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Expanding the Use of an SS18-SSX Antibody for Molecular Assays in Synovial Sarcoma

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Abstract: Synovial sarcoma is an aggressive malignancy that generally affects adolescents and young adults and is characterized by high rates of recurrence and metastasis, with a 10-year survival rate of about 50%. The fusion oncoprotein SS18-SSX, the product of a pathognomonic chromosomal translocation t(X;18), is the oncogenic driver of this sarcoma, disrupting differentiation through widespread epigenetic dysregulation. Experimental research into SS18-SSX biology has been limited by the lack of an antibody that specifically detects the endogenous fusion oncoprotein as opposed to its native SS18 or SSX components. Recently, a rabbit monoclonal antibody was developed and made commercially available, which specifically detects the fusion junction site epitope of SS18-SSX as found in at least 95% of synovial sarcomas. Here, we characterize a suite of molecular biology assays using this new antibody, both confirming existing and reporting on novel applications. We demonstrate its high sensitivity and specificity for synovial sarcoma diagnosis on patient samples through positive immunohistochemical staining on synovial sarcoma, tissue microarray, and full face sections. In addition, we demonstrate detection of the human SS18-SSX protein when expressed in a genetically engineered mouse model of synovial sarcoma. We also demonstrate nuclear staining of SS18-SSX in synovial sarcoma cells using immunofluorescence, and visualize the interaction between SS18-SSX and the BAF complex member BRG1 through a proximity ligation assay. Lastly, we confirm the interaction between SS18-SSX and promoter regions of target genes through chromatin immunoprecipitation. This antibody represents a breakthrough in sarcoma research and has value in multiple applications to expand the knowledge of synovial sarcoma biology.

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Synovial sarcoma is a soft tissue malignancy, which primarily affects adolescents and young adults, and generally arises in the limbs and extremities.[1,2] This aggressive cancer comprises 5-10% of all soft tissue sarcomas, and its treatment relies mainly on surgical resection of the local tumor supported by (neo)adjuvant radiation.[3,4] The disease is characterized by a pathognomonic in-frame chromosomal translocation event between chromosomes X and 18, t(X;18)(p11;q11) that leads to the expression of the fusion transcript *SS18*/*SSX* encoding the chimeric oncoprotein, SS18-SSX.[5] The translocation typically causes the last 8 amino acids of SS18 to be replaced by the C-terminal 78 amino acids of either SSX1 or SSX2.[6] SS18 is ubiquitously expressed across many tissue types, while SSX expression is limited to the testes.[5] SS18 is a known subunit of the mammalian SWI/SNF complex (specifically, its canonical BRG1/BRM-associated factor (cBAF) and non canonical GLTSCR1/like-containing BAF (gBAF) forms), a chromatin remodelling complex that contains mutated components in approximately 20% of all human cancers.[7–9] One of SS18-SSX's modes of action is to compete with wild-type SS18 for incorporation into the different BAF complexes and disrupt the relative abundance of these complexes, as demonstrated by recent experiments utilizing a conditional mouse model.[10] SS18-SSX–containing BAF complexes can be retargeted to other chromatin sites, causing aberrant-gene activation by opposing polycomb-mediated repression.[11] Other studies have implicated SS18-SSX interactions with additional epigenetic regulatory proteins and complexes, including the noncanonical polycomb group repressor complex (PRC1.1), the DNA binding protein ATF2, and transcriptional corepressor TLE1.[12,13] Together, these findings affirm the fusion oncoprotein's ability to cause widespread epigenetic dysregulation in the affected cells.

Attempts to detect the protein used in vitro assays have faced challenges. Studies utilizing anti-SS18 antibodies need to account for the concurrent presence of typically large amounts of wild-type SS18, as the fusion oncoprotein retains 410 out of the 418 amino acids present in full-length SS18. Antibodies directed against the SSX

region often do not display high specificity as SSX is an intrinsically disordered protein, with epitopes that have proven difficult to target. Instead, attempts to disentangle the roles of SS18-SSX from SS18 have often relied on the knockdown of the fusion oncoprotein using *shRNA* directed against the *SSX* regions, an approach limited to cell line models.[11] Immunoprecipitation-based studies have transfected epitope-tagged versions of SS18-SSX (eg, TAP, HA) to pull it down specifically and identify interacting partners.[7,12] This method is also limited to transfectable cell lines, typically nonsynovial sarcoma cell lines that probably do not accurately recapitulate the epigenetic and transcriptomic landscape in the human disease.

In 2020, a rabbit monoclonal antibody that specifically detects the fusion epitope of the endogenous human SS18-SSX protein became commercially available (Cell Signalling Technology, #72364, Danvers, MA, USA). An initial report characterized its application in immunohistochemistry, co-immunoprecipitation, and chromatin immunoprecipitation in several cell lines and in primary patient tumour tissue.[14] In the current study, we confirm these uses, but also expand on the molecular biology applications for this antibody and the different types of samples where it can be used to detect SS18-SSX protein expression.

MATERIALS AND METHODS

Western Blot

Five different synovial sarcoma cell lines were used: HSSYII (RIKEN, Saitama, Japan), SYO-1 (Dr. Akira Kawai, National Cancer Centre Hospital, Tokyo, Japan), Yamato (Dr. K. Itoh, Osaka Medical Center for Cancer and Cardiovascular Diseases, Japan), MoJo (Dr. K. Jones, University of Utah, Salt Lake City, UT), and Fuji (Dr. Kazuo Nagashima, Hokkaido University School of Medicine, Sapporo, Japan). U2OS, an osteosarcoma cell line, was used as a nonsynovial sarcoma control. All cell lines were maintained in RPMI-1640 medium with 10% fetal bovine serum and grown at 37°C with 5% CO_2.

Cells were grown for 72h and protein lysate was collected on ice using RIPA buffer supplemented with a protease inhibitor (Sigma-Aldrich, #5892970001, St Louis, MI, USA). Lysates were quantified by Pierce™ BCA Protein Assay Kit (Thermo Scientific, #23225, Waltham, MA, USA) and 20 µg was used for Western blot experiments. Gel blots were incubated overnight with SS18-SSX antibody (dilution 1:2000; Cell Signaling Technology, #72364, Danvers, MA, USA), followed by 1-hour incubation with goat anti-rabbit secondary antibody (dilution 1:15000; LI-COR Biosciences, #926-68071, Lincoln, NE, USA). Blots were visualized using the Odyssey Infrared System (LI-COR Biosciences, Lincoln, NE, USA).

Immunofluorescence

HSSYII and U2OS cells were seeded in 8-well chamber slides and grown for 24h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were then blocked with 1% BSA and incubated overnight at 4°C with SS18-SSX antibody (dilution 1:1000). Cells were incubated with goat anti-rabbit secondary antibody (dilution 1:800; Invitrogen, #A11012, Waltham, MA, USA) for 1 hour and mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI). The slide was visualized using ZEISS Axio Scan (ZEISS, Oberkochen, Germany).

Proximity Ligation Assay

Proximity ligation assay (PLA) was performed using the Duolink® In Situ Red Starter Kit Mouse/Rabbit (Sigma-Aldrich, DUO-92101, St Louis, MI, USA).

HSSYII and U2OS cells were seeded in 8-well chamber slides and grown for 24h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking with the provided buffer, cells were coincubated with SS18-SSX antibody (dilution 1:1000) and BRG1 primary mouse antibody (dilution 1:500; Novus Biologicals, MA5-31550, Littleton, CO, USA) overnight. After washing off the unbound primary antibody the next day, cells were incubated with PLA probes at 37°C for 1 h. Subsequently, they were incubated with ligation reagent for 1 h, followed by the amplification reagent for 100 min. At this stage, cells were coincubated with goat anti-mouse secondary antibody (dilution 1:800; Invitrogen, #A-11001, Waltham, MA, USA). Slides were then dried and mounted with Vectashield containing DAPI. Slides were visualized using ZEISS Axio Scan (ZEISS, Oberkochen, Germany).

Immunohistochemistry

A tissue microarray (TMA) was constructed using select formalin-fixed, paraffin-embedded (FFPE) patient surgical excision specimens from Vancouver General Hospital archived between 2007 and 2020: 37 synovial sarcoma cases and 11 normal or malignant control cases (Table 1). All synovial sarcoma cases were molecularly confirmed for *SS18-SSX* expression by fluorescence in situ hybridization (FISH) or NanoString nCounter Sarcoma Fusion CodeSet assay.[15] Further histologic subtyping revealed 27 monophasic, 7 biphasic, and 3 poorly differentiated synovial sarcoma cases. All cases were incorporated into the TMA as 2 cores of 0.6 mm diameter each.

In addition, 33 fresh frozen synovial sarcoma samples were obtained from Lund University Hospital,

TABLE 1. List of the Nonsynovial Sarcoma Control Tissues Included in Tissue Microarray (Fig. 3A), Each Represented by Duplicate Cores

Normal Tissue	Malignant Tissue
Skeletal muscle	Myxoid liposarcoma
Ovarian stroma	Epithelioid sarcoma
Breast glandular tissue	Ewing sarcoma
Testis	Sarcomatoid mesothelioma
	Sarcomatoid renal cell carcinoma
	Clear cell sarcoma
	Dedifferentiated liposarcoma

In addition, the TMA contained 27 monophasic, 7 biphasic and 3 poorly differentiated synovial sarcomas, each also represented in duplicate.

portions of which were subsequently formalin-fixed and paraffin-embedded. All cases had prior confirmation of *SS18-SSX* gene rearrangements through NanoString, reverse-transcription polymerase chain reaction (RT-PCR), or conventional cytogenetics during their clinical diagnostic work-up. Histologic subtyping revealed 26 monophasic, 6 biphasic, and 1 poorly differentiated synovial sarcomas. The clinicopathological features of these cases are described in Table 2.

Mouse model: formalin-fixed, paraffin-embedded tissue was obtained by dissecting and processing tumors growing in a genetically engineered conditional mouse model that reliably generates de novo synovial sarcoma tumors.[16] Briefly, this model contains a human *SS18-SSX2* construct that is conditionally expressed in Myf5+ myogenic precursor cells.

SS18-SSX immunohistochemistry was performed on 4 μm full face sections of formalin-fixed, paraffin-embedded tissues (N = 70 synovial sarcoma cases) using the Leica Bond RX (Leica Biosystems, Buffalo Grove, IL, USA) with the following conditions: heat-induced epitope retrieval using citrate-based BOND Epitope Retrieval Solution 1 (Leica Biosystems, Buffalo Grove, IL, USA) for 20 min; incubation of the SS18-SSX antibody (dilution 1:300) for 15 min at 37°C; and visualization of the fusion oncoprotein using the BOND Polymer Refine Detection kit (Leica Biosystems, DS9800, Buffalo Grove, IL, USA), which utilizes a 3,3′-diaminobenzidine (DAB) chromogen and hematoxylin counterstain.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the ChIP-IT® Express Enzymatic

Kit (Active Motif, #53009, Carlsbad, CA, USA).

HSSYII and SYO-1 cells were grown to confluence in 15 cm dishes. Chromatin was cross-linked using 1% formaldehyde for 10 min, followed by glycine solution for 5 min to stop the reaction. Cells were then scraped into ice-cold PBS containing PMSF and centrifuged before incubation in lysis buffer for 45 min. Cells were then physically homogenized on ice to obtain nuclear lysates, which were incubated with digestion buffer for 5 min at 37°C, followed by incubation with the provided Enzymatic Shearing Cocktail for an optimized timepoint. Fifty microliters of the sheared lysate was used to confirm

TABLE 2. Whole-Section Analyses of Synovial Sarcoma Cases from Lund University Hospital.

Case No.	Subtype	IHC	Molecular Confirmation	Specific *SS18-SSX* Exonic Fusion
1	Monophasic	+	Karyotype	
2	Monophasic	+	RT-PCR	*SS18 exon 10-SSX1 exon 6*
3	Monophasic	+	RT-PCR	*SS18 exon 10-SSX2 exon 6*
4	Monophasic	+	Karyotype	
5	Monophasic	+	FISH	
6	Monophasic	Negative	NanoString	*SS18 exon 10-SSX1 exon 4*
7	Monophasic	+	Karyotype	
8	PDSS	+	FISH	
9	Monophasic	+	FISH	
10	Monophasic	+	FISH	
11	Monophasic	+	RT-PCR	*SS18 exon 10-SSX2 exon 6*
12	Biphasic	+	RT-PCR	*SS18 exon 10-SSX1 exon 6*
13	Monophasic	+	FISH	
14	Monophasic	+	Karyotype	
15	Monophasic	+	FISH	
16	Monophasic	+	Karyotype	
17	Monophasic	+	Karyotype	
18	Monophasic	+	Karyotype	
19	Monophasic	+	RT-PCR	*SS18 exon 10-SSX2 exon 6*
20	Biphasic	Negative	NanoString	*SS18 exon 10-SSX1 exon 4*
21	Monophasic	Negative	NanoString	*SS18 exon 10-SSX2 exon 4*
22	Monophasic	+	RT-PCR	*SS18 exon 10-SSX2 exon 6*
23	Biphasic	+	RT-PCR	*SS18 exon 10-SSX1 exon 6*
24	Monophasic	+	RT-PCR	*SS18 exon 10-SSX1 exon 6*
25	Monophasic	+	RT-PCR	*SS18 exon 10-SSX2 exon 6*
26	Biphasic	+	RT-PCR	*SS18 exon 10-SSX2 exon 6*
27	Monophasic	Negative	RT-PCR/Sanger	*SS18 exon 9-SSX1 exon 7*
28	Monophasic	+	RT-PCR	*SS18 exon 10-SSX1 exon 6*
29	Monophasic	+	RT-PCR	*SS18 exon 10-SSX1 exon 6*
30	Biphasic	+	RT-PCR	*SS18 exon 10-SSX2 exon 6*
31	Biphasic	+	RT-PCR	*SS18 exon 10-SSX1 exon 6*
32	Monophasic	Negative	NanoString	*SS18 exon 10-SSX2 exon 6*
33	Monophasic	+	RT-PCR	*SS18 exon 10-SSX2 exon 6*

The table identifies the SS18-SSX IHC results for 33 samples from Lund University Hospital. The third column shows the type of clinical-grade assay performed to determine the presence of *SS18* gene arrangements and/or *SS18-SSX* translocation. For all cases that were negative for SS18-SSX by IHC (which recognizes only the *SS18 exon 10-SSX exon 6* Junctional Epitope), specific exon fusion partners were determined by exonic splice junction-specific NanoString probes or by sanger sequencing of RT-PCR products. Positive cases with RT-PCR confirmation are also listed as containing either the *SSX1* or the *SSX2* variant.

FISH indicates fluorescence in situ hybridization.

TABLE 3. Primer Sequences Used for ChIP-qPCR

Gene Promoter	Sense	Antisense
EGR1	TAGGGTGCAGGATGGAGGT	AAGCAGGAAGCCCTAATATGGCAG
CDKN2A	ACGAGGCACCTTGGAAACAGGTAT	AGAACGTGGCTTTAAGGTCTGGGA
SOX2	GAGAAGGGCGTGAGAGAGTG	AAACAGCCAGTGCAGGAGTT
Negative control	Active Motif, #71001, Carlsbad, CA, USA	Active Motif, #71001, Carlsbad, CA, USA

shearing efficiency and quantify DNA concentration. Ten micrograms of chromatin from each cell line was immunoprecipitated with either 2 μg of SS18-SSX antibody or control rabbit IgG antibody (Cell Signaling Technology, #2729, Danvers, MA, USA). Chromatin was then subjected to reverse cross-linking and proteinase K treatment, and subsequently stored at −20°C before qPCR assays.

Quantitative PCR (qPCR)

Chromatin lysates were subjected to column-based DNA clean-up (BioBasic, BS664, Markham, ON, Canada). Pre-IP input lysate from each cell line was used to generate a standard curve by creating 5 samples of 10-fold dilutions with known amounts of chromatin (50-0.005 ng). Two microliters of each sample was mixed with 8 μL of Fast SYBR™ Green Master Mix (Sigma-Aldrich, #4385612, St Louis, MI, USA) and the respective primer pairs, and subsequently run on an ABI ViiA7 qPCR system. Samples were run in triplicates. For downstream analyses, the chromatin amount present per sample was derived from input-generated standard curves and fold enrichment was calculated for SS18-SSX immunoprecipitation against IgG control. Primer sequences designed to target the promoter sequences are listed in Table 3.[13,17]

RESULTS

Western Blot

The antibody directed against the junctional epitope of the chimaeric oncoprotein detected SS18-SSX proteins in all 5 synovial sarcoma cell lines, and not in the osteosarcoma U2OS cell line (Fig. 1). As previously demonstrated, it has affinity for both the SS18-SSX1 (expressed in HSSYII, Yamato, and MoJo cell lines) and SS18-SSX2 (expressed in SYO1 and Fuji cell lines) proteins, both of which contain an identical fusion junction site as the product of *SS18 exon 10* being spliced to *exon 6 of SSX1 or SSX2*.[14] The presence of two bands at 65 and 75kD respectively is attributed to an alternative splicing event resulting in the exclusion of the amino acids encoded by *exon 8* in *SS18*, an event that also occurs with wild-type *SS18*.[18]

Immunofluorescence and Proximity Ligation Assay (PLA)

One novel application in our study was to observe *in situ* fluorescent staining of fixed synovial sarcoma cells. We observed punctate staining of SS18-SSX throughout the nuclei of synovial sarcoma cells, but not in osteosarcoma control cells (Fig. 2A).

PLA detects close physical proximity between target proteins, by conjugating two protein-specific antibodies (derived from two different species) to oligonucleotides that, if their targets are in proximity, can subsequently be ligated and amplified using fluorophore-labelled primer sequences. An interaction within 40 nm is observed as a positive signal that can be detected by fluorescent microscopy.[19] PLA performed in HSSYII cells visually confirms close proximity between SS18-SSX and BRG1, a member of the canonical BAF complex that others have shown to interact with SS18-SSX through co-immunoprecipitation (Fig. 2B).[11,14] This interaction was present in all synovial sarcoma cells abundantly throughout the nucleus, indicating a strong association between SS18-SSX and BRG1.

Immunohistochemistry (IHC)

By SS18–SSX IHC, 64 of the 70 (91%) synovial sarcoma samples analyzed showed strong, positive intense nuclear staining, while all control samples stained negative with minimal background (Fig. 3 and Table 2). As all samples had confirmed *SS18* gene rearrangements during their original diagnostic work-up, we sought to validate the presence of *SS18-SSX* fusion transcripts in the IHC-negative cases. NanoString analyses or RT-PCR followed by Sanger sequencing revealed the presence of alternative fusion junction sites in 5 of the 6 cases, among which 4 spliced *SS18 exon 10* to *SSX exon 4* rather than to the more common *SSX exon 6* recognized by the antibody (Fig. 3C&G). There was also a single instance of a novel fusion event between *SS18 exon 9* and *SSX1 exon 7* (Fig. 3D&H). This confirms that the antibody is indeed specific to the canonical fusion

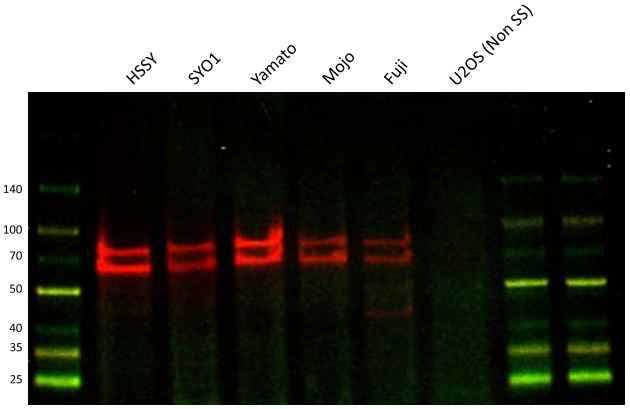

FIGURE 1. Western blot detection of SS18-SSX protein in 5 distinct synovial sarcoma cell lines containing the canonical fusion junction site. Protein bands are observed at 65 and 75 kD. Non-SS indicates non synovial sarcoma. full color online

junction between *SS18 exon 10* and *SSX exon 6*. Despite containing this fusion site, the remaining sample was IHC-negative, a result that occurred in the context of a profound degree of freezing artifacts (Fig. 3E&I).

A major breakthrough in synovial sarcoma research has been the generation of genetically engineered mouse models that are able to produce synovial sarcoma tumors by conditional expression of the human *SS18-SSX2* gene in a murine genomic background.[16] First described in 2007, variations of this model have since been used to examine the role and composition of SS18-SSX in various BAF complexes.[10] We therefore tested the antibody on mouse model formalin-fixed, paraffin-embedded tissue, which yielded strong, positive nuclear staining in synovial sarcoma tumors excised from mouse limbs (Fig. 4). Nerve tissue within the monophasic tumor and blood vessels within the biphasic tumor served as internal negative controls (Fig. 4A and B).

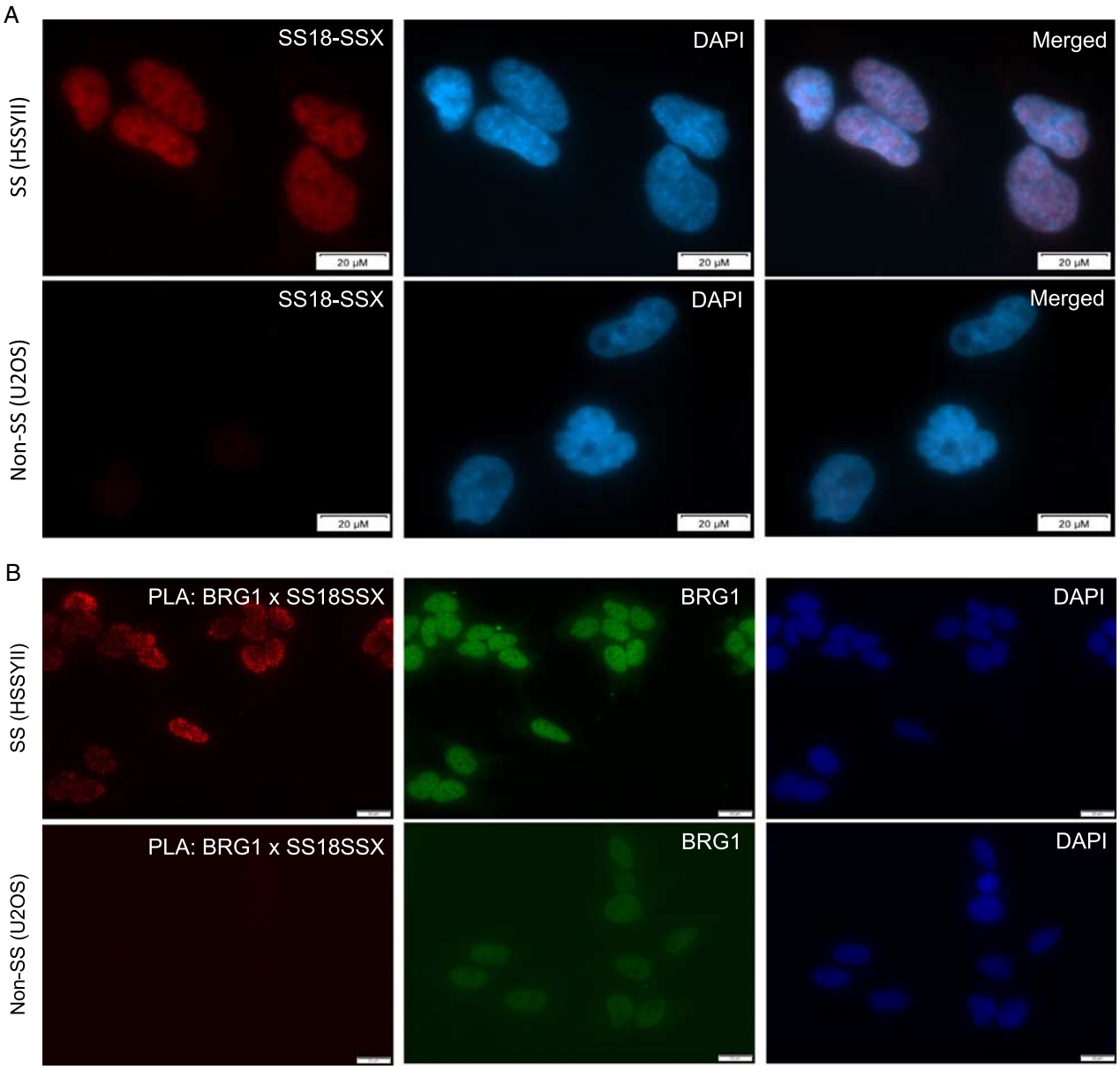

FIGURE 2. A Immunofluorescence images of SS18-SSX. Left column: SS18-SSX staining, using an anti-rabbit secondary antibody. Middle column: nuclear counterstaining by 4',6-diamidino-2-phenylindole (DAPI). Last column: merged images. Top row: synovial sarcoma cell line HSSYII. Bottom row: osteosarcoma cell line, U2OS. B Proximity ligation assay between SS18-SSX and BRG1. First column: Red dots indicate close proximity between SS18-SSX and BRG1 proteins within the cells' nuclei. Middle column: single-channel immunofluorescence staining of BRG1 protein. Last column: nuclear counterstain with DAPI. Top row: positive PLA staining in the HSSYII synovial sarcoma cell line. Bottom row: PLA assay performed in the osteosarcoma cell line, U2OS. PLA indicates proximity ligation assay.

Chromatin immunoprecipitation (ChIP)

In view of the role of SS18-SSX in epigenetic dysregulation, we performed ChIP experiments in 2 synovial sarcoma cell lines followed by qPCR analysis (Fig. 5). We observed enrichment of specific promoter regions of known target gene loci—*EGR1, SOX2, CDKN2A*—as compared to negative control loci. These results are congruent with previous ChIP experiments in cell lines

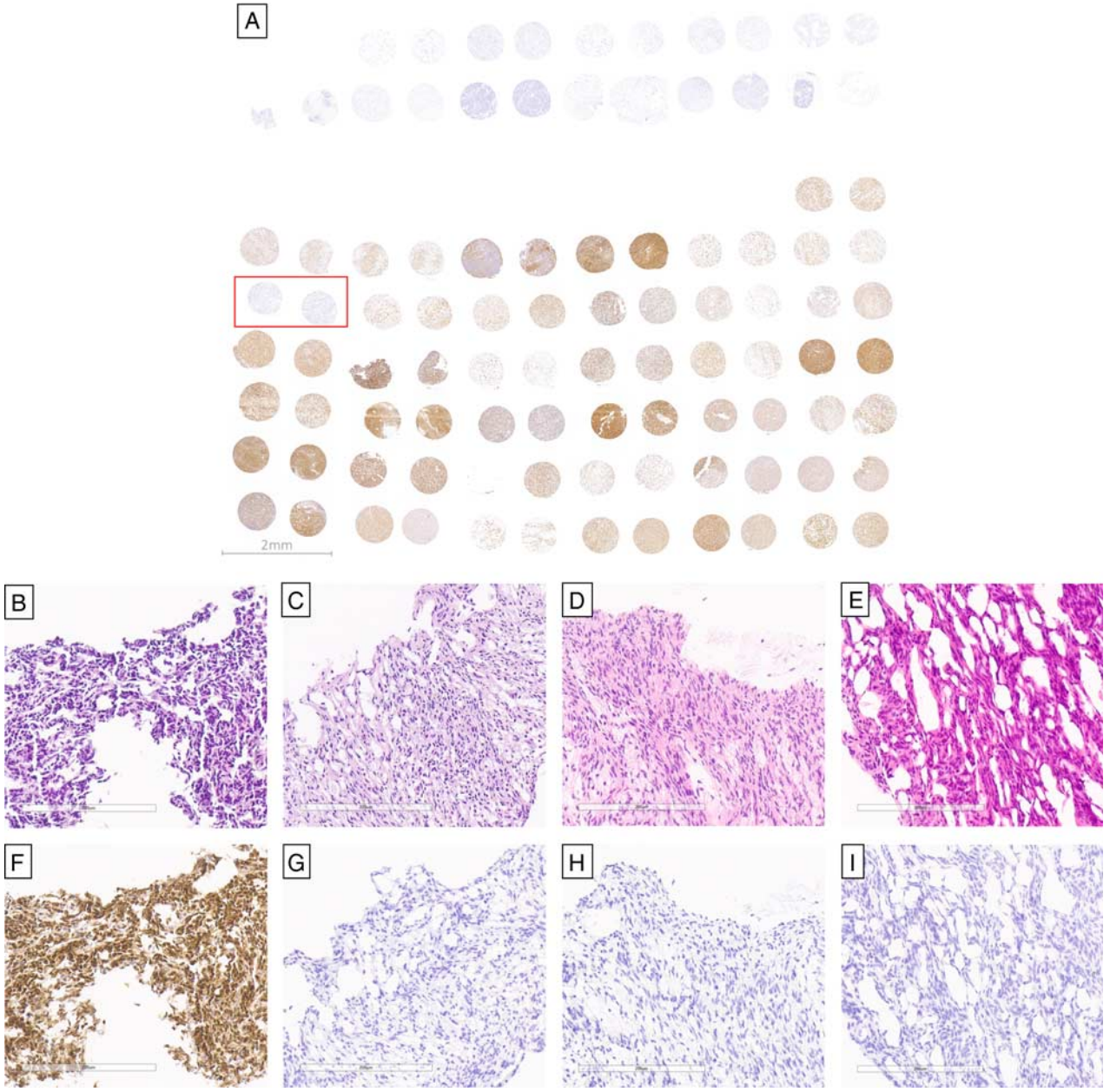

FIGURE 3. Confirmatory IHC for the SS18-SSX junction-specific antibody. A Tissue microarrays: 36 out of 37 synovial sarcoma samples showed positive nuclear staining for SS18-SSX. Each sample is represented by duplicate cores. One synovial sarcoma sample (red box) tested negative for IHC; this case carried an alternative *SSX exon 4* fusion point not recognized by the *SS18 exon 10-SSX exon 6* junction-specific antibody. The top 2 rows show negative staining for SS18-SSX on nonsynovial sarcoma formalin-fixed, paraffin-embedded control tissues, including malignant and nonmalignant samples (Table 1). B-I Whole section images of cases from Lund University Hospital stained for IHC (Table 2). B H&E, and F representative image of positive IHC staining, which contains the canonical fusion junction (*SS18 exon 10-SSX exon 6*) (Case 28). C H&E, and G negative IHC of case 6, which contains a fusion site between *SS18 exon 10* and *SSX1 exon 4*. D H&E, and H) negative IHC of case 27, which contains the alternative fusion site between *SS18 exon 9* and *SSX1 exon 7*. E H&E, and I negative IHC of case 32 presenting with a freezing artifact. H&E indicates hematoxylin-eosin; IHC, immunohistochemistry. full color online

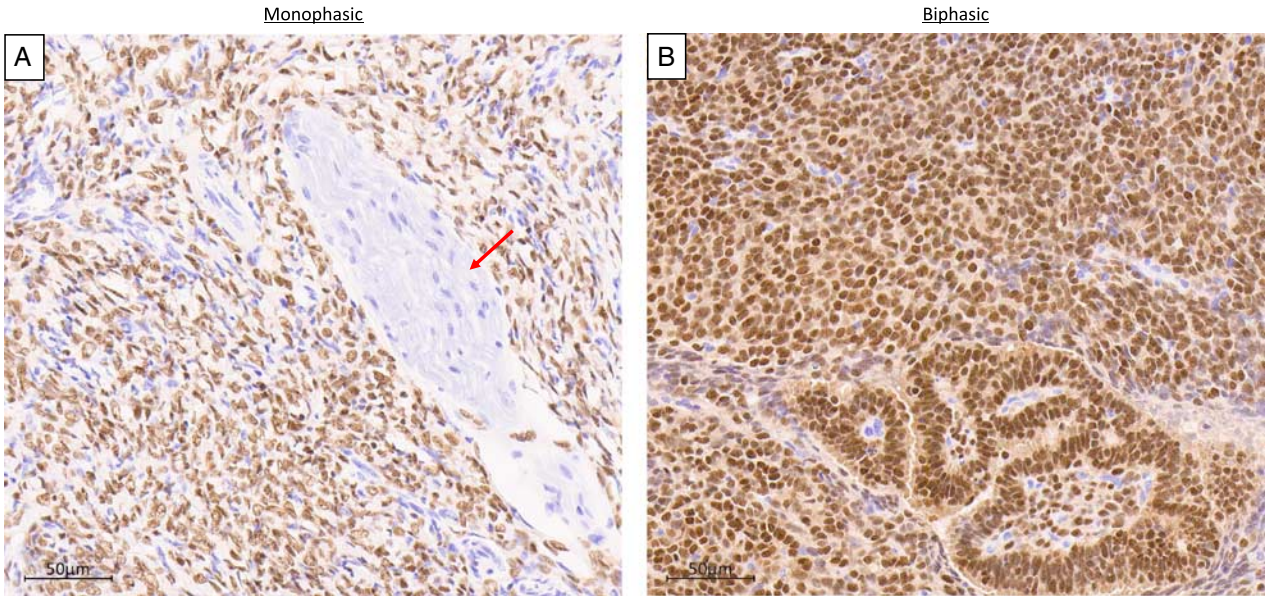

FIGURE 4. Novel IHC application on formalin-fixed, paraffin-embedded tumor tissue taken from tumors growing in the limbs of genetically engineered mice conditionally expressing the human SS18-SSX protein in *Myf5*+ cells. A Tumor tissue from a monophasic synovial sarcoma. Red arrow indicates negative IHC staining of a peripheral nerve. B Tumor tissue from a biphasic synovial sarcoma. Blood vessels interspersed within the tumor regions stain negative on IHC. IHC, immunohistochemistry. full color online

engineered to express epitope-tagged SS18-SSX.[13,17] These data add on to a previous report that showed enrichment in the promoter region of *TLE1* and *BCL2* using ChIP-seq.[14]

DISCUSSION

The current study presents a suite of applications for the recently published antibody that, by virtue of being directed against the junctional epitope of the fusion

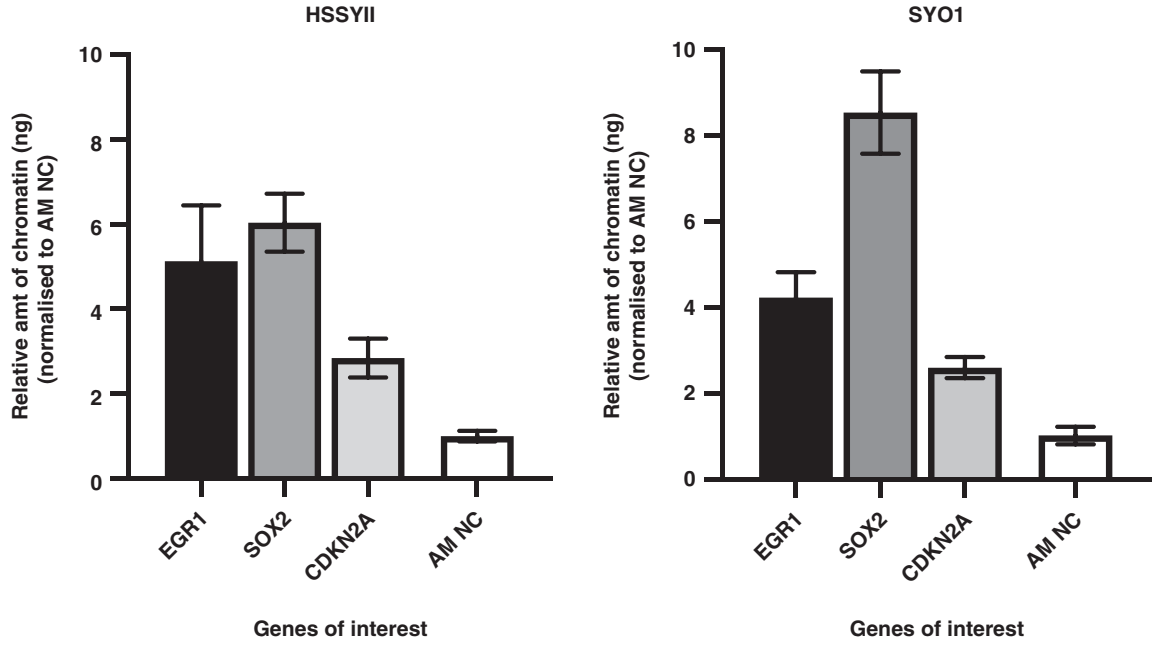

FIGURE 5. ChIP enrichment at SS18-SSX target gene loci. ChIP was performed using 10 µg of chromatin and 2 µg of antibody (Active Motif, #53009, Carlsbad, CA, USA). The indicated target gene promoter regions were enriched via quantitative PCR using validated primers against promoter regions. AM NC indicates Active Motif Negative Control (#71001, Carlsbad, CA, USA). The relative amount of chromatin for target gene loci was normalized to AM NC. Left: HSSYII synovial sarcoma cell line; right: SYO-1 synovial sarcoma cell line. AM NC indicates active motif negative control; ChIP, chromatin immunoprecipitation.

oncoprotein, is completely specific for SS18-SSX. The original report introducing the commercially available antibody had demonstrated its sensitivity and specificity for the fusion oncoprotein.[14] In this study, we confirmed its applicability in Western Blot and IHC in human tissue samples alongside its use in ChIP in promoter regions known to interact with SS18-SSX. We expanded its documented value to immunofluorescence and proximity ligation assay applications, while also establishing its ability to specifically detect the human protein in a murine background using tissues from genetically engineered synovial sarcoma conditional mouse models.

Our study included 70 synovial sarcoma samples, 64 of which were positive cases (91%) by SS18-SSX IHC. This is concordant with published literature across 8 studies, which have recorded a total of 303 positive IHC results from 326 (93%) synovial sarcoma samples, with sensitivity ranging from 86 to 100% in their respective studies.[14,20–26] Only one study attributed negative samples to a rare alternative fusion splice site, [23] while others alluded to technical factors arising from tissue handling.[20,22,24] Although we identified one technical false negative attributable to a freezing artifact, we were able to demonstrate that most cases are biological true negatives, where the fusion epitope is different from the one recognized by the diagnostic antibody.

Currently, molecular confirmation of a positive diagnosis of synovial sarcoma relies on RNA- or DNA-directed molecular techniques to detect the *SS18-SSX* fusion transcript and/or the t(X;18) chromosomal translocation.[27] Interphase fluorescence in situ hybridization is commonly used on formalin-fixed, paraffin-embedded samples to identify rearrangements of the *SS18* and *SSX* genes. Further information comes from techniques including RT-PCR (sometimes supported by Sanger sequencing) to identify the involved exons, or by hybrid capture or anchored multiplex sequencing strategies.[27] Our lab has developed a NanoString assay designed to specifically identify and quantify the presence of multiple known possible fusion junctions in translocation-associated sarcomas within a single experimental setup.[15] Compared to IHC tests, however, these molecular techniques require relatively expensive equipment, reagents, and trained personnel with sometimes lengthy turnaround. As an IHC-based biomarker, TLE1 is highly sensitive for a diagnosis of synovial sarcoma.[28] However, its imperfect specificity (~80%) requires extra caution to rule out certain sarcomas and histologic mimics.[29,30] Two independent studies have found the SS18-SSX antibody to be more sensitive and specific than fluorescence in situ hybridization for the diagnosis of synovial sarcoma.[22,31] As such, the SS18-SSX antibody represents a more specific, fast, and inexpensive tool to identify positive cases using existing formalin-fixed, paraffin-embedded material and pathology laboratory workflows. A similar strategy has also been developed for the diagnosis of alveolar rhabdomyosarcoma by Azorsa et al, wherein they detail the use of a PAX3-FOXO1-specific antibody with high degree of sensitivity and specificity for that fusion oncoprotein.[32]

One significant advantage of using the SS18-SSX antibody is that approximately 95% of all synovial sarcomas harbor the same fusion junction point, regardless of whether *-SSX1 or -SSX2* is the 3′ partner.[5] This is in contrast with many other translocation-associated sarcomas with multiple partners and/or alternative exonic fusion junction points that would compromise the sensitivity of any single chimeric junction-directed antibody for diagnostic use.[27,33] As seen with our negative IHC cases, the antibody facilitates detection of alternative or novel fusion junction sites in synovial sarcoma patients by flagging cases for RNA sequencing. While previous studies have only reported single instances of alternative fusions ,[23] our NanoString analyses confirmed the occurrence of a *SSX1 exon 4* fusion partner in 4 of our (IHC-negative) synovial sarcoma cases.

While co-immunoprecipitation experiments detect protein-protein interactions, PLA in addition provides in situ spatial information on a single slide. Previous PLA experiments in synovial sarcoma cells relied on antibodies against wild-type SS18 [34]; the current study confirms the proximity interactions are made by the SS18-SSX chimeric oncoprotein and not by native SS18. Such studies can validate these interactions on human or mouse formalin-fixed, paraffin-embedded tissue with minimal tissue consumption.

The conditional mouse model of synovial sarcoma used herein produces tumors with staining patterns matching those of human surgical excision specimens, with monophasic and biphasic features. As a credentialled in vivo model of the disease, this provides the opportunity to track temporal progression of synovial sarcoma, as opposed to the single advanced timepoint (in tumor evolution) represented in patient surgical samples. Thus, the antibody could be used in mice with earlier stages of the disease to detect endogenous SS18-SSX levels, interacting mouse proteins and chromatin elements, as well as to flag early precursor lesions comprising very few incipient synovial sarcoma cells.

As the antibody can specifically precipitate native SS18-SSX protein, its applications could be expanded to explore other interacting protein partners through mass spectrometry analyses to complement existing studies that have been limited to epitope-tagged cell lines. Similarly, chromatin derived from immunoprecipitation can be sent for sequencing, expanding knowledge of the genomic regions targeted by SS18-SSX. Overall, the existence of this specific SS18-SSX antibody will facilitate broader analyses of synovial sarcoma biology and oncogenic progression, providing an excellent tool to advance the study of this enigmatic disease.

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