Pro-Inflammatory Homeobox Gene, ISX, Regulates Tumor growth and Survival in Hepatocellular Carcinoma

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Abstract

Chronic inflammation drives initiation of hepatocellular carcinoma (HCC) but the underlying mechanisms linking inflammation and tumor formation remain obscure. In this study, we compared the expression of IL-6 (IL6) and CYCLIN D1 (CCND1) with the IL-6 induced homeobox gene ISX (intestine specific homeobox) in 119 paired specimens of HCC and adjacent normal tissues, and also in paired specimens from 11 non-HCC patients. In pathological analysis, ISX exhibited a tumor-specific expression pattern and a high correlation to patient survival time, tumor size, tumor number and progression stage. Enforced expression of ISX accelerated cell proliferation and tumorigenic activity in hepatoma cells through CCND1 induction. In contrast, shRNA-mediated attenuation of ISX in hepatoma cells decreased cell proliferation and malignant transformation in vitro and in vivo. A high positive correlation existed in human hepatoma tumors between ISX and CCND1 expression. Together, our results highlight ISX as an important regulator in hepatoma progression with significant potential as a prognostic and therapeutic target in HCC.
Introduction

Hepatocellular carcinoma (HCC), the fifth most commonly occurring cancer and the third leading cause of cancer-related deaths every year worldwide, has a multiple step progression with a high evidence of association with chronic inflammation exposure induced by environmental toxin intake and/or viral infection, such as HBV or HCV (1). The chronic inflammation often interacts with innate and adaptive immune responses, and the detailed regulatory mechanism leading to HCC tumor formation is unclear to date.

D-type cyclins are the major regulators governing G1 progression to S phase in response to mitogenic and oncogenic signals and serve as markers for human malignancies (2, 3). Through binding with and activation of their associated cyclin-dependent kinases, CDK4 and CDK6, cyclin D-CDK complexes phosphorylate the retinoblastoma tumor suppressor gene products, pRB, and pRB-related proteins, p130 and p107 (4, 5). This phosphorylation aborts growth-inhibitory functions of pRB, which leads to release of the E2F1 transcription factors and allows induction of E2F1-target genes that are required for progressing into S phase (6). The growth-promoting functions and deregulation expression of D cyclins is a driving force toward increased tumor proliferation and transforming activity in several human cancers (2, 7). D cyclin overexpression in human cancers is driven by several mechanisms including transcriptional activation, genome alteration, post-transcriptional regulation, and post-translational protein stabilization (2, 3). Furthermore, several mouse models have also shown that cyclin D1 can cooperate with other
oncogenes to transform cells (8, 9). The oncogenic characterization of cyclin D1 explored in cancer models and human cancers suggests that cyclin D1 and its associated CDKs’ activity may be a potential therapeutic target (3).

Homeobox genes, a superfamily of transcription factors with homeo domains, play an essential role in controlling cell growth, differentiation, and morphogenesis during early embryonic development (10). Deregulation of homeobox genes appears to increase cell survival and proliferation, and inhibit cell differentiation (11, 12). Recently, many homeobox genes were found to be aberrantly expressed in a wide variety of human tumor masses (13). Isx (Intestine-specific homeobox) is a newly identified pair-family homeobox transcription factor, which shows a intestine-specific expression pattern in both adult and fetal intestines (14). Phylogenetic analysis showed that the homeo domain of Isx belongs to the Paired sub-family and is homologous to Pax3, Pax7, and Prrx1. Targeted disruption of Isx in mice revealed that Isx was required for intestine-specific regulation of the high density lipoprotein (HDL) receptor and cholesterol transporter scavenger receptor class B, Type 1 (SRB1), for vitamin A metabolism (14).

In this study, we identified Isx as a pro-inflammatory cytokine-induced homeobox gene, which is ectopically expressed in hepatocellular carcinoma (HCC). By directly binding to the cyclin D1 promoter, Isx regulated cellular cyclin D1 expression which further increased cell proliferation and transforming activity. Furthermore, both Isx and cyclin D1 were upregulated in both hepatoma
tumor cell lines and tumor masses. The cellular function and tumor-specific expression pattern suggests that Isx is an important activator in proliferation and tumor formation in hepatocellular carcinoma.
Materials and Methods

Patients

This study assessed a total of 123 HCC patients from July 2004 to November 2009 from multiple medical center (Chung Ho Memory Hospital (89 HCC) and Changhua Christian Hospital (34 HCC)) with HBV and/or HCV infection and 11 non-HCC (4 infected with HBV/HCV and 7 non-infected with HBV or HCV) patients were enrolled into the Isx study, 119 of whom had adequate follow-up data for analysis. Experiments of human samples have been approved by Institutional Review Boards of Kaohsiung Medical University (Kaohsiung, Taiwan). (15)

Cell lines

HCC cell lines (Hep G2, Hep 3B, SK-Hep1, Huh-7 and HA22T) were obtained from ATCC and sub cultured and maintained according to ATCC’s protocol.

Western Blot, Luciferase Reporter Assays and Immunohistochemical Staining Analysis

Western blot, Luciferase Reporter Assays and immunohistochemical (fluorescence) staining analysis were done as previously described (15).

Q-PCR, Chromatin Immunoprecipitation (ChIP) and EMSA

The above assays were analyzed as previous described (15).

Tumorigenic assay of Isx in soft agar and nude mice assay

Cells ($10^4$ or $5 \times 10^3$) in 1-mL culture medium were mixed with an equal volume of 0.6% of top agar and plated onto 60-mm dishes with 0.5% bottom agar (15). More detail processes could be found in Supplemental Methods and Materials.

Statistical analysis

The quantitative variables are presented as means and standard deviations. Statistical differences were determined with 2-sample t-test. Pearson’s correlations were used to determine the correlation coefficient between expression levels of Isx and Cyclin D1. Statistical analysis of categorical
variables was carried out using chi-square analysis, one-way ANOVA and Fisher exact analysis. P values <0.05 were considered statistically significant.
Results

Patient characteristics

Isx is an ectopically expressed homeobox gene found in hepatoma microarray analysis (16); however, the correlation between clinical outcome of HCC and cellular function of Isx is unknown to date. In order to characterize the regulatory effect and clinical outcome of Isx in HCC, 123 HCC patients from two medical centers were enrolled into the Isx cohort study from July 2004 to November 2009. Of these, 119 had adequate follow-up data for analysis. The baseline characteristics of HCC and non-HCC patients were compared between groups with non-HCC, low and high Isx expression and the results are shown in Table 1. The overall survival time of all patients was 48 months. There were significant associations in terms of albumin (p = 0.0040), bilirubin (p = 0.0282), ac sugar (p = 0.0281), tumor size (p < 0.0001), number of tumors (p < 0.0001), and tumor grade (p < 0.0001), but not in age, sex, GOT, GPT, alkaline phosphatase, α-fetoprotein, γ-GT, cholesterol, and triglycerides (Table 1).

The Isx expression pattern was then detected in HCC tumor samples by immunohistochemistry staining with anti-Isx polyclonal antibody (Fig. 1A). Isx (brown, yellow arrows) showed a tumor-specific expression pattern in HCC tumor mass (left panel) compared with the adjacent normal liver tissue (black star) and was detected in both cytoplasm (red arrow) and nuclei (blank arrow) in tumor cells (right panel). Isx mRNA expression in HCC liver samples (81 patients with high Isx expression and 38 patients with low Isx expression) was significantly upregulated
compared with non-HCC patients analyzed with one-way ANOVA (Fig. 1B; p < 0.001). Analysis of the survival curves of HCC patients found that HCC patients with lower Isx expression had a significantly longer survival time than those with higher Isx expression after liver excision surgery (Fig. 1C, p = 0.0238). Isx expression seems to correlate with the expression of cyclin D1, an important cell cycle regulator. Three HCC tumor species (with HBV and HCV infection) and six different hepatoma cell lines were found to have high ectopic Isx protein expression compared with normal liver tissue (with no HBV/HCV infection) (Fig. 1D). The Isx and cyclin D1 mRNA expression in HCC tumor specimens from 99 HCC patients and 11 non-HCC liver biopsies showed that the Isx mRNA expression was highly associated with cyclin D1 expression in all patients (R = 0.7415, p < 0.0001, Fig. 1E). These results suggest that Isx plays an important regulatory role in HCC progression and patients’ survival. The highly correlated expression between Isx and cyclin D1 also suggests that Isx could be a predictive marker for cyclin D1 expression and hepatoma growth.

**Pro-inflammatory cytokines induced Isx expression through NF-κB signaling pathway**

To explore the association of Isx expression with inflammatory cytokines in HCC tumor formation, the pro-inflammatory cytokines elevated in serum or liver tissue of hepatoma patients in previous studies (17-19) were first used to treat six HCC cells (Hep G2, Hep 3B, SK-Hep1, Huh 7, PLC/PRF/5, and HA22T) to evaluate the induction activity of Isx. Pro-inflammatory cytokines, such as IL-6, IL-1, and TNF-α, were shown to be able to increase the expression of Isx mRNA at
both the lower and higher concentrations used (5 and 20 ng/ml, respectively) for 8 hours (Fig. 2A). Higher, but not lower, doses of IL-8, IL-1β and TNFβ also were shown to increase Isx mRNA expression. In order to further clarify the regulatory signaling of Isx by these cytokines, the Isx promoter region was sub-cloned into a luciferase assay system. IL-6, an elevated cytokine in serum sample of HCC patients (20) showed a dose-dependent regulatory effect on the promoter activity of Isx (Fig. 2B and C). Also, IL-6 (10 ng/ml) was shown to increase Isx protein and cyclin D1 expression in most of hepatoma cell lines except Huh 7 as well as NF-κB signaling (phosph-p65 and IκBα) activation (Fig. 2 D). E2F1 in Isx transfected cells, such as Hep G2, SK-Hep 1 and Huh 7, also showed an increase in protein level. Further, the Hep G2 cells treated with IL-6 (10 ng/ml) were separately co-incubated with different kinase-specific inhibitors to evaluate the transcriptional activation and the Isx expression was analyzed with Western blotting. The activated Isx protein expression induced by IL-6 treatment was aborted dose-dependently by a NF-κB specific inhibitor, BAY 11-7085 (5 μM and 25 μM) (Fig. 2E). The expression correlation between Isx and IL-6 was also analyzed in HCC patients. Isx mRNA showed high correlated expression with IL-6 mRNA (R²=0.80) (Supplemental Fig. 1). These results showed that IL-6 transcriptionally activated Isx expression through the activation of NF-κB signaling.

The transcriptional regulatory elements induced by IL-6 on Isx promoter (Fig. 3A) were then analyzed by promoter assay and chromatin immunoprecipitation (ChIP). IL-6 significantly increased Isx promoter-driven luciferase activity (3945 ± 253) until the promoter sequence had been
deleted to a length shorter than 220 bp (Fig. 3B). Different segments of oligonucleotides between -320 to -220 bp in the Isx promoter region were then synthesized to determine the NF-κB (p65) binding affinity by electrophoresis mobility shift assay (EMSA) in vitro. The nuclear proteins of IL-6-treated cells showed high binding affinity and were supershifted by the addition of an anti-p65 antibody in the -294 to -318 bp region on the Isx promoter (Fig. 3C). Deletion of this NF-κB binding element showed abortion of the luciferase activity induced by IL-6 (Fig. 3B). Further, the Isx promoter region (-320 to -220 bp) could be pulled down (3.91 folds) in p65 (Rel A) immunoprecipitates treated with IL-6 compared with vehicle-treated cells (Fig. 3D). This promoter binding activity of p65 induced by IL-6 was abated when the cells were treated with NF-κB-specific inhibitor, BAY 11-7085 (5 μM), MAPK kinase inhibitor (U0126, 5 μM) and JAK/STAT2 inhibitor (AG490, 20 μM). These results suggested that signals (MAPK and JAK/STAT2) activated by IL-6 could activate Isx mRNA expression through increasing NF-κB (p65) binding activity on Isx promoter. However, NF-κB signaling activated by IL-6 regulated Isx expression at both the transcriptional and translational level.

Moreover, dietary Vitamin A intake has been shown to regulate intestinal Isx expression (3), and its metabolites are suggested to play a role in tumorigenesis. We next examined the potential role of vitamin A and its related metabolites on IL-6-induced Isx expression. Higher levels of retinoid X receptor (RXR) binding to the Isx promoter were detected in hepatoma cells than those noted in normal hepatocytes which showed no detectable expression of Isx (Fig. 3E). Interestingly, while
IL-6 treatment increased Isx expression in hepatoma cells, the levels of RXRα’s binding to the Isx promoter were significantly reduced (ranging from 52 to 58%) in IL-6-treated hepatocytes, SK-Hep1 cells and, to a lesser degree, in HepG2 cells (18% reduction) (Fig. 3F). These results suggested that the Isx expression induced by IL-6 was a retinoid–independent regulatory pathway.

**Isx expression enhanced cell proliferation in hepatocellular carcinoma cell lines**

To characterize the cellular function of Isx, wild Isx and different truncated proteins tagged with GFP were transfected and characterized in Hep G2 cells. Overexpressed Isx protein (green) was mainly detected in nuclei (blue), but Isx protein with the deleted homeobox domain was detected in whole cells including cytoplasm and nuclei, with loss of its original nuclear localization (Fig. 4A). Interestingly, the GFP protein fused with the Isx homeobox domain showed a nuclear translocation pattern similar to that of the wild Isx protein (Fig. 4A). The cell proliferation activity of Isx in Hep G2 cells was then determined with cell growth curves and [H³] thymidine incorporation analysis. Hep G2 cells transfected with Isx or GFP with Isx homeo domain showed a higher proliferation rate than that of cells transfected with mock only or Isx protein without the homeo domain (Fig. 4B). Also, overexpressed Isx increased [H³] thymidine incorporation activity 4.9 fold in comparison to mock transfected Hep G2 cells after 16 hours incubation (Fig. 4C). The increase in proliferation activity induced by Isx was further observed in cell cycle analysis with flow cytometry. The Hep G2 cells transfected with Isx showed an increase in sub-G1 (1.6%–4.2%) and S phase cell cycle population (20.3%–30.5%) and a decrease in G1 (42.8%–39.6%) and G2/M (25.7%–18.9%) cell
cycle population compared with mock transfected only (Fig. 4D). These results suggested that Isx increased cell proliferation to speed up cell cycle progression from G1 to S phase despite an increase in sub-G1 apoptotic cells.

**Isx up-regulated cyclin D1 and E2F1 expression in HCC cells**

To determine the direct targets regulated by Isx, established regulators of the G1/S transition were monitored by Western blot analysis. As shown in Fig. 5A, overexpression of Isx in Hep G2 cells increased D cyclins (1 and 3) and E2F1 protein expression and cells transfected with Isx containing a deleted homeobox domain showed reduced activation effect on D cyclins and E2F1 expression. The activation effects of cyclin D1 and E2F1 by Isx were further confirmed in five hepatoma cell lines. Cyclin D1 in Isx transfected cells except Huh 7 showed an increase in protein expression level (Fig. 5B). E2F1 in Isx transfected cells, such as Hep G2, SK-Hep 1 and Huh 7, also showed an increase in protein level. The regulatory effect of Isx on these effected G1/S regulators was further addressed by promoter assay. E2F1 (-1630 to +30 bp) and cyclin D1 (-1721 to +21 bp) promoter regions amplified from human placental genome by PCR were sub-cloned into a luciferase expression system. Hep G2 cells transfected with Isx protein significantly increased luciferase activity driven by cyclin D1 and E2F1 promoter, respectively, as compared with mock-transfected cells (Fig. 5C). This result further confirmed that Isx protein activated cyclin D1 and E2F1 expression transcriptionally. To clarify the possible regulatory site of Isx, the cyclin D1 promoter and different truncated promoter regions of cyclin D1 (Fig. 5D, upper panel) were
sub-cloned into luciferase reporter constructs and transfected into Hep G2 cells to evaluate the transcriptional activation effects of Isx. As shown in Fig. 5D (lower panel), Isx upregulated the luciferase activity driven by the cyclin D1 promoter and the upregulated luciferase activity induced by Isx expression decreased to the mock-transfected level when the cyclin D1 promoter region was shorter than -260 bp. This result suggested the transcriptional regulation of Isx on cyclin D1 promoter was through the binding sequence between -260 to -297 bp.

**Isx increased cyclin D1 expression through directly binding to the cyclin D1 promoter in Vitro and in Vivo**

To further delineate the regulatory elements of Isx on the cyclin D1 promoter region, the different regions of oligonucleotide between -260 and -297 bp in cyclin D1 promoter were synthesized for EMSA analysis *in vitro*. The nuclear proteins extracted from Hep G2 cells transfected with Isx could specifically bind to the region between -260 and -272 bp and the binding complex also showed a super shift (arrow) of the anti-Isx antibody *in vitro* (Fig. 5E). The cyclin D1 promoter fragment (-197 to -330bp) bonded by Isx was detected by ChIP assay *in vivo* and showed an increase up to 11.6-fold compared with mock transfected cells (Fig. 5F). The binding activity of Isx on cyclin D1 promoter abated after NF-κB inhibitor (BAY 11-7085, 5 μM) treatment (Fig 5F). Interestingly, MAPK (ERK1/2) inhibitor (PD98059, 50 μM) also showed a decrease to baseline on the cyclin D1 promoter binding activity of Isx (Fig. 5F). The regulatory effect of Isx on cyclin D1 was also determined in HCC patients. Isx (green) showed highly expression localization with cyclin
D1(red) and Ki67(pink), a cell proliferation marker) (Supplemental Fig. 2). These results showed that Isx directly bound to cyclin D1 promoter and activated cyclin D1 (E2F1) expression transcriptionally.

**Isx expression was essential for cyclin D1 expression and proliferation activity in hepatocellular carcinoma cells**

To further evaluate the essential role of Isx on cell proliferation and tumorigenic activity, two sequence-specific shRNAis of Isx were transfected into four types of hepatoma cells with ectopic Isx expression (Hep G2, Hep 3B, SK-Hep1, and HA22T) and the knock-down efficiency in these hepatoma cells was examined by Western blot (Supplemental Fig. 3 and Fig. 6A). Isx protein expression was decreased 85% in Hep G2 cells but the knock down was less efficient in Hep 3B cells. These hepatoma cells with Isx knock down were first examined to determine the cell proliferation activity by [H\(^3\)] thymidine incorporation assay. The [H\(^3\)] thymidine incorporation rate in Isx knocked down cells showed a significant decrease of 62%, 56%, 54%, and 68%, respectively, in thymidine incorporation activity compared with those that were mock-transfected (Fig. 6B).

Further, many G1/S transition regulators, such as cyclin D1 (84%), CDK4 (62%), and E2F1 (86%), were significantly downregulated in Isx shRNAi cells (Fig. 6C and Supplemental Fig. 4). Some apoptotic factors were also shown to elevated in Isx shRNAi cells (Supplemental Fig. 5). Other tumor suppressors, such as p19 and p21, did not show any regulatory effect.

**Isx expression was critical for hepatoma tumorigenic activity**

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Following the determination of cellular proliferation, the transformational and tumorigenic activity of Isx shRNAi cells were then determined with a foci formation on soft agar anchorage independent assay \textit{in vitro} and tumor growth in nude mice \textit{in vivo}. As shown in Fig. 6D, overexpression of Isx increased the transforming activity (54%) in Isx transfected cells compared with mock-transfected Hep G2 cells; however, Isx shRNAi cells showed a significant decrease in transforming activity (84%) in comparison with mock-transfected cells \textit{in vitro}. Further, Hep G2 (Fig. 6E) and Hep 3B cells (Fig. 6F) with overexpressed Isx showed an increase in tumor size and tumor growth activity in nude mice \textit{in vivo} compared with mock-transfected cells and Isx shRNAi cells showed a significant decrease in tumor size and tumor growth activity compared with mock-transfected cells. These results suggest that Isx plays an essential role in promoting the transforming and tumorigenic activity of hepatoma cells.
Discussion

In this study, we identified Isx as a proto-oncogenic homeobox gene specifically overexpressed in hepatocellular carcinoma (HCC) tumor cells. Isx, an IL-6-induced homeobox gene, upregulated cyclin D1 and E2F1 expression through the NF-κB signaling pathway. Through direct binding to the cyclin D1 promoter, Isx increased cellular cyclin D1 expression through which it regulated the cell proliferation and transforming activity of HCC tumor cells in vitro and in vivo. The tumor-specific expression pattern, highly correlated to survival time, tumor progression, and cyclin D1 (IL-6) expression in HCC patients, suggested that Isx plays an essential role in hepatoma tumor formation and prognosis.

HCC is a chronic liver disease that occurs as a multistep process characterized by progressive accumulation of genetic alterations causing aberrant growth, malignant transformation, and metastasis (21). Homeobox genes, essential regulatory transcription factors for multiple body plan development (10), have been found to be deregulated in many malignancies and are thought to be potential oncogenes (11). Few homeobox genes have been found to be involved in HCC development, excluding Hox, Prox1, and CDX2; however, most of them are expressed both in normal and tumor cells, and are thought to act as tumor suppressors in the liver (22-24). In this study, we found Isx, in particular, ectopically expressed in tumor cells of HCC tumor mass, but not normal cells, and highly correlated to cyclin D1 expression in HCC tumor cells. This tumor-specific pattern and the gene silencing results suggest that Isx is an important proto-oncogenic protein in
HCC development. Isx, a gut specific transcription factor, was a putative repressor for intestinal SRBI and BCMO1 expression that consequently repressed vitamin A production and downstream retinoic acid (RA) signaling (3, 14). Retinoid-induced signaling is important in regulating cell growth, differentiation, and development (25), and found to abnormally express and highly associated with tumor development in many malignances, including HCC (26). Deficiency in retinol and RXRα expression were found in tumor cells, but not the adjacent normal hepatocytes in HCC patients and animal model, which was adversely to Isx expression in HCC patients (27).

Also, our study suggested that IL-6-induced Isx expression in hepatoma cells was independent of the retinoid signaling. These results appeared to be at variance with the previous studies showing that dietary vitamin A intake was able to increase intestinal Isx expression (3). IL-6 might regulate Vitamin A metabolism via Isx expression, but this is, at present, uncertain in the case of HCC. More studies would be needed to elucidate the regulatory effect. Further, the regulatory activity on Vitamin A by ectopic Isx expression is still unclear, although defects of vitamin A metabolism have been observed in Isx knockout mice.

In Isx-/- mice, the cell growth regulatory and transforming activity was not observed in tissue development and the offspring were born in the expected numbers and appeared healthy for at least 1 year (14). This regulatory effect of Isx on proliferation and the tumor-specific ectopic expression pattern in HCC tumor cells suggests a malignant genetic alteration profile induced by chronic inflammation exists in HCC tumor cells. This malignant alteration circumvents activated Isx
expression and a redundant regulatory effect in the intestine aborted the cellular proliferation effects.

The genomic alterations at different stages are still unclear. In this study, ectopic Isx expression was detected in HCC patients, and some non-HCC patients infected with HBV and/or HCV (with no hepatitis) also showed the decreasing trend in the level of Isx mRNA expression, although the difference did not reach not statistical significance. Isx expression in different liver disease stages was still unclear, because the limitation in sample collection and the detailed regulatory mechanism of Isx in HCC tumorigenesis needs further investigation.

Chronic inflammation induced by hepatitis B virus, hepatitis C virus, and alcohol are always correlated to HCC development and serves as the most common factor involved in HCC progression (28, 29). Many pro-inflammatory cytokines, such as TNFα, IL-1 and IL-6, are found at high serum concentrations in HCC patients; however, the pathological role of these cytokines in HCC development is unclear to date (18, 20). In this study, we demonstrated that Isx was showed high expression correlation to IL-6 in HCC patients and was an activated proto-oncogene through inflammatory cytokines (IL-6 or TNFα) that then subsequently regulate downstream cell cycle regulators, such as cyclin D1 and E2F1, to progress to cell proliferation and transformation in HCC. This result provides a positive linkage between inflammation and HCC development. IL-6, one of the pro-inflammatory cytokines, has been reported to have high expression levels in HCC patients (19, 20) and plays an important regulatory role in HCC development (18). Many viral proteins from HBV or HCV, and also alcohol abuse, all increase the IL-6 expression level in liver cells (28, 30).
IL-6 activates many signaling pathways to transform cellular responses, including JAK/STATs, MAPK, and AKT/PI3K (17, 31), and, through activating JAK/STAT3 signaling, NF-κB and downstream genes, such as Isx, are consequently activated to mediate cell survival and G1 to S cell cycle transition. In this study, one potential IκB binding site (RNNYYCC) on the Isx promoter region (-294 to -318 bp) was found to respond to NF-κB regulatory activation in Isx expression. The results of this element deletion on the Isx promoter showed that this potential IκB element was essential to NF-κB activation. Also, from the mouse model, increased IL-6 production has also been implicated in the pathogenesis of HCC (32). Further, in liver regeneration, IL-6 regulates cellular proliferation through the activation of cyclin D1 expression in hepatocytes, which provides a model for cyclin D1 activation by pro-inflammatory cytokines in HCC development (33).

In summary, in this study we found that Isx was an important tumor-specific proto-oncogenic homeobox gene. Through direct regulation of cyclin D1 and E2F1 expression, Isx regulated the proliferation of tumor cells and their transforming activity, and it appears worthwhile to further investigate its cellular function in HCC.
Acknowledgements

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References
17. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of
Table 1. Baseline characteristics of 119 hepatocellular carcinoma (HCC) patient comparing to 11 non-hepatocellular carcinoma patients

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<td>&lt;0.0001**</td>
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<td>&lt;0.0001**</td>
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ALT, alanine aminotransferase; *p values were calculated by Fisher exact test; **p<0.05

Patients: 123 HCC patients from two medical centers [Chung Ho Memorial Hospital (89 HCC) and Changhua Christian Hospital (34 HCC)] with HBV and/or HCV infection and 11 non-HCC
Figure Legends

Figure 1. Ectopic Isx showed a tumor-specific expression pattern and significantly decreased survival time in hepatocellular carcinoma. A, Isx expression showed a tumor-specific expression pattern in hepatocellular carcinoma tumor species. Isx, brown color; hepatocellular carcinoma, yellow arrow. Black star (*), area of normal hepatocytes. Isx (cytoplasm), red arrow; Isx (nuclear), black arrow. B, Isx mRNA significantly expressed in hepatocellular carcinoma (HCC) species. C, high Isx mRNA expression in HCC decreased survival time in HCC prognosis analyzed by Kaplan-Meier survival analysis. D, ectopic expression of Isx highly associated with cyclin D1 expression in HCC cell lines and patients. HCC1, 2, and 3, HCC tumor mass. Hep G2, Hep 3B, SK-Hep1, Huh 7, PLC/PRF/5, and HA22T, HCC tumor cell lines. E, the mRNA expression of cyclin D1 in non-HCC and HCC tumor species showed highly correlated expression with Isx mRNA expression.

Figure 2. Inflammatory cytokines induced Isx expression. A, cytokines and chemokines induced Isx mRNA expression in Hep G2 cells. **, p < 0.005; ***, p < 0.001. B, IL-6 induced Isx promoter-driven luciferase activity in Hep G2 cells. ***, p < 0.001. C, IL-6 showed a dose-dependent activation on Isx promoter-driven luciferase activity in Hep G2 cells. ***, p < 0.001. D, IL-6 activates Isx protein expression and NF-κB signaling. E, Specific inhibitors for NF-κB signaling pathway abated the activation of Isx mRNA (bottom panel) and protein expression(up panel) induced by IL-6 (10ng/ml). *, p < 0.001, compared with vehicle treatment. #,
p < 0.001, comparing to IL-6. (n=3, means ± SD)

Figure 3. Pro-inflammatory cytokine, IL-6, transcriptionally increased Isx expression through NF-κB activation. A, Illustration of serious Isx promoter deletion constructs. B, Isx promoter assay induced by IL-6 treatment. ***, p < 0.001. C, IL-6 increased NF-κB (p65) binding activity on different oligonucleotides of Isx promoter. V: nuclear proteins extracted from vehicle treated cells. D, NF-κB-specific inhibitor, BAY 11-7085(5 μM), abated the Isx promoter binding activity of NF-κB (p65) induced by IL-6. ***, p < 0.001. E, Hepatoma showed higher binding activity of retinoid X receptors (RXRs) on the Isx promoter than hepatocyte. ***, p<0.001. F, IL-6 decreased Isx promoter binding activity of RXRs in hepatocyte and hepatoma. *, p<0.05. (n=3, means ± SD)

Figure 4. Overexpressed Isx increased cell cycle progression in Hep G2 cells. A, GFP fused Isx expression pattern in Hep G2 cells. Upper panel, truncated construct maps of Isx. Lower panel, the cellular localization of Isx and truncated Isx proteins. Isx, green; F-actin, red; nuclei, nuclei, blue (DAPI). B, Isx overexpression increased cell proliferation and cell growth. *, p < 0.001. C, Isx increased cellular thymidine incorporation activity. *, p < 0.001. D, cell population analysis in different cell cycle phases of cells transfected with different Isx truncated protein. * and #, p < 0.001, compared to mock-transfected only. (n=3, means ± SD)

Figure 5. Isx increased cyclin D1 expression through direct promoter binding. A, Over expressed Isx increased the expression of cyclin D1 and E2F1. Arrow head, truncated Isx expression. B, Isx expression increased cyclin D1 and E2F1 expression in hepatoma cells. Arrow, E2F1. C, Isx
transcriptionally activated cyclin D1 and E2F1 promoter activity. ***, p < 0.001. D, Isx transcriptionally activated cyclin D1 promoter activity. Upper panel, series of deletion constructs of cyclin D1 promoter. Lower panel, relative luciferase activity. E, Isx nuclear extract from Hep G2 cells specifically binds the oligonucleotides of the cyclin D1 promoter region (-260-272bp) in vitro. The DNA binding activity of Isx nuclear extract was analyzed with electrophoresis mobility shift assay (EMSA). F, Isx directly bonded to the promoter region of cyclin D1 determined with chromosome immunoprecipitation assay (ChIP) in vivo. Upper panel, agarose gel electrophoresis analysis of ChIP. Arrow head, cyclin D1 promoter region (-197 to -330bp) Positive, 10% of input control; negative, no antibody. Lower panel, the quantitative statistical analysis of cyclin D1 promoter binding activity of Isx was determined by chromosome immunoprecipitation assay (ChIP). *, p < 0.001, compared with Isx-transfected only. (n=3, means ± SD)

Figure 6. Isx expression was essential for proliferation and transforming activity in hepatocellular carcinoma cells. A, Isx expression in Hep G2 cells was knocked down with two sequence-specific shRNAi constructs. B, hepatoma cells transfected with Isx shRNAi constructs showed a decrease in [H³] thymidine incorporation activity. *, p < 0.001. C, the cyclin D1 and E2F1 expression was decreased in Isx shRNAi cells. D, Isx shRNAi cells showed a decrease in tumor transforming activity in vitro. * and #, p < 0.001 compared with vector group. E and F, Isx shRNAi cells (Hep G2 and Hep 3B) showed a decrease in tumorigenic activity in vivo. ***, p < 0.001 compared to vector-transfected group. A and b, p < 0.001 compared to mock-transfected group. (n=3, means ± SD)
SD.
Figure 4; Hsu et al.

A

Anti-EGFP  actin  DAPI  DIC  Merged

Vector

lsx

△HD- lsx

Isx-HD

B

Cell Number (x10^4)

0  12  24  36  48  60 (hours)

C

Vector  lsx  △HD- lsx  HD(lsx)-GFP

CPM

D

Sub-G1  G1  S  G2/M

Counts

Hep G2- Vector  Hep G2- Isx

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Pro-Inflammatory Homeobox Gene, ISX, Regulates Tumor growth and Survival in Hepatocellular Carcinoma

Shih-Hsien Hsu, Li-Ting Wang, King-Teh Lee, et al.

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