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Integrative Analysis of SIX1 and Cancer Stem Cell Markers in Hepatocellular Carcinoma

Hepatoselüler Karsinomda SIX1 ve Kanser Kök Hücre Belirteçlerinin Bütünleştirici Analizi

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Öz

Giriş ve Amaç: SIX1 transkripsiyon faktörünün çeşitli memeli tümörlerinde yüksek ifade edildiği ve kanser kök hücre karakterinde rol oynadığı bilinmektedir. Bununla birlikte, HCC kanser kök hücrelerinde SIX1 transkripsiyon faktörünün rolü hala belirsizdir. Bu çalışmanın amacı, EMT-uyarıcı SIX1 ve kanser kök hücre belirteçlerinin ifade profillerinin in-vitro ve in-vivo analizlerde değerlendirilmesidir.

Gereç ve Yöntemler: SIX1 anlatımı, SNU398 HCC hücre hattında shRNA transdüksiyonu ile baskılandı. Tumor-küre formasyonu kanser kök hücre çalışmalarında kullanılan önemli bir analiz yöntemidir. SIX1-bağımlı kanser kök hücre belirteçleri PROM1, EPCAM ve OCT4 gen anlatım profilleri tümör-küre modelinde RT-qPCR ile değerlendirildi. Siroz ve HCC doku örneklerindeki transkriptom verilerinde genlerin anlatım ve korelasyon analizleri gerçekleştirildi.

Bulgular: Kanser kök hücre belirteçlerinin SIX1-baskılanmış anlatımının değerlendirilmesi sonucunda PROM1 ve EPCAM anlatımlarının anlamlı artmasına karşın OCT4 anlatımının anlamlı olarak azaldığı belirlendi. HCC doku transkriptom analizinde SIX1 anlatımı ile PROM1 ve EPCAM anlatımının ters korelasyon, OCT4 anlatımında pozitif korelasyon tespit edildi. İlginç olarak aynı gen ifadelerinin siroz örneklerinde OCT4 dışındaki diğer genlerin anlatımlarında farklı paternler saptanmıştır. SIX1, EPCAM, PROM1 ve OCT4 gen mRNA profillerinde siroz ve HCC numuneleri arasında anlamlı farklılık saptanmadı.

Sonuç: Kanser kök hücreleri kendi kendini yenileyebilen ve kanserin ilerlemesinde rol oynayan hücrelerdir. Çalışma sonucunda SIX1 ve kanser kök hücre ile ilişkili genlerin anlatım profillerinin aydınlatılması, HCC'nin moleküler yapısı hakkındaki bilgilerimizi geliştirmektedir.

Anahtar kelimeler: Hepatoselüler kanser, kök hücre, SIX 1transkripsiyon

Abstract

Objective: Transcription factor SIX1 aberrant expression has been shown in various mammalian tumors, and also recent studies indicated that SIX1 has a role in cancer stem cell properties. However, its roles in HCC cancer stem cell characteristics remain unclear. The aim of the study, to evaluate the EMT-inducer SIX1 and cancer stem cell markers expression profile in-vitro and in-vivo analyses.

Materials and Methods: SIX1 expression was suppressed by short hairpin RNA transduction in the SNU398 HCC cell line. Tumorsphere formation assay is a golden useful assay for cancer stem cell analysis. SIX1-dependent cancer stem cell markers PROM1, EPCAM, and OCT4 differential gene expression profiles were assessed in tumorsphere formation assay by RT-qPCR. Differential expression and correlation analyses were performed in transcriptome data in cirrhosis and HCC tissue samples.

Results: Assessment of SIX1-knockdown expression of target genes in tumorsphere formation assay revealed PROM1, EPCAM expressions were significantly up-regulated but OCT4 gene expression was significantly down-regulated. Conformably, PROM1 and EPCAM expressions were inversely but OCT4 expression was positively correlated in transcriptome arrays in HCC tissues. Interestingly, to the evaluation of the same gene expressions were

shown different patterns except, OCT4 in cirrhosis samples. The mRNA expression profiles did not change between cirrhosis and HCC samples in the SIX1, EPCAM, PROM1, and OCT4 gene expression profiles.
Conclusion: Cancer stem cells are self-renewable cell types and are responsible for cancer progression. Findings from this study highlight the SIX1 and cancer stemness-related genes expression correlations to improve our knowledge for HCC molecular signatures.
Key words: Hepatocellular cancer, stem cell, SIX 1 transcription

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and liver cancer is the fourth common cause of cancer-related death worldwide [1]. Despite the improvement of screening and diagnosis techniques, mortality continues to rise in HCC [2]. Multiple treatment options are available for HCC, including surgical resection or orthotopic liver transplantation are only feasible in early disease stage patients. Other treatment options include liver transplantation, radiofrequency ablation, trans-arterial chemoembolization, radioembolization and systemic targeted agent like sorafenib. Selection of a treatment procedure is based on tumor size, location, extrahepatic spread, and underlying liver function. [3, 4]. Generally, HCC patients diagnosed in advantages stages and the treatment options are limited [5]. For advanced stages, treatment is based on multi-kinase inhibitors like sorafenib or regorafenib, however, drugs have multiple side effects and only just adding a few months to patients' survival [6]. In this case, it is crucial to understand the genetic background of HCC. Cirrhosis is a well-established risk factor for HCC, around 80%–90% of HCC cases having underlying cirrhosis [7, 8]. Liver fibrosis generally causes first liver cirrhosis and secondly HCC, the connection between fibrosis and epithelial-mesenchymal transition (EMT) has been identified [9]. EMT re-activation effects fibrogenesis and carcinogenesis progression and showing rising cytokine levels in cirrhosis and late-stage HCC [10]. Furthermore, the EMT regulator transcription factors are relevant to the acquisition of cancer stem cell (CSC) characteristics and therapy resistance capacity the cancer cells. EMT process regulated by transcription factors such as ZEB1/2, TWIST, SNAI, and SIX1. Sine oculis homeobox 1 (SIX1) is considered as an oncofetal protein and its aberrant expression was detected in various mammalian cancers including HCC [11]. SIX1 up-regulation was detected in HCC tissues and associated with advanced tumor stage and poor overall survival [12]. The association between cancer stem cells and EMT properties are have been described [13]. Like other EMT-inducer transcription factors, SIX1 up-regulate CSC markers positive cell amounts in breast, colorectal, esophageal and pancreatic cancers. Cancer stem cells are generally identified by their potential for self-renewal and highly potential differentiation to establish the inherent cellular heterogeneity of the cancer. Although there is still no consensus on cancer stem cell phenotype in HCC, to identified the CSCs population of liver tumors some surface markers were identified such as CD133, CD13, EPCAM, CD90 and CD44 [14]. Previous studies have identified, hepatocellular carcinoma EPCAM positive cells have the tumor-initiating capacity and, CD133 and EPCAM overexpression were significantly correlated with poor overall survival in aggressive HCC [15, 16]. New meta-analysis identified that OCT4 expression was associated with poor survival in HCC [17].

The evaluation of CSC markers OCT4, CD133 (gene PROM1), and EPCAM expressions in the sphere-forming assay in SIX1 dependent. And also to understand the possible mechanism behind the regulation between cirrhosis and HCC tissue samples comparative analyses were explored the gene expression profile to generation to HCC. These findings may provide new references for the study on the gene expression profile of hepatocellular carcinoma, and added the new information to our knowledge to the developing new diagnostic strategies in the future.

2. Material and Methods

2.1. Production of Lentiviral Particles and Transduction to SNU398 Cell, Generation of SIX1-shRNA and Control Clones

Lentiviral particles were produced as follows: firstly, lentiviral SIX1 shRNA or control pLKO.1 plasmid was mixed with packaging plasmids pCMV-dR8.2 dvrp and pCMV-VSV-G at a ratio of 1.5:1.5:1 in 500 μl Optimem (Thermo Fisher Scientific, Rockford, IL, USA). Then, a second mixture consisting of the transfection agent PEI (Polysciences, Germany), which was added to 500 μl Optimem at a ratio of 1:3 (DNA μg: PEI μl), was prepared. The two mixtures were assembled in a single tube to generate a transfection reagent, which was used to transfect HEK293T cells after incubation for 20 min at room temperature. After 36-48 hours, viral particles were harvested from the supernatant of the transfected cells, filtered through 0.45 μm, and stored at -80∘C. SNU398 cells transduced with SIX1-shRNA and control-shRNA lentiviral particles in the presence of 8 μg/ml polybrene (#TR-1003-G, Sigma-Aldrich). 5 μg/ml puromycin (Thermo Fisher Scientific, #A1113802) was added to the cultures after 24 h for selecting stable shRNA and control clones.

2.2. Tumorsphere Formation Assay

Control-SNU398 and shSIX1-SNU398 cells were seeded at a concentration of 1.0×10^4 cells/ml into ultra-low attachment surface plates (Corning). These cells were cultured in DMEM (Gibco) supplemented with 50 ng/ml EGF (R&D), 10 ng/ml bFGF (R&D) and 2% B27 supplement (Gibco) at 37 °C in a humidified 5% CO2 atmosphere for 7 days.

2.3. Gene Expression and Quantitative Real-Time PCR (RT-qPCR)

Total RNA was isolated from shRNA-SNU398 and control-SNU398 with Nucleospin RNA plus isolation kit (Macharey-Nagel, #740984.250). Complementary DNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, #4368814) according to the manufacturer's instructions. The real-time quantitative PCR (RT-qPCR) reaction was performed using Maxima SYBR Green qPCR master mix (Thermo Fisher Scientific, #K0223), 0.4 µM primers and 50 ng cDNA were added in a final volume of 20 µL. PCR reaction was started by an initial denaturation of samples for 10 min at 95 °C followed 45 cycles with each cycle consisting of denaturation at 95 °C for 15s, annealing at 60 °C for the 30s and extension at 72 °C for 30s. Ct values were normalized to GAPDH and calibrated with Ct of controls. Relative gene expression was calculated by the ΔCt method. Control-SNU398 and shSIX1-SNU398 cells were analyzed by RT-qPCR by using the primers listed in Table 1.

Table 1. List of primers for real-time quantitative PCR

Gene	F (5'-3')	R (5'-3')
SIX1	AAAGGGAAGGAGAACAAGGATAG	AGCCTACATGATTACTGGGATTT
EPCAM	GGATCTGGATCCTGGTCAAAC	GCTATCACCACAACCACAATAAC
PROM1	CGGCTCTAATTTTTGCGGTA	TGTTGTGATGGGCTTGTCAT
OCT4	AGCGAACCAGTATCGAGAAC	TTACAGAACCACACTCGGAC
GAPDH	GGCTGAGAACGGGAAGCTTGTCAT	CAGCCTTCTCCATGGTGGTGAAGA

2.4. Bioinformatics and statistical analyses

The genes mRNA levels obtained from in GEO database with accession number GSE17548. Comparison of between different groups including *in-vitro* cell clones and cirrhotic, HCC tissues mRNA levels was tested with Student's t-test.

In statistical analyzing the correlation, data was performed with Pearson's correlation coefficient. A value of $P<0.05$ was considered to be statistically significant. Each experiment was performed at least three times and data are shown as the mean (±) SD.

3. Results

3.1. Assessment of SIX1 expression levels in HCC cell lines

Human HCC cell lines classified into two major groups according to hepatocyte lineage, epithelial and mesenchymal markers. The cells show epithelial characters that define as "*well*-differentiated", "*poorly differentiated*" cells overexpressed mesenchymal markers. HepG2, Hep3B, Huh7, and PLC/PRF/5 cells were characterized as epithelial, whereas SK-Hep1, SNU182, SNU423, SNU475, and SNU398 cells displayed mesenchymal phenotype [18]. According to the previous classification, the mesenchymal transcription factor SIX1 transcript expression was decreased in epithelial character cell lines HUH7, HEP3B, HEPG2 and PLC/PRF/5 HCC cell lines. The "*poorly differentiated*" cell lines SK-HEP1, SNU182, SNU475, and SNU398 were overexpressed, especially SNU398 the highest cell line. Only the SNU423 cell line expression profile was similar to the *well-differentiated* group cell line profile (Figure 1).

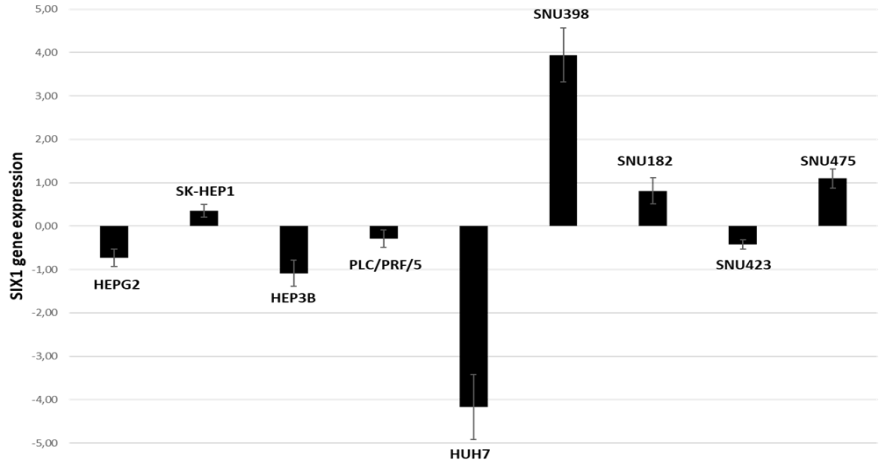


Figure 1. Expression of SIX1 in HCC cell lines. Bars represent relative gene expression levels of SIX1 calculated by the ΔCt method. The normalization gene is GAPDH.

3.2. HCC Cancer Stem Cell Markers OCT4, PROM1 and EPCAM expression in SIX1-dependent tumorsphere forming assay

After the determination of the SNU398 HCC cell line highly expressed SIX1 transcription factor, SIX1 expression was suppressed with lentiviral shSIX1 plasmid. For generated control clones cells transduced with pLKO.1 plasmid and both clones treated with puromycin to generate stable cell clones. The tumorsphere formation assay is a functional approach to assess cancer stem cell's self-renewable potential *in-vitro*. The self-renewable capacity is an important property of cancer stem cells. To determine the SIX1 role on the cancer stem cell markers expression, spheres generated with shSIX1-SNU398 and control-SNU398 cell clones. Control-SNU398 cell clone spheres are bigger than shSIX1-SNU398 cell spheres. In spheres, *SIX1* expression was nearly 8 fold and *OCT4* expression was 1.5 fold increased in shSIX1-SNU398 clones compare the control clones. Interestingly, *CD133* (*PROM1*) expression was approximately 8 fold and EPCAM expression was four-fold up-regulated in shSIX1-SNU398 clone compared to control clone cells (Figure 2).

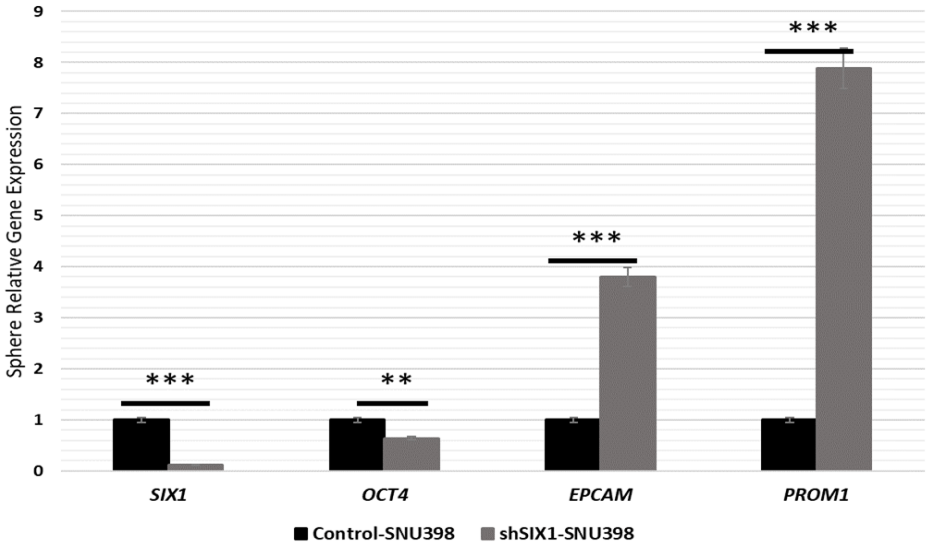


Figure 2. Examining the effect of SIX down-regulation on OCT4, CD133 (PROM1) and EPCAM gene expression in sphere-forming cells. The difference between groups was analyzed by t-test. **p < 0.01, ***p < 0.0001.

3.3. Assessment of correlation analyses between EMT-inducer SIX1 and CSC markers in cirrhosis and HCC tissue samples

Previous reports determined that HCC appeared against the background of cirrhosis [19]. After the determination of the differential expression profile of cancer stem cell markers in the SIX1 knockdown condition, their relationship with SIX1 was tested with OCT4, *PROM1*, and *EPCAM*. mRNA levels were analyzed for comparison in cirrhosis and HCC transcriptome arrays (GSE17548). Datasets contain 13 cirrhosis and HCC tissue samples from the same patients. In accordance with cell line data, a negative correlation between *SIX1* and *EPCAM* expression was found in tumor samples, on the contrary in cirrhosis tissues were positively correlated. The analysis between *SIX1* and *PROM1*, a negative correlation in tumor tissues was detected, but a significant positive correlation was observed in cirrhosis tissues. In contrast, *SIX1* and *OCT4* correlation analysis were not changed in cirrhosis and HCC tissues, it was similar and positively correlated (Table 2).

Table 2. Correlation analysis between SIX1 and OCT4, CD133, EPCAM in GSE17548 dataset.

Tissue Types	*N*	Gene ID	Correlation Coefficient *r*
HCC	13	*SIX1* / *EPCAM*	-0.25
Cirrhosis	13	*SIX1* / *EPCAM*	0.16
HCC	13	*SIX1* / *PROM1*	-0.34
Cirrhosis	13	*SIX1* / *PROM1*	0.62*
HCC	13	*SIX1* / *OCT4*	0.40
Cirrhosis	13	*SIX1* / *OCT4*	0.36

*Correlation between SIX1 and EPCAM, PROM1 and OCT4 are analyzed by Pearson's Correlation Coefficient, respectively. N, number of patients, * indicate a significant positive correlation (p<0.05).*

3.4. EMT marker SIX1 and CSC markers transcriptome profiles in cirrhosis and HCC tissue samples

Previous reports have been identified cancer stem cell markers and SIX1 up-regulated in tumor tissues compare to non-cancerous tissues [12, 17, 20]. After examined to the differential expression which is important for HCC cancer progression genes, *SIX1*, *PROM1*, *EPCAM*, and *OCT4*, to further analyses were studied the gene expression profiles in cirrhosis and HCC tumor tissues using by GEO database. The GSE17548 dataset was used to analyze the mRNA levels which include the same patient's cirrhosis and HCC tumor tissue samples. The expression profile was showed that *SIX1*, *PROM1*, *CD133*, and *EPCAM* were expressed both cirrhosis and HCC samples, however, the significant difference was not detected in each gene expression pattern compared to cirrhosis and HCC tumor samples (Figure 3).

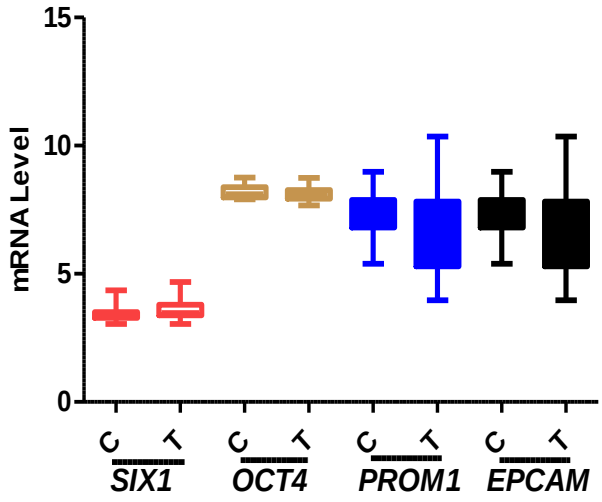


Figure 3. The analysis of the same patient SIX1, OCT4, PROM1 and EPCAM mRNA level in cirrhosis and HCC tissue samples. The mRNA levels were obtained from GSE17548.

4. Discussion

Although liver cancer ranks seventh in terms of incidence, it is the fourth most common cause of cancer death worldwide [1]. The HCC is the highest subgroup of liver cancer and HCC arises in the background of a cirrhotic liver [21]. The diagnosis and treatment options are limited for HCC patients and associated with poor survival results [22]. Due to these reasons to understand the genetic changes of HCC carcinogenesis enlightened the disease molecular mechanisms.

EMT process is important for cancer progression and one of the EMT inducer SIX1 was identified associated with poor survival in HCC. EMT transcription factors and cancer stem cells connection have been identified with previous papers [23]. There are no consensus CSC biomarkers identified for HCC in the literature yet, but some CSC markers are noteworthy. In the recent meta-analysis, HCC cancer stem cell marker OCT4 was identified as an important prognostic biomarker for HCC. The EpCAM positive HCC cells displayed hepatic cancer stem cell features and these cells were capable of initiating highly invasive HCC in NOD/SCID mice [16]. CD133, encoded by the *PROM1* gene, is known as liver cancer stem cell marker and also was overexpressed in liver cancer and its expression negatively associated with prognosis [24, 25]. In this study, the SIX1 transcript profile was tested in HCC cell lines and determined SNU398 cell line has the highest transcript level. SIX1 and HCC cancer stem cell markers OCT4, PROM1, and EPCAM gene expression profiles were tested in SIX1-dependent *in-vitro* and also cirrhosis and HCC tissue transcriptome arrays.

The sphere mRNA analysis of OCT4, PROM1 and EPCAM showed that PROM1 and EPCAM expressions were up-regulated only OCT4 expression was suppressed in SIX1 suppressed conditions in HCC cell clones. Although cancer stem cell markers EPCAM and PROM1 expressions raised the sphere-forming ability of the SIX1 knockdown clones were decreased (data not shown). Next, for testing the gene expression correlation between SIX1 and OCT4, PROM1, EPCAM in cirrhosis and HCC tissue samples, the GEO database was used. The results of correlation analyses between SIX1 and cancer stem cell markers were positively correlated in cirrhosis tissues. Interestingly, correlation analyses between SIX1 and cancer stem cell markers showed that SIX1 between PROM1 and SIX1 between EPCAM were negatively correlated. Only OCT4 was positively correlated with SIX1 in both cirrhosis and HCC tissue samples. HCC tissue expression correlation pattern was similar to in-vitro cell experiments. Finally, data comparison of cirrhosis and HCC same patient tissue samples mRNA levels showed no significant expression difference in all gene sets.

HCC progression is a multi-step process and up to now, understanding the mechanism of HCC prognosis in genetic background is still limited. Suppression of SIX1 expression affects the cancer stem cell markers expression profile and this pattern was similar in *in-vitro* and *in-vivo* analyses. In addition, EMT marker SIX1 and cancer stem cell markers expression profiles were changed between cirrhosis and HCC samples. On the other hand, each gene expression profile was not differentially changed in both tissues. Identifying gene profiles and their correlations, which are important for HCC progression, will help understand the molecular mechanism of liver cancer and develop potential therapies for liver cancer.

Declaration of Conflicting Interests

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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