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Review

The S100 proteins for screening and prognostic grading of bladder cancer

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Summary. The S100 gene family, which is composed of at least 24 members carrying the Ca^{2+} binding EF-hand motif, has been implicated in both intracellular and extracellular functions, including enzyme activities, immune responses, cytoskeleton dynamics, Ca^{2+} homeostasis, cell growth and cell differentiation. Altered S100 protein levels are associated with a broad range of diseases, including cardiomyopathy, inflammatory and immune disorders, neurodegenerative disorders and cancer. Although the precise role of S100 protein in carcinogenesis is poorly understood, it seems that formation of homo- and hetero-dimers, binding of Ca^{2+} and interaction with effector molecules are essential for the development and progression of many cancers. Several studies have suggested that S100 proteins promote cancer progression and metastasis through cell survival and apoptosis pathways. In animal models of bladder cancer, several S100 proteins are differentially expressed in bladder tumors relative to normal urothelium. In human bladder cancer, overexpression of S100A4, S100A8 or S100A11 are associated with stage progression, invasion, metastasis and poor survival. This review summarizes these findings and evaluates their implications for human bladder cancer management.

Key words: Bladder, Urinary tract, Neoplasia, Transitional cell caricnoma, Urothelial carcinoma, S100 proteins, Proteomics, Biomarkers

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Introduction

The S100 protein family includes more than 20 entities, all of which are identified only in vertebrates, sharing a common structure carrying the Ca^{2+}-binding EF-hand motif (Marenholz et al., 2004, 2006). S100 proteins (SP) are low molecular weight, acidic peptides of 9-13 kDa with two distinct EF-hands. Interestingly, of the 24 human SP, 19 family members are tightly clustered at chromosome locus 1q21. The other five SP are found at chromosome 4p16 (S100P), 5q13 (S100Z), 7q22-q31 (S100A11P), 21q22 (S100B) and Xp22 (S100G) (Marenholz et al., 2006). Their gene structure is highly conserved, generally comprising three exons and two introns, with the first exon noncoding. The mouse and rat have 17 and 14 proteins, respectively; with clustering that is analogous to human SP. The mouse has a cluster of 13 SP family members in region F1-F2 of mouse chromosome 3 (Marenholz et al., 2004). The rat has 12 family members in region q34 of rat chromosome 2 (Table 1). Most SP show cell-specific expression patterns, suggesting that different SP have distinct organ-specific functions (Donato, 2003). Many SP can form homo- and heterodimers, which seem important for their functional diversification. The calcium-dependent signaling roles of the SP are essential for each of their functions. The N-terminal EF-hand, sometimes referred as a 'pseudo-canonical' or 'half' EF-hand, binds calcium with a weak affinity ($K_d \approx$ 200-500 mM) (Donato, 1986). The C-terminal EF-hand is the more conserved EF-hand domain and binds calcium with 100 times greater affinity ($K_d \approx$ 10-50 mM) than the N-terminal EF-hand (Donato, 1986). Upon calcium binding, SP undergo a conformational change and a hydrophobic cleft forms in each monomer. The hydrophobic cleft is important for calcium-dependent recognition of S100 target proteins (Donato, 1999, 2003). In addition to calcium, many SP

bind Zn^{2+} and Cu^{2+} with high affinity. These divalent cations may influence SP intra- and extracellular functions (Marenholz et al., 2004). SP are multi-functional signaling proteins involved in numerous cellular functions, such as protein phosphorylation, enzyme activation, interaction with cytoskeletal components, and calcium homeostasis (Donato, 2003). Moreover, SP are regulators of many cellular processes such as cell growth, cell cycle progression, differentiation, transcription and secretion (Donato, 2003). The functions of SP in tumorigenesis and tumor progression have not been clearly elucidated, but recent reports have suggested that SP regulation of the signaling pathways may promote tumor progression.

Bladder cancer is the sixth most common malignancy in developed countries. It ranks as the fourth and ninth most frequently diagnosed cancer in men and women, respectively, in the United States. It is estimated that about 61,420 cases will be newly diagnosed in the USA and 13,060 patients will die from disseminated urothelial carcinoma in 2006. Most bladder tumors are transitional cell carcinomas (TCCs). However, squamous cell carcinoma is the predominant histologic type of bladder cancer in Middle Eastern countries (Davidson and Cheng, 2006; Jemal et al., 2006). Bladder tumors, including some preinvasive lesions, carry a substantial number of genetic alterations at the time of diagnosis. Losses of heterozygosity (LOH) at 1q, 3p, 4q, 6q, 8p, 9p, 9q, 10q, 11p, 13q, 14q, 17p, 18q and 21q have been observed in human bladder cancers, implying the possible involvement of tumor suppressor genes harbored in those chromosomal regions (Kroft and Oyasu, 1994; Knowles, 1995; Brandau and Bohle, 2001; Mazzucchelli et al., 2005). Bladder tumorigenesis is associated with losses from chromosomes 9, 13 and 17, including inactivation of CDKN2 (p16) at 9p, of Rb at 13q14 and of p53 at 17p11. With recent advances in molecular biology and novel technologies, several biomarkers have emerged as important adjuncts in the diagnosis of lesions of the bladder. When used in conjunction with careful histologic examination, immunohistochemistry can be a valuable aid in classifying tumors presenting in the bladder and mesenchymal lesions of the bladder and in establishing the urothelial origin of a metastatic tumor. In addition, a number of biomarkers may prove to be important indicators of prognosis or response to chemotherapy and tumor progression and recurrence or progression (Bollito

Table 1. S100 protein family members in human, mouse and rat and their expression and function in bladder tumors.

Gene name	Chromsome Location	Orthologous mouse gene	Chromsome location	Orthologous rat gene	Chromsome location	Expression in bladder cancer
S100A1	1q21	S100a1	3F1-F2	S100a1	2q34	underexpression in mouse and rat (unpublished data)
S100A2	1q21	S100a2 (S100L)	3F1-F2			-----
S100A3	1q21	S100a3	3F1-F2	S100a3	2q34	overexpression in mouse (unpublished data)
S100A4	1q21	S100a4	3F1-F2	S100a4	2q34	overexpression associated with stage progression, metastasis and poor survival in human (Davies et al., 2002) overexpression associated with metastasis in rat (Levett et al., 2002) overexpression in rat (unpublished data)
S100A5	1q21	S100a5	3F1-F2	S100a5	2q34	underexpression in mouse (unpublished data)
S100A6	1q21	S100a6	3F1-F2	S100a6	2q34	-----
S100A7	1q21	S100a7	3F1-F2			overexpression in human bladder SCC (Ostergaard et al., 1997, 1999)
S100A7A	1q21					-----
S100A7L2	1q21					-----
S100A7P1	1q21					-----
S100A7P2	1q21					-----
S100A8	1q21	S100a8	3F1-F2	S100a8	2q34	overexpression associated with aggressive, invasive bladder tumors in human (Tolson et al., 2006). overexpression in mouse and rat (unpublished data)
S100A9	1q21	S100a9	3F1-F2	S100a9	2q34	overexpression in mouse and rat (unpublished data)
S100A10	1q21	S100a10	3F1-F2	S100a10	2q34	overexpression in mouse and rat (unpublished data)
S100A11	1q21	S100a11	3E-F	S100a11	2q34	underexpression associated with progression and poor survival in human (Memon et al., 2005).
S100A11P	7q22-q31					-----
S100A12	1q21					-----
S100A13	1q21	S100a13	3F1-F2	S100a13	2q34	-----
S100A14	1q21	S100a14	3F2	LOC686203	2q34	-----
S100A16	1q21	S100a16	3F1-F2	S100a16	2q34	-----
S100B	21q22	S100b	10C1	S100b	20p12	-----
S100G	Xp22	S100g	XF4	S100g	Xq21	overexpression in mouse and rat (unpublished data)
S100P	4p16					-----
S100Z	5q13	S100z (predicted)	13D1			-----

et al., 2005; Emerson and Cheng, 2005; Kyroudi-Voulgari et al., 2005; Iczkowski and Butler, 2006). Numerous markers have been identified that correlate to some extent with tumor stage and prognosis. However, the power of many of these markers in predicting the clinical outcome of individual tumors is limited, and alternative markers are still needed for detection and prognostic grading of the disease.

Due to the propensity of superficial lesions to recur following transurethral resection, even after intravesical instillation of antineoplastic agents, frequent follow-up is required. Because of the highly aggressive nature of transitional cell carcinoma in situ and invasive nonpapillary bladder carcinoma, there has been an ongoing search for reliable screening tests for bladder carcinoma, to allow their detection as early as possible. Cystoscopy is invasive and costly, and urinary cytology has relatively low sensitivity. Therefore, investigators have long sought urine-based markers to detect new or recurrent transitional cell carcinoma. Furthermore, there has been considerable interest in identifying biologic indicators of individual tumor aggressiveness. In this review, we summarize the recent literatures regarding SP molecular testing for the detection and grading of bladder cancer.

S100 expression in bladder and other cancers

Many reports have described how SP are involved in the progression of various cancers. Overexpressed SP in colon and breast cancer include S100A1, S100A4, S100A6, S100A7, and S100B (Bronckart et al., 2001; Emberley et al., 2003; Hsieh et al., 2003; Jenkinson et al., 2004). S100A2 is underexpressed in squamous cell carcinoma and may be a tumor suppressor gene (Nagy et al., 2001). Loss of S100A2 and increased expression of S100A4 have been implicated in prostate tumor progression (Gupta et al., 2003). Decreased expression of S100A8 and S100A9 has also been found in esophageal squamous cell carcinoma (Luo et al., 2004). S100C mRNA is significantly underexpressed in invasive bladder tumors (T1-T4) when compared with superficial tumors (Ta). Low expression of S100C is associated with poor survival in patients with bladder cancer (Memon et al., 2005). Using microarray analysis, SP are often differentially expressed in rat and mouse bladder cancer models. In mouse urothelial carcinoma, seven S100 protein family members have altered expression. There is underexpression of S100A1 and S100A5 and overexpression of S100A3, S100A8, S100A9, S100A10 and S100G. In rat urothelial carcinoma, seven S100 protein family members show altered expression: underexpression of S100A1, and overexpression of S100A4, S100A8, S100A9, S100A10 and S100G (unpublished data).

S100A4

The expression of S100A4, also known as Mts1, is strongly correlated with the development of an aggressively metastatic phenotype. Ebralidze et al. first reported that the S100A4 mRNA expression level correlates with the metastatic potential of several tumor cell lines (Ebralidze et al., 1989). S100A4 expression has been reported in several cancer types both in human and animal models. Upregulated S100A4 mRNA is associated with metastasis in animal breast cancer models and with poor patient survival in humans. Transfection of the rat (Davies et al., 1993) or human (Lloyd et al., 1998) S100A4 gene in a nonmetastasizing rat mammary cancer cell line has been documented to result in metastasis from the mammary gland to the lung. S100A4 overexpression has been reported in several cancer types, including the breast (Rudland et al., 2000), bladder (Davies et al., 2002), colorectal (Gongoll et al., 2002), and gastric (Yonemura et al., 2000). Expression of S100A4 in malignant cells is associated with reduced patient survival and poor prognosis. In a recent report, S100A4 protein added to the extracellular space triggers invasion and metastasis cascades in tumor cells, emphasizing the important role of S100A4 in the tumor-stroma interaction (Schmidt-Hansen et al., 2004a,b). Together, these data suggest a specific role for S100A4 in invasion. S100A4 may also promote metastasis survival by angiogenic effects (Ambartsumian et al., 2001). In a highly metastatic Lewis lung carcinoma cell line, inhibition of S100A4 by antisense RNA resulted in decreased cell motility and invasiveness (Takenaga et al., 1997).

The mechanism of S100A4 function, however, has remained largely unknown. Several proteins in the cytoskeleton required for motility are known to interact with S100A4. Interaction of S100A4 with F-actin and bundles of actin filaments causes disorganization of the filaments, apparently due to the cross-linking activity of S100A4 (Watanabe et al., 1993). S100A4 also regulates cell cytoskeleton dynamics by interacting with myosin heavy chains II-A (Kriajevska et al., 1994; Li et al., 2003) and II-B (Kriajevska et al., 1998; Li et al., 2003). Regulation of the tropomyosin-actin association is thought to require interaction between S100A4 and tropomyosin isoform 2 (Takenaga et al., 1994).

S100A4 also has been found to bind the C-terminal regulatory domain of p53 and to inhibit phosphorylation of p53 by protein kinase C. This binding interferes with the DNA binding activity of p53. Furthermore, downregulation of p53 target gene transcription (p21/WAF, bax, thrombospondin-1, and mdm-2) and upregulation of the pro-apoptotic gene Bax is observed when S100A4 is induced in cell lines expressing wild type p53. Gene products of p21/WAF, thrombospondin-1, mdm2 and S100A4 cooperate with wild type p53 for apoptosis induction. The ability of S100A4 to enhance p53-dependent apoptosis may accelerate the loss of tumor cells bearing wild type p53 and allow clonal expansion of cells with mutant p53. In this way, S100A4 may contribute to the development of a more aggressive phenotype during tumor progression (Grigorian et al.,

2001). S100A4 may thus favor a metastasizing phenotype by enhancing myosin-actin motility and eliminating less aggressive cells with wild-type p53 (Chen et al., 2001).

Some clinical studies have found that when S100A4 is upregulated in bladder cancer, it is associated with an increased incidence of metastasis and reduced survival. Analysis of S100A4 protein expression emerged as the only independent predictor of the development of distant metastates versus metastasis-free survival in a multivariate analysis of 108 consecutive patients treated for transitional cell bladder cancer with preoperative radiotherapy and cystectomy (Agerbaek et al., 2006). S100A4 protein is more frequently observed in invasive bladder tumours than in non-invasive tumours. Moreover, in invasive tumors, S100A4 staining is usually strongest in invasive regions and in single infiltrating tumor cells. There are statistically significant associations between increased S100A4 expression and both metastasis and reduced survival (Davies et al., 2002). S100A4 overexpression in bladder cancer is also detected in rodent models. MYU-3L cells produce rapidly growing, invasive tumors in the bladder wall of rats, but they fail to metastasize. However, transfected MYU-3L cells overexpressing S100A4 produce metastases to the para-aortic lymph nodes and lungs. Expression of S100A4 protein is stronger and more consistent in the metastases (Levett et al., 2002).

S100A7

S100A7, also known as psoriasin, was described more than 10 years ago as an mRNA highly up-regulated in the lesions of psoriatic skin (Madsen et al., 1991). S100A7 functions as a transglutaminase substrate or cornified envelope precursor, signal transduction protein, chemokine, and antibacterial protein in normal epidermis. S100A7 is chemotactic for CD4+ cells in vitro (Jinquan et al., 1996). It is also markedly increased in healing wounds, inflammatory dermatoses, hyperproliferative skin diseases and skin cancer (Eckert and Lee, 2006). In skin, S100A7 is expressed in epithelial cells but not in stromal cells. S100A7 expression is predominantly observed in squamous cell carcinoma (Fukuzawa et al., 2006), including squamous cell carcinoma of the bladder (Celis et al., 1996), but may also be found in non-squamous tumors, such as cutaneous melanoma (Brouard et al., 2002), breast carcinoma (Krop et al., 2005), and gastric carcinoma (El-Rifai et al., 2002). S100A7 expression is more frequently seen in ductal carcinoma in situ (DCIS) than in invasive breast carcinoma (IBC). Its expression, however, is associated with poor prognosis both in DCIS and in IBC. Highly expressed S100A7 appears to play a role in breast tumor progression, and is associated with increased angiogenesis and adverse clinical outcomes in breast cancer (Krop et al., 2005).

Among bladder cancers, S100A7 protein is only expressed in squamous cell carcinoma. Immunohistochemical staining of bladder squamous cell carcinomas demonstrates that the S100A7 positive cells are confined to the squamous pearls. Urine from patients with squamous cell bladder carcinoma can be demonstrated to contain S100A7 by 2-D gel immunoblotting. Thus, S100A7 is a potential marker for squamous cell carcinoma of the bladder (Celis et al., 1996). Well differentiated squamous cell carcinomas of the bladder also express keratins, psoriasis-associated fatty acid-binding protein (PA-FABP) and galectin 7, in addition to psoriasin (S100A7). In comparison, poorly differentiated squamous cell bladder carcinoma does not express keratin 10, and such tumors are characterized by decreased expression of keratin 14, psoriasin (S100A7), PA-FABP, galectin 7, and stratifin (14-3-3 sigma). In one study, all bladder squamous cell carcinomas shed psoriasin (S100A7) into the urine, supporting the contention that this protein, alone or in combination with other polypeptides, may be a useful marker for the early detection of these lesions (Ostergaard et al., 1997). Proteomic technology has been employed to identify urinary markers for bladder squamous cell carcinoma markers in the urine, with a blind and systematic analysis of the protein profiles of fresh tumors, their secreted proteins and the patient's urine. It has been found that psoriasin (S100A7) is synthesized by the more differentiated cells in the tumor, whereas some poorly differentiated tumors do not express this protein at all. Nevertheless, since two-dimensional polyacrylamide gel electrophoresis immunoblotting of urine from bladder squamous cell carcinoma patients has consistently detected psoriasin (S100A7) in their urine specimens, this marker may be valuable for detection or followup after treatment of these lesions (Ostergaard et al., 1999).

S100A8/S100A9

S100A8 and S100A9 form homo- and heterodimers and are frequently co-expressed. In normal epidermis, S100A8 and S100A9 are expressed at very low levels. Calprotectin (S100A8/A9), a heterodimer of the two calcium-binding proteins S100A8 and S100A9, is secreted by neutrophils. It has emerged as an important pro-inflammatory mediator in acute and chronic inflammation. The S100A8/A9 heterodimer acts as a chemotactic molecule constitutively expressed by neutrophils, activated monocytes, and macrophages. Elevated levels of S100A8 and S100A9 proteins are found in sera from patients with a wide variety of illnesses associated with chronic inflammation, including rheumatoid arthritis, SLE, multiple sclerosis, cystic fibrosis, chronic inflammatory bowel diseases, and psoriasis (Gebhardt et al., 2006). More recently, increased S100A8 and S100A9 levels have also been detected in various human cancers. S100A8 and S100A9 have been demonstrated to exhibit strong up-regulation in advanced stages of skin cancer in mice and in humans. Recent clinical and experimental data suggest

that changes in expression or function of S100 proteins may be a key step in cancer development. Significant S100A8 and S100A9 up-regulation was found in breast, lung, gastric, colorectal, pancreatic, and prostate cancer, while down-regulation was detected in esophageal squamous cell carcinomas. Furthermore, altered S100A9 expression in adenocarcinoma of breast, lung, and thyroid correlates with poor tumor differentiation (Gebhardt et al., 2006).

Few studies have been conducted on the expression of S100A8/A9 in bladder tumors. One study of 12 invasive bladder cancer tissue biopsies paired with normal bladder samples from the same patients showed that S100A8 (MRP-8, calgranulin A) is highly expressed in tumor cells relative to normal urothelium in 50% of the samples. S100A8/S100A9, when fully characterized, may emerge as an important new marker of aggressive, invasive bladder carcinoma (Tolson et al., 2006).

S100A11/S100C

S100A11 (S100C, calgizarrin) is present in the basal and spinous layers of normal epidermis and participates in cytoskeleton assembly and dynamics. This protein is present in the nucleus and cytoplasm of basal cells but is associated with the plasma membrane in spinous cells (Broome et al., 2003). An S100A11 homodimer interacts with two annexin I molecules and forms an annexin-S100A11 heterotetramer. The calcium-regulated $(\text{annexin I/S100A11})_2$ heterotetramer probably plays a role in the organization of membrane fusion events (Rety et al., 2000); it appears to regulate and facilitate plasma membrane remodeling during terminal differentiation. S100A11 is dramatically downregulated in immortalized human fibroblasts compared with unaltered primary fibroblasts in culture. Microinjection of anti-S100A11 antibody into normal confluent quiescent cells has been shown to induce DNA synthesis (Sakaguchi et al., 2000). S100A11 has also been implicated in Ca^{2+}-induced growth inhibition of human keratinocytes in culture (Sakaguchi et al., 2003). S100A11 is overexpressed in ApcMin adenomas and ApcMin gastrointestinal tumors. Expression of S100A11 is also increased in human colorectal cancer cell lines, in adenocarcinomas regardless of tumor stage (Reichling et al., 2005; Melle et al., 2006) and in thyroid adenomas and carcinomas (Torres-Cabala et al., 2004).

A synthetic partial peptide of S100A11, S100A11/C, is cytotoxic to cultured fibroblasts when introduced into the cells. This induction of apoptotic cell death is independent of p53, p21WAF1/CIP1, and caspase activity (Makino et al., 2004). Expression of S100A11/C also markedly increases after addition of TGF-ß1 to the cultures. DNA synthesis is inhibited by S100A11/C in a dose-dependent manner in the absence of TGF-ß1 (Miyazaki et al., 2004). On exposure of the cells to TGF-ß1, S100A11/C is phosphorylated, bound to nucleolin, and transferred to the nucleus, causing induction of $p21^{WAF1/CIP1}$ and $p15^{INK4B}$ through activation of Sp1. TGF-ß1-induced growth inhibition is almost completely eliminated when S100A11 is functionally sequestered (Sakaguchi et al., 2004).These results indicate that the S100A11/C-mediated pathway is essential for the growth inhibition and apoptosis induced by TGF-ß1.

The expression of S100A11/C in bladder tumors has been reported in a single study. A significant downregulation of S100A11 was found in Grade 3 as compared with Grade 1 bladder cancer cell lines. There is a significantly lower mRNA expression of S100A11/C in bladder carcinomas invasive into the lamina propria (stage T1) and in more deeply invasive tumors (stages T2 to T4), than in superficial noninvasive tumors (Ta). A negative correlation also is found between S100A11/C and histopathologic grade. Importantly, the loss of S100A11/C expression is associated with poor survival in patients with bladder cancer (Memon et al., 2005).

Conclusions and future insights

The S100 protein family is involved in a large network of calcium-dependent and independent protein-protein interactions involved in the regulation of cell cycle progression, cell growth, differentiation, secretion and cytoskeletal organization. Although altered expression of S100 protein family members is regarded as being associated with malignancy, the mechanisms by which they promote an invasive phenotype or contribute to poor prognosis and poor survival are not well established. S100A4 influences cancer metastasis by remodeling of the extracellular matrix and cellular motility. Overexpression of S100A7 and S100A8/A9 in tumors plays a role in tumor differentiation and progression. Expression of S100A11 is essential for apoptosis and loss of S100A11 expression is associated with poor survival in cancer patients. So far, few studies have examined the expression of S100 protein family members in bladder tumors, and published reports are limited to only a few members of S100 family, specifically S100A4, A7, A8/A9 and A11. These studies have indicated that the afore-mentioned S100 family members are potential bladder cancers markers that may be useful in screening for bladder cancer, and for predicting tumor progression, prognosis, and even the effects of the therapy. Microarray analysis on experimental bladder cancers has revealed that, in addition to these S100 family members, other S100 family members including S100A1, S100A3, S100A5, S100A8/S100A9, S100A10 and S100G also differentially expressed in bladder cancers as compared to normal urothelium. The S100 protein family members exert many different effects through their interactions with cell surface receptors and intracellular molecules. These effects may contribute to their roles in bladder cancer progression and metastasis with associated poor patient survival. Identification and characterization of the molecular mechanisms by which S100 protein family members regulate their effectors will provide the biochemical foundation for understanding the biologic

roles of S100 proteins, and will provide important new insights into the molecular basis for the invasive behavior of tumor cells. This mechanistic information can be used to evaluate the utility of S100 proteins as diagnostic markers and as targets for novel therapies.

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