

# Sperm-Associated Antigen 9, a Novel Biomarker for Early Detection of Breast Cancer

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## Abstract

To date, there have been no tumor biomarkers validated and incorporated into oncologic practice for the early diagnosis of breast cancer. Recently, we showed that sperm-associated antigen 9 (SPAG9), a member of cancer testis (CT) antigen family, is associated with ovarian carcinomas. In the present study, we investigated SPAG9 expression and humoral immune response in breast cancer. We further evaluated the diagnostic potential of autoantibodies to SPAG9 protein in various stages, grades, and histotypes of breast cancer. We analyzed the association of SPAG9 immunoreactivity score (IRS) with predicted risk of breast cancer recurrence over 10 years. Our reverse transcription-PCR and immunohistochemical analyses revealed SPAG9 expression in 88% breast cancer specimens independent of tumor stages and grades. Further, the humoral immune response against SPAG9 was detected in 80% breast cancer patients

with SPAG9-expressing tumors. The linear regression modeling predicted a direct relationship between presence of lymphovascular invasion and high SPAG9 IRS, whereas the univariate and multivariate logistic regression models predicted a strong association of SPAG9 IRS with tumor grade. Further, our data indicated a significant higher trend of SPAG9 IRS with the predicted high risk of breast cancer recurrence. The present investigation reports for the first time SPAG9 expression and humoral immune response in early stages and low-grade breast cancer. Although our data indicated that autoantibodies against SPAG9 represent a promising approach for the development of biomarker, further large-scale validation studies are required to establish its potential use in early diagnosis and monitoring of breast cancer recurrence. (Cancer Epidemiol Biomarkers Prev 2009;18(2):630-9)

## Introduction

Breast cancer is the most common malignancy in women and is the second major cause of cancer-related mortality (1), with a high mortality rate of one in eight women in India (2). To reduce the mortality rate, various approaches, including mammography and development of molecularly targeted therapeutic drugs, have been in practice, but metastatic disease is eventually fatal. In developing countries, most of the cases arise due to limited awareness and nonavailability of effective screening systems (3). In many cases, cancer is not diagnosed and treated until cancer cells have already invaded surrounding tissues and metastasized throughout the body. Detecting cancers at their earlier stages means that current or future treatment modalities might have a higher likelihood of a true cure.

Biomarkers are important tools for cancer detection and monitoring. Several promising breast cancer-associated antigens have been cloned by serologic screening of cDNA expression libraries (4). Yet, no

biomarker, whether individual autoantigens or panels of antigens developed using antibody-based methods, has been validated and incorporated in routine oncologic practice for the early diagnosis of breast cancer (4). In this regard, CA 15-3 and CA 27.29 are the two established markers for breast cancer known to be expressed in <10% of early disease and in ~75% of advanced-disease patients. Neither is recommended for routine use in diagnosing breast cancer (5). Thus, there is an urgent need of noninvasive serum biomarkers for early detection of breast cancer. Instead of targeting serum antigens as biomarkers, autoantibodies generated against tumor antigens are being explored (6). In this regard, several tumor-associated antigens have been reported for their humoral response in various human cancers. Autoantibodies against p53, p62, ING1 (tumor suppressor), NY-BR-62, and NY-BR-85 with low frequency range have been reported in breast cancer (4, 7, 8). Because tumor-associated antigens are self-proteins and are not much immunogenic, further efforts are being made to identify immunogenic tumor antigens.

Cancer testis (CT) antigens are the unique class of tumor antigens with normal expression restricted in the testis and in various cancers but not in adult somatic tissues (6). Therefore, CT antigens become ideal targets for diagnostics and cancer vaccine, considering that several of them can elicit spontaneous cellular and/or humoral immune responses in cancer patients (9). In this regard, antibody response against a well-known CT antigen NY-ESO-1 is reported to be 4% in primary breast

Received 7/10/08; revised 10/22/08; accepted 11/10/08; published OnlineFirst 02/03/2009.

**Grant support:** Cancer Research Program, Associated Cancer Center for Immunotherapy, Department of Biotechnology, Government of India.

**Note:** Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

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doi:10.1158/1055-9965.EPI-08-0629

cancer patients (10). Autoantibodies against CT antigen SCP-1 and SSX-2 were detected in 6% and 1%, respectively, of primary breast cancer patients (11).

Recently, we showed the humoral immune response against sperm-associated antigen 9 (SPAG9) in 67% of epithelial ovarian cancer patients (12). We also showed high *in vivo* immunogenicity against SPAG9 in early stages (I and II) of various histotypes in epithelial ovarian cancer. In addition, gene expression microarray analysis of breast tumors (13) showed the expression of *SPAG9* gene with other important much-studied genes. In the present study, we evaluated the SPAG9 expression and humoral response in breast cancer patients. We also investigated SPAG9 association with various tumor stages and histologic grades. The key finding of the study is that SPAG9 expression and humoral immune response is detected in early stages (I and II) and in low-grade (grade 1) breast cancer patients, suggesting that SPAG9 is a potential target for development of diagnostic and therapeutic targets in breast carcinoma. In addition, there was a distinct pattern of higher SPAG9 immunoreactivity score (IRS), which was significantly associated with predicted risk of recurrence of breast cancer. Our study lays a foundation for further investigation into large-scale validation for providing early detection and treatment for breast cancer.

## Materials and Methods

**Tissue and Serum Specimens.** Breast tumor tissues and serum samples were obtained from cancer patients [94 infiltrating ductal carcinoma (IDC), 2 ductal carcinoma *in situ* (DCIS), and 4 invasive lobular carcinoma (ILC)] who underwent surgery at Research and Referral, Army Hospital under the protocol approved by Institutional Ethics Committee. The specimens were used after obtaining the written informed consent from each patient. The adjacent noncancerous tissue (ANCT) specimens were also collected from these patients [88 matched ANCT IDC, 2 matched ANCT DCIS, and 2 matched ANCT ILC]. It is important to point out that these tissues cannot be regarded as healthy normal. Pathologic reports for patient's tissue were provided by the organization. Tumor tissues were immediately fixed in formalin and processed for immunohistochemistry. All tumor specimens and ANCT samples were used for reverse transcription-PCR (RT-PCR) analysis and immunohistochemistry.

**Total RNA Extraction and Preparation of cDNA.** Total RNA extraction was done using the TRI reagent (Ambion) according to the manufacturer's protocol. RNA was dissolved in diethyl pyrocarbonate-treated water and concentration was determined. cDNA was synthesized from RNA using FastLane Cell cDNA kits (Qiagen). RT-PCR was done using cDNA as a template and following SPAG9 primers (forward 5'-GACAGA-GATGATTCGGGCATCACGAGAAAA-3' and reverse 5'-CTAAGTTGATGACCCATTATTACCTCGACTG-3'). Subsequently, 40 amplification cycles [1 cycle of denaturation at 94°C for 2 min, 40 cycles: denaturation at 94°C for 45 s; annealing at 52°C for 45 s; extension at 72°C for 2 min; and a final elongation cycle at 72°C for 7 min] were carried out for each specimen. The PCR

products were analyzed on 1% agarose gels and photographed under UV light. RT-PCR for  $\beta$ -actin mRNA expression was done as an internal control. Subsequently, the PCR product was subcloned into TOPO vector using TOPO kit (Invitrogen/Life Technologies) and the nucleotide sequence was confirmed by automated DNA sequencing.

**Immunohistochemistry.** Breast cancer tissue specimens were analyzed for localization of SPAG9 protein using anti-SPAG9 antibody or control IgG as described earlier (12). Briefly, paraffin-embedded tissue sections (4  $\mu$ m) on glass slides were heated at 60°C for 20 min and then deparaffinized with xylene and ethanol. Following deparaffinization, sections were rehydrated and then incubated with 1% H<sub>2</sub>O<sub>2</sub> for 10 min to remove endogenous peroxidase activity. Sections were incubated with 10% normal goat serum for 30 min to block nonspecific binding sites. Specimens were incubated with anti-SPAG9 antibody or control IgG at 4°C for overnight in humid chamber. Subsequently, the sections were washed thrice with PBS and incubated for 1 h at room temperature with secondary antibody of horseradish peroxidase-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories). Paraffin-embedded sections of ANCT were also processed as described above. To confirm the grades of the breast tumor, tissue sections were also incubated with Ki-67 antibody (Serotec). The sections were incubated with anti-Ki-67 antibodies at dilution of 1:20 at 4°C overnight in humid chamber. After washing, antigen-bound primary antibodies were detected by horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Reactivity in the tissue specimens was visualized using chromogen 0.05% 3,3'-diaminobenzidine (Sigma-Aldrich), counterstained with hematoxylin solution, mounted with DPX, and observed under Nikon Eclipse E 400 microscope (Nikon).

**Evaluation of Immunostaining of SPAG9 Expression.** We assessed the immunostaining of SPAG9 by counting >500 cells from 5 random fields of each specimen under  $\times 400$  magnification in the best-stained tumor area of each section as described earlier (14). SPAG9 IRS was defined as the percentage of stained breast tissue cells. We considered a distinct positive immunoreactivity in a specimen showing >10% of cancer cells stained for SPAG9 protein.

**ELISA.** The recombinant SPAG9 protein was expressed and purified (15) as described in Supplementary Data. As shown in Supplementary Fig. S1, the recombinant protein was purified to >95% purity when analyzed by SDS-PAGE. These results indicated that purified full-length SPAG9 protein could be used as an antigen for the detection of SPAG9 antibodies to monitor the immune response against SPAG9 in patients with breast cancer. To detect anti-SPAG9 antibodies in the sera of breast cancer patients, ELISA was carried out as described earlier (16). Sera from 100 breast cancer patients and 50 normal healthy donors were examined for SPAG9 antibody. Briefly, purified recombinant SPAG9 protein was coated at 100 ng/well in coating buffer (pH 9.6; 15 mmol/L Na<sub>2</sub>CO<sub>3</sub>, 35 mmol/L NaHCO<sub>3</sub> in distilled water) into 96-well microtiter plates (Nunc) at 4°C for overnight. The plates were washed with

PBS-0.05% Tween 20 and blocked in PBS-0.05% Tween 20 and 1% nonfat dry milk for 1 h at 37°C. After blocking, serial dilutions of cancer patient's serum in PBS-0.05% Tween 20 and 1% nonfat dry milk were added and incubated for 2 h at room temperature. Plates were washed and incubated at room temperature for 1 h with horseradish peroxidase-conjugated mouse anti-human IgG (Jackson ImmunoResearch Laboratories). Secondary antibody binding was followed by washes with PBS-0.05% Tween 20. The signal was developed using 0.1% orthophenylenediamine in 50 mmol/L citrate phosphate (pH 5.0) with 0.06% H<sub>2</sub>O<sub>2</sub> as the substrate. To stop the reaction, 5 N H<sub>2</sub>SO<sub>4</sub> (50 µL) was added in each well and the absorbance was determined at 492 nm with 620 nm as reference filter. Results for patient's serum dilution (1:100) were accepted with estimated ELISA titers above the mean  $\pm$  2 SD of the healthy sera. All breast cancer samples and normal healthy donor samples were tested in duplicates and the mean was used for data analysis. A subset of samples has been reassayed for five times in every ELISA plate for quality control. The intraassay and interassay coefficients of variation were 3.1% and 5.3%, respectively.

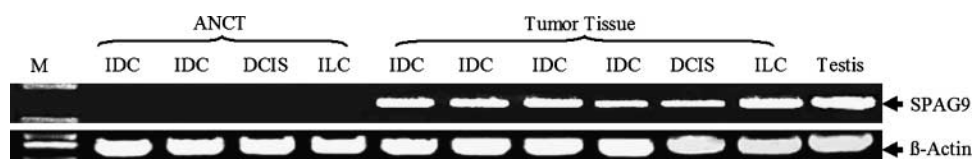
**Statistical Analysis.** Study analysis included information regarding (a) SPAG9 IRS scores as a continuous and dichotomous variable (positive/negative), (b) lymph node status as a continuous and dichotomous variable (positive/negative), (c) estrogen receptor status (positive/negative), (d) progesterone receptor status (positive/negative), (e) histologic grade [well differentiated (grade 1), intermediate (grade 2), and poorly differentiated (grade 3)], and (f) lymphovascular invasion (presence/absence). Relationship between categorical variables were compared by performing Pearson's  $\chi^2$  test, unpaired Student's *t* test, and Kruskal-Wallis test using SPSS 16.0 statistical software package (SPSS). Results were expressed as mean  $\pm$  SE. Independent variables were used in univariate and multivariate linear and logistic regression models to predict SPAG9 expression in breast cancer patients. *P* < 0.05 was considered statistically significant. To evaluate the predicted risk of breast cancer recurrence over 10 years, information on patient and tumor characteristics was subjected to the Adjuvant! Online Version 8 program (17).

## Results

**Expression of SPAG9 mRNA in Breast Cancer Tissues.** We first investigated the SPAG9 expression by RT-PCR. mRNA expression of SPAG9 was found in 87%

(82 of 94) in IDC, 100% (2 of 2) in DCIS, and 100% (4 of 4) in ILC and in testis. One hundred percent (1 of 1) of stage I, 88% (38 of 42) of stage II, 83% (40 of 48) of stage III, and 100% (3 of 3) of stage IV breast cancer patients were positive for the presence of SPAG9 mRNA. Further, 88 matched ANCT of IDC, 2 matched ANCT of DCIS, and 2 matched ANCT of ILC did not show SPAG9 expression. The amplicon size in tumor was the same as in testis (Fig. 1). The PCR product was confirmed as SPAG9 by automated sequencing. The association between SPAG9 expression and clinicopathologic features is shown in Table 1. SPAG9 expression was found in 90% early stages (I and II) and 84% late stages (III and IV) of breast cancer tissues. In addition, SPAG9 expression was detected in 97% (35 of 36) grade 1, 90% (40 of 44) grade 2, and 50% (7 of 14) grade 3 breast cancer tissues. The statistical analysis revealed that SPAG9 expression was independent of tumor stages, indicating no correlation between tumor stages and SPAG9 expression (*P* = 0.272) using Pearson's  $\chi^2$  test (Table 1). However, SPAG9 expression was significantly associated among the grades using Pearson's  $\chi^2$  test (*P* < 0.0001). No significant difference was observed in the expression of SPAG9 among stages I to IV by multiple comparisons with Kruskal-Wallis test (*P* = 0.173).

**Immunohistochemical Staining of Breast Cancer.** A panel of 100 breast cancer tissues and ANCT specimens were studied for the presence of SPAG9 protein expression by immunohistochemistry. A distinct positive immunoreactivity (>10% of cancer cells were stained) for SPAG9 protein was seen in 88% patient tissues studied based on IRS. Representative photomicrograph of SPAG9 reactivity in various histotypes of breast cancer tissues and ANCT are shown in Fig. 2. The immunohistochemical staining results indicated that SPAG9 expression was localized in the cytoplasm of tumor cells in the tissue section of IDC (Fig. 2A-D), ILC (Fig. 2I), and DCIS (Fig. 2K). However, no SPAG9 reactivity was observed with control IgG in serial sections of IDC tumor tissue sections (Fig. 2E-H), ILC (Fig. 2J), and DCIS (Fig. 2L). Furthermore, no positive immunoreactivity was observed in ANCT specimens (Fig. 2M-P). The statistical analysis revealed that SPAG9 protein expression was independent of tumor stages, indicating no correlation between tumor stages and SPAG9 expression (*P* = 0.272) using Pearson's  $\chi^2$  test (Table 1). Further SPAG9 immunoreactivity was investigated in various grades of breast cancer tissues. The SPAG9 reactivity in tumor cells was higher in grade 1 (Fig. 3A) compared with grades 2 (Fig. 3B) and 3 (Fig. 3C), respectively. The statistical analysis revealed that SPAG9 protein expression was



**Figure 1.** SPAG9 expression analysis in human breast cancer tissues. RT-PCR analysis of SPAG9 mRNA expression using SPAG9-specific primers yielded ~1.25-kb product. Representative samples showing SPAG9 expression in IDC, DCIS, and ILC and in normal testis (positive control). However, matched ANCT of IDC, DCIS, and ILC showed no SPAG9 expression.  $\beta$ -Actin gene expression was used as an internal control.

**Table 1. SPAG9 expression, humoral immune response, and clinicopathologic characteristics of breast cancer**

Pathologic and clinical features	SPAG9 expression [positive/tested (%)]	
	RT-PCR/immunohistochemistry	ELISA
All tumors	88/100 (88)	80/100 (80)
Early stages	39/43 (90)	38/43 (88)
I	1/1 (100)	1/1 (100)
II	38/42 (88)	37/42 (88)
Late stages	43/51 (84)	36/51 (70)
III	40/48 (83)	34/48 (70)
IV	3/3 (100)	2/3 (66)
Grades		
1	35/36 (97)	28/36 (78)
2	40/44 (90)	37/44 (84)
3	7/14 (50)	9/14 (64)
Histotypes		
IDC	82/94 (87)	74/94 (78)
DCIS	2/2 (100)	2/2 (100)
ILC	4/4 (100)	4/4 (100)

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

Clinicopathologic features	Student's <i>t</i> test		Pearson's $\chi^2$ test		Kruskal-Wallis test	
	RT-PCR/ immunohistochemistry IRS	ELISA	RT-PCR/ immunohistochemistry IRS	ELISA	RT-PCR/ immunohistochemistry IRS	ELISA
Early and late stages	0.387*	0.577*	0.272*	0.203*	—	—
Stage I-IV	—	—	—	—	0.173*	0.961*
Grades 1 and 2	<0.0001 <sup>†</sup>	0.191*	—	—	—	—
Grades 1 and 3	<0.0001 <sup>†</sup>	<0.0001 <sup>†</sup>	—	—	—	—
Grades 2 and 3	0.002 <sup>†</sup>	0.01 <sup>†</sup>	—	—	—	—
Grades 1-3	—	—	<0.0001 <sup>†</sup>	0.118*	<0.0001 <sup>†</sup>	0.018 <sup>†</sup>

Linear regression model predicting high SPAG9 IRS (>70% cells)

Independent variables	Univariate <i>P</i>	Multivariate <i>P</i>
Lymphovascular invasion	0.003 <sup>†</sup>	0.046 <sup>†</sup>
No. positive lymph nodes	0.002 <sup>†</sup>	0.027 <sup>†</sup>

\*Statistically nonsignificant.

<sup>†</sup>*P* < 0.05, statistically significant.

significantly associated among the grades using Pearson's  $\chi^2$  test (*P* < 0.0001). In addition, tumor grades were assessed using cancer cell proliferation marker Ki-67 (18). Our results confirmed high expression of Ki-67 with increasing grades as shown in representative photomicrograph (Fig. 3B, D, and F).

**SPAG9 IRS and Its Association with Clinicopathologic Variables and Predicted Risk of Breast Cancer Recurrence.** SPAG9 IRS was defined as the percentage of SPAG9-stained carcinoma cells in breast cancer tissue. SPAG9 immunoreactivity was observed in 100% (1 of 1) of stage I, 88% (38 of 42) of stage II, 83% (40 of 48) of stage III, and 100% (3 of 3) of stage IV breast cancer patients. SPAG9 IRS with stages II to IV were  $61.8 \pm 2.7\%$ ,  $67.6 \pm 1.5\%$ , and  $64.7 \pm 5.0\%$  (mean  $\pm$  SE), respectively. However, no significant differences between SPAG9 IRS in early stages (I and II) and late stages (III and IV) was found by performing Student's *t* test (*P* = 0.387). Furthermore, there were no significant differences between SPAG9 IRS among stages I to IV using Kruskal-Wallis test (*P* = 0.173). Ninety-seven percent (35 of 36) of grade 1, 90% (40 of 44) of grade 2, and 50% (7 of 14) of grade 3 patients showed SPAG9 reactivity by

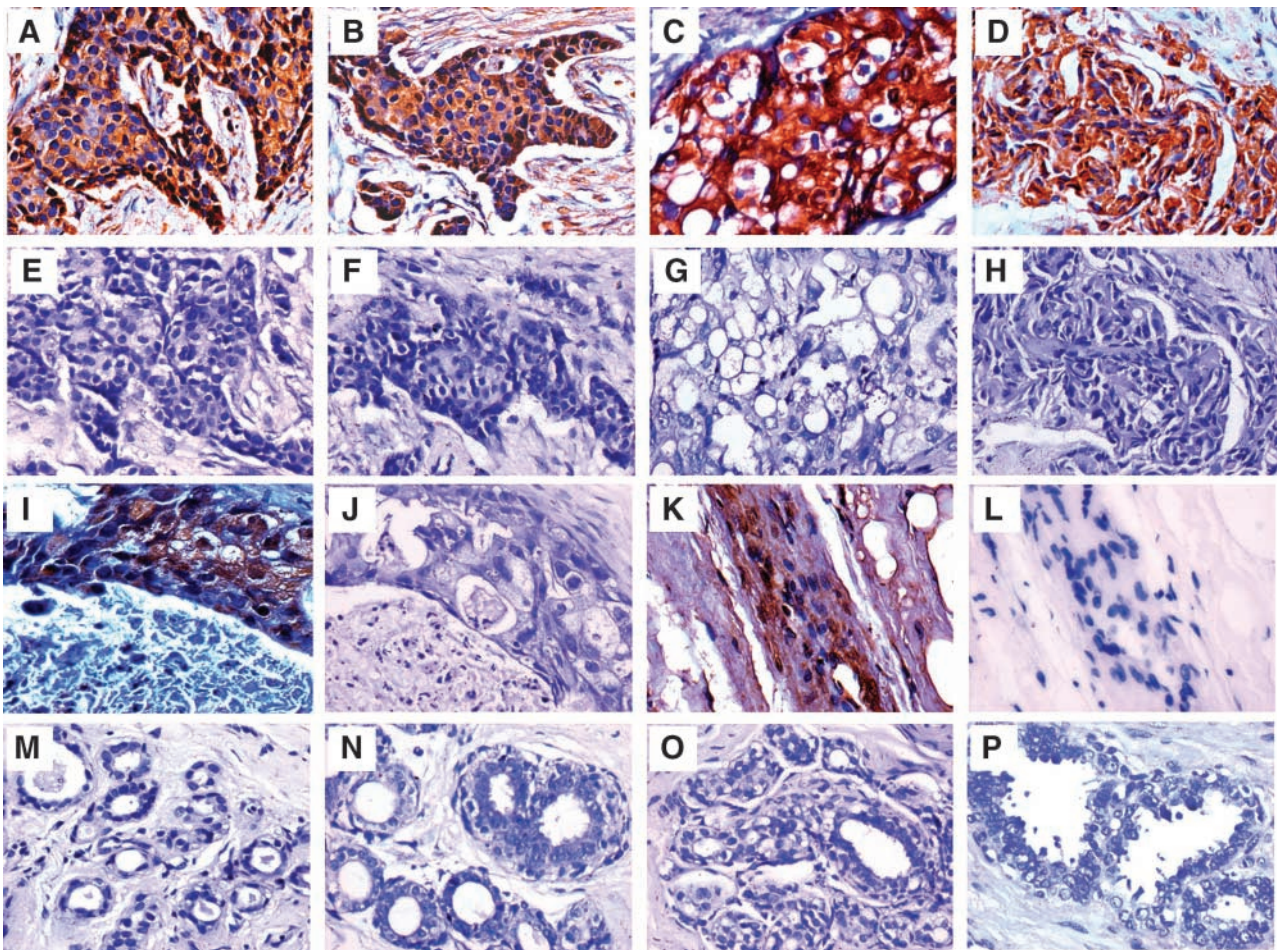
immunohistochemical analysis. SPAG9 IRS of breast cancer with grades 1 to 3 were  $72.60 \pm 1.6$ ,  $61.95 \pm 2.1$ , and  $45.00 \pm 5.7$  (mean  $\pm$  SE), respectively (Fig. 4A). SPAG9 IRS decreased with increasing grades of breast carcinoma. Statistical analysis revealed significant differences between SPAG9 IRS and grades 1 and 2 (*P* < 0.0001), grades 1 and 3 (*P* < 0.0001), and grades 2 and 3 (*P* = 0.002) by Student's *t* test. Furthermore, there were significant differences of SPAG9 IRS among grades 1 to 3 by multiple comparisons with Kruskal-Wallis test (*P* < 0.0001; Fig. 4A).

Further, association of SPAG9 IRS was examined with the various known risk factors of breast cancer patients such as tumor grade, nodal status, and clinical stages. Our data indicated that SPAG9 IRS was significantly associated among various grades of breast cancer using Pearson's  $\chi^2$  test (*P* < 0.0001; Fig. 4A). Based on SPAG9 IRS in breast tumors, three groups were formed for statistical analysis [low (20-50% cells), moderate (50-70% cells), and high (>70% cells)]. Moreover, the 10-year predicted risk of recurrence from breast cancer was calculated for each patient using the Adjuvant! Online program. There was statistically significant (Pearson's  $\chi^2$  test; *P* = 0.046) pattern of high SPAG9 IRS with predicted risk of breast

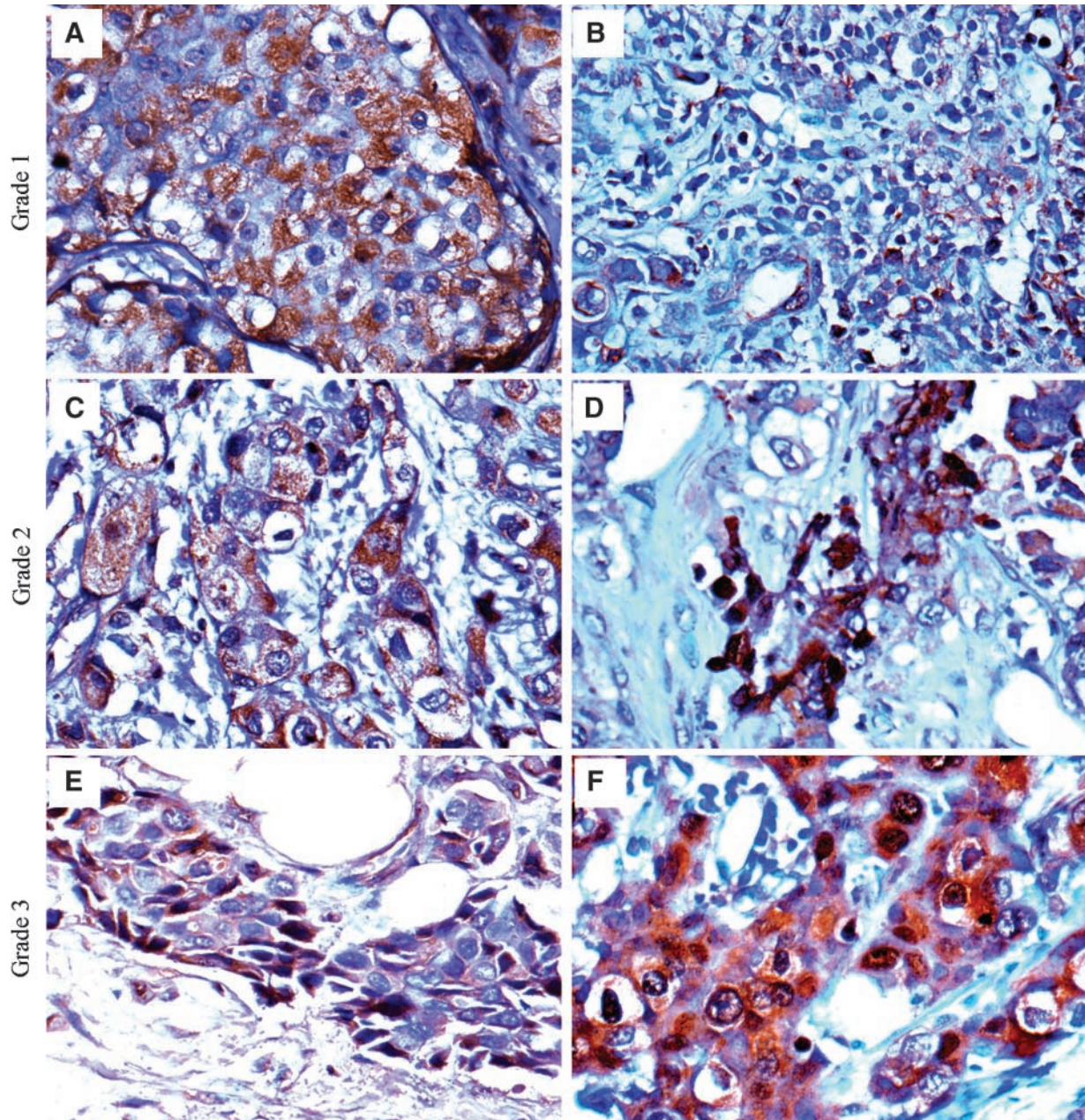
cancer recurrence (Fig. 4B). Furthermore, when examined using Pearson's  $\chi^2$  test, high SPAG9 IRS (>70% cells) showed strong correlation with presence of positive lymph nodes ( $P = 0.041$ ). In addition, presence of lymphovascular invasion and positive lymph nodes were significant in predicting high SPAG9 IRS (>70% cells) in both univariate and multivariate linear regression models (Table 1). Univariate and multivariate logistic regression models were used to predict the association between SPAG9 expression and testing variables such as histologic grade, tumor size, estrogen receptor status, and progesterone receptor status. Only tumor grade was significantly associated with the presence of SPAG9 expression ( $P < 0.0001$ ). Tumor size ( $P = 0.656$ ), estrogen receptor status ( $P = 0.553$ ), and progesterone receptor status ( $P = 0.179$ ) were not found to be statistically significant predictors of SPAG9 expression.

**Humoral Response against SPAG9.** Having established the SPAG9 expression in breast cancer, we performed ELISA to detect antibodies against SPAG9

in sera from breast cancer patients and healthy donors. We first established the basal signals in the ELISA system using sera from 50 healthy donors (mean  $\pm$  2 SD at  $A_{492\text{ nm}}$ ). Using the mean absorbance value of healthy donors as a cutoff value ( $0.175 \pm 0.08$ ), positive immunoreaction to SPAG9 was detected in 80% breast cancer patients with SPAG9-expressing tumor but not in the healthy donors (Fig. 5; Table 1). It is important to note that 88% (38 of 43) patients belonging to IDC histotypes of early-stage breast cancer (I and II) and 70% (36 of 51) later-stage (III and IV) showed humoral response against SPAG9. In different tumor grades, 78% (28 of 36) of grade 1, 84% (37 of 44) of grade 2, and 64% (9 of 14) of grade 3 revealed anti-SPAG9 antibodies. The association between anti-SPAG9 antibodies and clinicopathologic features is shown in Table 1. The statistical analysis revealed that anti-SPAG9 antibody response was independent of tumor stages and grades, indicating no correlation among tumor stages ( $P = 0.203$ ), grades ( $P = 0.118$ ), and humoral response using Pearson's  $\chi^2$  test. Furthermore, there was no significant difference



**Figure 2.** Immunohistochemical analysis of SPAG9 protein expression in various histotypes of breast cancer. Representative photomicrograph showing distinct cytoplasmic localization of SPAG9 protein in IDC (A-D), DCIS (I), and ILC (K) probed with anti-SPAG9 antibodies. No positive reactivity was observed in serial section of IDC (E-H), DCIS (J), and ILC (L) probed with control IgG. No SPAG9 immunoreactivity was observed in matched ANCT (M-P) probed with anti-SPAG9 antibody. Original magnification,  $\times 400$ ; objective,  $\times 40$ .



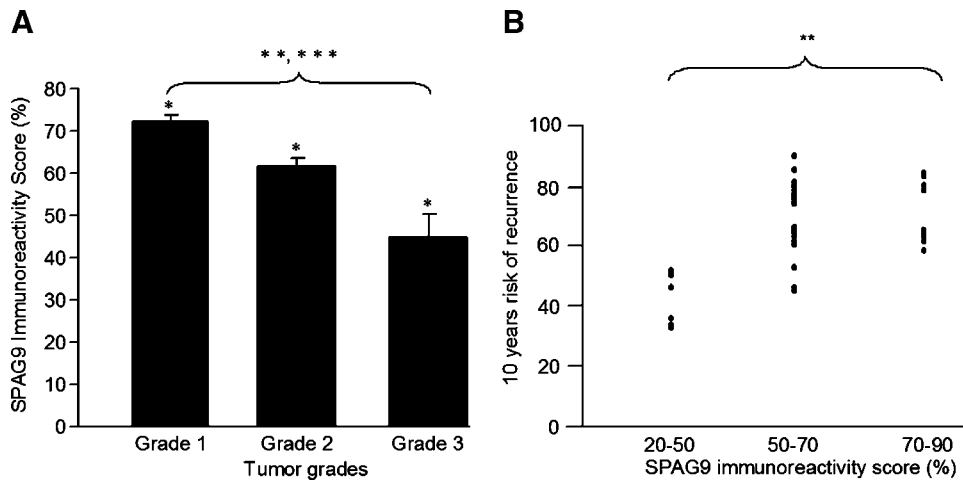
**Figure 3.** Immunohistochemical analysis of SPAG9 and Ki-67 protein expression in different grades of breast cancer. Cytoplasmic localization of SPAG9 protein in grades 1 (A), 2 (C), and 3 (E), respectively. Representative photomicrographs of grades 1 (B), 2 (D), and 3 (F) showing positive nuclear stained cells with antibodies against Ki-67. Original magnification,  $\times 400$ ; objective,  $\times 40$ .

among stages by multiple comparison with Kruskal-Wallis test ( $P = 0.961$ ). However, the statistical analysis revealed that there was significant difference between anti-SPAG9 antibodies in tumor grades 1 and 3 ( $P < 0.0001$ ) and tumor grades 2 and 3 ( $P = 0.01$ ) but not in tumor grades 1 and 2 ( $P = 0.191$ ) using Student's  $t$  test. Furthermore, there were significant differences of anti-SPAG9 antibodies among grades 1 to 3 by multiple comparison with Kruskal-Wallis test ( $P = 0.018$ ). Furthermore, no significant association was found

between SPAG9 antibodies and estrogen receptor status ( $P = 0.428$ ) and progesterone receptor status ( $P = 0.572$ ) using Pearson's  $\chi^2$  test.

### Discussion

In the past three decades, extensive research efforts have been put in identifying predictive serum tumor antigen markers for the development of diagnostic and therapeutic methods in breast cancer (19, 20). In this regard,



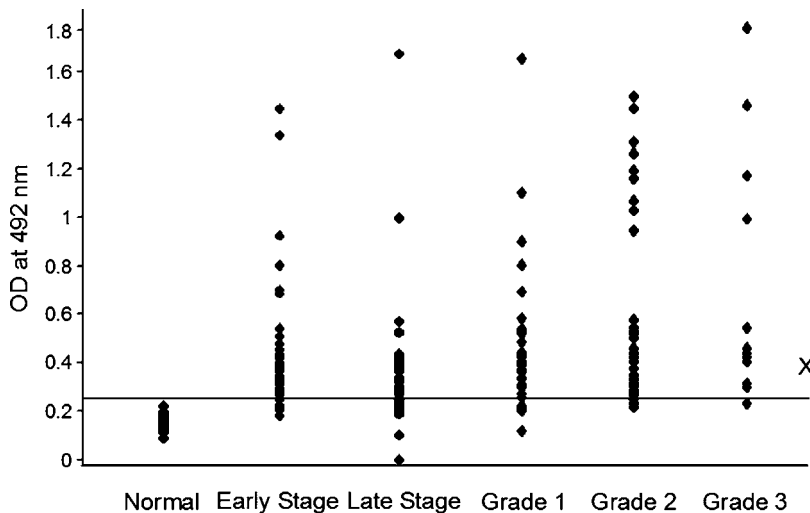
**Figure 4.** SPAG9 IRS and its association with pathologic grading and predicted risk of recurrence in breast cancer. **A.** Significant differences observed in SPAG9 IRS between grades 1 and 2 (\*,  $P < 0.0001$ ) and grades 1 and 3 (\*,  $P < 0.0001$ ) and grades 2 and 3 (\*,  $P = 0.002$ ) with unpaired Student's  $t$  test; \*\*,  $P < 0.0001$ , SPAG9 IRS was significantly associated among various grades of breast cancer using Pearson's  $\chi^2$  test; \*\*\*,  $P < 0.0001$ , significant differences of SPAG9 IRS among grades 1 to 3 by multiple comparisons with Kruskal-Wallis test. **B.** Based on SPAG9 IRS, breast cancer patients were grouped for statistical analysis into following groups: 20-50% IRS, 50-70% IRS, and 70-90% IRS. \*\*,  $P = 0.046$ , significant predicted risk of breast cancer recurrence in relation with SPAG9 IRS using Pearson's  $\chi^2$  test.

several serum tumor antigens such as CA 15-3, CA 27.29, CEA, BR 27.29, p53, HER-2/*neu*, BRCA1, BRCA2, c-myc, and MUC-1 have been reported for monitoring breast cancer patients (21). However, percentage of breast cancer patients expressing these tumor antigens in early stages (stages I and II) is not more than 20%, and no reports have been shown for the clinical utility of these tumor antigens in newly diagnosed breast cancer patients (5, 19). Various promising breast-associated autoantigens have been cloned using serologic screening of cDNA expression libraries or identified using proteomics, but no biomarker has been validated and incorporated into the routine diagnostic practice for breast cancer (4).

Emerging evidences have suggested that human tumors stimulate the production of autoantibodies that

could be used as noninvasive serologic markers for the early diagnosis and management of breast cancer (8, 22). In this regard, autoantibodies against subunit of replication protein A (RPA32) were found in 11% of patients with breast cancer including DCIS of the breast (23). Additional support for the possibility that autoantibodies can precede the clinical diagnosis of breast cancer was provided by various studies, which revealed the presence of antibodies at the time of the diagnosis of breast (23, 24). In addition, a recent study reported autoantibodies against a panel of antigens like p53, HER-2, MUC1, c-myc, and BRCA2, which ranged between 3% and 23% of primary invasive breast cancer sera (19).

Recently, we characterized *SPAG9* gene, a new member of the CT antigen family that was found to be expressed in ovarian malignancies (12). Furthermore, our



**Figure 5.** Analysis of SPAG9 antibodies in breast cancer patients. ELISA analysis of sera from breast cancer patients of different stages (early: I and II; late: III and IV) and grades (grades 1-3) and from healthy women donors. X, cutoff  $A_{492\text{ nm}}$  at mean  $\pm 2$  SD from healthy donors for positivity above or negativity below the line.  $P < 0.0001$ , significant difference in the seroreactivity between normal healthy women donors and breast cancer patients;  $P = 0.018$ , significant difference of SPAG9 antibodies among grades 1 to 3 by multiple comparison with Kruskal-Wallis test.

recent study showed the expression of SPAG9 in renal cell carcinoma patients and provided evidence for its role in cell migration, invasion, and tumor growth (25). Our earlier studies showed that SPAG9 functions as a scaffolding protein and interacts with c-Jun NH<sub>2</sub>-terminal kinase subgroup of mitogen-activated protein kinases (15, 26). The c-Jun NH<sub>2</sub>-terminal kinase-interacting proteins act by altering the signaling strength of their cognate mitogen-activated protein kinase module by regulating the signal amplitude and duration (27) and play an important regulatory role in cell survival, proliferation, apoptosis, and tumor development (15, 28). Thus far, various studies have reported the molecular interaction of scaffold proteins with which these proteins accelerate the kinetics of signaling by the respective mitogen-activated protein kinase signaling modules (29). In addition, recently various isoforms of SPAG9 have been reported (15, 30, 31), which are involved in the execution of specific mitogen-activated protein kinase signaling pathways and endothelial differentiation (32). It would be interesting to investigate the expression of relationship of SPAG9 isoforms with tumor development and progression of disease.

CT antigens represent a unique class of tumor antigens, which are expressed in a variety of cancerous tissues (6). In breast cancer patients, a recent study reported the expression of several CT antigens such as SCP-1 (65%), SSX-4 (65%), HOM-TES-85/CT-8 (47%), GAGE (26%), SSX-1 (20%), NY-ESO-1 (13%), MAGE-3 (11%), SSX-2 (8%), CT-10 (7%), MAGE-4 (4%), and CT-7 (1%; ref. 11). It is noteworthy that our results revealed high SPAG9 mRNA expression in early stages (90%) and low grade (97%) in breast cancer patients compared with other CT antigens. Further, we also found significant association of SPAG9 mRNA expression with low-grade tumor. In addition, there was no discrepancy in SPAG9 mRNA and protein expression in the tumor tissues. It was interesting to compare the SPAG9 protein expression with other CT antigens in breast cancer patients. A well-studied CT antigen NY-ESO-1 expression was detected only in 2% breast cancer specimens (20). Subsequently, another study showed NY-ESO-1 expression in 40% IDC specimens (33). A well-characterized CT antigen, MAGE-A4 expression was reported in 74% IDC specimens (33). A recent immunohistochemical study reported expression of CT antigen CT7 (MAGE-C1) in 18% of the cases of breast cancer (34). In contrast, SPAG9 protein expression was observed in 88% of breast cancer tissues analyzed irrespective of tumor histotypes, stages, and grades. It is important to mention here that SPAG9 IRS was significantly higher in grade 1 compared with grades 2 and 3 ( $P < 0.0001$ ) by Student's *t* test. This is one of the important criteria toward identifying tumor-specific protein targets for immunotherapy and development of cancer biomarker.

We were intrigued by our high SPAG9 IRS in low-grade and early-stage tumors. Our linear regression models analysis suggested significant relationship between high SPAG9 IRS (>70% cells) and presence of lymphovascular invasion and lymph nodes. This association suggests a possible, yet unproven, biological mechanism for the spread of breast cancer and recurrence. A recent report on high P7 protein expression (>20% cells) in primary breast cancer cells is suggestive of its association with cancer spread and recurrence (35).

Expression of KLF4 has also been reported to be associated with aggressive phenotype in early-stage IDC (36). The presence of a low molecular weight isoform of cyclin E also showed a strong association of its expression with stage I with aggressive phenotype of breast cancer (37). Our similar results may suggest that high SPAG9 expression may contribute in tumor invasion and aggressive phenotype. It is quite possible that the high SPAG9 expression in low-grade tumors may initiate parallel effector signaling pathways resulting in tumor spread and progression of disease, which needs to be further investigated. The mechanism of SPAG9 expression in breast cancer still remains to be determined. Furthermore, it is also of great importance that, as knowledge of cancer biology and therapy improves, early-stage and low-grade patients stand to benefit from this relevant molecular expression.

Although we evaluated the SPAG9 expression in breast cancer patients, we further investigated the *in vivo* immunogenicity of SPAG9 in these patients. Earlier, we reported the humoral immune response against SPAG9 in 67% of epithelial ovarian cancer patients (12). We also found high *in vivo* immunogenicity against SPAG9 in early stages of various histotypes in epithelial ovarian cancer. Our demonstration of *in vivo* immunogenicity in a significant proportion of breast cancer patients with SPAG9-expressing tumor is consistent with known immunogenicity of this antigen (12). It is important to note that 80% of the breast cancer patients with SPAG9-expressing tumors showed positive humoral immune response. The remaining breast cancer patients with SPAG9-expressing tumors did not have detectable SPAG9 antibodies by ELISA. It is possible that the presence of SPAG9 antibodies is dictated by the genetic background of the individual and that, 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. When we compared immune response against SPAG9 with the other known CT antigens, it is important to note that NY-ESO-1, SCP-1, and SSX-2 showed humoral immune response only in 4%, 6%, and 1% breast cancer patients, respectively (11). In yet another study, antibody response against CT antigen CTSP-1 was observed only in 16.6% of breast cancer patients (38). However, our study reveals that 88% of early-stage (I and II) cancer patients and 78% of low-grade (grade 1) cancer patients generated immune response against SPAG9, suggesting strong immunogenicity of SPAG9 protein in breast cancer patients. This is an important finding where significant proportion of early-stage (I and II) and low-grade (grade 1) breast cancer patients exhibited antibody response against SPAG9, supporting its potential role as a noninvasive serum biomarker for early diagnosis and cancer management. Our preliminary observations need to be validated in large number of breast cancer patients.

Metastasis is a complex multistep process that involves multiple tumor-host interactions. To survive, metastatic cancer cells must migrate from the primary tumor, invade into the lymphovascular system, and establish a new blood supply at their metastatic site. Our linear regression modeling analysis showed a strong



relationship between the presence of lymphovascular invasion and high SPAG9 IRS. The presence of high SPAG9 expression in early stages and grades might be responsible for the divergence of phenotypic expression and spread of breast cancer. It is possible that these changes are influenced by other tumor suppressor genes such as p53 and warrants further investigations. Interestingly, a cluster of 98 previously unidentified p53 target genes have been recently identified in breast cancer patients. These genes were implicated in novel aspects of p53 functions, with clinical relevance to p53-dependent tumorigenesis in primary breast cancer samples (39). Furthermore, they reported that most of the tumors with wild-type p53 showed low levels of p53 down-regulated genes compared with the tumors having p53 mutation (39). Further, their data revealed that p53-repressed genes such as antiapoptotic (*BCL2A1* and *TNFAIP8*) and *SPAG9* gene had high expression in p53-mutant tumors relative to p53 wild-type tumors. This report suggested for the first time that the dysregulation of p53 target genes was significantly linked to tumor spread within 5 years of diagnosis of breast cancer patients (39). Therefore, high SPAG9 expression characteristics in early-stage and low-grade tumors could be potentially used as a biomarker for tumor spread and progression of disease and warrants further investigation.

Our findings reveal that majority of various histotypes of breast cancer tissues expressed SPAG9 at both mRNA and protein levels. Our SPAG9 expression analysis suggested a strong relationship between the presence of lymphovascular invasion and high SPAG9 IRS. Furthermore, our data indicate a distinct pattern of high SPAG9 IRS in low-grade tumors, which is a predicted risk of breast cancer recurrence. Our study further relates SPAG9 *in vivo* immunogenicity with early-stage and low-grade breast cancer, suggesting SPAG9 as a candidate target for early diagnosis. Large-scale studies are required to determine the potential utility of the SPAG9 in guiding treatment decisions and following disease progression.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

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We thank Prof. A. Suroliya (Director, National Institute of Immunology, Aruna Asaf Ali Marg) for constant encouragement of this work and Dr. Vijay Kumar (International Centre for Genetic Engineering and Biotechnology) for critical reading of this article.

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