Sperm-Associated Antigen 9, a Novel Cancer Testis Antigen, Is a Potential Target for Immunotherapy in Epithelial Ovarian Cancer

Manoj Garg,[1] Dipak Chaurasiya,[1] Ritu Rana,[1] Nirmala Jagadish,[1] Deepika Kanojia,[1] Namrata Dudha,[1] Neha Kamran,[1] Sudha Salhan,[2] Amar Bhatnagar,[3] Sushma Suri,[2] Anju Gupta,[4] and Anil Suri[1]

Abstract

Purpose: Cancer testis antigens are a group of tumor antigens with gene expression restricted to male germ cells in the testis and in various cancerous tissues. Recently, we reported a novel testis-specific *sperm-associated antigen 9* (*SPAG9*) gene, a new member of the c-Jun NH_2-terminal kinase–interacting protein family, having functional role in sperm-egg fusion and mitogen-activated protein kinase signaling pathway. National Center for Biotechnology Information Blast searches revealed *SPAG9* nucleotide sequence similarities with expressed sequence tags of various cancerous tissues. In an effort to examine the clinical utility of SPAG9, we investigated the *SPAG9* mRNA and protein expression in epithelial ovarian cancer (EOC). Humoral immune response to SPAG9 was also evaluated in EOC patients.

Experimental Design: We determined the expression profile of *SPAG9* transcript by reverse transcription-PCR and RNA *in situ* hybridization and SPAG9 protein expression by immunohistochemistry in EOC specimens and human ovarian cancer cell lines. Using ELISA and Western blotting, we analyzed specific antibodies for SPAG9 in sera from patients with EOC.

Results: *SPAG9* mRNA and protein expression was detected in 90% of EOC tissues and in all three human ovarian cancer cell lines. Specific SPAG9 antibodies were detected in 67% of EOC patients and not in sera from healthy individuals.

Conclusions: Our findings indicate that SPAG9 is highly expressed in EOC and immunogenic in patients. Humoral immune response against SPAG9 in early stages of EOC suggests its important role in early diagnostics. These results collectively suggest that SPAG9, a novel member of cancer testis antigen family, could be a potential target for the development of diagnostic and therapeutic methods in EOC.

Ovarian cancer is the major cause of death from gynecologic malignancy. The overall mortality of ovarian cancer has remained unchanged despite new chemotherapeutic agents, which have significantly improved the 5-year survival rate (1). The main reason is lack of success in diagnosing ovarian cancer at an early stage, and therefore, the majority of patients with ovarian cancer are diagnosed only in the advanced stages. Detecting cancers at their earliest stages, even in the premalignant state, means that current or future treatment modalities might have a higher likelihood of a true cure. The lack of preventive methods, early diagnostic methods, and effective therapies to treat recurrent ovarian tumors points toward a pressing need for identification of molecular targets for diagnosis as well as therapy.

Cancer testis antigens represent a unique class of tumor antigens, which are expressed in a variety of cancerous tissues and are silent in normal tissues, except for the testis (2). A characteristic commonly shared by cancer testis antigens is, aside from the highly tissue-restricted expression profile, their likely correlation with tumor progression and immunogenicity in cancer patients. Forty-four cancer testis antigen genes or gene families have been identified to date (2, 3), but coordinated humoral and cellular immune responses have been reported for only a few cancer testis antigens, including NY-ESO-1, CAGE, and XAGE (4–6). Because of their broad expression in cancer and restricted expression in normal tissues and high immunogenicity, cancer testis antigens are the most attractive targets for cancer immunotherapy and serum biomarker.

Recently, we characterized a novel testis-specific *sperm-associated antigen 9* (*SPAG9*) gene (7), a new member of c-Jun NH_2-terminal kinase–interacting protein family (8) involved in molecular interactions during sperm-egg fusion and mitogen-activated protein kinase signaling pathway (8, 9). *SPAG9* is a single-copy gene mapped to human chromosome 17q21 (8), a region involved in amplification and expression of cancer-related genes as well as a hotspot for chromosomal aberrations in various cancers. Serologic analysis of ovarian tumor antigens revealed a bias toward antigens encoded on 17q21, suggesting

Authors' Affiliations: [1]Genes and Proteins Laboratory, National Institute of Immunology; [2]Department of Obstetrics and Gynecology; [3]Department of Cancer Surgery, Safdarjung Hospital and Vardhman Mahavir Medical College; and [4]Moolchand Hospital, New Delhi, India

Received 9/21/06; revised 11/21/06; accepted 12/6/07.

Grant support: Department of Biotechnology, Government of India.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Anil Suri, Genes and Proteins Laboratory, National Institute of Immunology, New Delhi 110067, India. Phone: 91-11-26703-700; Fax: 91-11-26-1621-25; E-mail: anil@nii.res.in.

doi:10.1158/1078-0432.CCR-06-2340

that elevated expression of genes encoded within this region of chromosome 17 is an important event in ovarian tumors (10). Gene expression microarray analysis of breast tumors (11), cervix carcinoma cells (12), dermatofibrosarcoma protuberans (13), and esophageal adenocarcinoma (14) reported *SPAG9* gene expression along with other important much-studied genes. Recently, the chromatin immunoprecipitation-paired-end ditag sequencing approach was used to identify novel p53 target genes. This study revealed that *SPAG9*, along with antiapoptotic genes (*BCL2A1* and *TNFAIP8*), is down-regulated in wild-type p53 tumors compared with the p53 mutant-type tumors (15). Further, the higher expression of these p53-repressed genes in p53 mutant-type tumors was shown to be linked to the development of distant metastasis in more aggressive tumors. Therefore, SPAG9 expression characteristics *in vivo* can potentially be used as molecular gauges of tumor aggressiveness and clinical outcome. In the present study, we analyzed the expression pattern of SPAG9 in different histotypes of epithelial ovarian cancer (EOC) tissues and ovarian cancer cell lines. SPAG9 antibodies were also examined in the patients suffering from different ovarian malignancies. Our results indicate that majority of cancer specimens revealed both *SPAG9* mRNA and protein expressions. In addition, we show the presence of specific humoral response against SPAG9 in EOC patients. Our study further relates SPAG9 immunogenicity with early stages of EOC, suggesting its important role in early diagnostics. These results collectively suggest that SPAG9, a novel member of cancer testis antigen family, could be a potential target for the development of diagnostic and therapeutic methods in EOC.

Materials and Methods

Tissue specimens. In the present study, a total of 30 EOC patients was examined. Tissue specimens were surgically obtained from patients undergoing surgery for EOC at the Safdarjung Hospital and Vardhman Mahavir Medical College (New Delhi, India) under the protocol approved by Institutional Ethics Committee. The written informed consent was obtained from each patient for the use of specimens in the present study. Pathologic reports were provided by the organization for tissue. Only 20 patient's tissue samples were available for reverse transcription-PCR (RT-PCR) analysis, immunohistochemistry, and RNA *in situ* hybridization (17 serous, 1 clear cell, and 2 mucinous adenocarcinoma). All tumors were immediately snap frozen and stored at −80°C for gene expression. Tumor tissues were also fixed in formalin and processed for immunohistochemistry and RNA *in situ* hybridization.

Cell lines and cell culture. Three human ovarian cancer cell lines, A-10, SKOV-6, and Caov-2, were used in this study. All the cancer cell lines were gifts from Dr. Kunle Odunsi (Roswell Park Cancer Institute, Buffalo, NY) and were grown in the recommended medium under standard conditions.

Total RNA extraction and preparation of cDNA. Total RNA was extracted from ovarian cancer cells and frozen tumor tissues using the TRI Reagent (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. Briefly, after phenol treatment and drying, RNA was dissolved in RNase-free water. The resulting RNA concentration was measured and the quality of the RNA was checked by electrophoresis on a 1% agarose gel. cDNA synthesis was carried out using FastLane Cell cDNA kits (Qiagen GmbH, Hilden, Germany). Subsequently, cDNA was used as a template for RT-PCR using SPAG9 primers (forward, 5′-GACAGAGATGATTCGGGCATCAC-GAGAAAA-3′; reverse, 5′-CTAAGTTGATGACCCATTATTATACCTC-GACTG-3′). The PCR products were electrophoresed in 1% agarose gels in the presence of ethidium bromide and photographed under UV light. β-Actin mRNA expression was examined by RT-PCR as an internal control. Finally, the PCR product was subcloned into TOPO vector using TOPO kit (Invitrogen, Life technologies, Carlsbad, CA) and the nucleotide sequence was confirmed by automated DNA sequencing.

SPAG9 antibodies and immunofluorescence microscopy. An expression plasmid comprising the prokaryotic expression vector pET28b(+) (Novagen, Madison, WI) and a cDNA encoding a complete open reading frame [comparable with 111 to 2,410 bp of the published human SPAG9 (7), amino acid residues from 1 to 766] of SPAG9 was constructed as described earlier (8). The plasmid pET28b-SPAG9 encoding a SPAG9 His_6-tagged fusion was transformed in *Escherichia coli* BL21 (DE3) cells by standard methods. Expression of recombinant His_6-tagged SPAG9 in bacterial culture was induced with 1 mmol/L isopropyl-L-thio-β-D-galactopyranoside at 37°C for 4 h. The recombinant SPAG9 protein was purified using Ni^{2+}-nitrilotriacetate resin (Qiagen, Chatsworth, CA) according to the manufacturer's instruction. Before immunization, the identity of the expressed recombinant protein was confirmed by microsequencing with tandem mass spectrometry (8). Polyclonal antibodies to recombinant SPAG9 were raised using alum as an adjuvant in rats. Further, rat serum IgG was isolated using Nab Protein G Spin Chromatography kit (Pierce, Rockford, IL) according to the manufacturer's protocol and used as anti-SPAG9 antibody for all our experiments. For immunofluorescence microscopy, ovarian cancer cells were harvested and processed for immunofluorescence assay as described earlier (8). Briefly, the anti-SPAG9 antibody was used as primary antibody to probe SPAG9 protein. After washing, cells were incubated with secondary antibody goat anti-rat IgG FITC conjugate (Jackson ImmunoResearch, West Grove, PA). The slides were finally washed and mounted in glycerol/PBS (9:1) and observed under Nikon Eclipse E 400 microscope (Nikon, Fukok, Japan).

Fluorescence-activated cell sorting analysis. Flow cytometric analysis was done on all the ovarian cancer cells as described before (8). Cells were harvested by trypsinization [0.5% trypsin (Sigma-Aldrich, St. Louis, MO) and 0.2% EDTA], washed twice with PBS, and fixed with 0.4% paraformaldehyde in PBS. Cells were incubated with anti-SPAG9 antibody followed by goat anti-rat IgG FITC conjugate. After the final wash, cells were resuspended in PBS and analyzed by flow cytometer (BD-LSR model, Becton Dickinson, San Jose, CA). Data acquisition and analysis was done using WinMDI (version 2.8) software. Cells stained with secondary antibody only were used to account for the background fluorescence.

Synthesis of riboprobes and* in situ *hybridization. The cDNA fragment from 1,789 to 2,152 bp of coding region of the published human SPAG9 (7) was PCR amplified and subcloned in pBluescript SK (−). Riboprobes were prepared using either T7 or T3 RNA polymerase for the antisense (complementary to endogenous mRNA) or sense (same as endogenous mRNA) *in vitro* transcript using Digoxigenin RNA Labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) as described earlier (7). Tissues from ovarian tumor (serous, clear cell, and mucinous adenocarcinoma) were probed using sense riboprobe (control) and antisense riboprobe (experimental) following the protocols supplied with Digoxigenin RNA Labeling kit.

Immunohistochemistry. Paraffin-embedded sections of normal testis (positive control) and ovarian tumor samples were analyzed for the localization of SPAG9 protein using anti-SPAG9 antibody. Sections (5 μm) were placed on glass slides, heated at 60°C for 20 min, and then deparaffinized with xylene and ethanol. Subsequently, the deparaffinized sections were rehydrated before incubation with 1% H_2O_2 for 10 min to remove endogenous peroxidase activity. Nonspecific binding sites were blocked with 10% normal goat serum for 30 min. Specimens were incubated at 4°C overnight with anti-SPAG9 antibody at a dilution of 1:25. After incubation, the sections were washed thrice with PBS. Subsequently, secondary antibody of

horseradish peroxidase–conjugated goat anti-rat IgG (Jackson ImmunoResearch) was applied and incubated for 1 h at room temperature. Specimens were visualized using chromogen 0.05% 3,3′-diaminobenzidine (Sigma-Aldrich) and counterstained with hematoxylin solution.

ELISA. Recombinant SPAG9 protein (8) at a concentration of 1 μg/mL in coating buffer [pH 9.6; 15 mmol/L Na_2CO_3, 35 mmol/L $NaHCO_3$ in distilled water] was adsorbed to 96-well microtiter plates (Nunc, Roskilde, Denmark) at 100 μL/well overnight at 4°C as described earlier (16). The plates were washed with PBS containing 0.05% Tween 20 and blocked in PBS with 5% normal goat serum for 1 h at 37°C. After washing, serial dilutions of cancer patient's serum in PBS with 5% normal goat serum were added and incubated for 2 h at room temperature. Plates were washed and incubated with horseradish peroxidase–conjugated goat anti-human IgG at room temperature for 1 h. The enzyme activation was carried out with 0.05% orthophenylenediamine in 50 mmol/L citrate phosphate (pH 5.0) with 0.06% hydrogen peroxide as the substrate. The reaction was stopped with 50 μL of 5 N H_2SO_4, and then, the absorbance was read at 492 nm with 620 nm as reference filter. Results for serum dilution (1:100) were accepted with estimated ELISA titers above the mean (+2 SD) of the healthy sera.

Gel electrophoresis and immunoblotting. For Western blotting, 0.5 μg of purified recombinant SPAG9 protein was resolved on 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences, Cowley, United Kingdom) according to the procedure described earlier (8). After the protein transfer, the membrane was cut into protein strips. The protein strips were blocked with 5% bovine serum albumin and incubated with patient's sera at a dilution of 1:100 for 2 h at room temperature. Further, strips were incubated with peroxidase-conjugated goat anti-human IgG for 2 h at room temperature. Immunoreactive bands were visualized using chromogen 0.05% 3,3′-diaminobenzidine. The protein content of the recombinant SPAG9 protein was determined by the bicinchoninic acid method as described in the manufacturer's protocol (BCA-1 kit for protein determination; Sigma-Aldrich) or by UV absorption at 280 nm.

Statistical analysis. The statistical difference between the antibody titers in EOC patients and healthy controls was compared and analyzed by using unpaired and paired Student's *t* test. A P value of <0.05 was considered significant.

Results

Expression of SPAG9 mRNA in ovarian cancer tissues and cell lines. In the present study, a total of 30 EOC patients was examined. Only 20 patient's tissue samples were available for RT-PCR analysis. As shown in Table 1, using 40 cycles of RT-PCR analysis, *SPAG9* mRNA was detected in 18 of 20 (90%) adenocarcinoma tissues irrespective of tumor histotypes. In various ovarian histotypes, *SPAG9* mRNA was observed in 15 of 17 (88%) serous adenocarcinoma, in 1 of 1 (100%) clear cell carcinoma, and in 2 of 2 (100%) mucinous adenocarcinoma (Table 1). The size of the PCR product in tumor was the same as in the ovarian cancer cells and in testis (Fig. 1A). The PCR product was confirmed as SPAG9 by nucleotide sequencing. The relationship between *SPAG9* mRNA expression and pathologic and clinical features is shown in Table 1. SPAG9 was detected in all the analyzed early-stage (I and II) and in 13 of 15 late-stage (III and IV) EOC tissues. Hence, *SPAG9* expression was independent of tumor stage, indicating no correlation between tumor stages and *SPAG9* expression.

Expression of SPAG9 protein in ovarian cancer cell lines. To determine the SPAG9 protein expression in ovarian cancer cells, A-10, SKOV-6, and Caov-2 cells were subjected to indirect immunofluorescence using the anti-SPAG9 antibodies. As shown in representative photomicrograph in Fig. 1B, strong SPAG9 immunoreactivity was observed in the ovarian cancer cells. Results indicated that, in fixed and permeabilized SKOV-6 ovarian cancer cells, SPAG9 expression was predominantly in the cytoplasm, whereas surface localization of SPAG9 was observed in live cells (Fig. 1B). Subsequently, these results were further confirmed by flow cytometric analysis (Fig. 1C). Side scatter versus forward scatter gate analysis was carried out for all the experiments to avoid debris. No or very low surface distribution (0.23% fluorescence intensity) was observed in the control cells, whereas 40.70% fluorescence intensity or displacement of fluorescence on *X* axis was observed in cells probed with anti-SPAG9 antibody, indicating surface localization of SPAG9 protein.

Table 1. SPAG9 expression (RT-PCR/immunohistochemistry), humoral response (ELISA/Western blot), and clinicopathologic features in EOC

Pathologic and clinical features	SPAG9 expression by RT-PCR/IHC (positive/tested)	ELISA/Western blot (positive/tested)
All tumors	18/20 (90%)	20/30 (67%)
FIGO stage		
I	1/1	2/2
IB	2/2	2/3
IC	1/1	0/1
II	1/1	1/2
Subtotal of FIGO stages I and II	5/5 (100%)	5/8 (62.5%)
III	4/5	7/9
IIIA	1/1	0/1
IIIC	1/1	1/2
IV	7/8	7/10
Subtotal of FIGO stages III and IV	13/15 (87%)	15/22 (68%)
Histology		
Adenocarcinoma		
Serous	15/17 (88%)	11/19 (58%)
Clear cell	1/1 (100%)	2/2 (100%)
Mucinous	2/2 (100%)	3/3 (100%)
Unclassified	—	4/6 (67%)

Abbreviations: FIGO, Fesddration Internationale des Gynaecologistes et Obstetristes; IHC, immunohistochemistry.

Expression of SPAG9 mRNA and protein in EOC tissues. *In situ* hybridization studies with digoxigenin-labeled riboprobes showed *SPAG9* mRNA expression in different subtypes of ovarian cancer tissues (Fig. 2). Antisense riboprobe hybridization resulted in chocolate brown reaction product, indicating the presence of *SPAG9* mRNA in ovarian cancer tissues as shown in Fig. 2D to F. As expected, the sense riboprobe failed to show any localization (Fig. 2G-I).

To investigate the SPAG9 protein expression in EOC, the panel of 20 EOC specimens and human testis tissue was analyzed by immunohistochemistry using anti-SPAG9 antibodies. The immunohistochemical studies revealed a distinct immunoreactivity of SPAG9 protein in human testis (Fig. 3A). The immunolocalization of SPAG9 protein was restricted to sperm head and not in any other cell types of seminiferous tubules. Among the analyzed EOC specimens, SPAG9 protein

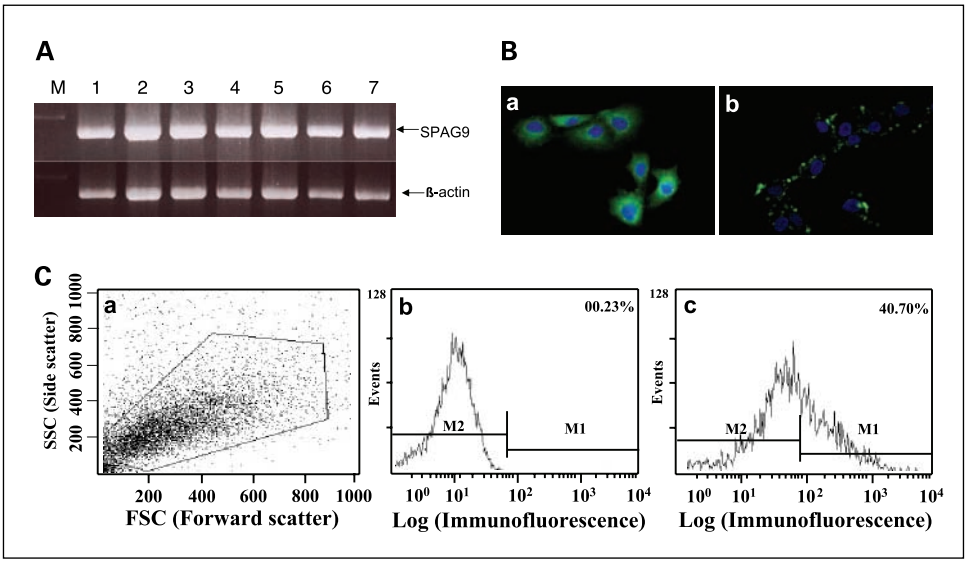

Fig. 1. *A,* RT-PCR analysis for SPAG9 mRNA expression yielded 1.2 kb product. mRNA from serous (*lane 1*), mucinous (*lane 2*), and clear cell carcinoma (*lane 3*) tissues; from three ovarian cancer cell lines, A-10 (*lane 4*), SKOV-6 (*lane 5*), and Caov-2 (*lane 6*); and from testis (*lane 7*). β-Actin gene expression was used as an internal control. *B,* evaluation of SPAG9 protein expression in ovarian cancer cells by indirect immunofluorescence microscopy. *a,* SKOV-6 cells revealed predominant cytoplasmic SPAG9 expression in fixed and permeabilized cell stained with anti-SPAG9 antibodies; *b,* surface localization of SPAG9 was observed in live SKOV-6 cells. Cells were stained with 4′,6-diamidino-2-phenylindole to show the nucleus. The fluorescence images were merged using Image-Pro Plus version 5.1. Original magnification, ×400; objective, 40×. *C,* fluorescence-activated cell sorting analysis of SPAG9 expression in SKOV-6 cells. *a,* side scatter (*SSC*) versus forward scatter (*FSC*) gate analysis; *b,* percentile fluorescence (displacement of fluorescence on *X axis*) of control cells stained with secondary antibody only; c, percentile fluorescence of SPAG9-expressing cells probed with anti-SPAG9 antibodies (*M1*). M1, gating of fluorescence-positive cells; M2, gating of fluorescence-negative cells.

expression was observed in 18 of 20 (90%) ovarian cancer samples (Table 1). Representative photomicrographs of SPAG9 staining in EOC tissue are shown in Fig. 3. As the results indicated, SPAG9 expression was observed in both the epithelial and stromal components of mucinous carcinoma tissues (Fig. 3B). In serous carcinoma samples, 15 of 17 displayed immunoreactivity throughout the tumor tissue section (Fig. 3C). In some tumors, SPAG9 staining was heterogeneous, with areas corresponding to invasive foci and regions with poor histologic differentiation displaying a stronger staining. Ovarian tissue section diagnosed with clear cell carcinoma also showed strong SPAG9 immunoreactivity (Fig. 3D).

Considering all 20 tissue samples analyzed by RT-PCR and immunohistochemistry in the present study, *SPAG9* mRNA and protein expression was observed in 18 of 20 (90%) patients of the tumors (Table 1). No positive immunostaining was observed in the absence of demonstrable mRNA expression by RT-PCR. All the *SPAG9* mRNA-positive specimens revealed a strong SPAG9 immunoreactivity in immunohistochemistry, and no discrepancy was observed between immunohistochemistry and RT-PCR results.

Antibody response to SPAG9 in ovarian cancer patients. Sera from 30 EOC patients were examined for SPAG9 antibody by ELISA. We first established the basal signals in the ELISA system using sera from 30 healthy donors (mean + 2 SD at $A_{492\ nm}$ = 0.1721 + 0.0600). Using the mean + 2 SD as the cutoff signal intensities at $A_{492\ nm}$, we found that high-titer IgG antibodies directed at SPAG9 protein were detectable by ELISA in EOC patients irrespective of disease stages (Fig. 4A). SPAG9 immunoreactivity was observed in 20 of 30 (67%) patients with different disease stages (Table 1). A demonstrable humoral response against SPAG9 was observed in 11 of 19 (58%) serous adenocarcinoma, in 2 of 2 (100%) clear cell carcinoma, and in 3 of 3 (100%) mucinous adenocarcinoma. Sera from 4 of the remaining 6 EOC patients, for which histotypes were not known, also showed immunoreactivity with recombinant SPAG9. It is interesting to note that 62.5% (5 of 8) of patients suffering from various histotypes with early stages of ovarian cancer (stages I and II) showed humoral response against SPAG9. Similarly, 68% (15 of 22) of patients suffering from later stages (stages III and IV) revealed *in vivo* immunogenicity against SPAG9 (Table 1).

Further, we evaluated the presence of SPAG9 antibody in the patient's serum by Western blotting. Specific SPAG9 antibodies were detected in sera from 20 of 30 (67%) ovarian cancer patients irrespective of malignant histotypes as shown in representative Western blot. However, no specific SPAG9 antibodies were detected in sera from healthy individuals (Fig. 4B). Further, the specificity of SPAG9 antibody from patient's sera was evaluated by carrying out neutralization experiment by including recombinant SPAG9 (15 μg/mL) in the incubation with the patient's serum, which resulted in loss of immunoreactivity with SPAG9 protein (Fig. 4B). Tumor stage information was available for 30 patients with adenocarcinoma (6 with stage I, 2 with stage II, 12 with stage III, and 10 with stage IV). As mentioned in Table 1, 62.5% of patients with stages I and II and 68% of patients with stages III and IV had SPAG9 antibodies, suggesting that the occurrence of antibodies was not a feature of advanced-stage disease.

SPAG9 gene expression and immune responses. Among the 30 patient's sera samples analyzed by ELISA, 67% of patients showed humoral response. Out of the total 30 patients, 20 patient's tissue samples were available for *SPAG9* expression analysis by RT-PCR and immunohistochemistry. Of these 20 tissue samples examined by RT-PCR and immunohistochemistry, both *SPAG9* mRNA and protein expressions were

observed in 18 of 20 (90%). SPAG9 antibody was present in 13 of 18 (72%) EOC patients with SPAG9-expressing tumors, suggesting that SPAG9 antibody was most likely generated as a consequence of *SPAG9* expression. The analysis of *SPAG9* expression and its humoral response in ovarian cancer patients of different histotypes and disease stages are presented in Table 1.

Discussion

Ovarian carcinoma remains the leading cause of cancer-related death due to lack of screening test for earlier detection of the disease when current therapies are most effective. Thus, improved methods are clearly needed to identify treatable, early-stage ovarian tumors. Numerous candidate ovarian cancer biomarkers have been identified to date by using cDNA microarrays (17, 18). However, for the vast majority of these genes, neither the expression pattern of the protein product nor its localization and function in the tumor tissues have been investigated. In this regard, we investigated the clinical utility of a well-characterized evolutionarily conserved sperm-specific SPAG9 protein, which acts as a scaffolding protein in mitogen-activated protein kinase signaling module during cell-cell interactions (8, 19, 20). In the present study, we analyzed the expression and *in vivo* immunogenicity of SPAG9 in EOC patients with its eventual application in the presymptomatic detection of ovarian cancer.

Cancer testis antigens are expressed in a variety of cancers with normal expression restricted to male germ cells in the testis but not in adult somatic tissues and, hence, represent ideal diagnostic and therapeutic targets. The present investigation reports for the first time SPAG9 expression and immunogenicity in EOC patients. The predominant SPAG9 expression in EOC patients was interesting when compared with other known potential cancer testis antigens. Sperm protein 17 mRNA has been reported to be expressed in 68% (17 of 25) of ovarian tumors, whereas immunohistochemical studies based on the results of the Northern blot analysis revealed that, in 12

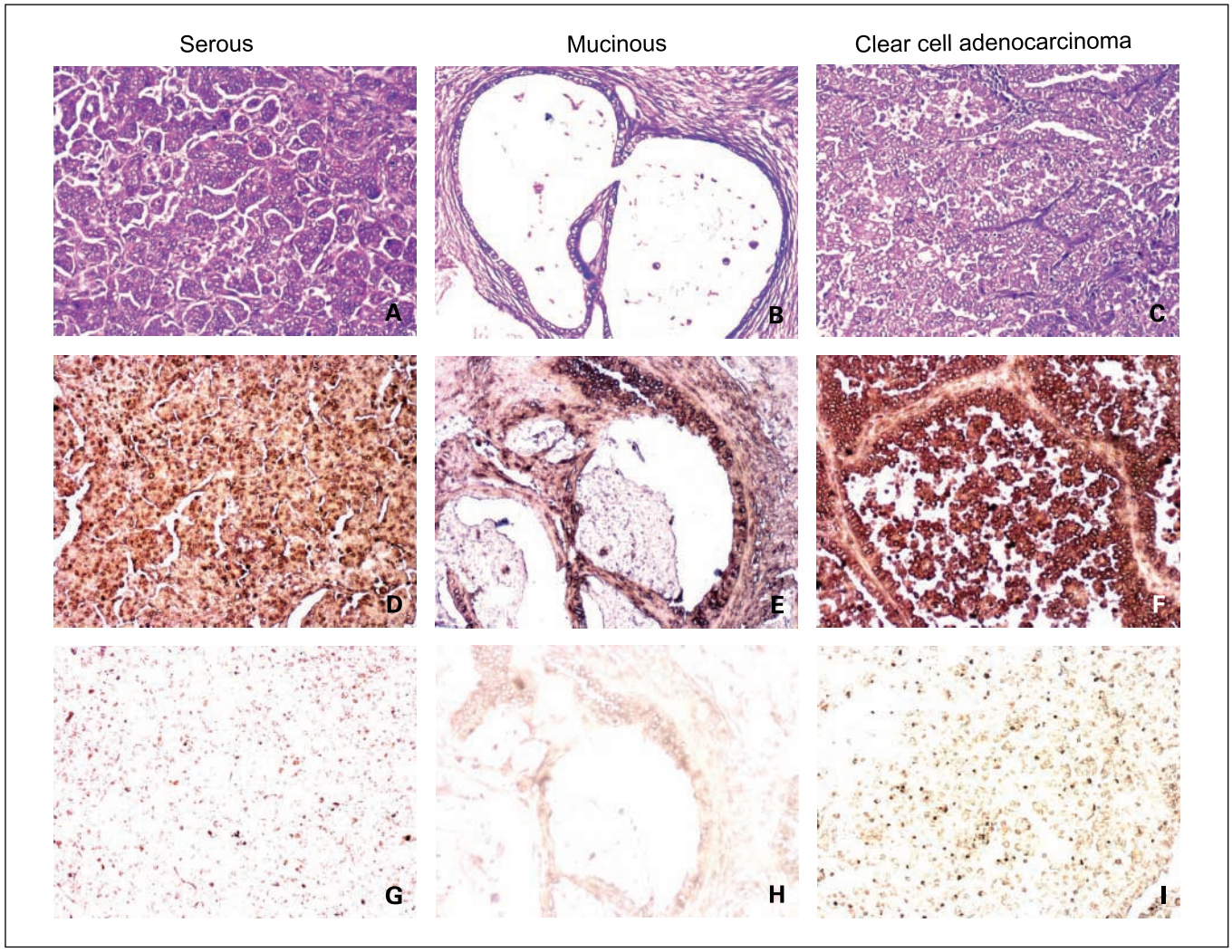

Fig. 2. *In situ* hybridization showing *SPAG9* expression in different subtypes of EOC tissue. Representative H&E-stained sections from serous (*A*), mucinous (*B*), and clear cell adenocarcinoma (*C*). Tissue sections from the same specimens were hybridized with digoxigenin-labeled SPAG9 antisense (*D*, serous; *E*, mucinous; *F*, clear cell adenocarcinoma) and sense (*G*, serous; *H*, mucinous; *I*, clear cell adenocarcinoma) riboprobes. Signal was developed using alkaline phosphatase nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Original magnification, ×200; objective, 20×.

paraffin-embedded ovarian tissue, 1 exhibited strong immunoreactivity, 4 showed moderate staining, 5 showed weak staining, 1 showed no staining, and 1 block had no tumor present. However, the authors did not elaborate on histotypes of EOC (21). SPAG9 expression was observed in both the epithelial and stromal components of mucinous carcinoma tissues, and the significance of this observation is not yet clear. Similar finding of stromal cell staining in few ovarian cancer samples was also reported earlier for sperm protein 17 expression (21). Another study on testis-specific synaptonemal complex protein-1 reported mRNA expression in only 15% of ovarian cancer specimens. However, immunohistochemical studies showed no detectable protein expression in synaptonemal complex protein-1 mRNA-positive ovarian tumor samples (22). A well-characterized cancer testis antigen NY-ESO-1 mRNA and protein expression was reported in 43% of EOC specimens (23). In contrast, *SPAG9* mRNA and protein expression was observed in (90%) of EOC tissues analyzed irrespective of disease stage and histotypes. This is one of the important criteria toward identifying tumor-specific protein targets for immunotherapy and development of cancer biomarker. Moreover, tumor stages have no effect on the frequency of SPAG9 expression in different malignant histotypes of epithelial ovarian tissues. The presence of SPAG9 expression in early-stage EOC tissues thereby ensures its important role as potential biomarker for early diagnosis of ovarian malignancies. Hence, SPAG9 is an important candidate molecule, which shows a considerable merit over other cancer testis antigens.

Our demonstration of humoral response in a significant proportion of EOC patients with SPAG9-expressing tumor is consistent with the known immunogenicity of this antigen. Our earlier studies have clearly shown that SPAG9 is highly immunogenic *in vivo*. Immunogenicity studies in rats showed the generation of antibodies against recombinant SPAG9 (8, 24). Subsequently, *SPAG9* DNA immunization studies in mice also showed specific immune response against SPAG9 (16). Recently, the immunogenic potential of recombinant SPAG9 protein was evaluated in nonhuman primate model (25) for its eventual implications in the development of human contraceptive vaccine. This is particularly relevant that similar humoral response against SPAG9 was observed in EOC patients. It is interesting to note that NY-ESO-1, the most immunogenic cancer testis antigen with well-documented spontaneous and vaccine-induced immunity (26), showed antibodies to NY-ESO-1 in only 12.5% of ovarian cancer patients (27). In another study, antibody response against synovial sarcoma on X chromosome 4 was observed in only 1 of 44 ovarian cancer patients (28). Similarly, no demonstrable antibody response was observed in synaptonemal complex protein-1–expressing ovarian cancer patients (22). However, the present study shows that SPAG9 antibody was detected in 67% of serum samples, indicating strong immunogenicity of SPAG9 protein in ovarian cancer patients. Furthermore, the expression of antibodies to NY-ESO-1, p53, HER2/neu, HOXB7, and MAGE family gene products is reported in late-stage ovarian cancer patients, and possible existence of these responses in a significant proportion of early-stage ovarian cancer patients remains unknown (2). Here, we showed that 62.5% of early-stage (I and II) and 68% of late-stage (III and IV) cancer patients generated significant proportion of immune response against SPAG9. This is an important finding where significant number (62.5%) of early-stage EOC patients exhibited strong antibody response against SPAG9 expression, supporting its potential role as a serum biomarker.

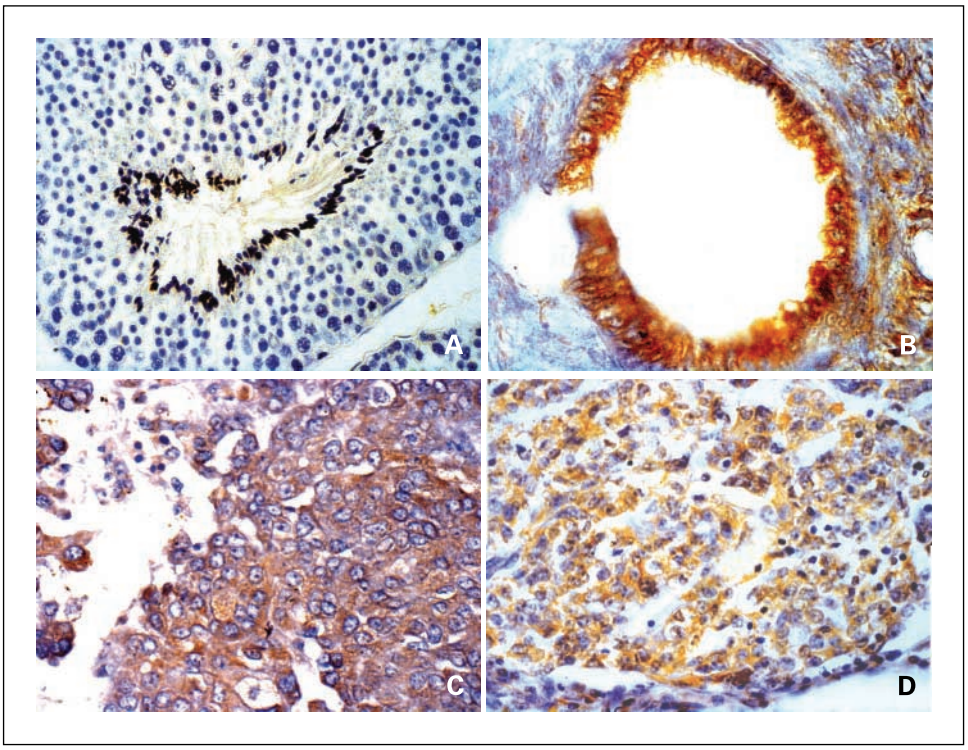

Fig. 3. Analysis of SPAG9 expression in EOC tissues. *A,* immunohistochemical staining of normal testis. SPAG9 protein was detected in the sperm head and not in any other cell type. *B* to *D,* cytoplasmic localization of SPAG9 protein was observed in mucinous (*B*), serous (*C*), and clear cell carcinoma (*D*). Original magnification, ×400; objective, 40×.

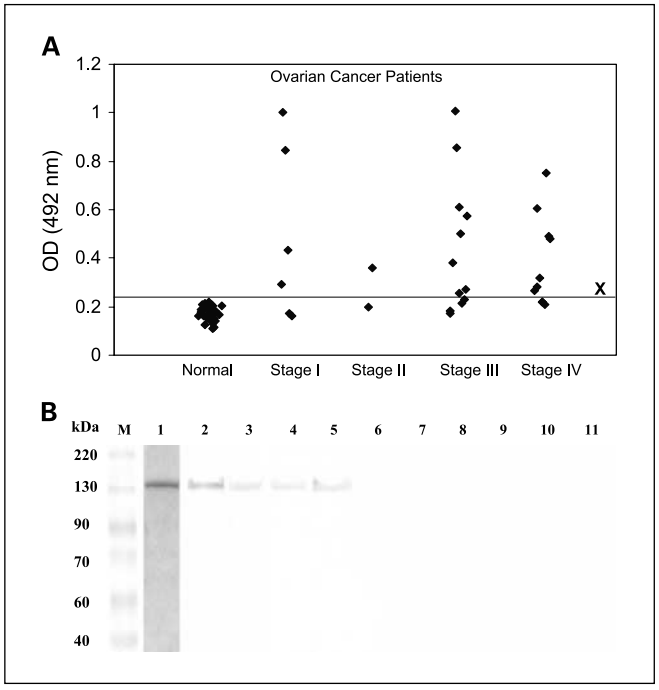

Fig. 4. Detection of SPAG9 antibody in serum from EOC patients. *A,* ELISA analysis of sera from healthy women and ovarian cancer patients from different stages. X, cutoff $A_{492\ nm}$ at mean + 2 SD from healthy donors for positivity above or negativity below the line. There is a significant difference in the seroreactivity between normal healthy donors and ovarian cancer patients ($P < 0.0001$). *B,* Western blotting analysis. Lane 1, affinity-purified recombinant SPAG9 stained with Coomassie brilliant blue; lane 2, immunoblot of recombinant SPAG9 shows a specific band of ~170 kDa with anti-SPAG9 antibody (positive control). Serum from serous (*lane 3*), mucinous (*lane 4*), and clear cell carcinoma (*lane 5*) showed specific immunoreactivity with recombinant SPAG9. Neutralization experiments were done by including recombinant SPAG9 (15 μg/mL) in the incubation with sera from serous (*lane 6*), mucinous (*lane 7*), and clear cell carcinoma (*lane 8*) patients, which resulted in loss of immunoreactivity. Sera from healthy individuals revealed no reactivity (*lanes 9-11*).

Yet, another important issue in tumorigenesis is tumor aggressiveness and development of distant metastasis in cancer patients. Identification of specific genetic markers that are associated with tumor aggressiveness may prove to be powerful new biomarkers to assess the progression of disease. A well-studied tumor suppressor gene, *p53*, which regulates the expression of genes involved in a variety of functions, including cell cycle arrest, DNA repair, and apoptosis (29), has been reported to be mutated in >50% of cancer patients (30). A recent study has shown that p53 down-regulated genes are expressed at lower levels in most of the p53 wild-type tumors relative to the p53 mutant-type tumors (15). The data further indicated the higher expression of p53-repressed genes, which included antiapoptotic genes (*BCL2A1* and *TNFAIP8*) and *SPAG9* gene in p53 mutant-type tumors. This was the first report where dysregulation of target genes was, in each case, significantly linked to the development of distant metastasis within 5 years of diagnosis of breast cancer patients (15). Therefore, SPAG9 represents an important molecule, whose expression characteristics in various cancer tissues could be potentially used as a molecular gauge of tumor aggressiveness and lower survival probability of cancer patients. Further studies are warranted to validate the clinical utility of SPAG9 in cancer diagnostics, tumor aggressiveness, and clinical outcome.

In conclusion, we have shown in the present study that majority of EOC tissues exhibited SPAG9 expression at both mRNA and protein levels. SPAG9-specific humoral response was found in various histotypes with different stages of ovarian cancer, indicating that SPAG9 is a promising candidate biomarker for early diagnostics, prognosis, and tumor aggressiveness. SPAG9 should be evaluated in additional studies, using large size samples, to confirm and expand our findings. In addition, the potential use of SPAG9 as a prognostic marker in tissue biopsies and in monitoring patients after surgery should explored.

Acknowledgments

We thank Professor A. Surolia (National Institute of Immunology, New Delhi, India) for his constant encouragement of this work and Dr. Kunle Odunsi for gifts of ovarian cancer cell lines.

References

1. Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. CA Cancer J Clin 2004;54:8–29.
2. Suri A. Cancer testis antigens—their importance in immunotherapy and in the early detection of cancer. Expert Opin Biol Ther 2006;6:379–89.
3. Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. Cancer Immun 2004;4:1.
4. Tureci O, Mack U, Luxemburger U, et al. Humoral immune responses of lung cancer patients against tumor antigen NY-ESO-1. Cancer Lett 2006;236:64–71.
5. Nakagawa K, Noguchi Y, Uenaka A, et al. XAGE-1 expression in non-small cell lung cancer and antibody response in patients. Clin Cancer Res 2005;11:5496–503.
6. Iwata T, Fujita T, Hirao N, et al. Frequent immune responses to a cancer/testis antigen, CAGE, in patients with microsatellite instability-positive endometrial cancer. Clin Cancer Res 2005;11:3949–57.
7. Shankar S, Mohapatra B, Suri A. Cloning of a novel human testis mRNA specifically expressed in testicular haploid germ cells, having unique palindromic sequences and encoding a leucine zipper dimerization motif. Biochem Biophys Res Commun 1998;243:561–5.
8. Jagadish N, Rana R, Selvi R, et al. Characterization of a novel human sperm-associated antigen 9 (SPAG9) having structural homology with c-Jun N-terminal kinase-interacting protein. Biochem J 2005;389:73–82.
9. Jagadish N, Rana R, Mishra D, Kumar M, Suri A. Sperm associated antigen 9 (SPAG9): a new member of c-Jun NH_2-terminal kinase (JNK) interacting protein exclusively expressed in testis. Keio J Med 2005;54:66–71.
10. Stone B, Schummer M, Paley PJ, et al. Serologic analysis of ovarian tumor antigens reveals a bias toward antigens encoded on 17q. Int J Cancer 2003;104:73–84.
11. Willis S, Hutchins AM, Hammet F, et al. Detailed gene copy number and RNA expression analysis of the 17q12-23 region in primary breast cancers. Genes Chromosomes Cancer 2003;36:382–92.
12. Kitahara O, Katagiri T, Tsunoda T, et al. Classification of sensitivity or resistance of cervical cancers to ionizing radiation according to expression profiles of 62 genes selected by cDNA microarray analysis. Neoplasia 2002;4:295–303.
13. Linn SC, West RB, Pollack JR, et al. Gene expression patterns and gene copy number changes in dermatofibrosarcoma protuberans. Am J Pathol 2003;163:2383–95.
14. Helm J, Enkemann SA, Coppola D, et al. Dedifferentiation precedes invasion in the progression from Barrett's metaplasia to esophageal adenocarcinoma. Clin Cancer Res 2005;11:2478–85.
15. Wei CL, Wu Q, Vega VB, et al. A global map of p53 transcription-factor binding sites in the human genome. Cell 2006;124:207–19.
16. Jagadish N, Rana R, Mishra D, Garg M, Selvi R, Suri A. Characterization of immune response in mice to plasmid DNA encoding human sperm associated antigen 9 (SPAG9). Vaccine 2006;24:3695–703.
17. Drapkin R, Hecht JL. The origins of ovarian cancer: hurdles and progress. Women's Oncol Rev 2002;2:261–8.
18. Terry KL, Sluss PM, Skates SJ, et al. Blood and urine markers for ovarian cancer: a comprehensive review. Dis Markers 2004;20:53–70.
19. Shankar S, Mohapatra B, Verma S, Selvi R, Jagadish N, Suri A. Isolation and characterization of a haploid germ cell specific sperm associated antigen 9 (SPAG9) from the baboon. Mol Reprod Dev 2004;69:186–93.

20. Jagadish N, Rana R, Selvi R, et al. Molecular cloning and characterization of the macaque sperm associated antigen 9 (SPAG9): an orthologue of human SPAG9 gene. Mol Reprod Dev 2005;71:58–66.

21. Straughn JM, Jr., Shaw DR, Guerrero A, et al. Expression of sperm protein 17 (Sp17) in ovarian cancer. Int J Cancer 2004;108:805–11.

22. Tammela J, Jungbluth AA, Qian F, et al. SCP-1 cancer/testis antigen is a prognostic indicator and a candidate target for immunotherapy in epithelial ovarian cancer. Cancer Immun 2004;4:10–6.

23. Odunsi K, Jungbluth AA, Stockert E, et al. NY-ESO-1 and LAGE-1 cancer-testis antigens are potential targets for immunotherapy in epithelial ovarian cancer. Cancer Res 2003;63:6076–83.

24. Jagadish N, Rana R, Mishra D, et al. Immunogenicity and contraceptive potential of recombinant human sperm associated antigen (SPAG9). J Reprod Immunol 2005;67:69–76.

25. Rana R, Jagadish N, Garg M, et al. Immunogenicity study of recombinant human sperm associated antigen 9 (SPAG9) in bonnet macaque (*Macaca radiata*). Hum Reprod 2006;21:2894–900.

26. Jager D, Stockert E, Karbach J, et al. Urine antibody against human cancer antigen NY-ESO-1. Cancer Immun 2002;2:10–8.

27. Stockert E, Jager E, Chen YT, et al. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. J Exp Med 1998;187:1349–54.

28. Hasegawa K, Koizumi F, Noguchi Y, et al. SSX expression in gynecological cancers and antibody response in patients. Cancer Immun 2004;4:16.

29. Vogelstein B, Lane D, Levine A. Surfing the p53 network. Nature 2000;408:307–10.

30. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science 1991;253:49–53.