Role of S100A3 in human hepatocellular carcinoma and the anticancer effect of sodium cantharidinate

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Abstract. The fifth most common cancer worldwide is hepatocellular carcinoma (HCC), which has an annual mortality rate of ~800,000. Although surgical procedures for HCC, such as hepatic resection and liver transplantation, have progressed and the outcomes of patients have improved, HCC is still characterized by frequent recurrence, even after liver transplantation. In the present study the expression of the protein coding gene, S100 calcium binding protein A3 (S100A3), was observed in 62 HCC tissues and tumor-surrounding tissues. The present study indicated that S100A3 activation was involved in tumorigenesis and tumor aggressiveness. The protein and mRNA expression levels of S100A3 in the human HCC cell line (HepG2) were investigated using western blotting and reverse transcription-quantitative polymerase chain reaction analysis, respectively. The function of sodium cantharidinate in inducing HCC cell apoptosis was also investigated. Sodium cantharidinate inhibited the protein and gene expression of S100A3 in HepG2 cells in vitro. These data suggested that S100A3 has an important role in human HCC. The present study indicates that the functional properties of sodium cantharidinate are promising for the development of a novel drug that may control the expression of S100A3 and improve the treatment of human HCC in the near future.

Introduction

Due to its incidence, hepatocellular carcinoma (HCC) is becoming the most common primary liver malignancy and is a leading cause of cancer-related mortality worldwide, with a mortality rate of ~800,000 per year (1). Surgical procedures for HCC have recently progressed with respect to hepatic resection and liver transplantation. However, limitations in treatment are evident in the frequency of recurrence observed, even after liver transplantation (2-5). There is, therefore, an urgency to develop novel approaches for HCC prevention and treatment. At present, chemotherapy is also a focus for tumor treatment (6).

The molecular targeting agent, sorafenib, is reported to improve survival rates and the outcomes of patients with unresectable or early stage HCC (7,8). Although other target agents are under investigation, sorafenib remains the only approved molecular targeted treatment for advanced HCC. Trials comparing novel agents in combination with sorafenib are ongoing. Evaluations of combined treatment with systemic targeted therapies and local therapy are undergoing investigation to further improve the outcomes of patient with HCC (9-11).

The S100 protein family has a wide range of biological functions that are associated with the regulation of cell proliferation, differentiation, apoptosis, Ca2+ homeostasis, energy metabolism, inflammation and migration/invasion. These processes involve interactions with multiple target proteins, including enzymes, cytoskeletal subunits, receptors, transcription factors and nucleic acids. The matricellular protein, S100A3, is expressed in various tissues and cell types (12-14). S100A3 protein is encoded by the S100A3 gene and contains two EF-hand calcium-binding motifs in humans (15-17). These motifs are connected by 10-12 residues, which form a critical ‘hinge’ region (loop 2) that is involved in target interactions. In the absence of a target, the majority of S100 proteins exhibit low Ca2+-binding affinity; however, when a target is bound, a 5- to 300-fold increase in Ca2+-binding affinity occurs (18-20).

Over the last decade, the importance of S100A3 as a biomarker for tumor progression has been recognized, although...
it is unclear whether S100A3 is important in HCC and whether it is possible to inhibit S100A3 with drug treatment. During this period, the treatment options for HCC have expanded to include additional chemotherapeutic agents and targeted therapies (21,22). In recent years, traditional Chinese medicine (TCM), or its extractions, have had a positive role in the treatment of cancer (23,24). However, it has not yet been elucidated whether TCM is able to inhibit the expression of S100A3 and prevent the symptoms of HCC. Sodium cantharidinate has been used in TCM (25-27). Previous studies have indicated that sodium cantharidinate may induce cell cycle arrest and trigger apoptosis in various types of tumor cells (28-33). The present study investigated whether S100A3 is important in HCC and whether sodium cantharidinate may be used to inhibit the expression of S100A3. The protein and mRNA expression levels of S100A3 in the human HCC cell line (HepG2) were investigated using western blotting and reverse transcription-quantitative polymerase chain reaction analysis, respectively. The function of sodium cantharidinate in inducing HCC cell apoptosis was also investigated. This research may provide novel targets for the treatment of HCC and thus further improve the outcomes of patient with HCC.

Materials and methods

Reagents. Sodium cantharidinate (C7632) was purchased from Meryer (Shanghai, China); MTT (4890-025) and RNase (H42490210) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Propidium iodide (PI) and an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit were purchased from BD Biosciences (556547; San Jose, CA, USA). Dulbecco's modified Eagle medium (DMEM; 10-013), trypsin (T3924), foetal bovine serum (FBS; 10270106), PBS (21-040-CVR), penicillin and streptomycin (SV30010) were obtained from Gibco (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Preparation of sodium cantharidinate. Sodium cantharidinate was dissolved in PBS (pH 7.2) to prepare a stock solution at a concentration of 1.0 mmol/l, which was stored at -20°C. Complete DMEM was added to dilute the sodium cantharidinate to the appropriate concentrations prior to use.

Patients and tissue specimens. A total of 62 patients, comprising 41 males and 21 females, with a mean age of 62.4 years (range, 34-78 years) were included in the present study. Human HCC tissue and tumor-surrounding tissue specimens were obtained by surgical resection at the Jilin University First Hospital (Changchun, China) between May 2012 and July 2013. The present study was approved by the Ethics Committee of Jilin University First Hospital and written informed consent was obtained from all patients. An experienced pathologist examined hematoxylin and eosin-stained sections from each tumor sample to confirm the histological diagnosis and assess the tumor content. Histological diagnoses, including the tumor differentiation and encapsulation, were made according to the guidelines proposed by the World Health Organization (34). The differentiation grade of the tumor and its clinical stage were recorded.

Effect of sodium cantharidinate on the viability of HepG2 cell lines. The inhibitory effect of sodium cantharidinate on the viability of HepG2 cells was detected by performing MTT assays, with all experimental steps performed according to the instructions of the kit. Cells were seeded on 96-well plates at a density of 5x10^4 cells/ml in complete DMEM in the presence or absence of sodium cantharidinate (5.0 µmol/l). Analysis was performed via MTT assay or apoptosis detection, as outlined.

Isolation of primary human hepatocytes (PHH). All procedures were performed in accordance with the provision of the Ethical Commission of Jilin University First Hospital. PHH were obtained from healthy peritumoral liver resection specimens from non-hepatitis B virus, non-hepatitis C virus and non-human immunodeficiency virus-infected patients undergoing partial hepatectomy for primary HCC or secondary metastatic lesions caused by other types of cancer. PHH were isolated and cultured as described previously (35).
cantharidinate (0.2, 1.0, 5.0 and 25.0 µmol/l), were incubated for 24-72 h. MTT (1 mg/ml) was added to each well, and the cells were for 4 h at 37 C. MTT solution was aspirated and 100 µl DMSO was added. Subsequently, 96-well plates were analyzed using a microplate spectrophotometer (Synergy H1; BioTek Instruments, Inc., Winooski, VT, USA) at 540 nm. Experiments were repeated in triplicate. The inhibition percentage was calculated as: Inhibition percentage=(1-the value in experimental group/the value in the control group) x100%.

Flow cytometry for measuring cell apoptosis. Annexin V-FITC and PI double staining flow cytometry analyses were employed. HepG2 cells were plated in 96-well plates containing 200 µl medium, at a density of 5x10⁴ cells/well. Induction of apoptosis in HepG2 cells was examined in the presence or absence of sodium cantharidinate (5.0 µmol/l). Following 48 h in culture, the cells were collected in 1.5 ml centrifuge tubes, washed three times with cold PBS and binding buffer, and stained with Annexin V-FITC and PI (Annexin V-FITC Apoptosis Detection kit; BD Biosciences, San Jose, CA, USA) for apoptosis detection. HepG2 cells in centrifuge tubes were resuspended in binding buffer. FITC-Annexin V (5 µl) was added to the tubes, which were incubated for 10 min at room temperature, followed by the addition of 5 µl PI. Samples were incubated with PI for a further 15 min at room temperature and immediately analyzed using a flow cytometer (FACScan; BD Biosciences) using FlowJo FACS analysis software 7.6 (Tree Star, Inc., Ashland, OR, USA). Cells in different portions represented the different cell states as follows: Upper right, late-apoptotic cells; lower left, viable cells; lower right, early-apoptotic cells, upper left, dead cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the HCC tissues and adjacent non-tumorous tissues or HepG2 cells using a Qiagen RNeasy Mini kit (Qiagen China Co., Ltd., Shanghai, China). Cultured HepG2 cells were scraped, lysed in Buffer RLT, and collected using cell scrapers (Thermo Fisher Scientific, Inc.). Human tissue samples were homogenized in Buffer RLT using pellet pestles (Sigma-Aldrich; Merck Millipore) and passed through a 27 G x1/2-inch needle. To remove lipid and tissue debris, the homogenates were centrifuged at 1,500 x g for 5 min at 4C, and the aqueous phase was collected. Following the RNeasy Mini kit instructions, 70% ethanol was added to cell lysates or the homogenates and the samples were mixed by pipetting prior to being transferred to columns. Following centrifugation for 2 min at 4 C and 12,000 x g, the flow-through extract was retained and stored on ice. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Waldbronn, Germany). cDNA was synthesized with Supermix II reverse transcriptase using random hexamer primers (TransGen Biotech, Beijing, China) according to the manufacturer’s instructions. Primers for S100A3 and GAPDH were designed and synthesized by Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China). An Applied Biosystems StepOnePlus Real-Time PCR system (Thermo Fisher Scientific Inc.) was used to determine the mRNA levels of S100A3 and GAPDH (internal control). Primers were designed as follows: S100A3, forward 5'-GAC the mRNA levels of S100A3 and GAPDH (internal control). A system (Thermo Fisher Scientific Inc.) was used to determine

| Variable                        | Male (n=41) | Female (n=21) |
|---------------------------------|-------------|---------------|
| Age, years (%)                  | 54.6        | 68.8          |
| Range                           | 34-76       | 51-78         |
| Minimum                         | 34 (2.4)    | 51 (9.5)      |
| Maximum                         | 76 (4.8)    | 78 (14.3)     |
| Grade of differentiation, n (%) | Poorly differentiated 20 (48.8) 12 (57.1) | Moderately differentiated 12 (29.3) 4 (19.1) | Highly differentiated 9 (21.9) 5 (23.8) |
| Clinical stage, n (%)           | I/II 14 (34.1) 7 (33.3) | III/IV 27 (65.9) 14 (66.7) |

Table I. Clinicopathological features of patients with hepatocellular carcinoma (n=62).

Western blotting. HepG2 and PHH cells were lysated and quantitated using a Boster protein quantitative kit (AR1110; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Subsequently, 50 µg protein were prepared in SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 50 mM DTT and 0.1% bromophenol blue) with a mixture of protease inhibitors and PhosSTOP Phosphatase Inhibitor (Roche Diagnostics, Basel, Switzerland). Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Roche Diagnostics). The membrane was blocked with 0.05% Tween-20 in PBS containing 5% skimmed milk and incubated overnight with the primary S100A3 antibody (WH0000274M1; 1:1,000; Sino Biological Inc., Beijing, China) at 4°C overnight. The membrane was subsequently washed three times in 0.05% Tween 20 in PBS and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (SA00001-2; 1:1,000; Proteintech Group, Inc., Wuhan, China) for 2 h at room temperature. Following washing, protein bands were visualized using an enhanced chemiluminescent system (Thermo Fisher Scientific, Inc.). The image analysis was conducted using Image J software 1.48u (National Institute of Health, NY, USA). β-actin antibody (4967; 1:1,000) was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Statistical analysis. All data were analyzed and assessed for significance using the Pearson omnibus normality test. All data are presented as the mean ± standard deviation. Mean values were compared using paired t-tests (two groups), followed by the Bonferroni correction for multiple comparison tests. P<0.05 was considered to indicate a statistically significant difference. All statistical tests were performed using Prism software (GraphPad Software 5.0, Inc., La Jolla, CA, USA).
Results

Clinicopathological features of patients. Experimental samples were obtained from patients who underwent surgical resection of human HCC with a range of grade differentiations. Expression levels of S100A3 in HCC tissues and adjacent non-tumorous tissues were recorded and analyzed. The demographics of the patients are shown in Table I, along with the clinical stages of the HCC and the grades of differentiation. There was an incidence of 34.1% for stage I/II and 65.9% for stage III/IV, respectively, in males. In females, the incidence of stage I/II was 33.3%, and stage III/IV was 66.7%, respectively. The incidence of grade differentiation was also recorded in patients. Poor differentiation was predominantly detected in patients, with incidence rates of 48.8% in males and 57.1% in females.

Expression of S100A3 in human HCC tissues and HepG2 cells. Immunohistochemical staining was performed to assess the protein expression of S100A3 in human HCC tissues and adjacent non-tumorous tissues. The results indicated that S100A3 was expressed in the cytoplasm of HCC tissues and adjacent non-tumorous tissues of the patients with HCC (Fig. 1A and B, respectively). The expression of S100A3 significantly increased in the HCC tissues ($P<0.001$; Fig. 1C), with expression predominantly detected in the tumor regions. The mean percentage of S100A3 in adjacent non-tumorous tissues and HCC tissues was 8 and 22%, respectively. The difference was significant ($P<0.001$; Fig. 1C). Total RNA (mRNA) was extracted from HCC tissues and adjacent non-tumorous tissues. qPCR was used to detect the expression of S100A3 mRNA. There was a significant difference between the S100A3 mRNA levels in the HCC and the adjacent non-tumorous tissues ($P<0.001$; Fig. 1D). These results were
consistent with the findings of immunohistochemical staining. The HepG2 cell line is a specific human HCC cell line that has often been used for mechanism studies in vitro (36). S100A3 expression in HepG2 cells was also investigated by western blotting. Fig. 1E indicated S100A3 expression was higher in HepG2 cells, as compared with PHH.

**Effect of sodium cantharidinate on the viability of HepG2 cells.** HepG2 cells were cultured in complete DMEM. The MTT assay revealed that sodium cantharidinate significantly inhibited the viability of HepG2 cells (P<0.001). The inhibitory effects of sodium cantharidinate on HepG2 were dose- and time-dependent within the ranges of 0.2-5.0 µmol/l and 24-72 h. Treatment with sodium cantharidinate at 5.0 µmol/l for 48 h elicited the greatest inhibitory effect. This dose inhibited the viability of HepG2 cells with an inhibitory rate exceeding 90%, compared with that of the blank control group (P<0.001). The time-effect curve is illustrated in Fig. 2.

**Annexin V-FITC assay.** An Annexin V-FITC and PI double staining assay was performed to explore whether sodium cantharidinate induced HepG2 cell apoptosis. HepG2 cells were treated with sodium cantharidinate at a concentration of 5.0 µmol/l for 48 h and were analyzed using flow cytometry. When compared with the control group (Fig. 3A), the numbers of early and late apoptotic cells markedly increased in the sodium cantharidinate-treated group (Fig. 3B). Statistical analysis demonstrated that the proportion of early and late apoptotic cells in the sodium cantharidinate-treated group reached 57.2%, which was significantly greater than the proportion observed in the control group (20.1%) (P<0.001; Fig. 3C). This finding indicated that sodium cantharidinate significantly induced the apoptosis of HepG2 cells.
Effect of sodium cantharidinate on the expression of S100A3 in HepG2 cells. HepG2 cell pellets were harvested for the detection of S100A3 gene and protein expression 48 h after incubation in the presence or absence of sodium cantharidinate (5.0 µmol/l). Fig. 4A indicates that the expression of S100A3 mRNA in the sodium cantharidinate-treated group significantly decreased (0.22-fold) when compared with the control group (P<0.001). The relative expression of S100A3 protein in HepG2 cells was investigated by western blotting (Fig. 4B). HepG2 cells expressed a large quantity of S100A3; however, a 6-fold decrease in protein expression was observed (normalized to β-actin) after treatment with 5.0 µmol/l sodium cantharidinate (Fig. 4C). Consistent with the q-PCR results, these findings demonstrated that sodium cantharidinate inhibited the mRNA and protein expression levels of S100A3. These results suggested that sodium cantharidinate may be able to inhibit the expression of S100A3 and therefore may have the ability to block tumor growth.

Discussion

Globally, HCC is a leading cause of cancer-related mortality, with incident rates still on the increase and an annual mortality rate of ~800,000 (1). Although advances in surgical procedures, including hepatic resection and liver transplantation, have aided the treatment of HCC, patient prognosis has only marginally improved and the frequency of recurrence is considerably high, even after liver transplantation (37). The pathogenesis of HCC remains unclear; however, it has generally become accepted that the genetic mutations that occur within normal cells are associated with carcinogenic risk factors, including environmental factors, smoking, frequent consumption of raw freshwater fish, hepatitis B virus infection and a family history of HCC (38). Treatments that target mutant genes are of clinical significance. S100A3, which is a structurally and functionally associated protein, is widely distributed in multiple tumor types (39). It has been indicated that dysregulated expression and function of S100A3 contributes to pathological conditions, including cancer metastasis and diseases associated with defective assembly (15).

It has previously been unclear whether S100A3 has an important role in HCC. The present study showed that mRNA and protein expression levels of S100A3 were significantly increased in HCC and that S100A3 expression in human HCC was significantly inhibited by sodium cantharidinate. Effective anti-cancer agents exhibit the desirable feature of potent cytotoxicity towards tumor cells while eliciting limited adverse effects on normal cells (40). Sodium cantharidinate may directly destroy the liver cancer cell lines, which provided the favorable theoretical basis for the application of treatment of primary liver cancer (41). The present study demonstrated that sodium cantharidinate exhibited inhibitory activity against S100A3 in human HCC. Sodium cantharidinate was able to inhibit the proliferation of HepG2 cells within the ranges of 0.2-5.0 µmol/l and 24-72 h. Furthermore, sodium cantharidinate reduced the mRNA and protein expression levels of S100A3 in human HepG2 cells and significantly induced apoptosis in HepG2 cells. To the best of our knowledge, this is the first study that has shown that mRNA and protein expression levels of S100A3 are downregulated by sodium cantharidinate.

In conclusion, the present study indicated S100A3 has an important role in human HCC occurrence and development. Sodium cantharidinate has the therapeutic ability to inhibit the expression of S100A3 in HepG2 cells and induce cellular apoptosis. The present study provides the theoretical basis for the application of sodium cantharidinate in the treatment of primary liver cancer.

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