Cancer Immunity, Vol. 3, p. 13 (9 October 2003) Submitted: 2 September 2003. Accepted: 2 September 2003.
Contributed by: LJ Old

SSX antigens as tumor vaccine targets in human sarcoma

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Keywords: human, sarcoma, tumor antigens, SSX, mRNA, RT-PCR

Abstract

The efficacy of current standard therapies for the treatment of sarcoma remains limited. With the aim of identifying target antigens relevant to the development of vaccine-based immunotherapy of sarcoma, we have addressed the relevance of tumor-specific antigens encoded by genes belonging to the *SSX* family as vaccine targets in sarcoma tumors. Expression of *SSX*-1 to -5 was analyzed in a collection of sarcoma tumors of diverse histological subtypes and in sarcoma cell lines. We found expression of at least one *SSX*-encoded antigen in 42% of sarcoma tumors, including 5 of 7 different histological subtypes, and in 50% of sarcoma cell lines. *SSX-1* was the most frequently expressed family member, followed by *SSX*-4, -2 and -5. Expression of *SSX-3* was detected in only one sample. Importantly, most SSX positive samples co-expressed more than one family member. In addition, assessment of CD8+ T cell recognition of HLA-A2+ SSX-2+ sarcoma cells showed that the latter were efficiently recognized and lysed by SSX-2-specific CTLs. The results of this study indicate that SSX antigens are relevant targets for the development of vaccine-based immunotherapy of sarcoma and encourage the start of vaccination trials using SSX-derived immunogens in sarcoma patients.

Introduction

Sarcomas are a heterogeneous group of malignant mesenchymal tumors that account for about 1% of human cancers (1) and include more than 50 histological types and subtypes (2, 3). These tumors are associated with a

high mortality, the overall one-year survival of sarcoma patients being of only 50% (1). The general efficacy of current standard therapies, including surgery and radiotherapy to control local recurrence, as well as of systemic chemotherapy in the case of metastatic disease, remains limited. On the other hand, the development of alternative immunotherapeutic approaches for the treatment of sarcomas has thus far been hampered by the lack of relevant antigenic targets.

Genes belonging to the *SSX* family were initially identified because of the involvement of two family members (*SSX*-1 and -2) as fusion partners of the *SYT* gene in the chromosomal translocation commonly found in synovial sarcoma (4, 5). Gene products encoded by the members of the *SSX* family belong to the group of the so-called cancer/testis antigens, non-mutated self-antigens whose expression is limited to germ cells in normal adult tissues and to cancers of various histological types. Because of their expression pattern, cancer/testis antigens appear to be ideal candidates for the development of generic cancer vaccines, as eliciting specific immune responses directed against this type of antigens would in principle only target cancer cells while sparing normal cells. A previous survey of the expression of the main *SSX* gene family members (*SSX*-1 through -5) in a large series of different human tumor types showed expression of several family members in a significant proportion of tumors, albeit the proportion of expressing tumors varied considerably depending on the particular histological type (6). For defined tumor types, expression of at least one of the *SSX* family members was frequently observed in some tumor types such as head and neck cancer (75%), ovarian cancer (50%), and malignant melanoma (43%), whereas other tumor types including a few cases of leiomyosarcoma were found not to express any *SSX* gene. Interestingly, expression of at least one of the 5 main *SSX* gene family members was recently reported in 16 of 17 osteosarcoma tumor samples (7). The aim of our study was to address the relevance of *SSX* gene products as tumor-vaccine targets for patients with different histological subtypes of sarcoma.

Results

We analyzed the expression of *SSX* gene family members in a collection of 26 sarcoma samples of diverse histological subtypes. These were as follows: gastrointestinal stromal tumor (3 cases), synovial sarcoma (2 cases), leiomyosarcoma (10 cases: 5 uterine and 5 at other sites), angiosarcoma (3 cases), malignant fibrous histiocytoma (4 cases), osteosarcoma (1 case) and liposarcoma (3 cases). In addition, the expression of *SSX* gene family members was assessed in a group of 8 sarcoma cell lines: SK-ES-1 (Ewing's sarcoma), SK-LMS-1 (leiomyosarcoma), SK-UT-1 (uterine leiomyosarcoma), SW 872 (liposarcoma), Saos-2 (osteosarcoma), HOS (osteosarcoma), MES-SA (uterine sarcoma) and HT-1080 (fibrosarcoma). Control melanoma cell lines SK-MEL-37 and SK-MEL-23, for which *SSX* gene family member expression has previously been described (8), as well as normal testicular tissue were used as internal controls.

The expression of *SSX* genes in tumor tissue samples or tumor cell lines was initially assessed using MEL40A and MEL40B primers that amplify *SSX*-1 to -5 cDNA (8). We found expression of *SSX* genes in 11 of the 26 sarcoma tumor samples (Table 1) and in 4 of the 8 sarcoma cell lines (Table 2) analyzed. Using a set of previously described gene-specific primers (8), we then analyzed the expression of individual *SSX* gene family members in SSX positive samples as defined by MEL40A/MEL40B amplification. Representative results are shown in Figure 1. As summarized in Table 3, *SSX-1* was expressed in 9 tumor samples and 4 cell lines, *SSX-2* was expressed in 5 tumor samples and 2 cell lines, *SSX-4* was expressed in 6 tumor samples and 3 cell lines, and *SSX-5* was expressed in 5 tumor samples and none of the cell lines. *SSX-3* was expressed in a single tumor sample. A single PCR product, corresponding to the previously described gene product, was observed in the case of *SSX*-1, -2 and -3. In contrast, a distinct band of lower molecular weight (279 bp, Figure 1) was observed for *SSX-4* in addition to the main PCR product (415 bp). This secondary PCR product was previously described (8) and corresponds to an *SSX-4* alternative splice variant that lacks the fifth exon of the coding region as

confirmed by sequencing of the purified PCR product (not shown). Similarly, a secondary PCR product (this time of higher molecular weight as compared to the main PCR product of 315 bp) was observed for *SSX-5*. This secondary PCR product has also been observed previously (9) and corresponds to an alternative splice variant resulting from the presence of the third exon as confirmed by sequencing of the purified RT-PCR products (data not shown).

Table 1. Expression of *SSX* genes in fresh sarcoma tumor samples.

Histological Subtype	Expression of *SSX* Genes (positive/total samples analyzed)
Gastrointestinal stromal tumor	1/3
Synovial sarcoma	0/2
Leiomyosarcoma:	5/10
uterine	4/5
other sites	1/5
Angiosarcoma	0/3
Malignant fibrous histiocytoma[a]	2/4
Osteosarcoma	1/1
Liposarcoma	2/3

[a]Poorly differentiated sarcoma.

Table 2. Expression of *SSX* genes in sarcoma cell lines.

Cell Line	Histological Subtype	Expression of *SSX* Genes
SK-ES-1	Ewing's sarcoma	-
SK-LMS-1	Leiomyosarcoma	+
SK-UT-1	Uterine leiomyosarcoma	-
SW 872	Liposarcoma	+
Saos-2	Osteosarcoma	+
HOS	Osteosarcoma	-
MES-SA	Uterine sarcoma	-
HT-1080	Fibrosarcoma	+

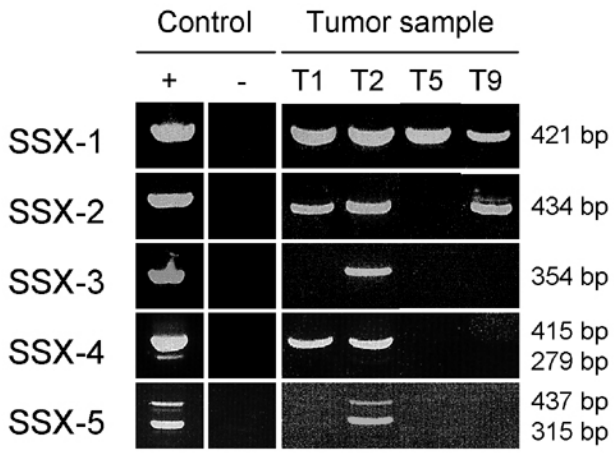

Figure 1. Representative results of the RT-PCR analysis of the expression of *SSX* genes in sarcoma tumor samples. Melanoma cell line SK-MEL-37 served as a positive control for the expression of *SSX*-1, -2, -4 and -5. Testis mRNA was used as a positive control of *SSX-3* expression. Melanoma cell line SK-MEL-23 was used as a negative control.

Table 3. Expression of *SSX* gene family members in sarcoma tumor samples and sarcoma cell lines.

	Histological Subtype	*SSX-1*	*SSX-2*	*SSX-3*	*SSX-4*	*SSX-5*
Tumor Samples						
T1	Uterine leiomyosarcoma	+++	++	-	++	-
T2	Uterine leiomyosarcoma	+++	+++	++	++	++
T3	Uterine leiomyosarcoma	+++	+	-	+	-
T4	Uterine leiomyosarcoma	++	-	-	-	+
T5	Leiomyosarcoma	+++	-	-	-	-
T6	Malignant fibrous histiocytoma[a]	+	-	-	-	++
T7	Malignant fibrous histiocytoma[a]	+	+++	-	+	+++
T8	Osteosarcoma	+	-	-	-	+
T9	Liposarcoma	++	++	-	-	-
T10	Liposarcoma	-	-	-	++	-
T11	Gastrointestinal stromal tumor	-	-	-	++	-
Cell Lines						
SK-LMS-1	Leiomyosarcoma	+	-	-	-	-
HT 1080	Fibrosarcoma	+++	-	-	+	-
SW 872	Liposarcoma	+	++	-	++	-
Saos-2	Osteosarcoma	+	+	-	+	-

[a]Poorly differentiated sarcoma.

Using CD8+ tumor-reactive T lymphocytes from a melanoma patient, we recently identified the first SSX-2-derived CD8+ T cell epitope (10, 11). The epitope identified corresponds to peptide SSX-2_{41-49} and is recognized by CD8+ T lymphocytes in the context of the HLA-A2 molecule, one of the most frequently expressed HLA class I alleles. Spontaneous specific CD8+ T cell responses to this epitope are frequently found in HLA-A2+ melanoma patients expressing *SSX-2* in their tumor lesions (12). To assess if SSX-2 antigen expression in sarcoma cells could result in killing by specific CTLs we tested the ability of SSX-2_{41-49}-specific CTLs to recognize and lyse SW 872 cells (HLA-A2+ SSX-2+, Table 2 and data not shown). As illustrated in Figure 2, and similarly to the results obtained using HLA-A2+ and SSX-2+ melanoma cells, SW 872 sarcoma cells were efficiently lysed by specific CTLs either in the presence or in the absence of peptide SSX-2_{41-49}, indicating recognition of the endogenous antigen. In contrast HLA-A2+ SSX-2- tumor cells were efficiently lysed only in the presence of exogenously added peptide.

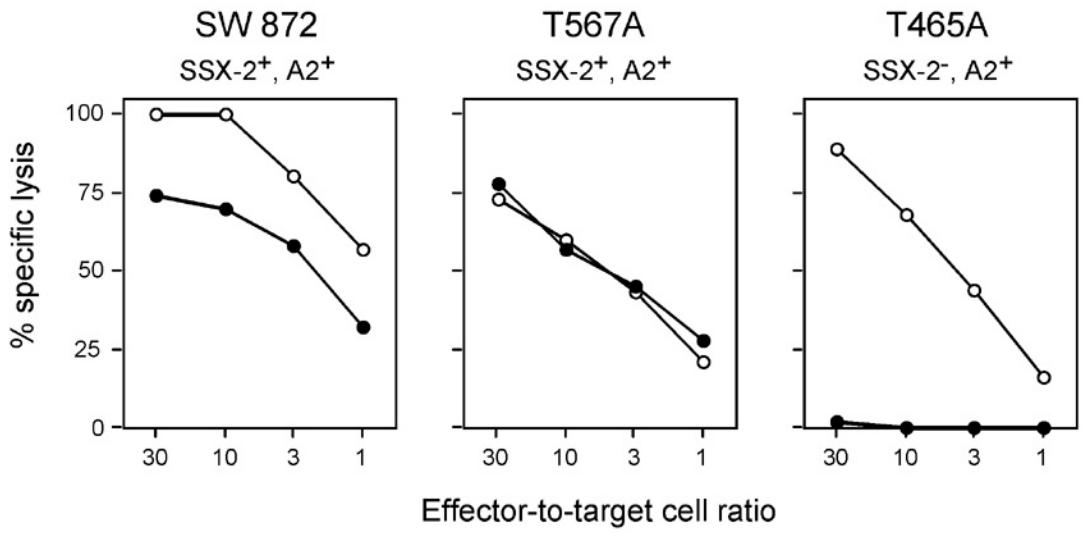

Figure 2. Recognition of the SW 872 sarcoma cell line by the SSX-2_{41-49}-specific clone D2.5. Recognition was assessed in a chromium release assay in the absence (closed circles) or in the presence (open circles) of exogenously added peptide SSX-2_{41-49} (1 µM) at the indicated effector to target cell ratio. Melanoma lines T567A and T465A were used as control target cells.

Discussion

We found expression of *SSX* genes in 42% of the sarcoma tumor samples and in 50% of the sarcoma cell lines analyzed, including 5 of 7 different histological subtypes examined (Tables 1 and 2). At variance with a previous report (6), we found *SSX* gene expression in leiomyosarcoma (5 of 10 tumor samples and 1 of 2 cell lines). Consistent with previous reports for other tumor types (7), a significant fraction of the positive sarcoma samples co-expressed more than one family member (Table 3). Of the five family members, *SSX-1* was the most frequently expressed, followed by *SSX*-4, -2 and -5. Interestingly, *SSX-3*, previously found to be expressed in osteosarcoma (7) but in none of the other tumor types analyzed (6), was expressed in one uterine leiomyosarcoma tumor sample.

Expression of *SSX* genes in synovial sarcoma was previously reported in 3 of 4 cases (6), and found to be independent of the *SYT*/*SSX* translocation. None of the 2 synovial sarcoma tumors analyzed in our study expressed full length SSX. The lack of full length mRNA, however, does not imply that *SSX* gene products are

not expressed in synovial sarcoma. Indeed, in most cases synovial sarcomas carry either the *SYT*/*SSX-1* or the *SYT*/*SSX-2* translocation involving expression of the part of the *SSX* gene located upstream of the translocation break point (4, 5). Thus, provided that a relevant epitope is located in this part of the protein, the *SSX* gene product involved in the translocation can be the target of specific immune responses. It is also noteworthy that the *SYT*/*SSX* junctional sequences encoded as a result of the molecular rearrangement may also serve as specific immune targets for synovial sarcoma (13).

Cancer/testis antigens are immunogenic proteins. Experimental evidence that *SSX* gene products can be spontaneously immunogenic in cancer patients has come from the analysis of spontaneous immune responses to the antigen HOM-MEL-40. HOM-MEL-40 was originally identified in a malignant melanoma patient by antibody screening of a tumor-derived cDNA expression library with autologous serum and shown to be identical to *SSX-2* (14). High-titer IgG antibodies to HOM-MEL-40 were found in 10% of melanoma patients (15). Using monoclonal CTLs specific for the HLA-A2-restricted SSX-2_{41-49} epitope identified previously, we demonstrate here that SSX-2 antigen expression in sarcoma cells can result in killing by specific CTLs. SSX-2_{41-49} is the only SSX-derived epitope identified to date. Considering the frequency of SSX antigen expression in sarcoma tumors, the identification of additional CD8+ and CD4+ T cell epitopes from SSX-2 and from other frequently expressed SSX antigens is of clear interest for the development of vaccine-based immunotherapy of sarcoma and is presently being pursued in our laboratory.

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 sarcoma and encourage the start of clinical trials of sarcoma patients using *SSX* gene product-derived immunogens for vaccinations. The identification of sarcoma-associated antigens that can potentially be recognized by cellular effectors of the immune system opens new avenues for antigen-directed, patient-specific, immunotherapy. Importantly, vaccination of sarcoma patients with SSX-derived immunogens is compatible with current standard therapy and could be started after surgery as an adjuvant therapy to prevent disease recurrence. Interestingly, the expression of *SSX* genes suggests that other cancer/testis antigens could also be frequently expressed in sarcoma, possibly extending the number of relevant targets as well as the proportion of sarcoma patients eligible for immunotherapy.

Acknowledgements

M.A. and D.V. are recipients of grants from the Cancer Antigen Discovery Collaborative program of the Cancer Research Institute, New York. C.S.H. is supported by a grant from The Breast Cancer Alliance Inc. We would like to thank Mary Hesdorffer for help in the collection of patients' samples.

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Materials and methods

Study patients

Tumor tissues were obtained during routine surgery at the Presbyterian Hospital, Columbia University, New York, NY. Tissues were preserved in RNAlater (Sigma, St Louis, MO) and stored at -20°C. Human sarcoma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The study was approved by the local ethical review board. Patients had given their written informed consent for the use of blood and tissue for research purposes.

RT-PCR analyses

Total cellular RNA was prepared from frozen tissue specimens or tumor cell lines using a Nucleospin® RNA II extraction kit (Macherey-Nagel, Düren, Germany). cDNA synthesis was performed using Promega Reverse Transcription System A3500 (Madison, WI) and cDNA integrity was tested by amplification of beta-actin in a 35-cycle PCR reaction. SSX antigen mRNA expression in tumor tissue samples or tumor cell lines was assessed using previously described oligonucleotide primers (8). RT-PCR analyses and semiquantitative assessment of mRNA expression were performed using melanoma cell line SK-Mel-37 as reference for *SSX*-1, -2, -4 and -5 and testis tissue for *SSX-3*. Expression levels were scored as follows: +++, 50-200%; ++, 10-50% and +, 1-10% of the levels found in the reference.

Antigen recognition assay

Antigen recognition was assessed in a chromium-release assay using the sarcoma cell line SW 872 (HLA-A2+ SSX-2+) as target and the melanoma cell lines T567A (HLA-A2+ SSX-2+) and T465A (HLA-A2+ SSX-2-) as internal controls (the melanoma cell lines were kindly provided by Dr. D. Rimoldi, LICR, Lausanne Branch, Switzerland). A previously derived CD8+ T cell monoclonal population specific for the HLA-A2-restricted CD8+ T cell epitope SSX-2_{41-49} (clone D2.5) was used as effector. Briefly, tumor cells were labeled with ^{51}Cr for 1 hr at 37°C and washed three times. Labeled tumor cells were mixed together with effector cells at the indicated effector to target cell ratio, either in the absence or in the presence of peptide SSX-2_{41-49}. Chromium release was measured in the supernatant harvested after a 4 hr incubation at 37°C. The percent specific lysis was calculated as: 100 x [(experimental - spontaneous release)/(total - spontaneous release)].

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Cancer Immunology Research

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Cancer Immun 2003;3:.