Article

Cancer Testis Antigens and Immunotherapy: Expression of PRAME and NY-ESO-1 Predicts Survival in Soft Tissue Sarcoma

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Abstract: (1) Background: *PRAME*, *NY-ESO-1* and *SSX2* are cancer testis antigens (CTAs), which in normal tissues are expressed in testicular germ cells with re-expression in numerous cancer types. Their ability to elicit humoral and cellular immune responses have rendered them promising targets for cancer immunotherapy, but they have never been studied in a large and well-characterized cohort soft tissue sarcomas (STS). (2) Methods: On protein level, we examined *PRAME*, *NY-ESO-1* and *SSX2* expression in tumour tissues of 249 STS using immunohistochemistry. We correlated expression levels with clinicopathological parameters including Tumour-infiltrating lymphocyte (TIL) counts, grading and long- term survival. (3) Results: Expression of *PRAME*, *NY-ESO-1* and *SSX2* was observed in 25 (10%), 19 (8%), and 11 (4%) of 249 specimens with distinct patterns for histo subtypes. Expression of *PRAME* was associated with shorter patient survival (p=0.005) and higher grade (G2 vs G3, p=0.001) while *NY-ESO-1* expression was correlated with more favourable survival (p=0.037) and low grade (G2 vs G3, p=0.029). Both *PRAME* and *NY-ESO-1* expression was more frequent in STS with low TILs counts. (4) Conclusions: CTAs *PRAME, NY-ESO-1* and *SSX2* show distinct expression patterns in different STS subtypes. These results may guide future immunotherapeutic approaches in STS.

Keywords: soft tissue sarcoma, human, cancer/testis antigens, PRAME, NY-ESO-1, SSX2, biomarker, tumor infiltrating lymphocytes, immunohistochemistry

1. Introduction

Soft tissue sarcomas (STS) are a heterogenous group of rare malignant tumours originating from the soft tissues that can occur in different parts of the human body. While treatment of localised disease is based on radical resection, evidence from a limited number of trials supports the use of perioperative systemic therapy in high-risk tumours [1,2]. Therapeutic options, however, are limited, and in a recent trial, a histology-tailored regimen was inferior to standard chemotherapy with epirubicin and ifosfamide [2]. Immunotherapy, which has been successfully introduced for several cancers, might be an option to individualise therapy of sarcoma patients [3].

In normal tissues, expression of cancer-testis antigens (CTAs) is restricted to germ cells and trophoblasts. They are aberrantly expressed in various types of cancer. Following their identification in malignant melanoma, CTAs have been demonstrated in carcinomas of various sites such as lung, ovary, urinary bladder, liver and other organs [4]. Several CTAs such as MAGE, NY-ESO-1 and PRAME, are expressed in various sarcoma subtypes, such as synovial sarcoma [5-11], myxoid/round cell liposarcoma [6,11-16] and other soft tissues sarcomas. CTAs can be highly immunogenic and trigger immune responses in the autologous host. Consequently CTAs are considered potential targets for immunotherapy of cancer [17].

Tumor-infiltrating lymphocytes (TILs) belong to the microenvironment of malignant tumours; they are an essential part of the human body's own reaction to the tumour and they are thought to enhance the tumour response to certain chemotherapeutics [18]. Consequently, high TIL counts have been associated with a more favourable prognosis following neoadjuvant treatment of high-risk soft tissue sarcomas [19]. Furthermore, following their extraction and in vitro expansion, TILs can be applied in adoptive T-cell therapies [20].

In the present study, we analysed the expression of CTAs NY-ESO-1, PRAME and SSX2 as well as the presence of TILs in a large and well-characterized cohort of high-risk soft-tissue sarcoma patients with long-term follow-up and correlate our findings with clinical tumour characteristics and survival data.

We show that PRAME, NY-ESO-1 and SSX2 display distinct expression patterns in different STS subtypes. PRAME and NY-ESO-1 expression levels are correlated to patient survival and tumour grade in opposing ways while presence of either PRAME or NY-ESO-1 is correlated with low TILs counts. These results may guide future immunotherapeutic approaches in STS.

2. Results

2.1 Patient cohort

The patients' age at the time of diagnosis ranged from 18 to 79 years (median age 53 years, female n = 125, male n = 124). Tumour locations and histological subtypes are shown in **2.3 Cancer-Testis** Antigens PRAME and NY-ESO-1 are expressed more frequently in soft tissue sarcomas with low counts of tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes (TILs) were found in 193 (78%) of 249 specimens. Examples of immunohistochemistry staining of TILs are depicted in **Figure 1** and detailed results for TILs with respect to histologic subtypes are shown in **Table 2.**

Table 1. Only patients with intermediate (n = 118) or high grade (n = 131) sarcomas were included in this analysis.

2.2 Cancer-Testis Antigens show distinct expression patterns in sarcoma subtypes

Expression of PRAME, NY-ESO-1 and SSX2 was observed in 25 (10%), 19 (8%), and 11 (4%) of 249 specimens (248 specimens for SSX), respectively. Examples of immunohistochemistry staining for CTAs are shown in 2.3 Cancer-Testis Antigens PRAME and NY-ESO-1 are expressed more frequently in soft tissue sarcomas with low counts of tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes (TILs) were found in 193 (78%) of 249 specimens. Examples of immunohistochemistry staining of TILs are depicted in **Figure 1** and detailed results for TILs with respect to histologic subtypes are shown in **Table 2**.

Table 1, and detailed results of TMA analysis with respect to histologic subtypes are shown in **Table 2**. Expression levels for all CTAs are shown in **Table A1**.

2.3 Cancer-Testis Antigens PRAME and NY-ESO-1 are expressed more frequently in soft tissue sarcomas with low counts of tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes (TILs) were found in 193 (78%) of 249 specimens. Examples of immunohistochemistry staining of TILs are depicted in **Figure 1** and detailed results for TILs with respect to histologic subtypes are shown in **Table 2**.

Table 1: Patient Characteristic. UPS Undifferentiated pleomorphic sarcoma; SFT Solitary fibrous tumour; MPNST Malignant peripheral nerve sheath tumour

		n	%
Total		249	100
Sex	male	124	50
	female	125	50
Histological subtype	UPS	82	33
	Leiomyosarcoma	50	20
	Synovial sarcoma	28	11
	Dedifferentiated Liposarcoma	47	19
	Angiosarcoma	9	4
	MPNST	13	5
	other	20	8
Location	Extremities	85	34
	Retroperitoneal	54	22
	Abdominal/visceral	42	17
	Trunk	60	24
	Other	8	3
Surgical margins	R0/R1	202	81
	R2 or no resection	47	19
Grading	intermediate	118	47
	high	131	53
Size	<50 mm	20	8
	50-79 mm	61	24
	80-120 mm	64	26
	>120 mm	76	31
	Missing	28	11

Table 2: Cancer testis antigen expression and TILs according to tumour entity: The table shows the number and percentage of patients with high antigen expression and high tumour-infiltrating lymphocyte (TIL) infiltration. UPS: Undifferentiated pleomorphic sarcoma; DDLPS: Dedifferentiated Liposarcoma; SFT: Solitary fibrous tumour; MPNST: Malignant peripheral nerve sheath tumour. [a] 81 of 82 specimens were analysed for SSX2 expression; HPF: high power field

	Total	*PRAME*		*NY-ESO-1*		*SSX2*		TIL	
Histologic subtype	**n**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
UPS	82 [a]	6	7%	2	2%	3	4%	71	87%
Leiomyosarcoma	50	3	6%	1	2%	0	0%	33	66%
Synovial Sarcoma	28	3	11%	12	43%	7	25%	13	46%
DDLPS	47	2	4%	3	6%	0	0%	39	83%
Angiosarcoma	9	3	33%	1	11%	0	0%	8	89%
MPNST	13	5	38%	0	0%	1	8%	11	85%
other	20	3	15%	0	0%	0	0%	18	90%
Total	249 [a]	25	10%	19	8%	11	4%	193	78%

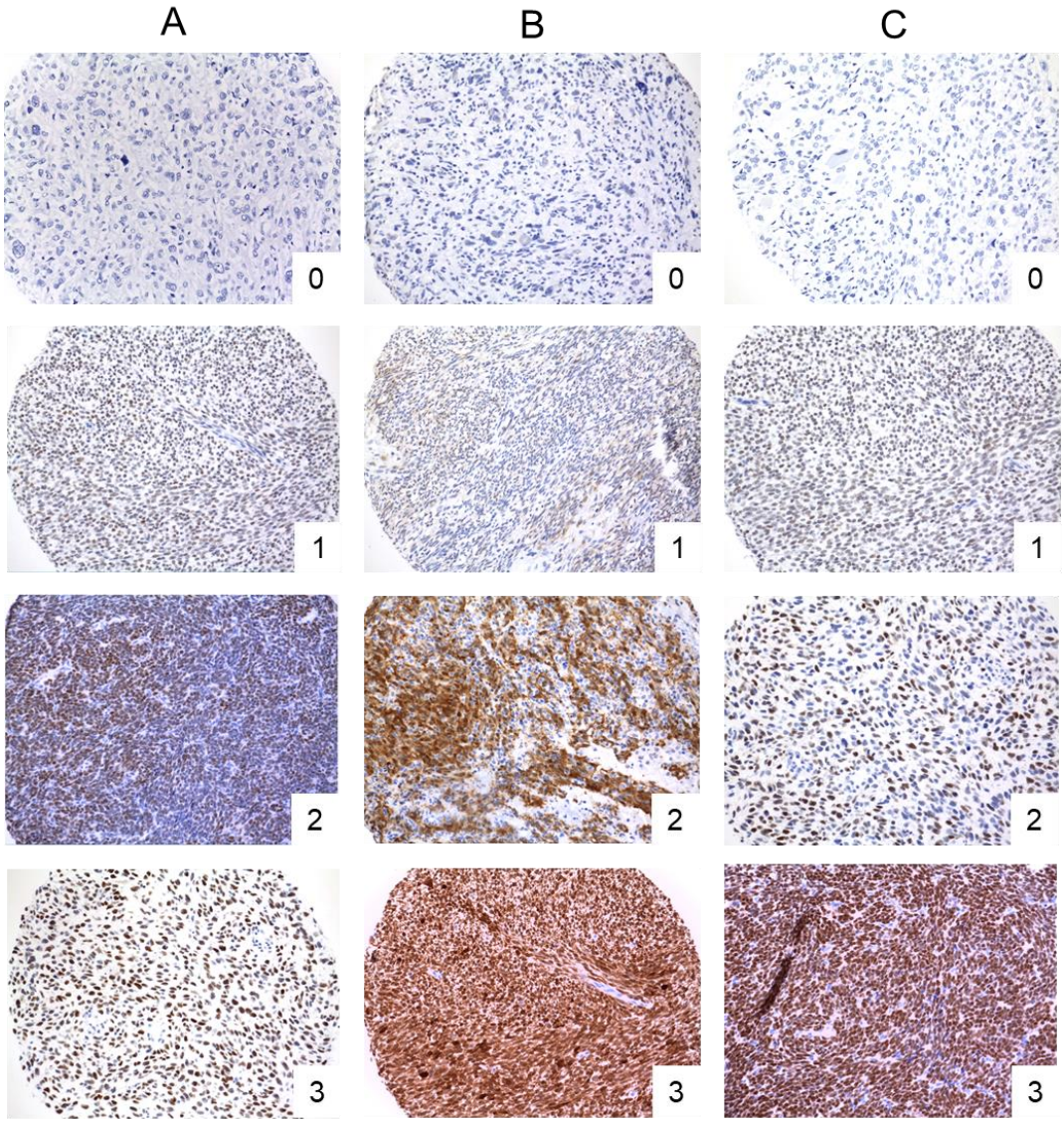


Figure 1: Immunhistochemical staining of tissue microarrays for (A) PRAME, (B) NY-ESO-1 and (C) SSX2. Numbers represent semiquantitative scoring of immunostaining: 0, negative; 1, weak; 2, moderate and 3, strongly positive. Magnification 20x.

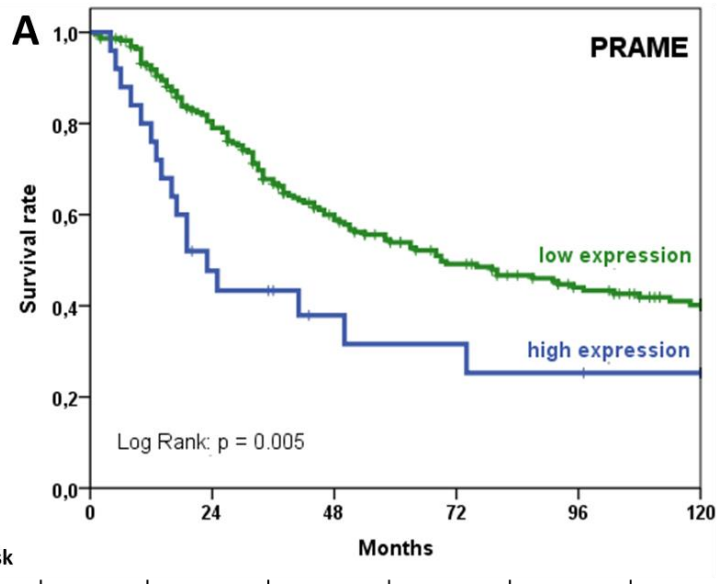
PRAME expression tended to be more frequent in tumours with low numbers of TILs (9/56, 16.1%) as compared to tumours with high TILs (16/193, 8.3%, p = 0.126). *NY-ESO-1* expression was three times more frequent in tumours with low numbers of TILs (9/56, 16.1%) as compared to tumours with high TILs (10/193, 5.2%, p = 0.018).

2.4 Expression of PRAME and NY-ESO-1 is associated with grading in opposing ways

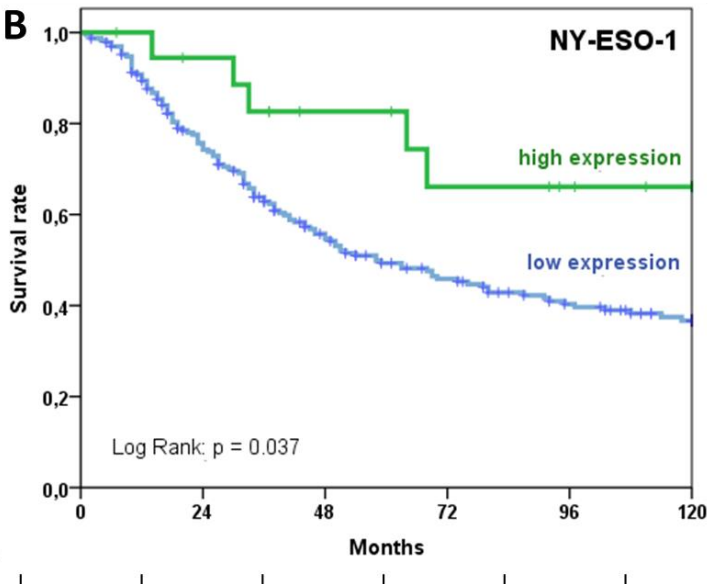
Expression of *PRAME* was correlated with higher grading (G2 vs G3, p=0.001), while *NY-ESO-1* expression was seen more frequently in lower grading (G2 vs G3, p=0.029). The low frequency of *SSX2* expression did not allow for a meaningful statistical analysis.

2.5 PRAME expression is prognostic of shorter survival while NY-ESO-1 predicts a more favourable prognosis

Median duration of follow-up was 40 months for all patients, 96 months for 110 patients still alive and 31 months for 139 patients who had died. Patients with *PRAME*-positive tumours showed statistically significant shorter median survival (23.0 (13.5 - 32.5) months vs. 60.0 (66.6 – 78.9) months, p=0.005), while high *NY-ESO-1* expression was associated with improved survival (> 120 months vs. 68.0 (49.2 – 86.8) months, p=0.037). The low frequency of *SSX2* expression did not allow for a meaningful statistical analysis. A Kaplan Mayer analysis of overall survival is shown in **Figure 2**.



Patients at risk						
Low expression	224	167	111	82	63	47
High expression	25	11	6	5	4	3



Patients at risk						
High expression	19	16	11	8	6	4
Low expression	230	162	106	79	61	46

Figure 2: Univariate analysis of overall survival is depicted as Kaplan-Meier curves stratified according to the expression of (**A**) PRAME and (**B**) NY-ESO-1.

3. Discussion

In this study, we analysed the expression of CTAs *PRAME*, *NY-ESO-1* and *SSX2* as well as the presence of tumour-infiltrating lymphocytes (TILs) in a well-characterized cohort of high-grade soft tissue sarcoma patients. We found the expression of *PRAME* and the presence of a high number of TILs to be associated with shorter survival, while *NY-ESO-1* expression was more frequent in patients with a more favourable prognosis. Furthermore, the expression of *PRAME* was associated with higher grade and a lower number of TILs while *NY-ESO-1* expression was correlated with lower grade and low TILs. To our knowledge, the present study of 249 cases is the largest study of CTAs in soft tissue sarcoma demonstrating a prognostic significance of these markers.

Significant *PRAME* overexpression has been described in uterine carcinosarcoma, synovial sarcoma and multifocal leiomyosarcoma while other sarcoma subtypes appear to express *PRAME* less frequently [21]. In the present study, we found *PRAME* expression in undifferentiated pleomorphic sarcoma (UPS), malignant peripheral nerve sheath tumour (MPNST), synovial sarcoma, leiomyosarcoma (LMS), angiosarcoma, dedifferentiated liposarcoma (DDLPS), uterine carcinosarcoma and rhabdomyosarcoma, making *PRAME* a potential target for immunotherapy in these histological subtypes. Moreover, in our mixed cohort of intermediate and high grade sarcomas, *PRAME* expression was prognostic for unfavourable survival, which was not the case in the studies of liposarcoma, leiomyosarcoma and synovial sarcoma subgroups by others [9,21]. Our results, therefore, might help to establish *PRAME* expression as a prognostic marker and valuable target antigen in soft tissue sarcoma. The fact that we demonstrated *PRAME* expression in only 3 of 28 synovial sarcomas compared to 100% overexpression in the gene expression study by Roszik et al. [21] might be due to different methods, detection of *PRAME* on protein level and scoring evaluation. Interestingly high expression of *PRAME* was described as a negative prognostic marker in breast carcinoma [22] and was associated with the epithelial mesenchymal transition (EMT) which support our findings.

Currently, *PRAME* is a target antigen in a clinical phase I trial evaluating multi tumour-associated antigen-specific cytotoxic T lymphocytes in rhabdomyosarcoma (NCT02239861, TACTASOM). Given that *PRAME* expression was negatively correlated with lymphocyte infiltration in our study and that *PRAME* has been described to downregulate antigen-presentation [21], effective strategies for T-cell recruitment and activation will be needed. These may include a boost of MHC class I expression using, e.g., demethylating drugs and other approaches [23], combinatorial therapy with NK cells [24] or checkpoint inhibitors [25].

NY-ESO-1 has been studied extensively in various cancers. We found it to be expressed in almost half of synovial sarcomas and rare cases of UPS, LMS, DDLPS and angiosarcoma. This essentially confirms the results of previous studies, although expression in synovial sarcoma has been demonstrated to be as high as 76% to 80% [10,26,27]. In our cohort of soft tissues sarcoma patients, *NY-ESO-1* expression was associated with lower grade and it was predictive of more favourable survival as shown before in a series of high-grade soft tissues sarcoma by Kakimoto *et al*. [26]. Interestingly, in non-small-cell lung cancer high *NY-ESO-1* expression was associated with poor prognosis [28], while in epithelial ovarian cancer [29] and breast cancer [30] where no relationship with survival has been found. These discrepancies may be caused by the variability of specific interactions between different *NY-ESO-1*-expressing tumours and their microenvironment including altered expression of tumour antigens, epitope spreading and the induction of immunosuppressive cells [31]. Furthermore, *NY-ESO-1* is highly immunogenic and expression might stimulate T-cell response in some tumours.

Widespread expression across various cancer types has made *NY-ESO-1* an attractive target for different immunotherapeutic strategies such adoptive cell transfer, cancer vaccines and immune checkpoint inhibition. While its strong immunogenic nature implies that therapies directed against *NY-ESO-1* may also boost the natural immune response, its restricted expression in normal tissue presumably limits off-target toxicities [31].

In an early trial using genetically engineered lymphocytes reactive to *NY-ESO-1*, the group of Rosenberg at the U.S. National Cancer Institute demonstrated objective tumour regression in patients

with metastatic synovial sarcoma [32]. Their adoptive cell transfer approach has later been refined by others, demonstrating durable tumour responses in synovial sarcoma using engineered CD^{8+} *NY-ESO-1*^{c259} T-cells [33]. The study continues to recruit patients to a follow-up cohort (NCT01343043) while myxoid, round cell liposarcoma patients can be treated with *NY-ESO-1*^{c259} T cells in a parallel trial (NCT02992743). Interestingly, in our study, *NY-ESO-1* was expressed three times more often in tumours with low TILs (16% vs. 5%, p = 0.018). A suppression of $CD3^+$ T-lymphocytes has been described in malignant melanoma expressing *NY-ESO-1*, although its mechanism remains unclear [34]. In a previous analysis of our cohort, PD-L1 positive STS were enriched for PD-1 positivity [35], pointing to an actual PD-L1 interaction with PD-1 positive TILs in STS, which is a prerequisite for patients' eligibility for checkpoint inhibitor therapy. It appears, however, that non-T-cell inflamed tumours, which are resistant to PD-1/PD-L1 inhibitors, can still be treated with adoptive T-cell based immunotherapy [36].

Furthermore, the inter- and intra-tumour heterogeneity of *NY-ESO-1* expression needs to be addressed when it is employed as a target antigen: demethylation, e.g., may both increase *NY-ESO-1* expression and induce specific $CD8^+$ immune responses [37,38]. Lastly, in another example of adoptive cell transfer, *NY-ESO-1* is one of multiple target antigens in the tumour-associated antigen (TAA)-specific cytotoxic T lymphocytes (TACTASOM, NCT02239861) trial in rhabdomyosarcoma.

As for tumour vaccines, an autologous dendritic cell vaccine (ADKV) loaded with allogeneic tumour lysate with expression of CTAs *NY-ESO-1* and *MAGE-A3* is currently evaluated in a Russian phase II trial (NCT01883518). Finally, in a combinatorial approach assuming synergistic immunotherapeutic effects, a *NY-ESO-1* pulsed dendritic cell vaccination is tested in combination with the PD-L1 inhibitor atezolizumab vs atezolizumab alone in synovial or myxoid/round cell liposarcoma (NCT02609984) [39]. Several other trials applying adoptive T-cell therapies in combination with immune checkpoint inhibitors are open for advanced solid tumours, which includes sarcomas [31].

Low expression of *SSX2* did not allow for a meaningful statistical analysis in this study. It may, however, be a target in synovial sarcomas, which were *SSX2*-positive in 25% in our study and which are targeted *via* SSX2-directed cytotoxic T-lymphocytes in the TACTASOM trial (NCT02239861).

4. Materials and Methods

4.1 Patients

A total of 249 cases with high-risk soft-tissue sarcomas were retrieved from the archives of the department of pathology of Ludwig-Maximilians-Universität Munich, Germany. s Patients were diagnosed and treated between 1989 and 2012 at the Sarcoma Centre of the same institution. Data on clinical parameters, including sex, age and primary site, were extracted from the original pathology reports and the database of the previously published EORTC-STBSG 62961 trial (NCT00003052) [40]. Nearly all patients underwent surgical resection of the tumour and about two-thirds received additional radiotherapy. Patients were followed in outpatient clinics or by contacting their general practitioner, and clinical data were updated until December 2019. The study has been approved by the institutional review board at Ludwig-Maximilians-Universität Munich (No. 20-127).

4.2 Histopathology, tissue microarray construction and immunohistochemistry

For tissue microarray (TMA) assembly, representative tumor areas were marked on H&E stained slides of formalin-fixed, paraffin-embedded tumour samples from all patients and two 0.6 mm punch biopsies were taken of each sample. Samples from tonsils were added as controls.

Immunohistochemical staining was done on 5 µm TMA sections according to standard procedures. Antibodies against *NY-ESO-1*, *PRAME* and *SSX2* were obtained and used for the analysis as detailed in **Table 3**. All assays were performed on a Leica Bond-3 automated stainer platform (Leica, Buffalo Groves, IL). Before application of the primary, heat-based antigen retrieval was performed employing a high pH buffer (ER2, Leica). A polymeric secondary kit (Refine, Leica) was used to detect the primary. Immunostaining was evaluated and scored semi-quantitatively by

two authors (M.A. and T.K.) on a four-tier scale: 0, negative; 1, weak; 2, moderate and 3, strongly positive, that was reduced to a 2-tier system (0—low, versus 1/2/3—high for PRAME and SSX2, 0/1/2 – low, versus 3, high for NY-ESO-1) for statistical analysis. In case of discrepancies in the scoring results of both researchers, consent was built after individual re-evaluation of each sample. Researchers scoring the TMAs were blinded to the clinical data.

Table 3: Antibodies used for immunohistochemistry staining. ER2: Epitope Retrieval Solution 2.

Antigen	Product No.	Supplier	Clone	Dilution	Pre-treatment
NY-ESO-1	SC-53869	Santa Cruz	E978	1:100	ER2
PRAME	ab219650	Abcam	EPR20330	1:1000	ER2
SSX2	AMAb91141	Atlas Antibodies	CL3202	1:3000	ER2

Tumour-infiltrating Lymphocytes (TILs) were counted per high power field (HPF, 400x magnification) in H&E stained TMA slides. Samples with counts of at least 4 lymphocytes per HPF were considered positive.

4.3 Statistical Analysis

Survival was calculated from the date when sarcoma was first diagnosed. Overall survival (patients' death without regarding the cause of death) was used as the endpoint for estimating prognosis. Survival curves were calculated by the Kaplan–Meier method. The log-rank test was used to assess differences in survival. Significant and independent predictors of disease-specific survival were identified by Cox proportional hazard analysis. The stepwise procedure was set to a threshold of 0.05. All statistical analyses were performed using SPSS 20.0 (SPSS, Chicago, IL) software. Statistical significance was defined as a p value < 0.05.

5. Conclusions

CTAs *PRAME*, *NY-ESO-1* and *SSX2* show distinct expression patterns in different STS subtypes. *PRAME* and *NY-ESO-1* expression levels are correlated to patient survival and tumour grading in opposing ways while both CTAs are more frequent in tumours with low TIL counts. These results may guide future immunotherapeutic approaches in STS.

Author Contributions: Conceptualization, Markus Albertsmeier, Achim A. Jungbluth and Thomas Knösel; Data curation, Rolf D. Issels, Eric Kampmann and Gabriele Schubert-Fritschle; Formal analysis, Markus Albertsmeier, Annelore Altendorf-Hofmann and Thomas Knösel; Investigation, Markus Albertsmeier, Annelore Altendorf-Hofmann, Rolf D. Issels, Gabriele Schubert-Fritschle and Thomas Knösel; Methodology, Markus Albertsmeier, Annelore Altendorf-Hofmann, Eric Kampmann, Achim A. Jungbluth and Thomas Knösel; Project administration, Markus Albertsmeier, Eric Kampmann and Thomas Knösel; Resources, Lars H. Lindner, Rolf D. Issels, Martin K. Angele, Thomas Kirchner and Thomas Knösel; Software, Annelore Altendorf-Hofmann and Gabriele Schubert-Fritschle; Supervision, Lars H. Lindner, Hans-Roland Dürr, Martin K. Angele and Thomas Kirchner; Visualization, Markus Albertsmeier and Annelore Altendorf-Hofmann; Writing – original draft, Markus Albertsmeier; Writing – review & editing, Lars H. Lindner, Hans-Roland Dürr, Martin K. Angele, Achim A. Jungbluth and Thomas Knösel. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1: Frequency of levels of expression for different Cancer Testis Antigens: 0, negative; 1, weak; 2, moderate and 3, strongly positive.

Expression	PRAME		NY-ESO-1		SSX2	
	n	%	n	%	n	%
0	224	90.0	214	85.9	237	95.6
1	6	2.4	8	3.2	2	0.8
2	8	3.2	8	3.2	4	1.6
3	11	4.4	19	7.6	5	2.0
Total	249	100.0	249	100.0	248	100.0
Missing					1	

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