] S. R. Puli et al. "Staging accuracy of esophageal cancer by endoscopic ultrasound: a meta-analysis and systematic review". In: *World journal of gastroenterology: WJG* 14.10 (2008), p. 1479.

[157] M. J. Wiersema and L. M. Wiersema. "High-resolution 25-megahertz ultrasonography of the gastrointestinal wall: histologic correlates". In: *Gastrointestinal endoscopy* 39.4 (1993), pp. 499–504.

[158] P. R. Pfau et al. "The role and clinical value of EUS in a multimodality esophageal carcinoma staging program with CT and positron emission tomography". In: *Gastrointestinal endoscopy* 65.3 (2007), pp. 377–384.

[159] S. Takashima et al. "Carcinoma of the esophagus: CT vs MR imaging in determining resectability." In: *AJR. American journal of roentgenology* 156.2 (1991), pp. 297–302.

[160] J Vickers. "Role of endoscopic ultrasound in the preoperative assessment of patients with oesophageal cancer." In: *Annals of the Royal College of Surgeons of England* 80.4 (1998), p. 233.

[161] T. W. Rice, E. H. Blackstone, and V. W. Rusch. "of the AJCC Cancer Staging Manual: esophagus and esophagogastric junction". In: *Annals of surgical oncology* 17.7 (2010), pp. 1721–1724.

[162] T. Program. *Esophageal Cancer*. 2014. URL: `http://top.ucsf.edu/conditions--procedures/esophageal-cancer.aspx`.

[163] J. Skowronek, T. Piotrowski, and G. Zwierzchowski. "Palliative treatment by high–dose-rate intraluminal brachytherapy in patients with advanced esophageal cancer". In: *Brachytherapy* 3.2 (2004), pp. 87–94.

[164] C. E. Reed. "Comparison of different treatments for unresectable esophageal cancer". In: *World journal of surgery* 19.6 (1995), pp. 828–835.

[165] J. Müller et al. "Surgical therapy of oesophageal carcinoma". In: *British Journal of Surgery* 77.8 (1990), pp. 845–857.

[166] A. Herskovic et al. "Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus". In: *New England Journal of Medicine* 326.24 (1992), pp. 1593–1598.

[167] L. E. Gaspar et al. "A phase III study of external beam radiation, brachytherapy and concurrent chemotherapy in localized cancer of the esophagus (RTOG 92-07): Preliminary toxicity report". In: *International Journal of Radiation Oncology* Biology* Physics* 37.3 (1997), pp. 593–599.

[168] J. T. Leung and R. Kuan. "Brachytherapy in oesophageal carcinoma". In: *Australasian radiology* 39.4 (1995), pp. 375–378.

[169] L Bhatt, S Tirmazy, and S Sothi. "Intraluminal high-dose-rate brachytherapy for palliation of dysphagia in cancer of the esophagus: initial experience at a single UK center". In: *Diseases of the Esophagus* 26.1 (2013), pp. 57–60.

[170] E. Rosenblatt et al. "Adding external beam to intra-luminal brachytherapy improves palliation in obstructive squamous cell oesophageal cancer: a prospective multi-centre randomized trial of the International Atomic Energy Agency". In: *Radiotherapy and oncology* 97.3 (2010), pp. 488–494.

[171] M. Y. Homs et al. "Predictors of outcome of single-dose brachytherapy for the palliation of dysphagia from esophageal cancer". In: *Brachytherapy* 5.1 (2006), pp. 41–48.

[172] A. Yorozu, T. Dokiya, and Y. Oki. "High-dose-rate brachytherapy boost following concurrent chemoradiotherapy for esophageal carcinoma". In: *International Journal of Radiation Oncology* Biology* Physics* 45.2 (1999), pp. 271–275.

[173] T. Vuong et al. "The safety and usefulness of high-dose-rate endoluminal brachytherapy as a boost in the treatment of patients with esophageal cancer with external beam radiation with or without chemotherapy". In: *International Journal of Radiation Oncology* Biology* Physics* 63.3 (2005), pp. 758–764.

[174] T. Helleday et al. "DNA repair pathways as targets for cancer therapy". In: *Nature Reviews Cancer* 8.3 (2008), pp. 193–204.

[175] R. W. Johnstone, A. A. Ruefli, and S. W. Lowe. "Apoptosis: a link between cancer genetics and chemotherapy". In: *Cell* 108.2 (2002), pp. 153–164.

[176] A. L. Kovac. "Benefits and risks of newer treatments for chemotherapy-induced and postoperative nausea and vomiting". In: *Drug safety* 26.4 (2003), pp. 227–259.

[177] Y Hishikawa et al. "High-dose-rate intraluminal brachytherapy for esophageal cancer: 10 years experience in Hyogo College of Medicine". In: *Radiotherapy and Oncology* 21.2 (1991), pp. 107–114.

[178] R. K. Sur et al. "Radiation therapy of esophageal cancer: role of high dose rate brachytherapy". In: *International Journal of Radiation Oncology* Biology* Physics* 22.5 (1992), pp. 1043–1046.

[179] C. A. F. Joslin, A Flynn, and E. J. Hall. *Principles and practice of brachytherapy: using afterloading systems*. Arnold, 2001.

[180] D. Baltas, L. Sakelliou, and N. Zamboglou. *The physics of modern brachytherapy for oncology*. CRC Press, 2006.

[181] T. Flynn and B. Thomadsen. "Sealed Sources in Radiotherapy". In: *Practical Radiotherapy: Physics and Equipment* (2009), p. 257.

[182] M Andrássy, Y Niatsetsky, and J Pérez-Calatayud. "Co-60 versus Ir-192 in HDR brachytherapy: Scientific and technological comparison". In: *Rev. Fis. Med* 13 (2012), pp. 125–130.

[183] E. B. Podgorsak. *Radiation physics for medical physicists*. Springer Science & Business Media, 2010.

[184] S. Lettmaier and V. Strnad. "Intraluminal brachytherapy in oesophageal cancer: defining its role and introducing the technique". In: *Journal of contemporary brachytherapy* 6.2 (2014), p. 236.

[185] P. Hoskin and C. Coyle. *Radiotherapy in Practice-Brachytherapy*. Oxford University Press, 2011.

[186] M. Kadhim et al. "Non-targeted effects of ionising radiationâĂŤimplications for low dose risk". In: *Mutation Research/Reviews in Mutation Research* 752.2 (2013), pp. 84–98.

[187] W. F. Morgan. "Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro". In: *Radiation research* 159.5 (2003), pp. 567–580.

[188] M. M. Shareef et al. "Role of tumor necrosis factor-α and TRAIL in high-dose radiation–induced bystander signaling in lung adenocarcinoma". In: *Cancer research* 67.24 (2007), pp. 11811–11820.

[189] S. A. Lorimore, P. J. Coates, and E. G. Wright. "Radiation-induced genomic instability and bystander effects: inter-related nontargeted effects of exposure to ionizing radiation". In: *Oncogene* 22.45 (2003), pp. 7058–7069.

[190] C. Mothersill, M. Moriarty, and C. Seymour. "Radiotherapy and the potential exploitation of bystander effects". In: *International Journal of Radiation Oncology* Biology* Physics* 58.2 (2004), pp. 575–579.

[191] L. A. Loeb. "A mutator phenotype in cancer". In: *Cancer research* 61.8 (2001), pp. 3230–3239.

[192] M. Mancuso et al. "Oncogenic bystander radiation effects in Patched heterozygous mouse cerebellum". In: *Proceedings of the National Academy of Sciences* 105.34 (2008), pp. 12445–12450.

[193] O. V. Belyakov et al. "Biological effects in unirradiated human tissue induced by radiation damage up to 1 mm away". In: *Proceedings of the National Academy of Sciences of the United States of America* 102.40 (2005), pp. 14203–14208.

[194] W. F. Morgan and M. B. Sowa. "Effects of ionizing radiation in nonirradiated cells". In: *Proceedings of the National Academy of Sciences of the United States of America* 102.40 (2005), pp. 14127–14128.

[195] B. Surinov, V. Isaeva, and N. Dukhova. "Postirradiation volatile secretions of mice: syngeneic and allogeneic immune and behavioral effects". In: *Bulletin of experimental biology and medicine* 138.10 (2004), pp. 384–386.

Part I: Clinical Assays

Chapter 2

The involvement of serum serotonin levels producing radiation-induced bystander effects for an in vivo assay with fractionated high dose-rate (HDR) brachytherapy.

Christine Pinho*, Raimond Wong, Ranjan K. Sur, Joseph E.Hayward Colin Seymour, and Carmel Mothersill

* Author Christine Pinho has changed her surname legally from Pinho (maiden name) to Hanu (married name).

This clinical study was originally designed and ethics approval (REB # 06-193) was successfully obtained by Dr. Lorna Ryan, Dr. Raimond Wong, Dr. Ranjan Sur, Dr. Carmel Mothersill, and Dr. Colin Seymour. Further revisions to ethics approval and annual renewals were performed primarily by the author of this thesis and reviewed by the Principal Investigator, Dr. Raimond Wong. Patient recruitment was performed by Dr. Ranjan Sur. The author of this thesis consented patients, administered patient demographic questionnaires, and collected patient data throughout the duration of the study. Brachytherapy treatment planning procedures were carried out by the JCC Medical Physic staff, amongst them were Dr. Thomas Farrell and Dr. Joseph Hayward. Sample collection, sample transportation and clonogenic assays were all performed by the author. Data and statistical analyses were performed by the author. The manuscript was written by the first author and edited by Dr. Mothersill, Dr. Seymour, Dr. Wong, and Dr. Hayward. This is an Accepted Manuscript of an article published by Taylor & Francis in International Journal of Radiation Biology on October 2012, available online: http://www.ncbi.nlm.nih.gov/pubmed/?term=pinho+the+involvement+of+serotonin DOI: 10.3109/09553002.2012.715794.

International Journal of Radiation Biology, October 2012; 88(10): 791–797

ISSN 0955-3002 print / ISSN 1362-3095 online DOI: 10.3109/09553002.2012.715794

informa healthcare

The involvement of serum serotonin levels producing radiation-induced bystander effects for an in vivo assay with fractionated high dose-rate (HDR) brachytherapy

Christine Pinho[1], Raimond Wong[2], Ranjan K. Sur[2], Joseph E. Hayward[1,2], Thomas J. Farrell[1,2], Colin Seymour[1] & Carmel Mothersill[1]

[1]*Medical Physics & Applied Radiation Department, McMaster University, Hamilton, ON, and* [2]*Department of Oncology, Juravinski Cancer Centre, Hamilton, ON, Canada*

Abstract

Purpose: **The primary goal of this investigation was to observe whether measurable levels of bystander factor(s) can be detected in esophageal carcinoma patients' urine samples taken after undergoing high dose rate (HDR) intraluminal brachytherapy (ILBT). However, a small pilot study was developed to evaluate whether serotonin [5-Hydroxytryptamine (5-HT)] serum levels play an active role in the mechanisms of radiation-induced bystander effects (RIBE) at high doses.**

Materials and methods: **In the present study, a colony-forming in vivo assay was developed and used for the detection of non- targeted effects. Samples of urine were collected from five esophageal carcinoma patients undergoing fractionated HDR-ILBT. To observe whether 5-HT modulates the bystander effect at higher doses, different batches of foetal bovine serum (FBS) and 5-HT were tested on the same urine samples before and after brachytherapy.**

Results: **Some of our data suggests statistically significant evidence for serotonin playing an active role as a signalling molecule at higher doses when patients underwent HDR-ILBT.**

Conclusion: **However, a more thorough investigation, with a larger sample size, is warranted before serotonin can be known to play a role in bystander effects at this particular dose range and treatment regime.**

Keywords: Bystander effect, serotonin (5-HT), high dose-rate (HDR) brachytherapy, esophageal carcinoma, urine

Introduction

Bystander effects have been studied extensively over the years (Nagasawa and Little 1992, Prise et al. 1998, Mothersill and Seymour 1998, 2003, Mothersill et al. 2006, Poon et al. 2007, Zhou et al. 2009), but, to date, the exact nature of this phenomenon is not well understood. However, a number of publications suggest there may be several pathways involved (Morgan 2003, Hei et al. 2008, Blyth and Sykes 2011). For instance, there are reviews (Morgan 2003, Hei et al. 2008, Mothersill and Seymour 2012) highlighting that the induction of a bystander effect may be a result of different cellular pathways, where one pathway produces the bystander signal(s) and the other responds to the signal(s). One signalling candidate was emphasized by Azzam et al. (2003) in a review paper stressing the importance of reactive oxygen species role in bystander effects. Another signalling candidate, of great interest for the present study and known to play a role at the low dose range is serotonin [5-Hydroxytryptamine (5-HT)] (Saroya et al. 2009).

5-HT, a neurotransmitter, is produced within the central nervous system. Outside of this system, the amine is produced within the enterochromaffin (EC) cells found in the gastrointestinal (GI) and respiratory tract (Hansen and Skadhauge 1997, Gustafsson et al. 2005). Poon et al. (2007) suggested that the presence of 5-HT is needed to modulate the bystander effect. In this report, the HPV-G cell line, when irradiated, resulted in the 5-HT levels being depleted from the medium by the amine attaching to the HPV-G cell membrane receptors (5-HT type 3 receptors). Other work attempting to analyse the root cause of inter-laboratory variability for in vitro medium-transfer bystander effects, found a correlation between the concentration of serotonin in foetal bovine serum (FBS) batches and the induction of bystander effects (Mothersill et al. 2010). It was observed that a 5-HT serum level higher than 25 ng/ml would result in the production of a bystander signal. In vivo work with zebrafish when injected with a 5-HT inhibitor, reserpine, actually blocked the bystander effect whereas untreated reserpine fish resulted in a bystander response (Saroya et al. 2009).

The bystander phenomenon is generally observed as a result of low LET radiation and particularly at low doses (0.01–0.5 Gy). From reviewing the literature, it becomes apparent that the intentional exposure to high doses of

radiation, such as therapeutic radiation in cancer patients, has not been studied as extensively as lower doses have been for radiation-induced bystander effects (RIBE) (Mothersill and Seymour 2001, Morgan 2003). Moreover, in the published literature, in vitro experiments are more typically studied than in vivo irradiated bystander experiments on humans. In the present pilot study, our group was investigating in vivo RIBE in high dose rate (HDR) intraluminal brachytherapy (ILBT), for esophageal carcinoma patients. This radiotherapy modality is characterised by the placement of a radionuclide source alongside a cancerous tumor resulting in an advantageous high tumor to normal tissue ratio, which consequently leads to a high local tumor control and low exposure to neighbouring normal tissues (Devlin 2006). A colony-forming in vivo assay was used for the detection of non-targeted radiation effects in urine samples collected from esophageal carcinoma patients undergoing fractionated HDR-ILBT. To our knowledge, this study is the first of its kind.

The primary goal of this investigation was to observe whether esophageal carcinoma patients, undergoing HDR-ILBT, will have a bystander factor(s) measurable in their urine samples post-treatment. Secondary objectives were to test different batches of FBS and 5-HT on the same urine samples before and after brachytherapy to evaluate whether 5-HT serum levels play an active role in the mechanisms of RIBE at high doses. The hypothesis being tested in this paper is that urine samples taken following HDR-ILBT will pass the bystander signal to non-irradiated HPV-G reporter cells; however, different batches of FBS may modulate the endpoints observed due to various concentrations of 5-HT being present in the serum batches.

Materials and methods

Study design

In this pilot study, five esophageal carcinoma patients underwent HDR-ILBT with a total dose of 1800 cGy in three fractions. All patients received 600 cGy per HDR fraction, and the dose-rate ranged anywhere between 4200 cGy/h to 10,300 cGy/h. All patients had inoperable tumors, and HDR-ILBT was given with a remote afterloading HDR unit (Varisource HDR, Nucleotron, Varian International, USA) administrating a source, ^{192}Ir. The exact method for source placement with catheters is described by Sur et al. (1998). All patients enrolled were diagnosed with either squamous cell carcinoma (SCC) or adenocarcinoma (ACE) and were recruited from March 2011 to February 2012. Informed consent was given by all patients participating in the study, and ethics approval was obtained from the Hamilton Health Sciences Faculty of Health Sciences (HHS/FHS) research ethics board (REB # 06-193). Eligibility criteria for this study were based on a clinical diagnosis of esophageal carcinoma and patients were undergoing three fractions of HDR-ILBT. Exclusion criteria for this study were patients undergoing concurrent chemotherapy, external beam radiotherapy, or both during HDR-ILBT treatments. Additionally, immunodeficiency patients with HIV, AIDS, and hepatitis, and patients diagnosed with cervical esophageal carcinoma or liver damage, such as cirrhosis, were deemed ineligible to participate in the study.

Sample collection

Urine samples were obtained from patients at each fraction of HDR-ILBT before and after treatment. Samples were placed in a 70 ml sealed sterile container (Sarstedt, Montreal, QC, Canada) and placed in a collection holder on ice immediately following excretion to stabilize heat-labile proteins and maintain the integrity of the sample (Holland et al. 2003). All samples were shipped on ice to the laboratory and were processed within 8 h of excretion.

Cell culture

The HPV-G reporter cell line used for the assays was derived from human kertinocytes, which are a very resistant cell line immortalized with the human papillomavirus (Pirisi et al. 1988). RPMI-1640 culture medium was used for all experiments, and the constituents supplemented were 10% FBS (Invitrogen, Burlington, ON, Canada), 5 ml of 10,000 units of penicillin and 10,000 µg/ml of streptomycin (Gibco, Burlington, ON), 5 ml of 200 mM L-Glutamine (Gibco, Burlington, ON), 0.5 µg/ml hydrocortisone (Sigma-Aldrich, Oakville, ON), and 20 ml of 1M Hepes for pH regulation. All experiments were performed in a biosafety level 2 laboratory within laminar flow cabinets. HPV-G cell stocks were seeded and maintained within T75 flasks with 30 ml of RPMI, and the cells were subcultured when they reached 80–100% confluency using a 1:1 solution of 0.25% trypsin and 1 mM EDTA at 37°C for 8 min. All cells were incubated at 37°C and 5% carbon dioxide in air.

Serum samples

Two serum batch numbers were tested and both FBS were purchased from Invitrogen, Burlington, ON, Canada. One FBS batch tested had sufficient levels of 5-HT, a concentration of 88 ± 4 ng/ml, and is denoted bystander FBS, whereas, the other FBS batch tested had insufficient levels of 5-HT, a concentration of 5 ± 1 ng/ml, and was denoted non-bystander FBS throughout this paper. Moreover, the non-bystander FBS was exposed to laboratory light for 3 h prior to supplementing into the culture medium due to previous findings (Mothersill et al. 2010). The 5-HT serum levels for each batch were measured by Mothersill et al. (2010) with a 5-HT ELISA kit from past work. Both serum batches were screened by the suppliers to ensure the serum could maintain cell growth and all quality control requirements were met. From a previous study, it was determined that 5-HT is highly sensitive to light and as a precaution all bystander experiments were performed in minimal light. Additionally, all media and serum bottles were shielded from light with aluminium foil.

Clonogenic assay

A colony-forming in vivo assay, originally developed by Seymour and Mothersill (2006) for the detection of non-targeted effects in blood samples for radiotherapy patients, was adapted and tailored for urine samples. A total of 24 h prior to plating T25 reporters, a medium change was performed and on the eve of HDR treatments 700 cells were seeded in T25 flasks with 5 ml of RPMI culture medium. Treatments were performed early morning and urine samples were transported and processed shortly after. It is worth mentioning that, while

designing the protocol, various toxicity tests were performed with urine samples to determine the appropriate concentration of urine to add to reporter flasks. However, these are not shown in this paper. For all patients, a 10× dilution factor was observed to be the optimal concentration tested and was used for all experiments. Triplicate flasks were made containing 1 ml of diluted pre- and post-treatment urine samples. To determine whether different 5-HT serum levels had an effect with the in vivo colony-forming assay, the non-bystander and bystander FBS were tested on the same urine samples before and after brachytherapy. Furthermore, since the non-bystander FBS was believed to be missing sufficient concentrations of 5-HT needed to induce a bystander response, then an extra set of flasks with non-bystander FBS was set up and supplemented with 5-HT. The non-bystander FBS + 5-HT group was then compared to the bystander FBS group to observe whether there was a similar response. Controls were unirradiated human urine samples, standard plating efficiency, a diluted medium control, and 5-HT control. A control for the additional 1 ml volume of diluted medium (diluted with sterile distilled water) was set up to ensure that the additional 1 ml volume of diluted urine with medium did not result in an effect relative to the standard plating efficiency. Recipient flasks were kept in a humidified incubator at 37°C with 5% CO_2 in air for 8–10 days. Afterwards, the flasks were stained with 20% carbol fuchsin (VWR, Bridgeport, NJ, USA) and colonies with 50 cells or more were counted and scored only.

Preparation of 5-HT solutions

Serotonin (5-hydroxytryptamine) was purchased (Sigma-Aldrich, Oakville, ON) and stock solutions were prepared and sterilized by filtering with a 0.22 µm Naglene filter, then serially diluted to achieve appropriate concentration. Afterwards, the desired concentration – 2 µg/ml – was added to reporter culture flasks individually, which was a 0.1 ml volume to the 5 ml of culture medium. A control for the 5-HT was set up for each patient and fraction of treatment, this was to ensure that the 5-HT alone did not result in an effect relative to the standard plating efficiency.

Statistical analysis

All flasks were set up in triplicate for each patient and fraction of brachytherapy. The statistcal differences between the triplicates were expressed as the standard error of the mean (±SEM). Significance was determined by two different statistical tests being performed, paired *t*-test and analysis of variance (ANOVA) followed by Fisher's least square difference (LSD) (OriginPro 8.5). Both analyses revealed different responses. The paired *t*-test was used to determine whether there was a bystander response to the treatment regime for each patient, whereas, the ANOVA analysis was used to observe how the mean cell survival changed between post-treatment and control samples when testing different serum batches and 5-HT. This was performed to analyse whether the treatment had a greater change in clonogenic survival than would be expected by chance, a confidence interval of 95% and $p < 0.05$ was selected to be statistically significant.

Results

Figures 1, 2, and 3 show the percentage change in clonogenic survival (%) for reporters between post-treatment and control

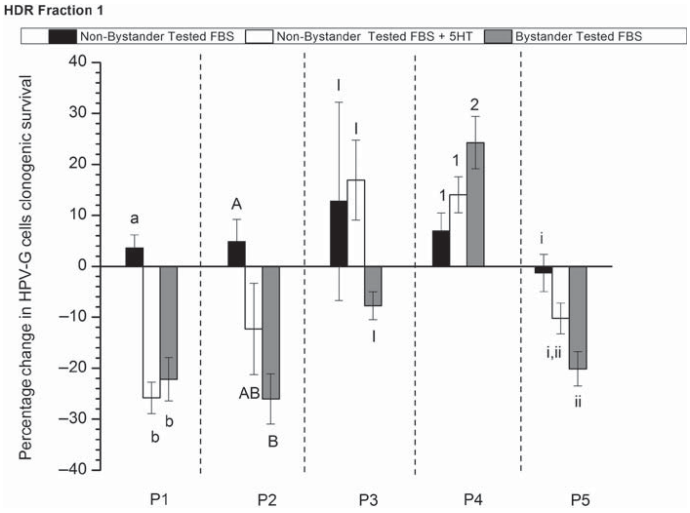


Figure 1. Indicated in the graph is the percentage change in reporters' clonogenic survival (%) that were exposed to post-treatment samples and compared to pre-treatment controls at fraction 1. For all five patients, separate ANOVA analyses were performed to determine whether there are significant changes or similarities in cell survival caused by the level of 5-HT present in the serum batches. Lettering, roman numerals, and numbers indicated significant or similar changes between the three groups (non-bystander FBS, non-bystander FBS + 5-HT, and bystander FBS). All data was normalized to the keratinocytes exposed to control urine samples. For each patient, absolute controls for keratinocytes, serotonin controls and urine pre-treatment controls were set up at each fraction of treatment. The mean plating efficiency for HPV-G absolute controls – both non-bystander and bystander FBS – and serotonin controls were 34.4 ± 4.9, 33.8 ± 5.2, 34.4 ± 3.6%, respectively. All values are mean + SEM for $n = 3$.

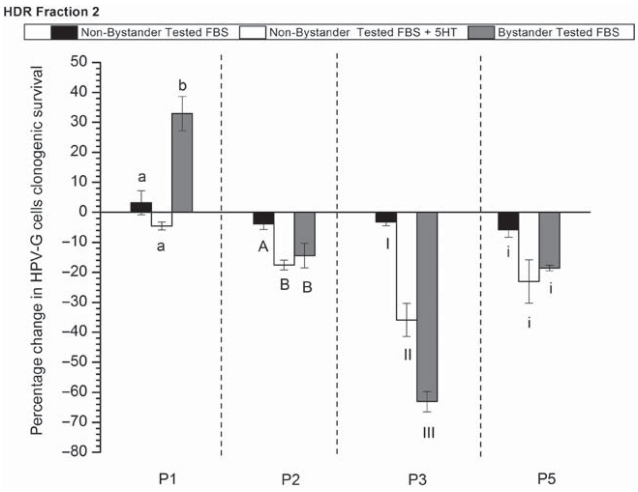


Figure 2. Shown above is the percentage change in reporters' clonogenic survival (%) that were exposed to post-treatment samples and compared to pre-treatment controls at fraction 2. Similar to fraction 1, separate ANOVA analyses were performed on each patient to determine whether there are significant changes or similarities in cell survival caused by the level of 5-HT present in the serum batches. Lettering, roman numerals, and numbers indicated significant or similar changes between the three groups (non-bystander FBS, non-bystander + 5-HT FBS, and bystander FBS). The mean plating efficiency for HPV-G absolute controls — both non-bystander and bystander FBS — and serotonin controls were 37.9 ± 7.6, 38.2 ± 6.6, 39.3 ± 4.7%, respectively. All values are mean + SEM for $n = 3$.

urine samples at fractions 1, 2, and, 3, respectively, when testing the non-bystander, bystander, and non-bystander + 5-HT serum groups. As illustrated in Figure 1, for patient 1, the reporters exposed to the non-bystander FBS did not have a significant change between the post-treatment and control urine samples, there was an insignificant increase in survival to 3.5 ± 2.6%. However, when the reporters were exposed to the non-bystander FBS + 5HT and the bystander FBS there was a similar percentage change in survival between the post-treatment and control urine samples for both serum batches, there was a 25.8 ± 3.1% and 22.1 ± 4.2% reduction in cell survival, respectively. The latter results were statistically

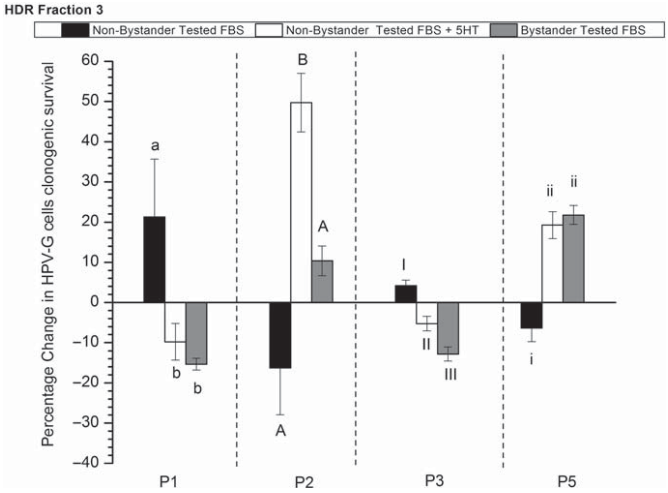


Figure 3. Shown above is the percentage change in reporters' clonogenic survival (%) that were exposed to post-treatment samples and compared to pre-treatment controls at fraction 3. Similar to fraction 1 and 2, separate ANOVA analyses were performed on each patient to determine whether there are significant changes or similarities in cell survival caused by the level of 5-HT present in the serum batches. Lettering, roman numerals, and numbers indicated significant or similar changes between the three groups (non-bystander FBS, non-bystander + 5-HT FBS, and bystander FBS). The mean plating efficiency for HPV-G absolute controls – both non-bystander and bystander FBS – and serotonin controls were 39.0 ± 7.7, 39.8 ± 6.6, 39.6 ± 4.2%, respectively. All values are mean + SEM for $n = 3$.

significant and are suggesting that the addition of the 5-HT had an effect on the non-bystander serum. Similar results are seen in Figure 1, for patient 5, there was an insignificant percentage change in the reporters survival, a reduction of $1.3 \pm 3.7\%$, when the non-bystander FBS was supplemented into the culture medium. However, when the non-bystander FBS + 5HT and bystander FBS were used there was a similar and significant response, there was a $10.2 \pm 3.0\%$ and $20.1 \pm 3.4\%$ reduction in cell survival, respectively. These results revealed statistically significant results suggesting thatthe addition of 5-HT to the non-bystander FBS modulated a response.

In Figure 2, for patient 2, the results are suggesting that 5-HT may have an effect since there is a small insignificant change, a $3.9 \pm 1.8\%$ reduction in cell survival, between the post-treatment and control samples for the non-bystander FBS group. Whereas, the non-bystander FBS + 5HT and bystander FBS shows a comparable and significant reduction in cell survival of $17.6 \pm 1.6\%$ and $14.4 \pm 4.1\%$, respectively. Furthermore, similar results discussed above for Figures 1 and 2 are seen in Figure 3 (refer to patients 1 and 5). The latter results revealed a statistically significant pattern which suggested that the response may be 5-HT dependent.

The same data above, however depicted in a different way, is represented in Tables I, II, and III showing the clonogenic survival (%) for reporter cells at fractions 1, 2, and 3 of brachytherapy, respectively. From Tables I, II, and III, although the majority did not reached statistical significance, there seems to be a numerical trend worthy of being noted. In Table I, for patient 1, there is a very small change in survival between pre- and post-treatment samples for the non-bystander serum, $79.5 \pm 3.9\%$ and $82.3 \pm 2.1\%$, respectively. When this same batch of serum is supplemented with 5HT, there seems to be a greater reduction in cell survival between control and treatment samples of $101.3 \pm 4.8\%$ and $75.2 \pm 3.1\%$, respectively. Also, the reporters exposed to the bystander FBS have nearly comparable cell survival for the control and treatment samples, $91.4 \pm 1.5\%$ and $71.2 \pm 3.9\%$, respectively, as the reporters exposed to the non-bystander FBS + 5HT.

Table I. The table indicates percentage (%) of clonogenic survival of exposed HPV-G cells to urine before and after fraction 1 of HDR treatment.

Urine samples	HDR brachytherapy fraction 1			
	Patient #	NBS FBS[a]	NBS FBS + 5HT[b]	BS FBS[c]
Control	1	79.5 ± 3.9	101.3 ± 4.8	91.4 ± 1.5
Treatment		82.3 ± 2.1	$75.2 \pm 3.1^*$	71.2 ± 3.9
Control	2	36.5 ± 4.5	48.0 ± 3.3	69.3 ± 3.3
Treatment		38.3 ± 1.6	42.1 ± 4.3	51.3 ± 3.4
Control	3	56.2 ± 1.2	52.1 ± 3.1	52.8 ± 1.8
Treatment		63.4 ± 10.9	60.9 ± 4.1	$48.7 \pm 1.4^*$
Control	4	71.8 ± 2.9	65.1 ± 1.9	55.0 ± 2.8
Treatment		76.8 ± 2.5	74.3 ± 2.3	68.6 ± 2.8
Control	5	74.4 ± 3.5	66.3 ± 2.1	77.7 ± 3.4
Treatment		73.4 ± 2.7	59.5 ± 2.0	62.1 ± 2.6

All values are mean + SEM for $n = 3$.
[a]Growth medium supplemented with non-bystander foetal bovine serum (NBS) FBS.
[b]Growth medium supplemented with non-bystander (NBS) FBS and 2 μg/ml of serotonin.
[c]Growth medium supplemented with bystander (BS) FBS.
*Significant difference ($p < 0.05$) from control by using a paired t-test.

Table II. The table indicates percentage (%) of clonogenic survival of exposed HPV-G cells to urine before and after fraction 2 of HDR treatment.

Urine samples	HDR brachytherapy fraction 2			
	Patient #	NBS FBS[a]	NBS FBS + 5HT[b]	BS FBS[c]
Control	1	55.7 ± 0.8	56.6 ± 0.9	57.8 ± 2.0
Treatment		57.5 ± 2.2	54.1 ± 0.8	$76.8 \pm 3.3^*$
Control	2	80.4 ± 0.3	76.3 ± 1.9	75.6 ± 2.6
Treatment		77.3 ± 1.5	62.9 ± 1.3	64.7 ± 3.1
Control	3	23.8 ± 2.5	31.9 ± 3.3	23.7 ± 2.9
Treatment		23.1 ± 0.3	20.5 ± 1.8	8.7 ± 0.8
Control	5	55.3 ± 2.0	65.6 ± 1.3	50.8 ± 2.4
Treatment		52.1 ± 1.4	50.5 ± 4.7	41.4 ± 0.5*

All values are mean + SEM for $n = 3$.
[a]Growth medium supplemented with non-bystander foetal bovine serum (NBS) FBS.
[b]Growth medium supplemented with non-bystander (NBS) FBS and 2 μg/ml of serotonin.
[c]Growth medium supplemented with bystander (BS) FBS.
*Significant difference ($p < 0.05$) from control by using a paired t-test.

A similar pattern above shown in Table I for patient 1 is demonstrated in Table II, for patients 2, 3, and 5. For these patients, there is very little change in survival between pre- and post-treatment samples for the FBS without adequate levels of 5HT, and when 5HT is added to the FBS then this same batch of serum seems to result in a larger change in survival between control and treatment samples. Also, interesting enough, the two batches of FBS tested, gave similar endpoints and nearly the same magnitude of change in survival when the 5-HT was supplemented into the FBS known to have insufficient concentrations of this molecule. The latter data depicted in tabular format reveals a pattern suggesting that 5-HT may modulate the bystander effect at higher doses for this reporter bioassay.

Discussion

The primary motivation behind this study was to determine if there exist bystander factor(s) passed through cancer patients' urine samples post-treatment, and to observe whether 5-HT plays a role in modulating the bystander effect at the high dose range. As highlighted above, some of the data from our experiments seems to be suggesting that 5-HT may play an active role as a signalling molecule at higher doses. However, due to the complexity of the samples being tested and the

Table III. The table indicates percentage (%) of clonogenic survival of exposed HPV-G cells to urine before and after fraction 3 of HDR treatment.

Urine samples	HDR brachytherapy fraction 3			
	Patient #	NBS FBS[a]	NBS FBS + 5HT[b]	BS FBS[c]
Control	1	39.1 ± 0.9	53.7 ± 4.8	64.1 ± 1.5
Treatment		47.5 ± 5.6	48.5 ± 2.5	$54.3 \pm 0.9^*$
Control	2	52.5 ± 4.3	45.0 ± 2.4	68.1 ± 2.4
Treatment		47.6 ± 9.6	67.4 ± 3.3	$75.1 \pm 2.5^*$
Control	3	75.3 ± 4.5	66.5 ± 1.3	75.7 ± 1.1
Treatment		78.4 ± 1.0	63.0 ± 1.2	$66.0 \pm 1.3^*$
Control	5	35.7 ± 2.2	36.2 ± 0.7	46.0 ± 2.2
Treatment		33.5 ± 1.2	$43.1 \pm 1.4^*$	56.0 ± 1.1

All values are mean + SEM for $n = 3$.
[a]Growth medium supplemented with non-bystander foetal bovine serum (NBS) FBS.
[b]Growth medium supplemented with non-bystander (NBS) FBS and 2 μg/ml of serotonin.
[c]Growth medium supplemented with bystander (BS) FBS.
*Significant difference ($p < 0.05$) from control by using a paired t-test.

substantial inter-patient variability found within this study, it is suggested that a larger sample size be used. Regardless, some of the significant results in this study suggest that there exists a response when the signalling amine is added to the reporter bioassay.

Presently, there are few papers confirming the role of 5-HT being able to modulate medium-transfer bystander experiments, except for some in vivo and in vitro work (Poon et al. 2007, Saroya et al. 2009, Mothersill et al. 2010). To our knowledge, no such work has been studied in urine samples for bystander effects along with testing the serotonin serum batch variability with this reporter bioassay. In this pilot study, it is unclear if these results would be significant with a larger sample size and clearly further work is warranted. From a brief look into the literature, 5-HT is metabolised in the liver or kidney by an enzyme, monoamine oxidase (MAO), into one of the main metabolites of 5-HT, which is the 5-hydroxyindoleacetic acid (5-HIAA) and this by-product is excreted in the urine (Bearcroft et al. 1998). However, there are traces of urinary serotonin found in 24-h urine samples collected from healthy individuals in a study by Feldman (1986). In the latter work, urine samples were collected every 24 h, and so, it is worth mentioning, that the urinary serotonin levels may not coincide with the present study since two samples – before and after brachytherapy – were collected within a 2-h span from each other. Therefore, it is not clear what level of serotonin is present within the urine samples. Future work will need to take into account other factors, such as dietary intake of the amino acid, tryptophan, which eventually metabolises to 5-HT (Keszthelyi et al. 2009) and measuring levels of urinary serotonin (Feldman 1986) before and after brachytherapy. Furthermore, a quarter of a century ago, Bodó and Benkö (1987) worked with in vivo mice, the mouse brains were exposed to gamma-irradiation alone or a mixture of both neutrons and gammas. After irradiation exposure, the level of MAO activity had increased in the liver of the mice following both gamma irradiation and neutron and gamma irradiations and it would be interesting to observe whether there is a similar effect in cancer patients following brachytherapy. Perhaps, this is an area worth pursuing for future work when adequate techniques are developed.

In the current study, there exists additional complexity in regard to analysing human samples and this occurs without considering the difficulty or concerns of testing urine samples with this particular endpoint. According to an excellent review by Munro (2009), there are a number of factors that need to be taken into account within the clinical environment when analysing bystander effects in cancer patients that underwent a particular radiotherapy regime. For instance, according to Munro (2009), there is the added complexity of no longer analysing tissue and cells in isolation. Instead, considerable inter-patient variability in clinical studies may result from variations in genotypes, phenotypes, and exposure to a number of environmental agents, such as smoking (Seymour and Mothersill 1997), throughout the individuals' lifespan before the onset of cancer (Munro 2009). In the radiobiology community, the existence of individual variation is to be expected which is evident by the number of predictive assays being developed or analysed in the clinical environment (Crompton et al. 1997, Widel et al. 2003, Schnarr et al. 2007, Munro 2009). Now, the main premise for a predictive assay stems from the fact that cancer patients' tumors response to treatments are in very different ways due to a number of genetic and lifestyle factors; this is evident in the following publications (Mothersill et al. 2001, Schnarr et al. 2007). Additionally, there is previous work with blood serum and biopsy samples from cancer and healthy individuals that have been studied for non-targeted radiation effects and these studies, also, observed individual variation (Mothersill et al. 2001, Seymour and Mothersill 2006). Furthermore, a study performed by Kadhim et al. (2010) also found inter-patient variability when studying delayed chromosome aberrations in human lymphocytes exposed to a target microbeam. From the literature (Masand and Gupta 1999), it is apparent that perhaps some of the individual variation with the 5-HT experiments can be attributed to whether patients are taking antidepressants such as selective serotonin reuptake inhibitors (SSRIs), which could be analogous to the 5-HT inhibitor, resprine, work with zebrafish mentioned above. However, from a brief look at the medications taken by this small subset of patients (data not shown), it is clear that the patients are not on any of these SSRIs for depression. Therefore, the individual variation, where the 5-HT has no effect or trend at higher doses for some patients at least, does not seem to be influenced by patients being on SSRIs when undergoing fractions of brachytherapy.

Aside from the human variability aspect, there is variation attributed to serum batches found within this study. These findings are similar to the work done at the low dose range producing RIBE with in vitro medium-transfer bystander experiments, as discussed earlier, relating a relationship between serotonin serum levels and the occurrence of RIBE (Mothersill et al. 2010). The latter study explained the justification for inter-laboratory variation with RIBE cell culture experiments, and the present work is suggesting a similar trend, however at higher doses. Evidently, further work needs to be done to elucidate whether bystander effects occur within radiotherapy, especially since, such a phenomenon maybe helpful in understanding the risks towards healthy surrounding tissues, along with understanding potential revisions to treatment planning if necessary (Mothersill et al. 2004). Therefore, RIBE should be studied more extensively in radiotherapy to understand the very nature of normal tissue effects throughout the individual and whether bystander effects play a role. This pilot study is a stepping stone for many avenues in future work to be done within the field of radiotherapy on non-targeted effects. However, until a more reliable technique is developed, with a less complex sample being utilized, then there lies a wide gap of uncertainty in whether RIBE are 5-HT dependent at higher doses. For this pilot study, future work will need to incorporate a larger sample size, taking measurements of urinary serotonin levels, and documenting patients' dietary intake of foods enriched with 5HT before a definitive conclusion can be made on whether 5HT has an effect at this therapeutic dose range and cancer treatment regime. Additionally, further in vivo work, for an array of cancer treatment modalities, is warranted to

facilitate our understanding of this non-targeted phenomena's relevance at therapeutic doses typically used for cancer treatments.

Acknowledgements

We acknowledge Varian International B.V. for funding part of this study. A special thanks to the brachytherapy and ambulance care staff for helping in sample collection at the Hamilton Health Sciences Juravinski cancer centre (JCC). We would also like to thank Emilia Timotin for all of her help with patient consents and sample collection at the JCC. We acknowledge and thank all the patients who took the time to participate in this study; without these individuals this research would not be possible. We also acknowledge the statistical advice and help from Dr Richard Smith when preparing this manuscript.

Declaration of interest

The authors report no declaration of interest. The authors alone are responsible for the content and writing of the paper.

References

Azzam EI, de Toledo SM, Little John B. 2003. Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. Oncogene 22:7050–7057.

Bearcroft CP, Perrett D, Farthing MJG. 1998. Postprandial plasma 5-hydroxytryptamine in diarrhoea predominant irritable bowel syndrome: A pilot study. Gut 42:42–46.

Blyth BJ, Sykes PJ. 2011. Radiation-induced bystander effects: what are they, and how relevant are they to human radiation exposures? Radiation Research 176:139–157.

Bodó K, Benkö G. 1987. Alterations in monoamine oxidase activity of the mouse brain and liver after mixed neutron-gamma irradiation. Acta Physiologica Hungarica 69:181–188.

Crompton NE, et al. 1997. Theory and practice of predictive assays in radiation therapy. Strahlentherapie Und Onkologie: Organ Der Deutschen Röntgengesellschaft. [et al] 173:58–67.

Devlin PM, editor. 2006. Brachytherapy: Applications and Techniques. 1st ed. Philadelphia, PA: Lippincott Williams, Wilkins.

Feldman JM. 1986. Urinary serotonin in the diagnosis of carcinoid tumors. Clinical Chemistry 32:840–844.

Gustafsson BI, et al. 2005. Long-term serotonin administration induces heart valve disease in rats. Circulation 111:1517–1522.

Hansen MB, Skadhauge E. 1997. Signal transduction pathways for serotonin as an intestinal secretagogue. Comparative Biochemistry and Physiology Part A: Physiology 118:283–290.

Hei TK, et al. 2008. Mechanism of radiation-induced bystander effects: A unifying model. The Journal of Pharmacy and Pharmacology 60:943–950.

Holland NT, et al. 2003. Biological sample collection and processing for molecular epidemiological studies. Mutation Research/Reviews in Mutation Research 543:217–234.

Kadhim MA, et al. 2010. Genomic instability after targeted irradiation of human lymphocytes: Evidence for inter-individual differences under bystander conditions. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 688:91–94.

Keszthelyi D, Troost FJ, Masclee AAM. 2009. Understanding the role of tryptophan and serotonin metabolism in gastrointestinal function. Neurogastroenterology and Motility: The Official Journal of the European Gastrointestinal Motility Society 21:1239–1249.

Masand PS, Gupta S. 1999. Selective serotonin-reuptake inhibitors: an update. Harvard Review of Psychiatry 7:69–84.

Morgan WF. 2003. Non-targeted and delayed effects of exposure to ionizing radiation: I. radiation-induced genomic instability and bystander effects in vitro. Radiation Research 159:567–580.

Mothersill C, Seymour C. 2001. Radiation-induced bystander effects: Past history and future directions. Radiation Research 155:759–767.

Mothersill C, Seymour CB. 1998. Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: Evidence for release during irradiation of a signal controlling survival into the medium. Radiation Research 149:256–262.

Mothersill C, et al. 2006. Communication of radiation-induced stress or bystander signals between fish in vivo. Environmental Science & Technology 40:6859–6864.

MotherSill C, Seymour C. 2012. Changing paradigms in radiobiology. Mutation Research/Reviews in Mutation Research 750:85–95.

Mothersill C, Moriarty M, Seymour CB. 2004. Radiotherapy and the potential exploitation of bystander effects. International Journal of Radiation Oncology Biology Physics 58:575–579.

Mothersill C, et al. 2001. Individual variation in the production of a 'bystander Signal' Following irradiation of primary cultures of normal human urothelium. Carcinogenesis 22:1465–1471.

Mothersill C, et al. 2010. Serum serotonin levels determine the magnitude and type of bystander effects in medium transfer experiments. Radiation Research 174:119–123.

Mothersill C, Seymour C. 2003. Radiation-induced bystander effects, carcinogenesis and models. Oncogene 22:7028–7033.

Munro AJ. 2009. Bystander effects and their implications for clinical radiotherapy. Journal of Radiological Protection: Official Journal of the Society for Radiological Protection 29:A133–142.

Nagasawa H, Little JB. 1992. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Research 52: 6394–6396.

Pirisi L, et al. 1988. Continuous cell lines with altered growth and differentiation properties originate after transfection of human keratinocytes with human papillomavirus type 16 DNA. Carcinogenesis 9:1573–1579.

Poon RCC, et al. 2007. Bystander effects of ionizing radiation can be modulated by signaling amines. Environmental Research 105:200–211.

Prise KM, et al. 1998. Studies of bystander effects in human fibroblasts using a charged particle microbeam. International Journal of Radiation Biology 74:793–798.

Saroya R, et al. 2009. Injection of resperpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: Confirmation in vivo of a role for serotonin in the mechanism. Dose-Response 8:317–330.

Schnarr K, et al. 2007. Individual radiosensitivity and its relevance to health physics. Dose-Response 5:333–348.

Seymour CB, Mothersill C. 1997. Delayed expression of lethal mutations and genomic instability in the progeny of human epithelial cells that survived in a bystander-killing environment. Radiation Oncology Investigations 5:106–110.

Seymour C, Mothersill C. 2006. Development of an in vivo assay for detection of non-targeted radiation effects. Dose-Response: A Publication of International Hormesis Society 4:277–282.

Sur RK, et al. 1998. Fractionated high dose rate intraluminal brachytherapy in palliation of advanced esophageal cancer. International Journal of Radiation Oncology, Biology, Physics 40:447–453.

Widel M, et al. 2003. Radiation-induced micronucleus frequency in peripheral blood lymphocytes is correlated with normal tissue damage in patients with cervical carcinoma undergoing radiotherapy. Radiation Research 159:713–721.

Zhou H, et al. 2009. Consequences of cytoplasmic irradiation: studies from microbeam. Journal of radiation research 50(Suppl A): A59–65.

Chapter 3

Assessing patient characteristics and radiation-induced non-targeted effects *in vivo* for high dose-rate (HDR) brachytherapy

Christine Pinho*, Emilia Timotin, Raimond Wong, Ranjan K. Sur, Joseph E.Hayward Colin Seymour, and Carmel Mothersill

* Author Christine Pinho has changed her surname legally from Pinho (maiden name) to Hanu (married name).

This clinical study was originally designed and ethics approval (REB # 06-193) was successfully obtained by Dr. Lorna Ryan, Dr. Raimond Wong, Dr. Ranjan Sur, Dr. Carmel Mothersill, and Dr. Colin Seymour. Further revisions to ethics approval and annual renewals were performed primarily by the author of this thesis and reviewed by the Principal Investigator, Dr. Raimond Wong. Patient recruitment and biopsy extractions were performed by Dr. Ranjan Sur. Health Sciences JCC brachytherapy nursing staff extracted blood samples from all patients. Both the author of this thesis and Emilia Timotin consented patients, administered patient demographic questionnaires, and collected patient data throughout the duration of the study. Brachytherapy treatment planning procedures were carried out by the JCC Medical Physic staff, amongst them were Dr. Thomas Farrell and Dr. Joseph Hayward. Blood serum extraction, sample collection, sample transportation, and clonogenic assays were all performed by the author. Data and statistical analyses were performed by the author. The manuscript was written by the first author and edited by Dr. Mothersill, Dr. Seymour, Dr. Wong, and Dr. Hayward. This is an Accepted Manuscript of an article published by Taylor & Francis in International Journal of Radiation Biology on October 2015, available online: http://www.ncbi.nlm.nih.gov/pubmed/26136084 DOI: 10.3109/09553002.2015.1068458.

International Journal of Radiation Biology, October 2015; 91(10): 786–794

ISSN 0955-3002 print / ISSN 1362-3095 online
DOI: 10.3109/09553002.2015.1068458

Taylor & Francis
Taylor & Francis Group

Assessing patient characteristics and radiation-induced non-targeted effects in vivo for high dose-rate (HDR) brachytherapy

Christine Pinho[1], Emilia Timotin[1], Raimond Wong[2], Ranjan K. Sur[2], Joseph E. Hayward[1,3], Thomas J. Farrell[1,3], Colin Seymour[1] & Carmel Mothersill[1]

Departments of [1]Medical Physics & Applied Radiation Sciences, [2]Oncology and [3]Radiology, McMaster University, Hamilton, ON, Canada

Abstract

Purpose: **To test whether blood, urine, and tissue based colony-forming assays are a useful clinical detection tool for assessing fractionated treatment responses and non-targeted radiation effects in bystander cells.**

***Materials and methods*: To assess patients' responses to radiation treatments, blood serum, urine, and an esophagus explant-based in vivo colony-forming assay were used from oesophageal carcinoma patients. These patients underwent three fractions of high dose rate (HDR) intraluminal brachytherapy (ILBT).**

Results: **Human keratinocyte reporters exposed to blood sera taken after the third fraction of brachytherapy had a significant increase in cloning efficiency compared to baseline samples ($p < 0.001$). Such results may suggest an induced radioresistance response in bystander cells. The data also revealed a clear inverse dose-rate effect during late treatment fractions for the blood sera data only. Patient characteristics such as gender had no statistically significant effect ($p > 0.05$). Large variability was observed among the patients' tissue samples, these colony-forming assays showed no significant changes throughout fractionated brachytherapy ($p > 0.05$).**

Conclusion: **Large inter-patient variability was found in the urine and tissue based assays, so these techniques were discontinued. However, the simple blood-based assay had much less variability. This technique may have future applications as a biological dosimeter to predict treatment outcome and assess non-targeted radiation effects.**

Keywords: Bystander effect, high dose-rate (HDR) brachytherapy, esophageal carcinoma, urine, blood, esophageal explants

Introduction

High dose-rate intraluminal brachytherapy (HDR-ILBT) has established itself as an effective treatment modality for patients diagnosed with advanced stages of esophageal cancer (Sur et al. 2002). Brachytherapy enables high doses of radiation to be delivered to the tumor to improve the patients' dysphagia scores, quality of life (Berry et al. 1989), has the advantage of providing conveniently fast outpatient procedures (Sur et al. 1998). A remote afterloading HDR unit is used for delivering high doses of gamma radiation, from an Iridium-192 (Ir^{192}) source, to the tumor site (Sur et al. 2002). A rapid drop-off of dose from the treatment site to surrounding normal tissues results in a very small risk of injury to nearby normal tissues (Yoshioka et al. 2013). Fractionated HDR-ILBT has shown to significantly improve dysphagia-free survival and longevity in comparison to other palliative modalities (Sur et al. 1998).

Dose to normal tissues typically restricts treatment planning protocols for radiotherapy modalities, as these are limited by normal tissues tolerance doses (Mothersill et al. 2004a). However, in vitro research has documented the biological implications of bystander factors being released into non-irradiated cells which has been shown in the literature to trigger a cell death response (Mothersill et al. 2004b). Consequently, non-targeted radiation effects can ultimately affect treatment planning protocols, as there is a possibility of much larger out-of-field effects in normal tissues than initially expected (Butterworth et al. 2013). Other work has suggested that radiation-induced bystander effects (RIBE) may provide insight into understanding the efficacy of radiotherapy, as bystander factors may enhance tumor cell killing (Boyd et al. 2008, Prise and O'Sullivan 2009, Butterworth et al. 2013). Currently, it is not fully understood whether the release of bystander signals into healthy surrounding tissues, near radiation fields, leads to unwanted damage in normal cells (Brenner et al. 2000, Hall and Wuu 2003, Mothersill and Seymour 2006, Boyd et al. 2008). Therefore we and others have extended the investigation of non-targeted radiation effects from an in vitro experimental approach (Mothersill and Seymour 1997, Prise et al. 1998, Lyng et al. 2000, Seymour and Mothersill, 2000) to the whole organism by using animal

(Morgan 2003, Chai and Hei 2008, Koturbash et al. 2008) and human models (Seymour and Mothersill, 2000, Mothersill et al. 2002, Marozik et al. 2007, Chai and Hei 2008).

For the past several years, non-targeted radiation effects such as clastogenic effects (Seymour and Mothersill 2006, Howe et al. 2009), RIBE (Emerit et al. 1995, Mothersill and Seymour 1997, Ryan et al. 2009) and adaptive responses have been well documented. Radiation-induced clastogenic effects are found in atomic bomb survivors (Pant and Kamada 1977), humans undergoing radiotherapy (Seymour and Mothersill 2006), and in the blood serum collected from Chernobyl liquidators (Marozik et al. 2007).

One of the earlier studies observing clastogenic effects was published by Goh and Sumner (1968); the study evaluated chromosomal aberrations in cultivated leukocytes treated with blood plasma taken from patients that underwent total body irradiation. The findings showed that blood plasma exposed to radiation increased the number of chromosome breaks in leukocytes compared to unirradiated samples. Similar clastogenic effects have also been reported by other investigators in the literature with humans (Hollowell and Littlefield 1968, Pant and Kamada 1977, Emerit et al. 1994, 1995, 1997) and animals (Faguet et al. 1984) following radiation exposure.

Earlier work focused on exploring the variability inherent in human urothelial tissue explants and their ability to express bystander signals in reporter cells (Mothersill et al. 2002). Signal production was found to be sex-specific and had a dependence on whether the participants had no existing malignancies. A gender discrepancy was observed, tissue samples harvested from female participants resulted in a higher reduction in cloning efficiency compared to males. Other researchers used a rodent model to assess non-targeted radiation effects within non-irradiated spleens following cranial radiation exposure (Koturbash et al. 2008). These authors found male mice to be more susceptible to bystander effects in comparison to females.

Another very important phenomenon associated with non-targeted radiation effects is induced radioresistance responses. This cell protective effect is not unique to radiation alone, rather it has been observed with acute hypoxia-induced stimuli within analogous systems and many different cell types (Michiels 2004). The induction of radioresistance responses were found in areas of high natural background radiation in Ramsar compared to control populations from regions of low background radiation (Mohammadi et al. 2006). Lymphocytes were extracted and exposed to 4 Gy of gamma radiation, and individuals residing in high natural radiation background areas had significantly higher DNA damage and repair than control groups (Mohammadi et al. 2006). Other investigators assessing fractionated X-ray treatments, found enhanced clonogenic survival following subsequent treatments in radiosensitive clones of human colorectal tumor cell lines (Qutob et al. 2006).

In the present study, non-targeted radiation effects were assessed with an in vivo-based assay for blood, urine, and esophageal biopsy samples taken before and after a fractionated brachytherapy regime. The primary motivation of this study was to explore radiation-induced bystander effects (RIBE) in blood, urine, and biopsy samples taken from esophageal cancer patients undergoing fractionated HDR-ILBT. Secondary objectives were to assess whether blood and urine samples pre-exposed to one treatment fraction of brachytherapy induces radioresistance, by stimulating an increase in reporter cells survival, during subsequent exposure to brachytherapy. Additionally, certain patient characteristics were assessed to determine whether these variables are influencing cell communicating signals that ultimately affects cell cloning capabilities.

Based upon previous in vitro studies (Boyden and Raaphorst 1999, Maguire et al. 2007), it is hypothesized that fractionated treatments will induce a cell communicating protective response in reporter cells exposed to patient samples taken following each fraction of brachytherapy. This work will contribute to the limited data available and further our understanding of non-targeted radiation effects in brachytherapy at therapeutic doses.

Materials and methods

Sample design

Blood, urine and biopsy samples were obtained from patients diagnosed with either esophageal adenocarcinoma (EA) or squamous cell carcinoma (SCC) undergoing HDR-ILBT between March 2011 and February 2012. The majority of patients were males diagnosed with esophageal adenocarcinoma (EA). Roughly 54% of the patients had stage III cancer and 26.7% had stage IV cancer, refer to Table I for patient characteristics and demographics. This research was carried out according to the Declaration of Helsinki with informed consent obtained from all participants, and ethics approval was obtained from the Hamilton Health Sciences

Table I. Patient demographics and clinical characteristics.

Patient characteristics	No. of patients ($n = 15$)
Mean age in years (SD)	69.3 (10.6)
Gender *n* (%)	
Male	11 (73.3%)
Female	4 (26.7%)
Type of cancer *n* (%)	
SCC	3 (20.0%)
EA	12 (80.0%)
Cancer staging *n* (%)	
Stage II	1 (6.7%)
Stage III	8 (53.3%)
Stage IV	4 (26.7%)
Not reported	2 (13.3%)
Tumor location *n* (%)	
GEJ	11 (73.3%)
Mid/Upper esophagus	1 (6.7%)
Above GEJ	3 (20.0%)
Metastases *n* (%)	
Yes	5 (33.3%)
No	8 (53.3%)
Not determined	1 (6.7%)
Smoking status *n* (%)	
Current	2 (13.3%)
Former	12 (80.0%)
Never	1 (6.7%)
Mean dose-rate (Gy/h) (SD)	
Fraction 1	59.4 (25.2)
Fraction 2	68.2 (23.4)
Fraction 3	65.4 (20.8)

Faculty of Health Sciences (HHS/FHS) research ethics board (REB # 06-193). In the present study, 24 patients were eligible for recruitment, however, only 11 men and four women, with a mean age of 69 years (age range, 57–90 years) participated in the study. Out of the 15 patients, two patients discontinued from the study after the first fraction of HDR-ILBT, one patient refused to undergo fraction 2 and 3 of brachytherapy, and one patient was deemed ineligible to participate after the first fraction of treatment by the attending physician. All patients received 600 cGy per HDR fraction prescribed 1 cm from the source axis to the esophageal planning volume with a remote afterloading HDR unit (Varisource HDR, Nucletron, Varian International, USA) administrating high doses of gamma radiation, by using a Ir^{192} source. The length of the treatment field is determined at the time of endoscopy which occurred right before the catheter is set in place on the day of brachytherapy. Appropriate margins were set based on clinical visual determination of the tumor where a 2 cm treatment margin was added proximal and distal to the tumor. The dose-rate ranged anywhere between 33.1 and 109.0 Gy/h. Further details on eligibility criterion has been described in a small pilot study published elsewhere (Pinho et al. 2012).

Sample collection

Blood and urine samples were collected at the start and end of each fraction of HDR-ILBT. Tissue specimens were biopsied from the tumor-free mucosa layer of the esophagus, proximal to the tumor site. A biopsy puncture technique was used to extract tissue specimens ranging in size from 1–2 mm^2. Biopsies of the esophagus were obtained prior to the first fraction of HDR-ILBT (baseline sample) and immediately following the final fraction of treatment (test sample).

Urine samples were placed in a 70 ml sterile container (Sarstedt, Montreal, QC, Canada) and peripheral blood samples in a 10 cc red lid serum Vacutainer containing no additive (BD Vacutainers, Fisher Scientific, Ottawa, ON, Canada). The samples were placed in a collection holder on ice immediately following extraction to maintain the integrity of the sample. For serum extraction, blood samples were centrifuged at 2000 rpm for 10 min and the serum was aliquoted into 5 ml sterile polypropylene tubes (Sarstedt, Montreal, QC, Canada). The serum was extracted from blood samples within 2 h of being collected from patients in the clinical trial laboratory at the Juravinski Cancer Centre (JCC). Biopsy samples were collected and transported in 15 ml sterile polypropylene tubes containing RPMI medium with a final concentration of 200 U/ml penicillin and 200 µg/ml streptomycin solution, 15 mM HEPES buffer, 1 µg/ml of Fungizone, 50 µg/ml of Nystatin, 0.5 µg/ml of hydrocortisone, and 2 mM of L-glutamine solution. Culture medium and supplements were obtained from Invitrogen Burlington Ontario. All samples were transported on ice to our research laboratory at McMaster University and were processed within 8 h of being collected.

Cell line

Human keratinocytes HPV-G cultures (Pirisi et al. 1988) were used as a reporter to determine whether bystander signals were being generated, following HDR-ILBT, in blood serum, urine and esophageal samples. The human keratinocyte reporter model has been widely accepted in a number of labs to have a well-characterized and stable bystander response over a large range of doses (Lyng et al. 2006, Mothersill et al. 2001, Ryan et al. 2008, Ahmad et al. 2013). The complete growth medium used for routine maintenance and colony-forming assays was RPMI-1640 with 10% foetal bovine serum (FBS) (Invitrogen, Burlington, ON, Canada), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Burlington, ON, Canada), 2 mM L-Glutamine (Gibco, Burlington, ON, Canada), 0.5 µg/ml of hydrocortisone (Sigma-Aldrich, Oakville, ON, Canada), and 15 mM of Hepes. All experiments were performed in a class II biosafety cabinet at McMaster University. Routine subculturing was performed on cell stocks reaching 80–100% confluency by using a 1:1 solution of 0.25% trypsin and 1 mM EDTA at 37°C for 8 min. Cell stocks were grown in 75 cm^2 flasks (T-75) filled with 30 ml of supplemented growth medium. Cell stocks and colony-forming experiments were incubated at 37°C and 5% carbon dioxide in air.

Tissue explants

Prior to fraction 1 and immediately following fraction 3, biopsies were taken as described above. Tissue dissections were not needed since three biopsies were taken at fraction 1 and 3. Each piece of tissue collected was approximately 1–2 mm^3 in size and these samples were aseptically plated in the center of 25 cm^2 flasks (T-25) filled with 4 ml of supplemented growth medium. The complete growth medium for clonogenic assays was similar to the tissue sample collection medium except for the exclusion of antimycotics and a final concentration of 100 U/ml penicillin and 100 µg/ml streptomycin solution (Gibco, Burlington, ON, Canada) was used. The esophageal explants were placed in the incubator at 37°C in 5% carbon dioxide in air for 48 h.

Clonogenic assay

Explant conditioned medium

For esophageal explants, HPV-G reporters were set up at a density of 500 cells per T-25 flask containing 4 ml of culture medium. Explant conditioned medium (ECM) was generated by incubating the esophageal explants in culture medium for 48 h as described above. After 48 h, a standard medium transfer was performed where the ECM was filtered with a 0.22 µm Nalgene filter (VWR Burlington, Ontario, Canada) and placed onto reporter cultures. Following medium transfers, reporters were grown in an incubator at 37°C with 5% carbon dioxide in air for 10–14 days. Once viable colonies had formed, the cells were stained with 20% carbol fuchsin (VWR, Burlington, Ontario, Canada) and colonies with ≥ 50 cells were scored. Biopsies taken at the start of fraction 1 prior to the patient undergoing HDR-ILBT were used as controls. Biopsies taken immediately following irradiation were treatment samples.

Blood serum

Seymour and Mothersill (2006) developed a blood serum in vivo colony-forming assay to assess human subject responses to radiation treatment. In the present

study, this technique was utilized on esophageal cancer patients undergoing fractionated brachytherapy. On the evening prior to patient treatments, human keratinocyte reporters were seeded at 300 cells per T-25 flask containing 5 ml of RPMI containing a final concentration of 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Burlington, ON, Canada), 2 mM L-Glutamine (Gibco, Burlington, ON, Canada), 0.5 µg/ml of hydrocortisone (Sigma-Aldrich, Oakville, ON, Canada), and 15 mM of Hepes. The FBS typically used in growth medium was substituted with 10% blood serum (0.5 ml per 5 ml of culture medium) collected before and after treatments. Treatments were performed early morning and blood serum was extracted at the JCC. All samples were then transported to McMaster University. The serum was added to the medium and then transferred to reporter flasks. Similar to explants clonogenics, reporters were incubated at 37°C with 5% CO_2 in air for 10–14 days, and then stained and colonies with ≥ 50 cells were scored.

Urine samples

The in vivo colony-forming assay with urine samples was developed by Pinho et al. (2012) and preliminary data were published in Pinho et al. (2012). These clonogenic assays were performed alongside biopsy and blood sample experiments. Human keratinocyte reporters were seeded with 700 cells in T-25 flasks in 5 ml of RPMI culture medium. Urine samples were diluted 10-fold and added to the flasks. 1 ml of diluted pre- and post-treatment urine samples were added to flasks. Control flasks with an additional 1 ml volume of diluted medium (diluted with sterile distilled water) was set up to ensure 1 ml volume of diluted urine with medium did not affect the colony-forming ability of reporters. Reporter cells were incubated at 37°C with 5% CO_2 in air for 10–14 days, and then stained and colonies with ≥ 50 cells were scored.

Statistical analysis

All reporter flasks were set up in triplicate for each sample and at every fraction of brachytherapy. Data presented in this paper display three measurements per patient at each treatment fraction. A Shapiro-Wilk normality test and Levene's tests found that the data violated the normality and equal variances conditions required for a parametric statistical analysis. When assessing whether blood and urine samples repeatedly taken from patients at various time-points throughout brachytherapy had a distinct treatment effect, an non-parametric Friedman's test with a post hoc Wilcoxon signed ranks test were performed. The p-values were adjusted with Bonferroni corrections to eliminate the chance of committing type I errors. When before and after treatment groups were compared for patient characteristics or tissue explant clonogenic assays, significance was determined by performing separate Wilcoxon signed rank tests. For the urine based colony-forming assay, there was a limited number of female patients able to give a sample. As a result, patients' cancer staging characteristics were analyzed only. The relationship between cell survival and dose-rate was assessed using a Spearman's correlation on the blood and urine samples. Statistically significant correlations were analyzed further with a linear regression analysis. A complete analysis on all 15 patients was not feasible for a number of reasons including patients leaving from the study, patients unable to give urine samples, and logistical difficulties associated with sample collection. All p-values less than 0.05 were considered statistically significant.

Results

Blood based colony-forming assay

Figure 1 shows the relationship between cloning efficiency and dose-rate administered at each fraction of brachytherapy. Patients undergoing fraction 2 and 3 of brachytherapy demonstrated a significant moderate positive relationship between cloning efficiency and dose-rate ($p < 0.05$*), whereas, fraction 1 had no such relationship. The association between cloning efficiency and dose-rate were assessed further with a linear regression analysis for fractions 2 and 3 of brachytherapy. A positive trend between cloning efficiency and dose-rate was observed for fractions 2 ($p < 0.05$*) and fractions 3 ($p < 0.001$**). This model indicates that 29.7% and 36.3% of the total variation with the cloning ability of non-irradiated keratinocytes can be explained by the dose-rate for fractions 2 and 3, respectively (Figure 1). The data is showing bystander reporters exposed to blood sera taken from cancer patients under-

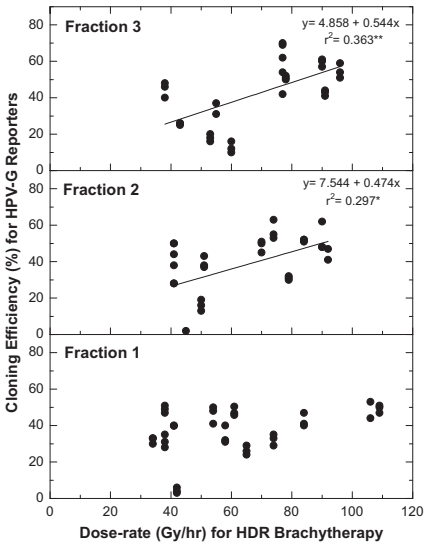


Figure 1. A statistically significant positive relationship between cloning efficiency (%) for HPV-G reporters and dose-rate was observed following fraction 2 and 3 of brachytherapy for blood sera samples taken from 11 patients. Outlined above are three measurements set-up per patient for each fraction of brachytherapy (n = 33). For treatment fractions illustrating a significant relationship between cloning efficiency and dose-rate, a linear regression model was used to determine whether the dose-rate variable contributed to the prediction of cloning capabilities of HPV-G reporters. Fraction 2 and 3 show a clear inverse dose-rate effect for HPV-G reporters exposed to blood sera taken following brachytherapy. * Indicates a p-value less than 0.05.

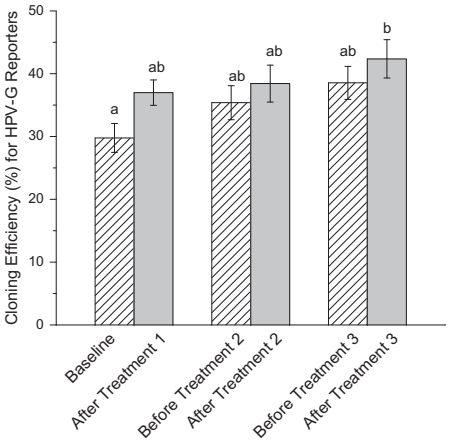


Figure 2. Illustrated in the graph is the colony-forming ability of HPV-G reporters (%) after exposure to blood serum samples before and after fractionated brachytherapy. Eleven patients had triplicate flask set-up at each treatment. All values are mean ± SEM for $n = 33$. Lettering indicates similarities and significance between the treatment groups.

going high dose-rate brachytherapy had a clear inverse dose-rate effect during late treatment fractions. The dose-rate variability observed across each of the fractions of brachytherapy can be explained in part by the patients' tumor size, but most likely the dose-rate differences between fractions is related closely with the decay parameters (i.e., source decay and source renewal), refer to Supplementary data in Figure 1, available online at http://informa healthcare.com/abs/doi/10.3109/09553002.2015.1068458.

When running the repeated measures analyses, brachytherapy revealed a statistically significant difference in cloning efficiency for HPV-G reporters treated with blood sera amongst the treatment fractions ($p < 0.001$*), refer to Figure 2. Although when examining each treatment fraction, it can be seen that samples taken before brachytherapy compared to post-treatment samples revealed no statistical changes in cloning efficiencies ($p > 0.05$). Rather a significant increase in the colony-forming ability of non-irradiated reporters was observed at the later part of brachytherapy. For instance, the final fraction of brachytherapy had a statistically significant increase in cloning efficiency by 12.60% relative to baseline samples ($p < 0.001$*). Whereas post-treatment samples at fractions 1 and 2 had an insignificant increase in cloning efficiency by 7.22% ($p = 0.705$) and 8.65% ($p = 0.210$) compared to baseline samples, respectively. To eliminate the chance of committing type I errors with Wilcoxon multiple pairwise comparisons when assessing treatment effects at various points in time, each p-value was adjusted with Bonferroni corrections refer to (Supplementary data in Table 1, available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068458).

When exploring the outcome of cloning efficiency in respect to gender for samples taken before and after treatment, these patient characteristics were found to have no significant influence on the growth of non-irradiated cells ($p > 0.05$), refer to Figure 3a. In contrast to gender differences, cancer staging showed a significant increase in cloning efficiencies for patients clinically diagnosed with stage III at fraction 1 of treatment and stage IV at fraction 2 of brachytherapy, as shown in Figure 3b.

Urine based colony-forming assay

The relationship between cell survival and dose-rate was also assessed for urine samples. For patients undergoing fraction 1, 2, and 3 of brachytherapy, there was no relationship between cell survival (%) and dose-rate (Figure 4).

Similar to the blood sample data, urine samples had a statistical difference in cell survival throughout the course of

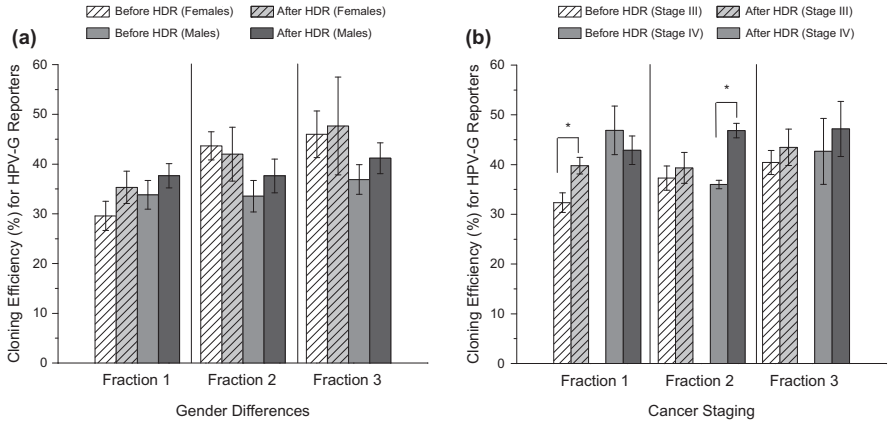


Figure 3. Gender differences (a) and cancer staging (b) were also assessed across each treatment group to explore whether any changes in cloning efficiency (%) were dependent on certain patient characteristics. For each fraction, separate Wilcoxon signed rank tests were performed to determine whether there are significant changes between males and females and cancer stage III and IV. Since 3 measurements were set-up per patient, $n = 33$ for males, $n = 12$ for females, $n = 24$ for patients diagnosed with cancer stage III and $n = 12$ for cancer stage IV. All values are mean ± SEM. * Indicates statistically significant difference from pre-treatment sample.

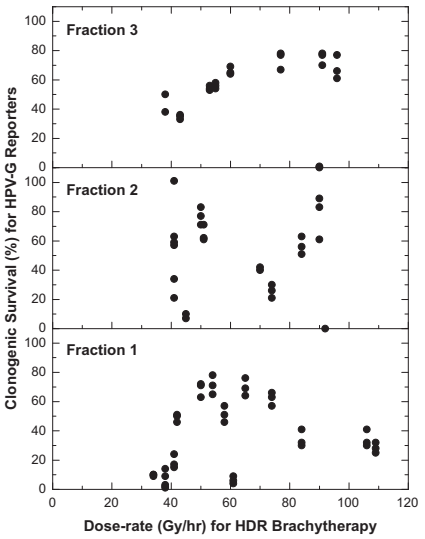


Figure 4. The relationship between cell survival (%) for HPV-G reporters and dose-rate of brachytherapy for urine samples are illustrated for fractions 1, 2, and 3. The sample size consisted of 11 patients. Outlined above are three measurements set-up per patient for each fraction of brachytherapy ($n = 33$). There were no statistically significant relationships found for fraction 1, 2, and 3.

brachytherapy ($p < 0.05$*), as shown in Figure 5 and Supplementary data in Table 2. available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068458 A significant increase in the survival of non-irradiated reporters was also observed at the later part of brachytherapy.

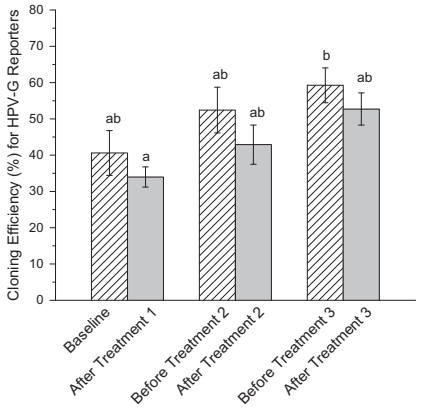


Figure 5. Illustrated in the graph is the cell survival (%) of HPV-G reporters after exposure to diluted urine samples before and after fractionated brachytherapy. Lettering indicates similarities and significance between the treatment groups. Eight patients had triplicate flask set-up for each fraction of brachytherapy. All values are mean ± SEM for $n = 24$.

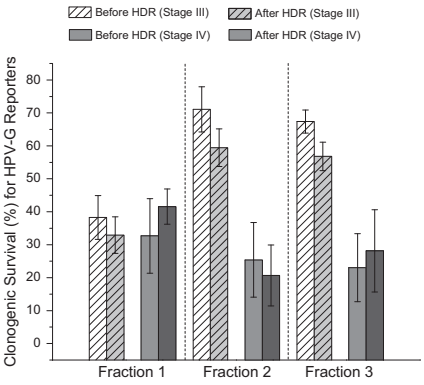


Figure 6. Cancer staging were also assessed across each treatment group to explore whether any changes in clonogenic survival (%) was dependent on certain patient characteristics. There were 8 and 4 patients clinically diagnosed with cancer stage III and IV, respectively. All values are mean ± SEM for $n = 24$ and $n = 12$ for cancer stage III and IV, respectively.

Instead of the effect occurring in the post-treatment samples at the final fraction of treatment, samples taken before the third fraction of brachytherapy had a significant increase in cell survival compared to post-treatment samples after the first fraction of brachytherapy ($p < 0.001$*). In Figure 5, it can also be seen that samples taken after fractions 1 and 2 had insignificant changes in cell survival compared to baseline samples. Similar to the blood sample results, these findings are suggesting that late treatment fractions of brachytherapy are inducing a radioresistance response in non-irradiated cells. Furthermore, cancer staging had no influence on the growth of non-irradiated cells ($p > 0.05$), however, the data reveals large variability for this endpoint (Figure 6).

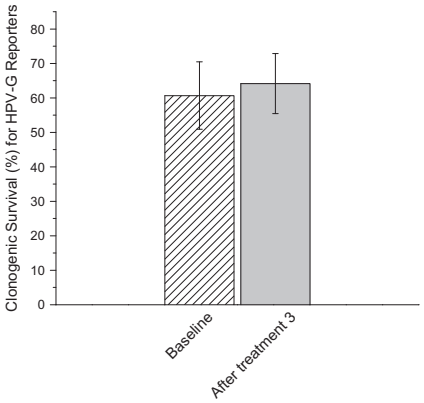


Figure 7. Shown above is the HPV-G reporters' clonogenic survival (%) after being exposed to explant conditioned medium with samples taken before the first fraction and immediately after the final fraction of brachytherapy. Six patients had triplicate flask set-up for each treatment. All values are mean ± SEM of $n = 18$.

Tissue explant based colony-forming assay

For the biopsy samples, tissues taken at baseline had a lower survival by 6.00% compared to samples taken immediately following the final treatment of brachytherapy ($p > 0.05$). These results reveal no indication of tissue explants generating bystander signals following brachytherapy.

Discussion

The primary objective of this study is to determine whether blood, urine, and tissue explant-based colony forming assays can be used to trace levels of bystander or protective signals being generated following brachytherapy treatments. A few patient characteristics were assessed to determine whether these factors might be influencing cell communicating signals and affecting the growth of non-irradiated cells.

Although the data is limited, the findings may suggest that cancer patients undergoing fractionated brachytherapy induced a radioresistance response for cells or tissues in close proximity to the irradiated tumor volume after undergoing treatment 3 compared to baseline samples. Such a response was observed in bystander cells exposed to blood sera and urine samples taken from esophageal cancer patients irradiated in vivo. Urine sample results had a similar trend as the blood serum data, although, these samples did not have a significant increase in cell survival when taken immediately after fraction 3. Instead, urine samples taken before fraction 3 had a significant increase in the reporters cell survival compared to post-treatment samples taken after the first fraction of brachytherapy.

In the literature, there is a considerable amount of research on cellular radioresistance responses performed in vitro (Thomas et al. 2013), once cells have been exposed to small acute doses or low acute dose-rates, initiating protective responses or enhanced repair processes. However, the induced radioresistance response is commonly triggered with doses below 1 Gy and dose-rates ranging from 0.18–2.43 Gy/min (Thomas et al. 2013). In the present study, irradiations occurred in vivo with a prescribed dose to the esophageal lumen of 600 cGy per HDR fraction and the average dose-rates per fraction were > 50 Gy/h. Our findings are suggesting an induced radioresistance response after subsequent treatment fractions. However, a characteristic of this phenomenon is that low doses below 1 Gy are required during in vitro irradiations. One possible explanation for the effect being triggered late into brachytherapy regimes with substantially higher doses and dose-rates, would be that circulating blood cells flowing through the tumor volume may have been directly irradiated with significantly lower doses of gamma radiation than the tumor itself. However, there is also a possibility that blood cells flowing nearby the tumor spend significantly less time in the radiation field and may have not been directly irradiated, rather the effect may be a systemic immune response (Mothersill and Seymour 2004). The response observed in the non-irradiated cells, incubated with supernatants of blood serum during the final fraction of brachytherapy, are presumably initiated as a result of neighboring cells receiving comparable doses to in vitro radiation studies.

Side-effects in radiotherapy regimes are primarily attributed to different patients having inherently unique radiosensitivities (Twardella and Chang-Claude 2002). One of the first promising studies assessing the RIBE and cancer patients' intrinsic radiosensitivities from blood samples was published by Howe et al. (2009). In this study, it was shown that lymphocyte cultures, taken from colorectal cancer patients, had a significant increase in radiosensitivity and its ability to produce bystander signals compared to cancer-free controls. Other studies detected bystander and radioprotective factors in the blood serum of Chernobyl accident survivors (Marozik et al. 2007) and cancer patients undergoing various fractions of radiotherapy (Seymour and Mothersill 2006), respectively.

Bystander signalling has been suggested to be associated with the activation of macrophages in mice (Lorimore et al. 2001). Recently researchers have shown that radiation stimulates the innate immune function (Manda et al. 2012, Multhoff and Radons 2012, Rödel et al. 2012, Mothersill 2013). With such high doses being prescribed at each fraction, further investigation with a macrophage Superoxide Dismutase (SOD) assay revealing innate immune function may be beneficial to test in future work (Johnston et al. 1978, Fukasawa et al. 1988). The superoxide anion (0_2^-) is a short-lived free radical that plays an essential role in immune responses (Johnston et al. 1978). Such a radical is commonly released from macrophages. Macrophages collected from blood and cultured using regular cell culture techniques would be one way of investigating whether this response is a systematic immune response. A more mechanistic approach to elucidate other cellular activities would be to assess reactive oxygen species (ROS) activity in the bystander cells by using the 2′,7′-Dichlorofluorescein (DCF) fluorescent probe (O'Dowd et al. 2006). This marker for ROS can be loaded into the human keratinocytes cells after exposure to medium supplemented with 10% blood serum taken from cancer patients undergoing brachytherapy. Past work has found that increased levels of fluorescence has been highly correlated with higher levels of ROS in bystander cells (O'Dowd et al. 2006).

From our experiments, the urine-based colony-forming assays showed substantial inter-patient variability relative to the blood based assay results. The urine-based assay had proved to be unreliable and was deemed unsuitable for further clinical work due to the large variation observed amongst patients and treatment fractions. However, the blood based assay had much less variability and revealed interesting findings that provides further insight on the previously published work (Seymour and Mothersill 2006, Marozik et al. 2007). Unlike previous in vitro work resulting in a lower ability to produce bystander signal(s) when tissue samples were harvested from males with a pre-existing malignancy (Mothersill et al. 2002), the present clinical study had no such influence on signal production for gender. Furthermore cancer staging had no observable influence on the growth of non-irradiated reporters in cancer patients undergoing brachytherapy for the urine samples, but there was a significant effect observed for cancer staging for blood serum data only. These inconsistent results are most likely attributed to the small sample size and would need to be assessed further with a larger sample.

The data also revealed a clear statistically significant inverse dose-rate response in bystander cells exposed to blood serum harvested immediately following brachytherapy at fractions 2 and 3. However, the urine samples had no such effect observed following fraction 2 and 3 treatments. An earlier study by Mitchell et al. (1979), observed an inverse dose-rate effect during in vitro radiations of HeLa cells exposed to dose-rates ranging from 0.37–1.54 Gy/h (Mitchell et al. 1979). These authors found that HeLa cells had an increase in cell death for lower dose-rates than higher ones. At certain dose-rates, HeLa cells progress through the cell cycle and become blocked in the radiosensitive G2 phase at lower dose-rates, resulting in enhanced cell killing.

One of the most problematic issues that should be addressed in this paper were the loss of valuable biopsy samples, due to patient contamination. Patient contaminations were attributed to opportunistic yeast infections such as *Candida albicans*, which are common in immunocompromised cancer patients as explained in Delsing et al. (2012). Due to the scarcity of data available on such infections for esophageal carcinoma patients (Chiou et al. 2000), these issues were originally overlooked at the start of the clinical study. Throughout the remainder of the sample collection, antimycotics (fungizone and nystatin) were supplemented in the collection medium prior to commencing the tissue explant colony-forming assays as a preventive measure. It is our recommendation for other investigators to either check for yeast infections or to supplement fungizone and nystatin for a short duration of time to avoid similar problems. As it stands, the tissue explant colony-forming assays led to inconclusive data and even more unanswered questions. Further investigations are warranted to assess the radiosensitivity and non-targeted radiations effects in nearby esophagus samples.

Another limitation associated with this study was the small sample size and the substantial inter-patient variation observed from one treatment fraction to the next. The limited sample size was attributed to difficulties with consenting patients for biopsy samples, as well as for all stages of treatment, and 3 weeks follow-ups (unreported data). The number of subjects required to achieve statistical power was conducted with a power analysis using G*Power software (Faul et al. 2007) with a power (1-β) set at 0.95 and $\alpha = 0.05$. Future work would need to have a sample size of 84 in order to determine whether the different treatment fractions changes in cell survival have reached statistical significance at the 0.05 level. In retrospect, a much simpler study design focusing on only one treatment fraction and blood serum samples alone would be more appropriate since esophageal cancer typically presents at advance stages where the diseases prognosis are quite poor (Sur et al. 1998).

In conclusion, this simple blood-based assay may have future implications as a clinical detection tool used to predict treatment outcome based upon certain patient characteristics, such as gender, cancer staging, and metastatic status. From these preliminary findings, a follow-up study with a larger sample size, including unirradiated cancer-free controls, and a thorough analysis of patient characteristics may shed light on whether this technique may be appropriate as a predictive assay for assessing radiation side-effects or treatment outcome. A follow-up study has been undertaken with a target sample size of 115 cancer patients and 15 healthy patients with a power (1-β) set at 0.95 and statistical significance level set to 0.05. This work will provide further insight on whether non-targeted radiation effects have relevance in HDR brachytherapy.

Acknowledgements

We wish to thank Varian International B.V. for supporting a portion of this work. We are grateful for the Hamilton Health Sciences JCC brachytherapy staff for assisting in sample collection. We acknowledge and thank all the patients who participated in this study. A special thanks to the Molecular Medicine laboratory at McMaster University and the JCC Clinical trials laboratory for centrifuge usage. All authors have read and approved this manuscript for submission, and have also read the journal's authorship agreement.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

References

Ahmad SB, McNeill FE, Byun SH, Prestwich WV, Mothersill C, Seymour C, Armstrong A, Fernandez C. 2013. Ultra-violet light emission from HPV-G cells irradiated with low let radiation from (90)Y; consequences for radiation induced bystander effects. Dose-Response 11:498:–Res

Berry B, Miller RR, Luoma A, Nelems B, Hay J, Flores AD. 1989. Pathologic findings in total esophagectomy specimens after intracavitary and external-beam radiotherapy. Cancer 64:1833–1837.

Boyden S, Raaphorst GP. 1999. Adaptive response and its variation in human normal and tumour cells. Int J Radiat Biol 75:865–873.

Boyd M, Sorensen A, McCluskey AG, Mairs RJ. 2008. Radiation quality-dependent bystander effects elicited by targeted radionuclides. J Pharm Pharmacol 60:951–958.

Brenner DJ, Curtis RE, Hall EJ, Ron E. 2000. Second malignancies in prostate carcinoma patients after radiotherapy compared with surgery. Cancer 88:398–406.

Butterworth KT, McMahon SJ, Hounsell AR, O'Sullivan JM, Prise KM. 2013. Bystander signalling: Exploring clinical relevance through new approaches and new models. Clin Oncol 25:586–592.

Chai Y, Hei TK. 2008. Radiation induced bystander effect in vivo. Acta Med Nagasaki 53:S65–S69.

Chiou CC, Groll AH, Walsh TJ. 2000. New drugs and novel targets for treatment of invasive fungal infections in patients with cancer. Oncologist 5:120–135.

Delsing CE, Bleeker-Rovers CP, van de Veerdonk FL, Tol J, Van der Meer JWM, Kullberg BJ, Netea MG. 2012. Association of esophageal candidiasis and squamous cell carcinoma. Med Mycol 1:5–8.

Emerit I, Levy A, Cernjavski L, Arutyunyan R, Oganesyan N, Pogosian A, Mejlumian H, Sarkisian T, Gulkandanian M, Quastel M. 1994. Transferable clastogenic activity in plasma from persons exposed as salvage personnel of the Chernobyl reactor. J Cancer Res Clin Oncol 120:558–561.

Emerit I, Oganesian N, Sarkisian T, Arutyunyan R, Pogosian A, Asrian K, Levy A, Cernjavski L. 1995. Clastogenic factors in the plasma of Chernobyl accident recovery workers: Anticlastogenic effect of *Ginkgo biloba* extract. Radiat Res 144:198–205.

Emerit I, Quastel M, Goldsmith J, Merkin L, Levy A, Cernjavski L, Alaoui-Youssefi A, Pogossian A, Riklis E. 1997. Clastogenic factors in the plasma of children exposed at Chernobyl. Mutat Res 373: 47–54.

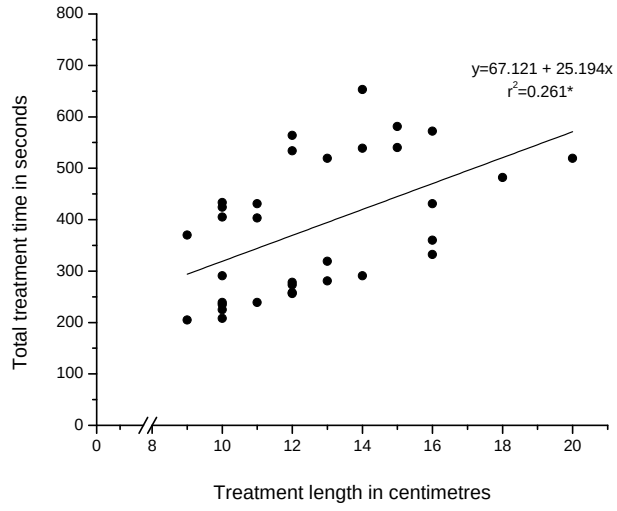
Faguet GB, Reichard SM, Welter DA. 1984. Radiation-induced clastogenic plasma factors. Cancer Genet Cytogenet 12:73–83.

Faul F, Erdfelder E, Lang AG, Buchner A. 2007. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 39:175–191.
Fukasawa M, Bryant SM, DiZerega GS. 1988. Superoxide anion production by postsurgical macrophages. J Surg Res 45:382–388.
Goh K, Sumner H. 1968. Breaks in normal human chromosomes: Are they induced by a transferable substance in the plasma of persons exposed to total-body irradiation? Radiat Res 35:171–181.
Hall EJ, Wuu CS. 2003. Radiation-induced second cancers: The impact of 3D-CRT and IMRT. Int J Radiat Oncol Biol Phys 56:83–88.
Hollowell JG Jr, Littlefield LG. 1968. Chromosome damage induced by plasma of x-rayed patients: An indirect effect of x-ray. Proc Soc Exp Biol Med 129:240–244.
Howe O, O'Sullivan J, Nolan B, Vaughan J, Gorman S, Clarke C, McClean B, Lyng FM. 2009. Do radiation-induced bystander effects correlate to the intrinsic radiosensitivity of individuals and have clinical significance? Radiat Res 171:521–529.
Johnston JR, Godzik C, Cohn Z. 1978. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. J Exp Med 148:115–129.
Koturbash I, Kutanzi K, Hendrickson K, Rodriguez-Juarez R, Kogosov D, Kovalchuk O. 2008. Radiation-induced bystander effects in vivo are sex specific. Mutat Res 642:28–36.
Lorimore SA, Coates PJ, Scobie GE, Milne G, Wright EG. 2001. Inflammatory-type responses after exposure to ionizing radiation in vivo: A mechanism for radiation-induced bystander effects? Oncogene 20:7085–7095.
Lyng FM, Maguire P, McClean B, Seymour C, Mothersill C. 2006. The involvement of calcium and MAP kinase signaling pathways in the production of radiation-induced bystander effects. Radiat Res 165:4005:at.
Lyng FM, Seymour CB, Mothersill C. 2000. Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. Br J Cancer 83:1223–1230.
Maguire P, Mothersill C, McClean B, Seymour CB, Lyng FM. 2007. Modulation of radiation responses by pre-exposure to irradiated cell conditioned medium. Radiat Res 167:485–492.
Manda K., Glasow A, Paape D, Hildebrandt G. 2012. Effects of ionizing radiation on the immune system with special emphasis on the interaction of dendritic and T cells. Front Oncol 2:102.
Marozik P, Mothersill C, Seymour CB, Mosse I, Melnov S. 2007. Bystander effects induced by serum from survivors of the Chernobyl accident. Exp Hematol 35:55–63.
Michiels C. 2004. Physiological and pathological responses to hypoxia. Am J Pathol 164:1875–1882.
Mitchell JB, Bedford JS, Bailey SM. 1979. Dose-rate effects in plateau-phase cultures of S3 HeLa and V79 cells. Radiat Res 79:552–567.
Mohammadi S, Taghavi-Dehaghani M, Gharaati MR, Masoomi R, Ghiassi-Nejad M. 2006. Adaptive response of blood lymphocytes of inhabitants residing in high background radiation areas of ramsar-micronuclei, apoptosis and comet assays. J Radiat Res 47:279–285.
Morgan WF. 2003. Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects in vivo, clastogenic factors and transgenerational effects. Radiat Res 159:581–596.
Mothersill C. 2013. Uncomfortable issues in radiation protection posed by low-dose radiobiology. Radiat Environ Biophys 52:293–298.
Mothersill C, Moriarty M, Seymour C. 2004a. Radiotherapy and the potential exploitation of bystander effects. Int J Radiat Oncol Biol Phys 58:575–579.
Mothersill C, O'Malley K, Seymour CB. 2002. Characterisation of a bystander effect induced in human tissue explant cultures by low LET radiation. Radiat Prot Dosimetry 99:163–167.
Mothersill C, Rea D, Wright EG, Lorimore SA, Murphy D, Seymour CB, O'Malley K. 2001. Individual variation in the production of a 'bystander signal' following irradiation of primary cultures of normal human urothelium. Carcinogenesis 22:1465cinog
Mothersill C, Seymour CB. 2006. Radiation-induced bystander and other non-targeted effects: Novel intervention points in cancer therapy? Curr Cancer Drug Targets 6:447–454.
Mothersill C, Seymour C. 1997. Survival of human epithelial cells irradiated with cobalt 60 as microcolonies or single cells. Int J Radiat Biol 72:597–606.
Mothersill C, Seymour CB. 2004. Radiation-induced bystander effects – implications for cancer. Nat Rev Cancer 4:158–164.
Mothersill C, Seymour RJ, Seymour CB. 2004b. Bystander effects in repair-deficient cell lines. Radiat Res 161:256–263.
Multhoff G, Radons J. 2012. Radiation, inflammation, and immune responses in cancer. Front Oncol 2:58.
O'Dowd C, Mothersill CE, Cairns MT, Austin B, McClean B, Lyng FM, Murphy JEJ. 2006. The release of bystander factor(s) from tissue explant cultures of rainbow trout (*Onchorhynchus mykiss*) after exposure to gamma radiation. Radiat Res 166:611–617.
Pant GS, Kamada N. 1977. Chromosome aberrations in normal leukocytes induced by the plasma of exposed individuals. Hiroshima J Med Sci 26:149–154.
Pinho C, Wong R, Sur RK, Hayward JE, Farrell TJ, Seymour C, Mothersill C. 2012. The involvement of serum serotonin levels producing radiation-induced bystander effects for an in vivo assay with fractionated high dose-rate (HDR) brachytherapy. Int J Radiat Biol 88:791–797.
Pirisi L, Creek KE, Doniger J, Dipaolo JA. 1988. Continuous cell lines with altered growth and differentiation properties originate after transfection of human keratinocytes with human papillomavirus type 16 DNA. Carcinogenesis 9:1573–1579.
Prise KM, Belyakov OV, Folkard M, Michael BD. 1998. Studies of bystander effects in human fibroblasts using a charged particle microbeam. Int J Radiat Biol 74:793–798.
Prise KM, O'Sullivan JM. 2009. Radiation-induced bystander signalling in cancer therapy. Nat Rev Cancer 9:351–360.
Qutob SS, Multani AS, Pathak S, McNamee JP, Bellier PV, Liu QY, Ng CE. 2006. Fractionated X-radiation treatment can elicit an inducible-like radioprotective response that is not dependent on the intrinsic cellular X-radiation resistance/sensitivity. Radiat Res 166:590–599.
Rödel F, Frey B, Gaipl U, Keilholz L, Fournier C, Manda K, Schöllnberger H, Hildebrandt G, Rödel C. 2012. Modulation of inflammatory immune reactions by low-dose ionizing radiation: Molecular mechanisms and clinical application. Curr Med Chem 19:1741–1750.
Ryan LA, Seymour CB, Joiner MC, Mothersill CE. 2009. Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. Int J Radiat Biol 85:87–95.
Ryan LA, Smith RW, Seymour CB, Mothersill CE. 2008. Dilution of irradiated cell conditioned medium and the bystander effect. Radiat Res 169:188iat R
Seymour CB, Mothersill C. 2000. Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve. Radiat Res 153:508–511.
Seymour C, Mothersill C. 2006. Development of an in vivo assay for detection of non-targeted radiation effects. Dose Response 4:277–282.
Sur RK, Donde B, Levin VC, Mannell A. 1998. Fractionated high dose rate intraluminal brachytherapy in palliation of advanced esophageal cancer. Int J Radiat Oncol Biol Phys 40:447–453.
Sur RK, Levin CV, Donde B, Sharma V. Miszczyk L, Nag S. 2002. Prospective randomized trial of HDR brachytherapy as a sole modality in palliation of advanced esophageal carcinoma–an International Atomic Energy Agency study. Int J Radiat Oncol Biol Phys 53: 127–133.
Thomas C, Martin J, Devic C, Bräuer-Krisch E, Diserbo M, Thariat J, Foray N. 2013. Impact of dose-rate on the low-dose hyper-radiosensitivity and induced radioresistance (HRS/IRR) response. Int J Radiat Biol 89:813–822.
Twardella D, Chang-Claude J. 2002. Studies on radiosensitivity from an epidemiological point of view – overview of methods and results. Radiother Oncol 62:249–260.
Yoshioka Y, Yoshida K, Yamazaki H, Nonomura N, Ogawa K. 2013. The emerging role of high-dose-rate (HDR) brachytherapy as monotherapy for prostate cancer. J Radiat Res 54:781–788.

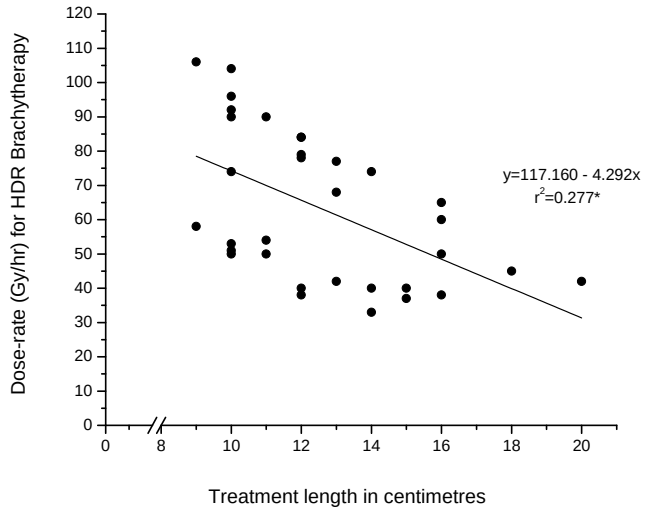
Supplementary material available online

Supplementary Tables 1 and 2 and Figure 1 available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068458.

3.1 Supplementary Information



(a)



(b)

Figure 3.1. As illustrated in graph 3.1(a) the relationship between total treatment time in seconds and tumour length treated in centimeters were assessed. Also, the relationship between dose-rate (Gy/h) and tumour length treated in centimeters are outlined in graph 3.1(b). These regression models are suggesting that nearly 27% of the dose-rate variability can be explained by the size of the tumour, as patients with larger tumours require a longer treatment time that ultimately effects the dose-rate. Therefore, the tumour length variable partly explains the dose-rate variability observed across each fraction of brachytherapy, since the irradiated length varied from one fraction to the next. Other factors that could explain the dose-rate variability are dependent on the iridum-192 source decay and source renewal. The data shown above were combined from fractions 1, 2, and 3 of brachytherapy.

Table 3.1. A comparison between HDR brachytherapy fractions cloning efficiency (%) for HPV-G exposed to blood sera

Brachytherapy fraction comparison	Mean cloning efficiency (%)	Unadjusted p-value	Bonferroni adjusted p-value
Baseline versus fraction 1 post-treatment	29.8 ± 2.3 versus 37.0 ± 2.0	0.047	0.705
Baseline versus fraction 2 pre-treatment	29.8 ± 2.3 versus 35.4 ± 2.7	0.114	1.000
Baseline versus fraction 2 post-treatment	29.8 ± 2.3 versus 38.4 ± 2.9	0.014	0.21
Baseline versus fraction 3 pre-treatment	29.8 ± 2.3 versus 38.5 ± 2.6	0.004	0.06
Baseline versus fraction 3 post-treatment	29.8 ± 2.3 versus 42.4 ± 3.0	<0.0001	<0.001**
Fraction 1 post-treatment versus fraction 2 pre-treatment	37.0 ± 2.0 versus 35.4 ± 2.7	0.610	1.000
Fraction 1 post-treatment versus fraction 2 post-treatment	37.0 ± 2.0 versus 38.4 ± 2.9	0.469	1.000
Fraction 1 post-treatment versus fraction 3 pre-treatment	37.0 ± 2.0 versus 38.5 ± 2.6	0.589	1.000
Fraction 1 post-treatment versus fraction 3 post-treatment	37.0 ± 2.0 versus 42.4 ± 3.0	0.053	0.795
Fraction 2 pre-treatment versus fraction 2 post-treatment	35.4 ± 2.7 versus 38.4 ± 2.9	0.034	0.510
Fraction 2 pre-treatment versus fraction 3 pre-treatment	35.4 ± 2.7 versus 38.5 ± 2.6	0.253	1.000
Fraction 2 pre-treatment versus fraction 3 post-treatment	35.4 ± 2.7 versus 42.4 ± 3.0	0.009	0.135
Fraction 2 post-treatment versus fraction 3 pre-treatment	38.4 ± 2.9 versus 38.5 ± 2.6	0.841	1.000
Fraction 2 post-treatment versus fraction 3 post-treatment	38.4 ± 2.9 versus 42.4 ± 3.0	0.065	0.975
Fraction 3 pre-treatment versus fraction 3 post-treatment	38.5 ± 2.6 versus 42.4 ± 3.0	0.038	0.570

Table 3.2. A comparison between HDR brachytherapy fractions cell survival (%) for HPV-G exposed to diluted urine

Brachytherapy fraction comparison	Mean cell survival (%)	Unadjusted p-value	Bonferroni adjusted p-value
Baseline versus fraction 1 post-treatment	40.6 ± 6.2 versus 34.0 ± 2.8	0.836	1.000
Baseline versus fraction 2 pre-treatment	40.6 ± 6.2 versus 52.4 ± 6.3	0.060	0.900
Baseline versus fraction 2 post-treatment	40.6 ± 6.2 versus 42.9 ± 5.4	0.280	1.000
Baseline versus fraction 3 pre-treatment	40.6 ± 6.2 versus 59.3 ± 4.8	0.028	0.420
Baseline versus fraction 3 post-treatment	40.6 ± 6.2 versus 52.7 ± 4.4	0.121	1.000
Fraction 1 post-treatment versus fraction 2 pre-treatment	34.0 ± 2.8 versus 52.4 ± 6.3	0.019	0.285
Fraction 1 post-treatment versus fraction 2 post-treatment	34.0 ± 2.8 versus 42.9 ± 5.4	0.049	0.735
Fraction 1 post-treatment versus fraction 3 pre-treatment	34.0 ± 2.8 versus 59.3 ± 4.8	0.003	0.045*
Fraction 1 post-treatment versus fraction 3 post-treatment	34.0 ± 2.8 versus 52.7 ± 4.4	0.012	0.180
Fraction 2 pre-treatment versus fraction 2 post-treatment	52.4 ± 6.2 versus 42.9 ± 5.4	0.048	0.720
Fraction 2 pre-treatment versus fraction 3 pre-treatment	52.4 ± 6.2 versus 59.3 ± 4.8	0.290	1.000
Fraction 2 pre-treatment versus fraction 3 post-treatment	52.4 ± 6.2 versus 52.7 ± 4.4	0.713	1.000
Fraction 2 post-treatment versus fraction 3 pre-treatment	42.9 ± 5.4 versus 59.3 ± 4.8	0.004	0.06
Fraction 2 post-treatment versus fraction 3 post-treatment	42.9 ± 5.4 versus 52.7 ± 4.4	0.106	1.000
Fraction 3 pre-treatment versus fraction 3 post-treatment	59.3 ± 4.8 versus 52.7 ± 4.4	0.780	1.000

Chapter 4

The influence of smoking on radiation-induced bystander signal production in esophageal cancer patients

Christine Hanu, Emilia Timotin, Raimond Wong, Ranjan K. Sur, Joseph E.Hayward Colin Seymour, and Carmel Mothersill

Revisions and annual renewals to research ethics board were primarily carried out by the author of this thesis and reviewed by the Principal Investigator, Dr. Raimond Wong. Patient recruitment were performed by Dr. Ranjan Sur and Emilia Timotin. The JCC brachytherapy nursing staff extracted blood samples from all patients. Emilia Timotin consented patients, administered patient demographic questionnaires and extracted blood serum throughout the duration of this phase II study. Brachytherapy treatment planning procedures were carried out by the JCC Medical Physic staff. Sample transportation and clonogenic assays were all performed by the author. Data and statistical analyses were performed by the author. The manuscript was written by the first author and edited by Dr. Mothersill, Dr. Seymour, Dr. Wong, and Dr. Hayward. This is an Accepted Manuscript of an article published by Elsevier in Environmental Research on January 2016, available online: http://www.ncbi.nlm.nih.gov/pubmed/26750714 DOI: 10.1016/j.envres.2015.12.030.

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

The influence of smoking on radiation-induced bystander signal production in esophageal cancer patients

C. Hanu [a,*], E. Timotin [a], R. Wong [b], R.K. Sur [b], J.E. Hayward [a,c], C.B. Seymour [a], C.E. Mothersill [a]

[a] Medical Physics & Applied Radiation Sciences, McMaster University, Hamilton, ON, Canada
[b] Department of Oncology, McMaster University, Hamilton, ON, Canada
[c] Department of Radiology, McMaster University, Hamilton, ON, Canada

ARTICLE INFO

Article history:
Received 4 December 2015
Received in revised form
21 December 2015
Accepted 24 December 2015

Keywords:
Bystander effect
Smoking status
High dose rate (HDR) brachytherapy
Esophageal carcinoma
Blood based colony-forming assay

ABSTRACT

The relevance of radiation-induced bystander effects in humans is unclear. Much of the existing data relate to cell lines but the effect of bystander signals in complex human tissues is unclear. A phase II clinical study was untaken, where blood sera from 60 patients along with 15 cancer-free volunteers were used to detect whether measurable bystander factor(s) could be found in the blood following high dose rate (HDR) brachytherapy. Overall, there was no significant change in bystander signal production (measured in a human keratinocyte reporter system) before and after one treatment fraction of HDR brachytherapy ($p > 0.05$). Further assessment of patient characteristics and environmental modifiable factors including smoking were also analyzed. Similar to previously published data, samples taken from smokers produced weaker signals compared to non-smokers ($p < 0.05$). Although the number of non-smoking subjects was low, there was a clear decrease in cloning efficiency observed in keratinocyte cultures for these patients that requires further study. This study found that samples taken from smokers do not produce bystander signals, whereas samples taken from non-smokers can produce such signals following HDR brachytherapy. These findings highlight the importance of studying the interactions of multiple stressors including environmental modifiers with radiation, since some factors such as smoking may elicit protection in tumor cells which could counteract the effectiveness of radiation therapy.

1. Introduction

Bystander effects have been readily assessed and documented within a number of cell lines (Lyng et al., 2000; Mothersill and Seymour, 1997; Prise et al., 1998; Seymour and Mothersill, 2000), however, this is not the case for humans (Prise and O'Sullivan, 2009). The relevance or implications of bystander effects in radiation carcinogenesis or radiotherapy are not fully understood. Further work is necessary to determine the carcinogenic risk to normal tissues, and tissues close to radiation fields along with elucidating whether the release of bystander factor(s) can be used to protect against radiation carcinogenesis or to enhance treatment planning options in radiotherapy (Boyd et al., 2008; Butterworth et al., 2013; Prise and O'Sullivan, 2009). Epidemiological studies, utilizing cancer registries, have found elevated secondary cancer risks in cancer survivors previously exposed to radiotherapy (Morton et al., 2014; Birgisson et al., 2005; Tubiana, 2009). More than 50% of cancers developed throughout a lifetime will require the patient to undergo some form of radiotherapy for curative or palliative treatment (Randal, 2000). It is imperative to explore whether bystander signal(s) can be detected in human samples following radiotherapy since it may shed further insights we are not yet aware of during treatment planning or as a preventive for adverse effects in healthy tissues.

This study is an extension of previously published work performed on 15 esophageal carcinoma patients undergoing high dose rate (HDR) brachytherapy (Pinho et al., 2015). In the pilot study, blood serum samples were harvested from patients and substituted in lieu of fetal bovine serum (FBS) in culture. Signals secreted into the medium from the blood sera were tested for strength by exposing them to a reporter cell line or well established reporter assay (Pinho et al., 2015). In this assay a decrease in colony forming ability in the reporters means a strong bystander signal and no effect or increased cloning efficiency means an growth stimulating, absent or weaker signal. Our pilot study revealed an insignificant increase in cell survival immediately following the first fraction of treatment, suggesting that a growth stimulating signal might be present but the results did not reach

* Corresponding author.
E-mail address: pinhoc@mcmaster.ca (C. Hanu).

http://dx.doi.org/10.1016/j.envres.2015.12.030
0013-9351/

statistical significance due to sample variation. Based upon our pilot work, we hypothesized that an increase in reporter cloning efficiency will occur immediately following the first fraction of brachytherapy (Pinho et al., 2015). Similar to the one-year pilot study, patient characteristics such as gender were assessed to determine whether these factors can lead to variations in the production of bystander signal(s) following exposure to HDR brachytherapy. In addition to gender, the smoking status of esophageal carcinoma patients were also assessed before and after treatment.

Our motivation for assessing certain patient characteristics stems from studies reporting individual variations in urothelium tissues irradiated *ex vivo* (Mothersill et al., 2001). Furthermore, bystander signal(s) seem to have a dependence on gender and smoking in normal urothelium tissue samples extracted from healthy patients. Samples taken from females, who were non-smokers, had a significant decrease in survival compared to tissues harvested from males that smoked (Mothersill et al., 2001) suggesting that gender and smoking status are important factors in determining human tissue response to radiation exposure. In contrast to human females being more likely to produce strong anti-growth bystander signals, male mice have been shown to emit stronger bystander signal(s) in comparison to females (Koturbash et al., 2008). Smoking is a risk factor that has been linked to the development of bladder and oral cancers. A by-product of tobacco and known carcinogen, nitrosamines, is often excreted in the urine (Lyng et al., 1995). A significant growth effect was observed in urothelial cultures exposed to nitrosamines. Removal of the carcinogen caused the explant proliferation to return to the control level indicating that constant exposure is necessary for the response. Ultimately smoking can result in pre-neoplastic changes in urothelium cells. Earlier work by Colucci et al. reported a substantially higher expression of p53 and exon mobility-shifts in normal oral mucosa cells taken from smokers compared to non-smokers. This work suggested that p53 mutations may be an early occurrence in oral cancer development (Colucci et al., 1997).

Individual variation in bystander signal production has not only been related to environmental factors, but also to genetic factors (Mothersill et al., 2005). For example, past work with C57BL/6 mice and CBA/Ca mice revealed that only the C57BL/6 mice have the ability to produce bystander signals due to certain genetic factors that influence pro-apoptotic and anti-apoptotic proteins (Mothersill et al., 2005) linked to immune system function (Lorimore et al., 2013). All of these studies above have shown that multicellular systems can be influenced by certain individual characteristics. In the present study, the influence of gender and smoking status on bystander signal(s) were investigated in cancer patients while undergoing HDR brachytherapy.

2. Methods

2.1. Sample design

This study is an extension of a recently published one-year pilot study performed in 2011 (Pinho et al., 2015). In the phase II study reported here, all patients were either clinically diagnosed with esophageal adenocarcinoma (EA) or squamous cell carcinoma (ESCC). Blood samples were collected from 60 cancers before and after a single fraction of brachytherapy from January 2014 to 2015. In the present study, 49 males and 11 females, with a mean age of 70.3 years (age range, 49–90 years) participated in the study. Out of the 60 patients, 2 patients were excluded from the analysis as they were clinically diagnosed with neuroendocrine carcinoma. Similar to the pilot study, the majority of patients were males (81.7%) diagnosed with esophageal adenocarcinoma (EA). Roughly 40.0% of the patients had stage III cancer and 31.7% had stage IV cancer, refer to Table 1 for patient characteristics and demographics. Blood samples were also collected from 15 cancer-free volunteers with a mean age of 43.7 years (age range, 26–60 years). All participants gave informed consent, and ethics approval was obtained from the Hamilton Health Sciences Faculty of Health Sciences (HHS/FHS) research ethics board (REB # 06-193). Patients undergoing other forms of radiotherapy and/or chemotherapy prior and during HDR brachytherapy were not eligible to participate in this study. Patients received either 600 cGy per HDR fraction or 800 cGy prescribed 1 cm from the source axis to the esophageal planning volume with a remote afterloading HDR unit (Varisource HDR, Nucletron, Varian International, USA). The dose rate ranged anywhere between 26.9 Gy/h and 111.9 Gy/h. Further details on eligibility criterion have been described in a small pilot study published elsewhere (Pinho et al., 2012, 2015).

Table 1
Patient demographics and clinical characteristics.

Patient characteristics	No. of patients ($n=60$)
Mean age in years (SD)	70.3 (10.6)
Gender N(%)	
Male	49 (81.7%)
Female	11 (18.3%)
Type of cancer N(%)	
SCC	4 (6.7%)
EA	50 (83.3%)
Not reported	4 (6.7%)
Cancer staging N(%)	
Stage I	1 (1.7%)
Stage II	9 (15.0%)
Stage III	24 (40.0%)
Stage IV	19 (31.7%)
Not reported	7 (11.7 %)
Tumor location N(%)	
GEJ	13 (21.7%)
Mid/Upper esophagus	10 (16.7%)
Above GEJ	33 (55.0%)
Not reported	4 (6.7 %)
Metastases N(%)	
Yes	34 (56.7%)
No	21 (35.0%)
Not determined	5 (8.3%)
Smoking status N(%)	
Active smoker	10 (16.7%)
History of smoking	26 (43.3%)
Non-smoker	12 (20.0%)
Not reported	12 (20.0%)
Mean dose rate (Gy/h) (SD)	
Fraction 1	62.1 (21.4)

2.2. Blood serum extraction

The blood samples were taken and put into Vacutainers for serum extraction (BD Vacutainers, Fisher Scientific, Ottawa, ON, Canada), centrifuged at 2000 rpm for 10 min, and the serum was frozen and stored at $-70\,^{\circ}C$ before use. The serum was filtered through Nalgene 0.22 μm filters in order to remove all residual cell components of the blood.

2.3. Reporter cell line

An immortal human keratinocyte cell line called HaCaT was kindly received from Dr. Orla Howe from the Dublin Institute of Technology (DIT). These cells were used as a reporter cell line for this clinical study since they have been documented to produce a strong bystander response to irradiated cell conditioned medium (ICCM) or medium from irradiated fish tissue explants (Mothersill and Seymour, 1998; Ryan et al., 2008; Furlong et al., 2013, 2015).

The complete growth medium used for routine maintenance and colony-forming assays was RPMI-1640 with 10% foetal bovine serum (FBS) (Invitrogen, Burlington, ON, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Burlington, ON, Canada), 2 mM L-Glutamine (Gibco, Burlington, ON, Canada), 0.5 μg/ml of hydrocortisone (Sigma-Aldrich, Oakville, ON), and 15 mM of Hepes. All experiments were performed in a class II biosafety cabinet at McMaster University. Routine subculturing was performed on cell stocks reaching 80–100% confluency by using a 1:1 solution of 0.25% trypsin and 1 mM EDTA at 37 °C for 8 min. Cell stocks were grown in 75 cm^2 flasks (T-75) filled with 30 ml of supplemented growth medium. Cell stocks and colony-forming experiments were incubated at 37 °C and 5% carbon dioxide in air.

2.4. Clonogenic reporter assay

Human keratinocyte reporters were seeded at 200 cells per well containing 3 ml of complete growth medium. The RPMI contained a final concentration of 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Burlington, ON, Canada), 2 mM L-Glutamine (Gibco, Burlington, ON, Canada), 0.5 μg/ml of hydrocortisone (Sigma-Aldrich, Oakville, ON, Canada), and 15 mM of Hepes. The FBS typically used in growth medium was substituted with 10% blood serum (0.3 mL per 3 mL of culture medium) collected before and after treatments. Treatments and blood sera extraction were performed at the Juravinski Cancer Centre. All frozen samples were then transported to McMaster University. The serum was added to medium and then transferred to reporter flasks. Reporters were incubated at 37 °C with 5% CO_2 in air for 10–14 days, and then stained and colonies with ≥50 cells were scored using the standard Puck and Marcus assay (Puck and Marcus, 1956).

2.5. Dysphagia scores

Roughly 90% of advanced esophageal cancer cases have an obstruction of their esophageal lumen (Perez and Brady, 2011). This obstruction leads to dysphagia, which is defined as a difficulty or pain when swallowing. There are varying degrees of dysphagia that can be quantified by using the scale developed by Mellow and Pinkas in 1985 (Mellow and Pinkas, 1985). The dysphagia score criteria used when assessing patients at their initial consult and at the end of their brachytherapy regimen are scored 0–4. Patients with no dysphagia symptoms are assigned a score of 0 since they have the ability to eat a normal diet. Patients with moderate dysphagia are assigned a score of 1 since they have the ability to eat some solid foods. A dysphagia score of 2 and 3 were assigned to patients presenting with poor passage of semi-solid foods and severe dysphagia where liquids can only be swallowed, respectively. Patients presenting with the inability to eat solid foods and swallow liquids at all received a score of 4.

2.6. Statistical analysis

Sixty patients consented to giving two blood samples, a baseline sample and another immediately following brachytherapy. All samples were set-up in triplicate. Normality and equal variances conditions required for a parametric statistical analysis were not fulfilled. Significance was determined by using a non-parametric Wilcoxon signed rank tests for paired data (blood samples taken before and after brachytherapy). Significance was also determined by using a Mann–Whitney U test for independent data (healthy controls versus cancer control samples, smoker versus non-smoker and male versus female groups). All p-values less than 0.05 were considered statistically significant.

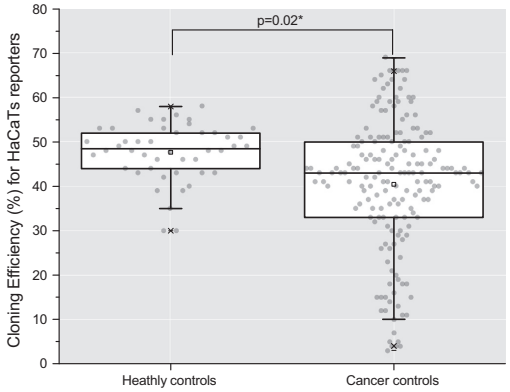


Fig. 1. Illustrated in the graph are the colony-forming ability of HaCaT reporters (%) after exposure to control samples of blood serum taken from cancer patients and cancer-free volunteers. 58 patients and 15 volunteers had three well-plates set-up per sample. All values are mean ± SEM for cancer controls $n=174$ and healthy controls $n=45$. * indicates a p-value less than 0.05.

3. Results

As illustrated in Fig. 1, cancer patients' baseline samples, taken prior to any irradiation exposure, had a statistically significant decrease in cloning efficiency compared to cancer-free control samples. Tumor bearing individuals appear to have cytotoxic soluble factors via the blood not seen in normal healthy individuals ($p < 0.05$). There were no observable treatment effects found in 60 esophageal cancer patients blood serum harvested after a single exposure of brachytherapy, since all reporter cells had an insignificant change between samples taken before and after treatment ($p > 0.05$). When comparing before and after treatment effects for gender, there was no significant influence of irradiation on signal production in either males or females ($p > 0.05$), refer to Table 2.

In contrast to gender, smoking status showed a significant change in the reporter assay, refer to Table 2 and Fig. 2. In this figure, there is a highly significant decrease in cloning efficiency percentage in samples taken from non-smokers after brachytherapy compared to baseline samples ($p < 0.001$). In Table 2, the data show that smokers (both history of smoking and active smokers) have a smaller decrease in cloning efficiency compared to non-smokers. In fact, the blood sera from smokers had an increase in cloning efficiency whereas non-smokers had a further reduction in

Table 2
Smoking status and gender differences influence on the cloning efficiency (%) for keratinocytes exposed to samples irradiated during brachytherapy.

Patient characteristics comparison	Mean cloning efficiency (%) after brachytherapy	No. of patients	p-value
Total males versus total females	38.7 ± 1.4 versus 45.4 ± 2.6	48 versus 10	0.663
Smokers versus non-smokers	40.7 ± 1.8 versus 35.2 ± 2.2	36 versus 12	0.02*
Male smokers versus male non-smokers	39.1 ± 1.8 versus 33.3 ± 3.7	33 versus 7	0.129
Female smokers versus female non-smokers	55.2 ± 6.6 versus 37.7 ± 1.2	3 versus 5	0.128
Active smoker versus non-smokers	43.9 ± 3.1 versus 35.2 ± 2.2	10 versus 12	0.017*
History of smoking versus non-smokers	39.4 ± 2.1 versus 35.2 ± 2.2	26 versus 12	0.051

* p-values less than 0.05 were considered significant.

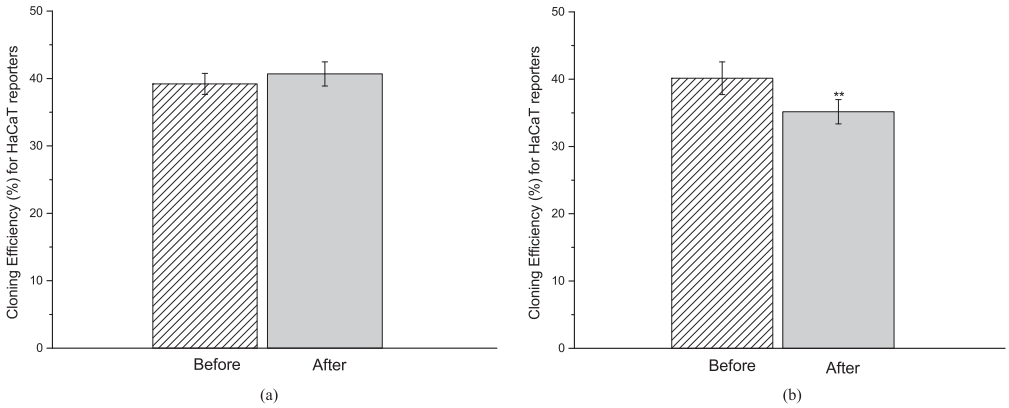


Fig. 2. Blood serum harvested before and after HDR brachytherapy were assessed with respect to the patients smoking status to explore whether any changes in cloning efficiency (%) were dependent on this particular environmental modifier. The cloning efficiency of human keratinocytes treated with blood serum harvested from smokers (a) and non-smokers (b) before and after treatments are illustrated above. All patients had three measurements set-up per sample, $n=82$ for smokers and $n=21$ for non-smokers. All values are mean $\pm$ SEM. ** indicates significance at a p-value <0.001.

cloning efficiency following brachytherapy ($p=0.02$). When analyzing the effect separately for gender, both male and female smokers had a higher cloning efficiency in comparison to male and female non-smokers. However, this trend was not statistically significant ($p > 0.05$). There is a significant difference in cloning efficiency percentage when comparing non-smokers to actively smoking patients undergoing HDR brachytherapy ($p=0.017$), refer to Table 2. Furthermore, patients with a history of smoking versus non-smokers showed a similar but less significant change in cloning efficiency ($p=0.051$), refer to Table 2.

In Fig. 3, this graph clearly highlights that HDR brachytherapy significantly improves the patients' dysphagia scores for all patients ($p < 0.001$) regardless of their smoking status. However, it should be noted that there seems to be an insignificant trend that non-smokers have more of an improvement in dysphagia scores at the end of treatment compared to actively smoking individuals and patients with a history of smoking. Non-smokers showed a 13% greater improvement in dysphagia scores compared to active smokers. Furthermore, these individuals can be ranked in the following order regarding their ability to improve after HDR brachytherapy: non-smokers (1.21 $\pm$0.13) >history of smoking (1.11 $\pm$0.10) >active smokers (1.07 $\pm$0.14). These improvements in dysphagia scores would be equivalent to less obstruction of the esophageal lumen by the tumor mass. The dysphagia score variability is displayed in Fig. 4 at initial consult and at the end of the HDR brachytherapy regimen. The cloning efficiency of human keratinocytes treated with blood serum harvested before and after treatments were assessed with respect to the patients' smoking status and treatment outcome (dysphagia scores) to explore whether smoking limits the effectiveness of HDR brachytherapy, refer to Fig. 4. Fig. 4 shows the variability in dysphagia scores at the start and end of treatment. These graphs show that blood sera harvested from active smokers have higher cloning efficiency and less improvement in dysphagia scores compared to non-smokers. Non-smokers had a dysphagia score of 2 or less after brachytherapy whereas smokers had 3 or less. Furthermore, the arrowhead in Fig. 4(b) highlights a group of active smokers producing increases in cloning efficiency in non-irradiated cells exposed to blood sera post-treatment and also have a poor dysphagia score after treatment.

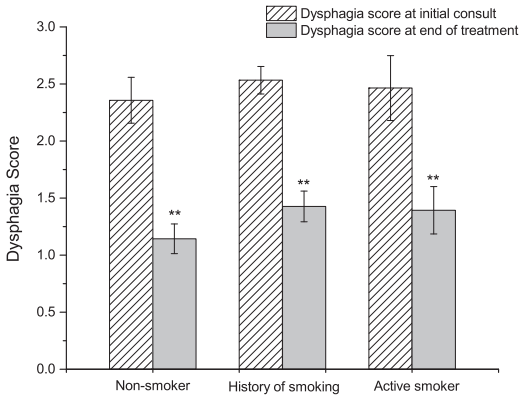
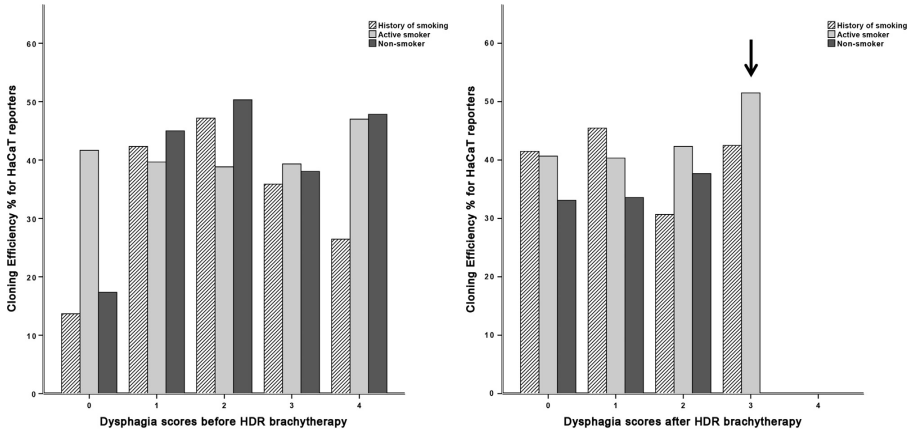


Fig. 3. Illustrated in the graph are the mean dysphagia scores assigned to each patient at their initial consult and at the end of their treatment regimen. These were assessed with respect to the patients' smoking status to explore whether smoking limits the effectiveness of HDR brachytherapy. All patients had three measurements set-up per sample, $n=73$ for history of smoking, $n=27$ for active smokers, and $n=36$ for non-smokers. All values are mean $\pm$SEM. ** indicates significance at a p-value <0.001.

4. Discussion

The primary objective of this ongoing study was to look at the production of radiation induced bystander signals in humans by developing a blood based assay. The study groups used were radiotherapy patients and volunteer controls but the assay may have wider applicability in the human population exposed in accident situations. The reason is that bystander signals are produced as a response to low as well as high dose exposure to radiation and are also modulated by factors such as other stressors or heavy metals (Wahab et al., 2008). In the present paper, the data shows that smoking status can modulate these bystander responses. While we are not sure yet whether producing bystander signals is a beneficial or adverse effect, it could be a useful "yes/no" indicator of radiation exposure or response, in the same way that the presence of clastogenic factors in the blood can persist in the A-bomb survivors and atomic veterans (Mothersill



(a) (b)

Fig. 4. The cloning efficiency of human keratinocytes treated with blood serum harvested before (a) and after (b) HDR brachytherapy were assessed with respect to the patients' smoking status and treatment outcome (dysphagia scores) to explore whether smoking limits the effectiveness of HDR brachytherapy. The arrowhead highlights a group of active smokers with an increase in cloning efficiency in non-irradiated cells and producing a poor dysphagia score after treatment. All patients had three measurements set-up per sample, $n=73$ for history of smoking, $n=27$ for active smokers, and $n=36$ for non-smokers.

and Seymour, 2001). The advantage of a blood based assay is that it is fast and minimally invasive.

As was found in the pilot work (Pinho et al., 2015), gender did not show significant differences in overall response of the reporter cells to the sera from irradiated individuals. There were no significant survival changes observed in the human keratinocytes when exposed to samples harvested from all 60 esophageal cancer patients following irradiation. It is plausible that the brachytherapy treatment was incapable of increasing the bystander signal overall in all patients, as it was already elevated/saturated by some stress-related factor associated with the cancer itself. However, smoking status appears to be an important modifiable environmental factor that may influence the production of bystander signal(s) following irradiation. Blood sera samples taken from smokers had a higher survival in the reporter assay than samples taken from non-smokers immediately after brachytherapy. Modern carcinogenesis theory suggests that cancer is a metabolic disease and involves changes in the cellular microenvironment which permits a cancer to develop (Delitto et al., 2015; Wang et al., 2015; Jin et al., 2014; Jędrzejas et al., 2012; Schuller, 2009; Rubin, 2002). Thus, smoking would be one factor contributing to a supportive microenvironment for the disease to develop and has been shown in these cohort of patients to be unable to produce bystander signals.

Cigarette smoking is one of the main risk factors for esophageal carcinoma (Reid et al., 2010), which is a malignancy that is expected to affect 19,180 new patients in the United States and Canada collectively this year (Canadian Cancer Statistics Advisory, 2015; American Cancer Society, 2015). Very little emphasis has been placed on smoking cessation while patients undergo treatments, since it is believed to have little to no effect on the overall treatment outcome. However, several studies are suggesting that smoking may limit the effectiveness of chemotherapy (Dresler, 2003) and radiation therapy (Chen et al., 2011; Fortin et al., 2009; Browman et al., 1993). Other work has shown that smoking can increase the risk of mortality in esophageal, pharynx, larynx, and lung cancer (Doll et al., 1994). Furthermore, unfavorable survival rates were observed in head and neck cancer patients that smoked during radiation therapy compared to non-smokers (Chen et al., 2011). Smoking is also a known carcinogen affecting other tissues and not only the lung (Siana et al., 1989; Mayfield et al., 1998; Knight-Lozano et al., 2002; Lyng et al., 1995; Brennan et al., 1995; Sopori, 2002; Chao et al., 2000) although the mechanisms by which the systemic effects are produced is not clear.

Interestingly, urothelium biopsy samples harvested from smokers has been previously shown to influence bystander signal production in *ex vivo* experiments using tissue culture to study bystander effects (Mothersill et al., 2001). Bystander cells exposed to tissue samples harvested from smokers had a smaller reduction in cloning efficiency following irradiation than samples taken from non-smokers. Similar findings have been reported in this current study in esophageal cancer patients. Non-smokers had a statistically significant reduction in cloning efficiency compared to smokers following HDR brachytherapy. Moreover, other researchers have reported smoking to induce tumor radioresistance (Nordsmark et al., 2005; Overgaard, 2011), which is a common characteristic of esophageal cancer. One possible explanation for smoking induced tumor radioresistance would be that smoking has been shown to facilitate the formation of carboxyhemoglobin, which increases hypoxia levels (Lawther and Commins, 1970). Essentially the binding of carbon monoxide to hemoglobin produces carboxyhemoglobin, which ultimately has a substantially higher affinity than oxygen to hemoglobin. Smokers were reported to have roughly a 20% increase in the carboxyhemoglobin levels in comparison to non-smokers (Siemann et al., 1978). A prospective study by Hoff et al. investigated the ability of hemoglobin levels to predict hypoxia-induced radioresistance in head and neck cancer patients (Hoff et al., 2012). The authors found elevated levels of carboxyhemoglobin in head and neck cancer patients, which effectively diminished the oxygen supply in tumors. Other researchers found a tobacco-derived carcinogen, nitrosamines, with the ability to stimulate significant growth in urothelial cultures when present (Lyng et al., 1995).

Other researchers investigating head and neck cancer patients' undergoing radiotherapy have reported smoking-induced radioresistance from hypoxia by inhalation of carbon monoxide, which

has a substantially higher affinity to hemoglobin than oxygen (Hoff et al., 2012). It is possible that samples harvested from smokers produce pro-growth signals post-irradiation because cigarette smoking can alter the normal damage response in cells (Mothersill and Seymour, 2006). Consequentially smoking leads to less oxygen being released into the tissues, including tumor masses or perhaps nearby cells. The more hypoxic a tumor or normal cell is, the less sensitive it will be to radiation. Larger bystander effects have been reported in radiosensitive cell lines and this seems to become much more apparent when considering the p53 status of the cell line (Ryan et al., 2008, 2009). Higher p53 mutations have been reported in normal oral mucosa cells taken from smokers compared to non-smokers (Colucci et al., 1997). Because a significant response was observed while using a non-irradiated keratinocyte cell model, future work should be tailored to determine whether there is a link between smoking and radiation response in a wide range of human exposure situations including environmental exposures and planned low dose medical procedures. Usually radiation resistance is defined as less cell death following a given dose but when thinking about carcinogenesis less death could mean more damaged cells available to develop pre-cancer mutations.

In summary, this study shows that in a study of human patients exposed to radiation, smoking status is a key determinant of outcome. The suggested mechanism is that cigarette smoking interferes with damage response pathways mediated by radiation induced signaling pathways such as the bystander signaling mechanism. While the group used in this study was cancer patients, it is likely that the mechanisms hold for non-cancer patients as suggested by past studies by this group and others. As such, smokers may need to be regarded as a separate group in risk assessments in radiation protection. These findings highlight the importance of studying the interactions of multiple stressors including environmental modifiers combined with radiation.

Acknowledgments

This work was funded by the Natural Sciences and Engineering Research Council of Canada (Grant ID # RGPIN293153-12). We acknowledge the continued assistance of the Hamilton Health Sciences JCC brachytherapy staff during sample collection. We also thank the JCC Clinical trials laboratory for centrifuge usage and freezer storage. The authors thank Dr. Orla Howe from the Dublin Institute of Technology (DIT) for the HaCaT cells.

References

American Cancer Society, 2015. Cancer Facts & Figures 2015.
Birgisson, H., Påhlman, L., Gunnarsson, U., Glimelius, B., 2005. Occurrence of second cancers in patients treated with radiotherapy for rectal cancer. J. Clin. Oncol. 23 (25), 6126–6131.
Boyd, M., Sorensen, A., McCluskey, A.G., Mairs, R.J., 2008. Radiation quality-dependent bystander effects elicited by targeted radionuclides. J. Pharm. Pharmacol. 60.
Brennan, J.A., Boyle, J.O., Koch, W.M., Goodman, S.N., Hruban, R.H., Eby, Y.J., Couch, M.J., Forastiere, A.A., Sidransky, D., 1995. Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. New Engl. J. Med. 332 (11), 712–717.
Browman, G.P., Wong, G., Hodson, I., Sathya, J., Russell, R., McAlpine, L., Skingley, P., Levine, M.N., 1993. Influence of cigarette smoking on the efficacy of radiation therapy in head and neck cancer. New Engl. J. Med. 328 (3), 159–163.
Butterworth, K.T., McMahon, S.J., Hounsell, A.R., O'Sullivan, J.M., Prise, K.M., 2013. Bystander signalling: exploring clinical relevance through new approaches and new models. Clin. Oncol. 25.
Canadian Cancer Statistics Advisory, 2015. Canadian Cancer Statistics.
Chao, A., Thun, M.J., Jacobs, E.J., Henley, S.J., Rodriguez, C., Calle, E.E., 2000. Cigarette smoking and colorectal cancer mortality in the cancer prevention study ii. J. Natl. Cancer Inst. 92 (23), 1888–1896.
Chen, A.M., Chen, L.M., Vaughan, A., Sreeraman, R., Farwell, D.G., Luu, Q., Lau, D.H., Stuart, K., Purdy, J.A., Vijayakumar, S., 2011. Tobacco smoking during radiation therapy for head-and-neck cancer is associated with unfavorable outcome. Int. J. Radiat. Oncol. Biol. Phys. 79 (2), 414–419.
Colucci, S., El-Gehani, R., Flint, S., Mothersill, C., 1997. p53 mutations and protein expression in primary cultures of normal oral mucosa in smokers and non-smokers. Oral Oncol. 33 (4), 240–246.
Delitto, D., Zhang, D., Han, S., Black, B.S., Knowlton, A.E., Vlada, A.C., Sarosi, G.A., Behrns, K.E., Thomas, R.M., Lu, X., et al., 2015. Nicotine reduces survival via augmentation of paracrine hgf-met signaling in the pancreatic cancer microenvironment. Clin. Cancer Res., clincanres–1256.
Doll, R., Peto, R., Wheatley, K., Gray, R., Sutherland, I., 1994. Mortality in relation to smoking: 40 years' observations on male British doctors. BMJ 309 (6959), 901–911.
Dresler, C.M., 2003. Is it more important to quit smoking than which chemotherapy is used? Lung Cancer 39 (2), 119–124.
Fortin, A., Wang, C.S., Vigneault, É., 2009. Influence of smoking and alcohol drinking behaviors on treatment outcomes of patients with squamous cell carcinomas of the head and neck. Int. J. Radiat. Oncol. Biol. Phys. 74 (4), 1062–1069.
Furlong, H., Mothersill, C., Lyng, F.M., Howe, O., 2013. Apoptosis is signalled early by low doses of ionising radiation in a radiation-induced bystander effect. Mutat. Res./Fundam. Mol. Mech. Mutagen. 741, 35–43.
Furlong, H., Smith, R., Wang, J., Seymour, C., Mothersill, C., Howe, O., 2015. Identification of key proteins in human epithelial cells responding to bystander signals from irradiated trout skin. Dose-Response 13 (3) 1559325815597669.
Hoff, C.M., Grau, C., Overgaard, J., 2012. Effect of smoking on oxygen delivery and outcome in patients treated with radiotherapy for head and neck squamous cell carcinoma—a prospective study. Radiother. Oncol. 103 (1), 38–44.
Jędrzejas, M., Skowron, K., Czekaj, P., 2012. Stem cell niches exposed to tobacco smoke. Przegl. Lek. 69 (10), 1063–1073.
Jin, S., Deng, Y., Hao, J.-W., Li, Y., Liu, B., Yu, Y., Shi, F.-D., Zhou, Q.-H., 2014. Nk cell phenotypic modulation in lung cancer environment. PLOS One 9.
Knight-Lozano, C.A., Young, C.G., Burow, D.L., Hu, Z.Y., Uyeminami, D., Pinkerton, K. E., Ischiropoulos, H., Ballinger, S.W., 2002. Cigarette smoke exposure and hypercholesterolemia increase mitochondrial damage in cardiovascular tissues. Circulation 105 (7), 849–854.
Koturbash, Igor, Kutanzi, Kristy, Hendrickson, Karl, Rodriguez-Juarez, Rocio, Kogosov, Dmitry, Kovalchuk, Olga, 2008. Radiation-induced bystander effects in vivo are sex specific. Mutat. Res./Fundam. Mol. Mech. Mutagen. 642.
Lawther, P., Commins, B., 1970. Cigarette smoking and exposure to carbon monoxide. Ann. N.Y. Acad. Sci. 174 (1), 135–147.
Lorimore, S.A., Rastogi, S., Mukherjee, D., Coates, P.J., Wright, E.G., 2013. The influence of p53 functions on radiation-induced inflammatory bystander-type signaling in murine bone marrow. Radiat. Res. 179 (4), 406–415.
Lyng, F.M., de Feijter-Rupp, H.L., Hayashi, T., O'Malley, K., Murphy, D.M., Cottell, D.C., Trosko, J.E., Seymour, C.B., Mothersill, C., 1995. Effect of a tobacco-related nitrosamine on intercellular communication in human urothelial cells: a possible factor in smoking-related bladder carcinogenesis. Oncol. Res. 8 (9), 371–378.
Lyng, F.M., Seymour, C.B., Mothersill, C., 2000. Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. Br. J. Cancer 83.
Mayfield, L., Söderholm, G., Hallström, H., Kullendorff, B., Edwardsson, S., Bratthall, G., Brägger, U., Attström, R., 1998. Guided tissue regeneration for the treatment of intraosseous defects using a biabsorbable membrane a controlled clinical study. J. Clin. Periodontol. 25 (7), 585–595.
Mellow, M.H., Pinkas, H., 1985. Endoscopic laser therapy for malignancies affecting the esophagus and gastroesophageal junction: analysis of technical and functional efficacy. Arch. Intern. Med. 145 (8), 1443–1446.
Morton, L.M., Gilbert, E.S., Stovall, M., van Leeuwen, F.E., Dores, G.M., Lynch, C.F., Hall, P., Smith, S.A., Weathers, R.E., Storm, H.H., et al., 2014. Risk of esophageal cancer following radiotherapy for hodgkin lymphoma. Haematologica 99 (10), e193–e196.
Mothersill, C., Seymour, C., 1997. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. Int. J. Radiat. Biol. 71 (4), 421–427.
Mothersill, C., Seymour, C.B., 1998. Cell–cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. Radiat. Res. 149.
Mothersill, C., Seymour, C., 2001. Radiation-induced bystander effects: past history and future directions. Radiat. Res. 155 (6), 759–767.
Mothersill, C., Seymour, C., 2006. Radiation-induced bystander and other non-targeted effects: novel intervention points in cancer therapy?. Curr. Cancer Drug Targets 6 (5), 447–454.
Mothersill, Carmel, Rea, David, Wright, Eric G., Lorimore, Sally A., Murphy, Dennis, Seymour, Colin B., O'Malley, Kiaran, 2001. Individual variation in the production of a 'bystander signal' following irradiation of primary cultures of normal

human urothelium. Carcinogenesis 22.
Mothersill, C., Lyng, F., Seymour, C., Maguire, P., Lorimore, S., Wright, E., 2005. Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo. Radiat. Res. 163 (4), 391–399.
Nordsmark, M., Bentzen, S.M., Rudat, V., Brizel, D., Lartigau, E., Stadler, P., Becker, A., Adam, M., Molls, M., Dunst, J., et al., 2005. Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. Radiother. Oncol. 77 (1), 18–24.
Overgaard, J., 2011. Hypoxic modification of radiotherapy in squamous cell carcinoma of the head and neck—a systematic review and meta-analysis. Radiother. Oncol. 100 (1), 22–32.
Perez, C.A., Brady, L.W., 2011. Radiation Oncology: Management Decisions. Lippincott Williams & Wilkins, Philadelphia, PA.
Pinho, C., Wong, R., Sur, R.K., Hayward, J.E., Farrell, T.J., Seymour, C., Mothersill, C., 2012. The involvement of serum serotonin levels producing radiation-induced bystander effects for an in vivo assay with fractionated high dose-rate HDR brachytherapy. Int. J. Radiat. Biol. 88 (10), 791–797. http://dx.doi.org/10.3109/09553002.2012.715794.
Pinho, C., Timotin, E., Wong, R., Sur, R.K., Hayward, J.E., Farrell, T.J., Seymour, C., Mothersill, C., 2015. Assessing patient characteristics and radiation-induced non-targeted effects in vivo for high dose-rate (HDR) brachytherapy. Int. J. Radiat. Biol, 1–9, Taylor & Francis, http://dx.doi.org/10.3109/09553002.2015.1068458.
Prise, K.M., O'Sullivan, J.M., 2009. Radiation-induced bystander signalling in cancer therapy. Nat. Rev. Cancer 9.
Prise, K.M., Belyakov, O.V., Folkard, M., Michael, B.D., 1998. Studies of bystander effects in human fibroblasts using a charged particle microbeam. Int. J. Radiat. Biol. 74.
Puck, T.T., Marcus, P.I., 1956. Action of X-rays on mammalian cells. J. Exp. Med. 103 (5), 653–666.
Randal, J., 2000. Canada faces shortage of radiation therapists. J. Natl. Cancer Inst. 92.
Reid, B.J., Li, X., Galipeau, P.C., Vaughan, T.L., 2010. Barrett's oesophagus and oesophageal adenocarcinoma: time for a new synthesis. Nat. Rev. Cancer 10 (2), 87–101.
Rubin, H., 2002. Selective clonal expansion and microenvironmental permissiveness in tobacco carcinogenesis. Oncogene 21 (48), 7392–7411.
Ryan, L.A., Smith, R.W., Seymour, C.B., Mothersill, C.E., 2008. Dilution of irradiated cell conditioned medium and the bystander effect. Radiat. Res. 169 (2), 188–196. http://dx.doi.org/10.1667/RR1141.1.
Ryan, L.A., Seymour, C.B., Joiner, M.C., Mothersill, C.E., 2009. Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. Int. J. Radiat. Biol. 85 (1), 87–95. http://dx.doi.org/10.1080/09553000802635062.
Schuller, H.M., 2009. Is cancer triggered by altered signalling of nicotinic acetylcholine receptors? Nat. Rev. Cancer 9 (3), 195–205.
Seymour, C.B., Mothersill, C., 2000. Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve. Radiat. Res. 153 (5 Pt 1), 508–511.
Siana, J.E., Rex, S., Gottrup, F., 1989. The effect of cigarette smoking on wound healing. Scand. J. Plast. Reconstr. Surg. 23 (3), 207–209.
Siemann, D.W., Hill, R.P., Bush, R.S., 1978. Smoking: The influence of carboxyhemoglobin (hbco) on tumor oxygenation and response to radiation. Int. J. Radiat. Oncol. Biol. Phys. 4 (7), 657–662.
Sopori, M., 2002. Effects of cigarette smoke on the immune system. Nat. Rev. Immunol. 2 (5), 372–377.
Tubiana, M., 2009. Can we reduce the incidence of second primary malignancies occurring after radiotherapy? a critical review. Radiother. Oncol. 91 (1), 4–15.
Wahab, M., Nickless, E., Najar-MKacher, R., Parmentier, C., Podd, J., Rowland, R., 2008. Elevated chromosome translocation frequencies in New Zealand nuclear test veterans. Cytogenet. Gen. Res. 121 (2), 79–87.
Wang, C., Guan, S., Chen, X., Liu, B., Liu, F., Han, L., Nesa, E.U., Song, Q., Bao, C., Wang, X., et al., 2015. Clinical potential of mir-3651 as a novel prognostic biomarker for esophageal squamous cell cancer. Biochem. Biophys. Res. Commun. 465 (1), 30–34.

Part II: In vitro Assays

Chapter 5

Low-dose non-targeted radiation effects in human esophageal adenocarcinoma cell lines

Christine Hanu, Raimond Wong, Ranjan K. Sur, Joseph E.Hayward Colin Seymour, and Carmel Mothersill

The author and Dr. Mothersill designed these *in vitro* bystander medium-transfer experiments. The first author completed the cell preparation, performed cell irradiations, bystander medium transfer experiments, data collection and statistical analysis. The first author wrote the manuscript, which was edited by Dr. Mothersill, Dr. Seymour, Dr. Wong, and Dr. Hayward. This manuscript has been accepted subject to revision.

5.1 Abstract

Purpose: In this paper, we investigate non-targeted radiation effects in esophageal adenocarcinoma cell lines (OE19 and OE33) using human keratinocyte and colorectal cancer cell reporters following γ-ray exposure.

Materials & Methods: Both clonogenic assays and ratiometric calcium endpoints were used to check for the occurrence of bystander signals in reporter cells.

Results: We report data suggesting that γ-irradiation increases cell killing over the expected linear quadratic (LQ) model levels in the OE19 cell line exposed to doses below 1 Gy i.e. which may be suggestive to be a low HRS response to direct irradiation. Both EAC cell lines (OE19 and OE33) have the ability to produce bystander signals when irradiated cell conditioned medium (ICCM) is placed onto human keratinocyte reporters, but do not seem to be capable of responding to bystander signals when placed on their autologous reporters. Further work with human keratinocyte reporter models showed statistically significant intracellular calcium fluxes following exposure of the reporters to ICCM harvested from both OE33 and OE19 cells exposed to 0.5 Gy. Since bystander effects were also found at the 0.5Gy ICCM region, these data support other recent reports that these two phenomena can co-exist in the low dose region.

Conclusion: These experiments suggest that the OE19 and OE33 cell lines produce bystander signals in human keratinocyte reporter cells. However, the radiosensitivity of the EAC cell lines cannot be enhanced by the bystander response since both cell lines could not respond to bystander signals.

5.2 Introduction

Esophageal cancer is quite rare in comparison to other epithelial tumours. Only 1% of cancers originate from the esophagus throughout the United States and Canada [1, 2]. Although this disease may be rare in comparison to other cancers, the tumours are extremely aggressive and the adenocarcinomas are particularly refractive to treatment resulting in a very poor prognosis. Roughly 90% of esophageal cancer cases will result in death amongst US and Canadian adults [1, 2]. Such poor outcomes are mainly attributed to the disease being detected at advanced stages where curative

treatments are no longer an option but lack of effective treatment protocols even in early stage cancers is also a problem. The tumours tend to be very radioresistant [3, 4] meaning the use of radiotherapy even for mass reduction is limited. Optimizing treatment through application of novel mechanistic approaches is therefore attractive and in this paper we explore the possible relevance of two mechanisms - the radiation-induced bystander effect (RIBE) and low dose HRS which are associated with low dose response to ionizing radiation. While these are both associated with low dose exposure, they also are relevant as contributors to total dose response after higher doses [5–8].

To date, there are no other HRS or RIBE studies exploring responses in esophageal cancer cell lines. Our research group has also been the only one to investigate bystander effects in samples taken from esophageal cancer patients while undergoing HDR brachytherapy regimens [9, 10]. However many other studies have suggested that RIBE and HRS vary in normal and tumour cell lines [11–13] and in normal tissues from the same patient [14]. This means that understanding their mechanistic basis could open up new therapeutic approaches. For this reason, RIBE and other non-targeted radiation effects (NTE) have actively been studied by using cell [8, 12, 15–17], animal [18, 19] and human models [9, 10, 14, 20]. Several attempts have been made to isolate the bystander factor(s) responsible for increased cell death [15–17], since these may provide new targets for radiotherapy [20–22]. However there is still no clear evidence for any particular candidate "bystander factor" suggesting there may be multiple mechanisms.

Although RIBEs are cell line specific, the p53 pathway has been suggested to play a role in the transduction of bystander effects [23, 24]. Medium transfer mix/match protocols were used to study bystander effects in HCT 116 p53 wildtype and null and mutant cells. The studies showed that cells expressing both functional and non-functional p53 can generate bystander signals. However, in these studies functional p53 was needed for cells to respond to the signals [23] and the type of mutation was important [25]. In this present study, the two esophageal adenocarcinoma cell lines were tested which have mutated p53 whereas the human colon cancer (HCT 116 p53 wildtype) reporter cell line possesses functional p53. Based upon previous studies on bystander effects and p53 status [23, 24], it is hypothesized that both esophageal adenocarcinoma cell lines will be capable of producing bystander signals, however, these cells will not be able to respond due to their mutated p53 status.

Another very important phenomenon associated with non-targeted and low dose radiobiology is low-dose HRS/IRR [13, 26–29]. One of the earliest studies reporting HRS/IRR responses was published by Joiner and Denekamp, in 1986. These responses have been found in a number of tumour cell lines including glioblastoma, human prostate and colorectal carcinoma cell lines [13, 26–29] . This phenomenon is characterized by an abrupt shift from a sensitive low dose response to a more resistant one at higher doses [11, 28]. In a review by Martin et al. 2013, it was suggested that the increase cell killing at lower doses are a result of defective DNA damage response pathways but bystander signaling has also been implicated elsewhere [7, 32, 33]. In the present study, HRS/IRR responses were assessed along with bystander response in these two esophageal adenocarcinoma cell lines.

5.3 Methods and Materials

5.3.1 Cell lines

HCT 116 p53 +/+ cells were originally obtained as a gift from Dr. Robert Bristow from Princess Margaret Hospital, Toronto, ON, Canada. These cells are derived from a human colon cancer [34] and have been documented to have functional p53 [23, 34]. These cells have been proven to generate and respond to bystander signals [23, 24]. The HaCaT cell line was originally obtained as a gift from Dr. Orla Howe from the Dublin Institute of Technology (DIT). These cells are derived from normal human keratinocytes and have been documented to have point mutations in the p53 protein (His179Tyr, Asp281Gly, Arg282Trp) on both of its alleles [35]. They also have been shown to have a very stable bystander response to ICCM from donor cells or irradiated fish tissue explants [12, 16, 17, 36, 37]. The OE33 cells were obtained as a gift from Dr. Niamh Lynam-lennon from Trinity College Dublin, Ireland. These cells are derived from a primary tumour clinically diagnosed as a stage II esophageal adenocarcinoma. They have a missense mutation in TP53 where guanine has been substituted for adenine on codon 135 [38]. The OE19 cells were purchased from Sigma-Aldrich (Oakville, ON, Canada). These cells are derived from a primary tumour, clinically diagnosed as

a stage III esophageal adenocarcinoma. They have been reported to be mutant TP53 [38]. All cell lines were screened for mycoplasma and cleared prior to commencing experiments by using the Plasmotest^{TM} Mycoplasma detection kit (Catalog # rep-pt1), which was purchased from Cedarlane (Burlington, ON, Canada).

5.3.2 Cell culture

Cultures were performed in a biosafety level 2 (BSL2) laboratory within a laminar flow cabinet. All cell lines were grown in RPMI-1640 culture medium supplemented with 10% FBS (Invitrogen, Burlington, ON, Canada), 5 ml of 200 mM L-Glutamine (Gibco, Burlington, ON), and 25mM Hepes for pH regulation. Culture medium for experiments only were supplemented with 5 ml of 10,000 units of penicillin and $10,000$ µg/ml of streptomycin (Gibco, Burlington, ON). All cell stocks were maintained in T-75 flasks with 30 mL of growth medium. They were all routinely passaged until reaching a confluency between 80-100%. All cells were incubated at 37°C and 5% carbon dioxide in air.

5.3.3 Irradiations

All cells were irradiated in T-25 flasks by using a Cesium-137 (Cs-137) source (McMaster University, Hamilton, ON). A flask-to-source distance of 31 cm and dose-rate of 0.226 Gy/min were used during cell irradiations. All flasks were returned to the incubator immediately following irradiations.

5.3.4 Survival Curve Models

All flasks were set up at a density of 500 and 1000 cells per flask for OE33 and OE19, respectively. Flasks were irradiated at 0.05 Gy, 0.1Gy, 0.5Gy, 1Gy, 2Gy, and 5Gy 5 to 6 h after seeding cells. Following irradiations, all flasks were grown in an incubator at 37°C with 5% CO_2 in air for 8-14 days for the OE33 and 16-24 days for the OE19 cell lines. Once appropriate colonies had formed, the cells were stained with 20% carbol fuchsin (VWR, Bridgeport, NJ, USA) and colonies

with 50 cells or more were scored [39]. The data was used to generate a cell survival curve for both EAC cell lines with the survival fraction plotted as function of dose (Gy). All flasks were set-up in triplicate and all data points are mean survival fraction ± SEM for n=9. Both esophageal adenocarcinoma (EAC) cell lines were fitted to the linear quadratic (LQ) and induced repair (IR) survival models [27].

The LQ model is outline below:

$$S = e^{(-\alpha D - \beta D^2)} \tag{5.1}$$

Where the α represents the slope of the linear component of the model. While β represents the slope of the quadratic portion of the model.

The IR equation is outline below:

ï£ij

$$S = e^{(-\alpha r(1+\frac{\alpha s}{\alpha r}-1)e^{-D/D_c)}d-\beta D^2)} \tag{5.2}$$

Where the αr represents the slope of the resistant linear portion of the model. While αs represents the slope of the sensitive linear portion of the model.

5.3.5 Clonogenic Assay

All ICCM donor flasks were set up at a density of 300,000 cells per flask. The donor seeding density was chosen based upon a previous study [16] proving this cell concentration to be effective at producing a strong bystander signal. The seeding density for reporter cells varied for each cell line. Both OE33 and HCT 116 p53 +/+ reporters were set up using a seeding density of 500 cells per flask. OE19 cells required a higher density of 2000 cells per flask because of their low plating efficiency. HaCaTs were seeded at 300 cells per flask. Donors were irradiated at 0.5 Gy and 5 Gy 6 h after plating (colon cancer cell lines) and 15 h after plating (esophageal cells). Esophageal cell lines required a longer time for cells to adhere to the bottom of the flasks. Controls were unirradiated cells, irradiated medium without cells, and sham-irradiated cells. Following irradiations,

donors were returned to the incubator for 1 hour. Afterwards, irradiated cell conditioned medium (ICCM) or control cell conditioned medium (CCCM) was filtered with a 0.22 µm Naglene filter (VWR, Bridgeport, NJ, USA) and transferred from donor flasks to reporter flasks. Following medium transfer, reporter flasks were grown in an incubator at 37°C with 5% CO_2 in air until viable colonies with 50 cells or more were formed. The cells were stained with 20% carbol fuchsin (VWR, Bridgeport, NJ, USA) and colonies were scored.

5.3.6 Ratiometric calcium measurements

HaCaT reporters were used for ratiometric calcium measurements. Live cell calcium imaging was performed for the OE19 and OE33 CCCM, 0.5Gy ICCM, and 5Gy ICCM. HaCaT cells were seeded in glass bottom dishes coated with poly-d-lysine(MatTek corporation, Ashland, MA) at a density of approximately 100,000 cells per dish. The cells were incubated at 37°C and 5% CO_2 for 24 h before calcium measurements. Cells were washed three times with HankâĂŹs Balanced salt solution (HBSS) calcium buffer (invitrogen,ON) supplemented with 25mM of Hepes (invitrogen,ON). Cells were incubated with 1000 µl of 8.4 µM Fura-2 AM (Sigma-Aldrich, Oakville, ON) for 50 minutes at 37°C and 20 minutes at room temperature to aid in the de-esterification process [40, 41]. Prior to imaging, cells were rinsed three times and then 300 µl of imaging buffer was added to the dish. Equipment used for calcium measurements are Photon Technology International (PTI) lamp power supply, xenon arc lamp and random access monochromator with a liquid light guide that is coupled to an Olympus 1x81 microscope and computer. UV excitation wavelengths, 380 and 340nm, are used to excite the Fura-2 loaded into the cells. Images were taken for 13 minutes after adding 100 µl of either CCCM or ICCM from esophageal adenocarcinoma donors to reporter cells after a stable baseline was reached at roughly 80 seconds.

5.3.7 Statistical Analysis

All experiments with HaCaT, HCT 116 p53 +/+, and EAC reporters were analyzed with a one-way ANOVA. A bonferroni *post hoc* correction was used to determine significant differences

amongst the groups. Data are mean ± standard error. All p-values less than 0.05 were considered statistically significant.

5.4 Results

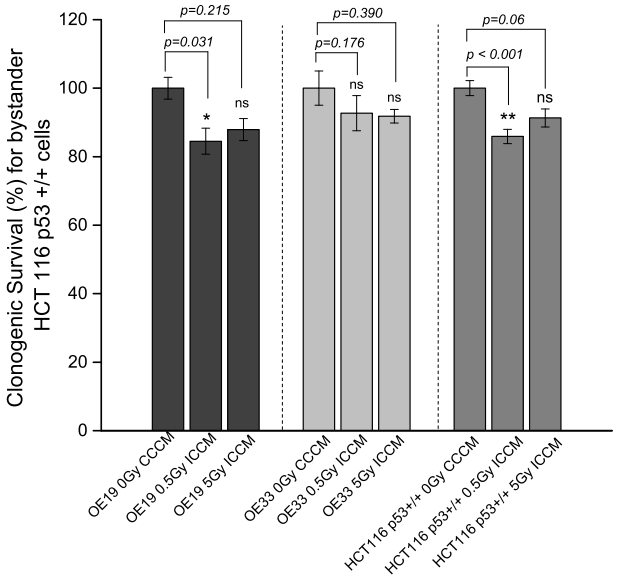


Figure 5.1. Cell Survival for HCT 116 p53 +/+ cells exposed to ICCM from OE19, OE33, and HCT 116 p53 +/+. Reporter cells were exposed to CCCM, 0.5 Gy ICCM, or 5 Gy ICCM. Separate ANOVAs were performed for each donor cell placing ICCM onto HCT 116p53+/+ reporters. * and ** indicates a significance change for cells exposed to ICCM compared to CCCM represented by p< 0.05 and p< 0.001, respectively. Data are mean ±SEM for *n=9*.

Figure 5.1 show a statistically significant reduction in HCT 116 p53 +/+ cell survival percentage when exposed to autologous 0.5 Gy ICCM ($p < 0.001$). The OE19 donors also produce a significant reduction in cell survival when 0.5 Gy ICCM was placed onto this reporter cell line (p=0.031). However the OE33 donor ICCM does not increase cell killing ($p > 0.05$).

Although there were no significant decreases in survival observed with the OE33 donors 0.5 Gy ICCM onto HCT 116 p53 +/+ reporters, there was a significant decrease in cell survival when the HaCaT reporter cells were used (p=0.005), refer to Figure 5.2. As illustrated in Figure 5.3, both esophageal adenocarcinoma cell lines were observed to have insignificant reductions in cell survival when transferring autologous bystander medium onto unirradiated cells ($p > 0.05$). To confirm whether there were bystander signals being produced in OE33 and OE19 cells, intracellular calcium measurements were done. Figure 5.4 illustrates a significant influx of calcium ions after exposure to autologous 0.5 Gy ICCM onto unirradiated human keratinocytes (p<0.001). Figure 5.5 shows a statistically significant increase of intracellular calcium in HaCaT reporters when exposed to bystander medium from OE33 and OE19 at the 0.5 Gy level only (p=0.042 and p=0.025, respectively). Similarly the OE33 and OE19 clonogenic assays revealed a statistically significant increase in cell death following exposure to bystander medium at 0.5 Gy level ($p < 0.05$),

as shown in Figure 5.2. Bystander cells exposed to 5 Gy ICCM had an insignificant decrease in cell survival ($p > 0.05$). Furthermore, there were no significant transients increases in intracellular calcium in bystander human keratinocytes receiving 5Gy ICCM from both the OE19 and OE33 donor cells ($p > 0.05$).

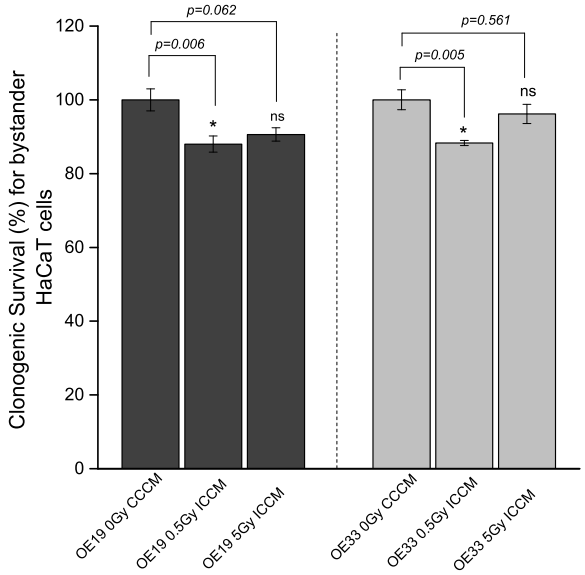


Figure 5.2. Cell survival for HaCaT cells exposed to irradiated ICCM from OE19 and OE33 donors. Reporter cells were exposed to CCCM, 0.5 Gy ICCM, and 5 Gy ICCM. Separate ANOVAs were performed for each donor cell placing ICCM onto HaCaT reporters. * and ** indicates a significance change for cells exposed to ICCM compared to CCCM represented by $p < 0.05$ and $p < 0.001$, respectively. Data are mean ±SEM for *n=9*.

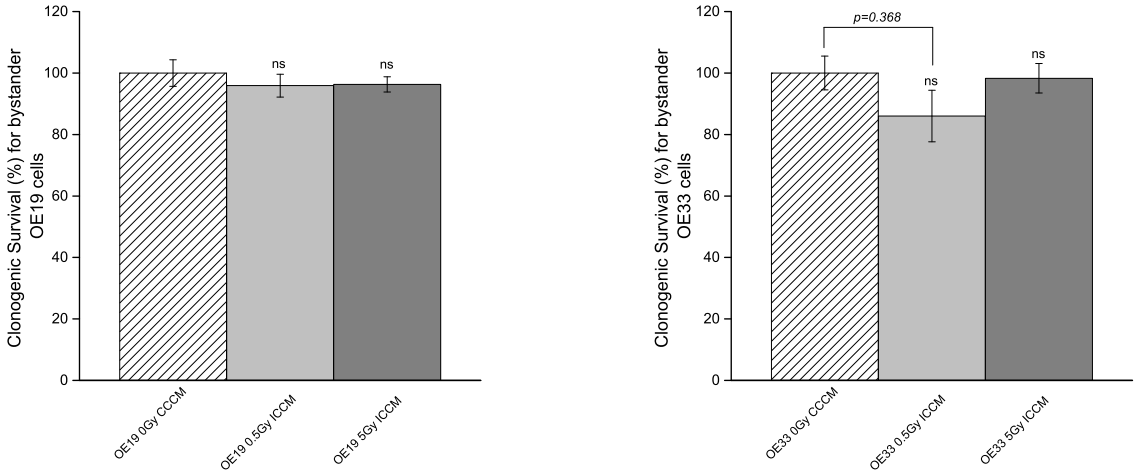


Figure 5.3. Cell survival for OE19 (a) and OE33 (b) reporters exposed to ICCM from OE19 and OE33 donors, respectively. Reporter cells were exposed CCCM, 0.5 Gy ICCM, and 5 Gy ICCM. Data are mean ± SEM for n=9.

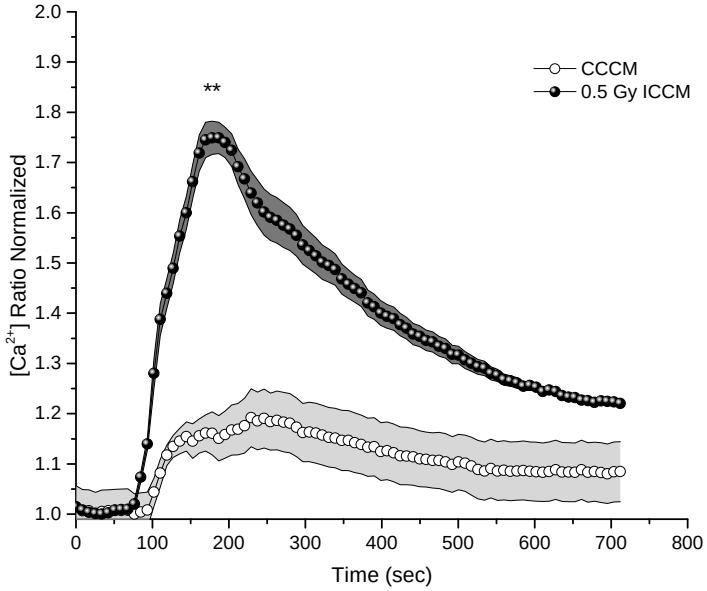


Figure 5.4. Intracellular calcium flux in HaCaT bystander cells exposed to 0.5Gy ICCM from autologous donors. 0.5Gy ICCM calcium ratios are significantly different from CCCM ($p < 0.05$). ** indicates a significance change for cells exposed to ICCM compared to CCCM represented by $p < 0.001$. Data are mean ±SEM for *n=10* cells. Lower and upper bounds are outline by the shaded error regions.

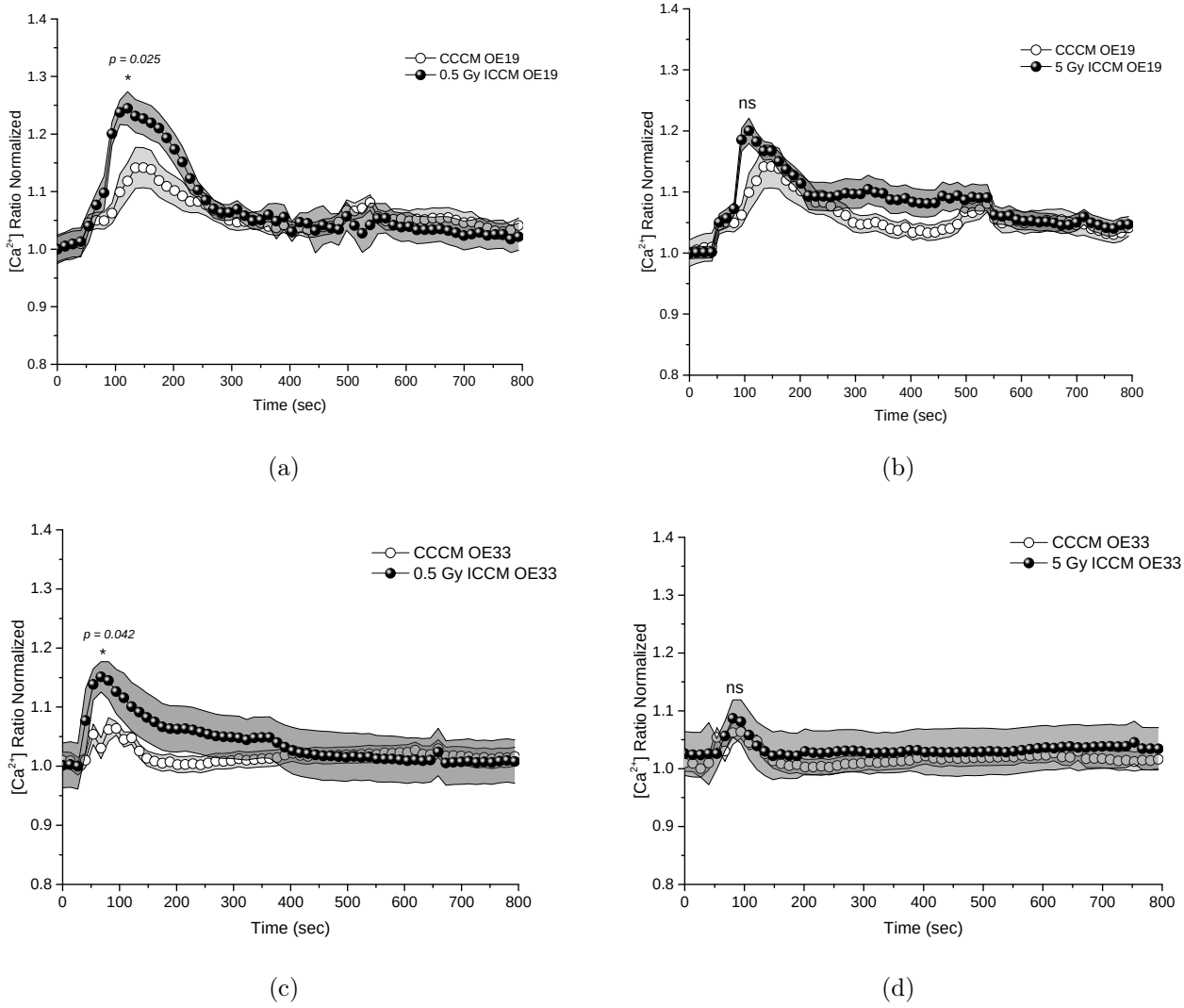
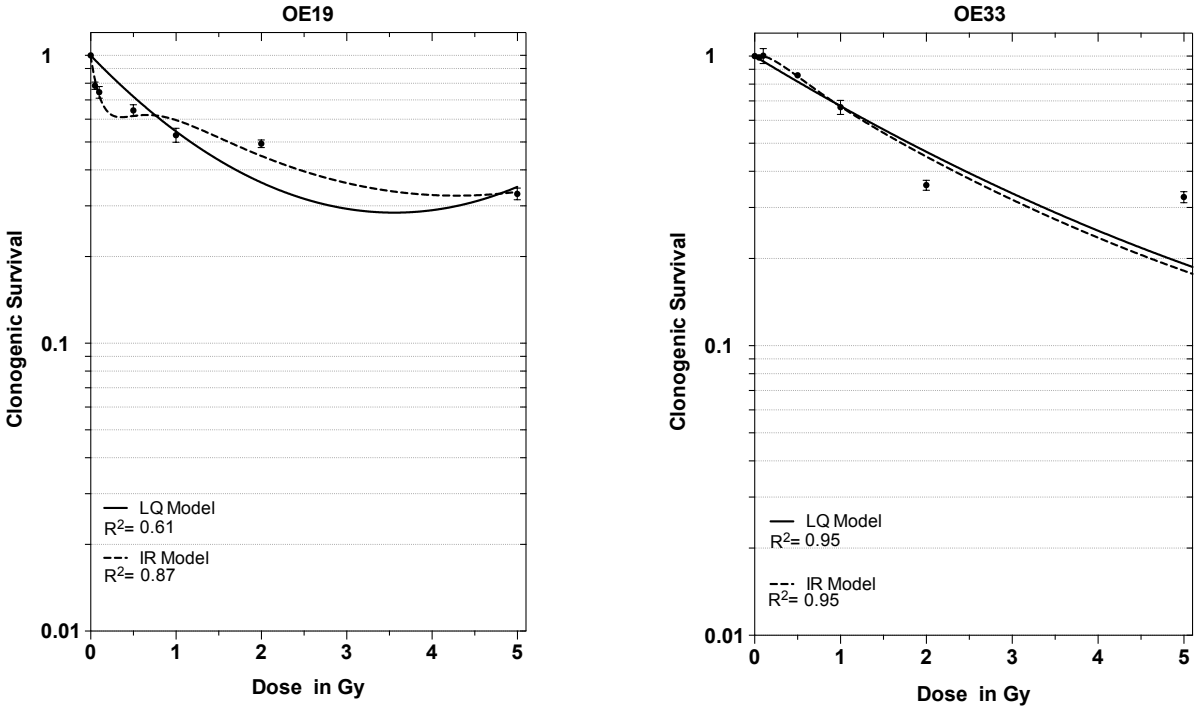


Figure 5.5. Intracellular calcium measurements used to monitor bystander signal generations in esophageal adenocarcinoma cell lines. HaCaT bystander cells were exposed to 0.5Gy and 5 Gy ICCM from OE19 (a-b) and OE33 (c-d) donor cells.* indicates a significance change for cells exposed to ICCM compared to CCCM represented by $p < 0.05$. Data are mean ±SEM for $n=5$ cells. Lower and upper bounds are outline by the shaded error regions.

Figure 6 shows the cell survival percentage of OE19 and OE33 cells as a function of radiation dose. The OE19 cell lines seem to fit the IR model better than the LQ model and reveal a clear increase in cell killing at doses below 1 Gy.



(a) (b)

Figure 5.6. The radiation response was assessed for OE19 (a) and OE33 (b) cell lines. Each graph is representing cell survival as a function of radiation dose. Data are mean ±SEM for *n=9*.

5.5 Discussion

In this study, bystander signal production was assessed in the OE33, OE19, and HCT 116 p53 +/+ cell lines using both the clonogenic assay and ratiometric calcium measurements. In agreement with previously published work by Mothersill et al. 2011 ([23]), this work has also shown HCT 116 p53 +/+ cells having the ability to generate and respond to bystander signals. The data in this paper suggest that both EAC cell lines were unable to respond to bystander signals ($p > 0.05$). However, both the human keratinocyte and colorectal cancer reporter cells detected that the OE19

cells produce bystander signals ($p < 0.05$). Data for the human keratinocyte reporter model also suggests that the OE33 cell lines have the ability to produce bystander signals ($p < 0.05$). However, the decrease in cell survival seen using HCT 116 p53 +/+ as reporters when harvesting ICCM from OE33 donors was not statistically significant ($p = 0.353$). There was a trend for decreased survival for 5Gy ICCM for both EAC cell lines, but these results did not reach statistical significance.

An influx of calcium ions following exposure to ICCM has been found to be an early indicator of apoptosis in human keratinocyte bystander cells [15]. Increases in the cellular calcium concentrations can result in openings in the outer membrane of the mitochondria leading to a leakage of cytochrome c [42]. Diffusion of cytochrome c into the endoplasmic reticulum can initiate further release of calcium that is uptake into the mitochondrial matrix [43]. Subsequently the mitochondria undergo depolarization and increase production of reactive oxygen species (ROS). These distinct cellular responses, mitochondrial depolarization [15, 44, 45], intracellular calcium fluxes, and elevated ROS production have been found in bystander cells following exposure to ICCM [15, 45]. In the present study, the calcium flux with ICCM harvested from HaCaT donors and placed onto autologous reporters has been shown as a "proof of principle" of the reliability of using this reporter model to monitor bystander effects [15]. Both EAC cell lines show a statistically significant intracellular calcium fluxes following exposure of the HaCaT reporters to 0.5Gy ICCM harvested from the OE33 and OE19 donors.

The association between wild type p53 expression and ability to respond to bystander signals, while clear in the HCT+/+ and -/- cells [23] cannot be universal because in 1993, Lehman and colleagues [35] discovered three different point mutations on both alleles-His179Tyr, Asp281Gly, and Arg282Trp-in the HaCaT human keratinocytes. The point mutation located on codon 281, Asp281Gly, has been described as a gain of function mutation linked to an alternative pathway to accomplish apoptosis [46]. In fact, a cell death response, in particular apoptosis, is frequently reported in bystander cells [12, 16, 17, 36]. Although the exact nature of the bystander response seems to be cell line specific and has yet to be made clear, the role of p53 is clearly complicated. The type of p53 mutation in the reporter cells may be important, since different types of p53 point mutations can affect the activity of this protein [47]. Unlike the point mutation found on codon

281 in HaCaTs which leads to an alternative apoptosis pathway, the OE33 point mutation has been documented on exon 5 (Cys135Tyr) resulting in non-functional p53 protein activity [48]. Furthermore, the frameshift mutation found in OE19 cells has been documented on exon 10 (928_930insA) and results in the expression of a truncated p53 protein that has been suggested to be partially functional [48]. Therefore this may explain the greater magnitude of cell death found in the HaCaT reporters exposed to ICCM harvested from EAC donors compared to the non-significant findings with the EAC reporters.

This work also reports a HRS/IRR response in the OE19 cell line. This phenomenon is common in radioresistant cells with a tendency to transition from a sensitive low dose response to a more resistant one at higher doses [11, 28]. The inherently radioresistant OE19 cell line showed a low HRS response below 1 Gy of γ-irradiation and bystander effects were also found at the 0.5Gy ICCM region. Other studies has shown that low dose HRS and bystander effects are not mutually exclusive [32, 33] including recent findings found by our research group with a radioresistant T98G glioma cell line [7]. However, these two phenomena has been shown to be independent in some cell lines such as the human fibroblast MSU 1.1 cell line [49] and human breast MCF7 cell lines [24, 50]. Only one other study has been published using radiation with the OE19 cell line but the doses used were 2 Gy and 4 Gy so no conclusions can be drawn concerning HRS/IRR as the doses were too high [51].

One possible explanation for the OE19 cells inducing low dose HRS whereas the OE33 do not may simply be because these cells possess only partial function of their p53 proteins. Other work has reported human A549 lung caricarcinoma and T98G human glioma cell lines have marked low dose HRS that are associated with p53-dependent apoptosis [50]. Another potential reason may be that the non-homologous end joining (NHEJ) repair is deficient in G1 cells and also the cells may accumulate in an early G2/M checkpoint arrest following irradiation for the OE19 cells. Other work has shown that NHEJ repair deficiency, the failure to arrest in cell cycle checkpoints, and p53-dependent apoptosis result in a marked HRS/IRR response in cells [52]. Marples and colleagues (2004) has reported that the induction of HRS depends on ineffective arrest in G2 following radiation damage [53]. In 2010, a study found that cells irradiated in either the G1 or S phase with 0.2 Gy induced a significant accumulation of the cells in G2-phase [54]. Most of the cells are predominately

in the G1 phase for both OE33 and OE19 cell lines. Approximately 30% and 50% of the cells are in the G0/G1-phase population for the OE33 and OE19 cell lines, respectively. Roughly 20% of the cells are in the G2/M-phase for both OE33 and OE19 cell lines [48]. Lynam-Lennon et al., (2010) have reported that irradiation with 2 Gy induced a significant accumulation of the cells in G2/M-phase post irradiation with a significant decrease in the proportion of cells in the G1 phase [4]. It is unclear whether a similar occurrence results at doses below 1 Gy of radiation in the OE33 and OE19 cell lines, but this would be one avenue worth investigating in future studies.

To date, there are very few established esophageal carcinoma cell lines [38]. One reason for this could be that only a small portion of tumour cells can be extracted from preoperative biopsies since larger biopsies pose a risk for gastrointestinal perforations. Furthermore, *in vivo* models using mice and rats have been reported to be unreliable simply because anatomically the gastroesophageal junction differs considerably from humans [38]. Only 16 EAC cell lines have been established up to the present time, but recently only 10 of these cells have been authenticated to originate from EAC [38]. A reliable panel of 10 EAC cell lines has recently been authenticated that include FLO-1, KYAE-1, SK-GT-4, OE19, OE33, JH-EsoAd1, OACP4C, OACM5.1, ESO26 and ESO51. In 2007, a cell line used as a model for EAC called TE-7 was authenticated to originate from esophageal squamous cell carcinoma [55]. Furthermore, this same researcher found that 4 out of the 14 TE series esophageal squamous cell carcinoma cell lines (TE-2, TE-3, TE-12, TE-14) share a similar genotype as the TE-7 cell lines [55]. Therefore, all 5 TE esophageal squamous cell carcinoma cell lines should be considered one single line. By assessing the HRS/IRR response and bystander effects with a range of lower and higher doses in the OE19, along with other esophageal cancer cell lines, it might be possible to determine whether HRS/IRR and bystander effects are consistently seen in esophageal cancer and what the likely impact of these on treatment outcome might be.

In 1988, Mothersill et al. developed an *in vitro* technique to assess the radiation response of normal and cancerous esophageal human tissue explants. The findings revealed a clear dose response between normal and tumour esophageal explants with less cell growth reduction in the cancerous tissues compared to the tissues harvested from normal segments of the esophagus following radiation exposure. Further work is necessary to test these low dose phenomena presented in this study on an

entire panel of esophageal cancer cell lines along with normal and tumour esophageal tissue explants in order to delineate the radiation responses amongst various esophageal cell lines and human tissues.

In summary, our results suggest that both OE19 and OE33 cells have the ability to produce bystander signals as measured by the clonogenic assay and the ratiometric calcium measurements. Using the HaCaT reporter cell lines we observed a significant reduction in cell survival for both EAC cell lines, whereas when the HCT 116 p53 +/+ cell line was used as a reporter no signal was detected using the OE33 donor cells. This highlights the importance of using a range of cell lines and reporters to determine the full picture when studying these low dose effects. The results also show a low HRS response in the human esophageal adenocarcinoma OE19 cell line. A full understanding of the dose response and underlying mechanisms is necessary before any conclusions can be drawn about the role, if any, of these low dose effects in radiotherapy or their potential value as novel targets for modulating therapeutic ratios.

5.6 References

[1] Canadian Cancer Statistics AdvisoryCommittee. *Canadian Cancer Statistics 2015*. Toronto, ON, 2015.

[2] American Cancer Society. *Cancer Facts & Figures 2015*. Atlanta, 2015.

[3] N. Lynam-Lennon et al. "Altered mitochondrial function and energy metabolism is associated with a radioresistant phenotype in oesophageal adenocarcinoma". In: 9.8 (2014). DOI: 10.1371/journal.pone.0100738.

[4] N. Lynam-Lennon et al. "Alterations in DNA repair efficiency are involved in the radioresistance of esophageal adenocarcinoma". In: *Radiation research* 174.6a (2010), pp. 703–711.

[5] K. S. Marples P. Lambin and B MC Joiner. "Low dose hyper-radiosensitivity and increased radioresistance in mammalian cells". In: *International journal of radiation biology* 71.6 (1997), pp. 721–735.

[6] M. C. Joiner et al. "Low-dose hypersensitivity: current status and possible mechanisms". In: *International Journal of Radiation Oncology* Biology* Physics* 49.2 (2001), pp. 379–389.

[7] S. C. B. Fernandez-Palomo C and C. Mothersill. "Inter-relationship between low-dose hyper-radiosensitivity and radiation-induced bystander effects in the human T98G glioma and the epithelial HaCaT cell line". In: *Accepted. Rad Res.* (2016).

[8] C. B. Seymour and C. Mothersill. "Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve". eng. In: *Radiation Research* 153.5 Pt 1 (May 2000), pp. 508–511. ISSN: 0033-7587.

[9] C. Pinho et al. "Assessing patient characteristics and radiation-induced non-targeted effects in vivo for high dose-rate (HDR) brachytherapy". In: *Int. J. Radiat. Biol* (2015), pp. 1–9. DOI: 10.3109/09553002.2015.1068458.

[10] C Hanu et al. "The influence of smoking on radiation-induced bystander signal production in esophageal cancer patients". In: *Environmental Research* (2016).

[11] B Marples and M. Joiner. "The response of Chinese hamster V79 cells to low radiation doses: Evidence of enhanced sensitivity of the whole cell population". In: *Radiation research* 133.1 (1993), pp. 41–51.

[12] L. A. Ryan et al. "Dilution of irradiated cell conditioned medium and the bystander effect". eng. In: *Radiation research* 169.2 (Feb. 2008), pp. 188–196. ISSN: 0033-7587. DOI: 10.1667/RR1141.1.

[13] B. G. Wouters, A. M. Sy, and L. D. Skarsgard. "Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity". In: *Radiation research* 146.4 (1996), pp. 399–413.

[14] C. Mothersill et al. "Individual variation in the production of abystander signal'following irradiation of primary cultures of normal human urothelium". In: *Carcinogenesis* 22.9 (2001), pp. 1465–1471.

[15] F. Lyng, C. Seymour, and C Mothersill. "Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis". In: *British journal of cancer* 83.9 (2000), p. 1223.

[16] C Mothersill and C Seymour. "Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells." In: *International journal of radiation biology* 71.4 (1997), pp. 421–427.

[17] C. Mothersill and C. Seymour. "Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium". In: *Radiation research* 149.3 (1998), pp. 256–262.

[18] S. A. Lorimore et al. "The influence of p53 functions on radiation-induced inflammatory bystander-type signaling in murine bone marrow". In: *Radiat. Res.* 179.4 (2013), pp. 406–415.

[19] C. Mothersill et al. "Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo". In: *Radiat. Res.* 163.4 (2005), pp. 391–399.

[20] O. Belyakov et al. "A proliferation-dependent bystander effect in primary porcine and human urothelial explants in response to targeted irradiation". In: *British Journal of Cancer* 88.5 (2003), pp. 767–774.

[21] K. Butterworth et al. "Bystander signalling: exploring clinical relevance through new approaches and new models". In: *Clinical Oncology* 25.10 (2013), pp. 586–592.

[22] K. M. Prise and J. M. O'Sullivan. "Radiation-induced bystander signalling in cancer therapy". In: *Nature Reviews Cancer* 9.5 (2009), pp. 351–360.

[23] C. Mothersill et al. "A role for p53 in the response of bystander cells to receipt of medium borne signals from irradiated cells". In: *International journal of radiation biology* 87.11 (2011), pp. 1120–1125.

[24] E. Kalanxhi and J. Dahle. "The role of serotonin and p53 status in the radiation-induced bystander effect". In: *International journal of radiation biology* 88.10 (2012), pp. 773–776.

[25] Le et al. "An observed effect of p53 status on the bystander response to radiation-induced cellular photon emission." In: *Manuscript submitted* (2015).

[26] L. Garcia et al. "Fitting the linear–quadratic model to detailed data sets for different dose ranges". In: *Physics in medicine and biology* 51.11 (2006), p. 2813.

[27] P Lambin et al. "Hypersensitivity of a human tumour cell line to very low radiation doses". In: *International journal of radiation biology* 63.5 (1993), pp. 639–650.

[28] L. A. Ryan et al. "Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response". In: *International journal of radiation biology* 85.1 (2009), pp. 87–95.

[29] B. Wouters and L. Skarsgard. "The response of a human tumor cell line to low radiation doses: evidence of enhanced sensitivity". In: *Radiation research* 138.1s (1994), S76–S80.

[30] M. Joiner and J Denekamp. "The effect of small radiation doses on mouse skin." In: *The British journal of cancer. Supplement* 7 (1986), p. 63.

[31] L. M. Martin et al. "Exposure to low dose ionising radiation: molecular and clinical consequences". In: *Cancer letters* 338.2 (2013), pp. 209–218.

[32] A.-C. Heuskin et al. "Low-dose hypersensitivity and bystander effect are not mutually exclusive in a549 lung carcinoma cells after irradiation with charged particles". In: *Radiation research* 180.5 (2013), pp. 491–498.

[33] O. Nuta and F. Darroudi. "The impact of the bystander effect on the low-dose hypersensitivity phenomenon". In: *Radiation and environmental biophysics* 47.2 (2008), pp. 265–274.

[34] F Bunz et al. "Requirement for p53 and p21 to sustain G2 arrest after DNA damage". In: *Science* 282.5393 (1998), pp. 1497–1501.

[35] T. A. Lehman et al. "p53 mutations in human immortalized epithelial cell lines". In: *Carcinogenesis* 14.5 (1993), pp. 833–839.

[36] H. Furlong et al. "Apoptosis is signalled early by low doses of ionising radiation in a radiation-induced bystander effect". In: *Mutat Res Fund Mol Mech Mut* 741 (2013), pp. 35–43. DOI: 10.1016/j.mrfmmm.2013.02.001.

[37] H. Furlong et al. "Identification of Key Proteins in Human Epithelial Cells Responding to Bystander Signals From Irradiated Trout Skin". In: *Dose-Response* 13.3 (2015), p. 1559325815597669.

[38] J. J. Boonstra, H. W. Tilanus, and W. N. Dinjens. "Translational research on esophageal adenocarcinoma: from cell line to clinic". In: *Diseases of the Esophagus* 28.1 (2015), pp. 90–96.

[39] T. T. Puck and P. I. Marcus. "Action of X-rays on mammalian cells". In: *The Journal of experimental medicine* 103.5 (1956), pp. 653–666.

[40] T. Bíró et al. "Distinct subpopulations in HaCaT cells as revealed by the characteristics of intracellular calcium release induced by phosphoinositide-coupled agonists". In: *Archives of dermatological research* 290.5 (1998), pp. 270–276.

[41] L Csernoch, J Hunyadi, and L Kovacs. "Calcium release activated calcium entry in a human skin derived cell line (HaCaT)". In: *Experimental dermatology* 9.3 (2000), pp. 200–205.

[42] M. P. Mattson and S. L. Chan. "Neuronal and glial calcium signaling in Alzheimer's disease". In: *Cell calcium* 34.4 (2003), pp. 385–397.

[43] K. Zhang and R. J. Kaufman. "From endoplasmic-reticulum stress to the inflammatory response". In: *Nature* 454.7203 (2008), pp. 455–462.

[44] D. R. Green and J. C. Reed. "Mitochondria and apoptosis". In: *Science* 281.5381 (1998), p. 1309.

[45] F. Lyng, C. Semour, and C Mothersill. "Early events in the apoptotic cascade initiated in cells treated with medium from the progeny of irradiated cells". In: *Radiation protection dosimetry* 99.1-4 (2002), pp. 169–172.

[46] M. He et al. "A mutant P53 can activate apoptosis through a mechanism distinct from those induced by wild type P53". In: *FEBS letters* 517.1 (2002), pp. 151–154.

[47] C. Rollenhagen and P. Chène. "Characterization of p53 mutants identified in human tumors with a missense mutation in the tetramerization domain". In: *International journal of cancer* 78.3 (1998), pp. 372–376.

[48] C. D. Fichter et al. "Occurrence of multipolar mitoses and association with Aurora-A/-B kinases and p53 mutations in aneuploid esophageal carcinoma cells". In: *BMC cell biology* 12.1 (2011), p. 1.

[49] C. Mothersill, C. Seymour, and M. Joiner. "Relationship between radiation-induced low-dose hypersensitivity and the bystander effect". In: *Radiation research* 157.5 (2002), pp. 526–532.

[50] L. Enns et al. "Low-Dose Radiation Hypersensitivity Is Associated With p53-Dependent Apoptosis11U. S. Department of Energy, University of California Lawrence Livermore National Laboratory contract W-7405-Eng-48 (KT Bogen), National Cancer Institute (Canada) grant 013104 (M. Weinfeld), Alberta Cancer Board Bridge and Pilot grant R-418 (AD Murtha), and US Department of Energy Low-Dose Radiation Research Program (KT Bogen)." In: *Molecular Cancer Research* 2.10 (2004), pp. 557–566.

[51] G. J. Hötte et al. "Radiation sensitivity of esophageal adenocarcinoma: the contribution of the RNA-binding protein RNPC1 and p21-mediated cell cycle arrest to radioresistance". In: *Radiation research* 177.3 (2012), pp. 272–279.

[52] C. Thomas et al. "Low-dose hyper-radiosensitivity of progressive and regressive cells isolated from a rat colon tumour: impact of DNA repair". In: *International Journal of Radiation Biology* 84.7 (2008), pp. 533–548.

[53] B. Marples et al. "Low-dose hyper-radiosensitivity: a consequence of ineffective cell cycle arrest of radiation-damaged G2-phase cells". In: *Radiation research* 161.3 (2004), pp. 247–255.

[54] M. Fernet et al. "Control of the G2/M checkpoints after exposure to low doses of ionising radiation: implications for hyper-radiosensitivity". In: *DNA repair* 9.1 (2010), pp. 48–57.

[55] J. J. Boonstra et al. "Mistaken identity of widely used esophageal adenocarcinoma cell line TE-7". In: *Cancer research* 67.17 (2007), pp. 7996–8001.

[56] C Mothersill et al. "Differential response of normal and tumour oesophageal explant cultures to radiation". In: *Acta Oncologica* 27.3 (1988), pp. 275–280.

Chapter 6

Serotonin is not involved in radiation-induced bystander signal production in OE19 or OE33 esophageal adenocarcinoma cell lines

Christine Hanu, Raimond Wong, Ranjan K. Sur, Joseph E.Hayward Colin Seymour, and Carmel Mothersill

The author and Dr. Mothersill designed these in vitro bystander medium-transfer experiments. The first author completed the cell preparation, performed cell irradiations, bystander medium transfer experiments, data collection and statistical analysis. The other authors participated in discussion and provided feedback on interpretation of the results.

6.1 Abstract

In this paper, we test whether serotonin dependent mechanisms are involved in bystander signal production and response in two esophageal adenocarcinoma cell lines (OE19 and OE33) using these cell lines or a positive control human colorectal cancer reporter cell line (HCT 116 p5+/+). Serotonin has been shown previously to be involved in generating bystander signals following γ-ray exposure in other human cell lines, where the 5-HT_3 receptor is involved and in clinical samples from esophageal carcinoma patients. We test the involvement of this signaling molecule in the bystander signal and response pathway in esophageal adenocarcinoma. We report data showing that serotonin is not required for bystander signal production in the HCT 116 p53 +/+, OE33, or OE19 cell lines. Serotonin caused a stimulatory effect in cell survival for the OE19 cell line, but this is not related to irradiated cell conditioned (ICCM) exposure. Furthermore,the OE19 produce bystander signals

but are unable to respond to bystander signals. The data suggest that whatever role serotonin may have in radiation response mechanisms is complex and cell line dependent. There may be multiple mechanisms involved in the bystander process in esophageal cancer cells.

6.2 Introduction

Over the past 20 years many potential signaling candidates have been reported to influence radiation-induced bystander signal generation and response pathways, one of which is serotonin. Serotonin (5-hydroxytryptamine, 5-HT) was successfully extracted from the enterochromaffin cells of rats in 1937 [1]. Since then this signaling amine has been found to have a very important role in chemotherapy-induced emesis, cancer development, and in bystander signal generation[2–5]. A number of studies over the last eight years [5–9] have reported the involvement of serotonin-dependent mechanisms in signal production and bystander response pathways. Micromolar levels of serotonin were found to be depleted from the medium of HPV-G human keratinocyte cultures during cell irradiation and it was shown that the serotonin had been bound by the 5-HT_3 receptors[5]. An inter-laboratory study showed that commercially available serum batches had variable concentrations of serotonin, which were directly correlated with the expression of the bystander effects in some but not all systems tested [10]. Another study found a 25ng/ml threshold level of serotonin was required to produce bystander signals in the HPV-G cell line but another human keratinocyte cell line (HaCaT) produced serotonin independent bystander effects [11]. Other studies have exploited the use of antagonist 5-HT_3 receptors to abolish the bystander effect in human keratinocyte cells, such as granisetron and ondansetron [5, 9]. The 5-HT_3 receptors are the only ligand-gated ion channels (LGIC)[12] belonging to the cys-loop superfamily. This family is characterized by 5 subunits around a central pore that is permeable to Ca^{2+}, K^+, and Na^+ ions. Binding of the 5-HT agonist to the 5-HT_3 receptors opens the gated ion channel allowing the influx of cations into a cell. This essentially alters the equilibrium and can either stimulate or inhibit a signaling pathway. Both 5-HT_3 receptors and an influx of Ca^{2+} into bystander cells have been shown to be contributing factors producing bystander effects in HPV-G human keratinocytes [5, 13]. The 5-HT_3 receptor requires nanomolar

concentrations of ondansetron and granisetron to be activated [14]. Both of these 5-HT_3 receptors antagonists have been commonly used as anti-emetics during chemo- and radiotherapy [4, 15].

Most serotonin mediated bystander effects have been tested by using HPV-G cultures [5–7, 9]. One study found that serotonin dependent mechanisms have a role in radiation-induced bystander signaling and response pathways in the MCF-7 breast cancer cells [8]. This same study also found that supplemented serotonin led to a bystander effect in HCT 116 p53-/- reporter cells exposed to 0.5 Gy ICCM harvested from HCT 116 p53+/+ cells, but had no effect when exposed to its autologous donors. Another study found that immortalized HaCaT human keratinocyte cells are not dependent on serotonin to induce bystander effects [11]. Both 5-HT and p53 pathways have been suggested to play a role in the induction of bystander effects [8, 16]. Medium transfer mix/match protocols were used to study bystander effects in HCT 116 p53 wildtype and null cells. The authors revealed that both functional and non-functional p53 are capable of generating bystander signals. However, functional p53 is needed in cells to respond to the bystander signals. The EAC cell lines tested in the experiments, described in this paper, have mutated p53 whereas the HCT 116 p53 +/+ cell line possesses functional p53. The work linking serotonin and bystander effects has mostly been done *in vitro* but preliminary clinical findings by this group, and reported in Chapter 2 of this thesis, suggested that serotonin may be involved in the production of bystander signals in a small group of esophageal cancer patients undergoing HDR bracytherapy [17].

The present study is an extension of both our previously published studies [17, 18] investigating the mechanisms underlying RIBE in EAC. The primary objective was to see if serotonin-dependent mechanisms were involved in bystander effects in EAC cell lines. Primary objectives were to investigate serotonin-dependent mechanisms in bystander effects for other epithelial cancer cells including esophageal adenocarcinoma. We also investigated a 5-HT_3 receptor antagonist, ondansetron, that is commonly used as an anti-emetic agent for chemotherapy- and radiotherapy-induced nausea and vomiting [19]. It is hypothesized that the addition of serotonin over the threshold of 25ng/mL will modulate a cell death bystander effect in human colon cancer bystander cells when exposed to ICCM from donor esophageal adenocarcinoma cells. When serotonin is combined with a selective 5-HT_3 antagonist, it is expected that the bystander response will be abolished.

6.3 Methods and Materials

6.3.1 Cell lines

HCT 116 p53 +/+ cells are derived from a human colon cancer [20]. They have the ability to produce and respond to bystander signals [16]. The OE33, OE19, and HCT 116 p53 +/+ cell lines were used as donors and reporter cells in mix and match protocols to tease out signal production and response functions. Both the OE19 and OE33 cell line are cells derived from primary tumours clinically diagnosed as stage II and III EAC, respectively. The OE33 cell lines have a doubling time of 30 hours [21] and have a p53 protein with a point mutation on codon 135 (cysteine→tyrosine) [22]. The OE19 cells have a rather long doubling time of 50 hours [23] and have been documented as p53 mutated [22]. All cell lines were screened for mycoplasma and cleared prior to commencing experiments by using the Plasmotest^{TM} Mycoplasma detection kit (Catalog # rep-pt1), which was purchased from Cedarlane (Burlington, ON, Canada).

6.3.2 Cell culture

Cell culture was performed in a biosafety level 2 (BSL2) laboratory within a laminar flow cabinet. All cell lines were grown in RPMI-1640 culture medium supplemented with 10% FBS (Invitrogen, Burlington, ON, Canada), 5 ml of 200 mM L-Glutamine (Gibco, Burlington, ON), and 25mM Hepes for pH regulation. Culture medium for experiments only was supplemented with 5 ml of antibiotic solution containing 10,000 units of penicillin and $10,000\,\mu g$/ml of streptomycin (Gibco, Burlington, ON). For serotonin medium-transfer bystander experiments, the background serum serotonin levels were destroyed when the FBS was exposed to laboratory light for 3 hours prior to supplementing into the culture medium. Past work by our research group [7] has shown that serotonin is highly sensitive to light and can be degraded from 89 ng/ml to 5 ng/ml after 3 hours of exposure to laboratory light. All cell stocks were maintained in T-75 flasks with 30 mL of growth medium. They were all routinely passaged upon reaching a subconfluent cell density of 80–100%. All cells were incubated at 37 °C and 5% carbon dioxide in air.

6.3.3 Preparation of serotonin and inhibitor solutions

Serotonin creatinine sulfate monohydrate and ondansetron (brand name Zofran) were purchased from Sigma-Aldrich, Oakville, ON, Canada. Serotonin stocks were initially dissolved in 0.1M HCl as recommended by the supplier. Further dilutions using double distilled water (ddH_2O) to reach a stock concentration of 20 mg/ml. Ondansetron hydrochloride (Sigma-Aldrich, Oakville, ON) (Sigma-Aldrich, Oakville, ON, Canada) stock solutions were prepared by diluting in ddH_2O to a stock concentration of 2 mg/ml. All stock concentrations were sterilized by filtration using a 0.22 µm Naglene filter prior to serial dilution. The stock solutions were serially diluted to produce the required concentration of each drug so that each drug solution could be added in a 0.1 ml volume to achieve a total volume of 5 ml per flask. An acid control was set-up since serotonin was initially dissolved in 0.1 M HCL. These acid controls showed no significant changes to survival compared to the control samples, refer to Figure A.8.

6.3.4 Irradiation

All cells were irradiated in T-25 flasks by using a Caesium-137 (Cs-137) source (McMaster University, Hamilton, ON). A flask-to-source distance of 31 cm and dose-rate of 0.226 Gy/min were used during cell irradiations. All flasks were returned to the incubator immediately following irradiations.

6.3.5 Clonogenic Assay

All donors were set up at a density of 300,000 cells per flask. The donor seeding density was chosen based upon previous studies [24] showing this cell concentration was maximally effective at producing a strong bystander signal. The seeding density for reporter cells varied for each cell line according to cloning efficiency of the controls. For both OE33 and HCT 116 p53 +/+ reporters a seeding density of 500 cells per flask was used. OE19 cells required a much higher density of 2000

cells per flask in order to produce enough viable colonies to score. Donors were irradiated to 0.5 Gy and 5 Gy 6 h after plating (colon cancer cell lines) and 15 h after plating (esophageal cancer cell lines). Esophageal cancer cell lines required a longer time for cells to adhere to the bottom of the flasks. Controls were unirradiated cells, irradiated medium without cells, sham-irradiated cells, 5-HT concentration controls and Ondansetron concentration controls. Following irradiations, donors were returned to the incubator for 1 hour. After that time, the culture medium was harvested as ICCM or control cell conditioned medium (CCCM). This was filtered and transferred from donor flasks to reporter flasks. Following medium transfer, reporter cells were grown in an incubator at 37 °C with 5% CO_2 in air for 8–14 days for both the OE33 and HCT116p53 wildtype cell lines. The OE19 specifically required a longer incubation time from 14–20 days in order to form viable colonies with 50 cells or more. Once appropriate sized colonies had formed, the cells were stained with 20% carbol fuchsin (VWR, Bridgeport, NJ, USA) and colonies with 50 cells or more were scored.

6.3.6 Statistical Analysis

Separate two-way ANOVA analyses with bonferroni *post hoc* correction were conducted for medium transfer experiments in the presence and absence of 5-HT3 antagonist inhibitor. A student t-test was used to highlight significant findings on bar graphs below for main effect factors. Data are mean ± standard error, unless otherwise stated. All p-values less than 0.05 were considered statistically significant.

6.4 Results

A two-way ANOVA was conducted to examine the effects of bystander medium exposure and serotonin concentrations on cell survival for human esophageal and colon cancer cell lines. There was no statistically significant interaction between 0.5 Gy and 5 Gy ICCM exposure and serotonin concentrations affecting the mean cell survival for all cell lines tested in this paper. This includes the OE33, OE19, and HCT 116 p53 +/+ cell lines, refer to Table 6.1. Therefore an analysis of the main effects for ICCM exposure and serotonin was performed, and the p-values are outlined in Table 6.2.

Table 6.1. Two-way ANOVA Table

Experimental system	Variables	p-value (No inhibitor)	p-value (Inhibitor)
HCT 116 p53 +/+ (donors/reporters)	ICCM	<0.001**	<0.001**
	Serotonin	0.021*	0.946
	Interaction[a]	0.338	0.805
HCT 116 p53 +/+ (reporters)	ICCM	0.004*	<0.001**
OE19 (donors)	Serotonin	0.813	0.716
	Interaction[a]	0.921	0.981
HCT 116 p53 +/+ (reporters)	ICCM	0.002*	0.154
OE33 (donors)	Serotonin	0.893	0.980
	Interaction[a]	0.992	0.944
OE33 (donors/reporters)	ICCM	0.203	0.007*
	Serotonin	0.439	0.818
	Interaction[a]	0.786	0.982
OE19 (donors/reporters)	ICCM	0.859	0.182
	Serotonin	< 0.001**	< 0.001**
	Interaction[a]	0.126	0.354

[a] Interaction between bystander medium and serotonin concentrations

Table 6.2. Multiple comparisons for bystander medium and serotonin concentrations

Experimental system	Variables	No inhibitor		Inhibitor	
		Mean difference (95 % CI)	p-value	Mean difference (95 % CI)	p-value
HCT 116 p53 +/+ (donors/reporters)					
	CCCM vs 0.5 Gy ICCM	17.39 (12.88, 21.89)	<0.001**	20.06 (14.62, 25.49)	<0.001**
	CCCM vs 5 Gy ICCM	12.14 (7.63, 16.64)	<0.001**	13.08 (7.65, 18.52)	0.007*
	No 5HT vs 10nM 5HT	4.78 (-0.97, 10.53)	0.165	0.04 (-6.97, 6.90)	1.000
	No 5HT vs 100nM 5HT	-0.96 (-6.71, 4.79)	1.000	-0.59 (-7.53, 6.34)	1.000
	No 5HT vs 1000nM 5HT	-1.15 (-6.90, 4.60)	1.000	-1.37 (-8.30, 5.56)	1.000
HCT 116 p53 +/+ (reporters) OE19 (donors)	CCCM vs 0.5 Gy ICCM	10.61 (3.03, 18.19)	0.003*	18.69 (11.45, 25.94)	< 0.001**
	CCCM vs 5 Gy ICCM	4.17 (-3.42, 11.57)	0.551	6.53 (-0.72, 13.78)	0.092
	No 5HT vs 10nM 5HT	3.41 (-6.27, 13.09)	1.000	3.67 (-5.59, 12.92)	1.000
	No 5HT vs 100nM 5HT	1.30 (-8.38, 10.98)	1.000	1.30 (-7.96, 10.55)	1.000
	No 5HT vs 1000nM 5HT	2.11 (-7.57, 11.79)	1.000	0.85 (-8.13, 9.83)	1.000
HCT 116 p53 +/+ (reporters) OE33 (donors)	CCCM vs 0.5 Gy ICCM	12.17 (4.03, 20.30)	0.001*	0.25 (-5.38, 5.88)	1.000
	CCCM vs 5 Gy ICCM	7.09 (1.05, 15.22)	0.109	-3.78(-9.41,1.85)	0.316
	No 5HT vs 10nM 5HT	0.59 (-9.80, 10.98)	1.000	0.22 (-6.97, 7.41)	1.000
	No 5HT vs 100nM 5HT	2.26 (-8.13, 12.65)	1.000	-0.70 (-7.41,6.97)	1.000
	No 5HT vs 1000nM 5HT	-1.48 (-11.87, 8.91)	1.000	-0.67 (-7.86, 6.52)	1.000
OE33 (donors/reporters)					
	CCCM vs 0.5 Gy ICCM	7.03 (-4.15, 18.21)	0.372	14.42 (2.73, 26.11)	0.01*
	CCCM vs 5 Gy ICCM	1.03 (-10.36, 12.42)	1.000	12.08 (0.39, 23.77)	0.04*
	No 5HT vs 10nM 5HT	6.70 (-7.57, 20.98)	1.000	2.74 (-12.18,17.66)	1.000
	No 5HT vs 100nM 5HT	-1.44 (-15.72, 12.83)	1.000	-2.04 (-16.58, 12.50)	1.000
	No 5HT vs 1000nM 5HT	2.59 (-11.68, 16.87)	1.000	-1.37 (-16.29, 13.55)	1.000
OE19 (donors/reporters)					
	CCCM vs 0.5 Gy ICCM	1.00 (-5.97, 7.97)	1.000	1.81 (-5.57, 9.18)	1.000
	CCCM vs 5 Gy ICCM	-0.56 (-7.52, 6.41)	1.000	-3.72 (-11.09,3.65)	0.665
	No 5HT vs 10nM 5HT	1.96 (-6.93, 10.86)	1.000	1.85 (-7.56, 11.26)	1.000
	No 5HT vs 100nM 5HT	-6.41 (-15.30, 2.49)	0.331	-7.37 (-16.78, 2.04)	0.225
	No 5HT vs 1000nM 5HT	-19.00 (-27.89, -10.11)	< 0.001**	-14.26 (-23.67, -4.85)	0.001*

* Significant less than 0.05

** Significant less than 0.001

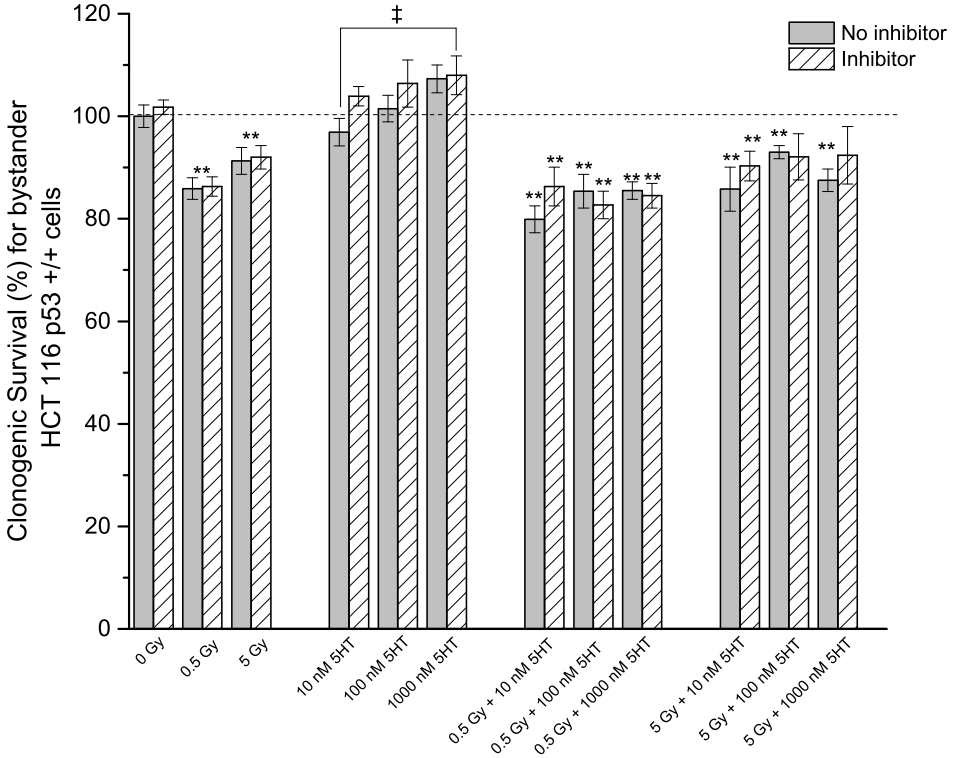


Figure 6.1. The cell survival for HCT 116 p53 +/+ reporters exposed to irradiated cell condition medium from autologous cells is shown above. Cells were exposed to bystander medium (0Gy CCCM, 0.5Gy ICCM or 5 Gy ICCM) in the presence of serotonin and its serotonin inhibitor in autologous reporter cells. * Indicates a significance change for cells exposed to ICCM compared to CCCM represented by p< 0.001. Double daggers indicates a significant difference for serotonin concentrations with a p< 0.05. Data are mean ±SEM for *n=9*.

For the HCT 116 p53 +/+ medium-transfer bystander experiments, there was a statistically significant difference in mean cell survival in the human colon cancer cells receiving ICCM harvested from directly irradiated cells (p<0.001), as shown in Table 6.2. Also, HCT 116 p53+/+ cells receiving serotonin have a statistical significance in cell survival (p=0.021), as shown in Table 6.2. A multiple comparison bonferroni test shows that bystander HCT 116 p53+/+ cells exposed to 0.5 Gy ICCM or 5 Gy ICCM was associated with a mean cell survival of 17.39 (95% CI, 12.99 to 21.79) and 12.14 (95% CI, 12.99 to 21.79) lower than control, respectively (p < 0.001), refer to Table 6.2. Serotonin alone seems to stimulate cell survival of HCT 116 p53 +/+ cells exposed to 1000nM of 5HT compared to 10 nM of 5-HT (p=0.040).

Both EAC cell lines were observed to have insignificant reductions in cell survival when

transferring ICCM onto autologous reporter cells ($p > 0.05$), as shown in 6.2(a) and 6.3(a). However, OE19 donor medium produced a significant reduction in cell survival when 0.5Gy ICCM was transferred onto human colorectal cancer cell lines ($p<0.05$), as shown in 6.2(b) and Table 6.2. When human colorectal cancer cells are reporters, there is more of effect observed at 0.5 Gy ICCM than 5Gy ICCM. Also, the presence of an inhibitor does seem to be effective at increasing the death effect at 0.5Gy ICCM, ($p < 0.001$), refer to Table 6.2. The difference between serotonin concentrations and its inhibitor was not statistically significant ($p=1.000$). Bystander human OE19 esophageal cancer cell lines had no statistically significant change in cell survival after exposure to 0.5 Gy and 5 Gy ICCM. However, there was a mean increase in cell survival of 19.00 and 14.26 in the presence of 1000 nM of serotonin and its inhibitor respectively ($p< 0.001$), refer to Table 6.2. Figure 6.2 shows the effect of exposed non-irradiated OE19 and HCT 116 p53 +/+ reporter cells to bystander medium and serotonin in the nanomolar range.

For the OE33 medium-transfer bystander experiments, there was a significant decrease in cell survival found in the HCT 116 p53 +/+ reporters cells at 0.5 Gy ICCM in the absence of a serotonin inhibitor ($p <0.05$), refer to Table 6.2. However, once the serotonin inhibitor was added there was no significant change in cell survival for 0.5 Gy ICCM for the HCT 116 p53 +/+ reporters. In contrast, there was a significant decrease in cell survival found in the OE33 reporters cells at 0.5 Gy ICCM and 5 Gy ICCM in the presence of a serotonin inhibitor ($p <0.05$), refer to Table 6.2. A three-way ANOVA was conducted to determine whether bystander medium exposure, serotonin concentration, and its inhibitor had an effect on the survival of non-irradiated bystander cells. There was no statistically significant three-way interaction between bystander medium, serotonin, and its inhibitor for both HCT 116 p53 +/+ and OE33 bystander cells, $p=0.987$ and $p=0.880$ respectively. There was no statistically significant change between CCCM and 0.5 Gy ICCM for OE33 and HCT 116 p53 +/+ cells receiving bystander medium from OE33 donor cells, $p=0.06$ and $p=0.202$ respectively. An independent t-test revealed no significant increase or decrease in cell survival in the presence of serotonin and its inhibitor, as shown in Figure 6.3.

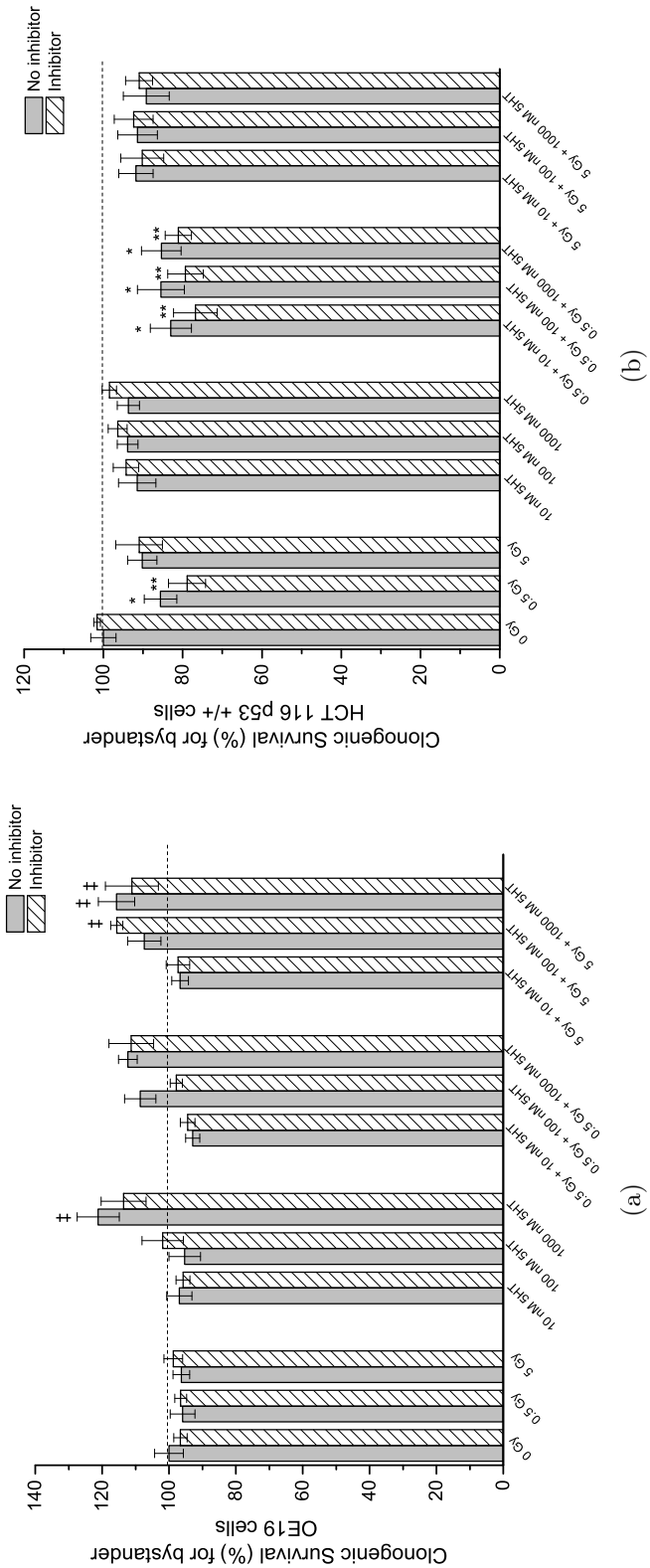


Figure 6.2. The cell survival for OE19 and HCT 116 p53 +/+ reporters exposed to irradiated cell condition medium from OE19 donor cells is shown above. Cells were exposed to 0Gy CCCM, 0.5Gy ICCM or 5 Gy ICCM in the presence of serotonin and its inhibitor for OE19 reporters (a) and HCT 116 p53 +/+ reporter cells (b). ** and * indicates a significance change for cells exposed to ICCM compared to CCCM represented by $p< 0.001$ and $p< 0.05$, respectively. Double daggers indicates a significant difference for serotonin concentrations compared to CCCM control with a $p< 0.001$. Data are mean ±SEM for $n=9$.

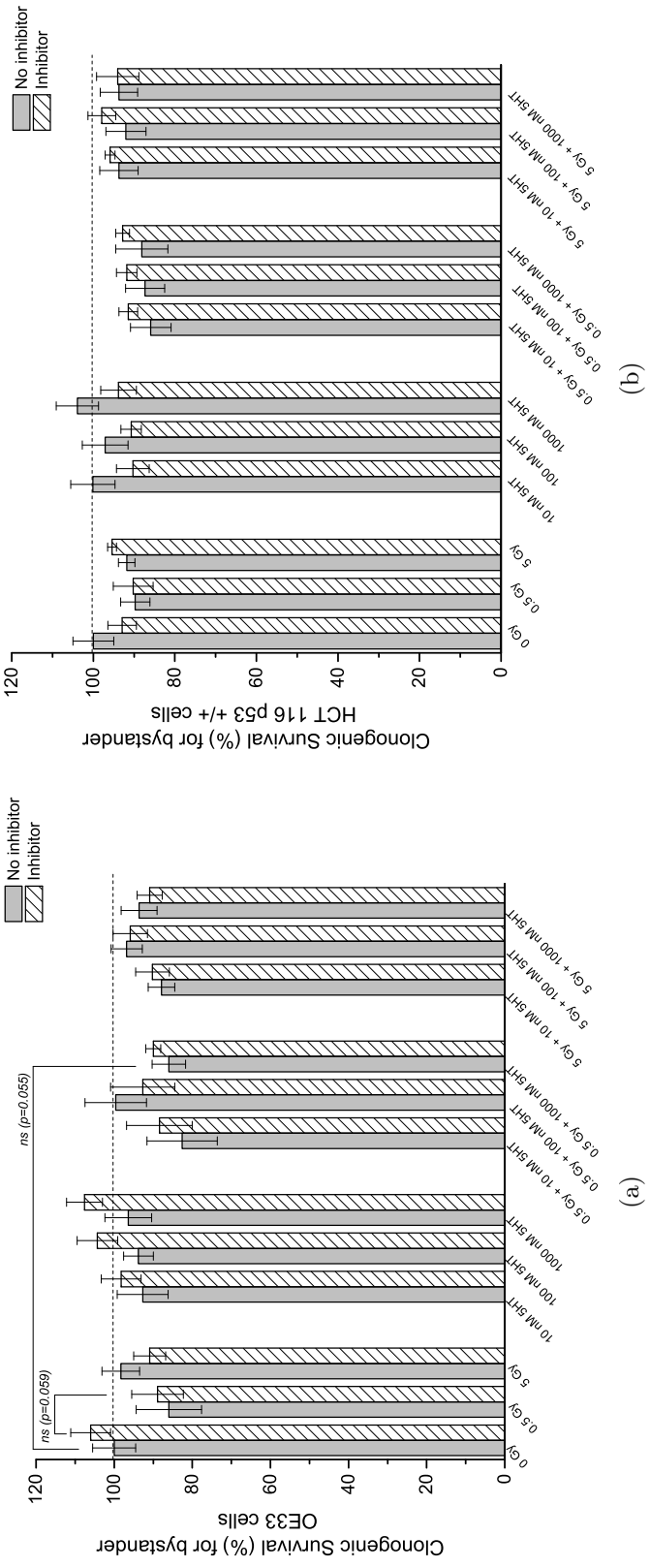


Figure 6.3. The cell survival for OE33 and HCT 116 p53 +/+ reporters exposed to irradiated cell condition medium from OE33 donor cells is shown above. Cells were exposed to 0Gy CCCM, 0.5Gy ICCM or 5 Gy ICCM in the presence of serotonin and its inhibitor for OE33 reporters (a) and HCT 116 p53 +/+ reporter cells (b). Data are mean ±SEM for $n=9$.

6.5 Discussion

In this study, bystander signal production and response was assessed in the OE33, OE19 and HCT 116 p53 +/+ cell lines using the clonogenic assay. The role of serotonin in the mechanism of radiation-induced bystander signaling and response pathways was also investigated. Three different concentrations of serotonin (10 nM, 100 nM, and 1000 nM) were investigated along with ondansetron, 5-HT_3 antagonist. The data in this paper suggest that OE19 cell line did not show a bystander effect. However, clonogenic survival of the human colorectal cancer reporter cell line was reduced when exposed to ICCM from the OE19 cells suggesting the latter have the ability to produce bystander signals ($p<0.05$) but cannot respond to them. There was a decrease in cell survival when bystander medium was transferred from OE33 donors to both HCT 116 p53 +/+ and OE33 reporters. However, the difference between CCCM and 0.5 Gy ICCM, was not statistically significant ($p= 0.06$). This suggests that these cells do not produce bystander signals. Further testing is warranted to determine whether the OE33 cells respond to bystander signals with other cell lines as donors since the p-value was almost significant. For all of the cell lines tested, there was no statistically significant interaction found between serotonin and ICCM ($p > 0.05$). Therefore, this would mean that serotonin does not play a mechanistic role in the induction of RIBEs in the human OE19 and OE33 esophageal cancer cell lines or in the human colorectal cancer cell line.

Serotonin alone caused a marked increase in cell survival at 1000 nM for the OE19 cells that received no radiation. Therefore, the serotonin effect observed seems to be completely unrelated to RIBE. This stimulatory effect was observed only with the OE19 cell line at a concentration of 1000 nM, which cannot be explained without further investigation. Past work with the HCT 116 p53 null cell line has shown a protective bystander effect at a serotonin concentration of 100 ng/ml [8], but this did not occur in its functional p53 partner cell line in the present study. Several studies have shown the involvement of serotonin mechanisms in bystander signal production and responses [5–9]. Also, a small subset of patients undergoing brachytherapy has shown that serotonin modulates bystander signal production in cohort of cancer patients [17]. In the present study, our hypothesis was proven to be incorrect since the addition of serotonin over the threshold of 25 ng/mL does not

affect bystander clonogenic cell death in the cultured esophageal cancer cell lines. Furthermore, the combination of serotonin with a selective 5-HT_3 antagonist did not have an effect on cell survival as predicted at the start of the study. Therefore, there may be multiple mechanisms involved in the bystander effect for esophageal cancer. One possible mechanism could be influenced by the p53 status of the esophageal cancer cell lines as has been shown with other cells [16].

In conclusion, our results suggest that the OE19 cells have the ability to produce bystander signals, as measured by the clonogenic assay, using colorectal cancer reporter cells but they do not respond to signals. Furthermore, bystander signal generation is not dependent on serotonin in any of the cell lines used. The results suggest that the role of serotonin in radiation-induced bystander effect biology is complex and cell line specific. There are bystander effects and serotonin induced proliferation but these two processes do not imply that one causes the other. We postulate that serotonin stimulates clonogenic survival in the OE19 cell line, but probably not due to ion gated channels since no real difference was observed in the presence or absence of the inhibitor. There may be multiple mechanisms involved in the bystander effect in esophageal cancer and p53 status may have a role in the bystander process.

6.6 References

[1] P. M. Whitaker-Azmitia. "The discovery of serotonin and its role in neuroscience". In: *Neuropsychopharmacology* 21 (1999), 2S–8S.

[2] P. J. Pauwels. "5-HT receptors and their ligands". In: *Neuropharmacology* 1083 (2003), p. 38.

[3] D Sarrouilhe et al. "Serotonin and cancer: what is the link?" In: *Current molecular medicine* 15.1 (2015), pp. 62–77.

[4] M. Aapro. "Granisetron: an update on its clinical use in the management of nausea and vomiting". In: *The oncologist* 9.6 (2004), pp. 673–686.

[5] R. Poon et al. "Bystander effects of ionizing radiation can be modulated by signaling amines". In: *Environmental research* 105.2 (2007), pp. 200–211.

[6] R. Saroya et al. "Injection of resperpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: confirmation in vivo of a role for serotonin in the mechanism". In: *Dose-Response* 8.3 (2010), dose–response.

[7] C. Mothersill et al. "Serum serotonin levels determine the magnitude and type of bystander effects in medium transfer experiments". In: *Radiation research* 174.1 (2010), pp. 119–123.

[8] E. Kalanxhi and J. Dahle. "The role of serotonin and p53 status in the radiation-induced bystander effect". In: *International journal of radiation biology* 88.10 (2012), pp. 773–776.

[9] J. Fazzari et al. "Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect". In: *International journal of radiation biology* 88.10 (2012), pp. 786–790.

[10] C. Mothersill et al. "A laboratory inter-comparison of the importance of serum serotonin levels in the measurement of a range of radiation-induced bystander effects: overview

of study and results presentation". In: *International journal of radiation biology* 88.10 (2012), pp. 763–769.

[11] F. M. Lyng et al. "The importance of serum serotonin levels in the measurement of radiation-induced bystander cell death in HaCaT cells". In: *International journal of radiation biology* 88.10 (2012), pp. 770–772.

[12] A. J. Thompson, L. Zhang, and S. C. Lummis. "The 5-HT3 receptor". In: *The Serotonin Receptors*. Springer, 2006, pp. 439–457.

[13] F. M. Lyng, C. Seymour, and C Mothersill. "Initiation of apoptosis in cells exposed to medium from the progeny of irradiated cells: a possible mechanism for bystander-induced genomic instability?" In: *Radiation research* 157.4 (2002), pp. 365–370.

[14] G. J. Kilpatrick et al. "1-(m-Chlorophenyl)-biguanide, a potent high affinity 5-HT 3 receptor agonist". In: *European journal of pharmacology* 182.1 (1990), pp. 193–197.

[15] A. L. Kovac. "Benefits and risks of newer treatments for chemotherapy-induced and postoperative nausea and vomiting". In: *Drug safety* 26.4 (2003), pp. 227–259.

[16] C. Mothersill et al. "A role for p53 in the response of bystander cells to receipt of medium borne signals from irradiated cells". In: *International journal of radiation biology* 87.11 (2011), pp. 1120–1125.

[17] C. Pinho et al. "The involvement of serum serotonin levels producing radiation-induced bystander effects for an in vivo assay with fractionated high dose-rate (HDR) brachytherapy". eng. In: *Int. J. Radiat. Biol* 88.10 (Oct. 2012), pp. 791–797. ISSN: 1362-3095. DOI: 10.3109/09553002.2012.715794.

[18] C. Pinho et al. "Assessing patient characteristics and radiation-induced non-targeted effects in vivo for high dose-rate (HDR) brachytherapy". In: *Int. J. Radiat. Biol* (2015), pp. 1–9. DOI: 10.3109/09553002.2015.1068458.

[19] J.-H. Ye, R. Ponnudurai, and R. Schaefer. "Ondansetron: A Selective 5-HT3 Receptor Antagonist and Its Applications in CNS-Related Disorders". In: *CNS drug reviews* 7.2 (2001), pp. 199–213.

[20] F Bunz et al. "Requirement for p53 and p21 to sustain G2 arrest after DNA damage". In: *Science* 282.5393 (1998), pp. 1497–1501.

[21] DSMZ. *Cell line: OE-33*. 2015. URL: `https://www.dsmz.de/catalogues/details/culture/ACC-706.html`.

[22] J. J. Boonstra et al. "Verification and unmasking of widely used human esophageal adenocarcinoma cell lines". In: *Journal of the National Cancer Institute* 102.4 (2010), pp. 271–274.

[23] DSMZ. *Cell line: OE-19*. 2015. URL: `https://www.dsmz.de/catalogues/details/culture/ACC-700.html`.

[24] C Mothersill and C Seymour. "Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells." In: *International journal of radiation biology* 71.4 (1997), pp. 421–427.

Chapter 7

Discussion and Conclusion

The work presented in this thesis describes the use of clinical and *in vitro* approaches to determine the existence and relevance of bystander effects in adenocarcinoma of the esophagus. The goal of this final chapter is to discuss my contribution to the field by giving brief overviews of the papers presented in this thesis. Some of the limitations encountered, along with possible solutions, have been addressed to suggest new avenues for future clinical work.

7.1 Radiation-induced bystander effects in esophageal tumours

There are no previous studies investigating esophageal cancer patients undergoing HDR brachytherapy and esophageal cell lines for bystander effects. Little is known about the significance of bystander and abscopal effects in radiotherapy modalities because there is high variability from patient to patient. There are no suitable bioassays or biomarkers for investigating radiation-induced bystander signaling and response pathways. The type of cancer being investigated in this thesis is often found at advanced stages with HDR brachytherapy and EBRT being the usual treatment options. Thus, this work adds to a very limited literature in an important field. Five questions were outlined at the end of chapter 1. These questions have been addressed throughout this PhD project and below is a summary of these findings and recap of the thesis hypotheses, refer to Table 7.1 and Table 7.2, respectively. In this section, the findings and significance of this thesis are addressed.

Table 7.1. Summary of results

Thesis Questions & Summary of Findings
1. Are bystander effects produced in brachytherapy patients?
• The results suggest they are produced in non-smokers [1]
• Non-smokers produced strong bystander signals
• Smokers produce weak or absent signals
2. Are they detectable in non-tumour samples such as blood and urine?
• The results suggest they are produced in both blood and urine samples [2, 3]
• Both samples produce an increase in human keratinocyte reporter cells at the final fraction of brachytherapy
3. Do they persist during fractionated treatments?
• The results suggest there is an adaptive response initiated in bystander cells [3]
• A significant response was not found during early exposures
• A marked increase in reporter cell survival was observed after multiple exposures of HDR brachytherapy compared to baseline samples
• A larger sample size is needed to validate these findings
4. Are RIBE in any way related to outcome, stage of the cancer, gender, lifestyle factors such as smoking status?
• The results suggests that bystander effects may be related to smoking status [1]
• Smoking status influences patients' responses to radiation
• Non-smokers had a greater improvement in dysphagia scores than actively smoking individuals
• For both gender and cancer staging the results were inconclusive due to the limited sample size
5. Are available esophageal cell lines useful for studying bystander effects and their underlying mechanisms such as the role of serotonin?
• The results suggests the OE33 and OE19 cells produce bystander signals
• Both esophageal cancer cell lines tested produced a significant reduction in cell survival in human keratinocyte reporters
• OE19 cells do not seem to be capable of responding to bystander signals when exposed to ICCM from their autologous donors
• The esophageal cancer cell lines tested did not require serotonin to produce bystander signals
• Serotonin has been shown to have a role in HDR brachytherapy bystander effect in a small cohort of esophageal cancer patients [2]

Table 7.2. Recap of Thesis Hypotheses

Thesis Hypotheses
H1: Fractionation will induce less cell death in reporter cells following each fraction of brachytherapy.
H2: Gender and smoking status will influence bystander effects
H3: Bystander effects will lead to a significant decrease in reporter cell survival
H4: Serotonin will modulate bystander effects in esophageal cancer
H5: Cell lines possessing mutated p53 will not respond to bystander signals

7.1.1 Part I: Clinical assays

A vast amount of research focuses on the investigation of bystander effects using *in vitro* models [4–9]. Most of the data concern work with established cell lines of limited applicability to *in vivo* situations involving complex tissues. The availability of a human tissue model is essential since it may aid in understanding the relevance of bystander effects (if any) in treatment planning, or help to identify novel therapeutic targets for limiting adverse effects in healthy tissues [10]. In the first part of this thesis, one study focused on fractionated HDR brachytherapy within a small group of esophageal cancer patients. Samples of blood serum, urine, and the esophagus tissues were harvested from esophageal cancer patients. The results demonstrated a significant increase in human keratinocyte cloning efficiency at the final fraction of treatment compared to baseline samples for both the blood serum and urine samples. These findings suggest the presence of factors in the blood and urine capable of inducing an increase in the reporter cloning efficiency, suggestive of a potential adaptive response. Based upon past work [11], the initial hypothesis concerning multiple bystander exposures has been shown to be correct and does not need to be revised (Table 7.2), but it does need to be validated with a much larger sample size. Furthermore, in a small subset of patients undergoing brachytherapy it was shown that serotonin modulates bystander signal production, however, serotonin was not consistently involved in experiments with cultured esophageal cancer cell lines. These findings will be discussed below in the next subsection.

The first pilot study found that blood serum samples produced consistent results in comparison

to the urine and esophageal biopsy samples. Therefore, blood serum samples were used for the subsequent clinical study to validate the production of bystander signals with a larger sample size along with cancer-free volunteers. In contrast to past work [12, 13], gender had no influence on bystander signal generation in this blood-based assay. However, these results do not disprove the hypothesis that females produce stronger bystander signals. Rather this was most likely attributed to the female sample size being too small to reach conclusive results. This thesis has found that smoking status influences patient responses to radiation treatments. These findings support past work with urothelium biopsy samples harvested from smokers [12]. The earlier study found that smokers had a smaller reduction in cloning efficiency following irradiation than samples taken from non-smokers. In the present study, samples taken from smokers produce a weak or absent bystander signal, whereas samples taken from non-smokers can produce strong bystander signals following HDR brachytherapy, see Table 7.2.

The work also showed an improvement in dysphagia scores at the end of treatment in non-smokers compared to actively smoking individuals. Similar results were found in head and neck cancer patients, smokers were more resistant to radiotherapy than non-smokers [14]. In the literature, it has been documented that smoking induces radioresistance by inducing hypoxia. Because cigarettes produce carbon monoxide, the oxygen atoms no longer bind to the hemoglobin, as efficiently as the carbon monoxide, leading to hypoxic conditions in tissues of smokers [14–16]. Tobacco-derived carcinogens- nitrosamines, have also been shown to stimulate growth in urothelial cultures [17]. Cigarettes contain many carcinogens and additives that can increase tumour growth and alter the normal damage response pathways in cells. One additive of particular interest for future work would be nicotine, since it has been documented in the literature to reverse the bystander effect by stimulating a higher cell survival in bystander cells receiving ICCM [18]. Moreover, nicotine has been shown to promote carcinogenesis and apoptosis resistance in a number of cells [19–22]. It is important to consider that nicotine may play a role in the induced radioresistance response because only one esophageal cancer patient in our pilot study was a non-smoker. Consequently, one possible explanation may be that nicotine causes resistance to radiation by promoting cell proliferation and inhibiting apoptosis. There is also a possibility that a combination of systemic nicotine levels

attained during smoking have a role in rendering esophageal cancer resistant to radiotherapy, since smoking increases hypoxia levels in tumours [14, 16].

7.1.2 Part II: *In vitro* assay

The second part of this thesis addressed question five of this thesis (i.e. Is esophageal cancer cells useful for studying bystander effects and their underlying mechanisms such as the role of serotonin?), refer to Table 7.1. This question could not be easily investigated by using clinical models. In this part the focus was on established EAC cell lines with the aim of determining whether these produced bystander effects. Using a well-established human keratinocyte HaCaT reporter model, significant cell killing effects could be measured in reporter cells exposed to medium harvested from OE33 and OE19 cell lines irradiated to 0.5 Gy. There was a trend for OE33 donor cells to produce a bystander effect using autologous cells but the p value was p=0.06 indicating that the effects were on the borderline of significance. The OE33 donor cell medium produced a decrease in cell survival for HCT 116 p53 +/+, but the p-values were insignificant (p=0.202). This highlights the importance of using a range of cell lines and reporters when testing these low dose bystander effects. Therefore, cell culture experiments performed in this thesis revealed that EAC lines are able to produce bystander signals, but the OE19 cell line cannot respond to the signals. The OE33 cells require further testing to determine whether they respond to bystander signals with other cell lines as donors since the p-value was almost significant.

In contrast to our clinical findings suggesting that bystander effects are modulated by serotonin within a small subset of patients, this does not seem to be the case with *in vitro* EAC medium transfer bystander experiments. It was clear that the two OE33 and OE19 cell lines were still able to generate bystander signals regardless of the presence or absence of serotonin. Past work by our research group [23] has shown that serotonin is highly sensitive to light and can be degraded from 89 ng/ml to 5 ng/ml after 3 hours of exposure to laboratory light. The background serum serotonin levels found in FBS were inactivated after exposure to laboratory light for 3 hours prior to supplementing into the culture medium for these experiments. The EAC cell lines revealed no

underlying serotonin requirement to producing bystander signals, there may be other underlying mechanisms involved in the bystander process in these cell lines. Most likely there are other multiple mechanisms that play a role in signal production in esophageal cancer. Part of the reason why the esophageal cancer cells cannot respond to bystander signals may be due to the mutated p53 status as shown by others in the literature [24, 25].

Another very important NTE observed in this thesis was a low dose HRS response in the OE19 cell line, which again was not observed in the OE33 cell lines. The OE19 cell line showed a low HRS response below 1 Gy of γ-irradiation. Only one other study has been published using radiation with the OE19 cell line, but the doses used were higher than 2 Gy [26] and thus were too high to show the HRS response. Bystander effects were also found at 0.5 Gy ICCM, which suggests that these two phenomena may coexist. This supports other studies that showed low dose HRS and bystander effects are not mutually exclusive [27, 28]. Earlier work on bystander effects has shown that HRS/IRR cannot exist in the same cell line, but the dose ranges tested were above the transition from HRS to IRR [24]. Recently published data suggests the phenomenon of IRR may involve suppression of HRS and bystander effects [29]. This means doses above the transition from HRS to IRR do not produce bystander effects or HRS responses. One possible reason for the OE19 but not OE33 cells inducing low dose HRS may have to do with the p53 status of the cell. HRS responses have been reported to undergo apoptosis in a p53-dependent manner [30]. The OE19 cell lines have mutated p53 that still has partial function, but the OE33 cells have non-functional p53 protein activity [31].

7.1.3 Study design limitations

One of the limitations associated with these clinical studies was the small sample size. The limited sample size was attributed to patients leaving the study, patients being unable to give samples, patient contaminated samples, and other problems such as weather affecting sample transport. In both clinical studies, the sample populations were not normally distributed which led to non-parametric statistical approaches being used for data analysis instead of the more robust

parametric tests. A power analysis using G*Power software [32] with a power (1-β) set at 0.95 and α=0.05 showed that the number of subjects required to achieve statistical power was a sample size of 84 and 115 for future fractionated and single exposure HDR brachytherapy studies, respectively (Figure A.5 and Figure A.6).

In addition to there being difficulties consenting patients for biopsy samples, some tissue samples were lost from patient derived yeast contamination. Pre-treating tissue samples with antimycotics were very successful and this needs to be considered in future clinical studies. Another study limitation was not being able to age match cancer-free volunteers to cancer control samples, however, both control groups were nil per os (NPO) after midnight until their samples were taken. There are contradictory data in the literature concerning gender related bystander signal variation [12, 13], part of this thesis was to confirm or rebut past work suggesting females produced stronger bystander signals [12]. However, the majority of the participants in the current study were older males, which made it difficult to answer part of this question. With hindsight, selecting a cancer that affects both males and females equally would be more likely to succeed in addressing this however the funding was specifically for esophageal cancer so this was not really possible. Another disadvantage was the loss of a valuable reporter cell model, (HPV-G human keratinocytes), due to a mycoplasma infection found in the frozen stocks during a routine check. There was no other option but to use an equally well-documented reporter human keratinocyte, called HaCaT [33–36], for the remaining experiments.

In vitro work alone typically looks at cells in isolation, where the growth of cells in a monolayer forces one side of the cells to adhere to the substrate with absolutely no opportunity for cellular contact on the other side. These environments are unnatural and are not normally found in tumours *in vivo*. Many different populations of cells compete and cooperate with each other surround tumour cells, whereas cultured cells lack these cellular complexities and have no inflammatory cells or vasculature [37]. The use of esophageal cancer cell lines makes the detection of bystander effects much simpler than *in vivo*, however, the tissue microenvironment is very important when extrapolating from cell lines to clinical settings. Therefore, these cell lines are not representative of esophageal adenocarcinoma patients enrolled within our clinical studies. Any cell culture investigation comes

with limitations when extrapolating back to human data since each cell line has been derived from a primary tumour taken from a single patient. However, the use of tumour cell lines has been reported to be advantageous for basic mechanistic studies because these cultures contain pure populations of tumour cells [38] and have an unlimited growth potential *in vitro* [39].

Outlined above are a few possible clinical limitations identified in this thesis. However, this does not invalidate some very important findings found during this project. One of the main finding is that bystander effects can be detected in patients with esophageal cancer. Additionally, smoking status is a key determinant of outcome; smokers produce weak or absent bystander signals compared to non-smokers. Furthermore, multiple exposures to bystander signals results in a cell survival increase during the pilot study. These findings may also have wider applicability in other human exposure situations including environmental exposures, accident situations, and planned low dose medical procedures. While there may be some limitations in this clinical work, these are not inherently bad since it provides a new opportunity to challenge other scientists to look more into the clinical relevance of bystander effects. Limitations are a part of science, and these study limitations will become another scientist's inspiration.

7.2 Future work

A new hypothesis for future work on smoking status is that nicotine promotes cell proliferation and radioresistance in human esophageal cancer cells. It is possible that samples harvested from smokers produce pro-growth signals post-irradiation because cigarette smoking can alter the normal damage response in cells [40]. Studies in the literature show nicotine has a role in cell proliferation, resistance to apoptosis, and radioresistance [20, 41]. Future clinical studies could continue to harvest blood serum and tissue samples to look further into the induced radioresistance responses observed following fractionation of brachytherapy. Future clinical studies could also investigate whether nicotine has a role in producing weak or absent bystander signals. Further *in vitro* work could also be carried out by using a panel of esophageal adenocarcinoma (FLO-1, KYAE-1, SK-GT-4, OE19, OE33, JH-EsoAd1, OACP4C, OACM5.1, ESO26 and ESO51) [37] and squamous cell carcinoma cell

lines (TE-1, TE-4, TE-5, TE-6, TE-7, TE-8, TE-9, TE-10, TE-11, TE-13) [42] recently authenticated in the literature.

Not all patients with advanced esophageal cancer respond similarly to radiation treatments, identification of subsets of patients who do or do not benefit from such a treatment is warranted in the future. By identifying certain environmental and genetic modifiers, radiotherapy regimens could be tailored more effectively. This thesis has made it clear that non-smokers produce (or respond to) bystander signals compared with smokers. Since humans are seldom exposed to one stressor at a time, testing a combination of multiple stressors along with radiation might significantly alter the therapeutic response. Future studies should focus on apoptotic protein expression in blood and tissue samples to predict individual susceptibility to bystander signal production during radiotherapy with a view to predicting treatment response (i.e. tumour shrinkage). Future work should also test bystander effects in the HRS dose region for OE19 and other esophageal cancer cell lines to elucidate the HRS/IRR response at lower radiation doses occurring in areas outside the treatment field. These future experiments may also clarify whether delivering doses in the HRS dose range (< 1 Gy) might enhance tumour sensitivity. This could have implications for radiotherapy and could help improve patient prognosis. In summary, it is recommended that these ideas, are investigated in future *in vivo* and *in vitro* projects to determine whether bystander effects in human cancer patients undergoing radiotherapy are harmful or beneficial.

7.3 Conclusion

This thesis presents the first work investigating radiation-induced bystander effects in esophageal adenocarcinoma using both cell models and human patients. The key contributions are:

1. Bystander signals are being generated in non-smoker cancer patients undergoing HDR brachytherapy and in EAC cell lines. Non-smokers have the ability to produce strong bystander signals whereas smokers produce a weak or absent signal.

2. Multiple exposures of brachytherapy suggest an induced adaptive response in non-irradiated

human keratinocytes.

3. The esophageal cancer cell lines tested were not serotonin dependent meaning there may be multiple mechanisms involved in the bystander effect.

4. A HRS response below 1 Gy of γ-irradiation was found in one esophageal cell line derived from an adenocarcinoma, which also showed bystander signal production in the same dose range.

Together, the studies in this thesis may contribute to the knowledge on bystander or even abscopal effects in radiotherapy. Since advanced stages of esophageal cancer is often treated with HDR brachytherapy and even EBRT, the information in the thesis adds to a very limited literature in an important field. Our findings with smoking status may have more general application in efforts to understand the wider implications of systemic smoking effects in carcinogenesis but also in radiotherapy response. This thesis motivates other research to identify the propagated soluble factors that promote radiation-induced signaling pathways in esophageal cancer, to develop risk based radiation exposure guidelines, and to develop clear bioassays and biomarkers for bystander effects.

7.4 References

[1] C Hanu et al. "The influence of smoking on radiation-induced bystander signal production in esophageal cancer patients". In: *Environmental Research* (2016).

[2] C. Pinho et al. "The involvement of serum serotonin levels producing radiation-induced bystander effects for an in vivo assay with fractionated high dose-rate (HDR) brachytherapy". eng. In: *Int. J. Radiat. Biol* 88.10 (Oct. 2012), pp. 791–797. ISSN: 1362-3095. DOI: 10.3109/09553002.2012.715794.

[3] C. Pinho et al. "Assessing patient characteristics and radiation-induced non-targeted effects in vivo for high dose-rate (HDR) brachytherapy". In: *Int. J. Radiat. Biol* (2015), pp. 1–9. DOI: 10.3109/09553002.2015.1068458.

[4] F. Lyng, C. Seymour, and C Mothersill. "Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis". In: *British journal of cancer* 83.9 (2000), p. 1223.

[5] C Mothersill and C Seymour. "Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells." In: *International journal of radiation biology* 71.4 (1997), pp. 421–427.

[6] K. Prise. "Studies of bystander effects in human fibroblasts using a charged particle microbeam". In: *International journal of radiation biology* 74.6 (1998), pp. 793–798.

[7] C. B. Seymour and C. Mothersill. "Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve". eng. In: *Radiation Research* 153.5 Pt 1 (May 2000), pp. 508–511. ISSN: 0033-7587.

[8] J. Fazzari et al. "Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect". In: *International journal of radiation biology* 88.10 (2012), pp. 786–790.

[9] F. Lyng, C. Semour, and C Mothersill. "Early events in the apoptotic cascade initiated in cells treated with medium from the progeny of irradiated cells". In: *Radiation protection dosimetry* 99.1-4 (2002), pp. 169–172.

[10] K. M. Prise and J. M. O'Sullivan. "Radiation-induced bystander signalling in cancer therapy". In: *Nature Reviews Cancer* 9.5 (2009), pp. 351–360.

[11] R. Iyer and B. E. Lehnert. "Alpha-particle-induced increases in the radioresistance of normal human bystander cells". In: *Radiation research* 157.1 (2002), pp. 3–7.

[12] C. Mothersill et al. "Individual variation in the production of abystander signal'following irradiation of primary cultures of normal human urothelium". In: *Carcinogenesis* 22.9 (2001), pp. 1465–1471.

[13] I. Koturbash et al. "Radiation-induced bystander effects in vivo are sex specific". In: *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 642.1 (2008), pp. 28–36.

[14] C. M. Hoff, C. Grau, and J. Overgaard. "Effect of smoking on oxygen delivery and outcome in patients treated with radiotherapy for head and neck squamous cell carcinoma–a prospective study". In: *Radiother Oncol.* 103.1 (2012), pp. 38–44. DOI: 10.1016/j.radonc.2012.01.011.

[15] D. W. Siemann, R. P. Hill, and R. S. Bush. "Smoking: The influence of carboxyhemoglobin (HbCO) on tumor oxygenation and response to radiation". In: *Int. J. Radiat. Oncol. Biol. Phys.* 4.7 (1978), pp. 657–662.

[16] P. Lawther and B. Commins. "Cigarette smoking and exposure to carbon monoxide". In: *Annals of the New York Academy of Sciences* 174.1 (1970), pp. 135–147.

[17] F. M. Lyng et al. "Effect of a tobacco-related nitrosamine on intercellular communication in human urothelial cells: a possible factor in smoking-related bladder carcinogenesis." In: *Oncology research* 8.9 (1995), pp. 371–378.

[18] R. Poon et al. "Bystander effects of ionizing radiation can be modulated by signaling amines". In: *Environmental research* 105.2 (2007), pp. 200–211.

[19] P. Dasgupta et al. "Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines". In: *International Journal of Cancer* 124.1 (2009), pp. 36–45.

[20] A. A. Khalil et al. "Nicotine enhances proliferation, migration, and radioresistance of human malignant glioma cells through EGFR activation". In: *Brain tumor pathology* 30.2 (2013), pp. 73–83.

[21] S. Wright, J. Zhong, and J. Larrick. "Inhibition of apoptosis as a mechanism of tumor promotion." In: *The FASEB journal* 8.9 (1994), pp. 654–660.

[22] S. A. Grando. "Connections of nicotine to cancer". In: *Nature Reviews Cancer* 14.6 (2014), pp. 419–429.

[23] C. Mothersill et al. "Serum serotonin levels determine the magnitude and type of bystander effects in medium transfer experiments". In: *Radiation research* 174.1 (2010), pp. 119–123.

[24] L. A. Ryan et al. "Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response". In: *International journal of radiation biology* 85.1 (2009), pp. 87–95.

[25] C. Mothersill, C. Seymour, and M. Joiner. "Relationship between radiation-induced low-dose hypersensitivity and the bystander effect". In: *Radiation research* 157.5 (2002), pp. 526–532.

[26] N. Lynam-Lennon et al. "MicroRNA-31 modulates tumour sensitivity to radiation in oesophageal adenocarcinoma". In: *Journal of molecular medicine* 90.12 (2012), pp. 1449–1458.

[27] A.-C. Heuskin et al. "Low-dose hypersensitivity and bystander effect are not mutually exclusive in a549 lung carcinoma cells after irradiation with charged particles". In: *Radiation research* 180.5 (2013), pp. 491–498.

[28] O. Nuta and F. Darroudi. "The impact of the bystander effect on the low-dose hypersensitivity phenomenon". In: *Radiation and environmental biophysics* 47.2 (2008), pp. 265–274.

[29] S. C. B. Fernandez-Palomo C and C. Mothersill. "Inter-relationship between low-dose hyper-radiosensitivity and radiation-induced bystander effects in the human T98G glioma and the epithelial HaCaT cell line". In: *Accepted. Rad Res.* (2016).

[30] L. Enns et al. "Low-Dose Radiation Hypersensitivity Is Associated With p53-Dependent Apoptosis11U. S. Department of Energy, University of California Lawrence Livermore National Laboratory contract W-7405-Eng-48 (KT Bogen), National Cancer Institute (Canada) grant 013104 (M. Weinfeld), Alberta Cancer Board Bridge and Pilot grant R-418 (AD Murtha), and US Department of Energy Low-Dose Radiation Research Program (KT Bogen)." In: *Molecular Cancer Research* 2.10 (2004), pp. 557–566.

[31] C. D. Fichter et al. "Occurrence of multipolar mitoses and association with Aurora-A/-B kinases and p53 mutations in aneuploid esophageal carcinoma cells". In: *BMC cell biology* 12.1 (2011), p. 1.

[32] F. Faul et al. "G* Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences". In: *Behavior research methods* 39.2 (2007), pp. 175–191.

[33] C. Mothersill and C. Seymour. "Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium". In: *Radiation research* 149.3 (1998), pp. 256–262.

[34] L. A. Ryan et al. "Dilution of irradiated cell conditioned medium and the bystander effect". eng. In: *Radiation research* 169.2 (Feb. 2008), pp. 188–196. ISSN: 0033-7587. DOI: 10.1667/RR1141.1.

[35] H. Furlong et al. "Apoptosis is signalled early by low doses of ionising radiation in a radiation-induced bystander effect". In: *Mutat Res Fund Mol Mech Mut* 741 (2013), pp. 35–43. DOI: 10.1016/j.mrfmmm.2013.02.001.

[36] H. Furlong et al. "Identification of Key Proteins in Human Epithelial Cells Responding to Bystander Signals From Irradiated Trout Skin". In: *Dose-Response* 13.3 (2015), p. 1559325815597669.

[37] J. J. Boonstra, H. W. Tilanus, and W. N. Dinjens. "Translational research on esophageal adenocarcinoma: from cell line to clinic". In: *Diseases of the Esophagus* 28.1 (2015), pp. 90–96.

[38] A. F. Gazdar, B. Gao, and J. D. Minna. "Lung cancer cell lines: Useless artifacts or invaluable tools for medical science?" In: *Lung Cancer* 68.3 (2010), pp. 309–318.

[39] J. R. Masters. "Human cancer cell lines: fact and fantasy". In: *Nature reviews Molecular cell biology* 1.3 (2000), pp. 233–236.

[40] C. Mothersill and C. Seymour. "Radiation-induced bystander effects: evidence for an adaptive response to low dose exposures?" In: *Dose-response* 4.4 (2006), dose–response.

[41] S. Wright et al. "Nicotine inhibition of apoptosis suggests a role in tumor promotion." In: *The FASEB Journal* 7.11 (1993), pp. 1045–1051.

[42] J. J. Boonstra et al. "Mistaken identity of widely used esophageal adenocarcinoma cell line TE-7". In: *Cancer research* 67.17 (2007), pp. 7996–8001.

Appendix A

Appendix

A.1 Cell lines

Table A.1. Cell Line Characteristics

Cell Name	Tissue	Disease	p53 status	Doubling Time
HPV-G	Skin	Normal (immortalized)	p53 suppressed	22h [1]
HaCaT	Skin	Normal (immortalized)	mutated	21h [2]
OE19	Esophagus	Esophageal adenocarcinoma stage III	mutated	50h[3]
OE33	Esophagus	Esophageal adenocarcinoma stage IIA	mutated	30h [4]
HCT116	Colon	Colon Cancer	wildtype	16-18h[5, 6]

A.1.1 Human keratinocyte cell lines

The HPV-G cells have been derived from a neonatal human foreskin [7] and obtained as a gift from J. Di Paolo, NIH, Bethedsa. They are an non-transformed human keratinocyte immortalized with the human papillomavirus 16, HPV16, to proliferate indefinitely in culture. HPV-G cells have a cobblestone morphology and has a doubling time of 22 hours [1]. These cells have their TP53 suppressed by E6 oncogene, but even so they still have 30% expression of their wild-type p53 protein [7].

The human HaCaTs keratinocytes were used as a reliable reporter cell model during our phase II clinical study. These cell lines are well-established and have been well-documented to produce bystander signals and have a very stable bystander response reported in the literature [1, 8, 9]. The

HaCaT cells are an non-transformed immortal human keratinocytes with a doubling time of 21 hours [2]. A deletion of the entire TP53 gene in one allele and a point mutation in second allele has rendered these cells to be p53 mutated. These cells were kindly sent by Dr. Orla Howe from the Dublin Institute of Technology (DIT).

A.1.2 Human esophageal and colon cancer cell lines

The OE33 cells are derived from a primary tumour, clinically diagnosed as a stage IIA EAC (Barrett's tumour), in a 73-year-old female patient. The OE33 cells were receive as a gift from Dr. Niamh Lynam-lennon from Trinity College Dublin, Ireland. These cell lines have a doubling time of 30 hours [4] and have a p53 protein with a point mutation on codon 135 (cysteine $\rightarrow$ tyrosine) [10]. The OE19 cells are derived from a primary tumour, clinically diagnosed as a stage III EAC, in a 72-year-old male patient. The OE19 cells have been documented to be inherently radioresistant [11], and were purchased from Sigma-Aldrich (Oakville, ON, Canada). These cell lines have a rather long doubling time of 50 hours [3] and have been documented as p53 mutated [10].

The HCT116 p53 wildtype was derived from a human colon cancer [12] and have been documented to have functional p53 [12, 13]. These cell lines have a doubling time of 16-18h[5, 6]. These cells were received as a gift from Dr. Robert Bristow from Princess Margaret Hospital, Toronto, ON, Canada. One very important reason for selecting this cancer cell line as a reporter model for our medium transfer bystander experiments was that these cells were found to have the ability to produce and respond to bystander signals [13, 14].

A.1.3 Mycoplasma sensor cells

This subsection outlines the importance of having routine detection methods in place for mycoplasma contamination. One of the smallest and simplest organisms are known as mycoplasma. These organisms lack a cell wall, which means that antibiotics are ineffective against these contaminants. Mycoplasma have often been referred to as a parasite since they take up residence inside cells which can alter the host cell's DNA synthesis, induce chromosomal aberrations, influence signal

transduction and promote cellular transformation[15]. Therefore, it is imperative that routine detection methods are put forth to prevent erroneous results from contaminated cell lines. Contamination cannot always be eliminated, but this is precisely the reason for having established and routine detection methods in place [16]. A brief overview of the PlasmoTest and mycoplasma sensor cell line is described below.

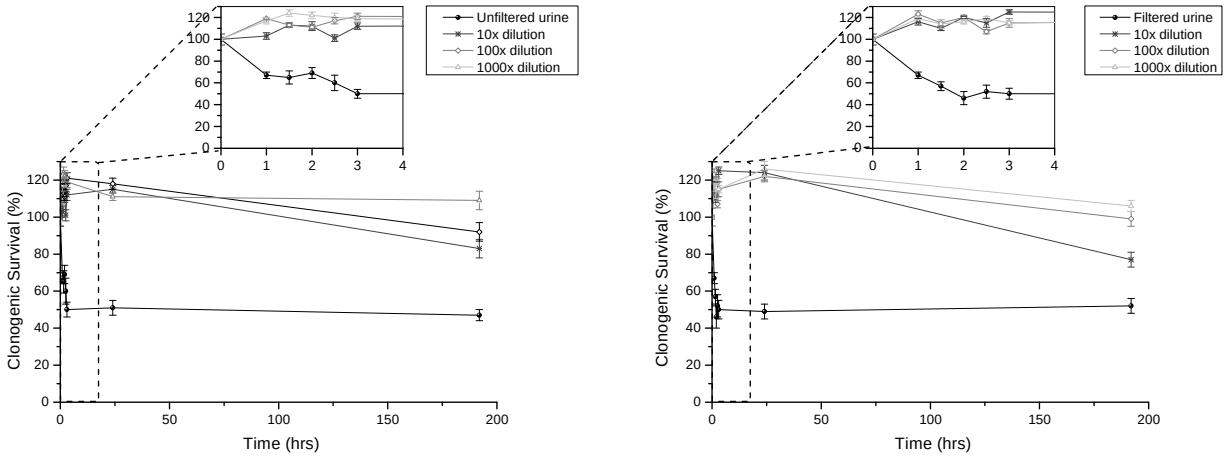
The Hek-Blue-2 cells are engineered human embryonic kidney cells (HEK 293) that have been transfected with the Toll-like Receptor 2 (TLR2) gene along with a number of genes involved in the recognition pathway of mycoplasma contaminants [17]. This cell line was included in the Plasmotest^{TM} Mycoplasma detection kit (Catalog # rep-pt1), which was purchased from Cedarlane (Burlington, ON, Canada). These cells are extremely sensitive to mycoplasmal lipoproteins, which makes them an ideal screening tool for mycoplasma infected cultures. The Plasmotest was utilized as a screening tool to eliminate the risk of using mycoplasma infected cell cultures for experiments.

The growth medium used for the routine maintenance of HEK-Blue-2 cells was Dulbecco's modified eagle medium (DMEM) with 4.5 g/L of glucose (Invitrogen, Burlington, ON, Canada). DMEM was supplemented with 10% foetal bovine serum (FBS) (Gibco, Burlington, ON, Canada), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen, Burlington, ON, Canada), 2mM L-Glutamine (Invitrogen, Burlington, ON, Canada), 100 µg/ml Normocin^{TM} and 1x HEK-Blue selection^{TM} mix. Normocin^{TM} is a patent antibiotic mix with the capabilities to combat mycoplasma, bacteria, and fungus contaminants in the HEK-Blue-2 cells. The HEK-Blue selection^{TM} mix is also a patent formulation consisting of many selective antibiotics required to maintain the transfected transgenes integrity. All cell cultures were performed in a class II biosafety cabinet at McMaster University to prevent the spread of the adenovirus agent that is contained within these cells. Routine subculturing was performed on cell stocks reaching 70–80% confluency by using a Falcon 18 mm cell scraper (VWR, Burlington, ON, Canada). Cell stocks were grown in 75cm^2 flasks (T-75) filled with 15 ml of supplemented growth medium. Cell stocks were incubated at 37°C and 5% carbon dioxide in air. The HEK-Blue-2 cell line was kept at a low passage number since a passage number higher than 30 was not guarantee to possess the same TLR2 gene expression necessary for mycoplasma detection.

The PlasmoTest^{TM} kit by InvivoGen was used to visually detect mycoplasma contamination in cell cultures. This colourimetric assay is highly robust and utilizes a cell line to signal the presence of mycoplasma contaminants in cultured cells. As previously mentioned above, the Hek-Blue-2 cells have been engineered to express the TLR2, which is a pathogen recognition receptor that detects the lipoproteins found surrounding mycoplasma [17].

Quarantine cell cultures were detached by using a cell scraper and centrifuged at 1500 rpm for 5 minutes. 500 µl of the supernatant was collected and transferred to a 5mL sterile tube. Samples were heated for 100 °C for 15 minutes to rid the samples of any external sources of phosphatases not secreted by the HEK-Blue-2 cells. External phosphatases will produce a false positive result. The HEK-Blue-2 detection medium is solubilized in 50 mL of ultra-pure water and then sterilized by using a 0.22 µm Nalgene filter. In a 96 well-plate, 50 µl of each heated sample was pipetted into their respective wells. 50 µl of the positive and negative controls were also placed into their respective wells. HEK-Blue-2 cells were scraped to form a suspension with pre-warmed detection medium. 50,000 cells were plated into each well containing a sample of supernatant to be tested and negative and positive controls. The well-plate was incubated at 37 °C and 5% carbon dioxide in air incubator for 16 to 20 hours. After incubation, the presence of Mycoplasma was detected by a change in the detection medium from pink to blue.

A.1.3.1 Urine toxicity testing



(a) (b)

Figure A.1. Urine samples were taken from 3 healthy individuals and used for toxicity testing to develop urine colony-forming assay. A 10 fold serial dilution was performed on the urine samples and supplemented onto HPV-G reporter cells. The urine samples were filtered or left unfiltered to assess whether filtration improved cell survival. The graphs are showing the clonogenic survival of HPV-G cells after being exposed to either concentrated or diluted unfiltered (a) and filtered urine samples (b) at various time points. 3 measurements were set-up per sample, *n=9*.

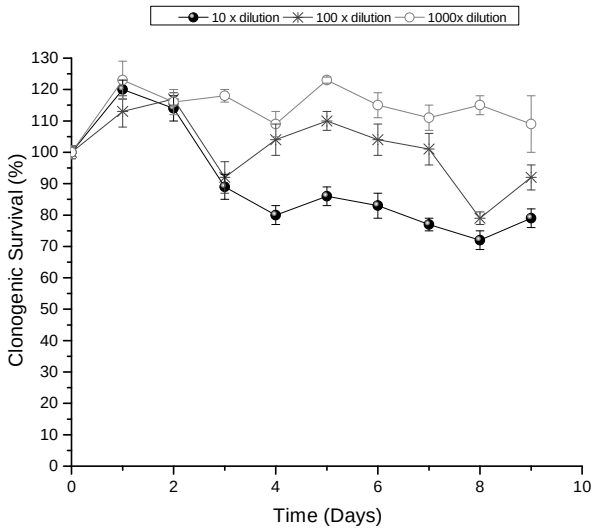


Figure A.2. A 10 fold serial dilution was performed with a urine sample taken from a healthy individual. Diluted urine samples were supplemented onto HPV-G reporter cells and survival was assessed over a span of 8 days.

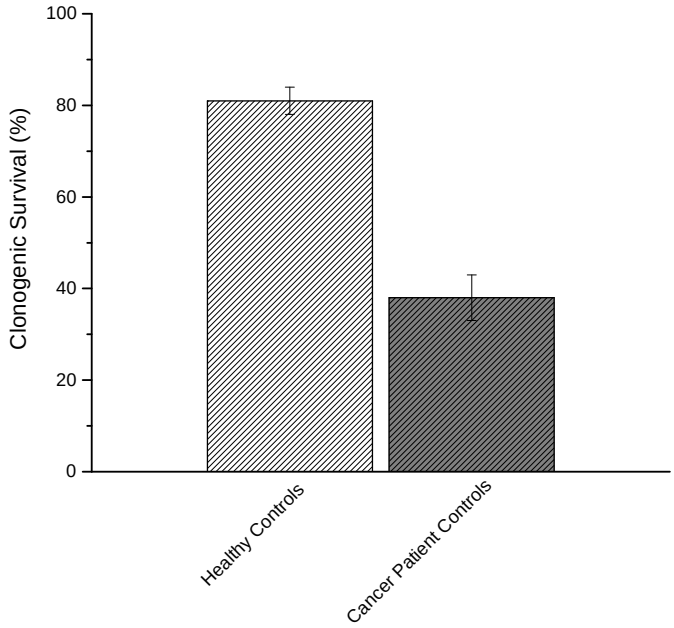


Figure A.3. The clonogenic survival (%) of HPV-G reporter cells exposed to healthy control urine samples and cancer patients baseline urine samples. There was a notable difference between 6 healthy control samples and 8 cancer patient urine samples, as illustrated in All values are mean ± SEM for *n=6* and *n=14* for healthy control and cancer patients baseline urine samples, respectively.

A.1.4 Ratiometric calcium

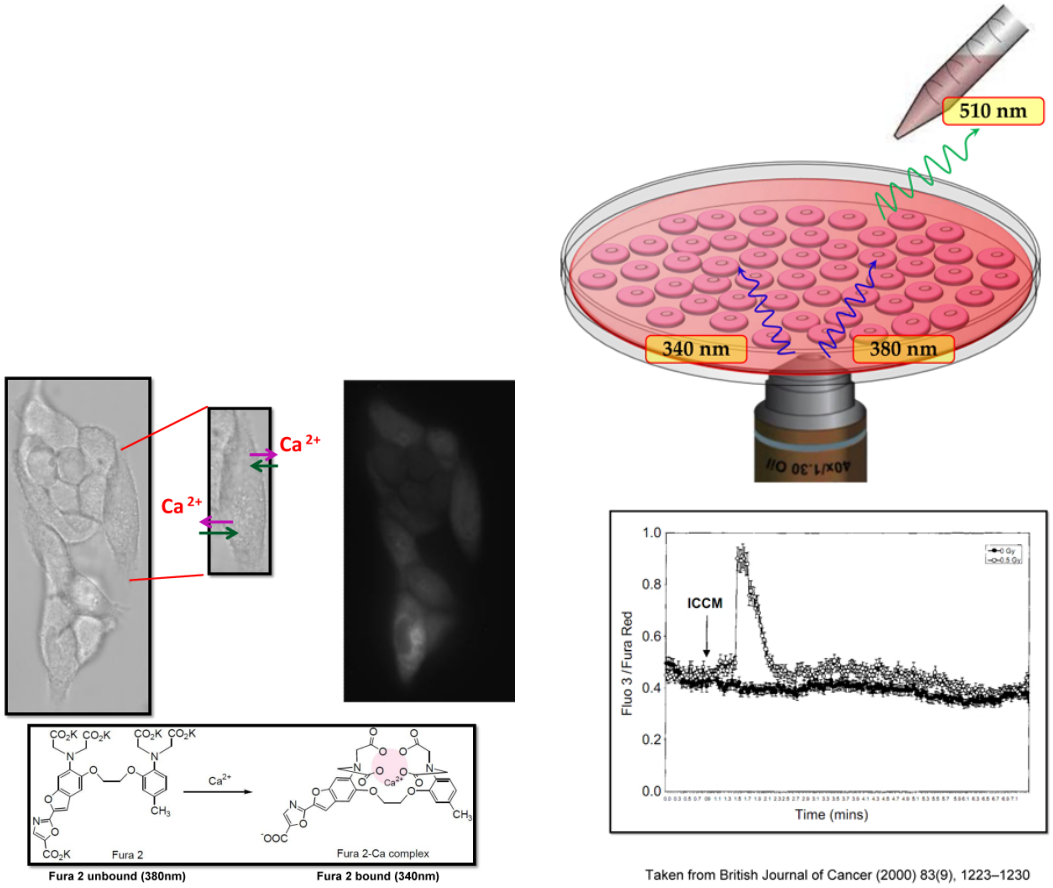
Intracellular calcium can be responsible for a number of cellular responses, including apoptosis and mitogenesis, and the intracellular concentrations are tightly regulated for this very reason[18]. For example, extracellular levels of calcium are roughly 3 to 4 mM whereas the intracellular calcium levels are kept below 100 nM[18]. Calcium movement into and out of a cell can result from a variety of stimuli. In particular, bystander medium, or mostly known as ICCM, is the stimulus of great importance for our research group when performing ratiometric calcium measurements. The movement of divalent calcium into a cell may occur through specific channels including LGICs and G protein-coupled receptors (GPCRs) [18]. Once intracellular calcium is taken into a cell a variety of responses may be elicited downstream, such as apoptosis[19]. There are several calcium binding proteins and organelles (ie. sarcoplasmic reticulum) that clear the cytosol of calcium after a response is no longer necessary, which is the reason why the response is often referred to as transient. In 1985, Grynkiewicz and colleagues found a way to measure calcium fluxes by use of certain calcium probes,

such as fura2. The authors referred to this method as Ratiometric calcium. Nearly one decade and a half later, Fiona Lyng and colleagues [19] adopted this technique to monitor bystander effects by measuring increases in the reporter cells' cytoplasmic calcium exposed to 0.5 Gy ICCM. Reporter cells displaying a distinct calcium influx would translate to an early indicator for apoptosis in cells.

Fura-2 acetoxymethyl (AM) ester is non-polar resulting in the calcium probe to be transported through the cell membrane into the cytosol [18]. The Fura2 AM ester groups are removed once inside the cell by intracellular esterases. This leads to the calcium probe becoming polar and unable to escape and travel back outside of the cell through the membrane. The calcium probe, Fura 2, consists of a flurophore region and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, (BAPTA) group that has a calcium sensitive region for binding [20]. BAPTA is a calcium chelator that selectively binds to calcium via carboxylic groups. Often times there is a fluorophore attached to the BAPTA group, which shifts the excitation from 380 nm to 340 nm once calcium has become bound to the BAPTA [21]. In this thesis, the calcium probe, Fura 2AM, is used to investigate the role of calcium cell signalling in RIBEs. Below is a detailed explanation of the necessary steps taken to measure intracellular calcium fluxes in bystander cells exposed to ICCM taken off of donor cells previously irradiated.

Mothersill's research group and others used HPV-G cultures as reporters to monitor calcium fluxes [19, 22]. Unfortunately, this cell line was lost by a mycoplasma infection in 2013, and as a result HaCaT cells were optimized for ratiometric calcium measurements. HaCaT cells were seeded in glass bottom dishes coated with poly-d-lysine(MatTek corporation, Ashland, MA) at a density of approximately 100,000 cells per dish. The cells were incubated at 37 ° C and 5% CO_2 for 24 h before calcium measurements. Cells were washed three times with Hank's Balanced salt solution (HBSS) calcium buffer (invitrogen,ON) supplemented with 25 mM of Hepes (invitrogen,ON). Cells were incubated with 1000 µl of 8.4 µM Fura-2 AM (Sigma-Aldrich, Oakville, ON) for 50 minutes at 37 ° C and 20 minutes at room temperature to aid in the de-esterification process as previously described by Biro and collaegues [23, 24]. Prior to imaging, cells were rinsed three times and then 300 µl of imaging buffer was added to the dish. Equipment used for calcium measurements are Photon Technology International (PTI) lamp power supply, xenon arc lamp and random access

monochromator with a liquid light guide that is coupled to an Olympus 1x81 microscope and computer. UV excitation wavelengths, 380 and 340nm, are used to excite the Fura-2 loaded into the cells. Images were taken for 13 minutes after adding100 µl of either CCCM or ICCM to reporter cells once a stable baseline was reached at roughly 80 seconds. Figure A.4 shows an illustration of a reporter cell line exposed to ICCM or CCCM medium during ratiometric calcium measurements.



(a) (b)

Figure A.4. The brightfield image with HaCaT human kerationcyte cultures is depicting a group of cells selected for calcium imaging. The fluorescence image of the cells after being loaded with a calcium probe, fura2 is excited at 380 nm is to the right of the brightfield image, as shown in (a). Unbound fura2 is excited with 380 nm UV wavelengths and calcium bound fura2 is excited with 340 nm UV wavelengths. Once calcium binds to the sensitive regions on the fura, the spectral properties of the fluorescent indicator changes and the UV excitation wavelength used is 340 nm. (b) is showing an illustration of a reporter cell line exposed to ICCM or CCCM medium and then excited with 340 and 380 nm wavelengths to activate bound and unbound fura2, respectively. Both excitation wavelengths emits a green 510 emission wavelengths.

A.2 Sample size calculations

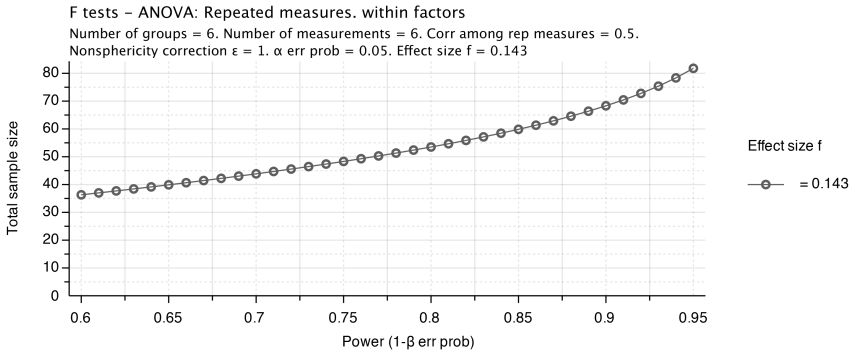


Figure A.5. A power analysis with G*Power software [25] for a repeated measure analysis found that with an α error probability (err prob) of 0.05 and power (1-β err prob) of 0.95 we would need a sample size of 84.

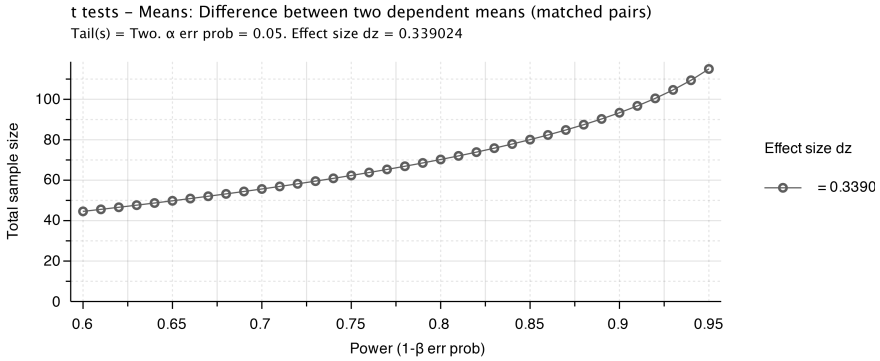
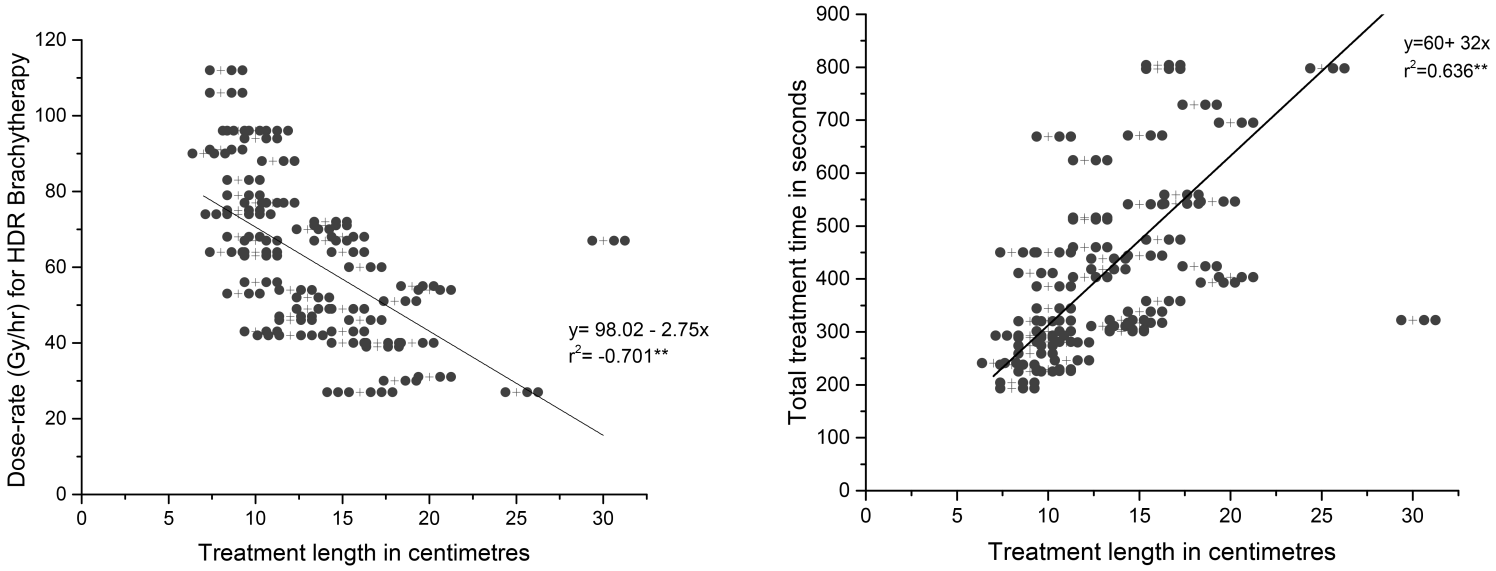


Figure A.6. In our phase II study, blood samples were collected before and after one single fraction of brachytherapy so a larger sample size could be obtained. The larger sample size would be used to generalize the data to a population of cancer patients undergoing brachytherapy. Power calculations were performed with G*Power software [25] by setting a power (1-β) at 0.95 and α=0.05. The mean difference and standard deviation obtained during the one-year pilot study was used to calculate the effect size of 0.339. We found that with an α err prob of 0.05 and power (1-β err prob) of 0.95 we would need a sample size of 115.

A.3 Supplementary information for phase II clinical study



(a) (b)

Figure A.7. As illustrated in graph A.7(a) the relationship between total treatment time in seconds and tumour length treated in centimeters were assessed. Also, the relationship between dose-rate (Gy/h) and tumour length treated in centimeters are outlined in graph A.7(b). These regression models are suggesting that nearly 60% to 70% of the dose-rate variability can be explained by the size of the tumour, as patients with larger tumours require a longer treatment time that ultimately effects the dose-rate.Other factors that could explain the dose-rate variability are dependent on the iridum-192 source decay and source renewal.

Acid control

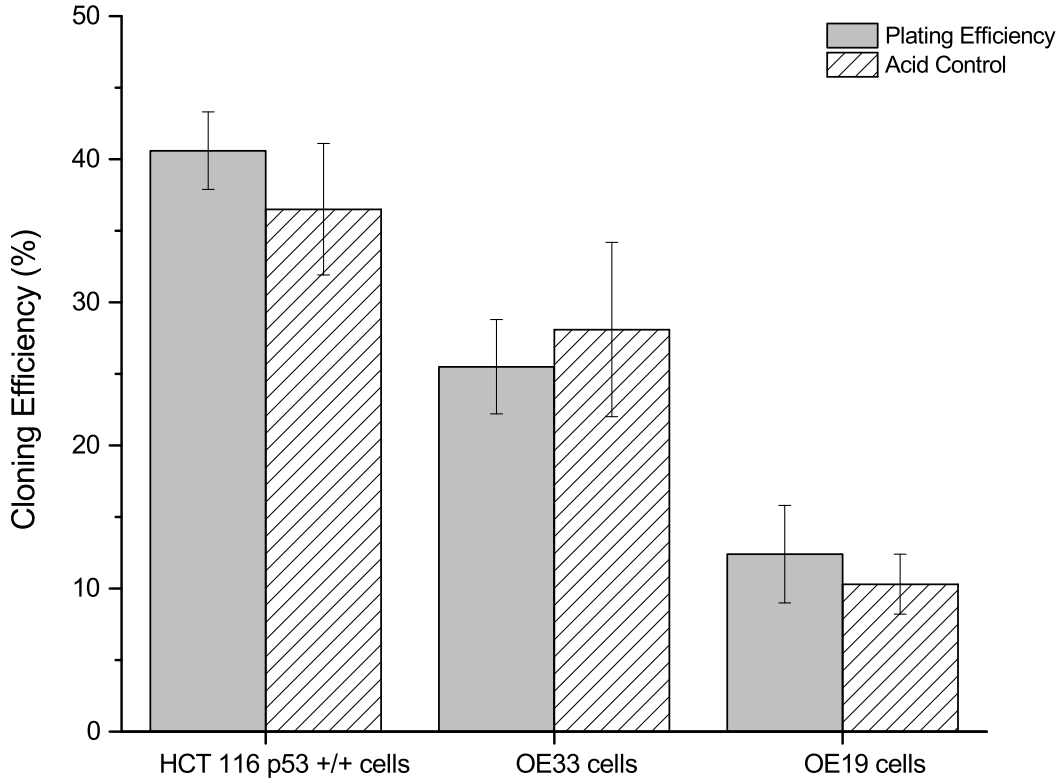


Figure A.8. An acid control was set-up since serotonin was initially dissolved in 0.1 M HCL. These acid controls showed no significant changes in cloning efficiency compared to the control samples for HCT 116 p53 +/+, OE33, and OE19 cells.

A.4 References

[1] C. Mothersill et al. "Serum serotonin levels determine the magnitude and type of bystander effects in medium transfer experiments". In: *Radiation research* 174.1 (2010), pp. 119–123.

[2] P. Boukamp et al. "Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line." In: *The Journal of cell biology* 106.3 (1988), pp. 761–771.

[3] DSMZ. *Cell line: OE-19*. 2015. URL: https://www.dsmz.de/catalogues/details/culture/ACC-700.html.

[4] DSMZ. *Cell line: OE-33*. 2015. URL: https://www.dsmz.de/catalogues/details/culture/ACC-706.html.

[5] Y. Zhou et al. "Transcriptional Regulation of Mitotic Genes by Camptothecin-induced DNA Damage Microarray Analysis of Dose-and Time-dependent Effects". In: *Cancer Research* 62.6 (2002), pp. 1688–1695.

[6] G. Li, C. Nelsen, and E. A. Hendrickson. "Ku86 is essential in human somatic cells". In: *Proceedings of the National Academy of Sciences* 99.2 (2002), pp. 832–837.

[7] L. Pirisi et al. "Continuous cell lines with altered growth and differentiation properties originate after transfection of human keratinocytes with human papillomavirus type 16 DNA". In: *Carcinogenesis* 9.9 (1988), pp. 1573–1579.

[8] C. Mothersill and C. Seymour. "Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium". In: *Radiation research* 149.3 (1998), pp. 256–262.

[9] L. A. Ryan et al. "Dilution of irradiated cell conditioned medium and the bystander effect". eng. In: *Radiation research* 169.2 (Feb. 2008), pp. 188–196. ISSN: 0033-7587. DOI: 10.1667/RR1141.1.

[10] J. J. Boonstra et al. "Verification and unmasking of widely used human esophageal adenocarcinoma cell lines". In: *Journal of the National Cancer Institute* 102.4 (2010), pp. 271–274.

[11] N. Lynam-Lennon et al. "MicroRNA-31 modulates tumour sensitivity to radiation in oesophageal adenocarcinoma". In: *Journal of molecular medicine* 90.12 (2012), pp. 1449–1458.

[12] F Bunz et al. "Requirement for p53 and p21 to sustain G2 arrest after DNA damage". In: *Science* 282.5393 (1998), pp. 1497–1501.

[13] C. Mothersill et al. "A role for p53 in the response of bystander cells to receipt of medium borne signals from irradiated cells". In: *International journal of radiation biology* 87.11 (2011), pp. 1120–1125.

[14] E. Kalanxhi and J. Dahle. "The role of serotonin and p53 status in the radiation-induced bystander effect". In: *International journal of radiation biology* 88.10 (2012), pp. 773–776.

[15] H. G. Drexler and C. C. Uphoff. "Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention". In: *Cytotechnology* 39.2 (2002), pp. 75–90.

[16] L. Nikfarjam and P. Farzaneh. "Prevention and detection of Mycoplasma contamination in cell culture". In: *Cell Journal (Yakhteh)* 13.4 (2012), p. 203.

[17] InvivoGen. *PlasmoTest^{TM} Mycoplasma Detection Kit Method based on the activation of Toll-Like Receptor 2 for the detection of Mycoplasma contaminations of cell lines*. 2015. URL: `http://www.invivogen.com/PDF/PlasmoTest_rep_pt1_TDS.pdf`.

[18] J. Howland. "The Encyclopedia of Molecular Biology". In: *Biochemical Education* (1995).

[19] F. Lyng, C. Seymour, and C Mothersill. "Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis". In: *British journal of cancer* 83.9 (2000), p. 1223.

[20] J. R. Lakowicz. *Principles of fluorescence spectroscopy*. Springer Science & Business Media, 2013.

[21] R Haughland. *Handbook of Fluorescent Compounds and Research Products, Molecular Probes*. 2002.

[22] J. Fazzari et al. "Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect". In: *International journal of radiation biology* 88.10 (2012), pp. 786–790.

[23] T. Bíró et al. "Distinct subpopulations in HaCaT cells as revealed by the characteristics of intracellular calcium release induced by phosphoinositide-coupled agonists". In: *Archives of dermatological research* 290.5 (1998), pp. 270–276.

[24] L Csernoch, J Hunyadi, and L Kovacs. "Calcium release activated calcium entry in a human skin derived cell line (HaCaT)". In: *Experimental dermatology* 9.3 (2000), pp. 200–205.

[25] F. Faul et al. "G* Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences". In: *Behavior research methods* 39.2 (2007), pp. 175–191.

A.5 Clinical data

A.5.1 Pilot study

Patients	Blood_BeforeT1	Blood_AfterT1	Blood_Before T2	Blood_After T2	Blood_Before T3	Blood_After T3	Blood_Follow up	BiopsyT1	BiopsyT3
1.00	59	47.00	.	.	.	.	.	.	.
1.00	56	51.00	.	.	.	.	.	.	.
1.00	62	50.00	.	.	.	.	.	.	.
2.00	20	46.80	40.00	28.00	37.00	26.00	45.00	.	.
2.00	23	50.40	51.00	38.00	33.00	25.00	48.00	.	.
2.00	23	45.80	34.00	28.00	37.00	26.00	.	.	.
3.00	26	39.80	50.00	50.00	49.00	48.00	45.00	.	.
3.00	23	40.00	55.00	44.00	39.00	40.00	37.00	.	.
3.00	23	40.00	50.00	50.00	46.00	46.00	41.00	.	.
4.00	21	35.00	47.00	52.00	43.00	51.00	.	17	62
4.00	15	28.00	60.00	51.00	48.00	59.00	.	29	49
4.00	14	31.00	42.00	52.00	55.00	54.00	.	32	31
5.00	24	33.00	39.00	48.00	55.00	70.00	.	8	15
5.00	27	30.00	48.00	62.00	56.00	70.00	.	9	9
5.00	27	33.00	50.00	48.00	58.00	69.00	.	0	23
6.00	32	51.00	49.00	55.00	39.00	62.00	.	51	76
6.00	34	47.00	52.00	53.00	22.00	42.00	.	38	44
6.00	36	49.00	43.00	63.00	36.00	54.00	.	38	37
7.00	36	26.00	.	.	.	.	.	.	.
7.00	43	24.00	.	.	.	.	.	.	.
7.00	43	29.00	.	.	.	.	.	.	.
8.00	60	47.00	26.00	30.00	48.00	50.00	43.00	.	.
8.00	48	41.00	24.00	32.00	51.00	52.00	39.00	.	.
8.00	43	40.00	29.00	31.00	59.00	51.00	42.00	.	.

Patients	Blood_BeforeT1	Blood_AfterT1	Blood_Before T2	Blood_After T2	Blood_Before T3	Blood_After T3	Blood_Follow up	BiopsyT1	BiopsyT3
9.00	.	.	.	.	.	.	.	.	.
9.00	.	.	.	.	.	.	.	.	.
9.00	.	.	.	.	.	.	.	.	.
10.00	52	53.00	38.00	47.00	65.00	60.00	35.00	.	.
10.00	51	44.00	33.00	47.00	58.00	61.00	34.00	.	.
10.00	58	44.00	37.00	41.00	46.00	57.00	37.00	.	.
11.00	25	41.00	15.00	19.00	36.00	18.00	35.50	100	83
11.00	30	50.00	17.00	13.00	27.00	20.00	.	115	101
11.00	39	48.00	19.00	16.00	32.00	16.00	.	85	101
12.00	28	31.00	34.00	37.00	28.00	43.00	41.80	67	80
12.00	30	32.00	41.00	43.00	32.00	44.00	.	82	94
12.00	33	40.00	36.00	38.00	29.00	41.00	.	81	74
13.00	10	3.00	.00	2.00	7.00	10.00	.	.	.
13.00	12	4.00	1.00	2.00	8.00	12.00	.	.	.
13.00	11	6.00	.00	2.00	6.00	16.00	.	.	.
14.00	.	.	.	.	.	.	.	.	.
14.00	.	.	.	.	.	.	.	.	.
14.00	.	.	.	.	.	.	.	.	.
15.00	32	29.00	34.00	45.00	27.00	37.00	.	123	109
15.00	25	33.00	38.00	50.00	29.00	37.00	.	123	101
15.00	27	35.00	36.00	51.00	31.00	31.00	.	98	105

Patients	Urine_Before...	Urine_AfterT1	Urine_Before...	Urine_AfterT2	Urine_BeforeT3	Urine_AfterT3	Urine_Foll...	UrineSurvival_BeforeT1	UrineSurvival_AfterT1	UrineSurvival_BeforeT2	UrineSurvival_AfterT2	UrineSurvival_BeforeT3	UrineSurvival_AfterT3	UrineSurvival_Followup
1.00	5.00	14.00	.	.	.	.	.	9.00	28.00	.	.	.	.	.
1.00	9.00	17.00	.	.	.	.	.	17.00	32.00	.	.	.	.	.
1.00	7.00	13.00	.	.	.	.	.	13.00	25.00	.	.	.	.	.
2.00	14.00	2.00	50.00	17.00	32.00	19.00	41.00	26.00	4.00	99.00	34.00	61.00	36.00	77.00
2.00	12.00	3.00	48.00	11.00	32.00	18.00	40.00	21.00	6.00	97.00	21.00	60.00	35.00	76.00
2.00	12.00	5.00	42.00	30.00	24.00	18.00	42.00	22.00	9.00	83.00	59.00	45.00	33.00	79.00
3.00	14.00	8.00	54.00	29.00	48.00	26.00	25.00	26.00	15.00	109.00	57.00	91.00	50.00	47.00
3.00	14.00	13.00	39.00	51.00	49.00	26.00	28.00	27.00	24.00	77.00	101.00	92.00	50.00	52.00
3.00	23.00	9.00	48.00	32.00	45.00	20.00	27.00	42.00	17.00	96.00	63.00	84.00	38.00	51.00
4.00	4.00	9.00	43.00	37.00	48.00	43.90	.	4.00	9.00	75.00	63.00	78.00	77.00	.
4.00	2.00	3.00	33.00	32.00	.	.	.	5.00	9.00	57.00	56.00	73.00	66.00	.
4.00	2.00	4.00	28.00	30.00	.	.	.	7.00	14.00	48.00	51.00	73.00	61.00	.
5.00	2.00	4.00	48.00	52.00	.	.	.	5.00	10.00	83.00	89.00	.	.	.
5.00	3.00	4.00	52.00	35.00	.	.	.	7.00	9.00	90.00	61.00	.	.	.
5.00	3.00	4.00	55.00	48.00	.	.	.	8.00	10.00	95.00	83.00	.	.	.
6.00	1.00	1.00	10.00	13.00	30.00	50.00	.	3.00	2.00	17.00	21.00	46.00	77.00	.
6.00	3.00	1.00	6.00	16.00	42.00	50.00	.	9.00	3.00	11.00	26.00	65.00	78.00	.
6.00	3.00	.00	13.00	18.00	45.00	43.00	.	8.00	1.00	22.00	30.00	70.00	67.00	.
7.00	31.00	35.00	.	.	.	.	.	68.00	76.00	.	.	.	.	.
7.00	36.00	30.00	.	.	.	.	.	79.00	64.00	.	.	.	.	.
7.00	29.00	32.00	.	.	.	.	.	63.00	69.00	.	.	.	.	.
8.00	3.00	20.00	.	.	.	.	.	7.00	41.00	.	.	.	.	.
8.00	3.00	15.00	.	.	.	.	.	7.00	32.00	.	.	.	.	.
8.00	4.00	14.00	.	.	.	.	.	8.00	30.00	.	.	.	.	.

Patients	Urine_Before...	Urine_AfterT1	Urine_Before...	Urine_AfterT2	Urine_BeforeT3	Urine_AfterT3	Urine_Foll...	UrineSurvival_BeforeT1	UrineSurvival_AfterT1	UrineSurvival_BeforeT2	UrineSurvival_AfterT2	UrineSurvival_BeforeT3	UrineSurvival_AfterT3	UrineSurvival_Followup
9.00	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9.00	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9.00	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10.00	3.00	20.00	.00	.00	.00	.00	.	7.00	41.00	.00	.00	.00	.00	.
10.00	3.00	15.00	.00	.00	.00	.00	.	7.00	32.00	.40	.00	.00	.00	.
10.00	4.00	14.00	.00	.00	.00	.00	.	8.00	30.00	.00	.00	.00	.80	.
11.00	33.00	26.00	20.00	27.00	22.00	19.00	38.00	91.00	71.00	62.00	83.00	62.00	53.00	121.00
11.00	34.00	23.00	18.00	23.00	23.00	20.00	33.00	94.00	65.00	55.00	71.00	63.00	56.00	105.00
11.00	32.00	28.00	18.00	25.00	24.00	19.00	.	89.00	78.00	57.00	77.00	67.00	54.00	.00
12.00	26.00	22.00	30.00	26.00	27.00	29.00	26.00	67.00	57.00	71.00	61.00	72.00	78.00	63.00
12.00	25.00	18.00	32.00	30.00	24.00	26.00	26.00	65.00	46.00	76.00	71.00	64.00	70.00	61.00
12.00	29.00	20.00	34.00	26.00	26.00	29.00	26.00	76.00	51.00	80.00	62.00	69.00	77.00	62.00
13.00	16.00	15.00	6.00	3.00	39.00	34.00	6.00	53.00	50.00	21.00	10.00	74.00	65.00	20.00
13.00	15.00	14.00	6.00	3.00	41.00	33.00	5.00	50.00	46.00	20.00	10.00	78.00	64.00	17.00
13.00	17.00	15.00	9.00	2.00	39.00	36.00	4.00	56.00	51.00	30.00	7.00	75.00	69.00	15.00
14.00	17.00	23.00	.	.	.	.	.	52.00	71.00	.	.	.	.	.
14.00	19.00	20.00	.	.	.	.	.	61.00	63.00	.	.	.	.	.
14.00	17.00	23.00	.	.	.	.	.	53.00	72.00	.	.	.	.	.
15.00	26.00	21.00	23.00	19.00	14.00	19.00	8.00	81.00	66.00	47.00	40.00	42.00	56.00	30.00
15.00	23.00	20.00	24.00	20.00	17.00	19.00	10.00	71.00	63.00	50.00	42.00	49.00	54.00	34.00
15.00	26.00	18.00	27.00	20.00	16.00	20.00	10.00	81.00	57.00	55.00	42.00	47.00	58.00	35.00

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	Lymph
1.00	Male	68	EA	Former	Stage IV	GEJ	M1	Nx
1.00	Male	68	EA	Former	Stage IV	GEJ	M1	Nx
1.00	Male	68	EA	Former	Stage IV	GEJ	M1	Nx
2.00	Female	59	EA	Current	Stage III	GEJ	M0	N1
2.00	Female	59	EA	Current	Stage III	GEJ	M0	N1
2.00	Female	59	EA	Current	Stage III	GEJ	M0	N1
3.00	Male	60	EA	Former	Stage III	GEJ	M0	N1
3.00	Male	60	EA	Former	Stage III	GEJ	M0	N1
3.00	Male	60	EA	Former	Stage III	GEJ	M0	N1
4.00	Male	59	EA	Former	Stage II	GEJ	M0	N1
4.00	Male	59	EA	Former	Stage II	GEJ	M0	N1
4.00	Male	59	EA	Former	Stage II	GEJ	M0	N1
5.00	Female	75	EA	Former	Stage III	Above GEJ	M0	N1
5.00	Female	75	EA	Former	Stage III	Above GEJ	M0	N1
5.00	Female	75	EA	Former	Stage III	Above GEJ	M0	N1
6.00	Male	60	EA	Former	Stage III	GEJ	M0	N0
6.00	Male	60	EA	Former	Stage III	GEJ	M0	N0
6.00	Male	60	EA	Former	Stage III	GEJ	M0	N0
7.00	Female	73	SCC	Current	Stage III	Above GEJ	M0	N1
7.00	Female	73	SCC	Current	Stage III	Above GEJ	M0	N1
7.00	Female	73	SCC	Current	Stage III	Above GEJ	M0	N1
8.00	Male	85	EA	Former	Stage III	GEJ	M0	N1
8.00	Male	85	EA	Former	Stage III	GEJ	M0	N1
8.00	Male	85	EA	Former	Stage III	GEJ	M0	N1

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	Lymph
9.00	Male	59	EA	Former	Stage IV	GEJ	M1	N1
9.00	Male	59	EA	Former	Stage IV	GEJ	M1	N1
9.00	Male	59	EA	Former	Stage IV	GEJ	M1	N1
10.00	Male	57	EA	Former	Stage IV	GEJ	M1	N1
10.00	Male	57	EA	Former	Stage IV	GEJ	M1	N1
10.00	Male	57	EA	Former	Stage IV	GEJ	M1	N1
11.00	Male	70	EA	Former	Stage III	GEJ	M0	N1
11.00	Male	70	EA	Former	Stage III	GEJ	M0	N1
11.00	Male	70	EA	Former	Stage III	GEJ	M0	N1
12.00	Male	81	EA	Former	Stage III	GEJ	M0	N0
12.00	Male	81	EA	Former	Stage III	GEJ	M0	N0
12.00	Male	81	EA	Former	Stage III	GEJ	M0	N0
13.00	Male	66	SCC	Former	.	Mid	M0	N0
13.00	Male	66	SCC	Former	.	Mid	M0	N0
13.00	Male	66	SCC	Former	.	Mid	M0	N0
14.00	Female	90	SCC	Never	.	Above GEJ	.	N1
14.00	Female	90	SCC	Never	.	Above GEJ	.	N1
14.00	Female	90	SCC	Never	.	Above GEJ	.	N1
15.00	Male	77	EA	Former	Stage IV	GEJ	M1	N1
15.00	Male	77	EA	Former	Stage IV	GEJ	M1	N1
15.00	Male	77	EA	Former	Stage IV	GEJ	M1	N1

Patients	Treatment_lengthT1	Treatment_lengthT2	Treatment_lengthT3	DoseRate1	DoseRate2	DoseRate3	Time1	Time2	Time3
1.00	.	.	.	109	.	.	198	.	.
1.00	.	.	.	109	.	.	198	.	.
1.00	.	.	.	109	.	.	198	.	.
2.00	16.00	14.00	13.00	61	41	43	353	526	508
2.00	16.00	14.00	13.00	61	41	43	353	526	508
2.00	16.00	14.00	13.00	61	41	43	353	526	508
3.00	15.00	14.00	15.00	41	41	38	528	526	570
3.00	15.00	14.00	15.00	41	41	38	528	526	570
3.00	15.00	14.00	15.00	41	41	38	528	526	570
4.00	12.00	12.00	10.00	38	84	96	564	256	225
4.00	12.00	12.00	10.00	38	84	96	564	256	225
4.00	12.00	12.00	10.00	38	84	96	564	256	225
5.00	14.00	11.00	13.00	34	90	77	641	239	281
5.00	14.00	11.00	13.00	34	90	77	641	239	281
5.00	14.00	11.00	13.00	34	90	77	641	239	281
6.00	12.00	14.00	13.00	38	74	77	569	291	281
6.00	12.00	14.00	13.00	38	74	77	569	291	281
6.00	12.00	14.00	13.00	38	74	77	569	291	281
7.00	16.00	.	.	65	.	.	332	.	.
7.00	16.00	.	.	65	.	.	332	.	.
7.00	16.00	.	.	65	.	.	332	.	.
8.00	12.00	12.00	12.00	84	79	78	258	273	278
8.00	12.00	12.00	12.00	84	79	78	258	273	278
8.00	12.00	12.00	12.00	84	79	78	258	273	278

Patients	Treatment_lengthT1	Treatment_lengthT2	Treatment_lengthT3	DoseRate1	DoseRate2	DoseRate3	Time1	Time2	Time3
9.00	10.00	.	.	.	.	.	.	.	.
9.00	10.00	.	.	.	.	.	.	.	.
9.00	10.00	.	.	.	.	.	.	.	.
10.00	9.00	10.00	10.00	106	92	90	205	235	239
10.00	9.00	10.00	10.00	106	92	90	205	235	239
10.00	9.00	10.00	10.00	106	92	90	205	235	239
11.00	11.00	11.00	10.00	54	50	53	403	431	405
11.00	11.00	11.00	10.00	54	50	53	403	431	405
11.00	11.00	11.00	10.00	54	50	53	403	431	405
12.00	9.00	10.00	10.00	58	51	91	370	424	238
12.00	9.00	10.00	10.00	58	51	91	370	424	238
12.00	9.00	10.00	10.00	58	51	91	370	424	238
13.00	20.00	18.00	16.00	42	45	60	519	482	360
13.00	20.00	18.00	16.00	42	45	60	519	482	360
13.00	20.00	18.00	16.00	42	45	60	519	482	360
14.00	16.00	.	.	50	.	.	431	.	.
14.00	16.00	.	.	50	.	.	431	.	.
14.00	16.00	.	.	50	.	.	431	.	.
15.00	10.00	10.00	13.00	74	70	55	291	308	391
15.00	10.00	10.00	13.00	74	70	55	291	308	391
15.00	10.00	10.00	13.00	74	70	55	291	308	391

A.5.2 Phase II clinical study

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	DoseRate	Dose	Time	Treatment_length	Cloning_before	Cloning_after	Dysphagia_start	Dysphagia_end
1.00	1	79	2	3	1	1	1	68	6.00	320	9.00	55.00	36.00	3.00	2.00
1.00	1	79	2	3	1	1	1	68	6.00	320	9.00	57.00	37.00	3.00	2.00
1.00	1	79	2	3	1	1	1	68	6.00	320	9.00	56.00	36.00	3.00	2.00
2.00	2	77	2	3	2	3	2	40	6.00	542	17.00	60.00	53.00	3.00	1.00
2.00	2	77	2	3	2	3	2	40	6.00	542	17.00	58.00	54.00	3.00	1.00
2.00	2	77	2	3	2	3	2	40	6.00	542	17.00	64.00	53.00	3.00	1.00
3.00	2	67	2	1	3	3	2	63	6.00	344	10.00	37.00	40.00	4.00	2.00
3.00	2	67	2	1	3	3	2	63	6.00	344	10.00	26.00	43.00	4.00	2.00
3.00	2	67	2	1	3	3	2	63	6.00	344	10.00	24.00	40.00	4.00	2.00
4.00	2	60	2	3	2	2	1	54	6.00	403	12.00	3.00	12.00	3.00	1.00
4.00	2	60	2	3	2	2	1	54	6.00	403	12.00	5.00	1.00	3.00	1.00
4.00	2	60	2	3	2	2	1	54	6.00	403	12.00	11.00	.00	3.00	1.00
5.00	2	79	2	1	3	4	2	49	6.00	438	13.00	12.00	19.00	4.00	3.00
5.00	2	79	2	1	3	4	2	49	6.00	438	13.00	15.00	16.00	4.00	3.00
5.00	2	79	2	1	3	4	2	49	6.00	438	13.00	11.00	23.00	4.00	3.00
6.00	1	60	2	2	2	1	1	47	6.00	460	12.00	66.00	74.00	4.00	2.00
6.00	1	60	2	2	2	1	1	47	6.00	460	12.00	57.00	66.00	4.00	2.00
6.00	1	60	2	2	2	1	1	47	6.00	460	12.00	60.00	71.00	4.00	2.00
7.00	2	71	2	1	.	.	.	96	6.00	226	.	59.00	73.00	3.00	3.00
7.00	2	71	2	1	.	.	.	96	6.00	226	.	62.00	66.00	3.00	3.00
7.00	2	71	2	1	.	.	.	96	6.00	226	.	59.00	74.00	3.00	3.00
8.00	2	72	2	1	3	1	2	96	6.00	226	10.00	46.00	37.00	2.00	1.00
8.00	2	72	2	1	3	1	2	96	6.00	226	10.00	44.00	43.00	2.00	1.00
8.00	2	72	2	1	3	1	2	96	6.00	226	10.00	51.00	43.00	2.00	1.00

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	DoseRate	Dose	Time	Treatment_length	Cloning_before	Cloning_after	Dysphagia_start	Dysphagia_end
9.00	2	75	2	1	3	3	1	72	6.00	301	14.00	43.00	50.00	2.00	1.00
9.00	2	75	2	1	3	3	1	72	6.00	301	14.00	44.00	48.00	2.00	1.00
9.00	2	75	2	1	3	3	1	72	6.00	301	14.00	43.00	48.00	2.00	1.00
10.00	2	87	2	2	2	3	1	106	6.00	204	8.00	35.00	51.00	3.00	.
10.00	2	87	2	2	2	3	1	106	6.00	204	8.00	39.00	49.00	3.00	.
10.00	2	87	2	2	2	3	1	106	6.00	204	8.00	44.00	47.00	3.00	.
11.00	2	.	2	.	3	3	2	64	6.00	338	15.00	43.00	34.00	3.00	1.00
11.00	2	.	2	.	3	3	2	64	6.00	338	15.00	49.00	36.00	3.00	1.00
11.00	2	.	2	.	3	3	2	64	6.00	338	15.00	43.00	37.00	3.00	1.00
12.00	2	73	2	1	3	3	2	60	6.00	358	16.00	53.00	59.00	1.00	.00
12.00	2	73	2	1	3	3	2	60	6.00	358	16.00	44.00	65.00	1.00	.00
12.00	2	73	2	1	3	3	2	60	6.00	358	16.00	47.00	55.00	1.00	.00
14.00	2	65	2	.	2	4	1	75	6.00	289	9.00	45.00	59.00	3.00	1.00
14.00	2	65	2	.	2	4	1	75	6.00	289	9.00	43.00	54.00	3.00	1.00
14.00	2	65	2	.	2	4	1	75	6.00	289	9.00	48.00	55.00	3.00	1.00
15.00	2	87	2	.	2	3	1	90	6.00	241	7.00	31.00	40.00	2.00	2.00
15.00	2	87	2	.	2	3	1	90	6.00	241	7.00	33.00	39.00	2.00	2.00
15.00	2	87	2	.	2	3	1	90	6.00	241	7.00	33.00	39.00	2.00	2.00
16.00	2	59	2	.	3	2	2	49	6.00	444	15.00	42.00	36.00	4.00	3.00
16.00	2	59	2	.	3	2	2	49	6.00	444	15.00	41.00	33.00	4.00	3.00
16.00	2	59	2	.	3	2	2	49	6.00	444	15.00	48.00	31.00	4.00	3.00
17.00	2	59	2	1	2	4	1	46	6.00	474	16.00	11.00	.00	.00	.00
17.00	2	59	2	1	2	4	1	46	6.00	474	16.00	13.00	.00	.00	.00
17.00	2	59	2	1	2	4	1	46	6.00	474	16.00	17.00	.00	.00	.00

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	DoseRate	Dose	Time	Treatment_length	Cloning_before	Cloning_after	Dysphagia_start	Dysphagia_end
18.00	2	62	2	2	2	1	1	52	6.00	418	13.00	64.00	61.00	2.00	1.00
18.00	2	62	2	2	2	1	1	52	6.00	418	13.00	65.00	59.00	2.00	1.00
18.00	2	62	2	2	2	1	1	52	6.00	418	13.00	62.00	66.00	2.00	1.00
19.00	2	80	2	1	2	3	1	39	6.00	559	17.00	44.00	60.00	2.00	1.00
19.00	2	80	2	1	2	3	1	39	6.00	559	17.00	46.00	55.00	2.00	1.00
19.00	2	80	2	1	2	3	1	39	6.00	559	17.00	43.00	63.00	2.00	1.00
20.00	2	.	2	.	2	4	1	27	6.00	798	25.00	66.00	51.00	3.00	1.00
20.00	2	.	2	.	2	4	1	27	6.00	798	25.00	64.00	56.00	3.00	1.00
20.00	2	.	2	.	2	4	1	27	6.00	798	25.00	66.00	50.00	3.00	1.00
21.00	2	67	2	2	1	5	1	31	6.00	695	20.00	60.00	59.00	4.00	3.00
21.00	2	67	2	2	1	5	1	31	6.00	695	20.00	66.00	53.00	4.00	3.00
21.00	2	67	2	2	1	5	1	31	6.00	695	20.00	60.00	56.00	4.00	3.00
22.00	2	75	2	1	3	4	2	56	6.00	386	10.00	69.00	56.00	2.00	2.00
22.00	2	75	2	1	3	4	2	56	6.00	386	10.00	66.00	52.00	2.00	2.00
22.00	2	75	2	1	3	4	2	56	6.00	386	10.00	63.00	57.00	2.00	2.00
23.00	1	77	2	1	4	4	1	40	6.00	541	15.00	58.00	75.00	3.00	3.00
23.00	1	77	2	1	4	4	1	40	6.00	541	15.00	59.00	69.00	3.00	3.00
23.00	1	77	2	1	4	4	1	40	6.00	541	15.00	59.00	70.00	3.00	3.00
24.00	2	77	2	1	3	4	2	30	6.00	729	18.00	39.00	39.00	2.00	.00
24.00	2	77	2	1	3	4	2	30	6.00	729	18.00	43.00	41.00	2.00	.00
24.00	2	77	2	1	3	4	2	30	6.00	729	18.00	43.00	44.00	2.00	.00
25.00	2	84	2	1	1	4	1	42	6.00	516	12.00	39.00	49.00	2.00	.00
25.00	2	84	2	1	1	4	1	42	6.00	516	12.00	45.00	51.00	2.00	.00
25.00	2	84	2	1	1	4	1	42	6.00	516	12.00	47.00	44.00	2.00	.00

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	DoseRate	Dose	Time	Treatment_length	Cloning_before	Cloning_after	Dysphagia_start	Dysphagia_end
26.00	2	58	2	1	1	4	1	54	6.00	403	20.00	40.00	41.00	3.00	.
26.00	2	58	2	1	1	4	1	54	6.00	403	20.00	44.00	42.00	3.00	.
26.00	2	58	2	1	1	4	1	54	6.00	403	20.00	37.00	48.00	3.00	.
27.00	2	51	2	3	2	4	1	68	6.00	317	15.00	56.00	44.00	2.00	2.00
27.00	2	51	2	3	2	4	1	68	6.00	317	15.00	49.00	41.00	2.00	2.00
27.00	2	51	2	3	2	4	1	68	6.00	317	15.00	46.00	37.00	2.00	2.00
28.00	2	.	2	.	3	2	2	88	6.00	246	11.00	36.00	31.00	4.00	4.00
28.00	2	.	2	.	3	2	2	88	6.00	246	11.00	40.00	32.00	4.00	4.00
28.00	2	.	2	.	3	2	2	88	6.00	246	11.00	50.00	38.00	4.00	4.00
29.00	1	71	2	3	3	1	2	96	8.00	301	10.00	26.00	41.00	3.00	2.00
29.00	1	71	2	3	3	1	2	96	8.00	301	10.00	29.00	34.00	3.00	2.00
29.00	1	71	2	3	3	1	2	96	8.00	301	10.00	27.00	36.00	3.00	2.00
30.00	2	66	2	2	3	4	2	55	6.00	393	19.00	52.00	46.00	4.00	3.00
30.00	2	66	2	2	3	4	2	55	6.00	393	19.00	50.00	45.00	4.00	3.00
30.00	2	66	2	2	3	4	2	55	6.00	393	19.00	43.00	50.00	4.00	3.00
31.00	2	81	2	1	3	4	2	112	6.00	193	8.00	45.00	41.00	3.00	3.00
31.00	2	81	2	1	3	4	2	112	6.00	193	8.00	50.00	45.00	3.00	3.00
31.00	2	81	2	1	3	4	2	112	6.00	193	8.00	46.00	43.00	3.00	3.00
32.00	2	49	2	1	2	4	1	67	6.00	322	30.00	53.00	57.00	2.00	.00
32.00	2	49	2	1	2	4	1	67	6.00	322	30.00	51.00	49.00	2.00	.00
32.00	2	49	2	1	2	4	1	67	6.00	322	30.00	41.00	56.00	2.00	.00
33.00	1	51	2	.	2	1	1	77	6.00	280	11.00	52.00	42.00	4.00	2.00
33.00	1	51	2	.	2	1	1	77	6.00	280	11.00	50.00	48.00	4.00	2.00
33.00	1	51	2	1	2	1	1	77	6.00	280	11.00	51.00	42.00	4.00	2.00

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	DoseRate	Dose	Time	Treatment_length	Cloning_before	Cloning_after	Dysphagia_start	Dysphagia_end
34.00	2	74	2	1	2	4	1	83	6.00	259	9.00	36.00	38.00	4.00	3.00
34.00	2	74	2	1	2	4	1	83	6.00	259	9.00	32.00	45.00	4.00	3.00
34.00	2	74	2	1	2	4	1	83	6.00	259	9.00	33.00	43.00	4.00	3.00
35.00	2	75	2	1	3	1	2	51	6.00	424	18.00	48.00	47.00	2.00	2.00
35.00	2	75	2	1	3	1	2	51	6.00	424	18.00	47.00	53.00	2.00	2.00
35.00	2	75	2	1	3	1	2	51	6.00	424	18.00	51.00	54.00	2.00	2.00
36.00	2	63	2	3	1	1	1	91	6.00	238	8.00	49.00	49.00	1.00	.00
36.00	2	63	2	3	1	1	1	91	6.00	238	8.00	52.00	42.00	1.00	.00
36.00	2	63	2	3	1	1	1	91	6.00	238	8.00	48.00	53.00	1.00	.00
37.00	2	52	2	1	2	4	1	77	6.00	281	10.00	43.00	44.00	2.00	.00
37.00	2	52	2	1	2	4	1	77	6.00	281	10.00	43.00	53.00	2.00	.00
37.00	2	52	2	1	2	4	1	77	6.00	281	10.00	41.00	55.00	2.00	.00
38.00	2	69	.	.	.	.	.	.	.	.	.	36.00	33.00	.	.
38.00	2	69	.	.	.	.	.	.	.	.	.	39.00	28.00	.	.
38.00	2	69	.	.	.	.	.	.	.	.	.	37.00	29.00	.	.
40.00	1	77	2	1	3	4	2	79	6.00	274	9.00	30.00	24.00	3.00	.
40.00	1	77	2	1	3	4	2	79	6.00	274	9.00	27.00	30.00	3.00	.
40.00	1	77	2	1	3	4	2	79	6.00	274	9.00	30.00	31.00	3.00	.
41.00	2	90	2	1	2	4	1	74	6.00	293	9.00	45.00	37.00	2.00	.00
41.00	2	90	2	1	2	4	1	74	6.00	293	9.00	41.00	34.00	2.00	.00
41.00	2	90	2	1	2	4	1	74	6.00	293	9.00	53.00	39.00	2.00	.00
42.00	2	64	2	2	3	2	2	74	6.00	293	9.00	12.00	14.00	2.00	1.00
42.00	2	64	2	2	3	2	2	74	6.00	293	9.00	15.00	17.00	2.00	1.00
42.00	2	64	2	2	3	2	2	74	6.00	293	9.00	15.00	28.00	2.00	1.00

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	DoseRate	Dose	Time	Treatment_length	Cloning_before	Cloning_after	Dysphagia_start	Dysphagia_end
43.00	2	74	.	.	.	.	.	.	.	.	.	35.00	44.00	.	.
43.00	2	74	.	.	.	.	.	.	.	.	.	43.00	49.00	.	.
43.00	2	74	.	.	.	.	.	.	.	.	.	42.00	52.00	.	.
44.00	1	74	1	.	2	2	1	64	8.00	450	10.00	47.00	40.00	1.00	1.00
44.00	1	74	1	.	2	2	1	64	8.00	450	10.00	44.00	41.00	1.00	1.00
44.00	1	74	1	.	2	2	1	64	8.00	450	10.00	43.00	44.00	1.00	1.00
45.00	2	60	2	3	1	4	1	42	6.00	512	12.00	45.00	41.00	4.00	1.00
45.00	2	60	2	3	1	4	1	42	6.00	512	12.00	44.00	35.00	4.00	1.00
45.00	2	60	2	3	1	4	1	42	6.00	512	12.00	51.00	43.00	4.00	1.00
46.00	2	66	.	1	.	6	1	53	6.00	411	9.00	40.00	43.00	1.00	1.00
46.00	2	66	.	1	.	6	1	53	6.00	411	9.00	33.00	46.00	1.00	1.00
46.00	2	66	.	1	.	6	1	53	6.00	411	9.00	37.00	43.00	1.00	1.00
47.00	2	83	1	2	1	3	1	43	8.00	669	10.00	45.00	31.00	.00	.00
47.00	2	83	1	2	1	3	1	43	8.00	669	10.00	39.00	34.00	.00	.00
47.00	2	83	1	2	1	3	1	43	8.00	669	10.00	41.00	43.00	.00	.00
48.00	2	.	2	2	2	4	1	64	8.00	450	8.00	38.00	48.00	1.00	.00
48.00	2	.	2	2	2	4	1	64	8.00	450	8.00	40.00	47.00	1.00	.00
48.00	2	.	2	2	2	4	1	64	8.00	450	8.00	41.00	41.00	1.00	.00
49.00	1	.	2	3	2	1	1	46	8.00	624	12.00	52.00	44.00	3.00	1.00
49.00	1	.	2	3	2	1	1	46	8.00	624	12.00	41.00	35.00	3.00	1.00
49.00	1	.	2	3	2	1	1	46	8.00	624	12.00	33.00	32.00	3.00	1.00
50.00	1	.	1	3	2	2	2	43	8.00	671	15.00	51.00	41.00	4.00	2.00
50.00	1	.	1	3	2	2	2	43	8.00	671	15.00	50.00	49.00	4.00	2.00
50.00	1	.	1	3	2	2	2	43	8.00	671	15.00	46.00	42.00	4.00	2.00

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	DoseRate	Dose	Time	Treatment_length	Cloning_before	Cloning_after	Dysphagia_start	Dysphagia_end
51.00	2	.	2	1	.	4	.	27	6.00	797	16.00	7.00	14.00	3.00	2.00
51.00	2	.	2	1	.	4	.	27	6.00	797	16.00	10.00	13.00	3.00	2.00
51.00	2	.	2	1	.	4	.	27	6.00	797	16.00	4.00	12.00	3.00	2.00
52.00	2	.	2	2	.	1	1	27	6.00	804	16.00	16.00	13.00	4.00	2.00
52.00	2	.	2	2	.	1	1	27	6.00	804	16.00	14.00	14.00	4.00	2.00
52.00	2	.	2	2	.	1	1	27	6.00	804	16.00	20.00	16.00	4.00	2.00
53.00	2	.	.	1	.	.	.	.	6.00	.	.	31.00	8.00	4.00	2.00
53.00	2	.	.	1	.	.	.	.	6.00	.	.	28.00	11.00	4.00	2.00
53.00	2	.	.	1	.	.	.	.	6.00	.	.	35.00	7.00	4.00	2.00
54.00	1	.	1	3	3	3	2	94	6.00	229	10.00	43.00	35.00	1.00	.00
54.00	1	.	1	3	3	3	2	94	6.00	229	10.00	41.00	36.00	1.00	.00
54.00	1	.	1	3	3	3	2	94	6.00	229	10.00	37.00	32.00	1.00	.00
55.00	2	.	2	1	1	2	1	71	6.00	304	14.00	4.00	8.00	3.00	3.00
55.00	2	.	2	1	1	2	1	71	6.00	304	14.00	4.00	8.00	3.00	3.00
55.00	2	.	2	1	1	2	1	71	6.00	304	14.00	5.00	9.00	3.00	3.00
56.00	2	.	2	2	3	1	2	67	6.00	322	14.00	44.00	37.00	1.00	1.00
56.00	2	.	2	2	3	1	2	67	6.00	322	14.00	35.00	40.00	1.00	1.00
56.00	2	.	2	2	3	1	2	67	6.00	322	14.00	40.00	41.00	1.00	1.00
57.00	2	.	2	1	3	1	2	96	6.00	225	9.00	44.00	32.00	3.00	1.00
57.00	2	.	2	1	3	1	2	96	6.00	225	9.00	40.00	36.00	3.00	1.00
57.00	2	.	2	1	3	1	2	96	6.00	225	9.00	38.00	35.00	3.00	1.00
58.00	2	.	2	1	2	2	1	70	6.00	311	13.00	18.00	12.00	4.00	2.00
58.00	2	.	2	1	2	2	1	70	6.00	311	13.00	19.00	12.00	4.00	2.00
58.00	2	.	2	1	2	2	1	70	6.00	311	13.00	15.00	10.00	4.00	2.00

Patients	Gender	Age	Cancer	Smoker	Stage	Location	Metastasis	DoseRate	Dose	Time	Treatment_length	Cloning_before	Cloning_after	Dysphagia_start	Dysphagia_end
59.00	2	.	2	3	2	4	2	40	6.00	546	19.00	18.00	19.00	.00	.00
59.00	2	.	2	3	2	4	2	40	6.00	546	19.00	13.00	13.00	.00	.00
59.00	2	.	2	3	2	4	2	40	6.00	546	19.00	21.00	19.00	.00	.00
60.00	2	.	2	3	2	1	1	67	6.00	321	10.00	33.00	31.00	3.00	2.00
60.00	2	.	2	3	2	1	1	67	6.00	321	10.00	41.00	28.00	3.00	2.00
60.00	2	.	2	3	2	1	1	67	6.00	321	10.00	34.00	32.00	3.00	2.00

PatientID	CE	Controls	Age	Gender
1.00	30.00	Healthy co...	48.00	Female
1.00	35.00	Healthy co...	48.00	Female
1.00	30.00	Healthy co...	48.00	Female
2.00	43.00	Healthy co...	51.00	Female
2.00	39.00	Healthy co...	51.00	Female
2.00	39.00	Healthy co...	51.00	Female
3.00	42.00	Healthy co...	50.00	Female
3.00	46.00	Healthy co...	50.00	Female
3.00	40.00	Healthy co...	50.00	Female
4.00	56.00	Healthy co...	35.00	Female
4.00	52.00	Healthy co...	35.00	Female
4.00	53.00	Healthy co...	35.00	Female
5.00	44.00	Healthy co...	26.00	Male
5.00	46.00	Healthy co...	26.00	Male
5.00	43.00	Healthy co...	26.00	Male
6.00	47.00	Healthy co...	38.00	Female
6.00	54.00	Healthy co...	38.00	Female
6.00	46.00	Healthy co...	38.00	Female
7.00	48.00	Healthy co...	41.00	Female
7.00	50.00	Healthy co...	41.00	Female
7.00	52.00	Healthy co...	41.00	Female
8.00	55.00	Healthy co...	53.00	Female
8.00	48.00	Healthy co...	53.00	Female
8.00	55.00	Healthy co...	53.00	Female

PatientID	CE	Controls	Age	Gender
9.00	49.00	Healthy co...	26.00	Male
9.00	50.00	Healthy co...	26.00	Male
9.00	48.00	Healthy co...	26.00	Male
10.00	55.00	Healthy co...	51.00	Female
10.00	58.00	Healthy co...	51.00	Female
10.00	43.00	Healthy co...	51.00	Female
11.00	52.00	Healthy co...	48.00	Male
11.00	57.00	Healthy co...	48.00	Male
11.00	48.00	Healthy co...	48.00	Male
12.00	50.00	Healthy co...	55.00	Female
12.00	49.00	Healthy co...	55.00	Female
12.00	47.00	Healthy co...	55.00	Female
13.00	51.00	Healthy co...	26.00	Female
13.00	49.00	Healthy co...	26.00	Female
13.00	53.00	Healthy co...	26.00	Female
14.00	51.00	Healthy co...	60.00	Female
14.00	53.00	Healthy co...	60.00	Female
14.00	53.00	Healthy co...	60.00	Female
15.00	43.00	Healthy co...	48.00	Female
15.00	44.00	Healthy co...	48.00	Female
15.00	50.00	Healthy co...	48.00	Female

A.6 *In vitro* data

Raw counts

Survival curve HPVG	PE 1	PE 2	0	0.1	0.5	1	2	5	10
	188.0	177.0	171.0	158.0	124.0	96.0	206.0	101.0	51.0
	166.0	162.0	161.0	153.0	141.0	91.0	187.0	87.0	63.0
	173.0	156.0	188.0	148.0	138.0	97.0	193.0	65.0	19.0
	199.0	186.0	183.0	164.0	122.0	90.0	212.0	90.0	40.0
	184.0	181.0	189.0	152.0	131.0	83.0	202.0	111.0	23.0
	176.0	193.0	191.0	173.0	139.0	84.0	199.0	88.0	12.0
	165.0	174.0	165.0	159.0	144.0	72.0	210.0	99.0	73.0
	151.0	168.0	178	155.0	147.0	75.0	212.0	89.0	62.0
	159.0	166.0	154	162.0	135.0	89.0	197.0	91.0	44.0
Mean PE (PE1 and PE2)	0.35		0.35	0.32	0.27	0.17	0.13	0.05	0.01
Cells Plated	500	500	500	500	500	500	1500	2000	5000
Individual PE	37.6	35.4	34.2	31.6	24.8	19.2	13.7	5.1	1.0
	33.2	32.4	32.2	30.6	28.2	18.2	12.5	4.4	1.3
	34.6	31.2	37.6	29.6	27.6	19.4	12.9	3.3	0.4
	39.8	37.2	36.6	32.8	24.4	18.0	14.1	4.5	0.8
	36.8	36.2	37.8	30.4	26.2	16.6	13.5	5.6	0.5
	35.2	38.6	38.2	34.6	27.8	16.8	13.3	4.4	0.2
	33.0	34.8	33.0	31.8	28.8	14.4	14.0	5.0	1.5
	30.2	33.6	35.6	31.0	29.4	15.0	14.1	4.5	1.2
	31.8	33.2	30.8	32.4	27.0	17.8	13.1	4.6	0.9
Normalised to mean PE	108.3	102.0	97.4	90.0	70.6	54.7	39.1	14.4	2.9
	95.6	93.3	91.7	87.2	80.3	51.8	35.5	12.4	3.6
	99.7	89.9	107.1	84.3	78.6	55.3	36.6	9.3	1.1
	114.7	107.2	104.2	93.4	69.5	51.3	40.3	12.8	2.3
	106.0	104.3	107.7	86.6	74.6	47.3	38.4	15.8	1.3
	101.4	111.2	108.8	98.5	79.2	47.8	37.8	12.5	0.7
	95.1	100.3	94.0	90.6	82.0	41.0	39.9	14.1	4.2
	87.0	96.8	101.4	88.3	83.7	42.7	40.3	12.7	3.5
	91.6	95.6	87.7	92.3	76.9	50.7	37.4	13.0	2.5
Mean Clonogenic Survival	100.0		100.0	90.1	77.3	49.2	38.4	13.0	2.4
Std Error	1.8		2.6	1.4	1.6	1.6	0.6	0.6	0.4

Survival curve HaCaT	PE 1	PE 2	0	0.1	0.5	1	2	5	10
	196.0	193.0	184.0	184.0	128.0	91.0	171.0	91.0	21.0
	182.0	199.0	202.0	191.0	140.0	88.0	187.0	74.0	42.0
	198.0	187.0	188.0	175.0	138.0	96.0	193.0	55.0	33.0
	178.0	180.0	173.0	168.0	131.0	100.0	202.0	63.0	22.0
	171.0	173.0	186.0	173.0	140.0	89.0	217.0	84.0	29.0
	175.0	189.0	192.0	186.0	144.0	105.0	230.0	79.0	12.0
	199.0	189.0	217.0	177.0	129.0	111.0	193.0	99.0	33.0
	201.0	211.0	199.0	161.0	137.0	113.0	199.0	100.0	46.0
	225.0	203.0	202	180.0	133.0	101.0	201.0	82.0	31.0
Mean PE (PE1 and PE2)	0.38		0.39	0.37	0.27	0.18	0.12	0.04	0.01
Cells Plated	500	500	500	500	500	500	1500	2000	5000
Individual PE	39.2	38.6	36.8	36.8	25.6	18.2	11.4	4.6	0.4
	36.4	39.8	40.4	38.2	28.0	17.6	12.5	3.7	0.8
	39.6	37.4	37.6	35.0	27.6	19.2	12.9	2.8	0.7
	35.6	36.0	34.6	33.6	26.2	20.0	13.5	3.2	0.4
	34.2	34.6	37.2	34.6	28.0	17.8	14.5	4.2	0.6
	35.0	37.8	38.4	37.2	28.8	21.0	15.3	4.0	0.2
	39.8	37.8	43.4	35.4	25.8	22.2	12.9	5.0	0.7
	40.2	42.2	39.8	32.2	27.4	22.6	13.3	5.0	0.9
	45.0	40.6	40.4	36.0	26.6	20.2	13.4	4.1	0.6
Normalised to mean PE	102.3	100.7	95.0	95.0	66.1	47.0	29.4	11.7	1.1
	95.0	103.9	104.3	98.6	72.3	45.4	32.2	9.6	2.2
	103.3	97.6	97.1	90.4	71.3	49.6	33.2	7.1	1.7
	92.9	93.9	89.3	86.7	67.6	51.6	34.8	8.1	1.1
	89.2	90.3	96.0	89.3	72.3	46.0	37.3	10.8	1.5
	91.3	98.6	99.1	96.0	74.4	54.2	39.6	10.2	0.6
	103.9	98.6	112.0	91.4	66.6	57.3	33.2	12.8	1.7
	104.9	110.1	102.8	83.1	70.7	58.3	34.3	12.9	2.4
	117.4	105.9	104.3	92.9	68.7	52.2	34.6	10.6	1.6
Mean Clonogenic Survival	100.0		100.0	91.5	70.0	51.3	34.3	10.4	1.5
Std Error	1.7		2.2	1.6	1.0	1.6	1.0	0.7	0.2

Survival curve HCT116 p53+/+	PE 1	PE 2	0	0.05	0.1	0.5	1	2	5	10
	225	215	260	245	232	155	115	290	261	20
	249	234	274	266	207	127	124	227	175	20
	258	289	253	215	227	189	98	303	227	26
	235	283	244	302	174	190	139	261	395	27
	228	251	297	291	211	209	112	277	300	32
	244	290	235	313	196	191	175	321	282	27
	221	232	244	260	232	188	88	176	298	12
	236	249	253	289	244	203	101	154	291	36
	247	220	276	297	259	188	113	142	304	40
Mean PE (PE1 and PE2)	0.5		0.5	0.6	0.4	0.4	0.2	0.2	0.1	0.0
Cells Plated	500.0	500.0	500.0	500.0	500.0	500.0	500.0	1500.0	2500.0	5000.0
Individual PE	45.0	43.0	52.0	49.0	46.4	31.0	23.0	19.3	10.4	0.4
	49.8	46.8	54.8	53.2	41.4	25.4	24.8	15.1	7.0	0.4
	51.6	57.8	50.6	43.0	45.4	37.8	19.6	20.2	9.1	0.5
	47.0	56.6	48.8	60.4	34.8	38.0	27.8	17.4	15.8	0.5
	45.6	50.2	59.4	58.2	42.2	41.8	22.4	18.5	12.0	0.6
	48.8	58.0	47.0	62.6	39.2	38.2	35.0	21.4	11.3	0.5
	44.2	46.4	48.8	52.0	46.4	37.6	17.6	11.7	11.9	0.2
	47.2	49.8	50.6	57.8	48.8	40.6	20.2	10.3	11.6	0.7
	49.4	44.0	55.2	59.4	51.8	37.6	22.6	9.5	12.2	0.8
Normalised to mean PE	91.9	87.8	100.2	94.4	89.4	59.7	44.3	37.2	20.1	0.8
	101.7	95.6	105.6	102.5	79.8	48.9	47.8	29.2	13.5	0.8
	105.4	118.1	97.5	82.8	87.5	72.8	37.8	38.9	17.5	1.0
	96.0	115.6	94.0	116.4	67.0	73.2	53.6	33.5	30.4	1.0
	93.1	102.5	114.4	112.1	81.3	80.5	43.2	35.6	23.1	1.2
	99.7	118.5	90.5	120.6	75.5	73.6	67.4	41.2	21.7	1.0
	90.3	94.8	94.0	100.2	89.4	72.4	33.9	22.6	23.0	0.5
	96.4	101.7	97.5	111.3	94.0	78.2	38.9	19.8	22.4	1.4
	100.9	89.9	106.3	114.4	99.8	72.4	43.5	18.2	23.4	1.5
Mean Clonogenic Survival	100.0		100.0	106.1	84.8	70.2	45.6	30.7	21.7	1.0
Std Error	2.2		2.5	4.0	3.3	3.3	3.3	2.9	1.5	0.1

Survival curve OE19	PE 1	PE 2	0	0.05	0.1	0.5	1	2	5
	75.0	93.0	104.0	78.0	62.0	70.0	56.0	95.0	94.0
	98.0	126.0	109.0	84.0	83.0	79.0	62.0	117.0	152.0
	97.0	124.0	101.0	85.0	79.0	61.0	63.0	109.0	134.0
	108.0	107.0	106.0	89.0	78.0	50.0	40.0	109.0	134.0
	128.0	116.0	101.0	75.0	74.0	68.0	74.0	100.0	105.0
	106.0	118.0	112.0	95.0	100.0	69.0	55.0	124.0	117.0
	94.0	112.0	118.0	88.0	91.0	80.0	49.0	101.0	123.0
	117.0	123.0	102.0	90.0	70.0	67.0	58.0	100.0	122.0
	117.0	96.0	111.0	73.0	81.0	77.0	53.0	97.0	135.0
Mean PE (PE1 and PE2)	0.109		0.107	0.084	0.080	0.069	0.057	0.053	0.035
Cells Plated	1000		1000	1000	1000	1000	1000	2000	3500
Individual PE	7.5	9.3	10.4	7.8	6.2	7.0	5.6	4.8	2.7
	9.8	12.6	10.9	8.4	8.3	7.9	6.2	5.9	4.3
	9.7	12.4	10.1	8.5	7.9	6.1	6.3	5.5	3.8
	10.8	10.7	10.6	8.9	7.8	5.0	4.0	5.5	3.8
	12.8	11.6	10.1	7.5	7.4	6.8	7.4	5.0	3.0
	10.6	11.8	11.2	9.5	10.0	6.9	5.5	6.2	3.3
	9.4	11.2	11.8	8.8	9.1	8.0	4.9	5.1	3.5
	11.7	12.3	10.2	9.0	7.0	6.7	5.8	5.0	3.5
	11.7	9.6	11.1	7.3	8.1	7.7	5.3	4.9	3.9
Normalised to mean PE	69.1	85.6	97.1	72.8	57.9	65.4	52.3	44.3	25.1
	90.2	116.0	101.8	78.4	77.5	73.8	57.9	54.6	40.5
	89.3	114.2	94.3	79.4	73.8	57.0	58.8	50.9	35.7
	99.4	98.5	99.0	83.1	72.8	46.7	37.3	50.9	35.7
	117.9	106.8	94.3	70.0	69.1	63.5	69.1	46.7	28.0
	97.6	108.6	104.6	88.7	93.4	64.4	51.3	57.9	31.2
	86.5	103.1	110.2	82.2	85.0	74.7	45.7	47.1	32.8
	107.7	113.2	95.2	84.0	65.4	62.6	54.1	46.7	32.5
	107.7	88.4	103.6	68.2	75.6	71.9	49.5	45.3	36.0
Mean Clonogenic Survival	100.0		100.0	78.5	74.5	64.4	52.9	49.4	33.8
Std Error	3.1		1.8	2.3	3.5	2.9	3.0	1.5	4.6

A	B	C	D	E	F	G	H	I	J	K
Survival curve OE33	**PE 1**	**PE 2**	**0**	**0.05**	**0.1**	**0.5**	**1**	**2**	**5**	**10**
	129.0	139.0	140.0	142.0	160.0	129.0	106.0	143.0	190.0	22.0
	131.0	90.0	149.0	139.0	141.0	122.0	93.0	156.0	172.0	44.0
	139.0	137.0	142.0	145.0	131.0	119.0	88.0	164.0	200.0	72.0
	145.0	151.0	155.0	136.0	172.0	117.0	111.0	173.0	221.0	33.0
	136.0	138.0	168.0	144.0	138.0	112.0	101.0	179.0	199.0	56.0
	171.0	177.0	175.0	151.0	151.0	124.0	113.0	181.0	212.0	84.0
	166.0	151.0	183.0	158.0	120.0	129.0	99.0	193.0	202.0	57.0
	121.0	155.0	134.0	137.0	121.0	127.0	91.0	189.0	221.0	46.0
	130.0	144.0	154.0	149.0	125.0	119.0	89.0	164.0	277.0	42.0
Mean PE (PE1 and PE2)	0.28		0.31	0.29	0.28	0.24	0.20	0.11	0.08	0.01
Cells Plated	**500**	**500**	**500**	**500**	**500**	**500**	**500**	**1500**	**2000**	**6000**
Individual PE	25.8	27.8	28.0	28.4	32.0	25.8	21.2	9.5	9.5	0.4
	26.2	18.0	29.8	27.8	28.2	24.4	18.6	10.4	8.6	0.9
	27.8	27.4	28.4	29.0	26.2	23.8	17.6	10.9	10.0	1.4
	29.0	30.2	31.0	27.2	34.4	23.4	22.2	11.5	11.1	0.7
	27.2	27.6	33.6	28.8	27.6	22.4	20.2	11.9	10.0	1.1
	34.2	35.4	35.0	30.2	30.2	24.8	22.6	12.1	10.6	1.7
	33.2	30.2	36.6	31.6	24.0	25.8	19.8	12.9	10.1	1.1
	24.2	31.0	26.8	27.4	24.2	25.4	18.2	12.6	11.1	0.9
	26.0	28.8	30.8	29.8	25.0	23.8	17.8	10.9	13.9	0.8
Normalised to mean PE	91.1	98.1	90.0	91.3	102.9	82.9	68.1	30.6	30.5	1.4
	92.5	63.5	95.8	89.4	90.6	78.4	59.8	33.4	27.6	2.8
	98.1	96.7	91.3	93.2	84.2	76.5	56.6	35.1	32.1	4.6
	102.4	106.6	99.6	87.4	110.6	75.2	71.4	37.1	35.5	2.1
	96.0	97.4	108.0	92.6	88.7	72.0	64.9	38.4	32.0	3.6
	120.7	124.9	112.5	97.1	97.1	79.7	72.6	38.8	34.1	5.4
	117.2	106.6	117.6	101.6	77.1	82.9	63.6	41.4	32.5	3.7
	85.4	109.4	86.1	88.1	77.8	81.6	58.5	40.5	35.5	3.0
	91.8	101.6	99.0	95.8	80.4	76.5	57.2	35.1	44.5	2.7
Mean Clonogenic Survival	100.0		100.0	92.9	89.9	78.4	63.6	36.7	33.8	3.3
Std Error	3.3		3.6	1.5	3.9	1.2	2.0	1.2	1.6	0.4

OE33/HaCaTs Bystander Experiment	**PE 1**	**PE 2**		**0Gy**	**irradiated medium**	**sham**	**0.5Gy**	**5Gy**
Trials 1 to 3	195	190		161	163	188	168	187
	178	183		195	168	183	164	167
	167	158		183	177	182	165	187
	157	165		182	159	165	167	187
300 cells seeded	163	195		190	155	161	173	167
	164	176		214	146	167	165	197
	157	181		182	176	199	164	177
	178	195		188	158	183	195	176
	155	194		207	179	154	160	183

OE19/HaCaTs Bystander Experiment	**PE 1**	**PE 2**		**0Gy**	**irradiated medium**	**sham**	**0.5Gy**	**5Gy**
Trials 1 to 3	153	160		195	207	188	147	189
	150	159		184	183	183	159	159
	142	148		194	152	182	151	160
	179	171		165	175	165	166	168
	148	164		200	150	161	170	169
	156	188		157	166	167	171	158
	176	177		164	207	199	189	159
	142	154		194	179	183	144	165
	163	147		191	175	154	141	170

OE33	irradiated medium	sham	0Gy	4ng/ml/0Gy	40ng/ml/0Gy	400ng/ml/0Gy	0Gy/zofran	4ng/ml/0Gy/zofran	40ng/ml/0Gy/zofran	400ng/ml/0Gy/zofran
Bystander	100	86	129	122	139	157	137	141	122	172
Trials 1 to 3	120	92	172	138	143	139	135	133	155	148
	118	123	148	149	141	127	124	142	163	139
500 cells seeded	143	122	114	122	115	118	138	127	124	147
	152	140	138	112	137	126	166	112	146	153
	168	134	115	80	107	102	168	130	137	150
	223	261	243	211	222	230	252	239	240	288
	249	233	241	249	207	263	239	226	247	237
	234	247	226	229	214	217	258	255	260	232
			0.5Gy	**4ng/ml/0.5Gy**	**40ng/ml/0.5Gy**	**400ng/ml/0.5Gy**	**0.5Gy/zofran**	**4ng/ml/0.5Gy/zofran**	**40ng/ml/0.5Gy/zofran**	**400ng/ml/0.5Gy/zofran**
			107	108	176	118	112	116	123	110
			104	115	162	124	131	122	101	93
			112	107	150	118	104	106	130	107
			96	97	97	99	111	101	117	106
			98	67	101	108	127	92	103	102
			96	88	108	82	93	99	111	112
			232	227	244	234	222	243	252	266
			248	238	259	240	228	256	223	270
			254	251	231	210	247	249	283	250
			5 Gy	**4ng/ml/5Gy**	**40ng/ml/5Gy**	**400ng/ml/5Gy**	**5Gy/zofran**	**4ng/ml/5Gy/zofran**	**40ng/ml/5Gy/zofran**	**400ng/ml/5Gy/zofran**
			141	133	131	121	126	131	129	106
			138	135	122	149	129	120	114	117
			156	122	145	136	143	113	126	111
			151	107	101	141	111	100	133	108
			125	101	119	134	105	99	134	127
			149	99	123	127	109	107	93	135
			199	251	220	206	211	233	245	217
			205	244	291	212	215	235	272	237
			238	219	243	224	246	222	239	244

OE33/HCT116p53	irradiated medium	sham	0Gy	4ng/ml/0Gy	40ng/ml/0Gy	400ng/ml/0Gy	0Gy/zofran	4ng/ml/0Gy/zofran	40ng/ml/0Gy/zofran	400ng/ml/0Gy/zofran
Bystander	223	305	283	281	305	304	252	239	240	288
Trials 1 to 3	249	312	322	302	278	299	239	226	247	237
	234	267	284	352	278	301	258	255	260	232
500 cells seeded	351	261	241	221	247	339	256	254	291	336
	299	233	237	228	225	235	244	241	269	233
	236	247	266	269	216	234	273	322	273	329
700 cells seeded	387	421	477	477	447	475	438	450	426	376
	382	428	421	454	485	509	450	354	339	375
	397	469	516	460	492	465	420	387	379	403
			0.5Gy	**4ng/ml/0.5Gy**	**40ng/ml/0.5Gy**	**400ng/ml/0.5Gy**	**0.5Gy/zofran**	**4ng/ml/0.5Gy/zofran**	**40ng/ml/0.5Gy/zofran**	**400ng/ml/0.5Gy/zofran**
		500 cells seeded	232	227	244	234	222	243	252	266
			248	238	259	240	228	256	223	270
			254	251	231	210	247	249	283	250
		500 cells seeded	265	214	227	237	253	296	276	263
			244	211	217	212	250	287	285	260
			227	212	219	202	226	281	296	277
		700 cells seeded	415	461	373	450	424	347	370	417
			394	390	481	449	474	402	368	406
			453	433	422	491	444	377	398	392
			5 Gy	**4ng/ml/5Gy**	**40ng/ml/5Gy**	**400ng/ml/5Gy**	**5Gy/zofran**	**4ng/ml/5Gy/zofran**	**40ng/ml/5Gy/zofran**	**400ng/ml/5Gy/zofran**
		500 cells seeded	297	299	320	311	274	312	292	205
			275	303	312	322	268	284	302	202
			231	254	302	317	271	266	299	267
		500 cells seeded	267	217	240	221	279	273	286	311
			270	240	224	244	288	280	234	293
			262	228	204	234	292	287	248	276
		700 cells seeded	376	398	338	436	416	390	396	462
			386	450	415	341	387	376	448	400
			391	460	410	382	386	399	452	441

OE19	irradiated medium	sham	0Gy	4ng/ml/0Gy	40ng/ml/0Gy	400ng/ml/0Gy	0Gy/zofran	4ng/ml/0Gy/zofran	40ng/ml/0Gy/zofran	400ng/ml/0Gy/zofran
Bystander	323	327	355	394	388	395	376	325	313	325
Trials 1 to 3	349	336	302	368	328	367	398	298	328	354
	358	368	329	421	397	383	331	346	270	355
2000 cells seeded	316	346	353	300	265	506	362	368	399	512
	308	333	347	309	358	501	352	344	418	435
	328	325	357	312	340	490	359	329	447	467
	415	404	450	357	378	435	348	402	394	444
	436	405	405	351	322	513	333	378	443	403
	394	398	423	390	389	434	349	391	373	479
			0.5Gy	**4ng/ml/0.5Gy**	**40ng/ml/0.5Gy**	**400ng/ml/0.5Gy**	**0.5Gy/zofran**	**4ng/ml/0.5Gy/zofran**	**40ng/ml/0.5Gy/zofran**	**400ng/ml/0.5Gy/zofran**
			349	307	408	431	353	315	322	322
			344	362	361	334	367	341	359	328
			311	322	342	377	324	319	363	333
			341	334	425	375	355	324	355	529
			331	326	490	366	367	344	379	431
			429	366	413	386	339	352	391	414
			352	350	379	448	393	374	345	421
			344	348	367	485	362	398	376	411
			383	371	422	526	345	361	358	507
			5 Gy	**4ng/ml/5Gy**	**40ng/ml/5Gy**	**400ng/ml/5Gy**	**5Gy/zofran**	**4ng/ml/5Gy/zofran**	**40ng/ml/5Gy/zofran**	**400ng/ml/5Gy/zofran**
			361	378	401	412	389	383	429	496
			361	343	412	496	401	398	403	501
			399	352	445	421	413	377	429	562
			377	383	437	512	362	395	405	352
			377	390	306	436	353	359	439	330
			347	382	482	394	323	312	461	353
			313	309	383	474	349	316	410	360
			334	327	353	361	332	301	447	410
			329	348	348	336	356	391	417	325

OE19/HCT116p53	irradiated medium	sham	0Gy	4ng/ml/0Gy	40ng/ml/0Gy	400ng/ml/0Gy	0Gy/zofran	4ng/ml/0Gy/zofran	40ng/ml/0Gy/zofran	400ng/ml/0Gy/zofran
Bystander	133	116	126	102	113	100	109	116	112	107
Trials 1 to 3	157	120	120	106	122	101	111	92	115	103
	139	114	105	114	113	99	114	103	99	120
300 cells seeded	175	178	195	191	193	192	186	187	196	190
	181	164	219	174	192	163	182	197	184	196
	179	156	199	202	176	203	180	172	171	187
	187	189	201	111	181	186	211	183	189	198
	121	199	157	185	167	191	218	194	190	201
	181	203	194	201	154	204	245	199	213	201
			0.5Gy	**4ng/ml/0.5Gy**	**40ng/ml/0.5Gy**	**400ng/ml/0.5Gy**	**0.5Gy/zofran**	**4ng/ml/0.5Gy/zofran**	**40ng/ml/0.5Gy/zofran**	**400ng/ml/0.5Gy/zofran**
			101	77	62	80	108	62	64	88
			95	68	77	77	115	71	83	86
			70	89	85	86	105	73	74	73
			166	161	166	178	112	166	171	185
			153	169	180	160	119	165	171	167
			164	173	186	154	134	177	181	177
			195	202	191	187	151	174	163	163
			184	204	199	203	157	193	154	166
			193	153	201	208	161	125	179	150
			5 Gy	**4ng/ml/5Gy**	**40ng/ml/5Gy**	**400ng/ml/5Gy**	**5Gy/zofran**	**4ng/ml/5Gy/zofran**	**40ng/ml/5Gy/zofran**	**400ng/ml/5Gy/zofran**
			101	97	112	76	65	84	75	81
			112	104	81	74	87	85	93	101
			124	116	78	82	91	73	99	96
			169	173	184	198	213	184	181	193
			147	187	195	184	190	193	222	184
			146	146	202	196	187	199	193	176
			164	148	162	206	198	203	192	197
			198	219	211	199	193	201	181	194
			193	206	193	191	207	196	203	186

HCT116p53++_5HT	irradiated medium	sham	0Gy	4ng/ml/0Gy	40ng/ml/0Gy	400ng/ml/0Gy	0Gy/zofran	4ng/ml/0Gy/zofran	40ng/ml/0Gy/zofran	400ng/ml/0Gy/zofran
Bystander	185	162	192	164	178	189	178	193	191	217
Trials 1 to 3	198	173	179	194	182	187	193	190	178	221
	169	158	195	189	184	204	190	211	204	216
500 cells seeded	163	189	180	184	188	209	196	193	183	166
	188	192	174	200	177	199	202	187	191	175
	180	158	179	177	211	207	189	201	174	188
	131	161	171	180	197	218	190	190	255	213
	171	193	205	170	201	190	178	195	223	199
	149	181	202	167	184	197	191	183	185	218
			0.5Gy	**4ng/ml/0.5Gy**	**40ng/ml/0.5Gy**	**400ng/ml/0.5Gy**	**0.5Gy/zofran**	**4ng/ml/0.5Gy/zofran**	**40ng/ml/0.5Gy/zofran**	**400ng/ml/0.5Gy/zofran**
			179	177	182	175	151	176	149	163
			163	158	133	170	160	169	147	147
			150	170	166	159	169	154	156	175
			158	140	144	152	163	138	173	151
			157	151	140	153	144	129	145	162
			145	143	160	160	155	140	134	131
			175	136	183	163	159	176	143	155
			135	136	148	139	180	190	181	176
			178	129	176	163	167	175	159	156
			5Gy	**4ng/ml/5Gy**	**40ng/ml/5Gy**	**400ng/ml/5Gy**	**5Gy/zofran**	**4ng/ml/5Gy/zofran**	**40ng/ml/5Gy/zofran**	**400ng/ml/5Gy/zofran**
			175	122	160	172	186	151	183	133
			159	145	180	142	167	182	171	163
			181	153	178	161	178	189	193	113
			196	141	175	177	191	154	189	198
			161	177	171	163	173	168	192	176
			176	162	174	150	170	156	118	188
			155	198	184	180	165	189	161	204
			163	186	172	167	160	150	151	202
			157	155	166	155	150	166	187	173