

SSX expression in gynecological cancers and antibody response in patients

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The SSX genes are members of the cancer-testis (CT) antigen family. SSX2, the prototype SSX gene, was found by serological analysis of antigens by recombinant expression cloning (SEREX). Since little is known about SSX expression in gynecological malignancies, we investigated SSX mRNA expression in 115 gynecological cancer specimens and 25 normal control samples by RT-PCR. We also tested the humoral immune response to SSX2 and SSX4 using recombinant proteins. We found relatively high SSX4 mRNA expression in endometrial cancer (24%), ovarian cancer (13%), and cervical cancer (20%). In contrast, SSX1 and SSX2 mRNA expression was detected in not more than 4% of the gynecological cancer specimens analysed. No SSX mRNA expression was found in 25 normal specimens. Two gynecological cancer patients had antibodies against SSX4, whereas no antibody reactivity to SSX4 was found in the sera of 40 normal individuals. This suggests that SSX4 is a potential target for cancer vaccines in gynecological cancer patients.

Introduction

Endometrial, ovarian, and cervical cancers are the most common gynecological malignancies. The prognosis is usually favorable for gynecological cancers in early stages. However, the only therapeutic options for advanced gynecological cancers are chemotherapy and radiotherapy, and the effects of these modalities are limited (1, 2, 3, 4). Since the discovery of human tumor antigens which are recognized by T-lymphocytes (5), immunotherapy has received particular attention as a new modality for treating cancer (6). Cancer-testis (CT) antigens constitute a category of tumor antigens that are expressed at varying frequencies in tumors and in the male germ cells in normal testis tissue. Based on their restricted expression in normal tissues and on their immunogenicity in cancer patients, CT antigens are the ideal targets for cancer vaccines (7). Several clinical trials involving MAGE and NY-ESO-1 peptides are ongoing, mostly involving metastatic melanoma patients (8, 9, 10).

The HOM-MEL-40 antigen encoded by the SSX2 gene was originally found in melanoma using autologous patient serum (11). Five SSX genes were isolated by immunoscreening or PCR cloning (12), and were fully characterized and shown to be located on chromosome X. Recently, four additional complete genes, SSX6, 7, 8 and 9, as well as 10 pseudogenes were identified by genomic library screening and database mining (13). The normal testis expresses SSX1, 2, 3, 4, 5, and 7, but not 6, 8, or 9 (13). In tumors, SSX1, 2, and 4 are expressed at varying

frequencies, whereas SSX3, 5, and 6 are rarely expressed. In addition, no expression of SSX7, 8, or 9 has been observed (13). The SSX family shares nucleotide homology ranging from 88% to 95%, and amino acid homology ranging from 77% to 91% (12).

Although the expression frequency of various SSX genes has been determined in a variety of cancers (14, 15, 16, 17, 18, 19), little is known about their expression in gynecological cancers. In the present study, we investigated SSX1, SSX2, and SSX4 mRNA expression in 115 gynecological cancer specimens. SSX4 was the most frequently expressed gene and a serum antibody response to it was seen in some patients.

Results

SSX mRNA expression in gynecological tumors

SSX1, SSX2, and SSX4 mRNA expression was investigated in 115 gynecological tumors, including 50 endometrial, 40 ovarian, and 25 cervical cancers, and in 25 normal tissues, including 10 endometria, 10 ovaries, and 5 uterine cervixes by RT-PCR using specific primers. As shown in Figure 1, SSX expression was observed in tumors, but not in normal tissues. Nucleotide sequencing confirmed the PCR products as being SSX1, SSX2, and SSX4 (data not shown). Table 1 summarizes the RT-PCR results. SSX4 mRNA expression was observed in 12/50 (24%) endometrial cancers, 5/40 (13%) ovarian cancers, and 5/25 (20%) cervical cancers, whereas SSX1 and SSX2 mRNA expression was detected in not more than 4% of the tumor samples analysed. Co-expression of SSX mRNA was then examined, and one of two SSX1 mRNA positive tumors and all four SSX2 mRNA positive tumors were found to co-express SSX4 mRNA.

No relationship was observed between the expression of SSX1, SSX2, and SSX4 mRNA and any clinical and pathological parameter including age, histological type and grade, tumor invasion, lymph node metastasis and clinical stage, or patient survival for any type of cancer (data not shown).

Antibody response to SSX in gynecological cancer patients

Using recombinant proteins, we analyzed sera from 131 patients with gynecological cancers, including 59 endometrial, 44 ovarian, and 38 cervical cancers, for antibodies against SSX2

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and SSX4 by ELISA. As shown in Figure 2, antibodies against SSX4 were found in the sera from two patients, one with ovarian cancer and the other with cervical cancer. No SSX1 or SSX2 mRNA expression was detected in the tumor samples from these two patients. No antibodies against SSX2 were found in the 131 patient sera analysed. Also, no antibodies against either SSX2 or SSX4 were detected in the sera of 40 healthy volunteers.

Discussion

In this study, we observed SSX4 mRNA expression in 12/50 (24%) endometrial cancers, 5/40 (13%) ovarian cancers, and 5/25 (20%) cervical cancers in Japanese patients. SSX1 and SSX2 mRNA expression were observed in not more than 4% of these tumors. Moreover, we discovered antibody responses against SSX4 but not SSX2 recombinant protein in sera from 1/44 ovarian and 1/38 cervical cancer patients, but 0/59 endometrial cancer patients. No antibody against SSX4 was detected in the sera from 40 healthy donors. SSX1 recombinant protein was not available for use in this study.

There are few reports on SSX gene expression in gynecological tumors (14). SSX1 and SSX2 mRNA expression were previously observed in 1/8 (12.5%) endometrial cancers, while SSX4 mRNA expression was observed in 1/8 (12.5%) endometrial cancers and 6/12 (50%) ovarian cancers in a group of Caucasian patients (14). The difference in frequency between this study and the afore-mentioned study might be due to the small number of samples analysed in the latter. Alternatively, it could be due to the difference between Japanese and Caucasian populations. Differing results among ethnic groups were also observed for the expression of the CT antigen NY-ESO-1 in gynecological tumors. In our analysis of 20 endometrial, 20 ovarian, and 20 cervical cancers, we observed NY-ESO-1 mRNA expression in only 1 case each of endometrial and cervical cancer by conventional RT-PCR, but in none of the ovarian cancer samples analyzed (data not shown), in contrast to another study which found that it was expressed in 30% of ovarian cancer samples from a group of Caucasian patients (20).

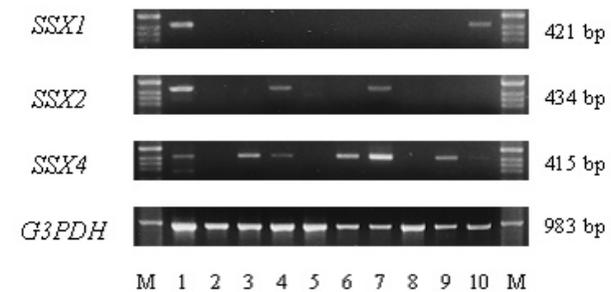
A survey of sera from 131 gynecological cancer patients showed antibodies against SSX4, but not SSX2, in two patients. One was a stage IV ovarian cancer patient, and the other a stage II cervical cancer patient with para-aortic lymph node metastasis. These two patients showed no mRNA expression of SSX1 or SSX2 in their tumors. No cross-reaction was observed with SSX4 antibody against SSX2. D. Jäger *et al.* (21) also found anti-SSX antibodies in cancer patient sera. Among the CT antigens, NY-ESO-1 appeared to be the most antigenic in terms of antibody production (22). Reactivity to NY-ESO-1, but not to other cancer-testis antigens, was observed for 4/32 (12.5%) Caucasian ovarian cancer patients (22). We previously reported that antibody against NY-ESO-1 was observed in 9/72 (12.5%) patients with G3 transitional cell carcinomas (23) and 10/140 (7.1%) patients with stage D prostate cancers (24) in a group of Japanese patients.

Due to their expression being restricted to normal testis tissue and a variety of tumors, cancer-testis antigens are the ideal targets for cancer vaccines (25). Clinical trials involving MAGE, NY-ESO-1, and other antigens are ongoing (8, 9, 10). Gynecological cancers have frequently been found to express SSX4 mRNA, and some patients exhibited a humoral immune response to SSX4. Therefore, SSX4 is a candidate for a vaccine against gynecological cancers. Peptide SSX2₄₁₋₄₉ (KASEKIFYV) is recognized by CD8 in association with HLA-A2, suggesting a new candidate peptide for immunotherapy (26). The SSX4

Table 1
SSX mRNA expression in gynecological cancers

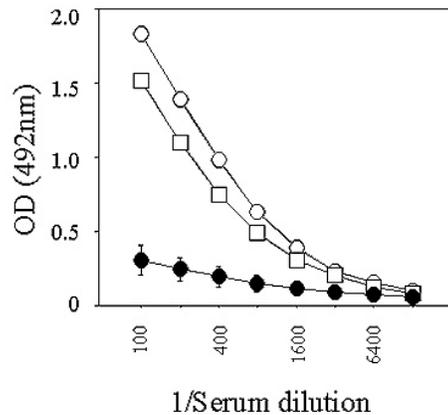
	Endometrial cancer	Ovarian cancer	Cervical cancer
SSX1	1/50 (2%)	0/40 (0%)	1/25 (4%)
SSX2	2/50 (4%)	1/40 (3%)	1/25 (4%)
SSX4	12/50 (24%)	5/40 (13%)	5/25 (20%)

Figure 1



Representative RT-PCR analysis for the expression of SSX mRNA. Normal testis (lane 1), normal endometrium (lane 2), endometrial cancers (lanes 3-4), normal ovary (lane 5), ovarian cancers (lanes 6-7), normal cervix (lane 8), and cervical cancers (lanes 9-10) are shown.

Figure 2



Representative ELISA responses against SSX4 recombinant protein. The open squares correspond to serum from cervical cancer patient CC27, and the open circles to serum from ovarian cancer patient OC9. The closed circles correspond to the mean value for sera from 40 healthy volunteers, and the error bars to ± 1 SD.

peptide corresponding to SSX2₄₁₋₄₉ differs by 2 amino acids from KASEKIFYV. In the case of the MAGE-A1 and MAGE-A3 tumor antigens, two homologous but not identical peptides derived from the same region constitute two independent epitopes (27). Nevertheless, this predicted SSX4 epitope will need to be confirmed experimentally, and it should allow us to analyze the ability of SSX4-specific CD8 T cells to kill tumor targets.

In conclusion, *SSX1*, *SSX2*, and *SSX4* mRNA expression were observed in gynecological malignancies, with *SSX4* being the most frequently expressed. In addition, an antibody response against *SSX4* was found in some gynecological cancer patients. *SSX4* is therefore a potential target for a cancer vaccine against gynecological cancers.

Abbreviations

CT, cancer-testis

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Materials and methods

Tissue samples and sera

Tumor specimens (50 endometrial carcinomas, 40 ovarian carcinomas, and 25 cervical carcinomas) and adjacent normal tissues (10 endometria, 10 ovaries, and 5 uterine cervixes) were obtained surgically and kept in a freezer at -80°C pending RNA extraction. Sera from 59 endometrial, 44 ovarian, and 38 cervical carcinoma patients and 40 healthy volunteers (28 females and 12 males) were used. The tissue samples and sera were obtained from the Department of Obstetrics and Gynecology of the Okayama University Graduate School of Medicine and Dentistry with informed consent. This study was conducted in accordance with the guidelines of the Ethical Committee at that institution.

RT-PCR

Total RNA was isolated from frozen tumor specimens using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and 2 µg of total RNA was reverse-transcribed into single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase (Ready-To-Go™ You-Prime First-Strand Beads, Amersham Pharmacia, Piscataway, NJ, USA) and oligo(dT)₁₅ primer. Complementary

DNA was tested for integrity by amplifying *G3PDH* transcripts in a 30-cycle reaction. The primers and PCR conditions have been described previously (12).

DNA sequencing

The PCR products were cloned into the pCR2.1 vector using an Original TA Cloning® Kit (Invitrogen, San Diego, CA, USA). An ABI 310 DNA Sequencer (Perkin-Elmer, Foster City, CA, USA) was used to determine the nucleotide sequence.

Clinical and pathological information

All data, including age, histological type and grade, tumor invasion, lymph node metastasis, and clinical stage, were obtained from the clinical and pathological records.

Statistical analysis

Statistical analysis was performed by the chi-squared and Fisher's exact tests. A value of $P < 0.05$ was considered to be statistically significant.

ELISA

Recombinant SSX2 and SSX4 proteins (kindly provided by Dr. Yao-Tseng Chen, 100 µl/well) at 1 µg/ml in coating buffer were added to 96-well plates and incubated overnight at 4°C. The plates were then washed with 0.05% Tween 20/PBS and blocked with 100 µl/well of 5% FCS/PBS for 1 h at room temperature. After washing, the sera (100 µl/well), serially diluted with 5% FCS/PBS, was added and the plates incubated for 2 h at room temperature. After a third wash, diluted goat antihuman IgG (100 µl/well) labeled with peroxidase (MBL, Nagoya, Japan) was added and the plates incubated for 1 h at room temperature. After another washing, substrate solution was added to each well and the plates were incubated for 15 min at room temperature. Finally, 3 M H₂SO₄ was added and the plates were read using a Benchmark microplate reader (BIO-RAD, Hercules, CA, USA).

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