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Expression of FGFR3 and FGFR4 and clinical risk factors associated with progression-free survival in synovial sarcoma^{☆,☆☆}

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Summary

Although rare, synovial sarcoma (SS) is one of the most common soft tissue sarcomas affecting young adults. To investigate potential tumor markers related to synovial sarcoma prognosis, we carried out a single-institution retrospective analysis of 103 patients diagnosed with SS between 1980 and 2009. Clinical outcome data were obtained from medical records, and archived tissue samples were used to evaluate the relationship between progression-free survival (PFS) and several prognostic factors, including tumor expression of FGFR3 and FGFR4. No associations were found between PFS and gender, body mass index, tumor site, *SS18-SSX* translocation, or FGFR4 expression. As seen in previous studies, age at diagnosis (<35, 63% versus ≥35 years, 31% 10-year PFS; $P = .033$), histologic subtype (biphasic, 75% versus monophasic 34% 10-year PFS; $P = .034$), and tumor size (<5 cm, 70% versus >5 cm, 22% 10-year PFS; $P < .0001$) were associated with PFS in SS patients. In addition, in a subset of patients with available archived tumor samples taken prior to chemotherapy or radiation ($n = 34$), higher FGFR3 expression was associated with improved PFS ($P = .030$). To the best of our knowledge, this is the largest study of SS to date to suggest a potential clinical role for FGFR3. While small numbers make this investigation somewhat exploratory, the findings merit future investigation on a larger scale.

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Keywords

Local recurrence; Metastasis; Soft tissue sarcoma; Tissue microarray

1. Introduction

Synovial sarcoma (SS) is a rare but aggressive soft tissue sarcoma found most often in young adults. A US population-based study of patients with SS diagnosed between 1983 and 2005 found an overall five-year, cancer-specific survival rate of 80% in the pediatric group, but only 60% in adults [1]. Other studies show an even lower progression-free survival (PFS) among adults diagnosed with SS [2].

SS is molecularly associated with a t(X;18) translocation, resulting in a fusion protein combining SS18 with SXX1, SXX2, or rarely SXX4 [3–5]. While tumor resection margin, histologic grade, mitotic activity, histologic subtype, and *SS18-SSX* genotype have all been associated with outcome, the most consistent prognostic factors have been age at diagnosis and primary tumor size [6]. The *FGFR4* Arg388Gly polymorphism associated with prolonged activation of the receptor [7], as well as *FGFR4* RNA expression level and mutations in tumors, have been related to more aggressive disease and poor prognosis in a variety of soft tissue sarcomas [8–10]. FGFR3, best known for its role in regulating bone length, acts by inducing apoptosis and senescence in chondrocytes [11]. Ishibe et al found elevated expression of several fibroblast growth factor receptors, including FGFR3, FGFR4, and their ligands, in SS cell lines and tissues and also demonstrated that inhibiting these receptors in vitro, as well as in vivo, reduced SS growth [12]. Based on these prior studies, we evaluated the association of FGFR3 and FGFR4 protein expression and PFS in a population of patients with SS.

2. Materials and methods

2.1. Patients

The University of Minnesota Orthopedic Tumor Database was used to identify patients diagnosed with SS at the Fairview–University of Minnesota Medical Center between 1980 and 2009. This screening identified 103 patients. Deidentified patient data were extracted from medical records, including place of residence, gender, age and body mass index (BMI) at or near time of diagnosis, tumor site, tumor size, histological subtype, the presence of metastases at diagnosis, treatment, and follow-up through October 2010. Tumor size information was obtained from computed tomography or magnetic resonance imaging scans, if available, and from ultrasound or physical examination size estimates if not. A histological subtype had been assigned in most cases but was “not specified” or classified as “pleomorphic” in 17 tumors. The primary tumor site was noted as upper extremity, lower extremity, or trunk (which included extremity girdles such as hip, shoulder, and axilla). Available pathologic slides (n = 51) were reviewed by a single pathologist with experience in soft tissue tumors (J.C.M.) to confirm the diagnosis. If we were unable to obtain a histologic subtype diagnosis from medical records or if the diagnosis was disputed, the newly reviewed diagnosis was used for analyses.

2.2. *SS18-SSX1* and *SS18-SSX2* PCR

To evaluate the presence of *SS18-SSX1* and *SS18-SSX2* in patients with available archived tumor tissue, RNA was isolated from 53 formalin-fixed, paraffin-embedded (FFPE) SS tissue blocks using the Ambion FFPE RNA isolation kit (Ambion, Austin, TX). Total RNA was converted to cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad,

CA). Real-time reverse transcriptase polymerase chain reaction was performed on cDNA using TaqMan primers and probes (Applied Biosystems, Foster City, CA) specific for *SS18-SSX1* (Hs03024820_ft) and *SS18-SSX2* (Hs03024398_ft) for 40 cycles, and products were verified by gel electrophoresis. Additionally, reverse transcriptase polymerase chain reaction was carried out using primers previously designed and used for this application [13]. All *SS18-SSX* fusion transcripts were amplified and then processed with XMNI designed to specifically digest the *SS18-SSX2* fusion. Polymerase chain reaction products were then sequenced in the University of Minnesota's Biomedical Genomics Center to confirm results. If *SS18-SSX* status was either negative or ambiguous following these methods, samples were assessed by Mayo Clinic's anatomic molecular pathology lab using their standardized protocol for detecting *SS18-SSX* fusion transcripts. Samples reported as *SSX1* or *SSX2* had to agree by at least 2 of the methods above to be included in analyses (n = 40).

2.3. Tissue microarray construction and immunohistochemistry

Representative areas of SS with high tumor cell density were identified on hematoxylin and eosin-stained sections for 53 FFPE SS specimens. Tissue microarray (TMA) blocks consisting of quadruplicate 1.0 mm core samples were constructed with a manual tissue arayer (MTA-1, Beecher Inc, Sun Prairie, WI) and limited to 64 cores per recipient block. Two additional cores were obtained when possible for RNA studies as described above.

Unstained TMA sections (4 μm) were deparaffinized and rehydrated using standard methods. For antigen detection and identification, slides were incubated in 6.0 pH buffer (Reveal Decloaking reagent, Biocare Medical, Concord, CA) in a steamer for 30 minutes at 95 to 98°C, followed by a 20-minute cool-down period. Slides were rinsed in running tap water, followed by immersion in Tris-buffered saline (TBS; pH 7.4). Subsequent steps were automated using an immunohistochemical (IHC) staining platform (Nemesis, Biocare). Endogenous peroxidase activity was quenched by slide immersion in 3% hydrogen peroxide solution (Peroxidazed, Biocare) for 10 min followed by TBS rinse. A serum-free blocking solution (Background Sniper, Biocare) was placed on sections for 10 minutes. Blocking solution was removed, and slides were incubated in primary antibody diluted in 10% blocking solution/90% TBS for 30 minutes at room temperature: rabbit anti-human FGFR3 polyclonal antibody (Abcam, Cambridge, MA; 1:400); rabbit anti-human FGFR4 polyclonal antibody (Abcam; 1:400); mouse anti-human vimentin monoclonal antibody (clone Vim3B4, Ventana Medical Systems, Inc, Tucson, AZ). Following incubation, Novocastra Post-Primary Block (Leica Microsystems Inc, Buffalo Grove, IL) was applied for 30 minutes at room temperature and rinsed with TBS. Novolink Polymer (Leica) was then applied for 30 min at room temperature and rinsed with TBS. Diaminobenzidine (Covance, Dedham, MD) was applied for 5 min followed by TBS rinse and counterstained with CAT Hematoxylin (Biocare) for 5 minutes. Slides were then dehydrated and protected with cover slip.

2.4. Slide digitization, annotation, and IHC quantification

Digital images of IHC-stained TMA slides were obtained at 40 \times magnification (0.0625 μm^2 per raw image pixel) using a whole slide scanner (ScanScope CS, Aperio Technologies, Vista, CA) fitted with a 40 \times /0.75 Plan Apo objective lens (Olympus, Center Valley, PA). Images were saved in SVS format (Aperio) on a server equipped with server software (ImageServer, Aperio) and retrieved using file management software (Spectrum, Aperio). Eight square regions (10 000 μm^2 each) of representative tumor were annotated for each spot on the array. Immunohistochemical staining intensity within these regions was evaluated using Positive Pixel Count (v9, Aperio) image analysis software. Positive pixels (NTotal) in the brown (diaminobenzidine) colorimetric channel (intensity: 0-255) were further stratified into weak (NWP), moderate (NMP), and strong intensities (NSP) using

upper-limit transmitted light intensity thresholds of 220, 175, and 100, respectively; where 255 is white and 0 is black. A metric of pixel intensity, IHC score, was defined as $[(1 \cdot \text{NWP}) + (2 \cdot \text{NMP}) + (3 \cdot \text{NSP})] / \text{NTotal} \cdot 100$ and averaged across all samples for each patient separately for FGFR3 and FGFR4. Positive vimentin staining was used as a control for viable tissue; regions of negative staining were excluded from image analysis (Fig. 1). Cores from one patient were excluded from final analysis because they were obtained from a metastasis two years after primary diagnosis, rather than a primary tumor.

2.5. Statistical methods

Patient demographics and clinical characteristics were summarized for all patients and those with adequate samples to determine *SS18-SSX* translocation. Comparisons were made between groups using χ^2 or Fisher exact tests, as appropriate. PFS was calculated from the date of diagnosis to the date of first known disease progression, relapse, or death, or was censored at the date of last contact to a maximum of 10 years for patients still alive without progressive disease. Variables considered for prediction of PFS included age at diagnosis (< 35 years, >35 years), gender, BMI (normal = <25, overweight = 25–29.9, obese = 30), tumor site (trunk, upper extremity, lower extremity), tumor size (< 5 cm, >5 cm), histology subtype (monophasic, biphasic), surgery type and treatment received, and FGFR3 and FGFR4 IHC score. Kaplan-Meier estimates and 95% confidence intervals (CI) for 5- and 10-year PFS were reported, and comparisons between groups were made using log-rank tests.

3. Results

A total of 103 patients were identified as having been diagnosed with SS in a 30-year period between 1980 and 2009 (patient characteristics summarized in the Table). The criteria for translocation status were met by 40 patients, with 60% having the *SS18-SSX1* translocation and 40% with the *SS18-SSX2* translocation. Of the characteristics assessed in this population, only gender and histological subtype had borderline relationships with translocation. *SS18-SSX1* was more prevalent in men ($P = .07$) and equally distributed between subtypes. *SS18-SSX2* was present in a higher percentage of monophasic SS samples ($P = .10$, Table 1).

In this population, the 5- and 10-year PFS estimates were 58% (95% CI: 47%-68%) and 50% (95% CI: 37%-61%), respectively (Fig. 2A). As expected, those patients diagnosed at 35 or older had significantly inferior PFS than those diagnosed before age 35, with 10-year PFS of 31% (95% CI: 14%-50%) and 63% (95% CI: 47%-76%), respectively ($P = .03$, Fig. 2B). Gender and BMI at time of diagnosis (Supplementary Figure 1) did not appear to be statistically significantly associated with PFS. Histologic subtype analysis revealed that patients with the monophasic subtype had inferior PFS than those diagnosed with the biphasic subtype ($P = .03$, Fig. 2C), with 34% (95% CI: 19%-50%) 10-year PFS for monophasic and 75% (95% CI: 54%-87%) PFS for biphasic. Additionally, patients with tumors that were >5 cm in greatest diameter had significantly inferior PFS than those with tumors measuring 5 cm or less, with 10-year PFS estimates of 22% (95% CI: 9%-39%) and 70% (95% CI: 45%-85%), respectively ($P < .0001$, Fig. 2D). Although there was a slight trend in location of primary tumors with PFS (upper extremity > lower extremity > trunk), the overall differences between these groups were not statistically significant ($P = .10$, Fig. 2E). In the 40 patients with translocation information available, there was no significant difference in PFS based on translocation type (*SS18-SSX1* versus *SS18-SSX2*, Fig. 2F).

A total of 47 patients had sufficient tissue available to calculate IHC scores based on FGFR3 and FGFR4 expression. Neither FGFR3 nor FGFR4 expression in tumors was statistically significantly related to any of the demographic or clinical factors we assessed

(Supplementary Table 1). Using the median IHC score to divide tumors into FGFR3 and FGFR4 high- and low-expression groups, we found no significant association between high versus low FGFR3 or FGFR4 expression and PFS when all cores passing quality check were included (Fig. 3A and 3C). However, only a subset ($n = 34$) of these tumor samples were taken from tumors resected prior to any chemotherapy or radiation treatment. In this subset, PFS in patients with primary tumors that expressed high levels of FGFR3 was significantly higher than those with lower IHC scores, with 61% (95% CI: 28%-82%) versus 17% (1%-51%) 10-year PFS, respectively ($P = .03$; Fig. 3B). FGFR4 expression in the tumor was not significantly associated with PFS, even after excluding cores of patients with prior exposure to chemotherapy and/or radiation (Fig. 3D).

4. Discussion

After evaluating demographic and clinical variables with outcome data from 103 patients diagnosed with SS at the Fairview University of Minnesota Medical Center between 1980 and 2009, we were able to address whether PFS in this patient population differed based on demographic and clinical characteristics found to be associated with survival in other studies of SS. Our results for age and tumor size were in agreement with a number of previous studies that found an association between inferior survival and older age at diagnosis [1,14–20] as well as primary tumor size >5 cm in largest diameter [1,2,14,15,20–27]. There was no evidence of an association with gender and PFS, and although we are the first to our knowledge to assess an association with BMI at diagnosis and PFS, we did not find a significant relationship.

Consistent with one prior study [21], we also found a significant association between monophasic subtype and inferior PFS. However, most of the studies to date have found either marginal or no association with monophasic or biphasic histologic subtype and outcome [1,14,16,18–20,22–26,28–30,32]. In our study, we also found that histological subtype was not significantly associated with fusion type. Other studies that carried out this comparison reported variable results; one prior study found no relationship between fusion and monophasic/biphasic subtype [31], two found marginally significant associations [29,30], while several other studies did find associations between these two factors [2,18,20,32]. In our study, we did not find an association between translocation variant and PFS; prior reports have had variable results for association of translocation with disease outcome [2,14,18,20,22,30–33].

A recent study demonstrated that transducing mouse myoblast cell lines with *SS18-SSX2* resulted in elevated *FGFR3* message compared to empty vector controls [27]. We detected FGFR3 and FGFR4 protein expression in a number of our SS tumor samples but found that this expression was not related to histological subtype or *SS18-SSX* translocation. These findings validate prior findings of *FGFR3* and *FGFR4* transcript expression and FGFR3 protein expression in SS frozen specimens and cell lines compared to other tumor types [12], although the previous study did not quantify differences in expression between *SS18-SSX1*– and *SS18-SSX2*–positive tumors. We did not find a significant difference in FGFR3 expression between monophasic and biphasic subtypes, concordant to the one prior study that assessed this relationship [12].

To our knowledge, this is the largest study that explores FGFR3 and FGFR4 protein expression in SS and its association with PFS. In our study, when post-treatment tumors were excluded, FGFR3 expression was associated with superior PFS. This is not the only malignancy to have improved prognosis associated with FGFR3 expression. In cervical cancer, one group found a general trend (albeit not significant) with improved disease-specific survival of patients with tumors expressing higher levels of *FGFR3b* mRNA [34].

In another study of patients with bladder cancer, while FGFR3 expression was not associated with survival, it was associated with earlier stage and lower-grade tumors [35]. The less prognostically dire phenotypes of these cancers might be explained by the inhibitory functions of FGFR3, as seen in prior studies where signaling through FGFR3 was shown to be important for inhibiting proliferation in developing chondrocytes [36,37].

FGFR4 appears to be important for growth and progression in another soft tissue sarcoma, pediatric rhabdomyosarcoma [9]. FGFR4 is not only elevated in rhabdomyosarcoma compared to normal muscle tissue [38], but the more aggressive PAX-FKHR-positive alveolar rhabdomyosarcoma subtype also appears to have elevated *FGFR4* message compared to fusion-negative rhabdomyosarcoma [39]. Additionally, one study found FGFR4 expression to be related to poor prognosis in humans and development of metastases in mice [9] and the FGFR4 Gly388Arg polymorphism, has been associated with poor prognosis in combined sarcoma patients [8]. In our study, we did not find an obvious association between FGFR4 expression and PFS in SS patients, although we cannot rule out lack of power to detect an association due to small sample size. It should be noted that in the present study, on average there were many fewer cells staining positive (including weak, moderate, and strong staining) for FGFR4 (8.2%) than FGFR3 (49.2%). *FGFR3* mRNA expression has been more consistently observed in SS tumors and cell lines than FGFR4 [12], and FGFR4 may therefore be less important in SS pathogenesis.

This study had several limitations. SS is a rare cancer, limiting sample size from a single institution. Patients also received diverse treatments (over the span of 30 years), and there may have been other unknown variables that influenced survival. The small sample size limited the ability to adjust for confounding factors. The effects of confounding and limited sample size might have restricted the ability to detect PFS differences according to FGFR3 or FGFR4 status for the whole population studied. We also used median cut-points of IHC data for analysis, but these may not be the biologically relevant cut-points for these receptors. This was a retrospective study relying on medical records at the University of Minnesota Medical Center for almost all of the collected information. Given the 30-year time period assessed, some information was not available for all patients, with many having incomplete follow-up. The analysis was also restricted to samples with available paraffin blocks to assess specific *SS18-SSX* translocation and FGFR3 and FGFR4 protein expression; it was further limited to samples with sufficient quality RNA to assess translocation.

In conclusion, our data support the previously identified prognostic factors in predicting PFS in SS, including histological subtype, tumor size, and age. Our study is the first to examine FGFR3 and FGFR4 tumor protein expression in relation to patient PFS. We found an association between elevated FGFR3 in SS tumors and improved PFS after excluding patients who had been treated with radiation or chemotherapy prior to excision of the tumor. This finding warrants further investigation in a larger sample of SS. Additionally, future investigations of mutations and splice variants in *FGFR3* might also render findings that account for prognostic differences in SS patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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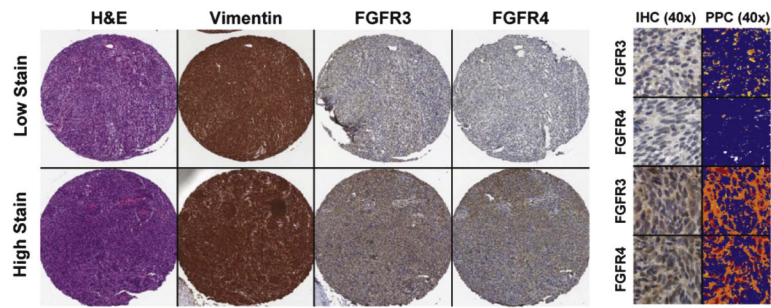


Fig. 1. Positive pixel count analysis of FGFR3 and FGFR4 with vimentin(+) regions. SS TMA spots show representative low and high staining patterns for FGFR3 and FGFR4 (images at 10× magnification). High magnification images (40×) on the right display IHC staining and the correlating image analysis markup. Pseudocolors represent each IHC staining intensity range: blue, no staining; yellow, weak staining; orange, medium staining; and red, strong staining.

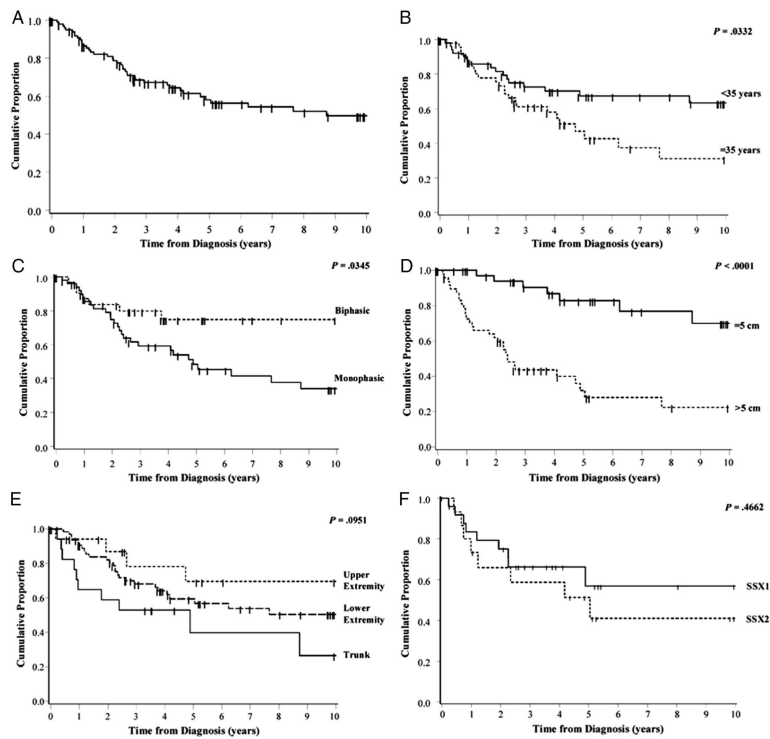


Fig. 2. Progression-free survival for SS patients diagnosed from 1980 to 2009. A, Ten-year PFS curves for all SS patients. B, By age at diagnosis ($n = 103$). C, By histological subtype, excluding 17 patients with unspecified or pleomorphic subtype ($n = 86$). D, By tumor size: >5 cm versus ≤ 5 cm, excluding 14 unknown. E, By tumor site: upper extremity, lower extremity, or trunk ($n = 103$). F, By translocation *SS18-SSX1* versus *SS18-SSX2* ($n = 40$) with cores available and quality RNA for detection of translocation.

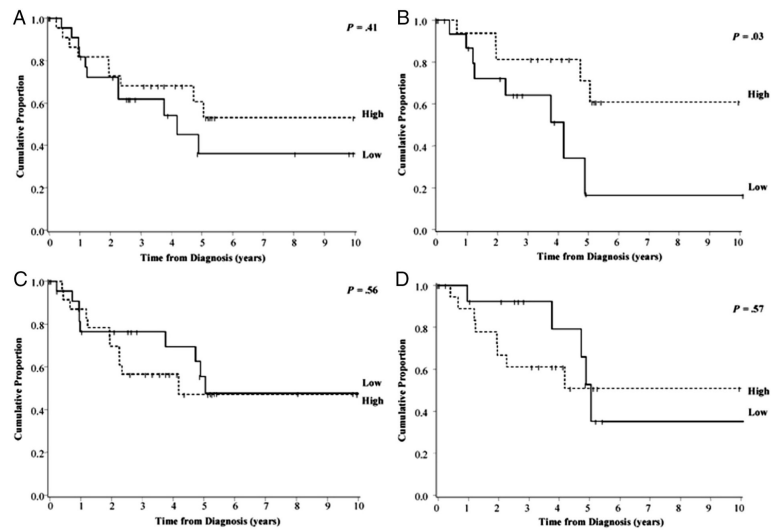


Fig. 3. Progression-free survival for SS patients diagnosed from 1980 to 2009 by levels of FGFR3 and FGFR4 expression in the tumor. Ten-year PFS curves for 47 SS patients based on high and low FGFR3 (A and B) and FGFR4 (C and D) expression. PFS was also examined after exclusion of tumors with prior exposure to radiation or chemotherapy (B and D).

Table

Characteristics of sarcoma patients diagnosed from 1980 to 2009, including subset with samples reporting either *SS18-SSX1* or *SS18-SSX2* translocation

Variable	All patients diagnosed (n = 103) n (%)	Patient with samples reporting <i>SS18</i>			<i>p</i> ^a
		Total (n = 40)	<i>SSX1</i> (n = 24, 60%)	<i>SSX2</i> (n = 16 (40%))	
Age at diagnosis					.24
35 y	54 (52%)	17 (43%)	12 (50%)	5 (31%)	
>35 y	49 (48%)	23 (58%)	12 (50%)	11 (69%)	
Sex					.07
Male	59 (57%)	22 (55%)	16 (67%)	6 (38%)	
Female	44 (43%)	18 (45%)	8 (33%)	10 (63%)	
BMI					.97
Normal (<25)	35 (34%)	11 (28%)	6 (25%)	3 (19%)	
Overweight (25-29.9)	31 (30%)	16 (40%)	10 (42%)	5 (31%)	
Obese (≥ 30)	14 (14%)	5 (13%)	3 (13%)	6 (38%)	
Unknown	23 (22%)	8 (20%)	5 (21%)	2 (13%)	
Site of tumor					.48
Trunk/non-extremity	17 (17%)	7 (18%)	5 (21%)	2 (13%)	
Upper extremity	18 (18%)	5 (13%)	4 (17%)	1 (6%)	
Lower extremity	68 (66%)	28 (70%)	15 (63%)	13 (81%)	
Tumor size, 5 cm cut point					.65
5 cm	40 (39%)	12 (30%)	6 (25%)	6 (38%)	
>5 cm	49 (48%)	26 (65%)	17 (71%)	9 (56%)	
Unknown	14 (14%)	2 (5%)	1 (4%)	1 (6%)	
Tumor size, 7 cm cut point					.67
7 cm	51 (50%)	22 (55%)	12 (50%)	10 (63%)	
>7 cm	38 (37%)	16 (40%)	11 (46%)	5 (31%)	
Unknown	14 (14%)	2 (5%)	1 (4%)	1 (6%)	
Histological subtype					.1
Monophasic	53 (52%)	23 (58%)	11 (46)	12 (75%)	
Biphasic	33 (32%)	17 (43%)	13 (54%)	4 (25%)	
Not specified	17 (17%)				
Metastatic disease at diagnosis					1.0
Yes	6 (6%)	2 (5%)	1 (4%)	1 (6%)	
No	97 (94%)	38 (95%)	23 (96%)	15 (94%)	
Surgery					
No surgery	2 (2%)				
Marginal excision	1 (1%)				
Excision/resection	81 (79%)				
Amputation	15 (15%)				
Unknown	4 (4%)				

	All patients diagnosed (n = 103)	Patient with samples reporting <i>SSI8</i>			<i>p</i> ^a
		Total (n = 40)	<i>SSX1</i> (n = 24, 60%)	<i>SSX2</i> (n = 16 (40%))	
Radiation					
Yes	69 (67%)				
No	28 (27%)				
Unknown	6 (6%)				
Chemotherapy					
Yes	37 (36%)				
No	63 (61%)				
Unknown	3 (3%)				
Tumor free post-surgery					
No	11 (11%)				
Yes	80 (78%)				
Unknown	12 (12%)				
Progression					
No	62 (60%)				
Local recurrence	9 (9%)				
Local relapse	3 (3%)				
Distant recurrence	26 (25%)				
Missing	3 (3%)				

NOTE. Percentages may not total 100% due to rounding.

^a χ^2 or Fisher exact test as appropriate.