Expression and Immunotherapeutic Targeting of the SSX Family of Cancer-Testis Antigens in Prostate Cancer

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Running Title: Expression of the SSX CTA in Prostate Cancer

Key Words: SSX, CTA, Prostate Cancer, 5-Aza-2'-deoxycytidine, Trichostatin A

Abbreviations: SSX, synovial sarcoma chromosome X breakpoint; CTA, cancer testis antigen; TAA, tumor associated antigen; RT-PCR, reverse transcription polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction; MHC, major histocompatibility complex; ELISPOT, enzyme-linked immunosorbent spot; HLA, human leukocyte antigen; 5-aza-dc, 5-Aza-2'-deoxycytidine; TSA, Trichostatin A; CTL, cytotoxic T lymphocyte; PAGE, polyacrylamide gel electrophoresis; IHC, immunohistochemistry; SFU, spot-forming units; EMA, epigenetic modifying agent
Abstract:

Recent FDA approval of the first immunotherapy for prostate cancer encourages efforts to improve immune targeting of this disease. The SSX proteins comprise a set of cancer-testis antigens (CTA) that are upregulated in MHC class I-deficient germline cells and in various types of advanced cancers with a poor prognosis. Humoral and cell-mediated immune responses to the SSX family member SSX2 can arise spontaneously in prostate cancer patients. Thus, in tumors that are MHC class I negative SSX2 and other SSX proteins may offer useful targets for tumor immunotherapy. In this study, we evaluated the expression of SSX family members in prostate cancer cell lines and tumor biopsies to identify which members might be most appropriate for immune targeting. We found that SSX2 was expressed most frequently in prostate cell lines, but that SSX1 and SSX5 were also expressed after treatment with DNA demethylating agents such as 5-aza-2’-deoxycytidine. Immunohistochemical analysis of microarrayed tissue biopsies confirmed a differential level of SSX protein expression in human prostate cancers. Notably, SSX expression in patient samples was restricted mainly to metastatic lesions in the absence of any expression in any primary prostate tumors examined. We determined that cross-reactive immune responses to a dominant HLA-A2-specific SSX epitope (p103-111) could be elicited by immunization of A2/DR1 transgenic mice with SSX vaccines. Our findings suggest that multiple SSX family members are expressed in metastatic prostate cancers which are amenable to simultaneous targeting.
Introduction:

Prostate cancer is the most frequently diagnosed and second leading cause of cancer-related death among American men, and a significant health concern worldwide (1). Organ-confined prostate cancer is initially treated by surgery or radiation therapy; however, approximately one-third of patients relapse, and another one-third of these patients will ultimately develop life-threatening, castrate-resistant tumors (1-3). Sipuleucel-T was recently FDA approved as the first immunotherapeutic vaccine shown to improve overall survival in patients with castrate-resistant prostate cancer (4). The success of this approach suggests that other simpler immunotherapies could be investigated targeting additional antigens, potentially with the goal of preventing the development of castrate-resistant metastatic disease.

Over the course of the last decade several prostate cancer antigens have been evaluated in clinical trials as immunotherapeutic targets for prostate cancer therapy including PSA (5, 6), PAP (7, 8), PSMA (9), and PSCA (10, 11). Although these protein targets are frequently expressed by prostate cancer cells, they are also expressed by normal prostate tissue and may not be critical for the survival of the tumor; hence their expression might be down-regulated by cancer cells during the course of immune targeting or disease progression (12-16). Thus, there remains a need to identify immune targets of prostate cancer that are highly expressed in metastatic disease and/or critical to the progression of the disease as simultaneous antigen targeting may be important to prevent the outgrowth of escape variants arising during the course of targeted therapy.
Cancer-testis antigens (CTA) are one class of tumor-associated antigen (TAA) upregulated in tumors of different histological origin, including prostate cancer, and especially prevalent in advanced disease (17-19). Demonstrated in some cases to be spontaneously immunogenic in cancer patients, these proteins are normally only expressed in germ cells of the testis (20-25). Due to the blood-testis barrier, a paucity of antigen-presenting cells (APC), and a lack of major histocompatibility (MHC) molecules on their surface, proteins exclusively expressed in germline tissue are considered immune-privileged (26, 27). Thus, the ectopic expression of CTA in cancer tissue makes these proteins ideal immunotherapeutic targets. NY-ESO-1 and the MAGE family of antigens have been the most extensively studied CTAs. Proteins of these families have been found to be preferentially expressed in metastases over primary tumor of various histological origin and are currently being evaluated as target antigens in clinical trials (28, 29).

Another superfamily of CTA thought to be attractive targets for cancer immunotherapy are the synovial sarcoma X chromosome breakpoint (SSX) proteins (30, 31). While the precise function of these proteins remains unknown, SSX expression has been associated with stem cell migration suggesting a potential biologically important role to the metastatic phenotype (32). The most investigated member, SSX2, has been shown to be expressed in prostate cancer lesions at the mRNA level (33), while expression of other SSX family members has been shown to be inducible with epigenetic modifying agents (EMA) in colon carcinoma cell lines (34). We have shown that SSX2 expression can be upregulated in prostate cancer cell lines upon treatment with 5-aza-2’-deoxycytidine (21). We have also shown that SSX2 antibodies and SSX2 peptide-
specific T cells can be found in the peripheral blood of some patients with prostate cancer, indicating that patients can have pre-existing immune responses to SSX2 (21, 35). Because the SSX antigens are highly immunogenic and essentially tumor-specific, these proteins might be more ideal as targets for immunotherapy than other prostate-associated antigens. However, the relative expression of specific SSX family members in prostate cancer cell lines and prostate tumor tissues remains unknown. In the current study we sought to identify which SSX proteins are expressed in prostate cancer cell lines and tissues and therefore potentially relevant target antigens for prostate cancer immunotherapy. We then next sought to determine whether it is possible to simultaneously target these SSX family members using immunotherapeutic vaccines.
Materials and Methods:

Cell Culture

Prostate cancer cell lines (LNCaP, 22-RV1, PC3, and DU-145) and non-cancer immortalized prostate epithelial cell lines (RWPE-1 and PREC-6) were grown in RPMI 1640 media supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 200U/mL penicillin/streptomycin, 1 mM sodium pyruvate, and 0.1μM β-mercaptoethanol. VCaP and LAPC4 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 5% glucose, 10% FCS, 200U/mL penicillin/streptomycin, 1 mM sodium pyruvate, and 0.1μM β-mercaptoethanol. Where indicated, cells were additionally cultured with 10μM 5-Aza-2'-deoxycytidine (5-aza-dc; Sigma, St. Louis, MO), 100nM Trichostatin A (TSA; Sigma), or both agents simultaneously for 72 hours. RNA was collected after treatment using the RNeasy Mini kit (Qiagen, Valencia, CA). Cell lines were obtained from ATCC (Manassas, VA), verified using polyphasic (genotypic and phenotypic) testing to confirm identity, and passaged in our laboratory for less than six months.

PCR, Reverse Transcription (RT)-PCR, and Quantitative RT-PCR Analysis

Gradient PCR was carried out to amplify DNA products from SSX image clones encoding SSX1, 2, 3, 4, 5, 6, 7, or 9 cDNAs (ATCC), without cross-amplification of other SSX family members. Image clones exclusively encoding SSX8 and SSX10 were not commercially available. Amplification of products with SSX8 and SSX10 primers in cell lines was consequently verified by direct DNA sequencing. SSX primers used for
PCR analysis were designed directly, or from published sequences (30, 34), and were commercially synthesized (Integrated DNA Technologies, San Diego, CA). PCR conditions were as follows: 95°C for 1 min. followed by 30 cycles of 95°C for 1 min., the specific annealing temperature for 1 min., and 72°C for 3 min. A final extension time was 10 min. at 72°C. Products were then separated and evaluated by agarose gel electrophoresis.

Reverse-transcription PCR using the One-Step RT-PCR kit (Qiagen) was carried out using RNA isolated from cell lines with the primers and annealing temperatures listed in Table 1 as well as β-actin control primers (actinA: 5’-TCATGAAGTGTGACGTTGACATCCGT-3’, actinB: 5’-CTTAGAAGCATTTCGCGTGCACGATG-3’) under the following PCR conditions: 50°C for 30 min., 95°C for 15 min., 35 cycles at 95°C for 1 min., specific annealing temperature for 1 min., and 72°C for 1 min; final extension for 10 min. at 72°C. For quantitative RT-PCR (qRT-PCR), 1 µg RNA was collected and reverse transcribed using qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer’s instructions. qRT-PCR was performed using PerfeCTa® SYBR® Green SuperMix for iQ(Quanta Biosciences, Inc.) according to the manufacturer’s instructions with 1µL of the cDNA synthesis reaction mixed with primers specific for each family member and the SuperMix. Samples were analyzed using a MyiQ™2 Two-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with annealing temperatures as shown in Table 1. All results were analyzed by the 2^ΔΔCt method (37) relative to the ribosomal protein P0 as a control gene (38): (P0 Forward: 5’-ACAATGCGACCATCTACAC; P0 Reverse:5’-GCAGACAGACACTGGCAAC).
Fold induction over vehicle treatment was calculated using the $2^{-\Delta \Delta Ct}$ method, as previously published (37).

**Western Blot**

Recombinant SSX GST-tagged proteins (SSX1-5) (Abnova, Walnut, CA) or prostatic acid phosphatase (PAP) protein (Fitzgerald, Acton, MA) were resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes using standard procedures. Membranes were probed with an SSX2 monoclonal antibody (Abnova, clone 1A4) or an SSX5 polyclonal antibody (Abnova, B01P).

**Immunohistochemistry**

Paraffin-embedded tissue sections were washed in xylene and rehydrated in ethanol, followed by antigen retrieval using Tris-EDTA buffer supplemented with 5mM CaCl$_2$, 0.5% Triton X-100 (pH 8.0), and 20µg/mL Proteinase K. After washing in PBS/0.1% Tween-20 and blocking in PBS/10%BSA, slides were stained with primary antibody overnight at 4°C. Samples were then stained with goat anti-mouse IgG secondary antibody (Sigma), washed, and stained using the LSAB System HRP kit (Dako, Carpinteria, CA) and DAB metal concentrate (Pierce, Rockford, IL). Slides were then counterstained with hematoxylin, mounted with coverslips, and imaged using an Olympus BX51 microscope (Olympus, Center Valley, PA) and SPOT RT analysis software (Diagnostic Instruments, Sterling Heights, MI).

**HLA-A2 T2 Binding Affinity Assays**
SSX nonamer peptides derived from the amino acid sequences of SSX1-9 and corresponding to SSX2 epitope p103-111, were evaluated for their predicted HLA-A2 affinity using the SYFPEITHI and BIMAS prediction algorithms (39, 40). The purity and identity of synthesized peptides was confirmed by mass spectrometry and gas chromatography (United Biochemical Research, Seattle, WA). In vitro HLA-A2 affinity for each peptide was determined by stabilization of HLA-A2 on TAP-deficient T2 cells as previously described (35, 41). Peptide-HLA-A2 stabilization was measured as relative mean fluorescent intensity (MFI), normalized to vehicle-pulsed T2 MFI, and all measurements were conducted in triplicate.

**Mice**

HLA-A2.01/HLA-DR1-expressing, murine MHC class I/II knock-out, transgenic (A2/DR1) mice on C57Bl/6 background were provided by Dr. François Lemonnier (42). Mice were maintained in microisolator cages under aseptic conditions and all experimental procedures were conducted under an IACUC-approved protocol.

**Immunization Studies**

Four to six week-old A2/DR1 mice were immunized subcutaneously with 100μg of an SSX peptide, or vehicle control in complete Freund’s adjuvant (Sigma). For DNA vaccination studies, mice were immunized every 14 days intradermally in the ear pinna with 100μg of either plasmid DNA vector control (pTVG4), or a plasmid DNA vaccine encoding SSX2 (pTVG-SSX2) (35). Mice were euthanized seven (peptide
immunization) or 14 (DNA immunization) days after the last immunization and splenocytes were isolated by centrifugation after red blood cell osmotic lysis.

**IFNγ ELISPOT**

IFNγ ELISPOT was performed according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN) as previously described (7, 35, 43). Cells were incubated for 48 hours in the presence of 2μg/mL of each individual SSX peptide, influenza matrix protein A peptide, 10 μg/mL concanavalin A (Calbiochem), or media alone, prior to IFNγ detection. Spots were counted with an automated plate reader (Autoimmun Diagnostika, Strassberg, Germany). The number of spots was corrected for the media alone negative control, and reported as the mean number of antigen-specific IFNγ spot-forming units (SFU) per 10⁶ splenocytes from triplicate samples.
RESULTS:

Identification of gene-specific primers for ten SSX family members

To evaluate mRNA expression of SSX family members, primers and annealing temperatures were first determined that would result in specific product amplification for each known family member. Using the published SSX gene-specific primers and annealing temperatures for PCR (30, 34), we observed cross-amplification of products from image clone DNA plasmids encoding different SSX family member cDNAs, particularly for the SSX4, 6, 7 and 9 primer pairs (data not shown). Based on these results, we designed new primers and conducted gradient PCR to establish conditions that would result in complete primer specificity. The primers and annealing temperatures used for these experiments are shown in Table 1, and the location of the published and re-designed primers within the aligned SSX sequences are shown in Supplementary Figure 1. Specific amplification was observed for all SSX primers (Figure 1). Image clones encoding SSX8 and SSX10 were not available; however SSX8 and SSX10 primer sets were tested independently for cross-amplification from the available image clones with no amplification observed (Figure 1B). These primers were also tested for PCR amplification from a human testis cDNA library. Products of the appropriate size were found for SSX8 but not SSX10 (Figure 1C). Amplified products of the predicted size for SSX8 were sequenced, confirming primer specificity and SSX8 expression in the testis.

SSX family members 1, 2, 3, 5, and 8 are expressed or inducible in prostate cell lines

Previous work by our group and others has shown that certain CTA, including SSX2, can be induced in cancer cell lines with EMA (21, 34). To determine which SSX
members could be potential targets for prostate cancer immunotherapy, three human androgen-dependent prostate cancer cell lines (LAPC4, LNCaP, VCaP), three androgen-independent cell lines (22-RV1, PC3, DU-145), and two immortalized prostate epithelial cell lines (RWPE-1 and PREC-E6) were cultured in vitro and treated with or without two different EMA. Specifically, cells were treated with a DNA methyltransferase inhibitor, 10μM 5-aza-dc, a histone deacetylase inhibitor, 100nM trichostatin A (TSA), or both agents simultaneously, while untreated controls were treated with vehicle alone. RNA was collected 72 hours after treatment for RT-PCR analysis (Figure 2). SSX1 was expressed at baseline only in untreated PC3 cells, but was frequently inducible following 5-aza-dc treatment. SSX2 was expressed at baseline in most of the cell lines and was inducible to varying degrees in the androgen-independent cells and the normal prostate epithelial cell lines. Additionally, in some instances SSX3, 5, and 8 were inducible with EMA treatment. In the case of SSX8, amplified products were gel-purified and sequenced, confirming SSX8 amplification in DU-145, LNCaP, and PREC-E6 cells, which to our knowledge is the first identification of SSX8 expression in any cancer cell line or tissue.

qRT-PCR was conducted with the same RNA samples in all cases where amplification was observed by RT-PCR. The expression patterns found by RT-PCR for SSX1, 2, 3, 5, and 8 were highly similar by qRT-PCR (Figure 3; SSX3, SSX8 not shown). TSA did not appear to significantly induce expression either alone or in combination with 5-aza-dc, suggesting that regulation of SSX gene expression is predominantly mediated, directly or indirectly, by changes in DNA methylation. Overall, these results demonstrate that SSX2 is the most commonly expressed family member in
prostate cancer cell lines, with higher (and more frequent) expression detected relative to other SSX family members. However SSX1 and SSX5 expression were frequently induced following treatment with a methylation inhibitor. Individual cell lines could also express multiple SSX family members.

**SSX proteins are expressed in human prostate cancer tissues**

We next sought to determine if the SSX family member proteins are expressed in human prostate tumors, and whether this expression is associated with stage of disease. Because the SSX proteins have highly similar amino acid sequences, the cross-reactivity to SSX1-5 GST-tagged recombinant proteins was evaluated by Western blot. Recombinant proteins for SSX family members 6-10 were not available and were thus not evaluated. We found that a monoclonal antibody, 1A4, recognized SSX2 and SSX3, while a murine polyclonal antibody, B01P, recognized SSX1, 3, 4, and 5, but not SSX2 (Figure 4A). These antibodies could also detect SSX protein expression in paraffin-embedded human testis tissue (Figure 4B). Using a paraffin-embedded tissue microarray, which included biopsy samples from 95 patients with varying stages of prostate cancer, 25 patients with high-grade prostatic intraepithelial neoplasia (HGPIN), or benign cores from 72 control patients, we evaluated SSX expression in cancer tissues by IHC. SSX expression was detected in some of these tissue specimens with differential staining observed depending on which SSX antibody was used (Figure 4C and Supplemental Figure 2). Given the cross-reactivity of these antibodies, the identification of specific SSX family members was not possible, but the presence of staining with 1A4 and not B01P in certain lesions, together with our findings in cell lines, suggested that some
tissues may express only SSX2 (among SSX1-SSX5). Staining with B01P, but not 1A4, suggested that other tissues may express one or more of the other SSX family members. Additionally, the nuclear SSX staining patterns varied among tissues; in some cases SSX expression was homogenous, while in other tissues the nuclear staining was punctate with expression detected in some cells but not adjacent tumor cells. Interestingly, SSX protein expression among tumors was found exclusively in metastatic lesions (5/22, 23%), and not in primary tumors (0/73, p <0.001, chi square, Table 2). A single benign tissue sample was scored positive, however, given predominantly cytoplasmic staining this might have been a staining artifact (Supplemental figure 2).

T cells specific for epitope p103-111 from SSX1 and SSX2 differentially recognize peptide p103-111 from other SSX proteins

Ultimately these studies were conducted to identify which SSX family members might be attractive targets for prostate cancer immunotherapy. The results above suggested that SSX1, 2, and 5, and possibly SSX3 and 8, may be the most relevant members expressed in prostate tumors. Because these proteins are highly homologous, we next wanted to determine whether they could be immunologically targeted following immunization with one SSX family member. To test this, we focused on SSX2 as the dominant SSX family member with highest expression among prostate cancer cell lines and restricted our analysis to a single HLA-A2-restricted epitope, SSX2 p103-111 (35). We previously identified that T cells specific for this immunodominant epitope can be detected in HLA-A2+ patients with advanced prostate cancer, and can lyse HLA-A2+ SSX2+ prostate cells (35). We therefore wanted to assess whether SSX2 p103-111-
specific T-cells could recognize the corresponding peptide derived from other relevant SSX family members. Using peptide-binding algorithms to predict HLA-A2 affinity, we found that most of the corresponding p103-111 peptides from the different family members were predicted to have moderate HLA-A2 affinity (Figure 5A). These peptides were then synthesized and evaluated for their in vitro HLA-A2 affinity (Figure 5B). We found that each SSX p103-111 peptide had affinity for HLA-A2, and this binding corresponded with the predicted binding algorithm scores. As a negative control, peptide p103-111 from SSX2 was modified (SSX2-IP) to abolish HLA-A2 binding.

To evaluate whether T cells specific for SSX2 p103-111 can recognize peptides derived from different SSX family members, we immunized HLA-A2 transgenic (A2/DR1) mice with an SSX2-encoding DNA plasmid vaccine (35). T cells specific for SSX2 p103-111 recognized p103-111 from SSX3/5/9 but no other family members (Figure 5C), suggesting that an SSX2 vaccine that elicits SSX2 p103-111 peptide-specific T cells could also be used to target SSX3, 5, or 9. Similarly, T cells from A2/DR1 mice immunized directly with peptide p103-111 from SSX1 or SSX2 were evaluated for cross-reactive immune responses by IFNg ELISPOT (Figure 5D). Again we found that T cells specific for SSX2 p103-111 could recognize peptide p103-111 from SSX3/5/9, while T cells elicited by immunization with SSX1 p103-111 cross-recognized peptides p103-111 from SSX4, SSX6/8, and SSX7. Together, these results show that multiple SSX family members can be expressed in prostate cancer and could potentially be targeted simultaneously using immunotherapeutic vaccines.
**Discussion:**

SSX2 mRNA expression has previously been reported in human prostate tumor tissues (21, 33, 44). Initially, Tureci *et al.* observed SSX2 expression in 5 of 25 prostate cancer lesions by RT-PCR (44), and later found SSX1, 2, 4 and 5 expression in a number of different cancer tissues, however, SSX2 was the only member found in prostate cancer lesions (33). When SSX6-9 were subsequently identified, it was found that SSX1, 2, 4, 5, and 6 could be induced with EMA treatment in colon cancer cell lines (34). Prior to our study, a comprehensive study evaluating SSX family member mRNA and protein expression had not been conducted for prostate cancer cell lines or tumor tissues.

In this report we have identified primers that specifically amplify individual SSX family members. These primers were used to evaluate SSX expression in prostate cancer cell lines by RT-PCR and qRT-PCR. We found that SSX2 mRNA transcripts were commonly expressed in prostate cancer cell lines at baseline, while SSX1 and SSX5 were frequently inducible with EMA. To a lesser extent SSX3 and SSX8 were also induced (4/8 and 3/8 cell lines, respectively). Using SSX antibodies that recognize different SSX family members, we found that tumors can have differential SSX protein expression, and expression is essentially restricted to metastatic lesions. We also found that T cells specific for an HLA-A2-restricted SSX2 immunodominant epitope can recognize the corresponding p103-111 peptide derived from other SSX family members. Cross-reactivity of SSX2 p103-specific T cells was observed for the corresponding p103-111 peptide from SSX3/5/9, while SSX1 p103-111-specific T cells appear to recognize p103 from SSX6/8 and perhaps SSX4 and SSX7.
In contrast to previous reports, we found that published primers for SSX4, 6, 7, and 9 led to amplification of other family member transcripts. In our hands SSX4 primers previously reported amplified products from SSX2, 3, and 4 cDNA templates at the annealing temperatures published for SSX4, while SSX6, 7, and 9 primers cross-amplified products from the SSX7 and SSX9 cDNA templates (data not shown). We also observed cross-amplification of SSX7 and SSX9 transcripts using the published SSX5 primers. We report here a set of primers and annealing temperatures specific to each family member that eliminated cross-amplification (Table 1, Supplementary Figure 1). We believe that cross-amplification with the original SSX4 primers is a significant finding due to the reported expression of this family member in cell lines and tumor tissues in numerous studies. Because we found these primers to cross-amplify SSX2, which is the most frequently reported family member in cancer cell lines and tissues, it could be that some reports of the frequency of SSX4 expression in various tumors are inaccurate.

The observed patterns of SSX family member expression and induction from our studies suggest underlying epigenetic marks in different cell lines that mediate responsiveness to EMA agents. Differences in promoter methylation of CpG islands, methylation of intragene cysteines, or alterations in chromatin folding due to acetylation or methylation of histone residues could all contribute to variations in SSX family member expression. From our studies, the largest induction of transcription was found after DNA methyltransferase inhibition with 5-aza-dc treatment, which is not surprising in light of previous findings that hypomethylation events arise early in prostate cancer tumorigenesis (45-47). Although the methylation status of CpG islands have not been
reported for the \textit{SSX} gene family members, whole genome bisulfite sequencing data from cancer cell lines is available online through the UCSC Genome browser (48). There appear to be no documented methylation sites or CpG islands in the promoter region of SSX2, 3, 4, 6, 7, 8, or 9, even far upstream of the start site or within intragene regions. Methylated sites are found in the promoter region of SSX1, and in an intragene region of SSX5 that could account for differences in baseline expression or inducibility. Other regulation sites or mechanisms may be present to account for the increased expression of SSX2, 3, and 8 in some cell lines after EMA treatment. In two of the three cell lines showing SSX8 inducibility, expression is only present with combined 5-aza-dc and TSA treatment, suggesting that chromatin remodeling may play a role in transcriptional regulation of this family member. Future studies will assess methylation patterns found in \textit{SSX} genes at the DNA level before and after EMA treatment.

We also demonstrate for the first time SSX protein expression in human prostate cancer tissues. We observed different patterns of SSX family member protein expression in prostate tumors in terms of member and homogeneity of staining. SSX expression was found almost exclusively in metastatic prostate cancer lesions as compared to primary prostate cancer lesions (p < 0.001, chi square). Expression identified here in bone, lymph node, and brain metastases suggest that SSX protein expression is inherent to metastatic disease independent of tissue site. Interestingly, primary tumor biopsies from patients with metastases (stage IV) also were SSX-negative, while metastatic tissues from 5 of 22 patients were SSX-positive (p = 0.027, chi square), further associating SSX expression specifically with metastatic disease. These results correspond to our previous studies looking at the frequencies of SSX2 p103-111 peptide-specific T cells in the PBMCs of
patients with prostate cancer by tetramer analysis (35). We found that patients with advanced disease had significantly higher frequencies of SSX-specific T cells compared to healthy donors and patients with early stage disease, further suggesting that these antigens may be associated with advanced prostate cancer. These findings may or may not suggest a biological importance of SSX expression in metastatic disease progression.

The expression of multiple SSX family members in some metastatic prostate cancer lesions suggests that SSX immunotherapy may need to consider targeting several SSX proteins simultaneously. We have previously identified an immunodominant SSX2 epitope (p103-111) that can elicit epitope-specific T cells in transgenic A2/DR1 mice immunized with a plasmid vaccine encoding SSX2 (35). Cross-reactivity of SSX2 p103-specific T cells from DNA or peptide-immunized mice was observed for the corresponding p103-111 peptide from SSX3/5/9, while SSX1 p103-111-specific T cells recognized p103 from SSX6/8, SSX4, and SSX7. Interestingly, while SSX antibodies were unable to fully distinguish between family members, our T-cell cross-reactivity results demonstrate that subtle differences in amino acids in the TCR-interacting portion of the epitopes conferred specificity for some members and cross-reactivity to others. These studies do not rule out the possibility that other SSX epitopes could be recognized among family members. Future work with T-cell directed therapies needs to consider whether SSX1, 2, and 5, as the most expressed SSX family members, will specifically be targeted for prostate cancer. Immune targeting could potentially be accomplished by immunizing with multiple plasmids encoding these members, or polytope vaccines could be designed encoding cross-reactive epitopes that will elicit T cells that can specifically recognize peptides from all SSX family members relevant to prostate cancer.
While SSX proteins may be expressed in advanced prostate tumors, it should also be noted that this expression may be heterogeneous as has been described for other CTA in various tumor types. This could potentially allow for antigen-loss variants during immune targeting with a vaccine. Antigen heterogeneity might be overcome with EMA treatment to up-regulate antigen expression in tumor tissue, however it should be noted that these EMA might have deleterious effects on immune function or may up-regulate antigen expression in normal tissues. Future work will need to assess the feasibility of combining these two treatments simultaneously to optimize tumor immunotherapy.
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**Figure and Table Legends**

**Table 1:**

*SSX family member sequence sources and primer design*

Shown are the GenBank® accession numbers for each SSX family member evaluated, the primer sequences and annealing temperatures used to amplify specific SSX products in PCR and RT-PCR studies, and the expected product size of each amplified family member transcript.

**Table 2:**

*SSX proteins are preferentially expressed in metastatic prostate cancer lesions.*

Shown are the numbers of patients in each disease stage with biopsy cores staining positive with either of the two SSX antibodies, or both antibodies simultaneously (“total SSX”) by tissue microarray IHC. Data are representative of two independent experiments. * (staining shown in Supplementary Figure 2).

**Figure 1:**

*PCR amplification of SSX gene products from plasmids expressing specific SSX family members.*

Panel A: Primer sets specific for each SSX family member were tested for PCR amplification using available image clone plasmids encoding different SSX family members as template DNA. SSX8 and SSX10 image clones were unavailable. Panel B:
SSX8 and SSX10 primer sets were tested against the available SSX image clones. Panel C: SSX2, 8, and 10 primer sets were evaluated by PCR amplification from human testis cDNA to verify specificity of SSX8 and SSX10 primers. Data in each panel is representative of two independent experiments.

**Figure 2:**

*RT-PCR analysis reveals SSX CTA expression in prostate cancer cell lines and inducible expression of SSX mRNA transcripts with epigenetic modifying agents.*

Three androgen-dependent and three androgen-independent prostate cancer cell lines, and two immortalized prostate epithelial cell lines, were evaluated for expression of SSX1-10 mRNA either in media alone without treatment (NT) or after treatment with 10μM 5-aza-dc (AZA), 100nM TSA (TSA), or treatment with both agents simultaneously (A+T). These results are representative of two independent studies.

**Figure 3:**

*Quantitative analysis of relative expression and fold induction of SSX 1, 2, and 5 mRNA in prostate cancer cell lines treated with epigenetic modifying agents.*

RNA isolated from prostate cancer cell lines showing SSX expression by RT-PCR was evaluated by qRT-PCR for expression relative to an internal control transcript (P0) (Panel A) and specific fold induction (Panel B) of SSX transcripts following EMA treatment. qRT-PCR was performed with primers specific for SSX1, 2, and 5, conducted in
triplicate and repeated in an independent experiment. Error bars represent the mean and
standard deviation of six wells from these two experiments. Comparison between groups
was made with a one-way analysis of variance followed by post hoc analysis with the
Tukey test. * indicates p<0.05 comparing with vehicle treatment (panel A) or with TSA
treatment (panel B).

Figure 4:

SSX proteins can be detected in tumor biopsy specimens from prostate cancer patients.

Panel A: 200 ng of GST-tagged recombinant SSX proteins (SSX1-5) or PAP protein
(negative control) were resolved by SDS-PAGE. Cross-recognition of SSX proteins was
determined by staining with mAb (1A4) or pAb (B01P) in Western blot. Panel B: These
antibodies were also evaluated for their recognition of SSX proteins in paraffin-
embedded testis tissue by IHC. An IgG isotype control was used as a primary antibody
negative control for staining specificity. Panel C: Tissue microarray slides were stained
using mAb 1A4 mAb, pAb B01P, or an IgG isotype control. Specimens were considered
positive for SSX expression if two blinded observers independently scored the cores
positive. Metastatic biopsy samples from three patients with differential SSX expression
are shown (Patient 1: bone metastasis; patient 2: brain metastasis; patient 3: lymph node
metastasis). Positive and negative staining are denoted by “+” and “-”, respectively.

Figure 5:
SSX1 and SSX2 p103-111-specific T cells exhibit cross-recognition for other SSX proteins

Panel A: Shown are the homologous amino acid sequences for SSX1-9 corresponding to the p103-111 epitope described previously for SSX2 (35). Highlighted amino acids represent non-identical residues compared to SSX2. Columns three and four show the predicted HLA-A2 affinity scores for each peptide using the SYFPEITHI (40) and BIMAS (39) prediction algorithms. Panel B: *In vitro* HLA-A2 affinity of p103-111 peptide from each family member at titrated peptide concentrations by T2 binding assay (MFI = mean fluorescence intensity). These results are representative of two independent experiments. Panel C: Splenocytes from individual A2/DR1 mice immunized with either DNA vaccine encoding SSX2 (pTVG-SSX2, n=9, ■) or vector control (pTVG4, n=3, ○) were analyzed to determine the frequency of peptide-specific T cells by IFNγ ELISPOT. Splenocytes were stimulated with one of each of the six p103-111 peptides from the different SSX family members, SSX2-IP, influenza matrix A peptide, or a ConA positive control. Each dot represents the frequency of peptide-specific IFNγ spot-forming units (SFU) from an individual mouse, measured in triplicate with background subtracted. Panel D: Splenocytes from individual A2/DR1 mice immunized with peptide p103-111 from either SSX1 (n=5, ■) or SSX2 (n=6, ●) or a vehicle control (n=4, △) were analyzed to determine the frequency of peptide-specific T cells by IFNγ ELISPOT as described above.
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<tr>
<th>Family Member</th>
<th>Accession Number</th>
<th>5' Primer Sequence</th>
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<td>SSX1</td>
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<td>SSX2</td>
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<td>Total Tissues</td>
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Figure 1

A

Primer Set

SSX1
SSX2
SSX3
SSX4
SSX5
SSX6
SSX7
SSX9

Image Clone

B

Image Clone

Primer Set

SSX8
SSX10

C

Primer Set

SSX2
SSX6
SSX10
Figure 2

Androgen-Dependent PCa Cell Lines

- LNCaP
- SSX
- SSX
- SSX
- SSX
- SSX
- SSX
- Acta

Androgen-Independent PCa Cell Lines

- 22-RV1
- Acta
- SSX
- SSX
- SSX
- SSX
- SSX
- SSX

Immortalized Prostate Epithelial Cell Lines

- RV1E1
- Acta
- SSX
- SSX
- SSX
- SSX
- SSX
- SSX

- TM4E6
- Acta
- SSX
- SSX
- SSX
- SSX
- SSX
- SSX

- DU-145
- Acta
- SSX
- SSX
- SSX
- SSX
- SSX
- SSX
Figure 5

A

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B

C

Stimulating Peptide Antigen

- pTVG4-SSX2 (n=9)
- pTVG4 (n=3)

D

- SSX1 (n=6)
- SSX2 (n=6)
- CFA (n=4)
Expression and Immunotherapeutic Targeting of the SSX Family of Cancer-Testis Antigens in Prostate Cancer

Heath A Smith, Robert J. Cronk, Joshua M. Lang, et al.

*Cancer Res* Published OnlineFirst August 31, 2011.

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