GENOMIC ORGANIZATION AND FUNCTIONAL STUDY OF A NOVEL TUMOUR SUPPRESSOR GENE – *OKL38*

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NATIONAL UNIVERSITY OF SINGAPORE
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Summary

Cancers are often associated with the mis-regulation of oncogenes and tumour suppressor genes. This thesis described the molecular cloning and characterization of OKL38, a candidate tumour suppressor gene. OKL38 is a novel pregnancy-induced growth inhibitor previously identified and isolated in our laboratory. The 18 kb OKL38 gene residing in chromosome 16q23.3 contained 8 exons and 7 introns with exon size ranging from 92 to 1270 bp, was cloned and sequenced. Four novel variants of OKL38 were identified of which 3 were cloned and characterized. OKL38 was ubiquitously expressed in all tissues, with abundant transcripts detected in ovary, kidney, liver and testis.

Concurrently, the rat OKL38 gene was cloned and characterized in order to establish a rat model to further unveil the functions and regulations of OKL38. Three novel rat OKL38 variants were identified, cloned and characterized. The tissues expression profiles of the rat OKL38 variants and the genomic structure of rat OKL38 were deciphered. Using this rat model, the regulatory effects of pregnancy and hCG treatment on OKL38 expression in both the rat mammary gland and ovary was determined and the results suggested that OKL38 may have a protective role against carcinogenesis.

Low level of OKL38 transcripts was detected in several breast cancer cell lines. In this study, loss or down-regulation of OKL38 mRNA was observed in 70% (14 of 20) of the kidney tumours using the cancer-profiling array (CPA) of paired normal/tumour cDNA. Western blot analysis revealed that OKL38 protein was undetectable in 78% (7 of

9 pairs) of kidney tumour tissues and immunohistological analysis showed that 64% (14 of 22) of kidney tumours either lost or under expressed OKL38 protein as compared to the adjacent normal tissue. The OKL38 protein was also lost or reduced in 64.2% (18 of 28) of the HCC as compared to adjacent benign tissues and in all liver cancer cell lines examined. Immunohistochemical analysis demonstrated that OKL38 protein was undetected in 41.3% (38 of 92) of HCC, while 39.1% (36 of 92) showed weak staining. The lost or reduced expression of OKL38 correlated with high tumour stages ($p=0.0042$) as determined by non-parametric trend analysis. Our findings suggest that OKL38 plays an important role in tumour progression and indicate the potential use of OKL38 as biomarker for kidney and liver cancer.

Beside its growth inhibitory role in MCF7 cells, the biochemical functions of OKL38 are unknown. However, over-expression of HuOKL38-eGFP recombinant protein in A498 and Chang liver cells lead to protein aggregation and cell death. The same phenomenon was observed when the RtOKL38-eGFP recombinant protein is over-expressed in BRL cells suggesting that the OKL38 protein may play an important role in regulating cell death. Importantly, deletion studies revealed that the 5' untranslated region of OKL38 spliced variants played a critical role in regulating translation of OKL38 mRNA. Our data suggest that the loss of this protein may lead to the development and/or progression of cancers. A better understanding of the function of OKL38 in normal and tumour tissue may lead to the development of new preventive and therapeutic modalities for cancers of the kidney, liver, ovary and breast.

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List of Abbreviations

α-MEM	Alpha Modified Eagle medium
ABL	adjacent benign liver
AdoMetDC	S-Adenosylmethionine decarboxylase
AFP	Alpha fetoprotein
AgNOR	silver-stained nucleolar organizer region
AIF	apoptosis-inducing factor
AIFL	AIF-like
AMID	AIF-homologous mitochondrion-associated inducer of death
AP	activator protein
ATF4	activating transcription factor 4
AUG	translation start codon
BHD	Birt-Hogg-Dubé
BRL	Buffalo rat liver
C/EBP	CCAAT/enhancer binding protein
CA	carbonic anhydrase
cAMP	cyclic adenosine monophosphate
CFTR	cystic fibrosis cellular stress response
CG	chorionic gonadotropin
CNP2	2',3'-cyclic nucleotide 3'-phosphodiesterase
CPA	Cancer Profiling Array
CUGBP1	CUG repeats binding protein 1
DEPC	diethyl pyrocarbonate
DMBA	7, 12-dimethylbenz (a) anthracene
DPE	downstream promoter element
DTT	dithiotreitol
eGFP	enhanced green florescence protein
EMBL	European Molecular Biology Laboratory
ES	embryonic stem
FAD	flavin adenine dinucleotide

FBS	fetal bovine serum
FGF	fibroblast growth factor
FH	fumarate hydratase
FSH	follicle-stimulating hormone
FSH-R	FSH receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gpUL4	cytomegalovirus early glycoprotein
GSH	glutathione
HBV	hepatitis B virus
HBx	hepatitis B virus X gene
HCC	hepatocellular carcinoma
hCG	human chorionic gonadotropin
HCV	hepatitis C virus
HGF	hepatocyte growth factors
HIF	hypoxia-inducible factor
Hi-Glu-DMEM	High glucose Dulbecco's Modified Eagle Medium
HLRCC	Hereditary Leiomyoma Renal Cell Carcinoma
hnRNP	ribonucleoprotein
HP	hydrogen peroxide
HPRC	Hereditary Papillary Renal Carcinoma
HSP	heat shock protein
IGF	Insulin-like Growth Factor
IGFBP	IGF binding protein
IK2	Ikaros factor
IL	interleukin
Inr	initiator
IP	intraperitoneal
IRE	iron-response element
IRES	internal ribosomal entry segment
ITAF	initiation trans-acting factor
LH	luteinizing hormone

LH-CG-R	lutropin-choriogonadotropin-receptor
LH-R	luteinizing hormone receptors
LOH	loss of heterozygosity
LSS	physiological laminar shear stresses
mdm2	murine double minute gene 2
MEN	mendonine
MI	microsatellite instability
MLS	mitochondrial localization sequence
MM	multiple myeloma
MTE	Multiple Tissue Expression
MTN	Multiple Tissue Northern
MZF	myeloid zinc finger protein
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
NKX25	homeodomain factor Nkx-2.5/Csx
NLS	nuclear localization sequence
ODC	ornithine decarboxylase
OKL38	Ovary Kidney Liver 38 kDa
ORF	open reading frame
PAH	polycyclic aromatic hydrogen
PBS	phosphate buffer saline
PCBP1	poly-(rC)-binding protein 1
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PERK	PKR-like ER kinase
PRG3	p53-responsive gene 3
PS	penicillin-streptomycin
PTB	polypyrimidine tract-binding
PTEN	phosphatase and tensin homologue deleted on chromosome 10
Pyr_redox	pyridine nucleotide-disulphide oxidoreductase

RACE	Rapid Amplification of cDNA Ends
RAR	retinoic acid receptor
RCC	renal cell carcinoma
ROS	reactive oxygen species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
sAUG	start AUG (translation start codon at main ORF)
SCID	severe combine immunodeficiency
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEAP	secreted alkaline phosphatase
Sp1	stimulatory protein 1
SSC	Sodium chloride-Sodium Citrate solution
TBH	t-butyl-hydrogen peroxide
TBST	Tris Buffer Saline Tween-20
TGF	transforming growth factor
TNM	tumour node metastasis
TNT	transcription and translation
Trx	thioredoxin
TSH	thyroid-stimulating hormone
TXNRD	thioredoxin reductase
uAUG	upstream AUG
unr	upstream of N-ras
uORF	upstream ORF
USF	upstream stimulating factors
UTR	untranslated region
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau

Publications

International Publications

Choon Kiat Ong, Chuan Young Ng, Caine Leong, Chee Pang Ng, Chye Sun Ong, Thi Thanh Tuyen Nguyen and Hung Huynh. **Structural Characterization of three Novel Rat OKL38 transcripts, their tissue distributions, and their regulation by human chorionic gonadotropin**. *Endocrinology*. 2004 Oct; 145(10): 4763-74.

Ong CK, Ng CY, Leong C, Ng CP, Foo KT, Tan PH, Huynh H. **Genomic structure of human OKL38 gene and its differential expression in kidney carcinogenesis**.
The Journal of Biological Chemistry. 2004 Jan 2; 279(1):743-54.

Huynh H, Ng CY, Ong CK, Lim KB, Chan TW. **Cloning and characterization of a novel pregnancy-induced growth inhibitor in mammary gland**.
Endocrinology. 2001 Aug; 142(8):3607-15.

Conference Papers

Choon Kiat Ong, Chuan Young Ng, Caine Leong, Chee Pang Ng, Chye Sun Ong, Thi Thanh Tuyen Nguyen and Hung Huynh. **Structural Characterization of three Novel Rat OKL38 transcripts, their tissue distributions, and their regulation by human chorionic gonadotropin**. American Association for Cancer Research 95th Annual Meeting, American Association for Cancer Research, Inc., Florida, Orlando, USA. (March 27-31, 2004).

Ong CK, Ng CY, Leong C, Ng CP, Foo KT, Tan PH, Huynh H. **Genomic structure of human OKL38 gene and its differential expression in kidney carcinogenesis.** American Association for Cancer Research 94th Annual Meeting, American Association for Cancer Research, Inc., Washington D.C., USA. (July 11-14, 2003).

Oral Presentations

Choon Kiat Ong, Caine Leong, Hung Thanh Nguyen, Puay Hoon Tan, Tan Van and Hung Huynh. **The role of 5' Untranslated region in translational suppression of OKL38 mRNA in hepatocellular carcinoma.**
- Selected for NUS-NMRC Young Scientist Award at the Combined SingHealth and NHG Scientific Meetings 2005 (04-06 Nov), Raffles City Convention Centre, Singapore.

Choon Kiat Ong, Chuan Young Ng, Caine Leong, Chee Pang Ng, Chye Sun Ong, Thi Thanh Tuyen Nguyen and Hung Huynh. **Structural Characterization of three Novel Rat OKL38 transcripts, their tissue distributions, and their regulation by human chorionic gonadotropin.**
- Awarded the 3rd prize at the 5th Combined Annual Scientific Meeting (CASM) 2004, National University of Singapore.

Ong CK, Ng CY, Leong C, Ng CP, Foo KT, Tan PH, Huynh H. **Genomic structure of human OKL38 gene and its differential expression in kidney carcinogenesis**.
- Awarded the 1st prize at the 3rd Annual Graduate Student Society-Faculty of Medicine (GSS-FOM) Meeting 2003, National University of Singapore.
- Selected for Young Investigator's Award (Basic Science) at the 14th Annual Scientific Meeting 2003, Singapore General Hospital.

CHAPTER 1: LITERATURE REVIEW

1.1 Functional Domains of Pyridine nucleotide-disulphide oxidoreductase and TrkA

Proteins are modular in nature and its putative functions could be predicted via dissecting the domains. Some proteins that are essential for cell survival may have been functionally conserved throughout evolution, while others may have diversified to fulfil the needs of the complexity of organisms. Two of the evolutionary conserved domains, the pyridine nucleotide-disulphide oxidoreductase (Pyr_redox) and TrkA domain are being described.

1.1.1 Proteins containing Pyridine nucleotide-disulphide oxidoreductase domain are bifunctional

The Pyr_redox family of proteins consists of both class I and class II oxidoreductase, and also NADH oxidases and peroxidases. This family of proteins includes the well-characterized glutathione reductase and thioredoxin reductase proteins, which carry this domain in addition to a pyridine dimerization domain (Williams, 1995). It has been shown that the Thioredoxin (Trx) and Glutathione (GSH) system are involved in a variety of redox-dependent processes such as DNA synthesis, antioxidant defense and regulation of cellular redox state (Holmgren et al., 1998; Halliwell, 1999; Sies, 1999). In addition, the Trx and GSH systems regulate the activities of various transcription factors, kinases and phosphatases, and they are implicated in the redox control of cell growth and death, transcription, cell signalling, and other processes (Rhee,

1999; Finkel, 2000). In the recent years, several proteins containing the oxidoreductase domain have been cloned and characterized. These genes include the apoptosis-inducing factor (AIF), p53-responsive gene 3 (PRG3)/ AIF-homologous mitochondrion-associated inducer of death (AMID) and the AIF-like (AIFL) gene (Daugas et al., 2000; Wu et al., 2002; Ohiro et al., 2002; Xie et al., 2005), of which the AIF has been extensively studied.

AIF is ubiquitously expressed in all tissues and cancer cell lines examined indicating that it could be an essential gene for important functions in the cell (Daugas et al., 2000). AIF is a phylogenetically old flavoprotein, which is confined to the mitochondrial intermembrane space in healthy cells (Cande et al., 2002). Upon lethal signaling, AIF would translocate from the mitochondria to the nucleus and induces apoptosis (Susin et al., 1999b; Joza et al., 2001; Ravagnan et al., 2001; Lipton and Bossy-Wetzel, 2002; Yu et al., 2002; Hansen and Nagley, 2003; Cregan et al., 2004; Cande et al., 2004;). Over-expression of AIF can cause apoptosis independent of caspases (Susin et al., 1999a; Susin et al., 1999b), although one recent study demonstrated that AIF over-expression was insufficient to induce apoptosis (Wu et al., 2002). In addition, AIF functions as a free radical scavenger (Klein et al., 2002) and plays a role in normal mitochondrial oxidative phosphorylation (Vahsen et al., 2004). A recent study demonstrated that AIF could suppress chemical stress-induced apoptosis involving reactive oxygen species (ROS) (Urbano et al., 2005). The precursor of mammalian AIF protein contains an N-terminal mitochondrial localization sequence (MLS, residues 1-100) and a large C-terminal portion (121-610) that share similarity with bacterial oxidoreductase (Susin et al., 1999b). The human mature AIF has very similar crystal structure to oxidoreductase (Ye et al., 2002) and displays NAD(P)H oxidase as well as

monodehydroascobate reductase activities (Miramar et al., 2001). The NADH oxidase activity of AIF is essential for maintaining the transformed state of colon cancer cells (Urbano et al., 2005). While studies have shown that the oxidoreductase function of AIF is not required for its apoptogenic function (Loeffler et al., 2001; Miramar et al., 2001). Similar to cytochrome c, AIF is a bifunctional protein with an electron acceptor/donor (oxidoreductase) function and a second apoptogenic function.

Heat shock proteins (HSP) are associated with AIF and may regulate its functions. The mitochondrial matrix protein HSP60 (heat shock protein 60) has an overlapping expression pattern to that of the AIF, as well as an identical subcellular distribution in histological section of human tissues (Daugas et al., 2000). HSP70 is known to show inhibitory properties to the apoptotic function of AIF (Ravagnan et al., 2001), suggesting that the HSP family of proteins may regulate the function of AIF *in vivo*.

Following the discovery of AIF, the homologs of this gene were identified namely, AMID/PRG3 (Wu et al., 2002; Ohiro et al., 2002; Wu et al., 2004) and AIFL (Xie et al., 2005). AIFL and AMID/PRG3, which are distantly related to AIF, have recently been described to exert a pro-apoptotic function. The AMID/PRG3 protein, like AIF, has significant homology with NADH oxidoreductase/flavoproteins from bacterial to mammalian species, but do not possess a recognizable MLS (Wu et al., 2002; Ohiro et al., 2002). Recent reports about the localization of AMID/PRG3 have been unclear (Wu et al., 2002; Ohiro et al., 2002). Wu *et al*. (2002) observed that AMID/PRG3 associate with the outer mitochondrial membrane and tissue distribution was undetectable in heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocyte; while expression

could be detected easily in colon cancer cell lines DLD and HCT116 and weak expression in B lymphoma cell line RPMI8226. On the other hand, Ohiro *et al*. (2002) reported the localization of AMID/PRG3 in the non-mitochondrial cytoplasm with high level of expression in heart, moderate level in liver and skeletal muscle, and low level in the placenta, lung, kidney and pancreas (Ohiro et al., 2002). Both groups have collectively shown that AMID/PRG3 expression is inducible by p53 (Ohiro et al., 2002; Wu et al., 2004) and down-regulated in tumour (Wu et al., 2004). Similar to AIF, the over-expression of AMID induces apoptosis in a caspase independent manner (Wu et al., 2002).

The recently characterized human AIFL, a homolog of AIF, contains a characteristic Rieske and a Pyr_redox domain (Xie et al., 2005). AIFL bears similar expression profiles to AIF and is predominantly localized to the mitochondria inner membrane. In addition, over-expression of AIFL induced apoptosis through the segment 1-190 containing the Rieske domain (Xie et al., 2005). AIFL is distinct from AIF in that induction of apoptosis is caspase-dependent (Xie et al., 2005). In all cases mentioned, the oxidoreductase domain in the AIF, AMID/PRG3 and AIFL does not seem to play a part in their apoptogenic function (Miramar et al., 2001; Loeffler et al., 2001; Wu et al., 2002; Ohiro et al., 2002; Xie et al., 2005).

A candidate tumour-suppressor gene, WW domain-containing oxidoreductase (WWOX/WOX1/FOR) has been reported to possess aberrant behaviours in breast, ovary, esophagus, lung, and liver cancers (Bednarek et al., 2000; Paige et al., 2001; Yakicier et al., 2001; Driouch et al., 2002; Kuroki et al., 2002; Yendamuri et al., 2003; Park et al., 2004). In addition to the oxidoreductase domain, the WWOX also contain two N-terminal

WW domains and a nuclear localization sequence (NLS) (Chang, 2002). Over-expression of the full-length or the WW domain of WWOX sensitizes the TNF-resistant Cos7 cells to TNF killing (Chang et al., 2001). All these examples seem to suggest that genes containing the oxidoreductase domain may be bifunctional; (i) involved in regulating cellular redox and (ii) regulating cell death.

1.1.2 Evolutionary conservation of potassium channels containing TrkA domain

Potassium (K^+) channels are ubiquitous in the animal and plant kingdoms and in yeast and bacteria exhibiting extraordinary heterogeneity among these organisms while preserving several salient features (Hille, 1992). These potassium channels contribute to the control of potassium flow, cell volume, release of hormones and transmitters, resting potential, and excitability of neurons and muscles (Jan and Jan, 1997). Two K^+ uptake systems exist in the bacteria, *Escherichia coli*; consisting of the inducible Kdp system and the Trk system, which is constitutively expressed (Rhoads et al., 1976). The Trk consists of a transmembrane protein named TrkH or TrkG, which is the actual K^+-translocating subunit, and the cytoplasmic membrane surface protein TrkA, which is a NAD^+ binding protein (Bossemeyer et al., 1989; Schlosser et al., 1993). The structure of TrkA protein consists of two similar halves: (i) N-terminal part of each TrkA half (residues 1-130 and 234-255, respectively) is similar to the complete NAD^+-binding domain of NAD^+-dependent dehydrogenases; (ii) C-terminal part of each TrkA half (residues 131-233 and 357-458, respectively) aligns with the first 100 residues of the catalytic domain of glyceraldehydes-3-phosphate dehydrogenase (Schlosser et al., 1993).

Jan and Jan (1997) have highlighted the similarity between the Trk system in bacteria and the voltage-gated potassium channels in mammals. The mammalian voltage-gated potassium channels consist of the transmembrane α-subunits and the cytoplasmic associating β-subunits. The β-subunits is postulated to play a role in regulating the channel activity according to the level of energy and/or reducing power of the cell (Jan and Jan, 1997). The β-subunits of the voltage-dependent K^+ channels seems to be the functional mammalian homologs of the bacteria TrkA protein. The β-subunits sequences show significant similarity to members of the NAD(P)H-dependent oxidoreductase superfamily (McCormack and McCormack, 1994), containing a potential NAD(P)H-binding motif but lacks membrane-spanning sequences (McCormack and McCormack, 1994; Scott et al., 1994; Rettig et al., 1994).

1.2 Translation control by 5' Untranslated region

Regulation of gene expression can be achieved at various levels, such as transcription, post-transcriptional processing, mRNA stability, translation, post-translational modifications and protein degradation. At the translation level, regulation of protein expression is crucial, especially in the absence of transcription as seen in early developmental stages or during cellular stress where general translational mechanism is inhibited. The presence of cis-elements in the 5' and 3'-untranslated regions (UTR) play an important role in determining the translational efficiency and the initiation site for protein synthesis. Some of these elements include upstream ORF (uORF), upstream AUG (uAUG), GC-content, iron-response element (IRE), internal ribosomal entry segment (IRES) among others. Together with the ribosomal scanning process, complex

translational regulatory mechanisms are achieved to ensure proper regulation of proteins involved in cellular functions.

1.2.1 Kozak sequence and translation initiation mechanisms

In higher eukaryotes, the ideal Kozak consensus sequence is CC**R**CCAUG**G**, with the most conserved nucleotides being **R** (A or G) at –3 and **G** at +4 (the A of the AUG codon is designated as +1) (Kozak, 1981; Kozak, 1984; Kozak, 1986). These Kozak sequences would generally be present at the AUG of the main ORF (95-97% of the cases), while only 43-63% of uAUG contain these consensus sequences (Meijer and Thomas, 2002). In addition, the presence of a hairpin, approximately 14nt downstream of the AUG is necessary for effective initiation of translation (Kozak, 1990).

The uAUGs can induce the formation of a translation-competent ribosome that may translate and (i) terminate and re-initiate, (ii) terminate and leave the mRNA, resulting in down-regulation of translation of the main ORF, or (iii) synthesize an N-terminally extended protein (Meijer and Thomas, 2002). The 'leaky scanning model' described that in the presence of uAUG, some of the ribosomes in the scanning initiation complex may skip the uAUG and initiate at the main ORF [start AUG (sAUG)] (Kozak, 1978). In the presence of uORF, the re-initiation model being a very inefficient mechanism is proposed. In this case, the 40S ribosomal subunit remains connected to the mRNA after termination at the uORF and resumes scanning. Initiation can only take place at the next AUG provided that the 40S subunit is reloaded with eIF2-GTP-Met-$tRNA_i$ (Miller and Hinnebusch, 1989).

1.2.2 Upstream ORF and AUG regulate translation of mRNAs

In most cases, 5'UTRs that enable efficient translation are short, have low GC content, are relative unstructured and do not contain uAUG codon (Kochetov et al., 1998). The leader sequences of approximately 90% of vertebrate mRNAs examined to date are between 10 and 200 bases long, yet two thirds of the mRNAs known to encode proto-oncogenes or factors related to cell proliferation contains atypical 5' untranslated regions which are more than 200 bases long and/or contain more than one AUG codon (Kozak, 1987; Kozak, 1991). In general, the presence of uAUGs in the 5'UTR would decrease the initiation efficiency on the sAUG preceding the main ORF (Morris and Geballe, 2000). A summary of the 5'UTRs discussed in the following text and their mechanisms of uORF-mediated control were illustrated in figure 1.1.

The uAUGs and uORFs have been demonstrated to play important roles in mediating translational control during cellular stress. One of the classical examples is the translation regulation of GCN4 transcription factor in the yeast, which is involved in amino acid synthesis (Hinnebusch, 1996). The 5'UTR of the GCN4 contains 590 nt and four uORFs (Fig. 1.1). Under normal conditions, the ribosomal subunits are capable of re-initiation upon reaching uAUG4, translate the uORF4, terminate and leave the mRNA, without translating the GCN4 ORF. When general protein synthesis is inhibited, only half of the ribosomal complexes are in time for re-initiation and termination at uORF4; while the rest will only be reloaded with eIF2-GTP-Met-tRNA$_i$ for initiation after scanning the remaining 150 nt of the 5'UTR, enabling the translation of the GCN4 ORF.

The translation of activating transcription factor 4 (ATF4) in the mammal is also inducible by stress (Harding et al., 2000). The mouse ATF4 5'UTR (272 nt) contains two

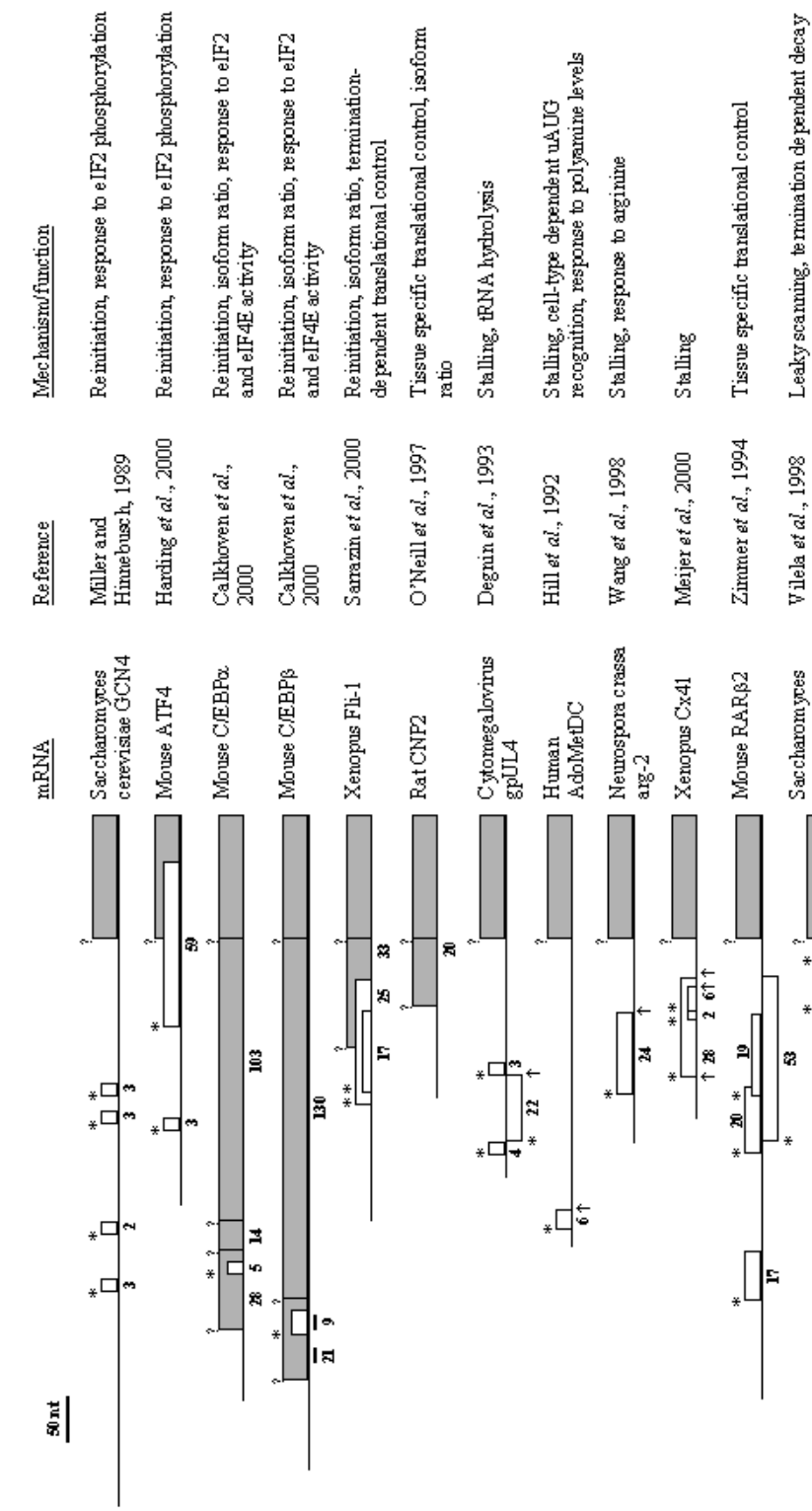


Figure 1.1: Schematic representation of uORF-containing 5'-UTRs. Summary of the 5'-UTRs under uORF-mediated control. Main ORFs are depicted by grey boxes, whereas uORFs are indicated by white boxes. The (*) and (?) indicate the various translational start sites. The numbers under the uORFs indicate the number of amino acids in the encoded peptides. Ribosomal stalling sites are represented with ascending arrows (↑). The lines under the 5'-UTR of C/EBPβ mark the CUGBP1 binding sites. (Adapted from Meijer and Thomas, 2002)

uORFs, of which the second uORF overlaps with the ATF4 ORF (Fig. 1.1). Stress activates the mammalian eIF2 kinases PERK (PKR-like ER kinase) and GCN2, which repress translation of most mRNAs by phosphorylating eIF2 but selectively increase translation of ATF4. Mutation in either of the 2 uAUGs leads to drastic increase in translational efficiency suggesting that these uAUGs possess a general translation suppression function.

Another regulatory function of uAUGs is to determine the initiation at different AUG codons, resulting in synthesis of proteins with N-terminal of different length. The CCAAT/enhancer binding protein (C/EBP) family of transcription factors, which are important for differentiation and cell proliferation. Both the C/EBPα and C/EBPβ mRNAs contain an uORF in a reading frame different from the C/EBP reading frame (Fig. 1.1). Four (C/EBPα) or three (C/EBPβ) potential translation initiation sites are present in the same reading frame, enabling the synthesis of several proteins with different N-terminal extension and an identical C-terminal DNA-binding domain (i.e. activating domain is missing from the shorter isoforms with truncated N-terminal). Therefore the isoforms ratio is crucial for the activity of this protein and is dependent on the presence of the out-of-frame uORF. The expression of the shorter isoforms is dependent on the re-initiation after the uORF translation (Calkhoven et al., 2000). The binding of CUG repeat binding protein 1 (CUGBP1) to the CUG repeats 5' to the uORF and the CCG repeats in the uORF of C/EBPβ stimulates the translation of truncated C/EBP isoforms, and therefore inhibiting C/EBP-dependent transcription of target gene (Timchenko et al., 1999). The translation of proto-oncogene *fli-1* is regulated by two uORFs, which determine the synthesis of two protein isoforms (i.e. 48 kDa and 51 kDa).

Initiation at the 48 kDa isoform AUG is stimulated by enabling re-initiation after the translation of the uORF (Sarrazin et al., 2000). Similar mechanism also regulates translation of the 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP2) mRNA (Fig. 1.1) (O'Neill et al., 1997).

Stalling of ribosomes at uORFs is another important mechanisms employed to regulate the translation efficiency of the main ORF of mRNA. Interestingly, the peptide sequences encoded by the uORFs, not the nucleotide sequences, are involved in the repression of translation of the human cytomegalovirus early glycoprotein (gpUL4) (Cao and Geballe, 1995), S-Adenosylmethionine decarboxylase (AdoMetDC) (Ruan et al., 1996), arg-2 (Fang et al., 2000), and *Xenopus lavis* connexin-41 (Meijer et al., 2000) mRNA (Fig. 1.1). Beside uORFs, stalling mechanism can be due to the presence of secondary structure (Ruegsegger et al., 2001) or adenine-rich element in the 5'UTR as in the case of PABP1 mRNA (Bag, 2001).

uORFs in the long 5'UTR of mouse retinoic acid receptor β2 (RARβ2) have been shown to be involve in the regulation of translation efficiency and localization-dependent translation (Zimmer et al., 1994; Reynolds et al., 1996). The translation of two uORFs in the yeast transcription factors YAP2 5'UTR stimulates the decay of the YAP2 mRNA (termination-dependent decay) (Vilela et al., 1999). These examples illustrate the versatility of uORFs or uAUGs in regulating translation of mRNA.

1.2.3 Internal ribosome entry segment and their cognate transacting factors orchestrate the regulation of 5'UTRs translation

Two distinct mechanisms, cap-dependent scanning and internal ribosome entry are involved in the translation initiation in eukaryotic cells. Internal translation initiation

requires the formation of a complex RNA structure element termed internal ribosome entry segment (IRES), which is a complex RNA scaffold containing multiple sites for interaction with components of the translational apparatus such as eIF4G and eIF4E (Kolupaeva et al., 1998; Lopez and Martinez-Salas, 2000; Martinez-Salas et al., 2001). IRES are usually located in the 5'UTR and may interact with trans-acting factors to recruit ribosome to initiate internal translation. It is estimated that about 10% of the genes may contain IRES and usually these genes are involved in control of cell growth and cell death (Stoneley and Willis, 2004). The IRES identified to date are generally GC-rich suggesting complex secondary structure. However, no common structure features have been found between IRES from different genes or closely related ones (Le Quesne et al., 2001; Stoneley and Willis, 2004; Jopling et al., 2004). It has been proposed that IRES functions as multiple structures modules and may also be involved in cap-dependent scanning mechanism (Stoneley et al., 2000; Le Quesne et al., 2001). RNA-RNA interactions between the 5' and 3' end of the IRES (Yaman et al., 2003) and presence of pseudoknot can inhibit its function (Le Quesne et al., 2001; Yaman et al., 2003). Primary sequence elements that are implicated in internal initiation have been identified in the IRESs of *Gtx, Rbm3 and fgf2* (Chappell et al., 2000a; Bonnal et al., 2003; Chappell and Mauro, 2003). The function of these elements in the context of IRES is still unclear, but may be involved in ribosome recruitment (Stoneley and Willis, 2004).

Factors that interact with IRESs known as internal initiation trans-acting factors (ITAFs) have been identified in recent years (Holcik and Korneluk, 2000; Mitchell et al., 2001; Evans et al., 2003; Holcik et al., 2003; Pickering et al., 2003). ITAFs are generally located in the nucleus and shuttling between the nucleus and cytoplasm may constitute to

its function with IRES. Two of the ITAFs, upstream of N-ras (unr) and polypyrimidine tract-binding (PTB) protein, form a specific RNA-protein complex with Apaf-1 IRES and collectively stimulate Apaf-1 internal initiation (Mitchell et al., 2001). A similar mechanism has also been documented in the Bag-1 IRES. The RNA chaperone activities of PTB and poly-(rC)-binding protein 1 (PCBP1) unwind a specific region of the Bag-1 IRES structure, thus stimulating its function (Pickering et al., 2003). ITAFs such as PTB can serve as a repressor of Bip internal initiation (Kim et al., 2000), suggesting that ITAFs, together with the IRESs could have either positive or negative regulatory effect on internal initiation (Stoneley and Willis, 2004). Others examples of ITAFs have been extensively described by Stoneley and Willis (2004).

Despite extensive research on IRESs and ITAFs, the molecular mechanism of IRES is still unclear. Yaman *et al* (2003) have proposed that the activity of cat-1 IRES is regulated by two sequential events. This involves an initial translation of a small uORF within the 5'UTR that cause structure remodeling of the IRES, leading to the attainment of an active IRES conformation; followed by the induction of cat-1 IRES activity, which is dependent on the phosphorylation of eIF2α. However, it is clear that IRES is implicated in the regulation of gene expression in diverse physiological states (i.e. amino acid starvation, cell-cycle arrest, programmed cell death, hypoxia, etc) enabling cells to respond to these conditions against the background of a general reduction in protein synthesis.

1.2.4 Cancer derived from mis-regulation of translation by 5'UTR

The translation initiation factor, eIF4E is associated with tumourigenesis as many mRNAs associated with growth contains highly structured 5'UTRs. Over-expression of

eIF4E relieves the translational repression imposed on structured 5'UTRs (Koromilas et al., 1992; Zimmer et al., 2000) and increased level is associated with a number of tumour types, including breast, head-and-neck, bladder, liver and colon cancer (Kevil et al., 1995; Rousseau et al., 1996; Nathan et al., 1997; DeFatta et al., 1999; Zimmer et al., 2000; Crew et al., 2000). The elevated level of eIF4E was suggested to increase the translation of oncogenes with highly structured 5'UTRs, such as c-myc and ornithine decarboxylase (ODC), and mRNAs that encode proteins involved in angiogenesis, such as fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) (Kevil et al., 1995; Rousseau et al., 1996; Nathan et al., 1997; DeFatta et al., 1999; Zimmer et al., 2000; Crew et al., 2000).

De-regulation of translation of mRNAs containing translation-inhibitory 5'UTRs often occurs by the production of alternate transcripts with shorter version of the 5'UTR that is often translated efficiently. Genes regulated in this manner include members of the transforming growth factor beta (TGF-β), BRCA1, and the murine double minute gene 2 (mdm2). The TGF-β1 expresses three transcripts, differing in their translational efficiencies due to the alternative length of their 5'UTRs, all are GC-rich and involved in the inhibition of translation of this mRNA (Kim et al., 1992; Romeo et al., 1993; Allison et al., 1998). The TGF-β3 mRNA has a long 1.1 kb 5'UTR that is inhibitory to translation with de-regulated expression being associated with disease. Many human breast cancer cell lines expressed a novel TGF-β3 transcript that possess a 5'UTR which is 870 nucleotides shorter than the longer version of 1.1 kb. This mRNA isoform with short 5'UTRs confer 7-fold higher translational efficiency, thus leading to cell transformation (Arrick et al., 1991; Arrick et al., 1994).

Over-expression of BRCA1 protein results in growth inhibition of tumours (Holt et al., 1996). BRCA1 mRNAs can be expressed from two promoters, α and β, resulting in alternative exon 1a and 1b. Incidentally, the ORF begins in exon 2 resulting in mRNAs with different 5'UTRs. BRCA1b transcripts were found to be expressed in breast cancer tissues but not in normal mammary glands, where the BRCA1a transcripts were predominantly expressed (Sobczak and Krzyzosiak, 2002). The BRCA1b mRNA was found to have a longer 5'UTRs with comparably stable secondary structure and uAUG, which together contribute to a 10-fold reduction in translational efficiency compared to BRCA1a (Sobczak and Krzyzosiak, 2002). Thus, loss of BRCA1 protein in breast cancer is partially due to the preferential expression and translational suppression of BRCA1b mRNA.

The Mdm2 gene encodes a nuclear phosphoprotein, which binds to p53 tumour-suppressor gene for destruction by the ubiquitin-dependent proteasome pathway. Two constitutively expressed transcripts of mdm2 were derived from alternative promoters. The longer transcript is inhibitory to scanning by ribosome, while the other variant, which is inducible by p53 contains a short 5'UTR (68 nt) that is translated more efficiently than the former (Landers et al., 1997). In Burkitt's lymphoma cells with wild type p53, over-expression of Mdm2 is due to enhanced translation, through up-regulation of mdm2 transcripts with short 5'UTR (Capoulade et al., 1998, 2001).

IRESs may be present in the long 5'UTRs and the mechanisms of IRES-regulated translation were described in *Section 1.2.3*. The ability of IRESs to function under condition of cell stress is particularly relevant for tumour progression. IRESs have been demonstrated to drive translation of angiogenic growth factors, such as FGF2 and VEGF

under hypoxic condition, when cap-dependent translation is compromised (Stein et al., 1998; Akiri et al., 1998; Huez et al., 1998; Townsend et al., 2003). In addition, transcription of VEGF could be activated by the transcription factor hypoxia-inducible factor 1 (HIF-1), which contains IRES that function during hypoxia (Lang et al., 2002). These cooperative regulations of protein translation during hypoxic condition promote tumour growth.

Aberrant translational regulation through an IRES-mediated mechanism has been identified in the human neoplasia multiple myeloma (MM). Significantly increased level of c-myc protein was found in MM cell lines and correlated to a single C to U transition in the c-myc 5'UTR (Paulin et al., 1996). The same mutation was also identified in 42% of bone marrow samples collected from MM patients (Chappell et al., 2000b). This mutation enhances the interaction between the ITAF, hnRNPK, and IRES, resulting in increased activity of the c-myc IRES (Paulin et al., 1998; Evans et al., 2003). Thus leading to over-expression of c-myc protein resulted in expansion of a plasma cell type in the bone marrow and osteolysis. These examples strongly illustrated the importance of 5'UTRs functions in regulating translation of genes related to cancers and their expression is associated to the progression of the disease.

1.3 Hepatocellular Carcinoma

The major risk factors and etiological agents responsible for development of hepatocellular carcinoma (HCC) in humans have been extensively identified and characterized. Among these include chronic infection with hepatitis B and C virus, exposure to aflatoxin B_1, and cirrhosis of any etiology. Generally, the neoplastic transformation of hepatocytes is the result of the accumulation of genetic damage during

the repetitive cellular proliferation that occurs in the injured liver in response to paracrine growth factor and cytokine stimulation. These genetic alteration including chromosomal deletion, rearrangements, aneuploidy, gene amplifications, and mutations, as well as epigenetic alteration, resulting in activation of proto-oncogenes and inactivation of tumour-suppressor genes, leading to cells acquiring autonomous growth potential. The mechanisms of hepatocarcinogenesis and potential biomarkers for HCC are briefly discussed in the following sections.

1.3.1 Epidemiology and Etiology of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) accounts for more than 90% of all primary liver cancers. HCC represents approximately 40% of all cancers in South-East Asia, Japan and Africa (Akriviadis et al., 1998), with much lower incidence rates found in Europe and the Americas (Mathers et al., 2002). High incidence of HCC is found in China, where men exhibit an incidence rate of approximately 36 cases per 100,000 populations (Parkin et al., 1999; Mathers et al., 2002). Extremely high incidences of HCC of 113 cases per 100,000 populations are observed among black males in Mozambique (Higginson, 1963). These observations strongly suggest that factors related to genetic background and/or environmental exposure contributes significantly to the incidence of this tumour among world population.

Estimates from the year 2000 indicate that liver cancer remains the fifth most common malignancy in men and the eighth in women worldwide with number of new cases estimated to be 564,000 per year (Bosch et al., 2004). Men are more prone to develop liver cancer compared to women. In the United State, ratio of male to female incidence is approximately 2:1 (Jemal et al., 2003), and world-wide is approximately

2.6:1 (Parkin et al., 1999). However, in high incidence countries or world region, the male to female incidence ratio can be as high as 8:1 (Simonetti et al., 1991). This consistently higher incidence in men than women suggest that sex hormone and/or their receptors may play a significant role in the development of primary liver tumours.

The etiology of human hepatocellular carcinoma is multi-factorial (Hassan et al., 2002). Causative factors such as exposure to naturally occurring carcinogens, industrial chemicals, pharmacologic agents, and various pollutants, which may contribute to the development of HCC have been identified (Grisham, 1995; Grisham, 1997). Exposure to hepatotoxic drugs and chemicals such as the radioactive compound thorium dioxide (used in imaging of blood vessels) (Andersson et al., 1994), vinyl chloride monomer (Du and Wang, 1998), pesticides (Austin et al., 1987), anabolic steroids and oestrogen (Vainio and Wilbourn, 1993); and inorganic arsenic compounds (Chen et al., 1992) can lead to the development of liver cancer.

Genetic liver diseases, mostly related to inborn errors of metabolism that result in the accumulation of metabolic products in hepatocytes, can lead to chronic hepatitis and cirrhosis, unfortunately, increases the risk of development of HCC (Hadchouel, 1994). These genetic conditions include hemochromotosis (Deugnier and Turlin, 2001), hereditary tyrosinemia (Russo and O'Regan, 1990), glycogen storage diseases (Bianchi, 1993), Wilson's disease (Wilkinson et al., 1983) and α1-antitrypsin deficiency (Propst et al., 1994).

Other factors such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and alcohol consumption contribute to the risk for development of HCC (International Agency for Research on Cancer, 1994c; Grisham, 1995; Grisham, 1997;

Schafer and Sorrell, 1999; Ince and Wands, 1999). Chronic infection with either HBV or HCV frequently results in chronic hepatitis and cirrhosis, which greatly increases the risk for the development of HCC in humans (100-fold to 200-fold) compared to uninfected individuals (International Agency for Research on Cancer, 1994b; Bailey and Brunt, 2002). Chronic alcohol consumption is associated with an elevated risk for HCC (Ikeda et al., 1993; Nalpas et al., 1995; Noda et al., 1996). Chronic liver damage caused by alcohol contributes secondarily to liver tumour formation (Simonetti et al., 1992). Ethanol appears to potentiate the hepatocarcinogenicity of chronic HBV and HCV infection (Nalpas et al., 1995; Noda et al., 1996). Besides alcohol consumption, tobacco smoking is another important risk factor (Trichopoulos et al., 1980; Doll, 1996).

Another major hepatocarcinogenic risk for human is the dietary consumption of aflatoxins B_1 in food grains contaminated with *Aspergillus flavus* and related fungi (International Agency for Research on Cancer, 1994c; Schafer and Sorrell, 1999; Ince and Wands, 1999). Rice or other grains under improper storage conditions (Zimmerman H.J., 1978; Zimmerman H.J., 1999) lead to the growth of these aflatoxin producing moulds. Aflatoxin B_1 is a potent, direct acting carcinogen in humans and chronic exposure leads inevitably to development of HCC (International Agency for Research on Cancer, 1994a). In common among all of these etiologic factors is the risk for development of chronic hepatitis and cirrhosis.

1.3.2 Molecular pathogenesis of hepatocellular carcinoma

Continuous intensive studies have shed light on the molecular mechanisms of hepatocarcinogenesis (Grisha, 2002; Feitelson et al., 2002; Thorgeirsson and Grisham, 2002; Coleman, 2003). Aneuploidy and abnormal chromosome numbers is a well-

documented feature of HCC. Approximately 40% of HCC exhibit aneuploid DNA content and numerical chromosomal abnormalities (Mise et al., 1998; Kato et al., 1998). The presence of aneuploidy in dysplastic lesions suggests that genetic abnormalities may be a significant determinant of neoplastic transformation in the liver (Terris et al., 1997). The majority of cultured cells derived from HCC contained either a sub-tetraploid chromosome number (64-99 chromosomes) or near diploid (Bardi et al., 1998; Parada et al., 1998). The chromosomal regions most frequently involved in structural rearrangements include 1p11, 1p22, 1p32, 1p34, 1p36, 1q10, 1q12, 1q25, 6q13-q15, 6q22-q25, 8q10, 16q24, and 17p11 (Grisha, 2002). In a particular study, chromosomes 4q, 8p and 16q were affected in HBV-related HCC, while 11q was affected in HCV-related HCC (Wong et al., 2000), indicating that specific genetic abnormalities can be attributed to specific etiological agents or mechanisms.

Chromosomal gains have been described in HCC involving 1p, 4q, 5p, 6q, 8p, 9p, 10q, 11q, 12q, 16q, 17p, and 19p, which may lead to gene amplification. Over-expression of proto-oncogenes may alter the intracellular signalling pathways leading to the unregulated cellular proliferation. These signalling pathways involve a number of positive mediators of cellular proliferation, including the protein products of the c-ras gene family, c-myc, c-fos and, various cyclins and cyclin-dependent kinases (Grisha, 2002). This is supported by recent studies that demonstrate pronounced gain in copy numbers of c-myc for all 20 HCCs evaluated (Raidl et al., 2004). Other studies showed that the c-myc gene was amplified in 31% (50/163) of HCC examined (Lee et al., 1988; Fukuda et al., 1988; Peng et al., 1993; Abou-Elella et al., 1996; Kawate et al., 1999a) and

over-expression of c-myc mRNA in more than 90% of HCC (Su et al., 1985; Zhang et al., 1990).

The hepatitis viral proteins displayed a pronounced effect on the mechanism of hepatocarcinogenesis (Feitelson et al., 2002). The ability of the HBx oncogenic protein to induce c-myc and other proto-oncogenes has been demonstrated using microarray analysis (Wu et al., 2001). The levels of expression of c-myc protein are also increased in chronic hepatitis (Tiniakos et al., 1993), and cirrhosis (Tiniakos et al., 1989; Gan et al., 1993). In addition, over-expression of c-myc in HCC could also be due to an up-regulation of the MAPK pathway (Grisha, 2002) and hypomethylation. The c-myc gene was found to be hypomethylated in 55% (15/27) of HCC and in significant percentage of cirrhotic liver adjacent to HCC (Nambu et al., 1987; Kaneko et al., 1985). These observations indicate that various mechanisms are involved in the genetic alteration of a single gene such as c-myc, leading to hepatocarcinogenesis. Interestingly, HCC due to HBV and HCV were also postulated to be an indirect result of enhanced hepatocytes turnover that occurs in an effort to replace infected cells that have been immunologically attacked (Block et al., 2003).

Major growth factors implicated in hepatocellular carcinoma including TGFα, insulin-like growth factor II (IGF-II), hepatocyte growth factors (HGF), and others were extensively described (Grisha, 2002; Feitelson et al., 2002; Coleman, 2003). More than 20 HCC associated genes have been identified and they are found either down- or up-regulated or mutated in HCC (Zeng et al., 2002). Using DNA microarrays, hundreds of genes involved in progression and metastasis of HCC have been identified (Ye et al., 2003; Patil et al., 2005). Over-expression of IGF-II and loss of IGF binding protein 3

(IGFBP3) were observed in a high proportion of HCC samples (Huynh et al., 2002). Over-production of pRb2/p130 in HepG2 cells led to growth suppression, cell cycle arrest at G0/G1, altered cell morphology, inhibition of *in vitro* colony formation and reduction in tumourigenicity in SCID mice (Huynh, 2004). These studies indicate that deregulation or functional loss of genes encoding growth factors leads to the development and progression of cancers.

Microsatellite instability (MI) is a specific form of genomic instability that is characterized by mutational alteration of simple repetitive sequences, including both insertional and deletional mutagenesis, typically resulting in frameshift mutations. (Coleman and Tsongalis, 2002). Microsatellite alteration involving the TGFβRII, M6P/IGFIIR, and BAX genes, which are critical for growth control and regulation of apoptosis, has been reported (Yakushiji et al., 1999). Microsatellite alteration involving at least two loci was detected in approximately 28% of the HCCs exhibiting MI (Horii et al., 1994; Salvucci et al., 1999), while others fail to identify MI in HCC (Kawate et al., 1999b; Rashid et al., 1999). These studies suggest that MI may be mechanistically involved with the molecular pathogenesis of a subset of HCC (Coleman and Tsongalis, 2002).

The loss or progressive loss of tumour-suppressor genes and other negative mediators of cellular proliferation have been implicated in the molecular pathogenesis of HCC (Elmore and Harris, 1998; Grisha, 2002; Feitelson et al., 2002; Staib et al., 2003; Coleman, 2003). These tumour-suppressor genes and tumour-suppressor-like genes include *p53, Rb1, p73, mdm2, APC, β-catenin, E-cadherin, PTEN, BRCA1, FHIT*, *WWOX* and others (Grisha, 2002). *E-cadherin* and *WWOX* are two of the potential

tumour-suppressor genes residing on chromosome 16q22 and 16q23, respectively (Niketeghad et al., 2001; Slagle et al., 1993; Park et al., 2004). The region 16q23 have been described as the second most active fragile site (FRA16D) in human the genome (Glover et al., 1984; Bednarek et al., 2000; Bednarek et al., 2001; Ludes-Meyers et al., 2003) and both E-cadherin and WWOX reside on or close to FRA16D. LOH of 16q correlates with metastasis and loss of these genes via LOH have been described (Slagle et al., 1993; Niketeghad et al., 2001; Park et al., 2004). E-cadherin functions as a receptor in adherent junctions and is essential for the maintenance of tissue architecture. Mutations in E-cadherin causes its inability to bind β-catenin, which leads to the accumulation of the latter in the cytoplasm and nucleus, resulting in constitutive Wnt activation and cell proliferation (Barth et al., 1997). In addition to LOH, hypermethylation of the E-cadherin promoter has been proposed as an alternative mechanism for Wnt activation in HCC (Kanai et al., 1997). The functions of WWOX in cancer have been described in *Section 1.1.1*. In view of all the evidences, HCC is a very complex disease and genetic alteration leading to unregulated cellular proliferation resulting in hepatocarcinogenesis.

1.3.3 Prognostic and diagnostic molecular markers for hepatocellular carcinoma

The molecular mechanisms of hepatocarcinogenesis are complex, constantly evolving and heterogeneous, which explains the generally poor treatment outcomes for HCC. The majority of patients with HCC have inoperable disease with very poor prognosis (Okuda et al., 1985). Five-year survival rate is limited to 25 to 50% after surgery (Colombo, 1992; Lai et al., 1995; Takenaka et al., 1996). Long-term survival is uncommon because of the frequent recurrence, metastasis or the development of new

primary tumours (Lai, 1994; Huguet, 2000). Current adjuvant or palliative treatment modalities have not effectively prolonged survival in HCC (Chan et al., 2000). Therefore, the understanding of the disease and continuous search for a cure, including the development of diagnostic/prognostic molecular markers for HCC is crucial (Qin and Tang, 2002; Qin and Tang, 2004; Marrero and Lok, 2004).

Conventionally, predictive factors for cancer relapse after surgical treatment consist of staging with the tumour node metastasis system (TNM) and grading by cellular differentiation; however, this cannot accurately predict the outcome of all HCC patients. The discovery and characterization of numerous HCC-associated genes serves as a platform for the identification of potential molecular biomarkers for HCC. Biomarkers are defined as indicators of cellular, biochemical, molecular, or genetic alterations by which normal or abnormal biological processes can be recognized (Srivastava and Gopal-Srivastava, 2002). Biomarkers for molecular biological sub-staging may provide an opportunity to identify those patients with the most aggressive forms of the disease. Besides the traditional tumour-associated markers, such as alpha-fetoprotein, with new advances in molecular biological techniques and cancer biology, many biomarkers related to invasion, metastasis, recurrence, and survival have been explored (Korn, 2001).

A large number of molecular factors have been shown to associate with the invasiveness of HCC, and provide prognostic significance. One aspect is to analyse the molecular markers for cellular malignancy, including DNA ploidy, cellular proliferation markers (PCNA, Ki-67, Mcm2, MIB1, MIA and CSE1L/CAS protein), nuclear morphology, cell cycle regulators (cyclin A, D and E, cdc2, p27, p73), oncogenes and their receptors (ras, c-myc, c-fos, HGF, c-met, and erb-B receptor family), tumour

suppressor (p53 gene and its related molecule mdm2), apoptosis related factors (Fas and FasL), as well as telomerase activity. The other aspect is to study the molecular factors involved in the process of HCC invasion and metastasis, which include adhesion molecules such as E-cadherin, catenins, CD44 variants, ICAM-1, laminin-5, osteopontin, proteinases responsible for the degradation of extracellular matrix (MMPs, uPA system), as well as angiogenesis regulators (VEGF, basic FGF, platelet-derived growth factor (PDGF), thrombospondin, angiogenin, pleiotroohin, endostatin, intratumour microvessel density). Another important aspect is to detect the transcripts of tumour associated antigen (such as AFP, MAGEs, and CK19), which have been proposed to be markers for metastatic recurrence of HCC (Qin and Tang, 2002; Qin and Tang, 2004). Factors that are involved in these biological processes and differentially expressed during the hepatocarcinogenesis may serve as potential diagnostic/ prognostic biomarkers for HCC.

The tumour suppressor gene p53 plays a central role in cellular responses, including cell-cycle arrest and cell death in response to DNA damage. Loss of p53 function can induce abnormal cell growth, increase cell survival, induce genetic instability, and drug resistance. Thus, p53 mutations are considered as strong marker candidates for predicting increased risk of local relapses, treatment failure, and overall disease-free survival in many kinds of human carcinomas, such as breast (Amornmarn et al., 2000; Blaszyk et al., 2000), colorectal (Diez et al., 2000; Kahlenberg et al., 2000), esophageal (Ireland et al., 2000), head and neck (Tamas et al., 2000), lung (Mitsudomi et al., 2000), and ovarian cancer (Shahin et al., 2000), as well as sarcoma (de Alava et al., 2000). However, many other reports failed to show the independent prognostic value of p53 in cancer of the tongue (Kantola et al., 2000), breast (Ferrero et al., 2000), stomach

(Kaye et al., 2000), lung (Schiller et al., 2001), ovarian (Gadducci et al., 2000), bladder (Fleshner et al., 2000), colorectal (Gallego et al., 2000), and non-Hodgkins's lymphoma (Nieder et al., 2001). Similar controversial results were observed in the case of p53 mutation and over-expression in HCC (Sugo et al., 1999; Heinze et al., 1999; Qin et al., 2002). The p53 protein functions as a transcription factor by shuttling between the nucleus and the cytoplasm. Qin *et al.* (2002) have also demonstrated that the 3-year and 5-year overall survival rate of HCC patients with positive p53 nuclear accumulation were much lower than those with negative p53 expression suggesting that the p53 mutation or nuclear accumulation of p53 expression could be a valuable marker for predicting the prognosis of HCC patients after resection. Other factors such as mdm2 that interacts and regulates p53 degradation may also potentially serve the same function as a molecular biomarker for HCC (Endo et al., 2000). The pathogenesis of HCC is a multi-gene process. Thus, the use of a single biomarker could not provide a reliable prediction. A combination of biomarkers may provide more accurate diagnosis/prognosis of the disease.

1.4 Renal Cell Carcinoma (RCC)

The genetic basis of kidney cancer has been relatively well established with the identification of genes involved in each histological type of tumour. These histological types include clear cell, Type I papillary RCC, Type II papillary RCC, and chromophobe RCC and oncocytoma. The genetic basis of these different kidney cancers and potential biomarkers for RCC will be highlighted.

1.4.1 Epidemiology and Etiology of Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the most common malignant tumour of the adult kidney, accounting for around 3% of human malignancies (Belldegrum and DeKernion, 1998). The incidence of RCC is increasing, and it is estimated that RCC accounts for 95,000 deaths per year worldwide (Vogelzang and Stadler, 1998). In the United States, kidney cancer affects nearly 32,000 individuals and is responsible for over 12,000 deaths each year (Linehan et al., 2001a; Landis et al., 1999). It is estimated that 200,000 individuals are living with kidney cancer in the U.S. Since 3 decades ago, kidney cancer has increased at a rate of about 2.5% per year in the U.S. with the highest rates of increase observed in African Americans. Between 1975 and 1995 RCC incidence rates increased annually 2.3% in white men, 3.1% in white women, 3.9% in black men and 4.3% in black women (Chow et al., 1999). Prognosis is significantly better in women than in men for clear cell RCC (Sejima and Miyagawa, 1999). The consistently higher incidences in women and better prognosis indicate that gender difference may influence the development of RCC.

Numerous studies have been performed to identify the risk factors for the development of RCC. Tobacco smoking, hypertension/anti-hypertensive medications, obesity, and family history have been identified as the major risk factors for the development of RCC. The population attributable risk for hypertension was 21%, obesity at 21 %, and smoking at 18%. Among all reported cases, at least 40% of the RCC cases were not explained by the risk factors studied (McLaughlin and Lipworth, 2000).

Kidney cancer is not a single disease but comprises of several cancers occurring in this organ (Chow et al., 1999; Linehan et al., 2001a; Linehan et al., 2002). The most

common histological types of kidney tumours are clear cell (75%), Type I papillary RCC (~5%), Type II papillary RCC (~10%), and chromophobe RCC and oncocytoma (~10%). These different types of kidney cancer may have markedly differing clinical courses leading to different responses in therapy and could be attributed to different genes (Linehan et al., 2001b; Linehan et al., 2002). However, approximately 3-5% of RCC cases are morphologically unclassified (Kovacs et al., 1997). This type of tumour is generally larger, more aggressive, high grade with unrecognisable cell types without typical renal cell epithelial components and is associated with poor clinical outcomes. The overall median survival in patients with unclassified RCC is only 4.3 months (Zisman et al., 2002).

1.4.2 Genetic basis of kidney cancer

The genetic basis of RCC was established by studying the hereditary forms of RCC. Genetic mapping and positional cloning enabled the identification of tumour suppressor gene(s) and oncogene(s) involved in the manifestation of RCC. Mutation or loss of these genes has also been demonstrated in the sporadic forms of RCC. To date, four types of inherited epithelial forms of renal carcinoma have been delineated clinically, and genes responsible for them have been characterized. Each disease has distinct clinical features, and is caused by mutation in a distinct gene. The four well-described types of inherited kidney cancer are von Hippel-Lindau (VHL), hereditary papillary renal carcinoma (HPRC), hereditary leiomyoma renal cell carcinoma (HLRCC) and Birt-Hogg-Dubé (BHD) syndrome. VHL is an inherited form of clear cell RCC, HPRC is a hereditary form of Type I papillary RCC, HLRCC is Type II papillary RCC and individuals affected with BHD are at risk of developing several types of renal

tumour, including chromophobe RCC and oncocytoma (Kirkali and Lekili, 2003; Linehan et al., 2003; Linehan and Zbar, 2004; Linehan et al., 2005).

1.4.2.1 von Hippel-Lindau (VHL)

Individuals with VHL are at risk for the development of tumours in a number of organs, including the kidneys, pancreas, adrenal glands, brain, spine, eye, and inner ear (Linehan et al., 2001; Linehan et al., 2002). Approximately 35% to 45% of individuals affected with VHL developed RCC of the clear cell histology (Glenn et al., 1990) and these are often early onset tumours. Partial deletion of the VHL gene or mutation that shorten VHL protein is often detected in the renal tumours derived from these patients (Linehan et al., 1995; Linehan et al., 2002; Lubensky et al., 1996). Walther *et al* (1995) estimated that as many as 600 microscopic clear cell neoplasma and 1,100 cysts may develop in an average VHL kidney and these tumours are malignant tumours that can metastasize.

Glenn (1992) and Latif *et al* (1993) reported the identification of the *VHL* gene, which resides on the chromosome 3p25. VHL mutations have been found in high percentages of VHL kindreds (nearly 100%) (Stolle et al., 1998), including intragenic mutation (frameshift, missense, nonsense, etc.), partial and complete deletions of the gene, splicing mechanism defects (Linehan et al., 2005), DNA hypermethylation (Herman et al., 1994), and chromosome 3 translocations (Koolen et al., 1998; Bodmer et al., 1998; Van Kessel et al., 1999). VHL germline mutation testing can now be used to identify at-risk individual affected by VHL. In sporadic non-inherited kidney cancer, mutations of the VHL gene were found in high percentage of patients with clear cell RCC, but not in tumour from patients with papillary RCC, chromophobe RCC or

oncocytoma (Gnarra et al., 1994; Shuin et al., 1994). These studies provided the initial foundation for genetic classification of kidney cancer.

VHL exists as a tumour suppressor gene and encodes for two protein isoforms due to translational initiation from two alternative in-frame AUGs (Schoenfeld et al., 1998; Iliopoulos et al., 1998; Blankenship et al., 1999). Both isoforms are capable of suppressing RCC growth *in vivo* and could shuttle to and forth between nucleus and cytoplasm, and associate with the endoplasmic reticulum and mitochondria (Iliopoulos et al., 1995; Duan et al., 1995; Lee et al., 1999a; Shiao et al., 2000; Schoenfeld et al., 2001; Groulx and Lee, 2002). VHL protein serves to target specific proteins such as members of the hypoxia-inducible factor (HIF) α family, for destruction. HIF1-α is a transcription factor that controls the transcription of a number of downstream genes, such as VEGF, PDGF, TGF-α, the glucose transporter (GLUT1) and erythropoietin. Loss of function or expression of the VHL protein leads to the accumulation of HIF1-α protein, with subsequent over-expression and increase level of these growth factors, which can promote renal cell growth and proliferation. The above mechanisms were extensively reviewed earlier (Kirkali and Lekili, 2003; Linehan et al., 2003; Linehan et al., 2005; Linehan and Zbar, 2004; Kaelin, Jr., 2004).

1.4.2.2 HPRC, HLRCC and BHD

HPRC is a hereditary cancer syndrome in which those affected are at risk of developing bilateral, multifocal Type I papillary RCC (Zbar et al., 1994; Zbar et al., 1995). This disease is highly penetrant and tumour is uniformly of Type I papillary histological type (Lubensky et al., 1999). HPRC patients are at risk of developing up to 3,000 tumours per kidney (Ornstein et al., 2000) and the kidney is the only organ that is

affected. These tumours usually appear later in life (in the fifth and sixth decade) and often are very well differentiated, malignant and can metastasize (Linehan et al., 2005).

Genetic linkage analysis located the gene responsible for HPRC to the long arm of chromosome 7, where the proto-oncogene, *c-Met* was found (Schmidt et al., 1997; Schmidt et al., 1998). Met encodes for the surface receptor for the ligand HGF. A germline mutation found in tyrosine kinase domain of the Met gene leads to constitutive activation of the receptor in papillary renal carcinoma (Schmidt et al., 1998). Mutations of the Met gene were also found in a subset of sporadic, non-inherited Type I papillary renal carcinoma.

Similarly, HLRCC is linked to the loss of fumarate hydratase (FH), which is a Krebs cycle enzyme (Tomlinson et al., 2002). Germline FH mutations were found in 31 of 35 HLRCC kindreds (Toro et al., 2003). Affected individuals are at risk of developing cutaneous and uterine leiomyomas and papillary RCC (Launonen et al., 2001). Kidney tumours derived from patients with HLRCC tend to be unilateral and solitary. They are primarily Type II papillary RCC; often very aggressive and appear to metastasize early (Linehan et al., 2005).

Mutations were suspected to be responsible for the inactivation of BHD gene, which may be a potential tumour suppressor gene (Nickerson et al., 2002; Linehan et al., 2003) localized on the short arm of chromosome 17 (Schmidt et al., 2001; Khoo et al., 2001). Individuals with BHD are at risk of developing fibrofolliculoma, pulmonary cysts and renal tumour (Birt et al., 1977; Toro et al., 1999; Zbar et al., 2002). BHD patients can develop renal tumours that are bilateral and multifocal; and are malignant and highly

metastastic. The renal tumours in BDH are predominantly chromophobe RCC, oncocytic neoplasms and oncocytoma (Pavlovich et al., 2002).

1.4.3 Molecular biomarkers for renal cell carcinoma

Patients with localized kidney cancer have an excellent prognosis as compared to those with advance disease. Five and 10-year survival is 95% for stage I, 88% and 81% for stage II, and 59% and 43% for stage III, respectively, while those presented with stage IV RCC have only 20% 5-year and 14% 10-year survival (Javidan et al., 1999). Complete surgical resection is considered to be the only effective treatment for patients with clinically localized RCC. However, the disease recurs postoperatively in 20%-40% of patients who undergo potentially curative nephrectomy (Belldegrum and DeKernion 1998). Accurate prediction of long-term cancer-free survival immediately after resection of clinically localized diseases, therefore, will be valuable for planning follow-up protocols and for identifying patients with a high risk of recurrence and for patients who would benefit the most from adjuvant therapy. Although conventional prognosis factors such as pathologic tumour stage and grading are useful, other novel prognostic parameters, including clinical, laboratory, and biomolecular factors, are needed to provide additional predictive value (Mejean et al., 2003).

Besides providing prognostic information (risk of recurrence or metastasis), tumour markers could also hold the key to targeted therapeutic intervention. Currently, many markers related to tumour proliferation, growth, angiogenesis, loss of cell adhesion and hypoxia-inducible factor among others, are being evaluated for their prognostic value. These biomarkers include proliferating cell nuclear antigen (PCNA), laminin, Ki-67, silver-stained nucleolar organizer region (AgNOR) counts, p53 mutations, over-

expressed growth factors, metallothionein, and increased micro-vessel density (Delahunt et al., 1993; Yoshida et al., 1994; MacLennan and Bostwick, 1995; Aaltomaa et al., 1997; Sejima and Miyagawa, 1999; Kirkali et al., 2001; Tuzel et al., 2001). However, none of these factors appears to be a better predictive prognostic marker than the usual staging and grading.

Other serum markers such as VEGF, interleukin (IL)-12, the soluble IL-2 receptor, and intercellular adhesion molecule-1 have been shown to have a prognostic value, but no specific serum markers for RCC are yet available (Hoffmann et al., 1999; Kallio et al., 2001). High levels of preoperative hCG β subunit may identify patients with increased risk of progression (Hotakainen et al., 2003), while high expression level of IGF-I receptor may predict poor survival among patients with RCC (Parker et al., 2002). Cadherin-6 has recently been shown to be a useful molecular marker to detect the circulating cancer cells disseminated from conventional RCC (Li et al., 2005). Similarly, CD70, a type II transmembrane protein belonging to tumour necrosis factor family, has been identified as a new specific tumour marker for clear cell RCC (Junker et al., 2005). Carbonic anhydrase (CA) 9, a member of the CA family thought to have a role in the regulation of intra- and extracellular pH during the period of hypoxia in tumour cells, have gained recent interest. Low CA9 staining was found to be an independent prognostic indicator of poor survival in patients with metastatic RCC and in low-grade and stage tumour (Bui et al., 2003; Soyupak et al., 2005).

Recently, Kim *et al* (2005) have demonstrated in patients with clear cell RCC that the use of 8 molecular markers in a prognostic model for survival is more accurate than a standard clinical model using the combination of stage, histological grade and

performance status. The markers used include Ki-67, p53, gelsolin, CA9, CA12, PTEN (phosphatase and tensin homologue deleted on chromosome 10), epithelial cell adhesion molecule and vimentin. The incorporation of molecular tumour marker into future staging systems is expected to revolutionize the approach to diagnosis and prognosis (Bui et al., 2001; Lam et al., 2005).

1.5 Human chorionic gonadotropin, Pregnancy and Cancer

The influence of hCG on cancer growth and development is highly controversial. On one hand, the hCG hormone has been demonstrated to confer protection against mammary carcinogenesis (Russo et al., 2005), altering the course and preventing the formation of granulosa cell tumour in CF-1 mice predisposed to tumour formation (Owens et al., 2002). On the other hand, hCG, particularly the β subunit, is over-expressed in a relatively high proportion of both the trophoblastic (i.e. hydatidiform mole, choriocarcinoma, placental site trophoblastic tumour, etc.) and non-trophoblastic tumours (i.e. bladder cancer, renal cancer, breast cancer, etc.) (Stenman et al., 2004). Elevated level of hCG β subunit is usually a sign of aggressive disease and is strongly associated with poor prognosis. Thus, it appears that the response of cancer to hCG may depend on the amount and timing of hCG application.

1.5.1 Functions of Human chorionic gonadotropin (hCG)

The glycoprotein hormone family comprises of chorionic gonadotropin (CG), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). All members are heterodimers consisting of an α and β subunit. The α subunit of 92 amino acids is common to all glycoprotein hormones. The biological

activity is determined by its β subunit, which displays extensive homology (80%) between the hCGβ and LHβ.

The CG hormone is essential for normal reproductive function (Pierce and Parsons, 1981) and acts to maintain high progesterone levels during pregnancy (Segaloff and Ascoli, 1993). Both the LH and CG stimulates the production of gonadal steroid hormones via its interaction with the lutropin-choriogonadotropin-receptor (LH-CG-R) present in the granulosa cells of the ovary and in the testicular Leydig cells (McFarland et al., 1989). Upon binding of CG to its receptor, adenylyl cyclase activity is elevated through an intracellular membrane-associated G protein-coupled mechanism that results in an increase in cAMP levels, leading to the synthesis of steroid and polypeptide hormones (McFarland et al., 1989). Jiang *et al*. (2002) have shown that hCG and inhibin induce histone acetylation in human breast epithelial cells, which could be an alternative route of gene regulation.

1.5.2 Protective effects of hCG and Pregnancy against mammary carcinogenesis

The protective effects of hCG and pregnancy against mammary carcinogenesis have been well established. Women who have their first pregnancy after 30 years of age have a 2-5 fold higher lifetime risk of developing breast cancer compared to those whose first pregnancy occurs before 20 years of age (MacMahon et al., 1970; Trichopoulos et al., 1983). The protective effect of pregnancy against the development of cancer is believed to be attributed by an increase in cellular differentiation and the reduced rate of cell proliferation in the mammary epithelium (Dickson and Lippman, 1995). Russo *et al* (1990a, 1990b, 1991) demonstrated that 7, 12-dimethylbenz (a) anthracene (DMBA)-

induced mammary carcinogenesis in the female rat is effectively inhibited by either pregnancy or human chorionic gonadotropin (hCG) treatment. The protective effect of hCG on the development of mammary tumours has been variously attributed to: induction of differentiation, inhibition of cell proliferation, increase in DNA repair capabilities, induction of inhibin by the mammary epithelium, activation of programmed cell death, and down-regulation of IGF-I and IGF-I receptors in mammary epithelial cells (Russo et al., 1990a; Russo et al., 1990b; Russo et al., 1991; Alvarado et al., 1994; Srivastava et al., 1997; Huynh, 1998).

Russo *et al* (2005) have also recently deciphered the genomic signatures that confer the long-term protection by pregnancy and hCG treatment against mammary carcinogenesis. The authors demonstrated that short-term treatment with recombinant hCG could induce the same genomic signature as the reproductive process, inhibiting not only the initiation but also the progression of mammary carcinogenesis, stopping the development of early lesions, such as intraductal proliferations and carcinoma *in situ*. These observations indicate that short-term hCG treatments provides a novel strategy in breast cancer prevention (Russo and Russo, 1994; Russo and Russo, 2000). Although much have been known about the effects of hCG on mammary gland, other important novel mechanisms involved in this complex process are yet to be discovered.

1.5.3 Granulosa cell tumours

The ovary is a dynamic organ that undergoes specific changes in response to the pituitary gonadotropins FSH and LH (Richards, 1994; Rinehart et al., 2004) and ovarian-derived factors to support follicle growth, ovulation, and luteinization. Ovarian granulosa cells undergo a complete differentiation process during the growth and maturation of

ovarian follicles. This process includes the acquisition of FSH-R in the early stages of follicular growth, and the induction of FSH-R and LH-CG-R in granulosa cells which is a critical step in reproductive physiology. Owen *et al* (2002) have demonstrated that administration of surges of hCG that mimic ovulatory levels of LH block the formation of granulosa cell tumours in genetically predisposed mice while triggering formation of luteomas. This switch of pathway from tumour to luteomas formation may be attributed to the sudden increase of hCG that is necessary for granulosa cell differentiation (i.e. during preovulatory). These observations suggest that surge in hCG may have protective function against granulosa cell carcinoma.

Granulosa cell tumours are rare and comprises of only 10% of all ovarian tumours (Cooke et al., 1995; Wynder et al., 1969). The clinical importance of this type of cancer was attributed to its potential for malignancy and recurrence (Wynder et al., 1969; Cooke et al., 1995; Fontanelli et al., 1998; Lee et al., 1999b). Altered expression of genes associated with granulosa cell tumours includes; inhibin (Lappohn et al., 1989; Burger et al., 1998; Robertson et al., 1999a; Robertson et al., 1999b), p53 (Gebhart et al., 2000), c-erB2, c-myc, and several others (King et al., 1996; Owens et al., 2002). Hormonal alterations also play a role in the development of granulosa cell tumours. Mice lacking the FSH-R and those that hypersecrete LH develop granulosa cell tumours, which could be due to elevated levels of gonadotropins (Risma et al., 1995; Danilovich et al., 2001). The complex interplay between hormonal regulation and gene alterations in granulosa cell tumours has provided a complex scenario to the disease process.

CHAPTER 2: INTRODUCTION

2.1 The isolation of OKL38

Growth factors and inhibitors play crucial roles in the growth regulation of cell proliferation and differentiation of mammary epithelial cells. Studies have shown that during pregnancy, with the onset of terminal differentiation, there is a dramatic decrease in the proliferation of the mammary epithelial cells. This is one of the hypothesized reasons for pregnancy protection of mammary gland against carcinogen insults. With the notion to isolate and characterize genes that are differentially regulated during pregnancy and potentially involved in this protection mechanisms, differential display was performed using mRNA from mammary gland of non-pregnant and 18-days pregnant rats. Among the 18 differentially expressed bands, a novel 450-bp DNA fragment, which was up-regulated in the pregnant animals, was identified. This novel gene is ubiquitously expressed in all rat tissues with the highest levels detected in the ovary, kidney and liver. The human cDNA of 1.6 kb was isolated from human ovarian library and later named OKL38 (Ovary Kidney Liver 38 kDa) (Huynh et al., 2001).

2.2 Pregnancy induced OKL38 expression

The expression of OKL38 in rat mammary gland at different stages of pregnancy and lactation was previously demonstrated (Huynh et al., 2001). OKL38 transcripts were up regulated during pregnancy and lactation in the rat mammary gland, with the highest level of expression during lactation, which coincided with maximal breast epithelial differentiation. The transcripts were also localized to the secretory epithelial cell of the

mammary gland during this period. These observations documented the existence of a certain degree of hormonal regulation in the expression of OKL38.

2.3 OKL38, a novel growth-inhibitor lost in cancer

Abnormal expression of the various growth factors and growth inhibitors has been implicated in tumourigenesis. Low levels of OKL38 transcripts are observed in various human breast cancer cell lines and barely detectable in DMBA-induced rat mammary tumours (Huynh et al., 2001). Transfection of MCF-7 cells with OKL38 cDNA resulted in growth inhibition *in vitro* and reduction in tumour formation *in vivo*, suggesting that *OKL38* may play a vital role in the growth regulation and differentiation of breast epithelial cells during pregnancy and tumourigenesis (Huynh et al., 2001). The molecular mechanisms by which *OKL38* exerts its role in growth inhibition and differentiation are still unknown.

2.4 Scope of this study

The novelty and significance of OKL38 in cancer prompt further investigations into the functions and regulations of this important gene. The objectives of this thesis include: (1) Cloning, sequencing and characterization of the human OKL38 gene; (2) Establishing a rat model to characterize the functions and regulations of OKL38 via cloning the rat homologs of OKL38; (3) Using the rat model to study the regulatory effect of hCG and pregnancy on OKL38 in both the mammary gland and ovary; (4) Investigating the expression profile of OKL38 in kidney and liver cancer, determining the potential use of OKL38 as a biomarker; (5) Identifying the mechanisms involved in the loss of OKL38 expression in cancer.

CHAPTER 3: MATERIALS AND METHODS

3.1 Reagents

Horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit secondary antibodies were purchased from Pierce, Rockford, Illinois. Chemiluminescent detection system was supplied from Amersham, Pharmacia Biotech, Arlington Heights, IL. Tissue culture dishes were purchased from Nunc Inc. Naperville, IL. High glucose Dulbecco's Modified Eagle Medium (Hi-Glu-DMEM), Alpha Modified Eagle medium (α-MEM), Fetal bovine serum (FBS), Lipofectamine™ 2000 reagent and penicillin-streptomycin (PS) were from Invitrogen, Carlsbad, CA. The human CG used in this study was purchased from Profasi®, Laboratoires Serono S. A., Aubonne, Switzerland.

3.2 Animals

Animal were maintained and treated according to the protocol approved by the institutional animal care committee. Twenty-four female Sprague-Dawley rats (fifty days old and weighing approximately 250g) were divided into 4 equal groups. Each group (n = 6) received daily intraperitoneal (IP) injection of either phosphate buffer saline (PBS) or 3 different doses of hCG (10, 20 and 40 UI in 200 µl of PBS) for 21 days. After the last injection, the rats were allowed to rest for an additional 2 days before they were sacrificed. The ovary, kidney, liver and mammary gland were harvested, frozen in liquid nitrogen and stored at –80^{o}C.

3.3 Probe labelling

All probes used for library screening as well as for Southern and Northern blot analyses were radioactively labelled with α [32p]deoxy-CTP (ICN, Costa Mesa, CA) using the Rediprime II DNA Labelling System (Amersham, Pharmacia Biotech, Arlington Heights, IL) as described by the manufacturer. Unincorporated nucleotides were removed using a nucleotide purification kit (Qiagen, GmbH, Hilden, Germany).

3.4 Screening, Subcloning and Sequencing of the human OKL38 gene

Two cosmid clones containing human *OKL38* gene were isolated from the human cosmid library (Clontech, Palo Alto, CA) employing the human OKL38 cDNA as probe (Huynh et al., 2001). Restriction digestion and Southern blot analysis were used to estimate the genomic DNA insert size and confirmation of their identity. Shotgun strategy was adopted to subclone the cosmid insert for sequencing. In brief, the cosmid clone was digested with various restricted enzymes, single or double digestion. The digested products were separated on an agarose gel and products less than 6 kb were cloned into pBluescript®SK (Stratagene, La Jolla, CA) vector with the sub-cloning map shown in figure 4.1A. All subclones and cosmid clones were subjected to complete sequencing via direct cosmid sequencing and the use of Template Generation System™ (Finnzymes, Finland) as recommended by the manufacturer. Sequencing of the 'CCCT' rich region resided in subclone Bs/X-1.1 (Fig. 4.1A) was attempted using the SequenceRx enhancer solution F (Gibco, Grand Island, NY). The GenomeWalker™ kit (Clontech, Palo Alto, CA) was used to clone and characterize the 3'-end of *OKL38* gene

using the GW-F forward primers (Table 2) as described by the manufacturer. All sequencing was performed using automated sequencing via the modified dideoxy chain termination using the BigDye™ version 3.0 (Applied Biosystem, Foster city, CA). All the DNA inserts concerned were sequenced to a redundancy of 3 times.

3.5 Rapid Amplification of cDNA Ends (RACE) Analysis

To establish the full-length cDNA of human OKL38, 5'RACE was performed using the SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer's direction with the following modifications: The secondary PCR of the primary RACE products was performed using Advantage® Genomic polymerase (Clontech, Palo Alto, CA). Gene-specific primer, GSP1R (Table 2) was designed based on the 5'end sequence of the previously isolated human OKL38 cDNA (Huynh et al., 2001), while GSP2R was designed based on the 5'end sequence of the GSP1R race fragment (Fig. 4.4A). The RACE products were identified using Southern blot analysis and subsequently cloned into pCR®-Blunt-II-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced using T7 and SP6 primers. Sequence analysis was carried out using Laser gene sequence analysis software (Dnastar Inc, Madison, WI).

The same strategy was also used to establish the full-length cDNA of rat OKL38. Gene-specific primer, 1R (Table 4) was designed based on the 5'end sequence of the isolated rat OKL38 cDNA. The RACE products were subsequently cloned into pCR®-Blunt-II-TOPO vector (Invitrogen, Carlsbad, CA) and 5 positive clones from each fragment were sequenced using T7 and SP6 primers. Sequence analysis was carried out as described above.

3.6 Primer Extension Study

To identify the transcriptional start site of OKL38 gene, primer extension study was performed. Two μM of ^{32}P-labelled primer (Pext1 on exon1 and Pext3 on exon3) (Table 2) were allowed to hybridized with 200 ng of human liver, kidney and prostate poly $(A)^+$ mRNA (Clontech, Palo Alto, CA). Touchdown methodology was employed to increase the stringency of hybridization with the following parameter: 85°C, 15 min; 84°C, 90 s; -0.1°C for every subsequent cycle to dock at a final temperature of 50°C. The hybridization mix was added to the reverse transcription mix to a total volume of 100 μl containing 5X Superscript buffer, 0.1 M DTT, 10 μM dNTP, 250 U Superscript II enzyme (Invitrogen, Carlsbad, CA), Rnasin (Promega, Madison, WI), diethyl pyrocarbonate water (DEPC-H_2O) and followed by extension for 1 hr. The reaction was terminated by heating to 70°C for 15 min, ethanol precipitated, dried, redissolved in formamide-dye mix, and run on a 6% denaturing gel. The fmol™ cycle sequencing kit (Promega, Madison, WI) was used to generate DNA ladder with subclone P-3.4 and S-5.5 (Fig. 4.1A) as template, and Pext1 and Pext3 as primers, respectively.

3.7 Cloning of OKL38 cDNAs

3.7.1 *Cloning cDNA of HuOKL38 - 1a, 2a and 2b cDNA*

Cloning of the full length cDNA of the HuOKL38-1a transcript was achieved using two PCR primers, forward primer 1F (nucleotide position 7-33) designed at the 5' end and reverse primer 1R (nucleotide position 1863-1889) (Table 2) designed at the 3'-end of the HuOKL38-1a cDNA. To clone the HuOKL38-2a and -2b cDNAs, forward primer 2F (nucleotide position 52 to 75 of -2a transcript) (Table 2) was used (Fig. 4.9A).

In all cases, the forward primer carried a *HindIII* and the reverse primer an *EcoRI* restriction site for directional cloning. To generate the cDNA template for PCR cloning, 1 μg of human liver mRNA was reversed transcribed using SuperScript™II reverse transcriptase (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. The template was amplified by PCR under the following conditions: denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min and 30 sec, and a final extension at 72°C for 5 min. PCR products of the correct size were cloned into pBluescript®SK (Stratagene, La Jolla, CA) for sequencing.

3.7.2 *Cloning of RtOKL38-2.0, -2.3 and -2.3A cDNAs*

Four rat OKL 38 cDNAs of approximately 1.1 kb were isolated from screening rat liver cDNA library using the human OKL38 cDNA as a probe. Cloning of the full length cDNA of the RtOKL38-2.0 transcript was achieved using two PCR primers, forward primer FL-2.0F (nucleotide position 12-34) designed at the 5' end and reverse primer FL-2.0R (nucleotide position 1937-1959) (Table 4) designed at the 3'-end of the 2.0 cDNA. To clone the RtOKL38-2.3 and -2.3A cDNAs, forward primer FL-2.3F (nucleotide position 6-30 of 2.3 transcript) (Table 4) was used instead of the FL-2.0F primer (Fig. 4.20). In all cases, the forward primer carried a *HindIII* and the reverse primer a *NotI* restriction site for directional cloning. To generate the cDNA template for PCR cloning, 1 μg of rat kidney poly(A)$^+$ mRNA was reversed transcribed using SuperScript™II reverse transcriptase (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. The template was amplified by PCR as described for cloning the human cDNAs and PCR products of the correct size were cloned into pBluescript®SK (Stratagene, La Jolla, CA,

USA) and sequenced. Two more internal primers, 2R and 3R (Table 4) were also utilized for sequencing.

Due to the low expression level of the RtOKL38-2.3A transcript, additional secondary PCR was performed for its cDNA isolation. After PCR amplification, the region below the 2.3 kb band (approximately at the 2.2 kb position on the agarose gel) was purified (Qiagen, GmbH, Hilden, Germany) and the extracted DNA was subjected to a second round of PCR amplification under the same conditions. A 2.2 kb fragment was obtained, cloned and sequenced as described above.

3.8 In Vitro Transcription and Translation (TNT) Study

3.8.1 Coupled TNT for human OKL38 variants

To verify the predicted molecular weight of the putative ORF of human OKL38, the TNT® Coupled Reticulocyte Lysate System (Promega, Madison, WI) was used to transcribe and translate the HuOKL38-1a, -2a, -2b and the previously cloned 1.6 kb cDNA according to the manufacturer's instruction. Briefly, the cloned cDNAs in pBluescript®SK (Stratagene, La Jolla, CA) were digested with *EcoRI* (New England Biolabs, Beverly, MA) and extracted twice with phenol/chloroform. The DNA was precipitated with ethanol, washed with 70% ethanol, air-dried and resuspended in H_2O. One μg of the digested plasmid was used for the *in vitro* TNT reaction. The synthesized protein was labelled with [^{35}S] L-Methionine (ICN, Costa Mesa, CA) and 5 μl of the reaction was loaded and electrophoresed on a 10% SDS-PAGE. The synthesized protein was transferred onto a nitrocellulose membrane and auto-radiographed to film.

The identity of the *in vitro* synthesized protein was confirmed via Western blot using rabbit anti-Human OKL38 antibodies.

3.8.2 Coupled TNT for rat OKL38 variants

To verify the predicted molecular weight of the putative ORF of rat OKL38 variants, the TNT® Coupled Reticulocyte Lysate System (Promega, Madison, WI) was used to transcribe and translate the RtOKL38-2.0, -2.3 and -2.3A cDNAs according to the manufacturer's instruction. Same TNT procedures were performed as described for the human OKL38 cDNAs with some modification: The constructs were digested with *NotI* and 0.4 pmol of the digested plasmid was used for the TNT reaction. Western blot analysis was performed on the TNT products using rabbit anti-Rat OKL38 antibodies.

3.9 Multiple Tissue Expression Array, Northern Blot Analysis and Cancer Profiling Array

The Multiple Tissue Expression (MTE) and Cancer Profiling Array (CPA) were purchased from Clontech (Clontech, Palo Alto, CA) and the human Northern blots were from Invitrogen (Invitrogen, Carlsbad, CA). Both the arrays and blots were hybridized with a 700 bp *SmaI* human OKL38 probe (936–1685 nt of HuOKL38-1a) that detects all the different variants. Ubiquitin (provided by manufacturer) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; American Type Culture Collection, Manassas, VA) were used for normalizing the arrays and Northern blottings, respectively. Pre-hybridization of the arrays and blots were performed at 50^{o}C for 1 hour in ExpressHyb™ (Clontech, Palo Alto, CA) with sheared salmon testis DNA added to a final concentration of 0.1 mg/ml. The denatured radiolabelled probe was mixed directly into the pre-

hybridization solution and hybridised overnight at 50°C. After hybridisation, the arrays were washed 3 times at 55°C in solution 1 (2x SSC, 0.5% SDS) for 30 min each time, repeated with solution 2 (0.2x SSC, 0.5% SDS) and a final rinse in 2x SSC at room temperature. Blots were exposed to a phosphor-imager. As for the Northern blot analysis, washing was performed at 45°C using solution 1 and 2. mRNA levels were determined by densitometric scanning of autoradiographs and normalised with GAPDH.

Total RNA for Northern blot analysis was extracted from rat tissues and organs using Trizol Reagent (Invitrogen, Carlsbad, CA). The poly $(A)^+$ mRNA was isolated from the indicated organs and tissues of female rats using the Oligotex mRNA Kit as described by the manufacturer (QIAGEN, GmbH, Hilden, Germany). Northern blot analysis was performed on poly $(A)^+$ mRNA or total RNA as described (Huynh et al., 1993). Blots were hybridized with: 1) rat OKL38 probe (nucleotide position 926-2004) that detects all three variants; 2) variant-specific probe (nucleotide position 6-395 of RtOKL38-2.3 transcript), which detects only the RtOKL38-2.3 and -2.2 transcripts; or 3) GAPDH. The mRNA levels were determined by densitometric scanning of autoradiographs and normalised with GAPDH.

3.10 Semi-quantitative RT-PCR of OKL38 variants

3.10.1 One-Step RT-PCR for detection of human OKL38 variants

One μg of total mRNA was used as template for One Step RT-PCR (Qiagen, GmbH, Hilden, Germany) adopting the procedure recommended by the manufacturer. To study the expression of the HuOKL38-1a transcript, variant-specific forward primer PCR-1F (nucleotide position 1-28) and a common reverse primer PCR-1R (Table 2) were

designed for RT-PCR. To determine the expression of HuOKL38-2a, -2b and -2c transcripts, RT-PCR was performed using the forward primer PCR-2F and reverse primer PCR-1R (Table 2). Total amount of OKL38 transcripts in the tissue samples were detected using a pair of primers, E7T-F and E8T-R (Table 2), which were designed in exon 7 and 8, respectively. The specificity of the designed primers and PCR conditions were optimised using the three cloned cDNAs, HuOKL38-1a, -2a and -2b as templates. A pair of tubulin primers, TubF (5' AACGTCAAGACGGCCGTGT 3') and TubR (5' GACAGAGGCAAACTGAGCAC 3'), which amplify a 400-bp fragment of tubulin cDNA, was used for normalization. The temperature regime of One Step RT-PCR was performed as follows: 50^{o}C for 30 min; 95^{o}C for 15 min; followed by (29-38) cycles of 94^{o}C for 1 min, 60^{o}C for 1 min, 72^{o}C for 1 min, and final extension at 72^{o}C for 5 min. The amplified products were separated on a 2.0% agarose gel.

To determine the abundance of HuOKL38-1a, -2a, -2b and -2c transcripts in HCC and ABL, 36 PCR cycles were used, while 29 cycles were used for the detection of the total OKL38 transcripts. Optimal cycles were determined for the detection of total OKL38 transcripts in transfected Chang Liver cells to avoid the undesired amplification of DNA constructs present in the RNA extracts. Combination of 1a/2aORF and 2bORF extracted RNA was used as the template for optimization and the cycles used were 17, 20, 23, 26, 29 & 32. One step RT-PCR was performed as described above for the set of reaction with reverse transcription. The reverse transcriptase enzyme activity was removed for the set "without reverse transcription" (Fig. 4.37F) by heating at 95 ^{o}C for 15 minutes and cooled, before adding to the RT-PCR reaction.

3.10.2 One-Step RT-PCR for detection of rat OKL38 variants

100 ng of poly(A)$^+$ mRNA were used as template for One Step RT-PCR (Qiagen, GmbH, Hilden, Germany) adopting the procedure recommended by manufacturer. To study the expression of the RtOKL38-2.0 transcript, variant-specific forward primer RTPCR-2.0F (nucleotide position 24-44) and a common reverse primer 2R (Table 4) were designed for RT-PCR. To determine the expression of RtOKL38-2.3 and 2.3A transcripts, RT-PCR was performed using the forward primer FL2.3F and reverse primer 2R (Table 4). The specificity of the designed primers and PCR conditions were optimised using the three cloned cDNAs, RtOKL38-2.0, -2.3 and -2.3A as templates. To optimise the number of cycles for RT-PCR in order to detect the 3 different variants and to prevent an amplification plateau for any one transcript, kidney mRNA (expressing high level of all three transcripts) was amplified through 23, 25, 27, 29, 31 and 33 cycles. The optimal cycles for detection of the RtOKL38-2.0 transcript were 25, and 31 were the optimal cycles for RtOKL38-2.3 and -2.3A transcripts. A pair of tubulin primers, TubF (5' AACGTCAAGACGGCCGTGT 3') and TubR (5' GACAGAGGCAAACTGAGCAC 3'), which amplify a 400-bp fragment of rat tubulin cDNA, was used for normalization. The One Step RT-PCR was performed as follows: reverse transcription at 50 ^oC for 30 min; activating *Taq* Polymerase at 95 ^oC for 15 min; followed by 25 (for RtOKL38-2.0 transcript) or 31 (for RtOKL38-2.3 and -2.3A transcripts) cycles of 94 ^oC for 1 min, 60 ^oC for 1 min, 72 ^oC for 1 min, and final extension at 72 ^oC for 5 min. The amplified products were separated on a 2.0 % agarose gel.

3.11 OKL38 Antibodies, Western Blot Analysis and Immunohistochemical Analysis

Rabbit polyclonal OKL38 antibodies were raised against the human OKL38 specific peptide: CAVEWGTPDPSSCGAQ (amino acid position 200-214); and rat OKL38 specific peptide: QMMRDQSILSPSPYEGYRSLPEHQ (amino acid position 343 to 366). Affinity purified rabbit anti-human OKL38 antibody was diluted in Tris buffer saline (TBS, 20 mM Tris, 200 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBST) at a final concentration of 1μg/ml. Western blot analysis was performed by incubating the blots with 1:2000 anti-OKL38 antibody overnight at 4^{o}C and washed 3 times with TBST, 15 min each. Subsequently, the blots were incubated with 1:7500 horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody for 1 hour. After washing 3 times with TBST, 15 min each, the blots were then visualized with a chemiluminescent detection system as described by the manufacturer.

For immunohistochemical analysis, 5 μM thick sections were cut, de-waxed in xylene, and then rehydrated as described (Huynh et al., 2002). Antigen retrival was performed by boiling the slides in 10 mM citrate buffer pH 6.0 for 20 min. Endogenous peroxidase activity was block by 3% hydrogen peroxide in methanol for 30 min. After two washes of Tris Buffer Saline (20mM Tris, 200 mM NaCl, pH 7.6) (TBS), the sections were pre-incubated with 5% skim milk in TBS containing 0.1% Tween-20 (TBST) for 15 min to reduce non-specific background staining. The section were washed twice with TBST for 5 min and incubated overnight at 4^{o}C, with purified primary antisera against human OKL38. Immunohistochemistry was performed using the streptavidin-

biotin peroxidase complex method according to manufacturer's instructions (Lab Vision, Fremont, CA) using AEC as the chromogen.

3.12 In situ hybridisation

To determine the cell-specific expression of OKL38, *in situ* hybridization was performed using sections derived from mammary tissues and ovaries of controls and 20 IU hCG-treated rats. To generate sense and anti-sense rat OKL38 probes, a 542-bp (nucleotides position 925-1466) of rat OKL38 cDNA (GenBank accession no. AY081218) was cloned into the pBluescript®SK plasmid. The plasmid construct was linearized and digoxigenin-labeled sense and anti-sense RNA probes were synthesized using the digoxigenin RNA labeling kit (Roche Molecular Biochemicals, Indianapolis, IN) as described by the manufacturer. Fresh tissues were treated with PBS containing 0.5% active DEPC for 10 min, embedded in Jung-tissue freezing medium (Leica Instruments, Nussloch, Germany) and stored at – 80 ^{o}C. Serial 7- to 8- μm sections were heated at 50 ^{o}C for 2 min to immobilise the tissue onto the slide. To minimize nonspecific background caused by lipid vesicles, the sections were delipidized for 5 min in chloroform, dried at room temperature and fixed in PBS containing 4% paraformaldehyde. Prehybridization, hybridization, washing and immunological detection were performed as described by Braissant and Wahli (1998).

3.13 Generation of OKL38-eGFP-pcDNA3.0 Construct

3.13.1 Generating human OKL38-eGFP-pcDNA3.0 construct

The HuOKL38-1a cDNA contained an ORF of 477 amino acids. To fuse this OKL38 protein to the eGFP via PCR, 4 primers namely 477-F, 477-eGFP-R, eGFP-F and eGFP-R (Table 2) were designed. Two separate PCR reactions were performed using the primer 477-F and 477-eGFP-R to amplify the ORF of OKL38 and primers eGFP-F and eGFP-R to amplify the ORF of eGFP. The amplified products from the two PCR reactions were then mixed and reamplified using primers 477-F and eGFP-R. The PCR reaction was performed as follows: 95^{o}C for 5 min; followed by 25 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 2 min, and final extension at 72^{o}C for 5 min. The recombinant products were cloned into pCR®-Blunt-II-TOPO vector (Invitrogen, Carlsbad, CA), screened for orientation and subsequently cloned into pcDNA3.0 (Invitrogen, Carlsbad, CA) mammalian expression vector. The OKL38-eGFP-pcDNA3.0 construct was fully sequenced and endotoxin free plasmid for transfection was prepared using Maxi-prep kit (Qiagen, GmbH, Hilden, Germany). The positive control eGFP-pcDNA3.0 was constructed with the same strategy using only the eGFP-F and eGFP-R primers for PCR cloning.

3.13.2 Generating Sense RtOKL38-eGFP- and eGFP-Antisense-RtOKL38-pcDNA3.0 construct

The rat OKL38 cDNA contained an ORF of 478 amino acids. The same strategy for generating HuOKL38-eGFP was used to generate the RtOKL38-eGFP. To fuse the rat OKL38 protein to the eGFP, PCR was performed using 4 primers namely 478-F, 478-

eGFP-R, eGFP-F and eGFP-R (Table 4). Two separate PCR reactions were performed using the primer 478-F and 478-eGFP-R to amplify the ORF of OKL38 and primers eGFP-F and eGFP-R to amplify the ORF of eGFP. The amplified products from the two PCR reactions were then mixed and reamplified using primers 478-F and eGFP-R. The PCR reaction was performed as follows: 95^{o}C for 5 min; followed by 25 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 2 min, and final extension at 72^{o}C for 5 min. The recombinant products were cloned into pCR®-Blunt-II-TOPO vector (Invitrogen, Carlsbad, CA), screened for orientation and subsequently cloned into pcDNA3.0 (Invitrogen, Carlsbad, CA) mammalian expression vector. As for the eGFP-Antisense-RtOKL38-pcDNA3.0 construct, the rat OKL38 ORF was inserted in the reverse order into the C-terminal of eGFP protein from the eGFP-pcDNA3.0 construct. All the three constructs were was fully sequenced and endotoxin free plasmid for transfection was prepared using Maxi-prep kit (Qiagen, GmbH, Hilden, Germany).

3.14 Generation of 5' and 3'UTR of HuOKL38-1a, -2a & -2b pcDNA3.0 Constructs

To determine the regulatory functions of 5' & 3'UTR of OKL38 variants, constructs of the 3 variants with or without UTRs were generated and cloned into the mammalian expression vector pcDNA3.0. The intact OKL38 ORF was used as the reporter gene for expression study. The full-length of HuOKL38-1a, 2a & 2b cDNAs were cloned into mammalian expression vector pcDNA3.0 as described in *Section 3.7.1*. To generate the 1a and 2a5'UTR constructs, forward primer 1F and 2F, and a common reverse primer ORF-R, were used for PCR amplification with the 1aFL and 2aFL constructs as template, respectively (Fig. 4.36). 1a/2a 3'UTR and ORF constructs were

amplified using a common forward primer, 1aORF-F and the reverse primers 1R and ORF-R, respectively. In these instances, the 1aFL construct was used as the template (Fig. 4.36). Similarly, the 2b5'UTR construct was amplified using forward primers, 2F and the reverse primer, ORF-R. The 2b3'UTR and ORF constructs were amplified using the common forward primer, 2bORF-F and the reverse primers, 1R and ORF-R, respectively (Fig. 4.36). The latter 3 fragments were amplified using the 2bFL construct as template. The amplified products were cloned into pCR®-Blunt-II-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced. Clones with the correct sequence were subcloned into pcDNA3.0 vector at the *HindIII* and *EcoRI* restriction site and sequenced again. All primers used for cloning were presented in Table 2.

3.15 Generation of HuOKL38-1a & -2a 5'UTRs-deleted pcDNA3.0 and pSEAP2-Control Vector constructs

To determine the regulatory region that is involved in the down regulation of OKL38 translation, HuOKL38-1a and –2a 5'UTRs-deleted constructs in two different reporter systems were generated (Fig. 4.38). The level of protein expression is determined by western blot analysis for those construct carrying the intact OKL38 ORF as reporter gene, while the secreted alkaline phosphatase (SEAP) detection system was used for those constructs carrying the SEAP ORF. The 1aD1, 1aD2, 2aD1, 2aD2 and 2aD3 pcDNA3.0 constructed were generated via PCR using the respective forward primers and the common reverse primer, ORF-R, as described in Table 2. Protein expressions from these constructs were detected by Western blot analysis. The same constructs carrying SEAP as the reporter gene, including 2aD4, were cloned adopting similar strategy, using the common reverse primer, UTR-R instead of the ORF-R. The E4

SEAP-CV construct was generated by annealing 2 long primers, E4-F and E4-R, and subsequently cloned in to the pSEAP CV. The levels of SEAP can be detected in the tissue culture medium using the Great EscAPe SEAP™ Detection Kit as described by the manufacturer (Clontech, Palo Alto, CA).

3.16 Screening for mutation in OKL38 gene

The OKL38 cDNA consisting of exon 3-8 was amplified from total RNA of HCC (5, 6, 8, 10 & 17) and liver cancer cell lines (HepG2, Hep3B, Chang Liver and PLC/PRF5). Exon 3-7 was amplified using forward primer, 1F and reverse primer, E81R, which reside in exon 3 and 8, respectively. Exon 8 was amplified using forward primer, E7TF and reverse primer, 1R, which reside in exon 7 and 8, respectively. The amplified DNA fragment were separated on a 1.2 % agarose gel and extracted using the Gel Extraction Kit (Qiagen, GmbH, Hilden, Germany). Sequencing was performed with internal primers, E82R, E81F & E61R and the 4 PCR primers (Table 2) using automated sequencing via dideoxy chain termination using the BigDye™ version 3.0 (Applied Biosystems, Foster City, CA). The sequencing results were aligned using the software Dnastar (LaserGene) and the results were tabulated in table 6.

3.17 Cell Culture and Transfection

Human kidney (A498) and liver (Chang liver) cancer cells, were maintained as monolayer cultures at 37^{o}C (5% CO_2) in α-MEM plus phenol red, supplement with 10% FBS and 1% PS. A498 cells were seeded at 2x10^5 in 100mm culture dishes containing cover slip swab with ethanol and grow to 70% confluence prior to transfection. Cells were transfected with 10 μg of either eGFP-pcDNA3.0 or OKL38-eGFP-pcDNA3.0 or

OKL38-pcDNA3.0 plasmid DNA and 12 µl of Lipofectamine™ 2000 reagent following manufacturer's recommendations. Each cover slip was removed at 24, 48, 72, 96 and 120 hours post-transfection using a sterile forceps. The cover slip with cells were fixed with 10% formalin, washed with PBS and mount on to slide for observation using microscope (Olympus) equipped with epifluorescence optics and appropriate filters for FITC.

Buffalo Rat Liver (BRL) cells were maintained as monolayer cultures at 37^{o}C (5% CO_2) in Hi-Glu-DMEM plus phenol red, supplement with 10% FBS, 1% PS. BRL cells were seeded at 2x10^5 in 100mm culture dishes containing cover slip swab with ethanol and grow to 70% confluence prior to transfection. Cells were transfected with 10 µg of eGFP-, RtOKL38-eGFP- or eGFP-Antisense-RtOKL38-pcDNA3.0 plasmid DNA and the cover slips were treated and observed as described above.

The human liver cancer cell lines HepG2, Hep3B, Chang Liver and PLC/PRF5 cells were obtained from American Type Culture Collection, Manassas, VA and maintained as cultures at 37 ^{o}C (5% CO_2) in Hi-Glu-DMEM plus phenol red, supplement with 10 % FBS and 1% PS. Cells were grown to about 80-90 % confluence prior to total protein and RNA extraction.

To determine the regulation of 5'UTRs, Chang Liver cells were seeded at 2.5 X 10^5 in 100-mm culture dishes and grown to 80-90 % confluence prior to transfection. Cells were transfected with 1 pmole of endotoxin-free plasmid DNA constructs and Lipofectamine™ 2000 reagent following the manufacturer's recommendations. The cells were harvested 24 hours post-transfection, divided into two portions for protein and RNA extraction. Endotoxin-free plasmids used for transfection were prepared using the Maxi-prep kit (Qiagen, GmbH, Hilden, Germany).

3.18 Patients and Tissue Samples

Prior written informed consent was obtained from all patients and the study received ethics board approval at the National Cancer Centre of Singapore, the Singapore General Hospital as well as the Binh Dan Hospital, Ho Chi Minh City, Vietnam.

Tissue samples were obtained intra-operatively from tumours and adjacent non-tumour kidney tissues during resection for kidney tumour at the Singapore General Hospital. The samples were snap frozen in liquid nitrogen and stored at –80^{o}C until analysis. A similar set of samples was fixed in 10% formalin and paraffin embedded. The HCC tissues samples were obtained intra-operatively from tumours and adjacent benign liver tissue during liver resection for HCC in 92 patients at the Singapore General Hospital and Binh Dan Hospital. 28 of 92 resected samples had single tumour and 64 of 92 had two tumours. Twenty-four tumours and its adjacent liver tissues were snap frozen in liquid nitrogen and stored at –80^{o}C until analysis. The samples were fixed in 10% formalin and paraffin embedded. The diagnosis of HCC was confirmed histologically in all cases. The kidney samples used in this study were also treated the same way. All the kidney tumours were of the clear cell histological type.

3.19 Staging and scoring in HCC

Staging of tumours was performed using the TNM system (Spiessl B et al., 1992). In addition, every tumour was examined macroscopically and microscopically for the presence of capsule formation, satellite, multiplicity and necrosis. Dysplasia and cirrhosis in the surrounding liver tissue were noted. 27 of 92 tumours were associated with cirrhosis. Within 27 cirrhotic HCCs, 24 of them had dysplastic tissue. Multifocality was defined as multiple

small uniformly sized tumours that were likely represented independent primary tumour (Ishak KG et al., 2001). This is distinguished from satellites, which were defined as tumour nodules, smaller than main tumour mass, located within a maximum distance of 2 cm. The term multiplicity was used for both multifocal tumours and for multiple intra-hepatic metastasis from a single primary tumour that were situated further than 2 cm from the edge of the main tumour mass.

The scoring for immunostainning in HCC using OKL38 antibody was described below. Tumour sections were considered negative if staining was absent or present in less than 5% of tumour cells. The immunostainning was scored using the formula IS = (i+1) * PI as described (Claudio et al., 2002), where i = intensity of staining varying between 1+ and 5+, and PI = % of positive cells. At least 20 high-power fields were chosen randomly, and 2000 cells were counted. Weak, Average and strong staining of OKL38 expression in carcinoma cells was considered when the IS were in between 20 to 100, 104 to 240 and 244 to 400 respectively.

3.20 Computational and statistical Analysis

Sequence identity and the ORF prediction were done using analysis software from the National Centre for Biotechnology Information (NCBI). The CLUSTAL W v1.82 program (EMBL) was used to perform the multiple sequence alignment and homology studies between rat, mouse and human OKL38. The predicted amino acid sequences were analysed using: i) cPfam CDS-Conserved Domain Search (NCBI) for conserved domain detection, ii) SignalP (Nielsen and Krogh, 1998) for analysing the present of signal peptide, iii) SOSUI (Hirokawa et al., 1998) and Tmpred (Hofmann and Stoffel, 1993) program for possible transmembrane region detection and iv) NetNGlyc 1.0 program

(Ricort and Binoux, 2004) and NetOGlyc 3.1 program (www.cbs.dtu.dk/services/netoglyc) (Julenius et al., 2005) were employed to detect possible glycosylation sites. The putative promoter region was analysed using the MatInspectorV2.2 (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl), CpG Island Searcher (http://www.uscnorris.com/cpgislands/cpg.cgi) (Takai and Jones, 2002) and Repeat Masker (http://ftp.genome.washington.edu/RM/RepeatMasker.html).

Statistical Analysis: Chi-square test was used to test the relationship between sex and OKL-38 expression in tumours, and between age and OKL-38 expression in tumours. Nonparametric trend analysis was used to investigate the correlation between tumour stage and OKL-38 expression in tumours. Log-rank test was used for assessing the equality of survivor functions. All these analysis were performed by means of STATA (StataCorp, 2001. STATA Statistical Software: Release 7.0. Stata Corporation, College Station, TX USA). Statistical significance was established at $p<0.05$. Survival time of those with HCC was calculated from the date of surgery to date of death. The Kaplan-Meier method was used to estimate survival curves (Altman et al., 2000). The log-rank test with one degree of freedom was used to test between OKL38 positive and negative staining groups and the hazard ratio and corresponding 95% confident interval calculated.

CHAPTER 4: RESULTS

4.1 Cloning, Sequencing and Characterization of the human OKL38 gene

The established human OKL38 variants sequence and the sequence data from the human genomic clone provided a wealth of information for deciphering the genomic organization of the human OKL38 gene. Analysis of the entire OKL38 gene will allows the elucidation of the relationship between the exon-intron arrangement, protein structural domains, and between alternative splicing and translational regulation. Furthermore, these works established the foundation for future studies on the promoter(s) of OKL38 gene as a prerequisite enabling us to unravel the mechanisms of activation, control and regulation of this cancer related gene.

4.1.1 Screening and sequencing of human OKL38 gene

In an attempt to isolate the human OKL38 gene, a genomic cosmid library was screened employing the 1.6 kb OKL38 cDNA as the probe. Two cosmid clones containing human OKL38 gene were isolated during the screening process and their identity were determined via Southern blotting. The clone containing a longer insert of approximately 35 kb was selected for sequencing. Direct sequencing of the insert ends in the cosmid clones indicated that approximately 100 bp was missing from the 3'end of OKL38 exon 8. A latter 2.5 kb genomic DNA fragment containing this missing end was subsequently cloned using the GenomeWalker™ kit. The cloning strategy is as shown in

figure 4.1A. The sequence information was deposited in Genbank with the accession number AF334780.

Residing in the subclone Bs/X-1.1 (Fig. 4.1A), an approximately 850 bp 'CCCT' rich region was identified. About 300 bp within this region could not be sequenced through current means. This region was part of intron 2, located 1.2 kb upstream of promoter P1 transcriptional start site (Fig. 4.2A). Another interesting region spanning ~500 bp was identified by Repeat Masker program as simple 'CA' repeats. Careful investigation showed that this region contained 4 long repeats with repeat 2 and 3, each 160 bp, aligned perfectly, while 1 and 4 partly conserved. This region contained approximately 70% of cytosine and adenine, and was localized 140 bp upstream of exon 2 (Fig. 4.2A). The functional significances of these unique regions are unknown at the present time.

4.1.2 Genomic structure of human OKL38 gene

Sequence comparison between the genomic and cDNA clones allowed the deciphering of the exon/intron junctions of the OKL38 gene. Each of the 5'-donor and 3'-acceptor splice sites conformed to the consensus sequences with the highly conserved, invariable GT/AG dinucleotides present at the immediate exon/intron boundaries (Table1). The human OKL38 gene spanned a genomic region of approximately 18 kb and contains 8 exons with sizes ranging from 92 bp to 1270 bp (Fig. 4.1B).

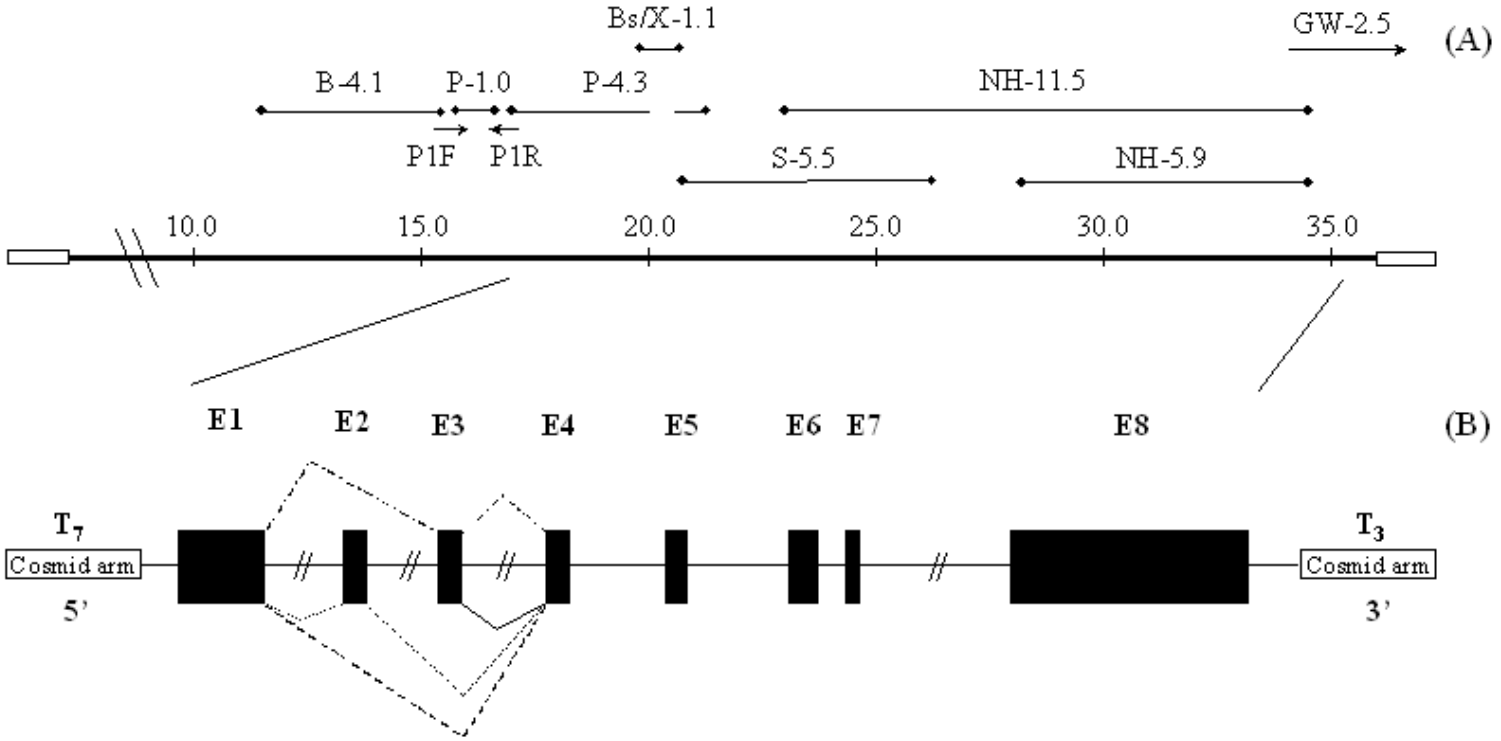


Figure 4.1: Cloning strategy and genomic organization of human OKL38 gene. (**A**) A schematic showing the contiguous map of OKL38 gene derived via restriction digestion with **B**: *BamHI*; **P**: *PstI*; **S**: *SacI*; **Bs/X**: *BstXI/XhoI* and **N/H**: *NotI/HindIII*. The **GW-2.5** fragment was cloned via genome walking, while P1F and P1R were fragments joined via direct cosmid sequencing. The subclone Bs/X-1.1 contains a 850 bp region of 'CCCT' repeats, which was cloned from subclone P-4.3. (**B**) The genomic organization of human OKL38 showing differential splicing at the 5' region of the gene. The various transcripts derived from differential splicing of exon 2 and 3 are shown.

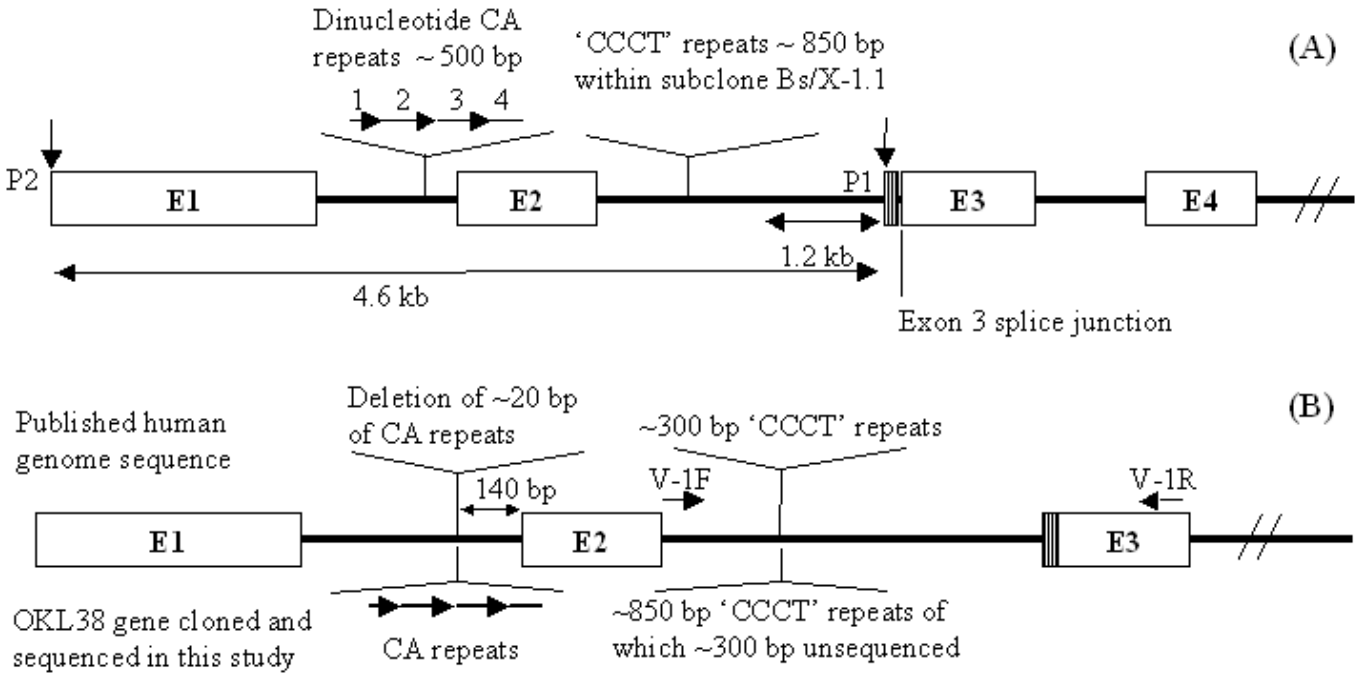


Figure 4.2: Sequence comparison and analysis of OKL38 gene. (**A**) Detailed features within the first 4 exons of human OKL38 gene. P1 and P2 represent the positions of promoter 1 and 2, respectively. (i) The distance between the 2 putative promoters, and (ii) the distance between promoter P1 and the 'CCCT' repeats are shown. Descending arrows indicate transcriptional start site. Note that the transcription start site is located 22 bp upstream of the exon 3 splice junction. (**B**) Our sequenced OKL38 gene showed two different regions when compared to that published by the human genome sequencing project. A longer 'CCCT' repeats region and a deletion of 20 bp of the 'CA' repeats are shown. Primers V-1F and V-1R were designed to verify the presence of longer 'CCCT' repeats in our sequenced OKL38 gene.

Table 1. Exon-Intron Junctions of human *OKL38* gene.

Exon No	Exon Size	5' splice donor	Intron Size (bp)	3' splice acceptor
1	440	CAGACAAGAG**gt**acgtcggc	1599	ccctctgt**ag**GTTCCTGCTA
2	160	CAGGACGAGG**gt**gaggaggg	~2.5kb	atccccac**ag**GGTAATGGGT
3	109	TGCCTGTCAG**gt**gagtgtcc	4270	cccactcc**ag**GTCCGCTGCC
4	99	ATCATTGTGG**gt**gagtgtca	1521	ctctcccc**ag**GTAACGGCCC
5	137	CCTGGACCAG**gt**gggtcagc	1172	cccctcc**ag**GACCTGGACT
6	192	AGCCTGGCAC**gt**gagtgggg	220	ttcctgc**ag**TCCATCGAAG
7	92	AGAAGCGAAG**gt**gaggccgc	3990	tccccaac**ag**AGGTCTTCGC
8	1270			

4.1.3 Discrepancy between the sequenced OKL38 and the Human Genome Sequencing Project

The human OKL38 genomic sequence established in this study was deposited in the GenBank before the completion of the Human Sequencing Project (Lander et al., 2001; Venter et al., 2001). BLAST analysis performed using the present sequence data against the published draft human sequence (Genomic contig: NT_024797.13) (NCBI) revealed that the OKL38 gene was localized to chromosome 16 (16q23.3). A comparison between the sequenced OKL38 gene with the published human genome using ClustalW delineated two regions of differences. An approximately 20 bp random deletion in the 'CA' repeats region and a 550 bp longer 'CCCT' rich region were observed in the sequenced OKL38 gene (Fig. 4.2B). To verify the presence of this longer 'CCCT' rich region in our cosmid clone, PCR was performed using a forward primer, V1-F, which resides immediately downstream of exon 2 and reverse primer, V1-R, which resides in exon 3 (Fig. 4.2B). A PCR product of ~2.5 kb was amplified instead of a 1.95 kb fragment, suggesting that the 850 bp 'CCCT' rich region in our clone is not an artefact of cloning. The presence of highly repetitive sequences and the discrepancy in sequence data may suggest that OKL38 lies in a chromosomal region of instability.

4.1.4 Identification of OKL38 putative promoters

Two putative promoters were identified via comparing the 5'RACE fragments with the cloned OKL38 genomic sequence (Fig. 4.2A). Analyzing the proximal 300 bp region of promoter P1 using MatInspector program identified several potential sites for the Ikaros factor (IK2), homeodomain factor Nkx-2.5/Csx (NKX25), upstream

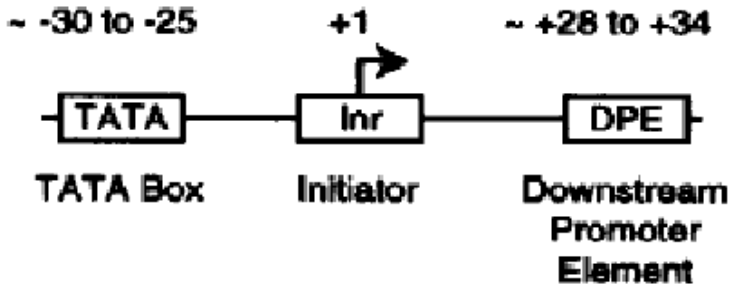
stimulating factors (USF), myeloid zinc finger protein (MZF1), Sp1 and AP-1, -2 and –4 (Fig. 4.3A). Using the CpG Island Searcher (Takai and Jones, 2002) (lower limit values 200 bp for length, 55% for GC content and 0.65 for observed CpG/expected CpG ratio), a CpG island of 215 bp located 746 bp upstream of the promoter P1 transcriptional start site was identified. No TATA or CAAT boxes were observed upstream of this initiation site. However, an alternative core promoter motif termed the initiator (Inr) (Javahery et al., 1994), which encompasses the initiation site, was identified (Fig. 4.3B). Interestingly, the sequence around this start site, **TCAGAGC** (position –2 to +5) matched the Inr consensus sequences except for the penultimate **G**, indicating that this core sequence is likely responsible for driving transcription from this site. Accordingly, a putative downstream promoter element (DPE), **ATGGGTG** (position +27 to + 33) was identified (Javahery et al., 1994) (Fig. 4.3B). Four of the seven nucleotides of this element were conserved (i.e. underlined nucleotides).

On the contrary, none of the basal transcription elements, such as TATA, CAAT, Inr or DPE were observed in promoter P2. But several potential sites for IK2, USF, myb and, many AP-1 and GATA1 were observed in the 300 bp of the proximal promoter region. A CpG island of 278 bp, located approximately 2.5 kb upstream of the putative initiation site was identified.

4.1.5 Establishment of full-length OKL38 variants via 5'-Rapid Amplification of cDNA Ends and Primer Extension Analysis

Establishing the variants full-length transcripts is an aspect of the effort to decipher the genomic organization of OKL38. The identification and cloning of the

(A)



(B)

Consensus sequence	T/C-T/C-A_{+1}-N-T/A-T/C-T/C	A/G-G-A/T-C-G-T-G
HuOKL38 promoter 1-Inr & DPE	TCAGAGC	ATGGGTG
Position	−2 to +5	+27 to + 33

Figure 4.3: Promoter P1 of human OKL38 gene. (A) A detailed map of putative transcription factors binding sites (Bold and italic) on promoter P1 of human OKL38 gene. Binding sites on the negative strand were highlighted in yellow with description below the promoter sequences, while those on positive strand were labeled above. Nucleotides in blue belong to exon 3 and the transcriptional start site at position +1 is colored red (A). (B) The consensus sequences and the location of the TATA box, Inr and DPE motifs are indicated. The putative Inr and DPE identified in human OKL38 promoter P1, their respective positions and conserved sequences (underlined) were shown.

OKL38 variants allow further characterization of their functions and regulations. To establish the full-length transcripts, 5'-Rapid Amplification of cDNA Ends (RACE) was performed using poly $(A)^+$ mRNA derived from human kidney tissues. Using GSP1R primer residing in exon 6, a PCR fragment of 443 bp, which consists of exon 3, 4 and 5 was amplified (Fig. 4.4A[ii] & B). The identity of this amplified fragment was verified by Southern blot analysis using exon 6 as the probe (Fig. 4.4C). Employing GSP2R primer residing in exon 3, a 494 bp fragment was amplified establishing exon 1 (Fig. 4.4A[iii] & D). This PCR product containing exon 1 was low in copy number (Fig. 4.4D) and could only be detected via Southern blot analysis using exon 3 as the probe (Fig. 4.4E). No fragment containing exon 2 was cloned in the 5' RACE experiment. To determine the possibility that the transcriptional start site was located in exon 1 and 3, primer extension studies was performed using Pext1 and Pext3 primer residing in the respective exons (Fig. 4.4A). The transcription start site preceding exon 3 could be observed from RNA derived from liver, kidney and prostate (Fig. 4.5) suggesting the presence of a cryptic promoter P1. A minor transcriptional start site at position –10 was also detected (Fig. 4.5). However, the transcriptional start site preceding exon 1 was not detectable by primer extension studies, suggesting low levels of exon 1 containing transcripts.

4.1.6 Tissue distribution of human OKL38

Differential expression of OKL38 in rat tissues, but not in the human, has previously been shown (Huynh et al., 2001). Human Multiple Tissues Expression (MTE) array was adopted to determine the global tissue distribution of human OKL38 (Fig. 4.6A). The MTE array showed that OKL38 was ubiquitously expressed in all the tissues

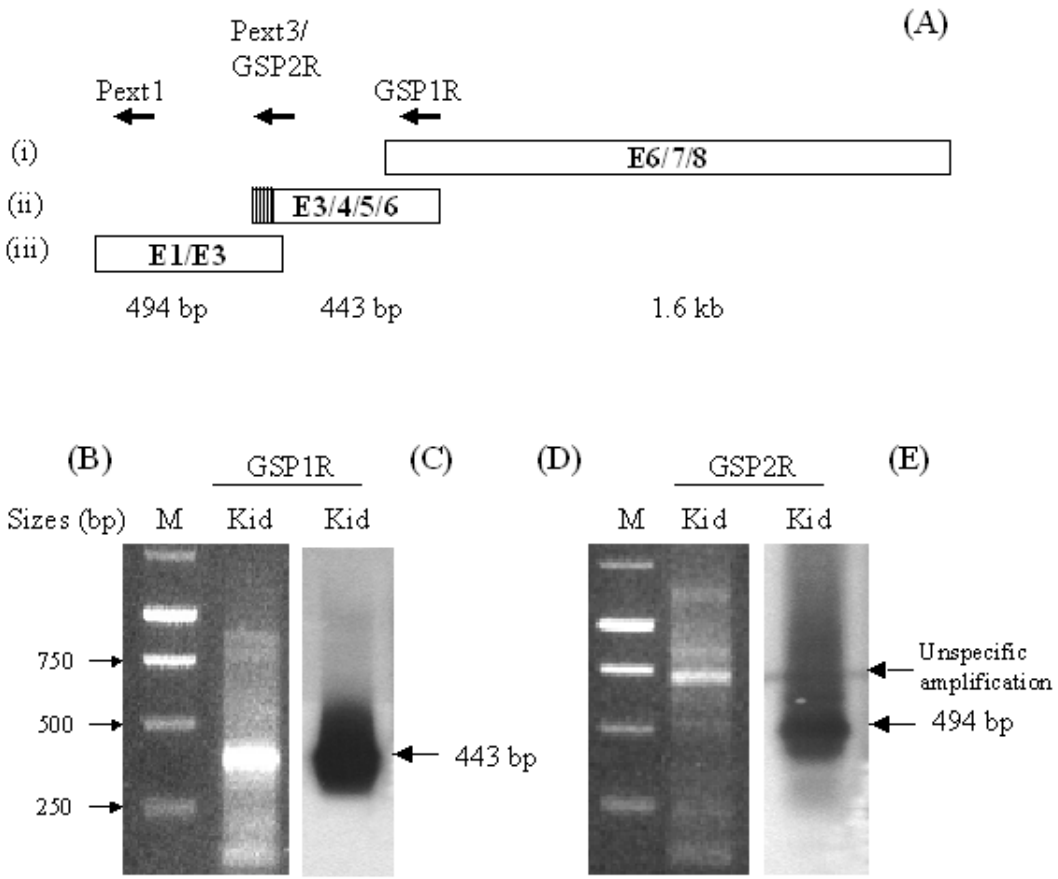


Figure 4.4: Schematic illustration of 5' RACE analysis of the human OKL38 cDNAs. (**A**) The 1.6 kb cDNA was cloned, which consists of only exon 6, 7 and 8. Gene-specific primer, GSP1R used in 5'RACE experiment established the exons 3, 4 and 5, while GSP2R established exon 1. 5'RACE PCR products of 443 bp (**B & C**) and 494 bp (**D & E**) were generated by GSPR1 and GSPR2, respectively. The amplified products were transferred to a nylon membrane and Southern blot analysis was performed as shown in (**C**) and (**E**) employing P^{32} radiolabelled cDNA cloned from exon 6 and 3, respectively. **Kid**: kidney mRNA was used as the template for 5'RACE studies.

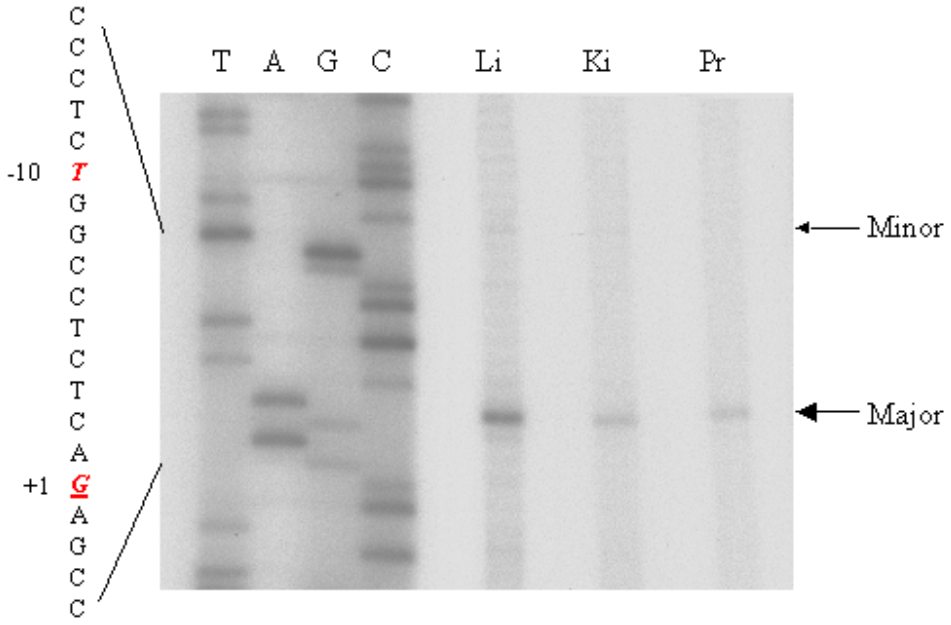
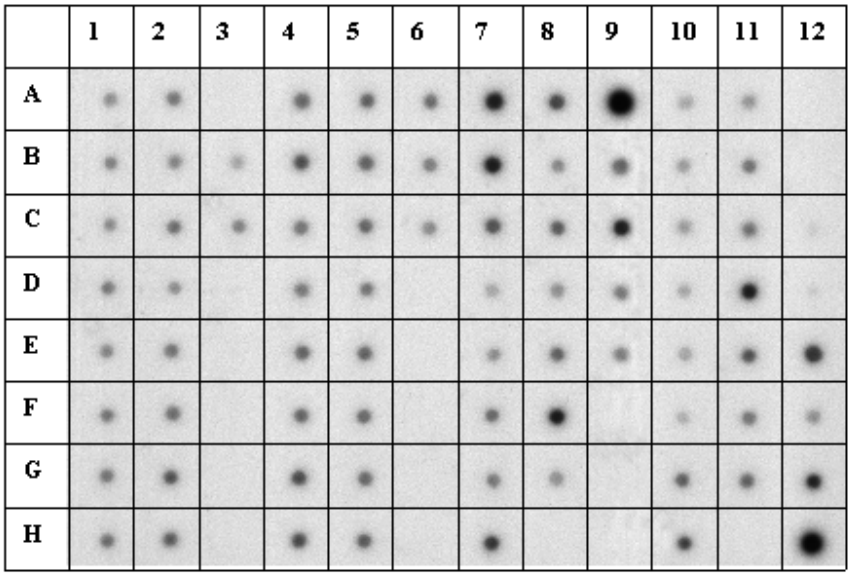


Figure 4.5: Primer extension analysis of the human OKL38. Two reverse primers, Pext1 and Pext3 were designed for primer extension analysis as illustrated in Figure 4.4A. Primer extension studies performed using **Li**: liver, **Ki**: kidney and **Pr**: Postate mRNA as templates and reverse primer Pext3. The nucleotides in red (G and T), indicate a major and a minor transcriptional start site, respectively. Note that Pext1 failed to obtain any extended product.



(A)

(B)

	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebellum, left		heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain	yeast total RNA
B	cerebral cortex	cerebellum, right	accumbens nucleus	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney	*E. coli* rRNA
D	parietal lobe	amygdala		atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver	*E. coli* DNA
E	occipital lobe	caudate nucleus		ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen	Poly r(A)
F	temporal lobe	hippo-campus		ventricle, right	ilocecum		lymph node	testis		Burkitt's lymphoma, Daudi	fetal thymus	human C_0t -1 DNA
G	p. g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone morrow	ovary		colorectal adeno-carcinoma, SW480	fetal lung	human DNA 100 ng
H	pons	putamen		apex of the heart	colon, ascending		trachea			lung carcinoma, A549		human DNA 500 ng

* paracentral gyrus

Figure 4.6: Global tissue distributions of human OKL38 transcripts via Multiple Tissue Expression (MTE) array. The MTE array was probed with a 700 bp human OKL38 ^{32}P-radiolabelled probe and the auto-radiograph was shown in (A). The identity of each cDNA dot is represented in the grid (B).

investigated, with high expression levels detected in the kidney (7:A), skeletal muscle (7:B), testis (8:F), liver (9:A), adrenal gland (9:C) and fetal liver (11:D) (Fig. 4.6B). Human Multiple Tissues Northern (MTN) blot analysis showed that transcripts of approximately 2.0 to 2.4 kb were ubiquitously expressed in all the 15 tissues investigated (Fig. 4.7). High expression of these transcripts in liver, kidney and testis confirmed the MTE array analysis. Larger putative transcripts ranging from 4.0 to 7.0 kb were also observed in the liver (Fig. 4.7A).

One step RT-PCR was devised to determine the differential expression of human OKL38 variants in various tissues. Four RT-PCR products of 443 bp, 373 bp, 533 bp and 482 bp corresponding to the HuOKL38-1a, -2a, -2b and -2c transcripts, respectively, were amplified (Fig. 4.8A-D). A RT-PCR product of 415 bp containing part of exon 7 and 8, represent total levels of OKL38 transcripts was amplified from kidney, liver and ovary (Fig. 4.8E). Figure 4.8A demonstrated that the primers designed for the one step RT-PCR were variant-specific and could identify the HuOKL38-1a, -2a and -2b variants. Southern blot analysis (Fig. 4.8D) was performed using exon 4 & 5 as probe and sequencing verified the identity of the amplified products. The 4 human OKL38 variants were expressed in all the three tissues investigated albeit at different levels. HuOKL38-1a possesses the highest level of expression as compared to the other 3 variants (i.e. fewer PCR cycles required for amplification of the HuOKL38-1a). Highest level of OKL38 transcripts was detected in the liver as compared to the kidney and ovary (Fig. 4.8B-E), which is in-line with the MTN and MTE results.

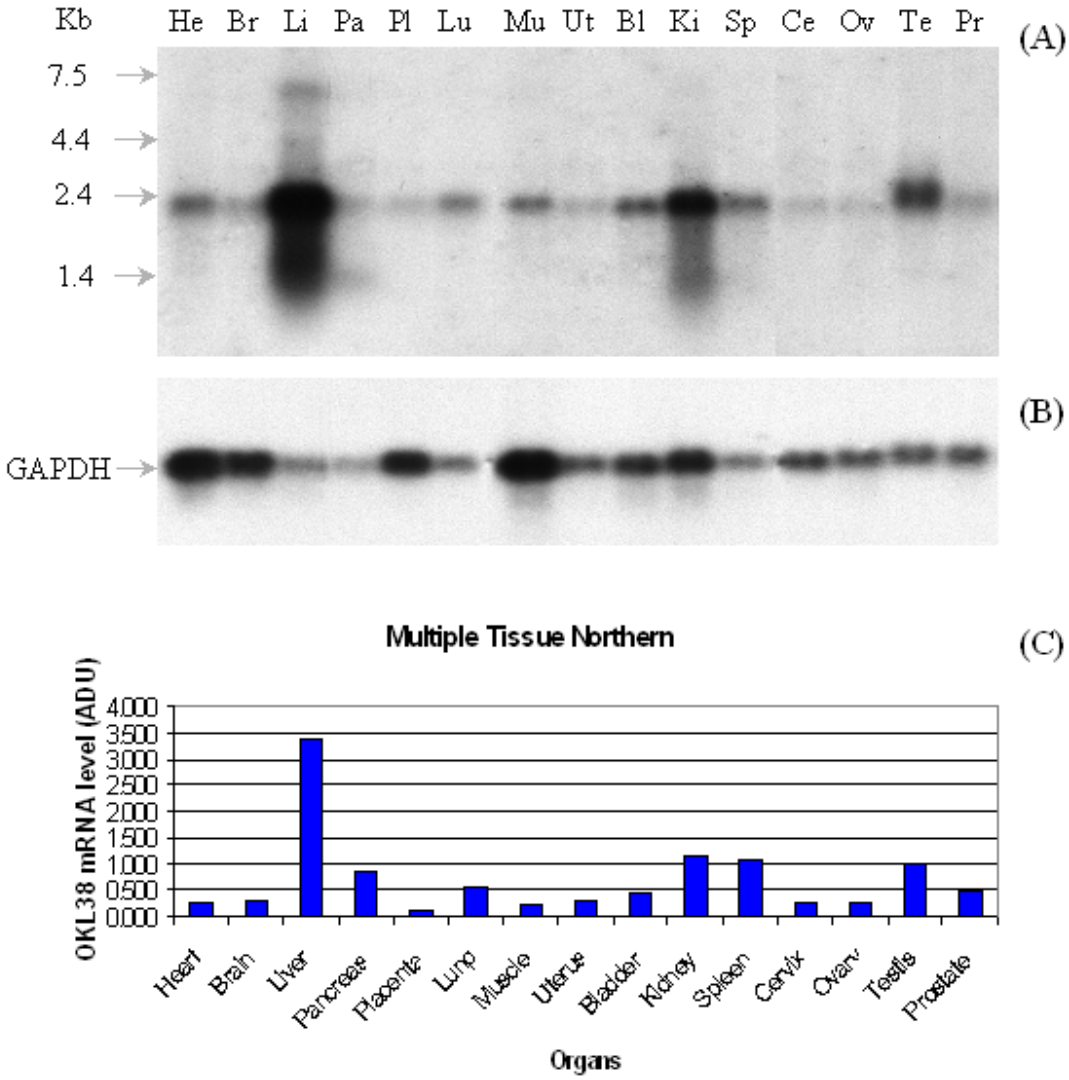


Figure 4.7: Tissue distribution of human OKL38 transcripts via Multiple Tissue Northern (MTN) blot analysis. The MTN blot analysis was performed using the 700 bp human OKL38 ^{32}P-radiolabelled probe which is specific for detection of all the OKL38 variants (A). The tissues represented were **He**: heart, **Br**: brain, **Li**: liver, **Pa**: pancreas, **Pl**: placenta, **Lu**: lung, **Mu**: muscle, **Ut**: uterus, **Bl**: bladder, **Ki**: kidney, **Sp**: spleen, **Ce**: cervix, **Ov**: ovary, **Te**: testis and **Pr**: prostate. The loading in each lane was normalized with GAPDH (**B**). The relative levels of OKL38 expression after GAPDH normalization was as shown in (C).

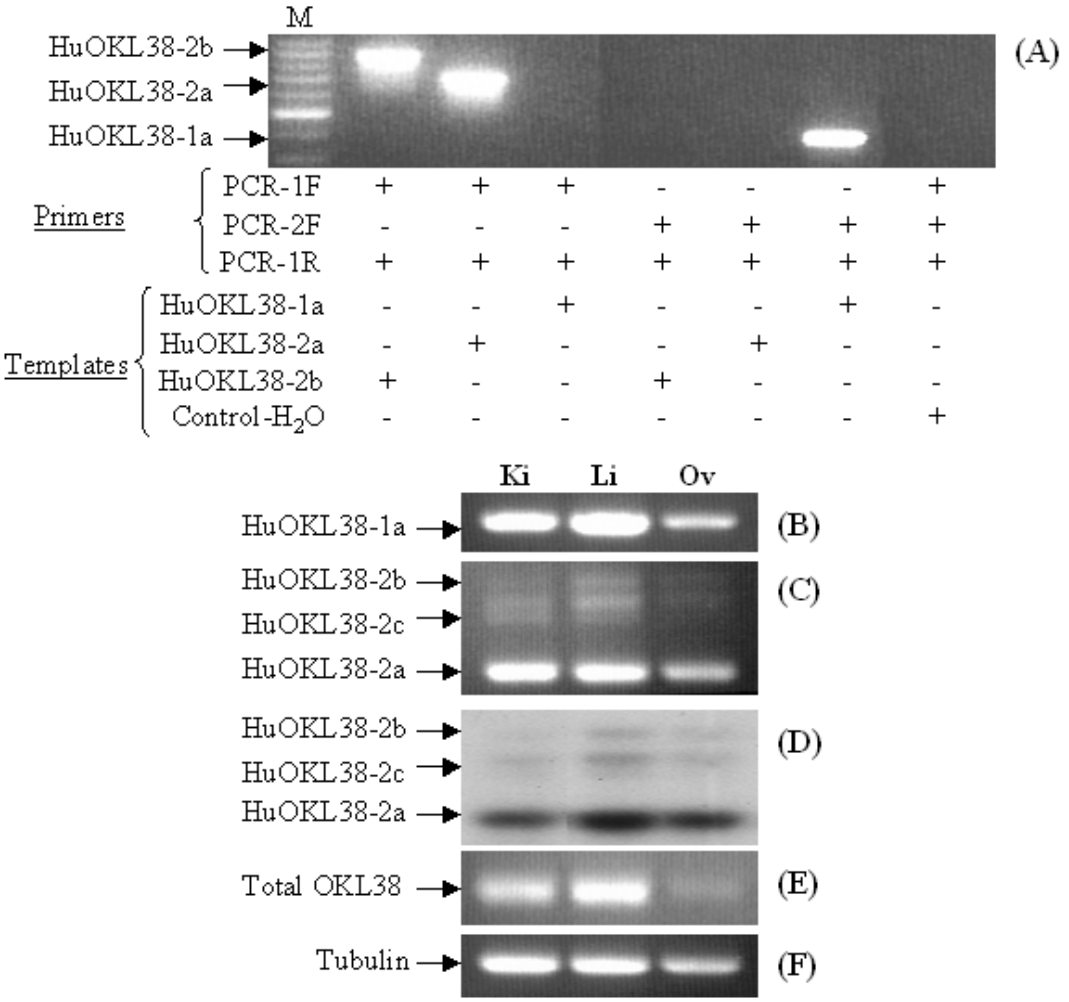


Figure 4.8: Detection and differential expression of human OKL38 variants in human tissues. (**A**) The specificity of each pair of primers was verified by PCR using the cloned OKL38 cDNAs. PCR was performed on 3 of the 4 variants of OKL38 cDNAs using a combination of 3 variant specific primers. Note that primers were specific for detection of all three variants. One step RT-PCR was performed using total RNA extracted from **Ki**: kidney, **Li**: liver and **Ov**: ovary (**B-F**). Five RT-PCR products of 443 bp, 373 bp, 533 bp, 482 bp and 415 bp corresponding to the HuOKL38-1a, -2a, -2b, -2c and total levels of transcripts, respectively. Number of PCR cycles used were 35 for HuOKL38-1a (**B**), 38 for HuOKL38-2a, -2b & -2c (**C&D**), 32 for detecting total levels of OKL38 (**E**) and 28 cycles for tubulin (**E**). The amplified products were separated on a 2.0% agarose gel and transferred onto nylon membrane. Southern blot analysis was performed using probe derived from exon 4, 5 and 6 (**D**).

4.1.7 Molecular Cloning of Human OKL38 cDNAs

Sequence comparison of the 5'RACE PCR products derived via employing primers GSP1R and GSP2R, and the cloned genomic sequence showed that the first 22 bp (position +1 to +22 at promoter P1; refer to figure 4.3A) is absent in exon 3 and was unique to the HuOKL38-1a variant. To clone the full length HuOKL38-1a, forward primer 1F (Table 2) that resided mostly within the 22 bp unique region of the GSP1R 5'RACE fragment and a common reverse primer, 1R (Table 2) were designed for RT-PCR cloning of the HuOKL38-1a transcript (Fig. 4.9A). Cloning of the HuOKL38-2a and 2b transcripts involves usage of forward primer 2F residing in exon 1 instead of 1F (Fig. 4.9A). Three PCR products of approximately 1.9, 2.2 and 2.4 kb were cloned and five identical clones of each cDNA were sequenced, which allowed establishment of 3 novel human OKL38 cDNAs of 1930, 2240 and 2400 bp, respectively. The 1.9 kb cDNA was annotated as HuOKL38-1a (GenBank Accession No. AY258068), the 2.2 kb cDNA as HuOKL38-2a (GenBank Accession No. AY258067) and the 2.4 kb cDNA as HuOKL38-2b (GenBank Accession No. AY258066).

The results from 5' RACE analysis indicate the presence of a transcript containing exon 3 (Fig. 4.4A). Expression studies using one-step RT-PCR and Southern blot analysis verified that this minor transcript (HuOKL38-2c) contained exon 1, 3, 4, 5 and 6 (Fig. 4.8). It is likely that this transcript also contains exon 7 and 8 as illustrated in figure 4.9A. However, due to the low expression of HuOKL38-2c in the liver and kidney RNA samples, we have not managed to clone the full-length sequence of this cDNA. The cloning of HuOKL38-1a, -2a & -2b variants confirmed the existence of these transcripts.

TABLE 2. Sequences of oligonucleotides used for RT-PCR ([a]), Cloning ([b]), Sequencing ([c]), Primer extension ([d]), PCR ([e]) and 5'RACE Primer ([f]).

Oligonucleotide	Sequence
[d] Pext1	5' CTGCTGACCCTGACCTTGTTCTAGA 3'
[d f] Pext3 / GSP2R	5' GGTCAGGGGAACACGGATCACAGAGTCC 3'
[f] GSP1R / PCR-1R	5' CGTAGAAGGGCATCAAAGAGCAGGG 3'
[a b] 1F	5' AAGCTTGGATCCCCACAGGGTAATGGGTGT 3'
[b] 1R	5' GAATTCTGGAAGGCGCAGGGCTGCAGGTCT 3'
[b] 2F	5' AAGCTTGGCAGGGAGGAAAGTCCACGTCT 3'
[a] PCR-1R	5' CGTAGAAGGGCATCAAAGAGCAGGG 3'
[a] PCR-2F	5' GGGAAGTGGAGACTGAGAGGCTGCTGC 3'
[b] 477-F	5' CACCATGAGCTCCTCCAGAAAGGACCA 3'
[b] 477-eGFP-R	5' CTCGCCCTTGCTCACCATGGGTGGCTTCCTGGTCTC 3'
[b] eGFP-F	5' ATGGTGAGCAAGGGCGAG 3'
[b] eGFP-R	5' TTACTTGTACAGCTCGTCCA 3'
[c] Seq1R	5' CTGGTCAGGAAGCCGCTCACCTGGAAGA 3'
[c] Seq2R	5' CAGTCTTCCTTGAAGCACAGCAGCTGGT 3'
[c] Seq3R	5' GATGGCGTGCTCCTTCCGGTGCTTCC 3'
[c] Seq4F / GW-F	5' GAGATGACCACATCCCTGCTGGATGC 3'
[c] Seq5F	5' GTGTTCAACCAGCTGCCCAAGATGC 3'
[c] P1F	5' CTGAGGAAGAGGGAGGCAAGAGACAGAG 3'
[c] P1R	5' GCTGATATGGGGAACTGTGGGGCGAGAC 3'
[e] V-1F	5' AGGCATCACCTCAGGCACAA 3'
[e] V-1R	5' CTGACAGGCAGTGGTGCAGGA 3'
[b] 1aORF-F	5'- AAGCTTGGATCCCCACAGGGTAATGGGTGT -3'
[b] ORF-R	5'- GAATTCTTAGGGTGGCTTCCTGGTCTCCTTCC -3'
[b] 2bORF-F	5'- AAGCTTCACCGTGGGATGGGGAA -3'
[b] 1aD1-F	5'- AAGCTTCTGTGATCCGTGTTCCC -3'
[b] 1aD2-F	5'- AAGCTTGGCCGGGCTCACT -3'
[b] 2aD1-F	5'- AAGCTTTGGAGGAAGGCCAGG -3'
[b] 2aD2-F	5'- AAGCTTCTCAAGCCCCAGCCTT -3'
[b] 2aD3-F	5'- AAGCTTCAAAACCACTGGGAAGC -3'
[b] 2aD4-F	5'- AAGCTTGGAGACTGAGAGGCTGCT -3'
[b] UTR-R	5'- GAATTCATGGCTGGTGGGGGGCTT -3'
[b] E4-F	5'- AGCTTAGGTCCGCTGCCAGCCCCAAGCCCCCCACCAGCCATG -3'
[b] E4-R	5'- AATTCATGGCTGGTGGGGGGCTTGGGGCTGGCAGCGGACCTA -3'
[a] E7TF	5'- GTCAAGGACTGGATGCAGAAGA -3'
[a] E8TR	5'- CAATGATGAGGACAGGGTCTGA -3'
[c] E81R	5'- TCTTCCAGGTGAGCGGCTTCCTGACCAG -3'
[c] E82R	5'- ACCAGCTGCTGTGCTTCAAGGAAGACTG -3'
[c] E81F	5'- GTGTTCAACCAGCTGCCCAAGATGC -3'
[c] E61R	5'- CCCTGCTCTTTGATGCCCTTCTACG -3'

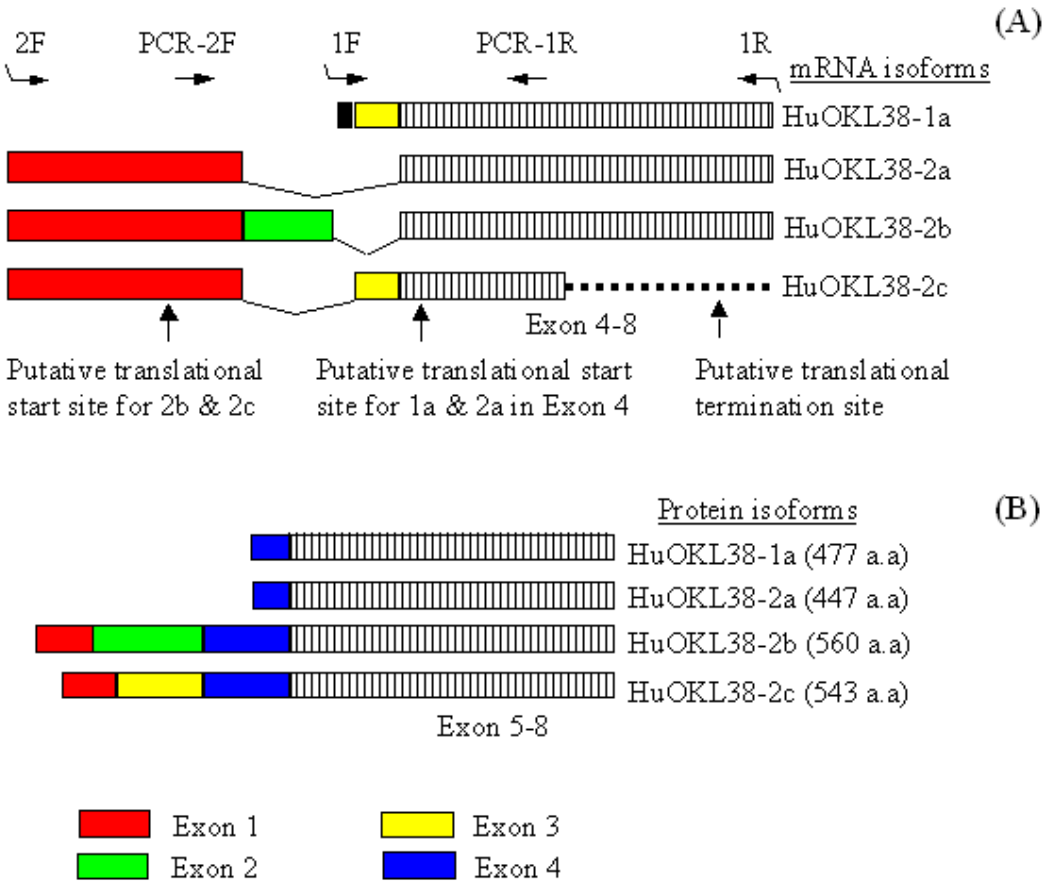


Figure 4.9: Splice variants of the human OKL38 gene. (A) Schematic showing the various OKL38 mRNA isoforms. HuOKL38-1a, -2a and -2b were cloned via RT-PCR using primers 1F, 2F and 1R, while HuOKL38-2c was verified via one-step RT-PCR using primer PCR-2F and PCR-1R. Primer 1F was used for specific amplification of HuOKL38-1a in one-step RT-PCR. Ascending arrows indicate the translational start and termination sites. The dotted line indicates putative cDNA region to be cloned in future studies. Putative protein isoforms derived from the mRNAs mentioned in (A) were shown in (B). The splicing events generated three different protein isoforms and different exons were represented by different coloured boxes.

4.1.8 Sequence analysis of Human OKL38 variants

Detailed sequence analysis of the human OKL38 variants would reveal useful information on the functions and regulations of the gene. The results from sequence analysis indicated that differential promoter usage and alternative splicing at the 5' region of *OKL38* gene were responsible for the variants existence. HuOKL38-1a transcript was derived from the usage of promoter P1, while HuOKL38-2a, -2b and -2c expression might be regulated via promoter P2. The HuOKL38-2a, -2b and -2c were derived by alternative splicing of exon 2 and 3, exon 3 and exon 2, respectively (Fig. 4.9A). The HuOKL38-1a and -2a variants harboured an ORF of 1431 base pairs. The conceptual translation of these cDNAs predicted a protein of 477 amino acids, with a calculated molecular mass of 52 kDa (Fig. 4.9B) and a predicted P. I. of 6.6. Two other protein isoforms with a molecular weight of 61 and 59 kDa were also predicted from HuOKL38-2b and -2c, respectively (Fig. 4.9B). *In vitro* Transcription and Translation study employing the cloned HuOKL38-1a, -2a, and -2b resulted in the synthesis of two proteins of approximately 52 and 61 kDa (Fig. 4.10A) and their identity was confirmed via Western blot analysis using anti-human OKL38 antibodies (Fig. 4.10B). The previously cloned OKL38 cDNA (GenBank Accession No. AF191740) showed the expression of a 38 kDa protein. Western blot analysis also detected low levels of the 38 kDa protein from these larger cDNA constructs suggesting that translation from an internal translation start codon (AUG) might exist (Fig. 4.10B). The predicted internal inframe start codon (AUG) and the molecular weight of the putative proteins in HuOKL38-1a were highlighted in figure 4.11. None of the predicted inframe ORF contains the Kozak's consensus sequence

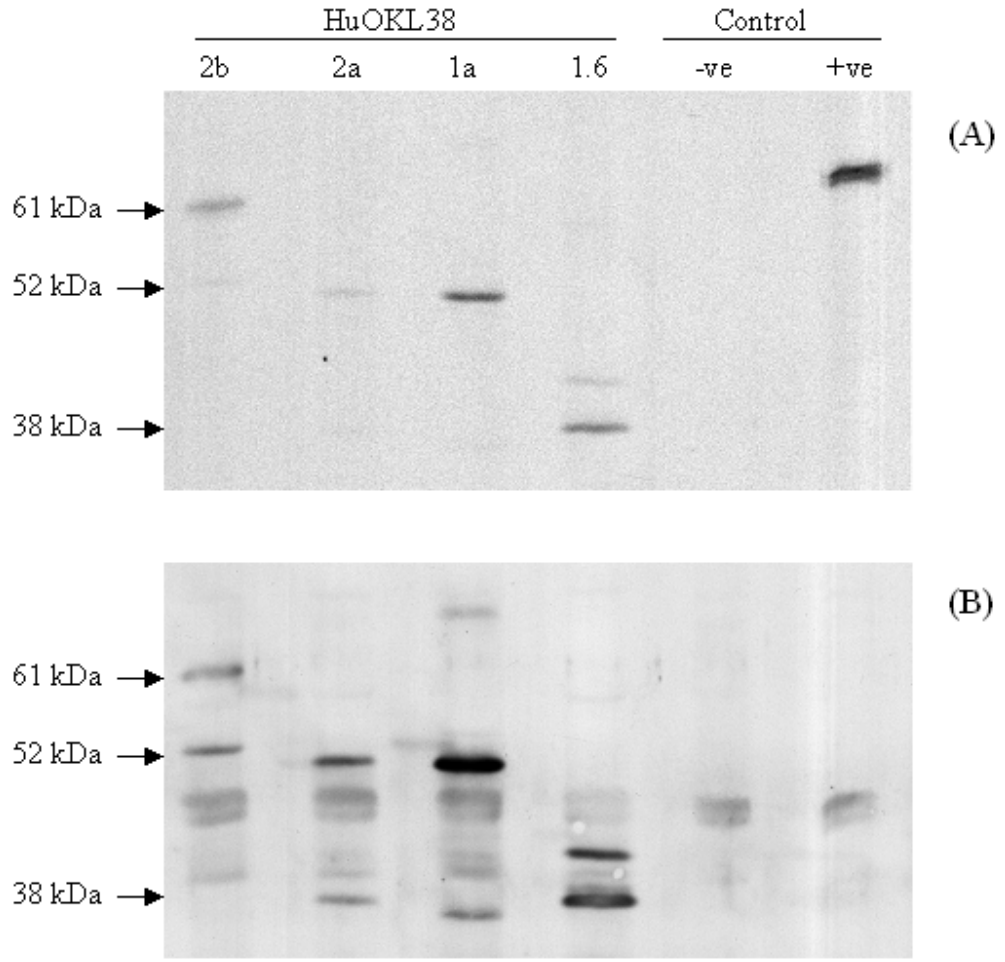
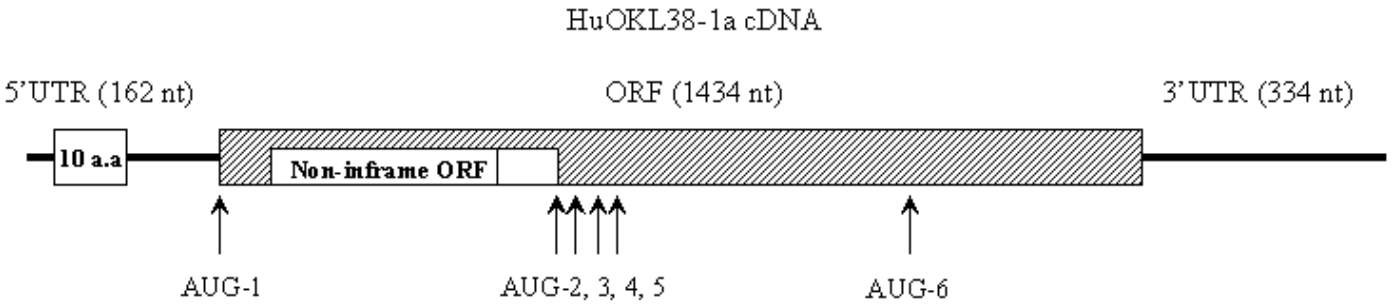


Figure 4.10: *In vitro* Transcription and Translation analysis (TNT) of OKL38 cDNAs. Radio-labelled (S^{35}) OKL38 proteins produced from cloned cDNAs variants using the TNT system as described in the *Materials and Methods*. The synthesized proteins were electrophoresed and fractionated on a 8% PAGE gel and transferred on to nitrocellulose membrane. Blots were exposed to the auto-radiograph and the S^{35} methionine-labelled OKL38 proteins are shown in (A). OKL38 proteins of various sizes were detected when identical blots were probed with rabbit anti-OKL38 antibodies (**B**).



(B)

In-frame AUG	Position (nt)	Kozak	Amino acids (a.a)	Protein size (kDa)
AUG - 1	163	No	477	51.99
AUG - 2	469	No	375	41.13
AUG - 3	574	No	340	37.22
AUG - 4	601	No	331	36.18
AUG - 5	634	No	320	34.89
AUG - 6	1156	No	146	16.24

Figure 4.11: Schematic representation of HuOKL38-1a. (**A**) Features of the HuOKL38-1a cDNA variant. The shaded box represents the main ORF, which encodes a protein of 52 kDa. A small non-inframe ORF with an internal AUG is shown within the main ORF. The arrows (AUG-2 to 6) indicates the positions of inframe internal translation start sites. An uORF of 10 amino acids is also shown in the 5'UTR of HuOKL38-1a. (**B**) Table summarizes the positions of the inframe internal AUGs and predicted size of the protein that they translate.

(Fig. 4.11B). An out of frame ORF (position 296-472 nt) was also identified within the 52 kDa ORF, which may play a role in internal translation initiation (Fig. 4.11A).

The human OKL38 cDNA variants derived through differential promoters usage and alternative splicing contained 5'UTRs of varying length (Fig. 4.12A). The 5'UTR of HuOKL38-1a is 162 bp in length, while the 5'UTRs of HuOKL38-2a and -2b are 472 bp and 383 bp in length, respectively. Small uORF of 10 amino acids was detected in the 5'UTR of HuOKL38-1a transcripts, while two uORFs of 21 and 30 amino acids were identified in the 5'UTRs of the HuOKL38-2a transcripts (Fig. 4.12A). Similarly, the 21 amino acids uORF was also present in the HuOKL38-2b transcripts, which resided in exon 1. The lower translation efficiency of HuOKL38-2a and -2b observed in the *in vitro* Transcription and Translation results (Fig. 4.10) suggest that these uORFs may play a role in translational suppression.

Similar to the previously cloned human OKL38 protein, no putative signal peptide was found in all three human OKL38 protein isoforms using the publicly available SignalP program (Nielsen and Krogh, 1998) and no potential glycosylation site was detected suggesting a protein of cytoplasmic distribution. Computational analysis of the predicted amino acid sequence using Pfam CDS-Conserved Domain Search (NCBI) detected two putative domains belonging to that of the pyridine nucleotide-disulphide oxidoreductase (Pyr_redox) and TrkA in all three human OKL38 isoforms (Fig. 4.12B). The TrkA domain (amino acid position 100-301) in OKL38 protein is 40% identical to the consensus sequences and is encoded by exon 6-8. The Pyr_redox domain (amino acid position 239-446) in OKL38 protein is 61.8 % identical to the consensus sequences and is

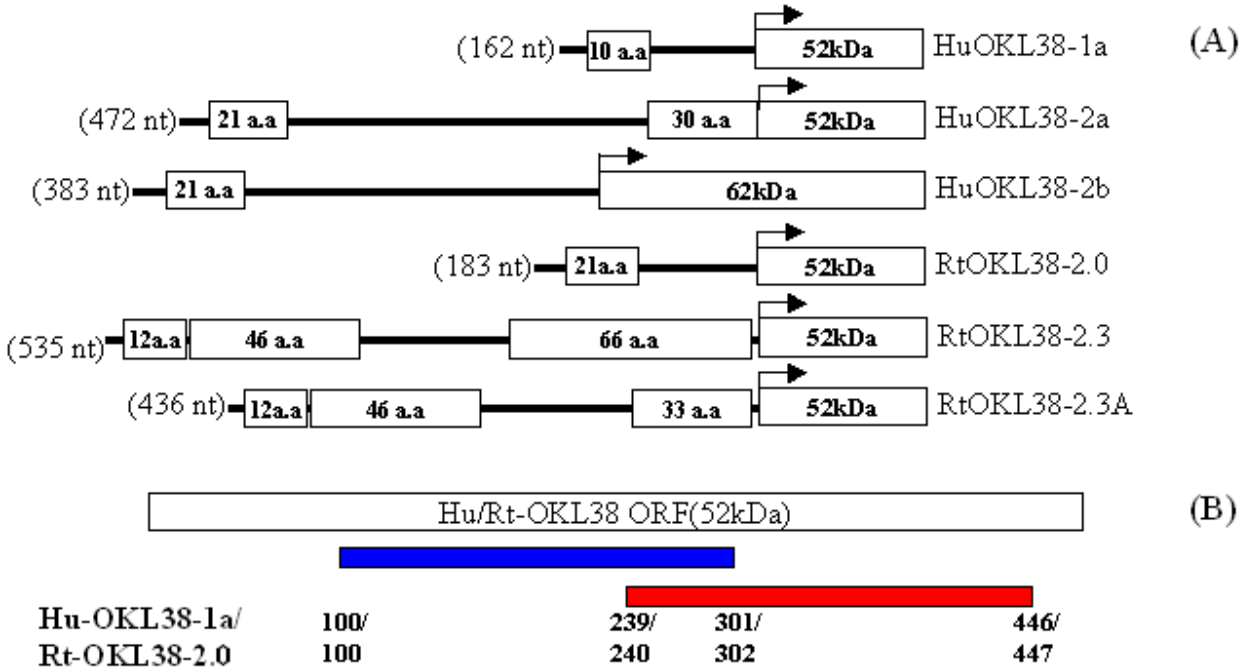


Figure 4.12. Schematic illustration of the similarities and differences between the human and rat OKL38. (A) The 5'UTRs (━━) and uORF (White box with size of protein indicated) of the human and rat OKL38 variants cloned in this study. The numbers on the left represent the length of 5'UTRs and the variants annotation on the right. Black forwarding arrows (┌►) indicate the translational start sites. (B) A graphical representation of the predicted TrkA (Red boxes) and Pyr_redox (Blue boxes) domain from human and rat OKL38, with position of amino acids indicated. Note that the TrkA domain partially overlaps with the Pyr_redox domain in OKL38 protein.

encoded by exon 8. The two domains overlaps each other by about 60 amino acids as shown in Figure 4.12B.

4.1.9 Over-expression of OKL38 is lethal to A498 and Chang Liver cells

Previous study demonstrated the growth inhibitory function of OKL38 suggesting its tumour suppressor role in breast cancer (Huynh et al., 2001). To further characterize the function of the full-length OKL38 protein (52 kDa), an OKL38-eGFP-pcDNA3.0 construct with a fused reporter gene eGFP to the C-terminal of OKL38 protein, was generated. Preliminary transfection studies showed that OKL38-eGFP recombinant protein formed aggregates in A498 kidney cancer cells as early as 24 hours post-transfection (Fig. 4.13B). None of the cells expressing the recombinant protein survived 96 hours post-transfection as compared to the eGFP positive control cells. The results showed that over-expression of OKL38 protein is lethal to A498 cells.

Seeming from the above observations, Chang liver cells were transiently transfected with the earlier constructs OKL38-eGFP-pcDNA3.0 and eGFP-pcDNA3.0 positive control plasmid (Fig. 4.14A-D). Similar to the observations in A489 kidney cancer cells, green aggregates were observed in the Chang liver cells 24 hours post-transfection (Fig. 4.14B). Rounding up of the OKL38-eGFP-transfected cells was observed and cell death was evident 48 hours post-transfection (Fig. 4.14C & D). After 5 days of culture, virtually no OKL38-eGFP-expressed cells were detected as compared to the controls.

To exclude the possibility that cell death was a consequence of misfolding of the large OKL38-eGFP recombinant protein, OKL38 without eGFP was constructed and

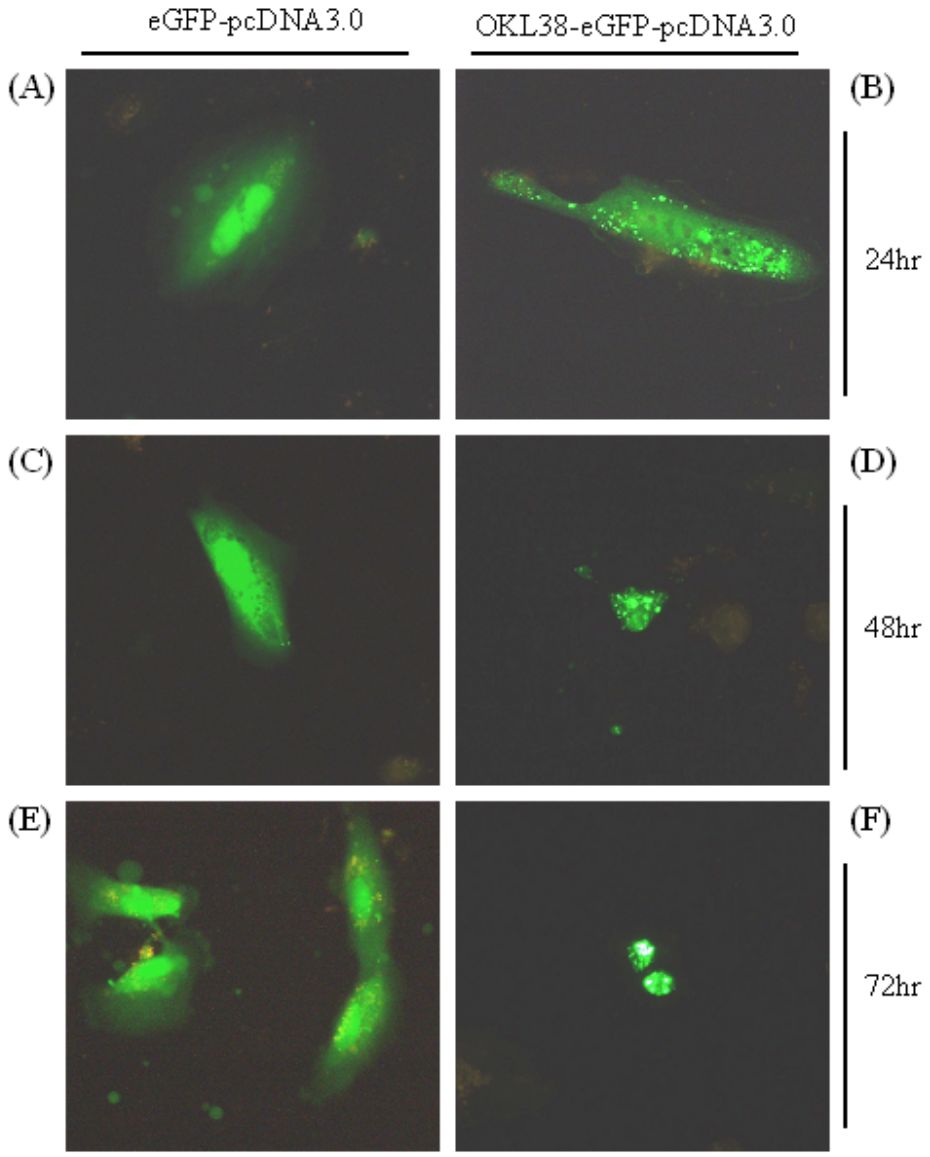


Figure 4.13: Transfection studies of HuOKL38-eGFP recombinant. A498 cells were transfected with control eGFP-pcDNA3.0 (**A**, **C** & **E**) and the OKL38-eGFP-pcDNA3.0 recombinant construct (**B**, **D** & **F**), as described in *Materials and Methods*. The transfected cells were harvested 24 hr (**A** & **B**), 48 hr (**C** & **D**) and 72 hr (**E** & **F**) post-transfection. Cells expressing OKL38-eGFP protein were visualized using microscope equipped with epifluorescence optics. Magnification X 800.

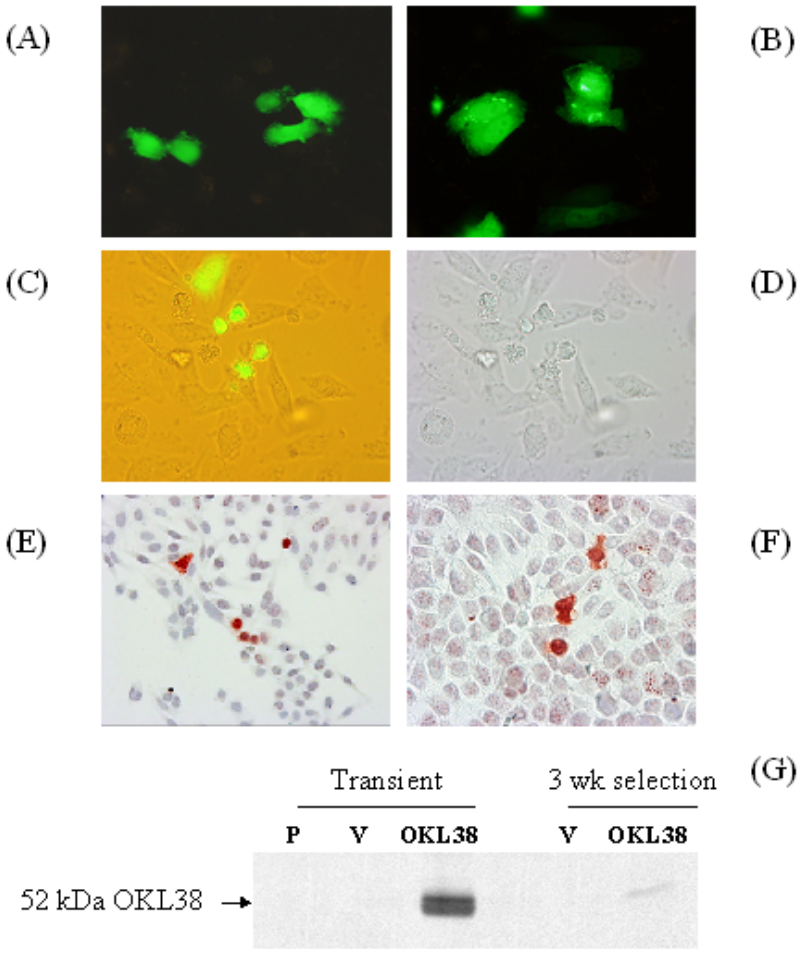


Figure 4.14: Morphology of OKL38 and OKL38-GFP-transfected Chang liver cells. Chang liver cells were transfected with plasmid containing eGFP (**A**), OKL38-eGFP (**B-D**), OKL38 (**E & F**) or pcDNA3.0 empty vector as described in *Materials and Methods*. After transfection, cells were grown in growth medium for 24 (**B**) & 48 (**A, C-F**) hrs. Cells expressing the eGFP were visualised under fluorescent microscope (Olympus BX60) equipped with an FITC filter (**A-C**). The same cells in (**C**) were also observed using phase contrast microscopy (**D**). Immunohistochemical staining using OKL38 antibody was performed to detect cells expressing high level of OKL38 protein (**E & F**). (NB: The transfected cells showed the same morphology regardless the presence of fused eGFP). Western blot analysis indicated the presence of OKL38 protein (52 kDa) 48 hr post-transfection and upon 3 weeks in selective media no protein was evident (**G**). **P**: Parental untransfected Chang Liver cells, **V**: Transfected with pcDNA3.0 control vector. Magnification: ×100 (E), ×200 (F) & ×400 (A-D).

transfected as described above. Immunohistochemical staining using OKL38 antibody showed that Chang Liver cells over-expressing the FL-ORF rounded up and were non-viable (Fig. 4.14E & F). Western Blot analysis showed that the transiently transfected Chang liver cells expressed a protein of approximately 52 kDa (Fig. 4.14G). However, viable cells selected under the presence of G418 had lost OKL38 expression, suggesting that over-expression of OKL38 is lethal to both A498 and Chang Liver cells (Fig. 4.13 & 4.14).

4.2 Cloning and Characterization of rat OKL38 gene

Animal models are valuable tools to researchers as they provide a platform for testing efficacy of drugs and *in vivo* characterization of novel genes. In order to develop a rat model for further characterization of *OKL38*'s role in growth, differentiation and tumourigenesis, the rat OKL38 gene was isolated and characterized. Deciphering the genomic structure and cloning of the rat OKL38 variants provide the foundation for future studies.

4.2.1 Genomic structure of Rat OKL38 gene

In silico data mining is widely used to harness the vast amount of information freely available in the Internet, especially with the completion of the human and other mammalian genome sequencing projects. Employing the NCBI BLAST algorithm, the sequence of the three cloned rat OKL38 cDNAs in this study were used to search against the Genbank sequence database. The rat OKL38 gene was mapped onto the rat chromosome 19q12 (WGS supercontig; NW_043217.1). Using the sequence information, the genomic structure of the rat OKL38 gene was deduced (Fig. 4.15), and the

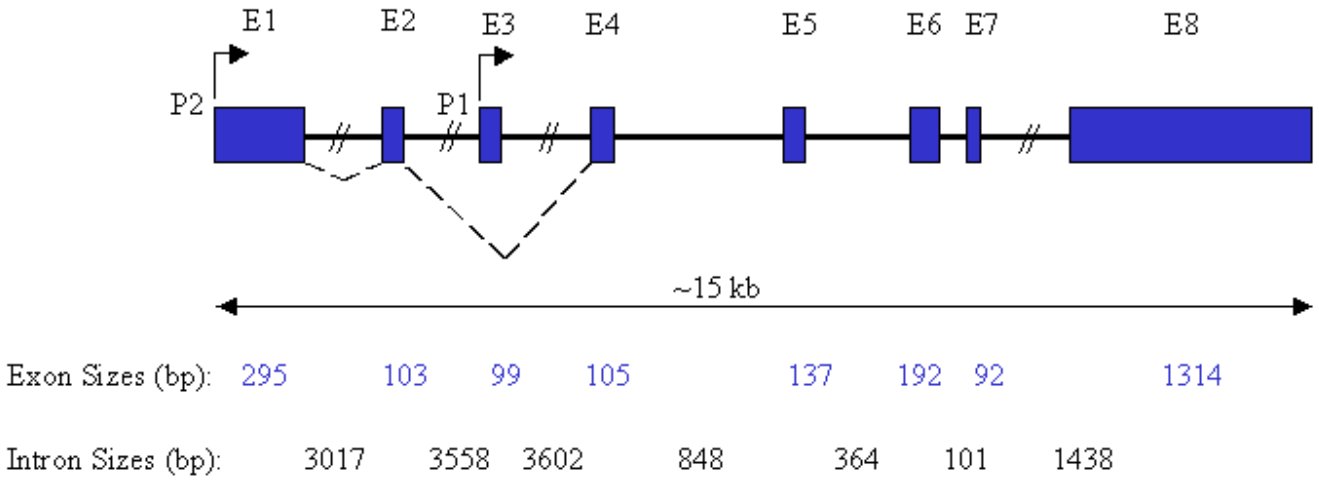


Figure 4.15: Genomic organization of rat OKL38 gene. Schematic of the genomic structure of rat OKL38 showing differential splicing at the 5' region of the gene. Forwarding arrows (↱) preceding 5'end of exon 1 and 3 denote the two putative promoters (P2 & P1) and the dotted line showed alternative splicing of exon 3. Exon numbers were indicated above the shaded boxes. The sizes of the exons and introns were indicated below the diagram.

TABLE 3. Exon-Intron Junctions of rat *OKL38* gene.

Exon No	Exon Size	5' splice donor	Intron Size (bp)	3' splice acceptor
1	295	GGACGGGCAG**gt**aagacaca	3017	tttgagac**ag**AATCTCTCAC
2	103	GGGATTTCAG**gt**gcgtgttg	3558	gttcttgc**ag**CAGGGCACTG
3	99	CGTCCATCAG**gt**gagcagtg	3602	tgttctcc**ag**GTCAGCTGCC
4	105	GTTATCATTG**gt**aagtgtct	848	gtctctgc**ag**GCAATGGTCC
5	137	CTTGGATCAG**gt**gggtctga	364	ccttcttc**ag**GACCTAGAAT
6	192	TGCCTGGCAT**gt**gagtgtcg	101	ttctctgc**ag**TCCATTGAAG
7	92	AGAAATGCAG**gt**aaggggac	1438	tcccttc**ag**AGGCCTCCGT
8	1314			

exon/intron boundaries were established. Each of the 5'-donor and 3'-acceptor splice sites conformed to the consensus sequences with the highly conserved, invariable GT/AG dinucleotides present at the immediate exon/intron boundaries (Table 3). The rat OKL38 gene spanned a genomic region of approximately 15 kb and comprised of 8 exons with sizes ranging from 92 bp to 1314 bp (Table 3).

The 5'RACE results and cloning of the rat OKL38 mRNA isoforms suggested that differential promoter usage and alternative splicing at the 5' region of *OKL38* gene might be responsible for the presence of the variants. Two putative promoters were also identified via 5'RACE analysis and sequence comparison in this study (Fig. 4.15). The RtOKL38-2.3 cDNA contained all the 8 exons, while the RtOKL38-2.3A cDNA was derived by alternative splicing of exon 3 (Fig. 4.15). Both the RtOKL38-2.3 and -2.3A transcripts were likely arrived as a result of upstream promoter (promoter P2) usage, while the cryptic promoter (promoter P1) in intron 2 might give rise to the RtOKL38-2.0 transcript, which only consists of exon 3 to 8 (Fig. 4.15). The results indicate that activities of promoter P2 are tissue-specific as the RtOKL38-2.3 and –2.3A variants could only be detected in the kidney (Fig. 4.16B & 4.19B).

4.2.2 Isolation of rat OKL38 cDNA

The cloning of human OKL38 cDNA (Accession No. AF191740) was previously reported to be cloned from a human ovarian cDNA library (Huynh et al., 2001). OKL38 transcripts were detected at high levels in the ovary, kidney and liver of rat (Huynh et al., 2001) and human as shown in this study. To facilitate the development of a rat model, it is necessary to isolate the rat OKL38 cDNA. The human OKL38 cDNA was used as a probe to screen rat liver cDNA library. Four positive plaques were isolated and further

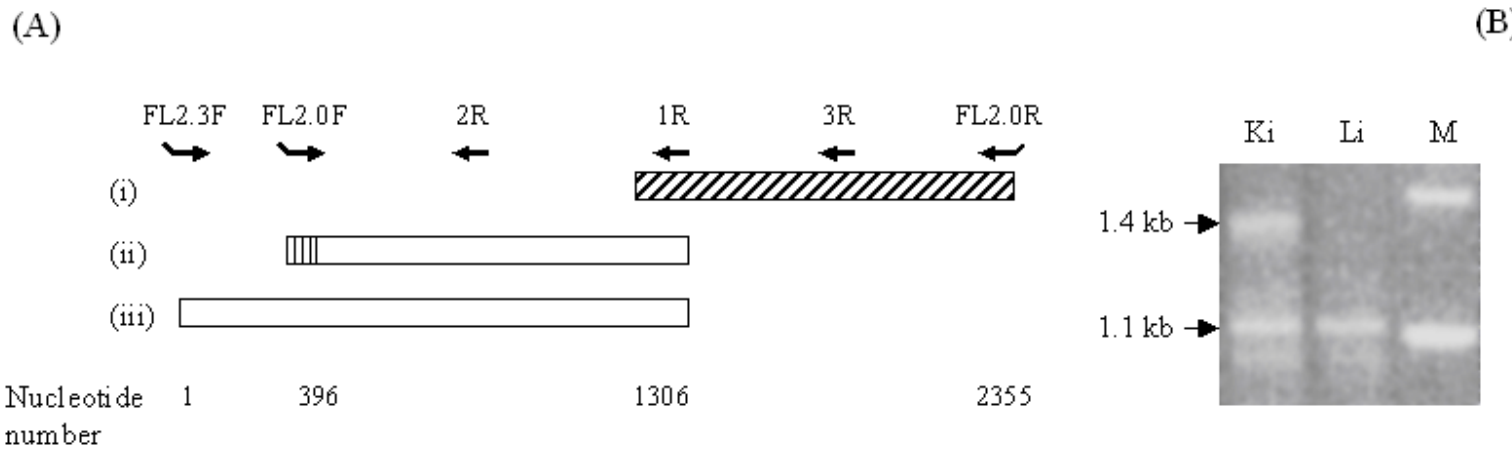


Figure 4.16: Schematic of 5' RACE analysis of the rat OKL38 cDNAs. (**A**) The shaded fragment was rat OKL38 cDNA obtained through screening (**i**), while the two non-shaded fragments, ~1.1 kb (**ii**) and ~1.4 kb (**iii**), corresponding to the 5'-end of RtOKL38-2.0 and -2.3 variants, were obtained from 5'RACE experiment using gene specific primer, 1R. The 5' end forward primers, FL-2.0F and FL-2.3F, together with the common most 3' end reverse primer, FL2.0R were designed for full-length amplification and cloning of the RtOKL38-2.0, -2.3 and -2.3A cDNAs. Internal primers, 3R and 2R, were designed for sequencing. (**B**) 5'RACE performed in kidney and liver gave rise to 1.1 and 1.4 kb PCR fragments. The lanes were: **Li**: liver; **Ki**: kidney and **M**: Molecular weight marker.

characterized with the longest insert of 1093 bp. Blast search with the non-redundant nucleotide database (GenBank) showed that the nucleotide sequence of the isolated rat OKL38 cDNA was 85% and 90% identical to the previously published human OKL38 cDNA (Huynh et al., 2001) and the mouse counterpart IMAGE:37844 (GenBank Accession No. BC022135), respectively.

4.2.3 Establishment of full-length cDNA of rat OKL38 variants

The Northern blot results showed that the rat OKL38 transcript is approximately 2 kb in size and the longest cDNA isolated via cDNA library screening was 1093 bp. To establish the full-length transcripts, 5'RACE was performed using poly $(A)^+$ mRNA derived from both rat liver and kidney tissues. Two major fragments of approximately 1.1 and 1.4 kb were obtained after secondary PCR amplification (Fig. 4.16B). The 1.1 kb fragment was detected in both liver and kidney, whereas the 1.4 kb fragment was only found in the kidney (Fig. 4.16B). Sequence alignment analysis using ClustalW program (EMBL) revealed that the 1.1 kb fragment from the liver was identical to that cloned from the kidney. The sequence of the 1.1 kb fragment aligned perfectly from the 3' end with the 1.4 kb fragment except for the first 43 nucleotides, which were found uniquely in the 5' end of the 1.1 kb fragment (Fig. 4.16A).

4.2.4 Tissue distribution of rat OKL38

To determine the tissue distribution of rat OKL38 transcripts, Northern blot analysis was performed using 5 μg of poly $(A)^+$ mRNA isolated from various rat tissues and hybridised with radio-labelled rat OKL38 probe. Transcripts of approximately 2.0 and 2.3 kb were detected in the kidney, while the 2.0 kb transcript was ubiquitously

expressed in liver, ovary, mammary gland and uterus (Fig. 4.17A). The RtOKL38-2.3 and -2.2 variants were differentially expressed in the kidney, which were detected by variants specific probe (Fig. 4.17B). Larger putative transcripts ranging from 4.0 to 9.0 kb were observed in both the liver and kidney (Fig. 4.17A). The overall expression pattern of rat OKL38 mRNA coincided with earlier published reports (Huynh et al., 2001).

To facilitate the differential tissues expression studies of rat OKL38 variants, variant-specific primers were designed and verified via PCR (Fig. 4.18). The distribution of rat OKL38 variants in various rat tissues was determined via One Step RT-PCR using variant-specific primers illustrated in figure 4.18A. The specificity of the designed primers for RT-PCR detection of rat OKL38 variants was verified via PCR (Fig. 4.18B). Three RT-PCR products of 432 bp, 791 bp and 690 bp were amplified and these corresponded to the RtOKL38-2.0, -2.3 and -2.3A transcripts, respectively (Fig. 4.19A-C). The RtOKL38-2.0 transcript was detected at high levels in the liver, kidney and ovary. The observation was in agreement with the earlier results from Northern blot analysis (Fig. 4.17A). Low levels of RtOKL38-2.0 transcript were detected in mammary gland and uterus (Fig. 4.19A). The RtOKL38-2.3 transcript was specifically detected in the kidney (Fig. 4.19B & C) and its identity was verified via direct sequencing and Southern blot analysis (Fig. 4.19C). Southern blot analysis revealed very low level of the RtOKL38-2.3 cDNA in the liver with no detectable expression in the ovary, mammary gland and uterus (Fig. 4.19B & C). A smaller fragment of 690 bp was repeatedly amplified in RNA sample derived from kidney (Fig. 4.19B & C) and this fragment probably belongs to the existing OKL38 cDNAs or another novel variant. To investigate

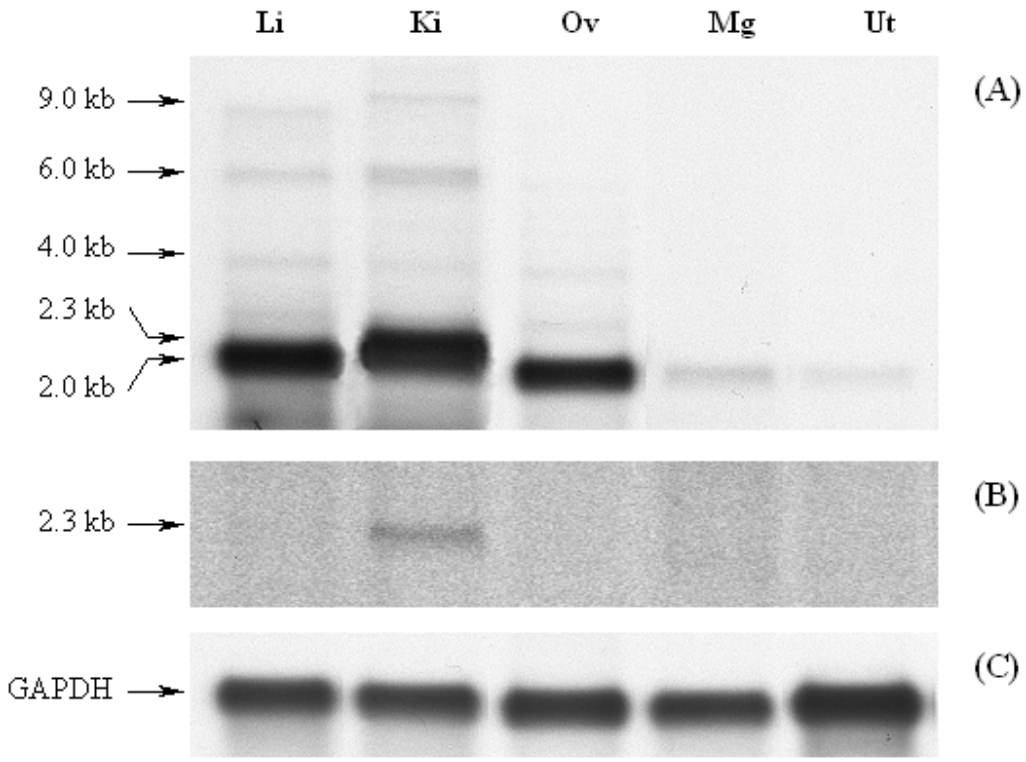


Figure 4.17: Tissue distribution of OKL38 transcripts in the rat via Northern blot analysis. Five μg of poly $(A)^+$ mRNA derived from indicated tissues were subjected to Northern blot analysis. Blots were hybridized with rat OKL38 cDNA probe that could specifically detects all rat OKL38 variants (**A**), variant specific cDNA probe that specifically detects only the RtOKL38-2.3 and -2.2 transcripts (**B**) and GAPDH cDNA (**C**). Larger transcripts ranging from 4.0 to 9.0 kb were also detected. Tissues are: **Li**: liver; **Ki**: kidney; **Ov**: ovary; **Mg**: mammary gland; and **Ut**: uterus.

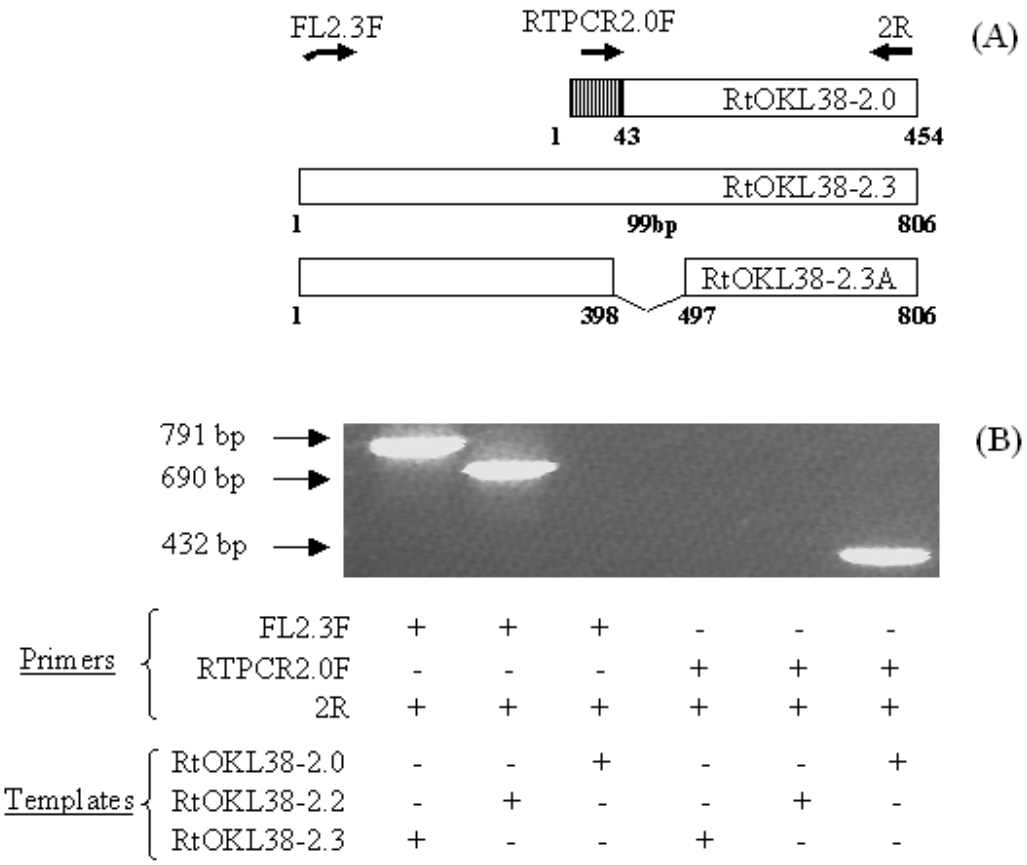


Figure 4.18: Schematic RT-PCR strategy for detection of rat OKL38 variants. (A) A detail illustration of the RT-PCR amplified products. The sequence from nucleotide position 1-43 of RtOKL38-2.0 was unique to this transcript and thus variant specific forward primer, RTPCR2.0F was designed in this region for expression study. Forward primer FL2.3F and the common reverse primer 2R were used to determine the expression of RtOKL38-2.3 and -2.3A. (B) Verification of primers specificity for one-step RT-PCR detection of rat OKL38 variants. Note that primers were specific for detection of all three variants. The 432 bp, 791 bp and 690 bp fragments correspond to RtOKL38-2.0, -2.3 and -2.3A variants, respectively. The amplified products were fractionated on a 2.0% agarose gel.

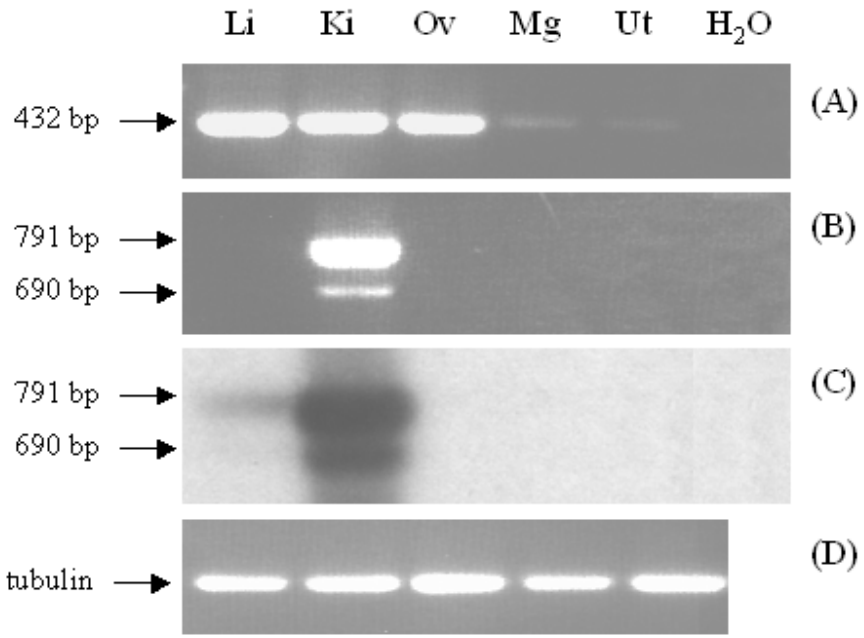


Figure 4.19: Expression of OKL38 variants in the rat tissues. Tissue distributions of rat OKL38 variants using RT-PCR were shown in (**A-C**). One step RT-PCR was performed using 100 ng of poly $(A)^+$ mRNA obtained from rat tissues. The 432 bp, 791 bp and 690 bp fragments correspond to RtOKL38-2.0, -2.3 and -2.3A variants, respectively. A pair of tubulin primers were used as internal controls (**D**). The amplified products were fractionated on a 2.0% agarose gel and transferred on to nylon membrane. Southern blot analysis was performed using RtOKL38-2.3 & -2.3A variants specific probe (**C**). Tissues are: **Li**: liver; **Ki**: kidney; **Ov**: ovary; **Mg**: mammary gland; and **Ut**: uterus.

this possibility, the 690 bp fragment was cloned and sequence analysis found that it belonged to an alternatively spliced form of the RtOKL38-2.3 variant, which was annotated as RtOKL38-2.3A (Fig. 4.18A).

4.2.5 Cloning of rat OKL38 variants

Using RT-PCR amplification, the full-length cDNA belonging to the 1.1 kb 5'RACE fragment was cloned using forward primer FL-2.0F (Table 4) residing within the unique 43 bp region of the 1.1 kb 5'RACE fragment and a reverse primer, FL-2.0R (Table 4) (Fig. 4.20). The cloning of the cDNA belonging to the 1.4 kb RACE fragment was amplified using forward primer FL-2.3F (Table 4) residing in the 5' end of the 1.4 kb fragment together with FL-2.0R primer (Fig. 4.20). The same set of primers was also used for the cloning of RtOKL38-2.3A variant. Since the RtOKL38-2.0, -2.3 and -2.3A transcripts were abundant in the kidney, these transcripts were subsequently cloned from the kidney. Three PCR products of 2.0, 2.2 and 2.3 kb were cloned and five identical clones from each were sequenced, which enabled the establishment of three rat OKL38 cDNAs of 2003 bp, 2256 bp and 2355 bp, respectively. The sizes of these three cDNAs were in agreement with the transcript sizes observed in Northern blot analysis (Fig. 4.17A & B). These three rat OKL38 cDNAs of sizes 2.0 kb, 2.2 kb and 2.3 kb were collectively annotated as RtOKL38-2.0 (GenBank Accession No. AY081218), RtOKL38-2.3A (GenBank Accession No. AF549442) and RtOKL38-2.3 (GenBank Accession No. AF549441), respectively. Full-length cloning of the three rat OKL38 variants and subsequent sequencing confirmed their existence.

TABLE 4. Sequences of oligonucleotides used for RT-PCR ([a]), Cloning ([b]), Sequencing ([c]) and 5'RACE ([d]).

Oligonucleotide	Sequence
[a] RTPCR2.0F	5' CTAATGGGCATCCTGTTCTTG 3'
[a c] 2R	5' CATCAAAGAGCAGGGCCACAGGACTC 3'
[a b] FL2.3F	5' GAAGCTTGGTAGCGTGCTTGTCTAGCATGC 3'
[b] FL2.0F	5' AAGCTTGGATCCCCAGGGCTAATGGGCAT 3'
[b] FL2.0R	5' GCGGCCGCAGATCTGATGGTGAAAGGTTA 3'
[c d] 1R	5' TGACAGCTCGTGGTGGACAAAAGGCA 3'
[c] 3R	5' AGGTGGCTTCCTGGTCTCCTTCTTCA 3'
[b] 478-F	5' CACCATGAGCTCCTGGAGGCAT 3'
[b] 478-eGFP-R	5' CTCGCCCTTGCTCACCATAGGTGGCTTCCTGGTCTC 3'
[b] eGFP-F	5' ATGGTGAGCAAGGGCGAG 3'
[b] eGFP-R	5' TTACTTGTACAGCTCGTCCA 3'

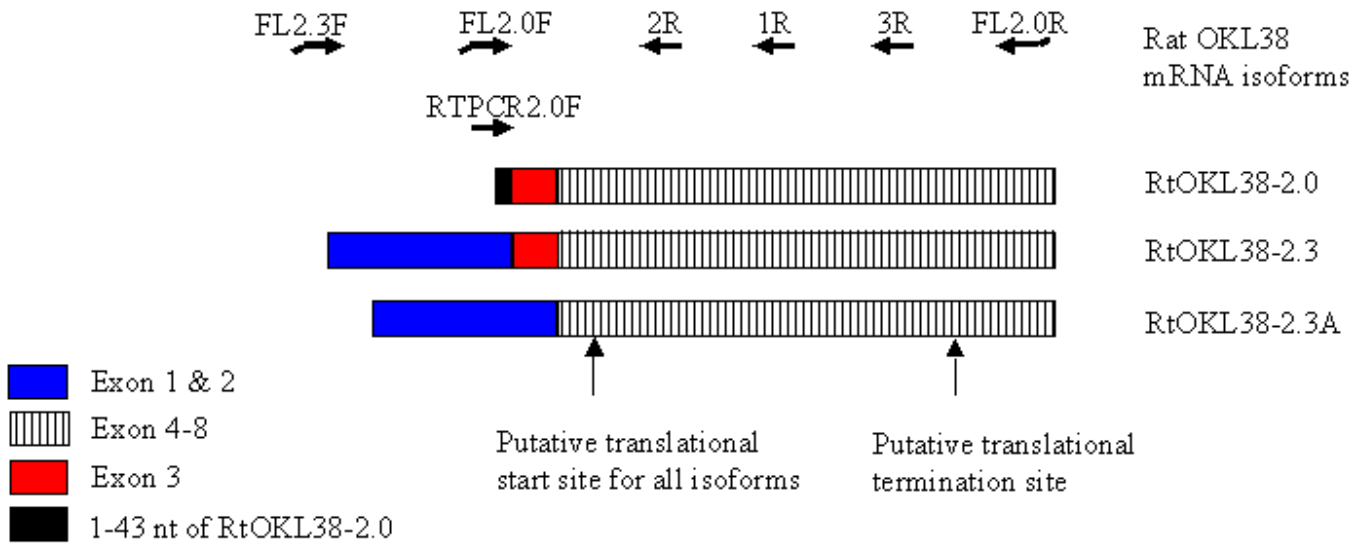


Figure 4.20: Schematic of rat OKL38 mRNA isoforms. Different coloured/shaded boxes denote the different exons present in each isoforms. Ascending arrows indicate the putative translational start and termination sites. The first shaded box of RtOKL38-2.0 indicates the first 43 nucleotides that lies in intron 2, which is unique to this transcript. Bold right and left facing arrows refers to primers employed for cloning, RT-PCR, sequencing and 5'RACE analysis.

4.2.6 Sequence analysis of rat OKL38 variants

The comparison of primary amino acid sequences from human, rat and mouse were illustrated in figure 4.21 using ClustalW (EMBL). Multiple sequence alignment study showed that the rat and mouse OKL38 (GenBank Accession No. BC022135) shared a high identity of 91% and 93% at the nucleotide and amino acid levels, respectively (Fig. 4.21). The identity between the rat and human OKL38 was only 80% at the nucleotide and 85% at the amino acid levels, respectively (Fig. 4.21).

The RtOKL38-2.0 cDNA harboured an ORF of 1434 base pairs with a conceptual translated protein of 478 amino acids, having a calculated molecular mass of 52 kDa and a predicted P. I. of 6.83. *In vitro* Transcription and Translation studies using the RtOKL38-2.0, -2.3 and -2.3A cDNAs resulted in the synthesis of an approximately 52 kDa protein (Fig. 4.22A) and its identity was confirmed by Western blot analysis using anti-OKL38 antibodies (Fig. 4.22B).

Computational analysis of the predicted amino acid sequence using Pfam CDS-Conserved Domain Search (NCBI) detected two putative domains belonging to that of the pyridine nucleotide-disulphide oxidoreductase (Pyr_redox) and TrkA (Fig. 4.12B & 4.21). The Pyr_redox domain was also present in glutathione reductase, thioredoxin reductase, AIF, AIFL and PRG3/AMID (Fig. 4.23). The glutathione reductase and thioredoxin reductase carry an additional Dimerization domain, whereas the AIFL contain an additional Rieske domain (Fig. 4.23). Similar to that observed in human, the rat OKL38 Pyr_redox domain is resided in exon 8, while the TrkA domain is encoded by exon 6-8. The hydrophobicity average of rat OKL38 was 0.200209, and was predicted to be a cytosolic protein using the SOSUI (Hirokawa et al., 1998) and Tmpred (Hofmann

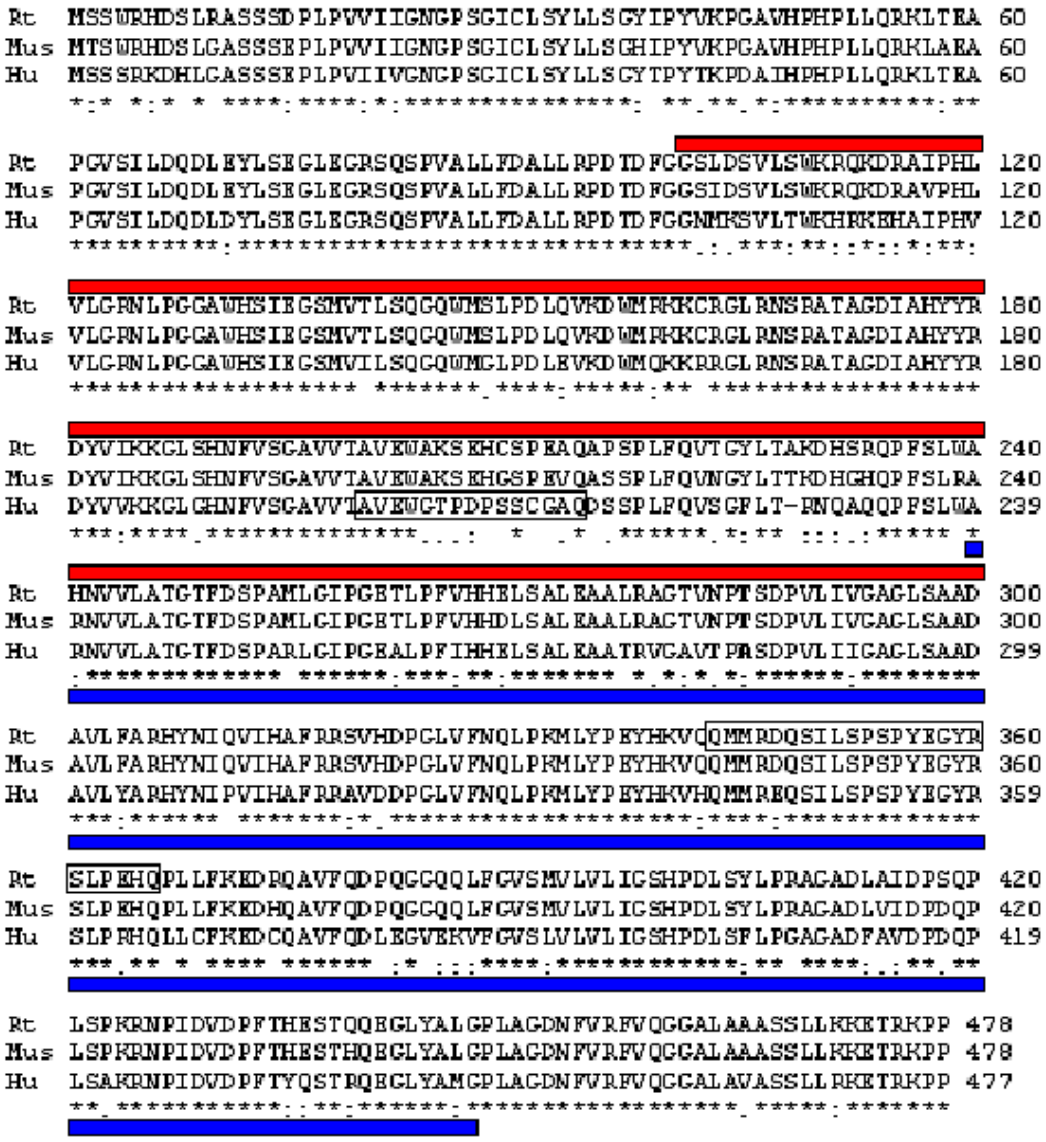


Figure 4.21: Multiple sequence alignment of OKL38 protein. The primary amino acid sequence of OKL38 from rat (Rt), mouse (Mus) and human (Hu) were aligned using ClustalW. Asterisk (*) represented the amino acids conserved in all the three species, whereas dots (:.) signified sequences partially conserved. The red boxes above the sequence represented the region of the TrkA domain, while the Pyr_redox domain is indicated by blue boxes below the sequence. The putative N-linked and O-linked glycosylation sites detected in the rat and mouse OKL38 at Asparagine-283 and Threonine-285 were in bold. Boxed amino acid sequence corresponded to peptides employed for antibody production.

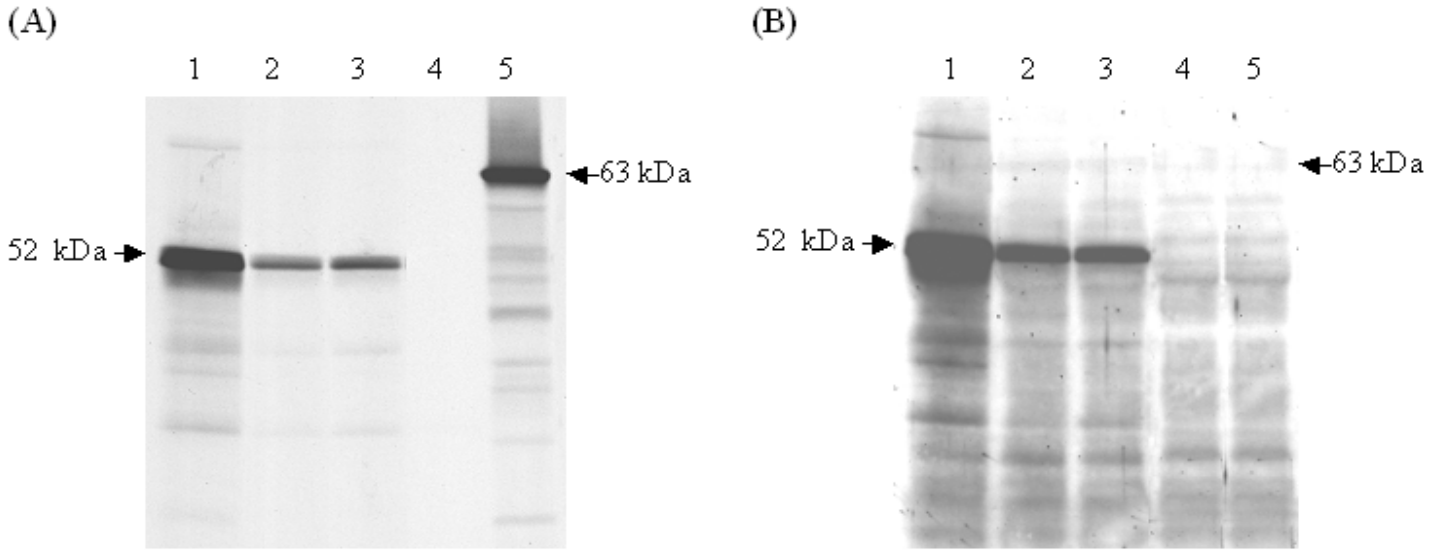


Figure 4.22: *In vitro* Transcription and Translation studies of rat OKL38 cDNAs. *In vitro* transcription and translation was performed using [^{35}S] L-Methionine and cDNA template from the three rat OKL38 variants. Lane consists of RtOKL38-2.0 (lane 1), -2.3 (lane 2) and -2.3A (lane 3) cDNA as template, H_2O as the negative control (lane 4) and luciferase construct as a positive control (lane 5). (**A**) Synthesized proteins were fractionated on 14% SDS-PAGE gel, transferred on to nitrocellulose membrane and exposed to Kodak film overnight. (**B**) Western blot analysis using anti-rat OKL38 antibody was performed on the same blot and a protein of approximately 52 kDa was detected.

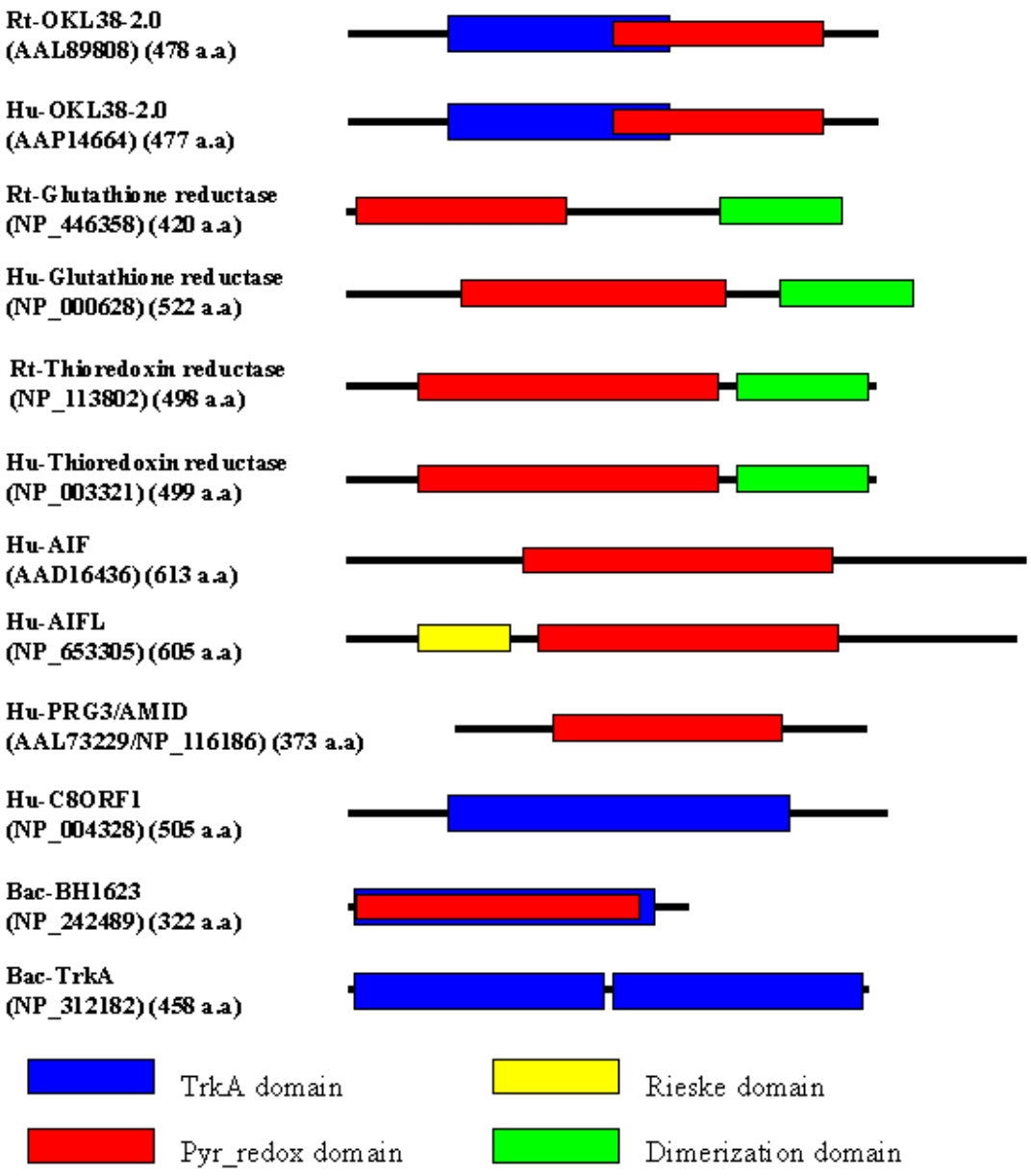


Figure 4.23: A schematic of OKL38 related proteins. The human and rat OKL38 contained both the Pyr_redox (Red boxes) and the TrkA (Blue boxes) domain, while the Dimerization (Green boxes) domain was only found in the thioredoxin and glutathione reductase. Both the Pyr_redox and Rieske (Yellow box) domains were found in the AIFL protein. The accession number and sizes of proteins were indicated on the left of the diagram. NB: The TrkA domain partially overlaps the Pyr_redox domain in OKL38 protein.

and Stoffel, 1993) programs. However, two putative N-linked and O-linked glycosylation sites at Asparagine-283 and Threonine-285, respectively, were identified using the NetOGlyc 3.1 program (Julenius et al., 2005) (Fig. 4.21). These two glycosylation sites were not conserved in the human OKL38 protein (Fig. 4.21).

Sequence alignment studies of RtOKL38-2.0, -2.3 and -2.3A cDNAs showed that the three variants shared the same 3'end and the only differences were observed at the 5' end. Inspecting the 5'UTR of the three RtOKL38 cDNAs revealed the presence of an in-frame stop codon (UGA) 18 bp upstream of the predicted translational start site (AUG). Despite the differences in the 5' end, the RtOKL38-2.0, -2.3 and -2.3A variants possess identical ORF. In-frame small upstream ORFs (uORFs) that encodes for 66 and 33 amino acids were detected in the 5'UTR of the RtOKL38-2.3 and -2.3A variants, respectively (Fig. 4.12A). Two other uORFs of 12 and 46 amino acids were also detected in the 5'UTRs of these two rat OKL38 variants. A small uORF of 21 amino acids was identified within the 5'UTR of RtOKL38-2.0 (Fig. 4.12A). These observations suggest the possible involvement of uORF and uAUG in translation regulation.

4.2.7 Functional expression of rat OKL38 protein

In this study, the cellular localization and function(s) of rat OKL38 protein was investigated. RtOKL38-eGFP-pcDNA3.0 construct, which fused the reporter gene eGFP to the C-terminal of full-length rat OKL38 protein, was constructed. Preliminary transfection studies showed that RtOKL38-eGFP fusion protein was localized to the cytosol, resulting in protein aggregates in BRL cells as early as 24 hours post-transfection (Fig. 4.24A). Cells expressing the fusion protein gradually rounded up and almost all the RtOKL38-eGFP expressing cells disintegrated 120 hours post-transfection. On the other

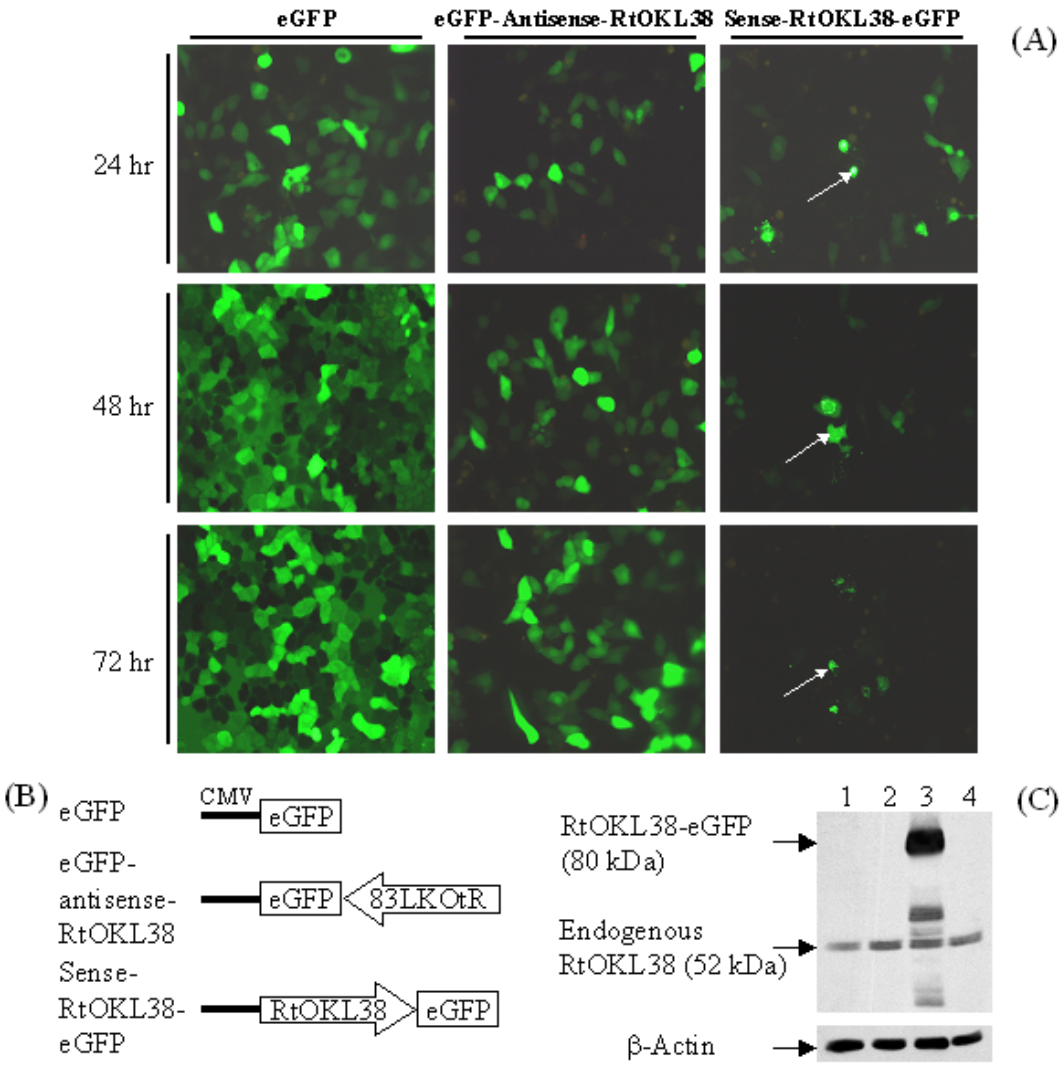


Figure 4.24: Transfection studies of RtOKL38-eGFP recombinant DNA constructs. Buffalo rat liver (BRL) cells were transfected with controlled eGFP, eGFP-antisense-RtOKL38 and Sense-RtOKL38-eGFP recombinant constructs. The transfected cells were harvested at 24 hr, 48 hr and 72 hr post-transfection (**A**). Cells expressing Sense-RtOKL38-eGFP protein were visualized using a fluorescent microscope. White arrows indicate the RtOKL38-eGFP recombinant protein form speckles and eventually lead to cell death. The three constructs cloned in the pcDNA3.0 vector backbone used for this transfection is as shown in (**B**). (**C**) Western blot analysis verifying the presence of RtOKL38-eGFP recombinant protein (Lane3). Untransfected BRL cells in lane 1 and BRL cells transfected with empty vector, Sense-RtOKL38-eGFP and eGFP-antisense-RtOKL38 in lane 2, 3 and 4 respectively. Magnification X 200.

hand, cell growth was observed in the cells transfected with the control eGFP or eGFP-antisense-RtOKL38 constructs. The presence of the fusion protein (RtOKL38-eGFP) was verified via Western blot analysis performed on the transfected cell using the anti rat OKL38 antibody (Fig. 4.24C). These observations indicate that over-expression of OKL38 protein was probably lethal to BRL cells.

4.3 Pregnancy and human chorionic gonadotropin (hCG) regulate OKL38 expressions

The ability of early pregnancy and hCG treatment to protect the breast against mammary carcinogenesis has been well documented (Russo et al., 1990b; Russo et al., 1990a; Russo et al., 1991). Previous studies have demonstrated that OKL38 is differentially regulated in the rat mammary gland throughout the course of pregnancy (Huynh et al., 2001). Since CG production by placenta increases during pregnancy, the effects of hCG on the regulation of OKL38 in rat mammary gland and ovary were investigated.

4.3.1 Rat OKL38 protein is up regulated during pregnancy

OKL38 transcripts were up-regulated in the rat mammary gland during pregnancy (Huynh et al., 2001). In the present study, the effect of pregnancy on rat OKL38 protein in the mammary gland was determined. Western blot analysis showed that OKL38 protein was highly expressed in the mammary glands of the pregnant and lactating rats, while the protein was barely detectable in the non-pregnant mammary glands (Fig. 4.25A). The level of OKL38 protein expression returned to basal levels during

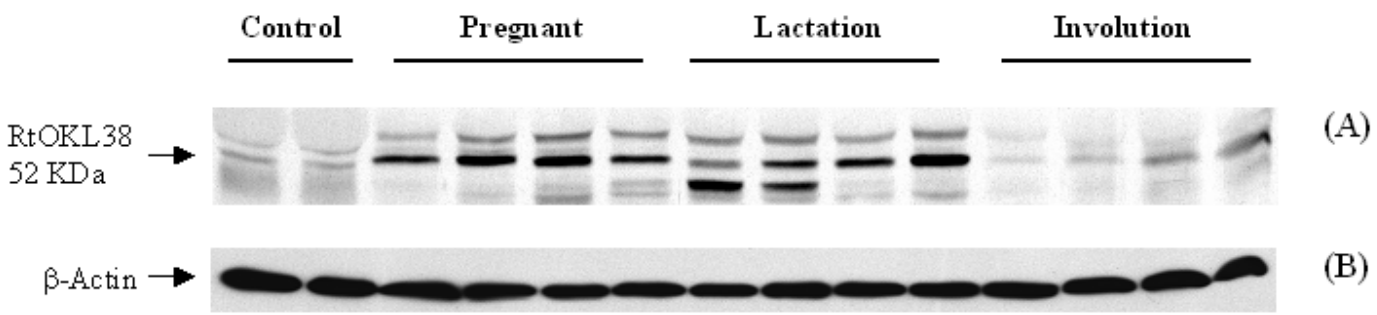


Figure 4.25: Expression profile of OKL38 protein in the rat mammary gland during the progression of pregnancy. (**A**) Total protein from the mammary gland of individual animals in each group (i.e. Control, Pregnant, Lactation and Involution) were used for Western blot analysis. 100 μg of total protein was fractionated on a 11% SDS-PAGE gel and blotted onto nitrocellulose membrane. Anti-rat OKL38 antibody detected several rat OKL38 isoforms in the mammary gland. The β-actin indicates equal loading in all lanes (**B**).

involution. Several OKL38 protein isoforms were evident in the rat mammary tissue suggesting the probable presence of OKL38 variants.

4.3.2 Human chorionic gonadotropin induce the expression of rat OKL38

The onset and advancement of pregnancy up-regulated OKL38 expression in the mammary gland, it was therefore important to determine whether pregnancy hormones such as CG was able to up-regulate OKL38. Total RNA derived from ovary and mammary gland of hCG-treated rats was used for Northern blot analysis. OKL38 transcript was significantly increased in a dose-dependent manner by hCG treatment, while low levels of transcript was observed in the mammary gland (Fig. 4.26A) and ovary (Fig. 4.26C) of vehicle-treated rats. In the ovary, hCG also induced the expression of OKL38 protein (Fig. 4.27A). Interestingly, the 52 kDa OKL38 isoform was only detected in the ovary suggesting the tissue specific nature of the various isoforms (Fig. 4.27A). Unlike the ovary and the mammary gland, OKL38 expression in the liver and kidney was not influenced by hCG (data not shown).

In situ hybridization analysis was performed to determine the cell specific expression of OKL38 in both mammary gland and ovary following hCG treatment. Low levels of OKL38 transcript expression in undifferentiated mammary epithelial cells of control rats had previously been demonstrated (Huynh et al., 2001). In this study, cryo-sections derived from mammary tissues and ovaries of controls and hCG-treated rats (20 IU/day) were hybridised with a sense and anti-sense OKL38 RNA dioxigenin-labeled probes. Intense signal was observed in the differentiated secretory epithelial cells of the mammary gland (Fig. 4.28B). In contrast, little or no signal was detected in the

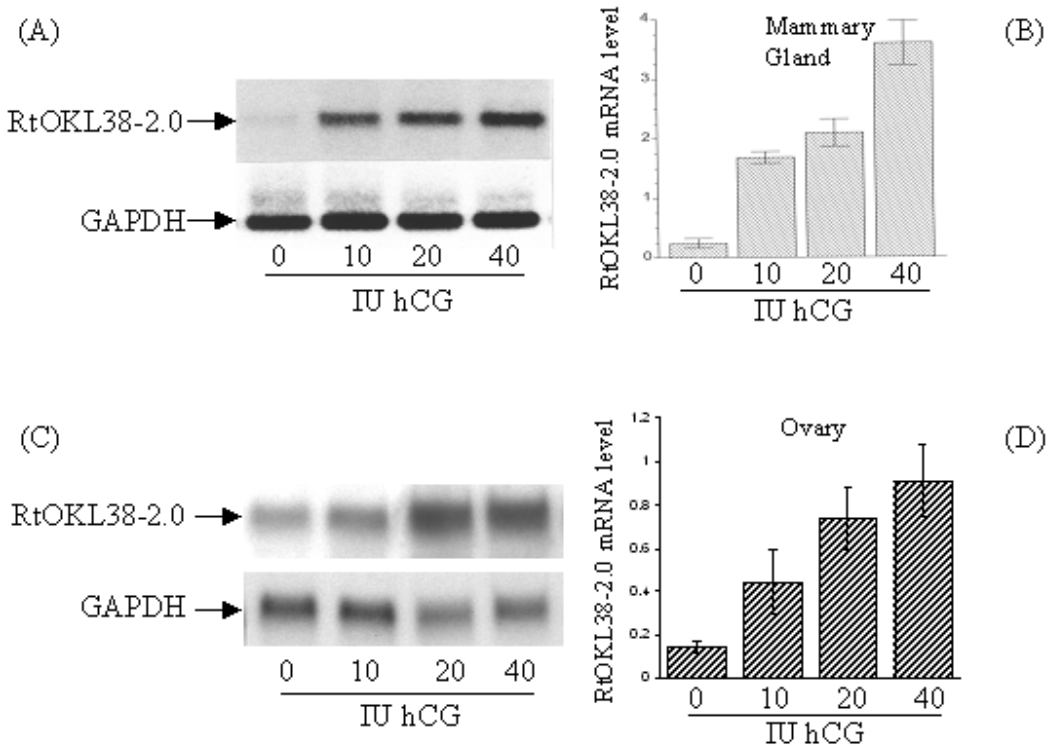


Figure 4.26: Effect of human chorionic gonadotropin on the expression levels of OKL38 transcripts in the rat mammary glands and ovaries. Female rats were treated with indicated concentrations of hCG over a period of 3 weeks to emulate pregnancy in rat. Five μg of poly $(A)^+$ mRNA derived from mammary tissues (**A**) and 50 μg of total RNA derived from ovary (**C**) were subjected to Northern blot analysis. Blots were hybridized with OKL38 and GAPDH cDNAs. Densitometric scanning of the OKL38 mRNA band in mammary gland (**B**) and ovary (**D**) after being normalized to the levels of GAPDH mRNA is shown. The results were derived from 6 independent animals from each group as described in the *Materials and Methods*.

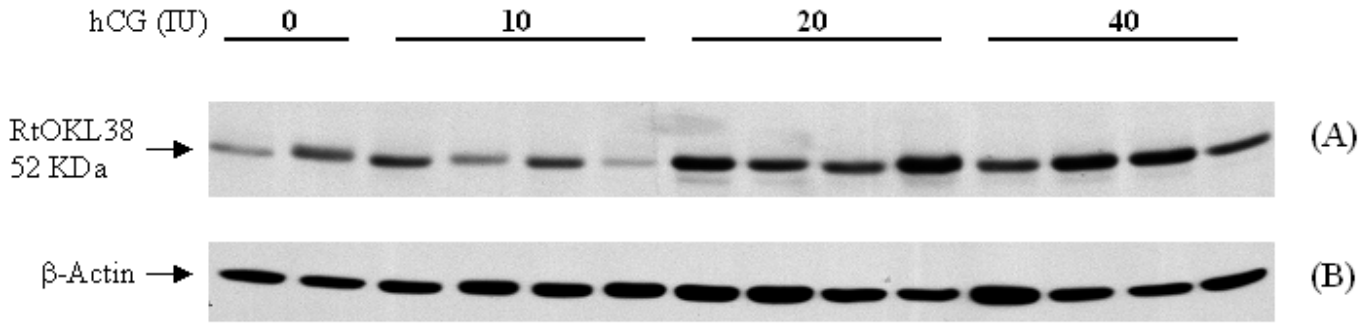


Figure 4.27: Effect of human chorionic gonadotropin on OKL38 protein expression in the rat ovaries. Expression of OKL38 protein in ovaries of rat treated with different concentration of hCG (**A**). Total protein from whole ovaries of rat treated with indicated concentration of hCG (i.e. PBS, 10U, 20U and 40U) were extracted for Western blot analysis. 100 μg of total protein from the indicated tissues were fractionated on 11% SDS-PAGE gel, blot onto nitrocellulose membrane and probed with anti-RtOKL38 antibody. A 52 kDa rat OKL38 protein was detected in the rat ovary. The β-actin indicates equal loading in all lanes (**B**).

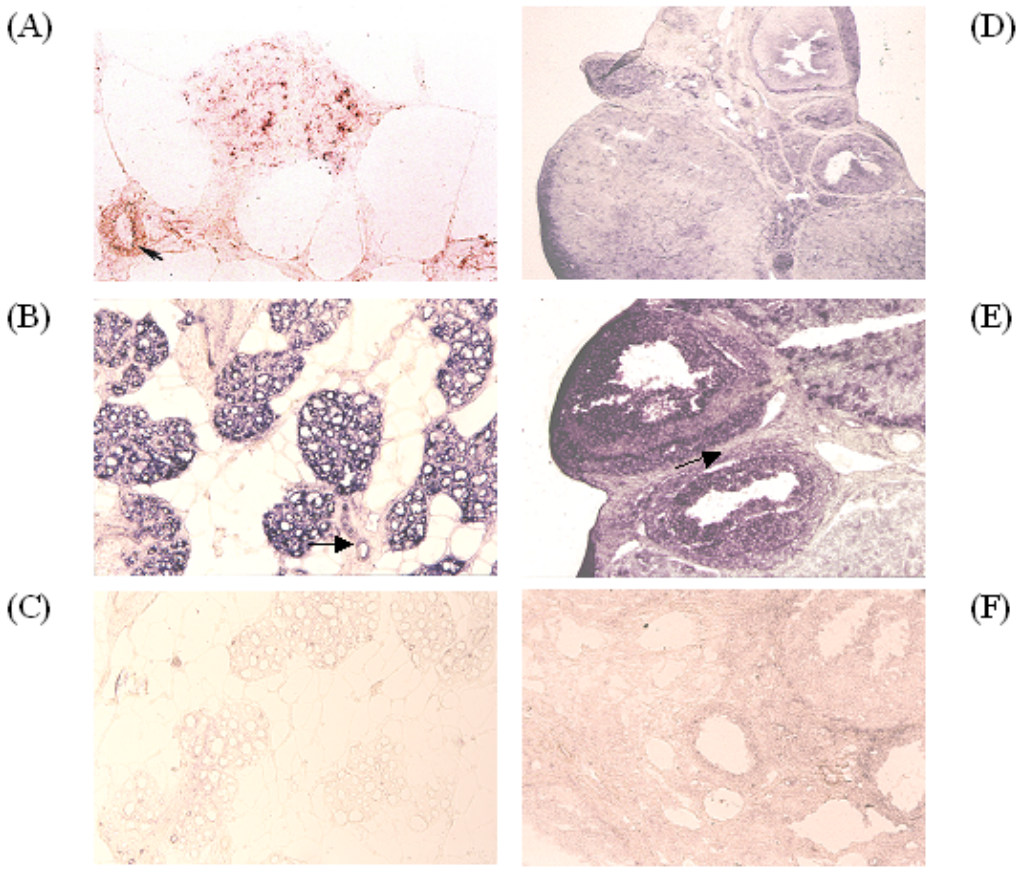


Figure 4.28: *In situ* hybridization with sense and antisense RNA probes for OKL38 expression in rat mammary gland and ovary. Sections derived from mammary and ovary of untreated (**A & D**) and rats treated with 20 IU of hCG (**B & E**) were subjected to *in situ* hybridization using a sense control (**C & F**) and anti-sense OKL38 probe (**A, B, D & E**). Low levels of OKL38 mRNA were detected in alveolar epithelial cells of the mammary gland from untreated rat (**A**). (**B**) High levels of OKL38 mRNA were detected in the secretory epithelial cells of mammary tissue derived from rats treated with 20 IU of hCG. No staining signal was detected in the connective tissue or blood vessels (**arrow, A & B**). In the ovary, higher levels of OKL38 mRNA were detected in the granulosa cells of the ovaries derived from hCG-treated animals (**E**) as compared to those of vehicle-treated animals (**D**). No staining signal was detected in the blood vessels and the connective tissues between the ovaries (**arrow, E**). Sense OKL38 probe showed no staining signal in the mammary gland and ovary of hCG-treated rat (**C & F**). Note that hCG-treated animals have larger ovaries due to enlargement of corpora lutea. Magnification, X400 (**A**), X100 (**B & C**) and X40 (**D, E & F**).

undifferentiated control mammary gland, blood vessels and connective tissues (Fig. 4.28A & B, indicated by arrow). Low OKL38 signal was detected in the granulosa cells of the ovary in the control vehicle-treated rats (Fig. 4.28D). Expression of OKL38 was greatly increased in the large corpora lutea and granulosa cells in the follicles of hCG-treated rats (Fig. 4.28E). No signal was detected in the connective tissues of the ovary (Fig. 4.28E, indicated by arrow). The control sense OKL38 RNA probe produces background signals in the mammary tissue (Fig. 4.28C) and ovary (Fig. 4.28F).

4.4 OKL38 and Cancer

Genes that are differentially expressed or lost in cancers are of great interest to scientists. The expression profiles of these genes are closely related to their functions (i.e. oncogenes or tumour-suppressor genes) and may serve as useful biomarkers for diagnosis and/or prognosis of cancers. These cancer-related genes usually play important roles in cell cycle, angiogenesis, apoptosis, proliferation, growth and differentiation. OKL38 has been implicated in regulating cell growth and differentiation, and tumourigenesis (Huynh et al., 2001). Thus, the distribution of human OKL38 variants in various human normal/ tumour tissues and cancerous cell lines were of particular interest.

4.4.1 Expression of human OKL38 variants in Kidney, Liver and ovarian Tissues and cell lines

A preliminary study of OKL38 expression in various tumours and cancer cell lines was performed. The expression of OKL38 variants in tumour and adjacent benign tissue, and cancer cell lines were determined via One Step RT-PCR using variant-specific primers (Fig 4.29). Southern blot analysis (Fig. 4.29C) was performed using exon 4 & 5

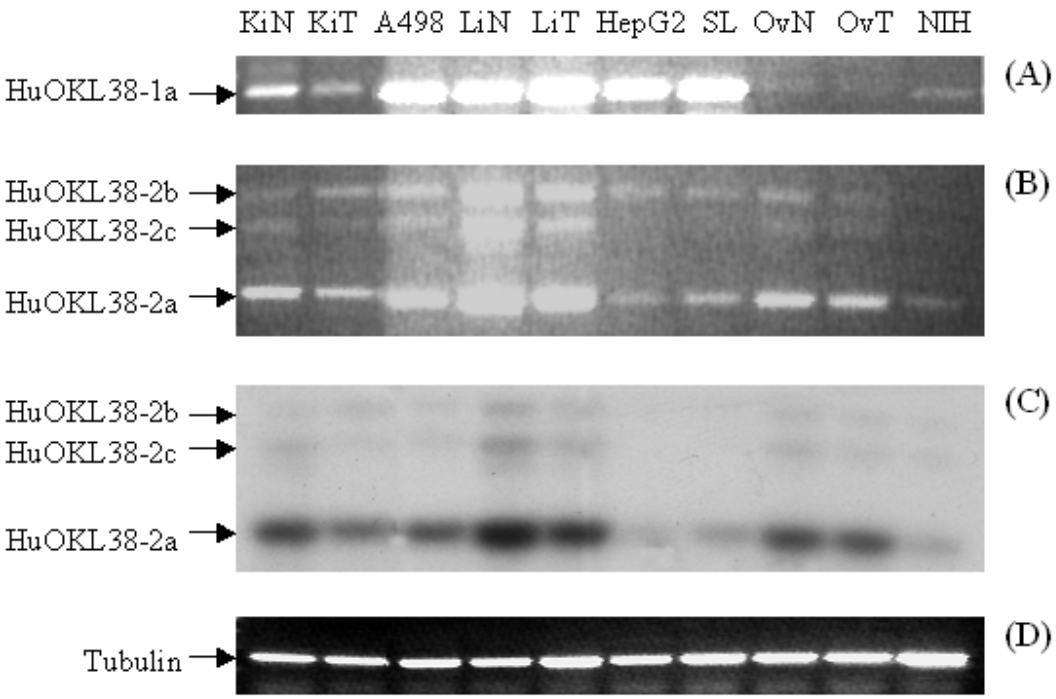


Figure 4.29: Differential expression of human OKL38 variants in normal(N)/tumor(T) tissues and cell lines. One step RT-PCR was performed using total RNA extracted from **Li**: liver, **Ki**: kidney and **Ov**: ovary tumor and adjacent normal tissue and cell line, **A498**: kidney, **HepG2**: liver, **SL**: a subline of HepG2, and **NIH**: ovarian (**A-D**). Four RT-PCR products of 443 bp, 373 bp, 533 bp and 482 bp corresponding to the HuOKL38-1a, -2a, -2b and -2c transcripts, respectively. RT-PCR was performed using primers, 1F and PCR-1R (**B**), primers, PCR-2F and PCR-1R (**C**), and a pair of tubulin primers (**E**) as internal control. The amplified products were separated on a 2.0% agarose gel and transferred onto nylon membrane. Southern blot analysis was performed using probe derived from exon 4, 5 and 6 (**D**).

as probe and sequencing verified the identity of the amplified products. In general the normal kidney, liver and ovarian tissues seemed to express similar level of OKL38 transcripts as compared to the adjacent tumour tissues. All the cancer cell lines showed lower expression of OKL38 transcripts compared to their corresponding normal tissue, except for the A498 kidney cancer cell line (Fig. 4.29). However, more paired tissue samples and cell lines are needed in order to determine the significant differences in the expression level of each variant in normal and cancerous tissues.

4.4.2 Loss of OKL38 expression in Kidney Cancer

Cancer Profiling Array (CPA) was used to determine the expression of OKL38 mRNA transcripts in paired normal/ tumour kidney. The CPA blot was probed with a pan-OKL38 variants probe (Fig. 4.30A). The results showed that 70% (14/20) of the kidney tumours expressed lower levels of OKL38 transcripts as compared to adjacent normal tissues (Fig. 4.30B).

The OKL38 mRNA transcripts were generally down-regulated in the kidney tumours, which led to investigating the possibility of reduction of OKL38 protein in the tumours. Western blot analysis was performed using rabbit anti-human OKL38 antibody on 9 paired normal/ tumour kidney tissues (Fig. 4.31A). The results showed that OKL38 protein was undetected in 78% (7/9) of the tumour kidney tissues as compared to their adjacent normal tissues. The endogenous 61 kDa, 52 kDa and 38 kDa isoforms of human OKL38 protein were also detected by Western blot analysis (Fig. 4.31A), which is in agreement with the earlier *in vitro* Transcription and Translation results (Fig. 4.10). Low levels of OKL38 protein was detected in the A498 kidney cancer cell line, contrary to the RT-PCR results in which high amounts of OKL38 transcripts were detected (Fig. 4.29).

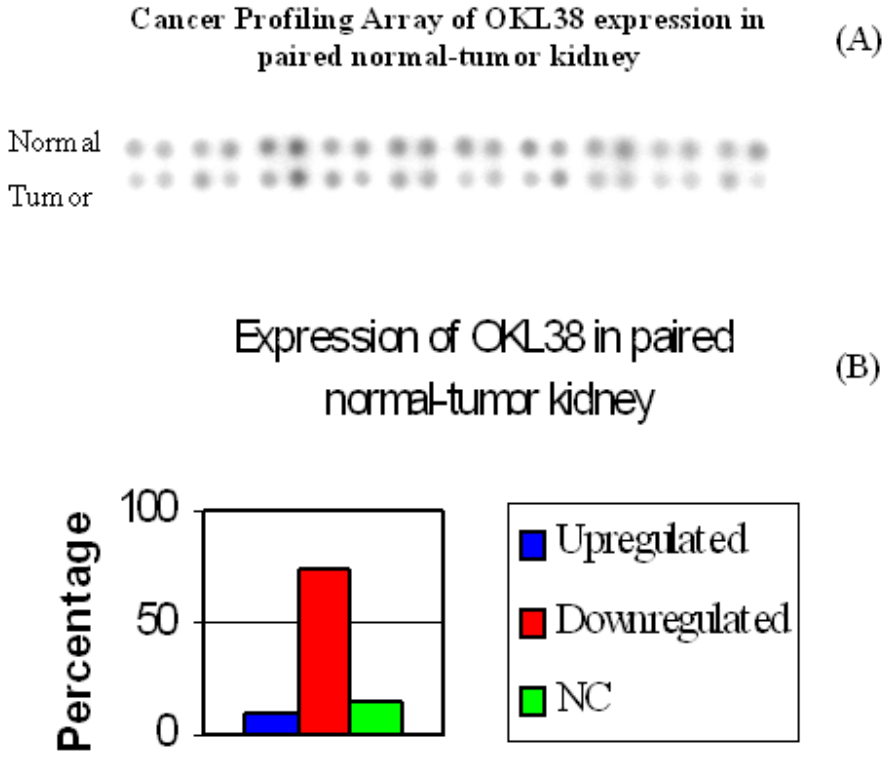


Figure 4.30: Cancer Profiling Array of human OKL38 in normal/ tumor kidney. The CPA was probed with a 700 bp human OKL38 *SmaI* probe as described under *Materials and Methods*. Note that the OKL38 transcript was generally down-regulated in kidney tumor samples (**A**). The relative density of OKL38 expression in tumor and adjacent normal tissues were compared and expressed as a percentage of total sample examined (**B**). The figure legend on the right indicates up-regulation, down-regulation and no observable change.

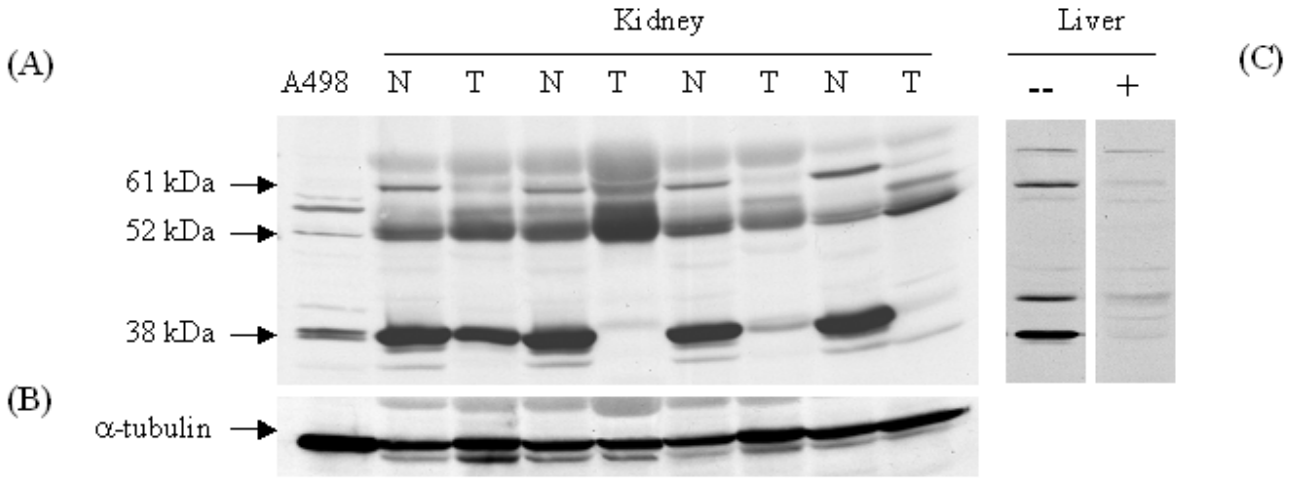


Figure 4.31: Expression of OKL38 protein in kidney tumor and adjacent normal tissue. (**A**) Western blot analysis showing the expression of OKL38 proteins in kidney cancer cells (**A498**) and paired normal (**N**)/ tumor (**T**) kidney tissues. The loading was normalized to the level of α-tubulin shown in (**B**). Note that three OKL38 protein isoforms with molecular weight of 61, 52 and 38 kDa were detected. The specificity of anti-OKL38 antibody is shown in (**C**) with Western blot analysis. The nitrocellulose membrane with liver total protein were blotted with OKL38 antibody **C** (--) and OKL38 antibody preadsorbed with 50X excess antigen peptide **C** (+). Note that the signal was reduced to background levels when preadsorbed antibody was used indicating that the antibody for OKL38 was specific for Western blot analysis.

The 61 kDa and 38 kDa proteins were differentially down-regulated in all the kidney tumour tissues (Fig. 4.31A).

Immunohistochemical analysis was performed on 22 paired normal/ tumour kidney tissues using the above-mentioned OKL38 antibodies (Fig. 4.32). OKL38 protein was undetected in 64% (14/22) of the kidney tumours examined, which supported the observation in Western blot analysis. All normal kidney epithelial cells were positive for OKL38 protein, except the Bowman's capsules (arrow) and the stroma (Fig. 4.32A), while no immunostaining of the tumour cell were observed (Fig. 4.32B). The OKL38 antibody preadsorbed with 50X excess antigen peptide showed negative signal in Western blot analysis (Fig. 4.31C) and Immunohistochemical analysis (4.32C&D) demonstrating the specificity of anti-human OKL38 antibody used in this study.

4.4.3 Loss of OKL38 protein in Liver Cancer of higher stages

The high levels of OKL38 transcripts in liver, low levels in breast cancer cell lines (Huynh et al., 2001), and loss of OKL38 protein in kidney tumour and cell line prompted the investigation of its expression in hepatocellular carcinoma (HCC). The abundance of OKL38 gene was investigated in 28-paired of adjacent benign liver (ABL) tissue and HCC, and 4 liver cancer cell lines (HepG2, Hep3B, Chang Liver and PLC/PRF5) by Western blotting (Fig. 4.33A). The 38 kDa OKL38 protein isoform was readily detected in the human liver, whereas the other isoforms (i.e. 52 and 61 kDa) were only detectable upon extended exposure. Loss of OKL38 protein was observed in 42.8% (12 of 28) of the HCCs and in all liver cancer cell lines examined. Low levels of OKL38 were also detected in 21.4% (6 of 28) of the cancerous tissues compared to ABL tissues (Fig. 4.33A).

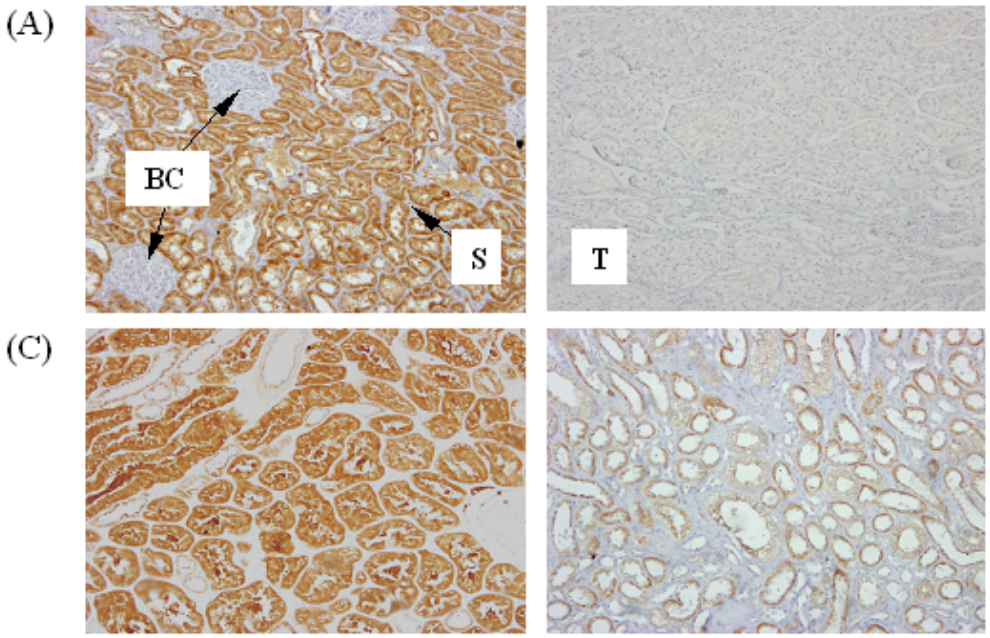


Figure 4.32: Immunohistochemical detection of OKL38 protein in kidney tumor and adjacent normal tissue. Immunohistochemistry studies performed on normal (**A**) and kidney tumor (**B**) tissues showed differential staining indicating loss of OKL38 protein in tumor tissue. Note that the Bowman's capsules (**BC**), the stroma (**S**) and the tumor (**T**) cells were not stained. The specificity of anti-OKL38 antibody is as shown in (**C** & **D**) with immunohistochemistry. Slide with normal kidney were probed with OKL38 antibody (**C**) and OKL38 antibody preadsorbed with 50X excess antigen peptide (**D**). Signal was reduced to background levels when preadsorbed antibody was used indicating that the antibody for OKL38 was specific for immunohistochemical study.

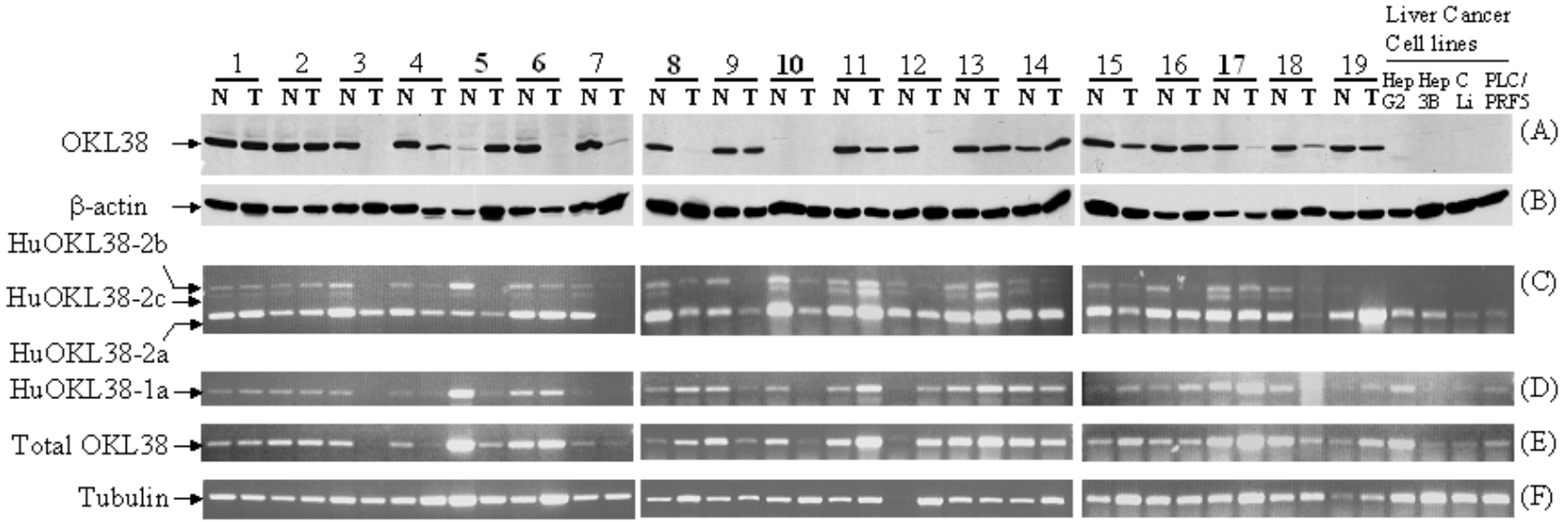


Figure 4.33: Expression of OKL38 in HCC and adjacent normal tissue. OKL38 transcripts and protein in 19 of the 28 paired liver tumor and adjacent normal tissue samples from patients, and 4 liver cancer cell lines, **HepG2**, **Hep3B**, Chang Liver (**C Li**) and **PLC/PRF5** were shown. (**A**) Western blot analysis showing the expression of OKL38 protein (i.e. 38 kDa) in paired normal(N)/ tumor(T) liver tissues and 4 liver cancer cell lines. The loading amount being normalized to the level of β-actin was shown in (**B**). One step RT-PCR using total RNA extracted from the liver tissue samples and cell lines showing the expression of HuOKL38-2b, -2c, -2a, -1a variants & the total levels of all the transcripts in the corresponding samples (**C**, **D** & **E**). Tubulin was used as internal control for one-step RT-PCR (**E**). Five of the patients selected for full-length sequencing of OKL38 cDNA were indicated in bold. Note that OKL38 protein was absent in tumor 3, 6, 7, 8, 10, 12, 17 and the 4 liver cancer cell lines.

One step RT-PCR was performed to determine the abundance of total OKL38 transcripts and its individual variants in 19 of the 28 patient paired-HCC samples. The results showed that OKL38 transcripts were down-regulated or lost in 37 % (7 of 19) and over-expressed in 16% (3 of 19) of the HCC samples investigated (Fig. 4.33C-E). No significant differences in the levels of OKL38 transcripts were detected in the rest (9 of 19) of the HCC samples (Fig. 4.33C-E). Relatively high levels of OKL38 transcripts were detected in the liver cancer cell lines, especially in the HepG2 cells, while no protein was observed (Fig. 4.33).

To identify the cell-type responsible for the loss of OKL38 and verify its potential use as biomarker, a total of 92 HCC and their ABL tissues were examined by immunohistochemistry. Figure 4.34A shows that intense staining was observed in the cytoplasm of adjacent benign hepatocytes. Hepatocytes within cirrhotic nodules were stained with OKL38 antibody. Approximately 41.3% (38 of 92) of HCCs examined were negative for OKL38 protein (Table 5A). Weak and patchy staining for OKL38 as shown in figure 4.34B, was observed in 39.1% (36 of 92) of HCC compared with ABL tissue (Table 5A). Bile duct epithelial cells and blood vessels were uniformly negative, as were the fibrovascular stroma within cirrhotic livers (Fig. 4.34B-D). Similar results as observed in the kidney cancer, OKL38 protein is also lost in HCC. Intense cytoplasmic staining was found in dysplasic or benign hepatocytes.

Table 5B summarized the tumour stage and the immunohistochemical analysis of OKL38 in human HCC. Loss or weak, patchy expression of OKL38 was found to occur at higher frequencies in stage 3 and 4 tumours, while most of stage 1 and part of stage 2 tumours still retained high levels of OKL38 expression. Significant relationship was found

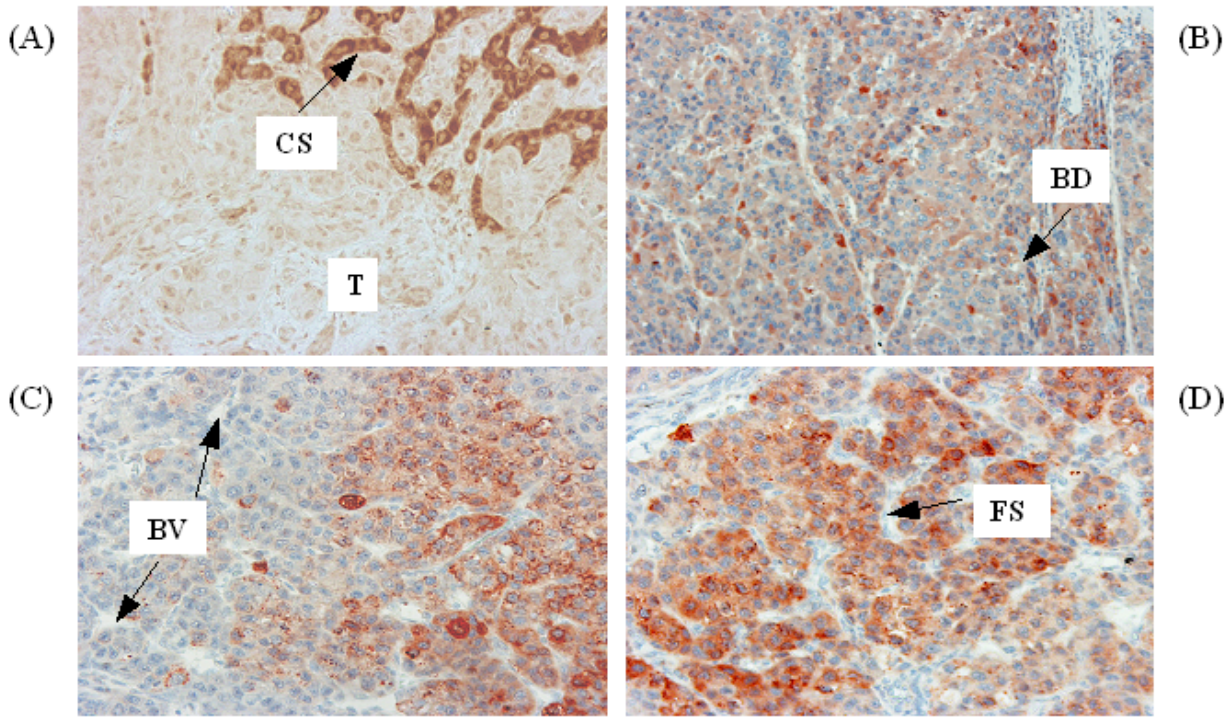


Figure 4.34: Immunolocalization of OKL38 in HCC and adjacent non-neoplastic tissues. Human HCC tumours and adjacent non-tumour livers were collected and paraffin-embedded. Five µm sections were subjected to immunohistochemical analysis using rabbit anti-human OKL38 antibody. (**A**) Strong positive cytoplasmic staining (**CS**) was observed in adjacent benign hepatocytes and complete negative staining was observed in the tumor (**T**). Weak and patchy staining in tumor tissue is indicated in (**B**); the average and strong staining in HCC was indicated in (**C**) and (**D**), respectively. Negative staining was observed in bile duct (**BD**), blood vessels (**BV**) and fibrostroma (**FS**). Original x400.

Table 5: Frequency of distributions of Clinico-pathological factors (92 patients)

A

Samples	OKL-38 Staining	Distribution of OKL-38 expression
HCC	➢ Negative	38/92 (41.3%)
	➢ Weak and patchy	36/92 (39.1%)
	➢ Average	5/92 (5.4%)
	➢ Strong positive	13/92 (14.1%)
Benign Liver	➢ Positive, very strong	92/92 (100%)
	➢ diffused	
Cirrhotic liver	➢ Positive, very strong	27/27 (100%)
	➢ diffused	

B

OKL 38 Staining	Stages			
	I	II	III	IV
Negative	0	20/50	8/22	10/12
Weak and patchy positive	2/8	20/50	14/22	2/12
Strong positive	6/8	10/50	0	0

C

Variables	n	%	no of deaths (%)
Sex			
Male	75	81.5	17/75 (26.7)
Female	17	18.5	7/17 (41.1%)
Age (year)			
<60	37	40.2	6/37 (16.2%)
>60	55	59.8	18/55 (32.7%)
Tumour stage			
I	8	8.7	0
II	50	54.3	14/50 (28%)
III	22	23.9	6/22 (27.3%)
IV	12	13	4/12 (33.3%)

between stages of HCC and the loss of OKL38 expression (Non-parametric trend analysis: $Chi^2(1) = 8.1796$, P = 0.0042). Comparison of the survival between those with positive and negative OKL38 staining suggested no correlation between OKL38 expression and overall survival in curative resection HCC (Log-rank test: $Chi^2(5) = 3.92$, P = 0.5613). Age ($Chi^2(5) = 1.9273$, P = 0.859) and sex ($Chi^2(5) = 6.2945$, P = 0.279) of the patients appear not to be significantly correlated to OKL38 expression (Table 5C).

4.5 Post-transcriptional regulation of OKL38 protein translation

Understanding the regulations of any genes is as crucial as characterization of its functions. This study and previous works (Huynh et al., 2001) have highlighted the significance of OKL38 in tumourigenesis rendering unveiling the mechanisms of OKL38 regulations imperative. Since OKL38 is differentially lost in kidney and liver cancers, deciphering the regulatory mechanisms of this gene is of particular interest.

4.5.1 The ORF of OKL38 is intact in the liver cancer cell line and HCC

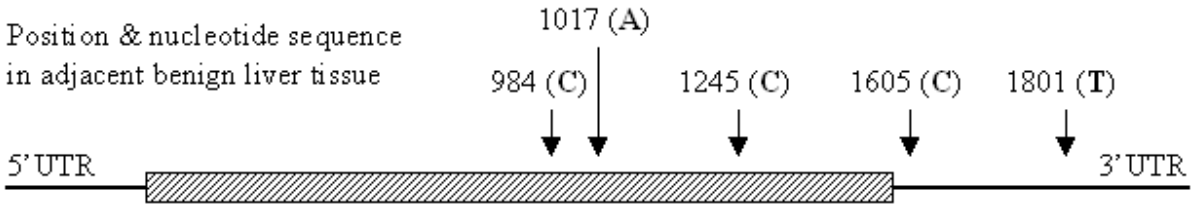
Approximately half of the paired-HCC samples showed no significant difference in the levels of OKL38 transcripts expression between the ABL tissues and HCC (Fig. 4.33). Comparing the expression profile between the OKL38 protein and its transcripts, 42 % (8 of 19) of the paired liver normal/tumour's OKL38 transcripts levels did not correlate with the protein expression (Fig. 4.33). OKL38 transcripts were detected in all four cancer cell lines but the protein was absent (Fig. 4.33). Similar phenomenon was observed in the A498 kidney cancer cell (Fig. 4.29 & 4.31). Based on the above observations, we postulated that the discrepancy between mRNA and protein levels could be due to (1) mutations within the coding region leading to frame shift or change of

amino acid sequence, which resulted in loss of protein expression or protein produced not detectable by anti-OKL38 antibody; (2) the UTRs may be involved in the translation regulation of OKL38 protein expression.

To examine the first possibility, HuOKL38-1a variants which consist of exon 3 to 8 was amplified by RT-PCR from 4 liver cancer cell lines and 5 paired HCC tissues that had low or undetectable level of OKL38 protein but high levels of OKL38 transcripts. Direct sequencing was performed using internal primers (Table 2) and the results were summarized in Figure 4.35. Sequence analysis showed that the 52 kDa ORF of OKL38 was intact in all 4 liver cancer cell lines and in 5 paired HCC and their ABL tissues. Three sites of silent mutation were detected in the ORF; A→G at position 1017 bp in both Hep3B and PLC/PRF5 cells, C→A at position 1245 bp in HepG2 cells and a probable single allele mutation (i.e. double peak detected in the sequencing results-C/G) detected in patient 10 tumour sample at position 984 bp. A cytosine was deleted at position 1605 bp in the Chang liver cell, and a T→C mutation at position 1801 bp in the 3'UTR of OKL38 gene from both the PLC/PRF5 and Chang Liver cell lines. The positions described above are with reference to published HuOKL38-1a variant. The results suggest that frame-shift mutation may not be the culprit for down-regulation or silencing the expression of OKL38 protein in HCC.

4.5.2 5'UTR of OKL38 variants suppress the translation of its mRNAs

The earlier results from mutation studies indicated that the alternative hypothesis might be true, where the UTRs might be involved in translational down-regulation of OKL38 protein in the liver cancer cell lines and HCC samples. The roles of UTRs in translational regulation of OKL38 mRNAs were investigated. HuOKL38-1a and -2a



HepG2	--	--	**A**	--	--
Hep3B	--	**G**	--	--	--
PLC/PRF5	--	**G**	--	--	**C**
Chang Liver	--	--	--	**Deleted**	**C**
Patient 10 N	--	--	--	--	--
Patient 10 T	**S**	**R**	--	--	**Y**

Figure 4.35: Mutation analysis of OKL38 in exon 3 to 8. A schematic of HuOKL38-1a variant containing exon 3 to 8. The nucleotide positions and sequences above the descending arrows were from the published HuOKL38-1a (GenBank Accession No.: AY258068) cDNA. The black lines and shaded box represent the UTRs and ORF, respectively. The table lists the position where mutation is detected. Dash lines in the table represented nucleotides that remained unchanged or not mutated. (S=C/G; R=A/G & Y=C/T).

variants share the same ORF that encodes a 52 kDa protein, while the HuOKL38-2b variant translates into a larger protein of 62 kDa. The three OKL38 variants differ in the 5'UTRs but share the same 3'UTR. Using OKL38 protein as a reporter gene, constructs with and without the different UTRs were generated by PCR from these variants and cloned into the mammalian expression vector, pcDNA3.0 (Fig. 4.36). *In vitro* Transcription and Translation was performed for all the cloned constructs and differential expression of S^{35}-radio-labeled OKL38 protein was shown in Figure 4.37A. The same blot was probed with anti-human OKL38 antibodies to verify its identity (Fig. 4.37B). The same set of constructs as shown in figure 4.36 were transfected into Chang Liver cells to further verify the *In vitro* Transcription and Translation results (Fig. 4.37C).

As shown in Figure 4.37A-C, all the constructs that contained the 5'UTRs (i.e. 1aFL, 2aFL, 1a5'UTR, 2a5'UTR, 2bFL & 2b5'UTR) expressed lower levels of OKL38 protein as compared to those without the 5'UTRs (i.e. 1a/2aORF, 1a/2a3'UTR, 2b3'UTR & 2bORF). The 5'UTRs derived from HuOKL38-2a and -2b variants seem to exert strong translation suppression activities, while that from HuOKL38-1a appeared slightly weaker. Comparing 1aFL, 1a5'UTR and 1a/2a3'UTR suggest that the 3'UTR of OKL38 seems to possess some translation enhancing activities (Fig. 4.37A-C).

To ensure that the translation suppression of the OKL38 5'UTRs observed in figure 4.37C was not due to different levels of transcription, half of the transfected Chang Liver cells were used for RNA extraction and the total OKL38 transcripts were determined using one step RT-PCR (Fig. 4.37D). Similar level of OKL38 transcripts were detected in all the Chang Liver cells transfected with constructs carrying OKL38 cDNA and no transcripts were detected in cells transfected with control vector (Fig.

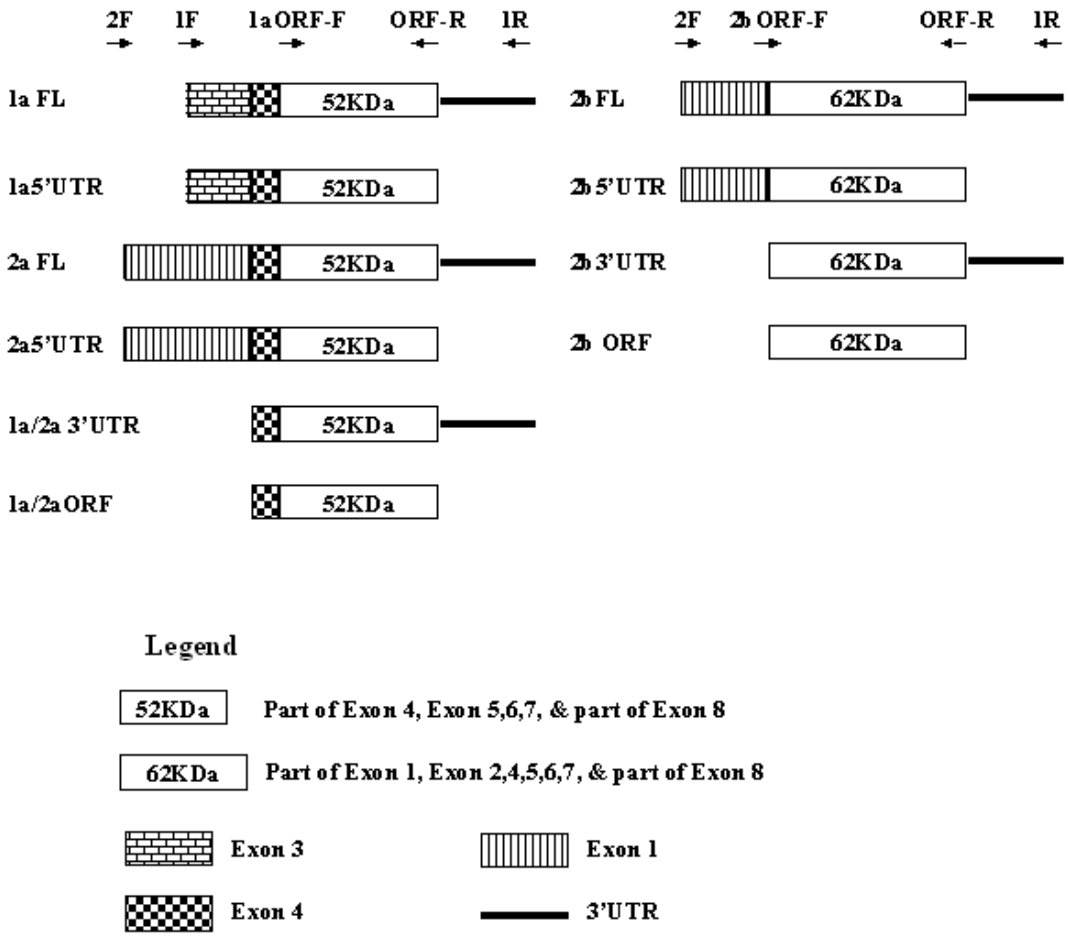


Figure 4.36: Schematic of the various constructs harbouring 5' and 3' UTRs from the HuOKL38-1a, -2a & -2b variants. Top forward and reverse facing arrows denote primers designed for PCR cloning of the various constructs and boxes of different shades indicate the different regions. The OKL38 ORF is employed as a reporter in these constructs and were cloned into mammalian expression vector, pcDNA3.0.

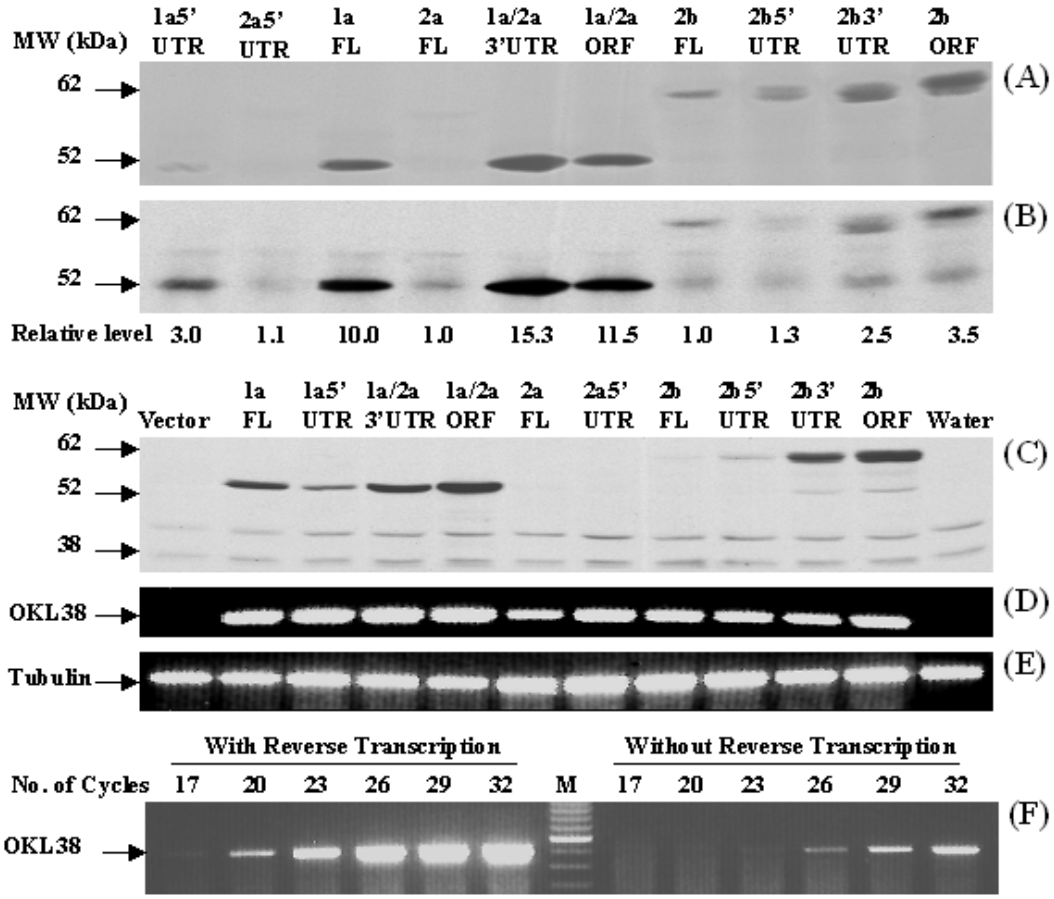


Figure 4.37: Regulation of OKL38 expression by 5' and 3' UTRs of HuOKL38-1a, 2a & 2b variants. Radiolabelled OKL38 proteins from various constructs using the TNT system were separated on a PAGE gel, transferred onto a nitrocellulose membrane and exposed to autoradiograph (**A**). The same blots were probed with rabbit anti-OKL38 antibodies and the expression of OKL38 is as shown in (**B**). The relative levels of protein expression were calculated via employing 2aFL and 2bFL as basal level for the 52 and 62 kDa OKL38 protein, respectively. (**C**) Western blot analysis of total protein extracted from Chang Liver cells transfected with the constructs shown in figure 4.36. Half of the transfected cells in (**C**) were used for RNA extraction and one step RT-PCR was performed for detection of total OKL38 transcripts (**D**) and tubulin (**E**) as internal control. (**F**) To determine the optimum cycles (22 cycles) used for detection of total OKL38 transcripts in the transfected Chang Liver cells without undesired amplification of contaminated DNA constructs.

4.37D). Equal levels of OKL38 transcripts were found in the transfected cells and the difference observed in the OKL38 protein level was due to the presence of the 5'UTRs. The desire minimum PCR cycles of 22 were determined for detection of OKL38 transcripts in the transfected Chang Liver cells without undesirable amplification of the possibly contaminated DNA constructs (Fig. 4.37F).

To further characterize the regions that are involved in translational suppression of OKL38 protein, several deleted 5'UTRs of the HuOKL38-1a and 2a variants were generated by PCR as illustrated in figure 4.38. These deleted constructs uses either the OKL38 or the SEAP protein as the reporter gene to determine the level of translation. The various 5'UTRs deleted constructs (pcDNA-3.0) carrying the OKL38 as the reporter gene were transfected into Chang liver cells and Western blot analysis was performed on the protein extracted from these transfected cells (Fig. 4.39A). In addition, these deleted OKL38 5'UTRs, including two additional fragments (2aD4 and E4) (Fig. 4.38), were subsequently cloned into the pSEAP2-CV and transfected into both Chang liver and PLC/PRF5 liver cancer cell to confirm the translation suppressive effect of the OKL38 5'UTRs observed ealier (Fig. 4.39A).

The deletion of the first 45 bp from the HuOKL38-1a 5'UTR (1aD1) allowed recovery of the translational efficiency to more than 60 % and 40 % in the OKL38 (Fig. 4.39A) and SEAP (Fig. 4.39B) reporter system, respectively, providing evidence that the regions between –156nt to –111nt of the 5'UTR is crucial for translational suppression. Interestingly, a small uORF of 10 amino acids with internal translation initiation codon in this region was identified (Fig. 4.40A). Subsequent deletion of the UTR increased the

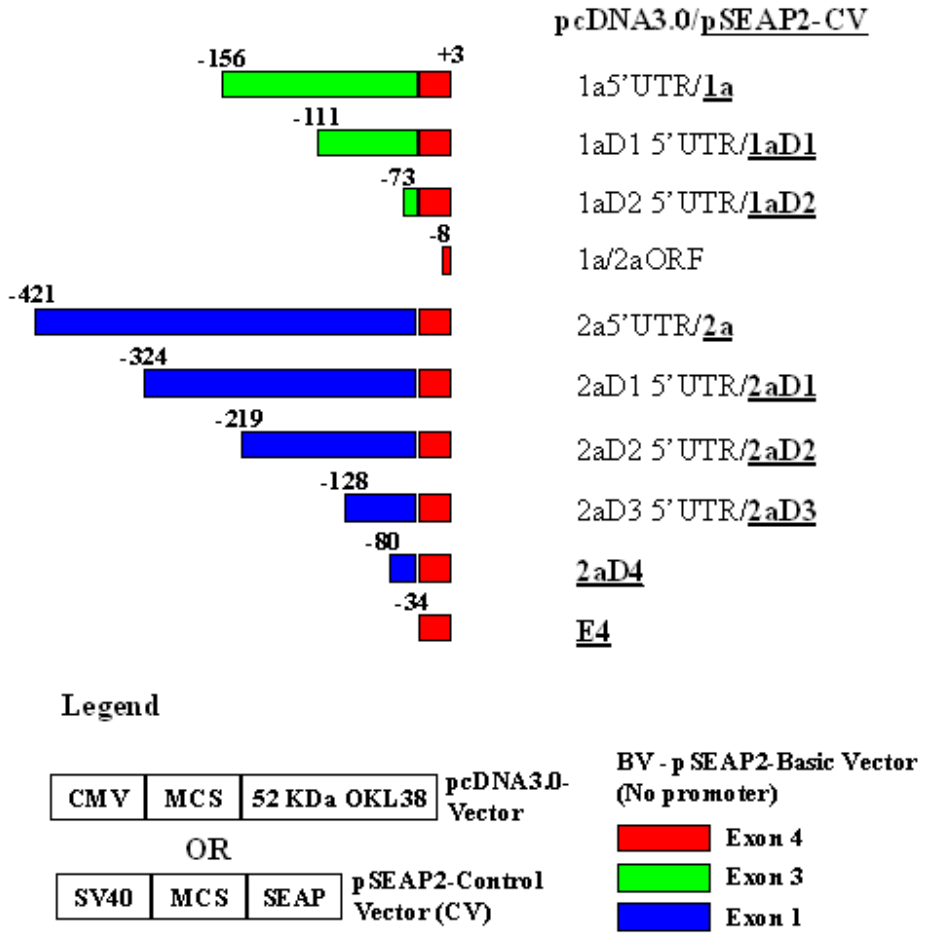


Figure 4.38: Schematic showing the 5'-deletion subclones of the 5'UTRs of HuOKL38-1a & -2a variants. The 5'-deleted constructs were cloned into two different reporter systems. Constructs underlined with bold faced were cloned into the multiple cloning site (MCS) of pSEAP2-CV, which uses the SEAP reported system. 1a5'UTR, 1aD1 5'UTR, 1aD2 5'UTR, 1a/2aORF, 2a 5'UTR, 2aD1 5'UTR, 2aD2 5'UTR and 2aD3 5'UTR were cloned into pcDNA3.0, which uses the cloned OKL38 as the reporter. Numbers above the bars indicate the various length of UTRs with respect to the translation start site (AUG as +1).

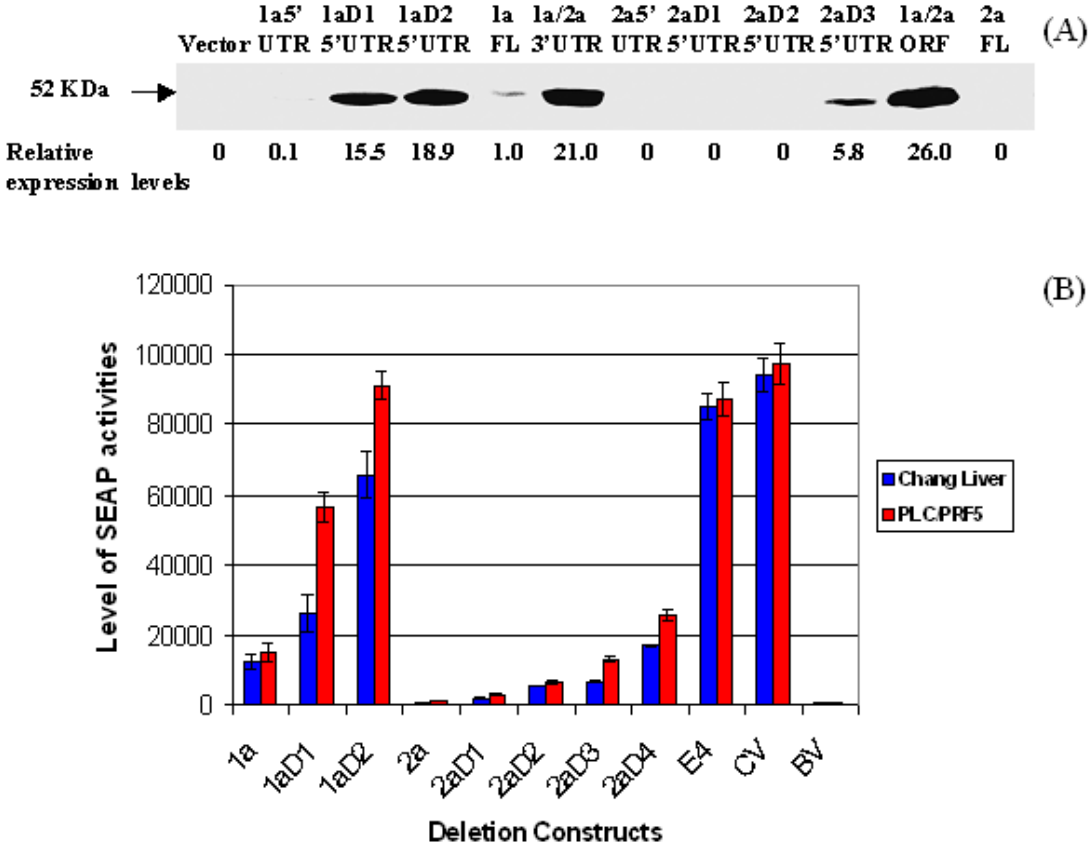


Figure 4.39: Regulation of OKL38 expression by 5'UTRs deletion constructs of HuOKL38-1a & -2a variants. The 5'UTR-deleted constructs described in figure 4.28 were transfected into Chang liver and PLC/PRF5 cells. (A) The deletion constructs cloned into pcDNA3.0 were transfected into Chang Liver cells and total protein was extracted 24 hr post-transfection. Western blot analysis was performed using anti-human OKL38 antibodies. The relative levels of OKL38 protein as compared to 1aFL is as indicated. The pSEAP2-CV 5'UTR deletion constructs were transfected into Chang Liver and PLC/PRF5 cells. The conditioned media was harvested 24 hr post-transfection, assayed for SEAP activities and results of 3 independent experiments are as shown in (B).

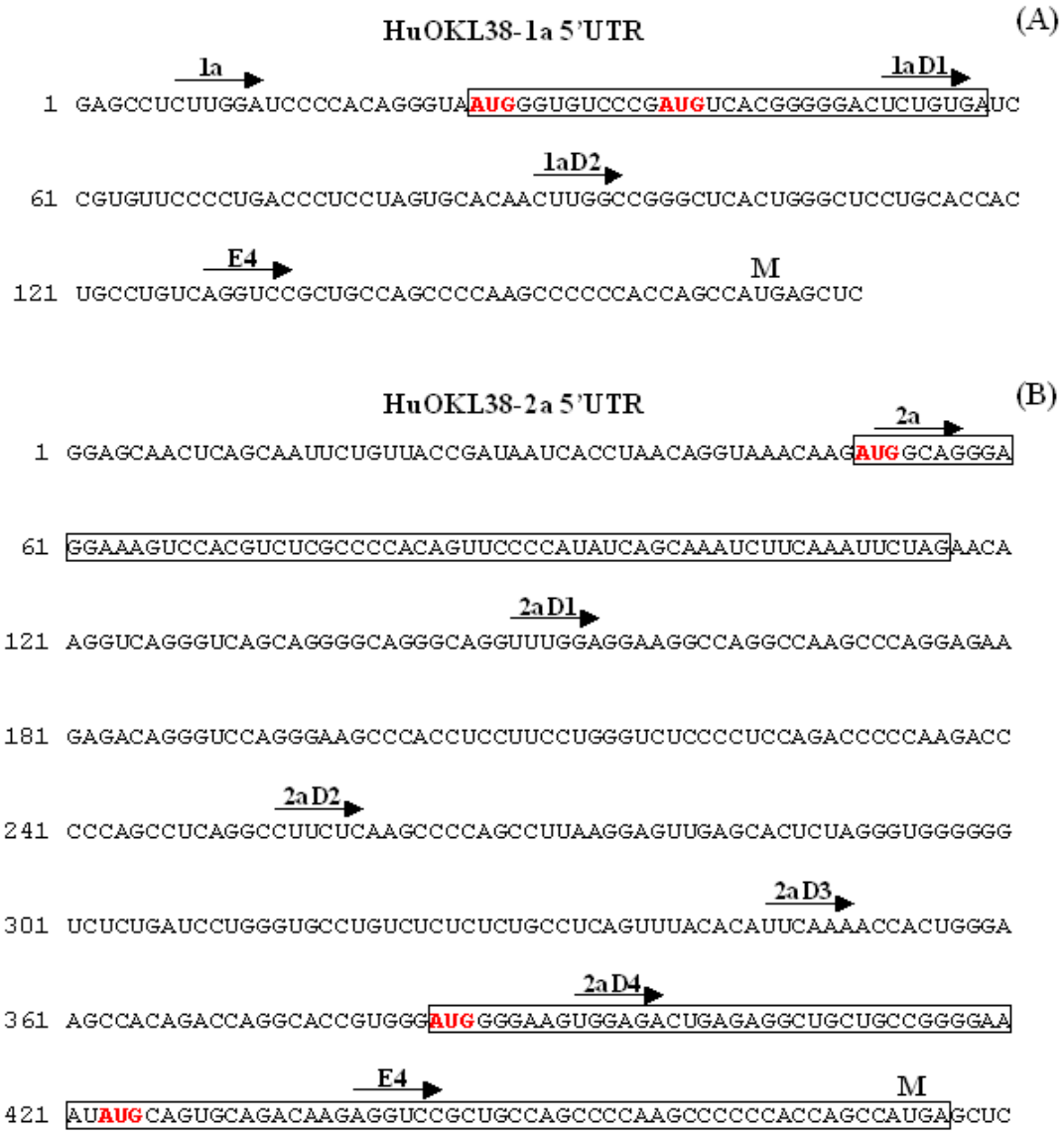


Figure 4.40: Detailed illustration of HuOKL38-1a and -2a 5'UTRs. Bold labels with arrow indicate the position of 5'UTR-deleted constructs described in figure 4.38. The small uORFs described in figure 4.12A were boxed and its translational start codon (AUG) were highlighted in red. M indicates the translational start site of the OKL38 protein. NB: The small uORF within the HuOKL38-1a (A) and -2a (B) 5'UTR have two inframe start codons.

translational efficiency of the OKL38 protein indicating that other factors such as structured 5'UTR might be involved in translational regulation.

The deletion of the HuOKL38-2a 5'UTR up to –34nt (E4) led to a surge in translational efficiency, which was comparable to the positive control (Fig. 4.39A & B). This demonstrated that almost the whole of HuOKL38-2a 5'UTR, from –421nt to –34nt, effectively suppressed the translation of OKL38 protein in both reporter systems. This region coincidentally corresponded to exon 1 of the OKL38 gene. There is no significant difference in the levels of translation between 2aD2 (-219nt) and 2aD3 (-128nt), whereas the construct 2aD4 (-80nt) showed a significant increase in translational efficiency in both the Chang Liver and PLC/PRF5 cells (Fig. 4.39B). This relief of translational suppression may be attributed to a small uORF detected within the region from –128nt to –80nt (Fig. 4.40B). This uORF have an internal translation initiation site (AUG) between –80nt (2aD4) and –34nt (E4) (Fig. 4.40B), which led to a sudden increase in translational efficiency upon deletion (Fig. 4.39B). This deletion is instrumental to the observed increase in translational efficiency. Collectively, the differential levels of translational efficiency as shown provided evidence that the second AUG in the uORF might play a very important role in translational suppression.

CHAPTER 5: DISCUSSION

5.1 The Human OKL38 gene

5.1.1 Features in OKL38 gene

The importance of OKL38 gene in cancer had previously been demonstrated (Huynh et al., 2001) and further reinforced in this study. The human *OKL38* gene was cloned, sequenced and characterized to decipher the function(s) and regulation(s) of this potential tumour suppressor. The human *OKL38* gene reported in this study was cloned and sequenced in advance of the published human genome sequence (Lander et al., 2001; Venter et al., 2001). The sequence data from this study showed two regions of mismatch and was verified to contain a 550 bp longer 'CCCT' repeats as compared to the published human sequence. The 300 bp unsequenced region in these repeats can be confidently assumed to contain 'CCCT' repeats as the same signals (CCCT) in the sequencing electropherogram diminishes toward the center of this region (data not shown). The functional importance of the 'CCCT' repeats in the human *OKL38* is unknown at the present time. However, the instability of 'CCCT' repeats was highlighted in several reports suggesting that the different repeats length was due to genomic instability (Kosteas et al., 1997; Fiskerstrand et al., 1999). Fiskerstand *et al*. (1999) have shown that specific regions in intron 2 of the rat preprotachykinin-A gene contains a 128 bp of 'CCCT' tandem repeats domain. This domain could support reporter gene expression in mouse embryonic stem (ES) cells that have been induced to differentiate but not in the undifferentiated ES cells. This would suggest that this region functions as a highly

restrictive enhancer and may be associated with differentiation. Similarly, the 'CCCT' repeats located in intron 2 of the OKL38 gene may associate with differentiation as postulated in the previous report (Huynh et al., 2001). The 'CCCT' repeats is located 1.2 kb upstream of the promoter P1 initiation site and it is likely that this region functions as a restrictive enhancer for P1 promoter (Fig. 4.2).

An approximately 500 bp of dinucleotide 'CA' repeats is localized to intron 1 of *OKL38* gene (Fig. 4.2). 'CA' repeats are widespread throughout the human genome, representing ~0.25% of the human genome. They are the most common dinucleotide polymorphism among the microsatellite DNA fraction (Lander et al., 2001). Human genes with polymorphic intronic 'CA' repeats includes the human interferon-γ gene (Awad et al., 1999), the epidermal growth factor receptor gene (Han and Nakamura, 1998) and the cystic fibrosis cellular stress response *(CFTR)* gene (Mateu et al., 1999) among others. An approximately 20 bp random deletion in the 'CA' repeats region was found in the sequenced *OKL38* gene suggesting polymorphism in this region. Recent report by Hui *et al.* (2003) have highlighted a discovery that dinucleotide 'CA' repeats of variable length found in the intron 13 of human eNOS gene promotes intron removal. They have shown that the ribonucleoprotein (hnRNP) L, a member of the hnRNP family, is the major factor that binds specifically and in a length-dependent manner to the 'CA'-repeat enhancer. Although the polymorphic nature of this region in the human *OKL38* gene has not been established, the intronic 'CA' repeats in the intron 1 of human *OKL38* gene may serve as docking sites for hnRNP L factor to bind and regulate the differential splicing of this gene. Evidence to this can be drawn from the alternative splicing occurring at the 5' end of *OKL38* gene, where the 'CA' repeats are present.

The genomic organization of human *OKL38* gene and its splicing pattern have been established in this study. The splicing pattern bears similarity to that of the human and mouse thioredoxin reductase 1 (TXNRD1) gene (Osborne and Tonissen, 2001). Differential promoter usage and alternative splicing at the 5'-region of the TXNRD1 are highly similar to that of the OKL38 gene, except that the gene contains 16 exons as compared to *OKL38* gene, which consists of 8 exons. The similarity in splicing pattern of TXNRD1 and OKL38 gene, coupled to the presence of a Pyr_redox domain in both genes suggests that OKL38 may be a new member of this family.

5.1.2 Promoters of human OKL38 gene

Several putative transcription factors binding sites have been identified within the 300 bp upstream region of the P1 transcription start site. A CpG island of 215 bp, located 746 bp precedes this TATA-less promoter upstream of promoter P1 initiation site. CpG islands are frequently associated with 5'-regulatory sequences of housekeeping genes and genes with a more tissue-restricted pattern of expression (Gardiner-Garden and Frommer, 1987). This characteristic was reflected in the expression profile of OKL38 in both the human and rat, where the transcripts are ubiquitously expressed in all tissues with significantly high expression in liver, kidney, ovary and testis (Fig. 4.6-8, 4.17 & 4.19). Putative Initiator (Inr) with the consensus Py-Py-A_{+1}-N-T/A-Py-Py where A_{+1} is the transcriptional start site (Burke and Kadonaga, 1997) was identified in promoter P1 suggesting that this core sequence is likely responsible for driving transcription (Fig. 4.3). Additional support for this conclusion is the presence of a putative downstream promoter element (DPE) in promoter P1 that is also found in about 20% of the TATA-less promoter containing Inr (Burke and Kadonaga, 1997).

Transcription factors binding sites identified within promoter P1 appears to differ from those observed in the promoter P2, suggesting that they may be differentially regulated. Interestingly, no typical promoter elements such as TATA or CAAT box, Inr or DPE were identified in the putative promoter P2 suggesting that other novel elements might regulate this promoter. This observation might help to explain the seemingly low levels of promoter P2 activities, where low levels of transcripts (i.e. HuOKL38-2a, -2b & -2c) were produced from this promoter. Further characterization of these promoters is needed to establish their regulatory functions.

Three full-length OKL38 variants were cloned in this study. The HuOKL38-1a and HuOKL38-2a mRNA isoforms are expressed from different promoters; thus, possess different 5'UTR (Fig. 4.9). These two mRNA isoforms encode for the same ORF (477 amino acids) verified by TNT analysis (Fig. 4.10). The lethal effect of this OKL38 protein isoform suggests that this protein has to be regulated in a precise and controlled manner. The observation bears similarity to that of the p53 protein, as abnormal regulation of this gene causes the cells to become cancerous or lethal to its own survival (Smith et al., 2003). Therefore, the use of 2 different promoters to produce 2 mRNA isoforms coding for the same protein is far from redundant. The intrinsic regulation of OKL38 protein at the translational level with possible cis-regulatory elements in the 5' UTR was demonstrated in this study.

5.1.3 The origin of human OKL38 isoforms

Sequence analysis of the cloned OKL38 variants showed that the 1st AUG on the HuOKL38-2b and -2c transcripts conform to the Kozak's consensus sequence (Kozak, 1981; Kozak, 1984; Kozak, 1986), except HuOKL38-1a and -2a. Interestingly, all the

internal in-frame AUGs identified in HuOKL38-1a, which were also present in all the other human OKL38 variants, does not conform to the Kozak's sequence (Fig. 4.11). The efficacy of translating OKL38 appeared to be independent of Kozak's sequence (Fig. 4.37A-C). The 5'UTRs in this instance played a more significant role in controlling the efficiency of translation of OKL38 protein.

Several OKL38 isoforms (61 kDa, 52 kDa and a doublet 38 kDa) were detected by Western blot analysis (Fig. 4.10 & 4.31). The 61 kDa OKL38 isoform might have been translated from HuOKL38-2b or -2c transcripts, while the 52 kDa protein is most probably derived from HuOKL38-1a or 2a transcripts. However, we do not exclude the possibility of internal translational initiation at in-frame AUG in the mRNAs of HuOKL38-2b and -2c variants. Small non-inframe ORFs were detected within the first AUG and the internal AUG (i.e. between AUG-a and AUG-1) in both the HuOKL38-2b and -2c transcripts (Fig. 5.1), which may recruit the translation machinery and reinitiate translation as described for the C/EBP family of transcripts, Fli-1 and CNP2 (*Section 1.2.2, Literature Review*). Similarly, the 38 kDa doublet might have derived from usage of internal translation start site with the same mechanism as small non-inframe ORFs were also observed within the 52kDa ORF (Fig. 4.11). Although no cleavage sites for caspase1 to 10, enterokinase, granzymeB and thrombin were found within the full-length OKL38 protein using ExPASy peptide cutter (Gasteiger E et al., 2005), post-translational processing of full-length protein by other endogenous peptidases could also be responsible for OKL38 protein isoforms observed in the present study.

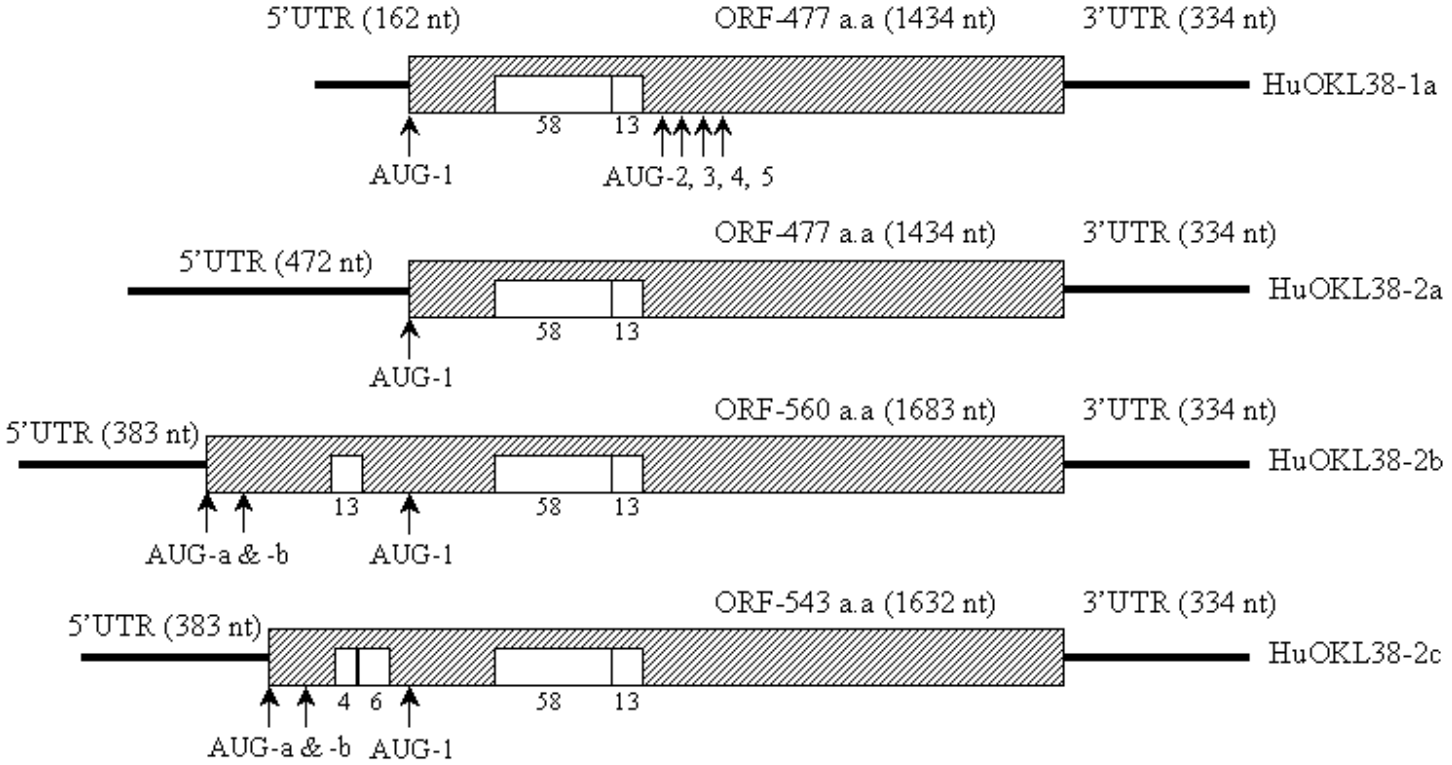


Figure 5.1: Schematic of HuOKL38-1a, -2a, -2b & -2c illustrating the positions of putative internal translation start sites. The shaded box represents the main ORF, which encode a protein of 52 kDa by HuOKL38-1a & -2a and proteins of 61 and 59 kDa encoded by HuOKL38-2b & -2c, respectively. Small non-inframe ORFs were represented by white boxes and the sizes were indicated below each box. The arrows (AUG-2 to 6 & -b) indicates the positions of inframe internal translation start sites. The length of 5' and 3'-UTRs and the main ORFs were indicated in the brackets.

5.1.4 OKL38, a putative bi-functional protein

Proteins are modular in nature and are generally comprising of several domains of rather diverse functions. The various isoforms of OKL38 may contain only certain domains but serve different functions in the cells. The cloned HuOKL38-1a encoded a longer protein of 52 kDa as compared to the previously cloned OKL38 cDNA (Huynh et al., 2001). The presence of a TrkA and Pyr_redox domain in OKL38 protein suggests that the gene may belong to the larger family of pyridine nucleotide-disulphide oxidoreductases and may share similar functions from this family of proteins. These proteins include Trx reductase and GSH reductase, and most probably also include AIF, AMID/PRG3 and AIFL (Fig. 4.23).

In addition to the Pyr_redox domain, the Trx reductase and GSH reductase contained a dimerization domain, which functions in protein-protein interaction. Similarly, AIFL contained a Rieske domain in addition to its Pyr_redox domain (Fig. 4.23). The cell death associated function of OKL38 demonstrated in this study was similar to those observed in over-expression of AIF, AMID/PRG3 and AIFL in cell lines. The oxidoreductase function in the AIF, AMID/PRG3 and AIFL is not required for its apoptogenic function (Miramar et al., 2001; Loeffler et al., 2001; Wu et al., 2002; Ohiro et al., 2002; Xie et al., 2005). The N-terminal portion of AIF and the Rieske domain in AIFL were sufficient to induce apoptosis (Loeffler et al., 2001; Miramar et al., 2001; Xie et al., 2005) suggesting that the Pyr_redox domain in OKL38 may be redundant for its cell death function. This indicates that the TrkA domain in the OKL38 may be sufficient to induce cell death.

The oxidoreductase function of Pyr_redox domain in the AIF protein and its role as free radical scavenger had previously been demonstrated (Klein et al., 2002). The Pyr_redox in OKL38 may play an important role in protecting the cells against oxidative stress via mopping up free radicals generated through respiratory processes. This postulation was further supported by the results from microarray analysis which showed the OKL38 was one of the genes that was induced in MCF7 cells by hydrogen peroxide, mendonine and t-butyl-hydrogen peroxide treatment (Chuang et al., 2002). On the other hand, up-regulation OKL38 by these oxidative stress-inducing agents might contribute to the cell death signalling pathways. The roles of OKL38 during oxidative stress are still unclear at the present moment. These observations seem to suggest that genes containing the oxidoreductase domain may be bifunctional, regulating cellular redox and cell death.

5.1.5 OKL38 - An ancient protein with diversified functions

Evolution has a mysterious way of shaping proteins and their functions. The conserved domain search program (NCBI) detected a TrkA domain in the full-length OKL38 protein and this domain belongs to a flavoprotein involved in bacterial K^+ transport. The TrkA gene has been described in several prokaryotes such as *Escherichia coli* and *Vibrio vulnificus* (Hamann et al., 1987; Chen et al., 2004). The mammalian counterpart was suggested to be the β subunits of voltage-gated potassium channel (Jan and Jan, 1997). In the *E. coli*, the TrkA protein contains two internal repeats each with an NAD(H)-binding motif, and the purified TrkA protein binds NAD(H) (Schlosser et al., 1993) (Fig. 4.23). The eukaryotic voltage-gated potassium channel may contain four β subunits, each with sequence similarity to the NAD(P)H-binding domain of oxidoreductase, similar to TrkA (Jan and Jan, 1997). Pyr_redox domain consists of a

small NADH-binding domain within a larger FAD-binding domain. These observations suggest that both the TrkA and Pyr_redox domains identified in the OKL38 protein might bind onto substrate such as NAD(P)H, NADH or FAD. Comparing the TrkA domain from *E. coli* and the OKL38 protein, the TrkA and Pyr_redox domain in OKL38 seem to be evolved from the two TrkA domain repeats observed in *E.coli*. One of TrkA domain from the TrkA protein in *E. coli* appears to be conserved in OKL38, while the second TrkA domain seems to have evolved into a Pyr_redox domain (Fig. 4.23). The over-expression of TrkA resulted in protein aggregates (Hamann et al., 1987), further illustrating the similarity observed between TrkA and OKL38 protein. However, there is no evidence available showing that OKL38 is a component of the mammalian potassium transport system. These observations point toward the possibility that OKL38 might have evolved from an ancient protein that had undergone gene duplication and evolved separately from their potassium channel β subunits cousins, to be functionally diverse.

5.2 Rat is a suitable model to study OKL38 gene

5.2.1 OKL38 is highly conserved in human, rat and mouse

Multiple sequence alignment (EMBL) delineates the regions of similarity for OKL38 protein from rat, mouse and human (Fig. 4.21). The OKL38 protein was highly conserved in all the three species suggesting the importance of this protein in cellular functions such as growth and differentiation. The Pyr_redox domain identified in human OKL38 was common to all three species. This domain is also present in the well-characterized GSH reductase and Trx reductase (Fig. 4.23). BLAST search using the deduced amino acid sequence against the non-redundant database identified several

proteins of unknown functions such as human C8ORF1 (GenBank Accession No. NP_004328) and the bacteria conserved protein, BH1623 (GenBank Accession No. NP_242489) from *Bacillus halodurans* (Fig. 4.23). The C8ORF1 is a predicted protein from the human chromosome 8, with an identity of 49% and a similarity of 62% to rat OKL38 protein. An unknown conserved protein, BH1623 from *B. halodurans*, shows a lower homology to that of the rat OKL38 with an identity of 39% and a similarity of 44%. Results from sequence comparison studies seem to advocate that the C8ORF1, BH1623, AIF, AMID/PRG3, AIFL, Trx reductase, GSH reductase and OKL38 may belong to the larger family of pyridine nucleotide-disulphide oxidoreductases.

5.2.2 Genomic organization of rat OKL38 gene

The genomic organizations of the human and the rat OKL38 gene have been deciphered in this study. The genomic structure of both the human and rat OKL38 gene bears similarity with each containing 8 exons with exon 5, 6 and 7 possessing identical sizes (Fig. 4.1B & 4.15). These three exons encoded almost the whole of the putative TrkA domain. The putative pyridine nucleotide-disulphide oxidoreductase domain resides in exon 8, the last exon in both species. The predicted OKL38 spliced junction in both species contained the GT/AG dinucleotides consensus sequences. Differential promoter usage and alternative splicing were utilized by both the human and rat to regulate OKL38 transcription. Recent evolution events may have fine-tuned the regulation of OKL38 in each individual species as the splicing pattern appear different, but preserving the protein and gene structure.

5.2.3 The presence of small upstream ORF (uORF)

Three OKL38 cDNA variants from each of the human and rat were identified and cloned in this study. The NCBI annotation project predicted the human OKL38 to be about the same length as the RtOKL38-2.0. Two mouse OKL38 cDNAs of 1982 bp (GenBank Accession No. BC022135) and 2383 bp (GenBank Accession No. BC006032) have also been reported. Sequence analysis of these cloned rat OKL38 cDNAs with those from the human and mouse showed that the transcripts contained different 5'UTRs. Small uORF detected in the 5'UTR of the RtOKL38-2.0, -2.3 and -2.3A variants may be involved in post-transcriptional regulation of protein synthesis, as they are often used to down-modulate the production of potent proteins such as cytokines, growth factors, protein kinases and transcription factors (Kozak, 2002). The cytotoxicity of OKL38 when over-expressed and the existence of different mRNA isoforms that encoded for the same protein suggest that translational regulatory cis-elements may exist in the 5'UTR of the mRNA to fine-tune the regulation of OKL38 protein. The presence of uORFs in the 5'UTRs of human and rat OKL38 variants suggests that they may have similar translational regulatory control mechanism (Fig. 4.12A).

5.2.4 Rat OKL38 isoforms and tissue distribution

Four human OKL38 variants were identified of which three were cloned, while only three variants were cloned from the rat. None of the OKL38 variants identified so far contain all the eight exons with the exception to RtOKL38-2.3, which was identified in this report. The RtOKL38-2.0 and HuOKL38-1a transcript consists of exon 3-8 with both having an approximate size of 2 kb. The RtOKL38-2.0 and HuOKL38-1a transcripts

encodes for proteins of 478 and 477 amino acids, respectively. Both proteins differ only in one amino acid and possess a high homology of 85% (Fig. 4.21).

The expression profiles of all the OKL38 variants were quite different in the human and rat except for HuOKL38-1a and RtOKL38-2.0, which showed similar tissues distributions in both species. Generally, both RtOKL38-2.0 and HuOKL38-1a are highly expressed in the liver, kidney and ovary of the respective species (Fig. 4.6-8 & 4.17-19). These transcripts were derived for promoter P1 of the human and rat OKL38 gene (Fig. 4.8 & 4.19) indicating that the promoter P1 of both species was regulated in similar manner. The discrepancy in OKL38 expression in the ovary of the human and rat is most probably attributed to the non-homogenous human ovarian tissue used in the RT-PCR and MTN, which was further supported by the *in situ* hybridization results showing that OKL38 transcripts were observed to be highly expressed in the granulosa cells of the rat ovaries. However, one does not rule out the possibilities where the levels of OKL38 differ in both human and rat ovaries.

Beside the HuOKL38-1a and RtOKL38-2.0 variants, differences were prominent in the other OKL38 variants from both species. The HuOKL38-2b and -2c variants encodes for proteins of 61 and 59 kDa, respectively, while only a single 52 kDa protein is encoded by the rat OKL38 variants. Interestingly, sequence comparison studies performed between the human and rat OKL38 variants showed that only the coding region for the 52 kDa protein was conserved and contained two putative domains, the Pyr_redox and the TrkA (Fig. 4.21). Over-expression of either rat or human OKL38-eGFP recombinant protein in cell lines localized the protein to the cytoplasm and subsequently leading to cell death (Fig. 4.13, 4.14 & 4.24) suggesting a conserved

function. No alignment was observed in the 5' UTR of the other OKL38 variants (i.e. HuOKL38-2a, -2b & -2c; RtOKL38-2.3 & -2.3A) in the two species indicating that regulation of these variants could be species specific.

Several larger transcripts ranging from 4.0 to 9.0 kb were detected in the rat liver and kidney, and human liver tissues by Northern Blot analysis (Fig. 4.7 & 4.17). In the present study, these larger transcripts were not characterized. 5'RACE analysis did not amplify any larger transcripts other than the 1.1 and 1.4 kb fragments from the rat kidney cDNA library. However, one does not exclude the possibility that they were derived as a result of differential splicing at the 3' end of the gene or other rare OKL38 variants expressed at extremely low levels.

The similarities in the genomic structures, cDNAs and proteins between the human and rat OKL38 gene indicate that the results observed in rat can be extrapolated to human counterparts. In general, the rat may serve as a suitable model to address most of the questions concerning the functions and regulations of OKL38.

5.3 Breast Cancer and regulation of OKL38 by human chorionic gonadotropin (hCG)

5.3.1 Pregnancy and hCG induced expression of OKL38 in rat mammary gland

Regulation of OKL38 during pregnancy in rat mammary glands has previously been demonstrated (Huynh et al., 2001). In this study, results from RT-PCR and Northern blot analysis showed that only the RtOKL38-2.0 variant was induced by hCG in the mammary and ovary. Low levels of OKL38 expression were detected in the mammary

epithelial cells of non-pregnant rat where the mammary gland is highly undifferentiated (Fig. 4.25 & 4.28A). The expression of OKL38 protein in mammary gland was observed to be high during pregnancy where the epithelial cells undergoes differentiation and throughout lactation, a stage where the breast acquired fully differentiated condition (Russo et al., 2005). After weaning, all the secretory units of the breast regressed and the mammary epithelial cells undergo apoptosis and remodulation (Russo et al., 1992). At this stage, the OKL38 expression returned to basal level as observed in figure 4.25. The close relationship between the degree of mammary epithelial differentiation and OKL38 expression advocate the involvement of this protein in maintaining mammary epithelial cell differentiation. This postulation was further supported by the fact that OKL38 is highly expressed in the liver and kidney, which is characterized by relatively low cellular turnover and extensive differentiation.

Previous *in vitro* and *in vivo* studies demonstrated the growth inhibitory properties of OKL38 (Huynh et al., 2001) and coupled to the lost of transcript observed in several breast cancer cell lines lists its importance in the process of mammary tumourigenesis. Pregnancy and hCG treatment have been shown to protect the breast from carcinogenesis (Russo et al., 1990b; Russo et al., 1990a; Russo et al., 1991). Here, hCG and pregnancy were demonstrated to induce OKL38 expression in mammary epithelial cells from which breast cancer derive. Taking these observations together, it is tempting to speculate that OKL38 may be one of the downstream effector proteins involved in pregnancy and hCG induced protection of the breast against carcinogenesis.

5.3.2 OKL38 is up-regulated by hCG in the rat ovary

The ability of hCG to induce OKL38 expression in the granulosa cells and the corpus lutes have been demonstrated. CG levels rise during the advancement of pregnancy and recombinant hCG has been shown to induce enlargement of the rat ovary due to the formation of the corpora lutea (Russo I. H., personal communication). In the ovary, hCG binds to the granulosa cells of the ovary through LH-CG-R, a seven transmembrane G-protein-coupled-receptor; this induces ovulation and maintains the corpus luteum that is essential for maintenance of pregnancy until the placenta becomes fully functional (Leung and Steele, 1992; Stouffer, 2003). The above observations suggested that the induction of OKL38 expression by hCG could be mediated through the LH-CG-R signalling pathway. Since CG is required for maintenance of progesterone hormone production during pregnancy (Devoto et al., 2002), it is also possible that hCG-induced OKL38 expression is mediated through progesterone production. Although no cAMP responsive element or progesterone responsive element were detected within the 1 kb region of putative promoter P1 (RtOKL38-2.0), other cis-elements such as AP1, AP4, NFKappaB, and CEBPβ were detected using the program MatInspector V2.2. These putative transcription factors binding sites may contribute to the induction of OKL38 by hCG.

Ovarian cancers derived from the granulosa cell are rare with an incident frequency of approximately 1-2 % and the recurrence of this cancer after resection is very high (Amsterdam and Selvaraj, 1997). Transgenic mice hypersecreting LH led to elevated serum levels of estrogens, androgens, progesterone, corticosteroids and prolactin (Renehan et al., 2004; Keri et al., 2000; Kero et al., 2000; Mann et al., 1999), and lead to

eventual formation of granulosa cell tumours (Risma et al., 1995; Owens et al., 2001). Surprisingly, these transgenic mice treated with ovulatory doses of hCG prevented the formation of granulosa tumours and instead induce the formation of a differentiated luteoma (Owens et al., 2002). Since OKL38 is inducible by hCG in the granulosa cell and is postulated to function in maintaining cellular differentiation, it is possible that hCG induced the formation of differentiated luteoma in the LH transgenic mice via up regulation of OKL38. The above postulation may be over simplified as other hormones and genes were also involved in orchestrating the effects of pathways switching (Owens et al., 2002), from granulosa cell tumour to differentiated luteoma formation. Investigating the effects of hCG on expression of OKL38 in these LH transgenic mice will shed some light on the functions of OKL38 in granulosa cell tumours.

5.4 Kidney Tumour and Hepatocellular Carcinoma (HCC)

5.4.1 Novel biomarkers for kidney tumour and HCC

Failure to detect cancers at an early stage is the main reason for unsuccessful treatment of operable diseases. The ability to predict outcomes of the disease will enable customized treatments and therapeutic intervention. Thus, identifying and development of useful prognostic and diagnostic biomarkers is an important field in cancer biology. OKL38 has been shown to be highly expressed in the liver and kidney. The OKL38 mRNA transcripts were down-regulated in several breast cancer cell lines and DMBA-induced mammary tumour (Huynh et al., 2001). These preliminary studies and the growth inhibitory property of OKL38 advocate the potential of this gene as a biomarker for kidney and liver cancers.

All the kidney tumour samples used in this study (except CPA) were of the clear cell histological type, which is the most common form of RCC. The results from CPA, immunohistochemistry and Western blot analysis indicate that OKL38 was down-regulated in the kidney tumour at both the mRNA and protein levels. Although the OKL38 transcripts were marginally down-regulated in some of the kidney tumour as compared to the adjacent benign tissue, the protein were lost in the tumours as shown by the immunohistochemical staining and Western blot analysis (Fig. 4.31 & 4.32). Interestingly, both the 38 and 61 kDa OKL38 isoforms were lost in the kidney tumour and the 61 kDa isoform seem to be a more effective biomarker for differentiating normal from tumour tissue than the 38 kDa isoform as shown in Figure 4.31A. Immunohistochemistry analysis failed to detect OKL38 protein in 64% (14/22) of the kidney tumour most probably attributed to the loss of the 38 and 61 kDa isoforms in the majority of the tumours. Due to the insufficiency of patient tissue samples, it is not possible at this time to provide any statistically significant conclusion. However, these preliminary results seem encouraging that the various isoforms of OKL38 proteins may serve as important biomarkers for detection of kidney cancer, especially of the clear cell histological type. Both larger patient sample size and diseases outcome information are necessary to further address this issue.

In the liver, the 52 and 61 kDa OKL38 isoforms are expressed at low levels while the 38 kDa OKL38 isoform is readily detected using the same antibody, suggesting that the various isoforms maybe tissue specific. Due to the complexity of the disease, the present TNM system and grading by cellular differentiation cannot accurately predict the disease outcome of all HCC patients. Loss or absence of OKL38 was observed in 64.2 %

of the HCC examined as compared to its ABL tissue. The loss of OKL38 protein in HCC was correlated with the stages of the disease (p=0.0042), indicating the potential use of OKL38 expression as a biomarker to improve the present system of tumour staging. OKL38 may be used concurrently with other HCC markers such as DNA ploidy, cellular proliferation markers, nuclear morphology, cell cycle regulators, oncogenes and their receptors, tumour suppressor, apoptosis related factors, telomerase activity, adhesion molecules, proteinases, angiogenesis regulators and tumour associated antigen (Qin and Tang, 2004), to refine the TNM staging system for HCC. The fine tuned system may include more sub-groups increasing the accuracy of prognosis. However, no significant correlation was established between OKL38 expression and the survival of patients. OKL38 is localized intracellularly and significant loss of the protein is only detectable in HCC of higher tumour stage indicating that it may not be a useful diagnostic biomarker for earlier detection of HCC. Since the 38 kDa OKL38 isoform is differentially lost in the liver tumour, it is important to determine the origin of this isoform.

5.4.2 Mechanisms of OKL38 down regulation in cancer

Over-expression of the OKL38 protein in A498, Chang liver and BRL cells led to cell rounding and subsequently cell death. This effect could be attributed to cell cycle arrest and apoptosis (Wang et al., 2004). These observations led to the speculation that OKL38 may play an important role in regulating cell death in tumour and loss of the protein may lead to the development and/or progression of the cancer as a result of altered equilibrium between cell death and survival. Thus, revealing the regulation of OKL38 in cancers is of immense importance. As observed in these series of studies, both transcriptions and translations were involved in the down-regulation of OKL38 in

cancers. These observations include (1) low levels of OKL38 transcripts were detected in breast cancer cell lines (Huynh et al., 2001); (2) lower levels of OKL38 transcripts were detected in the kidney tumours compared to adjacent benign tissue (Fig. 4.30); (3) OKL38 transcripts were lost or down-regulated in 37% of the HCC investigated (Fig. 4.33); (4) 42 % (8 of 19) of the paired ABL/HCC, 4 liver (Fig. 4.33) and 1 kidney cancer cell lines' OKL38 transcripts levels did not correlate with the protein expression.

5.4.2.1 Hypermethylation and Loss of Heterozygosity potentially down regulate OKL38 expression in HCC

Many possible mechanisms could be involved in the down-regulation or lost of OKL38 protein in tumours. Hypermethylation at the CpG islands in the promoter region has been known to silence tumour suppressor genes (Toyooka et al., 2001). A CpG island has been identified in each of the OKL38 promoters, which may be involved in silencing of the OKL38 gene in cancers. The OKL38 gene is localized to chromosome 16q23.3, which is susceptible to LOH in a variety of tumours (Larsen et al., 2000). This region has been described as the second most active fragile site (FRA16D) in the human genome (Bednarek et al., 2001; Bednarek et al., 2000; Glover et al., 1984; Ludes-Meyers et al., 2003) and LOH of two potential tumour suppressor genes (E-cadherin and WWOX) resided in this region of HCC have been described (Niketeghad et al., 2001; Slagle et al., 1993; Park et al., 2004). It is possible from here that this mechanism may play a role in down-regulating the OKL38 transcripts in some of the HCC investigated. On the contrary, Riou *et al.* (2002) have recently investigated the expression of gene residing on chromosome 16q23.3-24.1 in HCC and have identified that OKL38 was over-expressed in HCC. In this study, only 3 of the 19 paired ABL/HCC samples showed significantly

higher levels of OKL38 mRNA in HCC tissues, while approximately 50% of these cases showed no significant differences in the transcript levels. These observations may indicate that loss of OKL38 protein occurred only in a subset of HCC.

5.4.2.2 Down-regulation of OKL38 translation by small upstream open reading frame and long structured 5'UTRs in cancers

Aberrant expression of growth-related genes by translational mechanisms makes a significant contribution to cell transformation. These mechanisms of translational control of growth factors and proto-oncogene expression have been extensively reviewed (Willis, 1999; Clemens, 2004; Dua et al., 2001; Pickering and Willis, 2005). The discrepancy between the OKL38 transcripts and protein levels was observed in HCC, which prompt the investigation into the molecular mechanisms responsible for the absence of OKL38 protein in HCC. The intact ORF of the OKL38 transcripts (exon3-8) was observed in 5 patients and 4 liver cancer cell lines suggesting that the loss of OKL38 protein in HCC was not due to mutation or deletion (Fig. 4.35). The 5'UTRs of all the three OKL38 variants (HuOKL38-1a, -2a & -2b) derived through differential promoter usage and alternative splicing have been demonstrated to suppress the translation of OKL38 protein in both TNT system and transfected cells (Fig. 4.37). These observations suggested that besides transcriptional regulation of OKL38 transcripts, post-transcriptional regulation also played an important role in regulating OKL38 protein expression.

Two thirds of the mRNAs that encodes for proto-oncogenes or factors related to cell proliferation contains atypical 5' untranslated regions which are more than 200 bases long and/or have more than one AUG codon (Kozak, 1987; Kozak, 1991). It was reported that upstream open reading frames (uORF) found in 5'UTRs of the bcl2 gene play a role

in translational suppression (Harigai et al., 1996). An uORF of 10 amino acids was detected in HuOKL38-1a transcripts, while two uORF of 21 and 30 amino acids were identified in the 5'UTRs of the HuOKL38-2a transcripts. Similarly, the 21 amino acids uORF was also present in the HuOKL38-2b transcripts. The disruption of some of these small uORFs has lead to increase translational efficiency of the OKL38 protein (Fig. 4.40), which supported the potential role of these uORFs in translational suppression in OKL38 mRNAs.

The presence of these small uORFs may be insufficient to explain the differential lost of OKL38 expression in kidney and liver tumour and, cancer cell lines despite the presence of OKL38 transcripts with intact ORF. It is possible that other mechanisms may be responsible for the differential loss of OKL38 protein in HCC. Besides the uORFs, the long structured 5'UTRs of the HuOKL38-2a and -2b transcripts may contain IRES, interacting with other ITAFs, to suppress the translation of OKL38 protein. Important genes such as MDM2, TGFβ and Bcl-2 expressed variants with long 5'UTRs differing in length, exhibiting translational repression. The translation suppression effect by long structured 5'UTRs and uORF have been reported (Willis, 1999) and reviewed in s*ection 1.2*. RNA binding protein such as HuR has also been shown to bind to the IRES present in the 5'UTR of p27, leading to down-regulation of the translation of p27 (Kullmann et al., 2002). ITAFs such as hnRNPK, La autoantigen, PTB protein and others may bind to the structured 5'UTR to down-regulate translation of OKL38 mRNA. At this junction, it is possible that similar mechanisms involving RNA-binding protein/RNA complex, together with the 5'UTRs might suppress the translation of OKL38 mRNA. These ITAFs may be over-expressed in cancer cell lines and tumours leading to differential

suppression of translation. Experiments are under way to identify possible RNA binding protein in the 5'UTRs of OKL38 transcripts.

The OKL38 antibody used in this study has been demonstrated to be specific for Western and immunohistochemical analysis (Fig. 4.31 & 4.32). Only the 38 kDa isoform of the OKL38 protein was readily detected in the ABL tissue, in contrast to the 52 kDa isoform cloned and characterized in this study. Beside internal initiation mechanisms as discussed in section 5.1.3, one does not rule out the possibility that the 38 kDa isoform might have been processed from the 52 and 62 kDa isoforms. The cancer cell lines used in this study may not have the necessary mechanism to process these exogenous larger isoforms as observed in the transfection study. In view of all these observations, the OKL38 gene may be differentially down-regulated in cancer at the transcriptional and translational levels, and probably also via post-translational modification of the pre-OKL38 protein.

5.5 OKL38 – A stress related protein

In recent years, the use of micro-array platform to study the transcriptome is becoming very common. Profiling the genes expression during certain treatments or developments may reveal the biological functions or pathways involved. OKL38 have been shown to be significantly induced or suppressed by chemicals such as hydrogen peroxide (HP), mendonine (MEN), t-butyl-hydrogen peroxide (TBH), phthalate esters and polycyclic aromatic hydrocarbon (PAH) (Chuang et al., 2002; Liu et al., 2005; Mahadevan et al., 2005). Physiological laminar shear stresses (LSS), which act as differentiative stimuli on endothelial cell, induced the expression of OKL38 transcripts (Wasserman et al., 2002). The differential expressions of OKL38 transcripts in these

treatments suggest that the gene may be involved in many physiological and cellular processes.

Phthalate esters could induce male reproductive tract abnormalities in male rats exposed gestationally (Fisher, 2004; Fisher et al., 2003). OKL38 has been reported to be one of the genes that were down-regulated in the fetal testis of the rat following *in utero* exposure to a panel of developmentally toxic phthalates (Liu et al., 2005). Liu *et al.* (2005) have also identified the pathways disrupted by these compounds, which include cholesterol transport, steroidogenesis, intracellular lipid and cholesterol homeostasis, insulin signaling, transcriptional regulation, and oxidative stress. It is likely that OKL38 may be regulated by one of these pathways and plays an important role in male reproductive tract development. The suppression of OKL38 transcripts by phthalate could be due to the down-regulation of LH-R (Liu et al., 2005), which is the common receptor for both LH and hCG. In this study, OKL38 was shown to be inducible by hCG in the rat mammary gland and ovary. In the testis, the CG may play a role in maintaining OKL38 expression and phthalate-induced down-regulation of LH-R may consequently down-regulate OKL38 expression. Coincidentally, all the genes involved in oxidative stress were also down-regulated in the phthalate exposed rat testis further supported the postulation that OKL38 is involved in oxidative stress.

OKL38 is up-regulated by oxidative stress induced by chemical compounds such as HP, MEM and TBH (Chuang et al., 2002). Similarly, physiological LSS also induced the expression of OKL38 transcripts in cultured human umbilical vein endothelial cells (Wasserman et al., 2002). LSS induced many of the genes involved with antioxidant functions such as thioredoxin reductase and Cu/Zn superoxide dismutase, which play a

role in maintaining low levels of intracellular reactive oxygen species generated by LSS. The coordinated regulation of a diverse set of antioxidant effectors and the presence of a Pyr_redox domain in OKL38 protein strongly highlights the protective role of this protein in oxidative stress induced cellular damage.

Other than oxidative stress, OKL38 is also induced by PAH (i.e. Benzo[a]pyrene) which is a common compound present in urban dust (Mahadevan et al., 2005). Exposure to PAHs can target the expression of regulatory proteins involved in activation, detoxification and DNA repair, thus altering the ability of the cells to maintain genomic integrity. OKL38 may function as a tumour suppressor to protect and prevent the transformation to cancer cell during exposure to PAHs. Evidences from these studies demonstrated that OKL38 is differentially regulated by cellular stress and it might serve an important role in re-establishing equilibrium of the cellular environment.

CHAPTER 6: CONCLUSIONS AND FUTURE STUDIES

In conclusion, the human *OKL38* gene has been cloned, sequenced and the genomic organization revealed. Several important regions that may play important roles in the regulation of *OKL38* gene are identified. In addition, three of the novel human OKL38 cDNAs are cloned and partially characterized. Several lines of evidences indicate that OKL38 is a conserved essential protein and may belong to the Pyr_redox family of proteins. The rat model was established with the cloning of three rat OKL38 cDNAs, deciphering its genomic structure and tissues distribution. The effects of hCG on OKL38 expression in the rat mammary tissue and ovary was demonstrated using this model. The positive correlation between hCG-induced OKL38 expression and hCG-protection against mammary carcinogenesis suggests that OKL38 may function as the downstream effector protein of hCG-mediated protection against mammary carcinogenesis by maintaining cellular differentiation.

The present data showed that OKL38 protein is down-regulated in a high proportion of kidney and liver tumours indicating that the protein may play a crucial role in the progression of the disease. Loss of OKL38 protein in a high proportion of primary HCCs, which positively correlate with tumour grade indicate that OKL38 might be a useful marker for refining the TNM staging system. Results from this study points towards the vital role of OKL38 in the survival of cancer cells and its loss in HCC may permit the cancer cells to escape the normal cell death mechanisms leading to uncontrolled growth. In view of these observations, induction of OKL38 translation may serve as a therapeutic alternative or adjuvant in combating HCC and be worth further investigation.

Although much was known about OKL38 in this study, the functions and regulations of this gene are far from being understood. Characterization of the putative promoters will enable us to understand the regulation of OKL38 at the transcription level, which may reflect its intrinsic functions, as several chemical agents were involved in the induction and repression of the transcripts. The rat OKL38 promoter P2 seems to be active only in kidney making it an interesting candidate for the study of kidney tissue specific promoter. Further characterization of this promoter may lead to the development of vectors useful for kidney specific targeted gene expression.

In-depth studies of the 5'UTRs and the non-inframe ORFs in the main ORF (52 kDa) is crucial to decipher the loss of the 38 kDa isoforms in both the liver and kidney tumours. Isoform-specific antibodies can be raised against the various OKL38 isoforms and exploit expression studies in tumour, including the kidney, ovarian and breast cancer. Small uORFs and non-inframe ORFs are known to lead to internal translation initiation during cellular stress such as nutrient deficiency. Since OKL38 expression is closely related to cellular stress, similar translational machinery may be involved and characterization of the 5'UTRs may reveal novel isoforms of OKL38 involved in cellular stress. Finally, the generation of an OKL38 knockout animal seems tempting, which will advance our understanding of the functions of this important gene.

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