Targeted disruption of S100P suppresses tumor cell growth by down-regulation of cyclin D1 and CDK2 in human hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide. The number of cases of HCC has continued to increase in recent decades. Previous studies have suggested that S100P, a member of the S100P calcium-binding protein family, is aberrantly regulated in several malignant neoplasms. However, the underlying molecular mechanisms of the dysregulation of S100P remain to be elucidated. To investigate biological effects of S100P on hepatocarcinogenesis, aberrant expression of S100P was investigated by immunohistochemistry (IHC), Western blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) in HCC tissues and cell lines. Endogenous expression of S100P was disrupted by the RNA interference-mediated protein knockdown method in the human Hep3B liver cancer cell line. Then, cell growth and cellular apoptosis were compared with control siRNA transfectants. The effects of S100P-silencing on the major components of cell cycle regulation were assessed by Western blot analysis. As results, elevated levels of S100P were observed in the HCC tissues compared to the corresponding normal tissues. Targeted disruption of S100P suppressed cell growth and augmented cellular apoptosis. In addition, inhibition of S100P resulted in the down-regulation of cyclin D1 and CDK2. In conclusion, this study showed over-expression of S100P in HCC. The aberrant regulation of S100P in HCC might activate cyclin D1 and CDK expression and contribute to the mitogenic potential of tumor cells during HCC carcinogenesis. These findings provide information that suggests new therapeutic strategies for the treatment of liver cancer.

Introduction

Human hepatocellular carcinoma (HCC) is one of the most common neoplasms. HCC is currently the third leading cause of cancer death worldwide (1). Most patients with HCC have a poor prognosis and die within several months after the diagnosis (2,3). Recent studies have found that genetic alterations of tumor suppressor genes or oncogenes such as p53, β-catenin and AXIN are involved in hepatocarcinogenesis (4-6); however, the frequency of mutations, of these genes, appears to be very low in patients with HCC. Furthermore, it is unclear how these genetic changes correspond to the clinical characteristics of individual patients with HCC. Therefore, the mechanisms underlying the carcinogenesis of HCC remain unclear.

The S100 proteins belong to the EF-hand superfamily of Ca2+-binding proteins and include: S100A2, S100A4, S100A6, S100A7, S100B and S100P. These proteins are mediated by Ca2+-dependent signal transduction pathways involved in the regulation of the cell cycle, growth, differentiation and metabolism (7-10). The S100 proteins have been functionally associated with a variety of neurological, cardiac and neoplastic diseases. The S100 calcium-binding protein P (S100P), a 95-amino acid protein, was first purified from the placenta with limited cell distribution in other tissues (11,12); it is one of the latest members of the S100 family identified and consists of Ca2+-binding proteins of the EF-hand type (9,13). The functions of the members of this family of proteins vary widely. The primary function of S100P is to mediate the Ca2+-dependent signal transduction cascades involved in cell growth and differentiation, cell cycle regulation and metabolic control (8,10). Many studies have shown that S100P plays a role in carcinogenesis (14-16), and its expression has been shown to increase in several malignancies including those of the colon (17), breast (18), pancreas (19), prostate (20) and lung (21). In pancreatic cancer, S100P is over-expressed due
to hypomethylation of specific genes (22). In prostate cancer, S100P expression is regulated by androgens (20) and interleukin-6 (23). In gastric cancer cell lines, S100P expression has been shown to be induced by retinoic acid (24). In breast cancer cell lines, S100P levels have been associated with immortalization of cells (18). In colon cancer cell lines, S100P expression has been correlated with resistance to chemotherapy (25). In lung cancer, S100P expression has been correlated with decreased patient survival (26). It has been shown that S100P can induce anchorage-independent tumor cell growth in vitro and improve tumor growth in a xenograft model. These results suggested that S100P functionally participates in the control of tumorigenic potential in vivo (15). However, despite these observations, it is not clear what the specific molecular events are that underly the aberrant regulation of S100P in most cancers, including HCC.

The aim of this study was to investigate the biological role of S100P in HCC development and progression. The expression of S100P in HCC was compared to corresponding normal hepatocytes. In addition, S100P expression was disrupted by siRNA and the regulatory mechanisms of the cell cycle component, in particular the G1/S transition of the Hep3B cells, was evaluated.

**Materials and methods**

**Tissue samples.** Five frozen human HCCs and their corresponding normal tissues were obtained from Korean patients, and samples were randomly selected for this study. Approval was obtained from the institutional review board of The Catholic University of Korea, College of Medicine. Informed consent was provided according to the Declaration of Helsinki.

**Cell culture and reagents.** Hep3B, SNU182, SNU354, SNU387, SNU449 and SNU475 cells were purchased from Korean Cell Line Bank (KCLB; Seoul, Korea) and THLE-3, HepG2, PLC/PRF/5 and CHANG cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), respectively. Each cell line was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), respectively. Cells were trypsinized, and 1640 medium supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA) with 0.5 mg/ml of streptomycin and 1 mg/ml of penicillin was added. Each cell line was grown in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) with 0.5% fetal bovine serum (Hyclone, Logan, UT, USA) for 48 h, followed by 3 days of growth in serum-free medium. After growth, the cells were transfected with siRNA targeting S100P.

**Reverse transcriptase-polymerase chain reaction (RT-PCR).** Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration was determined by NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA, USA). Next, 1 μg of total RNA was reverse transcribed by Superscript II enzyme (Gibco-BRL, Gaithersburg, MD, USA) with 0.5 μg oligo(dT) (Amersham Biosciences, Piscataway, NJ, USA). The reaction mixture was incubated at 42°C for 60 min and then at 72°C for 15 min. To normalize differences in the amount of total cDNA added to each reaction, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression was used as an endogenous control. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 58°C for 5 sec, 72°C for 5 sec. S100P and GAPDH transcripts were measured using polyacrylamide gel electrophoresis.

**Western blot analysis.** The following antibodies were used: anti-S100P (BD Biosciences, San Jose, CA, USA), anti-PARP, anti-p15/16/21, anti-cyclinD1/D3 (Cell Signaling Technology, Danvers, MA, USA), anti-α-tubulin (Sigma), anti-CDK2 (Santa Cruz Biototechnology, Santa Cruz, CA, USA), anti-mouse IgG (Amersham Biosciences), anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA, USA) and ECL plus Western blotting detection system (Amersham Biosciences) were used to detect the bound antibodies. The intensity of the Western blot bands was quantified using LAS 3000 (Fuji Photo Film Co., Tokyo, Japan).

**Immunohistochemistry of the S100P.** For the immunohistochemical analysis, we used HCC tissue micro array (TMA) slides. The S100P primary antibody (1:25 dilution) was used in the IHC. Diaminobenzidine (DAB; Dako, Copenhagen, Denmark) was used as a chromogen, and the slides were then lightly counterstained with Mayer's hematoxylin. Scoring of the TMA was performed independently by two pathologists. In the event of disagreement, the two reached a consensus by jointly re-evaluating the TMA using a multi-head microscope. The intensity of the immunostaining was graded as: negative, weak positive, 1+ (moderate), 2+ and 3+ (strong). If the number of immunostained cells was less than 10%, the sample was considered negative.

**MTS assay for cell proliferation.** Proliferation of cells was measured at indicated time with the use of Cell Titer96 Aqueous One solution cell proliferation assay (MTS; Promega, Madison, WI, USA), according to the manufacturer’s instructions. Briefly, Hep3B cells were seeded in 12-well flat-bottomed microtiter plates at a density of 2×10^4 cells/well. The cells were transfected with siRNA targeting S100P or scramble siRNA, and then cells were allowed to grow for indicated time points and were then incubated with MTS for 1 h at 37°C in a humidified 5% CO₂ atmosphere. The absorbance was read at 490 nm by VICTOR3™ Multilabel Plate Readers (Perkin-Elmer, Waltham, MA, USA). All measurements were performed in triplicate and each experiment was repeated at least three times.
Cell cycle and apoptosis analysis. After transfection, the Hep3B cells (2x10⁵) were harvested by trypsinization at 48 h after transfection, and then washed with 1X PBS, followed by fixation in 70% alcohol for 1 day at -20°C. After fixation, the cells were washed with ice cold 1X PBS twice and incubated for 30 min in PBS containing RNase A (10 mg/ml) at 37°C. After the RNase A treatment, the nuclei were stained with 5 mg/ml of propidium iodide (PI) before their DNA content was measured using a flow cytometer (BD Biosciences). The Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) was used to quantify the level of apoptosis in the samples. Briefly, the cells were trypsinized, washed twice with cold PBS and resuspended in 1X binding buffer at a concentration of 1x10⁶ cells/ml, and 100 μl of the solution (1x10⁶ cells) was transferred to a 5 ml culture tube and 5 μl of Annexin V-FITC solution was added; PI (10 μl) was added to each tube and the cells were analyzed by flow cytometry.

Statistical analysis. Each experiment was performed at least three times. The data are presented as the mean ± standard errors for the number of experiments. Statistical significance was defined by the results of the unpaired Student’s t-test at a p<0.05 and the Chi-square test for the immunohistochemistry. 

Results

S100P expression in hepatocellular carcinoma. Accumulating evidence has shown that S100P is functionally associated with a variety of neoplasms; however, its role in human hepatocellular carcinoma has not yet been determined. Thus, in order to investigate aberrant expression of S100P in HCC, we measured and compared the expression levels of S100P in both liver cancer cell lines and human HCC tissues. In the liver cancer cell lines, when we assessed the expression of S100P by RT-PCR and Western blot analysis, most of the cancer cell lines derived from HCC showed a higher expression of S100P when compared to THLE-3 cells that were immortalized from normal hepatocytes and used as normal control cells for comparison. In the normal cells, S100P was not detectable by either RT-PCR or Western blot analysis. In addition, for the HepG2 cells, established from hepatoblastoma, there was a high expression of S100P (Fig. 1A). In order to confirm the aberrant expression of S100P in the HCCs, the differential expression of S100P was determined in five selective human HCC tissues. As shown in Fig. 1B, S100P was observed to be over-expressed in the tumor tissues compared to the corresponding normal tissues.

The protein expression of S100P was further validated by immunostaining the HCC specimens with S100P antibody; the results are summarized in Fig. 2 and Table I. As summarized in Table I, 19 out of the 25 HCCs tested (76%) resulted in moderate or strong positive staining with the S100P antibody, while 14 out of the 25 (56%) normal hepatocytes showed negative or weak expression of S100P in the adjacent normal tissues. The higher rate of positive (44%) staining in the normal tissues could be attributed to background disease affecting the normal tissues such as chronic hepatitis or liver cirrhosis. Fig. 2 shows a representative image of normal hepatocytes (Fig. 2A and C) and HCC tissues (Fig. 2B and D). These results suggest that S100P is dysregulated in HCCs and may contribute to HCC formation or progression.

Targeted disruption of S100P suppresses neoplastic activity of Hep3B cells. To explain the biological consequence of dysregulation of S100P expression, we targeted S100P by the RNA interference-mediated protein knockdown method, in Hep3B human HCC cells. S100P gene silencing was evaluated by siRNA targeting of S100P, and the efficiency of S100P-targeted siRNA-mediated down-regulation of S100P mRNA was assessed by RT-PCR. The results showed that both 50 and 100 nM of the S100P siRNA could effectively knock down the expression of S100P in the Hep3B cells compared to the non-silencing (scrambled)
Figure 2. Representative image of immunohistochemistry for S100P in human HCCs and adjacent normal tissues. Immunostaining of HCC and adjacent normal tissues was carried with S100P antibody. The representative images of normal hepatocytes with magnification (A), x100 and (C), x200, and HCC with magnification (B), x100 and (D), x200 were developed with DAB chromogen and counterstained with hematoxylin.

Figure 3. Targeted disruption of endogenous S100P suppresses the growth rate of Hep3B cells. (A), RT-PCR and Western blot analysis showing the silencing of endogenous S100P mRNA and protein levels in Hep3B cells after transfection with the indicated concentration of S100P specific siRNA (S100P siRNA), but not with scrambled siRNA (scr) or no treatment (none). (B), Cell growth rates of Hep3B cells transiently transfected with scrambled siRNA (scr) and S100P specific siRNA (S100P siRNA). The cells were seeded on 24-well plates at an equal density and cultured for the indicated time before the number of each cell type was evaluated by MTS assay. The data are presented as mean ± standard error for three experiments (unpaired Student's t-test, *p<0.05 vs. control).
siRNA. This observation was confirmed at the protein level by Western blot analysis, which showed that S100P-targeted siRNA could silence protein expression of S100P compared to a negative control siRNA (Fig. 3A). To determine the biological effect of the disruption of S100P expression, the growth rate of the Hep3B cells was determined by MTS assay. As shown in Fig. 3B, silencing of S100P significantly decreased the cell growth rate at both the 50 and 100 nM siRNA concentrations compared to cells treated with the control siRNA. This anti-mitogenic effect on Hep3 cells could be partially explained by disturbance of the cell cycle regulation or augmentation of cellular senescence and apoptosis. In addition, a similar result was observed in the cell viability assay (data not shown).

Next, we examined the effect of S100P, in the Hep3B cells, on cellular apoptosis. As shown in Fig. 4A, when the cells were almost completely knocked down, the cells displayed apoptosis by increasing the cleavage form of poly ADP-ribose polymerase (PARP), a hallmark of apoptosis. Similar results were obtained by flow cytometric analysis, measuring the Annexin V staining of cells of the siRNA transfectants. The flow cytometric analysis with propidium iodide (PI) and Annexin V staining, for the dead cells or apoptotic cells, indicated that both early and late stages of apoptosis (M2) were enhanced in the S100P-siRNA transfectant cells. As shown in Fig. 4B, a significant induction of apoptotic cells was seen in the S100P-siRNA transfectants compared to the control groups (none or scr siRNA treatment). The apoptotic

Figure 4. Increase in the basal level of apoptosis in the Hep3B cells after silencing endogenous S100P. (A), Endogenous S100P, in the Hep3B cells, was knocked down by S100P specific siRNA (S100P siRNA). The cleavage form of PARP, a hallmark of apoptosis, was assessed by Western blotting with antibody for PARP. For normalization of protein expression, α-tubulin was used as an internal control. (B), Silencing of endogenous S100P increased the basal apoptosis in the Hep3B cells. The Hep3B cells were transiently transfected with scrambled siRNA (scr) or S100P specific siRNA (S100P-siRNA) and stained with Annexin V and propidium iodide and then quantified by fluorescence-activated cell sorter (FACS) analysis. The population shown in the figure is the total distribution of cells. Significant induction of basal apoptosis was observed in the Hep3B cells after silencing of endogenous S100P at both concentrations. Data were obtained in three independent experiments.
cells (right in dot-plot graphs of Fig. 4B) were increased from 30.2 to 40.6% in the 50 and 100 nM S100P-siRNA treatment groups compared to no treatment (12.5%) or scr siRNA (16.9%) treatment.

**Regulatory mechanisms of S100P on G1/S cell cycle transition.** The fact that the suppression of S100P elicits regression of Hep3B cell growth implies that S100P is involved in the regulation of the cell cycle. In addition, FACS analysis with PI staining of cells showed a decrease in the proportion of cells in the S-phase in the S100P-siRNA transfectants (data not shown). Because cyclin-dependent kinases (CDKs), CDK inhibitors (CDKIs) and cyclins are the core components of the cell cycle clock, we next examined the effects of S100P suppression on these regulatory components in the G1/S phase of the cell cycle. In the G1/S phase transition of the cell cycle, it has been well-established that negative cell cycle regulators such as p21^WAF1/CIP1, p15^INK4B, p16^INK4A and p27^Kip1 are the key modulators that suppress cyclin D1/CDK4, 6 or cyclin E/CDK2 complexes (27-29). When these negative cell cycle modulators were examined in the S100P-siRNA transfectants, no significant changes of protein expression, α-tubulin was used as an internal control. Data are representative of two independent experiments.

### Discussion

S100P is a member of the S100 family that consists of Ca\(^{2+}\)-binding proteins, of the EF-hand type, with at least 20 members (9,13). This protein was first purified from the placenta (12), and has been associated with a large number of diseases by both intracellular and extracellular mechanisms (14). In addition, the S100P protein has been recently considered a potential biomarker of cancer due to its frequent expression in different types of tumor tissues (15,30). However, despite the accumulating evidence supporting aberrant cell cycle regulation and tumorigenic potential of S100P, in many solid tumors, there is no information on its role in human HCC. Much of the experimental data have been obtained by S100P-transfected cells *in vitro* and *in vivo* as well as from DNA microarray studies (14,15,31-33). We have noted that S100P expression has been shown to gradually increase from dysplasia, precancerous lesions, to HCC based on our previous findings, which suggests large-scale molecular changes from the dysplastic nodule to HCC by expression profiling (34). In addition, these results suggest that dysregulation of S100P might be associated with HCC progression and/or formation. In the present study, the findings showed up-regulation of S100P in HCCs; in addition, direct-targeting of RNA interference-mediated protein knockdown of S100P abolished the mitogenic properties of Hep3B cancer cells.

The analysis of endogenous expression of S100P in liver cancer cell lines revealed no detectable mRNA in the THLE-3 cells, which were derived from normal human hepatocytes. However, the cancer cells expressed S100P in both liver cancer cell lines and HCC tissues (Fig. 1). As summarized in Table I, the immunohistochemistry analysis of 25 HCCs showed that 19 out of the 25 tested cases resulted in positive findings (76%) in the HCCs; however, 11 cases (44%) were also positive in the normal tissues. This high rate of positive findings in normal tissues could be partially explained by the presence of background disease such as chronic hepatitis or liver cirrhosis.

With regard to the biological activity of S100P during tumor progression, there are several lines of evidence that indicate that S100P acts as a proliferative and prosurvival factor in several types of cancer cells including NIH-3T3 cells (35), breast cancer cells (36) and pancreatic cancer cells (14). Our results also showed aberrant expression of S100P in HCCs. To determine the biological role of S100P in HCC, RNA interference-mediated gene silencing was employed. The silencing of endogenous S100P, in the Hep3B cells, resulted in the regression of cell growth (Fig. 3) and increased the basal levels of apoptotic cells (Fig. 4). This result is consistent with previous findings that suggest that S100P might act as an aggressiveness factor in HCC cells by facilitating cell growth and conferring resistance to basal apoptosis.

Accumulating evidence has suggested that S100 proteins have regulatory roles associated with cell cycle transition (7,37). In the G1/S phase transition of the cell cycle, it is well-known that negative cell cycle regulators such as p21^WAF1/CIP1, p15^INK4B, p16^INK4A and p27^Kip1 are the key modulators responsible for suppressing cyclin D1/CDK4, 6 or cyclin E/CDK2 complexes (27-29). Thus to identify the underlying mechanism associated with the neoplastic potential of S100P...
aberrant regulation, we investigated the regulatory role of S100P with regard to the key components of the G1/S cell cycle transition. During the regulation of the cell cycle, p21WAF1/CIP1 inhibits the CDK/cyclin2 complex that induces hypophosphorylation of the Rb protein. In addition, cyclin D1 is also involved in regulating the Rb phosphorylation status in association with CDK4/6. As shown in Fig. 5, disruption of S100P simultaneously suppressed the expression of cyclin D1 and CDK2 in the Hep3B cells. This implies that aberrant regulation of S100P might augment two key modulators of the cell cycle exerting synergistic effects on cell cycle transition and may result in uncontrolled cell cycle activity during the development of HCC. Although these findings do not fully explain the mechanisms of S100P that are associated with the cell cycle, it is possible that S100P has a mitogenic role as a cell cycle regulator in Hep3B human hepatocellular carcinoma cells.

According to a recent study, addition of exogenous S100P stimulated cell proliferation and survival via the receptor for activated glycation end products (RAGE). The activation of RAGE is known to phosphorylate Erk and stimulates NF-κB (35). Erk activation is universally associated with stimulation of cell growth and NF-κB activation is frequently associated with increased cell survival. Thus, we investigated whether silencing of endogenous S100P by siRNA affected these molecules. However, we observed no significant change in these molecules at either the phosphorylation or protein expression levels (data not shown). Since little is known about the interaction of S100P and Erk 1/2, and the NF-κB signaling pathway, additional studies are necessary to fully understand the intracellular mechanisms of S100P in liver cancer cells.

In summary, the results of this study showed overexpression of S100P in both cancer cell lines and human HCCs. In addition, disruption of S100P in human HCC cell lines suppressed tumor cell growth by the transcriptional inhibition of CDK2 and cyclin D1. This is the first report that S100P is dysregulated in human HCC, and to suggest that S100P might contribute to tumor cell growth and survival. The current data suggest that S100P might be associated with the development of the aggressiveness in HCCs.

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References


