**Sperm associated antigen 9 (SPAG9) expression and humoral response in benign and malignant salivary gland tumors**

Sumit Agarwal¹, Deepak Parashar¹, Namita Gupta¹, Nirmala Jagadish¹, Alok Thakar², Vaishali Suri², Rajive Kumar³, Anju Gupta⁴, Abdul S Ansari⁵, Nirmal Kumar Lohiya⁵, and Anil Suri¹,*

¹Cancer Microarray; Genes and Proteins Laboratory; National Institute of Immunology; Aruna Asaf Ali Marg; New Delhi, India; ²Department of Otorhinolaryngology; All India Institute of Medical Sciences; New Delhi, India; ³Institute of Rotary Cancer Hospital; All India Institute of Medical Sciences; New Delhi, India; ⁴NMC Imaging and Diagnostic Centre; Vidyasagar Institute of Mental Health and Neuro-Sciences; New Delhi, India; ⁵Department of Zoology; Centre for Advanced Studies; University of Rajasthan; Jaipur, India

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Salivary gland cancers are highly aggressive epithelial tumor associated with metastatic potential and high mortality. The tumors are biologically diverse and are of various histotypes. Besides, the detection and diagnosis is a major problem of salivary gland cancer for available treatment modalities. In the present study, we have investigated the association of sperm associated antigen 9 (SPAG9) expression with salivary gland tumor (SGT). Clinical specimens of benign (n = 16) and malignant tumors (n = 86) were examined for the SPAG9 expression. In addition, the sera and adjacent non-cancerous tissues (n = 72) from available patients were obtained. Our *in situ* RNA hybridization and immunohistochemistry (IHC) analysis revealed significant difference (p = 0.0001) in SPAG9 gene and protein expression in benign (63%) and malignant tumor (84%) specimens. Further, significant association was also observed between SPAG9 expression and malignant tumors (P = 0.05). A cut-off value of >10% cells expressing SPAG9 protein designated as positive in IHC, predicted presence of malignant SGT with 83.72% sensitivity, 100% specificity, 100% PPV and 83.72% NPV. Humoral response against SPAG9 protein was generated in 68% of SGT patients. A cut-off value of 0.212 OD for anti-SPAG9 antibodies in ELISA predicted presence of malignant SGT with 69.23% sensitivity, 100% specificity, 100% PPV and 78.94% NPV. Collectively, our data suggests that the majority of SGT show significant difference and association among benign and malignant tumors for SPAG9 gene and protein expression and also exhibit humoral response against SPAG9 protein. Hence, SPAG9 may be developed as a biomarker for detection and diagnosis of salivary gland tumors.

**Introduction**

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries.¹,² In developing nations, it has been reported that 1 in 4 cancers in male occur in head and neck region and accounts for 30% of all cancers.³ Importantly, salivary gland cancer accounts for 3–5% of total head and neck cancer ⁴,⁵ and of wide histological and biological diversity.⁶ SGT could be benign and malignant type. The chances for malignant transformation of benign SGT vary from 1.9% to 23.3% which is a concern for oncologist all over the world. In addition, it has been well documented that pleomorphic adenoma, which is classified as benign tumor, is aneuploid, and invades normal adjacent tissue and metastasize at distant sites after certain period of time.⁷ While benign tumors are of pleomorphic and Warthin’s tumors, the malignant tumors are classified as mucoepidermoid, adenoid cystic, acinic cell, clear cell, basal cell carcinoma, adenocarcinoma not otherwise mentioned (NOS), squamous cell carcinoma, salivary duct carcinoma, myoepithelial carcinoma and polymorphous low grade adenocarcinoma based on their origin.⁸–¹⁰ Such morphological and biological diversity of SGT, makes it more challenging to diagnose and classify these tumors.⁵ Nevertheless, about 70% of all the SGT are of parotid gland origin followed by submandibular and sublingual glands.¹⁰ Besides, the heterogeneity within the tumor of the same patient does pose problem for pathologist to classify the origin of such tumors.¹¹ Prognosis of SGT varies according to histologic type and stage. A combination of radiation therapy and surgery is usually applied to treat malignant tumors. However, such treatments are often associated with disfigurement and loss of glandular function with undesirable side effects.¹¹ Hence, there is a need for a novel biomarker for the early detection and diagnosis of SGT, for better cancer management and treatment modalities.

Recently, a unique group of protein family designated as cancer testis (CT) antigens has been reported in various malignancies.¹ The restricted or no expression of CT antigens in normal tissues and their antigenic properties in cancer patients makes
these molecules an ideal choice as biomarker for early detection and diagnosis and therapeutic vaccines. Although, a few CT antigens like B antigen (BAGE), G antigen-1/2 (GAGE-1/2), helicase antigen (HAGE) and melanoma associated antigen-1 (MAGE-1) have been reported to be expressed in various types of SGT, none of the molecules have been found suitable in clinical management of such cases. Recently, we and others have demonstrated expression and association of SPAG9, a new member of CT antigen family, with various clinicopathological characteristics of tumors, especially in early stages in epithelial ovarian cancer, renal cell carcinoma, thyroid cancer, cervical carcinoma, breast cancer, colorectal carcinoma, bladder cancer, endometrial cancer, non-small cell lung cancer and astrocytoma. Besides, a strong humoral response against SPAG9 has also been demonstrated in various malignancies suggesting its potential usage as a serum based cancer biomarker.

Early detection of SGT would be essential for more effective clinical management leading to improved quality of life and increased survival rate. The present study was initiated to undertake a more comprehensive analysis of SPAG9 expression in SGT specimens in the context of clinic-pathological parameters, i.e., histo-pathological characteristics. We also investigated the humoral response against SPAG9 in various stages and histotypes of SGT patients. Our results suggest that SPAG9 may be used as a novel diagnostic biomarker for early detection of SGT, thus, may be useful in better management of SGT patients.

**Results**

**SPAG9 gene expression in SGT patients**

The SPAG9 gene expression was investigated by RT-PCR in SGT tissue along with available matched ANCT specimens (Fig. 1). The data revealed that 80% (82 of 102) of tumor specimens showed SPAG9 gene expression irrespective of benign, malignant tumor, stages, and various histotypes (Table 1). No mRNA was detected in matched ANCT specimens. The human testis cDNA was used as a positive control for SPAG9 gene expression. Our SPAG9 gene expression analysis (Table 1) revealed that SPAG9 transcript was detected in 63% (10 of 16) of benign tumors, 93% (13 of 14) of malignant stage I, 88% (15 of 17) of stage II, 75% (24 of 32) of stage III and 87% (20 of 23) of stage IV. Based on TNM classification, SPAG9 expression was detected in 81% (25 of 31) of SGT specimens positive with lymph node involvement as compared to 85% (47 of 55) of specimens negative with lymph node involvement. Furthermore, based on histological disease classification, 63% (10 of 16) of pleomorphic benign tumors, 90% (27 of 30) of mucoepidermoid, 83% (10 of 12) of adenoid cystic, 80% (4 of 5) of acinic cell, 88% (14 of 16) of clear cell, 80% (4 of 5) of basal cell, 70% (7 of 10) of adenocarcinoma not otherwise specified (NOS) and 75% (6 of 8) of polymorphic low grade adenocarcinoma specimens showed SPAG9 mRNA expression as depicted in Figure 1 and Table 1.

**Validation of SPAG9 gene and protein expression in SGT patients**

SPAG9 gene expression was also determined in serial SGT tumor specimen sections by *in situ* RNA hybridization studies using *in vitro* synthesized riboprobes and by IHC. Our *in situ* RNA hybridization studies employing antisense riboprobes confirmed SPAG9 gene expression in 93% (13 of 14) of malignant stage I, 88% (15 of 17) of stage II, 75% (24 of 32) of stage III and 87% (20 of 23) of stage IV tumors (Fig. 2). Based on TNM classification, SPAG9 expression was detected in 81% (25 of 31) of specimens positive with lymph node involvement as compared to 85% (47 of 55) of specimens negative with lymph node involvement (Table 1). However, as expected sense riboprobes failed to show SPAG9 gene expression in any of the serial tissue specimen sections as depicted in Figure 2.

SPAG9 protein expression was further confirmed by IHC in serial SGT tissue sections of benign tumors, various stages of malignant tumors and different histotypes and available matched ANCT specimens. SPAG9 protein expression was found in 80% (>10% of cells found positive for SPAG9 protein expression) of SGT specimens, whereas no expression was detected in 72 paired available matched ANCT specimens as shown in Figure 3. SGT specimens were also probed with control IgG which failed to show any immunoreactivity against SPAG9 protein (Fig. 3). As depicted in Table 1, SPAG9 protein expression was found in 63% (10 of 16) of benign tumors, 93% (13 of 14) of malignant stage I, 88% (15 of 17) of stage II, 75% (24 of 32) of stage III and 87% (20 of 23) of stage IV tumors. In addition, 81% (25 of 31) of specimens found positive for lymph node involvement showed SPAG9 protein expression as compared to 85% (47 of 55) of
Table 1. SPAG9 expression, humoral response and clinicopathological characteristics of salivary gland tumor

| Clinicopathological features | IHC (%) of tissue | RT-PCR (%) of tissue | ELISA (%) of serum |
|------------------------------|-------------------|----------------------|-------------------|
| Total parotid gland tumor specimens | 82/102 (80) | 82/102 (80) | 42/62 (68) |
| Adjacent normal cancerous tissues | 0/72 (0%) | 0/72 (0%) | — |
| Benign tumors: Pleomorphic adenoma | 10/16 (63) | 10/16 (63) | 6/10 (60) |
| Total malignant tumors | 72/86 (84) | 72/86 (84) | 36/52 (69) |
| Malignant tumor stages | — | — | — |
| Malignant stage I & II | 13/14 (93) | 13/14 (93) | 9/10 (90) |
| Malignant stage III & IV | 20/23 (87) | 20/23 (87) | 8/9 (89) |
| Malignant stage I & II | 24/32 (75) | 24/32 (75) | 11/23 (48) |
| Malignant stage III & IV | 20/23 (87) | 20/23 (87) | 8/9 (89) |

| SPAG9 expression, humoral response and clinicopathological characteristics of salivary gland tumor |
|---------------------------------------------------------------|
| Statistical analysis (p values of different test used in this study) |
| Clinicopathological features | Pearson’s χ² test | Mann–Whitney U-test | Kruskal–Wallis test |
|------------------------------|-------------------|----------------------|-------------------|
| | RT-PCR/IHC | ELISA | RT-PCR/IHC | ELISA | RT-PCR/IHC | ELISA |
| Tumor stage I and II | 0.665* | 0.531* | 0.548* | 0.413* | — | — |
| Tumor stage II and III | 0.274* | 0.086* | 0.165* | 0.509* | — | — |
| Tumor stage III and IV | 0.274* | 0.033* | 0.094* | 0.107* | — | — |
| Benign and malignant | < 0.050 | 0.302* | < 0.0001 | < 0.0001 | — | — |
| Lymph node involvement in malignant tumor | 0.562* | 0.598* | 0.878* | 0.858* | — | — |
| Tumor stage I, II, III and IV | — | — | — | — | 0.183* | 0.505* |
| Histotypes MEC, AdCC, ACC, CCC, BCAC, ANOS and PLGA | — | — | — | — | 0.977* | 0.798* |

* Statistically non-significant

Statistical analysis (p values of different test used in this study)

Specimens negative for lymph node involvement. Based on SPAG9 IRS, we observed significant difference between benign [IRS = 38.40 ± 5.36] and malignant tissue [IRS = 69.30 ± 1.98; (p = 0.0001)] by Mann–Whitney U-test (Fig. 5). In addition, significant association of SPAG9 protein expression was found between benign and malignant tumor (p = 0.05) using Pearson’s χ² test. However, while comparing the SPAG9 protein expression between malignant stage I (IRS = 68.69 ± 4.38) & II IRS = 71.00 ± 5.31; (p = 0.548), stage II (IRS = 71.00 ± 5.31) & III IRS = 63.20 ± 3.74; (p = 0.165) and stage III (IRS = 63.20 ± 3.74) & IV IRS = 75.70 ± 2.17; (p = 0.094), no significant difference was observed using Mann–Whitney U-test (Fig. 5). We further analyzed SPAG9 expression among different stages and various malignant histotypes using Kruskal–Wallis test. We did not find any significant difference among different malignant stages (p = 0.183) or in various histotypes (p = 0.977). In addition, no association was found between SPAG9 protein expression among malignant stage I & II (p = 0.665), stage II & III (p = 0.274) and stage III & IV (p = 0.274) by using Pearson’s χ² test. Furthermore, there was no significant difference (p = 0.878) between lymph node positive and negative salivary gland cancer patients as determined by Mann–Whitney U-test. Also, no significant association (p = 0.562) was found between SPAG9 protein expression and lymph node involvement in SGT specimens by Pearson’s χ² test. Thus, the above studies distinctly revealed no discrepancy between SPAG9 gene and protein expression. All SGT specimens found positive in in situ RNA hybridization and RT-PCR experiments were also found positive for SPAG9 protein expression in IHC analysis. However, no significant association was found in SPAG9 protein expression among various malignant stages.

Various histotypes of SGT specimens showed SPAG9 protein expression in 63% [(10 of 16); IRS = 38.40 ± 5.36] of pleomorphic benign tumors, 90% [(27 of 30); IRS = 69.19 ± 3.19] of mucoepidermoid, 83% [(10 of 12); IRS = 67.30 ± 6.30] of adenoid cystic, 80% [(4 of 5); IRS = 70 ± 3.58] of acinic cell, 88% [(14 of 16); IRS = 70.92 ± 4.86] of clear cell, 80% [(4 of 5); IRS = 73.50 ± 11.30] of basal cell, 70% [(7 of 10); IRS = 68.80 ± 7.27] of adenocarcinoma NOS and 75% [(6 of 8); IRS = 67.60 ± 6.07] of polymorphous low grade adenocarcinoma as given in Table 1 and illustrated in Figures 4 and 5. However, control IgG showed no SPAG9 immuno-staining in
Figure 2. Analysis of SPAG9 gene expression in SGT patients by *in situ* RNA hybridization. Representative images of H&E staining for benign, malignant stage I, II, III, and IV tumors are shown in left panel. The serial tissue sections probed with anti-sense riboprobes resulted in violet blue color as shown in the middle panel, whereas no hybridization was observed when probed with sense riboprobes as shown in the right panel. Original magnification: x200; objective: x20.
any of the SGT histotypes under investigation. Thus, our data showed no discrepancy within the results obtained from RT-PCR and IHC studies as detailed in Table 1.

Statistical analysis for sensitivity and specificity of SPAG9 expression was evaluated at cut-off value of > 10% SPAG9 positive cells per 500 in 5 random fields in IHC of serial SGT tissue sections of malignant tumors, stage I (T1) and benign tumors. SPAG9 positive cells predicted presence of malignant SGT with 83.72% sensitivity, 100% specificity, 100% PPV and 83.72% NPV. In stage I (T1) tumors, SPAG9 positive cells predicted presence of malignant SGT with 92.85% sensitivity, 100% specificity, 100% PPV and 98.63% NPV, whereas in benign tumors, SPAG9 expressing cells predicted SGT with sensitivity, specificity, PPV and NPV of 62.5%, 100%, 100%, and 92.3%. These observations may suggest that SPAG9 expression may predict malignant SGT.

Humoral response against SPAG9 protein in SGT patients

The circulating anti-SPAG9 antibodies were investigated in sera of 62 available SGT patients employing ELISA. Initially, we established a cut-off value of 0.212 [Mean + 2 SD; 0.137 + 0.074] optical density (OD) for circulating anti-SPAG9 antibodies using sera from 60 healthy donors. A higher value obtained from the patient serum above cut-off value was designated as positive for anti-SPAG9 antibodies whereas those below it as negative. Our data revealed that 68% (42 of 62) of SGT patients...
Figure 4. Validation of SPAG9 protein expression in various histotypes of malignant SGT by immunohistochemistry. (A) Top panel showing the cytosturc- 
ture of representative specimens of benign pleomorphic adenoma and various malignant histotypes such as mucoepidermoid carcinoma, adenoid cystic 
carcinoma and acinic cell carcinoma of SGT stained with H&E. Middle panel shows cytoplasmic localization of SPAG9 protein expression in the represen-
tative specimens of various histotypes of SGT probed with anti-SPAG9 antibody. Bottom panel depicts no immunoreactivity for SPAG9 protein in various 
histotypes of SGT specimens when probed with control IgG. (B) Top panel showing the cytostucture of representative specimens of various malignant 
histotypes such as clear cell carcinoma, basal cell carcinoma, adenocarcinoma not otherwise specified and polymorphous low grade adenocarcinoma of 
SGT stained with H&E. Middle panel shows cytoplasmic localization of SPAG9 protein expression in the representative specimens probed with anti-
SPAG9 antibody. Bottom panel depicts no immunoreactivity for SPAG9 protein when probed with control IgG. Original magnification: x400; objective: 
x40.
generated humoral response against SPAG9 protein. Interestingly, we found that these patients with anti-SPAG9 antibodies were also found to be positive for SPAG9 gene and protein expression. As shown in Table 1 and depicted in Figure 6A, our data revealed that humoral response generated against SPAG9 was found in 60% (6 of 10) of benign, 90% (9 of 10) of malignant stage I, 80% (8 of 10) of stage II, 48% (11 of 23) of stage III and 89% (8 of 9) of stage IV tumors. It is important to note that 74% (14 of 19) of malignant SGT patients with positive lymph node involvement generated anti-SPAG9 antibodies as compared to 67% (22 of 33) of SGT patients with negative lymph node involvement. In addition, the patient’s sera of different histotypes also revealed anti-SPAG9 antibodies which demonstrated 60% (6 of 10) of pleomorphic benign tumor patients, 72% (13 of 18) of mucoepidermoid, 63% (5 of 8) of adenoid cystic, 67% (2 of 3) of acinic cell, 71% (5 of 7) of clear cell, 80% (4 of 5) of basal cell, 63% (5 of 8) of adenocarcinoma NOS and 67% (2 of 3) of polymorphous low grade adenocarcinoma patients (Fig. 6A). We observed a significant difference (p=0.0001) among benign and malignant tumor patient sera found positive for circulating anti-SPAG9 antibodies using Mann–Whitney U-test. However, no significant association was found between benign and malignant tumors (p = 0.302) by Pearson’s χ² test. Similarly, no significant difference was found between stage I & II (p = 0.413), stage II & III (p=0.509), stage III & IV (p = 0.107) and between lymph node involved (p=0.858) malignant tumors as assessed by Mann–Whitney U-test. However, a significant association was found between stage III & IV samples (p = 0.033) as determined by Pearson’s χ² test. Kruskal–Wallis test revealed no significant difference in circulating anti-SPAG9 antibodies among various malignant stages (p = 0.505) of SGT and different malignant histotypes (p = 0.798).

Statistical analysis for sensitivity and specificity of circulating anti-SPAG9 antibody was performed in sera of benign tumor, stage I (T1) tumor and malignant SGT. It is important to mention, that a cut-off value of 0.212 OD for anti-SPAG9 antibodies predicted presence of malignant SGT with 69.23% sensitivity, 100% specificity, 100% PPV, and 78.94% NPV. In stage I (T1) tumors, SPAG9 antibodies predicted presence of malignant SGT with 90% sensitivity, 100% specificity, 100% PPV, and 98.36% NPV. In benign tumors, presence of SPAG9 antibody predicted SGT with 60% sensitivity, 100% specificity, 100% PPV, and 93.75% NPV. Further the presence of anti-SPAG9 antibodies in the sera of SGT patients was also confirmed by Western blot analysis as shown in Figure 6B. Immuno-reactivity against SPAG9 protein was detected in the sera of benign and malignant SGT of tumor stages and histotypes as compared to sera from 60 normal healthy donors which showed no immune-reactivity. Furthermore, the specificity of immuno-reactivity of patient’s sera against recombinant SPAG9 protein was confirmed in neutralization assays which showed complete loss of immuno-reactivity with SPAG9 protein (Fig. 6B). Likewise pre-incubation of anti-SPAG9 antibody with SPAG9 recombinant protein (15μg/mL) led to complete loss of immune-reactivity as depicted in Figure 6C.

Discussion

Salivary gland cancers are aggressive malignant tumors with higher metastatic rates leading to cancer related-deaths. So far, little is known about the pathogenesis of salivary gland cancer. Besides, their morphological and biological diversity also poses problem for diagnosis and treatment of SGT. Several biomarkers such as HER-2, mutated p53, ras-p21, cyclinD1, C-kit, vascular endothelial growth factor (VEGF), sphingosine kinase-1 (SPHK-1) and astrocyte elevated gene-1 (AEG-1) have been shown to be associated with SGT samples. However, none of these biomarkers have been included in clinical management and thus,
have little or no clinical relevance. Therefore, there is a pressing need to identify new biomarkers for early detection and diagnosis for better management of SGT patients. In this context, the unique family of proteins designated as CT antigens have been considered to be of clinical relevance due to their expression in various types of malignancies and high immunogenicity in cancer patients. Moreover, it has been well documented that CT antigens have restricted or no expression in somatic tissues except in testis during gametogenesis. Hence, CT antigens represent a novel target for developing diagnostic and therapeutic candidate molecules. Therefore, in the present study, we analyzed a CT antigen, SPAG9 expression and humoral response in various stages and histotypes of SGT patients.

To best of our knowledge, our investigation is the first study reporting SPAG9 gene and protein expression in SGT patients when compared to other CT antigens reported earlier. Based on SPAG9 IRSs, we observed significant difference among ANCT, benign and malignant SGT specimens and a significant association of SPAG9 protein expression between benign and malignant tumor specimens. Our data revealed that majority of malignant tumor patients showed SPAG9 expression as compared to benign tumor patients indicating a possible role of SPAG9 in SGT carcinogenesis. Recently, AEG-1 has been reported to be upregulated in salivary gland cancer as compared to ANCT specimens and was found to be significantly associated with advanced stage tumors and TNM classification. Earlier reports on HER-2 and VEGF also revealed up-regulation in malignant SGT which closely matched with clinical stages and lymph node metastasis. A similar result from data among ANCT, benign and malignant SGTs, suggest that high SPAG9 expression may contribute to malignant SGT development and needs further investigation.

![Figure 6. Humoral response against SPAG9 protein in SGT patients.](image)

(A) ELISA-based analysis of sera from available 62 SGT patients of benign, different stages (I, II, III and IV) and various histotypes of malignant tumor and from 60 normal healthy donors was carried out to determine the presence of anti-SPAG9 antibodies. The horizontal line X indicates the cutoff value 0.212 (0.137 ± 0.074) of 60 normal healthy donors at A492nm. SGT patients were designated as positive for the presence of anti-SPAG9 antibodies above X line and negative below the X line. (B) Western blotting analyses. Lane 1, affinity-purified recombinant SPAG9 stained with Coomassie brilliant blue; lane 2, immuno-blots of recombinant SPAG9 shows a specific band of 170 kDa with anti-SPAG9 antibody (positive control). Serum from patient having benign [PA, (lane 3 and lane 8)], malignant tumor stage I (lane 4), stage II (lane 5), stage III (lane 6), stage IV (lane 7), MEC (lane 9), AdCC (lane 10), ACC (lane 11), CC (lane 12), BCAC (lane 13), ANOS (lane 14), PLGA (lane 15) showed specific immunoreactivity against recombinant SPAG9 protein. Neutralization experiments were done by pre-incubating recombinant SPAG9 protein (15μg/mL) with sera from SGT patients which resulted in complete loss of immunoreactivity (lane 16). Sera from healthy individuals revealed no reactivity (lane 17). M-molecular weight marker. (C) Neutralization studies in serial tissue sections of benign and malignant stages of SGT. Neutralization studies were carried out by pre-incubating anti-SPAG9 antibody generated in rat with recombinant SPAG9 protein (15μg/mL) and used for probing endogenous SPAG9 protein in the serial tissue sections of SGT patients. Left panel shows the representative images of the H&E stained sections of malignant tumor stage I, II, III, IV. Middle panel depicts the SPAG9 protein expression probed with anti-SPAG9 antibodies. Right panel shows complete loss of SPAG9 localization when probed with neutralized sera.
**SPAG9** gene expression was observed in 63% benign tumor and 84% of malignant tumors which included various types of histotypes (Table 1). In contrast, earlier study on a CT antigen, HAGE mRNA was shown to be expressed only in 39% benign tumors (11 of 28) and in 50% malignant tumors (8 of 16). It was also demonstrated that BAGE, GAGE-1/2, and MAGE-1 mRNA were detected only in 13% (2 of 16), 25% (4 of 16) and 13% (2 of 16) of tumor specimens respectively.\(^1\) Interestingly, SPAG9 protein expression was observed in all the *SPAG9* mRNA positive tumor specimens irrespective of stages and histotypes of SGT. Recently, a study has shown an expression of MCM3, a proliferation marker in SGT by immunohistochemical analysis with 74.3% sensitivity and 93.3% specificity between malignant and benign SGT.\(^2\) A recent study on the expression of *SPAG9* has predicted presence of malignant endometrium with 74% sensitivity, 83% specificity, 88% PPV, and 64.5% NPV.\(^3\) In our study, SPAG9 protein expression in IHC predicted presence of malignant SGT with 83.72% sensitivity, 100% specificity, 100% PPV, and 83.72% NPV. Further, in a recent study, *SPAG9* overexpression was correlated with tumor stages in human hepatocellular carcinoma tissues ($p < 0.001$).\(^4\) However, we did not find any correlation between the different tumor stages and *SPAG9* expression ($p = 0.183$). To our best of knowledge, none of the earlier reports on CT antigens have validated their protein expression in SGT specimens. Thus, the present study has laid the foundation where *SPAG9* expression was shown to be associated with malignant tumors which may be used as a potential biomarker in diagnosis and therapy.

Earlier studies have reported that CT antigens are highly immunogenic and elicit immune response in cancer patients.\(^5\) Recently, we reported humoral response against SPAG9 protein in 67% of epithelial ovarian cancer,\(^6\) 77% of renal cell carcinoma,\(^7\) 78% of thyroid cancer,\(^8\) 80% of cervical cancer,\(^9\) 80% of breast cancer,\(^10\) 70% of colorectal cancer\(^11\) and 77% of bladder cancer patients.\(^12\) Further, a recent study on endometrial cancer also demonstrated a strong humoral response against SPAG9 protein in 72% of the cancer patients which supports the present findings.\(^13\) More recently, we have reported a humoral response against yet another CT antigen, A-kinase anchor protein 4 (AKAP4) in 65% of ovarian cancer,\(^14\) 94% breast cancer\(^15\) and 86% cervical cancer\(^16\) patients. Till now, no other CT antigen has been reported to show humoral response in SGT patients. CT antigens such as HAGE, BAGE, GAGE-1/2 and MAGE-1 have only been validated for mRNA expression\(^1\) and not for the protein expression. In this context, our investigation is a first report showing that *SPAG9* is highly immunogenic and elicits humoral response against SPAG9 protein in 68% of SGT patients found positive for *SPAG9* mRNA and protein expression. We have put forth evidence that majority of SGT patients revealed circulating antibodies against *SPAG9*. Statistical analysis revealed that circulating anti-SPAG9 antibodies predicted presence of malignant SGT with 69.23% sensitivity, 100% specificity, 100% PPV and 78.94% NPV. Whereas, in sera from benign SGT, SPAG9 antibodies predicted presence of SGT with 60% sensitivity, 100% specificity, 100% PPV and 93.75% NPV. Thus, our data on humoral response in SGT patients suggest that *SPAG9* could be a promising biomarker candidate for early detection and diagnosis of SGT and warrants further investigation at large scale.

The aberrant CT antigen expression is apparently associated with deregulated cellular growth and migration.\(^5\) Our earlier studies on MAPK (mitogen-activated protein kinase) interaction suggested that SPAG9 acts as a scaffolding protein which interacts with JNK (c-Jun N-terminal kinase) signaling module especially with JNK2 and JNK3.\(^17\) Since, JNK signaling interactions play a crucial role in cellular proliferation, differentiation, apoptosis, cellular transformation and tumor cell growth,\(^3\) *SPAG9* over expression in SGT could alter the efficiency and specificity of MAPK signaling leading to neoplastic growth of SGT. However, the underlying mechanisms associated with *SPAG9* expression during cancer are still unclear. In our recent attempt to understand the potential role of *SPAG9* in cancer progression, plasmid driven gene silencing approach was employed, which demonstrated that ablation of SPAG9 protein in bladder cancer cells resulted in cell cycle arrest in G0-G1 phase.\(^2\) The G1 arrest was characterized by upregulation of p16 and p21 and downregulation of cyclins and cyclin-dependent kinase\(^2\) indicating a potential role of *SPAG9* in cancer growth and cellular proliferation.

Collectively, our data demonstrated that majority of SGT patients exhibit *SPAG9* expression and elicit humoral response against SPAG9 protein in the sera irrespective of the stages and the histotypes of SGT. *SPAG9* expression was associated with malignant state of SGT patients and exhibited significantly higher antibody response in malignant tumor patients as compared to benign tumor patients. Here we are reporting the possibility of developing serum based biomarker for better cancer management of SGT patients. Further studies are warranted to investigate the association of *SPAG9* in large number of SGT patients.

**Materials and Methods**

**Patient specimens**

The present investigation was carried out in tissue specimens and sera sample of SGT patients who underwent surgical resection at All India Institute of Medical Sciences, New Delhi. Based on histopathological reports, the tissue specimens included 16 benign and 86 malignant specimens. Benign tumors included 16 pleomorphic adenoma specimens. Malignant tumor specimens included 30 mucoepidermoid, 12 adenoid cystic, 5 acinic cell, 16 clear cell, 5 basal cell carcinoma, 10 adenocarcinoma NOS, 8 polymorphous low grade adenocarcinoma and 72 matched available adjacent non-cancerous tissues (ANCT). Based on stages of malignant SGT, specimens included 14 stage I (T1), 17 stage T II, 32 stage T III and 23 stage T IV. Tumor specimens were collected during routine surgery in 5% buffered formaldehyde prepared in diethylpyrocarbonate (DEPC) water and processed for **IHC** and **in situ** RNA hybridization studies. In addition, a portion of tumor tissue specimens were also collected in **RNAlater** (Sigma-Aldrich, St. Louis, MO) and stored at –80°C until use.
for SPAG9 gene expression analysis. Peripheral blood from 62 available patients and 60 normal healthy subjects were also obtained. All the sera samples thus obtained from SGT and normal healthy donors were stored at −80°C until further investigations. All investigations were carried out after obtaining ethical clearances from Institute ethics committee (IEC), All India Institute of Medical Sciences, and Institutional human ethics committee (IHEC), National Institute of Immunology, New Delhi. Duly signed consent forms were also obtained from all the patients under investigation. Histological characteristics of the tissue samples were reviewed and confirmed by two independent pathologists by microscopic observation of hematoxylin and eosin (H&E) stained tissue sections.

Analysis of SPAG9 gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR)

SPAG9 gene expression was examined by extracting total RNA from the frozen SGT tissues employing RNaseasy mini kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer’s instructions. Subsequently, cDNA was synthesized using 500 ng of total RNA and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). SPAG9 gene specific primers Forward 5'-GAATTCGATCAGGAACTTAAGGA-3', Reverse 5'-GTTACCCCTGTTTTCTCGTGCCACCTGGACACCTTGCAA-3' were used to carry out RT-PCR using 50 ng cDNA as a template as described earlier.15 β-actin specific primers (forward: 5'-ATCTGGCACCA-CACCTTCTACACATTGAAGCTGCG-3' and reverse: 5'-CGTCACTCTCCTGCTTGCTATCACACTCTGC-3') were used as an internal control. The PCR amplicon was analyzed on 1% agarose gel and was sub-cloned into TOPO vector (Invitrogen, Life Technologies, Carlsbad, CA) for further studies.

In situ RNA hybridization

Serial SGT tissue sections were subjected to in situ RNA hybridization experiments to examine the expression of SPAG9 transcripts as described earlier.15 Antisense (complementary to endogenous mRNA) as well as sense (same as endogenous mRNA) riboprobes were synthesized in vitro using T7 or T3 RNA polymerases respectively and DIG RNA Labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) respectively. The riboprobes were used to hybridize SPAG9 transcripts in serial SGT tissue sections and visualized using nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate [(NBT/BCIP) as substrate, Roche Diagnostics GmbH, Mannheim, Germany] and images were captured by using Nikon Eclipse E400 microscope (Nikon, Fukok, Japan).

Immunohistochemistry (IHC)

The SPAG9 protein expression in SGT serial tissue sections was validated by IHC as described earlier.15 Briefly, the SGT tissue sections (4 μm) were deparaffinized and subsequently rehydrated using different gradients of alcohol and probed with anti-SPAG9 antibody or control IgG by incubating at 4°C for overnight in humid chamber. After washing thrice with phosphate buffer saline (PBS)-0.05% Tween20, the tissue sections were incubated with horseradish peroxidase-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized using chromogen, 0.05% 3,3'-diaminobenzidine [(DAB), Sigma-Aldrich, St. Louis, MO] counterstained with hematoxylin stain and mounted with 1,3-diyethyl-8-phenylxan-thine [(DPX), Sigma-Aldrich, St. Louis, MO]. The images of tissue sections were captured using Nikon Eclipse E 400 microscope (Nikon, Fukok, Japan).

SPAG9 Immuno-reactive Score (IRS)

The immune-stained SGT tissue sections were examined by two senior pathologists by counting five random fields (> 500 cells) under x400 magnification as described earlier.19 SGT tissue sections distinctly showing SPAG9 protein expression in > 10% of cells were considered as positive.

Detection of circulating anti-SPAG9 antibodies in SGT patients

The circulating anti-SPAG9 antibodies were detected in the sera of the SGT patients employing ELISA and by Western blotting as described earlier.15 In order to check the specificity of the circulating anti-SPAG9 antibodies, neutralization experiments were performed as described earlier.19 Briefly, sera of the SGT patients were pre-incubated with SPAG9 recombinant protein and subsequently, were used for probing recombinant SPAG9 by Western blotting as described earlier.19

Statistical analysis

Statistical data analyses were performed using SPSS 20.0 software package (SPSS Inc, Chicago, IL, USA). Mann–Whitney U-test was done to determine the statistical difference of SPAG9 gene and protein expression within, benign and malignant and among various tumor stages. The statistical significance between the humoral response in benign, malignant SGT and healthy donors was assessed by unpaired Mann–Whitney U-test. In addition, Mann–Whitney U-test was also performed to find the difference between lymph node positive and negative salivary gland cancer patients. The Pearson’s χ² test was used to examine the association of SPAG9 expression and humoral response among benign and malignant stages. The Kruskal–Wallis test was used to analyze the difference in SPAG9 expression and humoral response among different SGT malignant stages (I, II, III, IV) and various tumor histotypes [Mucopidermoid carcinoma (MEC), Adenoid cystic carcinoma (AdCC), Acinic cell carcinoma (ACC), Clear cell carcinoma (CCC), Basal cell adenoacarcinoma (BCAC), Adenocarcinoma not otherwise specified (ANOS), Polymorphous low grade adenocarcinoma (PLGA)]. A p value of less than 0.05 was considered statistically significant. The diagnostic accuracy of the tests was evaluated by using Bayesian statistics such as sensitivity [TP/(TP+FN)], specificity [TN/(TN+FP)], Positive Predictive Value [TP/(TP+FP)] and Negative Predictive Value [TN/(TN+FN)] where abbreviations stand for, True Positive (TP), True Negative (TN), False Negative (FN) and False Positive (FP) cases for SPAG9 protein expression and anti-SPAG9 antibodies in control and tumor patients.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
SA, DP and NG carried out all the experiments, prepared figures and drafted the manuscript. NJ, ASA, NKL, AG and VS participated in data analysis and interpretation of results. AG and VS pathologists performed histopathology examination of all the clinical specimens used in this investigation. AT and RK senior surgeons provided clinical samples and clinicopathological data from the hospital for this study. As designed the study, participated in data analysis and interpretation of results. All authors read and approved the manuscript. We acknowledge Dr V. Kumar, Senior Staff Scientist, International Center for Genetic Engineering and Biotechnology, New Delhi, India for critical reading and editing of this manuscript.

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