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Bladder cancer contributes significantly to the overall human cancer burden being the second most common urological tumors with a high recurrence rate and poor prognosis [Jemal et al., 2011]. Majority of the bladder cancers (~90%) are transitional cell carcinomas (TCC) and approximately 80% of these are nonmuscle invasive or superficially invasive tumors [Knowles, 2006]. Almost 20% of newly diagnosed TCC cases are more aggressive tumors with muscle invasion. Bladder tumor grades are associated with tumor stages and almost all superficially invasive tumors are of low grade, whereas nearly all muscle-invasive tumors are of high grade [Knowles, 2006]. The frequent recurrence of superficially invasive tumors and distant metastasis of muscle-invasive tumors are major problem for both patients and urologists. Currently, bladder cancer patients are monitored for cancer recurrence and progression by cystoscopy and urine cytology [Cheng et al., 2000]. Cystoscopy is the major tool for diagnosing bladder cancer, but it is associated with high cost and patient discomfort due to its invasive nature. In contrast, urine cytology has a poor sensitivity in low grade bladder tumors.

Development of TCC of the bladder is a highly complex process and involves various biological and functional molecules. A better understanding of the molecular mechanisms involved in bladder carcinogenesis and cancer progression would help to identify a number of molecular markers for bladder cancer having a potential diagnostic and prognostic value. An improved ability to accurately predict which patients will recur and ultimately progress to invasive and metastatic disease would greatly enhance the bladder cancer treatment. Thus, there is a great need to identify new tumor specific biomarkers for early diagnosis and prediction of bladder TCC for an appropriate cancer management of the patients. It is certain that there is a growing interest in identifying new diagnostic strategies and tools to complement standard histopathology to determine the presence of cancer cells in tissues [Bast et al., 2005]. In particular, an urgent requirement exists for biomarkers useful for early cancer detection, accurate pretreatment staging, prediction of response to treatment, and monitoring of disease progression [Basil et al., 2006].
5.1 Cancer-testis (CT) antigen

Cancer-testis (CT) antigens are protein antigens with normal expression restricted to adult testicular germ cells, and yet are aberrantly activated and expressed in various cancerous tissues [Suri, 2006]. Till today it is not known whether the expression of CT antigen is static or changes over the progression of the disease due to the genetic instability and heterogeneous protein expression in tumor cells. More than 100 CT antigen genes have been identified and reported in the literature [Caballero and Chen, 2009]. With the tumor-associated expression pattern, CT antigens have become a prime focus for early detection strategies against cancer in recent years. A subset of CT antigens has been shown to generate spontaneous humoral and cell-mediated immune responses in cancer patients, which increases the possibility that these antigens could be future cancer vaccine targets [Caballero and Chen, 2009]. This indicated that CT antigens are promising candidates for cancer immunotherapy and have become a major focus for the development of vaccine-based clinical trials in recent years. In addition, these antigens can also be used as biomarkers for early detection of cancers. Although a number of CT antigens have been discovered and has been suggested to be associated with the human malignancy, the complexity of carcinogenesis mechanism reflects on the need to establish compelling new criteria for validating their real applicability in cancer screening and therapeutics [Simpson et al., 2005].

5.2 Sperm associated antigen 9 (SPAG9), a novel CT antigen is associated with cancer

_Sperm associated antigen 9 (SPAG9)_ is a recently characterized gene from human testis—a new member of CT antigen family [Shankar et al., 1998]. SPAG9 belongs to JNK interacting protein family [Jagadish et al., 2005] involved in molecular interactions during sperm-egg fusion and MAPK signaling pathway [Jagadish et al., 2005]. _SPAG9_ is a single copy gene containing 19 exons, mapped on human chromosome 17q21.33, a region involved in gene amplification in various cancers. SPAG9 functions as a scaffolding protein for binding to JNKs that play an important regulatory role in several physiologic processes, including cell survival, proliferation, apoptosis and tumor
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development [Qi and Elion, 2005]. MAPKs interaction studies demonstrated that SPAG9 interacted with higher binding affinity to JNK3 and JNK2 compared with JNK1. However, the deleted mutant without JNK binding domain (JBD) failed to show any interaction with the MAPKs [Jagadish et al., 2005]. This interaction is interesting in view of the important regulatory role played by MAPKs in cell survival, apoptosis, tumor development, and embryonic morphogenesis [Kelkar et al., 2000]. Since the role of SPAG9 as scaffolding protein in MAPK module is well established [Jagadish et al., 2005] it may be possible for SPAG9 to facilitate the assembly and activation of MAPK signaling modules in tumorigenesis.

Recent studies demonstrated SPAG9 expression and its association with clinicopathological characteristics of tumors in epithelial ovarian cancer [Garg et al., 2007], renal cell carcinoma [Garg et al., 2008], breast cancer [Kanojia et al., 2009], cervical carcinoma [Garg et al., 2009a], thyroid cancer [Garg et al., 2009c] and colorectal carcinoma [Kanojia et al., 2011]. These findings suggest that SPAG9 may have a role in early spread of disease. Furthermore, SPAG9 expression was also found to be associated with circulating anti-SPAG9 antibodies in early stages and in low grade of breast cancer [Kanojia et al., 2009] and cervical cancer patients [Garg et al., 2009a] suggesting its potential use in early detection of disease. In addition, recently SPAG9 association was also found with tumor growth, migration and invasion in renal cell carcinoma [Garg et al., 2008], cervical carcinoma [Garg et al., 2009b] and colorectal carcinoma [Kanojia et al., 2011]. In this support, a study demonstrated that the JNK pathway plays a vital role in tumor-promoting functions in various types of cancer cells [Kim et al., 2005a]. Thus, overexpression of SPAG9 in cancerous tissue may alter the kinetics of MAPK signaling pathways, supporting cell proliferation, invasion, and migration.

5.3 SPAG9 gene expression in TCC tissue specimens

The restricted pattern of CT gene expression prompted a parallel search for additional CT antigen genes by mRNA expression profiling, namely by searching for genes that are expressed in testis and/or cancer, but not in other normal tissues. CT genes in TCC tissue specimens are among the least studied antigens. In the search for antigenic
targets for a bladder cancer vaccine, various tumor associated antigens have been studied but only limited data is available regarding the expression pattern of CT antigens in bladder cancer and their relationship with clinicopathologic characteristics.

In the present study, \textit{SPAG9} expression in the human bladder TCC tissue specimens was investigated. \textit{SPAG9} mRNA expression was detected in bladder TCC only but not in ANCT specimens. This predominant \textit{SPAG9} gene expression in TCC patients was found to be interesting when compared with other known potential CT antigens. Characterization of the expression of \textit{MAGE-A} gene family in bladder cancer has demonstrated that members of the \textit{MAGE-A} family are frequently expressed in bladder tumors at a level above the normal bladder tissue [Fradet et al., 2006]. Gene expression of members of \textit{MAGE-A} CT antigen family such as \textit{MAGE-A1} (56.9%) [Yin et al., 2011], \textit{MAGE-A3} (30%) [Picard et al., 2007], \textit{MAGE-A4} (33%) [Picard et al., 2007], \textit{MAGE-A8} (56%) [Picard et al., 2007], \textit{MAGE-A9} (54%) [Picard et al., 2007], \textit{MAGE-A10} (60%) [Schultz-Thater et al., 2010] and \textit{MAGE-A12} (51%) [Yin et al., 2011] have been reported in bladder tumor specimens. Another well-characterized CT antigen \textit{NY-ESO-1} mRNA expression was also studied in TCC specimens. A study has reported \textit{NY-ESO-1} gene expression in only 45% of the TCC specimens. However, other less studied CT antigens have also demonstrated their gene expression in TCC specimens such as 47% of \textit{LAGE-1} [Sharma et al., 2006], 52.9% of \textit{cTAGE-1} and 49% of \textit{c-TAGE-2} [Yin et al., 2011] mRNA expression. In contrast using RT-PCR, \textit{SPAG9} mRNA expression was observed in majority of TCC tissues (81%) analyzed irrespective of tumor stages and grades, but not in ANCT specimens. This is the most important criteria for identifying tumor-specific target antigen for development of cancer biomarker associated with the cancer progression. However, despite the majority of bladder TCC patients revealed \textit{SPAG9} expression, no significant association between \textit{SPAG9} gene expression and clinicopathological characteristics such as tumor stage and grade was observed. The predominant \textit{SPAG9} expression in patients with superficially invasive tumors (82%) ensures the significant role of \textit{SPAG9} and its association with the early spread and progression of bladder cancer. In contrast, the other CT antigens were reported to be less frequently expressed such as expression of \textit{NY-ESO-1} in 8%, \textit{LAGE-1} in 8% and \textit{BAGE} in 21% of the superficially invasive bladder tumors [Sharma et al., 2006]. Cancer relevant
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overexpression of a gene is an important feature and a prerequisite of a targeted gene for cancer biomarker. After establishing *SPAG9* mRNA expression by RT-PCR analysis, we also confirmed *SPAG9* gene expression at a single-cell level by *in situ* RNA hybridization in 81% of TCC specimens. This implies the potential clinical and therapeutic relevance of *SPAG9* in TCC.

5.4 *SPAG9* protein expression in TCC tissue specimens

The *SPAG9* gene expression was validated in terms of the protein expression by IHC to address its function and association with disease progression. Based on *SPAG9* specificity and frequency of expression in bladder tumor tissues and not in ANCT, *SPAG9* may be a potential candidate target for therapeutic use. In the present investigation, *SPAG9* protein expression was compared with the expression of the other well known CT antigens in the bladder tumors analyzed by IHC. The *SPAG9* expression was observed in a significant proportion of TCC patients when compared with other known CT antigens. Large number of TCC patients [81% (101/125)] showed *SPAG9* protein expression by IHC; however, no ANCT specimens had *SPAG9* protein expression demonstrating the specificity of *SPAG9* in tumor tissues. In contrast, protein expression of very few CT antigens has been reported in literature. A study has shown the MAGE-A9 and MAGE-A4 protein expression in 42% and 32% of the bladder tumor samples respectively [Picard et al., 2007]. Another well known CT antigen, NY-ESO-1 has shown cytoplasmic staining only in 30% of the bladder tumor cases [Sharma et al., 2006]. The protein expression of less common CT antigens has also been reported; GAGE, CT7-33 and CT10 demonstrated protein expression in 30%, 30% and 20% of the bladder cancer specimens [Fradet et al., 2006]. Bladder cancers of higher grade are likely to proceed towards the muscle invasion stage, which is directly related to mortality. In contrast, if tumor is confined to the lamina propria i.e., superficially invasive tumors, can be cured in the majority of the patients with localized therapies [Messing, 2002]. Our data showed that 82% of superficially invasive bladder TCC specimens had *SPAG9* protein, therefore valuable in the detection of this group of tumors indicating its potential in effective cancer treatment strategies.
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SPAG9 immunoreactive score (IRS) was assigned to define SPAG9 immunolocalization in carcinoma cells in TCC tissue. SPAG9 positive immunoreactivity was designated when >10% of the tumor cells revealed distinct SPAG9 protein expression. It was interesting to observe that SPAG9 expression was heterogeneous in terms of number of cells expressing SPAG9 protein in TCC tissue specimens. Based on SPAG9 IRS in bladder TCC specimens, we observed significant difference between the superficially invasive and muscle-invasive tumors; and low and high grade tumors indicating the significance and association of SPAG9 protein with the tumor stages and grades indicating its clinical utility in bladder cancer treatment management and strategies.

Our finding of SPAG9 protein expression in all of the SPAG9 mRNA-positive TCC specimens indicated that there was no discrepancy between gene and protein expression. We demonstrated SPAG9 protein expression, especially in nonmuscle invasive or superficially invasive stages of TCC which is an important step towards developing a tumor detection biomarker. High SPAG9 protein expression in early stages and low grade tumors indicate and support the potential role of SPAG9 in cellular proliferation and growth of tumor cells, which results in tumor spread and progression of disease.

5.5 Humoral immune response against SPAG9 in bladder TCC patients

CT antigens are predominantly expressed in normal gametogenic tissues as well as in different histological types of tumors and hence represent an ideal diagnostic and therapeutic candidate [Suri, 2006]. CT antigens are considered as tumour specific which could be targeted for immunotherapy and antigen-based target vaccines. The blood-testis barrier [Bart et al., 2002] prevents the immune system from contacting with CT antigen gene products. In addition to this, germ cells do not express HLA class I molecules [Fiszer and Kurpisz, 1998], so they cannot present their expressed proteins to the immune system. For these reasons, the immune system never comes into contact with these proteins and recognizes them as "non-self". CT antigens are known to generate spontaneous humoral and cellular immune responses in vivo [Old, 2001]; therefore tumor
antigen-associated antibody response could play an important role in the diagnosis and prognosis of the cancer. It is well known that the immune system of the cancer patient can be used to detect abnormalities in structure, function, intracellular location, and other alterations of cellular molecules involved in tumorigenesis [Tan, 2001]. This can be demonstrated by generation of either humoral or cell mediated immune responses and could be the earliest sign of tumorigenesis. The use of autoantibodies against specific tumor antigens for early cancer detection could be more useful and beneficial in cancer management, because the immune system can respond and amplify even a low-abundance antigen by generating a very specific and sensitive antibody response.

We analyzed sera of bladder TCC patients for humoral response against SPAG9. We demonstrated generation of humoral response against SPAG9 in a significant number of TCC patients consistent with the known immunogenicity of this antigen. In an analysis of 125 cases of bladder TCC, 95% bladder TCC patients with SPAG9 mRNA-positive tumors had generated humoral response against SPAG9: no patients with SPAG9 mRNA-negative tumors had SPAG9 antibody. However, the remaining 5% bladder TCC patients with SPAG9-expressing tumors did not have detectable SPAG9 antibodies. It is possible that the presence of SPAG9 antibodies is dictated by the genetic background of the individual. Physiologically, there may be patient's responders and patient's nonresponders. Within the responder population, the specific makeup of the cancer may be contributing to the SPAG9 antibodies. The generation of antibodies against SPAG9 may be regarded as a signal that indicates the presence of the tumor in the host. In terms of antibody frequency, SPAG9 appears to have high immunogenic potential in bladder cancer. This is an important finding where significant number of superficially invasive bladder cancer patients (78%) exhibited strong immune response against SPAG9 protein, supporting its potential role as a serum biomarker for early detection for better cure and surgical intervention.

5.6 Role of SPAG9 in bladder tumorigenesis

Our recent studies have focused on the expression and association of SPAG9 with various human malignancies, but the expression and role in bladder tumorigenesis was
not investigated. We examined the \textit{SPAG9} expression and showed that \textit{SPAG9} expression was observed in all of the bladder cancer cell lines of different histological types [Figure 4.8]. The \textit{SPAG9} expression was also established in the majority of patient's tumor specimens independent of stage and grade. This suggests that \textit{SPAG9} expression is an early event in the development of bladder cancer.

Identification of appropriate target antigens is the first and most crucial step in the successful development of antigen-specific immunotherapy. In this regard, the cell surface compartment is of substantial interest in identification of tumour specific proteins for developing therapeutic targets for the cancer treatment. Interestingly, our flow cytometric data analysis revealed that all bladder cancer cells showed cell surface localization of \textit{SPAG9}. Hence, these findings suggest that \textit{SPAG9} protein expression may have important implications in developing antibody-based therapy or drug-based therapy for better cancer management programmes.

Cancer is a disease of accumulation of clonal cells. Abnormal cell proliferation is necessary for tumorigenesis, which results in tumor cell growth and thus tumor burden. Indeed, the goal of most current cancer therapy is to reduce the number of tumor cells and to prevent their further accumulation. Therefore, we first investigated the effect of \textit{SPAG9} knockdown on cellular proliferation in high grade invasive bladder cancer cell UM-UC-3. To test whether \textit{SPAG9} positively regulates proliferation and survival of bladder cancer cells, we used the mammalian vector-based RNA interference technique to inhibit \textit{SPAG9} expression in UM-UC-3 cells. We demonstrated that ablation of \textit{SPAG9} protein expression was distinctly associated with cell growth inhibition in the cancer cells. In addition, \textit{SPAG9} shRNA transfected cells accumulated in G0-G1 phase compared with scrambled shRNA transfected cells [Figure 4.13]. This indicated that \textit{SPAG9} knockdown affects cell cycle and induced growth arrest in bladder cancer cells. These observations suggest that \textit{SPAG9} play a vital role in bladder cancer cell survival. We have shown here for the first time that \textit{SPAG9}, a novel member of the CT antigen family, plays important role in the survival of human bladder cancer cells \textit{in vitro}. 
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Migration and invasion of bladder cancer cells remains a major clinical problem due to the lack of effective specific therapies; therefore to investigate the cellular consequences of SPAG9 loss, we knocked down its expression in UM-UC-3 cells. We observed an association between SPAG9 expression and the acquisition of a migratory and invasive phenotype of bladder cancer cells. We demonstrated in in vitro assays that down-regulation of SPAG9 was able to inhibit cell migration and invasion of UM-UC-3 cells. The fact that shRNA against SPAG9 inhibit cancer cell proliferation or its role is implicated in cell survival in vitro; and preserved its strong antitumor effects which is indicated from the specific suppression of the cancer cell migration and invasion suggests that SPAG9 expression contributes significantly in enhancing the ability of bladder cancer cells to metastasize to other tissues or organs.

Finally, we propose that the SPAG9 expression, observed during tumor progression from a noninvasive to highly invasive stage, might be a crucial event participating in the acquisition of the invasive phenotype of bladder cancer. Therefore, the SPAG9 might be dispensable for the initiation of the tumor and also may be critical for the invasiveness during the dynamic progression of bladder cancer. Thus, SPAG9 could be a prospective biomarker and potential therapeutic target for future interventions to increase the life expectancy of the bladder cancer patients.