

Expression of S100 Protein Family Members in the Pathogenesis of Bladder Tumors

RUI SHENG YAO¹, ANTONIO LOPEZ-BELTRAN², GREGORY T. MACLENNAN³,
RODOLFO MONTIRONI⁴, JOHN N. EBLE⁵ and LIANG CHENG^{5,6}

¹Department of Surgery and The Alvin J. Siteman Cancer Center,
Washington University School of Medicine, St. Louis, MO, U.S.A.;

²Department of Pathology, Cordoba University, Cordoba, Spain;

³Department of Pathology, Case Western Reserve University, Cleveland, Ohio, U.S.A.;

⁴Institute of Pathological Anatomy and Histopathology, School of Medicine,
Polytechnic University of the Marche Region (Ancona), United Hospitals, Ancona, Italy;

Departments of ⁵Pathology and Laboratory Medicine, and ⁶Urology,
Indiana University School of Medicine, Indianapolis, IN, U.S.A.

Abstract. *The S100 proteins act as multifunctional signaling factors that are involved in the regulation of diverse cellular processes. To explore the involvement of S100 genes in bladder cancers, S100 gene expressions were systematically evaluated at the RNA level by microarray and real-time PCR. Total RNAs were obtained from 4-hydroxybutyl(butyl)nitrosamine (OH-BBN)-induced mouse and rat bladder cancers, human bladder cancers and matched normal bladder urothelium. Microarray analysis was performed on mouse and rat bladder cancers; real-time PCR was performed in mouse, rat and human bladder cancers and their matched normal urothelium for confirmation. Microarray analysis revealed that 9 and 6 members of the S100 gene family were differentially expressed in mouse and rat bladder cancers, respectively. Thirteen members of the S100 gene family were confirmed by real-time PCR to be differentially expressed in human bladder cancers, with overexpression of S100A2, S100A3, S100A5, S100A7, S100A8, S100A9, S100A14, S100A15, S100A16 and S100P, and underexpression of S100A1, S100A4 and S100B. S100A1, S100A3, S100A8, S100A9, S100A14, S100A15 and S100A16 showed similar patterns of differential expression in bladder cancers from mouse, rat and human. To our knowledge this is the first report of systematic evaluation of S100 gene*

expressions in bladder cancers. Our results indicate that differential expression of S100 gene family members is characteristic of bladder cancers and these genes may play important roles in bladder tumorigenesis and progression.

Bladder cancer is the sixth most common malignancy in developed countries. It ranks as the fourth and ninth most common cancer in men and women in the United States, respectively. It is estimated that about 61,420 cases will be newly diagnosed in the USA and 13,060 patients were expected to die from disseminated disease in 2006 (1). Most bladder tumors are transitional cell carcinomas (TCCs). In contrast, squamous cell carcinoma of the bladder is the predominant bladder cancer in Middle Eastern countries, a finding that is probably related to the prevalence of bilharziasis in that part of the world (2). Approximately 80% of bladder cancers are noninvasive papillary transitional-cell carcinomas, which respond favorably to surgical resection. However, 50-70% of patients experience tumor recurrence and 15% of bladder tumors will progress and invade the bladder muscle. Patients with progressive and muscle-invasive cancers have a significantly reduced 5-year survival rate, often associated with the development of metastases following the failure of conventional treatments (3, 4). Of patients initially presenting with muscle-invasive TCC, 50% will relapse with metastatic disease (5).

There are two primary chemically induced models of urinary bladder cancer in rodents. Both employ repeated intragastric administration of 4-hydroxybutyl(butyl) nitrosamine (OH-BBN) to induce bladder cancers in either mice or rats. The bladder cancers typically have a mixed histology, showing elements of both transitional and squamous cells (6, 7). These carcinogen-induced tumors have previously been shown to have certain of the

Correspondence to: Liang Cheng, MD, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, 350 West 11th Street, Clarian Pathology Laboratory Room 4010, Indianapolis, IN 46202, U.S.A. Tel: +1 317 491 6442, Fax: +1 317 491 6419, e-mail: lcheng@iupui.edu

Key Words: Bladder, urinary tract, neoplasia, transitional cell carcinoma, urothelial carcinoma, S100 proteins, proteomics, biomarkers.

alterations observed in human bladder cancer including overexpression of EGFR, survivin (8) and various cyclins, as well as decreased expression of the FHIT gene due to hypermethylation (9).

S100 proteins, a family of calcium-binding proteins, are multi-functional signaling proteins that are involved in numerous intra- and extracellular functions, such as protein phosphorylation, enzyme activation, interaction with cytoskeletal components and calcium homeostasis. In addition, it is currently thought that S100 proteins are involved in the regulation of many cellular processes such as cell growth, cell cycle progression, differentiation, transcription and secretion (10, 11). Deregulated expression of several members of the S100 protein family, including S100A2, S100A4, S100A6, S100A7, S100A11, S100P and S100B, has been reported in association with the progression of various human cancer (10, 11). In an immunohistochemical study of S100A4 expression in human bladder tumors, 28% of tumors were noted to show moderate or strong staining (12). Furthermore, overexpression of S100A4 was strongly associated with stage progression, development of metastases and poor survival in this study (12). In another study of immunohistochemical expression of S100 proteins in a broad spectrum of normal tissues and common cancers, a single case of transitional cell carcinoma of the bladder showed increased expression of S100A2, A6 and A11, but no staining for S100A8 and A9 (13). Expression of S100A7 protein in bladder cancer is reportedly limited to squamous cell carcinomas (SCC). Since S100A7 can be detected in the urine of patients with bladder SCC by 2-D gel immunoblotting, S100A7 is a potential marker for bladder SCC (14). Other investigators have observed that downregulation of S100A11 expression is associated with bladder cancer progression and poor survival (15).

Although there is considerable evidence that S100 proteins may play important roles in various types of cancer, there has been no comprehensive analysis to date concerning the expression of the S100 family of proteins in bladder cancer. In the present study, we systematically evaluated S100 gene family expression in bladder tumors with combined microarray analysis. In the course of these experiments, we observed that mRNA levels of several members of the S100 family are differentially expressed in mice, rats and humans when comparing bladder cancers with normal bladder urothelium.

Materials and Methods

Bladder tumor samples. Male B6D2F1 (C57Bl/6 x DBA/2 F1) mice and Female Fischer-344 rats were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN, USA). At 56 days of age, mice received the first of 12 weekly gavage treatments with OH-BBN (TCI America, Portland, OR, USA) (7.5 mg/gavage, once a week). Rats began receiving OH-BBN, 150 mg/gavage, 2x/week at

49 days of age, and the gavages continued for 8 weeks. The animals were observed daily, weighed weekly, examined for palpable urinary bladder tumors weekly and sacrificed 8 months following the first OH-BBN treatment (unless sacrificed earlier because of a large palpable bladder mass). Bladder tumors were removed and frozen for subsequent molecular assays. A portion of each tumor was fixed and processed for routine paraffin embedding, cut into 5 µm sections and mounted for H&E staining for histopathology. All bladder tumors evaluated in this study were urothelial carcinoma. Normal bladder tissue for comparison, including normal bladder epithelium, was procured from age-matched controls.

With the approval of the Indiana University and Purdue University Institutional Board Review Committee, ten pairs of surgical specimens of transitional cell carcinoma and their matched adjacent normal tissue were obtained from patients at the Indiana University School of Medicine (Indianapolis, Indiana); these patients had been diagnosed with invasive urothelial carcinoma and had undergone cystectomy. All patients had high-grade and advanced stage (pT2 or above) urothelial carcinoma. The samples were snap frozen in liquid nitrogen and remained in the vapor phase of that medium until they were subsequently analyzed.

RNA extraction. Total RNA from normal bladder urothelium and bladder tumors was isolated by Trizol (Invitrogen, Carlsbad, CA, USA) and purified using the RNeasy Mini Kit and RNase-free DNase Set (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocols.

RNA amplification and array analysis. *In vitro* transcription-based RNA amplification was performed on each sample. cDNA for each sample was synthesized using a Superscript cDNA Synthesis Kit (Invitrogen) and a T7-(dT)24 primer: 5'-GGCCAGTGAATT GTAATACGACT-CACTATAGGGAGGCGG-(dT)24-3'. The cDNA was cleaned using phase-lock gels (Fisher Cat ID E0032005101) and phenol/chloroform extraction. Biotin-labeled cRNA was then transcribed *in vitro* from cDNA using a BioArray High Yield RNA Transcript Labeling Kit (ENZO Biochem, New York, NY, USA) and purified again using the RNeasy Mini Kit. The labeled cRNA was applied to the Affymetrix Mu74Av2 and Rat 230 2.0 GeneChips (Affymetrix, Santa Clara, CA, USA) for mouse and rat, respectively, according to the manufacturer's recommendations. Every gene or EST is represented by a probe set consisting of approximately 16 probe pairs (oligonucleotides) of 25-mer oligonucleotides. One sequence of a probe pair represents the complementary strand of the target sequence while the other has a one base pair mismatch at the central base pair position. This mismatch sequence serves as an internal control for specificity of hybridization. The relative expression is reported as the average difference of the fluorescence intensity values between the perfect match and the mismatch oligonucleotides, resulting in the "average difference" value.

Detection of S100 genes expressions in bladder tumors by real-time PCR. The primer pairs of S100 family members were designed by Primer3 according to standard criteria (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Since not all the sequences of S100 family members for mice and rats are available in the NCBI GenBank, only 14 pairs of primers were designed for the mouse and rat studies. For human, 20 pairs of primers were designed according to the sequences provided in GenBank. Two

Table I. S100 gene family expression in mouse and rat bladder cancers detected by Affymetrix microarray.

Accession#	Mouse				Accession#	Rat			
	Expression					Expression			
	Genes	Epithelia	Tumor	Fold		Genes	Epithelia	Tumor	Fold
AF087469	S100a5	1088	210	-5.2**	AF140232	S100a6	138676	124393	-1.1
AF087687	S100a1	12384	2305	-5.4**	AI228548	S100a1	996	695	-1.4
X66449	S100a6	84834	82874	-1.0	NM_053681	S100a3	270	410	1.5
AF028071	S100g	155	483	3.1*	AI406499	S100a16	27169	30273	1.1
AF004941	S100a3	1506	5585	3.7*	BG378926	S100a11	77473	90614	1.2
U41341	S100a11	24689	52327	2.1**	NM_053587	S100a9	151	848	5.6**
M16465	S100a10	6362	29002	4.6*	NM_012618	S100a4	22713	61429	2.7**
M36579	S100a4	1467	4353	3.0*	NM_031114	S100a10	13052	26888	2.1**
X99921	S100a13	3688	6588	1.8	NM_012521	S100g	130	6627	50.9*
M83218	S100a8	254	2271	9.0*	NM_053822	S100a8	39	245	6.4*
AV293396	S100a14	4706	7157	1.5	NM_013191	S100b	168	362	2.2*
M83219	S100a9	55	1810	32.9*	BF548433	S100a5	1248	1508	1.2
					AW141940	S100a13	1465	1900	1.3

* $p < 0.05$; ** $p < 0.01$.

micrograms of total RNA per sample were used to synthesize cDNA in a total volume of 25 μ l using the Super-Script First-Strand Synthesis system for real-time PCR (Invitrogen, Carlsbad, CA, USA). cDNA generated from each sample was then diluted to 1000 μ l for real-time PCR. The real-time PCR assay was performed using the Bio-Rad iQ SYBR Green Super Mix kit (Bio-Rad, Hercules, CA, USA). One microliter of cDNA was added to a 25 μ l total volume reaction mixture containing water, SYBR Green Super Mix, and primers. Data were collected and analyzed on the Bio-Rad iCycler version 2.033. The internal standard values (GAPDH for mouse and β -actin for rat and human), a reflection of the number of cycles needed to reach a threshold of fluorescence, were subtracted from the cycle value for the individual gene whose expression was being assessed. In total, 14, 14 and 20 S100 family members were detected in mouse, rat and human bladder cancers, respectively.

Results

In order to detect the expression of S100 genes in bladder cancers, we performed an array analysis to compare the bladder cancers with the age-paired normal urothelium. We evaluated bladder cancers and normal urothelium of mice and rats for differential expression of members of the S100 gene family. The criteria for differential gene expression are fold change ≥ 2 and $p < 0.05$. Table I lists all the S100 genes present in the Genechips. Twelve and 13 members of S100 gene family were found in the mouse and rat Genechips, respectively. In mouse bladder cancers, 9 members of the S100 gene family were found to be differentially expressed, with 2 genes (A1 and A5) underexpressed and 7 genes (A3, A4, A8, A9, A10, A11 and G) overexpressed (Figure 1A and Table I). In rat bladder cancers, we found that 6 members of the S100 gene family differentially overexpressed (A4, A8, A9, A10, B

and G) (Figure 1A and Table I). S100 A4, A8, A9, A10 and G were found to be differentially expressed in both mouse and rat bladder cancers.

In order to confirm the array results, we systematically performed real-time PCR for S100 gene family members to detect their expression in bladder tumors. Comparing the real-time PCR results with the array data, we found a concordance of S100 expression between array and real-time PCR data in the great majority of cases, with $\sim 78\%$ (7/9) and $\sim 83\%$ (5/6) confirmation rate by real-time PCR in mouse and rat, respectively (Figure 1C and Table II).

We also analyzed the expression of S100 family members in bladder cancers by real-time PCR. In total, 14, 14 and 20 members of S100 gene family were detected by real-time PCR in mouse, rat and human bladder tumors, respectively. At least 6 members of the S100 gene family (A1, A3, A8, A9, A14, A15 and A16) were confirmed to have similar differential expression in bladder cancers, whether from mice, rats or humans (Table II). Thirteen members of the S100 gene family were confirmed by real-time PCR to be differentially expressed in human bladder tumors and 8 genes were also found to be differentially expressed in either mouse or rat bladder tumors or both. Interestingly, S100 A4 and A5 were underexpressed and overexpressed in human bladder tumors, respectively, but were oppositely expressed in mouse and rat bladder tumors (Table II).

Discussion

S100 family proteins are calcium-binding proteins that share EF-hand helix-loop-helix domains that are important for their function. A significant number of reports have indicated that

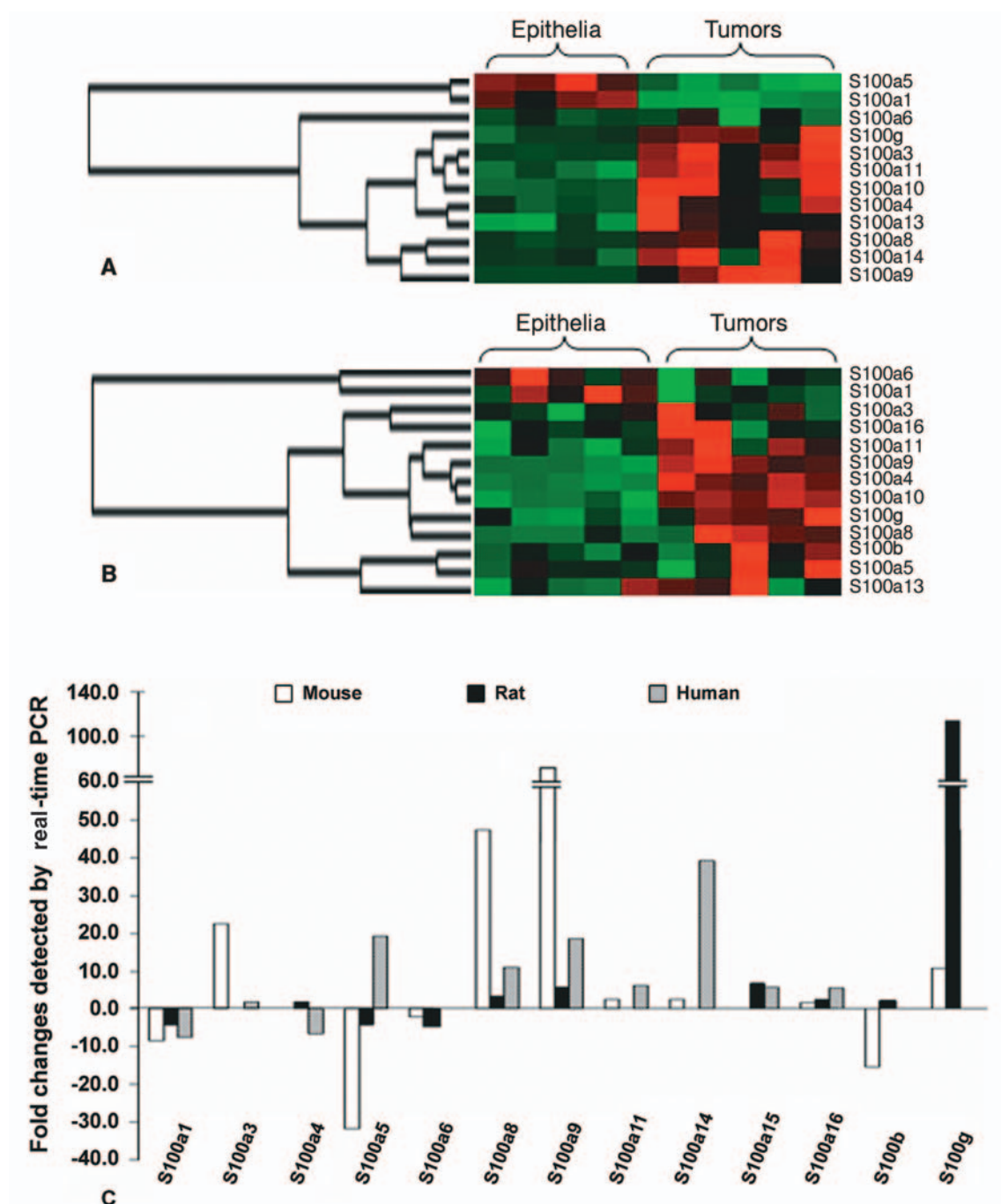


Figure 1. Expression of S100 family members in bladder cancers detected by microarray and real-time PCR. A, B: Differential expression of S100 genes examined by microarray in mouse (A) and rat (B). C: Differential expression of S100 genes examined by real-time PCR.

several S100 proteins are involved in the progression of bladder cancer. Overexpressed S100 proteins in various cancers include S100A1, S100A4, S100A6, S100A7, and S100B (16-19). S100A2 has been found to be significantly underexpressed in at least one type of cancer and has been postulated to be a tumor suppressor (20). Loss of S100A2 and increased

expression of S100A4 have been implicated in prostate tumor progression (21). Reduced expression of S100A8 and S100A9 has also been found in esophageal squamous cell carcinoma (22). S100A11 (S100C) mRNA is significantly underexpressed in invasive bladder tumors (stages T1-T4) compared with superficial tumors (stage Ta) and diminished expression of

Table II. *S100* gene expression in bladder cancers detected by microarray and real-time PCR.

	Fold changes, $p < 0.05$				
	Mouse		Rat		Human
	Array	RT-PCR	Array	RT-PCR	RT-PCR
S100A1	-5.4	-8.4	N/C	-4.0	-7.5
S100A2	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	5.3
S100A3	3.7	22.8	N/C	N/C	2.0
S100A4	3.0	N/C	2.7	1.9	-6.7
S100A5	-5.2	-31.8	N/C	-4.0	19.2
S100A6	N/C	-2.1	N/C	-4.8	N/C
S100A7	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	207.5
S100A8	9.0	47.5	6.4	3.4	11.0
S100A9	32.9	72.5	5.6	5.9	18.7
S100A10	4.6	N/C	2.1	N/C	N/C
S100A11	2.1	2.4	N/C	N/C	N/C
S100A12	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	N/C
S100A13	N/C	N/C	N/C	N/C	N/C
S100A14	N/C	2.6	<i>n/a</i>	<i>n/a</i>	39.4
S100A15	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	6.7	5.8
S100A16	<i>n/a</i>	1.5	N/C	2.5	3.5
S100B	<i>n/a</i>	-15.4	2.2	2.1	-4.1
S100G	3.1	10.8	50.9	133.0	N/C
S100P	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	14.2
S100Z	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	N/C

n/a: Not available in genechips for array and no sequences available in NCBI GenBank for RT-PCT detections.

N/C: No gene expression changes in array analysis or RT-PCR detections.

S100C is associated with poor survival in patients with bladder cancer (15). In this study, we began by systematically analyzing the differential expression of S100 genes in mouse, rat and human bladder cancers at the mRNA level by both microarray analysis and real-time PCR. Microarray comparison of bladder tumors and normal bladder urothelium samples of rat and mouse showed significantly increased expression of several members of the S100 family in tumors as compared to expression levels in normal urothelium. Eleven S100 family members (A1, A3, A4, A5, A8, A9, A10, A11, A13, A14 and G) and 8 S100 family members (A1, A4, A8, A9, A10, A11, B and G) were differentially expressed in mouse and rat bladder tumors, respectively (Table I). Further analysis by Real-time PCR on both mouse and rat bladder tumors showed substantial concordance with the microarray data. Real-time PCR evaluation of human bladder tumors revealed that at least six S100 family members showed differential expression that was similar in tumors from mice, rats, and humans (Table II). These data suggest that members of the S100 gene family play significant roles in the pathogenesis and progression of bladder cancer.

There are several possible reasons for the close association of the S100 gene family with the development of bladder cancer and its progression in humans. First, chromosome 1q

has been known to exhibit chromosomal rearrangements in various human tumors, including gains of 1q21-q24 in bladder cancers (23-25). Second, the chromosome region 1q21-q22 contains a high density of CpG islands. Hypermethylation of CpG islands has been shown to be a common mechanism for the inactivation of tumor suppressor genes and is found in a wide range of tumor types. Third, the results of our present study indicate that of 16 S100 family members that are clustered at 1q21, 12 show significant differential expression in human bladder cancers. Finally, we have presented proof that S100 gene dysfunction is closely correlated with bladder tumorigenesis in rodent bladder cancer models.

Increased expression of S100A4 has been correlated with worsened prognosis and the development of metastases in human carcinomas, including bladder cancer (12, 26, 27). The mechanism of S100A4 function has remained largely unknown. Several proteins involved in cellular cytoskeleton and motility, such as F-actin, have been shown to interact with S100A4 (28). S100A4 regulates cell cytoskeleton dynamics by interacting with myosin heavy chain II-A and II-B (29, 30). Regulation of tropomyosin-actin association is thought to be influenced by interaction between S100A4 and tropomyosin isoform 2 (31). S100A4 has also been found to bind to the C-terminal regulatory domain of p53 and inhibits phosphorylation of p53 by protein kinase C. In this way, S100A4 contributes to the development of a more aggressive phenotype during tumor progression (32). Myosin and p53 are likely to be intracellular targets of S100A4. S100A4 may facilitate metastasis by influencing the function of p53 as well as through its interaction with myosin (33). It has been reported that S100A1 reduced the S100A4 inhibition of nonmuscle myosin A self-association and phosphorylation *in vitro* and also reduced S100A4 induced cell growth and motility in soft agar and metastasis *in vivo*. These results show that interactions between different S100 proteins can affect the biological behavior of some cancers and that the presence of S100A1 protein in carcinoma cells may modulate the effects of S100A4 on their metastatic potential (34). Our results confirm that S100A1 is underexpressed in bladder cancers in mice, rats and humans, and S100A4 is overexpressed in bladder cancers in mice and rats. Our results indicate that S100A4 mRNA is underexpressed in human bladder cancers, findings that are contrary to previous reports of overexpression of S100A4 protein in these tumors, but similar to the findings in human esophageal squamous cell carcinoma (12, 26, 27, 35). The reasons for inconsistent expression of S100A4 at the mRNA and protein levels have not been resolved and probably reflect the complexity involved in the modulation of translation from mRNA to protein in the process of tumorigenesis. The interactions of S100A4 with other proteins, including different S100 proteins, such as S100A1 (34), may influence S100A4 expression at both the mRNA and protein levels, with consequent effects on bladder tumorigenesis.

S100A8 and S100A9 form homo- and heterodimers and are frequently co-expressed. Increased S100A8 and S100A9 levels have also been detected in various human cancers. S100A8 and S100A9 exhibit strong up-regulation in advanced-stage skin cancers in mice and humans. Few studies have been conducted on S100A8/A9 expression in bladder tumors. One study has shown S100A8 to be highly expressed in tumor cells in contrast to normal urothelium in 50% (6 of 12) invasive bladder cancers (36). VEGF-A, TGF and TNF released by primary tumor cells induce the expression of S100A8 and S100A9 by lung endothelium and myeloid cells, thereby facilitating the homing of tumor cells to premetastatic sites within lung parenchyma. S100A8 and S100A9 also increase the motility of circulating cancer cells by p38-mediated activation of pseudopodia, thereby facilitating invasion. Neutralizing anti-S100A8 and anti-S100A9 antibodies blocked the morphological changes and reduced the colonization by tumor cells in the lungs dramatically. This indicated that blocking S100A8 and S100A9 expression at the premetastatic stage could inhibit the migration of disseminating cancer cells (37). S100A8/A9 induced the activation of NF- κ B and increased phosphorylation of p38 and p44/42 MAP kinases. Extracellular S100A8/A9 stimulated migration of benign prostatic cells *in vitro* (38). The present study provides the first proof that S100A8 and S100A9 are significantly overexpressed in bladder cancers of rats, mice and humans.

Conflicting results have been reported for S100A11. S100A11 has been implicated in growth inhibition of human fibroblasts (39, 40). In normal cells, it appears that S100A11 is phosphorylated and preferentially accumulated in the nucleus, inhibiting DNA synthesis, whereas in immortalized cells it is not phosphorylated and remains in the cytoplasm (39, 40). Exogenous S100A11 remarkably inhibited DNA synthesis of HeLa cells with increase of p16^{Ink4a} and p21^{Waf1}. These findings suggest that S100A11 is a strong candidate for a tumor suppressor gene, but information concerning its expression in normal human tissues or common tumors is limited. Down-regulation of S100A11 has been associated with disease progression and poor survival in bladder cancer (15). However, some studies have shown that S100A11 is overexpressed in many tumors, including early pancreatic cancer (41) large cell lymphoma (42) prostate cancer (43) and breast cancer (13) S100A11 gene expression was clearly up-regulated in gastric cancer specimens from patients with lymph node metastases relative to those from patients without lymph node metastases (44). In accordance with these latter reports, the present study showed that S100A11 is overexpressed in bladder cancers of mice and humans.

S100A7 functions as a transglutaminase substrate/cornified envelope precursor, signal transduction protein, chemokine and antibacterial protein in normal epidermis. Several studies have indicated that S100A7 expression is predominantly related to squamous cell carcinomas (45), bladder squamous

cell carcinomas (14) and may also be found in non-squamous tumors, such as cutaneous melanoma (46), breast cancer (47) and gastric cancer (48). High expression levels of S100A7 are associated with increased angiogenesis and worsened clinical outcome in breast cancer and play a role in breast tumor progression (47). In bladder cancers, S100A7 protein expression has so far only been found in squamous cell carcinoma. Immunohistochemical studies on bladder SCCs show that S100A7 positive cells are confined mainly to the "squamous pearls". S100A7 protein can be detected in the urine of patients with SCC of the bladder by 2-D gel immunoblotting, indicating that S100A7 is a potential biomarker for bladder SCC (14).

Our study is the first to report the detection of significant differential expression of S100A1, S100A2, S100A3, S100A5, S100A14, S100A16 and S100P genes in human bladder cancer. Several of the genes were also found to be differentially expressed in rodent bladder cancer models (Table II). Although the exact roles played by these genes in bladder tumorigenesis remain unknown, the differential expression of S100 genes may influence tumorigenesis by regulation of protein phosphorylation and through their influences on cytoskeletal dynamics, calcium homeostasis, enzyme activities, transcription factors, cell growth and differentiation, cell cycle and apoptosis (49, 50). It has been reported that S100A1 stimulates Ndr, a nuclear serine/threonine protein kinase important in the regulation of cell division and cell morphology (51). Inhibition of expression of S100A1 in PC12 cells also results in a decrease in cell proliferation rate (52). It has been hypothesized that S100A2 is a potential tumor suppressor and that it is down-regulated in lung squamous cell carcinoma and breast cancer *via* hypermethylation of the promoter at CpG sites (53). However, recent studies have shown that S100A2 is highly expressed in non-small cell lung cancer (54), gastric cancer (48) and lymphoma (18), creating a more complex picture of the role of S100A2 in carcinogenesis. The pattern of underexpression of S100A1 and overexpression of S100A2 in the bladder cancers that we evaluated suggests that S100A2 overexpression may be an early tumorigenic event.

In addition to their intracellular functions, several S100 proteins, such as S100A4, S100A8, S100A9, S100I2, S100A13 and S100B, are secreted and act in a cytokine-like manner. The mechanism of secretion remains unknown (10, 50, 55). S100A4 may be also released from both tumor and stromal cells, by an undetermined mechanism. There is a strong possibility these proteins can be detected in urine of bladder cancer patients or tumor-bearing animals. Information accumulated in the past few years implicates S100A4 in the regulation of cancer invasiveness and metastasis; however, information concerning the role of S100 proteins in bladder cancer is very limited. The specific molecular functions of S100 proteins in bladder cancer warrant further investigation.

Traditional methods for the diagnosis of bladder cancer – cytology and voided urine cytology – can achieve specificities of 90-100%; however, cytology is invasive and may be also inconclusive at times, and urine cytology suffers from low sensitivity, particularly in detection of low-grade disease. Without question, molecular diagnostic markers are urgently needed to improve the detection of bladder cancer and survival of patients. Using sensitive and specific molecular techniques we have detected several members of S100 family that are differentially expressed in bladder cancers of mice, rats and humans. Further comparative studies are needed to assess the utility of S100 proteins as markers of bladder cancer. However the present experiments define the possibility of identifying tumor related markers which may prove useful in the detection of bladder cancer and may be potential targets for molecular-based therapeutic strategies in the prevention and/or management of bladder cancer.

References

- Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C and Thun MJ: Cancer statistics, 2006. *CA Cancer J Clin* 56: 106-130, 2006.
- Ghoneim MA, el-Mekresh MM, el-Baz MA, el-Attar IA and Ashamalla A: Radical cystectomy for carcinoma of the bladder: critical evaluation of the results in 1,026 cases. *J Urol* 158: 393-399, 1997.
- Droller MJ: Bladder cancer: state-of-the-art care. *CA Cancer J Clin* 48: 269-284, 1998.
- Cheng L, Weaver AL, Leibovich BC, Ramnani DM, Neumann RM, Sherer BG, Nehra A, Zincke H and Bostwick DG: Predicting the survival of bladder carcinoma patients treated with radical cystectomy. *Cancer* 88: 2326-2332, 2000.
- Droller MJ: Markers in bladder cancer – issues to consider. *J Urol* 160: 2009-2010, 1998.
- Grubbs CJ, Lubet RA, Koki AT, Leahy KM, Masferrer JL, Steele VE, Kelloff GJ, Hill DL and Seibert K: Celecoxib inhibits *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female Fischer-344 rats. *Cancer Res* 60: 5599-5602, 2000.
- Grubbs CJ, Moon RC, Squire RA, Farrow GM, Stinson SF, Goodman DG, Brown CC and Sporn MB: 13-*cis*-Retinoic acid: inhibition of bladder carcinogenesis induced in rats by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Science* 198: 743-744, 1977.
- Lubet RA, Huebner K, Fong LY, Altieri DC, Steele VE, Kopelovich L, Kavanaugh C, Juliana MM, Soong SJ and Grubbs CJ: 4-Hydroxybutyl(butyl)nitrosamine-induced urinary bladder cancers in mice: characterization of FHIT and survivin expression and chemopreventive effects of indomethacin. *Carcinogenesis* 26: 571-578, 2005.
- Iliopoulos D, Guler G, Han SY, Johnston D, Druck T, McCorkell KA, Palazzo J, McCue PA, Baffa R and Huebner K: Fragile genes as biomarkers: epigenetic control of WWOX and FHIT in lung, breast and bladder cancer. *Oncogene* 24: 1625-1633, 2005.
- Marenholz I, Heizmann CW and Fritz G: S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). *Biochem Biophys Res Commun* 322: 1111-1122, 2004.
- Heizmann CW, Fritz G and Schafer BW: S100 proteins: structure, functions and pathology. *Front Biosci* 7: 1356-1368, 2002.
- Davies BR, O'Donnell M, Durkan GC, Rudland PS, Barraclough R, Neal DE and Mellon JK: Expression of S100A4 protein is associated with metastasis and reduced survival in human bladder cancer. *J Pathol* 196: 292-299, 2002.
- Cross SS, Hamdy FC, Deloulme JC and 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* 46: 256-269, 2005.
- Celis JE, Rasmussen HH, Vorum H, Madsen P, Honore B, Wolf H and Orntoft TF: Bladder squamous cell carcinomas express psoriasin and externalize it to the urine. *J Urol* 155: 2105-2112, 1996.
- Memon AA, Sorensen BS, Meldgaard P, Fokdal L, Thykjaer T and Nexø E: Down-regulation of S100C is associated with bladder cancer progression and poor survival. *Clin Cancer Res* 11: 606-611, 2005.
- Bronckart Y, Decaestecker C, Nagy N, Harper L, Schafer BW, Salmon I, Pochet R, Kiss R and Heizman CW: Development and progression of malignancy in human colon tissues are correlated with expression of specific Ca²⁺-binding S100 proteins. *Histol Histopathol* 16: 707-712, 2001.
- Jenkinson SR, Barraclough R, West CR and Rudland PS: S100A4 regulates cell motility and invasion in an *in vitro* model for breast cancer metastasis. *Br J Cancer* 90: 253-262, 2004.
- Hsieh HL, Schafer BW, Sasaki N and Heizmann CW: Expression analysis of S100 proteins and RAGE in human tumors using tissue microarrays. *Biochem Biophys Res Commun* 307: 375-381, 2003.
- Emberley ED, Niu Y, Leygue E, Tomes L, Gietz RD, Murphy LC and Watson PH: Psoriasin interacts with Jab1 and influences breast cancer progression. *Cancer Res* 63: 1954-1961, 2003.
- Nagy N, Brenner C, Markadieu N, Chaboteaux C, Camby I, Schafer BW, Pochet R, Heizmann CW, Salmon I, Kiss R *et al*: S100A2, a putative tumor suppressor gene, regulates *in vitro* squamous cell carcinoma migration. *Lab Invest* 81: 599-612, 2001.
- Gupta S, Hussain T, MacLennan GT, Fu P, Patel J and Mukhtar H: Differential expression of S100A2 and S100A4 during progression of human prostate adenocarcinoma. *J Clin Oncol* 21: 106-112, 2003.
- Luo A, Kong J, Hu G, Liew CC, Xiong M, Wang X, Ji J, Wang T, Zhi H, Wu M *et al*: Discovery of Ca²⁺-relevant and differentiation-associated genes downregulated in esophageal squamous cell carcinoma using cDNA microarray. *Oncogene* 23: 1291-1299, 2004.
- Qin SL, Chen XJ, Xu X, Shou JZ, Bi XG, Ji L, Han YL, Cai Y, Wei F, Ma JH *et al*: Detection of chromosomal alterations in bladder transitional cell carcinomas from Northern China by comparative genomic hybridization. *Cancer Lett* 238: 230-239, 2006.
- Richter J, Jiang F, Gorog JP, Sartorius G, Egenter C, Gasser TC, Moch H, Mihatsch MJ and Sauter G: Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. *Cancer Res* 57: 2860-2864, 1997.
- Simon R, Burger H, Brinkschmidt C, Bocker W, Hertle L and Terpe HJ: Chromosomal aberrations associated with invasion in papillary superficial bladder cancer. *J Pathol* 185: 345-351, 1998.

- 26 Levett D, Flecknell PA, Rudland PS, Barraclough R, Neal DE, Mellon JK and Davies BR: Transfection of S100A4 produces metastatic variants of an orthotopic model of bladder cancer. *Am J Pathol* 160: 693-700, 2002.
- 27 Agerbaek M, Alsner J, Marcussen N, Lundbeck F and Von Der Maase H: Focal S100A4 protein expression is an independent predictor of development of metastatic disease in cystectomized bladder cancer patients. *Eur Urol* 50: 777-785, 2006.
- 28 Watanabe Y, Usada N, Minami H, Morita T, Tsugane S, Ishikawa R, Kohama K, Tomida Y and Hidaka H: Calvasculin, as a factor affecting the microfilament assemblies in rat fibroblasts transfected by src gene. *FEBS Lett* 324: 51-55, 1993.
- 29 Li ZH, Spektor A, Varlamova O and Bresnick AR: Mts1 regulates the assembly of nonmuscle myosin-IIA. *Biochemistry* 42: 14258-14266, 2003.
- 30 Kriajevska M, Tarabykina S, Bronstein I, Maitland N, Lomonosov M, Hansen K, Georgiev G and Lukanidin E: Metastasis-associated Mts1 (S100A4) protein modulates protein kinase C phosphorylation of the heavy chain of nonmuscle myosin. *J Biol Chem* 273: 9852-9856, 1998.
- 31 Takenaga K, Nakamura Y, Sakiyama S, Hasegawa Y, Sato K and Endo H: Binding of pEL98 protein, an S100-related calcium-binding protein, to nonmuscle tropomyosin. *J Cell Biol* 124: 757-768, 1994.
- 32 Grigorian M, Andresen S, Tulchinsky E, Kriajevska M, Carlberg C, Kruse C, Cohn M, Ambartsumian N, Christensen A, Selivanova G *et al*: Tumor suppressor p53 protein is a new target for the metastasis-associated Mts1/S100A4 protein: functional consequences of their interaction. *J Biol Chem* 276: 22699-22708, 2001.
- 33 Chen H, Fernig DG, Rudland PS, Sparks A, Wilkinson MC and Barraclough R: Binding to intracellular targets of the metastasis-inducing protein, S100A4 (p9Ka). *Biochem Biophys Res Commun* 286: 1212-1217, 2001.
- 34 Wang G, Zhang S, Fernig DG, Martin-Fernandez M, Rudland PS and Barraclough R: Mutually antagonistic actions of S100A4 and S100A1 on normal and metastatic phenotypes. *Oncogene* 24: 1445-1454, 2005.
- 35 Ji J, Zhao L, Wang X, Zhou C, Ding F, Su L, Zhang C, Mao X, Wu M and Liu Z: Differential expression of S100 gene family in human esophageal squamous cell carcinoma. *J Cancer Res Clin Oncol* 130: 480-486, 2004.
- 36 Tolson JP, Flad T, Gnau V, Dihazi H, Hennenlotter J, Beck A, Mueller GA, Kuczyk M and Mueller CA: Differential detection of S100A8 in transitional cell carcinoma of the bladder by pair wise tissue proteomic and immunohistochemical analysis. *Proteomics* 6: 697-708, 2006.
- 37 Hiratsuka S, Watanabe A, Aburatani H and Maru Y: Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat Cell Biol* 8: 1369-1375, 2006.
- 38 Hermani A, De Servi B, Medunjanin S, Tessier PA and Mayer D: S100A8 and S100A9 activate MAP kinase and NF-kappaB signaling pathways and trigger translocation of RAGE in human prostate cancer cells. *Exp Cell Res* 312: 184-197, 2006.
- 39 Sakaguchi M, Miyazaki M, Inoue Y, Tsuji T, Kouchi H, Tanaka T, Yamada H and Namba M: Relationship between contact inhibition and intranuclear S100C of normal human fibroblasts. *J Cell Biol* 149: 1193-1206, 2000.
- 40 Sakaguchi M, Tsuji T, Inoue Y, Miyazaki M, Namba M, Yamada H and Tanaka T: Loss of nuclear localization of the S100C protein in immortalized human fibroblasts. *Radiat Res* 155: 208-214, 2001.
- 41 Ohuchida K, Mizumoto K, Ohhashi S, Yamaguchi H, Konomi H, Nagai E, Yamaguchi K, Tsuneyoshi M and Tanaka M: S100A11, a putative tumor suppressor gene, is overexpressed in pancreatic carcinogenesis. *Clin Cancer Res* 12: 5417-5422, 2006.
- 42 Rust R, Visser L, van der Leij J, Harms G, Blokzijl T, Deloulme JC, van der Vlies P, Kamps W, Kok K, Lim M *et al*: High expression of calcium-binding proteins, S100A10, S100A11 and CALM2 in anaplastic large cell lymphoma. *Br J Haematol* 131: 596-608, 2005.
- 43 Rehman I, Azzouzi AR, Cross SS, Deloulme JC, Catto JW, Wylde N, Larre S, Champigneulle J and Hamdy FC: Dysregulated expression of S100A11 (calcizzarin) in prostate cancer and precursor lesions. *Hum Pathol* 35: 1385-1391, 2004.
- 44 Mori M, Shimada H, Gunji Y, Matsubara H, Hayashi H, Nimura Y, Kato M, Takiguchi M, Ochiai T and Seki N: S100A11 gene identified by in-house cDNA microarray as an accurate predictor of lymph node metastases of gastric cancer. *Oncol Rep* 11: 1287-1293, 2004.
- 45 Fukuzawa H, Kiyoshima T, Kobayashi I, Ozeki S and Sakai H: Transcription promoter activity of the human S100A7 gene in oral squamous cell carcinoma cell lines. *Biochim Biophys Acta* 1759: 171-176, 2006.
- 46 Brouard MC, Saurat JH, Ghanem G and Siegenthaler G: Urinary excretion of epidermal-type fatty acid-binding protein and S100A7 protein in patients with cutaneous melanoma. *Melanoma Res* 12: 627-631, 2002.
- 47 Krop I, Marz A, Carlsson H, Li X, Bloushtain-Qimron N, Hu M, Gelman R, Sabel MS, Schnitt S, Ramaswamy S *et al*: A putative role for psoriasin in breast tumor progression. *Cancer Res* 65: 11326-11334, 2005.
- 48 El-Rifai W, Moskaluk CA, Abdrabbo MK, Harper J, Yoshida C, Riggins GJ, Frierson HF Jr and Powell SM: Gastric cancers overexpress S100A calcium-binding proteins. *Cancer Res* 62: 6823-6826, 2002.
- 49 Donato R: Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta* 1450: 191-231, 1999.
- 50 Donato R: Intracellular and extracellular roles of S100 proteins. *Microsc Res Tech* 60: 540-551, 2003.
- 51 Millward TA, Heizmann CW, Schafer BW and Hemmings BA: Calcium regulation of Ndr protein kinase mediated by S100 calcium-binding proteins. *EMBO J* 17: 5913-5922, 1998.
- 52 Zimmer DB, Cornwall EH, Reynolds PD and Donald CM: S100A1 regulates neurite organization, tubulin levels, and proliferation in PC12 cells. *J Biol Chem* 273: 4705-4711, 1998.
- 53 Wicki R, Franz C, Scholl FA, Heizmann CW and Schafer BW: Repression of the candidate tumor suppressor gene S100A2 in breast cancer is mediated by site-specific hypermethylation. *Cell Calcium* 22: 243-254, 1997.
- 54 Heighway J, Knapp T, Boyce L, Brennand S, Field JK, Betticher DC, Ratschiller D, Gugger M, Donovan M, Lasek A *et al*: Expression profiling of primary non-small cell lung cancer for target identification. *Oncogene* 21: 7749-7763, 2002.
- 55 Marenholz I, Lovering RC and Heizmann CW: An update of the S100 nomenclature. *Biochim Biophys Acta* 26: 26, 2006.

Received May 25, 2007

Accepted June 4, 2007