

## Expression of Synovial Sarcoma X (SSX) Antigens in Epithelial Ovarian Cancer and Identification of SSX-4 Epitopes Recognized by CD4<sup>+</sup> T Cells

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**Abstract** **Purpose:** Synovial sarcoma X (SSX) breakpoint genes are expressed in a variety of cancers but not in normal tissues, except for testis, and are potential targets for immunotherapy. The aims of this study were to determine the expression and immunogenicity of these antigens in patients with epithelial ovarian cancer (EOC). **Experimental Design:** SSX-1-, SSX-2-, and SSX-4-specific reverse transcription-PCR were done on a panel of EOC specimens. Sera from a subgroup of the patients were tested for SSX-2 and SSX-4 antibody by ELISA and recombinant antigen expression on yeast surface (RAYS). *In vitro* stimulation of peripheral blood mononuclear cells from a patient bearing SSX-4-expressing tumor with a pool of long peptides spanning the protein sequence was used for assessment of SSX-4-specific CD4<sup>+</sup> T cells recognizing distinct antigenic sequences restricted by HLA class II alleles. **Results:** Our results indicate expression of SSX-1, SSX-2, and SSX-4 in 2.5%, 10%, and 16% of 120 EOC specimens, respectively. When all three SSX antigens are considered, aberrant expression was found in 26% of ovarian tumors. Antibodies to SSX-2 and SSX-4 were detectable by ELISA and RAYS in two patients. SSX-4-specific CD4<sup>+</sup> T cells recognizing two previously undescribed SSX-4-derived T-cell epitopes in association with HLA-DR (SSX-4: 51-70 and SSX-4: 61-180) were identified. **Conclusions:** Our study shows aberrant expression of SSX antigens in a proportion of patients with EOC. The evidence of humoral immunity to SSX-2 and SSX-4, and SSX-4-specific CD4<sup>+</sup> T cells among circulating lymphocytes in patients with antigen expressing EOC suggest that these antigens are attractive targets for specific immunotherapy in EOC.

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancies. There are >23,000 cases annually in the United States, and 14,000 women can be expected to die from the disease in 2005 (1). The majority of patients are diagnosed at an advanced stage, and despite modest improvements in survival with the use of adjuvant chemotherapy with platinum/paclitaxel-based chemotherapy,

overall survival for patients with advanced EOC remains poor (2). The poor survival in advanced disease is due to late diagnosis as well as lack of effective strategies for the prevention of recurrence in complete responders. Thus, there is a need to develop additional therapeutic approaches, such as immunotherapy for the management of this disease. The development of immunotherapeutic strategies in EOC requires the identification of tumor antigens expressed in these tumors and the design of vaccination strategies that may result in the generation of tumor antigen-specific T cells that could afford durable protection.

As a consequence of advances in the approaches for analyzing humoral (3) and cellular (4) immune reactivity to cancer in the context of the autologous host, an increasing number of tumor antigens recognized by autologous CD8<sup>+</sup> T cells (5) and/or antibodies (6) have been identified. Among the tumor antigens identified to date, the "cancer-testis" (CT) antigens are a distinct and unique class of differentiation antigens. The criteria for placing antigens in this category are based on several characteristic features (7, 8): (a) predominant expression in germ cells of the testis and generally not in other normal tissues, (b) expression in a proportion of malignant tumors of different histologic types, (c) expression in malignancies in a lineage nonspecific fashion, (d) often mapping of the gene on the X chromosome, (e) often members of

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Received 8/30/05; revised 10/18/05; accepted 10/26/05.

**Grant support:** Cancer Research Institute/Ludwig Institute for Cancer Research Cancer Vaccine Collaborative Grant and Anna-Marie Kellen Clinical Investigator Award of the Cancer Research Institute, NY (K. Odunsi).

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doi:10.1158/1078-0432.CCR-05-1902

multigene families. The members of this family of antigens continue to expand and include the synovial sarcoma X (SSX) breakpoint multigene family (9). The SSX gene family was initially identified as fusion partners to the SYT gene in synovial sarcomas harboring the t(X;18) translocation (10). There are currently nine known SSX genes, all of which map to chromosome X (11). The known SSX family members encode 188 amino acid-long proteins with identities ranging from 70% to 90% (12). Five SSX genes were initially isolated by immunoscreening or PCR cloning (12), and the remaining four complete genes, SSX-6, SSX-7, SSX-8, and SSX-9, as well as SSX-10 pseudogenes were identified by genomic library screening and database mining (11). The normal testis expresses SSX-1, SSX-2, SSX-3, SSX-4, SSX-5, and SSX-7 but not SSX-6, SSX-8, or SSX-9 (11). In tumors, SSX-1, SSX-2, SSX-4, and SSX-5 are expressed at varying frequencies, whereas SSX-3 and SSX-6 are rarely expressed (11, 13–15). In addition, no expression of SSX-7, SSX-8, or SSX-9 has been observed (11). All of these properties suggest that the SSX tumor antigens may be promising targets for immunotherapy.

Although CD8<sup>+</sup> T cells represent the major effector arm of the antitumor immune response, a growing body of evidence indicates that CD4<sup>+</sup> T cells play a pivotal role in orchestrating these responses (16–19). The multiple roles of antigen-specific CD4<sup>+</sup> T cells include the provision of help to CD8<sup>+</sup> T cells during the primary and secondary immune responses (20–22), direct cytotoxicity, and activation of B cells for production of tumor antigen-specific antibodies. Therefore, the identification of CD4<sup>+</sup> T-cell epitopes towards which spontaneous responses arise provides opportunities to characterize the molecular mechanisms of responses to tumor antigens and analyze the clinical significance of such responses in immunotherapy trials. In this regard, by analyzing CD4<sup>+</sup> T-cell responses to SSX-2- and SSX-4-derived sequences in circulating lymphocytes from antigen expressing melanoma patients, we have previously identified epitopes restricted by several MHC class II alleles (23–26). In this study, we have analyzed a large panel of patients with EOC for expression and spontaneous humoral immunity to SSX-1, SSX-2, and SSX-4 and examined the relationship between SSX expression and clinicopathologic outcome. In addition, we have analyzed the CD4<sup>+</sup> T-cell response to SSX-4, using circulating lymphocytes from an ovarian cancer patient bearing an antigen-expressing tumor. This allowed the identification of two CD4<sup>+</sup> T-cell epitopes mapping to the 51-70 and 161-180 regions of the protein and recognized by specific T cells in association with HLA-DR. Together, the findings described indicate that SSX antigens are promising targets for specific immunotherapy of EOC.

## Materials and Methods

**Patients and specimens.** Flash frozen tissue specimens were obtained from patients undergoing debulking surgery for epithelial ovarian cancer at the Roswell Park Cancer Institute, Buffalo, NY between 1995 and 2002. All tissue specimens were collected under an approved protocol from the Institutional Review Board. All tissue specimens were classified according to WHO criteria (27). Optimal surgical debulking was defined by residual individual tumor nodules measuring  $\leq 1$  cm in diameter. All patients received platinum- and paclitaxel-based first-line chemotherapy. The duration of disease-free survival was amount of time between the completion of chemotherapy and the first recurrence (if a complete response had been achieved). Overall survival was

defined by the interval between diagnosis and death. Data were censored at the last follow-up for patients with no evidence of recurrence or progression. Peripheral blood was obtained from healthy donors (New York City Blood Bank, New York, NY) and ovarian cancer patients (Roswell Park Cancer Institute) upon informed consent. Culture medium for lymphocytes was Iscove's modified Dulbecco's medium supplemented with 8% heat-inactivated pooled human serum (CTL medium), recombinant human interleukin-2 (GlaxoSmithKline, Geneva, Switzerland), and recombinant human interleukin-7 (R&D Systems, Inc., Minneapolis, MN).

**Cell lines.** Five ovarian cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and grown in the recommended media under standard conditions. These were SVOV3, OVCA-429, OVCA-432, SK-OV-3, and OVCAR-3. Homozygous EBV-transformed cell lines were obtained from the National Marrow Donor Program/American Society for Histocompatibility and Immunogenetics Cell Repository (access via <http://www.ashi-hla.org>). EBV-transformed cell lines were maintained in Iscove's modified Dulbecco's medium (Life Technologies/Invitrogen Corp., Rockville, MD) supplemented with 10% heat-inactivated FCS.

**Analysis of SSX gene expression.** Total tissue RNA was isolated from frozen tumor tissues and from ovarian cancer cell lines, using the TRIReagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturers' protocol. 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 done to analyze expression of SSX-1, SSX-2, and SSX-4. Integrity of cDNA was tested by amplification of glyceraldehyde-3-phosphate dehydrogenase in a 35-cycle PCR reaction. mRNA expression of SSX genes in tumor tissue samples or tumor cell lines was assessed using previously described oligonucleotide primers and conditions (12). The PCR products were 421 bp for SSX-1, 434 bp for SSX-2, and two alternatively spliced variants of 415 and 279 bp for SSX-4 and were visualized by ethidium bromide staining after separation over a 1.5% agarose gel.

**ELISA.** Recombinant SSX-2- and SSX-4-truncated proteins (28) at a concentration of 1  $\mu$ g/mL in coating buffer [15 mmol/L Na<sub>2</sub>CO<sub>3</sub>, 30 mmol/L NaHCO<sub>3</sub> (pH 9.6), with 0.02% NaN<sub>3</sub>] were adsorbed to TC microwell plates 60  $\times$  10 (Nunc, Roskilde, Denmark) at 10  $\mu$ L/well overnight at 4°C. Plates were washed with PBS and blocked overnight at 4°C with 10  $\mu$ L/well of 2% bovine serum albumin/PBS. After washing, 10  $\mu$ L/well of serum dilutions in 2% bovine serum albumin were added and incubated for 2 hours at room temperature. Plates were washed, and 10  $\mu$ L/well diluted secondary antibody/2% bovine serum albumin were added (goat anti-human IgG-AP; Southern Biotechnology, Birmingham, AL) and incubated for 1 hour at room temperature. Plates were washed, incubated with 10  $\mu$ L/well of substrate solution (Attophase substrate; JBL Scientific, San Louis Obispo, CA) for 25 minutes at room temperature, and immediately read (Cyto-Fluor 2350; Millipore, Bedford, MA). Sera were tested over a range of 4-fold dilutions from 1:100 to 1:100,000, as described previously (28).

**Recombinant antigen expression on yeast surface for the detection of serologic immune responses to SSX-2 and SSX-4.** Plasmid construction for SSX-2 and SSX-4, yeast transformation, and antibody detection were done as previously described (29). Briefly, yeast containing pYD1 as a control and yeast containing either pYD1-SSX-2 or pYD1-SSX-4 were incubated at room temperature with 100  $\mu$ L of preabsorbed serum (1:100 dilution) for 30 minutes. After washing, secondary biotinylated anti-human-IgG Fc- $\gamma$ -specific serum (Dianova, Hamburg, Germany) diluted 1:200 was added and incubated for 30 minutes at room temperature with occasional agitation. Antibody binding was detected using a biotinylated anti-murine IgG Fc- $\gamma$ -specific serum (Dianova) in the same manner as described above. Finally, labeled yeast cell suspensions were analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany). For each sample, 30,000 cells were collected. The ratio between the intensity of the signal measured on antigen expressing (induced) and noninduced (pYD1) yeast was

calculated for each individual serum sample. A sample was considered to be positive if this ratio was  $\geq 2$ .

**Molecular HLA-DRB1, DRB3, DRB4, and DPB1 typing.** HLA typing was done at the HLA typing laboratory of the Roswell Park Cancer Institute using sequence-specific primer pairs obtained from Genovision (30).

**Generation of SSX-4-specific CD4<sup>+</sup> T cells and antigen recognition assays.** *In vitro* stimulation of SSX-4-specific T cells was conducted as described previously (24). CD4<sup>+</sup> T cells were stimulated with autologous antigen-presenting cells in the presence of a pool of partially overlapping peptides spanning the entire SSX-4 protein sequence (2  $\mu$ mol/L each). The culture was tested 2 weeks later using the same peptides, as indicated. CD4<sup>+</sup> T cells secreting IFN- $\gamma$  in response to peptide stimulation were isolated using the cytokine secretion detection kit (Miltenyi Biotec, Auburn, CA) and cloned by limiting dilution culture as described (24). Clones were subsequently expanded by periodic (3-4 weeks) stimulation under the same conditions. For assessment of fine specificity of antigen recognition and restriction, T cells (10,000) were incubated in the absence or presence of peptides and of antibodies directed against different MHC class II molecules. Where indicated, antigen-presenting cells (EBV-B cells, 10,000 per well) preincubated or not with antigen and extensively washed were added. After 24 hours of incubation at 37°C, culture supernatants were collected, and the content of IFN- $\gamma$  was determined by ELISA (Biosource International, Camarillo, CA). Monocyte-derived dendritic cells were prepared from CD14<sup>+</sup> monocytes isolated from peripheral blood mononuclear cells by magnetic cell sorting using miniMACS (Miltenyi Biotec) and cultured in CTL medium containing 1,000 units/mL of recombinant human granulocyte macrophage colony-stimulating factor and 1,000 units/mL of recombinant human interleukin-4 (R&D Systems) during 6 days. SSX-4 protein was produced as detailed previously (24). Where indicated, dendritic cells were incubated with proteins for 12 hours and washed before their use in antigen recognition assay.

**Statistical analysis.** All statistical analyses were done with SPSS software (31). Statistical correlations were calculated using Pearson's *R*. The distribution of SSX expression and clinical outcome was analyzed by the  $\chi^2$  test. Estimated survival distributions were calculated by the method of Kaplan and Meier (32), and tests of significance with respect to survival distributions were based on the log-rank test (33). No adjustments were made for multiple comparisons.

## Results

**Study population.** The characteristics of the study population are presented in Table 1. The median age of the study population was 64 years (range, 26-91 years), and the median duration of follow-up was 22 months (range, 0.4-119 months). As expected, the majority of patients presented with grade 3 tumors (88%), at stage IIIC (72%), and with serous histology (70%). A complete response to therapy was achieved in 70 of the 123 patients (57%); a partial response was achieved in 45 patients (37%), whereas the remaining patients had no response. The median survival for all patients was 45 months [confidence interval (CI), 34-57 months], whereas the median disease-free survival, excluding patients with persistent/progressive disease after initial therapy, was 27 months (CI, 19-29 months). The estimated 5-year disease-free survival and overall survival (for the entire study population) were 32% (CI, 18-46%) and 37% (CI, 25-49%), respectively.

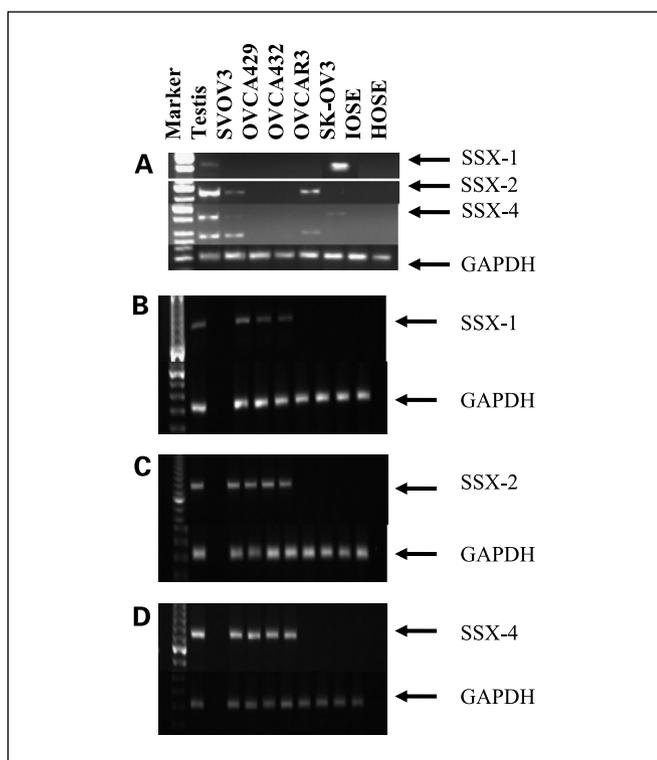
**Tumor expression of SSX-1, SSX-2, and SSX-4.** Expression of SSX-1, SSX-2, and SSX-4 mRNA in ovarian cancer cell lines, and epithelial ovarian tumor specimens was investigated by reverse transcription-PCR (Fig. 1). Intensities of PCR products

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 reverse transcription-PCR from the same tumor specimen. Cases with very low transcript levels, which were not reproducibly positive, were not regarded as positive. SSX-1 and SSX-2 yielded expected bands of 421 and 434 bp, respectively. For SSX-4, a distinct band of lower molecular weight (279 bp; Fig. 1) was observed in addition to the main PCR product (415 bp). This secondary PCR product was previously described (12) and corresponds to an SSX-4

**Table 1. Patient characteristics**

Characteristics	
Evaluable patients	123
Age (median/range), y	64 (26-91)
Follow-up (median/range), mo*	22 (0.4-119)
FIGO stage (%)	
IA	3 (2)
IB	1 (1)
IC	6 (5)
IIB	3 (2)
IIC	6 (5)
III	2 (2)
IIIA	1 (1)
IIIB	3 (2)
IIIC	88 (72)
IV	10 (8)
Histology (%)	
Papillary serous	86 (70)
Clear cell	8 (7)
Endometrioid	8 (7)
Mucinous	3 (2)
Undifferentiated	4 (3)
Others (transitional, mixed, carcinosarcoma)	14 (11)
Grade (%)	
1	5 (4)
2	10 (8)
3	107 (88)
Response to frontline therapy (%)	
Complete response	70 (57)
Partial response	45 (37)
Progression	3 (2)
Unknown	5 (4)
Recurrences (%)	
Recurrence/persistent disease	87 (70)
Current status (%)	
Alive, NED	40 (33)
Alive with disease	24 (19)
Dead of disease	59 (48)
CT antigen status by reverse transcription-PCR (%)	
SSX-1 positive	3/118 (2.5)
SSX-2 positive	12/122 (10)
SSX-4 positive	19/120 (16)
Any SSX	31/120 (26)

\*Median survival for all patients: 45 months (CI, 34-57 months).



**Fig. 1.** Detection of SSX gene expression in human ovarian cell lines and ovarian cancer patients by reverse transcription-PCR. *A*, reverse transcription-PCR with primers *SSX-1* yielded an expected band of 421 bp in normal testis; cancer cell lines SK-OV3, whereas other ovarian cancer cell lines SVOV3, OVCA432, OVCA429, and OVCAR3 and normal epithelial ovarian cell line IOSE and HOSE were negative. Reverse transcription-PCR with primers *SSX-2* yielded an expected band of 434 bp in normal testis; cancer cell lines SVOV3 and OVCAR3, whereas other ovarian cancer cell lines OVCA429, OVCA432, and SK-OV3 and normal epithelial ovarian cell line IOSE and HOSE were negative. Reverse transcription-PCR with primers *SSX-4* yielding two expected bands of 415 and 279 bp in normal testis. Cancer cell lines SVOV3 and SK-OV3 (both are very weak) correspond to two splice variants of *SSX-4* transcripts. Cancer cell line OVCAR3 only had one band of 279 bp that corresponds to the alternatively spliced variant of *SSX-4*, whereas other ovarian cancer cell lines OVCA432 and OVCA429 and normal epithelial ovarian cell line IOSE and HOSE were negative. *B*, reverse transcription-PCR analysis of *SSX-1* expression in ovarian cancer patients. *C*, reverse transcription-PCR analysis of *SSX-2* expression in ovarian cancer patients. *D*, reverse transcription-PCR analysis of *SSX-4* expression in ovarian cancer patients. Three positive and four negative for *SSX-1* and four positive and four negative for *SSX-2*, *SSX-4* ovarian cancer samples. Reverse transcription-PCR for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a positive control, yielding an expected band of 214 bp in all the samples.

alternative splice variant that lacks the fifth exon of the coding region as confirmed by sequencing of the purified PCR product (15). *SSX-1* was expressed by SK-OV3; *SSX-2* was expressed by SVOV3 and OVCAR3; and *SSX-4* was expressed by SVOV3 and SK-OV3 cell lines. The SK-OV-3 cell line showed dual expression of *SSX-1* and *SSX-4*, whereas the SVOV3 and OVCAR3 cell lines showed dual expression of *SSX-2* and *SSX-4* (Fig. 1). *SSX-1* mRNA expression was detected in 3 of 118 (2.5%), *SSX-2* in 12 of 122 (10%), and *SSX-4* in 19 of 120 (16%) of tumor specimens. Overall, the expression of *SSX-1*, *SSX-2*, or *SSX-4* mRNA was found in 31 of 120 (26%) of EOC specimens (Table 1).

**Antibody response to SSX-2 and SSX-4 in ovarian cancer patients.** Serum samples from 109 of the 122 patients were analyzed by ELISA and recombinant antigen expression on yeast surface (RAYS) for *SSX-2* and *SSX-4* antibodies. These

sera consisted of preoperative and serial specimens obtained during patients' course of disease (range, 1-3 years). A demonstrable antibody response to *SSX-2* and *SSX-4* was found in two (2%) patients with coexpression of *SSX-2* and *SSX-4* in tumors. In the first patient, *SSX-2* antibody was detected by ELISA over a range 1:400 to 1:1,600 dilution, whereas *SSX-4* antibody was detected at 1:100 dilution. In the second patient, *SSX-2* and *SSX-4* antibodies were detected over a range of 1:6,400 to 1:25,000 dilution. Specific immune responses to *SSX-2* and *SSX-4* in the two patients were also detected by RAYS (ratio > 2) with serum dilutions of up to 1:10,000. The patients had stage IIIc and IV disease and had evidence of antibody response at the time of initial diagnosis. Both patients remain alive (one with disease and the other without evidence of disease), and antibody positive >5 years after initial therapy.

**Correlation of SSX expression with clinicopathologic characteristics.** There was no statistically significant difference in the distribution of the expression of any of the SSX antigens and histologic grade or stage (Table 2). Patients whose tumors expressed *SSX-4* had a median overall survival of 39 months (CI, 22-56 months) compared with 50 months (CI, 36-64 months) among patients whose tumors did not express *SSX-4* ( $P = 0.06$ , Fig. 2). There was also no statistically significant difference in the survival distribution of *SSX-2*-positive or *SSX-2*-negative patients. When patients whose tumors were positive for any of the three SSX antigens were compared with *SSX*-negative patients, there was no statistically significant difference in the survival distribution.

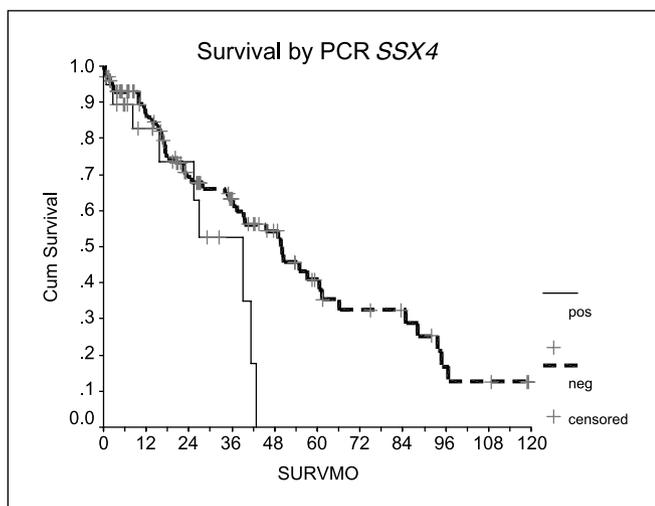
**Assessment of SSX-4-specific CD4<sup>+</sup> T cells in circulating lymphocytes of an ovarian cancer patient and identification of two SSX-4-derived CD4<sup>+</sup> T-cell epitopes.** Enriched CD4<sup>+</sup> T cells from peripheral blood mononuclear cell samples from an ovarian cancer patient with *SSX-4*-expressing tumor were stimulated *in vitro* with a peptide mix containing 20 amino acid-long peptides spanning the *SSX-4* protein sequence and overlapping by 10 amino acids. The presence of specific CD4<sup>+</sup> T

**Table 2.** Correlation between SSX mRNA expression and clinicopathologic features in EOC

Pathologic and clinical features	SSX-1 positive	SSX-2 positive	SSX-4 positive
All tumors ( $N = 123$ ) *	3 (3%)	12 (10%)	19 (16%)
Age (median/range)	72 (68,75)	63 (38,75)	65 (46,81)
Tumor grade			
G1	—	—	—
G2	—	2	1
G3	3	10	18
FIGO stage			
Ia	—	1	—
II	—	—	—
IIIc	3	10	18
IV	—	1	1

NOTE: There were no statistically significant differences in survival distributions based on expression of *SSX-1*, *SSX-2*, and *SSX-4* mRNA.

\*The expression of any of the SSX mRNA was found in 31 of 120 (26%) of EOC specimens.

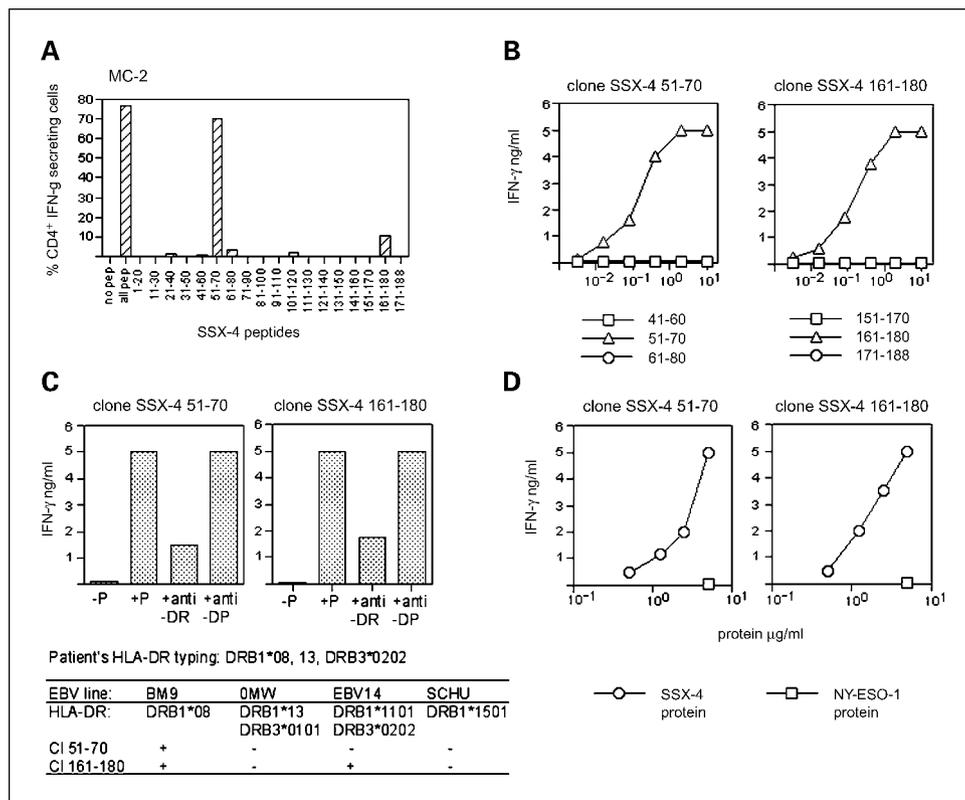


**Fig. 2.** Kaplan-Meier estimates of overall survival in EOC patients according to tumor expression of *SSX-4*.

cells was assessed on day 7 after stimulation by intracellular staining with cytokine-specific antibodies upon stimulation in the absence or presence of the *SSX-4* peptide mix. A low response was detected (0.04% IFN- $\gamma$  secreting CD4<sup>+</sup> T cells with *SSX-4* peptides versus <0.01% without peptides), and to amplify this response, the culture was stimulated again with the *SSX-4* peptide mix. After the second stimulation, the culture contained 0.29% IFN- $\gamma$  secreting CD4<sup>+</sup> T cells with *SSX-4* peptides versus 0.01% without peptides. *SSX-4*-specific CD4<sup>+</sup> T cells were isolated from a fraction of the culture by IFN- $\gamma$  secretion-guided magnetic cell sorting and either stimulated as

a polyclonal population or cloned under limiting dilution conditions. Assessment of reactivity of the IFN- $\gamma$ -producing cells enriched polyclonal culture to single peptides in the mixture revealed that the majority of *SSX-4*-specific cells in the culture (69.9%) were directed against peptide *SSX-4*: 51-70. A lower reactivity was also detected in the case of peptides *SSX-4*: 41-60 and 61-80. The reactivity toward these peptides could be explained by the presence in these peptides of partial sequences encoding the epitope(s) contained in peptide 51-70. A sizable fraction of cells in the culture (10.6%) was directed against a distinct peptide, *SSX-4*: 161-180. Finally, lower proportions of cells in the culture (1.3% and 1.8%) recognized peptides *SSX-4*: 21-40 and *SSX-4*: 101-120, respectively (Fig. 3A). This low reactivity likely corresponds to the presence, in these peptides, of distinct epitopes recognized by a small fraction of *SSX-4*-specific T cells in the culture.

We obtained clonal CD4<sup>+</sup> T-cell populations specific for the two major activities (*SSX-4*: 51-70 and 161-180). An example of recognition of *SSX-4* active peptides by the corresponding specific clonal populations is shown in Fig. 3B. In both cases, no recognition of neighboring peptides in the mix was detected. To identify the restriction element(s) used by *SSX-4*-specific CD4<sup>+</sup> T cells, recognition of peptides *SSX-4*: 51-70 and 161-180 was carried out in the presence of antibodies that specifically block the recognition of antigens restricted by different MHC class II elements. As illustrated in Fig. 3C, anti-HLA-DR antibodies significantly decreased the ability of *SSX-4*-specific CD4<sup>+</sup> T cells to recognize both peptides *SSX-4*: 51-70 and 161-180. In contrast, no significant inhibition was observed using anti-HLA-DP (Fig. 3C) or anti-HLA-DQ antibodies (data not shown). To establish the HLA-DR presenting alleles, we analyzed the HLA-DR alleles of the patient and then assessed



**Fig. 3.** *A*, the activity of individual *SSX-4* peptides was assessed after the second cycle of *in vitro* stimulation with the *SSX-4* peptide pool. Values at least 3-fold higher than baseline (no peptide) were considered significant. *B*, recognition of *SSX-4* peptides by specific CD4<sup>+</sup> T-cell clones was assessed in the presence of graded peptide dilutions. For clones specific for each of the identified sequences, antigen recognition was assessed for the corresponding active peptide as well as for neighboring overlapping peptides. The concentration of IFN- $\gamma$  in the culture supernatant was assessed by ELISA after 24 hours of culture. *C*, to determine the MHC class II restricting element, peptide recognition was assessed either in the absence or presence of anti-HLA-DR, anti-HLA-DP, and anti-HLA-DQ antibodies. The MHC class II restricting allele was determined by assessing the ability of molecularly typed EBV-B cells to present *SSX-4* peptides to the corresponding CD4<sup>+</sup> T-cell clones. *D*, the ability of monocyte-derived dendritic cells to process the *SSX-4* protein and present the relevant antigenic sequences to specific CD4<sup>+</sup> T cells was assessed after 12 hours of incubation of dendritic cells with soluble recombinant *SSX-4* protein at the indicated dose. Recombinant NY-ESO-1 protein was used as internal control.

the ability of partially matched homozygous EBV lines to present each of the peptides to the corresponding specific CD4<sup>+</sup> T-cell clone. The patient expressed *DRB1\*08*, *DRB1\*13*, and *DRB3\*0202*. For SSX-4: 51-70, presentation was obtained using the BM9 line that expresses *DRB1\*08* but by none of the lines sharing other DR alleles with the patient. Thus, in this case, presentation was unambiguously restricted by *DRB1\*08*. In contrast, peptide SSX-4: 161-180 was presented to specific CD4<sup>+</sup> T cells by both the BM9 line and the EBV14 line but not by other lines. EBV14 shared the *DRB3\*0202* allele with the patient. This brought us to the conclusion that both the *DRB1\*08* and *DRB3\*0202* alleles were able to present the SSX-4: 161-180 epitope to specific CD4<sup>+</sup> T cells. Interestingly, serologic cross-reactivity has been previously shown for these HLA-DR molecules, suggesting a high level of structural similarity, in good agreement with their ability to bind and present identical peptides to CD4<sup>+</sup> T cells. In our previous study assessing SSX-4-specific CD4<sup>+</sup> T cells in melanoma patients, we also detected reactivity toward peptide 51-70, although in that case, recognition by specific CD4<sup>+</sup> T cells was restricted by a different HLA-DR allele (*DRB1\*0701*) compared with our present study. Peptide 161-180 was cross-recognized by *HLA-DPB1\*1001*-restricted CD4<sup>+</sup> T cells from one of the patients in that study, although to a lesser extent compared with the peptide 151-170 that most likely contained the entire *HLA-DPB1\*1001* epitope. It is also noteworthy that although one of the patients in our previous study expressed the *DRB3\*0202* allele, no reactivity toward peptide 161-180 was detected for this patient. Together, this indicates that (a) the SSX-4 reactive peptides identified in this study likely contain multiple overlapping epitopes that can be recognized by SSX-4-specific CD4<sup>+</sup> T cells in association with different MHC-class II alleles; (b) depending on the relative frequency of CD4<sup>+</sup> T cells specific for a given SSX-4 peptides in a given patient expressing the appropriate allele, responses could not always be detectable. We then assessed the ability of SSX-4-specific CD4<sup>+</sup> T cells specific for the identified epitopes to recognize the SSX-4 native antigen. As illustrated in Fig. 3D, autologous dendritic cells from the patient were able to efficiently process the recombinant SSX-4 protein produced in *Escherichia coli* and present the relevant epitopes to the corresponding SSX-4-specific CD4<sup>+</sup> T-cell clones, in a dose-dependent fashion. In contrast, the clones were not significantly stimulated by the NY-ESO-1 protein, produced similarly in *E. coli*, used as an internal control. These results show that the T-cell epitopes recognized by SSX-4-specific CD4<sup>+</sup> T cells from the ovarian cancer patient are efficiently processed and presented by professional antigen-presenting cells.

## Discussion

There is increasing evidence that the immune system has the ability to recognize tumor-associated antigens expressed in human malignancies and to induce antigen-specific humoral and cellular immune responses to these targets. In epithelial ovarian cancer, 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 improved survival of patients with the disease (34). Because these infiltrating T cells are likely to be tumor antigen specific, it will be important to identify targets that could be used to

augment and/or induce the antitumor immune response. In the current study, we have undertaken a comprehensive analysis of a large panel of ovarian tumors and cell lines for the expression of CT antigens belonging to the SSX family. Our results indicate aberrant expression of SSX-1, SSX-2, and SSX-4 in 2.5%, 10%, and 16% of EOC specimens, respectively. When all three SSX antigens are considered, aberrant expression was found in ~26% of ovarian tumors. In a previous report, 6 of 12 (50%) EOC specimens showed expression of SSX-4 mRNA (13). In a more recent study, 0 of 40 (0%), 1 of 40 (3%), and 5 of 40 (13%) of EOC specimens showed expression of SSX-1, SSX-2, and SSX-4 mRNA, respectively (35). Taken together with our data, these studies indicate that a significant subset of tumors of EOC patients aberrantly express SSX-2 or SSX-4.

Expression of none of the SSX antigens in our study correlated with clinicopathologic characteristics (histologic type, tumor grade, recurrence, and survival) of tumor. In our previous analysis of other CT antigens, we showed that NY-ESO-1 expression did not correlate with clinical outcome (36), whereas the expression of another CT antigen, SCP-1 correlated with worse survival (37). The significance of these findings is not immediately obvious. In synovial sarcoma, SYT-SSX fusion proteins are believed to play an important role in tumor development (38). In addition, expression of SSX genes has been recently reported in human mesenchymal stem cells (39). Interestingly, in these cells, SSX colocalizes with proteins involved in cell adhesion, motility, and extracellular matrix interaction, such as matrix metalloproteinase 2 and vimentin. In addition, it was found that the migration of melanoma cells, which expresses SSX, matrix metalloproteinase 2, and vimentin, decreases when SSX is down-regulated, suggesting that SSX may play a functional role in normal stem cell migration and potentially a similar function in cancer cell metastases. Thus, although the lack of correlation found between SSX expression and clinical outcome reported here may argue against a role of SSX antigens in tumor progression, it may also simply reflect the fact that the majority of the patients (Table 1) had advanced-stage disease.

In an effort to study the immunogenicity of the SSX antigens in EOC, we studied spontaneous humoral immune responses to SSX-2 and SSX-4 using the complimentary strategies of ELISA and RAYS. The latter approach has the advantage that recombinant proteins can be expressed on the cell surface in a more naturally folded, partially glycosylated manner (40, 41). Therefore, antibody responses directed primarily against conformational epitopes that would not be detected by the conventional ELISA may be more readily detected by RAYS due to the improved accessibility of the epitopes when the antigen is displayed on the yeast surface. Both techniques yielded equivalent results and showed spontaneous antibody in two patients that showed unusually favorable clinical course. Although we have not screened normal healthy individuals for SSX-2 and SSX-4 antibodies in the current study, a previous report indicated no evidence of humoral immune response in 70 normal individuals to a panel of seven CT antigens, including SSX-2 (28). Consistent with our findings, spontaneous SSX-2 antibody response was infrequent, occurring in 1 of 234 cancer patients examined (28).

For one ovarian cancer patient bearing an SSX-4-expressing tumor, we could also assess the presence of SSX-4-specific CD4<sup>+</sup> T cells among circulating lymphocytes. Our analysis

allowed us to detect and isolate, for this patient, SSX-4-specific CD4<sup>+</sup> T cells recognizing two previously undescribed SSX-4-derived T-cell epitopes. The first was contained within peptide SSX-4: 51-70 and was recognized by specific CD4<sup>+</sup> T cells in association with *HLA-DRB1\*08*, whereas the second was contained within peptide SSX-4: 161-180 and was presented by two of the HLA class II molecules of the patient, *DRB1\*08* and *DRB3\*0202*. Importantly, in both cases specific CD4<sup>+</sup> T-cell clones were able to efficiently recognize the native SSX-4 antigen in the form of recombinant protein after processing and presentation by autologous professional antigen-presenting cells. These data show that consistent with our previous findings in melanoma patients (24, 26), T cells specific for SSX-derived sequences and able to recognize the native antigen are present among circulating lymphocytes of ovarian cancer patients and could likely be stimulated through vaccination with SSX-derived immunogens.

In summary, the data reported in the present study indicate that SSX gene products are relevant targets for the development of immunotherapeutic approaches for the treatment of ovarian cancer and encourage the development of clinical trials of EOC patients using SSX gene product-derived immunogens for vaccinations. In addition, the approach used in this study for the analysis of SSX-4-specific T-cell responses and epitope identification will be useful for monitoring SSX-4-specific T-cell responses in future immunotherapy trials. However, because CT-antigen expression may be the result of the activation of part, or parts, of a coordinated gene expression program, rather than being independent events (42), further studies are warranted to determine the expression of additional CT antigens in EOC to extend the number of relevant targets, as well as the proportion of patients that could be eligible for immunotherapy.

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**Article on Synovial Sarcoma X Antigens in Epithelial Ovarian Cancer**

In the article on Synovial Sarcoma X Antigens in the January 15, 2006 issue of *Clinical Cancer Research*, the name of an author, Sacha Gnjatic, was misspelled.

Valmori D, Qian F, Ayyoub M, et al: Expression of synovial sarcoma X (SSX) antigens in epithelial ovarian cancer and identification of SSX-4 epitopes recognized by CD4<sup>+</sup> T cells. *Clin Cancer Res* 2006;2:398-404.

# Clinical Cancer Research

## Expression of Synovial Sarcoma X (SSX) Antigens in Epithelial Ovarian Cancer and Identification of SSX-4 Epitopes Recognized by CD4<sup>+</sup> T Cells

Danila Valmori, Feng Qian, Maha Ayyoub, et al.

*Clin Cancer Res* 2006;12:398-404.

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