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Article

SCP-1 cancer/testis antigen is a prognostic indicator and a candidate target for immunotherapy in epithelial ovarian cancer

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SCP-1 is a novel tumor antigen that belongs to the growing family of cancer/testis (CT) antigens, and it is a potential target for immunotherapy. In an effort to determine the expression of *SCP-1* in epithelial ovarian cancer (EOC), one-step RT-PCR was performed with RNA from epithelial ovarian tumor tissues and with two normal ovarian surface epithelial cell lines. We used immunohistochemistry (IHC) to investigate SCP-1 expression in paraffin-fixed EOC samples and ELISA to test sera from a subgroup of patients for SCP-1 antibody. *SCP-1* was expressed in 15 out of 100 (15%) primary tumors, as determined by RT-PCR. The normal ovarian surface epithelial cell lines were negative for *SCP-1* expression, as were a panel of other normal tissues. None of the patients whose tumors were determined to be *SCP-1* positive by RT-PCR expressed the antigen by IHC or demonstrated a humoral immune response by ELISA. Tumors that expressed *SCP-1* mRNA tended to have a higher grade than those that did not ($P = 0.03$). There was a significant decrease in survival time ($P = 0.004$) for patients with *SCP-1* mRNA-positive tumors compared to those with *SCP-1* mRNA-negative tumors [median 25 mo, 95% confidence interval (CI) 0-56 mo; and median 97 mo, CI 32-162 mo, respectively]. The present study shows that *SCP-1* mRNA expression in patients with EOC is associated with a poorer chance of survival. These findings imply that further evaluation of SCP-1 as a potential target for vaccine therapy in EOC is warranted.

Introduction

EOC is the leading cause of gynecologic cancer deaths in the United States (1). While it is clear that most patients with EOC will respond to platinum- and paclitaxel-based chemotherapy, including complete responses, the relapse rate is approximately 85% within two years (2). Once relapse occurs, there is no known curative therapy, and management becomes primarily palliative. Thus, there is a need to develop additional therapeutic approaches for the management of this disease. A proposed strategy for minimizing the risk of recurrent disease is immunotherapy. Patients who demonstrate a complete response to frontline surgery and chemotherapy could be considered for immunotherapy, with the presumption that most do, in fact, have micrometastases. The development of successful immunotherapeutic strategies requires the identification and characterization of ovarian tumor-associated antigens that will be recognized by the host immune system, leading to tumor rejection. In EOC, there are few antigens other than CA-125 that are known to be associated with the disease. Therefore, there is a need to identify and characterize novel antigens that may be useful for an immunotherapy/vaccine approach toward this cancer (3).

As a consequence of recent advances in the approaches for analyzing humoral (4) and cellular (5) immune reactivity to cancer in the context of the autologous host, a number of tumor antigens recognized by CD8+ T cells (6) and/or antibodies (7) have been identified. A unique class of differentiation antigens, the CT antigens (8), are not expressed in normal tissues except for testis and, in some cases, placenta. This fact makes CT antigens especially attractive targets for specific immunotherapy of cancer. This family of antigens continues to expand and includes MAGE (9), GAGE/PAGE/XAGE (10, 11), NY-ESO-1/LAGE-1 (12, 13), SSX (14, 15, 16), SPANX (8, 17), TRAG-3 (18), BAGE (19), OY-TES-1 (20), CT17 (21), NY-BR-3 (22) and SCP-1 (23). The function of the majority of the CT antigens is currently unknown. The SCP-1 antigen (HOM-TES-14) is of particular interest because it is one of the few CT antigens with a known role in gamete development. It is a synaptonemal complex protein that is involved in chromosomal reduction during meiosis (24) and is expressed in a variable pattern in a number of human tumors (23). To our knowledge, *SCP-1* expression has not been studied in EOC. In a continuing effort to identify CT antigens that could be useful as targets for antigen-specific immunotherapy of EOC, we have analyzed a large panel of ovarian and peritoneal cancers for *SCP-1* expression. A secondary objective was to examine the relationship between *SCP-1* expression and clinical outcome.

Results

Study population

The characteristics of the study population are presented in Table 1. The median age of the study population was 63 yr (range 22-88 yr), and the median duration of follow-up was 24 mo (range 0.01-108 mo). As expected, the majority of patients presented with grade 3 tumors (84%), at stage IIIC (75%) and with serous histology (73%). A complete response to therapy was achieved in 52 of the 100 patients (52%), a partial response

was achieved in 41 patients (41%), and the remaining patients had no response. The median survival time for all patients was 57 mo (CI 26-88 mo), while the median disease-free survival time, excluding patients with persistent/progressive disease after initial therapy, was 21 mo (CI 17-24 mo).

Expression of *SCP-1* mRNA in EOC

Expression of *SCP-1* mRNA in ovarian cancer cell lines, normal ovary cell lines, a panel of normal tissues, and in epithelial ovarian tumor specimens was investigated by RT-PCR (Figure 1). The PCR products were of heterogeneous intensities, and some specimens yielded only faint amplicon bands. These were scored positive only if the result could be reproduced by a repeated RNA extraction and specific RT-PCR from the same tumor specimen. Cases with very low transcript levels, which were not reproducibly positive, were not regarded as positive. The *SCP-1* transcript was confirmed by sequencing the amplified PCR products. The normal ovarian surface epithelial cell lines, IOSE and HOSE, as well as the normal tissue panel of ovary, colon, spleen, thymus, leukocyte, prostate, and small intestine did not express *SCP-1* mRNA. Also, none of the four ovarian cancer cell lines, OVCA432, OVCA429, SKOV3, and OVCAR3, were positive for the antigen. *SCP-1* mRNA expression was detected in 15/100 (15%) of tumor specimens.

Immunohistochemical staining of SCP-1

SCP-1 exhibited intense immunohistochemical staining in testis (Figure 2), and the staining was restricted to germ cells. No reactivity in other structures of the testis was noted. The germ cells revealed staining that was present in the dividing cells and hence corresponded to the presence of the synaptonemal complex protein. None of the 15 *SCP-1* mRNA-positive tumor samples showed protein expression by IHC. An additional set of 30 specimens that were negative by RT-PCR also did not demonstrate immunohistochemical staining.

Antibody response to SCP-1 in ovarian cancer patients

A total of 15 serum samples were analyzed by ELISA for SCP-1 antibodies. The samples tested were from those patients who expressed *SCP-1*, as detected by RT-PCR. These sera consisted of preoperative and serial specimens obtained during the course of the patients' disease (range 1-3 yr). There was no demonstrable antibody response to SCP-1 in any of the 15 patients.

Correlation of *SCP-1* expression with clinical outcome

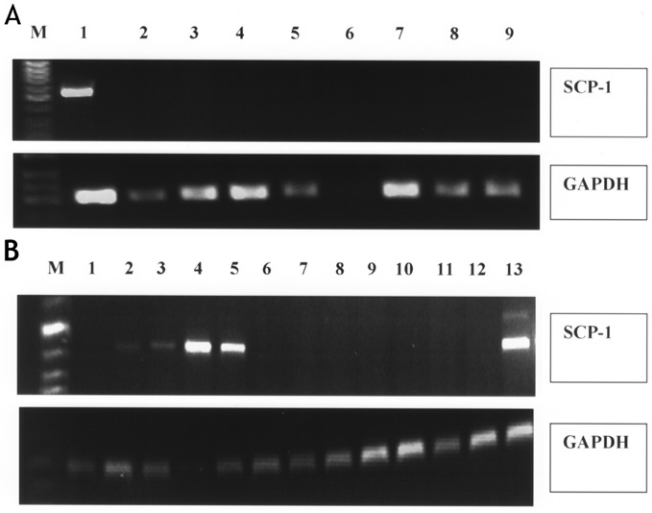
Our analysis of CT antigen expression and clinicopathological characteristics is presented in Table 2. Patients whose tumors expressed *SCP-1*, as detected by RT-PCR, had a median disease-free survival time of 14 mo (CI 11-17 mo), compared with 21 mo (CI 19-23 mo) for patients whose tumors did not express *SCP-1* mRNA ($P = 0.23$). Patients whose tumors expressed *SCP-1*, as detected by RT-PCR, had a significantly worse median overall survival time of 25 mo (CI 0-56 mo), compared to 97 mo (CI 32-162) for patients whose tumors did not express *SCP-1* mRNA ($P = 0.004$). A plot of the estimated survival curves for patients with *SCP-1* mRNA-positive and SCP-1 mRNA-negative tumors is shown in Figure 3. Patients with *SCP-1* mRNA-positive tumors tended

Table 1
Patient characteristics.

Characteristic	No. of Patients
Evaluable patients	100
Age (median, range)	62.3 (63.0, 22-88 years)
Follow-up [a] (median, range)	29.7 (24.1, 0.01-108 months)
FIGO stage:	
1a	4 (4%)
1b	1 (1%)
1c	3 (3%)
IIb	2 (2%)
IIc	4 (4%)
IIIa	1 (1%)
IIIb	2 (2%)
IIIc	76 (75%)
IV	7 (7%)
Histology:	
Papillary serous	73 (73%)
Clear cell	7 (7%)
Endometrioid	5 (5%)
Mucinous	2 (2%)
Undifferentiated	3 (3%)
Other (transitional, mixed, carcinosarcoma)	10 (10%)
Grade:	
1	7 (7%)
2	9 (9%)
3	84 (84%)
Response to frontline therapy:	
Complete response	52 (52%)
Partial response	41 (41%)
Progression	1 (1%)
Unknown	6 (6%)
Recurrences	
Recurrence/persistent disease	62 (62%)
Current status:	
Alive with no evidence of disease	35 (35%)
Alive with disease	31 (31%)
Died of disease	31 (31%)
Died from other causes	3 (3%)
CT Antigen status (RT-PCR)	
SCP-1 positive	15/100 (15%)

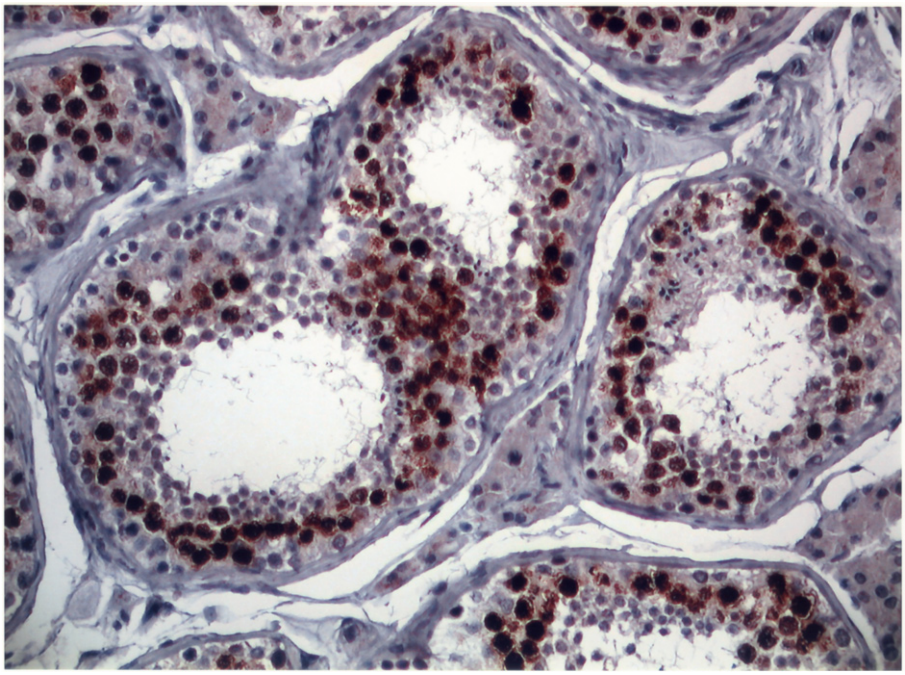
[a] The median survival for all patients was 57 months (CI 26-88 months).

Figure 1



RT-PCR analysis of *SCP-1* mRNA expression. (A) *SCP-1* mRNA expression in normal tissues. Testis (lane 1), followed by a panel of normal tissues: ovary (lane 2), colon (lane 3), spleen (lane 4), thymus (lane 5), negative control (lane 6), leukocyte (lane 7), prostate (lane 8), small intestine (lane 9). (B) Representative RT-PCR results of SCP-1 expression in EOC. Samples from left to right are EOC1-12. Lanes 2-5 are positive; lanes 1 and 6-12 are negative. Lane 13 is a positive control (testis).

Figure 2



IHC staining of SCP-1 in normal human testis. Positive testicular germ cells show diffuse cytoplasmic reactivity.

to have a tumor of higher grade than those patients who did not express *SCP-1* mRNA ($P = 0.03$). There were no significant differences in patients with *SCP-1* mRNA-positive tumors with regards to age, stage, or histology. Multivariate analysis for survival by stage, *SCP-1* mRNA expression, age, grade, and response showed that only tumor expression of *SCP-1* mRNA and partial response to chemotherapy were independent adverse prognostic variables ($P < 0.001$).

Discussion

The development of strategies for actively stimulating immunological rejection of tumors, previously an elusive goal, has been accelerated by our recently improved understanding of the molecular basis for immune recognition and immune regulation of cancer cells. It is now well known that the immune system has the ability to recognize tumor-associated antigens displayed on human malignancies and to direct cytotoxic responses to these targets. In EOC, support for the role of immune surveillance of tumors comes from recent observations that the presence of infiltrating T lymphocytes in tumors is associated with longer overall progression-free survival in patients (25). Since these infiltrating T cells are likely to be antigen-specific, it is important to identify targets that could be utilized to further augment and/or induce the antitumor immune response. In this regard, the CT antigens are ideal candidates for immunotherapy because most are expressed at high levels in male germ cells but otherwise have only aberrant expression in a variable proportion of a wide range of different cancer types. We previously found that *NY-ESO-1* is expressed in approximately 40% of EOCs (26). In the current study, we extend our observations on the expression of CT antigens in EOC.

We found that *SCP-1* expression was detected by RT-PCR in 15% of ovarian cancer cases, which is similar to the 14% of melanoma cases reported by Tureci *et al.* (23). In contrast, Luo *et al.* (27) reported that *SCP-1* was detected by RT-PCR in 6/21 (29%) of hepatocellular carcinoma specimens. In the report by Jager *et al.* (22), SCP-1 expression was detected by RT-PCR in 2/13 breast cancers, 4/5 renal cancers, 1/5 melanomas, 1/4 prostate cancers, and 1/4 transitional cell carcinomas, corresponding to a range of 15-80%. Finally, Mashino *et al.* (28) demonstrated a frequency of 1/46 (2%) for esophageal carcinoma, 24/102 (24%) for gastric carcinoma, 0/98 (0%) for colorectal carcinoma, and 44/129 (34%) for breast cancer.

Protein expression of SCP-1, as detected by IHC, has been investigated previously in pancreatic carcinoma (29) and hepatocellular carcinoma (27). In these reports, the anti-SCP-1 antibody, SC554, was used for IHC analysis of archived specimens. All 10 pancreatic adenocarcinoma specimens that were positive by RT-PCR demonstrated heterogeneous protein expression of SCP-1 (29). Similarly, in hepatocellular carcinoma, *SCP-1* expression at the mRNA level as detected by RT-PCR correlated well with the demonstration of SCP-1 positive tumor cells by immunohistology (27). The positive cells exhibited fine granular cytoplasmic staining, and some tumors demonstrated spotted nuclear reactivity. It is unclear why none of the ovarian carcinoma tumor specimens found to express *SCP-1* at the mRNA level were found to express it at the protein level. Since the antibody used in our study (SC554) stained normal human testes (positive control) appropriately and had previously been shown to stain tumor tissues (27, 29), our results are unlikely to be due to technical issues. It is possible that, at least in ovarian cancer, there is posttranslation modification of the SCP-1 protein such that the SC554 antibody cannot recognize changes in the epitope. It is also possible that the *SCP-1* gene is not even translated in ovarian tumor tissue. Clearly, additional studies would be needed to clarify the mechanisms of *SCP-1* expression in EOC.

The only study to investigate serum reactivity to SCP-1 in patients with cancer was reported by Tureci *et al.* (23). The authors found that 3/6 (50%) of breast cancer patients and 1/31 (3%) of renal cancer patients had an antibody response to SCP-1. Spontaneous humoral immune response to cancer-germline genes detected mostly range between 1-2% (*MAGE, SSX-2*) and 9.5% (*NY-ESO-1*) (8, 30, 31, 32). More recently, we found that 25% of patients with ovarian cancer who were positive by RT-PCR for *NY-ESO-1* or *LAGE-1* had a demonstrable antibody response (26). While the lack of spontaneous humoral immune response to SCP-1 in the current study could be due to the small number of patients with *SCP-1* expressing tumors available for analysis, the possibility of altered expression of the protein in EOC and subsequent altered antibody response may also explain this finding. Alternatively, the expression of the SCP-1 antigen, whether altered or not from the wild type, may not be expressed at a high enough level for the immune system to mount a detectable response.

It is of particular interest that we found the first evidence of a relationship between CT antigen expression and survival in EOC, independent of other known prognostic indicators. The median survival time of women whose tumors expressed *SCP-1* was significantly less than that of women whose tumors lacked expression (25 mo versus 97 mo, respectively, $P = 0.004$). This raises questions about the possible role of SCP-1 in tumorigenesis, invasion, and metastasis in EOC. While SCP-1 has a known role in gametogenesis, its role in cancer, along with the majority of CT antigens, is unknown. In addition, since we were unable to demonstrate evidence of a humoral immune response in patients with *SCP-1* mRNA-positive tumors, the relationship of any spontaneous immunity to SCP-1 to shortened survival time is not clear at this time. Nevertheless, the fact that the expression of SCP-1 is associated with poorer survival times would suggest that the antigen may play a role in tumor progression in EOC.

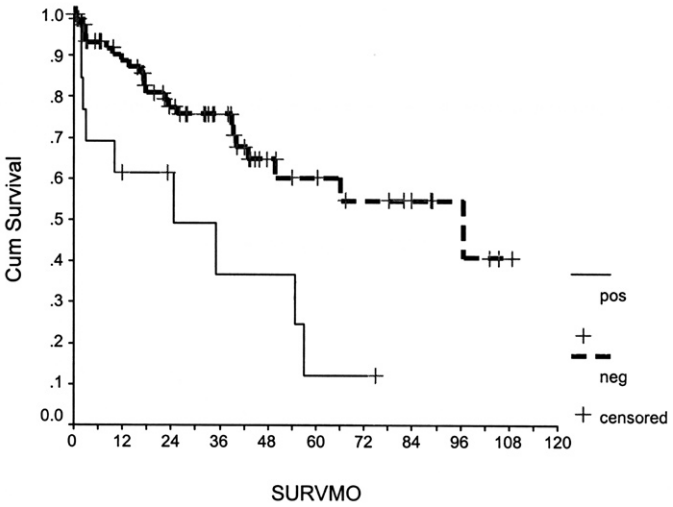
In summary, we conclude that further evaluation of SCP-1 as a potential target for vaccine therapy in EOC is warranted because of its restricted expression in normal tissues and its aberrant expression in tumor tissues. This antigen is of special interest among CT antigens because its expression is associated with a poorer clinical outcome in EOC. While the lack of protein expression detectable by IHC and the inability to demonstrate a spontaneous humoral immune response in EOC patients may raise questions about the suitability of SCP-1 as a vaccine target, the characterization of the protein translated in EOC, as well as the analysis of SCP-1 specific cytotoxic T cells in EOC, would shed additional light on the nature of any induced immune response in EOC. In addition, even if SCP-1 is poorly immunogenic, it should be possible to develop strategies to improve the immunogenicity of the antigen. Finally, the fact that SCP-1, like most other CT antigens, is expressed in only a small subset of patients with EOC, underlines the need to uncover additional antigenic targets for immunotherapy in this disease.

Table 2

Correlation between *SCP-1* expression and clinico-pathological features in EOC.

Pathological and Clinical Features	No. of Patients	
	SCP-1 Positive	*SCP-1* Negative
All tumors (n=100)	15 (15%)	85 (85%)
Age (median, range)	68 (68, 46-86)	61 (62, 22-88 yrs)
Tumor grade:		
1	-	6
2	4	5
3	11	74
FIGO stage:		
Ia	1	3
Ib	-	1
Ic	-	3
IIb	-	2
IIc	-	4
IIIa	-	1
IIIb	-	2
IIIc	13	63
IV	1	6
Histology:		
Papillary serous	13	60
Clear cell	-	6
Endometrioid	-	5
Mucinous	-	2
Undifferentiated	-	3
Other (transitional, mixed, carcinosarcoma)	1	9
Response:		
Complete response	7 (47%)	45 (53%)
Partial response	7 (47%)	33 (39%)
Total Recurrences/Persistent disease	9 (60%)	52 (61%)
Died of disease	9 (60%)	22 (26%)
Alive with disease	2 (13%)	29 (34%)
Alive with no evidence of disease	3 (20%)	32 (38%)

Figure 3



Kaplan-Meier estimates of overall survival in EOC patients according to *SCP-1* expression

Abbreviations

CI, confidence interval; CT, cancer/testis; EOC, epithelial ovarian cancer; IHC, immunohistochemistry

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Materials and methods

Patients and specimens

Flash-frozen tissue specimens were obtained from patients undergoing debulking surgery for EOC at the Roswell Park Cancer Institute, Buffalo, NY, USA, between 1995 and 2002. All tissue specimens were collected under a protocol approved by the Institutional Review Board (IRB). All pathology specimens were reviewed in our institution, and tumors were classified according to World Health Organization (WHO) criteria (33). In a subset of the patients, serum samples were available over extended periods of time during the course of disease. The medical records of the patients were also reviewed retrospectively in accordance with a protocol approved by the Institutional Review Board. The review included outpatient and in-patient treatment, including surgery and chemotherapy. Chemotherapy consisted of adjuvant carboplatin or cisplatin and paclitaxel in all patients. Study outcomes included overall survival time and time to progression, each measured from the time of definitive surgery. Progression was defined as objective evidence of recurrence, since all therapy was given in the adjuvant setting. The duration of overall survival was the interval between definitive surgery and death. Observation time was the interval between definitive surgery and last contact (death or last follow-up). Disease-free survival was the interval between definitive surgery and recurrence or death. Patients with persistent disease were excluded from the disease-free analyses.

Cell lines

The immortalized human normal ovarian surface epithelial cell lines, IOSE and HOSE, were grown in the recommended media under standard conditions. These were gifts from Dr. Nancy Auersperg (University of British Columbia, Vancouver, BC) and Dr. Sam Mok (Harvard University, Cambridge, MA, USA), respectively.

Total tissue RNA isolation

Total tissue RNA was isolated from frozen tumor tissues and from ovarian cancer cell lines using the TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. Potentially contaminating DNA was removed by treating with RNase-free DNase I (Boehringer-Mannheim, Mannheim, Germany). After phenol treatment and drying, RNA was dissolved in RNase-free water. The resulting RNA concentration was measured spectrophotometrically (GeneQuant; Amersham Pharmacia Biotech, Ltd., Cambridge, UK), and the quality of the RNA was checked by electrophoresis on a 1% agarose gel.

RT-PCR analysis of *SCP-1* expression

Two micrograms of each RNA sample were subjected to cDNA synthesis using the Ready-To-Go first strand synthesis kit (Pharmacia, Uppsala, Sweden). PCR was subsequently performed to analyze *SCP-1* expression. A 564-bp-long *SCP-1* specific PCR product was amplified using *SCP-1* specific sense 5'-GTACAGCAGAAAGCAAGC-AACTGAATG-3' and antisense 5'-GAAGGAACTGCTTTAGAATCCAATTTCC-3' primers. Glyceraldehyde -3-phosphodehydrogenase (GAPDH) specific sense 5'-GCTTCCCGTTCCTCAATTTTGAAG-3' and antisense 5'-ATGGGAAGGTGAAGG-TCGGAG-3' primers were used to obtain a 195-bp PCR product as a control. PCR was performed in a PTC-100® thermal cycler (MJ Research, Inc., MA, USA) and included a 60-min incubation at 50°C for reverse transcription and 15 min for enzyme inactivation at 95°C, followed by 30 cycles of PCR. Each PCR cycle consists of a 1

min denaturation at 94°C, followed by a 30-second annealing at 55°C and a 90-second extension at 72°C. After the last cycle, the final extension step was at 72°C for 10 min. The PCR products were visualized by ethidium bromide staining after separation in a 1.5% agarose gel.

IHC

Tumor specimens were fixed with buffered formalin and embedded in paraffin. Sections (5 μm) were placed on glass slides, heated at 60°C for 20 min, and then deparaffinized with xylene and ethanol. For antigen retrieval, tumor specimens mounted on glass slides were immersed into preheated antigen retrieval solution (DAKO high pH solution; Capinteria, CA, USA) for 20 min and allowed to cool for 20 min at room temperature. After the endogenous peroxidase was inactivated, mAb to SCP-1 (clone SC554) was added at a concentration of 2.5 μg/ml and incubated overnight at 4°C. SC554 was described previously (27). The primary antibody was detected with a biotinylated antimouse IgG (DAKO, Capinteria, CA, USA). Diaminobenzidine tetrahydrochloride was then added for development for 10 min, followed by counterstaining with hematoxylin solution. Negative control slides omitting the primary antibody were included in all assays. Positive control slides were testicular tissue with preserved spermatogenesis.

ELISA

Recombinant SCP-1 truncated proteins (30) at a concentration of 1 μg/ml in coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6, with 0.02% NaN_3) were adsorbed to TC microwell plates 60 x 10 (Nunc, Roskilde, Denmark) at 10 μl/well overnight at 4°C. Plates were washed with PBS and blocked overnight at 4°C with 10 μl/well of 2% BSA in PBS. After washing, 10 μl/well of serum dilutions in 2% BSA was added to the plates, which were then incubated for 2 h at room temperature. Plates were washed and 10 μl/well diluted secondary antibody in 2% BSA was added (goat antihuman IgG-AP; Southern Biotechnology, Birmingham, AL, USA), and incubated for 1 h at room temperature. Plates were washed, incubated with 10 μl/well of substrate solution (Attophose substrate; JBL Scientific, San Louis Obispo, CA, USA) for 25 min at room temperature, and immediately read (CytoFluor 2350; Millipore, Bedford, MA, USA). Sera were tested over a range of 4-fold dilutions from 1:100 to 1:100,000, as described previously (30).

Statistical analysis

All statistical analyses were performed with SPSS software (34). Statistical correlations were calculated using Pearson's *r*. The distribution of *SCP-1* expression and clinical outcome was analyzed by the chi-squared test. Estimated survival distributions were calculated by the Kaplan-Meier method (35), and tests of significance with respect to survival distributions were based on the log-rank test (36). No adjustments were made for multiple comparisons.

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