Genomic Structure of Human OKL38 Gene and Its Differential Expression in Kidney Carcinogenesis

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We previously demonstrated the growth inhibitory property of OKL38 and its possible roles in mammary carcinogenesis. To further understand the regulation and roles of OKL38 in tumorigenesis we proceeded to clone and characterize the human OKL38 gene and three of its variants with transcripts of 1.9, 2.2, and 2.4 kb. The human OKL38 gene spans ~18 kb and contains 8 exons and 7 introns with exon size ranging from 92 to 1270 bp. RT-PCR and sequence analysis suggest that different transcripts were arrived through differential promoter usage and alternate splicing. Multiple Tissue Expression array (MTE) and Multiple Tissue Northern blot (MTN) indicated that OKL38 was ubiquitously expressed in all tissues with high expression in liver, kidney, and testis. The cancer profiling array (CPA) of paired normal/tumor cDNA showed that OKL38 mRNA was down-regulated in 70% (14 of 20) of kidney tumors. Western analysis revealed that the OKL38 protein was undetectable in 78% (7 of 9 pairs) of kidney tumor tissues. Immunohistological analysis showed that 64% (14 of 22) of kidney tumors were either lost or underexpressed OKL38 protein compared with the adjacent normal tissue. A transfection study using OKL38-eGFP recombinant construct showed that overexpression of the 52 kDa OKL38 protein in A498 cells resulted in growth inhibition and cell death. This study demonstrates the complex genomic structure of the OKL38 gene and its growth inhibitory and cytotoxic properties. Our data suggest the potential use of OKL38 in diagnosis, prognosis, and/or treatment of kidney cancer.
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 of 60 °C. The touchdown method was employed to increase the stringency of hybridization. The template was amplified by PCR under the following conditions: denaturing gel. The fmol DNA ladder with subclone P-3.4 and S-5.5 (Fig. 1A) was digested with various restriction enzymes, 5′-end of the HuOKL38-1a cDNA. To verify the genomic structure of the HuOKL38 gene, variant-specific primer, GSP2R was designed based on the 5′-end sequence of the GSP1R RACE fragment (Fig. 3). The RACE products were identified using Southern blot analysis. In all cases, the forward primer carried a HindIII and the reverse primer an EcoRI restriction site for directional cloning cDNA amplification kit (Clontech) according to the manufacturer’s direction with the following modification: The secondary PCR of the primary RACE products was performed using Advantage® Genomic polymerase (Clontech). The gene-specific primer, OKL38 probe (936 –1863 bp) was used instead of the forward primer PCR-2F (nucleotide positions 339 –1521 bp of human liver, kidney, and prostate poly(A)/H11001 were allowed to hybridized with 200 ng of total mRNA. 1.6-kb cDNA according to the manufacturer’s instructions. Briefly, the cloned cDNAs in Bluescript®SK (Stratagene) were digested with EcoRI (New England Biolabs, Beverly, MA) and purified twice by phenol/chloroform extraction. The DNA was precipitated with ethanol, washed with 70% ethanol, air-dried, and resuspended in H2O. 1 μg of the digested plasmid was used for the TNT reaction. The synthesized protein was labeled with [35S]methionine (ICN), and 5 μl of the reaction were loaded and electrophoresed on a 10% SDS-PAGE. The synthesized protein was transferred onto a nitrocellulose membrane and exposed to film. To confirm the identity of the in vitro synthesized protein, Western blot analysis was performed on the TNT products using rabbit anti-OKL38 antibody.

**Multiple Tissues Expression (MTE) Array, Northern Blot Analysis, and Cancer Profiling Array (CPA)—**The MTE and CPA were purchased from Clontech and the human Northern blots were from Invitrogen. Both the arrays and blots were hybridized with a 700-bp Small human OKL38 probe (936 –1685 nt of HuOKL38-1a) that detects all the variants. Ubiquitin (provided by the manufacturer) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; American Type Culture Collection, Manassas, VA) were used for normalizing arrays and Northern blots, respectively. Pre-hybridization of the arrays and blots were performed at 50 °C for 1 h in ExpressHyb™ (Clontech) with sheared salmon testis DNA added to a final concentration of 0.1 mg/ml. The denatured radiolabeled probe was mixed directly into the pre-hybridization solution and hybridized overnight at 50 °C. After hybridization, the arrays were washed three times at 55 °C in solution 1 (2× SSC, 0.5% SDS) for 30 min each time, repeated with solution 2 (0.2× SSC, 0.5% SDS), and a final rinse in 2× SSC at room temperature. Blots were exposed to a phosphorimager overnight. For the Northern blot analysis, washing was performed at 45 °C using solutions 1 and 2. mRNA levels were determined by densitometric scanning of autoradiographs and normalized to the level of GAPDH.

**Semi-quantitative RT-PCR of OKL38 Variants—**1 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 primer forward primer PCR-1F (nucleotide positions 1–25) and a common reverse primer PCR-1R (nucleotide positions 339–443 of HuOKL38-1a) (Table II) 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 (nucleotide positions 387–415) and reverse primer PCR-1R (Table II). The specificity of the designed primers and PCR conditions were optimized using the three cloned cDNAs, HuOKL38-1a, -2a, and -2b as templates. A pair of tubulin primers, TubF (5′-AAAGCTAGAAGCCGCGCG-3′) and TubR (5′-GACAGAG-GCAAACTGAGAC-3′), which amplify a 400-bp fragment of tubulin DNA, was used for normalization. The One Step RT-PCR was performed as follows: 50 °C for 30 min; 95 °C for 15 min; followed by 38 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 5 min. The amplified products were separated on a 2.0% agarose gel.

**Patients and Tissue Samples—**Tissue samples were obtained intraoperatively from patients with colorectal cancers and adjacent non-tumor kidney tissues during resection for kidney tumor at the Singapore General Hospital. The samples were snap frozen in liquid nitrogen and stored at –80 °C until analysis. A similar set of samples was fixed in 10% formalin and paraffin-embedded. Prior written informed consent was obtained from all patients, and the study received ethics board approval at the National Cancer Centre Singapore as well as the Singapore General Hospital.

**OKL38 Antibody and Western Blot Analysis—**Rabbit polyclonal OKL38 antibody was raised against the OKL38-specific peptide: CAVEWTDPDSGCGAG (amino acid positions 200–214). Affinity-purified anti-OKL38 antibody bound to 20×10^6 OKL38-positive cells was coupled to a 63-kDa Protein A/G agarose column (Invitrogen) according to the manufacturer’s instructions. The column was washed extensively with 0.5% SDS to remove unbound proteins. The eluted proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with the OKL38 antibody, followed by an anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase. The signal was detected using a chemiluminescent substrate (Pierce).
rified rabbit anti-human OKL38 antibody was diluted in Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBST) at a final concentration of 1 μg/ml. Western analysis was performed by incubating the blots with 1:2000 anti-OKL38 antibody or anti-OKL38 antibody preadsorbed with 50 μg antigen peptide (control for antibody specificity) overnight at 4°C. Immunoblotting was performed by incubating the blots with 1:2000 anti-OKL38 antibody or anti-OKL38 antibody preadsorbed with 50 μg antigen peptide (control for antibody specificity) overnight at 4°C. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methanol for 30 min. After two washes of TBS, the sections were preincubated with 5% skim milk in TBS containing 0.1% Tween-20 for 15 min to reduce nonspecific background staining. The sections were washed twice with TBS for 5 min and incubated overnight at 4°C with purified primary antisera against human OKL38 or section were washed twice with TBST for 5 min and incubated overnight at 4°C with purified primary antisera against human OKL38 or farered rabbit anti-human OKL38 antibody was diluted in Tris-buffered saline (TBS, 20 mM Tris, 200 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBST) at a final concentration of 1 μg/ml. Western analysis was performed by incubating the blots with 1:2000 anti-OKL38 antibody or anti-OKL38 antibody preadsorbed with 50 μg antigen peptide (control for antibody specificity) overnight at 4°C and washed three times with TBST, 15 min each. Subsequently, the blots were incubated with 1:7500 horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody for 1 h. After washing three times with TBST, 15 min each, the blots were then visualized with a chemiluminescent detection system (Amersham Biosciences) as described by the manufacturer.

**Immunohistochemical Analysis**—5-μm thick sections were cut, dewaxed in xylene, and then rehydrated as described (5). Antigen retrieval was performed by boiling the slides in 10 mM citrate buffer pH 6.0 for 20 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methanol for 30 min. After two washes of TBS, the sections were preincubated with 5% skim milk in TBS containing 0.1% Tween-20 for 15 min to reduce nonspecific background staining. The sections were washed twice with TBST for 5 min and incubated overnight at 4°C, with purified primary antisera against human OKL38 or antisera preadsorbed with 50 μg antigen peptide to serve as a control for antibody specificity. Immunohistochemistry was performed using the streptavidin-biotin peroxidase complex method according to the manufacturer’s instructions (Lab Vision, Fremont, CA) using AEC as the chromogen.

Generating OKL38-eGFP-pcDNA3.0 Construct—The HuOKL38-1a cDNA contained an open reading frame (ORF) of 477 amino acids. To clone containing an insert of ~35 kb. Both clones contained the OKL38 gene as determined by Southern blotting and sequencing. Direct cosmid sequencing of the insert ends indicate that ~100 bp were missing from the 3′-end of OKL38 exon 8. A 2.5-kb fragment containing this missing end was cloned using the GenomeWalker™ kit. The cloning map was shown in Fig. 1A. The sequence information was deposited in GenBank™ with the accession number AF334780.

Comparing the sequenced genomic clone with the sequences of the cloned cDNAs, the exon/intron junctions of the OKL38 gene were identified. 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 I). The human OKL38 gene spanned a genomic region of ~18 kb and contains 8 exons with sizes ranging from 92 to 1270 bp (Fig. 1B).

In the process of sequencing, an ~850 bp CCCT-rich region residing in the subclone BsX-1.1 (Fig. 1A), of which 300 bp could not be sequenced even after the SequenceRx enhancer solution F (Invitrogen) had been used, was identified. This region was part of the intron 2, located 1.2-kb upstream of the promoter P1 transcriptional start site (Fig. 2A). Another interesting region spanning ~500 bp was identified with the Repeat Masker program as simple CA repeats. Careful investigation showed that this region contained 11 alternate repeats of 26

| Table II | Sequences of oligonucleotides used for RT-PCR primer, cloning, primer, sequencing primer, variant-specific primer, and 5’RACE primer |
|----------|----------------------------------------------------------------------------------------------------------------------------------|
| Oligonucleotide | Sequence |
| Pext1 | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| Pext3/GSP2R | 5‘GGTCAAGGGGAGACGATACAGAGTTCC-3’ |
| GSP1R/PCR-1R | 5‘CGTAAAGGGGACTCAAGAGGAGG-3’ |
| MF | 5‘AATCTGGATCCCCAGGTTAATGCTTCTG-3’ |
| 1F | 5‘GAATCTGGCAAGGGAGG-3’ |
| 2F | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| PCR-1R | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| PCR-2F | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| 477-F | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| 477-eGFP-R | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| eGFP-F | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| eGFP-R | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| Seq1R | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| Seq2R | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| Seq2R | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| Seq3R | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| Seq4F/GW-F | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| Seq5F | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| P1F | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| P1R | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| V-1F | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
| V-1R | 5‘CTGTCGACCTGACCTTGTTCTAGA-3’ |
and 28 nucleotides, interrupted by three conserved 26-nucleotide repeats. These repeats could be arranged to form four long repeats with repeats 2 and 3, each 160 bp, aligned perfectly, while 1 and 4 are partly conserved. This region contained 70% cytosine and adenine, and was localized 140-bp upstream of exon 2 (Fig. 2A). The functional significance of these unique regions is unknown at the present time.

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), which was prone to loss of heterozygosity (LOH) in a variety of tumors (7–10). Comparing our sequenced OKL38 gene with the published human genome using ClustalW delineated two different regions. A 20-bp random deletion in the CA repeats region and a 550-bp longer CCCT-rich region were observed in our sequenced OKL38 gene (Fig. 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. A PCR product of ~2.5 kb was synthesized instead of a 1.95-kb fragment, suggesting that the 850 bp CCCT-rich region in our clone is not an artifact of cloning. These differences suggest regions of DNA instability.

**Sequence Analysis of the Two Putative Promoter Regions**

Two putative promoters were identified while comparing the 5′-RACE fragments with the cloned OKL38 genomic sequence (Fig. 2A). Analyzing the proximal 300-bp region upstream of promoter P1 using the MatInspector program identified several potential sites for the Ikaros factor (IK2), homeodomain factor
Cloning and Sequence Analysis of HuOKL38-1a, -2a, and -2b cDNAs—Sequence comparison of the GSP1R and GSP2R RACE products, and the cloned genomic sequence showed that the first 22 bp (positions +1 to +22 at promoter P1) did not belong to exon 3 and was unique to the HuOKL38-1a variant. To clone the full-length of the above cDNA, forward primer 1F (Table I) that resided mostly within the 22-bp unique region of the GSP1 RACE fragment and a common reverse primer, 1R (Table II) were designed for RT-PCR cloning of the HuOKL38-1a transcript. For cloning of the HuOKL38-2a and -2b transcripts, forward primer 2F residing in exon 1 was used instead of 1F (Fig. 4A). Three PCR products of ~1.9, 2.2, and 2.4 kb were cloned and five clones of each cDNA were sequenced, which enabled us to establish three novel human OKL38 cDNAs of 1930, 2240, and 2400 bp, respectively. The 1.9-kb cDNA was annotated as HuOKL38-1a (GenBankTM accession no. AY258068), the 2.2-kb cDNA as HuOKL38-2a (GenBankTM accession no. AY258067), and the 2.4-kb cDNA as HuOKL38-2b (GenBankTM accession no. AY258066).

The 5'-RACE analysis, one-step RT-PCR, and Southern blot analysis indicated the presence of another minor transcript (HuOKL38-2c), which contained exons 1, 3, 4, 5, and 6. This transcript most likely also contains exons 7 and 8. However, due to the low expression of this transcript in the liver and kidney RNA used, we have not managed to clone the full-length sequence of this cDNA. The cloning and one-step RT-PCR results confirmed the existence of OKL38 variants.

Sequence analysis indicated that differential promoter usage and alternative splicing at the 5’-region of OKL38 gene were responsible for the variants. The HuOKL38-1a transcript was arrived as a result of promoter P1 usage, while -2a, -2b, and -2c expression might be regulated by promoter P2. The HuOKL38-2a, -2b, and -2c were derived by alternative splicing of exons 2 and 3, exon 3 and exon 2, respectively (Fig. 4A). The HuOKL38-1a and -2a variants harbored 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. 4B) and a predicted pI 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. *In vitro* transcription and translation study using the three cDNAs, namely HuOKL38-1a, -2a, and -2b resulted in the synthesis of ~52 and 61 kDa proteins (Fig. 5A), and their identity was confirmed by Western blot analysis using anti-OKL38 antibodies (Fig. 5B). The previously cloned OKL38 cDNA (GenBankTM 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 internal translation start codon (ATG) might exist (Fig. 5B).

Similar to the previously cloned human OKL38 protein, no putative signal peptide was found in all the three human OKL38 protein isoforms using the publicly available SignalP program (6). Computational analysis of the predicted amino acid sequence using Pfam CDS-Conserved Domain Search
NCBI detected a putative domain belonging to that of the pyridine nucleotide-disulfide oxidoreductase (Pyr-redox) in all the three OKL38 isoforms, and the domain was encoded by exon 8.

Expression of OKL38 in Various Human Tissues—We have previously shown the differential expression of OKL38 in rat tissues, but not in the human (4). MTE array was adopted to determine the global tissue distribution of human OKL38 (Fig. 6A). The MTE array showed that OKL38 was ubiquitously expressed in all the tissues investigated, with high expression detected in the kidney (7A), skeletal muscle (7B), testis (8F), liver (9A), adrenal gland (9C), and fetal liver (11D) (Fig. 6B).

Northern blot analysis showed that transcripts of ~2.0–2.4 kb were ubiquitously expressed in all 15 tissues investigated (Fig. 6C and E). 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. 6C).

Preliminary Expression Study of Human OKL38 Variants in Kidney, Liver, and Ovarian Tissue and Cell Line via Semi-quantitative One Step RT-PCR—Because the OKL38 has been implicated in regulating cell growth, differentiation (4), and tumorigenesis, the distribution of human OKL38 variants in various human normal/tumor tissues and cancerous cell lines were determined via One Step RT-PCR using variant-specific primers (Fig. 4A). Four RT-PCR products of 443, 373, 533, and 482 bp corresponding to the HuOKL38-1a, -2a, -2b, and -2c transcripts, respectively, were amplified (Fig. 7, A–D).

Western blot analysis (Fig. 7C) was performed using exons 4 and 5 as probes, and sequencing verifies the identity of the amplified products. All the four variants were expressed in all tissues investigated. Liver appeared to express the highest level of all the transcripts compared with kidney and ovary. In general, the normal kidney, liver, and ovarian tissues seemed to express higher levels of OKL38 transcripts compared with the adjacent tumor tissues. All the cancer cell lines showed lower expression of OKL38 transcripts, except the A498 kidney cancer cell line. 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.

Expression of OKL38 Protein in Paired Normal/Tumor Kidney—To determine the expression of OKL38 mRNA transcripts in paired normal/tumor kidney, CPA was used. The blot was probed with 700-bp human OKL38 SmaI fragment, which could detect all OKL38 variants. Fig. 8 shows that 70% (14/20) of the kidney tumors expressed lower levels of OKL38 transcripts compared with the adjacent normal tissues. The OKL38 mRNA transcripts were generally down-regulated in the kidney tumors, and we proceeded to investigate the possibility of reduction of OKL38 protein in the tumors. Western blot analysis was performed using rabbit anti-human OKL38 antibody on nine paired normal/tumor kidney tissues. The results showed that OKL38 protein was undetected in 78% (7/9) of the tumor kidney tissues compared with their adjacent normal tissues. The endogenous 61, 52, and 38 kDa isoforms of human OKL38 protein were also detected by Western blot
analysis, which is in agreement with the TNT results. Low levels of OKL38 protein were detected in the A498 kidney cancer cell line, which is contrary to the RT-PCR results (Fig. 7). The 61 and 38 kDa proteins were differentially down-regulated in the kidney tumor tissues (Fig. 9A). When the OKL38 antibody was preadsorbed with 50× excess OKL38 antigen peptide, immunoreactive signal was drastically reduced or lost (Fig. 9, C and G).

Immunohistochemical study was performed on 22 paired normal/tumor kidney tissues using the same OKL38 antibodies. OKL38 protein was undetected in 64% (14/22) of the kidney tumors examined, which supported the observation in Western blot analysis. All normal kidney epithelial cells were positive for OKL38 protein, except the Bowman capsules (arrow) and the stroma (Fig. 9D), while no immunostaining of the tumor cell was observed (Fig. 9E).

Overexpression of OKL38 Protein Was Lethal to A498 Cells—Because the evidence suggests that OKL38 functions as a tumor suppressor protein, we proceeded to investigate its growth inhibitory function. Initially, the full-length cDNA HuOKL38-1a was cloned into pcDNA3.0 and transfected into A498 cells to generate stable cell lines. However, the transfected cells failed to propagate and eventually disintegrated (data not shown). Thus, we could not characterize these transfected cells. To study the function of the OKL38 protein, we generated an OKL38-eGFP-pcDNA3.0 construct, which fused the reporter gene eGFP to the C-terminal of OKL38 protein. Our preliminary transfection study shows that OKL38-eGFP recombinant protein formed an aggregation in the A498 cells as early as 24 h post-transfection (Fig. 10). None of the cells expressing the recombinant protein survived 96 h post-transfection compared with the eGFP-positive control cells. The results suggest that over-expression of OKL38 protein was lethal to A498 cells.

Our previous study showed that OKL38 was involved in tumorigenesis of the breast (4). To understand the functions and regulation of OKL38 gene, we cloned, sequenced, and characterized the human OKL38 gene. The human OKL38 gene reported in this study was cloned and sequenced before the human genome sequence was published. Our sequence data showed two regions of mismatch, and we have verified that our clone contained a 550-bp longer CCCT repeat compared with the published human sequence. We presume that the 300-bp unsequenced region of these repeats also contains CCCT repeats. The functional importance of the CCCT repeats in the human OKL38 is unknown at the present time. However, the instability of CCCT repeats is highlighted in several reports, suggesting that the different repeat lengths are correlated with DNA instability (12, 13). Fiskerstrand et al. (13) have also shown that the specific region in intron 2 of the rat prepro-tachykinin-A gene contained 128 bp of the CCCT tandem repeat domain. This domain is able to support reporter gene expression in mouse embryonic stem (ES) cells that have been induced to differentiate, but not in undifferentiated ES cells, suggesting that this region is a highly restrictive enhancer and may be associated with differentiation (13). Similarly, CCCT repeats located in intron 2 of the OKL38 gene may associate with differentiation, as we have postulated in our previous report (4). Since the CCCT repeats are located 1.2-kb upstream of the promoter P1 initiation site, it is likely that this region functions as an enhancer for the P1 promoter (Fig. 2A). Experiments are underway to examine this possibility.

An ~500-bp dinucleotide CA repeat is localized to intron 1 of the OKL38 gene (Fig. 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 (14). Human genes with poly-morphic intronic CA repeats include the human interferon-γ gene (15), the epidermal growth factor receptor gene (16), and the cystic fibrosis cellular stress response (CFTR) gene (17). A ~20-bp random deletion in the CA repeat region is found in our sequenced OKL38 gene suggesting polymorphism in this region. Hui et al. (18) reported a recent discovery that dinucleotide CA repeats of variable length found in intron 13 of the human eNOS gene promote 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, it is possible that the polymorphic intronic CA repeats in intron 1 of human OKL38 gene may serve as docking sites for the hnRNP L factor to bind and regulate differential splicing of this gene. This is also supported by the fact that alternative splicing occurs at the 5′-end of the OKL38 gene, where the CA repeats are present.

The genomic organization of the human OKL38 gene and its splicing pattern have been established in this study. The splicing pattern was very similar to that of the human and mouse thioredoxin reductase 1 (TXNRD1) gene (19). 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 compared with the OKL38 gene, which consists of 8 exons. The similarity in splicing pattern of the TXNRD1 and OKL38 genes suggests that the two genes may be regulated in a similar fashion, and they may belong to the same family. This postulation is further supported by the presence of a pyridine nucleotide-disulfide oxidoreductase (Pyr-redox) domain found in both genes.

Regulation of the OKL38 gene at promoter P1 could involve
Fig. 6. Tissue distribution of human OKL38 transcripts. A, MTE array was probed with a 700-bp human OKL38 SmaI-radiolabeled probe as described under “Experimental Procedures.” The identity of each cDNA dot is represented in the grid (B). C, MTN blot analysis was performed using the same probe, which recognizes all the OKL38 variants. The tissues are: 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; Pr, prostate. The amount in each lane is normalized with GAPDH (D). The relative level of OKL38 expression after GAPDH normalization is shown in E.
several factors. 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 but also of genes with a more tissue-restricted pattern of expression (20). Putative Initiator (Inr) with the consensus Py-Py-A1-N-T/A-Py-Py where A1 is the transcriptional start site (21) is identified in promoter P1 suggesting that this core sequence is likely responsible for driving transcription at this site. Additional support for this conclusion is the presence of a putative DPE that is found in about 20% of the TATA-less promoters containing Inr (21). Transcription factor binding sites identified within promoter P1 are slightly different than those observed in the promoter P2, suggesting that they may be differentially regulated. Interestingly, no typical promoter elements are identified in the putative promoter P2 suggesting that other novel sequences may regulate this promoter. Further characterization of these promoters is needed to establish their regulatory functions.

In this study, we have cloned the full-length of three OKL38 variants. The HuOKL38-1a and HuOKL38-2a mRNA isoforms, which are expressed from different promoters; therefore, they possess different 5'-UTR (Fig. 4). These two mRNA isoforms encode for the same ORF (477 amino acids) verified by TnT analysis (Fig. 5). The lethal effect of this OKL38 protein isoform suggests that this protein has to be regulated in a precise and controlled manner. Therefore, the use of 2 different pro-
motors to produce 2 mRNA isoforms coding for the same protein is far from redundant. The intrinsic regulation of OKL38 protein may be regulated at the translational level with possible regulatory elements in the 5'-UTR.

Sequence analysis of the cloned OKL38 variants show that all the 1st AUG on the transcripts conform to the Kozak sequence (22), except HuOKL38-2b and -2c. We have noticed in this study and the previous study (4) that there are several OKL38 protein isoforms (61, 52, and a doublet 38 kDa) detectable by Western blot analysis (Figs. 5 and 9). The 61-kDa OKL38 isoform may have been expressed by HuOKL38-2b or -2c transcripts, while the 52 kDa protein is most probably derived from HuOKL38-1a or 2a transcripts. The 38-kDa doublet may have derived from usage of an internal translation start site. An internal AUG translation initiation codon at 139 (nucleotide positions 574-576 of HuOKL38-1a), which conforms to the Kozak sequence, was identified and verified by TyT. However, the post-translational process could also be responsible for OKL38 protein isoforms observed in the present study. The various isoforms of OKL38 may serve different functions in cells. We are currently determining the functions of these isoforms.

The Pry-redox family of proteins consists of both class I and class II oxidoreductases, as well as 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 (23). 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 (24–26). 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 signaling, and other processes (27, 28). The cell death-associated function of OKL38 demonstrated in this study and the presence of a pyr-redox domain in OKL38 protein suggest that the gene may belong to the larger family of pyridine nucleotide-disulfide oxidoreductases and may share similar functions to this family of proteins.

In our previous study, we have shown that OKL38 mRNA transcripts are down-regulated in several breast cancer cell lines. In this study, we have investigated the expression of OKL38 transcripts and protein in kidney cancer. Our results show that OKL38 is down-regulated in the kidney tumor at both the mRNA and protein levels. The down-regulation of OKL38 expression may possibly be caused by point mutation in the ORF or region of regulatory elements. The silencing of OKL38 expression could also be due to hypermethylation at the CpG islands in the promoter region, which have been known to silence tumor suppressor genes (29). The OKL38 gene is localized to chromosome 16q23.3, which is susceptible to LOH in a variety of tumors (7–10). This mechanism may also be the cause of OKL38 down-regulation. The results suggest that the OKL38 protein may play a growth inhibitory or differentiation role in normal kidney epithelial cells.

The OKL38 cDNA HuOKL38-1a cloned in this study is longer and encoded a longer protein of 52 kDa compared with the previously cloned cDNA (4). Overexpression of this protein causes growth inhibition and also is lethal to the A498 cells. The observation is similar to that of the p53 protein, as abnor-
mal regulation of this gene causes the cells to become cancerous or lethal to its own survival (30). These results supported our previous postulation that OKL38 functions as a tumor suppressor gene and that this protein may regulate cell homeostasis through cell death. We are currently investigating the mechanisms of OKL38-induced cell death.

In conclusion, we have cloned, sequenced, and revealed the genomic organization of the human OKL38 gene. Several important regions that may play important roles in the regulation of the OKL38 gene are identified. In addition, we have cloned three of the novel OKL38 cDNAs and partially characterized them. Our present data show that OKL38 protein is down-regulated in a high proportion of kidney tumors, suggesting its potential use as a diagnostic or prognostic markers for kidney cancer. We postulate that OKL38 regulates the differentiation and proliferation of normal cells through the regulation of cell death. Loss of OKL38 protein disturbs the balance between cell growth, differentiation, and cell death in normal tissue, resulting in uncontrolled growth and formation of tumors. The present study provides the basic foundation for future studies on the role of OKL38 in differentiation, growth, and tumorigenesis.

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