

# SOX12 upregulation is associated with metastasis of hepatocellular carcinoma and increases CDK4 and IGF2BP1 expression

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**Abstract.** – **OBJECTIVE:** In this study, we studied the expression profile of SOX12 gene in different pathological stages of hepatocellular carcinoma (HCC) and explored the possible downstream genes related to its regulation of HCC invasion and migration.

**MATERIALS AND METHODS:** Bioinformatic data mining was performed in liver cancer cohort in TCGA. HepG2 cells were used as *in vitro* cell model to assess the effect of SOX12 on CDK4 and IGF2BP1 expression. The association between the expression of SOX12, CDK4 or IGF2BP1 and survival time in HCC patients was also assessed based on the data in TCGA.

**RESULTS:** The regional lymph node metastasis (N1) and distant metastasis (M1) cases had significantly increased SOX12 expression than the lymph node negative (N0) and distant metastasis negative (M0) cases respectively. CDK4 and IGF2BP1 were among the top 20 SOX12 co-upregulated genes in HCC, which have well characterized enhancing the effect on HCC cell growth, invasion, and metastasis. In HepG2 cells, knockdown of SOX12 reduced IGF2BP1 and CDK4 expression, while enforced SOX12 expression significantly enhanced their expression. Low SOX12 or CDK4 expression was associated with significantly better 3-year survival and better 5-year survival. Low IGF2BP1 expression was associated with significantly better 3-year survival in HCC patients.

**CONCLUSIONS:** SOX12 upregulation is associated with regional and distant metastasis of HCC. SOX12 can increase the expression of CDK4 and IGF2BP1, which confer malignant phenotypes to HCC. The expression of SOX12, IGF2BP1 or CDK4 might be potential clinical prognostic markers for HCC.

Key Words

SOX12, CDK4, IGF2BP1, Metastasis, Hepatocellular carcinoma.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and is also a leading cause of cancer-related death worldwide<sup>1</sup>. Local and distant metastases are indicators of poor prognosis and are also the main causes of cancer-related death<sup>2,3</sup>. Therefore, elucidating the molecular mechanisms underlying HCC metastasis is critical for identifying novel therapeutic targets.

SYR-related high mobility group (HMG) box (SOX) family proteins are a conserved group of transcriptional factors widely participate in both normal embryonic development and oncogenic processes<sup>4,5</sup>. Previous studies<sup>5,6</sup> found that the SOX factors have extraordinary regulations on cancer cell proliferation, migration, invasion and tumor metastasis in multiple types of cancer. SOX12 belongs to the SOXC group of SOX family, together with SOX4 and SOX11<sup>7</sup>. These three SOX members show overlapping expression patterns and molecular properties<sup>8</sup>. SOX12-null mice are viable and do not show obvious malformations due to the compensation of SOX4 and SOX11<sup>7</sup>. The oncogenic effect of SOXC group in HCC has been reported. Briefly, SOX4 contributes to hepato-carcinogenesis via inhibiting p53-mediated apoptosis<sup>9</sup>. SOX12 induces epithelial-mesenchymal transition (EMT) by trans-activating Twist1 expression and also enhances cancer cell invasion and metastasis via increasing the expression of fibroblast growth factor binding protein 1 (FGFBP1)<sup>10</sup>. Among the 16 SOX genes, SOX12 was identified as one of the most up-regulated genes in HCC compared to adjacent normal tissues<sup>10</sup>.

As a transcription factor, the downstream regulation of SOX12 in HCC is still not fully understood. In this paper, we further studied its expression profile in different pathological stages of HCC and explored the possible downstream genes related to its regulation of HCC invasion and migration.

## Materials and Methods

### **Data Mining in Liver Cancer Cohort in TCGA**

Gene expression in liver cancer cohort in TCGA was analyzed using the UCSC Xena browser (<http://xena.ucsc.edu/>). The genes co-expressed with SOX12 in liver cancer cohort in TCGA was analyzed using the cBioPortal for Cancer Genomics<sup>11,12</sup> and further verified using the UCSC Xena. The associations between the expression of SOX12, CDK4 or IGF2BP1 and survival of HCC patients were also assessed using the UCSC Xena.

### **Protein-Protein Interaction (PPI) Network**

The genes co-upregulated with SOX12 in liver cancer cohort in TCGA were loaded into the Search Tool for the Retrieval of Interacting Genes (STRING) (<http://string-db.org/>) database for analysis of PPI network. The evidence level was set to  $> 0.7$ , which indicates experimentally validated interactions.

### **Cellular SOX12 Expression Identification**

Immunostaining of the distribution and expression of SOX12 in HCC cell line HepG2 cells were reviewed in Human Protein Atlas, an online database offering the combination of transcriptomics and antibody-based proteomics for mapping the human proteome down to the single cell level<sup>13,14</sup>.

### **Cell Culture and Transfection**

Human HCC HepG2 cells were purchased from the American Type Culture Collection and were cultured in Dulbecco's Modified Eagle Medium (DMEM) at 37°C in a 5% CO<sub>2</sub> incubator. The medium was supplemented with 10% FBS, 100 µg/ml penicillin, and 100 µg/ml streptomycin. SOX12 lentiviral shRNA and lentiviral expression particles were obtained from Genecopoeia. HepG2 were infected with the lentiviral particles in the presence of Polybrene.

### **Quantitative Real-Time-PCR (QRT-PCR)**

24 h after infections, Total RNA was extracted from cell samples by using the High Pure RNA Isolation Kit (Bio-Rad, Hercules, CA, USA) and then was reversely transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Then, QRT-PCR was performed to detect SOX12 expression using TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) and the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative mRNA expression was normalized to GAPDH. The primers for SOX12 were: forward, 5'-CGCGATGGTGCAGCAGCG-3' and reverse, 5'-GCCACTGGTCCATGATCTTC-3'. Relative mRNA expression levels (fold changes) between groups were calculated using the  $2^{-\Delta\Delta CT}$  method.

### **Western Blotting**

Total protein were extracted from HepG2 cells 48 h after infection by using a lysis buffer. Then the samples were subjected a conventional western blotting as described previously<sup>15</sup>. The primary antibodies used include anti-SOX12 (SAB1411973, Sigma-Aldrich, Carlsbad, CA, USA), anti-CDK4 (ab137675, Abcam, Cambridge, MA, USA), anti-IGF2BP1 (ab82968, Abcam), and anti-β-actin (ab8226, Abcam). After incubation and washing, the membranes were further incubated with HRP conjugate secondary antibodies. The blot signals were visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA).

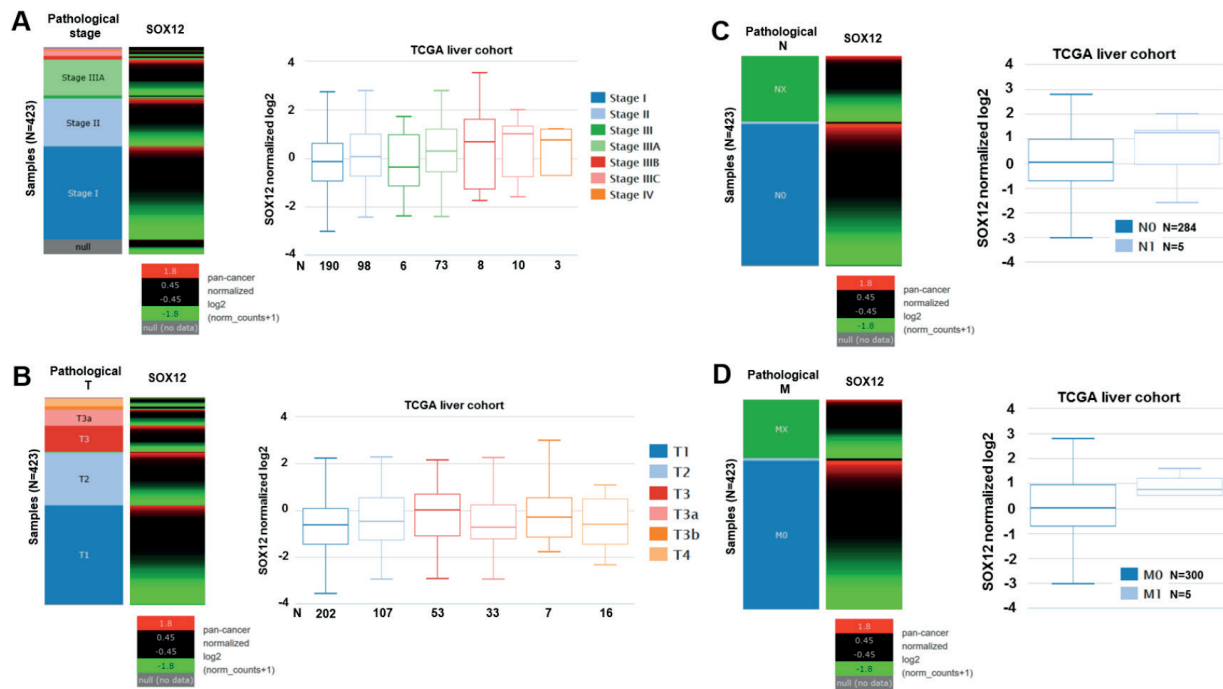
### **Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 6.0. The difference between groups was evaluated by unpaired, two-tailed Student's *t*-test. The difference between Kaplan-Meier curves were assessed using the log-rank test. The test statistics ( $\chi^2$ ) and *p*-value ( $\chi^2$  distribution) were given.  $p < 0.05$  indicates statistical significance.

## Results

### **SOX12 Upregulation is Associated with Regional and Distant Metastasis of HCC**

One recent study reported that SOX12 can promote metastasis of HCC<sup>10</sup>. In this study, we further characterized the expression profile of SOX12 in different stages of HCC. By using the data from liver cancer cohort in TCGA, we compared the expression profiles of SOX12 in different pathological stages (Figure 1A). The results showed



**Figure 1.** SOX12 upregulation is associated with regional and distant metastasis of HCC. **A-D**, Heat map (left) and bar chart (right) of SOX12 mRNA expression in different pathological stages (**A**), different T stages (**B**), different N stages (**C**) and different M stages (**D**) of HCC. The number of patients in different stages were listed below the bar chart. Analysis was performed using the UCSC Xena based on liver cancer cohort in TCGA database.

that SOX12 expression gradually increased with the development of pathological stages (Figure 1A). Then, we compared SOX12 expression in different T stages. The results showed similar SOX12 expression from T1 to T4 stages (Figure 1B). However, by comparing SOX12 expression between the cases with or without regional lymph node metastasis and between the cases with or without distant metastasis, we observed that the regional lymph node metastasis (N1) and distant metastasis (M1) cases had significantly increased SOX12 expression (Figure 1C-D).

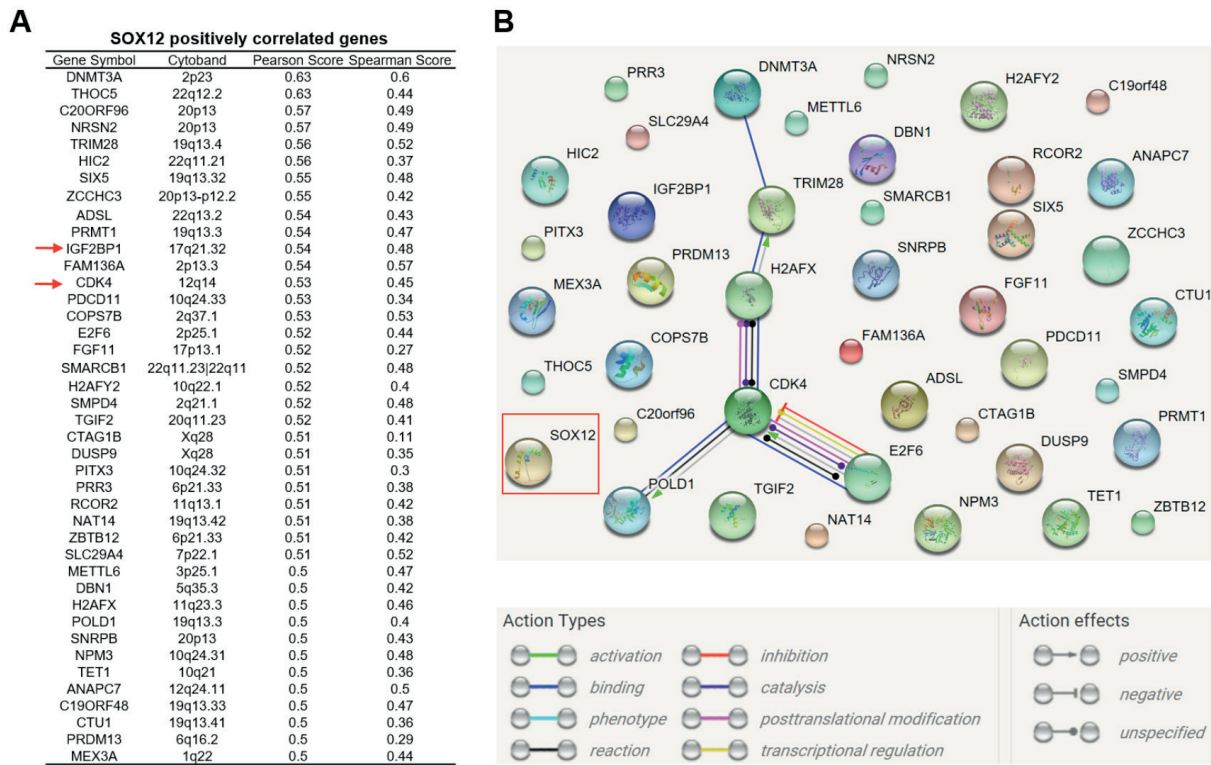
### ***SOX12 is Co-Upregulated with CDK4 and IGF2BP1 in HCC***

SOX12 can upregulate Twist1 and FGFBP1 in HCC, thereby promoting metastasis<sup>10</sup>. As a transcription factor, SOX12 might regulate expression of some other genes. Via data mining in liver cancer cohort in TCGA using cBioPortal for Cancer Genomics, we identified the most co-upregulated genes with SOX12 (Pearson's  $r \geq 0.5$ ) (Figure 2A). By performing PPI network analysis, we failed to identify any known interactions between SOX12 and the co-upregulated genes (Figure 2B). Interestingly, we found CDK4 and IGF2BP1 were among the top 20 co-upregulated genes in HCC (Figure

2A, red arrows), which have well characterized enhancing effect on HCC cell growth, invasion and metastasis<sup>16-18</sup>. Therefore, we decided to further investigate whether there is any regulative effect of SOX12 on CDK4 and IGF2BP1. By reviewing the heat map and performing regression analysis using the UCSC Xena, we further confirmed moderate level of co-upregulation between SOX12 and CDK4 (Figure 3A-B) and between SOX12 and IGF2BP1 (Figure 3A and C). SOX12 immunostaining in Human Protein Atlas showed that SOX12 has nucleoplasm expression in HepG2 cells (Figure 3D), which is consistent with its regulation at the transcriptional level. Then, HepG2 cells were infected with lentiviral SOX12 shRNA or lentiviral SOX12 expression particles respectively (Figure 3E and G). Following Western blotting data showed that knockdown of SOX12 reduced IGF2BP1 and CDK4 expression (Figure 3F), while enforced SOX12 expression significantly enhanced their expression (Figure 3H).

### ***Low SOX12, CDK4 or IGF2BP1 Expression is Associated with Better Survival Among HCC Patients***

Since metastasis is an indicator of poor survival, we further assessed whether the expression



**Figure 2.** PPI analysis of SOX12 and its co-upregulated genes in HCC. **A**, The most co-upregulated genes with SOX12 (Pearson's  $r \geq 0.5$ ) in liver cancer cohort in TCGA. Data was obtained by using cBioPortal for Cancer Genomics. **B**, PPI network among SOX12 and its co-upregulated genes in HCC.

of SOX12, CDK4 or IGF2BP1 is related to survival among the patients. In a two-group analysis by setting median gene expression as the cutoff, Kaplan-Meier curves and the log-rank tests showed that low SOX12 expression was associated with significantly better 3-year survival ( $p=0.008$ ) (Figure 4A) and better 5-year survival ( $p=0.008$ ) (Figure 4B). Low CDK4 expression was also associated with significantly better 3-year survival ( $p=0.005$ ) (Figure 4C) and better 5-year survival ( $p=0.007$ ) (Figure 4D). Low IGF2BP1 expression was associated with significantly better 3-year survival ( $p=0.018$ ) (Figure 4E). However, this association was not observed in 5-year survival ( $p=0.073$ ) (Figure 4F).

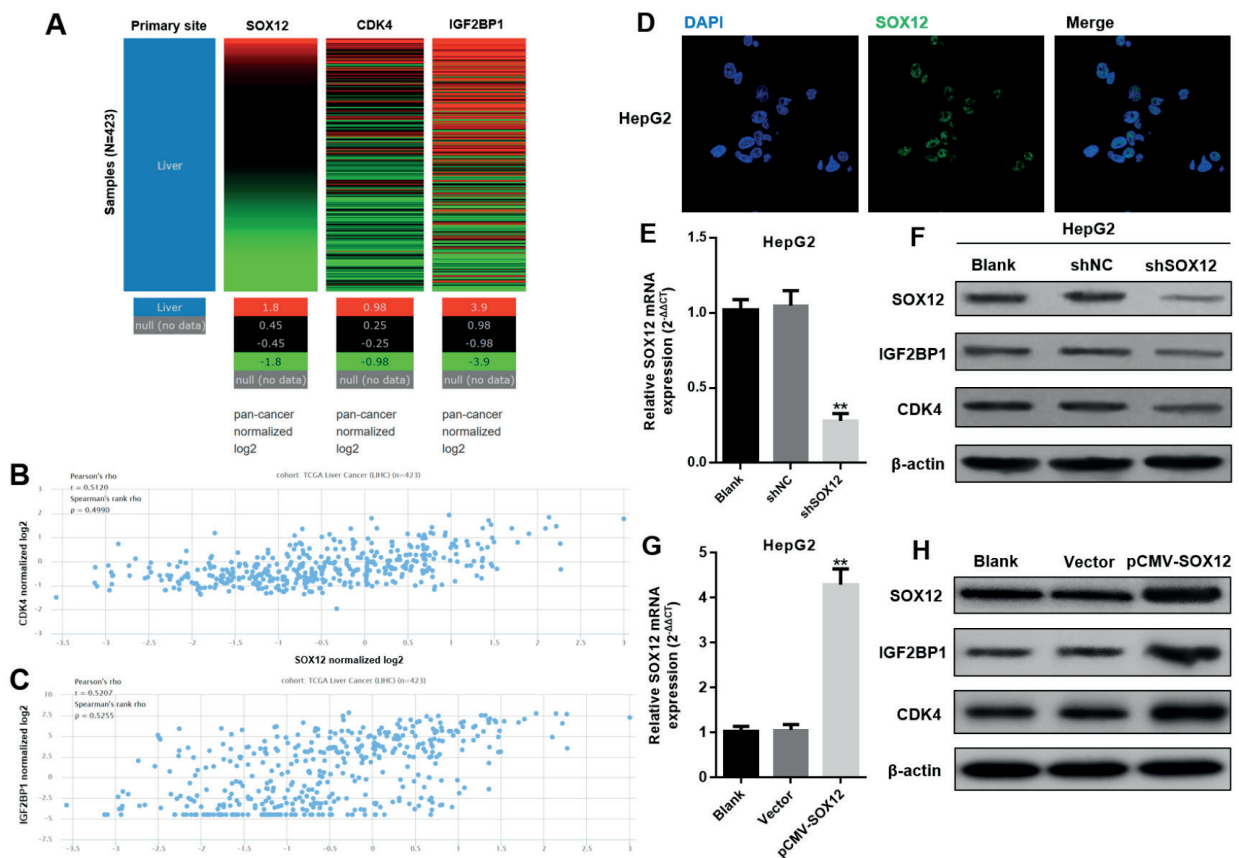
## Discussion

The regulative effect of SOX12 on cancer cell behaviors were mainly observed in colon cancer, breast cancer and HCC. In colon cancer, SOX12 acts as metastasis suppressor via modulating the WNT-TCF signaling pathway<sup>19</sup>. However, SOX12 can significantly upregulate MMP9 and Twist ex-

pression in breast cancer cells, thereby increasing their migration and invasion capability<sup>20</sup>. In HCC, SOX12 also functions as a metastasis enhancer via upregulating the expression of Twist1 and FGFBP1<sup>10</sup>. These findings suggest that the functional role of SOX12 might depend on the specific type of cancer. In this work, we further examined the expression profile of SOX12 in different pathological stages of HCC, based on the liver cancer cohort in TCGA. Our comparisons showed that SOX12 upregulation was associated with regional lymph node metastasis and distant metastasis.

Although Twist1 and FGFBP1 were identified as the downstream effectors of SOX12 in HCC, whether other genes are regulated by SOX12 is not clear. Our further data mining in TCGA database showed that CDK4 and IGF2BP1 were significantly co-expressed with SOX12, which have well characterized effect on HCC cell growth and invasion. CDK4 upregulation is associated with enhanced HCC cell proliferation and growth of xenografts<sup>16</sup>. Its upregulation might predict more malignant characteristics in human HCC tissues<sup>21</sup> and also a poor survival rate in the patients<sup>22</sup>. Palbociclib, a well-tolerated and selective CDK4/6





**Figure 3.** SOX12 increases CDK4 and IGF2BP1 expression in HCC cells. **A–C**, Heat map of SOX12, CDK4 and IGF2BP1 expression (A) and regression analysis of the correlation between SOX12 and CDK4 (B) and between SOX12 and IGF2BP1 (C). **D**, Immunostaining of SOX12 in HepG2 cells. Blue: DAPI; Green: SOX12. Images were obtained from Human Protein Atlas. **E** and **G**, QRT-PCR analysis of SOX12 mRNA expression 24 h after transfection of SOX12 shRNA (E) or SOX12 expression particles (G). **F** and **H**, Immunoblotting image of SOX12, IGF2BP1 and CDK4 expression 48 h after transfection of SOX12 shRNA (F) or SOX12 expression particles (H). \*\*,  $p < 0.01$ .

inhibitor, can suppress cell proliferation in human liver cancer cell lines by promoting a reversible cell cycle arrest<sup>17</sup>. As to IGF2BP1, it also plays an important role in malignant behaviors of HCC. Mechanistically, IGF2BP1 can bind and stabilize the c-MYC and MKI67 mRNAs and increase c-Myc and Ki-67 protein expression, two potent regulators of cell proliferation and apoptosis<sup>18</sup>. Knockdown of endogenous IGF2BP1 in HCC cells can significantly suppress cell growth, migration and invasiveness both *in vitro* and *in vivo*<sup>23–25</sup>. In HepG2 cells, we demonstrated that knockdown of SOX12 reduced IGF2BP1 and CDK4 expression, while enforced SOX12 expression significantly enhanced IGF2BP1 and CDK4 expression. In combination with previously research findings, we infer that IGF2BP1 and CDK4 are two important downstream effectors of SOX12 in HCC.

Although the oncogenic properties of IGF2BP1 and CDK4 in HCC were widely reported,

their prognostic values were still not quite clear. By data mining in liver cancer cohort in TCGA based on survival data of 365 patients, we observed that low SOX12 or CDK4 expression was associated with significantly better 3-year survival and also better 5-year survival, while low IGF2BP1 expression was associated with significantly better 3-year survival. Therefore, the expression of SOX12, IGF2BP1 or CDK4 might be potential clinical prognostic markers for HCC.

## Conclusions

SOX12 upregulation is associated with regional and distant metastasis of HCC. SOX12 can increase the expression of CDK4 and IGF2BP1, which confer malignant phenotypes to HCC. The expression of SOX12, IGF2BP1 or CDK4 might be potential clinical prognostic markers for HCC.

**Conflict of Interests**

The Authors declare that they have no conflict of interests.

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