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MOLECULAR AND FUNCTIONAL INVESTIGATION OF CANCER-TYPE AND LIVER-TYPE VARIANTS OF ORGANIC ANION TRANSPORTING POLYPEPTIDE 1B3

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MOLECULAR AND FUNCTIONAL INVESTIGATION OF CANCER-TYPE AND LIVER-TYPE VARIANTS OF ORGANIC ANION TRANSPORTING POLYPEPTIDE 1B3

DISSERTATION

A dissertation submitted in partial fulfillment

of the requirements for the degree of Doctor of Philosophy in the

College of Pharmacy

at the University of Kentucky

By

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Lexington, Kentucky

2015

ABSTRACT OF DISSERTATION

MOLECULAR AND FUNCTIONAL INVESTIGATION OF CANCER-TYPE AND LIVER-TYPE VARIANTS OF ORGANIC ANION TRANSPORTING POLYPEPTIDE 1B3

OATP1B3 belongs to the OATP (organic anion transporting polypeptides) superfamily, responsible for mediating the transport of various endogenous and xenobiotic substrates. OATP1B3 was initially reported to be expressed exclusively in the hepatocytes where it mediates the uptake of numerous endogenous substrates (e.g. bile acids, steroid hormone conjugates) and several clinically relevant drugs including anticancer drugs. Later, a number of studies reported that OATP1B3 is also frequently expressed in multiple types of cancers and may be associated with differing clinical outcomes. However, a detailed investigation on the expression, localization and functions of OATP1B3 expressed in cancer has been lacking. In this thesis work, we confirmed that colon and pancreatic cancer cells express a cancer-specific OATP1B3 variant (csOATP1B3), different from OATP1B3 wild-type (WT) expressed in the normal liver. The csOATP1B3 utilizes an alternative transcription initiation site and the translated product of csOATP1B3 lacks the first 28 amino acids at the N-terminus of OATP1B3 WT. Our results show that csOATP1B3 has modest uptake transporter functions and reduced plasma membrane localization compared to OATP1B3 WT. In our efforts to investigate the regulatory mechanism underlying the expression of csOATP1B3, we found that hypoxia inducible factor-1α (HIF-1α) may play a key role in the regulation of csOATP1B3 in colon and pancreatic cancer cells. In a separate study, we tested whether the N-terminal sequence of OATP1B3 WT plays an important role in the membrane trafficking. This is based on the observation that csOATP1B3 lacking the first 28 amino acids at N-terminus of OATP1B3 WT displays a predominantly cytoplasmic localization pattern. Using the constructs with N-terminal truncations and point mutations, we verified that the N-terminus of OATP1B3 WT contains important motifs in its membrane trafficking. In particular, the amino acids within a putative β-turn-forming tetrapeptide appear to be important in regulating the membrane trafficking of OATP1B3 WT. The findings from this thesis work provide important insights into the functional and clinical significance of OATP1B3 in cancer and normal liver.

KEYWORDS: OATP1B3, splicing variants, colon cancer, pancreatic cancer, hypoxia, membrane trafficking, normal liver

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Student's signature

January 12, 2015

Date

MOLECULAR AND FUNCTIONAL INVESTIGATION OF CANCER-TYPE AND LIVER-TYPE VARIANTS OF ORGANIC ANION TRANSPORTING POLYPEPTIDE 1B3

By

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DEDICATION

This work is dedicated to my parents and my wife.

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LIST OF ABBREVIATIONS

5'-RACE	Rapid Amplification of cDNA Ends
Asbt	Apical sodium-dependent bile acid transporter
CCK-8	Cholecystokinin-8
CRC	Colorectal cancer
DAB	3,3’-diaminobenzidine
DHEA-S	Dehydroepiandrosterone sulfate
E3S	Estrone-3-sulfate
Gd-EOB-DTPA	Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid
HCC	Hepatocellular Carcinoma
hCNT3	Human concentrative nucleoside transporter 3
HIF-1α	Hypoxia-inducible factor-1α
HNF 3β	Hepatocyte nuclear factor 3β
HRE	Hypoxia response element
hSVCT	Human sodium-dependent vitamin C transporter
LSC	Liquid Scintillation Counter
MBD2	Methyl-DNA Binding protein 2
MRP2	Multidrug resitance- associated protein 2
OATP1A2	Organic Anion Transporting polypeptide 1A2
OATP1B1	Organic Anion Transporting polypeptide 1B1
OATP1B3	Organic Anion Transporting polypeptide 1B3
OATP1C1	Organic Anion Transporting polypeptide 1C1
OATP2A1	Organic Anion Transporting polypeptide 2A1
OATP2B1	Organic Anion Transporting polypeptide 2B1
OATP3A1	Organic Anion Transporting polypeptide 3A1
OATP4A1	Organic Anion Transporting polypeptide 4A1
OATP4C1	Organic Anion Transporting polypeptide 4C1
OATP5A1	Organic Anion Transporting polypeptide 5A1
OATP6A1	Organic Anion Transporting polypeptide 6A1
OATPs	Organic Anion Transporting polypeptide
PDAC	Pancreatic ductal adenocarcinoma
PGE2	Prostaglandin E2
Pgp	P-glycoprotein
Pgp	P-glycoprotein
PK	Pharmacokinetics
PXR	Pregnane X receptor
RT-PCR	Reverse transcription-polymerase chain reaction
SCLC	Small cell lung cancer
SNPs	Single nucleotide polymorphisms
WT	Wild-type

Chapter 1

Rationale for Theis Work

(Each chapter of this thesis contains its own introduction and therefore this chapter is intended to provide a brief rationale for the entire thesis.)

The roles of efflux drug transporters in cancer therapy are extensively investigated, in particular due to their contribution to multi-drug resistance by expelling cancer drugs from cells. In contrast, the roles of uptake transporters in cancer therapy are not as well understood. The organic anion transporting polypeptides (OATPs, gene symbol *SLCO*) represent one of the major superfamilies of solute carriers and they are expressed in different tissues throughout the body. OATPs function as multispecific transmembrane carriers that can transport a structurally diverse array of endogenous and xenobiotic compounds. Substrates of OATPs include organic dyes, bile acids, prostaglandins, cyclic nucleotides, steroid hormone conjugates, thyroid hormones, drugs, and environmental toxins. OATPs are known to transport their substrates in a sodium-independent manner. Common structural features of the OATP superfamily include twelve transmembrane domains with intracellular amino and carboxy termini as well as a large extracellular loop between the ninth and tenth transmembrane regions [1, 2] .

Among 11 human OATP members, OATP1B3 was initially reported to be expressed exclusively in hepatocytes where it mediates the uptake of various endogenous substrates (e.g., conjugates of bile acids or steroid hormones) and clinically relevant drugs such as methotrexate, imatinib, SN-38, paclitaxel [3, 4]. Subsquent investigations have also reported that OATP1B3 is frequently expressed in multiple cancer tissues derived from the gastrointestinal tract, lungs, breast, pancreas, prostate, and testis, while no expression was detected in nonmalignant tissues of these organs [3, 5-7]. Although some investigations suggested a potential association between OATP1B3 expression and

differing clinical outcomes [8], a molecular and functional understanding of OATP1B3 in cancer was incomplete.

In majority of the previous reports, it was assumed that OATP1B3 detected in cancer is identical to that in the normal hepatocytes. These findings were based on the results from RT-PCR analyses amplifying different regions in the OATP1B3 transcript and immunodetection methods using antibodies against the C-terminal region of OATP1B3 protein [3, 5-7, 9]. In our previous report, we had noted that the immunohistochemical staining for OATP1B3 frequently showed a cytoplasmic pattern in clinical colon cancer tissues, in contrast to the membranous staining in normal hepatocytes [6]. This led us to hypothesize that cancer cells may express alternative form(s) of OATP1B3 transcript/protein, different from wild-type (WT) OATP1B3 expressed in normal hepatocytes.

This thesis consists of several chapters, each of which contains relevant background information, results and discussions for the molecular and functional investigations of OATP1B3 expressed in cancer and normal liver. Chapter 2 reviews the literature findings on the expression and functions of different OATPs in cancer. In Chapter 3, we report the identification of cancer-specific variant forms of OATP1B3 resulting from alternative splicing in colon and pancreatic cancer. Our results indicated that colon and pancreatic cancer cells express the cancer-specific variant of OATP1B3 (csOATP1B3) which has limited transport activity and reduced plasma membrane trafficking compared to WT OATP1B3 expressed in the normal liver. In Chapter 4, we investigated the mechanisms regulating the expression of csOATP1B3. Based on the initial clues from a report by Winter et al. [10] we investigated the role of hypoxia and the involvement of hypoxia inducible factor-1a (HIF-1α) in regulating the transcription of csOATP1B3. Our results showed that csOATP1B3, but not OATP1B3 WT is induced under ambient and chemical hypoxia. We

were also able to identify a functional hypoxia response element (HRE) located in the proximal upstream region that physically interacts with HIF-1α.

Despite intense interest in understanding the transporter functions of OATP1B3 WT and its role in drug-drug interactions, little has been known about the mechanisms regulating the cellular trafficking of OATP1B3. In Chapter 5, we investigated whether the N-terminal sequence of OATP1B3 WT contains important motifs responsible for its membrane trafficking. This is based on the observation that the OATP1B3 variant lacking the N-terminal 28 amino acids was localized predominantly in cytoplasm. Our results indicated that the truncation of N-terminal amino acids 12-23 leads to substantially reduced expression and membrane localization of OATP1B3. In particular, the amino acids within a putative β-turn-forming tetrapeptide appear to be important in regulating the membrane trafficking of OATP1B3 WT.

In summary, the findings from this thesis work provide a better molecular and functional understanding of cancer-type OATP1B3, which had been incorrectly presumed to be identical to OATP1B3 WT expressed in the liver. This thesis work also provides important insights into the regulation of cellular trafficking and expression of OATP1B3, potentially applicable to other members of the OATP family.

Chapter 2

Literature review: Role of Organic Anion Transporting Polypeptides (OATPs) in Cancer Therapy

(The contents of this chapter have been submitted for publication in the AAPS Journal, currently under revision).

2.1 Introduction

The Organic Anion Transporting Polypeptides (OATPs) represent an important superfamily of solute carriers expressed in various tissues throughout the body. The OATPs can mediate the bidirectional transport of a diverse array of endogenous and xenobiotic compounds, including organic dyes, bile acids, prostaglandins, cyclic nucleotides, steroid hormones and their conjugates, thyroid hormones, drugs and environmental toxins. The majority of OATP substrates are large amphipathic organic anions (molecular weights greater than 300 kDa), but OATPs can also transport cationic and neutral compounds. OATPs are known to transport their substrates in a sodium-independent manner. Common structural features of the OATP superfamily include twelve transmembrane domains with intracellular amino and carboxy termini as well as a large extracellular loop between the ninth and tenth transmembrane regions.

The OATP superfamily belongs to the *SLCO* gene family and is divided into six families based on sequence similarities [11]. Currently, 11 human OATPs are known with the OATP1 family being most well characterized. The OATP1 family includes four members, OATP1A2, OATP1B1, OATP1B3 and OATP1C1, which display broad and overlapping substrate specificity. The OATP2 family includes two members, OATP2A1 and OATP2B1, both of which display relatively narrow substrate specificity compared to other OATPs. The OATP4 family includes OATP4A1 and OATP4C1. The OATP3, OATP5 and OATP6

families include OATP3A1, OATP5A1 and OATP6A1, respectively. Orthologs of human OATPs are reported in other species, but there are cases that no single ortholog is shared between humans and animals. For example, human OATP1B1 and OATP1B3 have a single rodent ortholog of Oatp1b2 [11].

Early investigations primarily focused on the identification and characterization of OATPs expressed in various organs throughout the body and the roles of OATPs in determining the disposition of substrates *in vitro* and *in vivo*. Subsequent reports revealed that the expression of OATPs can be altered in different disease conditions including cancer, implying that OATPs may play a role in the development and progression of cancer as well as the disposition of anticancer drugs. In this chapter, we will summarize the recent progress delineating the role of OATPs in cancer therapy. We will first summarize the current understanding of the roles of OATPs on the disposition of anticancer drugs and the impact of their genetic polymorphisms on the expression and function of OATPs, as well as the use of animal models to study the role of OATPs in anticancer drug disposition. In the later section, we will provide an update on the current knowledge about OATPs expressed in cancer and their potential roles in cancer development, progression and treatment.

2.2 Role of OATPs in the disposition of substrates implicated in cancer therapy

OATPs can mediate the transport of a wide range of endogenous and xenobiotic substrates in various tissues. The endogenous substrates of OATPs include cyclic and linear peptides, prostaglandins, bile acids, steroid hormone and their conjugates and thyroid hormones. Diverse classes of drugs, including anticancer drugs are also substrates of OATPs [11, 12]. In particular, the transporters of the OATP1 family have a number of substrates relevant in cancer therapy (Table 2.1).

Endogenous steroid hormones have been implicated in enhancing the survival and proliferation of cancer cells. Estrone-3-sulfate (E3S) is a major form of circulating estrogens and serves as an important source of estrogenic activity in postmenopausal women, the population with a high incidence rate of hormone-dependent cancers. Due to its hydrophilic and charged nature, E3S is not readily permeable across the plasma membrane and it often relies on a transporter-mediated mechanism to enter the cells. Among the eleven human OATP members, seven (OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2B1, OATP3A1 and OATP4A1) are shown to transport E3S [4, 13]. These OATPs may facilitate the uptake of E3S not only in the major organs such as the liver, kidney, intestine and brain, but also in hormone-dependent cancers [13-15]. Similar to E3S, dehydroepiandrosterone sulfate (DHEA-S) is transported by multiple OATPs (OATP1A2, OATP1B1, OATP1B3 and OATP2B1) and may gain entry to cells via these transporters [4]. On the other hand, testosterone appears to be transported only by OATP1B3 [7, 16]. Many of these investigations however focused on the uptake of steroid hormones and their conjugates into cancer cells and the subsequent impact on cancer cell proliferation and related cellular pathways, rather than the disposition of hormonal substrates in the whole body.

In case of anticancer drugs handled by OATPs, a number of investigations have examined whether the expression/function of OATPs can influence the pharmacokinetic profiles of anticancer drugs, and consequently therapeutic effects and toxicities. The most well studied substrates include methotrexate, doxorubicin and taxanes (paclitaxel and docetaxel). For these drugs, multiple reports indicated that these agents are handled by OATP1A2, OATP1B1 and OATP1B3 [3, 17-19]. The results were obtained from various models, ranging from *in vitro* heterologous expression systems, *in vivo* preclinical knockout and humanized mouse models and clinical studies. Docetaxel is reported to be

a substrate of human OATP1B1 and OATP1B3 and rat and mouse Oatp1b2 *in vitro* [20, 21]. However, the pharmacokinetic profiles of docetaxel were not substantially altered in a cohort of patients harboring genetic variations associated with decreased OATP1B1 or OATP1B3 activity [22]. These results might be related to the presence of other uptake transporters with overlapping functions in the human liver. Indeed, a recent study using humanized transgenic mice indicated that elevated plasma levels of docetaxel observed in the knockout mice lacking Oatp1a/1b can be rescued by liver-specific expression of human OATP1B1, OATP1B3 or OATP1A2, confirming the relevance and overlapping nature of these OATP transporters in determining *in vivo* disposition of docetaxel [18]. When the involvement of OATPs in the handling of taxanes is considered, there was also a report indicating that taxanes are not handled by OATP1B3 [20]. These apparent discrepancies may be due to differences in the expression systems (*Xenopus laevis* oocytes vs HEK293). Other well-studied anticancer drugs handled by OATP1B1 and OATP1B3 are rapamycin (sirolimus) and SN-38 (7-ethyl-10-hydroxycamptothecin, an active metabolite of irinotecan) [20, 23-26]. Additionally, OATP1B1 transports CP-724,714 (a Her2 tyrosine kinase inhibitor), cis-diammine-chloro-cholylglycinate-platinum II (a bile-acid cisplatin derivative) and gimatecan (a camptothecin analog) [27-29]. Imatinib, used for leukemia therapy, is reported to be transported by OATP1A2 and OATP1B3 [30, 31]. Further investigations will be necessary to elucidate the clinical relevance of these transporters in influencing the *in vivo* disposition and therapeutic effects of these substrate drugs.

The magnetic resonance imaging agent gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) is also shown to be transported by OATP1B1 and OATP1B3 [32-34]. In a retrospective study involving 22 patients with hepatic cancer, high OATP1B3 expression levels were significantly correlated with increased uptake of Gd-

EOB-DTPA [32]. In individuals carrying certain genetic variations associated with decreased OATP1B1 activity, the liver enhancement by the gadolinium-based imaging agent was substantially attenuated [34]. These findings suggest that the activity of OATPs and genetic variations may be potential confounders leading to a reduced signal intensity in liver magnetic resonance imaging. However, a recent retrospective study took advantage of this relationship and attempted to use the signal intensity of Gd-EOB-DTPA as a marker for hepatic vascularity and OATP-related activity and potentially as a prognostic factor for patients with early-staged hepatic cancer [35]. Additional investigations will be important to evaluate the prognostic utility of these findings in improving the classification and management of early-staged hepatic cancer.

2.3 Impact of OATP polymorphisms on the pharmacokinetics of anticancer drugs

A number of naturally occurring single nucleotide polymorphisms (SNPs) in the genes encoding OATPs have been reported and extensively investigated for their impact on the expression and function of the corresponding OATPs and consequently on the disposition and efficacy of anticancer drugs. In particular, polymorphic variants of genes encoding OATP1A2, OATP1B1 and OATP1B3 have been reported to be clinically relevant. Comprehensive reviews on this topic are already available elsewhere [4]. Thus, only a brief summary and update involving the disposition of anticancer drugs is provided below.

For OATP1A2, the initial investigation was carried out using *Xenopus laevis* oocytes expressing genetic variants of OATP1A2 and methotrexate [17]. Four out of the twelve OATP1A2 variants examined displayed altered transport of E3S and methotrexate; the p.I13T (rs10841795) variant displayed enhanced transport activity, the p.R168C (rs11568564) and p.E172D (rs11568563) variants demonstrated decreased transport activity and the p.N278DEL (rs11568555) variant produced a nonfunctional protein. However, the impact of these OATP1A2 variants on the pharmacokinetics of methotrexate

in patients remains to be determined. More recently, a similar line of investigation was carried out with imatinib and genetic variations present in the coding and promoter regions of the gene encoding OATP1A2 [31]. The authors were able to identify polymorphic variations in the promoter region of OATP1A2 (−1105G>A (rs4148977) in linkage disequilibrium with −1032G>A (rs4148978), and −361G>A (rs3764043)) which correlated with the clearance of imatinib in patients with chronic myeloid leukemia [31]. Further clinical studies are required to confirm these findings in a larger group of patients and to verify whether the identified OATP1A2 variations correlate with therapeutic effects or toxicities of imatinib.

For OATP1B1, there are several commonly occurring SNPs and in particular, the OATP1B1*15 (harboring variations causing two amino acid substitutions, p.N130D (rs2306283) and p.V174A (rs4149056)) variant has been most extensively investigated. Compared to the wild-type OATP1B1 (OATP1B1*1a), the OATP1B1*15 displayed reduced uptake of SN-38 (an active metabolite of irinotecan), when tested in *in vitro* cell line models stably expressing OATP1B1 variant proteins [24]. The clinical relevance of these findings was subsequently validated by comparing the pharmacokinetics and toxicity profiles in patients receiving irinotecan therapy [36, 37]. Patients with the OATP1B1*15 genotype showed increased systemic exposure and toxicities of SN-38 compared to those with the wild-type OATP1B1 [36, 37]. Similar findings have been reported with patients treated with methotrexate. In particular, recent reports validating the relevance of the OATP1B1*15 genotype in determining the pharmacokinetics and toxicities of methotrexate were from genome-wide association studies or studies involving a large number of patients and cohorts from different institutions [38-40]. Continued investigations examining the prospective utility of the OATP1B1 genotypes in improving therapeutic effects and safety profiles of these anticancer drugs are warranted.

As a closely related member to OATP1B1, OATP1B3 displays substantial overlapping substrate specificity, yet harbors fewer genetic variations. Initially, the functional impact of three nonsynonymous SNPs of OATP1B3 (p.S112A (rs4149117), p.M233I (rs7311358) and p.G522C (rs72559743)) were examined using *in vitro* models [41]. However, none of these three SNPs were found to have a significant impact on the clearance and other pharmacokinetic parameters of paclitaxel in cancer patients [42]. For docetaxel, a potential association between OATP1B3 genotypes and toxicity (leukopenia/neutropenia) has been recently reported in patients [43, 44]. Additional investigations will be required to validate the clinical relevance of these findings.

2.4 Animal models to investigate the role of OATPs in the disposition of anticancer drugs

Given the increasingly recognized roles that OATPs play in determining the disposition of many drugs, it has become important to assess the potential of OATPs and their genetic variations as a source for variable drug disposition and response *in vivo*. In recent years a number of transgenic mouse models have been reported including the knockout mouse models lacking the orthologs of human OATP1A and OATP1B subfamily members and the humanized mouse models where human OATPs are introduced after deleting the genes for mouse orthologs.

In rodents, there is only one member of the Oatp1b subfamily and it is considered to be the closest ortholog for both human OATP1B1 and OATP1B3. The knockout mouse models lacking Oatp1b2 have been developed by three independent groups and have served as useful tools to delineate and extrapolate the *in vivo* relevance of both human OATP1B1 and OATP1B3 to the disposition of environmental toxins (e.g. phalloidin, microcystin-LR), statin drugs (e.g. cerivastatin, lovastatin, pravastatin, simvastatin) and antibiotics (e.g. rifampicin, rifamycin SV) [45-47]. Given the large overlap in tissue

distribution and substrate specificity within the OATP1 family, another transgenic mouse model deficient for all five established *Slco1a* and *Slco1b* genes (*Slco1a/1b*$^{-/-}$ mice) has been developed [48]. These mice lacking Oatp1a1, Oatp1a4, Oatp1a5, Oatp1a6 and Oatp1b2 displayed drastically reduced hepatic uptake of methotrexate, fexofenadine and paclitaxel and subsequently increased systemic exposure for all of these drugs [48, 49].

In addition, humanized transgenic mouse models expressing OATP1A2, OATP1B1 and OATP1B3, in the absence of the background expression of the mouse orthologs, have been developed and used to account for possible species-dependent differences between the mouse and human OATP orthologs. For example, a humanized OATP1B1 mouse model with liver-specific expression of OATP1B1 was generated and the disposition of methotrexate was investigated. The plasma concentrations of intravenously administered methotrexate in the humanized OATP1B1 transgenic mice were substantially lower than in the control animals [50]. In addition, the humanized OATP1B1 transgenic mice displayed a greater amount of methotrexate in the liver as well as a higher liver to plasma ratio of methotrexate than the control animals [50]. Similarly, transgenic humanized OATP1A/1B mouse models were generated with liver-specific expression of OATP1B1, OATP1B3 and OATP1A2 in an Oatp1a/1b knockout background. This model was utilized to show that paclitaxel, methotrexate, SN-38, docetaxel and doxorubcin are transported by OATP1A/1B *in vivo* [18, 19, 51, 52].

These findings have provided further insights regarding the contribution of OATP1A/1B transporters to the disposition, response and toxicity of anticancer drugs. Importantly, those investigations have provided the impetus for additional studies to elucidate the role of other OATPs and commonly observed polymorphisms in the pharmacokinetics and pharmacodynamics of anticancer drugs. Together, these tools will continue to provide a

useful guide to inform anticancer drug development and optimization of anticancer drug therapy.

2.5 OATPs expressed in cancer

There is a substantial body of evidence indicating that the expression of OATPs can be altered in various types of cancers. In this section we will summarize the current literature about different OATPs expressed in cancer tissues, their proposed functions and cancer-specific mechanisms of regulation.

2.5.1 OATP1A2 (Gene symbol, *SLCO1A2*)

OATP1A2 expression has been confirmed in several normal tissues and cell types including the blood-brain barrier, enterocytes, cholangiocytes and kidneys [53, 54]. OATP1A2 can mediate the transport of endogenous hormonal substrates (e.g. E3S, DHEA-S) and several cancer drugs (e.g. imatinib, paclitaxel, doxorubicin, docetaxel) (Table 2.1). Thus, a number of studies have examined the expression and functional impact of OATP1A2 in cancer. OATP1A2 expression was first reported in breast cancer and subsequently in additional types of cancer including colon, prostate and bone (Table 2.2) [55-59]. To date, the potential functional significance of OATP1A2 has been reported in breast cancer, but not in other types of cancer.

The expression of OATP1A2 mRNA and protein in breast cancer was first reported by Miki et al. [55]. The results from RT-PCR analyses indicated that OATP1A2 is expressed in human breast cancer tissues, but not in noncancerous breast, adipose tissues or stromal cells [55]. These findings are consistent with the reports showing that OATP1A2 mRNA was barely detectable in non-malignant mammary epithelial cells (below the level of quantification) [60]. Interestingly, Miki et al. also reported a significant correlation between the expression of OATP1A2 and the nuclear receptor PXR (pregnane X receptor)

providing possible insights into the regulation of OATP1A2 expression [55]. These findings were further validated in breast cancer cell line models [56] where treatment of T47-D cells with rifampicin (a well-known PXR activator) increased the expression of OATP1A2 and cellular uptake of E3S and promoted breast cancer cell proliferation *in vitro*. In line with these findings, a more recent study using mouse xenograft models reported that OATP1A2 may play a role in regulating *in vivo* tissue distribution of E3S and the growth of hormone-dependent breast cancer [14]. In the clinical setting, the expression of OATP1A2 combined with another transporter OCT6 in patients with triple negative breast cancer (which displays no detectable expression for estrogen receptor, progesterone receptor and Her2/neu and has very poor prognosis) was predictive of response to anthracycline/taxane-based neoadjuvant chemotherapy [61]. Further investigations will be required to evaluate the clinical relevance of these findings in patients with hormone-dependent or triple negative breast cancers.

Although detailed functional investigations have not been reported, the expression of OATP1A2 has been observed in other cancers. Arakawa et al. reported that OATP1A2 is expressed in human prostate cancer cell lines (LNCaP and 22Rv1) and facilitates the uptake of DHEA-S and enhances cancer cell growth under androgen-depleted conditions [58]. The levels of OATP1A2 mRNA were reported to be elevated in human osteosarcoma cell lines (HOS and MG-63) and human kidney cancer cells metastasized to bone tissue [59]. On the other hand, colon polyps and colon cancer tissues were reported to have reduced OATP1A2 mRNA levels compared to healthy colon tissue [57].

As noted above, the nuclear receptor PXR may play a role in regulating OATP1A2 expression in cancer [56]. A PXR response element present in the human OATP1A2 promoter was confirmed to have physical interactions with PXR and to play a role in the transactivation of OATP1A2 in breast cancer cells [56]. If OATP1A2 is eventually identified

to have an important role in cancer development, progression or therapy, its regulation through PXR may be a key relationship to investigate.

2.5.2 OATP1B1 (Gene symbol, *SLCO1B1*)

OATP1B1 is abundantly expressed in the normal liver and was initially considered to be liver-specific [62, 63]. OATP1B1 mediates the uptake of several endogenous substrates including hormones (E3S and DHEA-S) and anticancer drugs (rapamycin, SN-38, gimatecan, flavopiridol, docetaxel) (Table 2.1) (reviewed in [4] and [64]). Several investigations have been carried out to assess the expression levels and functions of OATP1B1 in cancer. To date, OATP1B1 is reported to be expressed in colon cancer, ovarian cancer and variably expressed in hepatocellular carcinoma (HCC).

A number of reports indicate that the expression of OATP1B1 is decreased in HCC compared to non-malignant liver. Since the first report on the decreased OATP1B1 protein levels in HCC cell lines [65], many subsequent investigations have reported similar findings using HCC tissue samples and cell lines [66-69]. However, there is also a report noting no significant reduction of OATP1B1 in HCC tissues compared to non-malignant liver tissues [70].

In contrast to the variable expression of OATP1B1 in HCC, OATP1B1 expression was reported to be elevated in cancers derived from nonhepatic tissues which normally do not express OATP1B1. For instance, OATP1B1 expression has been reported in colon polyps and colon cancer tissue [57] as well as ovarian cancer tissue samples and cell lines (SK-OV-3) [9]. In regards to its transport functions in cancer, OATP1B1 is implicated to play a role in paclitaxel uptake in ovarian cancer cells [9]. Overall, further investigations will be

necessary to examine the clinical significance of altered expression of OATP1B1 in cancers.

2.5.3 OATP1B3 (Gene symbol, *SLCO1B3*)

When OATP1B3 was cloned for the first time, it was found to be abundantly expressed in the normal liver, but not in any other non-malignant tissues [3]. This initial report also noted that OATP1B3 is expressed in multiple types of cancer. These findings have been confirmed by a number of subsequent investigations and OATP1B3 is arguably the most extensively investigated OATP member with regard to cancer-related alterations in expression. Recently there has been substantial progress in our understanding of cancer-specific expression of OATP1B3 including the identification of cancer-specific variants.

OATP1B3 mediates the transport of several endogenous substrates including hormones (E3S, DHEA-S, testosterone), cancer drugs (methotrexate, imatinib, paclitaxel, SN-38, rapamycin, docetaxel, doxorubicin), microcystins and MRI contrast agents (gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)) [32, 71]. Similar to the decreased expression of the closely related member OATP1B1 in HCC, OATP1B3 expression was shown to be decreased in primary and metastatic liver cancers [68, 70, 72]. It has been suggested that the decreased expression of OATP1B3 and OATP1B1 in hepatic cancer tissues may be related to poor differentiation status in the malignant tissues. On the other hand, OATP1B3 is frequently expressed in several cancerous tissues derived from the gastrointestinal tract, pancreas, lung, breast, prostate, and testes, all of which do not express any detectable level of OATP1B3 in noncancerous settings. This frequent expression of OATP1B3 across multiple cancer types prompted many investigators to probe the functional role of OATP1B3 in cancer.

The expression of OATP1B3 in multiple types of cancer was first reported by Abe et al. [3]. Specifically, northern blot analyses showed the expression of OATP1B3 in various established cancer cell lines (derived from the colon, stomach, pancreas, gall-bladder and lung) and clinical samples (from gastric, colon and pancreatic cancer) [3]. This finding was subsequently corroborated by multiple studies which utilized RT-PCR and immunohistochemical detection methods (Table 2.2). In human breast cancer, OATP1B3 was detected in approximately 50% of the clinical tissue samples examined by immunohistochemistry and OATP1B3 immunoreactivity was associated with a decreased risk of recurrence and improved prognosis [5]. The authors speculated that OATP1B3 overexpression may be associated with hormone-dependent growth mechanisms considering that OATP1B3 transports E3S. In line with these findings, subsequent studies have also suggested that OATP1B3 contributes to the growth of estrogen-dependent breast cancer [13, 15]. OATP1B3 is also reported to be expressed in prostate and colorectal cancers [7, 8, 16]. In reference to colorectal cancer, OATP1B3 is found to be expressed in the majority (56%) of clinical tissue specimens examined, and possibly associated with improved clinical outcomes [8]. During these investigations, it was also noted that the immunohistochemical staining for OATP1B3 showed predominantly a cytoplasmic pattern, clearly different from the membranous pattern in normal hepatocytes [6, 8].

Our laboratory has probed possible reasons for different localization patterns of OATP1B3 expressed in cancer cells vs normal hepatocytes. Through those efforts, we recently reported the presence of a cancer-specific OATP1B3 variant (csOATP1B3 or OATP1B3 V1) which utilizes an alternative transcription initiation site from wild-type (WT) OATP1B3 expressed in normal liver [73]. The protein translated from the longest open reading frame (ORF) lacks 28 amino acids at the N-terminus of OATP1B3 WT. Our

findings showed that in comparison to OATP1B3 WT, csOATP1B3 has defective plasma membrane trafficking, resulting in reduced transport activity of cholecystokinin-8 (a prototype OATP1B3 substrate) [73]. Similarly Nagai et al. reported that lung, colon and pancreatic cancer tissues and cell lines express an alternative OATP1B3 transcript [74]. However, this study proposed the four potential ORFs resulting in much shorter amino acid sequences than the ORF reported by our laboratory and another subsequent report [73, 75]. Currently, csOATP1B3 is found to be the major isoform expressed only in cancer cells, but not in normal tissues. Further studies are required to delineate the biological significance of csOATP1B3 in cancer, specifically whether csOATP1B3 confers any survival advantage or chemotherapy resistance to cancer cells.

With regard to the regulatory mechanisms for OATP1B3 expression, several investigations implicated the involvement of the liver-enriched transcription factor, hepatocyte nuclear factor 3β, (HNF3β) as well as epigenetic and hypoxia mediated mechanisms [70, 75-77]. The contribution of these reported mechanisms may vary depending on the tissue types and disease states. In an earlier report, Vavricka et al showed that HNF3β is responsible for the transcriptional repression of OATP1B3 expression in HCC [70]. A later study showed that epigenetic mechanisms by DNA methylation-dependent gene silencing are involved in the regulation of OATP1B3 in different cancer cell lines [76]. A more recent study showing that DNA methylation-dependent gene silencing involving the Methyl-DNA Binding protein 2 (MBD2) regulates the expression of csOATP1B3 confirmed the previous methylation-dependent gene silencing findings [75]. Our laboratory showed that csOATP1B3, but not OATP1B3 WT is transcriptionally activated by hypoxia. A functional hypoxia response element (HRE) within the promoter regions of csOATP1B3 was shown to physically interact with hypoxia inducible factor-1α (HIF-1α) in colon and pancreatic cancer cells [77]. We postulated that

hypoxia-induced regulation of csOATP1B3 may be operating in line with epigenetic regulation. This hypothesis is based on the observation that the HRE site proposed in our recent study [77] is located in close proximity to the potential methylation site [75]. Thus, we examined the effect of *in vitro* methylation on the transactivation of a reporter construct of the csOATP1B3 promoter containing the proposed HRE and methylation sites. Our results showed no substantial changes by *in vitro* methylation, suggesting that hypoxia and epigenetic mechanisms likely work independently in regulating the csOATP1B3 (unpublished data).

2.5.4 OATP1C1 (Gene symbol, *SLCO1C1*)

OATP1C1 was identified as a high affinity transporter for thyroid hormones in brain, Leydig cells of the testis [78] and ciliary bodies [79]. Thus far, no cancer drugs have been identified to be substrates of OATP1C1. A study by Liedauer et al. showed that OATP1C1 mRNA expression was detected in several human osteosarcomas, samples of metastases from kidney tumors and the highest expression was observed in aneurysmal bone cysts [59]. This is the only study to report the expression of OATP1C1 in cancer and the functional role of OATP1C1 in bone cancers is currently unknown.

2.5.5 OATP2A1 (Gene symbol, *SLCO2A1*)

OATP2A1 was initially cloned and identified as a prostaglandin transporter [80]. Currently, no cancer drugs have been reported to be substrates of OATP2A1. In non-cancerous tissues, OATP2A1 is expressed ubiquitously. Altered expression of OATP2A1 is reported in various types of cancers (Table 2.2). OATP2A1 expression is reported to be increased in breast cancer, HCC, cholangiocarcinoma and liver metastases from colon cancer [16, 81]. On the other hand, reduced OATP2A1 levels are reported in other types of cancer from colon, stomach, ovary, lung, and kidneys [82]. Interestingly, the reduced expression of OATP2A1 was linked with increased extracellular levels of the proinflammatory

prostaglandin E2 (PGE2) in colorectal cancer [82]. Higher extracellular levels of PGE2 may activate various signaling cascades in colorectal cancer by interacting with G protein-coupled receptors on the surface. Further investigations are required to better understand the impact of altered OATP2A1 expression in cancer.

2.5.6 OATP2B1 (Gene symbol, *SLCO2B1*)

OATP2B1 is a ubiquitously expressed uptake transporter that was initially cloned by Tamai et al. [83] (Table 2.2). OATP2B1 transports various substrates including steroid hormone conjugates, thyroid hormones, prostaglandins and other drugs. Although no cancer drugs are currently known to be substrates of OATP2B1, its altered expression is reported in different cancers (Table 2.2). In breast cancer, increased OATP2B1 expression was reported in clinical samples as well as cell lines and was correlated with high tumor grades, but not with an altered clinical outcomes [84]. OATP2B1 expression was also shown to be higher in bone cysts compared to osteosarcoma tissues [59]. Alternatively, Pressler et al. showed that OATP2B1 mRNA expression was lower in liver and pancreatic cancers compared to non-malignant tissues [85]. However, these findings have not been validated at the protein level. Currently, the role of OATP2B1 in cancer cells is not well understood and needs further investigation.

2.5.7 OATP3A1 (Gene symbol, *SLCO3A1)*

In non-malignant tissues, OATP3A1 is expressed ubiquitously where it transports various hormones, prostaglandins and drugs [4]. Altered expression of OATP3A1 is reported in various types of cancers (Table 2.2). Increased expression of OATP3A1 transcripts have been reported in multiple cancer cell lines [4, 83]. Higher OATP3A1 mRNA was detected in aneurysmal bone cysts as compared to osteosarcomas [59]. In breast cancer, OATP3A1 is localized both on the plasma membrane and in the cytoplasm [86]. Wleck et

al. showed that OATP3A1 levels were increased in primary and metastatic liver cancer [87]. Further studies are required to investigate the role of OATP3A1 expressed in cancer.

2.5.8 OATP4A1 (Gene symbol, *SLCO4A1)*

OATP4A1 is expressed ubiquitously and plays a role in the transport of several endogenous (hormones, prostaglandins and bile acids) and drug substrates (reviewed in [4]). Similar to OATP3A1, OATP4A1 is also overexpressed in multiple cancer cell lines, aneurysmal bone cysts, liver cancers and breast cancers [59, 81, 83, 87]. In colorectal neoplasia specimens, OATP4A1 mRNA levels were reported to be elevated [88]. Additionally, the authors suggested that increased expression of PGE2 transporting OATP2B1 and OATP4A1 may lead to decreased sensitivity to cyclic nucleotides in colorectal neoplasia. However further validation is required to better delineate the role of OATP4A1 in these conditions.

2.5.9 OATP4C1 (Gene symbol, *SLCO4C1)*

OATP4C1 expression in non-cancerous tissues is mainly limited to the kidney where it mediates the uptake of thyroid hormone, cAMP, cardiac glycosides, and methotrexate [89]. In breast cancer tissues and cell lines, Wleck et al. showed that elevated OATP4C1 mRNA levels were present [81]. However, little is known regarding the expression and role of OATP4C1 in other cancers.

2.5.10 OATP5A1 (Gene symbol, *SLCO5A1)*

OATP5A1 is reported to be expressed in the epithelial cells lining the mammary ducts [86]. To date, the tissue distribution and substrates of OATP5A1 are poorly understood. Different studies report the expression of OATP5A1 in cancers (Table 2.2). Immunohistochemical analyses confirmed that OATP5A1 is expressed on the membrane and in the cytoplasm of breast cancer cells [86]. OATP5A1 at both the mRNA and protein

levels was found to be upregulated in liver cancer [87] and small cell lung cancer (SCLC) [90]. As a potential marker of chemotherapy resistance, HEK-293 cells transfected with OATP5A1 showed resistance to satraplatin treatment [90]. Further studies may be warranted to elucidate the impact of OATP5A1 on tumor resistance and better understand the role OATP5A1 in cancer.

2.5.11 OATP6A1 (Gene symbol, *SLCO6A1)*

OATP6A1 was initially identified as a gonad-specific transporter expressed predominantly in the testes [91, 92]. Its expression is also reported in cancer tissues (lung esophageal, and bladder) and lung cancer cell lines [92]. However, little is currently known regarding the role of OATP6A1 expression in cancer.

2.6 Conclusions and Outlook

OATPs are expressed in multiple tissues and organs and mediate the transport of a wide range of substrates in a sodium-independent manner. It is increasingly recognized that OATPs play an important role in the disposition of substrates implicated in cancer therapy. In particular, the variable expression/activity of OATP1 family members and their genetic variations have been extensively investigated as a possible source for altered pharmacokinetics and pharmacodynamics of anticancer drugs. There is a long list of anticancer drugs recognized as OATP substrates, but further research is required to elucidate the *in vivo* relevance of these interactions. The increasing availability of transgenic mouse models is moving the field forward, yet further clinical validation in terms of disposition, response and toxicity to anticancer drugs will be crucial.

It is now well recognized that certain OATPs are differentially regulated in normal and cancer tissues. Certain OATPs differentially regulated in cancer may have pathogenic roles during cancer development and progression and potentially serve as therapeutic

targets. Further studies are necessary to obtain more comprehensive profiles of OATPs differentially regulated in cancer cells, along with a better understanding of molecular mechanisms underlying altered expression of OATPs in cancer. So far, many of the reports focused on the altered expression of certain OATPs and it will be important to clarify the functional implications of OATPs during the development and progression of cancers. Recently there has been progress in our understanding of the OATP1 family in terms of their expression, regulation and potential functions in cancer cells. With the wealth of provocative data generated, further studies are warranted to investigate the potential roles of other OATP family members expressed in cancer.

Table 2.1 Selected endogenous substrates, anticancer drugs and imaging agents transported by OATPs

OATPs	Substrates	Reference(s)
OATP1A2	**Endogenous hormones and conjugates**	
	Estrone-3-sulfate, DHEA-S	[93, 94]
	Anticancer drugs	
	Imatinib, Methotrexate, Paclitaxel, Doxorubicin, Docetaxel	[17-19, 30, 52]
OATP1B1	**Endogenous hormones and conjugates**	
	Estrone-3-sulfate, DHEA-S	[83, 95]
	Anticancer drugs	
	Methotrexate, Paclitaxel, Rapamycin, Flavopiridol, SN-38, Gimatecan, Doxorubicin, Docetaxel, CP-724,714 , Cis-diammine-chloro-cholylglycinate-platinum(II)	[3, 19, 96], 23, 24, 97] [18, 27, 29, 52] [28]
	Imaging agents	
	Gd-EOB-DTPA	[33]
OATP1B3	**Endogenous hormones and conjugates**	
	Estrone-3-sulfate, DHEA-S, Testosterone	[7, 95, 97]
	Anticancer drugs	
	Rapamycin, Methotrexate, Paclitaxel, Doxorubicin, Docetaxel, Imatinib, SN-38	[3, 19, 21, 23, 52] [18, 20, 21, 30]
	Imaging agents	
	Gd-EOB-DTPA	[32]

Table 2.2 Expression of OATPs in non-malignant and malignant tissues

OATPs	Non-malignant tissues	Malignant tissues and cells	
		Alterations	Detection method
OATP1A2	Blood-brain barrier [53] Enterocytes [54] Cholangiocytes [54] Kidney [98]	↑ in breast cancer [55, 56] ↓ in colon polyps and colon cancer [57] ↑ in prostate cancer cells [58] ↑ in bone cancer [59]	RT-PCR, IF RT-PCR qRT-PCR RT-PCR
OATP1B1	Liver [62, 63]	↓ in liver cancer [65-68, 85] ↑ in colon polyps and colon cancer [57] ↑ in ovarian cancer [9]	RT-PCR, IF, IB RT-PCR RT-PCR
OATP1B3	Liver [3, 99]	↓ in liver cancer [70] ↑ in colon cancer [6, 73, 74] ↑ in pancreatic cancer [73, 74, 100] ↑ in lung cancer [67] ↑ in prostate cancer [7, 16, 85, 101] ↑ in breast cancer [5] ↑ in testicular cancer [85] ↑ in ovarian cancer [9]	qRT-PCR, IB RT-PCR, qRT-PCR, IB, IHC RT-PCR, qRT-PCR, IB, IHC RT-PCR, qRT-PCR, IF qRT-PCR, IF qRT-PCR, IHC qRT-PCR, IF RT-PCR
OATP1C1	Brain [78] Testes [78] Ciliary body [79]	↑ in bone cancers [59]	RT-PCR

OATP2A1	Ubiquitous [102]	↑ in breast cancer [81] ↑ in liver cancer [87] ↑ in bone metastases from kidney cancer [59] ↓ in cancers of bowel, stomach, ovary, lung and kidney [82]	qRT-PCR qRT-PCR, IF qRT-PCR, IB RT-PCR
OATP2B1	Blood-brain barrier [103] Heart [104] Enterocytes [105] Liver [106] Placenta [107]	↑ in bone cancer [59] ↑ in breast cancers [81, 84] ↓ in liver and pancreatic cancers [85]	RT-PCR qRT-PCR, IF, IB qRT-PCR
OATP3A1	Ubiquitous [108]	↑ in bone cysts [59] ↑ in breast cancer tissues and cell lines [86] ↑ in primary and metastatic liver cancer [87] ↑ in cancer cell lines of multiple tissues [83]	RT-PCR qRT-PCR, IF, IHC qRT-PCR, IF RT-PCR
OATP4A1	Ubiquitous [83]	↑ in bone cysts [59] detected in breast cancer [81] ↑ in primary and metastatic liver cancer [87] detected in cancer cell lines of multiple tissues [83] ↑ in colon cancer [88]	RT-PCR RT-PCR qRT-PCR, IF RT-PCR qRT-PCR
OATP4C1	Kidney [89]	detected in breast cancer cell lines [81]	RT-PCR

OATP5A1	Lactiferous ducts of breast [86]	↑ in breast cancer [86] ↑ in primary and metastatic liver cancer [87] ↑ in small cell lung cancer [90]	qRT-PCR, IF, IHC qRT-PCR, IF qRT-PCR, IF
OATP6A1	Testes [91]	detected in lung, bladder and esophageal cancer [92]	RT-PCR

↑, increased; ↓, decreased; detection methods represented as reverse transcriptase-polymerase reaction (RT-PCR), quantitative RT-PCR (qRT-PCR), immunofluorescence (IF), immunoblotting (IB), immunohistochemistry (IHC)

Chapter 3

A cancer-specific variant of the *SLCO1B3* gene encodes a novel human organic anion transporting polypeptide 1B3 (OATP1B3) localized mainly in the cytoplasm of colon and pancreatic cancer cells

(The work in this chapter has been published in Molecular Pharmaceutics (2013) [73])

3.1 Introduction

Organic anion transporting polypeptide 1B3 (OATP1B3) belongs to the OATP superfamily that mediates transmembrane transport of endogenous and xenobiotic compounds in various organs [2]. OATP1B3 was initially reported to be expressed exclusively in hepatocytes, mediating the uptake of various endogenous substrates (e.g. conjugates of bile acids or steroid hormones) and clinically relevant drugs such as methotrexate and paclitaxel [109]. Several investigations including one from our own laboratory have shown that OATP1B3 is also expressed in cancer tissues derived from the gastrointestinal tract, breast, lung, pancreas and prostate, while expression was not detected in nonmalignant tissues of these organs [3] [5-7, 67, 110]. Although some reports suggested a possible association between OATP1B3 expression and differing clinical outcomes, a molecular and functional understanding of OATP1B3 in cancer has been incompletely investigated [5, 8].

In the majority of previous reports, it was assumed that OATP1B3 detected in cancer is identical to that in normal hepatocytes [3, 5-7, 9, 67]. These findings were based on results from RT-PCR analyses amplifying various regions in the OATP1B3 transcript and immunodetection methods using antibodies against the C-terminal region. In our previous study, we however noted that the immunohistochemical staining for OATP1B3 frequently showed a cytoplasmic pattern in clinical colon cancer tissues, in contrast to the

membranous staining in normal hepatocytes [6, 8]. This led us to hypothesize that cancer cells may express alternative form(s) of OATP1B3 transcript/protein, different from wild-type (WT) OATP1B3 expressed in normal hepatocytes. In our current study, we have identified multiple variant forms of OATP1B3 resulting from alternative splicing in colon and pancreatic cancer. In a similar line of work, Nagai et al. recently reported that colon, lung and pancreatic cancer tissues and cell lines express an alternative OATP1B3 transcript and its sequence is identical to the most prevalent variant form, OATP1B3 V1 identified from our current study [74]. Using the sequence information for this cancer-specific OATP1B3 isoform (Ct-OATP1B3 or OATP1B3 V1), Nagai et al. proposed the four putative open reading frame (ORF) sequences and reported the detection of GFP expression following transfection of the expression plasmids containing the proposed ORF sequences fused with GFP [74]. However, further investigation is necessary to determine whether any of the putative ORF sequences proposed by Nagai et al. is indeed translated into proteins in clinical cancer tissues or established cancer cell lines [74].

In this chapter, we report our findings on the presence of multiple OATP1B3 mRNA isoforms and further validation of the OATP1B3 V1 protein expression in clinical colon and pancreatic cancer tissues and established cell lines. Our immunohistochemical analyses support that colon and pancreatic cancer tissues express OATP1B3 V1 protein lacking 28 amino acids at the N-terminus. Compared to OATP1B3 WT, OATP1B3 V1 was found to undergo a differing extent of post-translational modifications, proteasomal degradation, and plasma membrane trafficking, and showed only limited transport activity toward cholecystokin-8 (CCK-8, a prototype OATP1B3 substrate).

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and Reagents

Peptide: N-glycosidase F (PNGase F) was purchased from New England Biolabs Inc. Sodium butyrate, ammonium sulfate, and rifampin were purchased from Sigma-Aldrich. Epoxomicin was synthesized and kindly provided by Dr. Kyung Bo Kim (University of Kentucky). [^{3}H]-Cholecystokinin-8 ([^{3}H]-CCK-8, 104.2 Ci/mmol, > 90% purity) was purchased from Perkin Elmer. Immunohistochemistry reagents were purchased from Biogenex.

3.2.2 5′-RACE (Rapid Amplification of cDNA Ends)

Total RNAs (1 μg) from 5 clinical colon cancer tissues (Biochain) and 8 colon cancer cell lines (CaCO2, HCT8, RKO, SW480, SW620, HCA7, HCT116, and DLD1) were converted to RACE-ready cDNA using the SMARTer™ RACE cDNA amplification kit (Clontech). Subsequently, reverse transcription-PCR (RT-PCR) was performed using the Advantage 2 PCR kit (Clontech), the universal primer provided in the SMARTer™ Race kit and primers designed to target OATP1B3 at exon 5 (5′-GCCACGAAGCATATTCCCCATGAAGACA-3′) or exon 10 (5′-CCGGCAACTGATTTGCTTTCGCAGAT-3′). Amplified products of different sizes were cloned into pCR2.1-TOPO (Invitrogen) and analyzed by direct sequencing.

3.2.3 Quantitative and Qualitative RT-PCR

Total RNAs (1 μg) from clinical cancer tissues (n=5 for colon and n=10 for pancreas) and multiple established cancer cell lines (n=8 for colon and n=6 pancreas) were converted to single-stranded cDNA using the SuperScript™ III cDNA synthesis kit (Invitrogen). For quantitative RT-PCR analyses of OATP1B3 WT or V1, primers were designed to amplify the regions unique to WT or V1. The following primer sequences were used; for WT, sense 5′-TCAAAGTCAAGGTGATCATT-3′ (exons 1 and 2) and antisense 5′-CCAAGAACATCTTGAATCCA-3′ (exons 2 and 3); for V1, sense 5′-AAAACTAGCAGATGTTCTTG-3′ (exons 2a and 3) and antisense 5′-

ATACCTATAATATCCCATGAAGAA-3′ (exons 4 and 5); for β–actin, sense 5′-GCATCCTCACCCTGAAGTAC-3′ and antisense 5′-GATAGCACAGCCTGGATAGC-3′. Quantitative RT-PCR was performed in duplicate using iCycler with the iQ SYBR-green Supermix (Bio-Rad). The condition of quantitative RT-PCR was as follows: annealing at 60°C with 40 cycles for OATP1B3 WT or V1; annealing at 65°C with 40 cycles for β–actin. In order to obtain the transcript copy numbers for OATP1B3 WT, V1 or β-actin, serial dilutions of the plasmids containing each amplicon were used as calibration samples. The specificity of each PCR run was verified by melting curve and agarose gel analyses as well as by confirming the mutually exclusive amplification of OATP1B3 WT or V1 using the respective primers.

In order to examine the presence of full-length transcripts of OATP1B3 WT or V1 spanning from the first to the last exons, qualitative RT-PCR was performed using Platinum Supermix (Invitrogen) and the following primers; for WT (exons 1-15, expected size of 2214 bp), sense 5′-GGATGGACTTGTTGCAGTTG-3′ and antisense 5′-TTAGTTGGCAGCAGCATTGT-3′; for V1 (exons 2a-15, expected size of 2077 bp), sense 5′-CAATGTATGGCCACGTTACT-3′ and antisense 5′-TTAGTTGGCAGCAGCATTGT-3′. Each PCR cycle consisted of a denaturation step at 94°C for 30 sec, a primer-annealing step at 55°C for 1 min, and an extension step at 72°C for 1 min. The PCR products were visualized on 1% agarose gel.

3.2.4 Antibodies

In addition to the polyclonal OATP1B3 antibody, SKT (epitope: the C-terminal peptide sequence SKTCNLDMQDNAAAN) validated previously,[2] the polyclonal OATP1B3 antibody MFL (epitope: the N-terminal sequence of OATP1B3 V1, MFLAALSFSY) was developed by immunizing a rabbit with the synthesized epitope sequence (Pocono Rabbit Farm & Laboratory, Inc.). The monoclonal Myc antibody (Cell Signaling) was used in

detecting the exogenously expressed OATP1B3 V1 or WT containing Myc tag sequence. β-actin, Na^+/K^+ ATPase and calnexin (Cell Signaling) were used as gel loading controls or a fractionation marker.

3.2.5 Immunohistochemistry

Paraffinized tissue sections of normal liver, colon and pancreatic cancer (US Biomax, Inc) were stained to detect the expression of OATP1B3 protein. Briefly, the tissue sections were subjected to the antigen retrieval using citrate buffer, followed by blocking with 2% BSA. Subsequently, the sections were incubated with polyclonal OATP1B3 antibodies, SKT or MFL, detecting the C-terminal or N-terminal sequence of OATP1B3 V1, respectively. The tissue sections were incubated with avidin-biotin blocking reagents, followed by incubation with biotinylated anti-rabbit IgG and streptavidin-HRP conjugates. The positive signals were visualized using 3,3'-diaminobenzidine (DAB). The nuclei were counterstained using hematoxylin. As a negative control, the same tissue sections were incubated with blocking buffer lacking the primary antibody. The specificity of MFL antibody was also validated using the antibody preincubated with 100 μg of antigenic peptide (ThermoFisher Scientific).

3.2.6 Cloning and Plasmid Construction

OATP1B3 WT-Myc/pCMV6-Entry, the expression plasmid containing the ORF of OATP1B3 WT and Myc tag sequence, was purchased from Origene. OATP1B3 V1-Myc/pCMV6-Entry, the expression plasmid containing the ORF of OATP1B3 V1 and Myc tag sequence was prepared by replacing the OATP1B3 WT sequence with the OATP1B3 V1-specific sequence cut from the cloned sequences of 5´-RACE products. An expression plasmid containing the exon 2a sequence in front of the ORF of OATP1B3 V1 sequence was also prepared for comparison purposes. All plasmids were verified by direct sequencing.

3.2.7 Cell Culture and Plasmid Transfection

Human cancer cell lines derived from colon cancer (CaCO2, HCT8, RKO, SW480, SW620, HCT116 and DLD1) and pancreatic cancer (AsPC-1, BxPC-3, Panc-1, MiaPaCa2, Capan-1 and Capan-2) were obtained from American Type Culture Collection (ATCC) and maintained in culture conditions recommended by ATCC. HCA7 cells were kindly provided by Dr. R. Coffey (Vanderbilt University) and maintained in DMEM supplemented with 10% FBS (HyClone). For further investigation of OATP1B3 V1 or WT expression and function, HCT116, HCT8 and Panc-1 cells were transiently transfected with the expression plasmids for OATP1B3 V1-Myc or WT-Myc as well as the empty vector, using Lipofectamine 2000 (Invitrogen) (for HCT116) or Fugene HD (Promega) (for HCT8 and Panc-1). Protein expression analysis, immunofluorescence microscopy and transport assays were typically carried out 48 hours after transfection.

3.2.8 Immunoblotting Analysis

Cell lysates were prepared in lysis buffer (10 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (Roche). In order to reduce the formation of protein aggregates, cell lysates were mixed with 4x Laemmli buffer and incubated for 30 min at room temperature. Equivalent amounts of protein from whole cell lysates were separated by SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated overnight with the following primary antibodies: OATP1B3 (SKT), Myc tag, β-actin, Na^+/K^+ ATPase, calnexin. The immunoreactive proteins were detected using a secondary antibody conjugated with horseradish peroxidase and an enhanced chemiluminescence substrate (Pierce).

In order to examine the extent of glycosylation of OATP1B3 V1-Myc and WT-Myc proteins, cell lysates were prepared following transient transfection of HCT116 with the corresponding constructs. OATP1B3 V1-Myc and WT-Myc proteins were pulled down

from total lysates prepared as described above using the Myc antibody and Protein G-agarose beads. For preclearing, 500 µg of total lysate was incubated with Protein G-agarose beads (Roche) for 30 min at 4 °C. After centrifugation, the precleared supernatant was collected and incubated with Myc antibody and Protein G-agarose beads overnight at 4 °C. After centrifugation, beads were washed five times with PBS (pH 7.4). Proteins bound to the beads were treated with PNGase F and subsequently incubated with Laemmli buffer for 30 min at room temperature. The resulting samples were analyzed by immunoblotting as described above.

To investigate the involvement of the proteasomal and lysosomal degradation pathways in handling of OATP1B3 V1 or WT, HCT116 cells were incubated with epoxomicin (50 nM, a proteasome inhibitor) or ammonium chloride (20 mM, a lysosomal inhibitor) for 6 hours following transient transfection of OATP1B3 V1 or WT. The cell lysates were subjected to immunoblotting as described above.

3.2.9 Transport Activity Assay Using Radiolabeled Cholecystokinin-8

In order to assess the transport activity of OATP1B3 V1, cellular uptake of CCK-8, a prototype OATP1B3 substrate, was measured following transient transfection. First, HCT116, HCT8 and Panc-1 cells were seeded onto a 12 well plate (2.5×10^5 cells per well) 24 hours before transfection. Subsequently the cells were transfected with the expression plasmids for OATP1B3 V1 or WT as well as the empty vector. Twenty-four hours after transfection, the cells were treated with 5 mM sodium butyrate overnight. On the day of the experiment, cells were washed three times with pre-warmed Opti-MEM (Invitrogen) at 37 °C. The uptake was initiated by adding 0.5 ml Opti-MEM containing ^{3}H-Cholecystokinin-8 (^{3}H-CCK-8, 0.01 µM) with or without rifampin (100 µM, an OATP1B3 inhibitor) to each well. After a 6 min incubation (verified to be in the linear range from a separate time-dependent uptake study) at 37°C, the uptake solution was removed and each well was

washed thrice in ice-cold PBS. The cells were lysed with 0.5 N NaOH on a shaker for 30 min. The lysates were normalized in 2.5 N HCl and 250 µl of lysate was added to 3 ml of LSC cocktail and radioactivity was measured using the TriCarb LSC counter (Perkin Elmer). The ^{3}H-CCK-8 uptake in each well was normalized to the protein amount measured using the BCA protein assay (Pierce). The protein expression of OATP1B3 V1 or WT in each experiment was also verified by immunoblotting analyses.

3.2.10 Cell Fractionation Using Surface Biotinylation

In order to further investigate subcellular localization of OATP1B3 V1, fractionation using surface biotinylation was carried out in HCT116 (expressing endogenous OATP1B3 V1) and HCT8 cells (following transient transfection with OATP1B3 V1-Myc or OATP1B3 WT-Myc). Briefly, cells were washed with ice-cold PBS-$Ca^{2+/}Mg^{2+}$ (138 mM NaCl, 2.7 mM KCl, 1mM $MgCl_2$, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$) and then treated with a membrane-impermeable biotinylating agent (1.5 mg/ml sulfo-NHS-SS-biotin, Pierce) at 4 °C for 1 hr. Subsequently, the cells were washed three times with ice-cold PBS-$Ca^{2+/}Mg^{2+}$ containing 100 mM glycine and incubated for 20 min at 4 °C with the same buffer to remove the remaining labeling agent. After washing, cells were disrupted with a lysis buffer containing protease inhibitors (Roche). Following centrifugation, streptavidin agarose beads (Pierce) were added to cell lysates and incubated for 1 hr at 4 °C. The beads were centrifuged and supernatant was collected as the cytoplasmic fraction. The beads were washed three times with ice-cold lysis buffer, and biotinylated proteins were released by incubation of the beads with Laemmli buffer for 30 min at room temperature. The biotinylated (membrane fractions) and cytoplasmic fractions were subjected to immunoblotting.

3.2.11 Immunofluorescence Microscopy

HCT8 cells were transfected with OATP1B3 WT or V1 (myc tagged and untagged) as described above. Twenty-four hours after transfection, cells were seeded onto 4 chamber

culture slides (BD Biosciences) and stabilized in growth media containing 5 mM sodium butyrate. Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde in ice-cold PBS for 30 min. The cells were then permeabilized with PBS containing 0.3% Triton X-100 for 20 min at room temperature and blocked using PBS containing 2% BSA for 1 hour at room temperature. The cells were incubated in the Myc tag antibody followed by incubation with a secondary antibody conjugated to Alexa fluor 488 or Alexa fluor 546 dye (Invitrogen). Nuclear DNA was stained with DAPI (Vector Laboratories Inc.). The immunofluorescence was detected using a Nikon (Eclipse Ti-U) fluorescence microscope.

3.2.12 Statistical Analysis

The results are expressed as mean ± SD. The statistical significance between groups in the transport studies was determined using one-way ANOVA followed by the Newman-Kuels test. P values of ≤ 0.05 were considered to be statistically significant. The calculations were done using GraphPad Prism 5.04.

3.3 RESULTS

3.3.1 Identification of OATP1B3 V1 as the Predominant Variant Expressed in Colon and Pancreatic Cancer

To examine whether cancer cells express OATP1B3 variants differing from the WT expressed in the normal liver, we performed 5′-RACE and RT-PCR using RNAs isolated from clinical colon cancer tissues and established colon cancer cell lines. Subsequent cloning and sequencing analyses revealed the identity of novel variants of OATP1B3. Among 16 OATP1B3 variants identified so far, OATP1B3 V1 was found to be most prevalent, detected in 10 out of 13 samples initially tested. The OATP1B3 V1 transcript is almost identical to the WT sequence, with exception to an alternatively spliced exonic

sequence, termed as exon 2a, in place of exons 1 and 2 in the WT transcript (Fig. 3.1A). The intron-exon organization of OATP1B3 WT and V1 is summarized in Table 3.1. All intron/exon boundaries are in accordance with the canonical splicing consensus motifs.

To determine the presence of the full-length transcript of OATP1B3 V1 or WT in clinical colon and pancreatic cancer tissues, we performed qualitative RT-PCR with primers amplifying the regions encompassing the entire ORFs for OATP1B3 V1 or WT (Fig. 3.1B). RT-PCR results indicated that majority of the tested clinical colon and pancreatic cancer samples express OATP1B3 V1, but not OATP1B3 WT. On the other hand, neither OATP1B3 V1 nor WT was detected in nonmalignant colon and pancreatic tissue samples, suggesting that OATP1B3 V1 is expressed in a cancer-specific manner. As expected, OATP1B3 WT was detected readily in normal liver control whereas V1 was not. Next, we further quantified the expression levels of OATP1B3 V1 and WT in colon and pancreatic cancer cell lines by quantitative RT-PCR (Fig. 3.1C). In line with the results obtained using clinical tissue samples (Fig. 3.1B), OATP1B3 V1 was detected in the majority of these cell lines at varying levels. In contrast, OATP1B3 WT was not detectable in any of the tested samples except the normal liver. Taken together, our data showed that colon and pancreatic cancer cells express OATP1B3 V1 containing the initial exonic sequence different from OATP1B3 WT.

Table 3.1. Intron-exon organization of OATP1B3 gene

Exonic boundaries of OATP1B3 WT and OATP1B3 V1 with the 5´ and 3´ splice sites are listed.

	OATP1B3 WT		OATP1B3 V1			
Exon	bases	length(bp)	bases	length(bp)	5' Splice site	3' Splice site
1	1-61	61			GTCAAGgtaaga	
2	62-210	149			TTCAAGgtagaa	taacagGTGATC
2a			1-135	135	TAGCAGgtaagg	ttcttcAGTTAC
3	211-352	142	136-277	142	AAATTGgtaact	ttttagATGTTC
4	353-485	133	278-410	133	GGGATAgtaagt	ttctagGAAATT
5	486-607	122	411-532	122	AAAAAGgtaaga	ttacagTTATAG
6	608-754	147	533-679	147	ATTTAGgtaacg	cgatagATTGTG
7	755-853	99	680-778	99	ATCTGAgtaagt	ttacagGTAGTT
8	854-1096	243	779-1021	243	TGACTGgtaggt	tcctagGCACTA
9	1097-1261	165	1022-1186	165	TGTTGGgtaaga	ctatagGTTTTT
10	1262-1457	196	1187-1382	196	TGATGGgtttgt	ttctagGAATCA
11	1458-1623	166	1383-1548	166	CATACAgtgagt	tttcagAAATAA
12	1624-1808	185	1549-1733	185	TGTGAAgtaagt	ttttagGTGTTT
13	1809-1873	65	1734-1798	65	CACTAGgtatga	tttcagGATTGT
14	1874-1991	118	1799-1916	118	TTTTGGgtaagt	ttgcagGAGGAA
15	1992-2712	721	1917-2637	721		tcacagAAGGGT

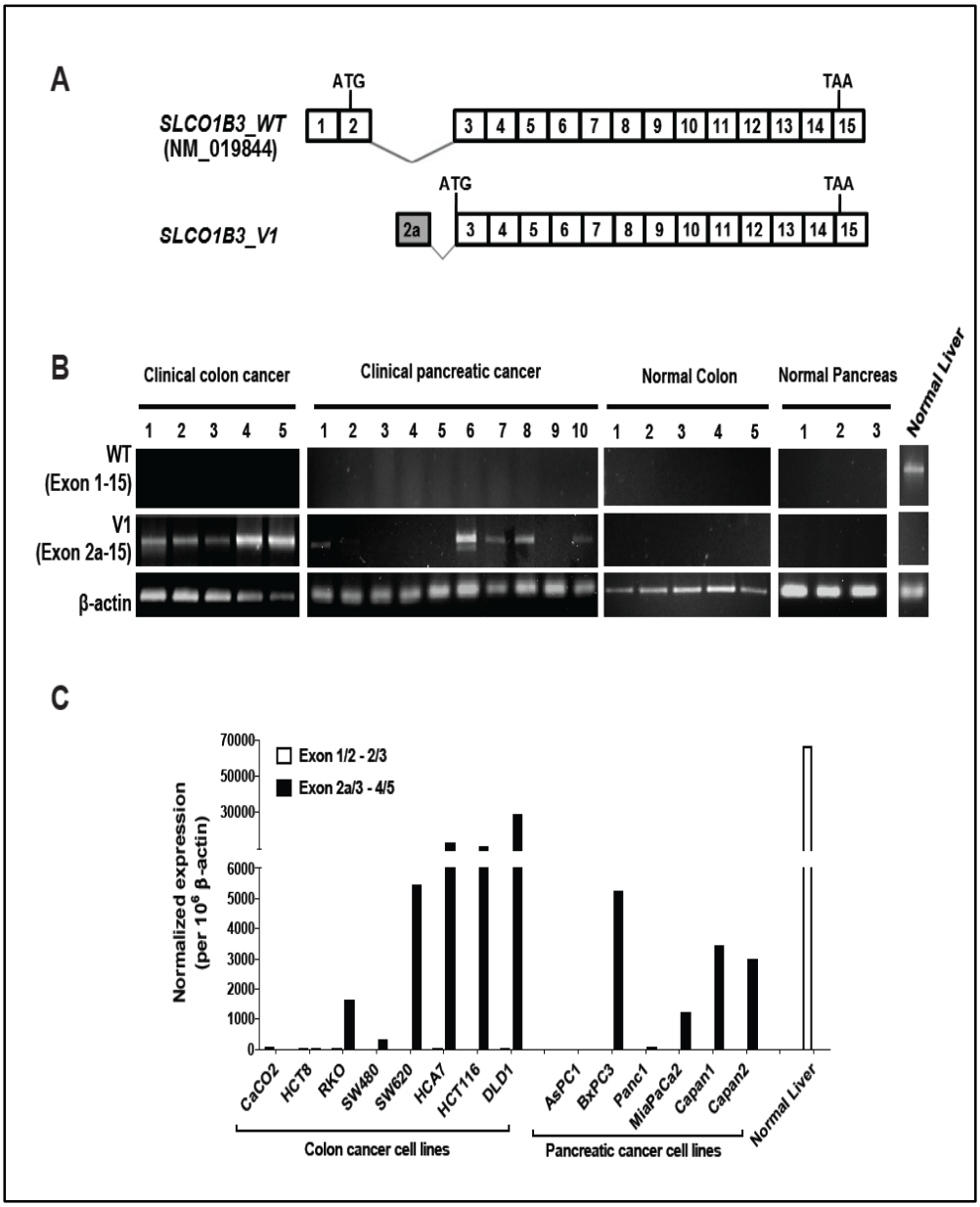

Figure 3. 1 Frequent expression of OATP1B3 V1, a novel splicing variant, in colon and pancreatic cancer

(A) Schematic representation of OATP1B3 V1 and WT transcripts. Exonic regions are shown in boxes and translation start and stop codons are marked. (B) Qualitative RT-PCR analyses show that OATP1B3 V1, but not WT is abundantly expressed in clinical colon and pancreatic cancer tissues. In contrast, normal liver tissue was shown to express OATP1B3 WT, but not V1. The primers used amplify the regions encompassing the first

and last exons of OATP1B3 V1 or WT. β-actin was used as a loading control. (C) Quantitative RT-PCR analyses show that OATP1B3 V1 is expressed at varying levels in the majority of colon and pancreatic cancer cell lines. In contrast, OATP1B3 WT was detected only in normal liver tissue. Data show the number of OATP1B3 V1 or WT transcript per 10^6 copies of β-actin.

3.3.2 Immunohistochemical Detection of OATP1B3 Variants in Colon and Pancreatic Cancer

After detecting the mRNA transcript of OATP1B3 variants in the majority of colon and pancreatic cancer tissues and cell lines, we set out to analyze the protein expression of OATP1B3 variants in clinical tissues. First, we searched for the possible ORF sequences of OATP1B3 variants (V1 ~ V16) using an ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). For OATP1B3 V1, the search results yielded many potential ORFs including the longest translated sequence of 674 amino acids. On the other hand, other variant forms of OATP1B3 (V2 ~ V16) have insertions or deletions (either in-frame or out-of-frame) at various locations, leading to substantial differences in the overall peptide sequences compared to OATP1B3 WT. Thus, based on the sequence similarity to OATP1B3 WT and relative abundance, we chose to focus our investigations on the V1 isoform. The longest translated product for OATP1B3 V1 utilizes the putative translation start site (underlined) at the beginning of exon 3 encoding the peptide sequence MFLAALSFSY at the N-terminus (Fig. 3.2A). The resulting protein is identical to OATP1B3 WT except lacking 28 amino acids at the N-terminus (Fig. 3.2B). Since our initial attempt to verify the protein sequence of OATP1B3 V1 using mass spectrometry was not successful, we developed an antibody detecting the N-terminal sequence (MFL epitope) of OATP1B3 V1 as an alternative way to verify the peptide sequence of OATP1B3 V1 (Fig. 3.2B). Our immunohistochemical analyses using two polyclonal OATP1B3 antibodies, MFL and SKT produced similar positive cytoplasmic staining patterns in colon and pancreatic cancer cells (Figs. 3.2C and D). Consistent with the previous findings, neither MFL nor SKT produced any positive staining in nonmalignant colon and pancreatic tissue sections (Fig. 3.2C and D). As a control, normal liver tissue sections were also stained with MFL or SKT (data not shown). Both SKT and MFL antibodies produced

immunopositive signals around the central veins, which were previously reported to express high levels of OATP1B3. However, differences in staining patterns were noted; SKT showed a membranous staining pattern while MFL showed a mainly cytoplasmic staining pattern. These differences were not entirely surprising given that the MFL epitope sequence is located in the first transmembrane domain of OATP1B3 WT and may not be readily accessible for antibody-mediated interactions. As another way of validating the specificity of MFL, normal liver and colon cancer sections were stained using neutralized MFL antibody and the results showed that peptide blocking led to substantially attenuated signals (data not shown). Overall, these results support that the predicted ORF sequence starting from exon 3 is indeed utilized for translation of the sequence starting with MFLAALSFSY.

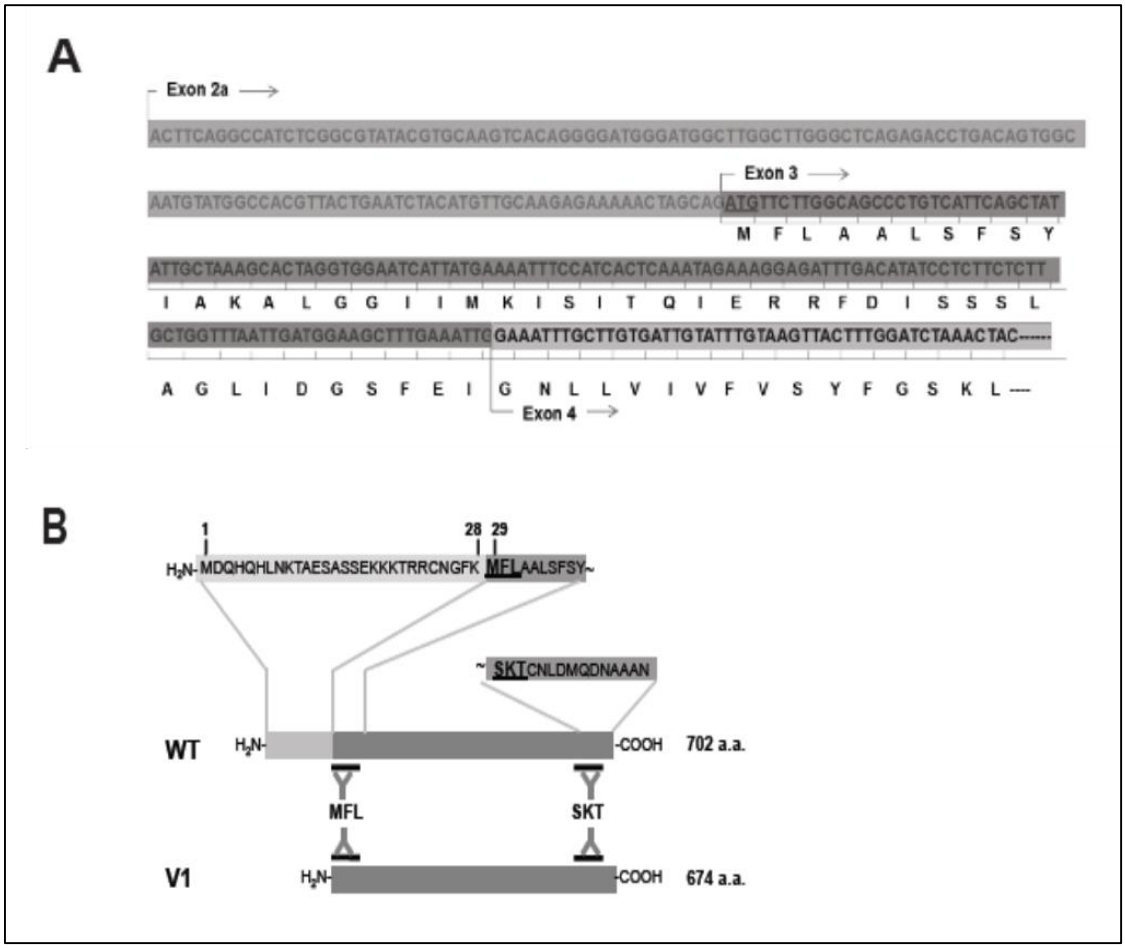

Figure 3. 2 Immunohistochemical staining of colon and pancreatic cancer tissue sections using the MFL or SKT antibodies generated against N- or C-terminal tails of OATP1B3 V1

(A) The sequences of OATP1B3 V1 transcript and translated product. Exons are indicated and the translation start site located at the beginning of exon 3 is underlined. (B) Schematic representation of epitope sequences used to develop the antibodies MFL and SKT. The MFL antibody was generated against MFLAALSFSY located at the N-terminal tail of OATP1B3 V1. The SKT antibody was generated against SKTCNLDMQDNAAAN located at the C-terminal tails of OATP1B3 V1 and WT.

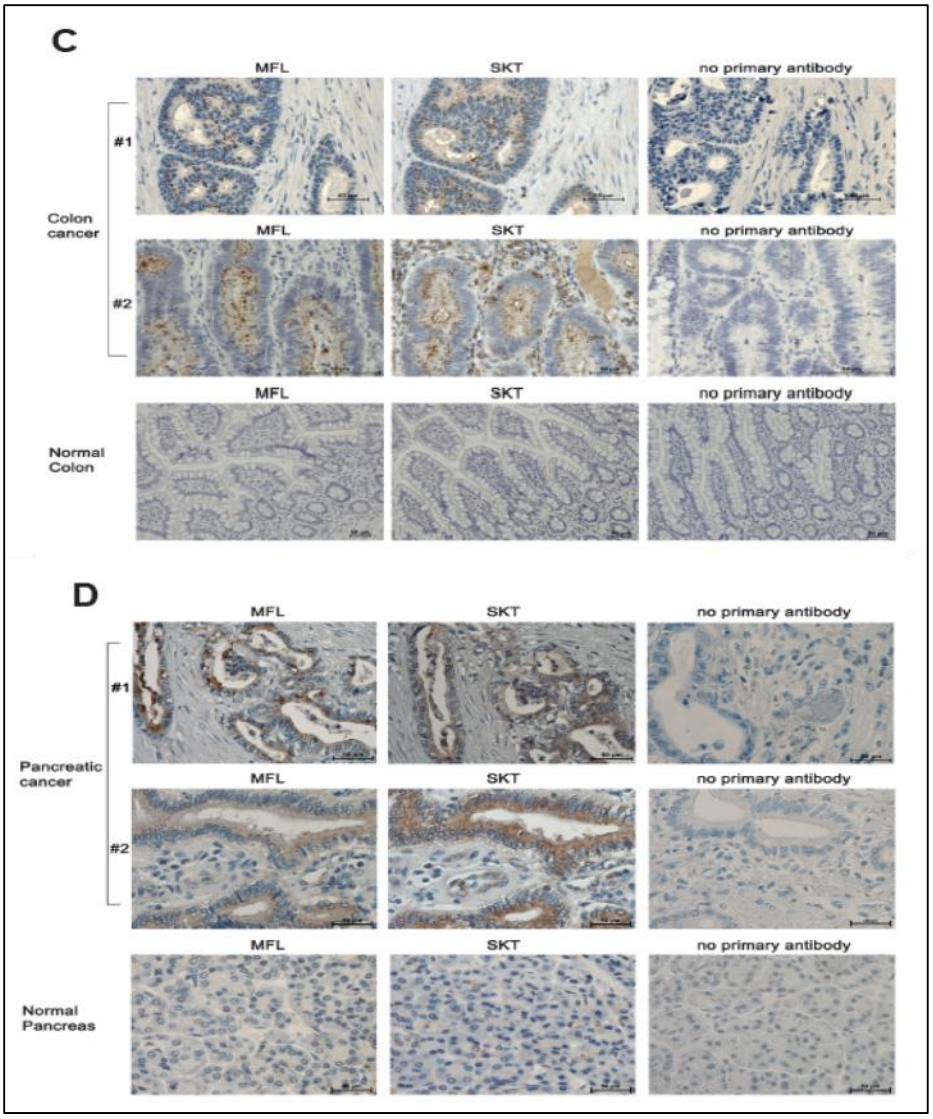

Figure 3. 3 Immunohistochemical staining of colon and pancreatic cancer tissue sections using the MFL or SKT antibodies generated against N- or C-terminal tails of OATP1B3 V1

(C & D) Both MFL and SKT antibodies generated similar positive staining patterns in tissue sections from colon cancer (C) and pancreatic cancer (D). Neither MFL nor SKT antibodies produced positive staining in non-malignant colon and pancreatic tissue sections. Cancer tissue sections following the exact procedure except omitting the primary antibody were included as negative controls.

3.3.3 Detection of OATP1B3 V1 in Colon and Pancreatic Cancer Cell Lines

In further investigating the expression and function of OATP1B3 V1, we utilized HCT116, HCT8 and Panc-1 cells expressing varying levels of OATP1B3 V1 (Fig. 3.1C). Amplification of OATP1B3 V1 using qualitative RT-PCR confirmed expression of the full-length V1 transcript in HCT116 cells. Additionally, it was noted that HCT116 expressed higher levels of V1 compared to HCT8 or Panc-1 cells (Fig. 3.3A). Immunoblotting analyses with the SKT antibody detected OATP1B3 V1 protein at approximately 75 kD and also demonstrated higher protein expression in HCT116 cells compared to HCT8 and Panc-1 cells (Fig. 3.3B). We have attempted to use the MFL antibody for immunoblotting, but our results indicated that the MFL antibody is not suitable due to high background and non-specific signals.

Using these cell line models, OATP1B3 V1 or WT was transiently expressed by transfecting the constructs containing the respective ORF and Myc-tag sequences. Initially, we also included a construct containing the exon 2a sequence prior to the OATP1B3 V1 ORF and Myc sequence. The resulting protein showed the same electromobility as that produced from the construct containing the ORF sequence starting from exon 3, further supporting the usage of the translation start site at the beginning of exon 3 (data not shown). Interestingly, our immunoblotting results indicated that the expression level of OATP1B3 V1 is much lower than that of WT in both HCT116 and Panc-1 cells despite the same amounts of OATP1B3 V1 or WT constructs used (Fig. 3.3C). Similar results were obtained in all three cell lines tested (HCT116, HCT8 and Panc-1 cells), even after further increasing the transfected amount of the constructs (data not shown). In addition, substantial size differences were noted between OATP1B3 V1 and WT. OATP1B3 V1-Myc was detected at molecular weight above 75 kD, whereas

OATP1B3 WT-Myc was detected as multiple bands with the most prominent band at approximately 120 kD (Fig. 3.3C).

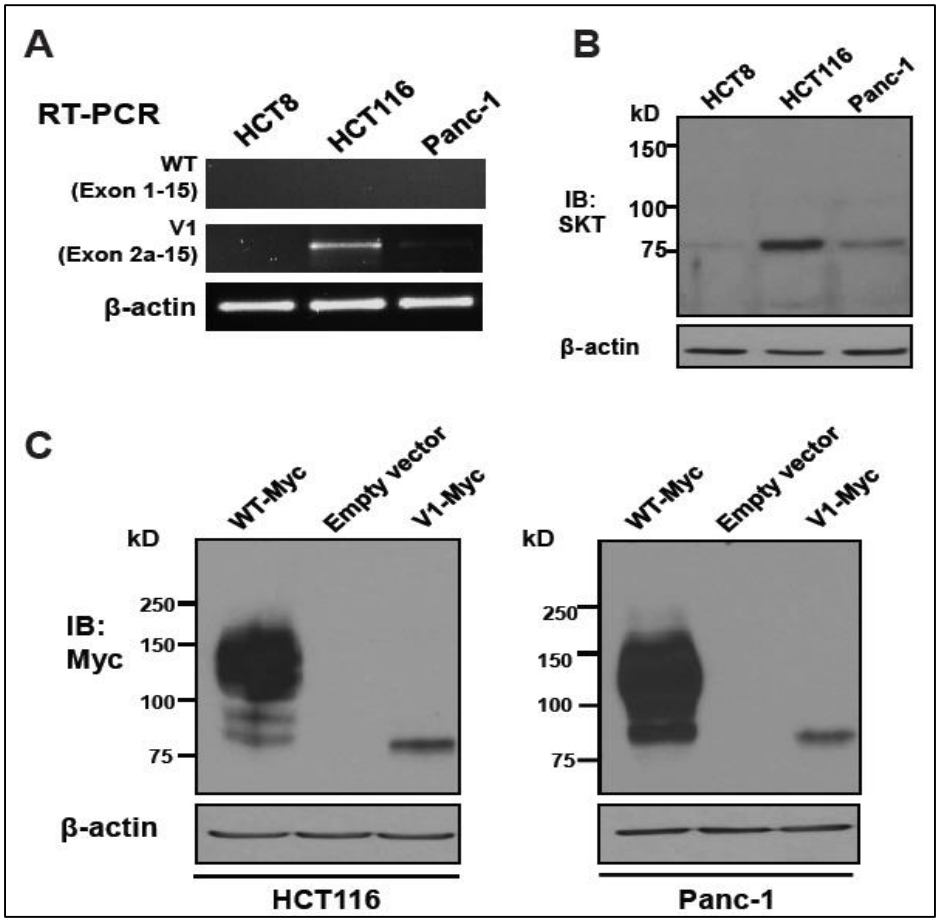

Figure 3. 4 Detection of OATP1B3 V1 in colon and pancreatic cancer cell lines

(A) Qualitative RT-PCR results show the expression of OATP1B3 V1 at varying levels, but no detectable WT in the cancer cell lines tested. (B) Immunoblotting analyses using SKT detect the expression of OATP1B3 V1 protein at varying levels in the cancer cell lines tested. (C) HCT116 and Panc-1 cells were transiently transfected with the constructs containing the ORF sequences of OATP1B3 V1 or WT fused with Myc tag as well as the empty vector. Immunoblotting analyses detected the expression of OATP1B3 V1 or WT proteins with differing electromobility and expression levels. β-actin was used as a loading control.

Post-translational modifications such as glycosylation are commonly observed for many membrane transporters including OATP1B3. Thus, we examined whether the observed differences in the electromobility of OATP1B3 V1 and WT are due to varying extents of glycosylation. Following PNGase F treatment, a substantial shift in molecular weight was seen for OATP1B3WT, which was detected at the expected size of approximately 75 kD (calculated from 702 amino acids with Myc tag sequence). A similar shift in molecular weight was also seen for OATP1B3 V1-Myc following the PNGase F treatment, but to a much lesser extent than OATP1B3 WT (Fig. 3.4A). Taken together, these results suggest that OATP1B3 V1 is subjected to a much lesser extent of glycosylation, compared to the WT protein.

Since the exogenous expression of OATP1B3 V1 was much lower than OATP1B3 WT (Fig. 3.3C), we investigated whether this low expression level of V1 was a result of decreased protein stability. Following transient transfection, HCT116 cells expressing OATP1B3 WT or V1 were treated with epoxomicin (a proteasome inhibitor) or ammonium chloride (a lysosomal inhibitor) (Fig. 3.4B). Epoxomicin treatment led to a substantial increase in the protein levels of OATP1B3 V1 (especially at higher molecular sizes), but not OATP1B3 WT. Ammonium chloride treatment had no impact on the protein levels of OATP1B3 V1 or WT. These results suggest that OATP1B3 V1 may be more susceptible to proteasomal degradation than OATP1B3 WT.

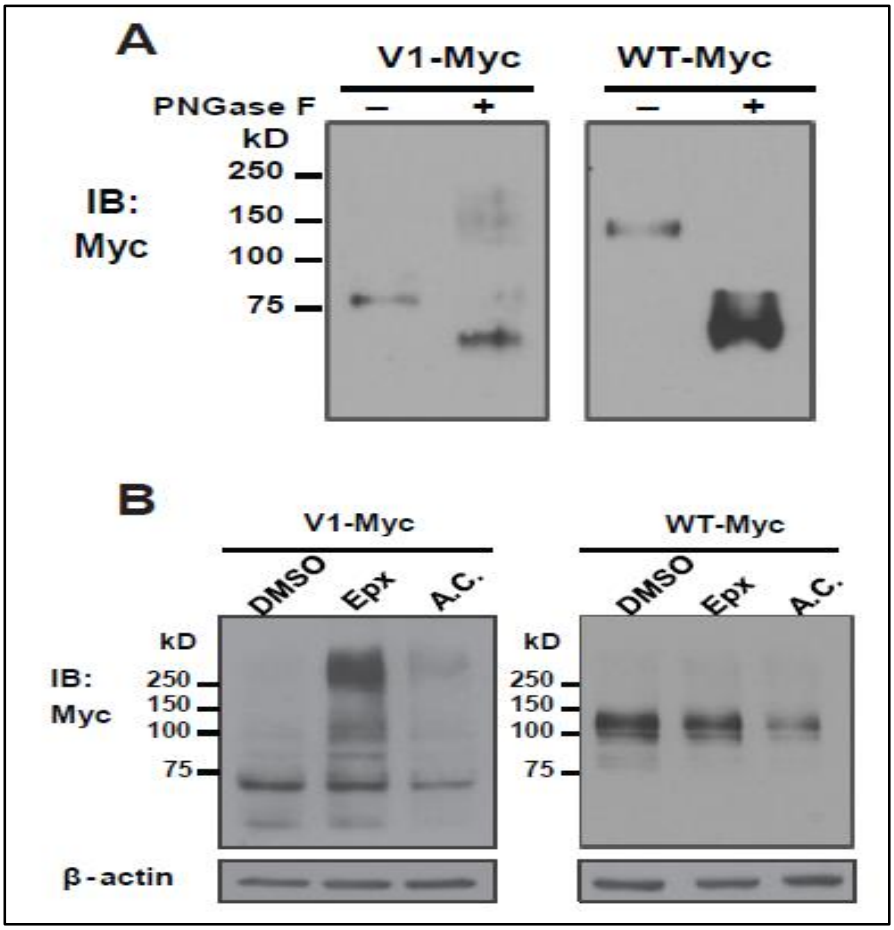

Figure 3. 5 Glycosylation and proteasomal degradation of OATP1B3 V1

(A) Immunoblotting analyses were performed using HCT116 lysates transiently transfected with the constructs, OATP1B3 V1-Myc or WT-Myc. The PNGase F treatment led to substantial shifts in the electromobility of OATP1B3 V1 and WT, but to a differing extent. (B) Immunoblotting analyses were performed using lysates prepared from HCT116 cells transiently transfected with the constructs, OATP1B3 V1-Myc or WT-Myc and subsequently treated with epoxomicin (Epx, a proteasomal inhibitor), ammonium chloride (A.C., a lysosomal inhibitors) or vehicle alone (DMSO). The epoxomicin treatment led to a marked increase of the immunoreactive signals of higher molecular sizes, consistent with accumulation of polyubiquitinated proteins. Neither epoxomicin nor ammonium chloride treatment influenced the expression of OATP1B3 WT.

3.3.4 Transporter Activity of OATP1B3 V1 in Colon and Pancreatic Cancer

To investigate whether OATP1B3 V1 functions as an uptake transporter in colon and pancreatic cancer, we performed cellular uptake studies using [^{3}H]-CCK-8 (a prototype OATP1B3 substrate) in three cell line models; HCT116 (high endogenous OATP1B3 V1 levels), HCT8 and Panc-1 (low endogenous OATP1B3 V1 levels). Transient expression of OATP1B3 V1 in these cell lines led to modest increases in the uptake of [^{3}H]-CCK-8; 1.4, 2.1 and 2.9-fold increase in HCT116, HCT8 and Panc-1, respectively (Fig. 3.5, upper panel). Rifampin, a known OATP1B3 inhibitor, effectively inhibited the uptake of [^{3}H]-CCK-8 in cells transfected with OATP1B3 V1, supporting that the increased [^{3}H]-CCK-8 uptake was likely mediated by OATP1B3-related transport activity. Interestingly, immunoblotting analysis of OATP1B3 V1 in HCT116, HCT8 or Panc-1 cells showed slightly different electromobility patterns, possibly related to a varying extent of post-translational modifications across different cell types (Fig. 3.5, lower panel). In order to compare the transport activity of OATP1B3 V1 to that of WT, we also examined the uptake of [^{3}H]-CCK-8 following transient transfection of OATP1B3 WT in the same cell line models. Considering our observations that the cellular expression levels of OATP1B3 WT are much higher than those of OATP1B3 V1 following the transfection of the same amount of constructs (Fig. 3.3C), we reduced the transfected amount of OATP1B3 WT construct up to 10-fold and verified that the intensity of immunoreactive signals detected using Myc antibody are comparable between OATP1B3 V1 and WT (data not shown). Our results indicated that transient expression of OATP1B3 WT markedly increases CCK-8 uptake in HCT116, HCT8 or Panc-1 cells (41.7, 26.8 and 29.5-fold increase, respectively). In separate experiments, we also examined whether endogenously expressed OATP1B3 V1 can mediate the uptake of [^{3}H]-CCK-8 using HCT116 cells (high endogenous OATP1B3 V1 levels) with rifampin. The results indicated that there is only a very minor decrease in

the CCK-8 uptake by rifampin; 2.0 ± 0.8 vs 1.6 ± 0.1 nmol/mg total protein. Taken together, our results suggest that OATP1B3 V1 has only a modest uptake transporter function in colon and pancreatic cancer compared to the OATP1B3 WT.

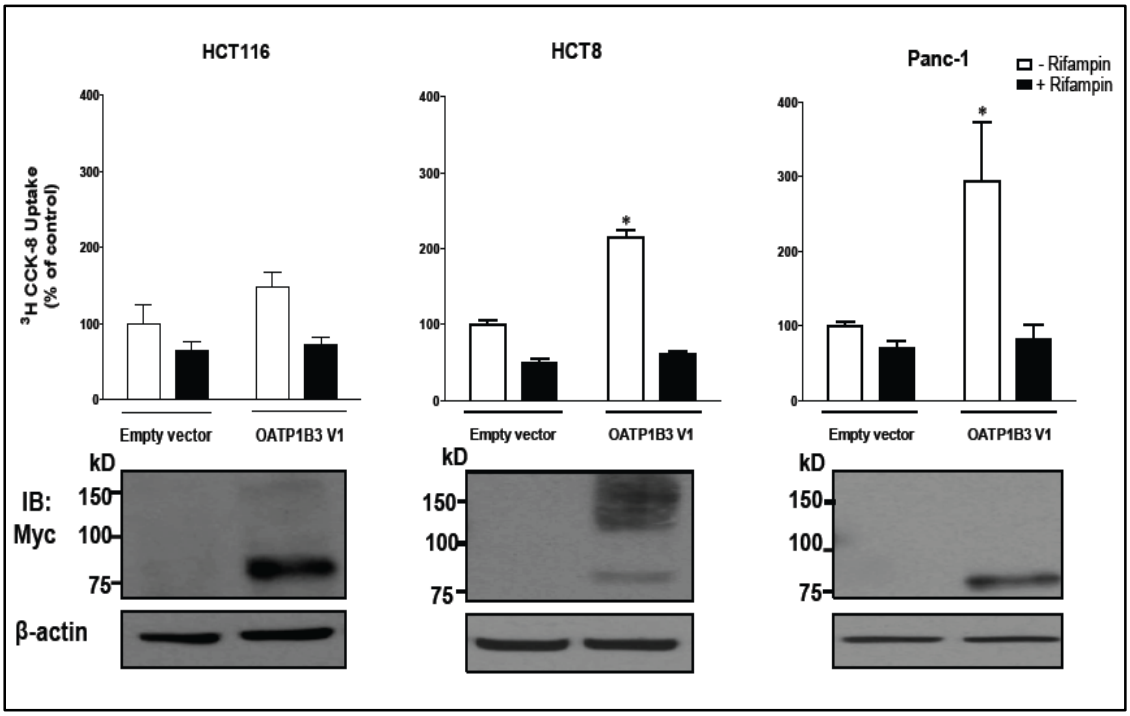

Figure 3. 6 OATP1B3 V1 shows only a modest increase in cellular uptake of CCK-8 in colon and pancreatic cancer cells

HCT116, HCT8 and Panc-1 cells were transiently transfected with OATP1B3 V1-Myc or empty vector. When cellular uptake of 3H CCK-8 was compared, the expression of OATP1B3 V1 led to only a modest (~2-3 fold) increase compared to the empty vector control. In the bottom panel shown are the immunoblotting results confirming the OATP1B3 V1 expression for each experiment. Data are represented as percentiles relative to the empty vector controls. Data are expressed as mean ± SD.

3.3.5 Subcellular Localization of OATP1B3 V1

To investigate whether the relative inefficiency of OATP1B3 V1 as a transporter was related to a defective membrane trafficking, we examined subcellular localization of OATP1B3 V1 by detecting OATP1B3 V1 in the cytoplasmic and membrane fractions prepared from HCT116 (to monitor endogenously expressed OATP1B3 V1) or HCT8 cells following transient transfection of OATP1B3 V1 (to monitor exogenously expressed OATP1B3 V1) using cell surface biotinylation. Our results indicated that OATP1B3 V1 (either endogenous or exogenous) is located abundantly in the cytoplasmic fraction although a small amount of OATP1B3 V1 was detected in the surface membrane fraction (Figs. 3.6A and B). These results are in contrast to OATP1B3 WT, which showed a much stronger signal in the surface membrane fraction than in the cytoplasmic fraction (Fig. 6B). Of note, OATP1B3 protein in the surface fraction displayed higher apparent molecular sizes than that in the cytoplasmic fraction (Figs. 3.6A and B). These results are consistent with a greater extent of post-translation modifications with matured proteins presented to the surface membrane. Fluorescence microscopy images further verified that OATP1B3 V1 (either Myc-tagged or untagged) is predominantly localized in the cytoplasm of HCT8 cells, while OATP1B3 WT was mainly localized on the plasma membrane (Fig. 3.6C).

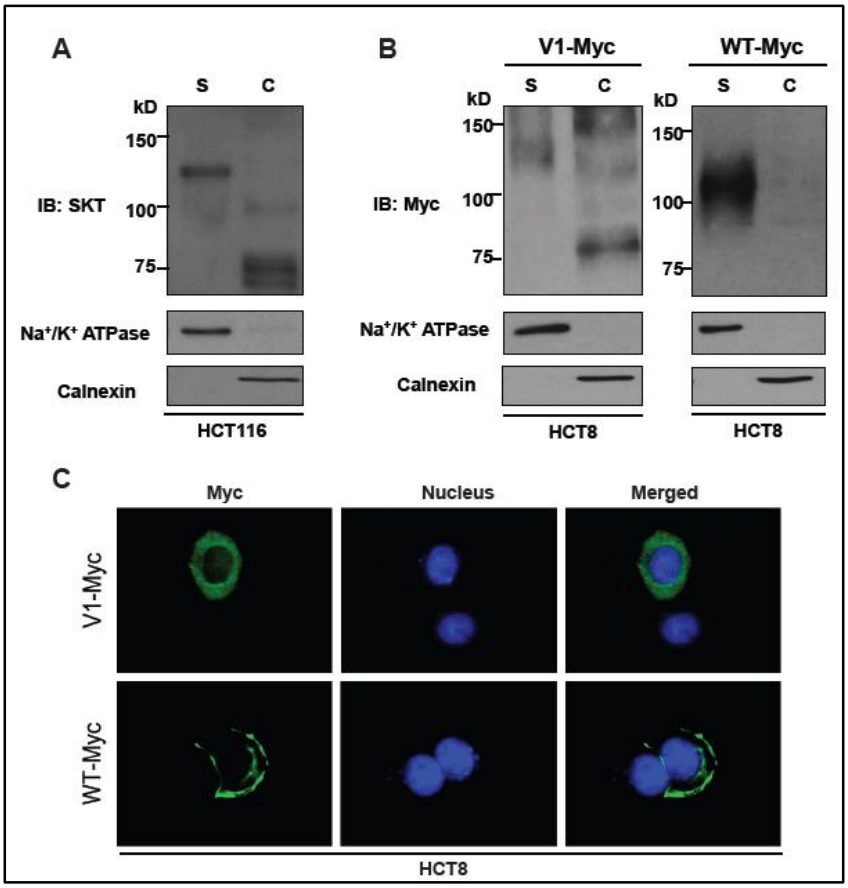

Figure 3. 7 Subcellular localization of OATP1B3 V1 in colon and pancreatic cancer cells

(A) Surface (S) and cytoplasmic (C) fractions were prepared using HCT116 cells expressing endogenous OATP1B3 V1. Immunoblotting analyses using SKT show that OATP1B3 V1 is present in both surface and cytoplasmic fractions. The immunoreactive band detected in the surface fraction showed a higher apparent molecular size than those in the cytoplasmic fraction, consistent with a greater extent of post-translational modifications. (B) HCT8 cells were transfected with the constructs OATP1B3 V1-Myc or WT-Myc. Similar to the results obtained with HCT116 cells, OATP1B3 V1-Myc was detected in both surface and cytoplasmic fractions with differing electromobility. In contrast, OATP1B3 WT-Myc showed much stronger signals in the surface fraction than in the cytoplasmic fraction, suggesting that OATP1B3 WT is mainly localized in the surface

membrane. (C) Immunofluorescent images were obtained in HCT8 cells transiently transfected with OATP1B3 V1-Myc or WT-Myc. Cells transfected with OATP1B3 V1-Myc showed the immunoreactive signals mainly localized in the cytoplasm. In contrast, cells transfected with OATP1B3 WT-Myc showed the immunoreactive signals mainly localized on the surface membrane.

3.4 DISCUSSION

Despite several reports on the ectopic expression of OATP1B3 across multiple types of cancer, it is unclear whether OATP1B3 expressed in cancer has biological functions similar to that in normal liver. In our current study, we demonstrate that colon and pancreatic cancer cells express variant forms of OATP1B3, different from OATP1B3 WT expressed in the normal liver. Using OATP1B3 V1, the most prevalent variant lacking 28 amino acids from its N-terminal tail, we show that OATP1B3 V1 has only limited transport activity and differs from OATP1B3 WT with regard to the extent of post-translational modifications and subcellular localization. While the mRNA sequence of OATP1B3 V1 is identical to the one recently reported by Nagai et al. [74], our results provide the first evidence that the molecular and functional properties of OATP1B3 V1 in cancer differ from those of OATP1B3 WT. These observed differences need to be taken into account when further investigating the biological and clinical significance of OATP1B3 variants in cancer.

Using the sequence information of the OATP1B3 V1 transcript, the longest translated product was predicted to be 674 amino acids. This product utilizes the translation start site located at the beginning of exon 3 and encodes MFLAALSFSY peptide at the N-terminus. The previous report by Nagai et al. proposed a translated product of 655 amino acids starting with MKISITQIE at its N-terminus [74]. This product proposed by Nagai et al. utilizes the translation start site located further downstream, but in frame with our proposed translation site (Fig. 3.2A). As a way of verifying the protein sequence of OATP1B3 V1, we initially attempted to utilize mass spectrometry. However, the results did not provide adequate protein sequence data, possibly related to the general difficulties associated with proteomic analyses of membrane-associated proteins [111]. As an alternative approach, we developed MFL, an OATP1B3 antibody targeting the N-terminal sequence of OATP1B3 V1. The immunohistochemical analyses showed that the staining patterns

using MFL were very similar to those obtained using SKT targeting the C-terminal sequence (Figs. 3.2C and D). These results provide the first evidence for the presence of the translated product containing the MFLAALSFSY peptide at the N-terminus in clinical colon and pancreatic cancer tissues. However, we cannot rule out the possibility that other translated products may exist including the translated products proposed by Nagai et al. [74] and those from additional OATP1B3 variants identified (data not shown). Of note, it should be mentioned that MFL may cross-react with OATP1B1, given the close similarity of the peptide sequences shared between OATP1B3 and OATP1B1 in the N-terminal region (80% sequence homology). However, the observed positive signals in our tissue sections are not likely due to OATP1B1 since several reports have indicated that OATP1B1 is not expressed in colon and pancreatic cancer [3] [4].

With regard to the transport activity, OATP1B3 V1 showed only limited activity for CCK-8 in colon and pancreatic cancer cells, in contrast to OATP1B3 WT which showed a much higher activity (Fig. 3.5). These findings are consistent with OATP1B3 V1 having less surface membrane localization compared to OATP1B3 WT (Fig. 3.6). So far little is known about the mechanisms by which OATP1B3 is localized to the plasma membrane. However, our results suggest that the missing 28 amino acids at the N-terminal tail of OATP1B3 may play an important role in membrane trafficking and transporter activity. Interestingly, a recent study reported that the variant form of human concentrative nucleotide transporter 3 (hCNT3, *SLC28A3*) missing the N-terminal tail is mainly located in the endoplasmic reticulum [112]. A follow-up investigation by the same group provided further insights into the motifs important for surface trafficking of hCNT3 [113]. In nonpolarized cells, acidic and hydrophobic motifs in the N-terminal tail of hCNT3 were important, whereas a putative β-turn domain was important in polarized cells. We were not able to locate similar motifs in the N-terminal tail sequence missing in OATP1B3 V1, but the investigations are on-

going to further narrow down sequence segments or motifs important for plasma membrane trafficking of OATP1B3 WT. Interestingly, our current results showed that the expression of OATP1B3 V1 leads to statistically significant increases in the CCK-8 uptake in HCT8 and Panc-1 cells (polarizable cell lines), but not in HCT116 cells (non-polarizable cell line) (Fig. 3.5). Thus, it remains to be determined if OATP1B3 variants are preferentially localized in certain intracellular organelles and whether localization patterns or transport activity of OATP variants would depend on the polarizing ability or status. Although we observed major differences in the membrane trafficking between OATP1B3 V1 and WT, it should be noted that alternative mechanisms (e.g. differences in post-translational modifications, mRNA/protein stability) may contribute to the limited transport activity of OATP1B3 V1.

In our current study, we observed that OATP1B3 V1 localized mainly in the cytoplasm has only limited transporter function in colon and pancreatic cancer. Although alternative splicing is increasingly recognized as a common event in eukaryotic cells, the abundance of OATP1B3 variants across multiple types of cancer argues for this protein being selectively generated for a purpose. Previously, we suggested that OATP1B3 may confer a survival advantage in colorectal cancer cells via p53-dependent pathways [6]. However, at that time, the presence of OATP1B3 V1 was not known and the studies were performed using the OATP1B3 WT sequence. We are currently investigating whether OATP1B3 V1 may have other biological functions, especially its potential as an antiapoptotic or prosurvival protein. In the literature, several reports indicate that membrane transporters may have functions independent of their transport activity. For example, P-glycoprotein (Pgp) encoded by the *MDR1* gene was shown to be localized intracellularly in several human leukemia, breast cancer and hepatoma cell lines [114, 115], [116, 117] and to serve as an anti-apoptotic protein in several types of cancers [118-123]. In particular, Pgp was

able to suppress apoptosis even in the absence of ATP-dependent drug efflux, suggesting that the antiapoptotic function of Pgp may be independent of its transporter activity [123].

Alternative splicing is increasingly recognized as a mechanism that generates protein and functional diversity and there are a number of reports on the splicing variants of membrane transporters. In particular, the expression of alternative mRNA transcripts of murine oatp1b2, an ortholog for both human OATP1B3 and OATP1B1, has been reported [56]. These alternative transcripts of oatp1b2 show quite different splicing patterns with human OATP1B3 variants, in that they share the same transcription start site as oatp1b2 WT, but lack the exonic sequences located in the middle region. The presence of alternative transcripts has also been reported with other human transporters such as OAT2 [124] and OATP3A1 [125]. However, the presence of cancer-specific variants appears to be unique for OATP1B3. In particular, no cancer-specific variants have been reported with OATP1B1 which shares more than 70% sequence homology and numerous substrates with OATP1B3. An effort to examine the mechanisms by which the cancer-specific expression of OATP1B3 variants is achieved may be warranted.

In conclusion, we report that colon and pancreatic cancer cells express variant forms of OATP1B3 mRNA and protein. OATP1B3 V1, the most prevalent form, appears to lack a full transport activity compared to OATP1B3 WT. Our results indicate that OATP1B3 V1 is located mainly in the cytoplasm and the extent of plasma membrane trafficking was much lower in OATP1B3 V1 than in WT. Although the biological significance of OATP1B3 variants in cancer is yet to be defined, our findings should be taken into consideration when further exploring the potential role of OATP1B3 expressed in cancer.

Chapter 4

Role of Hypoxia Inducible Factor-1α in the Regulation of the Cancer-Specific Variant of Organic Anion Transporting Polypeptide 1B3 (OATP1B3) in Colon and Pancreatic Cancer

(The work in this chapter has been published in Biochemical Pharmacoloy (2013) [77])

4.1 Introduction

Organic Anion Transporting Polypeptide 1B3 (OATP1B3) belongs to the superfamily of OATP transporters and mediates the hepatic uptake of various endogenous and xenobiotic compounds [2]. Although OATP1B3 was initially considered to be a liver-specific transporter, subsequent investigations revealed that OATP1B3 is also expressed in human cancers derived from multiple organs including colon, pancreas, prostate, breast and lung [3, 5-9, 67, 110, 126, 127]. Considering that OATP1B3 is not detected in any other non-malignant tissues except the liver, a question was raised whether or not OATP1B3 expression in cancer has any pathogenic and functional significance. Recently, our group and others reported the distinct identity of OATP1B3 expressed in cancer cells, namely cancer-specific OATP1B3 variants (csOATP1B3) that utilize an alternative transcription initiation site and lack the first two exons of the wild-type (WT) OATP1B3 expressed in the normal liver [73, 128]. Our previous investigations focused on csOATP1B3 V1, the most prevalent cancer-specific variant form and reported that csOATP1B3 V1 lacking the N-terminal 28 amino acids displays only a modest transport activity and a defective membrane trafficking compared to OATP1B3 WT [73]. While further investigations are under way to better understand the functional significance of csOATP1B3, the molecular mechanisms underlying the expression of csOATP1B3 may provide important clues about the role of csOATP1B3 in cancer.

Hypoxia arises from an imbalance between the supply and consumption of oxygen and it is recognized as a characteristic feature of locally advanced solid cancers [129-131]. In multiple types of cancers, hypoxia has been associated with adverse clinical outcomes including cancer cell invasion and metastasis [129-133]. Adaptation to the hypoxic environment involves changes in the expression of numerous genes encoding erythropoietic, vasoactive, and proangiogenic molecules and metabolic enzymes [131]. A crucial component in the induction of hypoxia-regulated genes is the hypoxia inducible factor-1 (HIF-1) complex, composed of HIF-1α and HIF-1β subunits. While the HIF-1β subunit (also known as aryl hydrocarbon nuclear translocator, ARNT) is constitutively expressed, the HIF-1α subunit is accumulated only under hypoxic exposure by escaping proteasome-mediated degradation [134, 135]. Under the hypoxic conditions, the HIF-1α/β heterodimer is translocated to the nucleus and binds to a specific *cis*-acting regulatory sequence referred to as the hypoxia response element (HRE) in target genes, thereby causing transcriptional activation.

Interestingly, a previous report by Winter et al. [10] suggested a possible link between hypoxia and the expression of OATP1B3 in cancer cells. In the process of defining an *in vivo* hypoxia gene signature (hypoxia metagene) in head and neck cancer, the authors found *SLCO1B3* encoding OATP1B3 among the highly upregulated genes under hypoxia [10]. This hypoxia metagene including *SLCO1B3* was associated with adverse outcomes in patients with head and neck cancer as well as breast cancer [10]. Since the presence of csOATP1B3 was not known at the time of publication of this report, it remained unknown whether hypoxia induces the expression of csOATP1B3, OATP1B3 WT or both.

Using colon and pancreatic cancer cells, our current study establishes that hypoxia induces the expression of csOATP1B3, but not OATP1B3 WT. In addition, we report that the csOATP1B3 promoter contains a functional HRE, which the HIF-1α protein binds to.

Selective knockdown of HIF-1α also decreased the basal expression level of csOATP1B3 and attenuated the extent of csOATP1B3 induction by hypoxia. Taken together, the current findings described in this chapter demonstrate that the transcription of csOATP1B3 is actively engaged during hypoxia, via a HIF-1α-dependent mechanism.

4.2 Materials and Methods

4.2.1 Cell culture and hypoxic treatments

Human cancer cell lines derived from colon (Caco-2, SW480, HCT-8, DLD-1, and HCT116) and pancreas (AsPC-1, MiaPaCa-2, and BxPC-3) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in the recommended conditions. For hypoxic treatment, cells at approximately 50% confluence were placed in a hypoxic chamber maintaining 1% O_2, 5% CO_2 and 94% N_2 or exposed to $CoCl_2$, a chemical inducer of HIF-1α for the time periods indicated. The medium was replaced every 24 h during hypoxic exposure.

4.2.2 RT-PCR

Single-stranded cDNA was synthesized from 1 µg of total RNA using the SuperScript™ III cDNA synthesis kit (Invitrogen, Carlsbad, CA). To examine the presence of full-length transcripts (spanning from the first to the last exons) of OATP1B3 WT or csOATP1B3 V1 in the resulting cDNA samples, RT-PCR was performed using Platinum® Supermix (Invitrogen, Carlsbad, CA) and the previously reported primers; OATP1B3 WT (exons 1-15, expected size: 2214 bp) or csOATP1B3 V1 (exons 2a-15, expected size: 2077 bp) [73]. In order to examine the effect of hypoxia on other OATP members closely related to OATP1B3, RT-PCR analyses of OATP1A2 and OATP1B1 transcripts were performed using the following primers; for OATP1A2, sense (5'-ATGGGAGAAACTGAGAAAAG-3') and anti-sense (5'- GCATGTTCTCTAATTCTGAA-3'); for OATP1B1, sense (5'-

TGAACACCGTTGGAATTGC-3') and anti-sense (5'-TCTCTATGAGATGTCACTGGAT-3'). PCR products were visualized on 1% agarose gel. Band intensities were densitometrically quantified using the Quantity One software (Bio-Rad, Hercules, CA) and normalized using the band intensities of the housekeeping control β-actin.

4.2.3 Immunoblotting analysis

Cell lysates were prepared in lysis buffer (10 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (Roche, Mannheim, Germany). To minimize the formation of protein aggregates, cell lysates were incubated at room temperature for 30 min after mixing with 4x Laemmli buffer. Equivalent amounts of total protein were resolved by SDS-PAGE and subsequently transferred to PVDF membranes. After blocking with 5% skim milk prepared in Tris-buffered saline containing 0.5% Tween 20, the membranes were incubated overnight with the following primary antibodies: OATP1B3 (SKT; generated against the C-terminal epitope and validated previously [73, 136]), HIF-1α (BD Biosciences, San Jose, CA), β-actin (Cell Signaling, Danvers, MA). The immunoreactive proteins were detected using a secondary antibody conjugated with horseradish peroxidase purchased from Cell Signaling (Danvers, MA) and an enhanced chemiluminescence substrate purchased from Pierce (Rockford, IL). Band intensities were densitometrically quantified using the Quantity One software (Bio-Rad, Hercules, CA) and normalized using the band intensities of the gel loading control β-actin.

4.2.4 Construction of plasmid vectors

The promoter region upstream of the first exon of OATP1B3 WT (−2023 to +100) or csOATP1B3 (−1853 to +151) was amplified by PCR using a BAC clone (RP11-269H12, Empire Genomics, Buffalo, NY) as a template. The amplified DNA was subcloned into pGL4.1 (Promega, Fitchburg, WI), and termed as OATP1B3 WT (-2023) and csOATP1B3

(-1853), respectively. The 5′-deletion constructs of the csOATP1B3 promoter were prepared by subcloning the PCR products using the specific primers into pGL4.1. The resulting constructs were termed as csOATP1B3 (-1053), csOATP1B3 (-553), csOATP1B3 (-263), respectively. The csOATP1B3 (-263)-mut-HRE construct was created by mutating the putative HRE located at +23 - +27 (from 5′-ACGTG-3′ to 5′-AAAAG-3′) using a site-directed mutagenesis kit obtained from Stratagene (Wilmington, DE). The sequences of each of the prepared plasmids was verified by direct sequencing.

4.2.5 Reporter assay

HCT-8 cells were plated onto 24 well plates and transfected with the reporter plasmid (360 ng/well) along with pRL-TK (40 ng/well, an internal control obtained from Promega, Fitchburg, WI). Twenty-four hours after transfection, the cells were exposed to either $CoCl_2$ (100 or 200 µM) or hypoxia (1% O_2) for another 24 h. After extensive washing with phosphate-buffered saline, cells were harvested and firefly and *Renilla* luciferase activities were determined using the Dual Luciferase Reporter Assay kit (Promega, Fitchburg, WI) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). The data were plotted using GraphPad Prism 5.04 (La Jolla, CA).

4.2.6 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from HCT-8 cells following the treatment with $CoCl_2$ (200 µM, 24 h) as reported previously [137]. Briefly, binding reactions were prepared in tubes containing 6 µg of nuclear extracts, 1.25 µg of Poly(dI-dC), 7.5 µl binding buffer (containing 20 mM HEPES, pH 7.9, 1 mM DTT, 0.1 mM EDTA, 50 mM KCl, 5 % glycerol, and 200 µg/µl bovine serum albumin) and water adjusted to make the final reaction volume of 20 µl. The binding reactions were carried out for 10 min on ice. Subsequently, the biotinylated, annealed probe containing the HRE of the csOATP1B3 promoter (5'-Biotin-triethyleneglycol-CATCTCGGCGTATACGTGCAAGTCACAG-3', synthesized by MWG

Biotech, Huntsville, AL) was added to the binding reaction mixtures and further incubated for 20 min at room temperature. For competition experiments, a 200-fold excess of the unlabeled, annealed probe was added to the reaction mixture. DNA-protein complexes were resolved by 4.5% polyacrylamide gel electrophoresis run at 4 °C. Signals from the biotinylated probe on the gel were detected using the EMSA gel shift Kit obtained from Affymetrix (Santa Clara, CA) according to the manufacturer's instructions. The sequences for the oligonucleotide probes with the intact HRE or mutated HRE sites as well as a consensus HRE probe (a positive control from the endothelin-1 gene [138]) were as follows; csOATP1B3 probe, 5′- CATCTCGGCGTATACGTGCAAGTCACAG-3′; csOATP1B3-mut-HRE probe, 5′-CATCTCGGCGTATAAAAGCAAGTCACAG-3′; consensus HRE probe, 5′-AGCTTGCCCTACGTGCTGTCTCAGA-3′.

4.2.7 siRNA-mediated knockdown of HIF-1α

Cells were transfected with siRNA duplexes targeting HIF-1α (pooled siRNA, Dharmacon, Lafayette, CO) or control scrambled siRNA (Santa Cruz biotechnology, Dallas, TX) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The mRNA and protein levels of HIF-1α and csOATP1B3 were analyzed 48 hours after transfection.

4.3 Results

4.3.1. Impact of hypoxia on the expression of csOATP1B3 in human colon and pancreatic cancer cell lines

We assessed a potential association between csOATP1B3 and HIF-1α by comparing the endogenous expression levels of csOATP1B3 V1 and HIF-1α in a panel of human colon and pancreatic cancer cell lines (Fig. 4.1). Our results indicated that the cell lines expressing detectable levels of csOATP1B3 V1 (i.e., DLD-1, HCT116, BxPC-3, MiaPaCa-

2) display HIF-1α protein accumulation. In contrast, the cell lines lacking csOATP1B3 V1 (i.e. Caco-2, SW480, HCT-8, AsPC-1) did not have any detectable levels of HIF-1α. This apparent association between csOATP1B3 and HIF-1α accumulation prompted us to further probe the involvement of HIF-1α in regulating the csOATP1B3 expression.

Using MiaPaCa-2 and HCT116 cell lines expressing detectable levels of csOATP1B3, we initially examined the effect of hypoxia (1% O_2) on csOATP1B3 expression levels. Our results show that hypoxia leads to a modest increase in csOATP1B3 levels in MiaPaCa2 cells, but not in HCT116 cells (Fig. 4.2A). Of note, OATP1B3 WT was detected in neither MiaPaCa-2 nor HCT116 cells, regardless of hypoxia treatment. In order to examine the cell line-dependency of the hypoxia effect on csOATP1B3 expression, we also performed similar experiments using HCT-8 cells. In HCT-8 cells, hypoxia markedly increased the levels of both HIF-1α and csOATP1B3 in a time-dependent manner (Fig. 4.2B). However, there were no hypoxia-induced changes in the levels of OATP1B1 or OATP1A2 in HCT-8 cells (Fig. 4.2B).

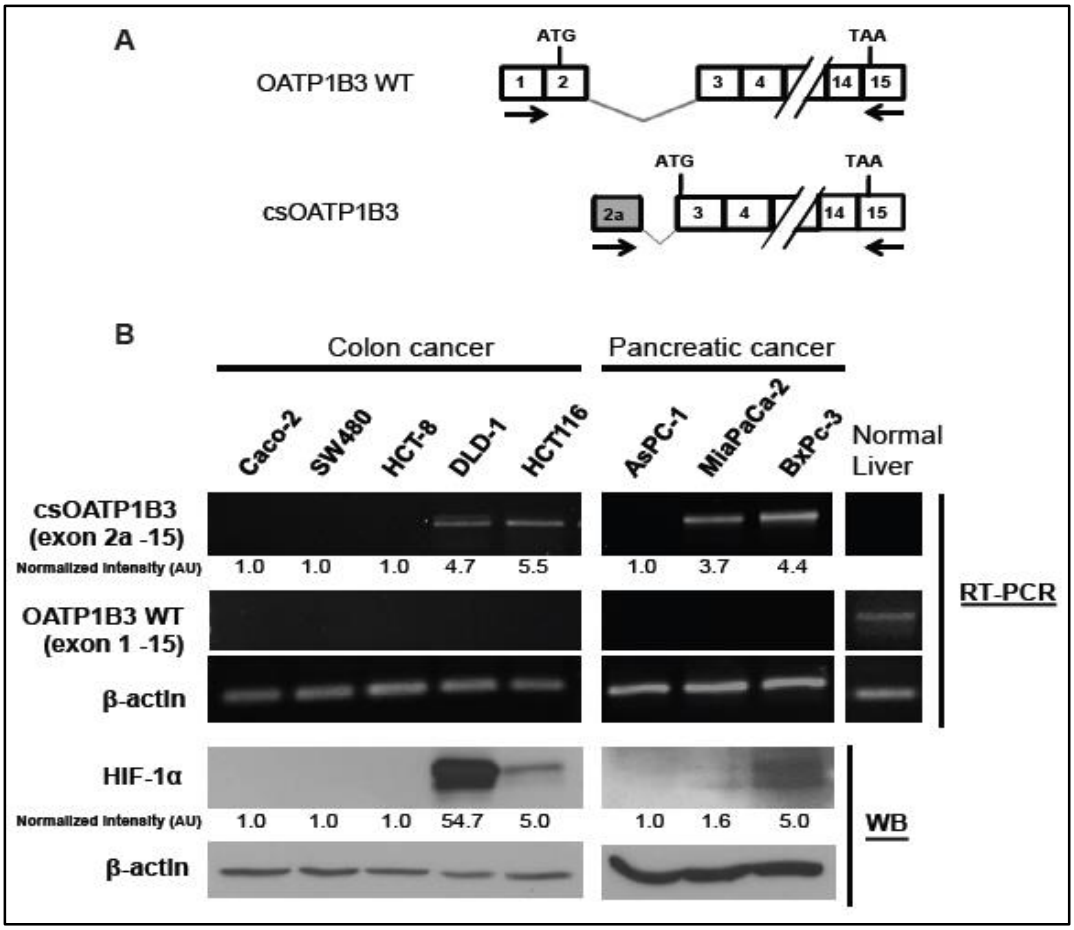

Figure 4. 1 Expression of csOATP1B3, OATP1B3 WT and HIF-1α in human colon and pancreatic cancer cell lines

(A) Schematic representation of the transcripts for csOATP1B3 V1 and OATP1B3 WT. The sequence of csOATP1B3 V1 differs from that of OATP1B3 WT, in that it contains the alternative exonic sequence (termed as exon 2a) instead of exons 1 and 2. Arrows represent the landing sites of primers used for amplification of OATP1B3 WT (exons 1-15) or csOATP1B3 (exons 2a-15). (B) RT-PCR and immunoblotting results showing varying levels of endogenous csOATP1B3 and HIF-1α expression in human colon (Caco-2, SW480, HCT-8, DLD-1, HCT116), pancreatic cell lines (AsPC-1, MiaPaCa-2, BxPC-3) and normal liver tissue. β-actin was used as a housekeeping control. Relative band intensities normalized to β-actin are represented as arbitrary units (AU).

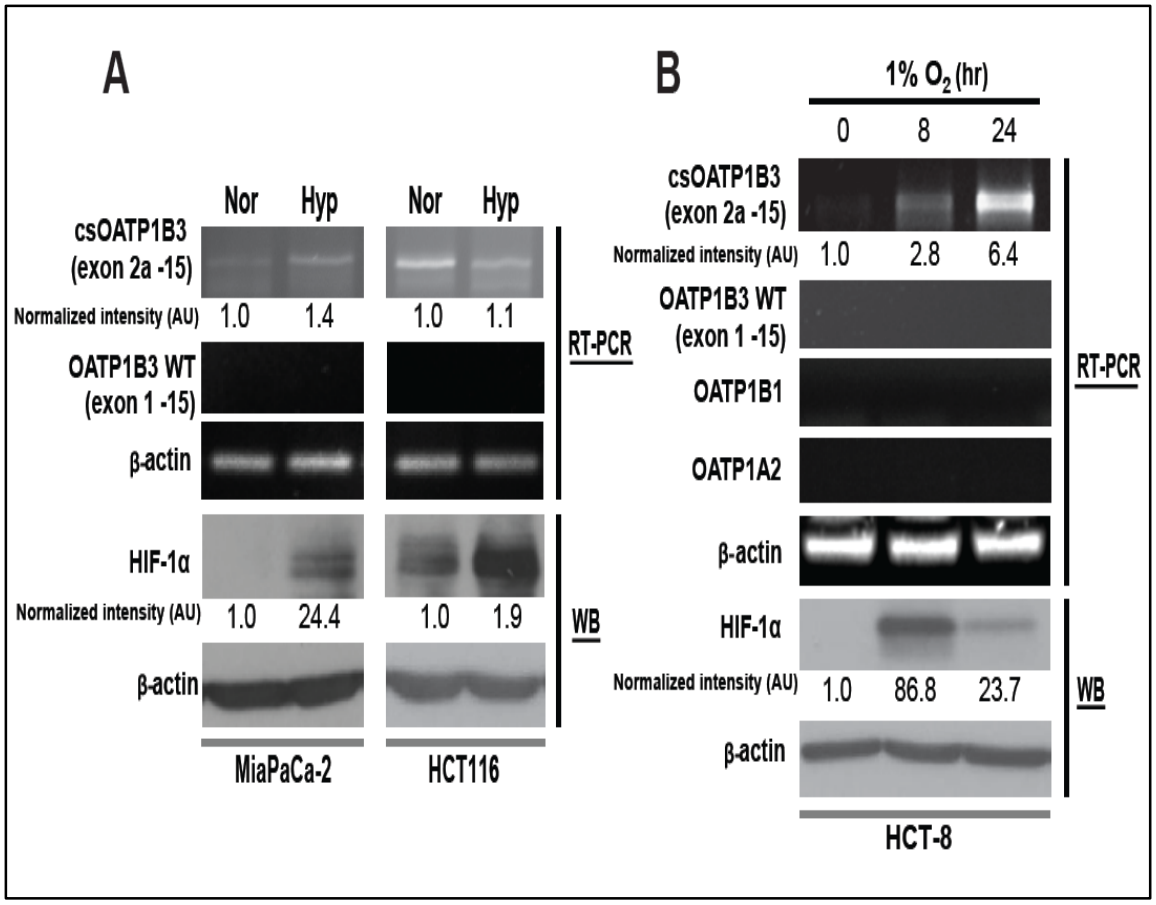

Figure 4. 2 Hypoxia leads to upregulation of csOATP1B3, but not OATP1B3 WT

(A) MiaPaCa-2 and HCT116 cells exposed to normoxia (Nor) or hypoxia (1% O_2, Hyp) for 48 h. (B) HCT-8 cells were exposed to hypoxia for 0, 8 and 24 h. RT-PCR analyses were performed to determine the expression level of csOATP1B3, OATP1B3 WT, OATP1B1 or OATP1A2. Immunoblotting analyses were performed to detect HIF-1α protein. β-actin was used as a housekeeping control Relative band intensities normalized to β-actin are represented as arbitrary units (AU).

4.3.2. Effects of hypoxia on the transactivation of csOATP1B3 promoter and identification of a functional HRE

Based on our results showing hypoxia-induced upregulation of csOATP1B3 in HCT-8 cells, we investigated whether the mechanism involves transcriptional activation. We performed *in silico* analyses of the csOATP1B3 promoter sequence, in search for any putative *cis*-acting elements responsive to hypoxia. Our results using the MatInspector database (Genomatix Software Inc, Ann Arbor, MI) indicated the presence of a putative HRE located within Exon 2a (Fig. 4.3A). In further validating the DNA sequences involved in the csOATP1B3 transactivation, we utilized a hypoxia mimetic agent $CoCl_2$ [139]. As expected, the exposure of HCT-8 cells to $CoCl_2$ led to a marked increase in the HIF-1α and csOATP1B3 levels, with no changes in the levels of OATP1B3 WT, OATP1B1 and OATP1A2 observed (Fig. 4.3B). These findings are consistent with those obtained using ambient hypoxia (Fig. 4.2B).

We then determined the effect of $CoCl_2$ treatment on the promoter activity of csOATP1B3 or OATP1B3 WT, using the reporter constructs of csOATP1B3 (-1853) and OATP1B3 WT (-2023) (Fig. 4.3A). The results indicated that $CoCl_2$ treatment increases the reporter activity of the csOATP1B3 (-1853), but not that of the OATP1B3 WT (-2023) (Fig. 4.3C). To further narrow down the *cis*-elements responsible for the hypoxia-mediated transactivation of csOATP1B3 promoter, we compared a series of 5′-end deletion mutants for their extent of activation by $CoCl_2$. Our results indicate that all three deletion mutants and the csOATP1B3 (-1853) construct were comparable in their extent of $CoCl_2$-induced transactivation (Fig. 4.3C). Using the smallest construct csOATP1B3 (-263), we prepared a construct where the putative HRE sequence is mutated. The csOATP1B3 (-263)-mut-HRE construct showed no increase in the reporter activity following the exposure to $CoCl_2$

treatment (Fig. 4.3C) or 1% O_2 (Fig. 4.3D). These results suggest that the putative HRE site may be responsible for the hypoxia-induced transactivation of csOATP1B3.

To determine whether HIF-1α indeed binds to the putative HRE in the csOATP1B3 promoter, EMSA was performed using the probe containing the putative HRE of csOATP1B3. Nuclear extracts isolated from HCT-8 cells treated with $CoCl_2$ (200 μM, 24 h) produced a unique band shift (Fig. 4.4, indicated by an arrow). This shift was competed away by unlabeled probe containing the intact HRE, but not by the unlabeled probe containing the mutated HRE (Fig. 4.4). As a positive control, a previously validated consensus HRE sequence from the endothelin-1 gene [138] was used and produced a similar band shift when incubated with the same nuclear extract. These results suggest that HIF-1α can physically interact with the HRE site in the csOATP1B3 promoter.

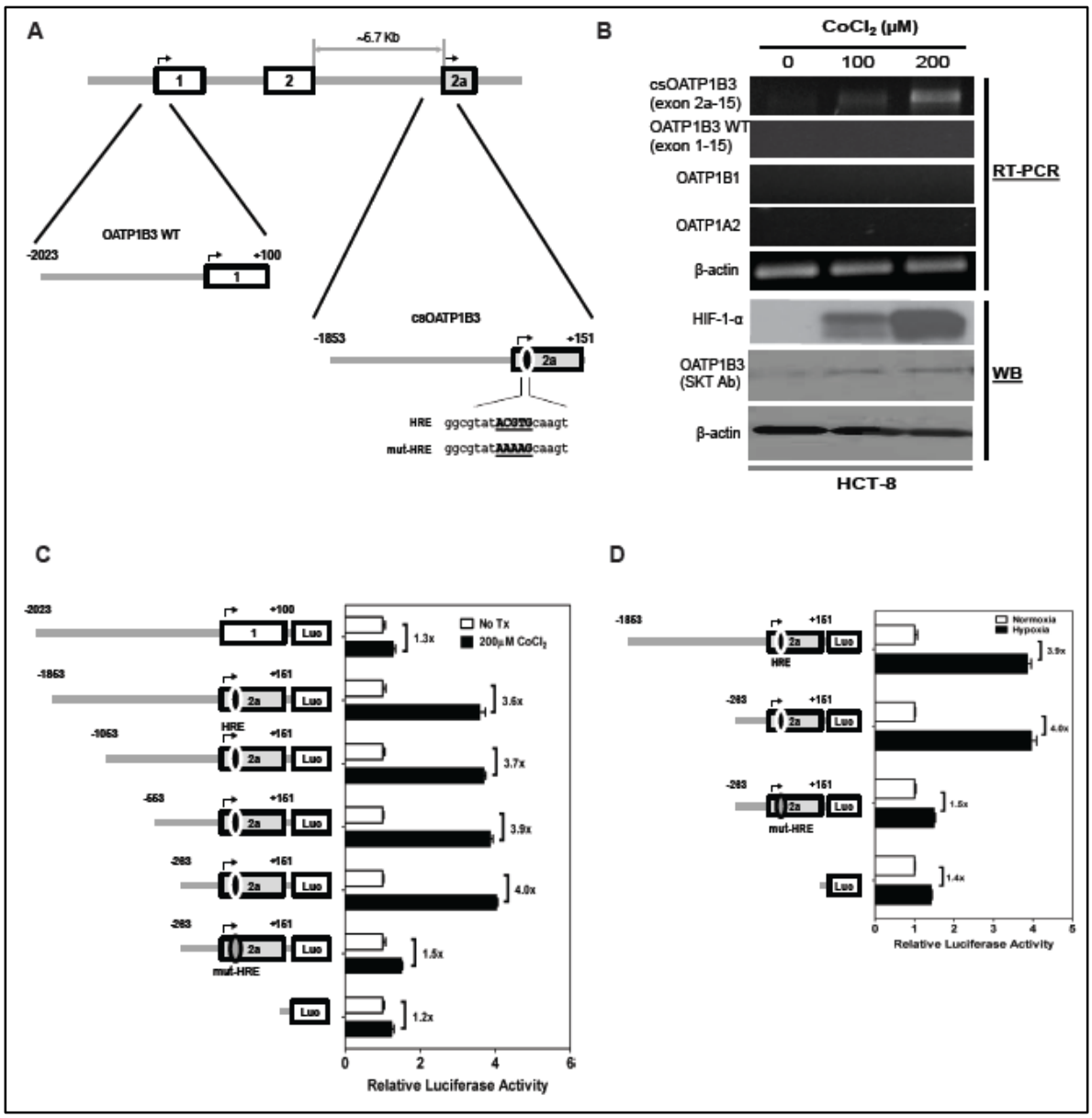

Figure 4. 3 Hypoxia-induced transactivation of csOATP1B3 promoter

(A) Schematic representation of the reporter constructs containing the promoter sequences for OATP1B3 WT or csOATP1B3. A consensus hypoxia-responsive element (HRE) present in exon 2a (underlined) is shown along with the mutated HRE sequence used in subsequent experiments. (B) Effect of $CoCl_2$, a hypoxia mimetic agent on the expression of csOATP1B3 and HIF-1α. HCT-8 cells exposed to $CoCl_2$ (100 or 200 μM, 24 h) were analyzed for the expression of csOATP1B3, OATP1B3 WT, OATP1B1, OATP1A2

and HIF-1α via RT-PCR and immunoblotting. (C) Effects of chemical hypoxia on the promoter activity using various reporter constructs of csOATP1B3 or OATP1B3 WT. The extent of $CoCl_2$-induced transactivation was compared among various promoter constructs following the transient transfection in HCT-8 cells and subsequent treatment with $CoCl_2$ (200 μM, 24 h). (D) Effects of ambient hypoxia on the promoter activity using various reporter constructs of csOATP1B3 promoter. The extent of hypoxia-induced transactivation was compared among various promoter constructs following the transient transfection in HCT-8 cells and subsequent exposure to 1% O_2 (24 h). Experiments were performed in triplicates, and their values are expressed as mean ± S.D.

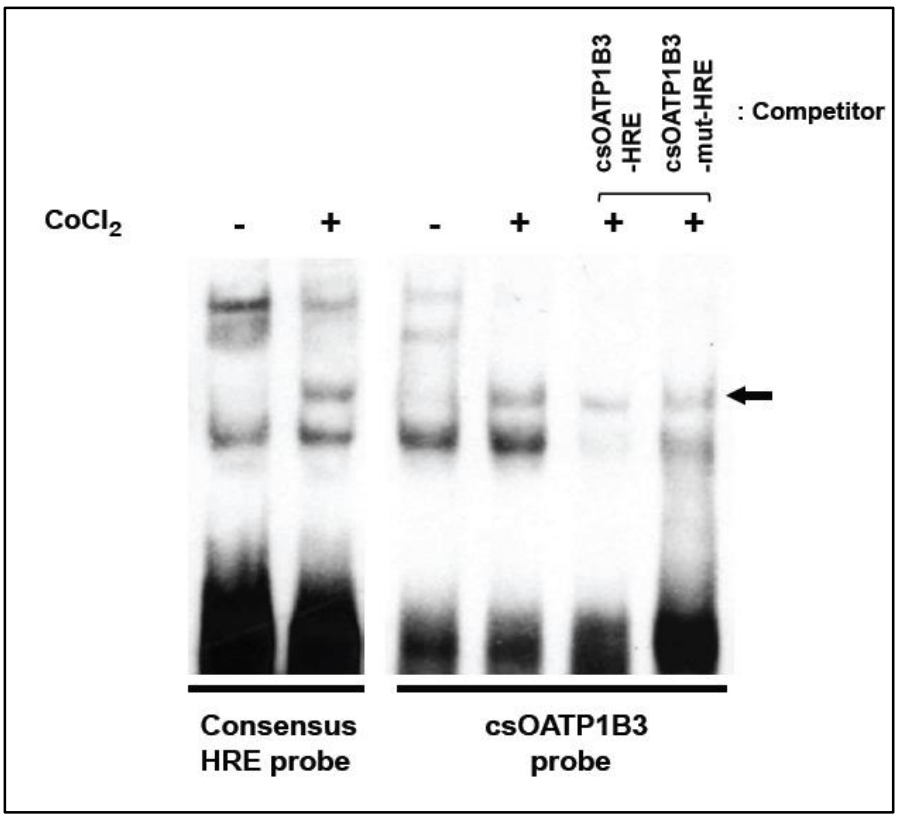

Figure 4. 4 Electrophoretic mobility shift assay (EMSA) results showing that HIF-1α binds to a hypoxia response element (HRE) in the csOATP1B3 promoter

Nuclear extracts from HCT-8 cells exposed to $CoCl_2$ (200 μM, 24 h) show a specific band shift when incubated with the biotinylated csOATP1B3-HRE probe or a consensus HRE probe (indicated by the arrow). The band shift was competed away by an excess of unlabeled csOATP1B3-HRE probe, but not by an excess of unlabeled csOATP1B3-mut-HRE probe where the consensus HRE is mutated.

4.3.3. Effects of HIF-1α knockdown on csOATP1B3 expression

In order to further validate the role of HIF-1α on csOATP1B3 expression, we examined the effect of siRNA-based HIF-1α knockdown on the csOATP1B3 levels in HCT-8 cells exposed to hypoxia and additional cell lines expressing basal levels of csOATP1B3. The extent of csOATP1B3 upregulation by $CoCl_2$ was substantially reduced in HCT-8 cells transfected with siRNA targeting HIF-1α, compared to those transfected with control siRNA (Fig. 5A). The siRNA-based HIF-1α knockdown also led to down-regulation of csOATP1B3 at both the mRNA and the protein levels in HCT116 cells expressing high basal levels of HIF-1α and csOATP1B3 (Fig. 5B). Similar results were obtained using additional cell lines (DLD-1 and BxPC-3 cells, data not shown).

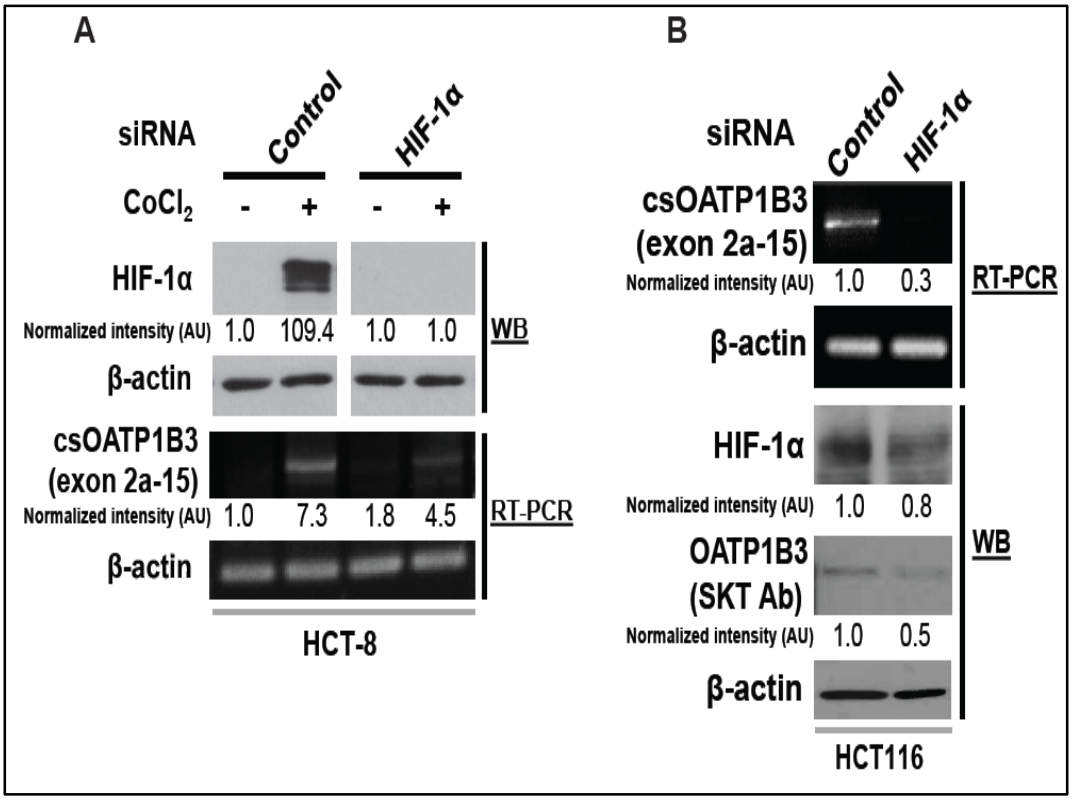

Figure 4. 5 HIF-1α knockdown decreases the expression of csOATP1B3

(A) The siRNA-based HIF-1α knockdown leads to a marked decrease in the extent of csOATP1B3 upregulation in HCT-8 cells exposed to $CoCl_2$. (B) The siRNA-based HIF-1α knockdown markedly decreases csOATP1B3 levels in HCT116 cells. β-actin was used as a housekeeping control Relative band intensities normalized to β-actin are represented as arbitrary units (AU).

4.4 Discussion

Alternative splicing is recognized as a common, yet important event of adding diversity in the proteomic world of eukaryotic cells. For an increasing number of membrane efflux and influx transporters including Oatp2 (the rodent ortholog of OATP1B3), the presence of alternatively spliced transcripts has been reported [125, 140-144]. Many of these splicing variant products have been detected in the organs or cell types normally expressing their WT transcripts. In contrast, the recently identified splicing variants of OATP1B3 are detected only in cancer settings and do not coexist with the WT OATP1B3 [73, 128]. These findings led us to hypothesize that the expression of csOATP1B3 may be influenced by oncogenic signaling pathways and may have potential roles in cancer development and progression. Following up on the previous reports suggesting a potential link between hypoxia and OATP1B3 expression in cancer [10, 145], our current study further investigated the regulatory mechanisms underlying hypoxia-induced upregulation of OATP1B3 in cancer. Our current findings provide evidence that hypoxia can induce the expression of csOATP1B3, but not OATP1B3 WT and that HIF-1α plays an important role in regulating csOATP1B3 expression in colon and pancreatic cancer cells. Further investigations would be necessary to address the relative importance of this HIF-1α-dependent mechanism on csOATP1B3 expression in clinical cancer tissues, in particular, in comparison to other reported mechanisms [76]. Our findings provide for the first time a mechanistic understanding of the csOATP1B3 regulation in colon and pancreatic cancer and underline the rationale for the investigation of functional and pathogenic significance of csOATP1B3.

HIF-1α is often accumulated in multiple types of human cancers derived from lung, breast, prostate and gastrointestinal tissues [45, 146-150]. For colon and pancreatic cancer, it has been reported that HIF-1α expression is predictive of poor prognosis and short disease-

free survival [151-154]. This association of HIF-1α with clinical outcomes has been often attributed to subsequent changes in downstream effectors important in tumor growth and survival as well as tumor metabolism [131, 148, 155]. Currently, it is not known whether csOATP1B3 serves as one of downstream effectors of the HIF-1α pathway, potentially providing tumor growth/survival advantages. In that regard, our laboratory had previously reported that OATP1B3 may serve as an antiapoptotic or prosurvival protein in colorectal cancer cells [6]. However, such findings were obtained using cancer cell line models stably transfected with OATP1B3 WT, as the presence of csOATP1B3 was not known at that time. Given the close sequence similarity (~96%) of the csOATP1B3 and OATP1B3 WT (amino acid sequence of csOATP1B3 V1 identical to OATP1B3 WT except missing N-terminal 28 amino acids), it is plausible that csOATP1B3 may confer apoptotic resistance or growth advantage in a similar manner to OATP1B3 WT. Supporting such a possibility, Ramachandran et al. [145] recently reported that selective knockdown of OATP1B3 expressed in colorectal cancer cell lines leads to a decrease in cell size and 3-dimensional spheroid volume. Similarly, Silvy et al. [156] reported that OATPs expressed in human melanoma cells may reduce the anticancer activity of cisplatin in a protein kinase C-dependent manner. Using clinical pancreatic cancer tissues and precancerous lesions, a recent report also suggested that OATP1B3 can serve as a potential diagnostic marker for early stage pancreatic cancer [127]. These findings are certainly intriguing and warrant further investigations to verify the functional roles and prognostic/predictive implications of csOATP1B3 in colon and pancreatic cancer.

Our current findings provide evidence for the involvement of HIF-1α in regulating csOATP1B3, but it is possible that other regulatory mechanisms may play a role in the expression of csOATP1B3. A previous report by Ichihara et al. demonstrated that OATP1B3 expression in cancer cell lines can be upregulated by the treatment with the

DNA-methylating agent, 5-aza-2'-deoxycytidine (5-Aza), suggesting the involvement of DNA-methylation based gene-silencing in the regulation of OATP1B3 expression [76]. We briefly examined whether the exposure of HCT-8 cells to 5-Aza induces the expression of csOATP1B3, OATP1B3 or both. Our results indicated that 5-Aza treatment leads to an increase in the level of csOATP1B3, but not OATP1B3 WT (unpublished data). Although not directly tested for the case of csOATP1B3 regulation, there exists evidence suggesting interplay among hypoxia, epigenetic modulation, and alternative splicing. For instance, it has been reported that hypoxia can influence cellular epigenetic status and that DNA demethylation can augment hypoxia-induced effects by positive auto-regulation of HIF-1α [157, 158]. A recent report also suggested that DNA methylation status of a HRE sequence can influence the interactions of HIF-1α with the HRE site, subsequently the hypoxic inducibility of the target gene [159]. More intriguingly, it was reported that HIF-1 proteins may regulate alternative splicing of many target genes [160]. For better understanding of csOATP1B3 regulation, further investigations are necessary to explore the interplay between different regulatory mechanisms.

In conclusion, we report for the first time that csOATP1B3, but not OATP1B3 WT, is transcriptionally regulated in response to hypoxia in colon and pancreatic cancer. Our results identified a functional HRE site in the csOATP1B3 promoter that physically associates with HIF-1α. Our data suggests that HIF-1α may play a key role in regulation of csOATP1B3 in colon and pancreatic cancer cells. Our findings may provide important clues about the functional and clinical significance of csOATP1B3 in colon and pancreatic cancer

Chapter 5

Roles of N-terminal motifs in plasma membrane trafficking of Organic Anion Transporting Polypeptide 1B3 (OATP1B3)

5.1 INTRODUCTION

Organic anion transporting polypeptide 1B3 (OATP1B3) belongs to the OATP (gene symbol *SLCO*) superfamily of solute carriers. OATP1B3 is abundantly expressed in the hepatocytes and mediates the transmembrane uptake of a variety of endogenous and xenobiotic substances. The endogenous substrates of OATP1B3 include bile salts, steroid hormone conjugates, thyroid hormone, peptides and natural toxins phalloidin and microcystin-LR [3, 4, 67]. OATP1B3 plays an important role in the disposition of several important therapeutic agents including cholesterol-lowering statins, anti-cancer drugs, antibiotics, cardiac glycosides and antidiabetics [4, 161]. In recent years, OATP1B3 is identified as an important determinant of transporter-mediated drug interactions [162].

Despite the increasingly recognized importance of OATP1B3 in drug therapy, little is known about the mechanisms regulating the intracellular trafficking of OATP1B3. Similar to other members of OATP superfamily, OATP1B3 is predicted to have 12 transmembrane domains (TMs) [2]. A previous study reported that TM10 of OATP1B3 is important for the recognition of the OATP1B3-specific substrate cholecystokinin-8 (CCK-8) [163]. These findings were based on the results obtained using a series of chimeric proteins between OATP1B3 and its closely related family member, OATP1B1 [163]. Other investigations probed the contribution of individual amino acids on the transport activity and membrane trafficking of OATP1B3 using the constructs harboring point mutations at several conserved positions (K41, K361, K399 and R580 located in the TMs 1, 7, 9 and11,

respectively) [164, 165]. Overall, our understanding of intracellular trafficking processes of OATP1B3 remains to be limited.

Our laboratory and others have recently reported the identity of a cancer-specific OATP1B3 variant which utilizes an alternative transcription initiation site and lacks the N-terminal 28 amino acids [73-75]. In contrast to the wild-type (WT) OATP1B3 expressed in normal liver, the cancer-specific variant was found to be localized predominantly in the cytoplasm [73]. These findings provided us an interesting clue that the N-terminus of OATP1B3 may play an important role in regulating its intracellular trafficking. Here, we set out to follow up on our previous observations and further identify N-terminal motifs important for regulation of the expression and membrane trafficking of OATP1B3. Utilizing the sequential truncation and point mutation approaches, we report that the N-terminal region at amino acid positions 14 to 17 with a putative β-turn-forming tetrapeptide is important for regulating the expression and membrane localization of OATP1B3.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and Reagents

Sodium butyrate and poly-lysine hydrobromide were purchased from Sigma-Aldrich. [^{3}H]-Cholecystokinin-8 ([^{3}H]-CCK-8, 104.2 Ci/nmol, >90% purity) was purchased from Perkin-Elmer.

5.2.2 *In-silico* analyses for potential signaling motifs at the N-terminal region of OATP1B3

In-silico algorithms such as PROSITE, CLOUDES, and BetaTPred were utilized to predict the potential amino acids motifs involved in cellular signaling and processing of OATP1B3[113]. The propensity of forming β-turn tetrapeptides (Δ scores) was calculated using the previously published method by Chou [166] .

5.2.3 Construction of expression plasmids for truncation and point mutants of OATP1B3 at the N-terminus

The expression plasmid for WT OATP1B3 containing the ORF sequence fused with Myc tag sequence at the C-terminus was purchased from Origene [73]. Truncation mutants of OATP1B3 were generated by swapping the incrementally truncated sequences which were PCR amplified with the restriction sites SgfI and PacI using the primers listed in Table 5.1. The insertion of the corresponding sequences (represented as Δ11, Δ23 and Δ28 respectively) was verified by direct sequencing. The expression plasmids for point mutants of OATP1B3 were prepared using QuikChange® site-directed mutagenesis (SDM) kit (Agilent) and the primers listed in Table 5.1. Direct sequencing was performed to verify whether the correct point mutation was introduced.

5.2.4 Cell culture and plasmid transfection

Human colon cancer cell lines HCT116 and HCT8 were purchased from American Type Culture Collection (ATCC). HEK293 cells were kindly provided by Dr. Markos Leggas (University of Kentucky). For expression and functional investigations, OATP1B3 and mutants were transiently transfected using Lipofectamine 2000 (Invitrogen) (for HCT116 and HEK293 cells) or Fugene HD (for HCT8 cells). Protein expression analyses and transport experiments were typically carried out 48 h after transfection. Initially comparable transfection efficiencies of the truncation and SDM constructs were verified by checking green fluorescence signals following co-transfection of a separate plasmid expressing green fluorescent protein (GFP).

Table 5.1: Primer sequences and combinations for generation of OATP1B3 truncation and point mutants

Construct	Sequence (5'-3')	Restriction site(s)
OATP1B3 Δ11	CGATGCGATCGCATGGAGTCAGCATCTT (Fw)	*Sgf1*
	CCATTGAATGATAAGGTTTGATTAATTAAACAGG(Rv)	*Pac1*
OATP1B3Δ23	CGATGCGATCGCATGTGCAATGGATTCA (Fw)	*Sgf1*
	CCATTGAATGATAAGGTTTGATTAATTAAACAGG(Rv)	*Pac1*
OATP1B3Δ28	CGATGCGATCGCATGTTCTTGGCAG (Fw)	*Sgf1*
	CCATTGAATGATAAGGTTTGATTAATTAAACAGG(Rv)	*Pac1*
OATP1B3^{16}AA^{17}	GCAGAGTCAGCATCT**G**CAG**C**GAAAAAGAAAACAAGACGC (Fw)	
	GCGTCTTGTTTTCTTTTTC**G**CTG**C**AGATGCTGACTCTGC (Rv)	
OATP1B3S13A	GAATAAAACAGCAGAG**G**CAGCATCTTCAGAGAAAAAG (Fw)	
	CTTTTTCTCTGAAGATGCTGCCTCTGCTGTTTTATTC (Rv)	
OATP1B3S15A	AAAACAGCAGAGTCAGCA**G**CTTCAGAGAAAAAGAAAAC (Fw)	
	GTTTTCTTTTTCTCTGAAGCTGCTGACTCTGCTGTTTT (Rv)	
OATP1B3S16A	CAGCAGAGTCAGCATCT**G**CAGAGAAAAAGAAAACAAG (Fw)	
	CTTGTTTTCTTTTTCTCTGCAGATGCTGACTCTGCTG (Rv)	

5.2.5 Immunoblotting analyses

Cell lysates were prepared using the lysis buffer (containing 10 mM Tris, 150 mM NaCl, 1% Sodium deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (cOmplete, Roche). Cell lysates were mixed with 4X Laemmli buffer and incubated at room temperature for 30 min. Cell lysates containing equivalent protein amounts were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking, the membranes were incubated overnight with a primary antibody against Myc tag or β-actin (Cell signaling). The immunoreactive proteins were detected using a secondary antibody conjugated with horseradish peroxidase and an enhanced chemiluminescence substrate (Pierce).

5.2.6 Radioactive uptake assay

[^{3}H]-Cholecystokinin-8 (CCK-8), a prototype OATP1B3 substrate was utilized to assess the transport activity of OATP1B3 WT and mutants, following transfection as described previously.[73] Briefly, MDCKII and HEK293 cells were seeded onto a 12 well plate (2.5 x 10^5 cells/well). After 24 h, the cells were transfected with OATP1B3 WT, its mutants and empty vector. The experiment was performed 24 h after transfection. Following three washes with pre-warmed Opti-MEM (Invitrogen), the uptake was initiated by adding 0.5 mL of Optim-MEM containing [^{3}H]-CCK-8 (0.01 μM). To further validate the involvement of OATP1B3 in the uptake of CCK-8, rifampin (an OATP1B3 inhibitor, 100 μM) [73] was added in an additional set of wells. The uptake was stopped at 5 min (verified to be in the linear range from a separate time-dependent uptake experiment) by rinsing the cells three times with ice-cold PBS. The cells were lysed with 0.5 N NaOH on a shaker for 30 min. The lysates were then neutralized with 2.5N HCl and 250 μL of lysate was added to 3 mL of LSC cocktail. Radioactivity was measured using TriCarb LSC counter (Perkin-Elmer).

The uptake of [^{3}H] CCK-8 in each well was normalized to the protein amount measured using the BCA protein assay kit (Pierce).

5.2.7 Cell surface biotinylation

In order to investigate the subcellular localization of OATP1B3 WT and mutants, surface biotinylation assays were performed in HEK293 cells, following transient transfection similar to the protocol described previously [73]. Briefly, cells were rinsed with ice-cold PBS-Ca^{2+}/Mg^{2+} and then treated with the membrane impermeable biotinylating agent (1.5 mg/mL sulfo-NHS-SS-biotin, Pierce) at 4^0C for 1h. Later, the cells were washed three times with ice-cold PBS-Ca^{2+}/Mg^{2+} containing 100 mM glycine and incubated at 4^0C for 20 min to remove the excess biotinylating agent. The cells were then lysed in lysis buffer containing protease inhibitors (Roche). After centrifugation, streptavidin agarose beads (Pierce) were incubated with cell lysates at 4^0C for 1 h. The beads were separated from the supernatant (cytoplasmic fraction) by centrifugation. The beads were washed three times with ice-cold lysis buffer and incubated in Laemmli buffer for 30 min to release the biotinylated (membrane) fraction.

5.2.8 Immunofluorescence assays

HEK293 cells were seeded onto 4 chamber culture slides (BD Biosciences). After twenty-four hours, cells were transfected with myc-tagged OATP1B3 WT, Δ11, Δ23, Δ28 truncation mutants and point mutants at the putative β-turn tetrapeptide site as described above. Twenty four hours after transfection, the cells were fixed with 4% paraformaldehyde in ice-cold PBS for 30 min. Later, the cells were permeabilized with PBS containing 0.3% Triton X-100 for 20 min at room temperature and blocked using PBS containing for 1 h at room temperature. The cells were then incubated with a primary antibody against the Myc tag (Cell Signaling), followed by a secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Nuclear DNA was stained using DAPI (Vector

Laboratories Inc.). The immunofluorescence signal was detected using a Nikon (Eclipse Ti-U) fluorescence microscope or Leica Confocal microscope (Leica TCS SP5).

5.2.9 Statistical Analyses

The results are presented as mean ± SD. In the transport studies, the statistical significance between groups was determined using one-way ANOVA followed by Newman-Kuels test. *P* values of ≤0.05 were considered to be statistically significant. The calculations were performed using GraphPad Prism 5.04.

5.3 RESULTS

5.3.1 Identification of putative amino acid signal sequences at the N-terminus of OATP1B3 WT involved in its plasma membrane trafficking

To probe the signal sequence(s) important for the plasma membrane localization of OATP1B3, we performed *in-silico* analyses for the N-terminus of OATP1B3. Using various prediction algorithms such as PROSITE, CLOUDES, and BetaTPred, we identified several putative consensus hits that may be involved in cellular signaling (Fig 5.1A). Based on the location of predicted consensus hits on the OATP1B3, we prepared three myc-tag-fused truncation mutants with the N-terminus of OATP1B3 incrementally deleted (represented as Δ11, Δ23, Δ28, respectively, Fig. 5.1B).

A

Amino acid position	Identified consenus hits	Prediction algorithms used
^{18}KKK^{20} & ^{22}RR^{23}	Positively charged amino acids	PROSITE
^{16}SEK^{18}	PKC phosphorylation site	PROSITE
^{14}ASSE^{17}	β-turn forming tetrapeptide	CLOUDES (Fuchs & Alix), BetaTPred
^{24}CNGF^{27}	β-turn forming tetrapeptide	CLOUDES (Fuchs & Alix), BetaTPred

B

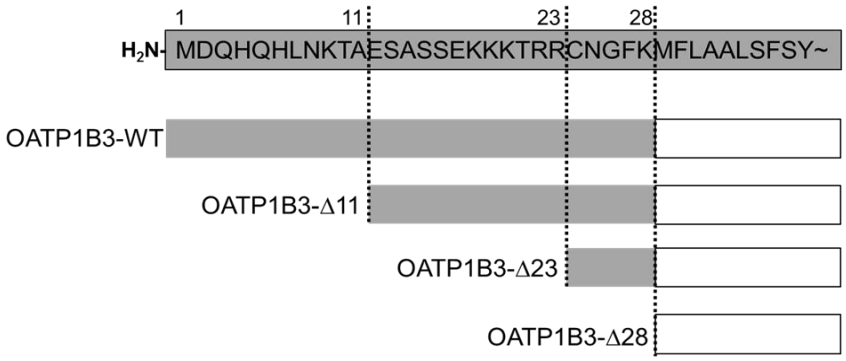

Figure 5. 1 In silico analyses of N-terminus of OATP1B3 wild-type (WT)

(A) Identification of putative amino acid motifs likely to be involved in cellular trafficking and localization signal of OATP1B3 by utilizing in silico algorithms. (B) Schematic representation of the prepared truncation mutants (myc-tag fused at C-terminus); deletion mutants of 11, 23 and 28 amino acids were represented as Δ11, Δ23 and Δ28, respectively.

5.3.2 Truncation of N-terminus leads to reduced plasma membrane localization of OATP1B3

Immunofluorescence imaging analyses indicated that the deletion of the first 11 amino acids at the N-terminus does not affect the plasma membrane localization in OATP1B3 (Fig. 5.2A). However, further deletion of 23 and 28 amino acids led to substantially reduced plasma membrane localization (Fig. 5.2A). Consistent results were also obtained using surface fractionation assays. While the truncation of the first 11 amino acids of OATP1B3 does not affect its surface expression level, the deletions of 23 and 28 amino acids at N-terminus result in the substantial reduction of OATP1B3 in the surface membrane fraction as well as in total lysates (Fig. 5.2B). These results suggest that important motif(s) regulating the surface localization and expression level of OATP1B3 may be present between amino acids 12 and 23.

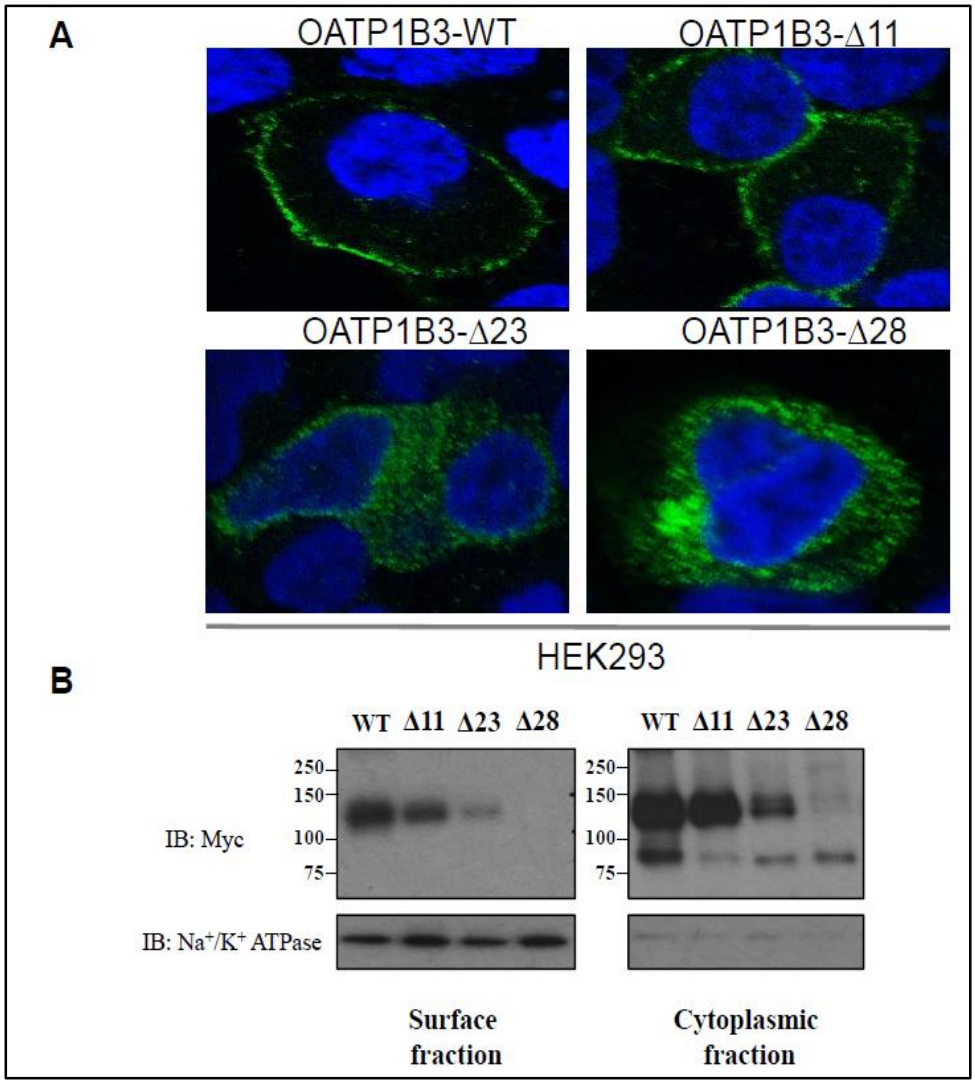

Figure 5. 2 Comparison of N-terminus truncation mutants of OATP1B3 WT

(A) Confocal microscopy analyses revealed that truncation of 23 and 28 amino acids at N-terminus of OATP1B3 led to reduced plasma membrane localization compared to OATP1B3 WT. (B) Immunoblotting analyses indicated that OATP1B3 Δ23 and Δ28 mutants have much reduced protein expression levels in surface and total fractions compared to OATP1B3 WT.

5.3.3 Comparison of point mutants at positively charged and putative phosphorylation sites located between amino acids 12 and 23 of OATP1B3

To further identify the important residues between amino acids 12 to 23, we utilized the SDM approach. Lysine and arginine residues were individually mutated to alanine at positions 18, 19, 20, 22 and 23 (represented as K18A, K19A, K20A, R22A and R23A respectively) and transiently expressed in HEK293 cells. The results from immunoblotting analyses of fractionated samples indicated that mutating the lysine or arginine sites at these positions did not affect the total protein expression or the surface levels as compared to OATP1B3 WT (Fig. 5.3). Similarly, mutating the serine sites at position 13, 15 and 16 (represented as S13A, S15A and S16A) did not impact total protein levels or surface expression as compared to OATP1B3 WT (Fig. 5.4). These findings suggest that lysine or serine residues at the amino positions 12 to 23 may not be important in regulating the expression or surface localization of OATP1B3.

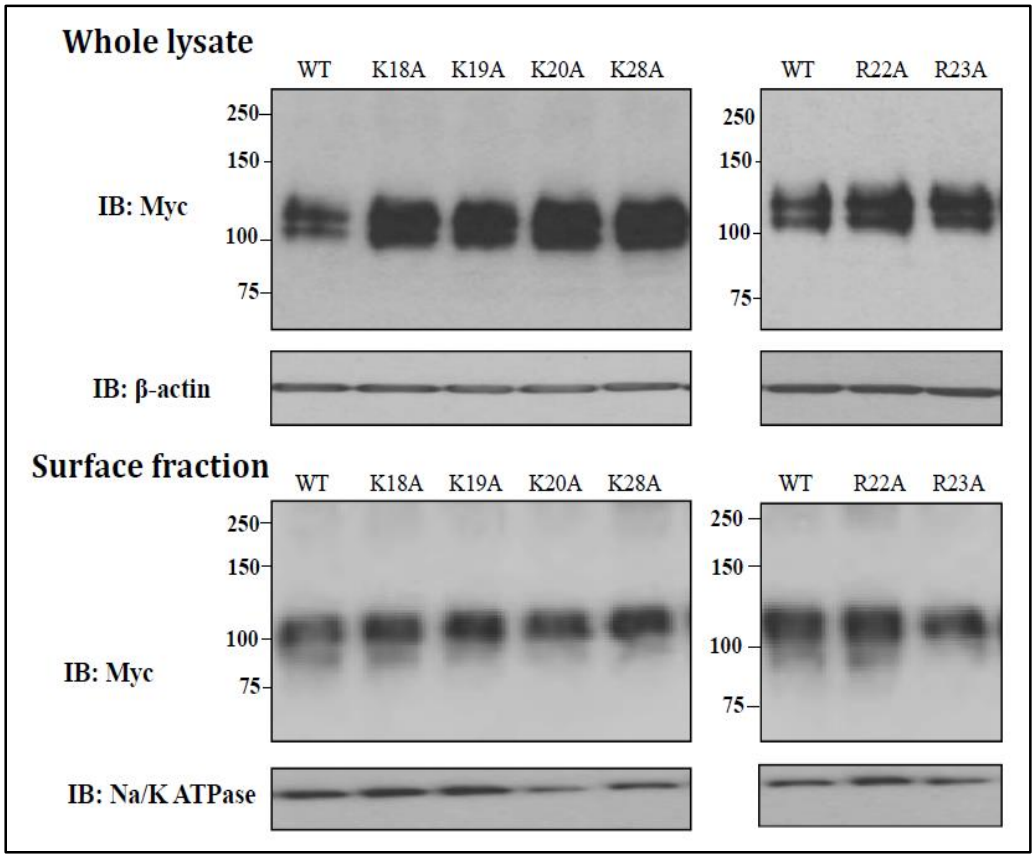

Figure 5.3 Comparison of point mutants of positively charged lysine or arginine residues located at the N-terminal region of OATP1B3 wildtype

Immunoblotting analyses reveal that the total and surface expression levels of point mutants at the N-terminal lysine and arginine residues are not substantially different from those of the OATP1B3 WT.

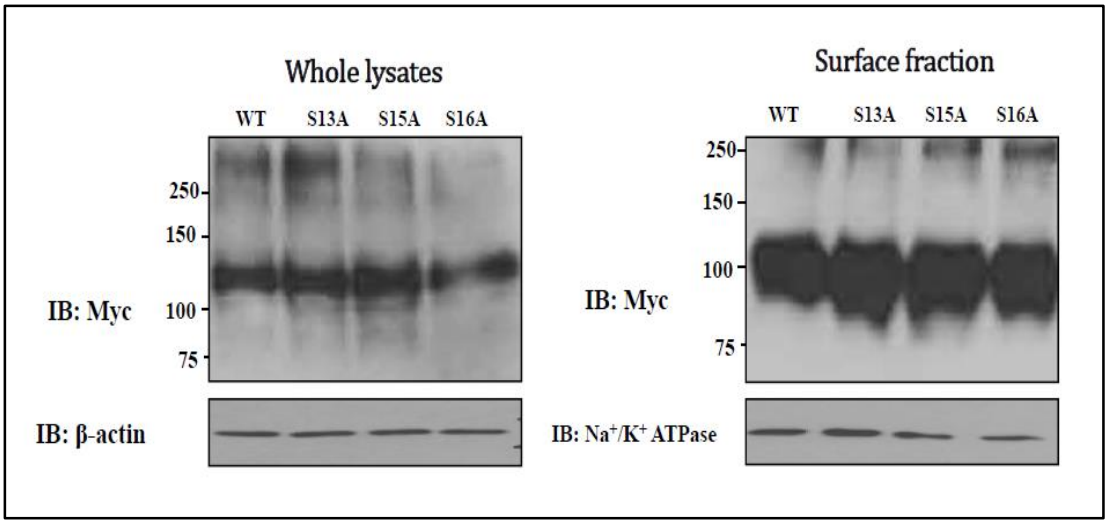

Figure 5. 4 Comparison of point mutants of putative phosphorylation sites located at the N-terminal region of OATP1B3 wildtype

Immunoblotting analyses reveal that the total and surface expression levels of point mutants at the N-terminal serine residues are not substantially different from those of the OATP1B3 WT.

5.3.4 Comparison of point mutants at the putative β-turn forming tetrapeptide site between amino acids 12 and 23 of OATP1B3

As a next step in interrogating the sequence(s) important in regulating OATP1B3 trafficking, we analyzed the β-turn forming propensity (Δ score) of the OATP1B3 WT sequence (^{14}ASSE^{17}) as well as mutants ^{14}ASaE^{17} and ^{14}ASaa^{17} (Fig. 5.5A). These mutated constructs were prepared by SDM and transiently transfected in HEK293 cells along with GFP (included to ensure comparable transfection efficiencies). The results from cell surface fractionation assays indicated that compared to OATP1B3 WT, the ^{14}ASaa^{17} mutant displays a significantly reduced expression level in the total lysate as well as in the surface membrane fraction (Fig. 5.5B). As expected, the ^{14}ASaa^{17} mutant was found to have a much reduced uptake of ^{3}H-CCK-8 compared to OATP1B3 WT.

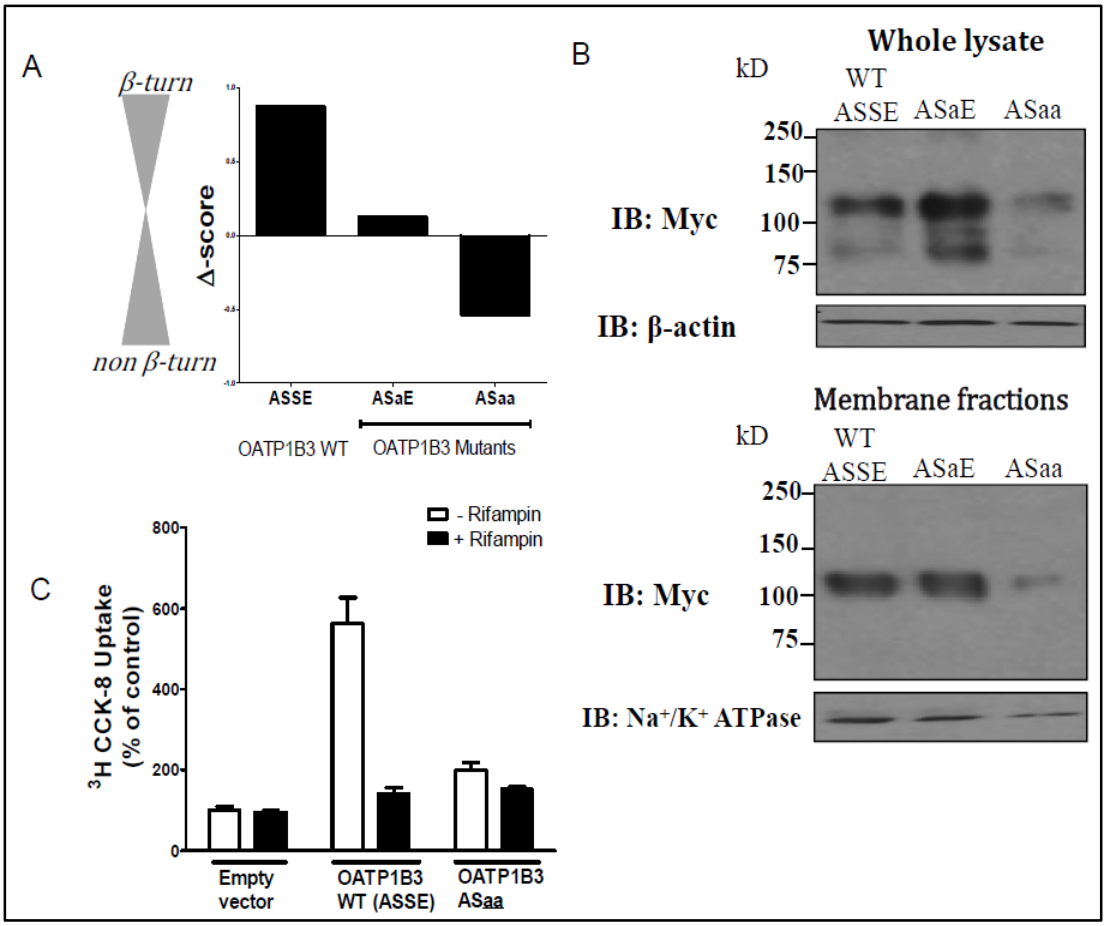

Figure 5.5 Comparison of point mutants at putative β-turn forming tetrapeptide sites at the N-terminus of OATP1B3

(A) *In silico* calculation using Chou algorithm revealed that the N-terminus of OATP1B3 WT contains a putative tetrapeptide (^{14}ASSE^{17}) with the highest Δ-score and propensity to form a β-turn. Sequential mutation of this position leads to substantial reduction in Δ-score. (B) Immunoblotting results showing that the ^{14}ASaa^{17} mutant at the putative β-turn forming sequence has lower total and surface expression levels than OATP1B3 WT. Na^+/K^+ ATPase was used as an internal control to ensure comparable surface membrane protein loading. (C) Comparison of cellular uptake of ^{3}H-CCK-8 in cells expressing ^{14}ASaa^{17} mutant and OATP1B3 WT in the presence and absence of rifampin (the inhibitor of

OATP1B3 transport activity). Data are represented as percentiles relative to the empty vector controls. Data are expressed as mean ± SD.

5.4 DISCUSSION

While the functional significance of OATP1B3 in hepatic drug disposition and overall drug response has been extensively investigated, little has been known about its mechanisms of expression and membrane localization. The findings from our current study for the first time identified the motifs at the N-terminus of OATP1B3 that may play a key role in regulating the membrane localization and expression of OATP1B3. Using sequential truncation and point mutation approaches, we report that the amino acid region between 12 to 23, specifically ^{14}ASSE^{17} with a high β-turn forming potential may be important. Our findings may provide important starting points in understanding the molecular processes of regulating intracellular trafficking of OATP1B3 and potentially other related members of the OATP family.

For certain OATP family members, the PDZ consensus sequences have been reported to play a role in regulating their membrane trafficking. However, no PDZ consensus sequences have been predicted for OATP1B3 and other members of the OATP1 family [167, 168]. Similar to our results supporting the involvement of a β-turn forming tetrapeptide (^{14}ASSE^{17}) in the membrane trafficking of OATP1B3, other studies have implicated the role of β-turn forming tetrapeptides in the membrane localization of transporters such as rat ileal apical sodium-dependent bile acid transporter (Asbt), human sodium-dependent vitamin C transporter (hSVCT1) and the human concentrative nucleoside transporter 3 (hCNT3) [113, 169-171]. In these previous investigations, disruption of the β-turn forming potential by SDM or deletion resulted in partial to complete mislocalization of these transporters [113, 169-171]. The prediction of the β-turn structures is currently dependent on mathematical calculation, partly due to the lack of state-of-art

technologies that can provide structural resolution. While the N-terminal β-turn structure that we propose in the current report remains to be structurally verified, our results do provide an interesting lead for further investigations on potential partnering proteins that may interact with OATP1B3 at the N-terminal region identified to form a β-turn.

Our results obtained from the point mutations at lysine, arginine and serine residues at the N-terminal region of OATP1B3 appear to indicate that these residues are not critically involved in the regulation of membrane trafficking or expression levels of OATP1B3 (Figs. 5.3 and 5.4). Interestingly, a recent report by Powell et al. indicated that OATP1B3-mediated transport activity can be impaired by protein kinase C (PKC) activation [172]. However, it needs to be investigated whether this effect occurs via direct phosphorylation of OATP1B3 and phosphorylation of other intermediary proteins. OATP2B1 is also reported to undergo PKC-mediated internalization [173]. However, specific motifs involved in the PKC-mediated internalization of OATP2B1 have not been identified.

In our current study, we observed that the N-terminal region of OATP1B3 may play a role in regulating not only protein localization, but also the expression level of OATP1B3. It is plausible that the processes regulating the localization and stability of OATP1B3 may be operating co-dependently. For instance, the *ABCB11* gene encoding bile salt export pump (BSEP) harbors naturally occurring mutations causing progressive familial intrahepatic cholestasis type 2 (PFIC2) [174]. These mutations (E297G and D482G) in BSEP led to the impairment of its surface trafficking as well as the reduced expression level. When the surface trafficking of BSEP was in part restored by 4-phenylbutyrate treatment, the authors observed an increased expression level of BSEP at the surface fraction and total lysates as well as the restoration of its transport activity [175].

In summary, we identified for the first time the N-terminal motifs of OATP1B3 important in regulating its expression and membrane localization. In particular, the amino acids within

a putative β-turn-forming tetrapeptide appear to be important. Our findings provide important starting points in further mechanistic investigations for the regulation of OATP1B3 trafficking and expression, potentially applicable to other OATP family members.

Chapter 6

Conclusions and future directions

(Each chapter of this thesis contains its own conclusion section and therefore this chapter is intended to provide a brief overview of the findings from this work.)

The results from this thesis work showed that colon and pancreatic cancer cells express a cancer-specific variant of OATP1B3 (csOATP1B3), which differs from OATP1B3 WT at its N-terminus (Chapter 3). Our findings also revealed that csOATP1B3 is regulated under hypoxia by a mechanism involving hypoxia-inducible factor 1α (Chapter 4). These findings add to the biological and mechanistic understanding of OATP1B3 in cancer. Our results highlight that the molecular entities and regulatory mechanisms of ectopically expressed proteins in cancer, including other OATP transporters may be dissimilar to those detected in the nonmalignant tissues. Careful investigations at the molecular level may lead to unexpected and exciting discoveries. We believe that these findings will help us better design future studies to investigate the clinical significance of csOATP1B3 in cancer patients. Some of the further investigations that may be designed are outlined below.

It would be important to investigate the functional impact of csOATP1B3 on the behavior of cancer cells. A previous report from our laboratory showed that OATP1B3 can confer cell survival advantages in colon cancer cells by p53-dependent mechanisms [6]. Because the presence of csOATP1B3 was not known previously, this particular experiment was carried out using cancer cells lines stably expressing the liver-type OATP1B3 [6]. To verify whether csOATP1B3 also confers cancer cell survival advantages in a similar manner to the liver-type OATP1B3, we carried out additional experiments using siRNA-based knockdown. Our preliminary results showed that the

knockdown of csOATP1B3 enhances cell death in colon cancer cells treated with oxaliplatin (data not shown). Further studies are required to elucidate the exact cellular mechanisms that provide cancer cell survival through csOATP1B3.

Recently, there have been multiple reports on the detection of OATP1B3 in multiple types of cancers [3, 5-7, 9, 67]. The majority of these reports utilized antibodies targeting the C-terminus epitope of OATP1B3 [5, 6, 8]. In future studies, it would be informative to obtain the expression patterns with antibodies targeting N-terminus of csOATP1B3 [73]. In addition, the investigation of intracellular locations of csOATP1B3 expressed in cytoplasm may shed light on the functional studies of csOATP1B3 in cancer.

More importantly, it would be important to gain a better understanding of the clinical significance of csOATP1B3. Future studies can be designed to examine the prognostic and predictive values of csOATP1B3 expression in clinical tissue samples (by examining any associations between the csOATP1B3 expression levels and clinical outcomes such as survival and response/resistance to chemotherapy). Depending on the results from these investigations, subsequent molecular investigations may be designed. For instance, if csOATP1B3 expression would be associated with resistance to chemotherapy, further investigations may be designed to interrogate the role of csOATP1B3 in conferring cell survival advantages. In fact, a very recent report by Teft et al. [124] utilized this approach and their findings suggest that OATP1B3 expression in cancer patients treated with irinotecan-based chemotherapy are associated with shorter progression-free survivals.

Another potential aspect is to examine the expression of csOATP1B3 in hepatic cancer tissues. The majority of research groups, including ours have reported the expression of csOATP1B3 expression in cancers derived from non-hepatic tissues. It would be interesting to determine whether csOATP1B3 is the predominant variant in other forms of cancers, especially in hepatocellular carcinoma (HCC). A previous report by Vavricka

et al. suggested that OATP1B3 mRNA levels were reduced in HCC [70]. Given that non-cancerous hepatocytes express only OATP1B3 WT, it would be interesting to investigate whether any csOATP1B3 is expressed in HCC and to compare the levels and functional impact of OATP1B3 WT and csOATP1B3.

Following up on our observations with csOATP1B3 in cancer, our results showed that the putative β-turn forming region at the N-terminus of OATP1B3 WT is important for its membrane localization and expression (Chapter 5). These studies provide foremost evidence regarding the role of the N-terminus in the expression and localization of OATP1B3. Further studies may be designed to determine whether there are any partnering proteins that interact with the N-terminal regions of OATP1B3 and play a role in the plasma membrane localization of OATP1B3 WT. One of the initial investigations could be aimed to understand the mechanisms of basolateral sorting of OATP1B3. Out of the different partnering proteins, AP-1B (from the AP family of clathrin adaptor proteins) is well-studied for its role in basolateral sorting [176]. Experiments that can be performed may include quantitative live imaging after selective knockdown of clathrin in cell line models expressing OATP1B3. These studies may assist in delineating whether the Golgi complex and clathrin mediated sorting mechanisms exist for OATP1B3.

Given our investigations focused on OATP1B3, it would be interesting to examine if these findings are applicable to the closely related family member, OATP1B1. Our *in silico* analyses revealed that the amino acid region at the N-terminus of OATP1B1 displays the highest propensity to form β-turn tetrapeptides. Studies may be designed to truncate the N-terminus and specifically mutate the β-turn tetrapeptide region of OATP1B1. Experiments including surface localization and transporter assays would demonstrate whether our findings may be applicable to other members of the OATP family.

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VITA

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EDUCATION AND TRAINING

- 2010 - 2014 PhD Candidate, Pharmaceutical Sciences,
  University of Kentucky, Lexington, KY
  Advisor: Wooin Lee, Ph.D.
- 2008 - 2010 Graduate student, School of Pharmacy
  University of Missouri- Kansas City, Kansas City, MO
- 2004 - 2008 B.S. in Pharmacy
  Sterling institute of Pharmacy, University of Mumbai, India

PROFESSIONAL POSITIONS

- 2010- 2014 *Graduate Research Assistant*, Department of Pharmaceutical Sciences, University of Kentucky College of Pharmacy, Lexington, KY
- 2010- 2011 *Graduate Teaching Assistant*, Department of Pharmaceutical Sciences, University of Kentucky College of Pharmacy, Lexington, KY
- 2011- 2011 *Summer Intern*, Drug Metabolism and Pharmacokinetics
  Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT
- 2008- 2010 *Graduate Research Assistant*, Department of Pharmaceutical Sciences, University of Missouri- Kansas City, Kansas City, MO

PUBLICATIONS

1. Hariharan S, **Thakkar N**, Mitra AK. Transporter-targeted drug delivery to the retina. *Retina Today* 4(4):57-62, 2009
2. **Thakkar N**, Kim K, Jang ER, Kim K, Han S, Lockhart C, Lee W. A cancer-specific variant of the SLCO1B3 gene encodes a novel human organic anion transporting polypeptide 1B3 (OATP1B3) localized mainly in the cytoplasm of colon and pancreatic cancer cells. *Molecular Pharmaceutics* 10:406−416, 2013
3. Han S*, Kim K*, **Thakkar N***, Kim D, Lee W. Role of Hypoxia Inducible Factor-1α in the regulation of the cancer-specific variant of organic anion transporting polypeptide

1B3 (OATP1B3) in colon and pancreatic cancer. *Biochemical Pharmacology* 86:816-823, 2013; (***, equally contributed**)

4. **Thakkar N** and Lee W. Cancer-specific OATP1B3 variant generated via alternative splicing: Lessons learned. *AAPS TPIFG newsletter*; 2:4, 24-26, 2013
5. Wang-Gillam A, **Thakkar N,** Lockhart A.C, Williams K, Baggstrom M, Naughton M, Suresh R, Ma C, Tan B, Lee W, Jiang X, Mwandoro T, Trull L, Belanger S, Creekmore A, Gao F, Fracasso P, Picus J. A phase I study of pegylated liposomal doxorubicin and temsirolimus in patients with refractory solid malignancies, *Cancer Chemotherapy and Pharmacology* 74, 2:419-426, 2014
6. Goff L.W, **Thakkar N,** Du L, Chan E, Tan B, Cardin D, McLeod H, Berlin J, Zehnbauer, Fournier C, Picus J, Wang-Gillam A, Lee W, Lockhart A.C. Thymidylate Synthase Genotype-Directed Chemotherapy for Patients with Gastric and Gastroesophageal Junction Cancers, *PLoS ONE*; 9(9):e107424. 2014
7. **Thakkar N**, Lockhart C, Lee W. The role of Organic Anion Transporting Polypeptides (OATPs) in cancer therapy. 'Invited Review Article' *The AAPS Journal*; Submitted September 2014, Under Revision

MANUSCRIPTS IN PREPARATION

8. **Thakkar N**, Lim YR, Machado D, Lee W. N-terminal motifs of OATP1B3 influence the protein stability and surface membrane trafficking.
9. Identification of novel, kinase-deficient variants of Discoidin Domain Receptor 2 (DDR2): differential expression in human esophageal cancer cell lines
10. Influence of epigenetic changes on OATP1A2 expression in gastrointestinal cancers

PROFESSIONAL AWARDS AND HONORS

- 2013 Drug development division pre-quals graduate student scholarship award
- 2013 Peter G. Glavinos Jr., Ph.D., Graduate travel award scholarship endowment
- 2013 Rho Chi Research day poster presentation; 2nd place award
- 2013 Outstanding Podia presentation; 2nd Place award, PGSRM, Iowa City, IA
- 2013-2014 University of Kentucky Graduate School Academic Year Fellowship

- 2014 Rho Chi Research day poster presentation; 2nd place award

LEADERSHIP ACTIVITIES

- 2006-2008 *College Representative,* Indian Pharmaceutical Association
- 2009-2010 *Vice President*, PGSRM (Pharmaceutics Graduate Student Research Meeting) committee at University of Missouri-Kansas City
- 2011-2012 *Vice Chair*, AAPS UK student chapter, University of Kentucky
- 2012- 2013 *Chair*, AAPS UK student chapter, University of Kentucky
- 2013- 2014 *Chair,* University of Kentucky PGSRM 2015 fundraising committee
- 2014- present *Student representative,* AAPS Drug transport focus group steering committee

SELECTED ABSTRACTS AND POSTER PRESENTATIONS

1. Eun Ryoung Jang, **Nilay Thakkar**, Donghern Kim, Kyunghwa Kim, Eun Y. Lee, Kyung-Bo Kim, Wooin Lee. Inhibition of the immunoproteasome subunit LMP2 by UK-101 leads to apoptosis and cell cycle arrest in pancreatic cancer. An abstract and poster presented at the Annual Meeting of American Association for Cancer Research (AACR) in Orlando, FL, 2011
2. **Nilay Thakkar**, Kyungbo Kim, Eunryoung Jang, Kyunghwa Kim, Songhee Han, Nipun Merchant, Craig Lockhart, Wooin Lee. Functional investigation of a novel cancer-specific OATP1B3 variant 1 (OATP1B3 V1) in colon and pancreatic cancer. An abstract and poster presented at the Annual Meeting of American Association for Cancer Research (AACR) in Washington, DC 2013
3. **Nilay Thakkar***, Songhee Han*, Kyungbo Kim*, Donghak Kim, Wooin Lee. Hypoxia-Inducible Factor 1α- mediated regulation of the Cancer-Specific Variant of Organic Anion Transporting Polypeptide 1B3 (csOATP1B3) in Colon and Pancreatic Cancer.

(* Contributed equally to this work). An abstract and poster presented at the 10th International ISSX Meeting in Toronto, ON, Canada 2013

4. **Nilay Thakkar,** Laura W. Goff, Liping Du, Emily Chan, Benjamin R. Tan, Dana B. Cardin, Howard L. McLeod, Jordan D. Berlin, Barbara Zehnbauer, Chloe Fournier, Joel Picus, Andrea Wang-Gillam, Wooin Lee, A. Craig Lockhart. Thymidylate Synthase Genotype-Directed Chemotherapy for Patients with Gastric and Gastroesophageal Junction Cancers. An abstract and poster presented at the 46th Annual Meeting of Pharmaceutics Graduate Student Meeting (PGSRM) in Chicago, IL, 2014
5. **Nilay Thakkar** and Wooin Lee. Role of N-terminal motifs of Organic Anion Transporting Polypeptide 1B3 (OATP1B3) in regulating its expression levels and surface membrane trafficking. A poster presented at the 13th Buffalo Pharmaceutics Symposium in Amherst, NY, 2014
6. **Nilay Thakkar**, Young-Ran Lim, Daniel Machado and Wooin Lee. Role of N-terminal motifs of Organic Anion Transporting Polypeptide 1B3 (OATP1B3) in regulating its expression levels and membrane trafficking. An abstract, poster and oral presentation at the 19th North American ISSX Meeting, San Francisco, CA, October 2014

ORAL PRESENTATIONS

1. Functional investigation of OATP1B3 variants in cancer. Oral presentation at the University of Kentucky, Pharmaceutical Sciences department seminar in Lexington, KY, June 2011
2. Functional investigation of novel csOATP1B3 variants in cancer. Oral presentation at the University of Kentucky, Pharmaceutical Sciences department seminar in Lexington, KY, March 2012

3. Cancer-specific OATP1B3 variant: Functions and trafficking mechanisms. Oral presentation at the University of Kentucky, Pharmaceutical Sciences department seminar in Lexington, KY, February 2013
4. Functional investigation of a novel cancer-specific OATP1B3 variant (csOATP1B3) in colon and pancreatic cancer. An abstract and oral presentation at the Annual Meeting of Pharmaceutics Graduate Student Research meeting in Iowa City, IA, 2013
5. Cancer-specific variant of OATP1B3: Functions, regulation and trafficking mechanisms. Oral presentation at the University of Kentucky, Pharmaceutical Sciences department seminar in Lexington, KY, October 2013
6. Hypoxia-Inducible Factor 1α- mediated regulation of the Cancer-Specific Variant of Organic Anion Transporting Polypeptide 1B3 (csOATP1B3) in Colon and Pancreatic Cancer. 4th Annual Symposium, University of Kentucky, College of Pharmacy in Lexington, KY, 2013
7. Applying basic science to personalize medicine in cancer. Elevator talk competition at University of Kentucky College of Pharmacy, Lexington, KY, 2013
8. Applying basic science to personalized medicine in cancer: Lessons learned. 3-Minute Thesis (3MT®) competition finalist at University of Kentucky, Lexington, KY, 2013
9. Role of N-terminal motifs of Organic Anion Transporting Polypeptide 1B3 (OATP1B3) in regulating its expression levels and membrane trafficking. An abstract and oral presentation at the 19th North American ISSX Meeting, San Francisco, CA, October 2014

PROFESSIONAL MEMBERSHIPS

- 2004-2008 Indian Pharmaceutical Association
- 2008 - present American Association of Pharmaceutical Scientists
- 2010 - present American Association for Cancer Research

- 2010 - present American Association for the Advancement of Science
- 2013- present International Society for the Study of Xenobiotics

Nilay Thakkar

Student's signature

January 12, 2015

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