RESEARCH ARTICLE

Sperm-Associated Antigen 9 is a Promising marker for Early Diagnosis of Endometrial Cancer

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Abstract

Background: Sperm-associated antigen 9 (SPAG9) has been recently proposed as a novel biomarker for early diagnosis of several human tumors, including ovarian, cervical and breast cancers. Its clinical value remains to be clarified for endometrial cancer (EC). In this study, we investigated the utility of serum SPAG9 levels in diagnosis of EC and its association with important clinicopathological parameters. Materials and Methods: This cross-sectional study was performed at a tertiary women's referral center in Ankara, Turkey. Preoperative serum samples were collected from patients surgically treated for endometrial cancer between June 2012-April 2013. Similar aged women with a biopsy proven benign endometrium were used as controls. Serum SPAG9 levels were measured with an enzyme-linked immunosorbent assay (ELISA) method and assessed for links with clinicopathological factors. Receiver operating characteristic (ROC) curve analysis was performed to assess power of SPAG9 levels for EC prediction. P values less than 0.05 were considered statistically significant. Results: A total of 63 women with EC and 27 with benign endometrium were included in the study. Mean age in the EC group was 58.7±1.1. Median SPAG9 levels in the EC and control groups were 18.3 (range, 12.7-53.8) and 14.1 (range, 4.3-65.3), respectively (p<0.001). A cut-off value of 17 ng/ml for SPAG9 predicted presence of malignant endometrium with 74% sensitivity and 83% specificity [Area under curve (AUC)=0.82, p<0.001]. SPAG9 levels did not demonstrate any significant association with histological type, FIGO stage, tumor grade, size, myometrial invasion, lymphovascular space invasion, cervical involvement, adnexal involvement, peritoneal cytology or lymph node status (all p>0.05). Conclusions: Testing for SPAG9 may be useful for early detection of EC in asymptomatic high-risk women. Its role in post-treatment follow-up and early detection of recurrence should be assessed in future trials.

Keywords: Endometrial cancer - sperm-associated antigen 9 - cancer testis antigens - early diagnosis

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Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy in developed countries with a reported mortality rate of 1.7-2.4 per 100,000 in 2008 (Jemal et al., 2011, Siegel et al., 2011). Markers for early detection of EC and prognosis prediction in treated patients have long been an area of interest, however to date, none of the proposed markers except CA125 gained acceptance in clinical settings (Chung et al., 2006, Yildiz et al., 2012).

Sperm-associated antigen 9 (SPAG9) is a newly discovered protein that belongs to cancer testis (CT) antigen family, and has significant functions in tumor development, cell growth, proliferation and apoptosis (Jagadish et al., 2005a, Yu et al., 2012). SPAG9 has been recently proposed as a novel biomarker for early diagnosis of several human tumors, including ovarian, cervical and breast cancer (Garg et al., 2007, Garg et al., 2009a, Kanojia et al., 2009). It is also expressed in endometrial cancer tissue, however its clinical value remains to be clarified (Yu et al., 2012). In this paper, we aimed to investigate the utility of serum SPAG9 levels in diagnosis of EC. We also assessed the associations between serum SPAG9 levels and clinicopathological risk factors in women with EC.

Materials and Methods

Study setting

This prospective cross-sectional study was performed between June 2012 and April 2013 at the gynecologic oncology department of Zekai Tahir Burak Women's Health Education and Research hospital in Ankara, Turkey. Institutional review board approval was obtained

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prior to patient recruitment.

Patient selection and serum collection

All participants provided written informed consent. Sixty-three women diagnosed with endometrial cancer within the study period were preoperatively included in the study group. Patients who received preoperative chemotherapy, radiotherapy or hormone therapy, who had a history of another malignancy or who did not accept to participate in the study were excluded. All women in the EC group underwent staging surgery including total abdominal hysterectomy, bilateral salpingo-oophorectomy (TAH-BSO), bilateral pelvic and paraaortic lymph node dissection and omentectomy. An experienced gynecologic pathologist analyzed pathologic specimens in a standardized fashion. Cases were staged according to International Federation of Gynecology and Obstetrics (FIGO) 2009 surgical staging system (Pecorelli, 2009).
A control group that included 27 women with a pathologically confirmed benign endometrium was constituted. These patients underwent TAH-BSO for benign indications (15 for myoma uteri, 10 for endometrial polyp and 2 for ovarian cyst). From each participant, 5 ml venous blood was drawn into a biochemistry tube. The tubes were centrifuged at 4000 rpm for 10 minutes. Serums were separated from the cellular components of blood, transferred to Eppendorf tubes, and stored at -80°C until analysis.

Measurement of SPAG9 in serum samples

A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) method (Human Sperm-associated antigen 9 ELISA Kit, Hangzhou, Eastbiopharm Co., Ltd., Hangzhou,China) was used for measuring SPAG9 concentrations in serum samples. Samples were processed according to the manufacturer's protocol. On the testing day, samples were allowed to thaw and were stabilized at room temperature. Initially, microspheres were incubated with standards, samples (40µl), controls (40µl) and SPAG9 detection antibody (10µl) in the 96-well microtiter filter plate for 60 minutes at 37°C. Streptavidin-HRP (50µl) solution was added to testing wells, and incubation was continued at 37°C for an additional 30 minutes. After the final wash, chromogen solutions (A and B) were added to wells and were again incubated for 10 minutes at 37°C. Reaction stopping solution (H_2SO_4) was then added to each well. Optical density measurements were made with 450 nm wavelengths, and corresponding sample concentrations were calculated. All serum samples from EC cases and controls were tested in duplicates, and the mean was used for final data analysis.

Data collection and statistical analysis

Data regarding clinical and pathological features were obtained from patient files and a hospital computer database. These features included patient age, body mass index (BMI), preoperative CA-125 level, tumor histology, FIGO stage, grade, size, myometrial invasion, lymphovascular space invasion, cervical and adnexal involvement, peritoneal washing cytology and status of the pelvic and paraaortic lymph nodes.

All statistical analyses were performed using SPSS 17.0 statistical software package (SPSS Inc., Chicago, Ill, USA). Continuous variables were expressed as mean ± standard deviation (SD), and categorical variables were expressed as number and percentage. Kolmogorov-Smirnov test was used to assess normal data distribution. Comparisons between groups were performed with Student's t-test, Mann-Whitney U, and Kruskal-Wallis tests, as appropriate. Receiver operating characteristic (ROC) curve was constructed for determining optimal cut-off points. We further combined SPAG9 and CA125 to assess the sensitivity and specificity of this combined approach in EC diagnosis. If any of the markers were above the predefined cut-off limit, the combined test was considered positive. P values less than 0.05 were considered statistically significant for all statistical analyses.

Results

A total of 90 patients [63 endometrial cancer (EC group) and 27 controls] were included in the study. None of the patients in the EC group had been diagnosed with a malignancy other than EC. Mean patient ages in EC and control groups were 58.6±9.1 and 54.8±7.9 years, respectively (p>0.05). Mean BMI in the EC and control groups were 31.2±4.2 kg/m^2 and 30.4±3.6 kg/m^2 (p>0.05). Median CA125 level in the EC group was 12.8 (range, 1.4-354) and in the control group it was 9.0 (range, 2.9-40) U/ml (p=0.008).

Median SPAG9 levels in the EC and control groups were 18.3 (range, 12.7-53.8) and 14.1 (range, 4.3-65.3), respectively (p<0.001) (Figure 1). SPAG9 levels were also

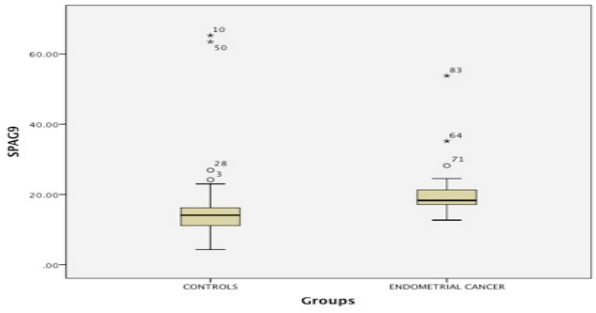

Figure 1. Boxplot Chart Demonstrating Sperm-Associated Antigen 9 (SPAG9) Levels in Controls and Endometrial Cancer (EC) Cases.

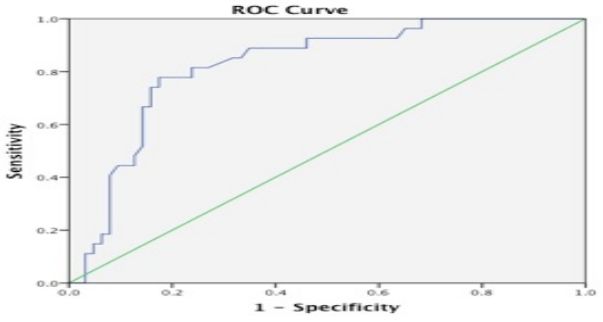

Figure 2. Receiver Operating Characteristic (ROC) Curve for Sperm-Associated Antigen 9 (SPAG9) in Endometrial Cancer Prediction.

significantly higher in FIGO stage I EC cases [median 16.3, range 12.7-44.7)] when compared to controls [median 14.1 (range, 4.3-65.3)] ($p<0.001$). Pathological features and serum SPAG9 levels in the EC group are presented in Table 1. Serum SPAG9 levels did not differ among divided groups according to histological type, FIGO stage, tumor grade, size, myometrial invasion, LVSI, cervical involvement, adnexal involvement, peritoneal cytology and lymph node status ($p>0.05$). According to their preoperative CA125 levels, 50 patients (79.3%) had CA125 <35 U/ml, and 13 patients (20.7%) had CA125 ≥35 U/ml. SPAG9 levels were similar between these groups (Table 1). SPAG9 levels were also similar between clinical types I and II (Table I).

ROC curve analysis was performed to determine optimal SPAG9 cut-off values in predicting EC (Figure 2). A cut-off value of 17 ng/ml for SPAG9 predicted presence of malignant endometrium with 74% sensitivity, 83% specificity, 88% positive predictive value (PPV), and 64.5% negative predictive value (NPV) [Area under curve (AUC)=0.82, $p<0.001$). SPAG9 and CA125 were combined as a single test with predefined cut-offs of 17 ng/ml and 35U/ml, respectively, and the test was considered positive when either one of the tests were above these cut-off values. According to this classification, 60 (66.7%) of the study patients had a positive combined test result, whereas 30 (33.3%) had a negative result. Sensitivity, specificity, PPV and NPV in EC detection of this model was 82.5%, 70.4%, 86.7% and 63.3%, respectively.

Table 1. Pathological Features, Sperm-Associated Antigen 9 (SPAG9) Levels in Endometrial Cancer Cases

Feature	N (%)	Serum SPAG9 a Median (Range) (ng/ml)	Serum SPAG9 a Mean ± SD (ng/ml)	P
Histological type				
Endometrioid	53 (84.1)	14.1 (4.3-65.3)	15.6±10.6	>0.05
Serous-papillary	6 (9.5)	14.8 (11.1-23.0)	15.3±4.3	
Clear-cell	4 (6.4)	14.2 (9.7-15.9)	13.5±6.7	
Clinical type				
Type I	53 (84.1)	14.1 (4.3-65.3)	15.6±10.6	>0.05
Type II	10 (15.9)	14.2 (9.7-23.0)	14.6±3.7	
FIGO stage				
I	42 (66.7)	14.0 (4.3-23.0)	13.6±4.9	>0.05
II	10 (15.9)	14.0 (9-65.3)	15.7±7.8	
III	11 (17.5)	14.8 (9.0-26.9)	23.1±8.3	
Tumor grade				
I	33 (52.4)	14.7 (4.3-63.5)	15.2±9.2	>0.05
II	18 (28.6)	13.6 (9-65.3)	17.01±12.8	
III	12 (19.0)	14.0 (4.9-26.9)	13.9±5.8	
Tumor size				
<2cm	24 (38.1)	14.8 (4.3-23.0)	14.4 ± 3.7	>0.05
≥2cm	39 (61.9)	13.6 (4.9-65.3)	16.1±12.1	
Myometrial invasion				
<1/2	42 (66.7)	14.5 (4.3-63.5)	15.08±8.3	>0.05
≥1/2	21 (33.3)	13.0 (4.9-65.3)	16.3±12.3	
LVSI b				
Negative	46 (73)	14.8 (4.3-65.3)	16.08±11.02	>0.05
Positive	17 (27)	12.8 (7.1-26.9)	13.9± 5.2	
Cervical involvement				
Negative	48 (76.2)	14.0 (4.3-24.2)	13.9±3.9	>0.05
Positive	15 (23.8)	14.3 (9.0-65.3)	20.7±8.3	
Adnexal involvement				
Negative	57 (90.5)	13.9 (4.3-65.3)	15.3±10.1	>0.05
Positive	6 (9.5)	15.6 (9.0-26.9)	17.1±7.1	
Peritoneal cytology				
Negative	60 (95.2)	14.0 (4.3-65.3)	13.4±4.9	>0.05
Positive	3 (4.8)	14.8 (9.0-16.5)	15.6±9.2	
Lymph node metastasis				
Negative	55 (87.3)	14.1(4.3-65.3)	13.1±6.8	>0.05
Only pelvic	5 (7.9)	16.5 (9.0-26.2)	15.9±5.7	
Paraaortic	3 (4.8)	13.6 (11.1-16.8)	15.6±9.2	
CA 125 level (U/ml)				
<35 U/ml	50 (79.3)	13.7 (9.0-17.7)	13.4±2.5	>0.05
≥35 U/ml	13 (20.7)	14.5 (4.3-65.3)	16.03±10.9	

[a] perm-associated antigen 9, [b]Lymphovascular space invasion

Discussion

Cancer testis (CT) antigens are a unique class of tumor antigens that are not expressed in normal tissues, except the testis (Suri, 2006). Their restricted gene expression in various malignancies has led the researchers to investigate their potential as a target for immunotherapeutic modalities, such as antigen based vaccination and antigen directed immunotherapy (Suri, 2006). Moreover, humoral and cellular immune responses to CT antigens have directed attention to their potential in early diagnosis of cancer (Suri, 2006, Garg et al., 2009a, Garg et al., 2009b, Kanojia et al., 2009).

Sperm-associated antigen 9 (SPAG9) is a recently discovered CT antigen and a new member of c-Jun NH2-terminal kinase (JNK) interacting protein family, with a functional role in sperm-egg fusion and mitogen-activated protein kinase signaling pathway (Jagadish et al., 2005a). SPAG9 gene is located at the chromosome 17q21, which is a region involved in amplification and expression of cancer-related genes (Jagadish et al., 2005b). Although it was originally thought to be expressed exclusively in testis, ongoing research revealed SPAG9 expression in numerous tumors originating from various sites and organs such as brain, thyroid, breast, lung, kidney, colon, ovary, cervix, and endometrium (Garg et al., 2008, Garg et al., 2009a, Garg et al., 2009b, Kanojia et al., 2009, Kanojia et al., 2011, Yu et al., 2012, Wang et al., 2013, Yi et al., 2013). In a study by Garg et al. (2007), SPAG9 expression and host immune responses were investigated in epithelial ovarian cancer (EOC) specimens and human ovarian cancer cell lines (Garg et al., 2007). Thirty EOC cases were included in this study. SPAG9 protein expression was observed in 18 of 20 (90%) cases. In 20 out of 30 (67%) cases, a demonstrable immune response was observed. Interestingly, 5 of 8 (62.5%) cases with early stage (FIGO stage I-II) disease showed humoral response to SPAG9. The authors concluded that SPAG9 was a significant biomarker for early detection of EOC (Garg et al., 2007). In a later study, SPAG9 expression was assessed in 66 cervical cancer cases and 54 adjacent non-cancerous tissues (Garg et al., 2009a). SPAG9 expression was observed in 54 of 66 (82%) of cervical cancer tissues, but not in non-cancerous tissues. Tissue expression was independent from disease stage, however it correlated with tumor grade. SPAG9 antibodies were detected in 80% of the cases, however there were no significant associations between humoral response and disease stage. In conclusion, taking into account its frequent expression and high potential of creating a host immune response, SPAG9 was suggested as a useful marker in detection of early stage cervical cancer (Garg et al., 2009a).

In a very recent study by Yu et al. (2012), SPAG9 in endometrial cancer was investigated for the first time in literature (Yu et al., 2012). The authors analyzed 47 cases with EC and 44 cases with a non-cancerous endometrium. Similar with previous studies, SPAG9 tissue expression was noted in EC tissues, but not in healthy adjacent tissues. They also found that SPAG9 was expressed in metastatic lymph nodes. Cases with grade 3 tumors had significantly higher SPAG9 levels in their serums, when compared to cases with grade 1-2 tumors (Yu et al., 2012). They stated that SPAG9 levels were similar between cases of endometrial hyperplasia and completely normal endometrium. However, serum SPAG9 values in EC cases (median 11.7 ng/ml, range 2.4-60.5 ng/ml) were significantly higher than the remaining benign cases (Yu et al., 2012). Finally, the authors concluded that SPAG9 was positively expressed in EC, and could serve as a marker in EC for diagnosis and treatment.

In our study, we investigated serum SPAG9 levels in women with endometrial cancer. Similar with the previous study by Yu et al. (2012), we found that SPAG9 levels were significantly higher in EC (Yu et al., 2012). We also found that FIGO stage I cases had higher SPAG9 levels than controls. This finding may be important for women undergoing endometrial evaluation for abnormal uterine bleeding, especially in the presence of equivocal endometrial biopsy results. Our results also suggested that SPAG9 might serve as a useful marker for early diagnosis of EC, in asymptomatic high-risk women such as Tamoxifen users, women with Lynch syndrome and BRCA mutation.

Differently from the previous study by Yu et al. (2012) we assessed associations between SPAG9 and all clinicopathological prognostic parameters (Yu et al., 2012). However, we did not observe significantly different SPAG9 levels according to risk factor groups. Although mean SPAG9 levels were higher in stage III EC and cases with cervical involvement, these differences did not reach a statistical significance. These data should be further assessed and clarified. It is well known that the two different clinical types of EC (Type I and II) have different biological behavior patterns. In this context, we compared SPAG9 levels between these two types. Although median SPAG9 levels did not demonstrate a statistically significant difference, these findings also should be further evaluated.

Combined use of SPAG9 and CA125 in EC detection was also investigated in this study. SPAG9 cut-off was set as 17ng/ml and CA125 cut-off was set as 35U/ml. Combined use of these tests raised the sensitivity to 82.5% from 74% in EC detection. However, specificity drop was also significant (70 *vs* 83%). Therefore, these two tests could be used in combination to increase sensitivity, in the expense of increased false positive tests and unnecessary interventions. A multimodality risk scoring system including clinical and laboratory variables could increase accuracy in EC detection.

In conclusion, SPAG9 is a promising marker for early detection of endometrial cancer. Future studies with larger sample sizes are needed to reach a final conclusion on its value in post-treatment monitoring and recurrence detection capability in women with EC. Considering the previous reports from the literature, SPAG9 is not a marker solely specific for EC detection. Accompanying malignancies of other organ systems should also be considered, especially in the presence of suspicious clinical findings.

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