Down-Regulation of S100C Is Associated with Bladder Cancer Progression and Poor Survival

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ABSTRACT

Purpose: The goal of this study was to identify proteins down-regulated during bladder cancer progression.

Experimental design: By using comparative proteome analysis and measurement of mRNA, we found a significant down-regulation of S100C, a member of the S100 family of proteins, in T24 (grade 3) as compared with RT4 (grade 1) bladder cancer cell lines. Moreover, quantification of the mRNA level revealed that decreased expression of the protein reflects a low level of transcription of the S100C gene. Based on this observation, we quantified the S100C mRNA expression level with real-time PCR in bladder cancer biopsy samples obtained from 88 patients followed for a median of 23 months (range, 1-97 months).

Results: We found a significantly lower mRNA expression of S100C in connective tissue invasive tumors (T1, \( P = 0.0030 \)) and muscle invasive tumors [(T2-T4), \( P < 0.0001 \)] compared with superficial tumors (Ta). A negative correlation between S100C and histopathologic grade (\( P = 0.0003 \)) was also observed. Furthermore, the papillary type showed higher expression of S100C than did the solid type of the tumor (\( P < 0.0001 \)). Importantly, we found that loss of S100C was associated with survival in bladder cancer patients (\( P = 0.0006 \)).

Conclusions: Our results show that low expression of S100C is associated with poor survival in patients with bladder cancer. Furthermore, loss of S100C in T1 as compared with Ta stage tumors emphasize that S100C expression is suppressed early during bladder cancer development.

INTRODUCTION

Bladder cancer is the fifth most common cancer in men and the ninth most common kind in women (1). Despite several attempts, it is difficult to predict tumor progression, optimal therapy, and clinical outcome (2). At presentation, 25% to 30% of bladder tumors are classified as muscle-invasive tumors, and thus associated with a significant risk of subsequent metastasis (30-60%; ref. 3). Patients with these tumors have a significantly reduced 5 years survival rate, often correlated with development of metastasis following the failure of conventional treatments. Tumor staging is considered to be one of the prognostic markers, but several markers including the presence of members of the S100 family have been suggested (4, 5).

The S100 family of \( \text{Ca}^{2+} \)-binding proteins comprises 19 members; each member exhibiting a unique expression pattern in human tissues (6). S100 proteins regulate numerous intracellular functions, which include protein phosphorylation, enzyme activation, interaction with cytoskeletal components, and calcium homeostasis (7). In addition, it is currently thought that S100 proteins are involved in the regulation of many cellular processes such as cell cycle progression and differentiation (8, 9). The association of S100 proteins with cancer development originates from the fact that an evolutionary conserved gene cluster of S100 was found on human chromosome 1q21 where several gene rearrangements during tumor development have been found (7, 10). S100 proteins, which are involved in tumor progression, include S100A1, S100A4, S100A6, S100A7, and S100B (11–14), whereas S100A2 has been postulated to be tumor suppressor (15). Loss of S100A2 and increased expression of S100A4 have been implicated in prostate tumor progression (16). Finally, decreased expression of S100A8 and S100A9 has been reported in esophageal squamous cell carcinoma (17). Thus, the balance of the expression levels of S100 proteins in the cells may be important in the regulation of normal cell growth.

S100C also called calgizzarin or S100A11 is an EF hand--type \( \text{Ca}^{2+} \)-binding protein. S100C is a less known member of the S100 family of proteins. A few studies have been carried out to determine the \textit{in vivo} and \textit{in vitro} functions of the S100C. For example, S100C has been implicated in growth inhibition of human fibroblasts (18, 19) and in \( \text{Ca}^{2+} \)-induced growth inhibition of human keratinocytes in culture (20). However, to date, the expression and the clinical significance of this important member of the S100 family have not been elucidated in bladder cancer. These observations led us to investigate the expression of S100C in bladder cancer biopsy samples, using quantitative real-time reverse transcription-PCR methods.

MATERIALS AND METHODS

Patients

Eighty-eight patients with primary bladder cancer were included. Biopsies were obtained by transurethral tumor resection. Tumor stage was assigned according to the Union Internationale Contre le Cancer Tumor-Node-Metastasis system (21).
Table 1  Clinical data

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>Ta</th>
<th>T1</th>
<th>T2-T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patient</td>
<td>21</td>
<td>18</td>
<td>49</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
<td>17</td>
<td>41</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>69</td>
<td>74</td>
<td>68</td>
</tr>
<tr>
<td>Range</td>
<td>53-88</td>
<td>60-88</td>
<td>54-83</td>
</tr>
</tbody>
</table>

Grading was done as described by Bergkvist et al. (22). The relationship among tumor stage, age, and sex distribution is presented in Table 1. Patients were divided into three groups depending on tumor stage: Ta, superficial tumors; T1, superficial invasive tumors; and T2-T4, muscle-invasive tumors. At the time of inclusion, 18 patients had received treatment in the form of radical radiotherapy, chemotherapy, or intravesical therapy with bacillus Calmette-Guerin.

cDNA was generated in a reverse transcription reaction where 0.1 μg RNA was mixed with 2.5 units AMW reverse transcriptase (Applied Biosystems) in a reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 1 unit/μl RNase inhibitor, 1 mmol/L deoxyribonucleoside triphosphate (dTTP, dATP, dGTP, and dCTP), 2.5 μmol/L 16 mer d(T)$_{16}$ primer, 50 mmol/L KCl, and 6.25 mmol/L MgCl$_2$ in a total volume of 20 μL (all reagents from Applied Biosystems). The reactions were incubated in a Perkin-Elmer 9700 thermocycler at 94°C for 90 seconds followed by 30 minutes at 42°C and finally at 94°C for 1 minute. Real-time PCR was done with the Lightcycler Syber Green I quantification kit (Roche) in LC glass capillaries with the following profile: initial heating to 94°C for 90 seconds followed by 40 PCR cycles of heating to 94°C, incubation for 5 seconds at the annealing temperature of 65°C, and incubation at 72°C for 10 seconds. Fluorescence data were collected and the mRNA quantified with Lightcycler software version 3.3 by using the second derivative method of quantification. All conditions for β-actin were the same as for S100C except that the annealing temperature was 68°C. A standard melting curve was used to check the quality of amplification, such as no primer dimer formation during PCR.

Preparation of Total RNA

Tumor samples or cultured cells were immediately placed in a denaturing solution [4 mol/L guanidine thiocyanate, 25 mmol/L sodium citrates (pH 7), 0.5% sarkosyl, and 0.1 mmol/L 2-mercaptoethanol] and stored at −80°C. Frozen biopsies (<20 mg) were homogenized by a Heidolph Diax 600 mixer. Total RNA was extracted from tissues as described by Chomczynski and Sacchi (23) and resuspended in diethyl pyrocarbonate-treated double distilled water. RNA was quantified using a UV spectrophotometer and samples were stored at −150°C.

Real-time Reverse Transcription-PCR

Measurement of mRNA was done by real-time reverse transcription-PCR Lightcycler instrument (Roche). The S100C primers, 5'-TGCACTCAGTCCCTGA-3' (sense) and 5'-AGGTGTTAGTGTGCT-3' (antisense), give rise to a PCR product of 313 bp. Specificity was verified by the size of the PCR product on agarose gel electrophoresis and nucleotide sequencing using a 310 genetic analyser (Applied Biosystems, Foster City, CA).

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A calibration curve composed of eight serial dilutions (1-0.0025 μg/μL RNA) derived from a pool of mRNA from the KLE cell line was included in each run together with positive and negative controls. The 0.1 μg/μL calibrator RNA and a sample without RNA were used as positive and negative controls, respectively. The results for each sample were read from the calibration curve and are shown as a ratio between target gene mRNA and β-actin mRNA.

Two-Dimensional PAGE

The cells were directly disrupted in urea lysis buffer [9 mol/L urea, 2% CHAPS, 1% DTT, and 0.8% (v/v) carrier ampholytes and sonicated on ice thrice for 2 seconds each. All protein extract were centrifuged at 15,000 × g for 30 minutes and the supernatants were used for analysis. The protein concentration was determined by the Bradford method (27). Samples were applied to the IPI strips, pH 4-10 (Amersham, Arlington Heights, IL), by in-gel rehydration using IPGphor (Pharmacia, Uppsala, Sweden) under constant 20 V for 12 hours. Isoelectric focusing was carried out by stepwise increase of voltage up to 12,000 V and continued at 12,000 V for 1 hour. Strips were incubated in the equilibration buffer [50 mmol/L Tris (pH 8.8), 6 mol/L urea, 30% glycerol, 2% SDS, and 1% DTT] for 15 minutes and in the same buffer with 4% IAA instead of 1% DTT for another 15 minutes. The equilibrated strips were placed on 10% to 18% gradient polyacrylamide gels and the separation continued at 100 V in the running buffer [25 mmol/L Tris (pH 8.8), 192 mmol/L glycine, and 0.1% SDS]. The experiment was repeated for different batches of cell lines at least six times.

Protein Visualization

The gels were fixed with 40% ethanol, 10% acetic acid for 1 hour, with 5% ethanol, and 5% acetic acid for 2 hours. The gels
were sensitized with 0.5 mol/L sodium acetate, 1% glutaraldehyde for 30 minutes and stained with ammonial silver solution (47 mmol/L silver nitrate, 0.33% ammonia, and 20 mmol/L sodium hydroxide) for 30 minutes. Proteins were developed with a solution of 0.01% citric acid, 0.1% formaldehyde, and the reaction was stopped with a solution of 5% Tris and 2% acetic acid. For in-gel digestion, the gels were fixed with 50% methanol, 12% acetic acid, and 1.85% formaldehyde for 1 hour and with 50% ethanol for 2 hours. The gels were pretreated with 0.02% sodium thiosulfate for 1 minute and impregnated with 0.2% silver nitrate and 2.8% formaldehyde for 30 minutes. After rinsing gels with deionized water, proteins were developed with a solution of 6% sodium carbonate and 1.85% formaldehyde, and the reaction was stopped with 50% methanol and 12% acetic acid.

### In-Gel Digestion

Protein spots were excised from the gel and transferred into microcentrifuge tubes and washed twice with deionized/filtered water, 50 mmol/L ammonium bicarbonate, and acetonitrile. Reduction and alkylation were carried out with 10 mmol/L DTT and 55 mmol/L IAA in 100 mmol/L ammonium bicarbonate, respectively. The gels were washed again with 50 mmol/L bicarbonate in 50% acetonitrile for 30 minutes, dried thoroughly, and incubated at 37°C for 16 hours in a solution of 50 ng/μL trypsin, 50 mmol/L ammonium bicarbonate, and 5 mmol/L CaCl2. After removal of the supernatant, the gel was incubated with 25 mmol/L ammonium bicarbonate in 50% acetonitrile, the supernatant was removed and the gels were then extracted twice with 5% formic acid in 50% acetonitrile. All extracts of a gel piece were pooled and dried in a vacuum centrifuge.

### Mass Spectrometry and Database Search

The dried peptides were resuspended in 0.5% trifluoroacetic acid and mixed with matrix solution saturated with α-cyano-4-hydrocinnamic acid. After drying the mixed sample, the mass of the peptide was determined using matrix-assisted laser desorption/ionization mass spectrometry (Voyager DE-STR, Applied Biosystems). Internal calibration was done with peptides from trypsin. Monoisotopic mass peaks were collected and applied to databases including Peptident (http://expasy.org/tools/peptident.html) and MS-fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) to identify the corresponding protein spots on two-dimensional gels (data not shown).

### Statistical Analysis

Nonparametric tests were used throughout this study. Two-sided Ps < 0.05 were considered significant. The Mann-Whitney U test and Kruskal-Wallis test were used to compare the expression of the S100C in clinical stages, histopathologic grades, tumor type, and size. Kaplan-Meier survival curves were used to estimate the survival of the patients. The log-rank test was used to compare the survivals. The significance of various variables for survival was analyzed by the Cox proportional hazards model in the multivariate analysis.

### RESULTS

#### Expression of S100C in Bladder Cancer Cell Lines

In order to identify down-regulated proteins during bladder cancer progression, comparative proteome analysis between two bladder cancer cell lines, RT4 (derived from grade 1) and T24 (derived from grade 3), were done using two-dimensional PAGE.

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**Table 2** Correlation between S100C mRNA levels and clinicopathologic variables of bladder cancer

<table>
<thead>
<tr>
<th>All tumors</th>
<th>Median*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total population</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>21</td>
<td>10.29</td>
</tr>
<tr>
<td>T1</td>
<td>18</td>
<td>2.616</td>
</tr>
<tr>
<td>T2-T4</td>
<td>49</td>
<td>1.393</td>
</tr>
<tr>
<td>Grades</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1 + 2</td>
<td>26</td>
<td>8.787</td>
</tr>
<tr>
<td>Grade 3 + 4</td>
<td>62</td>
<td>1.609</td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3 cm</td>
<td>50</td>
<td>2.913</td>
</tr>
<tr>
<td>&gt;3 cm</td>
<td>38</td>
<td>1.851</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary</td>
<td>38</td>
<td>7.581</td>
</tr>
<tr>
<td>Solid</td>
<td>37</td>
<td>1.316</td>
</tr>
<tr>
<td>Mixed</td>
<td>13</td>
<td>2.450</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.

*Median mRNA concentration of the S100C.
†Kruskal-Wallis Test.
‡Mann Whitney Test: NS, not significant.
Among the differentially expressed proteins, we found the most extensive and reproducible down-regulation of an 11-kDa protein spot in T24 compared with RT4 cells, in six independent experiments (Fig. 1A). This protein spot was identified as S100C (calgizzarin) by mass spectrometry. Furthermore, we found the mRNA expression of S100C to be high in RT4 cells compared with T24 (Fig. 1B).

Expression of S100C in Human Bladder Cancer

To evaluate the clinical impact of our observations we examined the mRNA expression of S100C in biopsies from 88 patients with bladder cancer (Table 2). The median level of S100C decreased 4-fold in connective tissue invasive tumors (T1, \( P = 0.0030 \)), whereas muscle-invasive tumors (T2-T4, \( P < 0.0001 \)) showed a 7-fold decrease in mRNA level compared with superficial tumors (Ta). No significant change was observed between T1 and T2-T4 mRNA levels (Fig. 2A). Among the different tumor types of bladder cancer, the papillary type tumor showed a significantly higher expression of S100C (Fig. 2B) compared with the solid type (\( P < 0.0001 \)). Furthermore, low expression of S100C correlated with higher histopathologic grades 3 + 4 (\( P = 0.0003 \)) of the tumor. No correlation was found between S100C expression and the size of the tumor.

Survival of Bladder Cancer Patients as a Function of S100C mRNA Expression

The median concentration of S100C mRNA observed for all the bladder tumors was selected as the cutoff limit and patients were categorized as low (below median) and high (above median). Using Kaplan-Meier survival curves, we observed a highly significant correlation between S100C mRNA expression and survival (\( P = 0.0006 \), log-rank test, \( \chi^2 = 11.67 \)). Patients with high S100C mRNA showed longer survival than patients with low levels of S100C mRNA (Fig. 3). Multivariate logistic regression analysis of the data obtained for 88 patients showed that S100C expression was not an independent factor of survival (\( P = 0.001 \) and 0.051, respectively).

DISCUSSION

We used the two-dimensional PAGE technique to investigate the down-regulated proteins in highly malignant bladder cancer. Because of the heterogeneous nature of human biopsy samples, our choice was to compare two bladder cancer cell lines RT4 and T24, which have been used as models of noninvasive and invasive bladder cancer, respectively (28–32). We found a significant down-regulation of the S100C protein level in the T24 cells. In agreement with this result, the mRNA coding for S100C was low in the T24 compared with the RT4 cells. The results suggest that

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**Table 3**  
Multivariate analysis (Cox regression) of different prognostic variables in patients with bladder cancer

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazards ratio (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100C expression</td>
<td>1.55 (0.78-3.09)</td>
<td>0.204</td>
</tr>
<tr>
<td>Stage</td>
<td>15.2 (2.91-78.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>Grade</td>
<td>2.54 (0.52-12.4)</td>
<td>0.247</td>
</tr>
<tr>
<td>Tumor type</td>
<td>0.92 (0.39-2.16)</td>
<td>0.850</td>
</tr>
<tr>
<td>Tumor size</td>
<td>1.93 (0.99-3.76)</td>
<td>0.051</td>
</tr>
</tbody>
</table>
S100C is down-regulated in more malignant bladder cells and that the down-regulation occurs on the transcriptional level.

Studies on biopsies from bladder cancer patients further confirmed a relation between the expression of S100C and the malignancy of the cancer. We found a significant down-regulation of S100C mRNA in muscle-invasive (T2-T4) and connective tissue—tissue-invasive tumors (T1) compared with non-invasive tumors (Ta). This suggests an early loss of S100C during tumor progression. Interestingly, our results revealed a significantly higher expression of S100C in the papillary type of bladder cancer compared with the solid or mixed types. The papillary type of bladder cancer is the least malignant tumor and rarely (<5%) invades or causes death (33, 34). These results further support the assumption that S100C expression may be involved in tumor suppression and better prognosis. Our results are in line with the few previous data reported on the expression of S100C. Kondo et al. report the down-regulation of S100C in various cancerous tissues compared with their normal counterparts (35). Furthermore, low expression of S100C has also been reported in immortalized human fibroblast cells as compared with normal fibroblasts (19). It is possible that loss of S100C may escalate cellular growth and proliferation. This assumption is supported by our observation of a strong correlation between poor survival of bladder cancer patients and low mRNA expression of S100C.

Various S100 proteins are involved in the malignant transformation of cells. One group of the proteins including S100A4 is observed in an increased concentration in malignant cells and has been implicated in malignant growth. For example, S100A4 has been shown to be involved in cell motility and invasion in an in vitro model for breast cancer metastasis (12). In addition, S100A4 expression correlated with invasion and metastasis in oral squamous cell carcinoma (36) as well as in gastric carcinoma (37).

Another group of S100 proteins including S100A2 has been suggested as tumor suppressor genes. For example, S100A2 expression is associated with better prognosis in squamous cell carcinoma of the esophagus (38) and is down-regulated in breast tumors and in a variety of other neoplasms (39–41).

Approximately 40% identity exists between S100C and S100A2 at the amino acid level (35). Furthermore, S100C is a key mediator of Ca2+ induced growth inhibition of human epidermal keratinocytes in culture (20). Our data support a tumor suppressor role for S100C in vivo thereby substantiating the close relationship between S100A2 and S100C.

Unfortunately, we could not expand our results by checking the protein expression of S100C in biopsy samples because no antibody is currently available. Nevertheless, our data strongly suggest early down-regulation of S100C during bladder cancer progression.

In conclusion, this study indicates that reduction of S100C expression is associated with increasing tumor aggressiveness and poor prognosis in bladder cancer patients.

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