Title	HNF1α and CDX2 transcriptional factors bind to cadherin-17 (CDH17) gene promoter and modulate its expression in hepatocellular carcinoma
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Citation	Journal Of Cellular Biochemistry, 2010, v. 111 n. 3, p. 618-626
Issued Date	2010
URL	http://hdl.handle.net/10722/129379
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Journal of Cellular Biochemistry

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Journal:	*Journal of Cellular Biochemistry*
Manuscript ID:	JCB-09-0616
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	09-Nov-2009
Complete List of Authors:	Zhu, Rui; The University of Hong Kong, Surgery Wong, Kwong-Fai; The University of Hong Kong, Surgery Lee, Nikki; The University of Hong Kong, Surgery Lee, Kai-Fai; The University of Hong Kong, Obstetrics and Gynaecology Luk, John; University of Hong Kong, Department of Surgery
Keywords:	Cadherin-17, Hepatocellular carcinomas, Hepatic nuclear factor 1α, Caudal-related homeobox 2, Promoter

HNF1α and CDX2 Transcriptional Factors bind to Cadherin-17 (CDH17) gene promoter and modulate its expression in Hepatocellular Carcinoma

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Running head: HNF1α and CDX2 regulate CDH17

Keywords: Cadherin-17; hepatocellular carcinomas; hepatic nuclear factor 1α; caudal-related homeobox 2; promoter

Number of Figures: 5

Number of Table: 1

ABSTRACT

Cadherin-17 (CDH17) is a novel adhesion molecule that expresses abundantly in fetal liver but is silenced in healthy adult. Recently, we have identified CDH17 is a cancer biomarker which is over-expressed in ~80% of hepatocellular carcinomas that associated with poor clinical outcomes. The present study aims to identify the transcription factor(s) and elucidate the regulatory mechanisms that drive CDH17 gene over-expression in human HCC. A 1-kb upstream sequence of CDH17 gene was cloned and the promoter activity was studied by luciferase reporter assay. By bioinformatics analysis, deletion and mutation assays, and chromatin immunoprecipitation studies, we identified hepatic nuclear factor 1α (HNF1α) and caudal-related homeobox 2 (CDX2) binding sites at the proximal (-81/+55 region relative to the transcriptional start site) promoter region which modulate the CDH17 promoter activities in two HCC cell lines (Hep3B and MHCC97L). Interestingly, the expression of CDH17 was high in mouse fetal livers (E13.5> E16.5> newborn> 3 weeks> adult) as well as in human HCC cell lines with higher metastatic potential (MHCC97L> Hep3B> MIHA), which is in concomitant with the expression levels of HNF1α and CDX2 mRNA and proteins. Furthermore, knockdown of HNF1α and CDX2 by small interfere RNA (siRNA) significantly decrease CDH17 transcript level in the transfected HCC cells. In sum, we identified -81/+55 region of CDH17 as minimal promoter region that is regulated by HNF1α and CDX2. The expression levels of these transcriptional factors as well as CDH17, may serve as biomarkers for HCC, and may be potential targets for cancer treatment.

INTRODUCTION

Hepatocellular carcinoma (HCC) ranks the fifth most common malignancy in the world [Llovet et al., 2003]. Hepatic resection and liver transplantation are the mainstays of surgical management for HCC, but the 5-year survival rate is still poor [Liu and Fan, 2006; Poon and Fan, 2004]. Early diagnosis and treatment of HCC remain challenging due to lack of highly specific and sensitive markers. The currently used serological tumor marker for HCC, α-fetoprotein (AFP), lacks specificity and sensitivity [Johnson, 2001].

Liver-Intestine cadherin (LI-cadherin), or CDH17 (cadherin-17) is a member of non-classical cadherin family [Jung et al., 2004] and located on chromosome 8q21.1 [Dantzig et al., 1994]. Beside the cell adhesion function, CDH17 was initially identified as a proton-dependent peptide transporter-1 in human colon adenocarcinoma Caco-2 cells [Dantzig et al., 1994]. CDH17 is over-expressed in gastric metaplasia and gastric carcinoma 42 associated with lymph node metastasis [Grotzinger et al., 2001; Kaposi-Novak et al., 2006; Ko et al., 2004; Ko et al., 2005], as well as in pancreatic carcinoma [Takamura et al., 2003]. Recently, our group has reported CDH17 is over-expressed in HCC and is associated with poor survival rate and a higher tumor recurrence [Wang et al., 2005; Wong et al., 2003]. In human, over-expression of CDH17 is frequently detected in ~80% HCC cases, but the corresponding regulatory mechanisms remain largely unclear.

Recently, CDX2 was found to play important role(s) in up-regulation of CDH17 expression in colorectal cancer cell lines and gastric adenocarcinoma [Hinoi et al., 2002; Ko et al., 2005], which was in turn was regulated by bone morphogenetic protein 2 (BMP2)

and BMP4 in gastric cancer cells [Barros et al., 2008].

HNF1 is one of the key transcriptional factors that regulate serum α-fetoprotein levels in hepatocellular carcinoma [Nakabayashi et al., 2004]. It is predominantly expressed in liver and kidney, and binds to the promoters of a variety of genes that are expressed exclusively in the liver including fibrinogen-α and -β, albumin, α-1-antitrypsin, liver-type pyruvate kinase, transthyretin, aldolase B, and hepatitis B virus large surface protein [Courtois et al., 1987; Xu et al., 2001]. Over-expression of HNF1α was found in well-differentiated HCC than in the surrounding non-HCC tissues [Wang et al., 1998].

In this study, we aimed to dissect the regulation of CDH17 expression in HCC and in mouse live during development, and functionally characterized two transcription factors (HNF1α and CDX2) on CDH17 expression. The specific regulation of these transcriptional factors was confirmed by RNAi approach in the transfected HCC cells.

MATERIALS AND METHODS

Plasmids, bacteria and reagents

The Dual-Luciferase® reporter assay system and the pGL3-Basic, pGL3-Control and pRL-TK reporter vectors were obtained from Promega (Madison, WI). *E. coli* DH5α competent cells were prepared by chemical method. Restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs (Ipswich, MA). Bradford reagent was purchased from Bio-Rad (Hercules, CA) and all other reagents were purchased from Sigma (St. Louis, MO).

Mouse liver and clinical HCC tissues

The use of animal in this study protocol was approved by the Using Live Animals for Teaching and Research (CULATR), The University of Hong Kong. Informed written consent was obtained from patients and the usage of clinical tissues for this study was vetted and approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Messenger RNA and protein lysates were extracted from BALB/c mouse livers at different stages: embryonic day 13.5 (E13.5), E16.5, new-borne (NB), postnatal (3 weeks), and adult (3 months).

Cloning of 5'-promoter region of the CDH17 gene

The promoter region of the human CDH17 gene (NM_004063.3) was predicted and analyzed using the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview). The

promoter fragment starting at -909bp and ending at +55bp was amplified by PCR using specific forward primer 5'-TAGAGAGTGGGCTGGGCTC-3' and reverse primer 5'-TGGTCGAGACTCTTGCTACG-3' and Pfu Turbo high-fidelity DNA polymerase (Stratagene, La Jolla, CA). In brief, PCR was carried out at 94 °C for 5 min and then 30 cycles at 94 °C for 40 s, 60 °C for 1 min, and 72 °C for 3 min and a final extension step at 72 °C for 10 min in a final reaction volume of 25 μl. The genomic DNA of HCC cell line MHCC97L was used as template and the CDH17 gene promoter was cloned by directional restriction cloning method [Lee et al., 2009; Wong et al., 2003].

Constructs of CDH17 mutant promoters

Another four deletion constructs (-686/+55, -332/+55, -81/+55 and -40/+55) were generated by PCR cloning with the following forward primers (5'-GAAGCCTTGACTTGAGAAAT-3', 5'-TGATCAAGTCTCCTGTGCT-3', 5'-AGTGGCTCTCGAAGAGCAAT-3', and 5'-TTTGACTGAAGCTGAAGGG-3'), respectively. These five CDH17 promoter constructs were cloned to the pGL3-Basic vector by XhoI/KpnI restriction digestions. For site-directed mutagenesis, mutated promoter constructs were generated using the QuickChange mutagenesis kit (Stratagene). All deletion and point-mutation constructs were sequenced with an ABI 3700 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA).

Cell Cultures and transfection

The human HCC cell lines MHCC97L and Hep3B and a normal immortalized human

hepatocyte cell line MIHA were cultured in Dulbecco's minimal essential medium (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 U/ml streptomycin and 2 mM L-glutamine at 37 °C and 5% CO_2. For transfection, cells were grown to about 90% confluence in a 24-well tissue culture plate and then added with 500 µl of serum-free medium containing 2 µl lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) mixed with 1 µg of purified plasmid cDNA and 0.1 µg of pRL-TK vector (the internal control reporter) per well. Followed by incubation at 37°C for 48 h, the promoter activity of the CDH17 gene was assessed by measuring luciferase activity using the Dual-Luciferase® reporter assay (Promega). The intensity of chemiluminscence was measured in a luminometer (Thermo, Waltham, MA). All experiments were performed in duplicate and repeated at least three times with positive (pGL3-Control) and negative (pGL3-Basic) controls.

Western blotting

Antibodies against CDX2 and β-actin were purchased from Millipore (Billerica, MA) and Sigma (St Loius, MO), respectively. Antibodies against CDH17, HNF1α and HNF1β were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein extracts from the liver of BALB/c mice at different developmental stages and from different cell lines MHCC97L, Hep3B and MIHA was prepared. In brief, cells were washed twice with ice-cold phosphate buffered saline (PBS) and then incubated in the lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40) on ice for 30 min. After centrifugation at 4 °C for 15 min at 14,000g, the supernatant (30 µg per lane) was collected and resolved on a 10% SDS-PAGE. Proteins

on the gel were then transferred onto a polyvinylidene fluoride membrane (0.4-μm pore size; Millipore). Non-specific protein binding onto the membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% non-fat milk at 4°C overnight [Lee et al., 2006]. After washing three times with TBST, the membrane was incubated with the primary antibodies (1:500 in TBST containing 5% non-fat milk) at room temperature for 1 h. After washing with TBST, bound antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies (1:20,000 dilution) and ECL reagent (Amersham Biosciences). The immunoreactive signals were developed on Kodak® Biomax™ MS film.

Quantitative PCR

Total RNA was isolated from cell cultures or frozen tissues using Trizol reagent (Invitrogen). Two micrograms of total RNA were used to synthesize first-strand cDNA using random hexamer primers and reverse transcriptase (Promega). A 5 μl aliquot of 1:10 diluted cDNA was mixed with 10 μl SYBR® GreenERTM qPCR superMix (Invitrogen) and 0.5 μM of the designated primers (Table 1). The thermal cycling conditions were 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. All reactions were done at least twice and the products were analyzed using the ABI Prism 7700 detection system (Applied Biosystems Inc.). Ribosomal 18S RNA was included in all reactions for internal house-keeping control and normalization.

Electromobility shift assay (EMSA)

Nuclear protein extracts from MHCC97L and Hep3B cells were prepared as described previously [Luk et al., 2004], and the amount of protein extract was quantified using the Bradford protein assay kit (Bio-Rad). Double-strand oligonucleotide probes were synthesized with annealing buffer (200 mM Tris-HCl;pH 9.0, 40mM $MgCl_2$, 1 M NaCl, 20 mM EDTA) and labeled with [γ-^{32}P] ATP (PerkinElmer, Waltham, MA) using the Ready-To-Go T4 Polynucleotide Kinase kit (Amersham BioSciences) with T4 polynucleotide kinase. Unincorporated nucleotides were removed by spin column in a Tris-EDTA buffer, pH 8.0. An aliquot (10 µg) of nuclear extract was incubated with or without 1µl unlabeled oligonucleotide probes or the previously described antibodies in 1x binding buffer (4% glycerol, 1 µg poly(dI–dC).(dI–dC), 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA and 0.5 mM DTT) in a 10 µl reaction volume for 20 min at room temperature. Radiolabeled probe was added to each reaction tube and the tubes were incubated for a further 20 min at room temperature. The reaction products were then resolved on a 5% non-denaturing polyacrylamide gel. The gels were dried under vacuum and autoradiographed on Kodak® Biomax™ MS films overnight at -70°C.

Chromatin Immunoprecipitation (ChIP) assay

Standard ChIP assays were performed with Millipore ChIP Assay kit according to the manufacturer's instructions. Briefly, approximately 1 x 10^6 cells were cross-linked using 1% formaldehyde at 37°C for 10 min, washed twice with PBS, and then resuspended in 200 µl SDS lysis buffer containing 1x protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and 10 mM of DTT. After incubation on ice for 10 min, the cells were

sonicated (Sonic Dismembrator Model 100, Fisher Scientific, Waltham, MA) four times at 20% maximum power for 10 s and the lysate was centrifuged at 4°C for 15 min at 14,000g. The resultant supernatant was diluted 1:10 with a dilution buffer containing a protease inhibitor. Twenty microliter of the diluted supernatant was collected as input control before immunoprecipitation. After precleared with 75 µL of salmon sperm DNA/protein A agarose (50% slurry) for 1 hour at 4°C with agitation, the immune complexes were incubated at 4°C overnight with either 2 µg of primary antibodies or IgG control, and then recovered with 60 µl of salmon sperm DNA/protein A agarose 50% slurry at 4°C for 1 hour. Precipitates were washed once sequentially with washing solutions provided by the manufacturer (Millipore), and finally with two washes of TE buffer. Chromatin complexes were incubated at room temperature for 15 min with 250 µl of elution buffer containing 1% SDS and 100mM sodium bicarbonate. The eluate was reverse cross-linked by heating at 65°C for 4 hours with 200 mM NaCl and 200 mg/ml proteinase K. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation and then amplified by PCR with forward primer 5'-GTGCTAAGTGTTGGGGGTACAA-3' and reverse primer 5'-TTGACTGAAGCTGAAGGGAGAG-3'. PCR was carried out at 94 °C for 5 min and then 30 cycles at 94 °C for 40 s, 60 °C for 1 min, and 72 °C for 1 min and a final extension step at 72 °C for 10 min in a final volume of 25 µl.

HNF1α and CDX2 siRNA knockdown

Small interference RNA (siRNA) was used to knockdown the expression of HNF1α and CDX2 in HCC cells (MHCC97L and Hep3B). RNA oligonucleotides were designed

according to the published sequences [Hinoi et al., 2005]. HCC cells were transfected with the designated siRNA duplex or scramble control using Lipofectamine™ 2000 (Invitrogen) as described above. After 48-hour incubation, both mRNA and protein extracts were purified for qPCR and Western blot analyses, respectively.

Statistical analysis

All results are represented as mean ± S.D. Statistical comparisons were performed by One-way ANOVA or paired Student's t-test using the SPSS statistical software package version 13.0 for Window (SPSS Inc., Chicago, IL) and $p<0.05$ was considered to be statistically significant.

RESULTS

Cloning and characterization of CDH17 gene promoter

CDH17 promoter region was amplified by PCR and cloned into pGL3-Basic reporter vector. The position and sequence of the primers used to generate the parental fragment and the four deletion constructs were shown in Figure 1A. Two bioinformatics programs, ALGGEN-PROMO (http://alggen.lsi.upc.es) and MatInspector (http://www.genomatix.de) were used to identify potential transcription factors at the -909/+55 promoter region and the results were compared to the transcription factor database TRANSFAC (version 8.3). A conserved CAAT box, but not the TATA-box, was found in the CDH17 gene promoter region. Interestingly, two putative transcription factors, HNF1 and CDX2 were identified. At this stage, the binding of HNF1α from HNF1β cannot be differentiated as they shared similar binding sequences. There are three putative binding sites for each HNF1 and CDX2 transcription factors in the CDH17 promoter, respectively. Figure 1B shows the luciferase reporter activities of different promoter deletion constructs when compared to the parental (-909/+55) control in two different HCC cell lines: MHCC97L ($CDH17^{high}$) and Hep3B ($CDH17^{low}$). The data indicated that CDX2 and HNF1α are two critical factors for the transcription of CDH17 because the luciferase activities decreased significantly from the pGL-81/+55Luc to pGL3-40/+55Luc. Both cell lines exhibited similar activity patterns although the signals were generally stronger in the MHCC97L cell line.

To confirm the functional roles of these transcriptional factors (TF), point mutations were introduced at the TF core binding sites in the CDH17 promoter sequence.

Reporter activities were markedly reduced for HNF1 mutant (-47/-45, $p<0.01$), followed by the CDX2 mutant (-91/-89, $p<0.05$) (Fig. 2). Mutations on other HNF1 and CDX2 binding sites did not drastically reduce promoter activities. Double mutations on the HNF1 (-47/-45) and CDX2 (-91/-89) binding sites reduced the CDH17 reporter activity to near basal level.

Binding of CDX2 and HNF1α to CDH17 promoter *in vitro* and *in vivo*

The binding of HNF1 and CDX2 to the CDH17 gene promoter in MHCC97L and Hep3B cells were studied by EMSA and ChIP assays. As shown in Fig. 3A, both the HNF1α and CDX2 wild-type (WT), but not the mutant probes were able to complexes with the HCC lysates. Further, addition of 10 and 50-fold excess of "cold" WT probes, but not the mutant probes, could displace or compete with the binding. Moreover, CDX2, HNF1α, but not HNF1β antibodies were able to retard the migration of the DNA-protein complex, suggesting the binding of the nuclear extract is specific. Meanwhile, the ChIP assay was performed to study TF-DNA interactions in vivo. After immunoprecipitation by designated antibodies, the recovered DNA was amplified by PCR using CDH17 promoter-specific primers spanning from -318 to -18 regions. Positive signals were detected by the HNF1α and CDX2, but not the HNF1β and isotype control (Fig. 3B).

Differential expression of CDH17, HNF1 and CDX in HCC and fetal mouse liver

Luciferase reporter assays revealed differential promoter activities in Hep3B and MHCC97L cell lines (Fig. 1B and Fig. 2). To study if the endogenous CDH17 transcript level correlates with the HNF1α and CDX2 expression levels, we determined the

expression of these transcripts in HCC cells and mouse livers at different developmental stages. MHCC97L which has a high metastatic potential also expressed a high level of CDH17 transcript as well as the CDX2 and HNF1α transcripts; whereas the primary HCC (Hep3B) and MIHA (immortalized normal human hepatocyte) expressed at a lower or undetectable levels of the gene (Fig. 4A).

Since CDH17 plays important roles during embryonic liver development, the expression of CDH17 and TFs levels in mouse liver at different development stages (E13.5, E16.5, newborn, 3 weeks and adult) were measured. Bioinformatics analysis of these transcriptional factor binding sites on CDH17 promoter between mouse, rat and human are highly conserved using ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), suggesting a conserved regulatory mechanism of CDH17 expression among different animal species (data not shown). It was found that CDH17 expressed abundantly in the early embryonic liver, but the expression of the gene becomes undetectable in late fetal stages and adult livers (Fig. 4A). Similar to the human HCC findings, the differential expression of CDH17 transcript in mouse liver largely correlated with the patterns of HNF1α and CDX2 levels. The result was supported by Western blot analysis of the CDH17, HNF1α and CDX2 protein levels (Fig. 4B). Interestingly, the decreases of CDH17 and HNF1a were more drastic in both human HCC cells and mouse livers, than the CDX2.

Knockdown of HNF1α and CDX2 suppress CDH17 expression

To demonstrate HNF1α and CDX2 regulate CDH17 expression, we used siRNA approach

to knockdown HNF1α and CDX2 expression and study the expression of CDH17 in MHCC97L cells (Fig. 5). RNAi-mediated targeting HNF1α or CDX2 lowered the mRNA level of the respective transcriptional factor (HNF1α or CDX2) by approximate 50%, when compared with the vehicle or scramble control. These down-regulations were accompanied with significant reduction of CDH17 transcript level in MHCC97L cells ($p < 0.05$). An additive effect of combined HNF1α or CDX2 RNAi treatment on CDH17 suppression was also observed, indicating a coordinate regulation of HNF1α and CDX2 in the cells.

DISCUSSION

Liver-intestine cadherin or cadherin-17 (CDH17) is expressed in the rat liver and intestinal epithelial cells [Angres et al., 2001], as well as in the embryonic mouse liver [Takamura et al., 2004], but no CDH17 expression was detected in normal liver tissue of mature humans and mice [Takamura et al., 2003]. CDH17 is involved in the maintenance of tissue integrity, tissue growth and differentiation [Berndorff et al., 1994]. Aberrant expression of CDH17 mRNA and protein was found in upper gastrointestinal malignancies including liver cancer [Wang et al., 2006; Wang et al., 2005; Wong et al., 2003]. In this study, we demonstrated that the expression of CDH17 is regulated by HNF1α and CDX2 transcriptional factors in HCC and mouse liver.

The human CDH17 gene promoter has a conserved CAAT box at the -65/-62 position, but lacks the TATA box, an initiator element in the predominant control region of typical eukaryotic genes [Suzuki et al., 2001]. In both primary and metastatic human HCC cell lines, the minimal CDH17 promoter (-81/-40) region contains one CDX2 and two HNF1 sites that mediated strong promoter activity in vitro. Further deletion of this minimal promoter region diminished the reporter activity to near basal levels. We also found that the transactivation activitiy of the pGL3-81/+55Luc construct was similar to longer constructs (up to -909 position). Although the deletion construct pGL3-332/+55Luc shown a decrease in promoter activity, the reason behind is still unknown. It is possible that an enhancer sequence is located at -686 and -332 region, and a repressor sequence is located at -32 to -81 region, thereby modulate the changes in promoter activity. As a result, the deletion construct pGL3-81/+55Luc shown a full-promoter activity. Yet, further

experiments are needed to demonstrate if these regulatory sequences play any significant role in vivo.

Mutation analysis identified a HNF1 site at -47/-45 position is crucial for CDH17 expression. To a lesser extent, CDX2 (-91/-89) regulates the CDH17 promoter activity in vitro. Furthermore, both EMSA and ChIP assays demonstrated binding of HNF1 and CDX2 to the CDH17 promoter sequences. HNF1α, but not the HNF1β isoform bound to the CDH17 promoter and the protein-DNA complex was recognized by HNF1α specific antibody. The temporal expression of HNF1α and CDX2 transcript in related to CDH17 expression was studied in human liver cell lines and mouse liver tissues. It was found that an elevated levels of HNF1α and CDX2 expression are closely associated with a higher expression of CDH17 in metastatic HCC cell line (MHCC97L >> Hep3B > MIHA) as well as in early embryonic mouse liver tissues [E13.5 > E16.5 > new born (NB)]. The role of HNF1α, and CDX2 on co-regulating the CDH17 gene expression was further demonstrated by iRNA suppression on CDX2 and HNF1α expression in HCC cells.

HNF1 and CDX2 are homeodomain transcription factors that regulate a number of intestine-specific genes including Lewis type 1 antigen synthase (β3Gal-T5) and UDP-glucuronosyltransferases (UGT) [Gregory et al., 2004; Isshiki et al., 2003]. CDX2 regulates CDH17 expression in colorectal cancer cell lines and gastric adenocarcinoma [Barros et al., 2008; Hinoi et al., 2002; Ko et al., 2005]. In this study, 3 putative CDX2-binding sites were found in the 1-kb CDH17 promoter region (Fig. 1A) and it is the -91/-89 site that contributed to CDH17 promoter activity and binding in HCC. In addition, we identified HNF1 homeobox A (HNF1α) that may play an important role in regulating

CDH17 transcription in human HCC as well as in mouse fetal liver. Although there are three putative HNF1-binding sites in the CDH17 promoter, deletion/mutation studies identified -47/-45 position is crucial in regulating CDH17 expression (Fig. 1B and Fig. 2).

HNF1α and HNF1β are two related transcriptional factors that bind to DNA as homo- or heterodimers. They were identified initially as liver-enriched transcription factors that were involved in the expression of several plasma proteins including α1-antitrypsin [Mendel and Crabtree, 1991]. However, no detectable HNF1β transcript and protein was found in the two HCC cell lines studied (data not shown), and HNF1β failed to bind to CDH17 promoter DNA sequence in EMSA or ChIP assays. These findings suggest that HNF1α, but not HNF1β is associated with the regulation of the CDH17 gene in HCC. Interestingly, HNF1β has recently been demonstrated to direct the expression of kidney-specific cadherin (cadherin-16) [Bai et al., 2002].

In conclusion, the present study provides the first evidence that HNF1α plays a critical role, together with CDX2, on the regulation of CDH17 gene expression in HCC as well as in early stages of fetal liver development in mice. Clinically, over-expression of CDH17 in HCC is associated with poor clinical outcomes; HNF1α and CDX2 in accompany with CDH17 may be potential therapeutic targets for disease diagnosis and treatment.

ACKNOWLEDGMENTS

The work was supported by a grant from the General Research Fund (HKU771607M) of the Research Grants Council of Hong Kong. We thank Dr. Jana Wo for the technical

assistance on microscopic surgery of fetal mouse liver and Dr. Arieh Bomzon for critical review and professional editing of the manuscript.

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FIGURE LEGENDS

Figure 1 Cloning and characterization of CDH17 gene promoter. **(A)** The nucleotide sequences of the 5'-flanking regions of human CDH17 gene and the putative transcriptional regulatory elements from -955 were depicted. The G at position +1 indicates the transcriptional initiation site based on the GenBank database. The translation start site (ATG) is underlined and the amino acid sequences are indicated by bold and capital letters above nucleotide sequences. The primer sequences used for making the parental fragment and the four deletion constructs were each marked with arrows (forward and reverse primers). (**B)** The promoter activity of CDH17 deletion constructs in Hep3B and MHCC97L cells was studied. The pGL-332/+55Luc and pGL-40/+55Luc constructs exhibited drastic reduction in luciferase activity ($p<0.05$ and $p<0.01$, respectively) when compared with the control fragment (pGL-909/+55Luc). The luciferase activity of the empty expression vector pGL3-Basic was assigned a value of 1 and the relative activities of each construct were normalized with the pRL-TK expression levels. Values are represented as mean ± S.D. from at least three independent experiments.

Figure 2 Mutation of Transcriptional factor binding sites on CDH17 promoter activity. The relative luciferase activities of mutated HNF1 and CDX2 sites in CDH17 promoter constructs () were studied in Hep3B and MHCC97L cells. The mutated HNF1 (filled oval) and/or CDX2 (filled rectangle) binding sites on CDH17 promoters were transfected into the HCC cells. Mutation at the CDX2 (-91 position) and/or HNF1 (-47 position) binding site(s) significantly reduced ($p<0.05$ and $p<0.01$, respectively) the

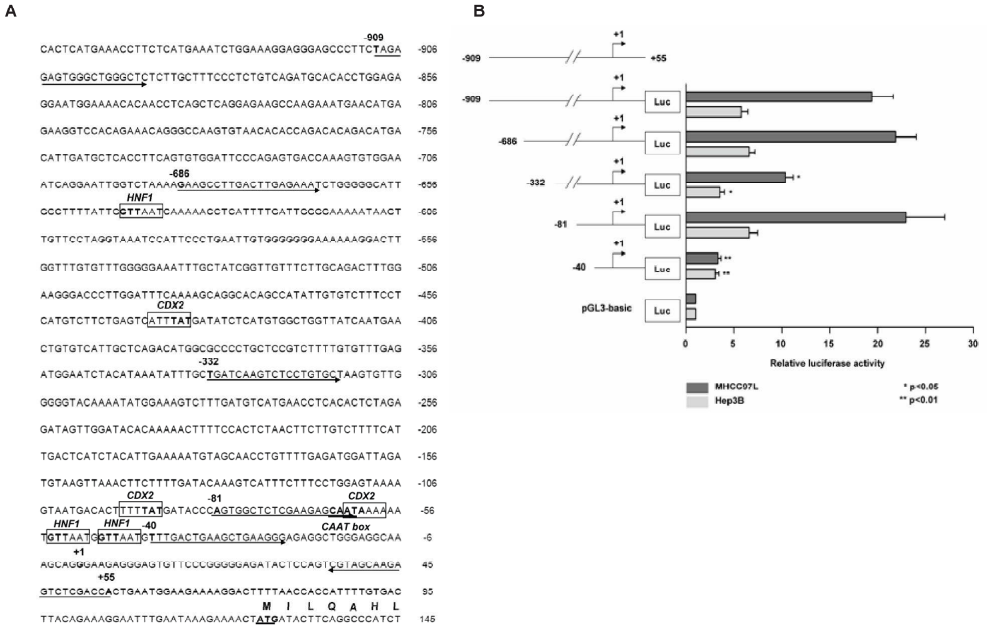
luciferase activity. Combined mutation of these two sites (-91 and -47) also resulted in a significant reduction in luciferase activity ($p<0.01$). Yet, no significant different in luciferase activity was found between single (HNF1) and double (HNF1 plus CDX2) sites mutation. Values are expressed as mean ± S.D. of at least three independent experiments.

Figure 3 Binding of HNF1 and CDX2 on CDH17 promoter sequence in vitro and in vivo. (A) Radiolabeled CDX2 and HNF1 probes (WT and mutant) were incubated with Hep3B and MHCC97L nuclear extracts and then resolved on a 5% polyacrylamide gel. The presence of CDH17-TF (either CDX2 or HNF1α) complexes suggests binding of TF and DNA in vitro. Unbound probes migrated to the bottom of gels. Addition of excessive (10X and 50X) specific WT (wt), but not mutant (mut) cold probes reduced the signal specifically. While the presence of specific antibodies binds to the TF-DNA complexes further retarded the migration of the complex in the gel (supershift). The sequences of the probes used were shown at the bottom. **(B)** ChIP assay was used to detect the presence of DNA-TF(CDX2 and HNF1α) complexes in MHCC97L and Hep3B cells in vivo. Antibodies against the CDX2, HNF1α, HNF1β, and IgG control, were used to immunoprecipitate the TF-DNA complexes. After recovery, the purified DNA was amplified by CDH17-specific PCR reaction. Input controls were genomic DNA extracted from MHCC97L and Hep3B cells without cross-linking and immunoprecipitation steps.

Figure 4 Expression of CDH17, HNF1α and CDX transcripts in human HCC cells and mouse liver. (A) Quantitative PCR was used to quantify the expression of CDH17,

HNF1α and CDX2 transcripts. The expression of CDH17, HNF1α and CDX2 transcripts were highest in human MHCC97L and lower in Hep3B and MIHA cells; while gradually decreases from E13.5, E16.5, new born (NB), 3 weeks old (3 wk) adult mouse liver. **(B)** Western blot analysis confirmed a high expression level of CDH17, HNF1α and CDX2 proteins in MHCC97L cells. A lower expression level was found in Hep3B and MIHA cells. The protein expression levels of CDH17 and HNF1α decrease drastically in mouse fetal liver from E135 until born. Yet, the expression of CDX2 protein remains constant through development. The expression of β-actin protein was used as loading control.

Figure 5 siRNA-mediated knockdown of HNF1α and CDX2 in HCC cells. (A) Targeting siRNA oligonucleotides against the HNF1α and CDX2 genes were transfected into the CDH17 expressing MHCC97L cells. Quantity of the CDH17, HNF1α and CDX2 transcripts was measured by real-time qPCR. Vector and scramble siRNA were included as controls in each experiments. Data were shown as mean ± S.D. from three independent experiments. * denotes statistically significantly different at $p<0.05$. **(B)** Western blotting demonstrated that CDH17 protein expression was down-regulated after siRNA against HNF1α and CDX2.



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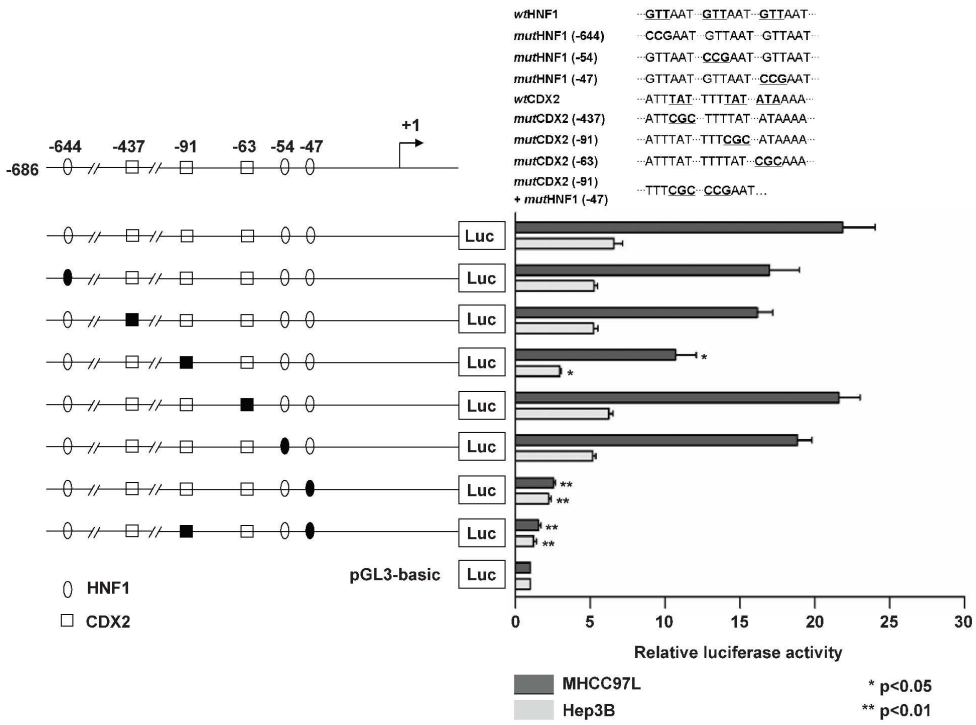

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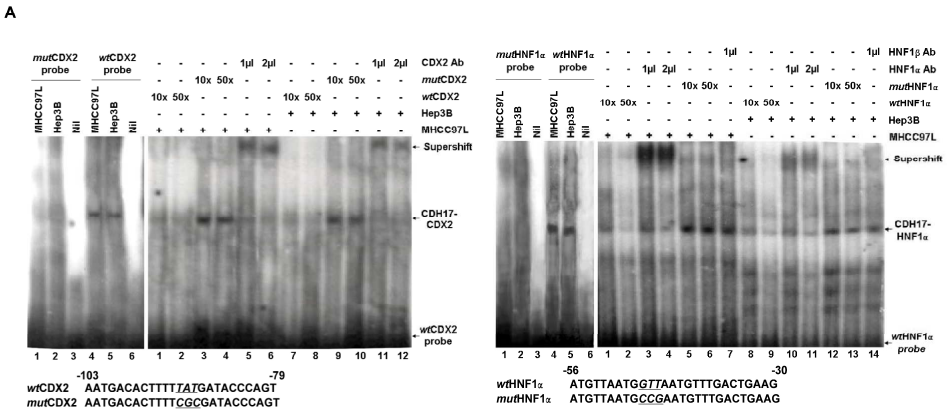

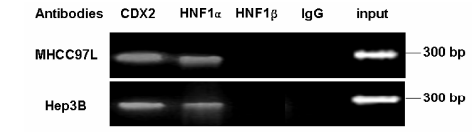

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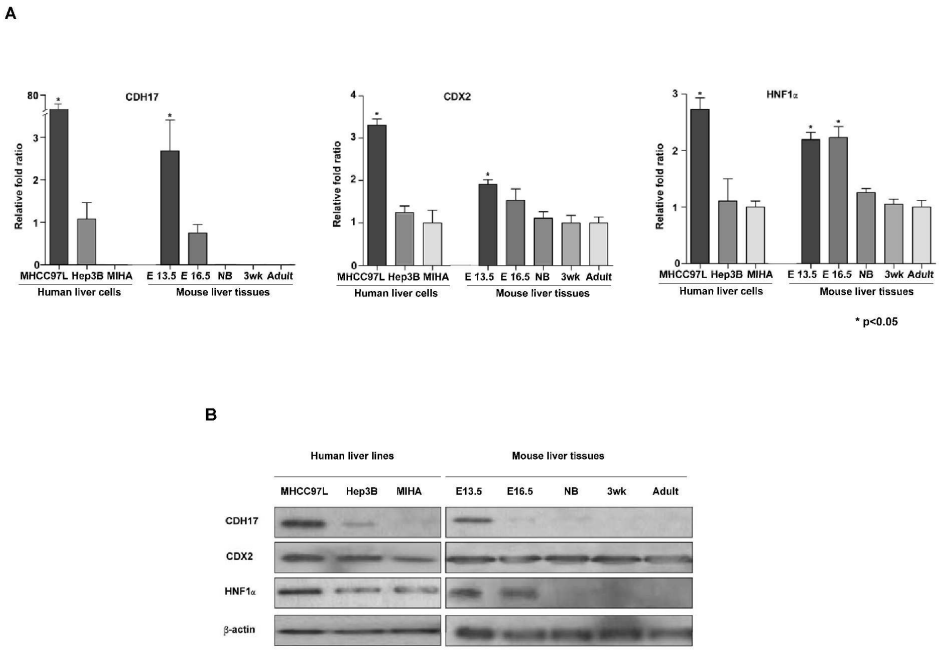

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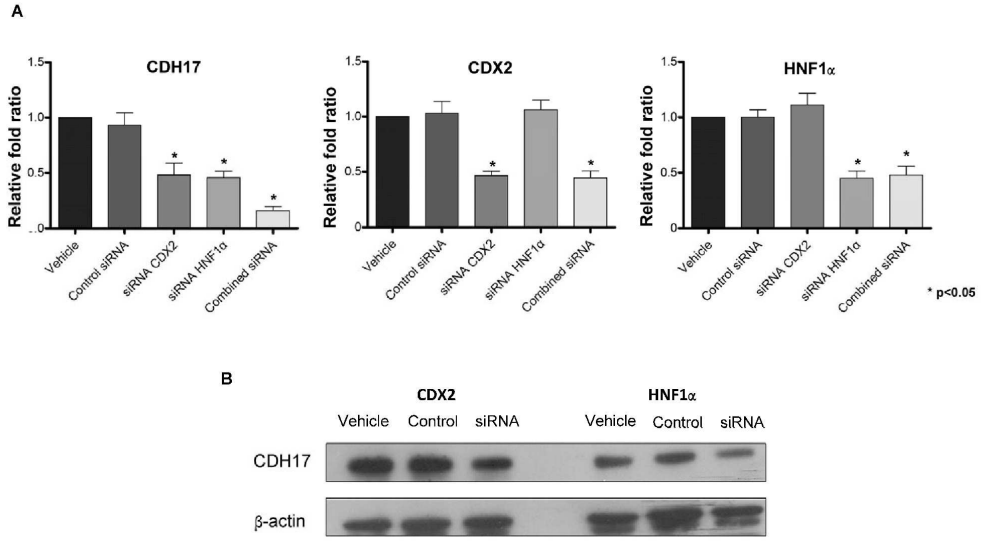

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Table 1 Primers used for real-time PCR for detection of transcripts in human liver cells and mouse liver tissues

Primer Name	Sequence	Product Size (bp)
Human		
CDH17 Forward	5'-GGGGGAGATACTCCAGTCGT-3'	167
CDH17 Reverse	5'-TCCAGTTGCCAAATAAAGCA-3'	
CDX2 Forward	5'-GAACCTGTGCGAGTGGATG-3'	158
CDX2 Reverse	5'-TCCTCCGGATGGTGATGTAG-3'	
HNF1α Forward	5'-CCATCCTCAAAGAGCTGGAG-3'	173
HNF1α Reverse	5'-CTGGTTGAGGCCAGTGGTAT-3'	
Mouse		
CDH17 Forward	5'-GGCCAAGAACCGAGTCAAGTC-3'	190
CDH17 Reverse	5'-CTCCATGAGAATCCAAGGCTG-3'	
CDX2 Forward	5'-CAAGGACGTGAGCATGTATCC-3'	106
CDX2 Reverse	5'-GTAACCACCGTAGTCCGGGTA-3'	
HNF1α Forward	5'-GACCTGACCGAGTTGCCTAAT-3'	103
HNF1α Reverse	5'-CCGGCTCTTTCAGAATGGGT-3'	