

SIX1 Down-Regulation Influence Drug Resistance, Epithelial-Mesenchymal Transcription Factors and Self-Renewal Capacity in Hepatocellular Carcinoma

Pelin Balcik Ercin (✉ pbalcik@gtu.edu.tr)

Gebze Technical University <https://orcid.org/0000-0002-3470-0393>

Arzu Aysan

Gebze Technical University: Gebze Teknik Universitesi

Nazli Salik

Gebze Technical University: Gebze Teknik Universitesi

Esmâ Erciyas

Gebze Technical University: Gebze Teknik Universitesi

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Abstract

Sine oculis homeoprotein 1 (SIX1) was discovered to exert an essential role in embryonic development and it was also identified to be re-activated in various types of mammalian cancer. Immunohistochemical and SIX1 gene expression analyses were performed to determine the prognostic role of SIX1 expression. SIX1 expression was suppressed by short hairpin RNA transduction in the SNU398 HCC cell line. The effects of SIX1 on proliferation, epithelial-mesenchymal transition, apoptosis, drug resistance, and sphere formation were assessed in SIX1 knock-down cells. The upregulated expression levels of SIX1 were revealed to be correlated with the stage of the disease in breast, colon and liver cancer, with liver cancer exhibiting the highest expression profile. SIX1 knockdown significantly affected the cell morphology, proliferation, downregulated the protein expression levels of ZEB1, ZEB2 and SNAI1 and upregulated the expression levels of TWIST1 in hepatocellular carcinoma cells. Furthermore, SIX1 knockdown cells were more sensitive to sorafenib treatment; however, the expression profile analysis of the drug resistance genes *ABCB1*, *ABCC1* and *ABCG2* did not explain this sensitivity. Finally, SIX1 knockdown cells were identified to have decreased CD90 levels and lost their sphere-forming ability, which is essential for cancer stem cell properties. Overall, these results indicated that SIX1 expression may be useful as a diagnostic marker for patients with HCC.

Introduction

Cancer is a major health problem worldwide, demonstrating ever increasing incidence rates [1]. Lung, breast, colorectum and liver cancers are the top four types of cancer responsible for the most cancer-related deaths [2]. Tumor metastasis, the process by which the primary cancer disseminates to a distant organ and develops into a metastatic lesion, continues to be the primary cause contributing to cancer-related deaths [3]. Epithelial-mesenchymal transition (EMT) is a fundamental mechanism of cell migration, which has been associated with poor survival and resistance to chemotherapy in numerous types of cancers [4]. The SNAI, TWIST and ZEB (ZEB1 and ZEB2) family of transcription factors are the most thoroughly studied mediators of EMT in human cancers; however, other studies have identified new transcription factors that induced EMT, such as sine oculis homeobox 1 (SIX1) [5]. The overexpression of SIX1 in cancer was first observed in 1998, and it has since been discovered to be associated with a poor prognosis in numerous types of cancer [6]. SIX1 was reported to affect the tumor progression through the regulation of proliferation, invasion and metastasis, genomic instability and the resistance to cell death [6, 7]. Notably, the EYA2 co-activator was identified to be required for the ability of SIX1 to mediate the prometastatic properties of cells in breast cancer [8]. In addition, SIX1 was previously demonstrated to mediate paclitaxel resistance in breast cancer cells [9]. Drug resistance, a high rate of self-renewal and the ability to differentiate are intrinsic properties of cancer stem cells (CSC), and it has been suggested that EMT and CSC properties are mechanistically related to each other in cells [10]. Interestingly, several recent studies have indicated that SIX1 served an important role in regulating CSC characteristics in leukemia, breast, colorectal, esophageal and pancreatic cancer cells [11, 12, 7, 13–15].

The present study aimed to determine the expression profile of SIX1 in various types of cancer, in which the highest expression levels were detected in liver cancer. Furthermore, the results revealed that the expression levels of SIX1 were upregulated, which subsequently affected the expression levels of EMT transcription factors, and the drug resistance and self-renewal capacity of Hepatocellular carcinoma (HCC).

Materials And Methods

Cell Lines and Culture Conditions

Hepatocellular carcinoma cell lines SNU398, SNU182, SNU475 were cultured in RPMI1640 supplemented with 10% fetal bovine serum. HCC cell lines HUH7, HEP3B, PLC/PRF/5 and liver cancer cell line HEPG2 were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere. The cell lines were obtained from Dr. Tamer Yagci and were previously used by Yagci et al [16]. All cell lines were routinely checked for mycoplasma contamination. The identities of the cell lines were validated by STR analysis.

Antibodies and Plasmids

The following antibodies and plasmids were used: anti-ZEB2 antibody (home-made monoclonal antibody, clone 6E5) [17, 18], rabbit polyclonal anti-SIX1 antibody (NOVUS, Seattle, WA) and mouse monoclonal SIX1, ZEB1, SNAI1, TWIST1, CK19, VIMENTIN, EYA2, EZRIN, c-PARP, β -ACTIN, CALNEXIN and ALFA-TUBULIN antibodies (Santa Cruz Biotechnology, Dallas, TX), cell signaling.shRNA lentiviral particles targeting human SIX1 (TRCN0000468669, Sigma-Aldrich, St. Louis, MO, USA), and a non-silencing control, (empty vector, pLKO.1, Addgene #8453), packaging plasmids pCMV-dR8.2 dvrp (Addgene #8455) and pCMV-VSV-G (Addgene #8454).

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 and transfection agent PEI (Polysciences, Germany) in Optimem medium (Thermo Fisher Scientific, Rockford, IL, USA). HEK293T was transfected and after 36-48 hours, viral particles were harvested from the supernatant. SNU398 cells transduced with SIX1-shRNA and control-shRNA lentiviral particles in the presence of 8 μ g/ml polybrene (#TR-1003-G, Sigma-Aldrich). 5 μ g/ml puromycin (Thermo Fisher Scientific, #A1113802) was added to the cultures after 24 h for selecting stable shRNA and control clones.

Proliferation Assay

The shSIX1-SNU398 and control-SNU398 cells were plated in 96-well plates at an initial density of 5×10^3 cells, with 100 μ L media per well. After 24 h of incubation, cell proliferation data were measured at

12, 24, 36, and 48h by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Thermo Fischer Scientific #M6494).

Western Blotting

Cells lysed in SDS lysis buffer (10% glycerol, 2% SDS in 62.5 mM Tris-HCl) including protease Inhibitor Cocktail (Roche). After the determination of protein concentration, an equal amount of protein from each lysate from was resolved by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, #IPVH00010). Membranes were incubated with primary antibodies at 4°C overnight, then were incubated with the secondary antibody for 2 hours at room temperature. Membranes were immersed in SuperSignal West Femto chemiluminescent reagent (Thermo, #34095) and the protein bands were visualized with the ChemiDocTMXRS system (Bio-Rad, CA).

Gene Expression Analysis

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 the 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). Ct values were normalized to GAPDH and calibrated with Ct of controls. Relative gene expression was calculated by the Δ Ct method. Control-SNU398 and shSIX1-SNU398 cells were analyzed by RT-qPCR by using the primers listed in Table SI.

Drug Resistance

Sorafenib (sc220125, Santa Cruz) was prepared according to the manufacturer's instructions. The density of 1.5×10^5 cells/well control-SNU398 and shSIX1-SNU398 were seeded into 6-well plates. 5 μ M sorafenib was added to the cells when cells reached 70% confluency. After treatment with sorafenib for 24 hours, cells were harvested, Western blotting and apoptosis analyses were performed. For apoptosis assay, cells were collected resuspended at a density of 1×10^6 cells/mL in 1X binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl_2) and stained with 5 μ L Annexin V-APC (BD) and 5 μ L 7AAD-PE (BD) for 15 min at room temperature. Samples were analyzed using Accuri C6 (BD) flow cytometer.

Sphere Formation Assay

Cells were seeded at a concentration of 1.0×10^3 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), 25 μ g/ml insulin (Gibco), and 2% B27 supplement (Gibco) at 37 °C in a humidified 5% CO_2 atmosphere for 7 days. After incubation, images of spheroids were acquired with a Leica DMI8 inverted microscope, and the diameter of the spheroids was determined.

Measurement of Cell Surface CD90 Expression with Flow Cytometry

Cells were resuspended at a density of 3×10^5 cells/ml in PBS and transferred into new tubes which were used for the control group and CD90 treated groups. After that, 5 μ l FCR (Miltenyi Biotech) and CD90 (Sony) antibody was added on the tubes and incubated at room temperature samples were analyzed using Accuri C6 (BD) flow cytometer.

Analyses of SIX1 Expression in Human Cancer Samples

The expression of SIX1 was assessed by RT-qPCR in commercial TissueScan qPCR Cancer Survey cDNA arrays I (Origene, #CSRT101). The array consists of 72 tumors and 24 non-malignant tissue samples from eight different primary organs (breast, colon, kidney, liver, ovary, thyroid, lung, and prostate) and also provides clinical information (Table S2). SIX1 expression in cDNA arrays was calculated by using the $\Delta\Delta C_t$ method (Log2) [19].

The same tumor types were analyzed in the Expression Project for Oncology (expO) datasets (GEO accession GSE2109). In this study R software was used for data manipulation, calculation and graphical display (<https://www.r-project.org>).

Immunohistochemistry was carried out with the Hepatocellular Carcinoma Tissue Array, HLiv-HCC150PG-01 (Biomax). SIX1 protein expression level was evaluated by integrating the percentage of positive tumor cells and the intensity of positive staining. Briefly, sections were scored as 0 (negative), 1 (bordering), 2 (weak), 3 (moderate) or 4 (strong), whereas the staining extent was scored according to the area percentages: 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%). The products of staining intensity and extent scores were the final staining scores (0–16). The groups with a score of 0 were excluded from the analyses and did not participate in the statistics.

Statistical Analyses

GraphPad Prism statistical software was used to analyze the significance of data. Gene expression levels in cell lines and TissueScan cDNA array were represented as mean +SD (standard deviation). Un-Paired Student's t-test was used for statistical analyses of data from gene and protein expression experiments in cell lines and tissue array. The differential transcription profile of expO datasets was analyzed with Kruskal-Wallis test followed by post-hoc Dunn's test [20]. Significant differences were denoted as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

SIX1 expression levels are upregulated in various types of cancer

Previous studies reported that the upregulation of SIX1 expression levels was associated with a poor prognosis in breast, lung, pancreatic, cervical, colorectal and ovarian cancers. Thus, the present study first analyzed the SIX1 expression pattern in liver, colon, breast, ovarian, kidney, lung, thyroid and prostate cancers using cDNA arrays; the array represented tissues from three non-malignant samples and nine tumor samples of eight different cancer types. Compared with the control tissues, a marked upregulation in SIX1 expression levels were observed in the liver, colon, breast, ovary, kidney, lung and prostate tumor tissues, while a slight downregulation was observed in thyroid cancer tissues (**Fig. 1a**). Interestingly, liver, breast and colon cancer tissues exhibiting high expression levels of SIX1 are very common, indicating that SIX1 upregulation may be associated with cancers with high mortality rates [2]. Thus, the analysis of SIX1 expression levels was further investigated using expression project for oncology (expO) datasets for tumor tissues of the liver, breast and colon histopathological types, which were grouped according to the pathological stage. The statistical analyses of the colon, liver, breast cancers transcriptional values were analyzed by Kruskal-Wallis test followed by post-doc Dunn's test. Although SIX1 expression was significantly increased in pathological stage 3 and 4 compared to pathological stage 1 in liver and colon cancer samples, a significant increase was detected only in stage 4 compared to stage 1 in breast cancer (**Fig. 1b**). Following these cDNA array results, the bioinformatics analysis also produced a similar SIX1 expression profile, with the highest expression levels detected in liver cancer.

The upregulation of SIX1 expression levels in liver cancer samples prompted the study into its expression levels with immunohistochemistry (IHC). The tissue array was treated with an anti-SIX1 antibody and analyzed according to the patient grade. Grade 1 and 3 IHC samples had significantly upregulated protein expression levels of SIX1 compared with the non-associated tissues (**Fig. 1c**). The IHC results also validated the bioinformatics findings. Taken together, these data suggested that the upregulation of the SIX1 mRNA and protein expression levels may be an important indicator of the progression of liver cancer.

SIX1 expression levels are detected in poorly differentiated HCC cell lines and SIX1 knockdown promotes the epithelial phenotype in the SNU398 cell line

Given the importance of the upregulation of SIX1 in numerous types of cancer, especially liver cancer, the role of the SIX1 transcription factor role in HCC was subsequently investigated. First, SIX1 and EYA2 mRNA expression levels were analyzed in well-differentiated HUH7, HEP3B, HEPG2 and PLC/PRF/5 cell lines and poorly differentiated SNU182, SNU475 and SNU398 liver cancer cell lines. SIX1 transcripts were observed in poorly differentiated cell lines, on the contrary, the EYA2 transcripts, which is required for SIX1 activation [21, 8], was detected in all liver cancer cell lines (**Fig. S1**).

Western blotting analysis identified that the poorly differentiated cell lines expressed the SIX1 protein, especially in SNU475, SNU398 and SNU182 cells. According to our Western blot result, SIX1 protein may be present in SNU 475 in the hyperphosphorylated state, causing it to lose its ability to bind DNA [22]. For this reason, SNU398 cells, which had the second highest SIX1 expression levels, were selected for further analysis (**Fig. 2a**). SIX1-shRNA-SNU398 (shSIX1-SNU398) and pKo.1-shRNA-SNU398 (control-SNU398)

stable clones were generated to determine the role of SIX1 in HCC cells (**Fig. 2b**). Changes in the SIX1 expression levels were identified to promote marked differences in the cellular morphology; for example, compared with the control-SNU398 clones, shSIX1-SNU398 cells exhibited a clustered distribution, which is an indicator of an epithelial phenotype (**Fig. 2c**). In order to explore the effects of SIX1 knockdown on the proliferation capacity, we analyzed shSIX1-SNU398 and control-SNU398 HCC cell clones cell proliferation by MTT assay. Our results from cell viability assays were in line with previous studies [23, 24], demonstrating that cellular proliferation is significantly inhibited in shSIX1-SNU398 at 24, 36 and 48 hours compared to control-SNU398 cells (**Fig. 2d**)

Knockdown of SIX1 expression affects the expression levels of EMT markers

Subsequently, the effect of SIX1 knockdown on *ZEB1*, *ZEB2*, *TWIST*, *EPCAM*, *VIM*, *CDH1*, *SOX2*, *ITGA5* and *ITGB1* expression levels was analyzed using RT-qPCR. Previous studies indicated that SIX1 promoted the $\alpha 5\beta 1$ integrin-mediated metastatic capability of cervical cancer cells, and SIX1 was discovered to bind to the regulatory element of SOX2 and control SOX2 expression levels in human glioma cell lines [25, 26]. Thus, whether SIX1 could affect the expression levels of EMT transcription factors and EMT markers in HCC was investigated. The results obtained in the present study were consistent with previous reports; for example, *ITGA5*, *ITGB1* and *SOX2* expression levels were discovered to be downregulated by ~0.4-fold in the SIX1 knockdown clones. The expression levels of *ZEB1*, *ZEB2* and *TWIST1* were downregulated in shSIX1-SNU398 cells compared to control clones. In addition, the results revealed that the expression levels of *VIM*, which is a mesenchymal marker, were downregulated, while the expression levels of *EPCAM*, which is an epithelial marker, were upregulated in shSIX1-SNU398 clones (**Fig. 3a**).

The western blotting results were consistent with the RT-qPCR data, suggesting that SIX1 may serve a role in the downregulation of *ZEB1*, *ZEB2*, *SNAI1* and vimentin expression levels, which are EMT transcription factors. Interestingly, *TWIST1* expression levels were downregulated at the mRNA level, while its protein expression levels were upregulated. However, the CK19 epithelial marker was significantly upregulated in the shSIX1-SNU398 clone. Interestingly, SIX1 silencing had no significant effect on *EYA2* expression at the protein level. The suppression of SIX1 expression also significantly downregulated the expression levels of the *EZRIN* protein, which is transcriptionally regulated by SIX1 and regulates cytoskeletal organization and adhesion [27] (**Fig. 3b**).

SIX1 knockdown upregulates c-PARP expression levels and the apoptotic rate following sorafenib treatment in HCC cells

EMT is a possible mechanistic basis for anti-cancer drug resistance [28]. Until now, to the best of our knowledge, the SIX1-dependent effects on drug treatment has only been investigated in gastric and breast cancer cells [9, 29]. To further determine the effect of SIX1 in HCC, shSIX1 and control cell clones were treated with sorafenib, which is a standard chemotherapeutic drug for HCC. To understand the effect of SIX1 knockdown on the drug resistance, flow cytometric analysis was performed and it was noted that SIX1 knockdown cells were more sensitive to sorafenib treatment. The apoptotic cell number in shSIX1 and control cell clones was 93.9% and 66.7%, respectively, and the knockdown of SIX1

expression significantly increased the number of apoptotic cells (**Fig. 4a**). Moreover, these findings were further confirmed by the subsequent protein changes. After exposure to sorafenib, the expression of cleaved PARP was increased in shSIX1-SNU398 cells compare to control-SNU398 and untreated cells (**Fig. 4b**). These results indicated that SIX1 expression may exert a significant influence on the chemotherapeutic drug response.

There are various molecular mechanisms involved in multidrug resistance, and ATP-binding cassette (ABC) transporters are a family of transporter proteins that are responsible for drug resistance [30]. Therefore, whether SIX1 expression exerted an effect on the expression levels of the drug resistance genes, *ABCG2*, *ABCC1* and *ABCB1*, was investigated. Interestingly, the expression levels of *ABCC1* and *ABCB1* did not markedly change, while the expression levels of *ABCG2* were markedly upregulated (**Fig. 4c**). However, this finding did not fully explain the role of SIX1 in the drug sensitivity mechanism. Furthermore, the role of SIX1 knockdown on the drug response and the expression levels of drug resistance-related genes were analyzed. SIX1 knockdown cells were more sensitive to sorafenib; however, the expression levels of drug-resistant associated genes were not changed or downregulated.

SIX1 expression levels are important for HCC cell clonogenicity capacities

CSCs have several biological properties that distinguish them from other cancer cells, such as resistance to treatment, evasion from cell death, dormancy and a self-renewal capacity [31]. To determine the role of SIX1 on the self-renewal capacity of HCC cell clones, a sphere formation assay was performed. shSIX1-SNU398 and control-SNU398 cells were capable of forming morphological spheres; however, the spheres of SIX1 knockdown cells were significantly reduced both the size and number of spheres formed. shSIX1-SNU398 sphere colony (> 200 μm) and control-SNU398 colony (< 65 μm) spheres are depicted in **Fig. 5a**. The control cells formed significantly increased numbers of spheres (>100 μm) compared with the SIX1 knockdown cells. Notably, following the second passage of sphere cells, shSIX1-SNU398 cell spheres did not retain the ability to self-renewal (**Fig. 5b**).

To validate the morphological data, the mRNA expression levels of *SIX1*, *ZEB2*, *VIM*, *KLF4* and *THY1* were analyzed in the control and SIX1 knockdown sphere-forming cells. The results revealed that *ZEB2*, *VIM*, *KLF4* and *THY1* gene expression levels were significantly downregulated in SIX1 knockdown sphere cells (**Fig. 5c**).

A recent study demonstrated that the CSC marker CD90, a THY1 gene product, was important for the cell cycle, migration, invasion and sphere forming ability of HCC cells [32]. The observed SIX1-dependent effects in the self-renewal capacity prompted the analysis of the CD90 cell population in shSIX1-SNU398 and control-SNU398 cells by flow cytometry; the knockdown of SIX1 significantly decreased the CD90⁺ subpopulation in shSIX1-SNU398 cell clones (65.5%) compared with the control-SNU398 cell clones (35.1%) (**Fig. 5d**).

Discussion

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, demonstrating a high mortality rate. Several studies have reported that the expression levels of SIX1 were upregulated in various types of mammalian cancer and that the overexpression of SIX1 lead to increased motility of cancer cells, including in breast, colorectal and hepatocellular carcinomas [33, 7, 34–40, 1]. In the present study, SIX1 expression levels were discovered to be upregulated in the different sub-types of cancer samples, except thyroid cancer in cDNA arrays analyses. A recent paper pointed out SIX1 level was positively associated with tumor size and lymph node metastasis in thyroid cancer [41]. Unfortunately, we tested thyroid expression profile in just cDNA array includes; 3 non-malignant, 6 papillary, 1 hurtle cell, 1 medullary (metastatic) carcinoma histologic types cancer tissues (Table S1) and the Ct value of metastatic thyroid sample was early. Future studies will be explained to more detailed the SIX1 expression role in thyroid cancer. The up-regulated SIX1 expression was detected especially in the liver, colon, and breast cancer tissues compared with the non-malignant tissues in cDNA analyses. The present study further explored the expression of SIX1 in the *expO* dataset according to the patient stage in three types of cancer (liver, colon and breast); interestingly, the expression levels of SIX1 were upregulated according to the patient stage. Thus, the upregulation of SIX1 expression levels appears to be important for liver, colon and breast cancers, which are all placed in the top five cancers responsible for cancer-related mortalities [2]. The findings of the present study are consistent with previous studies, and the reported SIX1 overexpression in our *in-silico* and *in-vivo* data suggested that SIX1 may be important for HCC progression.

Resistance to chemotherapy agents, limited available treatment options and high mortality rates are associated with the incidence of HCC, and prompted the present study to investigate the role of SIX1 in hepatocarcinogenesis in more detail. The results revealed that the SIX1 expression patterns were distinct in well and poorly differentiated HCC cell lines. Therefore, the expression levels of SIX1 in the SNU398 cell line, which naturally had high SIX1 expression levels, were knocked down, which promoted the downregulated of the expression levels of the mesenchymal marker Vimentin and an upregulation in the expression levels of epithelial markers, such as CK19 and EPCAM in cells. In addition, in accordance with these results, the cell morphology dramatically changed. The present study initially hoped to perform wound healing and colony-forming assays; however, these assays were not performed because of the changes in cell morphology were dependent on SIX1 suppression. SIX1 has been reported to control the expression of various genes that have significant roles in cancer progression, such as EZRIN in rhabdomyosarcoma, SOX2 in glioma and $\alpha 5\beta 1$ integrin in cervical cancer cells [38, 26, 25]. Consistent with these findings, the present study observed a decrease in the expression levels of EZRIN, SOX2, ITGA5 and ITGB1 in SIX1 knockdown cells. In addition, SIX1-dependent protein expression levels analysis revealed that the expression levels of the epithelial marker CK19 were significantly upregulated, while those of the mesenchymal marker Vimentin was markedly downregulated in HCC. Conversely, the expression levels of TWIST1 were significantly upregulated at the protein levels. Interestingly, EMT transcription factor analysis also revealed that the expression levels of the ZEB1, ZEB2 and SNAI1 transcription factors were significantly downregulated. In contrast to our results, the changes in the expression levels of the EMT markers ZEB1, ZEB2, SNAI2, TWIST1, TWIST2 and VIM in SIX1 overexpressing esophageal squamous carcinoma cell clones were not investigated [15].

The link between EMT and drug resistance prompted further investigations into the role of SIX1 in drug resistance. In the present study, SIX1 knockdown cells were discovered to be more sensitive to sorafenib treatment and influenced the c-PARP protein expression levels and apoptotic cell levels. SIX1 overexpression conferred resistance in breast cancer, while the suppression of SIX1 increased the sensitivity for a drug in gastric cancer cells [9, 29]. Transcription factors of the EMT process are known to mediate ATP binding cassette transporters, which have a role in the drug resistance mechanism [42]. Despite SIX1 knockdown cells being more sensitive to sorafenib treatment, the expression profiles of the ABC transporter members *ABCB1*, *ABCC1* and *ABCG2* did not explain the drug response. A possible explanation for this discrepancy may be that another signal transduction mechanism was involved in this process. For instance, a previous study reported that the NF- κ B signaling pathway, which serves a crucial role in the development of drug resistance, was activated by SIX1 and SIX2 [43, 44].

Cancer stem cells share numerous characteristics with normal stem cells, including their differentiation and self-renewal abilities, and their drug resistance properties [45]. Previous studies have demonstrated that the SIX1 transcription factor affected the properties of CSCs in a variety of mammalian cancer cells, except for in HCC. Therefore, after determining that SIX1 served a role in the drug resistance mechanism, the present study investigated the role of SIX1 on the self-renewal ability of HCC cells. The results revealed that fewer spheres with smaller diameters were formed in SIX1 knockdown cell clones compared with the control cell line, and more importantly, after the second passage, no sphere formation was observed. The gene expression analysis of spheres formed from the SIX1 knockdown cell line, the mRNA expression levels of *ZEB2*, *VIM*, *KLF4* and *THY1* were markedly downregulated compared with the control cell spheres. KLF4 transcription factor expression decreased in SIX1 knockdown spheres and a recent study pointed out the KLF4 transcription factor can induce a CSC-like phenotype in HCC [46]. Zhang *et al* [32] revealed that CD90, a gene product of *THY1* and a marker of HCC CSCs, promoted the sphere-forming ability in HCC. The results of the present study revealed that SIX1 suppression affected the CD90 positive cell count and the sphere-forming ability of the cells in HCC. Thus, these findings suggested that SIX1 may be used as a biomarker for liver cancer progression and may serve crucial roles in the drug resistance mechanism and cell renewal capacity in hepatocarcinogenesis. The future studies will be needed to test SIX1-dependent tumor stemness capacity in *in vivo* models of HCC and also additional studies will be required to assess EMT markers protein expression profile in SIX1-dependent HCC cell colonies.

Although the SIX1 protein was also expressed in the SNU475 and SNU182 cell lines, the present study only used the SNU398 cell line for further investigations, because the SIX1 protein is found in the hyperphosphorylated form in SNU475 cells, and a previous study indicated that the hyperphosphorylated form of the SIX1 protein had no DNA binding capacity [22]. The other HCC cell line, SNU182, only had slightly increased SIX1 expression levels, so it was not used to determine the SIX1-mediated effects in HCC cells. Notably, SIX1 silencing exerted no significant effect on the protein expression levels of EYA2, a SIX1 co-activator. These results indicated that the observed findings in the present study were through SIX1, not via EYA2.

In conclusion, the present study provided further information on the functional roles of SIX1 in hepatocarcinogenesis and also demonstrated the importance of SIX1, both as a biomarker and as a target for drug treatment.

Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

Author's contributions:

P.B-E. designed the study. P.B-E., A.A., N.S. and E.E. performed the experiments. P.B-E., A.A., N.S. and E.E. analyzed and interpreted the data. P.B-E, A.A. and N.S wrote the manuscript. All authors read and approved the final manuscript.

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Figures

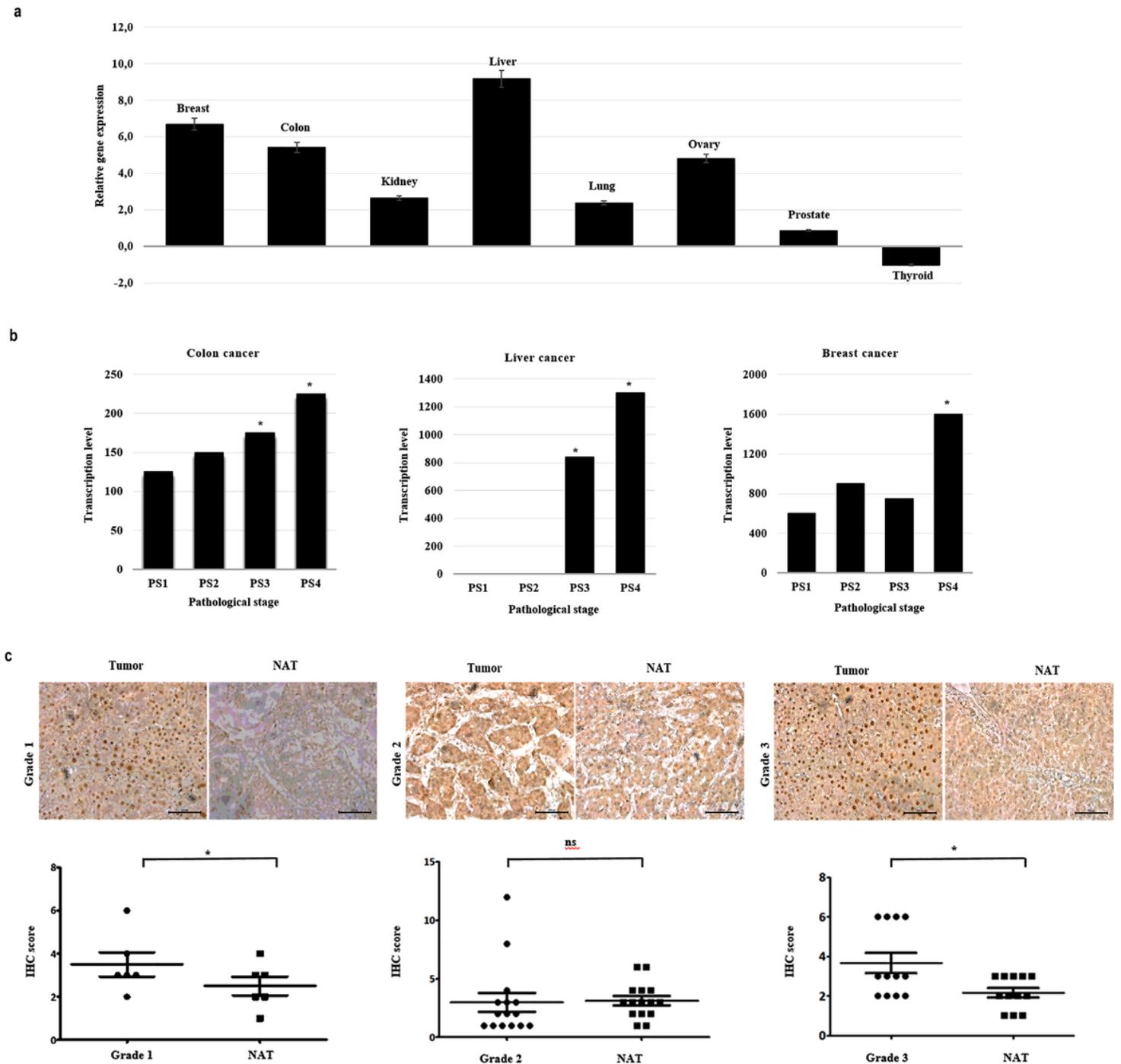


Figure 1

SIX1 gene expression levels both in vivo and using bioinformatic analysis in different types of cancer. a Analysis of the expression levels of SIX1 in cancer cDNA arrays representing tissues from three non-malignant and nine tumor samples from eight different types of cancer. Compared with the control tissue, the expression levels of SIX1 in the liver, colon, breast, ovary, kidney, lung and prostate tumor tissues were markedly upregulated, while they were slightly downregulated in thyroid cancer. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method. b mRNA transcription levels of SIX1 in colon, liver and breast cancers depending on the pathological stages using an Expression Project for Oncology (expO) dataset.

SIX1 expression was significantly upregulated in pathological stage 3 compared to pathological stage 1 in liver and colon cancer, and also SIX1 high expression was detected in stage 4 compared to stage 1 in all 3 types of cancer (*P<0.05). c SIX1 expression levels in tumor tissues were compared with normal tissues in three different stages of hepatocellular carcinoma. SIX1 expression levels in grade 1 (n=6) and grade 3 (n=20) were significantly different in the tumor tissues compared with the normal tissues. In contrast, no significant differences were observed in grade 2 tissues (n=12). NAT, non-associated tissue. *P<0.05, unpaired Student's t-test. Magnification, x40; scale bar, 200- μ m.

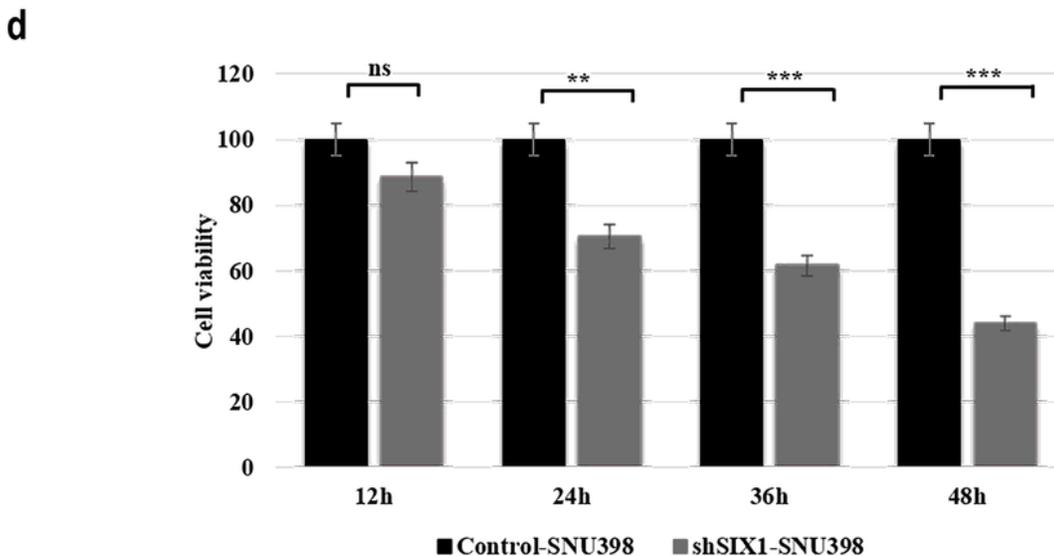
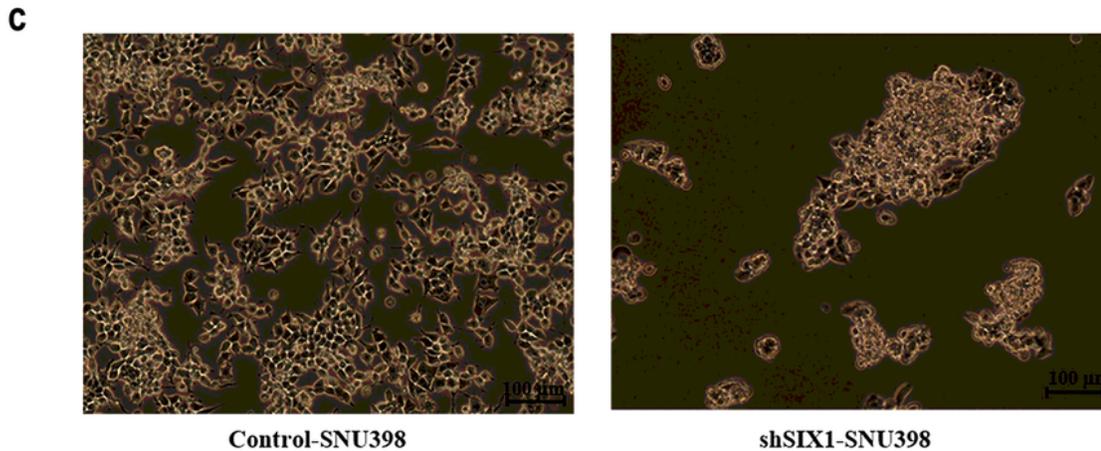
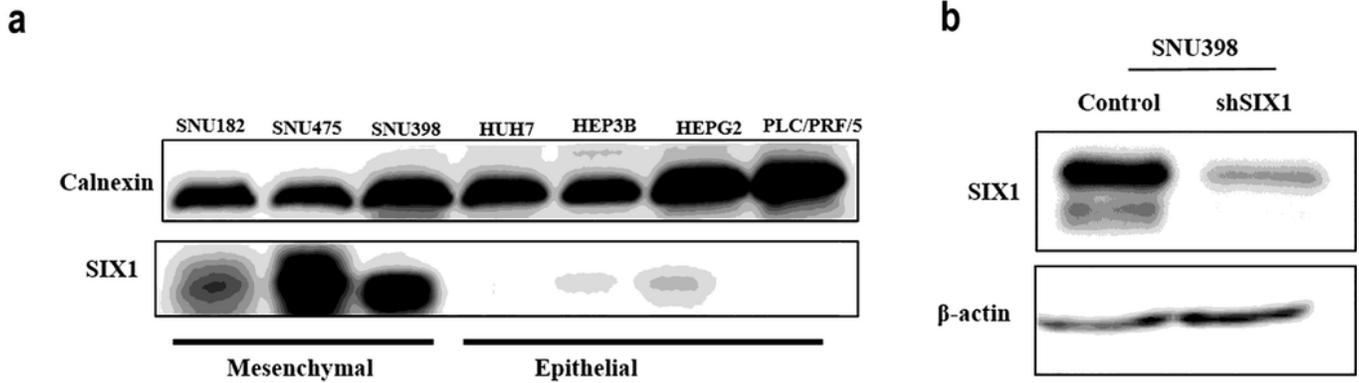


Figure 2

Expression levels of SIX1 in HCC cells and knockdown of SIX1 expression levels in the SNU398 cell line. a Expression levels of SIX1 in different HCC cell lines with mesenchymal and epithelial characteristics. b Transfection efficiency of the knockdown of the SIX1 gene in the SNU398 cell line was validated using western blotting. β -actin was used as the loading control. c Inhibition of SIX1 expression levels in the SNU398 cell line initiated a change in the cell morphology. The different cell morphologies of the shSIX1-SNU398 and control-SNU398 cells were observed under an inverted microscope. Magnification, x20; scale bar, 100- μ m. (D) Bars represent proliferation of cells with and without SIX1 expression as determined by MTT assay. The differences between groups were analyzed using a Student's t-test. ns, non-significant. *** $P < 0.001$, **** $P < 0.0001$.

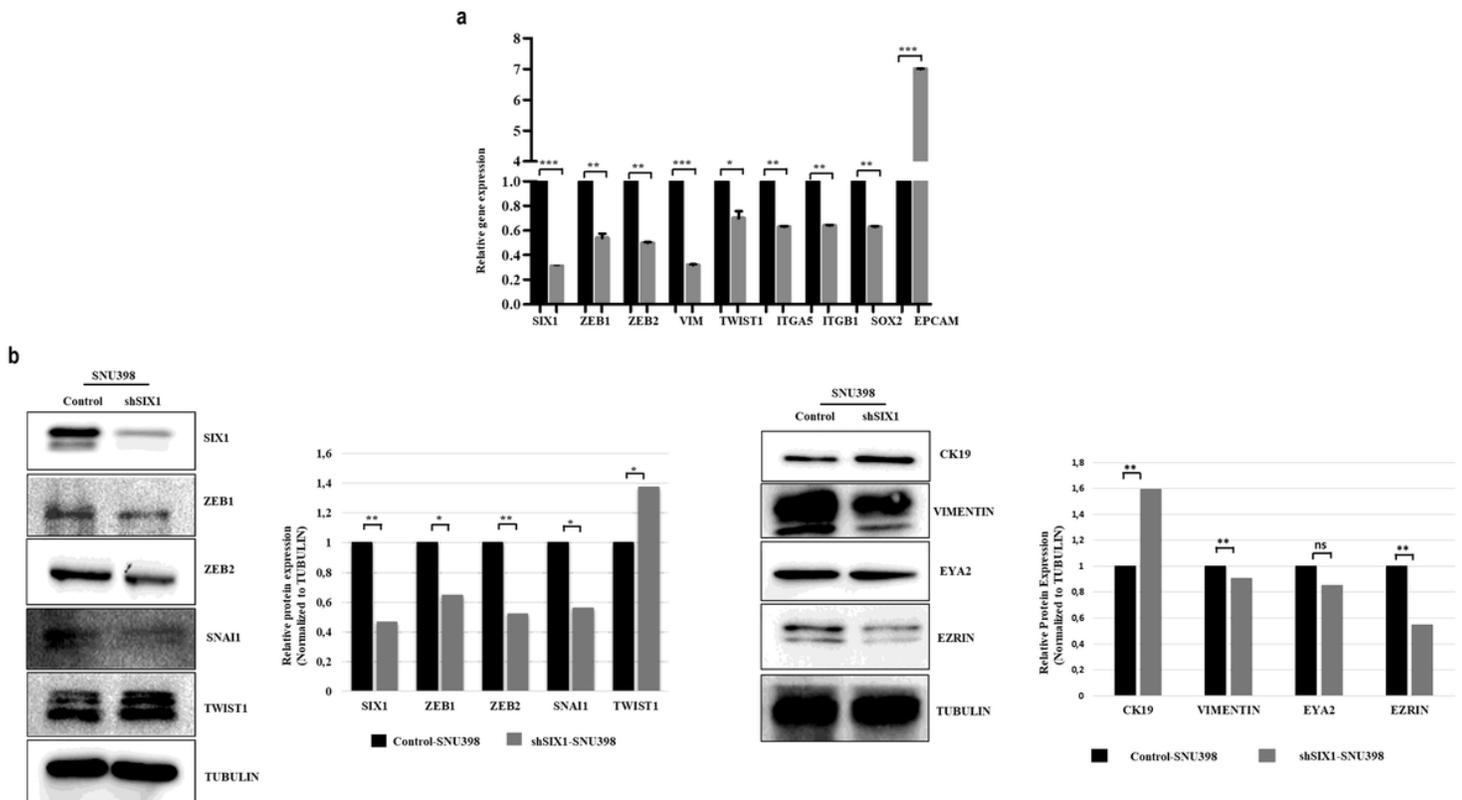


Figure 3

SIX1 knockdown regulates EMT mRNA and protein expression levels. a Effect of SIX1 knockdown on the expression levels of ZEB1, ZEB2 and EMT-related genes was analyzed using RT-qPCR. b Expression levels of EMT-related proteins in the control-SNU398 and shSIX1-SNU398 cells were analyzed using western blotting. α -tubulin served as the loading control. The differences between the groups were analyzed using a Student's t-test. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

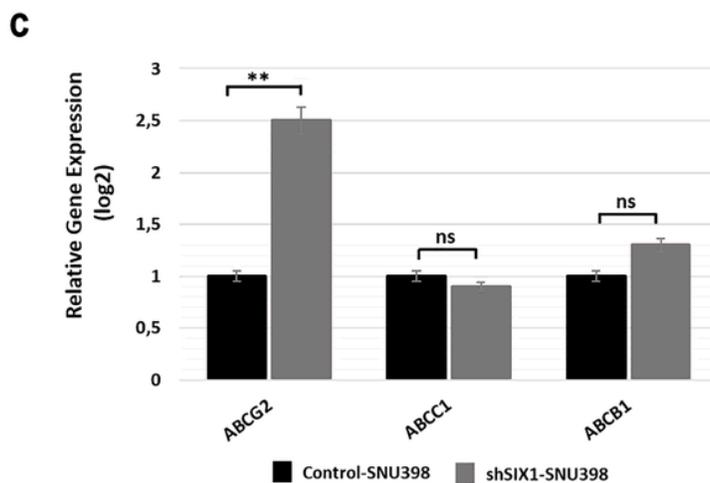
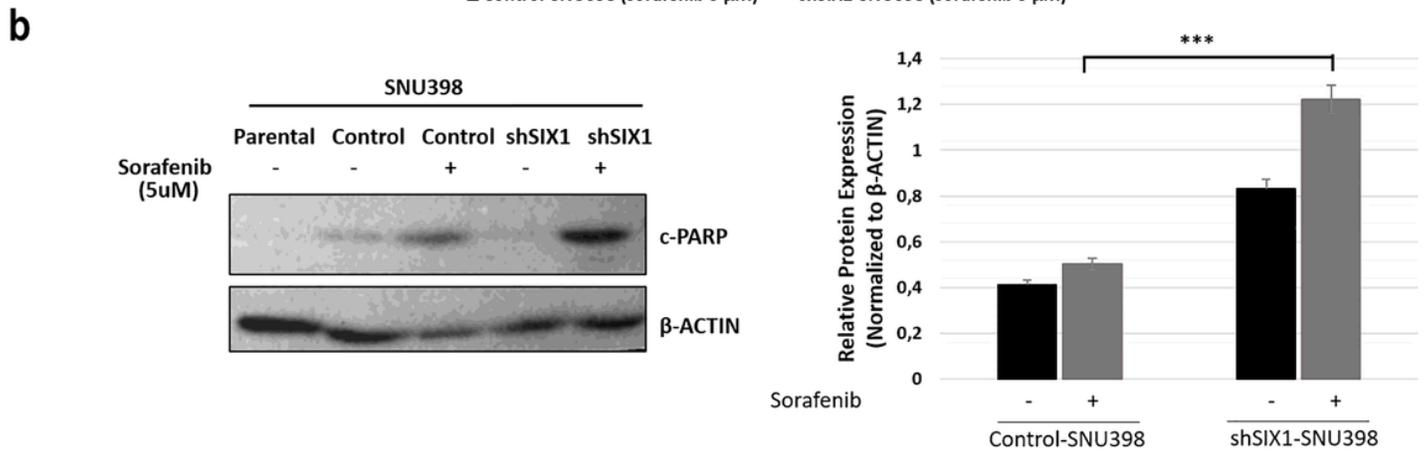
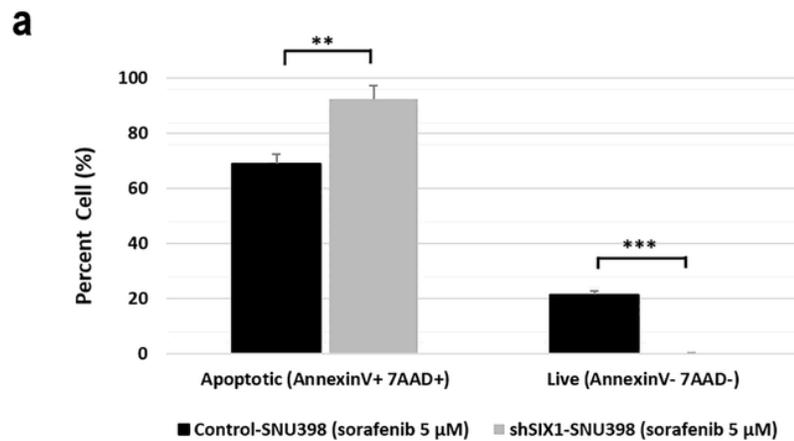


Figure 4

Effect of SIX1 knockdown on apoptosis and drug resistance in the presence of sorafenib. a The percentage of apoptotic and living cells are shown. Apoptotic cells were analyzed by a BD Accuri C6 flow cytometer. b Western blotting was used to investigate the expression levels of the c-PARP protein in SNU398 (parental), shSIX1-SNU398 and control-SNU398 cells. The c-PARP levels was increased in shSIX1-SNU398 cells compared to control-SNU398 cells presence of 5 μM sorafenib. β-actin was used as the loading control. c Effect of SIX1 knockdown in the presence of 5 μM sorafenib on the drug response

and the expression levels of drug resistance-related genes were analyzed using RT-qPCR.. **P<0.001, ***P<0.0001 .

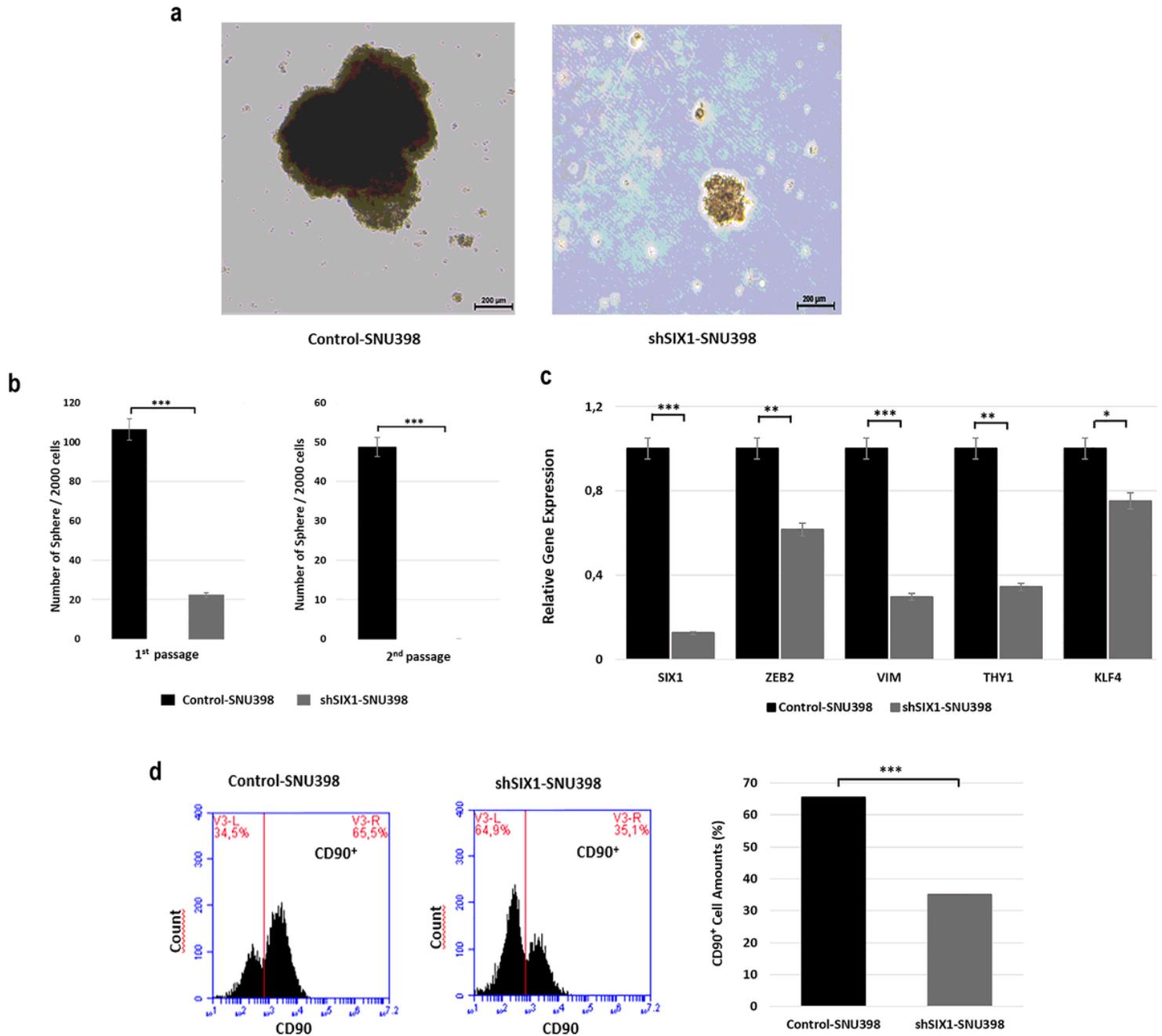


Figure 5

Determining the effect of SIX1 on sphere formation and self-renewal ability. a Sphere formation was measured in shSIX1- SNU398 and control-SNU398 cells. The sphere size decreased in the shSIX1-SNU398 cell line. b To determine the effect of SIX1 on the self-renewal capacity following the sphere formation assay, these cells were passaged two times. It was subsequently observed that the shSIX1-SNU398 cells were unable to self-renew. c RT-qPCR was used to analyze the expression levels of SIX1, ZEB2, VIM, KLF4, and THY1 genes in sphere-forming cells. The gene expression analysis revealed that the expression levels of SIX1, ZEB2, VIM, KLF4, and THY1 were significantly downregulated in shSIX1-SNU398 sphere-

forming cells. d CD90 cell population in shSIX1-SNU398 and control-SNU398 cells was analyzed using flow cytometry. The CD90+ subpopulation was decreased in shSIX1-SNU398 cells compared with the control-SNU398 cells. The differences between groups were analyzed using a Student's t-test. **P<0.01, ***P<0.0001. Magnification, x5; scale bars, 200- μ m.

Supplementary Files

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