ORIGINAL ARTICLE

S100A6 as a Potential Serum Prognostic Biomarker and Therapeutic Target in Gastric Cancer

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Abstract

Background Increased expression of S100A6 in many cancer tissues and its association with tumor behavior and patient prognosis were demonstrated, and there are no studies analyzing the serum levels of S100A6 in patients with gastric cancer (GC).

Aim Serum S100A6 levels were investigated as a marker of tumor aggressiveness in patients with GC, and the S100A6 gene was examined as a potential therapeutic target in GC.

Methods Serum S100A6 levels were detected in 103 GC patients and 72 healthy subjects by ELISA. Clinicopathological features of GC patients were analyzed in correlation to serum S100A6 levels. Two small interfering RNAs against S100A6 (siRNA1-S100A6 and siRNA2-S100A6) were generated and transfected into SGC7901 cells using pSUPER gfp-neo vector, and the effects of S100A6 knockdown on cell proliferation, invasion and apoptosis were evaluated in vitro. The effects of S100A6 silencing on tumor growth and metastasis were evaluated in vivo in a pseudo-metastatic GC nude mouse model.

Results Serum S100A6 levels were significantly higher in GC patients than in healthy controls ($P < 0.001$). Serum S100A6 levels were significantly correlated with lymph node metastasis, TNM stage, perineural invasion and vascular invasion. Serum S100A6 level was an independent predictor of overall survival. SiRNA-mediated silencing of S100A6 significantly induced apoptosis and decreased proliferation, clone formation and the invasiveness of GC SGC7901 cells in vitro and significantly reduced tumor volume and number in vivo ($P < 0.01$).

Conclusion Serum S100A6 level may serve as a potential prognostic biomarker in GC. Inhibition of S100A6 decreased the metastatic potential of GC cells.

Introduction

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide. It was predicted to be the eighth leading cause of death from all causes worldwide in the year 2010 [1, 2]. The prognosis of GC depends highly on the clinical and pathological stage at diagnosis. Surgical resection remains the mainstay of treatment and is very effective in early stage cancers. However, most GC cases are diagnosed at an advanced stage, when the prognosis is extremely poor. Surgery is the only potentially curative treatment for localized GC. Contemporary combination therapies for advanced GC, which consist of 5-fluorouracil (5-FU) and/or cisplatin, have shown response rates of 20–40 % with a median survival of 6–12 months [3]. The high mortality of GC can be attributed to a high incidence of serosal invasion, direct invasion into the adjacent organs, peritoneal seeding, lymph node metastasis and distant metastasis of GC [4]. The high death rate associated with this disease is related to the difficulty in detecting GC at an early stage. Although certain serum tumor markers such as AFP, CEA, CA19-9, CA72-4 and CA50 are

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currently used clinically in GC, they have shown poor effectiveness for the detection of early stage disease. Therefore, the identification of novel markers for GC detection is important.

S100A6, also known as calcyclin, is a calcium-binding protein belonging to the S100 family, which is localized to the cytoplasm and/or nucleus in a wide range of cell types [5]. S100A6 interacts with several target proteins, thereby regulating the dynamics of cytoskeleton constituents, cell growth and differentiation, and calcium homeostasis [6]. S100A6 expression is increased in a number of malignant tumors including neuroblastoma, papillary thyroid carcinoma, osteosarcoma, cholangiocarcinoma and pancreatic cancer [7–11]. Quantification of S100A6 mRNA is a promising tool for the diagnosis of pancreatic cancer, where S100A6 has been shown to be a promising therapeutic target [12]. S100A6 is overexpressed in GC and its detection was suggested as a promising tool for the diagnosis of GC [13]. Furthermore, a clear correlation between high S100A6 expression and various clinicopathological features, such as depth of wall invasion, positive lymph node involvement, liver metastasis, vascular invasion, and tumor-node metastasis stage was shown [14]. Although increased expression of S100A6 in many cancer tissues and its association with tumor behavior and patient prognosis were demonstrated, there are no studies analyzing the serum levels of S100A6 in patients with GC.

Inhibition of S100A6 was shown to decrease the proliferation and invasiveness of pancreatic cancer cells in vitro [12]. However, the function of S100A6 in GC has not been elucidated.

In the present study, we examined the serum level of S100A6 in patients with GC to determine its prognostic value and investigated the effects of S100A6 silencing on the proliferation and invasion of GC cells in vitro and in vivo.

Materials and Methods

Patients

A total of 103 consecutive patients with gastric adenocancer were enrolled in the study between January 2007 and January 2008. The diagnosis was confirmed in all patients by histopathologic examination of gastric resection specimens. Surgery consisted of subtotal or total gastrectomy and D2 (extended) lymph node dissection in all patients, except in cases of peritoneal or distant metastasis. Microdissected areas were assessed by an expert pathologist to estimate perineural and vascular invasion, depth of tumor invasion, lymph node metastasis and histopatholo-

gical grade. Control samples were collected from the physical examination center of the affiliated hospital of Qingdao University between January 2008 and December 2009. All participants underwent history taking, physical examination, routine blood and *H. pylori* IgG tests, chest X-ray, abdominal sonography or computed tomography, esophagogastroduodenoscopy, colonoscopy, sigmoidoscopy with stool hemoglobin or computed tomographic colonoscopy, and mammography or breast sonography in women and/or thyroid sonography. Control samples from patients with confirmed cancer, suspected cancer or inflammatory conditions requiring medical management were excluded, resulting in 72 control samples. Informed written consent was obtained before patient enrollment. The study was approved by the affiliated hospital of Qingdao University.

Assay of Serum S100A6 Levels

Blood samples were collected from fasting participants in the early morning before medical treatment or anesthesia. Peripheral blood was collected using 5-mL syringes and stored in SST II tubes (Becton–Dickinson, Franklin Lakes, NJ, USA) at room temperature for 1 h. Samples were centrifuged at $3,000g$ for 5 min. Supernatants were collected and stored at -80 °C. Serum S100A6 levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) and expressed as picograms per milliliter (pg/mL). The minimum detectable dose of S100A6 is typically <35 pg/mL. Intra-assay reproducibility was <8.5 % and inter-assay reproducibility was <10 %. All participants provided informed consent.

Cell Culture

The GC cell line SGC7901, which is characterized by S100A6 overexpression [14], was obtained from Prof. Chen Dong from the affiliated hospital of Qingdao University [15]. Cells were routinely grown as a monolayer in RPMI-1640 medium (GIBCO BRL, Carlsbad, CA), supplemented with 10 % (v/v) fetal calf serum (FCS, Gibco) and antibiotics at 37 °C in a humidified 5 % CO_2 atmosphere.

Animals

Nude mice aged 4–6 weeks and weighing 18–24 g were obtained from the Chinese Academy of Medical Sciences Cancer Institute. The certificate code was SSXK000004. Mice were housed in a specific pathogen-free (SPF) environment. Mice were treated in accordance with the Institutional Animal Care and Use Committee guidelines.

Reagents

All chemical reagents were purchased from Sigma Chemical Company (St Louis, MO, USA) unless otherwise specified. Plasmids for transfection experiments were purified using Qiagen's maxi kit (Valencia, CA, USA). Anti-S100A6 antibody was purchased from Proteintech Group, Wuhan, China.

siRNA Design

We used two S100A6-targeting small interfering RNAs (siRNA: target sequence, AAGCCCTCAAGGGCTGA AAAT for siRNA1-S100A6 and AAGCTGCAGGATGCT GAAATT for siRNA2-S100A6) and did BLAST searches to ensure the specificity of these siRNAs as reported previously [16]. To verify the specificity of the knockdown effect, we used a control siRNA provided by Qiagen.

Preparation of Recombinant Plasmids

Oligonucleotides (64 bp) were ligated into the mammalian expression vector pSUPER gfp-neo (OligoEngine) at the BglII and HindIII cloning sites. Recombinant siRNA1-S100A6-pSUPER gfp-neo and siRNA2-S100A6-pSUPER gfp-neo constructs were used to transform *Escherichia coli* DH5a, which were selected on ampicillin-agarose plates and verified by sequencing.

Transfection and Selection of Clones

SGC7901 cells were transfected with recombinant pSU-PER gfp-neo using the Lipofectin method. Briefly, cells were plated in six-well culture plates and grown to 70 % confluence. Growth medium was removed, and the cells were washed twice in serum-free Opti-MEM and then incubated for 5 h in 1 mL serum-free Opti-MEM with 10 μL Lipofectin reagent and 1 μg recombinant pSUPER gfp-neo with a target or pSUPER gfp-neo as control. At 24 h after transfection, the medium was replaced with normal growth medium, and after 48 h, each well was passaged into a 10-cm plate with growth medium containing G418 at 600 μg/mL. After 7–10 days, the cells were passaged and plated at 30–50 per plate. Single colonies were selected and the cloning procedure was repeated. We established six clones of S100A6 knockdown cells. The clonal cell lines were maintained in complete medium with G418 at 150 μg/mL.

Western Blotting

After transfection, cells were harvested by trypsinization and lysed in lysis buffer. Debris was sedimented by centrifugation for 5 min at 12,000*g*, and the supernatants were solubilized for 5 min at 100 °C in Laemmli's sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 100 mM dithiothreitol. The protein concentrations of the lysates were determined with a protein quantitation kit, and 40 mg of each sample was separated by 10 % SDS-PAGE. Separated polypeptides were then electrophoretically transferred to 0.2-mm nitrocellulose membranes. Membranes were blocked for 1 h in Tris-buffered saline–Tween containing 5 % (w/v) nonfat dried milk. The blots were then probed overnight with primary antibodies and developed using species-specific secondary and tertiary antisera. Immunoreactive material was detected by the enhanced chemiluminescence technique (Amersham).

Cell Viability Assay Using 3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide (MTT)

SGC7901 and SGC7901-gfp-neo cells and S100A6-suppressed SGC7901 cells (siRNA1-S100A6 and siRNA2-S100A6) were plated on 96-well plates at a density of 1×10^4 cells in a volume of 100 μL/well. After 24 h, 10 μL of MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (Sigma, Missouri, USA) was added and plates were incubated for 4 h at 37 °C. Cells were then lysed with the solubilization solution according to the manufacturer's instruction. Optic densities were determined at 560 and 650 nm as a reference wavelength. Cell numbers were calculated by cell dilution series.

Colony Formation Assay

Each clone and a control cell line (1×10^4 cells) were plated in 0.5 mL of RPMI 1640 medium containing 20 % FCS and 0.56 % methylcellulose in 24-well plates. Colony units were counted 14 days after plating.

Apoptosis Assay by ELISA

The Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA, USA) was used to detect apoptosis in SGC7901 cells exposed to different treatments according to the manufacturer's protocol. Briefly, SGC7901 cells were transiently transfected with pSUPER gfp-neo, siRNA1-S100A6-pSUPER gfp-neo and siRNA2-S100A6-pSUPER gfp-neo constructs for 72 h. After transfection, the cytoplasmic histone DNA fragments from SGC7901 cells were extracted and bound to an immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone DNA fragments. After addition of the substrate for peroxidase, the spectrophotometric absorbance of the samples was determined using the ULTRA Multifunctional Microplate Reader (TECAN) at 405 nm.

Cell Invasion Assay

SGC7901, SGC7901-gfp-neo and S100A6-silenced (siRNA1-S100A6 and siRNA2-S100A6) cells were seeded in BD Matrigel invasion chambers (BD Biosciences) at a density of 1×10^5 cells per well. The medium in the upper chamber was supplemented with 5 % FCS and the lower chamber contained medium with 10 % FCS. After 24 h, cells migrated into the lower chamber were stained and counted. Experiments were performed in triplicate and repeated twice.

In Vivo Growth Assay

SGC7901, SGC7901-gfp-neo and S100A6-silenced (siRNA1-S100A6 and siRNA2-S100A6) cells were resuspended in DMEM at a density of 1×10^7 viable cells/mL, and 1×10^6 viable cells were injected subcutaneously into the backs of nude mice. Tumor growth was evaluated every 2–3 days, and tumor diameter was measured using a caliper. The treatment was continued for 20 consecutive days.

In Vivo Metastasis Assay

All the experiments were performed under the approval of the Animal Experimentation Committee of Qingdao University. SPF athymic BALB/c male mice (4–6 weeks) were kept under sterile conditions in a laminar flow room in cages with filter bonnets and fed a sterilized mouse diet and water. SGC7901, SGC7901-gfp-neo and S100A6-silenced (siRNA1-S100A6 and siRNA2-S100A6) cells (1×10^6 cells/mouse) in 100 μL of PBS were injected into the peritoneal cavity using a 23-gauge needle. Twenty days later, mice were killed and the number of metastatic tumors was determined.

Statistical Analysis

All statistical procedures were performed with SPSS 11.0 (SPSS Inc., Chicago, Inc.). All data are presented as median values (interquartile range), and nonparametric analyses were used to assess differences. The Kruskal–Wallis analysis of variance (ANOVA) and the Mann–Whitney U test were used to evaluate differences between multiple groups and unpaired observations, respectively. Overall survival was measured from the date of initial surgery to the date of death, considering death from any cause as the end point, or the last date of data collection as the end point if no event was documented. Clinicopathologic factors known to be associated with prognosis were tested in univariate analysis. Multivariate analysis was performed using the Cox proportional hazards regression model (backward, stepwise) to assess the influence of each variable on survival. The significance of the in vitro results was determined using Student's t test (2-tailed). The significance of the in vivo metastasis results was determined using the Mann–Whitney U test. $P < 0.05$ was considered significant.

Results

Association Between Serum S100A6 Levels and Pathologic Variables in Gastric Cancer Patients

There were 40 (55.5 %) men and 32 (44.5 %) women in the control group. The mean serum S100A6 level in healthy controls was 19.83 ± 7.46 pg/mL. No significant differences in S100A6 levels were observed between the control men and women (men = 20.3 ± 7.8 pg/mL vs. women = 19.0 ± 7.6 pg/mL). Serum S100A6 levels in GC patients were significantly higher than those in healthy controls (52.96 ± 13.57 vs. 19.83 ± 7.46 pg/mL, respectively, $P < 0.001$). High S100A6 levels were detected in patients with lymph node metastasis ($P = 0.003$), perineural invasion ($P = 0.017$), vascular invasion ($P = 0.014$) and high TNM stage ($P = 0.026$) (Table 1). No correlation was found between S100A6 levels and age, sex, tumor localization and differentiation ($P > 0.05$). These results suggested that S100A6 levels are significantly correlated with disease prognosis in patients with GC.

Association Between Serum S100A6 Levels and Prognosis and Survival Rates in GC Patients

GC patients were divided into two groups according to serum S100A6 levels and based on a cutoff value of 27.29 pg/mL (the highest value in the controls) as follows: Group A ($n = 72$) included patients with serum S100A6 level ≥ 27.29 pg/mL and group B ($n = 31$) included those with a level <27.29 pg/mL. A log-rank test and Kaplan–Meier analysis were used to calculate the effect of S100A6 levels on survival. In the log-rank test, a significant correlation was observed between serum S100A6 levels and patient survival ($P = 0.0013$; Fig. 1). The results of univariate analysis showed that TNM stage ($P = 0.002$) and S100A6 levels ($P = 0.0001$) were significant factors affecting overall survival. In the multivariate regression analysis, TNM stage [hazard ratio (HR) 5.63; 95 % CI 2.28–19.37; $P = 0.026$], lymph node metastasis (HR 5.12; 95 % CI 2.13–17.56; $P = 0.038$) and S100A6 levels (HR 6.13; 95 % CI 2.94–19.36; $P = 0.012$) were significant independent factors for overall survival (Fig. 2).

Table 1 Relationship between S100A6 levels and pathologic variables in gastric cancer patients

Variable	n	S100A6 (pg/mL)	P value
Age (year, mean ± SD)			0.092
<45	16	54.4 ± 14.6	
45–65	56	53.2 ± 10.8	
>65	31	51.3 ± 13.5	
Sex			0.523
Male	73	53.8 ± 10.2	
Female	30	51.8 ± 13.1	
Tumor localization			0.485
Cardia/fundus	42	53.4 ± 16.6	
Corpus	10	52.4 ± 10.3	
Antrum	51	52.7 ± 12.4	
Tumor size			0.284
<4 cm	31	51.3 ± 12.9	
>4 cm	72	52.4 ± 11.5	
Differentiation			0.365
Well	17	51.5 ± 10.4	
Moderate	18	53.1 ± 10.2	
Poor	52	52.6 ± 10.8	
Undifferentiated	16	52.2 ± 13.3	
Lymph node metastasis			0.003
Yes	82	57.5 ± 14.8	
No	21	37.4 ± 10.2	
TNM stage			0.017
I/II (early GC)	48	36.5 ± 14.6	
III/IV (late GC)	55	58.3 ± 12.4	
Perineural invasion			0.014
Yes	81	56.5 ± 15.7	
No	22	33.8 ± 14.3	
Vascular invasion			0.026
Yes	79	57.2 ± 11.8	
No	24	40.4 ± 13.6	

Fig. 1 Kaplan–Meier survival curves for 103 patients with gastric cancer according to serum S100A6 levels. siRNA-S100A6-mediated silencing of S100A6. Six independent cloned cell lines were established from SGC7901 cells treated with siRNA1 and siRNA2 against S100A6 and named SGC7901/siRNA1-S100A6-pSUPER gfp-neo (siRNA1-S100A6) clones C1–C6 (Fig. 2a), SGC7901/siRNA2-S100A6-pSUPER gfp-neo (siRNA2-S100A6) clones C1–C6 and negative control SGC7901/pSUPER gfp-neo (Fig. 2b). Western blot analysis showed that S100A6 expression was almost completely inhibited in all the clones expressing siRNA1-S100A6 or siRNA2-S100A6 (Fig. 2). siRNA1-S100A6-C1 and siRNA1-S100A6-C2 were used for all subsequent analyses

S100A6 Depletion Reduces the Number of Colonies and Inhibits Cell Proliferation In Vitro

siRNA1-S100A6-mediated depletion of S100A6 resulted in a fourfold reduction in the number of colonies ($P < 0.01$; Fig. 3a). The mismatch control (pSUPER gfp-neo) did not affect SGC7901 clonogenicity.

Next, we examined the effect of siRNA1/2-S100A6 on SGC7901 proliferation in culture. Cells transfected with siRNA1-S100A6 and siRNA2-S100A6 showed significantly lower proliferation rates than cells transfected with pSUPER gfp-neo ($P < 0.01$) as determined by generalized estimating equation (Fig. 3b).
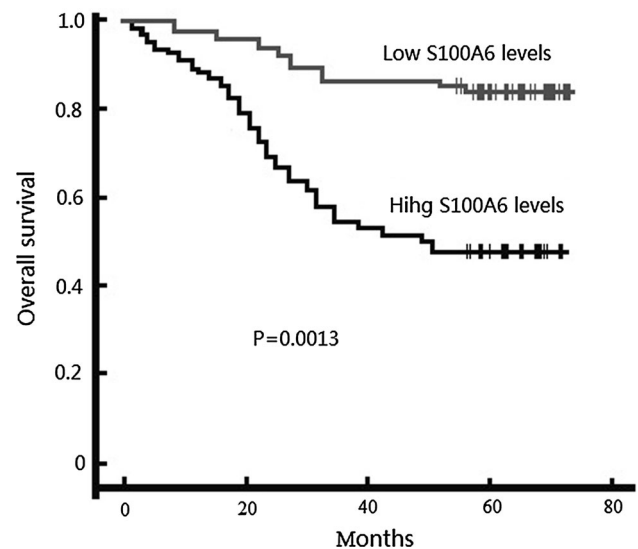
S100A6 Depletion Induces Apoptosis in SGC7901 Cells

S100A6 depletion by siRNA1-S100A6 or siRNA2-S100A6 transfection for 72 h induced apoptosis in SGC7901 cells as shown by histone DNA ELISA analysis (Fig. 3c). These results are consistent with those of the MTT and colony formation studies, suggesting that the loss of viable cells caused by S100A6 depletion is partly mediated by the induction of apoptotic cell death.

S100A6 Depletion Inhibits Invasion of SGC7901 Cells In Vitro

To examine the effect of S100A6 silencing on the capabilities of SGC7901 cells, we used an in vitro invasion assay. Cells transfected with siRNA1-S100A6 and siRNA2-S100A6, and SGC7901 and pSUPER gfp-neo controls were seeded in the upper chamber of a Matrigel-coated transwell plate in medium with reduced (5 %) FCS. After 24 h, cells migrated to the lower chamber containing a higher (10 %) FCS concentration were stained and

Fig. 2 S100A6 expression in cells transfected with different siRNA-S100A6 clones. S100A6 expression in cells transfected with siRNA1-S100A6 clones C1, C2, C3, C4, C5, C6 and siRNA2-S100A6 clones C1, C2, C3, C4, C5, C6, and in control cells (SGC7901 and pSUPER gfp-neo) was assessed by Western blotting
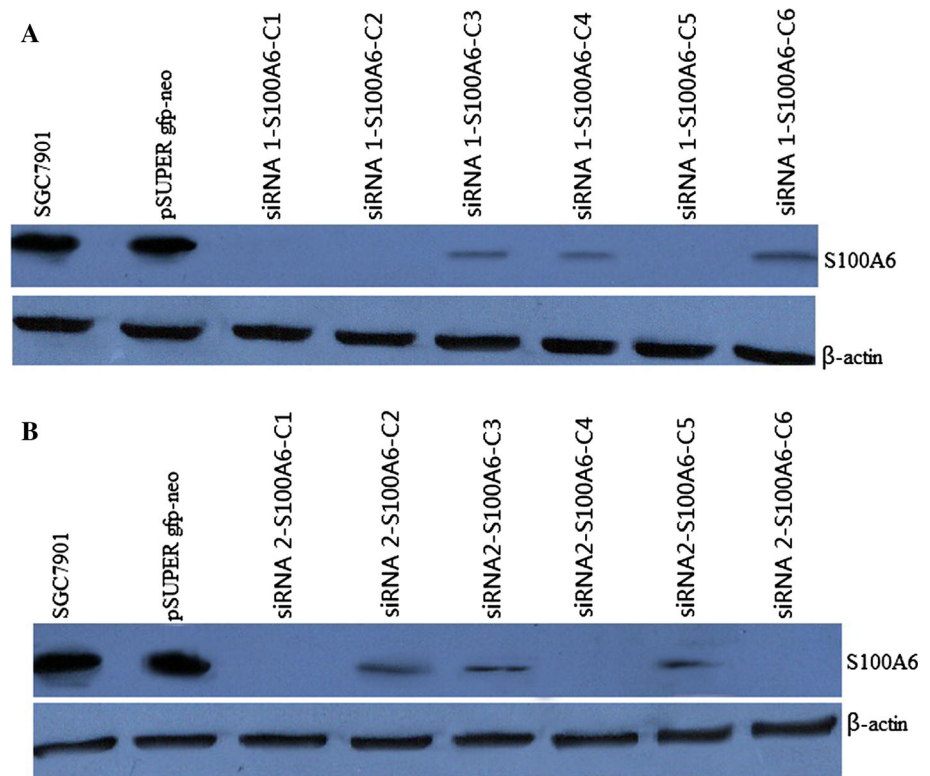
counted. In both siRNA1-S100A6 and siRNA2-S100A6 clones, invasion was significantly reduced compared to SGC7901 and pSUPER gfp-neo controls (Fig. 4). These data show that S100A6 depletion inhibits the invasion of GC cells in vitro.

In Vivo Inhibition of Tumor Growth

In in vivo experiments, two mice in the group carrying SGC7901 or pSUPER gfp-neo control tumors died before the end of the 3-week period, whereas all mice in the siRNA1-S100A6 and siRNA2-S100A6 groups were alive and exhibited a healthier appearance. As shown in Fig. 5, siRNA1-S100A6 and siRNA2-S100A6 transfection significantly reduced tumor volume by 63 and 67 %, respectively ($P < 0.01$), relative to the vehicle control.

S100A6 Depletion Inhibits Metastasis in Nude Mice

The effect of S100A6 silencing on the inhibition of invasion in vitro prompted us to examine its effect on metastasis using a nude mouse model in vivo. SGC7901 and SGC7901-gfp-neo cells and S100A6-suppressed cells (siRNA1-S100A6 and siRNA2-S100A6) (1×10^6 cells/mouse) in 100 μL of PBS were injected into the abdominal cavities using a 23-gauge needle. All mice were killed 20 days after cell inoculation. Figure 6 shows two representative mice carrying SGC7901-gfp-neo and siRNA1-S100A6-transfected tumors. At autopsy, in the SGC7901-gfp-neo mouse, metastatic tumors were found on the mesenterium and greater omentum. In the siRNA1-S100A6 mouse, tumors were very small and few. We counted the number of metastatic peritoneal tumors and showed that siRNA-S100A6 transfection significantly reduced the number of tumors (Fig. 6).

Discussion

To establish proper therapeutic modalities for GC, an accurate assessment of the factors affecting tumor progression and patient prognosis is critical. Recent studies have shown the correlation between tissue S100A6 expression and tumor invasion and metastasis in GC [14]. S100A6 expression was found to be higher in patients who had metastatic tumors compared with patients who had non-metastatic tumors [10–12, 16]. Wang et al. [14] showed that S100A6 plays an important role in the progression of GC and affects patient prognosis. Few studies have evaluated the prognostic impact of serum S100A6 levels in cancer patients. However, there are no published studies analyzing the relationship between serum S100A6 levels and prognosis in GC.

In the present study, we found that serum S100A6 levels were remarkably increased in patients with GC compared
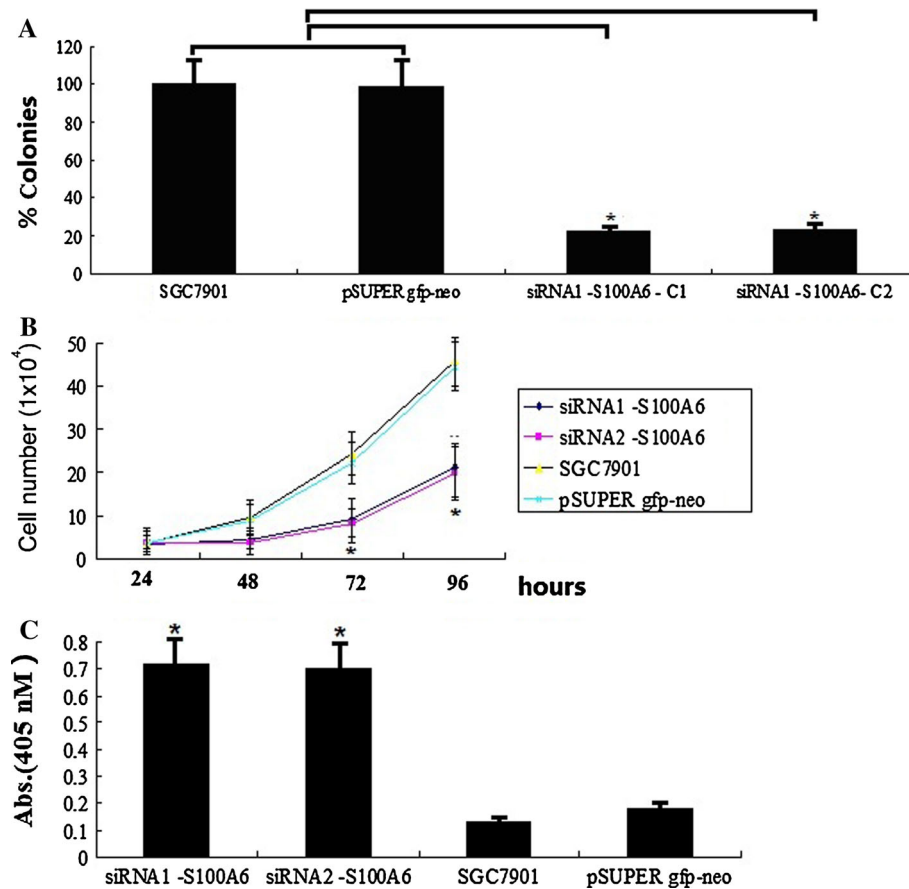
Fig. 3 S100A6 depletion inhibits colony formation and proliferation. **a** siRNA1-S100A6-C1, siRNA1-S100A6-C2 and pSUPER gfp-neo clones, and SGC7901 cells were plated at a density of 1×10^4 in 0.5 mL of RPMI 1640 medium containing 20 % FCS and 0.56 % methylcellulose in 24-well plates for 14 days. Colony numbers of siRNA1-S100A6-treated cells were significantly lower than those of the corresponding controls (*$P < 0.01$). **b** siRNA1-S100A6, siRNA2-S100A6 and pSUPER gfp-neo clones, and SGC7901 cells were plated at a density of 1×10^4, and cell proliferation was assessed at 24-h intervals for 96 h using the MTT assay. **c** Apoptotic cell death assessed by ELISA after 72 h of transient transfection of SGC7901 cells with siRNA1-S100A6 or siRNA2-S100A6. An increased apoptotic response was evident in the siRNA transfection group compared to the pSUPER gfp-neo control. Points, mean; *bars* SE (*$P < 0.01$)
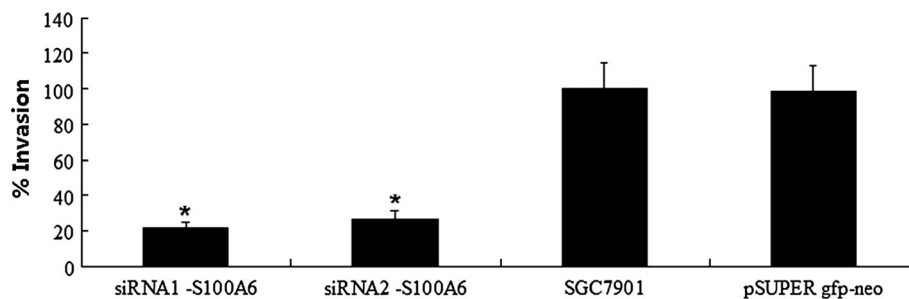

Fig. 4 S100A6 depletion inhibits the invasion of human SGC7901 cells. Cells were seeded in the upper chamber in medium supplemented with 5 % FCS. After 24 h, cells migrated to the lower chamber were stained and counted. In the lower chamber, medium supplemented with 10 % FCS was used as chemoattractant. Invasion of the SGC7901 cell line was set to 100. Results are reported as percent migration $\pm$ SD compared with SGC7901 cells. Experiments were performed twice in triplicate. *$P < 0.01$ versus SGC7901 control
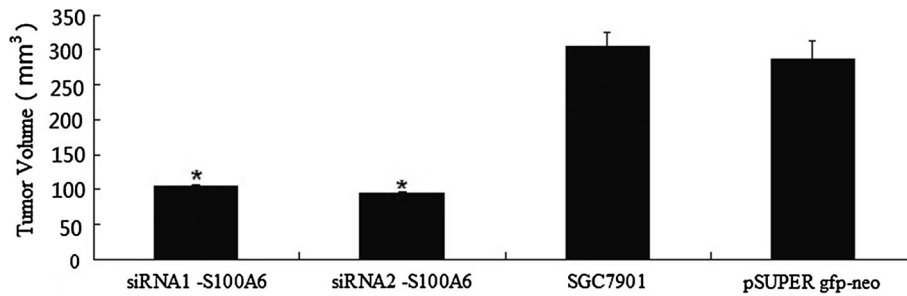
Fig. 5 Tumor growth curves. Mice received 1×10^7 viable cells by subcutaneous injection on day 0. These curves show tumor growth until day 20. siRNA1-S100A6 and siRNA2-S100A6 transfection significantly reduced tumor volume. *$P < 0.01$ versus SGC7901 control
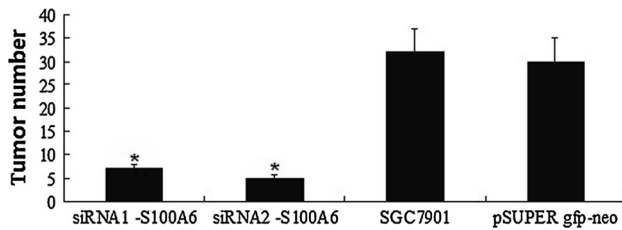

Fig. 6 The number of metastatic tumors in each group. Tumors were counted with the naked eye. The data represent the mean and SD ($n = 7$). *$P < 0.01$ versus SGC7901 control

with healthy controls. Further analysis showed that a high serum S100A6 level was associated with lymph node metastasis, TNM stage, perineural invasion and vascular invasion. No relationship between serum S100A6 levels and age, sex, and tumor differentiation, localization and size was observed. However, serum s100A6 level was correlated with patient prognosis and was an independent prognostic predictor for overall survival. These results suggest that serum S100A6 is a potential prognostic biomarker. Our findings supported the involvement of S100A6 expression in GC as determined by immunohistochemistry in a previous study [14].

Although overexpression of S100A6 is correlated with metastasis and prognosis in patients with GC, whether the S100A6 gene can be used as a therapeutic target in GC patients remains to be elucidated. Ohuchida et al. [12] used in vitro experiments and microarray analysis with RNA interference to evaluate the functional role of S100A6 and its potential as a therapeutic target for pancreatic cancer. Luo et al. [17] reported that knockdown of S100A6 expression inhibited cell adhesion and promoted cell migration and invasion in human osteosarcoma lines, and S100A6 overexpression enhanced cell adhesion and inhibited cell invasion. Tsoporis et al. [18] showed that S100A6 is induced in the myocardium post-infarction in vivo and in response to growth factors and inflammatory cytokines in vitro. Forced expression of S100A6 in cardiomyocytes affects the regulation of cardiac-specific gene expression in response to trophic stimulation. S100A6 has been proposed as a target for the inhibition of cell invasion and the promotion of apoptosis. In the present study, we showed that stable RNA interference-mediated silencing of the S100A6 gene decreased the invasiveness of GC cells, promoted apoptosis and reduced the growth and proliferative potential of human GC cells in vitro. In vivo systems using cells stably expressing siRNA against S100A6 are necessary to address the relative contribution of S100A6 to tumorigenesis versus metastasis. Nevertheless, our data provide evidence supporting a role for S100A6 in the proliferation, invasion and metastatic spread of GC cells and indicate that the S100A6 gene may be a therapeutic target for the treatment of GC. However, the exact mechanism or pathway underlying the function of S100A6 remains to be elucidated.

In conclusion, serum S100A6 levels may provide a useful prognostic biomarker for GC, and our findings suggest that this molecule should be considered as a therapeutic target.

Conflict of interest None.

References

1. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer.* 1999;83:18–29.
2. Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *Lancet.* 1997;349:1498–1504.
3. Meyerhardt JA, Fuchs CS. Chemotherapy options for gastric cancer. *Semin Radiat Oncol.* 2002;12:176–186.
4. Kwon HC, Kim SH, Oh SY, et al. Clinicopathologic significance of expression of nuclear factor-κB RelA and its target gene products in gastric cancer patients. *World J Gastroenterol.* 2012;18:4744–4750.
5. Cross SS, Hamdy FC, Deloulme JC, Rehman I. Expression of S100 proteins in normal human tissues and common cancers using tissue microarrays: S100A6, S100A8, S100A9 and S100A11 are all

overexpressed in common cancers. *Histopathology*. 2005;46:256–269.

6. Lesniak W, Slomnicki LP, Filipek A. S100A6-new facts and features. *Biochem Biophys Res Commun*. 2009;390:1087–1092.

7. Tonini GP, Fabretti G, Kuznicki J, et al. Gene expression and protein localisation of calcyclin, a calcium-binding protein of the S-100 family in fresh neuroblastomas. *Eur J Cancer*. 1995;31A:499–504.

8. Sofiadis A, Dinets A, Orre LM, et al. Proteomic study of thyroid tumors reveals frequent up-regulation of the Ca2+-binding protein S100A6 in papillary thyroid carcinoma. *Thyroid*. 2010;20:1067–1076.

9. Luu HH, Zhou L, Haydon RC, et al. Increased expression of S100A6 is associated with decreased metastasis and inhibition of cell migration and anchorage independent growth in human osteosarcoma. *Cancer Lett*. 2005;229:135–148.

10. Kim J, Kim J, Yoon S, et al. Choe I.S100A6 protein as a marker for differential diagnosis of cholangiocarcinoma from hepatocellular carcinoma. *Hepatol Res*. 2002;23:274.

11. Vimalachandran D, Greenhalf W, Thompson C, et al. High nuclear S100A6 (Calcyclin) is significantly associated with poor survival in pancreatic cancer patients. *Cancer Res*. 2005;65:3218–3225.

12. Ohuchida K, Mizumoto K, Ishikawa N, et al. The role of S100A6 in pancreatic cancer development and its clinical implication as a diagnostic marker and therapeutic target. *Clin Cancer Res*. 2005;11:7785–7793.

13. Yang YQ, Zhang LJ, Dong H, et al. Upregulated expression of S100A6 in human gastric cancer. *J Dig Dis*. 2007;8:186–193.

14. Wang XH, Zhang LH, Zhong XY, et al. S100A6 overexpression is associated with poor prognosis and is epigenetically up-regulated in gastric cancer. *Am J Pathol*. 2010;177:586–597.

15. Chen D, Jiao XL, Liu ZK, Zhang MS, Niu M. Knockdown of PLA2G2A sensitizes gastric cancer cells to 5-FU in vitro. *Eur Rev Med Pharmacol Sci*. 2013;17:1703–1708.

16. Ohuchida K, Mizumoto K, Ishikawa N, et al. The role of S100A6 in pancreatic cancer development and its clinical implication as a diagnostic marker and therapeutic target. *Clin Cancer Res*. 2005;11:7785–7793.

17. Luo X, Sharff KA, Chen J, He TC, Luu HH. S100A6 expression and function in human osteosarcoma. *Clin Orthop Relat Res*. 2008;466:2060–2070.

18. Tsoporis JN, Izhar S, Parker TG. Expression of S100A6 in cardiac myocytes limits apoptosis induced by tumor necrosis factor-alpha. *J Biol Chem*. 2008;283:30174–30183.