Upregulated expression of S100A6 in human gastric cancer

Yan Qing YANG, *, † Lan Jing ZHANG, † Hui DONG, † Chang Long JIANG, † Zheng Gang ZHU, ‡ Jian Xin WU, † Yun Lin WU, †, ‡ Jun Song HAN, † Hua Sheng XIAO, † Heng Jun GAO† & Qing Hua ZHANG*, †

*State Key Laboratory of Medical Genomics, Shanghai Institute of Hematology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China, †National Engineering Center for Biochip at Shanghai, Shanghai, China, ‡Chinese National Human Genome Center at Shanghai, Shanghai, China, Department of Surgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; and **Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

OBJECTIVE: The expression of S100A6 (calcyclin), a member of the S100 calcium binding protein family, is elevated in a number of malignant tumors, but there have been few reports about its expression in gastric cancer. The aim of this study was to investigate its expression regulations in human gastric cancer and noncancerous mucosa, and the response to chemotherapeutic drugs in the gastric cancer cell line.

MATERIALS AND METHODS: In one matched gastric cancer sample pair, the serial analysis of gene expression (SAGE) experiment was conducted to compare the gene expression profiles between cancerous and adjacent tissues. To detect the expression regulations among more cancerous tissues, microarrays were carried out and real-time RT-PCR was conducted to validate the results. At the protein level, Western blot and tissue microarray (TMA) examination were further used to verify S100A6 expression. The regulation detection of S100A6 with flurouracil and doxorubicin at the mRNA and protein level was performed in the SGC7901 cell line.

RESULTS: With the SAGE strategy, five times more S100A6 tags were identified in cancer tissues than in normal tissues. With the cDNA microarray, S100A6 was found to be significantly upregulated in 21 of 42 (50%) nonselective gastric cancers. In 10 other paired samples, the upregulation of S100A6 was consolidated with RT-PCR and Western blot analysis as well. A total of 14 endoscopy-sectioned gastric noncancerous lesions and corresponding normal gastric mucosa were also applied to profile the gene expression; both cDNA microarray and RT-PCR demonstrated no significant alterations of S100A6 at the mRNA level. TMA examination showed that 34 of 52 (65.4%) cancer samples were positively stained, while only 17 of 80 (21.3%) noncancerous lesions were positively detected and all nine normal mucosae were detected to be negative. An in vitro experiment showed that in the gastric cell line SGC-7901, S100A6 mRNA was detected to be upregulated from 24 to 72 h after treatment with 5 mg/L 5-flurouracil or 0.3 mg/L doxorubicin, and there were two wave upregulations of the S100A6 protein.

CONCLUSION: The observed regulated expression of S100A6 suggests that it is associated with gastric cancer tumorigenesis and quantitation of S100A6 is a promising tool for diagnosis of gastric cancer.

KEY WORDS: microarray, neoplasma of the stomach, S100A6, serial analysis of gene expression, tissue microarray.
INTRODUCTION

S100A6 (calcyclin) is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. S100 genes include at least 13 members which are located as a cluster on chromosome 1q21.1 S100A6 was first identified as its expression was upregulated in acute leukemia,2 and the protein was purified from Ehrlich ascites tumor cells.3 Like other S100 proteins, S100A6 functions as Ca2+ sensor in a way that is analogous to the prototypical sensors calmodulin and troponin C.4 S100A6 has been identified as participating in a lot of bioprocesses, including cell cycle, intracellular calcium homeostasis and signaling, ion transport, exocytosis of insulin from pancreatic cells, cytoskeleton rearrangement, and ubiquitinylated proteolytic degradation in a calcium–regulated manner through interaction with partner calcyclin-binding protein (CacyBP).5–10 S100A6 is expressed in fibroblasts and epithelial cells, neurons, Schwann cells, and subpopulations of astrocytes.11–13 The expression alteration of S100A6 has been reported to be related to several malignancies including osteosarcoma, pancreatic neoplasms, malignant melanoma, hepatocellular carcinoma,14–18 and colorectal adenocarcinoma tumorigenesis and invasion/metastasis,19,20 but opposite results have been reported for prostate cancer.21,22

Gastric carcinoma is still one of the most common malignancies and is the second most frequent cause of cancer-related deaths,23 but few S100A6 studies in gastric cancer have been reported. We designed this study to detect S100A6 expression regulations in human gastric cancer samples with an integrated methodology, and the regulation induced by chemotherapeutic drugs, so as to understand the biological meanings of S100A6 in gastric cancer, and make suggestions for further diagnostic studies and drug developments.

MATERIALS AND METHODS

Tissue samples

Tissue samples used in this study were obtained from the Ruijin and Xinhua Hospitals affiliated with the Shanghai Jiaotong University School of Medicine, and were divided into four groups: (A) 42 pairs of surgically resected gastric cancer tissue samples (including different pathologic types such as diffuse and intestinal types; and stages II to IV) and corresponding adjacent noncancerous mucosas were applied for cDNA microarray analysis; (B) one pair of tissue samples which was used in cross-platform microarrays and the serial analysis of gene expression (SAGE) experiment; (C) 10 pairs of tissue samples were used in later RT-PCR validation and Western blot testing to detect the protein expression; and (D) 14 pairs of endoscopy-sectioned noncancerous lesions (including atrophic gastritis, and gastric ulcers according to the international classification standard) and corresponding normal mucosa biopsies were pathologically examined and submitted to TMA and RT-PCR experiments. The total 66 pairs of representative samples described above were snap-frozen in liquid nitrogen and stored at −80°C until use for RNA extraction. For all the samples, the pathology examination results were provided by the department of gastroenterology and pathology at the corresponding hospital. Ethics approval for this study was granted by the Human Research Ethics Committee of the Shanghai Jiaotong University School of Medicine.

RNA preparation

Total RNA was extracted using Trizol (Invitrogen Inc., Carlsbad, CA, USA) and purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality of total RNA was determined with 2100 bioanalizer (Agilent Technologies, Santa Clara, CA, USA).

Serial analysis of gene expression (SAGE)

For one pair of cancerous and adjacent paracancerous tissue samples (20 µg total RNA) were submitted to SAGE analysis. SAGE library construction, tag extraction, and data analysis were performed as before.24 We generated two libraries and got 46 211 and 56 425 tags, representing 6964 and 8656 tag sequences for cancer and para-cancer samples, respectively.

Microarray assay

For 42 surgically sectioned gastric cancer and 14 endoscopy noncancerous lesion sample pairs, gene expression profiling studies with home-made cDNA microarrays were carried out. The microarray contained 13 824 probes (representing 11 203 UniGenes and 16 controls),25 and 50 µg total RNA was used to directly label Cy3-dCTP or Cy5-dCTP (GE Healthcare, Waukesha, WI, USA) for cancer samples or corresponding paracancerous sample RNA with reverse-transcription, respectively, as previously described.26,27 The hybridization was carried out once for each sample pair, except the one used in SAGE. More microarray comparisons were done with duplicates.

The paired samples used for the SAGE experiment were also conducted by microarray experiments with Affymetrix U133A Genechip (Affymetrix Inc, Santa
Clara, CA, USA) and Agilent Human Oligo Microarray (Agilent Technologies, Santa Clara, CA, USA) in addition to cDNA arrays. The reagents and protocols were obtained from the providers; 10 µg and 400 ng of each total RNA were used for labeling the affymetrix and Agilent arrays, respectively.

RT-PCR

RT-PCR was carried out to validate the microarray and SAGE results, and test the expression level in more samples. Five µg total RNA of each tested sample was used for reverse transcription in a total volume of 20 µL, and Superscript II (Invitrogen Inc., Carlsbad, CA, USA) and Oligo(dT) were applied. PCR primers were as follows:

S100A6 forward: ATTGCAAGGCTGATGGAAGACT
reverse: GTTCACCTCCTGGTCCTTGTTC

β-actin forward: TTGTTACAGGAAGTCCCTTGCC, and reverse: ATGCTATCACCTCCCCTGTGTG.

Quantitative PCR was performed in 25 µL with the SYBR premix Ex Taq Kit (Takara, Dalian, China) on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All RT-PCRs were performed in triplicate, and the results were normalized with the Ct values of β-actin.

Western blot analysis

Ten pairs of surgically resected and 14 pairs of endoscopy-sectioned samples were homogenized and prepared with the Mammalian Cell Extract Kit (BioVision Inc, Mountain View, CA, USA). Twenty µg of total protein was subjected to 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis according to standard protocol. The separated proteins were electrotransferred to the polyvinylidene difluoride filter (Millipore Corp, Bedford, MA, USA). The membrane was blocked with 5% fat-free milk powder overnight at 4°C, and incubated with primary antibodies for 2 h at room temperature, followed by 1 h incubation with horseradish peroxidase conjugated goat antimouse IgG as a secondary antibody (Sigma-Aldrich, Milwaukee, WI, USA) (1:4000). The primary antibodies included mouse antiserum against *E. coli* expressed recombinant human S100A6 protein (1:2000) or mouse antihuman S100A6 monoclonal antibody (Sigma) (1:4000), and mouse anti-β-actin monoclonal antibody (Abcam, Cambridge, UK) (1:2000). Signal detection was performed using an ECL® Plus Kit (GE Healthcare, Waukesha, WI, USA) according to the manufacturer’s instructions and developed with X-ray film (Kodak, Rochester, NY, USA).

Tissue microarrays (TMAs)

A TMA containing 141 tissues with duplicated dots was constructed from paraffin embedded samples. Each sample was re-examined for pathology validation before and after the TMA was constructed. There were 52 cancerous, 80 adjacent noncancerous lesions, and nine normal gastric mucosa tissues (no abnormal lesions observed under microscope) on the TMA with double spots for each tissue. The immunohistochemistry detection of S100A6 was performed as previously described.²⁸

S100A6 expression in gastric cancer cell line with drug treatment

The human gastric adenocarcinoma cell line SGC-7901 was provided by the Shanghai Institute for Biological Sciences of China, Academy of Sciences, and maintained at 37 °C in RPMI-1640 (Invitrogen Inc., Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (JRH biosciences, Melbourne, Australia) and antibiotics, under 5% CO₂ and saturated moisture. 5-flurouracil and doxorubicin were given to the cells, and the viability was detected using the 3-[4,5-dimethylthiazolzyl]-2,5-diphenyl tetrazolium bromide (MTT) method. The total RNA and protein were isolated at different time points and the RT-PCR and Western blot analysis were conducted using the procedures above, while the antisera were replaced with monoclonal antibodies.

Statistical analysis

χ² tests and Fisher’s exact probability tests were used to evaluate the relationship between alterations of S100A6 expression and sample types. All P-values were derived from two-tailed statistical tests, with a value of <0.05 denoting significance.

RESULTS

Cross platform detection of S100A6 expression in gastric cancer tissue

One paired sample of gastric adenocarcinoma and corresponding paracancerous mucosa were chosen to undergo SAGE analysis. Among the SAGE library datasets, S100A6 was mapped with a SAGE tag as CCCCCCTGGAT. The tag counts from the two libraries demonstrated the upregulated expression in cancer tissue against the adjacent noncancerous tissue (Table 1). A total of 46 211 tags from cancer sample were obtained, and there were 16 hits for S100A6 gene, among 56 425 tags from noncancerous sample; while only three hits were identified for S100A6 gene.
Regulated expression of S100A6 gene in gastric samples with cDNA microarrays

Home-made cDNA microarrays were applied in order to study the gene expression profiling in samples of 42 gastric cancers and 14 noncancerous gastric lesions. After data processing with locally weighted robust scatterplot smoothing (LOWESS) for normalization, S100A6 were detected to be upregulated in 21 out of 42 unclassified gastric cancer samples, with the criterion as two-fold changes. In 14 endoscopic noncancerous lesions, two samples displayed upregulated expression, while no regulation was detected in the rest of the 12 samples (Table 2).

For the 14 pairs of endoscopy-sectioned gastric lesion samples, quantitative RT-PCR to validate the expression regulations from the microarray data above was used. As shown in Fig. 2, RT-PCR revealed same results for 12 of 14 samples with the microarray, indicating that there were no changes for 11 samples and one up-regulation (for sample 13), with the exception of one upregulated sample (sample 7) with the microarray but no changes with RT-PCR, and one downregulated

Microarrays including the Oligo microarrays from Affymetrix and Agilent, and the home-made cDNA microarray were performed together with quantitative RT-PCR to compare the expression level of S100A6 in this sample pair. The global signal intensity of microarrays or the $\text{Ct}$ value of $\beta$-actin were used to normalize the expression level. S100A6 was detected to be upregulated across the methods at different scales, all demonstrated to be more than 1.5 times upregulated in cancerous tissue (Fig. 1). This coincided with the SAGE results.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Counts</th>
<th>Species</th>
<th>S100A6 tags*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric cancer</td>
<td>46 211</td>
<td>6964</td>
<td>16</td>
</tr>
<tr>
<td>Noncancerous</td>
<td>56 425</td>
<td>8656</td>
<td>3</td>
</tr>
</tbody>
</table>

* $P = 0.001$, S100A6 tag proportion in gastric cancer samples vs. noncancerous samples.

Table 1. Comparison of SAGE tags

The global signal intensity of microarrays or the $\text{Ct}$ value of $\beta$-actin were used to normalize the expression level. S100A6 was detected to be upregulated across the methods at different scales, all demonstrated to be more than 1.5 times upregulated in cancerous tissue (Fig. 1). This coincided with the SAGE results.

Microarrays including the Oligo microarrays from Affymetrix and Agilent, and the home-made cDNA microarray were performed together with quantitative RT-PCR to compare the expression level of S100A6 in this sample pair. The global signal intensity of microarrays or the $\text{Ct}$ value of $\beta$-actin were used to normalize the expression level. S100A6 was detected to be upregulated across the methods at different scales, all demonstrated to be more than 1.5 times upregulated in cancerous tissue (Fig. 1). This coincided with the SAGE results.

Table 2. S100A6 regulation detected with microarray examination

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Samples</th>
<th>Upregulated</th>
<th>Downregulated</th>
<th>No change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric cancer</td>
<td>42</td>
<td>21</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Noncancerous gastric lesion</td>
<td>14</td>
<td>2</td>
<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>

© 2007 The Authors
Journal compilation © 2007 Chinese Medical Association Shanghai Branch, Chinese Society of Gastroenterology and Blackwell Publishing Asia Pty Ltd.
sample (sample 9) with RT-PCR that showed no change on microarray.

Protein and gene expression comparison in gastric cancer samples

To further recognize the regulation of S100A6 in gastric cancer samples, another 10 pairs of surgically resected gastric cancer and adjacent noncancerous samples were chosen to test the regulations with real time RT-PCR and Western blot analysis. With the RT-PCR, it was found that S100A6 was upregulated in five of 10 (50%) tumor samples, which were in accord with the microarray results above. While the other three paired samples showed no regulation, two (20%) tumor samples appeared to be downregulated with RT-PCR analysis (Fig. 3a).

At the protein level, β-actin was used as an internal control to confirm equal amounts of protein loaded for Western blot analysis. S100A6 protein was detected to have higher expression levels in all tumor samples against the adjacent noncancerous samples, except for one which showed low but similar protein levels (Fig. 3b). The rate of detection of the S100A6 protein in endoscopy noncancerous samples was rather low (data not shown).

Immunohistochemical analysis of S100A6 expression with TMA examination

In order to examine the expression of S100A6 at the histological level, TMA analysis was performed. S100A6 was predominantly detected in the cytoplasm, while nuclear staining was also detectable for some samples. Nine normal mucosal tissues were all detected to be negative, 17 of 80 (21.3%) tumor adjacent noncancerous lesions were immunopositive, while 34 of 52 (65.4%) cancer tissues showed wildly and strongly positive stainings (Table 3 and Fig. 4). The results of S100A6 expression at the tissue level are summarized in Table 3, indicating that the proportion of S100A6 positivity could be associated with the progress of gastric carcinogenesis.

Table 3. Immunohistochemistry detection of S100A6 at the histological level

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Samples</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric cancer</td>
<td>52</td>
<td>18 (34.6)</td>
<td>34 (65.4)*</td>
</tr>
<tr>
<td>Noncancerous gastric lesion</td>
<td>80</td>
<td>63 (78.7)</td>
<td>17 (21.3)***</td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>9</td>
<td>9 (100)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* P = 0.00027 (gastric cancer vs. normal mucosa), ** P = 0.001 (gastric cancer samples vs. noncancerous gastric lesions), *** P > 0.05 (noncancerous gastric lesions vs. normal mucosa).
Upregulation of S100A6 in gastric cancer

DISCUSSION

As a member of the calcium-binding protein S100 family, S100A6 are localized in the cytoplasm and/or nucleus of a wide range of cells, and have been reported as being involved in the regulation of a number of cellular processes such as cell-cycle progression and differentiation. The association of S100A6 to gastric cancers is not unique. In pancreatic cancer, S100A6 has a higher expression at RNA and the protein level in malignant cells than in benign cells and non-neoplastic tissues, and has been considered as a therapeutic target. In prostate cancer, S100A6 expression in cancer cells is downregulated and a marker for the basal cells, which may be due to the promoter hyper-methylation. Expression of S100A6 has been detected as a new marker for cholangiocarcinoma rather than primary hepato-cellular carcinoma. The upregulated expression has also been detected in colorectal carcinoma. But so far, few studies of S100A6 in gastric cancer have been reported. In our experiment, we detected the upregulated expression of S100A6 in 50% of gastric cancer tissues with TMA, and only two of 14 noncancerous tissues were detected to have regulated expression. The further comparison of protein and mRNA expression in 10 more cancerous samples revealed that protein regulation was detected in more samples than with the RT-PCR method. Together with the results from TMA examination, immunohistochemistry could detect more positive results in both cancerous (65.4%) and noncancerous lesion samples (21.3%), and these findings suggest that the protein detection may be more sensitive than gene expression. S100A6 may be associated with the progression of gastric carcinogenesis, and could be used as a biomarker for gastric cancer detection. Though the calcyclin-binding protein (CacyBP) was reported to interact with S100A6, the expression of CacyBP was found not regulated in detected gastric cancer samples (data not shown), indicating that there are more regulation pathways for CacyBP function.

For gene-expression profiling detection, available strategies like SAGE, TMA, and massively parallel signature sequencing are useful for high throughput analysis. In our experiment, we carried out SAGE and TMAs to compare the gene expression profiles between gastric cancer and paracancerous samples. The data were consolidated with the findings of the downregulated expression of typical gastric function–related genes like lipase (LIPF), gastric intrinsic factor (GIF), pepsinogen, etc., and the upregulated expression of general cancer-related genes like matrix metalloproteinase 9 (MMP9), glutathione S-transferase pi (GSTP), heat shock protein DNAJ and oncogene JUN, and the RAS gene family member RAB22A (data not shown). The upregulation of the S100A6 after SAGE analysis is consistent with the results derived with Affymetrix and Agilent oligo microarrays, and the home-made cDNA microarray at different regulation scales, while RT-PCR indicated a similar regulation for the same sample (Fig. 1). In our work, the equivalent results drawn from the different microarray platforms, and validated with RT-PCR, were in accordance with the recent large-scale cross-platform validation works carried out by the MicroArray Quality Control (MAQC) team, which concluded that the microarray platforms could get similar gene expression regulation results based on reliable experiments. Our work also demonstrated...
that the home-made cDNA microarray was useful for regulated gene expression screening research by comparing with results with those drawn from commercially available arrays.26,27,33

To investigate S100A6’s potential as a therapeutic target and to understand the functions of S100A6 in the drug treatment of gastric carcinoma, we conducted an in vitro drug treatment of the gastric cell line SGC-7901 with the two clinically used chemotherapeutic drugs 5-fluorouracil and doxorubicin.14 As S100A6 participates in cell proliferation and the cell cycle, its expression is elevated in certain types of cancer cells, and inhibition of its expression might regulate cell proliferation.5,15,29 Both 5-fluorouracil and doxorubicin are widely used chemotherapeutics; they interfere with DNA replication and thus inhibit cell proliferation. So when the cells were treated with the drugs, the viability decreased as expected, and S100A6 expression was upregulated by a feedback mechanism. This could explain the 24 h delay of the mRNA upregulation of S100A6. This further suggests that S100A6 could be considered as a therapeutic target different from the mechanism of DNA replication in the case of 5-fluorouracil or doxorubicin treatment. The S100A6 protein was also regulated with drugs, but with a two-wave regulation pattern; the mechanism remains to be further explored.

In conclusion, our results have demonstrated that, for the first time, S100A6 is overexpressed in gastric cancer, and moderately expressed in noncancerous lesions. Further studies on the physiological role of S100A6 will help us to know more about S100A6 functions in gastric carcinogenesis. At this point, S100A6 might be used as a new diagnostic marker.

ACKNOWLEDGMENTS

This work was jointly supported by the Chinese National Key Program on Basic Research 973 (#2006CB910402), Shanghai Science and Technology Program (#05XD1414), and the Master Professor Project of the Shanghai Jiaotong University School of Medicine. The authors thank LB Lin, XN Zhang, Q Wang, YC Chen, Y Gao, Y Hu, and ZD Zhu of the Shanghai Biochip Center for their technical assistance.

REFERENCES


5 Calabretta B, Battini R, Kaczmarek L, de Riel JK, Baserga R. Molecular cloning of the cDNA for a growth factor-inducible
22 Rehman I, Cross SS, Azouzzi AR et al. S100A6 (Calcyclin) is a prostate basal cell marker absent in prostate cancer and its precursors. *Br J Cancer* 2004; 91: 739–44.