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Structure and Promoter Characterization of Aldo-Keto Reductase Family 1 B10 Gene

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Abstract

Aldo-keto reductase family 1 member B10 (*AKR1B10*) is overexpressed in human hepatocellular carcinoma, lung squamous carcinoma, and lung adenocarcinoma in smokers. Our recent studies have showed that *AKR1B10* plays a critical role in the growth and proliferation of cancer cells by detoxifying reactive carbonyls and regulating fatty acid biosynthesis. However, little is known about the regulatory mechanisms of *AKR1B10* expression. In this study, we determined the structure of *AKR1B10* gene and characterized its promoter. The results demonstrated that *AKR1B10* consists of 10 exons and 9 introns, stretching approximately 13.8 kb. A 5'-RACE study determined the transcriptional start site of *AKR1B10* at 320 bp upstream of the ATG translational start codon. A TATA-like (TAATAA) and a CAAT box are present from -145 to -140 bp and -193 to -190 bp upstream of the transcriptional start site, respectively. Motif analysis recognized multiple putative oncogenic and tumor suppressor protein binding sites in the *AKR1B10* promoter, including c-Ets-1, C/EBP, AP-1, and p53, but osmolytic response elements were not found. A -4,091 bp of the 5'-flanking fragment of the *AKR1B10* gene was capable of driving GFP and luciferase reporter gene expression in HepG2 cells derived from human hepatocellular carcinoma; progressive 5'-deletions revealed that a -255 bp fragment possesses full promoter activity.

Keywords

AKR1B10; Aldose reductase-like-1; ARL-1; promoter; and gene structure

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1. Introduction

Aldo-keto reductase family 1 member B10 (AKR1B10), also known as aldose reductase-like-1 (ARL-1), is a novel protein identified from human hepatocellular carcinoma (HCC) (Cao et al., 1998; Hyndman and Flynn, 1998). This protein belongs to the aldo-keto reductase superfamily, a protein cluster implicated in osmolytic regulation, carbonyl detoxification, cellular carcinogenesis, and cancer therapeutics (Ko et al., 1997; Lee et al., 2001; Crosas et al., 2003; Hyndman et al., 2003; Jin et al., 2006). *AKR1B10* is primarily expressed in the adrenal gland, colon and small intestine with low levels in the liver, thymus, prostate and testis (Cao et al., 1998; Hyndman and Flynn, 1998), but overexpressed in 54% of HCC, 84.4% of lung squamous cell carcinoma, and 29.2% of lung adenocarcinoma in smokers, potentially serving as a diagnostic and/or prognostic marker (Cao et al., 1998; Fukumoto et al., 2005; Penning, 2005).

AKR1B10 is a monomeric enzyme that efficiently catalyzes the reduction of carbonyls with NADPH as a co-enzyme (Cao et al., 1998; Gallego et al., 2007). This reaction converts highly reactive aldehydic and ketonic groups into hydroxy groups, protecting cells against carbonyl toxicity. Our recent studies have demonstrated that targeting expression of *AKR1B10* gene in colorectal cancer cells (HCT-8) and transformed human embryonic kidney cells (293T) significantly affected the cell proliferation, clonogenic growth, and susceptibility to reactive carbonyls, such as acrolein and crotonaldehyde (Yan et al., 2007; Zu et al., 2007). AKR1B10 also shows strong enzymatic activity toward all-*trans*-retinal, 9-*cis*-retinal, and 13-*cis*-retinal, converting them to retinols (Crosas et al., 2003). This reaction may diminish cellular retinoic acid, a signaling molecule regulating cell proliferation and differentiation (Dragnev et al., 2000; Penning, 2005).

In *H-ras* transformed human mammary epithelial cells and colorectal cancer cells (HCT-8), AKR1B10 affects *de novo* synthesis of fatty acids by mediating acetyl-CoA carboxylase- α (ACCA) degradation through the ubiquitination-proteasome pathway (Ma et al., 2008). ACCA is a rate-limiting enzyme in long chain fatty acid synthesis, catalyzing malonyl-CoA formation by ATP-dependent carboxylation of acetyl-CoA (Witters et al., 1994; Zang et al., 2005). Long chain fatty acids are the building blocks of biomembranes and the precursors of lipid second messengers, thus being critical to cell proliferation, migration, signal transduction, and intracellular trafficking (Manes et al., 1999; Simons and Toomre, 2000; Rouquette-Jazdanian et al., 2002; Swinnen et al., 2004; Swinnen et al., 2006). In breast cancer cells, ACCA knockdown induced by small interfering RNA resulted in cell cycle arrest and apoptosis (Chajes et al., 2006). Therefore, AKR1B10 may play a critical role in the development and progression of cancer through detoxifying intracellular cytotoxic carbonyls, mediating retinal metabolism, and regulating fatty acid biosynthesis. However, the regulatory mechanisms of *AKR1B10* expression remain unclear. This study determined *AKR1B10* gene structure and characterized its promoter, aiding in the understanding of its expression regulation.

2. Materials and Methods

2.1. Cell culture

HepG2 (human hepatocellular carcinoma) cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in RMPI 1640 medium (Hyclone, UT) containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C, 5% CO₂.

2.2. 5'-RACE (rapid amplification of cDNA ends)

Total RNA was extracted using Trizol[®] reagent (Invitrogen, CA) from HepG2 cells and hepatocellular carcinoma tissues (collected via Co-operative Human Tissue Network sponsored by National Cancer Institute) as previously described (Cao et al., 2005). Contaminated genomic DNA was removed by DNase I at 37°C for 30 min in 200 µl mixture containing 20 µg total RNA, 20 U RNase-free DNase I, and 50 U RNase inhibitor (Invitrogen, CA), followed by incubation with proteinase K (2.0 mg/ml in 0.5% SDS) and purification with phenol/chloroform extraction. The first strand cDNA was synthesized at 42°C for 1 hour in 40 µl mixture consisting of 10 pmol gene specific primer (refer to Figure 2), 200 µM dNTP, 10 µg total RNA, 8 µl 5 × reaction buffer, 4 µl 0.1 M DTT, and 400 U Superscript II[®] retrotranscriptase (RTase). The RNA strand was removed by RNase H (0.5 U/µl, final) at 55°C for 10 min, and cDNA was purified with GLASSMAX DNA isolation spin cartridge. Poly (dC) tail was added by terminal dNTP transferase (TdT, Invitrogen, CA) at 37°C for 10 min in reaction mixture composed of 10 U TdT and 200 µM dCTP, followed by heat inactivation at 65°C for 15 min. The dC-tailed cDNA was amplified in 50 µl PCR mix containing 5 µl dC-tailed cDNA, 10 pmol gene specific primer, 10 pmol anchor primer (Invitrogen, CA), 200 µM dNTP, and 2.5 U Taq DNA polymerase at cycling conditions: 94°C/30 sec, 45°C/30 sec, and 72°C/45 sec for 35 cycles. PCR products were examined in 1.0% agarose gel and subcloned using TA Cloning[®] kit (Invitrogen, CA). Plasmid DNAs were purified from 5 colonies each and submitted for sequencing analysis in the Center for Genetic Medicine at Northern University, Chicago, IL.

2.3. AKR1B10 gene structure

AKR1B10 gene structure was determined by blasting its cDNA (Cao et al., 1998) with genomic sequence in human genome database of National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>), following the GT-AG rule (Wieben, 2003).

2.4. Cloning of AKR1B10 promoter and construction of reporter gene vectors

A 4,411 bp fragment, containing 320 bp of 5'-untranslated region and 4,091 bp of 5'-flanking region, was amplified by PCR using human genomic DNA extracted from HepG2 cells as template and cloned into pGlow-TOPO vector (Invitrogen, CA), creating pAKR1B10/Glow-TOPO vector that expresses green fluorescent protein (GFP). Thereafter, *AKR1B10* promoter DNA fragment was released by *KpnI* and *BglII* and inserted into pGL4 (Promega, WI), constructing luciferase reporter vectors. Progressive 5'-end deletions of *AKR1B10* promoter were performed by enzyme digestion, where available, or PCR amplification and subcloning. Table I summarizes the primers used in the construction of 5'-deletion vectors. Constructs were verified by DNA sequencing.

2.5. Transient transfection and luciferase activity assays

HepG2 cells (5×10^4 /well) were plated in 24-well plates and incubated overnight. *AKR1B10* promoter-GFP (1.0 µg) or promoter-luciferase vectors (0.9 µg) plus CMV-driven β-galactosidase plasmid (0.1 µg, as an internal control) were transfected into HepG2 cells using ExGen 500 reagent (Fermentas, MD) at 3.3 µl ExGen 500 plus 1 µg plasmid DNA per well. The Glow-TOPO or pGL4 empty vector was used as a negative control. Forty-eight hours after transfection, cells were examined under fluorescent microscopy (for GFP) or harvested by scrapers, rinsed with PBS and lysed in 30 µl passive lysis buffer (Promega, WI). Luciferase activity was determined using luciferase reporter assay system (Promega, WI); and β-galactosidase activity was measured at 37°C for 5–10 min in 300 µl mixture containing 15 µl cell lysates and 150 µl 2 × β-galactosidase assay buffer (120 mM Na₂HPO₄ (pH 7.5), 2 mM MgCl₂, 100 mM β-mercaptoethanol, and 1.33 mg/ml *ortho*-nitrophenyl-β-galactoside). Reactions were stopped by the addition of 500 µl of 1 M Na₂CO₃; and enzymatic products,

chromophore *o*-nitrophenol, were detected at OD₄₂₀. Promoter activity was calibrated by dividing luciferase activity over β -galactosidase activity and expressed as fold over the pGL4 vector control.

2.6. Statistical analysis

Statistical analysis was performed using Student's *t* test with INSTAT statistical analysis package (Graph Pad Software, CA). Significance was defined as $p < 0.05$.

3. Results

3.1. AKR1B10 gene structure

The structure of *AKR1B10* gene was determined using cDNA sequence (Cao et al., 1998) and NCBI human genome database. As shown in Figure 1, *AKR1B10* consists of 10 exons and 9 introns. Exons range in length from 78–385 bp; and the first in-frame ATG codon is located in exon 1. Exon 10 encodes the -COOH terminus of AKR1B10 protein and contains a translational stop codon TGA and a 3'-untranslated region. A polyadenylation signal, AATAAA, is located 297 bp downstream of the TGA codon. *AKR1B10* introns have typical donor and acceptor sites, following the GT-AG rule; the whole *AKR1B10* gene spans approximately 13.8 kb. These *in silico* data are consistent with the variant with 1590 bp in the NCBI AceView web site (<http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/>) (Thierry-Mieg and Thierry-Mieg, 2006). *AKR1B10* gene structure is highly identical to *AKR1B1*, which stretches about 16.8 kb; exons 2–9 of these two genes are exactly same in length (Figure 1), indicating their evolutionary relationship. *AKR1B10* gene is located at 7q33 while *AKR1B1* is at 7q35 (NCBI database).

3.2. Transcriptional start site and regulatory motifs of AKR1B10 promoter

To characterize the *AKR1B10* promoter, we first determined its transcriptional start site. 5'-Ends of *AKR1B10* transcripts were amplified by 5'-RACE to determine the DNA sequences. As shown in Figure 2, the transcriptional start site of *AKR1B10* was mapped 320 bp upstream of the ATG translational start codon. The *AKR1B10* mRNA from HepG2 cells and HCC tissues exhibited a same transcription site.

A motif analysis of the *AKR1B10* promoter was performed using Motif Search Software (Bioinformatics Center, JP; <http://motif.genome.jp/>). As shown in Figure 3, putative TATA-like (TAATAATT) and CAAT (CAATGA) boxes are located at -145 to -140 bp and -193 to -190 bp upstream of the transcriptional start site, respectively. Other important putative promoter elements include a C/EBP binding site at -168 to -155 bp, an AP-1 element at -222 to -212 bp, a SP1 site at -333 to -324 bp, a CHOP-C/EBP- α site at -385 to -373 bp, a p53 binding motif at -666 to -657 bp, and an AP4 site at -793 to -784 bp. In addition, a putative c-Ets-1 binding site overlaps with a C/EBP consensus element at -99 to -80 bp. Osmolytic response elements were not found in this *AKR1B10* promoter region.

Interestingly, a complex microsatellite (TTCC)₂(TCCC)₅(TCCT)₇ (-604 to -549 bp) is present in a C and T enriched repeat region (-656 to -502 bp) of the *AKR1B10* promoter. Preliminary studies (unpublished data) suggested that this highly polymorphic complex microsatellite may associate with the prognosis of colorectal cancer.

3.3. Promoter activity of 5'-flanking fragment of AKR1B10 gene

To characterize the promoter activity, a 4,411 bp fragment, containing 320 bp of 5'-untranslated region and 4,091 bp of the 5'-flanking fragment of the *AKR1B10* gene, was subcloned into pGlow-TOPO vector to drive GFP expression. Figure 4 shows GFP expression in HepG2 cells, driven by this 4,411 bp fragment. To quantitatively measure the promoter activity, this 5'-

fragment and its progressive 5'-deletions were used to transiently drive luciferase reporter gene expression in HepG2 cells. The CMV-driven β -galactosidase vector was co-transfected as an internal control. Data indicated that a -255 bp fragment, containing most of the putative motifs identified, showed full promoter activity in the HepG2 cells; a further deletion to -21 bp completely abolished the promoter activity, suggesting -255 to -21 bp of the 5'-flanking region of the *AKR1B10* gene contains essential promoter components required for basal promoter activity (Figure 5). A significant decrease of promoter activity was observed in the -1,061 bp promoter-luciferase construct, indicating the presence of a repressor element between -1,061 and -804 bp.

4. Discussion

AKR1B10 is overexpressed in human cancers and promotes the growth and proliferation of cancer cells, thus being critical to the development and progression of cancer (Cao et al., 1998; Fukumoto et al., 2005; Yan et al., 2007; Zu et al., 2007; Ma et al., 2008). This study characterized the gene structure and promoter region of the *AKR1B10* and identified a novel complex microsatellite in its promoter region.

AKR1B10 possesses more than 70% amino acid sequence identity to and overlapping substrate specificity with *AKR1B1* (Cao et al., 1998). This study revealed a highly similar gene structure and very close chromosome loci between these two genes, suggesting their evolutionary relationship. However, the 5'-flanking region of the *AKR1B10* gene, which showed promoter activity in our studies, does not appear to have common features with *AKR1B1* (Wang et al., 1993). Unlike *AKR1B1*, the *AKR1B10* promoter does not have a typical TATA box although a TAATAA sequence is present from -145 to -140 bp upstream of the transcription start point. The lack of a typical TATA consensus sequence is a feature of the genes with a housekeeping function, such as *N-ras* and transforming growth factor- α (Hagiwara et al., 1991). In addition, osmotic regulatory elements, which regulate *AKR1B1* response to environmental osmolytic changes (Ko et al., 1997), were not found in the *AKR1B10* promoter, reflecting its distinct expression pattern (Cao et al., 1998). However, some important promoter elements were indeed recognized in the *AKR1B10* promoter, including: (a) an AP-1 consensus element, which responds to transcription factors composed of heterodimeric proteins from c-Fos, c-Jun, ATF, and JPD families (Troen et al., 2004; Bossis et al., 2005); (b) consensus motifs for C/EBP and CHOP-C/EBP- α , the transcription factors involved in a broad cellular responses, such as proliferation, growth and differentiation (Koshiishi et al., 2008); and (c) a c-Ets-1 proto-oncogenic transcription factor and a p53 tumor suppressor protein binding site (Muller-Tiemann et al., 1998; Yamaguchi et al., 2007). Considering the upregulation of the *AKR1B10* gene in some types of cancer tissues, one might postulate that the expression of *AKR1B10* is regulated directly by these oncogenic and tumor suppressor products.

A complex microsatellite (TTCC)₂(TCCC)₅(TCCT)₇, was found in a C and T enriched repetitive region (-656 to -502 bp) in the *AKR1B10* promoter. We have expanded the studies on its polymorphism in colorectal cancer, normal adjacent tissues, and normal donors. Preliminary data (unpublished) indicates that the polymorphism of this microsatellite may associate with colorectal cancer prognosis, being a potential biomarker.

In summary, we characterized the *AKR1B10* gene structure and promoter and recognized a number of important putative promoter elements that warrant further evaluations to fully understand the regulation of the *AKR1B10* expression. The similarities of the *AKR1B10* and *AKR1B1* gene structures suggest their evolutionary relationship, but the distinct promoter elements and tissue distributions of *AKR1B10* and *AKR1B1* transcripts suggest their difference in biological function that merits further investigation.

5. Conclusion

AKR1B10 gene, composed of 10 exons and 9 introns, is structurally identical to *AKR1B1*, but demonstrates distinct promoter components. The putative oncogenic and tumor suppressor protein binding sites in the *AKR1B10* promoter may regulate its tumor-specific expression, warranting a further study. A complex microsatellite is present in the *AKR1B10* promoter region, which may be a potential biomarker. A –255 bp of 5'-flanking fragment contains most of important promoter elements and possesses the full promoter activity, representing a targeting region for further elucidation of *AKR1B10* expression regulation in the cancer cells.

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Figure 1. Gene structures of *AKR1B10* and *AKR1B1*
A) Scaled representation of *AKR1B10* and *AKR1B1* genes. Open boxes indicate the exons numbered on the top. B) Nucleotide sequences in intron-exon borders. *AKR1B10* and *AKR1B1* genes both contain ten exons and nine introns following a typical GT/AG (bolded) rule at donor and acceptor sites.

Figure 2. Transcriptional start site of *AKR1B10* gene

The 5'-ends of *AKR1B10* transcripts were determined by 5'-RACE as described in the Section 2.2. PCR products (*upper*) and DNA sequenced (*lower*) are exhibited. The transcriptional start site is marked as +1. ATG translational start codon is bolded and primers used in 5'-RACE are underlined and orientated by arrows. RACE, rapid amplification of cDNA ends; H, HepG2 cells; T, hepatocellular carcinoma tissues; and M, DNA molecular marker.

-816 GCTGACTACC AACTAGTTAC AGCTGACTCC AATCAGGTGC GGATTTGACC ATCTCTGCAC
AP-4

-756 TGAACCTGCA ATTGCAACTT TTAA⁺AATTGA AACGCTGCTT CTCACCTCTC CTCCCTAGAC

-696 TCTATTTTAC AGAGGAAACC ATCACAGGGA AAGCAAGCCC TTGTTTTTTC TTCCTTCTTT
p53

-636 GCTTTGCTTT CTTTCTCTCC TTCCTTCTTT CTTTCCTTCC TCCTTCCCTC CCTCCCTCCC

-576 TCCTTCCTTC CTTCCCTCCT TCCTTCCTTC TTTCTTTTCT TCCTTCCTGC CTTCCCTTCT

-516 TTCTTTCTTT TTTTGTAGAC AGGGTCTCAC TGTCTGTCGC CCAGGCTGGA GTGCAGTGGC

-456 ATGATCTAGG CTCATTGCAA CCTCCGCCTC CCAGGTTCAA GTGATTCTCC TTTCTCAGCC

-396 TCCCAAGTAG CTGGGATTGC AGATACGTGC CCCACGCCC GGCTAATTTT TGTATTTTTA
CHOP-C/EBP-α

-336 GTAGAGGCAG GGTTTCACTA TGTTGGCCAG ACTTCTTTCC CTCCTCCTC CAACTCTTCT
SP1

-276 TTTCTTCCTT GCTGTCTCCC TCCCTACCTT CCAACTTTTG GCTGTGTTGA ATTTGTTGAC
AP-1

-216 TCATCCTGAA CAAGCAGAAA CTCCAATGAT ACTGTCACCT AAGAGAGAC TTTGACAAG
CAAT box C/EBP

-156 GTTGCCATGT TTAATAATTC ATTACAGAA TCCTCAATCT CACAAAAGCC TGGAATGGAT
c-Ets-1 TATA box

-96 TTCCCTGTTTG GTTATGGCAG GGAAGCTACT GTGGCCAGGC TCCAACCTCT CCCACTGTAG
C/EBP +1

-36 ATAGCAGCAG CTTTCTTAAG CAGCCTGTGG CCGGGC**A**CAG TAGCTCACAC CTGTAATCCC +24

AGCACTTTGG AAGGCCGAGG TGGGCGGATC ACCTGAGCTC AGGAGTTTGA GACCAGCCTG +84

..... CCAACC**ATGG** CCACGTTTGT +334

Figure 3. Putative motifs of the 5'-flanking region of *AKR1B10* gene

Important putative motifs are identified and underlined as indicated. The transcriptional start site is indicated as +1; the complex microsatellite is boxed; and the translational start codon is bolded.

Figure 4. Promoter activity of the 5'-flanking region of *AKR1B10* gene

HepG2 cells were transfected with pAKR1B10/Glow-TOPO and empty pGlow-TOPO vectors as described in the Section 2.5. Green fluorescent proteins were examined under fluorescent microscopy with excitation at 395 nm and emission at 509 nm (10 x objectives).

Figure 5. Progressive deletions and activity of *AKR1B10* promoter

AKR1B10 promoter-luciferase vectors with progressive 5'-deletions were constructed and transiently transfected into HepG2 cells as described in the Section 2.4. CMV-driven β -galactosidase vector was used as an internal control to correct the transfection efficiency. *Lines to the left* indicate *AKR1B10* promoter linked to luciferase (open box). *Bars on the right* denote luciferase activity, expressed by fold over empty vector after corrected for β -galactosidase activity.

Table I

Primer sequences used for promoter subcloning

Primer	Sequences	Location
P1	5'- CTAATCTGTCACCTTGGAGG - 3'	-4,091 bp
P2	5'- CTAAAAAAGATATCCCTTCTCACTGATTC - 3'	-2,067 bp
P2341	5'- AGGACCTCTG AACAACTGCG TG - 3'	-1,776 bp
P3056	5'- GAAGTATAAGATTTTCACTCATAG - 3'	-1,061 bp
P3862	5'- CCCTACCTTCCAAC TTTGGCTG - 3'	-255 bp
P320a (R) *	5'- GAAT CATTTCTGCA CCAACC - 3'	+320 bp

* Reverse primer, used to pair with all forward primers for amplification.