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Expression of cancer-testis genes in human hepatocellular carcinomas

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Abstract

Cancer-testis (CT) genes are expressed in a variety of human cancers, but not in normal tissues except for testis, and represent promising targets for immunotherapy and gene therapy. We investigated the expression of 10 CT genes (MAGE-1, MAGE-3, MAGE-4, GAGE, NY-ESO-1, SSX-1, HOM-MEL-40/SSX-2, SSX-4, HOM-TES-14/SCP-1, and HOM-TES-85) in 21 hepatocellular carcinoma (HCC) biopsy specimens. The most frequently expressed CT genes were SSX-1 and GAGE, which were found in 8/21 (38%) HCC samples, followed by HOM-TES-14/SCP-1 (6/21 or 29%), MAGE-3 (5/21 or 24%), HOM-TES-85 and MAGE-1 (4/21 or 19% each), whereas SSX-4 and HOM-MEL-40/SSX-2 were only expressed in 2/21 cases each, MAGE-4 in one case, and NY-ESO-1 not at all. Of the 21 HCC cases investigated, only four did not express any of the CT genes tested, 17 (81%) expressed at least one, 9 (43%) coexpressed two, four (19%) coexpressed four, three (14%) coexpressed five and one coexpressed 8 of the 10 CT genes tested. We conclude that a majority of HCC cases might be amenable to specific immunotherapeutic interventions. However, the identification of additional tumor-specific antigens with a frequent expression in HCCs is warranted to develop widely applicable, polyvalent HCC vaccines.

Introduction

Hepatocellular carcinoma is one of the most prevalent malignancies in East Asia (1). There are several established methods of treatment including surgery, ethanol injection therapy, transarterial embolization and chemotherapy. However, the prognosis for patients with HCC remains poor and new therapeutic strategies are actively pursued, one of the most popular being immunotherapy.

Active immunotherapy approaches using vaccines derived from defined antigens appear to be especially attractive to treat HCC, since such strategies might be employed in high-risk patients even before evident liver cancer has developed. A prerequisite for the development of specific vaccines is the existence and identification

of genes exclusively or preferentially expressed in malignant tissues, as compared to normal tissues. According to their expression pattern and the specificity of the immune responses they evoke, antigens expressed by human tumors can be classified into different groups (2). These include the so-called "shared tumor antigens", the differentiation antigens (including the idiotypes of B-cell lymphomas), the products of viral, mutated, differentially spliced, overexpressed and amplified genes, as well as common autoantigens expressed by the malignant cells of a tumor. Of these groups of antigens, the so-called "shared tumor antigens" appear to be the most promising targets for immunotherapeutic approaches in HCC.

It is enigmatic that all of the "shared tumor antigens" in humans that have been molecularly defined to date by cellular and serological techniques (3, 4) have in common their expression spectrum, with expression restricted to different types of cancers and normal testis. Therefore the term "cancer testis antigens" (CTA) has been coined to refer to them and the term "cancer testis genes" (CT genes) for the genes encoding them (5). Examples of CTAs are the T-cell defined MAGE (6), BAGE (7) and GAGE (8) antigens, as well as HOM-MEL-40/SSX-2 (9), the other SSX family members (10, 11), NY-ESO-1 (5), HOM-TES-15/SCP-1 (12) and HOM-TES-85 (13) which have been defined using SEREX, the serological identification of antigens by recombinant expression cloning (14).

There have been reports on the expression of the MAGE gene family members in HCCs (15, 16, 17, 18), but investigations encompassing the CT genes described more recently have not been reported. To investigate as broad as possible a spectrum of CT genes, we selected a panel of CT genes based on known correlated expression patterns [e.g. NY-ESO-1 (5) and LAGE (19)] and/or relatedness of genes and gene families [e.g. the MAGE family and related genes such as CT7/CT10 (20, 21) or DAM (22)]. Besides NY-ESO-1, we chose MAGE-1, MAGE-3 and MAGE-4 as representatives for the MAGE family of genes because they have been reported to be the most commonly expressed MAGE genes in cancer. In addition, SSX-1, SSX-2, and SSX-4 (the most commonly expressed members of the SSX gene family), SCP-1 and HOM-TES-85 were included in the study panel. HOM-TES-85 is a new 40 kDa CT antigen identified by screening a cDNA bank enriched for testis-specific transcripts with the serum of an allogeneic patient with seminoma (13). Our results show that the majority of human HCCs express at least one of the shared tumor antigens, thus rendering many patients eligible for trials involving tumor-specific strategies.

Results

Study population and validity of the experimental approach

Tumor specimens were assessed for cDNA integrity and only those in which both an 800 bp beta-actin and p53 product could be amplified were investigated. Therefore, four of the 25 HCC cases (cases # 14, 17, 18, and 19) had to be excluded from the analysis and only 21 HCC specimens could be investigated for the expression of the following ten CT genes: MAGE-1, MAGE-3, MAGE-4, GAGE, HOM-MEL-40/SSX-2, SSX-1, SSX-4, HOM-TES-14/SCP-1, HOM-TES-85 and NY-ESO-1. The characteristics of the patients are shown in Table 1. To exclude false positive PCR products due to small amounts of contaminating DNA in the RNA preparation, individual primers that correspond to sequences located in different exons of the gene were chosen. With the experimental conditions chosen, DNA generated no PCR products. Each RT-PCR experiment was performed in triplicate using the same poly(dT)-primed cDNA sample and the appropriate controls.

Table 1. Clinical and pathological characteristics of 21 HCC cases investigated[a].

Case No.	Gender	Age	Virus Type	Tumor Dimensions (cm)	Histological Grade	Stage	AFP (ng/ml)
1	M	62	HBV	5x2x3	poorly differentiated?	I/II	n.a.
2	M	41	HBV	4x2x3	well differentiated	I	50
3	M	52	HBV	2x1x3	well differentiated	I	n.a.
4	M	30	HBV	4.2x3x3	poorly differentiated	I	>1211
5	M	73	HBV	5x4x3	well differentiated	II	473
6	M	50	HBV	3x2x4	well differentiated	II	n.a.
7	M	41	-	n.a.	n.a.	n.a.	<50
8	M	29	HBV	2x2x1	well differentiated	I	<50
9	M	40	HBV	3x2x3	well differentiated	I	n.a.
10	M	57	HBV	3x3x3	poorly differentiated?	II	<50
11	F	16	HBV	8x6x3	poorly differentiated	III	>1600
12	M	65	HBV	6x6x5	moderately differentiated	I/II	380
13	M	47	HBV	11x10x7	well differentiated	II/III	>400
15	M	31	HBV	2.5x3x2	poorly differentiated	III	n.a.
16	M	?	HBV	4x2x2	poorly differentiated	III	<50
20	M	58	HBV	10x9.6x8	poorly differentiated	I/II	>400
21	M	47	HBV	3x2.1x2	well differentiated	I	10
22	M	45	HBV	5x3x3	moderately differentiated	I	<50
23	M	45	HBV	10x9x8	poorly differentiated	I/II	n.a.
24	M	46	-	4x4x4	adenocarcinoma	I	88
25	F	48	HBV	10x8x8	poorly differentiated	I	<50

[a]Abbreviation: n.a., not available.

Representative examples of RT-PCR results from HCC specimens are shown in Figure 1. Intensities of PCR products were found to be heterogeneous 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 PCR from the same tumor specimen. Cases with very low transcript levels, which were not reproducibly positive, were not considered as positive.

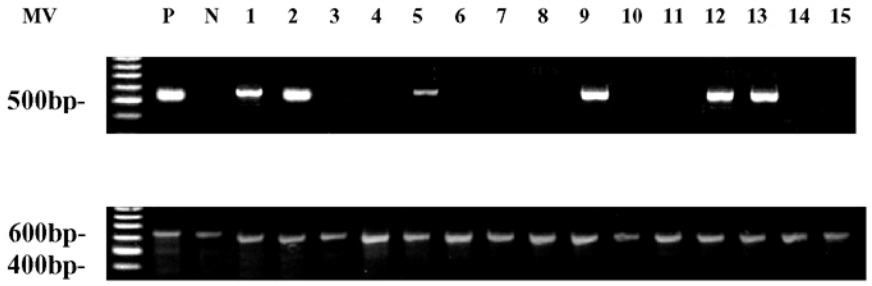

Figure 1. Representative RT-PCR results of the analysis of HOM-TES-14/SCP-1 expression in HCC. Samples (from left to right): P, testis (positive control); N, negative control; 1, HCC 1; 2, HCC 6; 3, HCC 25; 4, HCC 2; 5, HCC 4; 6, HCC 23; 7, HCC 8; 8, HCC 20; 9, HCC 22; 10, HCC 15; 11, HCC 13; 12, HCC 9; 13, HCC 16; 14, HCC 11; 15, HCC 12.

Expression of individual CT genes in human hepatocellular carcinoma

As shown in Table 2, NY-ESO-1 expression was undetectable in all 21 HCC specimens tested, MAGE-4 was expressed in only one HCC case, while HOM-MEL-40/SSX-2 and SSX-4 expression was observed in two cases each. An intermediate frequency of expression was observed for MAGE-1, HOM-TES-85, MAGE-3 and HOM-TES-14/SCP-1, which were expressed in four, four, five, and six cases respectively. The most frequently expressed CT genes in the HCC cases investigated in this study were GAGE and SSX-1, which were expressed in 8/21 (38%) of the cases.

Table 2. Expression of cancer testis genes by human hepatocellular carcinomas.

Case No.	MAGE-1	MAGE-3	MAGE-4	GAGE	NY-ESO-1	SSX-1	HOM-MEL-40	SSX-4	HOM-TES-14	HOM-TES-85
1	-	-	-	-	-	+	-	-	+	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	+	-
5	-	+	-	-	-	+	-	-	-	-
6	-	-	-	-	-	-	-	-	+	-
7	-	-	-	-	-	-	-	-	-	-
8	+	-	-	+	-	+	-	-	-	+
9	-	-	-	-	-	-	-	-	+	-
19	-	-	-	+	-	-	-	-	-	-
11	-	-	-	+	-	-	-	-	-	+
12	+	-	-	+	-	+	-	+	-	+
13	+	-	-	+	-	-	-	-	-	-
15	-	-	-	+	-	-	-	-	-	-
16	-	-	-	-	-	+	-	-	+	-
20	-	+	-	-	-	-	-	-	-	-
21	-	-	-	-	-	+	-	-	-	-
22	+	+	+	+	-	+	+	-	+	+
23	-	+	-	+	-	+	+	+	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	+	-	-	-	-	-	-	-	-
Proportion	**4/21**	**5/21**	**1/21**	**8/21**	**0/21**	**8/21**	**2/21**	**2/21**	**6/21**	**4/21**

Coexpression of multiple CT genes in human hepatocelular carcinoma

Of the 21 HCC cases studied, only four did not express all ten CT genes tested, 17 (81%) expressed at least one, 9 (43%) coexpressed at least two, four (19%) coexpressed at least four, and 3 (14%) coexpressed at least five CT genes (Figure 2). One case (case # 22) coexpressed 8 of the 10 CT genes tested.

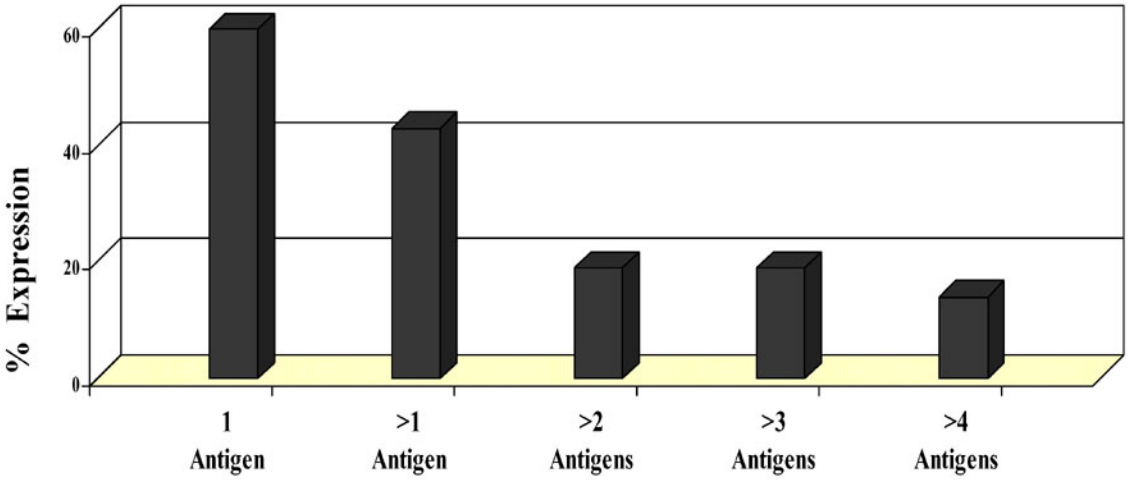

Figure 2. Coexpression of cancer-testis genes in hepatocellular carcinomas.

Detection of CT antigens in HCCs by immunofluorescence

There are no monoclonal antibodies available for immunohistology against the two CTAs expressed most frequently in HCC, i.e. GAGE and SSX-1. Our immunohistological analysis of CTA expression in HCC specimens thus had to be restricted to SCP-1. With the experimental conditions described, 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. A typical example is shown in Figure 3. While no SCP-1 protein could be demonstrated in some areas of the tumor, the majority of cells in the other areas stained positive.

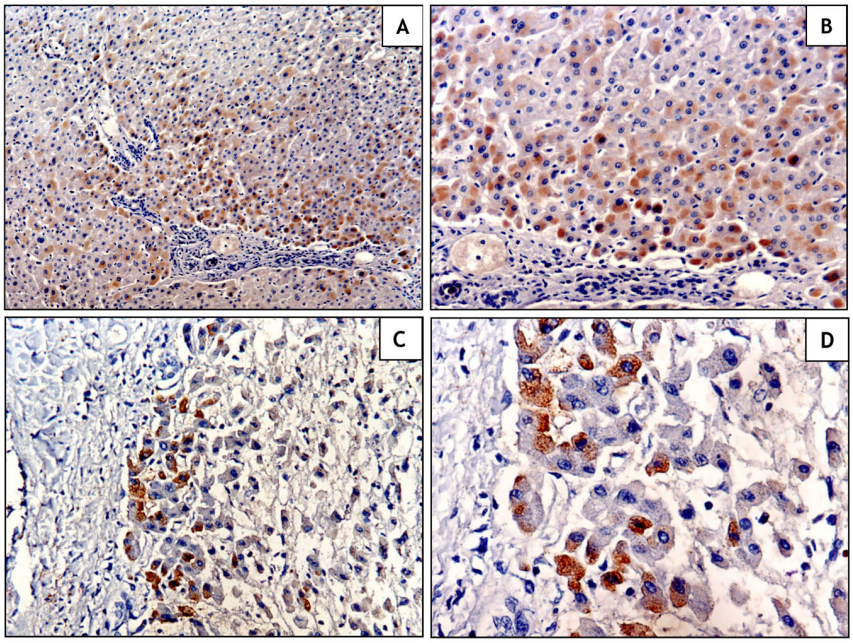

Figure 3. Immunohistological detection of HOM-TES-14/SCP-1 in a hepatocellular carcinoma. Positive carcinoma cells show a diffuse cytoplasmic reactivity.

Discussion

A wide range of human neoplastic tissues expresses CT genes. With regards to members of the MAGE gene family, several groups (15, 16, 17) have reported their expression in human HCC. The frequency of expression reported ranged from 15 to approx. 70% of the cases investigated. Similarly, the reported frequency of HCC cases expressing at least one MAGE gene ranged from 30% (18) to 88% (16). The expression of the MAGE family of genes in the HCC cases we studied is in the lower end of the reported ranges. The reason for these discrepancies is unclear. Patient selection and regional epidemiological differences might account for some of the variation. For instance, expression of MAGE genes has been reported to correlate with tumor size (18) and to be inversely correlated with hepatitis B surface (HBs) antigen positivity, a feature that was shared by all HCC cases included in this study, except for one case where this information was not available. While the primers used for the RT-PCR by the various groups were largely identical, the number of RT-PCR cycles varied (23) considerably. We chose 25 cycles because, under these conditions, cases that were found to express SCP-1 by RT-PCR also expressed the protein, as demonstrated by antigen-positive cells in immunohistology using the monoclonal SCP-1 antibody.

In our study, the SEREX-defined SSX-1 gene and the CTL-defined GAGE gene were the most frequently expressed in HCC, followed by the HOM-TES-14/SCP-1, MAGE-3, HOM-TES-85 and MAGE-1 genes. Thus, the SEREX-defined CTA significantly increased the number of HCC cases for which mono- or polyvalent CTA vaccines might be available. None of the CT genes assayed in this study was preferentially expressed in HCC. Rather, the absence of expression in certain types of tumors (e.g. the absence of NY-ESO-1 expression in gastric and colorectal cancers or lymphomas) seems to characterize a given CT gene better than the description of where it is expressed.

The function of most of the CT genes is unknown. Those for which the function is known are the HOM-TES-14/SCP-1 gene, which is involved in meiotic chromosome pairing (12, 24), the SSX genes, which contain a KRAB domain that has recently been shown to have a transcriptional repressor function (25), and HOM-TES-85, a novel member of the leucine zipper proteins which are involved in DNA binding and gene transcription (13).

While the mRNA levels of CT genes do not strictly correlate with the level of protein expression, we have detected SCP-1 protein in all cases of malignant tumor in which SCP-1 mRNA was found to be expressed (12). This was also true for the HCC cases investigated in this study; all RT-PCR positive samples also expressed the protein, as shown by immunohistology using a monoclonal anti-SCP-1 antibody. Immunohistology of the SCP-1 positive cases showed that the expression of this antigen in HCCs is not homogeneous throughout the tumor, but rather that there are areas that are completely negative interspersed with areas where the majority of the cells express the antigen (Figure 2).

From the data of our study it appears that about >80% of the patients with HCC would be eligible for specific immunotherapeutic approaches with at least one CT antigen in ways similar to those currently being evaluated in malignant melanomas (26, 27, 28), which express CT antigens at a similar frequency as HCC. While all patients with a tumor expressing a given CT antigen would be candidates for vaccine strategies using whole antigenic proteins, the percentage of patients eligible for peptide-specific vaccination would be much lower, since it requires antigenic peptides with binding motifs restricted to specific MHC alleles. Therefore, additional antigenic CT genes must be identified for human HCC, especially if the goal is to develop multivalent vaccines for a majority of patients. Since the expression of a CT antigen by a tumor is a prerequisite for a strong antibody response against the respective molecule in the patient's serum, we expected that the SEREX analysis using sera from HCC patients and an expression library derived from an HCC sample would identify new HCC-associated CTAs. However, no new CT genes were in such a study (29). An interesting aspect of this study was the fact that no hepatitis B virus-associated antigens were detected by the SEREX analysis of HCC, even though

the HCC sample which was the source of the cDNA library was HBV positive and the patient's serum contained antibodies against HBV. This suggests that the anti-HBV antibody response is directed against posttranslationally modified (e.g. glycosylated) epitopes of the HBV-associated antigens and that the immune response against HCCs covers a much broader spectrum of antigens than that which can be expressed by a bacterial expression system, which is restricted to the expression of proteins which are not modified posttranslationally.

Abbreviations

CT, cancer-testis; CTA, cancer-testis antigen; HCC, hepatocellular carcinoma; SEREX, serological identification of antigens by recombinant expression cloning

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Materials and methods

Patients and materials

The study was approved by the local ethical review board ("Ethikkommission der Ärztekammer des Saarlandes"). Tumor tissues were obtained during routine diagnostic or therapeutic procedures at the Nanning University Medical School, Nanning, China. All data including age, gender, stage and other clinical factors were obtained from the clinical and pathological records. Histological grading was done according to (30). HCC samples used for the RT-PCR analysis were checked microscopically for the presence of neoplastic tissue and the absence of contaminating normal liver tissue. Normal tissues were collected from autopsies of tumor-free patients. Recombinant DNA work was done with the official permission and according to the rules of the State Government of Saarland.

RT-PCR analysis

Twenty-one cases of surgically resected HCCs were included in this study. Total cellular RNA was extracted from frozen tissue specimens using guanidium-isothiocyanate, followed by an acidic phenol extraction and isopropanol precipitation as previously described (31). Total RNA (4 µg) was primed with an 18-nucleotide oligo(dT) and reverse-transcribed with Superscript II (Gibco BRL, Eggenstein, Germany) according to the manufacturer's instructions. The integrity of the cDNA thus obtained was tested by amplifying beta-actin transcripts in a 25 cycle PCR reaction as described elsewhere (12). For the PCR analysis of the expression of individual CTA gene transcripts, 1 µg first-strand cDNA was amplified with transcript-specific oligonucleotides (10 pmol) using 2 U AmpliTaq Gold (Perkin Elmer, Weiterstadt, Germany), 10 nMol of each dNTP (dATP, dTTP, dCTP, dGTP) and 1.67 mM $MgCl_2$ in a 30 µl reaction. The primers (MWG Biotech, Ebersberg, Germany) used for the RT-PCR analysis were as follows:

SX-1: 5'-cta aag cat cag aga aga gaa gc-3' and 5'-aga tct ctt att aat ctt ctc aga aa-3'; annealing temperature 56°C
HOM-MEL-40/SSX-2: 5'-gtg ctc aaa tac cag aga aga tc-3' and 5'-ttt tgg gtc cag atc tct cgt g-3'; annealing temperature 67°C
SSX-4: 5'-aaa tcg tct atg tgt ata tga agc t-3' and 5'-ggg tcg ctg atc tct tca taa-3'; annealing temperature 60°C
HOM-TES-14/SCP-1: 5'-gta cag cag aaa gca agc aac tga atg-3' and 5'-gaa gga act gct tta gaa tcc aat ttc c-3'; annealing temperature 60°C
HOM-TES-85: 5'-gga gag gct act caa gat gca gaa gc-3' and 5'-ctg agt gac tat gag atc tct ctg agt-3'; annealing temperature 60°C
NY-ESO-1: 5'-cac aca gga tcc atg gat gct gca gat ccg g-3' and 5'-cac aca aag ctt ggc tta gcg cct ctg ccc tg-3'; annealing temperature 60°C
MAGE-1: 5'-cgg ccg aag gaa cct gac cca g-3' and 5´-gct gga acc ctc act ggg ttg cc-3'; annealing temperature 58°C
MAGE-3: 5'-tgg agg acc aga ggc ccc c-3' and 5'-gga cga tta tca gga ggc ctg c-3'; annealing temperature 63°C
MAGE-4: 5'-gag cag aca ggc caa ccg-3' and 5'-aag gac tct gcg tca ggc-3'; annealing temperature 63°C
GAGE: 5'-aga cgc tac gta gag cct-3' and 5'-cca tca gga cca tct tca-3'; annealing temperature 52°C

Amplification was performed in a TRIO-Thermoblock (Biometra, Göttingen, Germany). After activation of AmpliTag Gold polymerase for 12 min at 94°C for hot-start induction, 25 cycles of PCR were performed (1 min at the annealing temperature indicated above, 2 min at 72°C and 1 min at 94°C), with a final elongation step at 72°C for 8 min. A 15 µl aliquot of each reaction was size-fractionated on a 2% agarose gel, visualized by ethidium bromide staining and the size of the product determined.

Immunohistology

Immunohistology was performed using the Ultra Sensitive TM S-P kit (Maxin Company, Shanghai, China) following the manufacturer's recommendations. Formalin-fixed sections of the biopsy material were deparaffinized and hydrated by serial incubations in 100, 90, 80 to 70% (v/v) xylene and graded alcohol. After extensive washing, endogenous peroxidase was blocked by treatment with 3% (v/v) H_2O_2. Microwave treatment was performed in citrate buffer for 5 min at 800 W. After blocking unspecific binding by preincubation of the slides with 1% (w/v) BSA in PBS for 1 hr at room temperature, the slides were incubated overnight at 4°C with monoclonal anti-SCP-1 antibody SC554 (IgG1kappa) diluted 1:10 in 1% BSA (w/v). The SC554 anti-SCP-1 monoclonal antibody had been obtained by fusion of SP2/0 cells with spleen cells from a BALB/c mouse immunized with a truncated fusion protein of SCP-1. SC554 binds to an epitope located between amino acids 20 and 64 of the SCP-1 molecule. Monoclonal MOPC-21 (IgG1kappa; Sigma) was used as a negative control. After washing with PBS, biotinylated rabbit anti-mouse antibody was added and the slides were incubated for 1 hr at room temperature. After another washing step, the slides were incubated for 15 min at 37°C with streptavidin diluted 1:300 in Tris-buffered saline. The reaction was developed with freshly prepared DAB for 2 to 5 min at room temperature, followed by hematoxylin counterstaining and embedding in Canada balsam.

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