CYB5D2 POSSESSES TUMOUR SUPPRESSING ACTIVITIES

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ABSTRACT

Loss of chromosome 17p is frequently observed in various cancers. One of the most commonly mutated targets p53 is on chromosome 17p13.1. However, studies have also reported loss of the 17p13.2 region in breast and medulloblastoma, thereby suggesting the residence of potential tumour suppressors in 17p13.2. Cytochrome b5 domain containing 2 (CYB5D2) is located on 17p13.2 implying CYB5D2 being a candidate tumour suppressor. CYB5D2 (neuferricin) belongs to the family of membrane associated progesterone receptors (MAPR). The archetypal member of the family, progesterone receptor membrane component 1 (PGRMC1), has been shown to play a role in domains independently of its function in mediating progesterone signalling. Consistent with this, CYB5D2 was reported to promote neurogenesis and inhibit the proliferation of Neuro2a cells. However, its role in tumorigenesis remains unknown.

To investigate the role of CYB5D2 in tumorigenesis, western blot analysis was performed on 20 matched clear cell renal cell carcinomas (ccRCC) and the adjacent non-tumour kidney (ANK) tissues; significant down-regulation of CYB5D2 was demonstrated in ccRCC in comparison to ANK tissues, an observation that was confirmed by immunohistochemistry (IHC) analysis of 9 pairs of ccRCC-ANK tissues. Ectopic expression of CYB5D2 inhibited the proliferation and the invasion of A498 ccRCC along with the inhibition of AKT activation. Collectively, the above results support the possibility of CYB5D2 being a potential tumour suppressor.

In support of the results obtained in ccRCC, we were able to show a significant reduction of CYB5D2 in cervical squamous carcinoma compared to normal cervical

tissues in our analysis of CYB5D2 expression in 35 cervical squamous tumours. In vitro, overexpression and knockdown of CYB5D2 inhibited and enhanced the invasion of HeLa cells, respectively. As a member of the MAPR family, CYB5D2 contains the signature motif of the family, the cytochrome b5 (cyt-b5) like heme/steroid binding domain. This domain is known for heme binding and research in our laboratory has shown the residue D86 being critical for heme association. Ssubstitution of D86 with G (D86G) abolished not only CYB5D2's ability to bind heme but also its capacity of inhibiting HeLa cell invasion. Taken together, we provide evidence that CYB5D2 possesses activities in suppressing tumorigenesis, at least for the tumorigenesis of ccRCC and cervical squamous carcinoma.

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LIST OF ABBREVIATIONS

Adc	Adenocarcinoma
ccRCC	Clear cell Renal Cell Carcinoma
CREB	c-AMP response element binding protein
CYB5D2	Cytochrome b5 domain containing 2
DPH	Diphthamide Biosynthesis Protein
EGFR	Epithelial Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
FHIT	Fragile Histidine Triad
FKHR	Forkhead family of transcription factor
GP	Gag-pol expression vector
GSK3β	Glycogen Synthase Kinase 3 β
HIFα	Hypoxia Inducible Factor α
HPV	Human Papillomavirus
HR	High Risk

IHC	Immunohistochemistry
LR	Low Risk
MAPR	Membrane-Associated Progesterone Receptor
mTOR	Mammalian Target of Rapamycin
NAT	Normal Adjacent kidney Tissue
OVCA1	Ovarian Cancer 1
PBS	Phosphate Based Saline
PDGF	Platelet Derived Growth Factor
PGRMC1	Progesterone Receptor Membrane Component 1
PI3K	Phosphatidylinositol-3 kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α
PIP3	Phosphatidylinostiol-3,4,5 triphosphate
PR	Progesterone Receptor
PTEN	Phosphatase and tensin homolog
pVHL	von Hippel Lindau Protein
RCC	Renal Cell Carcinoma

SCC	Squamous Cell Carcinoma
siRNA	small interfering RNA
TGFα	Transforming Growth Factor α
TMA	Tissue Microarray
TSC	Tuberous Sclerosis Complex
VEGF	Vascular Epithelial Growth Factor
VHL	von Hippel Lindau
VSV-G	Vesicular stomatitis virus G protein
XIAP	X-linked inhibitor of Apoptosis

I. INTRODUCTION

1.1 Renal Cell Carcinoma

Renal cell carcinoma affects more than 5,000 Canadians every year. It is the third most common form of genitourinary cancer, behind prostate and bladder (Jemal et al. 2011; Lee and Haber 2001). According to Canadian Cancer Society, there will be approximately 6,000 newly diagnosed cases of kidney cancer in 2013 with about 30% death rate. In the United State, the estimated number of RCC in 2013 will be 65,150 cases, with nearly 14,000 people dying from the disease (Siegel et al. 2013).

Renal cell carcinoma originates from the epithelium of the renal tubules (Cohen and McGovern 2005; Linehan et al. 2003). Based on the type of epithelial neoplasm and the location of the tumour, RCC is characterized into 6 different subgroups: clear cell, papillary, chromophobe, oncocytoma, collecting ducts, and unclassified (Motzer et al. 1996). Clear cell renal cell carcinoma (ccRCC) accounts for 75% of total RCC. The tumour usually arises from the proximal convoluted tubules (Levy et al. 1998; Nelson et al. 2007; Uchida et al., 2002) and is the most aggressive form of the RCC. The typical genetic abnormality of ccRCC is loss of the Von Hippel Lindau (VHL) tumour suppressor (Linehan et al. 2010; Nelson et al., 2007); the typical histological feature of the tumor is the presence of a clear cytoplasm, which arises from the fixation of tumour tissues, resulting in solubilisation of the high lipid contents present in the cytoplasm of ccRCC cells (Rini et al. 2009). Papillary RCC is the second most common type of RCC, comprising 10-15% of all RCC cases. This type of RCC is derived from distal convoluted

tubules. Compared to ccRCC, papillary RCC is less aggressive (Linehan et al., 2003; Nelson et al., 2007). Genetic mapping revealed that pro-oncogene MET located on chromosome 7 plays an essential role in the pathogenesis of papillary RCC (Linehan et al., 2003). Together chromophobe and oncocytoma account for 4 % of the RCC cases; these are benign tumours that are thought to originate from the collecting duct, specifically type A and B of intercalated cells respectively (Cohen and McGovern, 2005). Collecting duct RCC represents less than 1% of RCC (Nelson et al., 2007).

RCC is associated with several risk factors including cigarette smoking, obesity, hypertension, and genetics (Cohen and McGovern, 2005; Linehan, 2006; Linehan et al., 2007). Renal abnormalities such as end-stage renal failure, renal cystic disease, as well as tuberous sclerosis also increase the risk of RCC (Linehan et al., 2003). On the other hand, mutations in tumour suppressor genes on chromosome 3 have been implicated with RCC in many studies. The Fragile Histidine Triad (FHIT) gene resides at 3p12-p14 in which the t(3:8) translocation is involved in heredity of RCC (Clifford et al., 1998). Near the *FHIT* gene, regions of 3p21.2-p21.3 are also commonly mutated in RCC and lung cancer (Clifford et al., 1998). The *VHL* tumour suppressor gene is located on chromosome 3p25-p26, and individuals with single wild type *VHL* gene develop the conditions commonly known as VHL disease (Kim and Kaelin, 2004; Rini et al., 2009). The VHL protein (pVHL) promotes the degradation of hypoxia induce factor α (HIFα) through polyubiquitination (Brugarolas, 2007; Kondo and Kaelin, 2001). The absence of pVHL causes the accumulation of HIFα, which leads to up-regulation of downstream target genes including vascular epithelial growth factor (VEGF), erythropoietin, transforming

growth factor-α (TGF-α), and platelet-derived growth factor (PDGF) (Grandinetti and Goldspiel, 2007). Expressions of these proteins collectively facilitate angiogenesis, and proliferation and survival of ccRCC cells.

The prognosis of ccRCC is heavily dependent on its stage at the time of diagnosis. Due to the lack of symptoms, tumour is often metastasized in most patients at diagnosis (Brugarolas, 2007; Motzer et al., 1996). Research has shown that approximately 40-45% of ccRCC cases have metastasis at the time of admission. Moreover, 30% of the local tumour will develop metastases (Levy et al., 1998; Nelson et al., 2007; Uchida et al., 2002). While patients with local tumours confined in the kidney have 5 year survival rate of over 90%, the number declined drastically to 50-60% for those with locally advanced tumour. The 5 year survival rate for metastatic cases is 5-10% (Brugarolas, 2007). The symptoms and signs of RCC include flank pain, hematuria, and lump at the abdominal area (Nelson et al., 2007). At times, systemic symptoms may also present due to irregular hormone level released by the kidney. These include hypertension, hypercalcemia, and amyloidosis (Nelson et al., 2007). In the past decade, increasing cases of RCC have been diagnosed as a tribute to better medical imaging (Drucker, 2005). Frequent uses of ultrasound imaging, computed tomography, and magnetic resonance imaging have allowed detection of early RCC as well as distance metastasis and lymph node invasion (Drucker, 2005; Nelson et al., 2007).

Radical nephrectomy has been the conventional treatment for local tumour since late 20th century. Nonetheless, given the high incidence of metastasis in ccRCC, pharmacological agents have been developed to follow with surgical treatment to maximize the therapeutic

effect on RCC. While chemotherapy is effective against other cancers, RCC is highly resilient owning to the expression of multidrug resistant genes (Liu et al., 2001; Mickisch 1994). It was observed that occasionally the tumour elicits immune responses leading to spontaneous remission (Coppin et al., 2008). This observation become the foundation of immunotherapy using high dose interleukin 2 (IL-2) and interferon α for the treatment of advance and metastatic ccRCC (Grandinetti and Goldspiel, 2007; Prenen et al. 2009). Since 2005, treatments aimed for targeted therapy have emerged. The most notable ones are the multiple receptor tyrosine kinase inhibitors Sunitinib and Sorafenib (Thompson et al., 2009; Yang et al. 2003). These drugs interfere with Vascular Epithelial Growth Factor (VEGF), Platelet Derived Growth Factor (PDGF), and Ras/Raf/MEK pathways that are activated in ccRCC due to accumulation of HIFα (Costa and Drabkin, 2007). Another targeted approach for ccRCC is to inhibit the mammalian target of rapamycin (mTOR) pathway. mTOR activation is known to facilitate HIFα signalling through stabilization of the complex (Prenen et al., 2009). Although randomized phase II and III clinical trials have demonstrated the effectiveness of these novel targeted therapies on progression free survival, metastatic ccRCC remains as a fatal disease.

1.2 Cervical Cancer

As of 2008, cervical cancer was the third most common cancer in female worldwide, and accounts for 8% of cancer-related deaths (Jemal et al., 2011). Specifically, approximately 530,000 new cases are diagnosed every year, with over half of these patients dying from the disease (Jemal et al., 2011). Globally, the incidence of cervical cancer is disproportional, with approximately 80% of the cases occurred in developing countries. This is due to lack of educations, and for the most part, shortage of Papanicolaou tests for early diagnosis (de Martel et al., 2012; Hoory et al. 2008). Histologically, cervical cancer can be categorized into two major types: squamous cell carcinoma (SCC) and adenocarcinoma (AdC). Tumour of SCC develops from the squamous epithelia of the ectocervix. It accounts for 80-85% of total cervical cancers (Green et al., 2003; Tjalma et al. 2005). In contrast, AdC only represent 10% of overall cases and tumours arise from the glandular epithelia of the endocervix (Green et al., 2003).

Over the last 2 decades, Human Papillomavirus (HPV) has been recognized for its association with cervical cancer. To date, infection by HPV is widely accepted as the primary cause of the disease (de Freitas et al., 2012). Within the family of papillomavirus, 120 HPV have been identified. Among them, 40 are known to infect the genital area (de Freitas et al., 2012). Base on the virus's ability to integrate itself to the host genome, HPVs are divided into High-Risk (HR) or Low-Risk (LR) group with probability of genome integration at 1% and 0.1% respectively (Tsuda, 2003). Within HR-HPVs, HPV16 and HPV18 are the first and second most commonly identified virus occurring in cervical cancer (Clifford et al. 2003; Dürst et al. 1983). The structure of HPV is identified

as double stranded, closed circular DNA of approximately 8kb size with no envelope (Bravo et al. 2010). The genome of HPV encodes eight different proteins which are named according to the functional timing of these proteins during the virus's life cycle (Baker et al., 1987). In the reproductive infection of HPV, early (E) proteins are required for transcriptional regulation (You et al., 2004). Specifically, the E2 protein is critical in initiation of viral DNA replication and regulation of E6 and E7 expression (González Martín, 2007; Hoory et al., 2008). The late protein L1 and L2 are capsid proteins that are required for packaging of infectious viral particles (Evander et al. 1997; Qi et al., 1996; Roden et al. 1994). The proliferative infection on the other hand, is the critical event for the development of cervical cancer (González Martín, 2007). During this phase, the HPV DNA is integrated into the host genome. Usually, the circular DNA of HPV is disrupted at the E2 gene, resulting in an integration of HPV that has lost the function of E2 protein (Yu et al., 2005). Consequently, uncontrolled E6 and E7 expression due to absence of E2 leads to persistent cell proliferation (Boyer et al. 1996; González Martín, 2007; Münger et al., 2001; Scheffner et al. 1990; Tommasino et al., 2003). On one end, the E7 protein blocks the activity of cyclin-dependent kinase inhibitors p21 and p27, whereas the E6 protein inhibits p53 function through degradation by ubiquitination (González Martín, 2007). Collectively, the E6 and E7 proteins facilitate cell proliferation by preventing cell cycle arrest.

HPV 18 demonstrated 100% integration, only 75% of HPV 16 positive tumours reveal genome integration (Cullen et al. 1991; Ferenczy and Franco, 2002), thereby suggesting the requirement of other factors in the tumorigenesis of the cervix. One such factor is diet.

A study has suggested that frequent intake of foods containing high level of vitamin A, in particular, retinol, help reduce risk of organ-confined cervical cancer (Shannon et al., 2002). However, researchers weren't able to demonstrate the relationship between retinol food intake and metastatic disease. Moreover, diet high in vitamin B_1, B_2 and B_{12} is thought to decrease the persistency of HPV infection, possibly through their function in DNA methylation (Hernandez et al. 2003). In addition, foods rich in natural anti-oxidants are also protective against cervical cancer due to the ability of anti-oxidants to reduce DNA damages (Duarte-Franco and Franco, 2004). Another factor contributing to cervical cancer development are sex hormones. Estrogen is known to induce the immune system during infection (Sonnex, 1998); however, the effect of estrogen on cervical cancer is related to the stage of the infection and disease. During early HPV infection, estrogen is beneficial due to its stimulation of innate and humoral immunity. In contrast, estrogen may increase susceptibility to cervical cancer in persistent HPV infection (Brabin, 2002). It is known that squamous epithelia contain estrogen receptors (Monsonego and Magdelenat, 1991; Sonnex, 1998). Upon the activation of estrogen receptors, cells undergo proliferation. In conjunction with the anti-apoptoic effect of the HPV E6 and E7 proteins, incidence of pre-cancerous cervical intra-epithelial neoplasia progressing into invasive disease become more frequent in response to estrogen signalling.

The standard treatments for cervical cancer have been radiotherapy and chemotherapeutic agents such as cisplatin (Bonomi et al., 1985; Omura, 1994). Nonetheless, recurrences often occur in patients and the long term survival rate of patients with metastatic disease is only 9 months (Long et al., 2005). In an effort to improve the effectiveness of current

treatments, therapies that target specific molecular pathway of the cancer have been developed. A major group of drugs that have been implicated with high efficacy are treatments that target VEGF pathway. These include Bevacizumab, antibodies against VEGF, and Sunitinib, a tyrosine kinase inhibitor (Caceres-cortes et al., 2001; Ferrara et al. 2004; Presta et al., 1997). Angiogenesis is important in tumour formation. Without proper formation of blood vessel, tumour size is greatly limited (Folkman, 1971; Rahimi, 2012). Cetuximab and Gefitinib belong to another group of therapies which aim to inhibit the action of Epithelial Growth Factor Receptor (EGFR) (Fukuoka et al., 2003; Therapy, 2013). It is well established that activation of EGFR leads to tumorigenesis through activation of the PI3K/Akt pathway (Berg and Soreide, 2012). mTOR is also widely targeted owning to its involvement in cell proliferation, migration and survival (Strimpakos et al. 2009; Temkin et al. 2010; Zagouri et al. 2012). A novel approach in treating cervical cancer is the use of histone deacetylase inhibitors, Entinostat and Valproic acid (Isenberg et al., 2007; Ryan et al., 2005). Epigenetic silencing of tumour suppressor genes has been implicated as a mechanism for cancer development (Takai et al., 2011). Targeting histone deacetylase may decrease incidence of tumour suppressor repression.

1.3 Loss of Heterozygosity at Chromosome 17p

Deletion of allele on chromosome has been observed in various cancers. Specifically, incidences where loss of allele occurred on chromosome 17p have been reported in colorectal cancer (Sato et al. 1990), small cell lung cancer (De Smaele et al., 2004), bladder cancer (Phelan et al., 1998), breast cancer (Seitz et al., 2001) and ovarian cancer (Stack et al., 1995). This loss of heterozygosity that was detected repeatedly at chromosome 17p implies the existence of tumour suppressor genes. Consistent with this notion, the *TP53* gene which encodes one of the best known tumour suppressors is known to reside on 17p13.1 (Stack et al., 1995). In addition to the *TP53* locus, 17p13.3 is also frequently lost in breast and ovarian cancer (Phillips et al., 1996). The coding sequence of the region has been cloned and named OVCA1 or DPH2L by two different groups (Phillips et al., 1996; Schultz et al., 2002). Nonetheless, both studies were able to demonstrate that in over 80% of ovarian cancer cases, expression of OVCA1/DPH2L was reduced.

The prevalence of allele losses on chromosome 17p is not only due to the presence of TP53 at 17p13.1. Several studies have shown allelic alteration at 17p13.2 in cancer (Byun et al., 2003; Chuensumran et al., 2007; Stack et al., 1995). By combining yeast artificial chromosome with florescent in situ hybridization technique, mapping on chromosome 17p revealed a novel site that also exhibits high frequency of allele lost in 40 breast cancer patients (Stack et al., 1995). This new region lies downstream of TP53 with parts of the chromosomal band confined by 17p13.3 suggested new potential tumour suppressors residing in the region. Expanding on this notion, allelic imbalance is also

reported in intrahepatic cholangiocarcinoma (Chuensumran et al., 2007). Of the 52 patient exanimated, 16 of them indicated amplification at this region, whereas seven cases showed loss of heterozygosity. In addition, the disease prognosis was dependent on allele status with reduction linked to poor clinical outcomes (Chuensumran et al., 2007).

In addition to loss of heterozygosity, epigenetic modification is also shown to be involved in inactivation of tumour suppressors (Byun et al., 2003). It was reported that there is a high frequency of methylation on the promoter of the *XAF1* gene which is located on chromosome 17p.13.2. XAF1 is known to promote apoptosis by inhibiting the action of XIAP (X-linked inhibitor of apoptosis), an anti-apoptotic protein (Byun et al., 2003). Therefore silencing of XAF1 by hypermethylation was thought to be the primary contribution to the development of gastric tumours. Collectively, results from these studies suggest the emerging role of chromosome 17p13.2 in human cancer.

1.4 PI3K/Akt Pathway in Carcinogenesis

The PI3K/Akt pathway regulates cell growth and survival which are important aspects of cancer (Fresno Vara et al., 2004). Phosphatidylinositol-3 kinase (PI3K) is activated upon binding of growth factor to receptor protein tyrosine kinase. This results in the generation of second messenger phosphatidylinositol-3, 4, 5 triphosphate (PIP_3) that interacts and activates Akt (Bartholomeusz and Gonzalez-Angulo, 2012; Hassan et al., 2013). As a result of PIP_3 binding, Akt undergoes conformation change to expose two

phosphorylation sites (Fresno Vara et al., 2004). At the same time, Akt is also translocated to the inner side of the membrane and phosphorylated at serine-473 by mTOR2 (Jacinto et al., 2006; Sarbassov et al. 2005). Subsequently, PDK1 phosphorylates Akt at threonine-308 which results in full activation of Akt (Blume-Jensen & Hunter, 2001). This phosphorylation at T308 is thought to contribute to the stabilization of Akt in its active form.

The activated Akt interacts with various substrates to regulate process of apoptosis and cell cycle progression. Pro-apoptotic factor Bad is inactivated due to phosphorylation by Akt (Albert et al., 2002). On the other hand, Akt also inactivates the Forkhead family of transcription factor (FKHR) through phosphorylation. FKHR is known to induce the expression of pro-apoptotic Fas ligand (Testa and Bellacosa, 2001). In addition, Akt activates cyclic AMP response element-binding protein (CREB), which in term leads to expression of anti-apoptotic NF-κB (Porta and Figlin, 2009). In contrast, Akt promotes cell proliferation through its phosphorylation on glycogen synthase kinase-3 (GSK3), p27, and mTOR. GSK3 is inhibited by Akt (Fresno Vara et al., 2004). As the result, β-catenin accumulates and eventually translocates into the nucleus. Within the nucleus, β-catenin induces cell cycle progression by promoting expression of cyclin D1 (Diehl et al. 1998). Akt prevents p27 translocation out of the cytoplasm, thereby inhibits its anti-proliferative function (Viglietto et al. 2002). Lastly, Akt activates mTOR by inhibiting the formation of TSC1/2 complex (Porta and Figlin, 2009), thereby causing the activation and phosphorylation of $p70^{s6k}$ which lead to cell cycle progression.

The PI3K/Akt pathway is often altered in human cancers. Specifically, overexpression of Akt has been observed in ovarian, pancreas, breast and stomach tumours (Bellacosa et al., 1995; Cheng and Ruggeri, 1996). In a recent study by Sourbier *et al.* 2006, Akt was found to be constitutively active in both VHL positive and negative ccRCC cell lines (Sourbier et al., 2006). In consistent with this notion, the critical role of Akt activation in the progression of ccRCC is further supported by their animal work. By treating with a PI3K inhibitor, nude mice injected with ccRCC cells form significantly smaller tumours compared to those treated with control DMSO (Sourbier et al., 2006). Recently, a study has proposed a mechanism for constant activation of Akt (Carpten et al., 2007). It was identified that a point mutation replacing glutamic acid at position 17 with lysine occurred in the Pleckstrin homology domain of Akt. This allows the translocation of Akt to the membrane independent of PI3K activation (Carpten et al., 2007). In line with these results, amplification on PI3K was also reported in 50% of cervical cancer (Ma et al., 2000). It was found that *PIK3CA*, the gene encoding the p110α catalytic subunit of PI3K was overexpressed in 8 of 18 cervical tumours. The mechanism by which p110α is amplified is thought to be a result of somatic mutation on exon 9 and 20 of the *PIK3CA* gene (Hassan et al., 2013). In particular, exon 9 mutations are most often observed in cervical squamous carcinoma and colorectal cancer, whereas mutations on exon 20 are frequently seen in breast and ovarian cancer (Bartholomeusz and Gonzalez-Angulo, 2012). In addition to direct interference PI3K and Akt, loss of PTEN function is also frequently observed in prostate, breast, and endometrial cancer (Freihoff et al., 1999; McMenamin et al. 1999; Salvesen and MacDonald, 2001). PTEN is an upstream negative

regulator of the PI3K/Akt pathway. PTEN loss is most frequently due to somatic mutation, loss of heterozygosity, and epigenetic silencing (Garcia et al., 1999; Khan et al., 2004; Risinger et al., 1998; Julun Yang et al., 2010).

1.5 Membrane-Associated Progesterone Receptors

The membrane-associated progesterone receptors (MAPR) are a group of heme-binding proteins that belongs to the larger cytochrome b5 family (Mifsud and Bateman, 2002). Members of the MAPR family include CYB5D2 (neuferresin), neudesin, and progesterone receptor membrane component 1/2 (PGRMC1/2) (Kimura and Nakayama, 2012; Mifsud and Bateman, 2002). Each of the members contains a C-terminal cytochrome-b5-like heme/steroid binding domain, and shares high structural similarities (Mifsud and Bateman, 2002). Despite the homologies they share, the distribution of these proteins differs from one another. Neudesin and CYB5D2 (neuferrsein) have been shown to be a secretive protein, whereas PGRMC1/2 are single transmembrane proteins (Kimura and Nakayama, 2012). The activity of these proteins can be affected by the binding of heme to the cyb-b5-like heme binding domain. Heme has important functions in various biochemical reactions including molecule transportation and signalling transduction (Kimura and Nakayama, 2012). In support of this notion, the association of heme to haemoglobin and myoglobin in oxygen transport is a well-known process (Burmester et al., 2000). In addition, synthesis of steroids by cytochrome also requires heme (Black et al. 1994). It is clear that heme-binding proteins play role in a variety of physiological and

biological processes, however, beside their normal physiological functions; in line with this notion, MAPR family members have been shown to function in tumorigenesis.

Neudesin was identified by Kimura and colleagues (Kimura et al., 2005) while searching the mouse embryonic cDNA database. Both human and mouse neudesin contain heme-binding domain that share high homologies with PGRMC1 (Kimura et al., 2008). The mouse neudesin is highly homologous to human neudesin (Kimura and Nakayama, 2012). By mutating the amino acid sequence, the group was able to identify Tyr-81 and Tyr-87 as crucial residues required for heme-binding (Kimura et al., 2008). Similar to other members of the MAPR family, bioactivity of neudesin is dependent on heme binding. When associated with heme, neudesin possesses neurotrophic and neuroprotective effects (Kimura et al., 2008; Kimura et al., 2006; Kimura et al., 2005). In mice, the expression of neudesin is observed in brain, heart, lung and kidney (Kimura et al., 2006). Collectively, evidence suggests that neudesin is an important factor in the development of the nervous system. Studies have reported that under serum starvation, the addition of recombinant neudesin significantly increase the survival of primary cultured mouse neurons in a dose-dependent manner (Kimura et al., 2005). Phosphorylation of Erk1/2 and Akt was observed in mouse neurons in the presence of recombinant neudesin, suggesting the improvement of cell survival induced by recombinant neudesin being attributed to the activation of MAPK and PI3K pathways (Kimura et al., 2005). In consistent with this finding, knockdown of neudesin robustly reduced the proliferation of Neuro2a cells (Kimura et al., 2008).

The association of neudesin to cancer was first reported in a study that investigated the relationship between relative abundance of neudesin level and breast cancer (Neubauer et al., 2006). Progesterone receptors (PR) and estrogen receptors (ER) have been used as prognostic factor in breast cancer progression (Platet et al., 1998; Thompson et al., 1992). The presence of these two receptors on tumour often indicates good response to tamoxifen treatment. However, those with estrogen receptors alone are usually less responsive to the same dose (Jakesz et al., 2005; Ravdin and Green, 1992). In order to provide an explanation to this discrepancy, neudesin was found to be over-expressed in PR^+/ER^+ tumour compared to PR^-/ER^+ tumour (Neubauer et al., 2006), suggesting a role of neudesin in sensitizing PR^+/ER^+ breast tumours to tamoxifen; this would implies that neudesin has a negative impact on breast tumorigenesis. However, a recent study has shown that neudesin promotes breast cancer tumorigenesis (Han et al., 2012). By ectopically expressing neudesin in MCF-7 breast cancer cells, the invasiveness of cells significantly increased. Moreover, athymic nude mice with bilateral injection of neudesin overexpressing MCF-7 cells formed larger tumours when compared to mice injected with empty vector cells (Han et al., 2012). The PI3K/Akt pathway is often disrupted in many cancers owning to its contributions to cell proliferation (Fresno Vara et al., 2004). In line with this notion, the group also reported an increase in phosphorylated Akt and ERK in cells overexpressing neudesin in comparison to control cells (Han et al., 2012). The conflicting role of neudesin in breast cancer might be explained through its interaction with the P450 proteins (Debose-Boyd, 2007; Hughes et al., 2007; Rohe et al. 2009). Since P450 proteins are required for generation of active tamoxifen metabolites, the up-

regulation of neudesin in PR^+/ER^+ breast cancer may thus facilitate the tumour to tamoxifen-derived cytotoxicity. However, under other situations, neudesin may promote breast cancer progression via its roles in activating Akt and Erk. Therefore, neudesin may different impacts on breast cancer.

1.6 PGRMC1 and Cancer

PGRMC1 is the most investigated member of the MAPR family. PGRMC1 was first identified by Gredes et al. (Gerdes et al., 1998), and was named Human membrane progesterone receptor (Hpr6.6). Despite the fact that PGRMC1 was later found not to belong to progesterone receptor, it can still exert effect on progesterone signalling through binding of P450 proteins and PAIR-BP1 (Gerdes et al., 1998) thereby influences the development of cancer (Fresno Vara et al., 2004). In consistence with this notion, there are reports indicating the emerging role of PGRMC1 in the pathology of cancer. In a study reported by Hand and Carven (Hand and Craven, 2003), it was observed that oxidative stress induced a 4-fold higher phosphorylation on Akt in MCF-7 cells transfected with PGRMC1 in comparison to control cell. Although how PGRMC1 activates Akt was not investigated in the study by Hand and Carven (Hand and Craven, 2003). The mechanism became clear in recent study by Ahmed et al. (Ahmed et al. 2010) where they have shown that PGRMC1 associated with the epidermal growth factor receptor (EGFR) to regulate cell survival. By using small interfering RNA (siRNA) and immunofluorescence, they were able to demonstrate that knockdown of PGRMC1

reduced the plasma membrane staining of EGFR was reduced (Ahmed et al., 2010), indicating a role of PGRMC1 in stabilizing EGFR on the plasma membrane. It is possible that PGRMC1 induces Akt activation through the EGFR signalling, an event that is known to activate the PI3K/Akt pathway.

Consistent with its role in Akt activation, several papers have suggested a role for PGRMC1 in promoting tumorigenesis. In a recent study by Caven et al. (Ahmed et al., 2010), it was found that athymic nude mice implanted with A549 control cells developed tumours weighting 2.9 fold more than mice implanted with A549 cells in which PGRMC1 was knocked down (Ahmed et al., 2010). Furthermore, cells treated with PGRMC1 RNAi were not able to form colonies on soft agar in comparison to normal A549 cells (Ahmed et al., 2010). In line with these observations, PGRMC1 also takes part in breast cancer development. Human breast cancer MCF-7 cell transfected with Serine-180 to Alanine (S180A) mutated PGRMC1 showed an increase in cell proliferation under progesterone treatment (Neubauer et al., 2009). The role of Serine-180 in PGRMC1 function was further confirmed by another study where MFC-7 cell transfected with S180A PGRMC1 demonstrated enhance survival compared to cell ectopically expressing PGRMC1 with mutations at other residues, S56A, Y138F, and Y179F (Neubauer et al., 2008). The same group also observed increased expression of PGRMC1 in estrogen receptor α negative breast tumour (Neubauer et al., 2008). It is revealed that serine-180 is one of the phosphorylation sites of PGRMC1, and may be a location for potential protein interaction or signalling transduction (Cahill, 2007).

PGRMC1 is also able to enhance cell viability under various treatments by anti-neoplastic agents. Knockdown of PGRMC1 in MDA-MB-231 human breast cancer cell bi RNAi displayed increased susceptibility to doxorubicin (Crudden et al. 2006). Moreover PGRMC1-mediated enhancement of cell survival requires heme association, as infection of MDA-MB-231 with adenovirus expressing PGRMC1-D120G, a heme-binding defected PGRMC1 mutant, significantly reduced its ability to sustain cell survival (Crudden et al. 2006). In a separate study using ovcar-3 cell, over-expression of PGRMC1 reduced the percentage of apoptotic nuclei (Peluso et al., 2008). Collectively, accumulating evidence support a role of PGRMC1 in promoting tumorigenesis.

1.7 Current Understanding of CYB5D2

Our current understanding of CYB5D2 remains very limited. The protein was first reported by a group of Japanese scientists for its role in promoting neurogenesis and CYB5D2 was thus named as Neuferricin (Kimura et al. 2010). To determine the putative functions of CYB5D2, siRNA targeting CYB5D2 was introduced into Neuro2a neuroblastoma cells. It was revealed that CYB5D2 knockdown in Neuro2a cell showed elevation in cell proliferation and survival compared to control siRNA-treated Neuro2a cell (Kimura et al. 2010). Furthermore, reduction of CYB5D2 in Neuro2a cell induced a significantly higher expression of Bcl-2, but not of Bax and p53 (Kimura et al. 2010). Bcl-2 is known to suppress apoptosis, whereas Bax and p53 promote apoptosis (Ferlazzo et al., 2008). This evidence suggested that higher cell viability observed in CYB5D2

knockdown cell may be attributable to up-regulation of Bcl-2. Recently, our publication demonstrated the ability of CYB5D2 to confer HeLa cell survival under etoposide induced cytotoxicity (Xie et al., 2011). However, this increase in survivability does not involve core apoptosis process as major apoptotic proteins including caspase 3, caspase 9 and Bcl-xL level were comparable in CYB5D2 overexpressing and empty vector transfected HeLa cell (Xie et al., 2011).

II. HYPOTHESIS AND OBJECTIVES

The central hypothesis is that CYB5D2 possesses tumour suppression activity. The main objectives of my research are to investigate whether CYB5D2 reduces tumorigenesis. Specifically, I will examine the expression of CYB5D2 in primary cervical cancer and ccRCC, and investigate the effects of CYB5D2 on the tumorigenic activities of HeLa cervical cancer and A498 ccRCC cells.

III. MATERIAL AND MATHODS

3.1 Reagents

Agarose, ammonium per sulphate (APS), ampicillin, aprotinin, bovine serum albumin (BSA), Bromophenol blue, calcium chloride ($CaCl_2$), crystal violet, dimethyl sulfoxide (DMSO), dipotassium hydrogen orthophosphate (K_2HPO_4), disodium hydrogen, EDTA, ethidium bromide, GenElute Plasmid Miniprep kit, glycine, leupeptin, phenylmethylsulfonyl fluoride (PMSF), potassium acetate, potassium chloride, potassium dihydrogen orthophosphate (KH_2PO_4), sodium chloride (NaCl), sodium citrate, sodium dihydrogen orthophosphate (NaH_2PO_4), sodium dodecyl sulphate (SDS), sodium fluoride, sodium orthovanade (Na_3VO_4), TEMED, Tris, Triton X-100, Tween 20, β-glycerophosphate, and β-mecaptoethanol were purchased from Sigma, Oakville, ON. Hygromycin B and trypsin-EDTA were purchased from Invitrogen, Carlsbad, CA. 30% Acrylamide/Bis solution was purchased from Bio-Rad, Mississauga, ON. Methanol was purchased from Caledon Laboratories, Georgetown, ON. Reagent alcohol was purchased from Commercial Alcohols, Brampton, ON.

3.2 Cell lines and Antibodies

3.2.1 Cell Lines

A498 Human ccRCC cells, 293T Human embryonic kidney cells, and HeLa Human cervical cancer cells were purchased from American Type Culture Collection (ATCC).

A498 cells were cultured in Minimum Essential Medium (MEM); 293T and HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) in a tissue culture incubator set at 37^{o}C and 5% CO_2. All media were supplemented with 10% fetal bovine serum (FBS) (Sigma, Oakville, ON), and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA).

3.2.2 Antibodies

Antibodies used for western blotting and immunohistochemistry were rabbit anti-CYB5D2 (developed from our laboratory), goat anti-AKT (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho AKT (Cell Signalling, Danvers, CA), mouse anti-GSK3β (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho GSK3β (Cell Signalling, Danvers, CA), rabbit anti-P70S6K (Cell Signalling, Danvers, CA), rabbit anti-phospho P70S6K (Cell Signalling, Danvers, CA), and goat anti β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies for western blotting were anti-rabbit and anti-mouse IgG purchased from Amersham, and anti-goat IgG purchased from Santa Cruz Biotechnology. Secondary antibody for immunohistochemistry was purchased from Vector Laboratories (Burlingame, CA).

3.3 Generation of anti-CYB5D2 Antibody

3.3.1 Purification of GST Fusion Protein

Plasmid containing GST-CYB5D2 fusion cDNA was obtained from Dr. Fengxiang Wei. BL-21 competent cells were transformed with GST-CYB5D2 plasmid. In brief, 1 µL of the plasmid was added into 100 µL of BL-21 cells. Cells were transferred into 13 mL round bottom tube and incubated on ice for 30 minutes. Following incubation, cells were heat shocked in water bath at 42^{o}C for 90 seconds, then placed on ice for 2 minutes. 900 µL of Lysogeny Broth (LB) media was added to cells and shook at 250 rpm at 37^{o}C for 1 hour. After shaking, 100 µL of LB media with transformed BL-21 cells were spread on ampicillin plate using hockey stick made from glass pipette. The plate was incubated at 37^{o}C overnight. Single bacterial colony was picked from the plate and grew in 3 mL LB media with 100 µM ampicillin at 37^{o}C overnight while shaking at 250 rpm. 3 mL LB with bacteria were then added into 500 mL of fresh LB media with 100 µM ampicillin and shook at 250 rpm at 37^{o}C for bacterial growth. After 3 to 4 hours, absorbance of 1 mL of LB media was measured at 595 nm to determine the extent of growth. When the optical density (OD) reached to 1.0-1.2, IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to LB media to a final concentration of 0.2 mM for an overnight shaking at 250 rpm at room temperature to induce protein expression. In the same time, Glutathione Agarose beads (Sigma, Oakville, ON) was prepared according to manufacturer's instruction. In brief, 70 mg of dry agarose beads were measured and mixed with ddH_2O in 50 mL conical tube. The tube was inverted 3-4 times and left at 4^{o}C, overnight, to allow for maximum swelling. After overnight induction of protein expression, bacteria-containing medium was centrifuged at 4000 rpm, 4^{o}C for 15 minutes. The supernatant was discarded and the bacterial pellet was re-suspended in 10 mL 1x MTPBS (10x

MTPBS, NaCl 87.66g + Na_2HPO_4 22.71g + NaH_2PO_4 4.8g in ddH_2O to final volume of 1L, pH 7.3). On ice, bacterial solution was sonicated according to the following procedure: medium speed for a 10 second sonication and rest for 20 seconds, repeating this procedure 20 times. Following sonication, Triton X-100 was added to the solution to a final concentration of 1%, and the solution was centrifuged at 10,000 g, 4^{o}C for 20 minutes. The supernatant was collected and then incubated with pre-swelled glutathione agarose beads at 4^{o}C for 1 hour with gentle shaking. Beads along with solution were transferred to a column with 1cm diameter. Beads were then washed at 4^{o}C with 1x MTPBS-DTT (1x MTPBS with addition of 1 mM DTT). Absorbance of the eluate was measured at 280 nm. When OD was less than 0.05, 5 mM of glutathione (make fresh, 0.0154g + 10mL 50mM Tris-HCl pH 8.0 + 1mM DTT) was added to elute protein. 1 mL of the glutathione solution was added and collected each time for a total of 10 mL. Concentration of protein in each fraction was determined by measuring the absorbance at 595 nm using Bradford assay. Proteins were then dialyzed in 1x MTPBS at 4^{o}C overnight. Dialysis continued the following day, 1x MTPBS was changed every 2 to 3 hours for 3 to 4 times. Concentration of proteins was measured again at 595 nm. Proteins were stored in -80^{o}C fridge for future use.

3.3.2 Purification of anti-CYB5D2 Antibody

To generate antibody specific for CYB5D2, rabbit was immunized against CYB5D2 and serum was collected by Anthony Bruce. To separate immunoglobulin G (IgG) type antibody from serum, saturated ammonium sulphate ($(NH_4)_2SO_4$, Sigma, Oakville, ON)

was prepared by adding 950 mg of $(NH_4)_2SO_4$ to 1 L of ddH_2O and stirred at room temperature overnight. The solution was then kept at 4°C for at least 24 hours before use. Equal volume of cold saturated $(NH_4)_2SO_4$ was added drop-wise to cold rabbit serum while stirring at 4°C to give the solution a final concentration of 50% ammonium sulphate. The mixture was continued to stir at 4°C overnight, followed by centrifugation at 10000 g at 4°C. The supernatant was removed and the precipitated IgG pellet was re-suspended in 10% of original serum volume in 1x PBS. IgG was then dialyzed against PBS. PBS was changed 3 times. 0.3 g of Cyanogen bromide activated sepharose 4B beads (Sigma, Oakville, ON) was swollen in 15 mL of 10 mM HCl at room temperature for 30 minutes. Beads were then washed with 250 mL of 10 mM HCl in a column with 1 cm diameter. Coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was allowed to run through beads in the column to activate the protein binding site. Beads were then transferred to 15 mL conical tube with addition of 10 mg of GST-CYB5D2 fusion protein and incubated at 4°C overnight with gentle shaking. Following the incubation, beads were washed with 20 bed volumes of coupling buffer. Excessive protein coupling sites of the beads were blocked with 0.2 M glycine, pH 8.0 at 4°C for 16 hours with gentle shaking. Upon completion of blocking, beads were washed with 20 bed volumes of coupling buffer, followed by 20 volumes of sodium acetate buffer, pH 4.0, 20 volumes of coupling buffer, and then 10 volumes of 1X PBS; GST-CYB5D2 affinity column was then ready for purification of anti-CYB5D2 IgG by addition of the IgG solution to the column; the flow-through IgG solution was collected and re-applied to the column 3 times. Beads were then washed with PBS until OD measured at 280 nm below 0.02. Antibody was eluted by adding 10

mL of 0.1 M glycine, pH 3.0 at 1 mL each for 10 times. Each fraction of eluate was collected in 1.5 mL eppendorf tube containing pre-determined volume of 1.5M Tris-HCl, pH 8.8, to neutralize the eluate. Eluted antibodies were concentrated using spin column (Amicon Ultra-4 Centrifugal Filter Unit, Millipore, Billerica, MA) according to manufacturer's instruction. Briefly, 4 fractions of eluted antibodies (total of 4 mL) were applied to the column and centrifuged at 3270 g at 4^{o}C for 15 minutes. Concentrated antibodies were collected and concentration determined at 595 nm. Antibodies were stored in -80^{o}C fridge for future applications.

3.4 Cell and Primary ccRCC Tissue Protein Analysis

3.4.1 Kidney Tissue and Primary Tumour Tissue Lysate Preparation

Approximately 10 mg of primary ccRCC tumour and the respective normal adjacent tissue was cut. The samples were then homogenized using homogenizer in appropriate amount of lysis buffer. Lysate was prepared as described below.

3.4.2 Cell Lysate Preparation

Cell lysates were obtained by washing cells twice with cold phosphate buffered saline (PBS, 1.36M NaCl, 14.7 mM KH_2PO_4, 80 mM NaH_2PO_4, 26.8 mM KCl, pH 7.4). Cells were then collected in cold PBS using a cell scraper, followed by centrifugation at 2000 rpm (it is better to give g force), 4^{o}C for 5 minutes. PBS was removed by suction and the cell pellet was lysed in appropriate amount of cell lysis buffer (20 mM Tris-HCl pH 7.4,

150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate) containing 1 mM PMSF (phenylmethylsulfonyl fluoride), 2 µg/mL leupeptin, and 10 µg/mL aprotinin on ice for 30 minutes. Cell lysates were transferred into 1.5 mL eppendorf tube and centrifuged at 16400 rpm for 15 minutes at 4^{o}C. The supernatant was collected and the protein concentration was determined using Bradford assay (Bio-Rad, Mississauga, ON). Absorbance was measured at 595 nm using Ultrospec 2100 pro spectrophotometer (Biochrom Ltd, Cambridge, England). Lysates were stored in -80^{o}C fridge for future applications.

3.5 Western Blotting

A total of 100 µg of protein from cell or tissue lysate was prepared in 5x Protein Sample Buffer (PSB, 0.1 mM Tris pH 6.8, 5% SDS, 50% glycerol, 2% β-mercaptoethanol, 0.02% bromophenol blue) and diluted to final concentration of 1x PSB using ddH_2O. Prior to gel electrophoresis, protein samples were boiled for 3 minutes using a heat block. 12.5% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel was used to separate proteins at 50 mA in running buffer (20 mM Tris-HCl, 192 mM glycine, 1% SDS, diluted to final concentration of 1X running buffer by ddH_2O). Amersham hybond ECL nitrocellulose membrane (Amersham, Bair d'Urfe, QC) was prepared in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol) and proteins were then transferred onto the membrane at 260 mA for 75 minutes. 5% skim milk dry power in 1x

Tris Buffered Saline with 0.1% Tween 20 (TBST) was used to block the membrane for 1 hour at room temperature with shaking at 55 rpm. Membranes were then washed by 1x TBST for 3 times with shaking at 155 rpm, followed by overnight incubation at 4^{o}C using specific primary antibodies. Membranes were washed 3 times by 1x TBST before the incubation with appropriate secondary antibodies at room temperature for 1 hour. Membranes were subsequently washed by 1x TBST for 3 times and signals were detected using ECL Western Blotting kit (Amersham) and exposed onto Kodak X-OMAT X ray film. Primary antibodies were prepared as follows: anti-CYB5D2 (1:100) in 5% skim milk dry power in 1x TBST, anti-β actin (1:1000), anti-AKT (1:1000), anti-phospho AKT (1:500), anti-GSK3β (1:1000), anti-phospho GSK3β (1:1000), anti-P70S6K (1:1000), and anti-phospho P700S6K (1:1000) in 5% bovine serum albumin power in 1x TBST. Secondary antibodies were prepared as follows: anti-rabbit (1:3000), anti-mouse (1:3000), anti-goat (1:3000) in 5% skim milk dry power in 1x TBST.

3.6 Immunohistochemistry (IHC)

3.6.1 IHC Analysis of Primary Tissue Slides

Paraffin blocks containing primary ccRCC tumour and the respective normal adjacent kidney tissues were obtained from Brianne Hill. Tissue blocks were sent to the Core Histology Laboratory at McMaster University for preparation of slides. Tissue slides were deparaffinised by 3 successive incubations in xylene, each for 10 minutes. Slides were rehydrated in 100% ethanol (EtOH) twice, each time for 2 minutes, followed by 2 washes,

each for 2 minutes, in 70% EtOH. Slides were then washed in ddH_2O using a coplin jar for 2 times at 2 minutes each with shaking at 120 rpm. 30% hydrogen peroxide solution (H_2O_2, Sigma, Oakville, ON) was diluted using ddH_2O to the final concentration of 3% and applied to the slides for 15 minutes at room temperature to remove any trace of endogenous peroxidase activities. During H_2O_2 incubation, antigen retrieval buffer (Sodium Citrate Buffer, 10 mM sodium citrate, 0.05% tween 20, pH 6.0) was heated in a food steamer for 20 minutes. Slides were washed by 1x PBS at 120 rpm in a coplin jar, followed by incubation in pre-heated antigen retrieval buffer in food steamer for 20 minutes. Slides were cooled down in room temperature while still submerged in the buffer. Excess antigen retrieval buffer was removed by washing slides with 1x PBS. Slides were then incubated with blocking buffer (10% normal goat serum, 1% bovine serum albumin) for 1 hour at room temperature in a humid chamber. Slides were partially dried by shaking off remaining blocking buffer, followed by overnight incubation in humid chamber with primary antibody (anti-CYB5D2, 1:250) prepared in blocking buffer at 4^oC. Subsequently, slides were washed by 1x PBS and secondary anti-rabbit biotinylated antibody (1:200, Vectastain ABC kit, Vector Laboratories) was applied to slides for 1 hour at room temperature in humid chamber. This is followed by washing the slide with 1x PBS and incubation of ABC reagent for 1 hour at room temperature in humid chamber. ABC solution was prepared according to manufacturer's instruction (Vectastain ABC kit, Vector Laboratories) during secondary antibody incubation. In brief, 20 µL of reagent A was added into 1 mL of blocking buffer, followed by addition of 20 µL of reagent B. After incubation with ABC reagent, slides were washed with 1x PBS

and staining detected by addition of DAB solution for 90 seconds. DAB solution was prepared according to manufacturer's instruction (Vector Laboratories) just prior to use. Briefly, 1 drop of buffer solution, 4 drops of DAB, and 1 drop of Hydrogen Peroxide solution were added in successive sequence into 5 mL of ddH_2O. DAB solution was removed by ddH_2O and slides were incubated in ddH_2O for 5 minutes. Haematoxylin (Sigma, Oakville, ON) was used to counterstain slides for 10 seconds, followed by washing slides with running tap water and incubation in tap water for 5 minutes. Slides were then dehydrated in 70% EtOH for 2 incubations with each for 3 minutes, followed by 100% EtOH of the same procedure. Finally, slides were incubated in xylene for 3 minutes in 2 subsequent series. Coverslips were applied to slides using Cytoseal mounting solution (Richard Allan Scientific, Kalamazoo, MI).

3.6.2 IHC Analysis of Tissue Microarray Slide

Tissue microarray (TMA) slide, CR1001, was purchased from US Biomax, Rockville, MD. Patients' information was summarized (Table 1). TMA slide was baked at $55^{o}C$ in oven for 1 to 2 hours to melt the layer of paraffin. TMA slide was then stained following the procedure described above. Upon completion of staining, TMA slide was sent to the Advanced Optical Microscopy facility in Toronto, ON for scanning by ScanScope (Aperio Technologies, Vista, CA). Scanned image was analyzed using ImageScope software (Aperio Technologies, Vista, CA). Staining intensity of the tissue core was divided into 4 categories by the software: strong positive, positive, weak positive, and negative. Scores from each group were used to calculate the H-score using the formula:

$$H - Score = (\%weak \times 1 + \%postive \times 2 + \%strong \times 3 + 1) \times 100$$

% weak, positive, or strong = % weak, positive, or strong / total (weak + positive + strong + negative) (He, Ingram, Rybak, & Tang, 2010).

Table 1. Summary of data for patients with cervical cancer

Patients	Pathological diagnosis	Age	Grade
1	Endocervical type adenocarcinoma	42	1
2	Endocervical type adenocarcinoma	42	1
3	Endometrioid adenocarcinoma with squamous metaplasia	48	1
4	Endometrioid adenocarcinoma with squamous metaplasia	48	1
5	Endocervical type adenocarcinoma	52	1-2
6	Endocervical type adenocarcinoma	52	1-2
7	Endometrioid adenocarcinoma	32	1-2
8	Endometrioid adenocarcinoma	32	1-2
9	Instestinal type adenocarcinoma	72	2
10	Instestinal type adenocarcinoma	72	2
11	Endocervical type adenocarcinoma	43	2
12	Endocervical type adenocarcinoma	43	2
13	Clear cell adenocarcinoma	40	-
14	Clear cell adenocarcinoma	40	-
15	Instestinal type adenocarcinoma	51	2
16	Instestinal type adenocarcinoma	51	2-3
17	Endocervical type adenocarcinoma	50	2-3
18	Endocervical type adenocarcinoma	50	2-3
19	Instestinal type adenocarcinoma	34	2
20	Instestinal type adenocarcinoma	34	2
21	Adenocarcinoma	44	3
22	Adenocarcinoma	44	3
23	Adenocarcinoma	52	3
24	Adenocarcinoma	52	3
25	Adenocarcinoma	59	3
26	Adenocarcinoma	59	3
27	Endometrioid adenocarcinoma	26	3
28	Endometrioid adenocarcinoma	26	3
29	Adenocarcinoma (fibrous tissue and blood vessel)	32	-
30	Adenocarcinoma (fibrous tissue and blood vessel)	32	-
31	Adenosquamous carcinoma	43	-
32	Adenosquamous carcinoma	43	-
33	Adenosquamous carcinoma	64	-
34	Adenosquamous carcinoma	64	-
35	Adenosquamous carcinoma	38	-
36	Adenosquamous carcinoma	38	-
37	Adenosquamous carcinoma	54	-

38	Adenosquamous carcinoma	54	-
39	Adenosquamous carcinoma	43	-
40	Adenosquamous carcinoma	43	-
41	Squamous cell carcinoma	53	2
42	Squamous cell carcinoma	53	2
43	Squamous cell carcinoma	27	2
44	Squamous cell carcinoma	27	2
45	Squamous cell carcinoma	68	2-3
46	Squamous cell carcinoma	68	2-3
47	Squamous cell carcinoma	37	3
48	Squamous cell carcinoma	37	3
49	Squamous cell carcinoma	43	3
50	Squamous cell carcinoma	43	3
51	Squamous cell carcinoma	69	2
52	Squamous cell carcinoma with necrosis	69	2
53	Squamous cell carcinoma (sparse)	48	2
54	Squamous cell carcinoma	48	2
55	Squamous cell carcinoma	36	3
56	Squamous cell carcinoma	36	3
57	Squamous cell carcinoma	63	2
58	Squamous cell carcinoma	63	2
59	Squamous cell carcinoma	47	2
60	Squamous cell carcinoma	47	1-2
61	Squamous cell carcinoma	40	2
62	Squamous cell carcinoma	40	2
63	Squamous cell carcinoma	76	2
64	Squamous cell carcinoma	76	2
65	Squamous cell carcinoma	38	3
66	Squamous cell carcinoma (fibrous tissue and blood vessel)	38	-
67	Squamous cell carcinoma	36	2-3
68	Squamous cell carcinoma	36	2-3
69	Squamous cell carcinoma	62	3
70	Squamous cell carcinoma	62	3
71	Squamous cell carcinoma	51	3
72	Squamous cell carcinoma	51	3
73	Squamous cell carcinoma	32	3
74	Squamous cell carcinoma	32	3
75	Squamous cell carcinoma	58	3
76	Squamous cell carcinoma	58	3
77	Squamous cell carcinoma	27	3
78	Squamous cell carcinoma	27	3
79	Squamous cell carcinoma	39	2
80	Squamous cell carcinoma	39	3
81	Cancer adjacent normal cervical tissue	45	-
82	Cancer adjacent normal cervical tissue	45	-
83	Cancer adjacent normal cervical canals tissue	62	-
84	Cancer adjacent normal cervical canals tissue	62	-
85	Cancer adjacent normal cervical canals tissue	50	-

86	Cancer adjacent normal cervical canals tissue	50	-
87	Cancer adjacent normal cervical tissue of No 13	40	-
88	Cancer adjacent normal cervical tissue of No 13	40	-
89	Cancer adjacent normal cervical tissue (fibrous tissue and blood vessel)	60	-
90	Cancer adjacent normal cervical tissue	60	-
91	Normal cervical tissue	18	-
92	Normal cervical tissue	18	-
93	Normal cervical tissue	15	-
94	Normal cervical tissue	15	-
95	Normal cervical tissue (fibrous tissue and blood vessel)	21	-
96	Normal cervical tissue (fibrous tissue and blood vessel)	21	-
97	Normal cervical tissue (with hyperplasia of glandular epithelium)	21	-
98	Normal cervical tissue (with hyperplasia of glandular epithelium)	21	-
99	Normal cervical tissue (fibrous tissue and blood vessel)	19	-
100	Normal cervical tissue (fibrous tissue and blood vessel)	19	-

3.7 Generation of Stable Cell Line

Retroviral methodology was used to stably express the gene of interest into a mammalian cell line. Packaging of virus was achieved by transfecting 293T cells with the gag-pol expression vector (GP), envelope-expressing vector (VSV-G, vesicular stomatitis virus G protein) (Agilent Technologies, Mississauga, ON) and retroviral vector containing gene of interest. 293T cells were seeded the day before transfection to ensure optimal cell density. The transfection cocktail was prepared by adding 10 µg of each of pVPack-VSV-G, pVPack-GP and retroviral vectors into 50 µL of 2.5M $CaCl_2$ and H_2O to final volume of 500 µL. In a 13 mL round bottom tube, transfection cocktail was added into 500 µL of 2x Hepes Buffered Saline (HeBS, 0.28 M NaCl, 0.05M hepes, 1.5 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.1) in drop-wise fashion by p1000 pipette while shaking. The mixture of HeBS and transfection cocktail was allowed to stand in room temperature for 20 minutes, followed

by drop-wise addition of the HeBS mixture to a 293T plate containing 9 mL of DMEM media. For 10 to 15 drops of the HeBS mixture added, the plate was shook gently to maximize transfection efficiency. The plate of 293T cells was then incubated at 37^{o}C, 5% CO_2 for 12 hours. This was followed by a medium change and 48 hour incubation. Virus containing medium was then collected and filtered using 0.45 μm filter. Approximately 1 mL of media with virus was added to a plate of A498 cells and incubated for 90 minutes in cell culture incubator, while the remaining 9 mL of media were centrifuged at 20000 rpm at 4^{o}C. After centrifugation, supernatant was removed and the virus pellet was re-suspended in 1 mL of complete MEM media. The concentrated virus media was then re-introduced to the plate A498 cells and incubated at 37^{o}C, 5% CO_2 for 80 minutes. During the incubation period, plate was shook by hand every 20 minutes for maximum viral infection. Infected plate of A498 cells was topped by fresh complete MEM media to a final volume of 10 mL and grown in cell culture incubator for 24 hours. The virus containing media was replaced by fresh complete MEM media and the infected A498 cells were grown for 48 hours before addition of antibiotics, hygromycin B (1:400, 0.5 mg/mL)

3.8 Invasion Assay

Matrigel inserts with the pore size of 8 μm were purchased from BD Biosciences (Becton Dickson, Franklin Lakes, NJ). Both control and matrigel inserts were placed in a 24-well plate with serum free media for 2 hours at 37^{o}C, 5% CO_2 for rehydration. During this

period, 2 x 10^5 cells were re-suspended in 500 µL of serum free media. Following the rehydration, serum free media with cells was added into the insert (top chamber), while 0.5 mL of media containing 10% serum was added to the bottom chamber. The plate was incubated for 22 hours at 37°C, 5% CO_2. After incubation, remaining cells left on the upper surface of the membrane were washed off using cotton swaps. Invading cells on the bottom surface of the membrane were fixed with fixative solution (2% formaldehyde and 0.2% glutaraldehyde in PBS) for 10 minutes, followed by staining with crystal violet solution for 15 minutes for visualization. Membranes were cut from the insert and placed on glass slides. Coverslips were added using immersion oil. Images of membranes were taken using microscope. For each membrane, 5 random images were taken. Image J software (National Institute of Health, USA) was used to count cells. The following formula was used to determine percent invasion:

$$\%invasion = \left(\frac{number\ of\ cells\ migrated\ through\ matrigel\ membrane}{number\ of\ cells\ migrated\ through\ control\ membrane}\right) \times 100$$

3.9 Proliferation Assay

Respective cells were seeded in 6-well plates at density of 10^4 per well, followed by daily counting cells using hemocytometer under the microscope for 6 days, every 24 hours. The experiment was repeated 3 times.

3.10 Colonies Formation Assay

Respective cells were seeded at density of 1000, 5000, and 10000 cells per well in 6-well plates. Plates were incubated at 37^{o}C, 5% CO_2 for 7 days with a medium change every 2 days. By the end of incubation, media was removed by aspiration and cells were fixed by fixative solution for 10 minutes. Cells were then stained with crystal violet for 15 minutes, followed by washing of plates with tap water. Experiment was repeated 3 times.

3.11 Collection of Primary RCCs Tissues

Primary tumour and its respective normal adjacent kidney tissue were collected from total of 46 RCC patients at St. Joseph's Healthcare, Hamilton, ON. Collection of tissue was in compliance to regulation of Research Ethics Board and with patient's consent. Pathological examinations were performed by pathologists from the hospital of St. Joseph's Healthcare (Table 2.)

Table 2. The information for ccRCC patients

Patient	Age	Gender	Pathology	Fuhrman	Metastasis at Surgery	Tumour Size
9	54	M	Clear Cell RCC	1	No	3.5*3.6*2.5 cm
16	70	F	Clear Cell RCC	1	No	3.5*4*3 cm
21	51	M	Clear Cell RCC	1	No	4.5*3.3*3.4 cm
22	54	F	Clear Cell RCC	1	No	6.2*2.4*1.2 cm
26	41	F	Clear Cell RCC	1	No	6.2*5.0*3.0 cm
36	50	M	Clear Cell RCC	1	No	1.7*1.5*1.5 cm
50	45	F	Clear Cell RCC	1	No	2.5*2.8*2.2 cm
51	48	M	Clear Cell RCC	1		5 cm

53	53	F	Clear Cell RCC	1	No	5.6*4.5*2.5 cm
54	61	M	Clear Cell RCC	1	No	5*5.5*4.5 cm
12	38	M	Clear Cell RCC	2	No	2.9*2.5*2.6 cm
13	63	M	Clear Cell RCC	2	No	11.5*7.5*6.5 cm
14	77	M	Clear Cell RCC	2	No	6*6.5*6.5 cm
18	58	M	Clear Cell RCC	2	No	2*2*1.5 cm
19	58	M	Clear Cell RCC	2	No	5.4*4*4 cm
23	77	F	Clear Cell RCC	2	No	5.5*4.5*4.0 cm
30	76	M	Clear Cell RCC	2	Yes -bone	6.2*5*5.5 cm
31	68	F	Clear Cell RCC	2	No	3.5*3*2.5 cm
32	59	M	Clear Cell RCC	2	No	5.5*5.0 cm
33	49	F	Clear Cell RCC	2	No	5*4.9 cm
34	43	M	Clear Cell RCC	2	No	3 cm
37	83	F	Clear Cell RCC	2	No	8*7*6 cm
41	67	F	Clear Cell RCC	2	No	3.2 cm
43	N/A	F	Clear Cell RCC	2	No	2 cm
44	91	F	Clear Cell RCC	2	No	2.7*2.5*2.5 cm
45	72	F	Clear Cell RCC	2	No	3.5*3.0*3.0 cm
46	48	F	Clear Cell RCC	2	No	1 cm
48	75	M	Clear Cell RCC	2	No	6*4*4 cm
49	74	M	Clear Cell RCC	2	No	3.5*2.5 cm
52	62	M	Clear Cell RCC	2	No	
5	54	M	Clear Cell RCC	3	No	5.5*5*5 cm
10	48	M	Clear Cell RCC	3	No	6*4*4 cm
11	75	F	Clear Cell RCC	3	No	11*11*9 cm
15	63	M	Clear Cell RCC	3	No	4.5*4*4 cm
17	63	M	Clear Cell RCC	3	No	3.5*2.0*2.5 cm
24	48	M	Clear Cell RCC	3	No	13.5*11*7 cm
25	45	M	Clear Cell RCC	3	No	4.5*3.5*3.0 cm
27	68	M	Clear Cell RCC	3	No	6*5.5*4.5 cm
28	57	M	Clear Cell RCC	3	Yes	9*9*5.5 cm
35	68	M	Clear Cell RCC	3	No	4.5*4.5*3.6 cm
39	56	F	Clear Cell RCC	3	No	3 cm
40	71	M	Clear Cell RCC	3	No	12.5*10*6.5 cm
42	62	M	Clear Cell RCC	3	No	3 cm
47	62	M	Clear Cell RCC	3	No	3.5*3.5*2.0 cm
6	65	M	Clear Cell RCC	4	Yes -lung	8*6*5.5 cm
8	54	F	Clear Cell RCC	4	Yes	
29	55	M	Clear Cell RCC	4	No	13*8*9 cm
38			Clear Cell RCC			

IV. RESULTS

4.1 Reduction of CYB5D2 in ccRCC

CYB5D2 is expressed in various organs including the brain, heart, adrenal gland and kidney (Kimura et al., 2010). I conducted western blot analysis to investigate the status of CYB5D2 expression in 20 primary ccRCC samples (Fig 1). Of the 20 samples analyzed, robust reductions in CYB5D2 were clearly demonstrated in ccRCC in comparison to normal adjacent kidney tissue (NAT) (Fig 1). The intensity of the bands were quantified using the Image J analysis program (Fig 2), which showed the reduction of CYB5D2 in ccRCC being statistically significant (*p<0.0001). The specificity of our anti-CYB5D2 antibody was previously demonstrated (Xie et al., 2011). To further confirm the specificity of anti0-CYB5D2 antibody, I was able to show that addition of recombinant CYB5D2 competed out the antibody's ability to react with endogenous CYB5D2 in NAT lysates (Fig 3). The differential CYB5D2 levels detected in NAT versus the respective ccRCC tissues did not result from protein degradation during lysate preparation, as comparable level of actin were present across samples (Fig 1) and lysates for any pair of NAT-ccRCC tissues were prepared side-by-side. Collectively, we have demonstrated the expression of CYB5D2 in human kidney, an observation that is consistent with the presence of CYB5D2 in mouse kidney (Kimura et al., 2010). More importantly, we were able to show significant down-regulation of CYB5D2 in ccRCC in comparison to NAT.

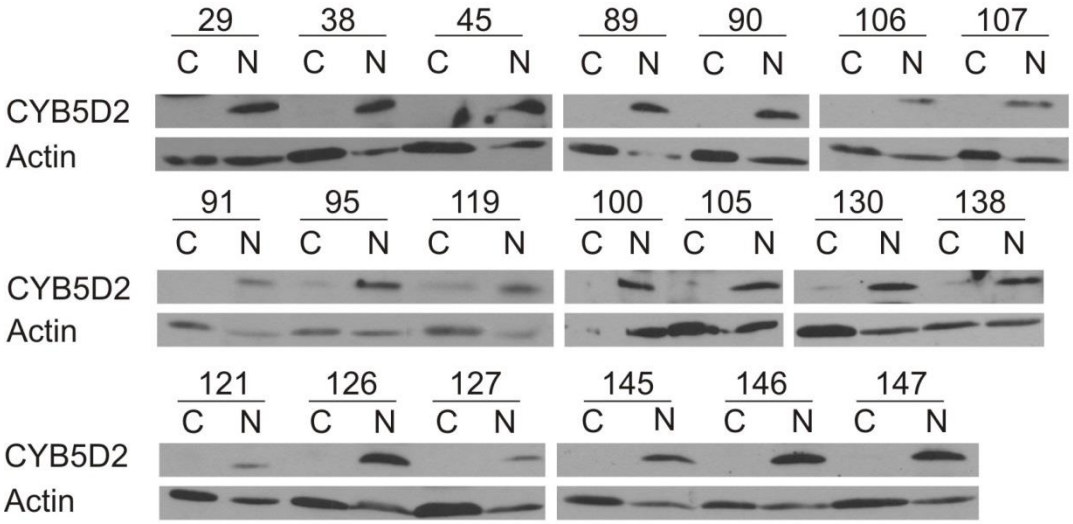

Figure 1. Reduction of CYB5D2 in ccRCC. Western blot analysis of CYB5D2 in the lysate of 20 ccRCC tissues (C) and adjacent non-tumor kidney tissues (N) is shown
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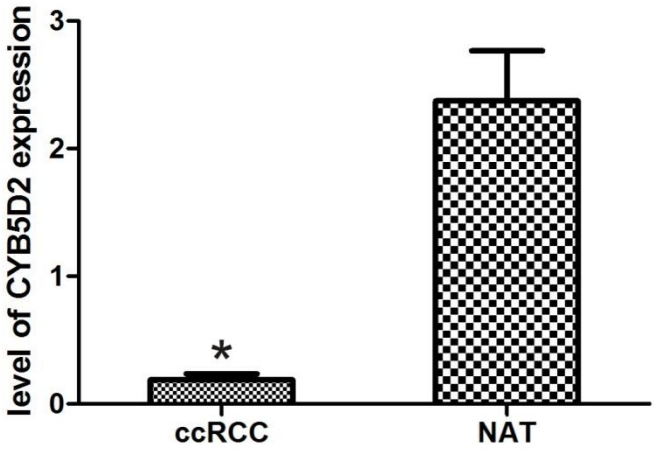

Figure 2. Quantification of western blot analysis of CYB5D2. CYB5D2 level in NAT and ccRCC were normalized to the respective actin. The relative CYB5D2 abundance is shown. * $p < 0.05$ in comparison to NAT (2-tailed student t-test).

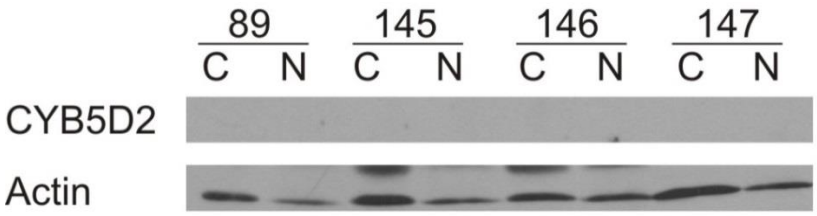

Figure 3. Anit-CYB5D2 antibody specifically recognizes CYB5D2. Western blot analysis of CYB5D2 in the indicated ccRCC (C) and NAT (N) lysates for the indicated patients was carried out using our anti-CYB5D2 antibody in the presence of recombinant CYB5D2 protein.

4.2 Expression of CYB5D2 in renal tubular epithelial cells

To confirm CYB5D2 reduction in ccRCC, I have analyzed CYB5D2 in 9 pairs of NAT and ccRCC (total of 18 samples) by immunohistochemistry (IHC) (Fig 4). In comparison to NAT, CYB5D2 was clearly reduced in ccRCC (Fig 4); the same pattern was observed in all samples analyzed. CYB5D2 was largely present in the epithelia of the renal proximal tubules (Fig 4). The detected CYB5D2 signals in NAT by IHC was most likely specific, as recombinant CYB5D2 but not GST was able to compete out the recognition of CYB5D2 by anti-CYB5D2 antibody (Fig 5). Since ccRCC is widely regarded to be originated from the tubular epithelia (Cohen and McGovern 2005; Linehan et al. 2003), the observed CYB5D2 expression in the renal proximal tubular epithelium supports the notion that reduction of CYB5D2 facilitates the pathogenesis of ccRCC.

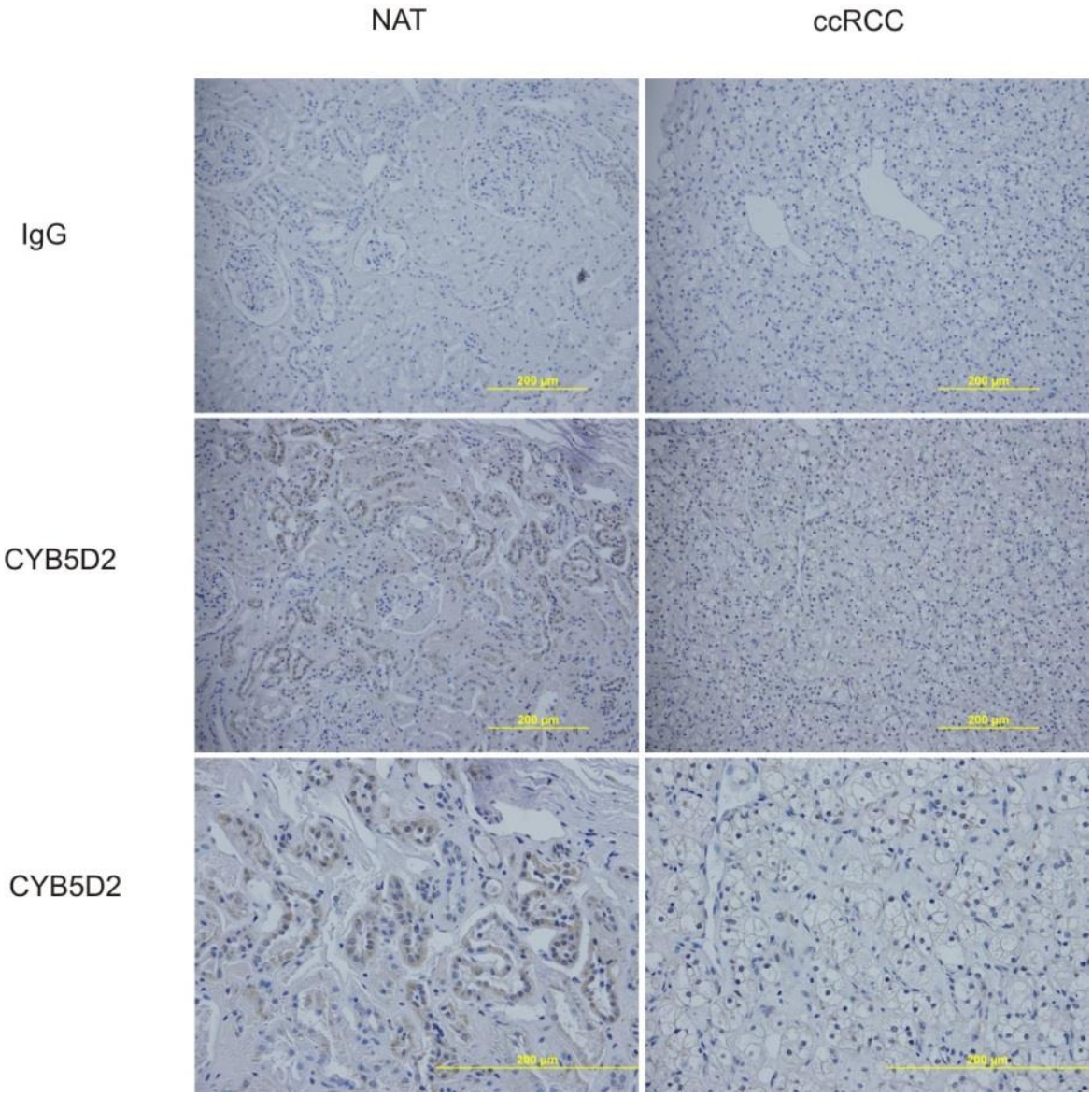

Figure 4. Expression of CYB5D2 in the tubular epithelial cells. Immunohistochemical (IHC) staining of CYB5D2 in ccRCC and the adjacent non-tumor kidney tissue (NAT) was performed using control IgG or anti-CYB5D2 IgG on 9 patients. Typical images from a signle patient are shown. Scale bars represent 200 µm. The images in the middle and bottom panels were from the same tissue, but acquired with different magnifications.

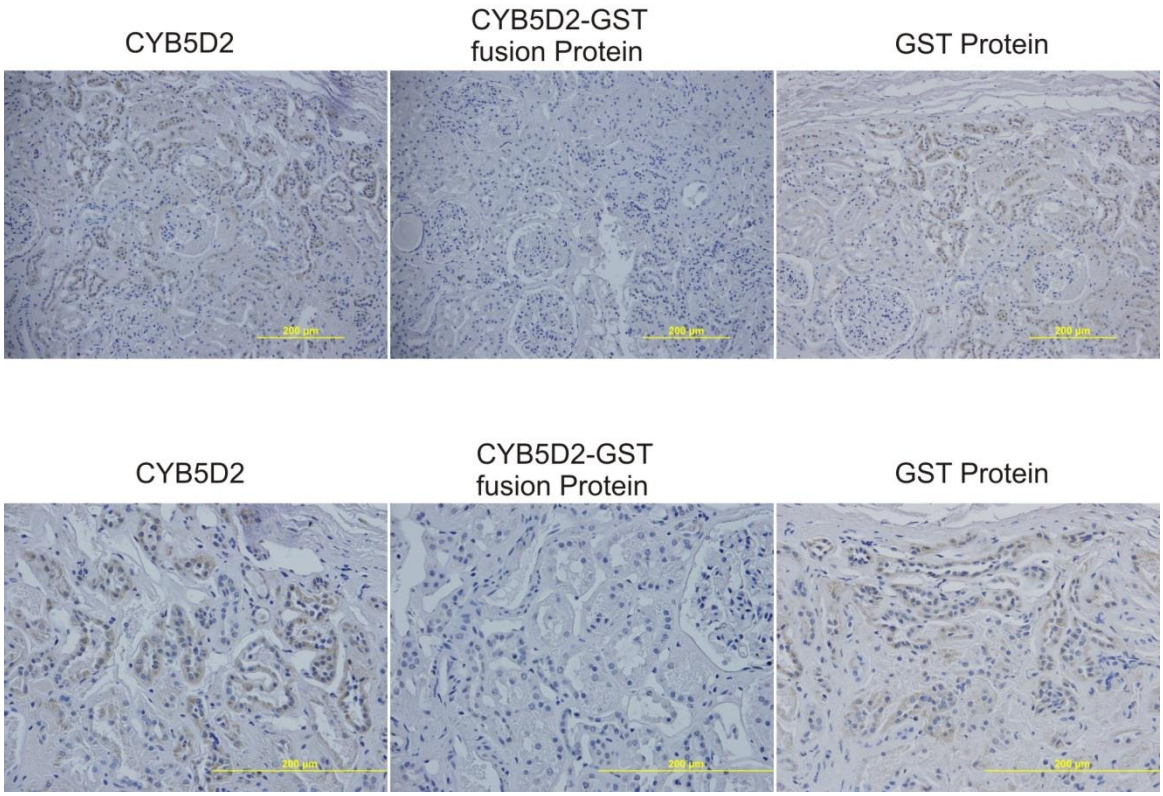

Figure 5. Anit-CYB5D2 antibody specifically recognizes CYB5D2 by IHC. Non-tumour kidney tissues were IHC stained for CYB5D2 in the presence of either recombinant CYB5D2 or GST as indicated. Anti-CYB5D2 antibody was used at 1:250 dilutions. Scale bars represent 200 μm.

4.3 Ectopic CYB5D2 Inhibits the Proliferation and the Invasion of A498 ccRCC Cells

The observed reduction of CYB5D2 in ccRCC supports the possibility of CYB5D2 suppressing ccRCC tumorigenesis. To examine this concept, A498 ccRCC cells were stably infected with empty vector (EV) or CYB5D2 (Fig 6). In comparison to A498 EV cells, A498 CYB5D2 cells proliferated at a reduced rate (Fig 7). Since invasion capacity

of cancer cells correlates with their metastatic potential (Copper et al., 1993), I have also analyzed the impact of ectopic CYB5D2 on A498 cell's invasion ability. While a slightly more than 50% of A498 EV cells were able to penetrate 8 μm matrigel membrane, approximately 30% of A498 CYB5D2 cells were able to do so (Fig 8), demonstrating that CYB5D2 reduces A498 cell's invasion capacity. Taken together, these results support the possibility that CYB5D2 possesses tumour suppression activity towards ccRCC.

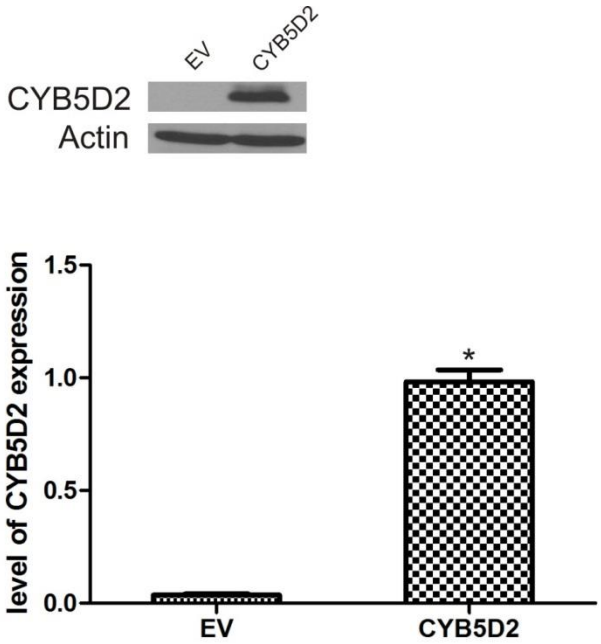

Figure 6. Ectopic expression of CYB5D2 in A498 ccRCC cells. A498 cells were stably infected with empty vector (EV) or CYB5D2; the presence of ectopic protein was confirmed (top panel). Western blot analysis using anti-CYB5D2 antibody was performed three times; typical images from a single repeat are included (top panel); the levels of CYB5D2 in EV and CYB5D2 stable lines were quantified (bottom panel) * $p<0.05$ in comparison to EV cells (2-tailed student *t* test).

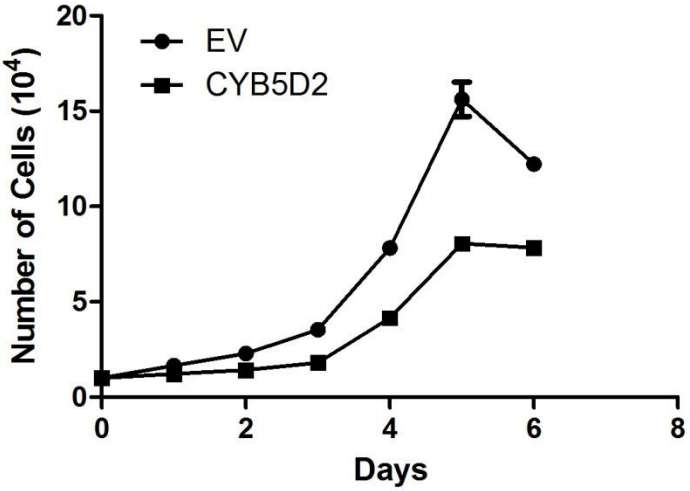

Figure 7. CYB5D2 inhibits A498 cell proliferation. 10^4 A498 EV or CYB5D2 stable cells were seeded in 6-well plates; cell numbers were determined daily using Hemocytometer over a period of 6 days.

4.4 Ectopic CYB5D2 Inhibits Akt Activation in A498 ccRCC Cells

Abnormal activation of the PI3K/Akt pathway makes major contributions to tumourigenesis. Alteration of this pathway occurs frequently in human cancers. Our discovery that CYB5D2 suppresses A498 cell proliferation suggests a possible role of CYB5D2 in regulation of Akt activation. In supporting this possibility, western blot analysis revealed a reduction in Akt phosphorylation in CYB5D2 overexpressing cells compared to EV cells (Fig 9). Moreover, phosphorylation of GSK-3β at S9, a well-regarded endogenous substrate of Akt, was also decreased in cells overexpressing CYB5D2. In addition, decrease in $p70^{S6K}$ phosphorylation was also observed in CYB5D2 overexpressing A498 cells (Fig 9). The phosphorylation of $p70^{S6K}$ on Thr-389 is mediated

by mTOR (Sanchez Canedo et al., 2010)and mTOR activation can be mediated by Akt. Collectively, the above observations support the possibility that CYB5D2 inhibits A498 cell proliferation via inhibiting Akt activation.

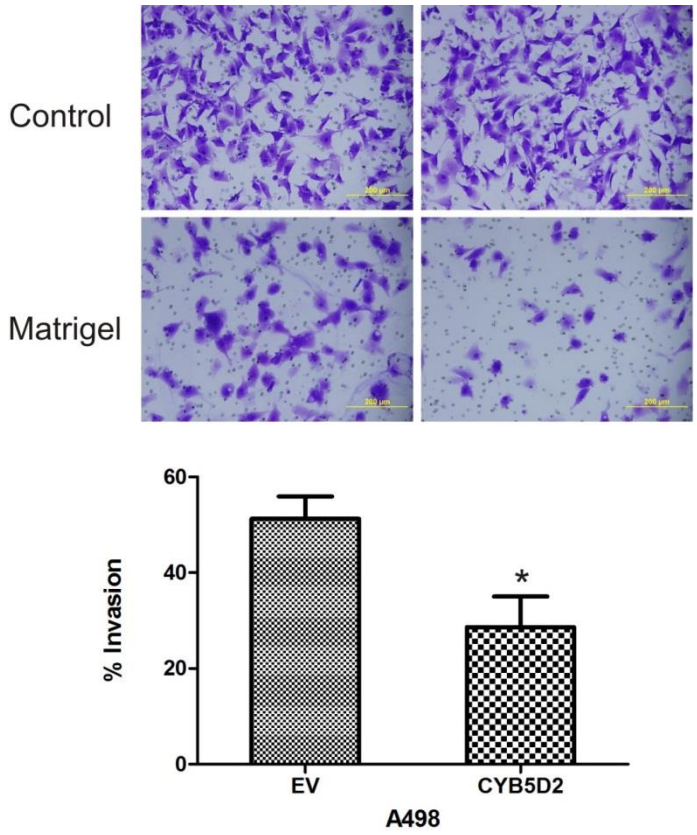

Figure 8. CYB5D2 inhibit A498 cell invasion. A498 EV and CYB5D2 cells were examined for their potential to cross through control or matrigel membrane. Experiments were repeated three times. Typical images from a single repeat are shown; scale bars represent 200 μm (top panel). The average percentages ± SE of invasive cells for the indicated lines are graphed. *p<0.05 (2-tailed student t test) in comparison to EV cells.

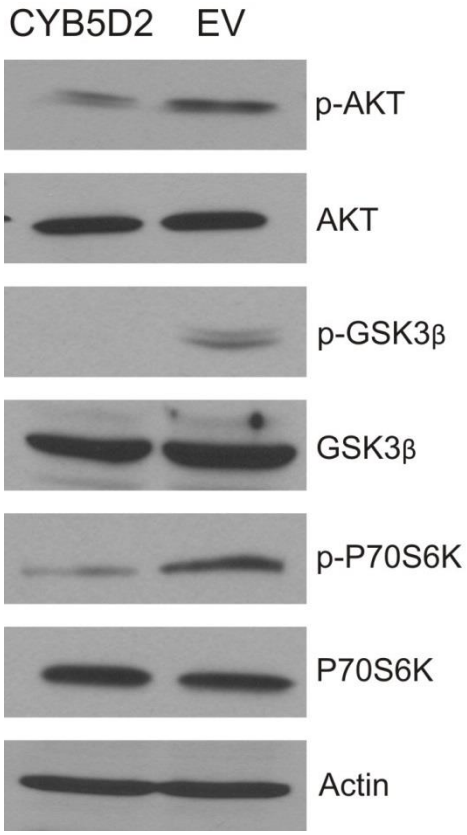

Figure 9. CYB5D2 inhibits Akt activation in A498 cells. A498 EV and CYB5D2 cells were analyzed by western blot for the indicated events. p-Akt: phosphorylation of Akt at S473; p-GSK3β: phosphorylation of GSK3β at S9; p-P70S6K: phosphorylation of P70S6K at T389. Experiments were repeated three times; typical images from a single repeat are shown.

4.5 CYB5D2 Inhibits HeLa Cell Invasion but not Proliferation

To determine whether CYB5D2-mediated inhibition of cell proliferation and invasion is unique to A498 cells, we generated HeLa EV and HeLa CYB5D2 lines (Fig 10). Both lines displayed a comparable proliferation potential based on the results obtained in colony formation assay (Fig 11A, B); these results were further supported by the similar

growth curves obtained for both cell lines (Fig 11C). Taken together, the above results demonstrate CYB5D2 being not inhibitory towards HeLa cell proliferation.

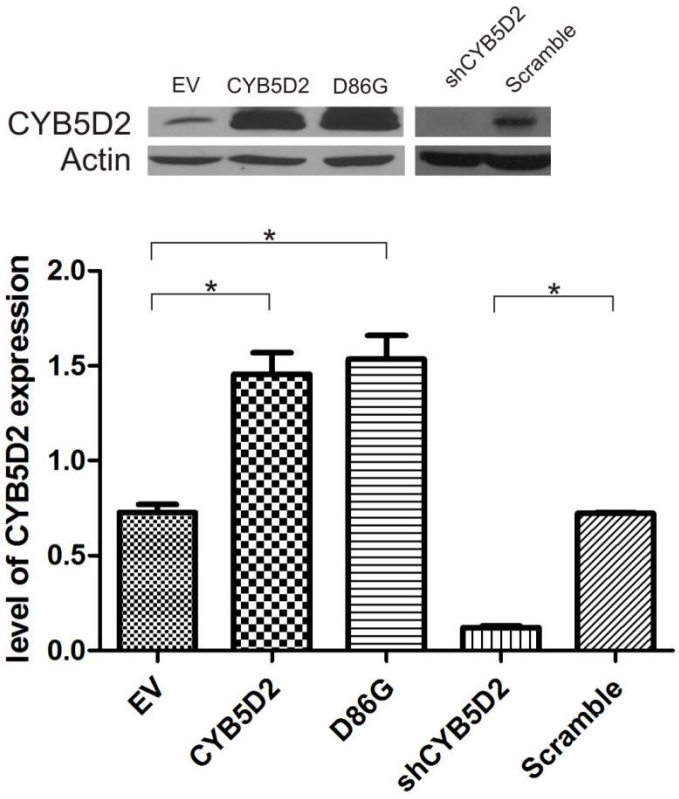

Figure 10. Ectopic expression of CYB5D2 in HeLa cervical cancer cells. HeLa cells were stably infected with empty vector (EV), CYB5D2, CYB5D2 (D86G) mutant, short hairpin RNA targeted CYB5D2 (shCYB5D2), and control short hairpin RNA (Scramble) as indicated (top panel). Western blot analysis using anti-CYB5D2 antibody was performed three times; typical images from a single repeat are included (top panel); the levels of CYB5D2 in the indicated lines were quantified (bottom panel) * $p<0.05$ (2-tailed student *t* test) in comparison to EV or control short hairpin RNA treated cells, respectively.

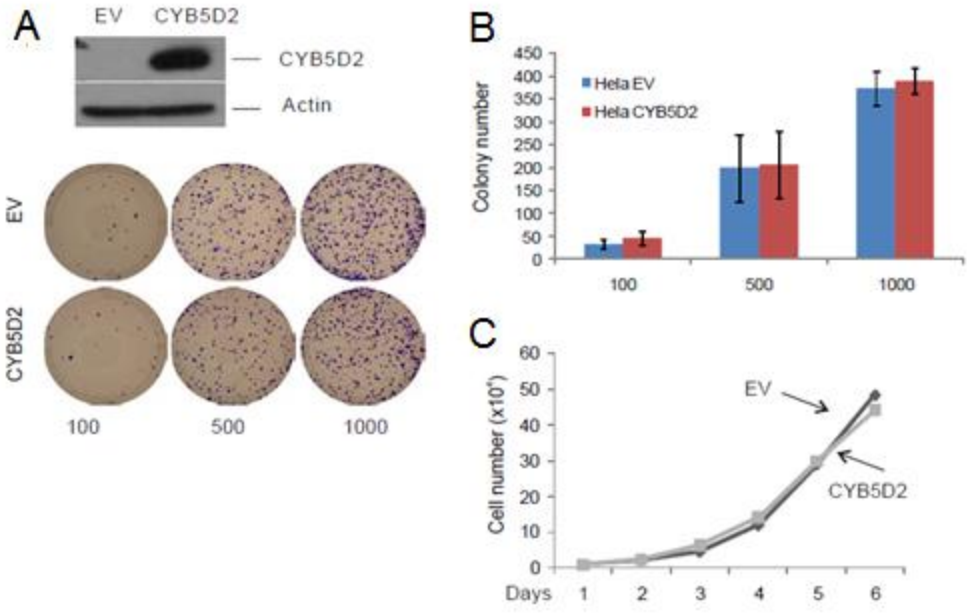

Figure 11. Ectopic CYB5D2 does not affect HeLa cell proliferation. (**A**) Western blot confirms expression of ectopic CYB5D2 in HeLa cells compared to empty vector (top panel). 100, 500, and 1000 cells were seeded into a 6-well plate followed by staining of cell colonies with 0.1% crystal violet after 2-3 weeks. (**B**) Quantification of experiment in panel A. (**C**) $2x10^3$ cells were seeded in a 6-well plate. Cell numbers were counted daily. Experiments were repeated 3 times, a representative data is shown

In our subsequent effort in the examination of whether CYB5D2 reduces HeLa cell's invasion capacity, I was able to show that in comparison to EV cells ectopic expression of CYB5D2 significantly reduced HeLa cell's invasion ability (Fig 12A). By taking advantage of previously established HeLa CYB5D2 knockdown cells (Fig 10) (Xie and Tang, unpublished work), it was observed that knockdown of CYB5D2 substantially enhanced HeLa cell's invasion capacity (Fig 12B).

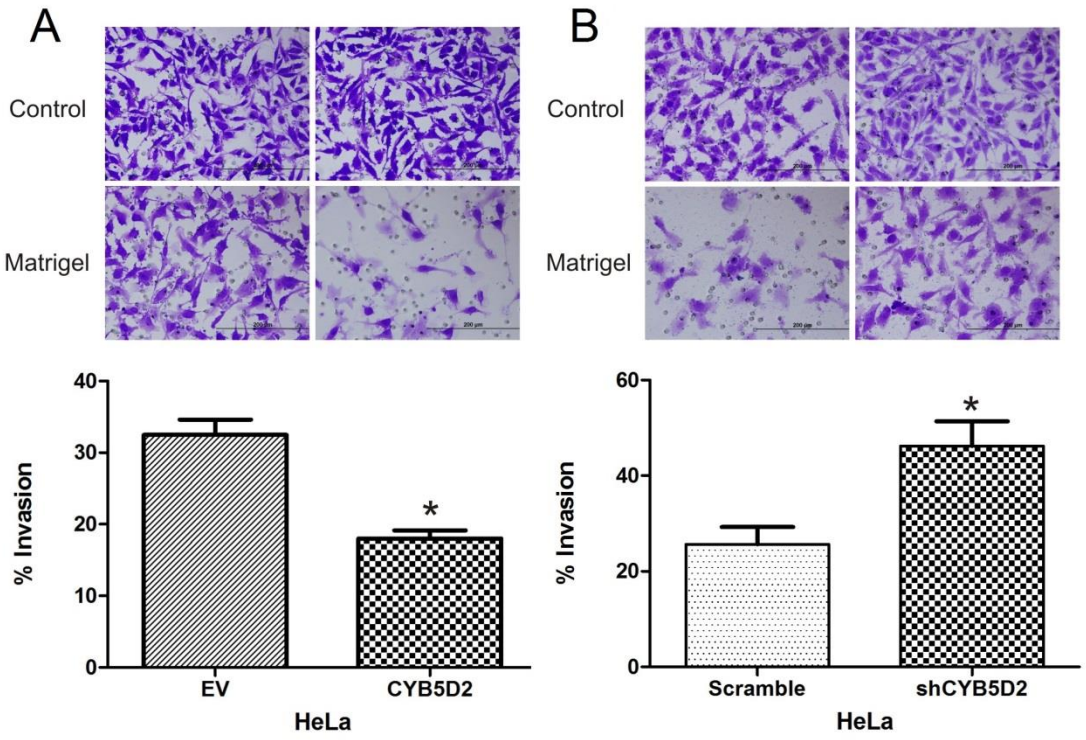

Figure 12. CYB5D2 inhibit HeLa cell invasion. (A) HeLa EV and CYB5D2 cells were examined for their potential to cross through control or matrigel membrane. Experiments were repeated three times. (B) CYB5D2 knockdown HeLa cells (shCYB5D2) and HeLa short hairpin scramble control cells (Scramble) were examined for their potential to cross through control or matrigel membrane. Experiments were repeated three times. Typical images from a single repeat are shown; scale bars represent 200 μm (top panel). The average percentages ± SE of invasive cells for the indicated lines are graphed. **p*<0.05 (2-tailed student t test) in comparison to EV cells.

A typical characteristic of the MAPR family members is their association with heme (Crudden et al., 2006; Kimura and Nakayama, 2012; Kimura et al., 2010). The most studied MARP family member PGRMC1 binds heme and heme-association contributes to the functions of PGRMC1 (Crudden et al. 2006). In accordance with this notion, CYB5D2 has been shown to bind heme (Kimura et al., 2010); research from our laboratory has demonstrated the requirement of the D86 residue for CYB5D2 to associate

with heme; substitution of D86 with 86G (D86G) rendered the CYB5D2 mutant heme-binding deficient (Bruce and Tang, unpublished observation). To examine a role of heme association in CYB5D2-mediated inhibition of HeLa cell invasion, HeLa cells ectopically expressing CYB5D2 (D86G) were established (Fig 10). In comparison to EV cells, HeLa CYB5D2 (D86G) cells were less invasive (Fig 13), demonstrating CYB5D2 inhibiting HeLa cell invasion independently of its heme association.

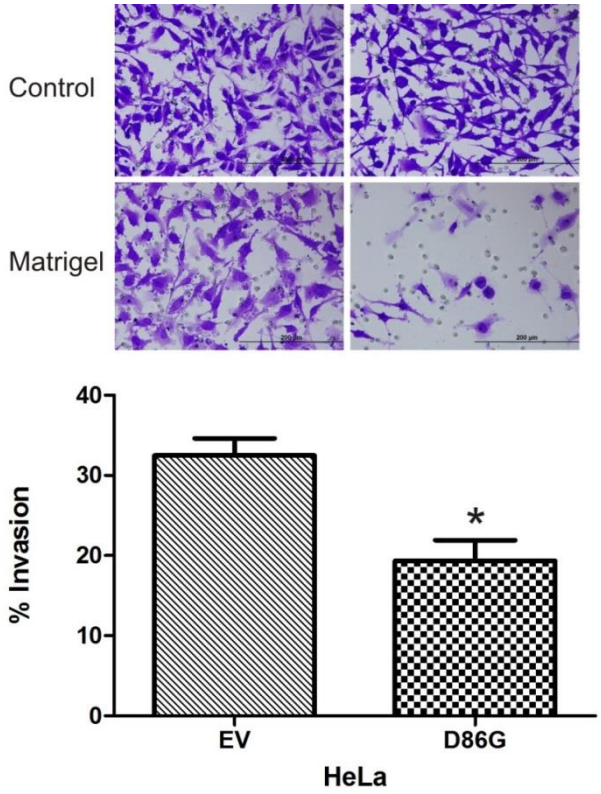

Figure 13. CYB5D2 inhibit HeLa cell invasion. HeLa EV and CYB5D2-D86G mutant cells were examined for their potential to cross through control or matrigel membrane. Experiments were repeated three times. Typical images from a single repeat are shown; scale bars represent 200 μm (top panel). The average percentages ± SE of invasive cells for the indicated lines are graphed. *p<0.05 (2-tailed student t test) in comparison to EV cells.

4.6 Reduction of CYB5D2 in Cervical Squamous Cell Carcinoma

Taken together, results from the above HeLa stable cell lines revealed that CYB5D2 suppresses cervical cancer progression. To consolidate this notion, I examined CYB5D2 expression in primary cervical cancer tissues using a tissue microarray (TMA) slide which contained 20 normal cervical tissues, 40 squamous cell carcinomas, and 40 adenosquamous carcinomas (Table 1). Comparing to normal cervical tissue (NCT), CYB5D2 levels were significantly reduced in squamous cell carcinoma (SCC) (Fig 14A, B). In 40 SCCs, 35 (87.5%) had reduction of CYB5D2 (Fig 14C), with 2 tumours displayed higher levels of staining and 3 tumours showed slightly lower staining in comparison to NCT (Fig 14C). While the staining of adenosquamous carcinoma was generally less than normal cervical tissue (Fig 14A, B, D), the reduction in CYB5D2 was not significant (Fig 14B). This was mainly due to 4 patients with more intense staining than the majority of patients (Fig 14D). Although statistical analysis excluding these 4 patients revealed a significant result, analysis using several programs was unable to point these samples as outliers. Thus, examination of a larger adenosquamous carcinoma population will be required to determine whether CYB5D2 is down-regulated in this type of cervical cancer. The IHC staining was specific, as the staining on normal human kidney could be effectively competed out by recombinant GST-CYB5D2 but not GST (Fig 5). Since SCC accounts for the vast majority of cervical cancer (de Freitas et al., 2012; González Martín, 2007), the above data collectively reveal the down-regulation of CYB5D2 in SCCs.

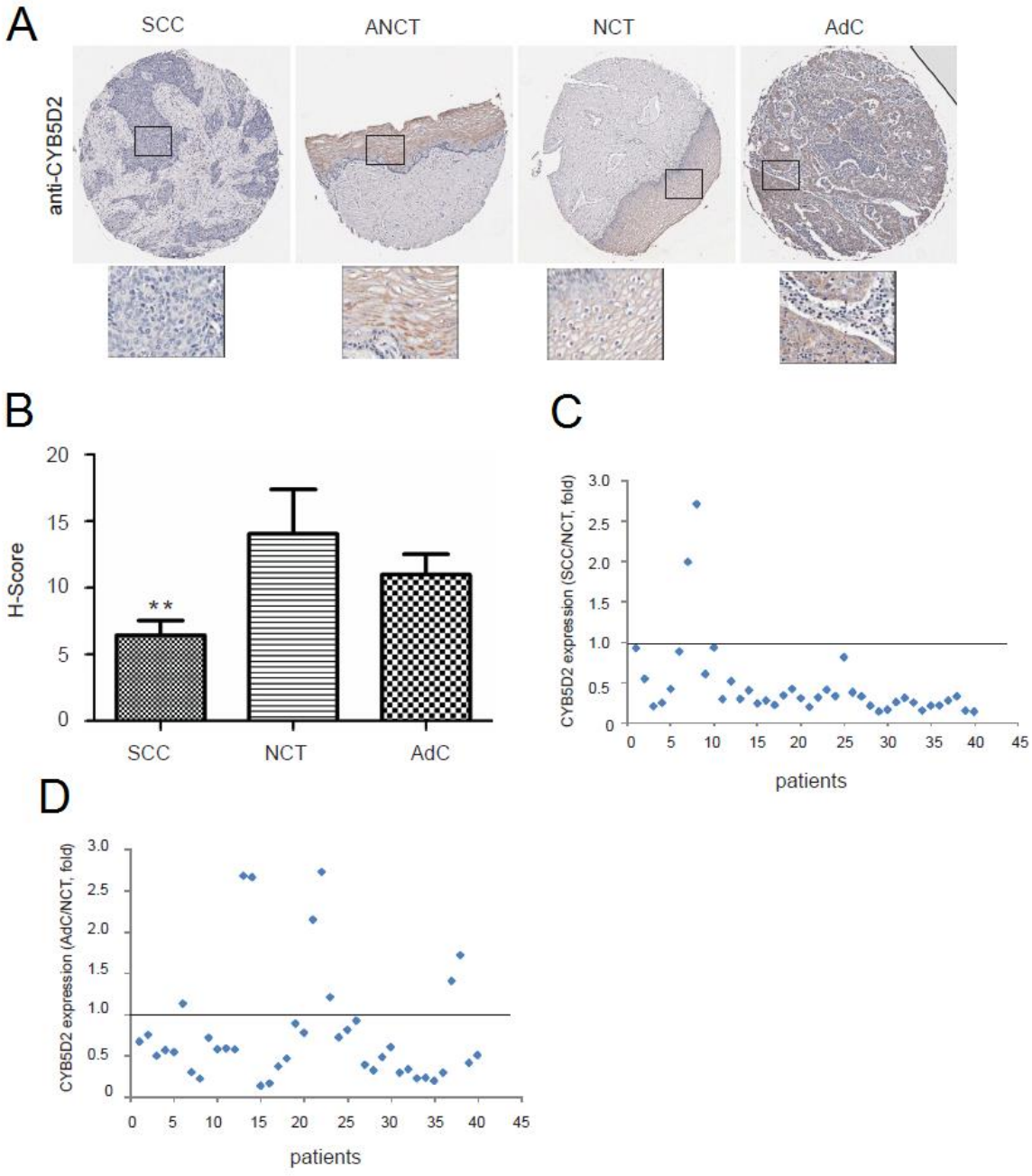

Figure 14. Reduction of CYB5D2 in squamous cell carcinoma. (A) typical images of squamous cell carcinoma (SCC), adjacent normal cervical tissue (ANCT), normal cervical tissue (NCT) and adenocarcinoma (AdC) are shown. Selected region was magnified 3-fold and presented under the typical images. (B) TMA slide was scanned using ScanScope and analyzed using ImageScope (Aperio). Staining intensity was

converted in to H-score using formula: H-Score = (% weak x 1 + % medium x 2 + % strong x 3 +1) x 100. Background signal was subtracted. Average H-score ± SD for SCC, NCT, and Adc were plotted. (** $p < 0.01$). (C)(D) CYB5D2 expression in SCC and AdC patients were expressed as fold changes to the levels of CYB5D2 in NCT (average H-Score). The distributions of changes are graphed. The horizontal line represents 1 fold or no changes and the dots below or above the lines are down-regulated or up-regulated respectively.

V. DISCUSSION

Clear cell renal cell carcinoma is the most aggressive form of RCC and also accounts for at least 75% of the total cases (Linehan et al., 2003; Motzer et al., 1996; Nelson et al., 2007). Despite the prevalence of lung, breast, colorectal, and bladder cancer among Canadians, the lethality of ccRCC remains the highest. The biggest factor contributing to the death rate of ccRCC is perhaps, its asymptomatic condition in the early stage. Without obvious warning signs, approximately half of the cases present with metastatic cancer at diagnosis. This in conjunction to ccRCC's notorious resistance to radiation and chemotherapy (Liu et al., 2001; Mickisch, 1994) is attributable to the high fatality rate that is associated with ccRCC.

Over the years, it has become clear that VHL is a major tumour suppressor for the pathogenesis of ccRCC. The molecular pathways regulated by the VHL-HIFα axis have been extensively studied, and several treatments were developed to specifically target the process, largely owning to the advancement in elucidation of the pathway. Despite these advances, metastatic ccRCC remains fatal. This situation is largely attributable to our limited understanding of ccRCC. While inactivation of VHL plays a critical role in the pathogenesis of ccRCC, it is evident that loss of VHL is not sufficient to cause ccRCC. VHL deficiency occurs frequently in renal cysts prior to ccRCC and mice with VHL being specifically knocked-out at the proximal tubule epithelium did not develop ccRCC. Collectively, other genetic abnormalities in addition to the loss of VHL are also required for ccRCC development. Our research here indicates that reduction in CYB5D2 is

potentially a genetic alteration that coordinates with the loss of VHL1 in promoting ccRCC tumourigensis.

5.1 Mechanism of CYB5D2 Reduction

In our investigation, we have observed the reduction of CYB5D2 in ccRCC, using both western blot analysis and IHC. In all 20 patients examined, CYB5D2 was reduced when compared to adjacent non-tumour kidney tissue. Similar results were obtained in the IHC analysis of 9 ccRCC cancer tissues. Even though the expression of CYB5D2 was demonstrated in brain, heart, adrenal gland and kidney (Kimura et al., 2010), there is currently lack of information regarding the involvement of CYB5D2 in tumorigenesis. While a larger cohort is still needed to increase the power of our study, our research offers a new perspective in ccRCC development. The loss of CYB5D2 in cancer may be attributed to its location on chromosome 17. The short arm of this chromosome is one of the most frequently lost regions in many cancers (Lee et al., 1990; Phelan et al., 1998; Phillips et al., 1996; Seitz et al., 2001), including the allelic deletion at the p53 gene detected in colorectal cancer (Baker et al., 1989). In addition, the region of 17p13.2 has also been implicated with prognostic value in the progression of intrahepatic cholangiocarcinoma (Chuensumran et al., 2007). CYB5D2 is located on 17p13.2; therefore its reduced expression that we observed here may be a result of allelic alteration commonly seen in chromosome 17p during tumorigenesis. In conjunction, we also detected CYB5D2 in cervical cancer. To our best knowledge, the expression of CYB5D2

has not been reported in the urogenital area. However, the expression of PGRMC1 has been observed in HeLa cells (Crudden et al., 2005). This is consistent with our observed presence of CYB5D2 in HeLa cells. Interestingly, CYB5D2 expression was reduced only in squamous cell carcinoma of the cervix in comparison to normal cervical tissue, an observation that is consistent with a publication showing the deletion of chromosome 17p in cervical cancer (Atkin and Baker, 1989). The reasons as to why the differential expression of CYB5D2 within subtypes of cervical carcinoma is currently unclear. Epigenetics has been shown to modulate gene expression to promote tumorigenesis. A typical example is the methylation of the *VHL* gene to down-regulate its expression in ccRCC (Herman et al., 1994). In line with this discussion, modification at the 17p13.2 locus was reported (Byun et al., 2003), suggesting epigenetic silencing as a possible factor leading to the reduction of CYB5D2 in ccRCC and cervical cancer.

5.2 CYB5D2 Mediates Tumour Suppression

In cancer, reduction in a protein expression implies its role in tumour suppressor (J.-H. Lee et al., 1990). In ccRCC, somatic mutation leading to inactivation of VHL gene is a well-established mechanism (Kim and Kaelin, 2004). Researchers are also able to demonstrate degradation of p53 protein in cervical cancer by the E6 protein of the HPV 16 (Tommasino et al., 2003; Tornesello et al., 2013). Base on this notion, the observed CYB5D2 loss in ccRCC patients may suggest the possible role of this protein as a tumour suppressor. The anti-tumour function of CYB5D2 is supported by the result showing here

that the invasiveness of A498 ccRCC cells and HeLa cervical cancer cells were reduced when CYB5D2 is overexpressed.

The process of epithelial to mesenchymal transition (EMT) is known to be the major driving force of cancer metastasis. During this event, cell adhesion complex is lost and individual cells acquired greater mobility (Guarino et al., 2007; Vuoriluoto et al., 2011). The hallmark of EMT is the loss of E-cadherin followed by the gain of N-cadherin, a process also known as cadherin-switch (Guarino et al., 2007; Yilmaz and Christofori, 2009). As a result of cadherin switch, predominant expression of N-cadherin facilitates stromal binding by tumour cells, leading to invasion (Hulit et al., 2007; Nieman et al., 1999; Seidel et al., 2004; Steeg, 2006; Thiery, 2002). Whether CYB5D2 influences EMT to reduce cell invasion remain unknown as we were not able to detect E-cadherin in A498 cell. This is in accordance with previous publication where A498 is shown to have no expression of E-cadherin (Tan et al., 1995). However, the possibility that CYB5D2 may inhibit metastasis by inhibiting EMT cannot be excluded. Expanding on the role of CYB5D2 in suppression of cell invasion, we also report inhibition of cell proliferation in A498 cell. However, in contrast to PGRMC1, which has been shown to induce tumorigenesis in lung and breast cancer through the activation of PI3K/Akt pathway (Fresno Vara et al., 2004; Neubauer et al., 2009; Wan et al., 2000), we observed reduction in Akt phosphorylation by CYB5D2 in A498 cells. Deregulation of Akt leading to its constant activation was observed in many cancers including ccRCC and cervical carcinoma. Here, we provided evidence suggesting that CYB5D2 possesses activity in anti-tumorigenesis.

5.3 Clinical Significances and Future Directions

Loss of VHL has long been regarded as the critical factor in ccRCC. However, deficiency in this gene is not sufficient in inducing ccRCC. Here we provide evidence suggesting an interesting theory where CYB5D2 may be a novel tumour suppressor in ccRCC. Whether it's by germ line mutation or sporadic occurrence, deletion of VHL is observed in most ccRCC cases; therefore it is important to expand the sample size to determine the prevalence of CYB5D2 loss in the ccRCC population. In addition, the anti-tumour function of CYB5D2 needs to be assessed *in* vivo. Tumorigenesis and cancer metastasis are events involving complex cellular mechanisms, thus animal models using CYB5D2 expressing cell lines are essential to further consolidate our findings. An interesting area of research is the relationship between VHL and CYB5D2. Our result indicated a major impact of CYB5D2 on ccRCC pathogenesis. On this basis, does VHL interact with CYB5D2 or do they function independently? These questions remain to be answered.

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