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# Regulation of AKR1B1 by thyroid hormone and its receptors

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ABSTRACT

The objective of this study was to identify genes regulated by thyroid hormone ($T_3$) mediated by its receptor (TR) and associated with tumorigenesis. The gene encoding aldo-keto reductase family 1, member B1 (AKR1B1), as previously identified by c-DNA microarray, is known to be up-regulated by $T_3$ treatment. Enzyme AKR1B1 was elevated roughly 3-fold in HepG2-TRα1 cells at the protein level and 4.6-fold increase at the mRNA level after 48 h $T_3$ treatment. Similar findings were obtained from thyroidectomized rats after $T_3$ application. To identify and localize the critical TR element (TRE), series deletion of the promoter mutant were constructed and electrophoretic mobility shift assays were carried out. The TRE on the *AKR1B1* promoter was localized to the −1099/−1028 region. Further, this study demonstrated that AKR1B1 over-expression in some types of hepatocellular carcinomas (HCCs) is TR-dependent and might play a crucial role in the development of HCC. Thus, $T_3$ regulates AKR1B1 gene expression via a TRE-dependant mechanism and associates liver cancer.

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## 1. Introduction

Thyroid hormone, 3,3′,5-triiodo-L-thyronine ($T_3$) has an important role in eukaryotic cell development, differentiation and metabolism (Privalsky, 2004). The function of $T_3$ is mediated by its nuclear thyroid hormone receptors (TRs). TRs are derived from two genes, TRα and TRβ, which are located on chromosomes 17 and 3, respectively (Yen, 2001; Zhang and Lazar, 2000). Each gene expresses at least two receptor isoforms, α1 and α2, and β1 and β2, as a result of alternate splicing of their primary transcripts (Yen, 2001). The gene regulating activity of TRs is mediated by binding to specific DNA sequences, known as thyroid hormone response elements (TREs), located at the promoter regions of $T_3$ target genes (Lazar, 1993; Yen, 2001). The transcriptional activity of TRs not only depends on $T_3$, but also on the type of TREs. In the absence of the $T_3$ ligand, TRs suppress expression of target genes that are positively regulated by $T_3$, a phenomenon known as transcriptional silencing (Yen, 2001). This process is believed to be mediated by interaction, via the ligand binding domain, between the receptor and transcriptional corepressors such as SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) (Koenig, 1998). Ligand binding induces dissociation of TRs from corepressors, causing recruitment of transcriptional coactivators such as steroid receptor coactivator (SRC) and subsequent activation of target gene expression (Huang et al., 2008; Weiss and Ramos, 2004).

Aldo-keto reductase (AKR) family 1, member B1 (AKR1B1) gene encodes a member of the AKR superfamily, which consists of more than 40 known enzymes and proteins. The AKR1B1, participates in glucose metabolism and plays a protective role against toxic aldehydes derived from lipid peroxidation and steroidogenesis that could affect cell growth/differentiation when accumulated (Lefrancois-Martinez et al., 2004). This member catalyzes the reduction of a number of aldehydes to the corresponding alcohol. AKR plays an important role in the regulation of hepatic peroxisome proliferator-activated receptor α (PPARα) phosphorylation and activity and lipid homeostasis (Qiu et al., 2008). However, little is known about the physiological role of this enzyme or of related AKRs in human tissues.

Previously, we utilized cDNA microarrays to identify genes induced by $T_3$ in HepG2-TR cells (Huang et al., 2006; Shih et al., 2004). The cDNA microarray analysis showed that $T_3$ stimulated *AKR1B1* expression aprroximately 5-fold (data not shown). AKR1B1 regulation by $T_3$ and TR has not been studied previously. Thus, this investigation focused on the AKR1B1 gene regulation by $T_3$, and further verified its significance with regards to over-expression in the human hepatocellular carcinoma (HCC).

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This study shows that $T_3$ up-regulates AKR1B1 expression in HCC cells. The promoter activity of the *AKR1B1* is induced by $T_3$. The promoter activity assays, electrophoretic mobility shift assays (EMSA) or chromatin immunoprecipitation assays (ChIP) were performed to locate the TRE. Finally, the possible physiological functions of such regulation were elucidated.

## 2. Materials and methods

### 2.1. Cell culture

The human hepatoma cell lines HepG2, its sub-lines, J7, and Huh7 were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. Serum was depleted of $T_3$ (Td) as described elsewhere (Samuels et al., 1988). Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$.

### 2.2. Immunoblot analysis

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel; the separated proteins were then transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ, USA) as described elsewhere (Chen et al., 2008a,b). Antibodies included goat polyclonal antibody to AKR1B1 (Santa Cruz Biotechnology, Santa Cruz, CA). C4, a mouse monoclonal antibody against TRα1 and TRβ1, was a gift from Sheue-yann Cheng (NCI, Bethesda, MD, USA).

### 2.3. Northern blot analysis

Total RNA was extracted from cells using TRIzol reagent. Equal quantities of total RNA (20 μg) were separated on a denaturing gel (1.2% agarose-formaldehyde gel) as described previously (Chen et al., 2008a,b). The gel was then blotted onto a nitrocellulose membrane and subjected to Northern blot analysis as described previously (Chen et al., 2008a,b).

### 2.4. Cloning the AKR1B1 5′-flanking region and promoter activity assay

Fragments of the *AKR1B1* promoter (nucleotides −1929 to +101) were amplified via PCR according to the published nucleotide sequence and then inserted into the pA3TK-Luc vector. The promoter construct sequence was verified by automated DNA sequencing. To measure the influence of $T_3$ on the transcriptional activity of the *AKR1B1* promoter, CV-1 cells ($1 \times 10^5$ per 12-well plate) were co-transfected via a lipofectamine protocol using 1.2 μg/well of pA3TK-Luc vector containing *AKR1B1* promoter sequences (Invitrogen Corp., Carlsbad, CA, USA). Cells were also co-transfected with 0.33 μg of the β-galactosidase expression vector, pSVβ plasmid (Clontech Laboratories, Inc., Palo Alto, CA) as described elsewhere (Tai et al., 2007).

### 2.5. Preparation of nuclear extracts

Nuclear extracts were prepared as previously described (Huang et al., 2006).

### 2.6. Electrophoretic mobility shift assay

The *AKR1B1* promoter fragment (−1099/−1028) was synthesized, annealed and labeled with [α-$^{32}$P] dCTP (3000 Ci/mmol; Amersham) via klenow enzyme for use as a probe. For the EMSA, an equal amount of *in vitro*-translated (Promega, Madison, WI, USA) TR, RXR protein (Chen et al., 2008a,b) was incubated with [α-$^{32}$P]-labeled *AKR1B1* fragments as described elsewhere (Lin et al., 2002).

### 2.7. ChIP

ChIP assays were performed to determine the interaction of TR with TRE on the *AKR1B1* promoter region. The HepG2-TRα1 cells were treated with or without 10 nM $T_3$ for 24 h, harvested and following cross-linked with 1% formaldehyde for 10 min. The reaction was terminated by the addition of 0.125 M glycine. Cells were washed 4× in ice-cold PBS, resuspended in RIPA lysis buffer (0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA) in the presence of protease inhibitors (1 mM each of PMSF, aprotinin, leupeptin), and sonicated to shear chromatin using a Misonix Sonicator 3000 Homogenizer (Mandel Scientific Company Inc., Guelph, Ontario, Canada). The sonicated DNA fragments were in the range of 100–1000 bp. The samples were pre-cleared with 60 μl protein A/G–Agarose (Santa Cruz) for 30 min at 4 °C. Complexes were immunoprecipitated with 2 μg anti-TR antibody (C4), RXRβ antibody (Santa Cruz) or anti-IgG antibody (R&D Systems, Inc. Minneapolis, MN). The fragment of 110 bp *AKR1B1* promoter containing the predicted TRE binding sites was detected by PCR with the primers: Forward 5′-TTTAGAGAGACCTGGTGCT-3′, Reverse 5′-TCTTTCCCCTAATTATGC-3′.

### 2.8. Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR)

Total cellular RNA was extracted by TRIzol as described elsewhere. Subsequently, cDNA was synthesized using the Superscript II kit for RT-PCR (Life Technologies) as described previously (Tai et al., 2007). Real-time Q-RT-PCR was conducted as described previously (Tai et al., 2007).

### 2.9. Over-expression of AKR1B1 protein in Huh 7 cells

Transfection of AKR1B1 cDNA into Huh 7 cells was performed using Lipofectamine Reagent (Invitrogen). After 24 h of incubation, cells were transferred to medium containing G418 for selection. After 2 weeks of selection, specific expression of the AKR1B1 was confirmed by Western blot analysis.

### 2.10. Experimental animals and human specimens

In accordance with techniques described elsewhere, thyroidectomy (Tx) was performed on two groups of 6-week-old male Sprague–Dawley (SD) rats ($n = 12$), and sham operations were performed on one group of 6-week-old male rats ($n = 6$) (Chen et al., 2008a,b). Each rat was given 1% calcium lactate in drinking water after surgery. Two weeks after surgery, each rat received intraperitoneal injection of $T_3$ at 10 μg/100 g body weight or control vehicle (2.5 mM NaOH in PBS) daily for 2 additional weeks. Rats were sacrificed at the end of the experiment, and their serum was tested for $T_3$ and thyroid stimulation hormone (TSH). The $T_3$ and TSH were assayed by radioimmunoassay (RIA) (Chopra et al., 1992) and immunoradiometric assay (IRMA) (Wians et al., 1988), respectively. All animal experiments in this study complied with National Institute of Health (NIH) guidelines and the Chang-Gung Institutional Animal Care and Use Committee Guide for Care and Use of Laboratory Animals.

Additionally, 90 patients with HCC diagnosed between 2000 and 2003 were consecutively selected for this study. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee at Chang-Gung Memorial Hospital (CGMH).

### 2.11. Statistical analysis

Statistical analysis was carried out with the help of the Statistical Package for Social Science, version 13.0 (SPSS, Inc. Chicago, IL, USA).

## 3. Results

### 3.1. Effects of $T_3$ on the abundance of AKR1B1 protein and mRNA in HepG2-TRα1 and -TRβ1 cell lines

$T_3$ induction of *AKR1B1* expression was verified by Q-RT-PCR (data not shown). To further examine the regulation of the *AKR1B1* gene by $T_3$, isogenic HepG2 cell lines that consistently express wild-type TRα1 (HepG2-TRα1#1, #2) and TRβ1 (HepG2-TRβ1) were utilized. As a control, HepG2 cells were transfected with the empty vector, yielding a cell line expressing the Neo protein (HepG2-Neo cells). The four HepG2 cell lines used in this study are referred to as HepG2-TRα1#1, -TRα1#2, -TRβ1, and -Neo where the TR protein was over-expressed 10-, 3- and 3.2-fold, respectively as compared to the HepG2-Neo control cell line (Fig. 1A). The expression of the AKR1B1 protein and mRNA were compared among the various HepG2 cell lines after incubation in the absence or presence of $T_3$ for various times (Figs. 1 and 2). The results indicated that $T_3$ significantly ($P < 0.05$) increased the amount of AKR1B1 protein in the HepG2-TRα1#1, #2 and -TRβ1 stable cell lines. The AKR1B1 levels were enhanced by roughly a factor of 1.2- to 1.8-fold following incubation of HepG2-TRα1#1 for 12 h. Further, the amount of AKR1B1 was increased around 1.2- to 2.1-fold following incubation of HepG2-TRα1#1, #2, and -TRβ1 cells with $T_3$ (1–100 nM) for 24 h. A slightly greater induction (1.5- to 3.0-fold) of AKR1B1 protein was observed when the cells were treated with $T_3$ for 48 h (Fig. 1B–D). However, non-transfected HepG2 cells, which do not express detectable TR, did not induce AKR1B1 protein expression (data not shown). Exposure of J7 cells, which expressed detectable endogenous TR proteins, from 1 to 10 nM $T_3$ for 24–48 h significantly ($P < 0.05$) increased AKR1B1 protein expression (10 nM $T_3$, 1.45-fold at 24 h and 1.81-fold at 48 h; Fig. 1F). These experimental results

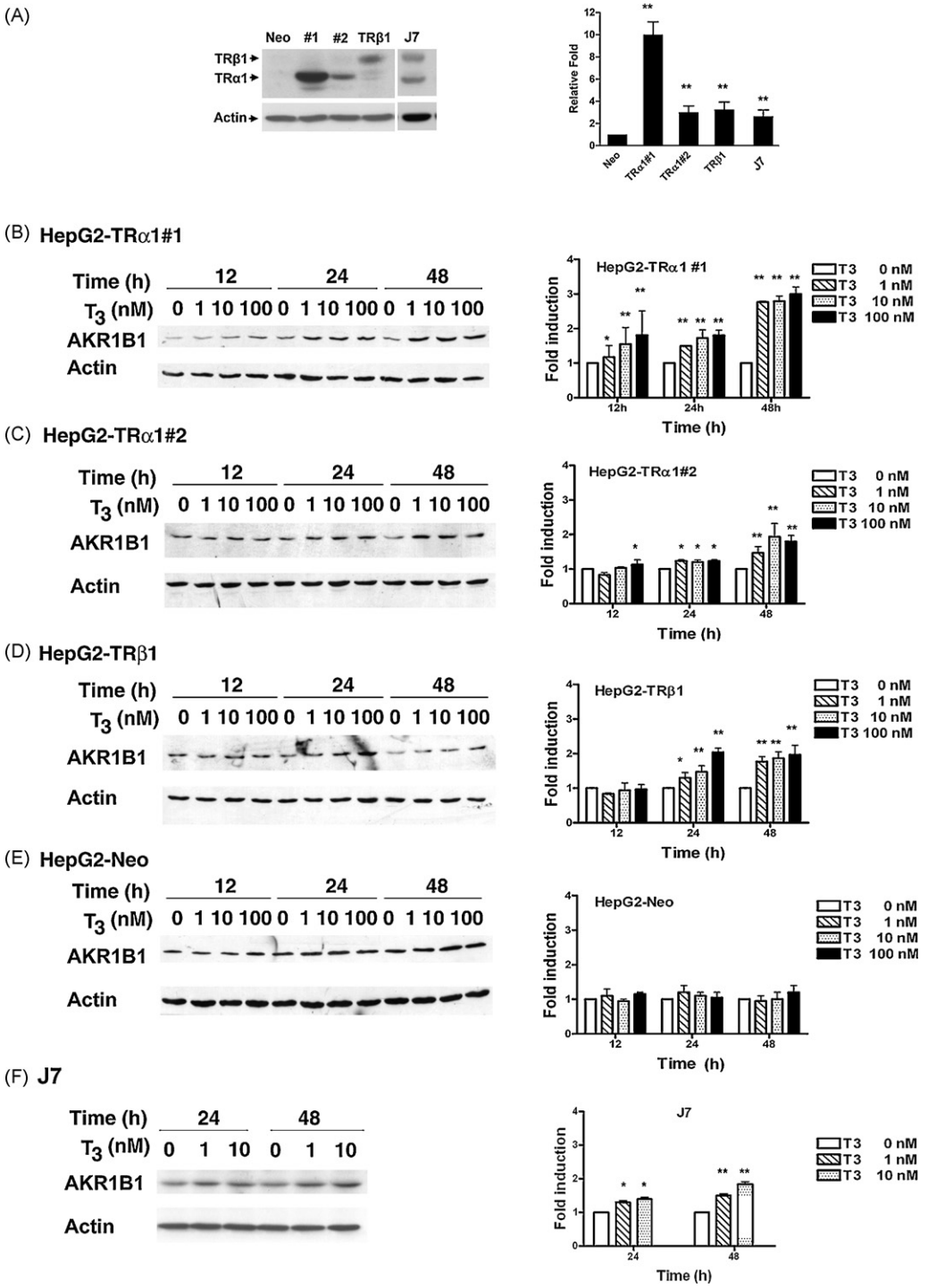



**Fig. 1.** Activation of AKR1B1 by $T_3$ in HepG2 cell lines analyzed at the protein level. Expression of TR protein in five cell lines (A). The HepG2-TRα1#1 (B), -TRα1#2 (C), -TRβ1 (D), -Neo (E), and J7 (F) cells were treated with or without 1–100 nM $T_3$ for 12, 24 and 48 h. Cell lysates (100 μg protein) were then subjected to immunoblot analysis with polyclonal antibodies against AKR1B1 (Santa Cruz). The position of the 36-kDa AKR1B1 protein is indicated. The intensities of AKR1B1 band in (B) to (F) were quantified and normalized to that of actin. Data are means ± SE of values from three independent experiments. Values are shown as fold induction of 12 h Td-control ($T_3$-depleted medium, 0 nM $T_3$), and differences were analyzed by Student *t*-test. $^*P < 0.05$; $^{**}P < 0.01$. The statistical significance is being compared to the 12 h Td-control.

demonstrate the receptor-dependent properties of $T_3$ stimulation.

The effect of $T_3$ on the expression of *AKR1B1* mRNA was examined by Northern blot analysis. A 1.4-kb *AKR1B1* mRNA was detected in all four cell lines examined (Fig. 2). Exposure of HepG2-TRα1#1, #2 and HepG2-TRβ1 cells to 10 and 100 nM $T_3$ for 12, 24 and 48 h, respectively, resulted in a time-dependent increase (1.2- to 4.6-fold) in the amount of *AKR1B1* mRNA. Incubating the HepG2-TRα1#1 cell line in 10 nM $T_3$ for 24 and 48 h increased *AKR1B1* levels 3.5- and 4.6-fold, respectively (Fig. 2A). However, when the HepG2-TRα1#2 cells were exposed to 10 nM $T_3$ for 24 h and 48 h, *AKR1B1* mRNA expression increased 3.0- and 3.2-fold, respectively. These results demonstrated that the level of AKR1B1 induction depends on the degree of expression of TR proteins. Additionally, the effect of $T_3$ on AKR1B1 protein or mRNA expression in HepG2-Neo cells was minimal (Figs. 1E, 2D).

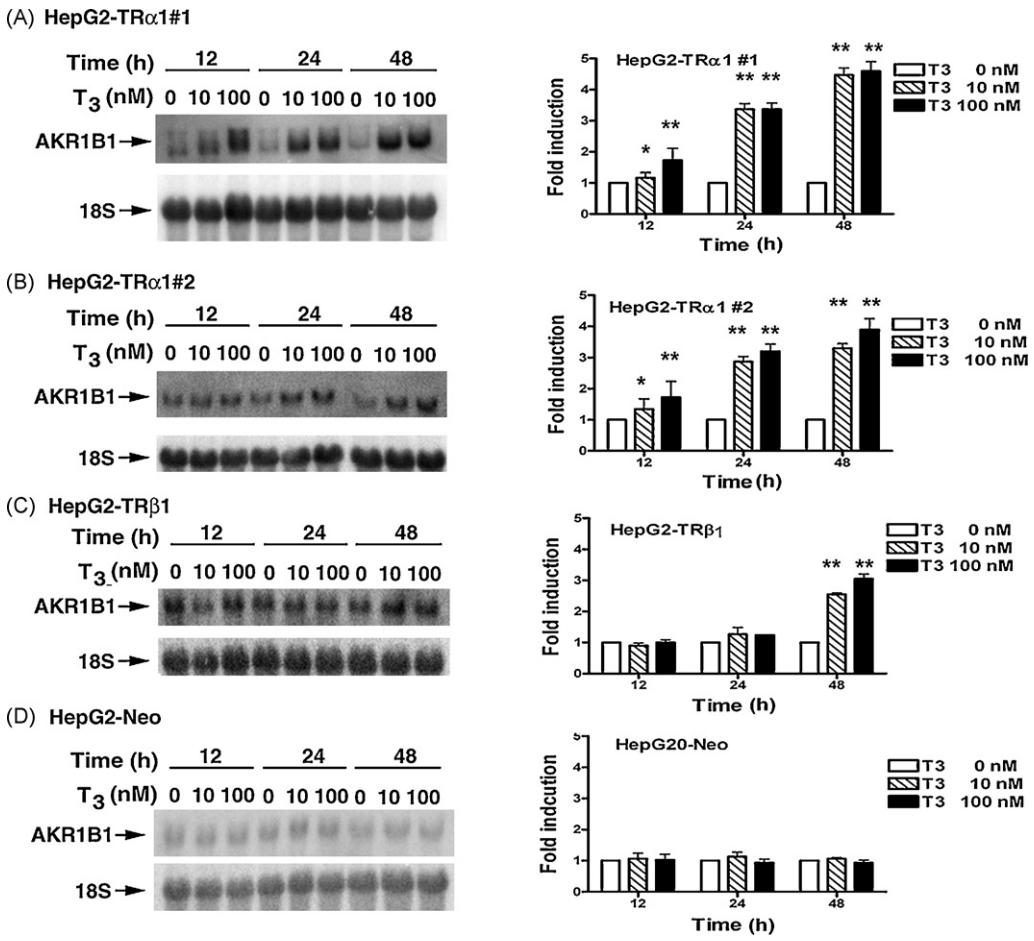



**Fig. 2.** Effects of $T_3$ on the *AKR1B1* mRNA level in HepG2 cell lines. (A) HepG2-TRα1#1, (B) HepG2-TRα1#2, (C) HepG2-TRβ1, and (D) HepG2-Neo cell lines were incubated for 12, 24 or 48 h in the absence or presence of $T_3$ (10, 100 nM). Total RNA was isolated and sequentially analyzed (20 μg per lane) by Northern blot with $^{32}$P-labeled cDNA probes of the genes encoding *AKR1B1* and *18S* ribosomal RNA. The positions of the 1.4-kb *AKR1B1* mRNA and the 1.9-kb *18S* ribosomal RNA are indicated. The intensities of the *AKR1B1* mRNA bands shown in (A) to (D) were quantified and normalized to that of *18S* ribosomal RNA. The extent of the $T_3$-induced elevation of *AKR1B1* transcripts was plotted against that of $T_3$-free control cells for each time point (shown on right). Values (means ± SE) are shown as fold induction of Td-control. Differences were examined by Student *t*-test. $^{**}P < 0.01$. The statistical significance is being compared to the 12 h Td-control.

Therefore, these results imply that the effect of $T_3$ on the expression of the AKR1B1 protein is mediated, at least in part, at the transcription level.

### 3.2. Effects of $T_3$ and cycloheximide on the abundance of AKR1B1 mRNA

To elucidate further the regulatory action of $T_3$ on AKR1B1 expression, a protein synthesis inhibitor, cycloheximide, was applied. Induction of *AKR1B1* mRNA expression by $T_3$ in the presence or absence of cycloheximide was observed in HepG2-TRα1#1 cells. The transcriptional response of *AKB1B1* mRNA to $T_3$ over 12 and 24 h periods was not significantly reduced in the presence of cycloheximide in HepG2-TRα1#1 cells (data not shown). This experimental data suggests that suppressing protein synthesis did not inhibit $T_3$-induced *AKR1B1* transcription. This lack of inhibiting effect suggests de novo protein synthesis may not be essential to activation of *AKR1B1* and that regulation may be direct.

### 3.3. Expression of AKR1B1 induced by $T_3$ at the transcriptional level

Promoter activity was carried out to confirm that $T_3$ regulation of *AKR1B1* expression occurred at the transcriptional level. The *AKR1B1* 5′-flanking region encompassing nucleotides −1929/+101 was cloned and then placed upstream of the luciferase reporter gene in pA3TK-Luc. Various deletion mutants were also prepared to localize the potential TRE. Using these reporter constructs, the effect of TR trans-activation by $T_3$ on the *AKR1B1* 5′-flanking regions was determined. Fig. 3 illustrates that transactivational activity of the −1929/+101 (+1 is relative to the transcriptional initiation site) containing the reporter construct was increased 11.32-fold in the presence of $T_3$ in CV-1 cells. When of the −1929/+101 was truncated to yield the −1929/−920, −1475/−920, −1431/−920, −1099/−920, and −1099/−1028 constructs, the $T_3$-induced activation was roughly 3.15- to 7.3-fold. Cells transfected with the −1099/−1028 construct still resulted in a 3.15-fold increase in transcriptional activity. Conversely, the activity of fragments −939/+101, −1431/−1253, −1250/−1091, −1031/−925 and −987/−920 (Fig. 3) was not induced by $T_3$. The −1099/−1028 fragment contains palindrome-like sequences (−1079/−1068). The reporter construct was prepared with mutated region in −1079/−1068. Subsequently, the *AKR1B1* activation by $T_3$ was diminished by mutation at the critical site of this region. Overall, these experimental data further indicate that the TR interaction site was localized in the −1079/−1068 region. Examining the nucleotide sequences in −1079/−1068 regions of the *AKR1B1* promoter revealed atypical TRE with sequences of −1079 (GAGTCA)(CTACCA) −1068. The above sequences are 58.3% homologous to the reported TRE consensus palindrome sequence (AGGTCA)

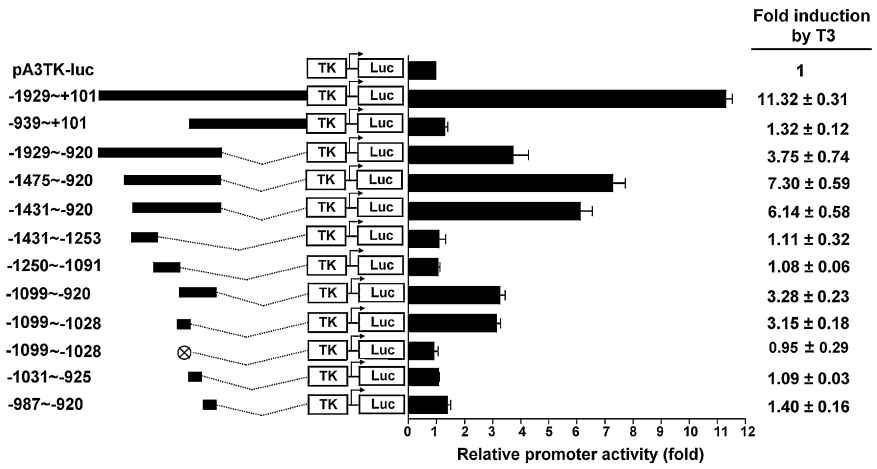



**Fig. 3.** $T_3$-dependent activation of *AKR1B1* promoter by TR. The CV-1 cells were transfected with luciferase reporter plasmid driven by a minimal thimidine kinase promoter and various fragments in *AKR1B1* 5′-flanking region along with vectors expressing TRβ and β-galactosidase (as a transfection efficiency control). Cells were then incubated for 24 h in the presence or absence of $T_3$ (10 nM) before harvesting to determine luciferase activity. The activity of luciferase was normalized to the activity of β-galactosidase. Various deletion mutants of *AKR1B1* 5′-flanking region were also generated and transfected. The mutant reporter construct was prepared with mutated region between −1099/−1028 indicated by (⊗). Data are means ± SE of values from three independent experiments, each performed in triplicate.

(TGACCT) (Nagaya et al., 1996). Taken together, our data indicate the activation of *AKR1B1* promoter activity by $T_3$ is mediated by TR/TRE.

### 3.4. Binding of TR to a −1099/−1028 fragment of the AKR1B1 promoter containing TRE

To confirm that −1099/−1028 function as TRE in the *AKR1B1* promoter, EMSA was conducted using the fragment as a probe. Reactions performed with [α-$^{32}$P] labeled −1099/−1028 fragment, T7 Quick Coupled Transcription/Translation (TNT) protein cannot bind to this fragment (Fig. 4A, lane 1). TRα1 or TRβ1 protein produced one predominant bands consisting of either a TRα1/TRα1 or TRβ1/TRβ1 homodimer (Fig. 4A, lanes 2 and 7). RXRα can barely form TR/RXRα complex in our experimental conditions. The specific TR/TRE complex could be competed by adding 2× or 5× molar excess of specific cold competitors (Fig. 4A, lanes 5–6), but not by non-specific cold competitors (Fig. 4A, lane 4). A similar result can be observed by using TRβ1 protein (Fig. 4A, lanes 7–11).

To examine whether intrinsic TR was recruited to the *AKR1B1* gene promoter in living cells, we employed ChIP assays as well as EMSA. When HepG2-TRα1 nuclear extracts were used (Fig. 4A, lanes 12–17), one predominant band was detected (Fig. 4A, lane 13) by using [α-$^{32}$P] labeled −1099 to −1028 fragment. However, the band was not detected in the probe only (lane 12). Adding *in vitro* translated RXRα or RXRβ (data not shown) protein did not produce an extra band (lane 14). Although the band could not be competed out by adding 5 times molar excess of non-specific cold probe (Fig. 4A, lane 15), it could be competed out by adding 2× or 5× specific cold probe (Fig. 4A, lanes 16–17). These experimental results suggest that the factor binding to the −1099 to −1028 on *AKR1B1* promoter is specific. Similar results were observed when ChIP assay was performed. Briefly, the results demonstrated that both TR and RXRβ were clearly recruited to the TRE binding site (Fig. 4B, lanes 12–13) whereas control IgG showed only background levels (Fig. 4B, lanes 3, 7, 11). However, a primer set for the negative control (Fig. 4B, lanes 2–5, human *GAPDH* gene) revealed no detective band. The positive control (Fig. 4B, human *furin* gene, lanes 6–9) (Chen et al., 2008a,b) showed detectable bands using RXR or TR antibodies. The findings from promoter assay, EMSA, and ChIP assays suggest that TR activates *AKR1B1* gene transcription by directly binding to the TRE located between −1099 and −1028.

### 3.5. In vivo expression of AKR1B1 protein induced by $T_3$

To determine the *in vivo* response of *AKR1B1* gene to $T_3$ treatment, two groups of 6-week-old male SD rats were thyroidectomized (Tx). Subsequently, the first group was injected with $T_3$ daily for 2 weeks (Tx + $T_3$), and a second control group (Tx) received no $T_3$ injections. Additionally, the third group received sham operations. After 2 weeks of Tx, the rats were sacrificed and their serum was collected for analysis of $T_3$ and TSH. The livers were dissected and protein extracted for Western blot analysis. The $T_3$ serum levels in the Tx group were 0.022-fold (12.3 ng/dl vs. 548.1 ng/dl) those in the group that underwent $T_3$ treatment (Tx + $T_3$). Levels of TSH in the Tx group were 67.2-fold (2.42 ng/ml vs. 0.036 ng/ml) those in the $T_3$-treated group. The $T_3$ and TSH serum levels in the sham group were 48.1 ng/dl and 0.197 ng/ml, respectively. Western blot analysis demonstrated that AKR1B1 protein levels were elevated 4.0-fold in the $T_3$ treated group (Tx + $T_3$), higher than in the Tx group (Fig. 5A). Furthermore, *AKR1B1* mRNA levels in the Tx + $T_3$ rat were 7.6-fold higher than that in the Tx group (Fig. 5B). *AKR1B1* mRNA expression in the three groups of rats was analyzed by agarose gel electrophoresis after Q-RT-PCR (Fig. 5B). The protein and RNA levels in sham-operated rats were 2.6- and 4.9-fold, respectively, higher than that in the Tx rats (Fig. 5). Further, the AKR1B1 protein and mRNA levels in the Tx + $T_3$ group was 57.7% and 54% higher, respectively, than those in the sham-operated group (Fig. 5). However, the thyroidectomy by itself did not change AKR1B1 expression levels (data not shown).

### 3.6. Ectopic over-expression of AKR1B1 did not promote cell proliferation, migration, or invasion in Huh 7 hepatoma cells

We over-expressed AKR1B1 protein in the Huh 7 cell line, which expresses low level AKR1B1 protein. The cell growth rate was determined. Fig. 6A shows that AKR1B1 expression in two Huh 7-AKR1B1 sub-lines (#1 and #2; Fig. 6A, lanes 3, 4) was 2–2.5-fold higher than that in control cells transfected with control vector (Fig. 6A, lanes 1, 2). To determine the effect of AKR1B1 over-expression in Huh 7 cells, cell proliferation was determined by cell number counting from 1 to 7 days. The AKR1B1-over-expressed cells had lower proliferation rates at 7th day than the cells transfected with control vector (Fig. 6A). However, the difference was not significant. Also, the migration, or invasion in the AKR1B1-over-expressed cells was unchanged (data not shown). The

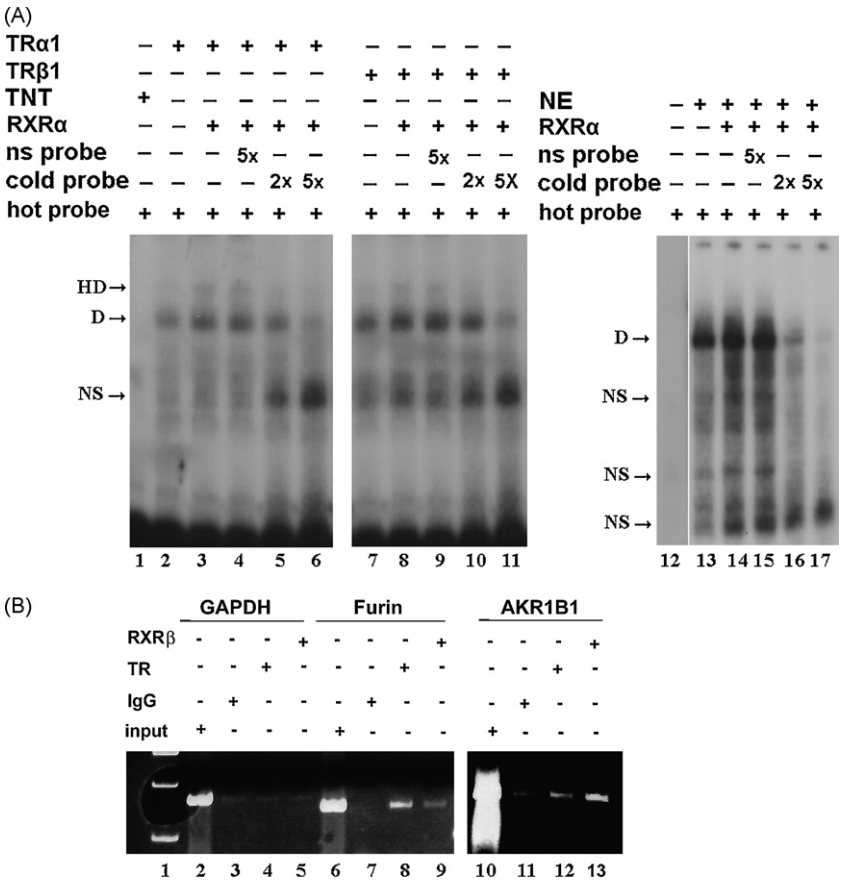



**Fig. 4.** Direct binding of TR proteins to the TRE spanning −1099 to −1028 fragments of the *AKR1B1* promoter. (A) *In vitro*-translated TRα1, TRβ1 (lanes 1–11) or nuclear extracts (NE, lanes 12–17) incubated with TRE, along with TNT *in vitro*-translated RXRα in a final volume of 20 μl. The mixture was reacted with approximately 100,000 cpm of [α-$^{32}$P] labeled oligonucleotide probes of the *AKR1B1* promoter (−1099 to −1028) for 40 min at room temperature. The *AKR1B1* promoter fragment −987 to −920 served as a non-specific control. ns: non-specific; TNT: T7 Quick Coupled Transcription/Translation; D: TR dimmer; HD: TR/RXR heterodimer. (B) ChIP assays demonstrated that TR together with RXR was recruited to the *AKR1B1* 5′-flanking region. The primer sets for *AKR1B1* TRE, positive control TRE (*furin*) and for negative control (*GAPDH*) were prepared. ChIP assay results were evaluated by PCR and gel electrophoresis. Representative results are shown. All ChIP assays were repeated at least three times. Lanes 2, 6, and 10 are 1% input; Lane 1 is a size maker.

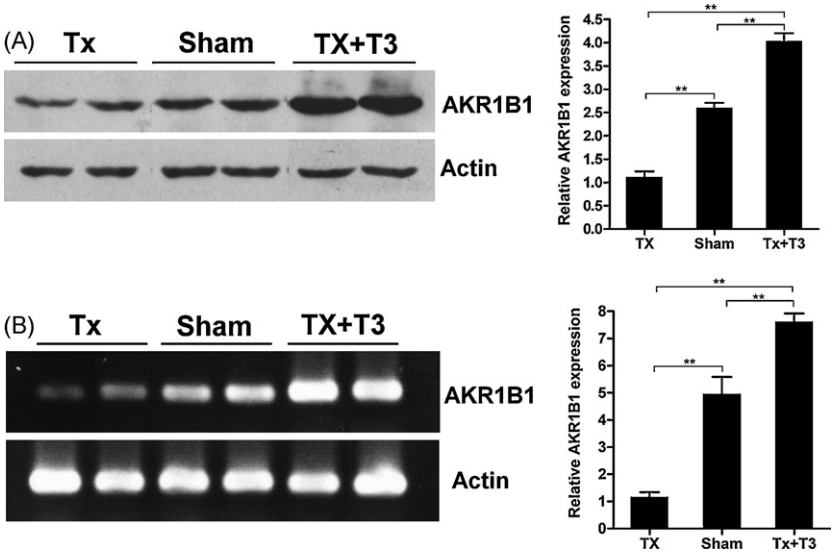



**Fig. 5.** Induction of AKR1B1 expression by thyroid hormone in rat liver. (A) Expression of AKR1B1 protein in Tx, sham, or Tx + $T_3$ male SD rat livers was determined by Western blot analysis. Intensities of AKR1B1 and actin bands on blots were quantified. (B) Expression of *AKR1B1* in Tx, sham and Tx + $T_3$ male SD rat liver was determined by Q-RT-PCR and analyzed by the agarose gel. Values (means ± SE) are shown as fold induction of control (Tx) group, and differences were examined by Student *t*-test. Data are means ± SE of values from two independent experiments ($n$ = 10 rats per group). $^{**}P < 0.01$.

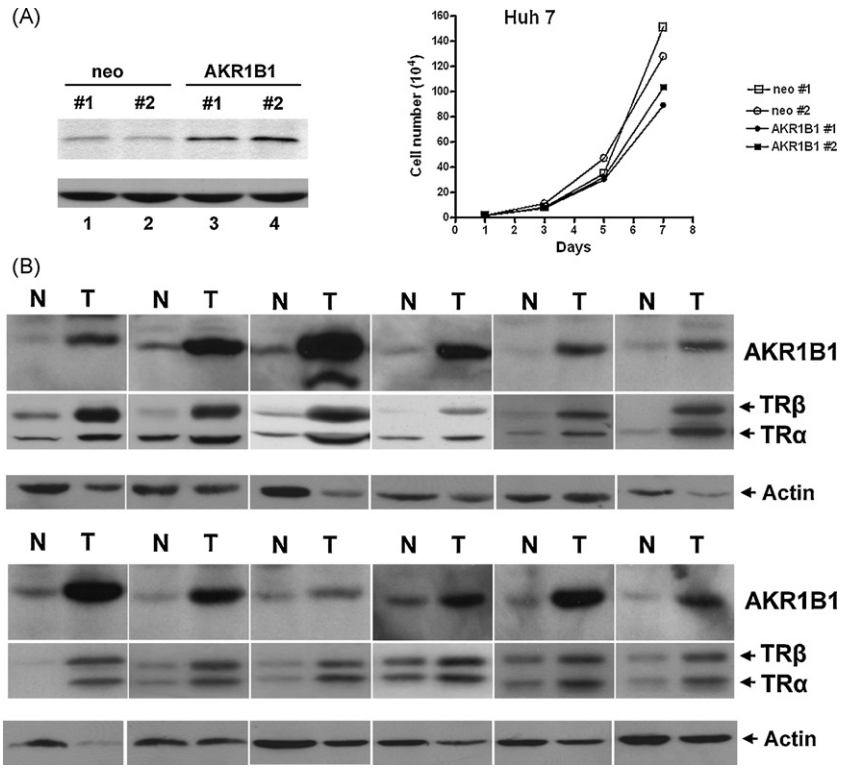



**Fig. 6.** Over-expression of AKR1B1 in human HCC cell line or tissues. (A) AKR1B1 was over-expressed in the Huh 7 cells and their growth rate was determined by cell counting. (B) The AKR1B1 and TR proteins were over-expressed in the 12-representative tumor tissues (T) compared with matched non-cancerous adjacent tissue (N) by Western blot analysis. Actin is used as an internal control. The figure shows a compilation of separate blots, pieced together.

significance of AKR1B1 over-expression, however, requires further study.

To further dissect the physiological significance of up-regulated AKR1B1 protein, the AKR1B1 expression in HCC and its clinicopathologic significance was investigated. In total, 90 patients with HCC were consecutively selected for this study. An equal amount (40 μg) of protein from each specimen was loaded for electrophoresis and Western blot analysis. Equal loading was confirmed by Coomassie blue staining after SDS-PAGE (data not shown). The AKR1B1 protein was detected in most of the tissues. The percentage of all paired-samples with up-regulated AKR1B1 was 23.3% (21/90) in the cancerous tissues, relative to the matched non-cancerous adjacent tissues. Additionally, both TRα1/TR(1 and AKR1B1 were increased in 13.3% (12/90) of the cancerous tissues. The results from 12 representative paired-HCC specimens are shown in Fig. 6B, indicating the increased AKR1B1 expression and the concomitantly increased TR protein expression in HCC tissues. After adjusted for other factors (age, gender, lymph nod invasion, and stages), tumor/normal (T/N) ratios of AKR1B1 expression was significantly and negatively associated with the presence of liver cirrhosis ($\beta = -1.002$; 95% CI of $\beta = -1.842$ to $-0.163$; $P = 0.020$). Stepwise regression model included only liver cirrhosis as an independent predictor ($\beta = -0.733$; 95% CI of $\beta = -1.300$ to $-0.166$; $P = 0.012$). Cox proportional hazard model was used for survival analysis. Therefore, AKR1B1 was excluded as a predictor for disease-free or overall survival.

## 4. Discussion

This study characterized *AKR1B1* gene up-regulation, previously identified in cDNA microarray screening for $T_3$-responsive genes in HepG2-TRα1 cells. However, the regulation of AKR1B1 expression by $T_3$ has not been reported. We attempted to elucidate the molecular mechanism of AKR1B1 regulation by $T_3$ in isogenic HepG2 cell lines. The results also revealed that $T_3$-induced AKR1B1 expression in human HCC cell lines resembles that observed *in vivo* animal studies. Moreover, the experimental data presented herein clearly demonstrate not only that the expression of *AKR1B1* mRNA is induced by $T_3$ but also identifies inverted palindrome-like TRE located between −1099 and −1028 on the *AKR1B1* promoter. In the current study, *in vivo* assay was established to determine the regulation of AKR1B1 in rats. The thyroidectomized rat, in which AKR1B1 expression levels in the Tx + $T_3$ rat were much higher than that in the Tx group. The experimental results indicate the $T_3$ induced AKR1B1 expression occurs *in vitro* and *in vivo*. Further, AKR1B1 expression levels were up-regulated in a portion of HCCs.

AKR1B1 was up-regulated in 23.3% of HCCs. Among the population, both TRα1/TR(1 and AKR1B1 were all increased in 13.3% of the cancerous tissues indicating the increased AKR1B1 expression and the concomitantly increased TR protein expression in HCC tissues. The human AKR1B1 plays a protective role against toxic aldehydes derived from lipid peroxidation and steroidogenesis that could affect cell growth when accumulated (Lefrancois-Martinez et al., 2004). Scuric et al. (1998) reported expression of human aldose reductase protein in all five tested HCCs, which suggests that this protein may play an important role in liver carcinogenesis. Zeindl-Eberhart et al. (2004) found several novel tumor-associated protein variants in human HCC that represent members of the aldo-keto reductase (AKR) superfamily. They concluded that AKR has strong potential as an immunohistochemical diagnostic marker of human HCC. Therefore, expression of aldo-keto reductase family of proteins in HCCs suggests that this protein may play a role in liver carcinogenesis. Interestingly, the over-expression of AKR1B1 was significantly and negatively associated with the presence of liver cirrhosis. However, the underlying mechanism or significance is currently unknown.

Additionally, AKR1B1-related protein, aldo-keto reductase family 1 B10 (AKR1B10), was also over-expressed in human HCC and non-small cell lung carcinoma, may facilitate cancer cell growth by detoxifying intracellular reactive carbonyls (Fukumoto et al., 2005; Li et al., 2008; Ma et al., 2008). AKR1B10 may also be over-expressed in two major types of uterine cancer, cervical cancer, and endometrial cancer (Yoshitake et al., 2007). AKR1B10 may regulate cell proliferation and cellular response to additional carbonyl stress, thus being a potential target for cancer intervention (Yan et al., 2007). AKR1B10 also acts as a retinaldehyde reductase *in vivo*. This activity may be relevant in controlling the first step of retinoic acid synthesis. Up-regulation of AKR1B10, resulting in retinoic acid depletion, may lead to cellular proliferation (Gallego et al., 2007). An *in vivo* study showed that administration of aldo reductase-small interfering RNA (siRNA) to nude mice bearing SW480 human colon adenocarcinoma cells completely arrested tumor progression (Tammali et al., 2006). A similar function or observations may apply to AKR1B1 that was over-expressed in some HCCs. Taken together, induction of aldose reductase gene expression during hepatocarcinogenesis may render cancer cells resistant to various toxic carbonyl compounds produced during metabolism (Takahashi et al., 1995).

Further, AKR1B1 is involved in the polyol pathway through which glucose is converted to sorbitol, and has been implicated in the etiology of diabetic complications. If mice are lacking AKR1B1, they showed hypercalciuria, hypercalcemia, and hypermagnesemia (Aida et al., 2000). Human AKR1B1 has been reported as a factor in the pathogenesis of diabetic complications (Chang et al., 2003). Variants in the AKR1B1 have also been indicated in the development of diabetic retinopathy and nephropathy (Donaghue et al., 2005). However, how this function correlated with tumorigenesis seen in HCC or lung cancer waits for further investigation.

AKR1B1 induction can also be mediated by $T_3$ indirect pathway and/or via other TR-independent pathways. After administration of androgen to castrated rats, it stimulated AKR1B1 expression. The results suggest that AKR expression is associated with some growth-related processes in re-growing rat prostate (Nishi et al., 2000).

In conclusion, this investigation demonstrates that ligand-activated TR directly transactivates *AKR1B1* gene expression. The TRE is localized between −1099 and −1028 of the *AKR1B1* promoter region. Although this phenomenon has been documented in a human tumor cell line, this regulation is similarly observed in SD rats model. AKR1B1 protein over-expression was observed in some HCCs indicating the possible tumor enhancing role of $T_3$/TR during hepatocarcinogenesis.

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