Basic Research

Identification of a Novel *MAN1A1-ROS1* Fusion Gene Through mRNA-based Screening for Tyrosine Kinase Gene Aberrations in a Patient with Leiomyosarcoma

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Abstract

Background Soft tissue sarcomas are a heterogeneous group of rare malignant tumors. Advanced soft tissue sarcomas have a poor prognosis, and effective systemic therapies have not been established. Tyrosine kinases are increasingly being used as therapeutic targets for a variety of cancers and soft tissue sarcomas. Although complex karyotype sarcomas typically tend to carry more potentially actionable genetic alterations than do translocation-associated sarcomas (fusion gene sarcomas), based on our database review, we found that leiomyosarcoma and malignant peripheral nerve sheath tumors have lower frequencies of potential targets than other nontranslocation soft tissue sarcomas. We theorized that both leiomyosarcoma and malignant peripheral nerve sheath tumors might be included in any unique translocations. Furthermore, if tyrosine kinase imbalances, especially fusion genes, occur in patients with leiomyosarcomas and malignant peripheral nerve sheath tumors, tyrosine kinase inhibitors might be a drug development target for this sarcoma. In this study, we used a tyrosine kinase screening system that could detect an imbalance in mRNA between 5'- and 3'-sides in tyrosine kinase genes to identify

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potential novel therapeutic tyrosine kinase targets for soft tissue sarcomas.

Questions/purposes   (1) Are there novel therapeutic tyrosine kinase targets in tumors from patients with soft tissue sarcomas that are detectable using mRNA screening focusing on imbalance expressions between the 5' and 3' end of the kinase domain? (2) Can potential targets be verified by RNA sequencing and reverse transcription PCR (RT-PCR)? (3) Will potential fusion gene(s) transform cells in in vitro assays? (4) Will tumors in mice that have an identified fusion gene respond to treatment with a therapeutic drug directed at that target?

Methods   We used mRNA screening to look for novel tyrosine kinase targets that might be of therapeutic potential. Using functional assays, we verified whether the identified fusion genes would be good therapeutic candidates for soft tissue sarcomas. Additionally, using in vivo assays, we assessed whether suppressing the fusion's kinase activity has therapeutic potential. Study eligibility was based on a patient having high-grade spindle cell and nontranslocation sarcomas, including leiomyosarcoma, malignant peripheral nerve sheath tumor, and high-grade myxofibrosarcoma. Between 2015 and 2019, of the 172 patients with soft tissue sarcomas treated with surgical resection at Juntendo University Hospital, 72 patients had high-grade nontranslocation sarcomas. The analysis was primarily for leiomyosarcoma and malignant peripheral nerve sheath tumors, and there was a limitation of analysis size (reagent limitations) totaling 24 samples at the start of the study. We collected additional samples from a sample bank at the Tokyo Medical and Dental University to increase the number of sarcomas to study. Therefore, in this study, a total of 15 leiomyosarcoma samples, five malignant peripheral nerve sheath tumors samples, and four high-grade myxofibrosarcoma samples were collected to achieve the sample size of 24 patients. To identify tyrosine kinase fusion genes, we designed a NanoString-based assay (NanoString Technologies Inc, Seattle, WA, USA) to query the expression balances regarding transcripts of 90 tyrosine kinases at two points: the 5' end of the kinase domain and within the kinase domain or 3' end of the kinase domain. The tumor's RNA was hybridized to the NanoString probes and analyzed for the expression ratios of outliers from the 3' to 5' end of the kinase domain. Presumed novel fusion events in these positive tumors that were defined by NanoString-based assays were confirmed tyrosine kinase fusion genes by RNA sequencing and confirmatory RT-PCR. Functional analyses consisting of in vitro and in vivo assays were also performed to elucidate whether the identified tyrosine kinase gene fusions were associated with oncogenic abilities and drug responses.

Results   We identified aberrant expression ratios regarding the 3' to 5' end of the kinase domain ratios in *ROS1* transcripts in a leiomyosarcoma in a 90-year-old woman. A novel *MAN1A1-ROS1* fusion gene was identified from her thigh tumor through RNA sequencing, which was confirmed with real-time PCR. In functional assays, *MAN1A1-ROS1* rearrangement revealed strong transforming potential in 3T3 cells. Moreover, in an in vivo assay, crizotinib, a *ROS1* inhibitor, markedly inhibited the growth of *MAN1A1-ROS1* rearrangement-induced transformed cells in a dose-dependent manner.

Conclusion We conducted tyrosine kinase screening to identify new therapeutic targets in soft tissue sarcomas. We found a novel *MAN1A1-ROS1* fusion gene that may be a therapeutic target in patients with leiomyosarcoma. This study demonstrates that the mRNA screening system may aid in the development of useful therapeutic options for soft tissue sarcomas.

Clinical Relevance If novel tyrosine fusions such as *MAN1A1-ROS1* fusion can be found in sarcomas from other patients, they could offer avenues for new molecular target therapies for sarcomas that currently do not have effective chemotherapeutic options. Therefore, the establishment of a screening system that includes both genomic and transcript analyses in the clinical setting is needed to verify our discoveries and take the developmental process of treatment to the next step.

Introduction

Soft tissue sarcomas (STSs) are a heterogeneous group of rare malignant tumors. The treatment is primarily surgical

resection with or without radiotherapy, but systemic chemotherapy with cytotoxic anticancer agents may improve the prognosis of STS [16, 18]. However, advanced STSs still have a poor prognosis, and effective systemic therapies have not been established. Therefore, identifying novel therapeutic targets that might identify effective therapies is desirable. Tyrosine kinases, especially tyrosine kinase fusion genes including *ALK*, *ROS1*, and *RET*, are increasingly being used as therapeutic targets for a variety of cancers [25, 28]. Recent reports revealed that tyrosine kinase inhibitors specific to *NTRK1-3* exhibit antitumor activity in a small population of STSs carrying the *NTRK* fusion gene [8, 14, 15]. Multitarget assays, mainly DNA-based, with next-generation sequencing have recently become common in clinical diagnostic laboratories in the cancer field [2, 27, 31]. However, DNA-based gene panel assays are sometimes inadequate to detect fusion genes, no matter how well-designed they are [2, 11, 12]. Therefore, multitarget assays with RNA-based systems may be a better alternative for detecting fusion genes.

Genetically, STSs are classified into two types. Translocation-associated sarcomas have been shown to have translocations, activating mutations, and simple gene copy number alterations [26]. These are associated with specific histologic subtypes and are believed to be involved in the oncogenic process in these sarcomas. The second type is complex karyotype (nontranslocation) sarcomas, including leiomyosarcoma and malignant peripheral nerve sheath tumor (MPNST). Unlike translocation-associated sarcomas, for which a potential target could be designed that focuses on these known lesions, these tumors have complex, numerous, and patternless genetic aberrations that make the identification of potential drug targets more difficult. Very few specific molecular events, including receptor tyrosine kinase fusion genes, have been defined in complex karyotype sarcomas [6, 16, 26]. With respect to potentially actionable genetic events, large-scale clinical sequencing revealed that one-third of STSs have actionable genetic events composed of mainly copy number alterations, including *MDM2* and *CDK4*, nonsynonymous mutations such as those for *PIK3CA* and *PTEN*, and a small number of receptor tyrosine kinase fusions [31]. Complex karyotype sarcomas tend to carry more potentially actionable genetic alterations than do translocation-associated sarcomas [31]. However, while reviewing the database regarding the frequencies of actionable genetic events in STSs, we found that leiomyosarcoma and MPNST have lower frequencies of potential targets than other nontranslocation STSs, although they have been classified as complex karyotype (nontranslocation) sarcomas [31]. Furthermore, we theorized that these findings might suggest that both leiomyosarcoma and MPNST are included in any unique translocations harboring tyrosine kinase fusion genes. Given this background, we conducted tyrosine kinase screening in complex karyotype sarcomas using NanoString assays (NanoString Technologies Inc, Seattle, WA, USA) to detect an imbalance in the expression of mRNA between the 5'- and 3'-sides in tyrosine kinase genes and to identify novel therapeutic tyrosine kinase targets including tyrosine kinase fusions in STSs, especially leiomyosarcoma and MPNST [28].

We therefore asked: (1) Are there novel therapeutic tyrosine kinase targets in tumors from patients with soft tissue sarcomas that are detectable using mRNA screening focusing on imbalance expressions between the 5' and 3' end of the kinase domain? (2) Can potential targets be verified by RNA sequencing and reverse transcription PCR (RT-PCR)? (3) Will potential fusion gene(s) transform cells in in vitro assays? (4) Will tumors in mice that have an identified fusion gene respond to treatment with a therapeutic drug directed at that target?

Materials and Methods

Experimental Overview and Primary/Secondary Study Endpoints

Our primary study goal was the identification of tyrosine kinase fusion genes in STSs (Fig. 1). To achieve this, we conducted NanoString-based transcriptome assay and analyzed 24 tumor samples with STSs, including 15 leiomyosarcomas, five MPNSTs, and four high-grade myxofibrosarcomas (Step 1). This assay screened the expression balances on the transcripts of 90 tyrosine kinases at two points: the 5' and 3' endpoints of the kinase domain. RNA sequencing and confirmatory RT-PCR validated the existance of tyrosine kinase fusion genes in presumed novel fusion events, identified by NanoString (Step 2). Our secondary study goals were the confirmation of the identified tyrosine kinase gene fusions that were associated with oncogenic abilities and drug responses (Fig. 1). Then, functional analyses consisting of in vitro (focus formation assay [Step 3]) and in vivo assays (xenograft tumor assays [Step 4]) were conducted.

Patient Sample

Data on 24 patients with STSs consisting of 15 patients with leiomyosarcomas, five with MPNSTs, and four with high-grade myxofibrosarcomas were collected and analyzed using a protocol approved by the institutional review boards at Juntendo University (No. 2018135) and Tokyo Medical and Dental University. Informed consent was obtained from the patients in this study (Table 1). Histologic subtypes were determined by two board-certified pathologists (TS, TH) based on histologic features, immunohistochemical staining, and the WHO

Overview of this study
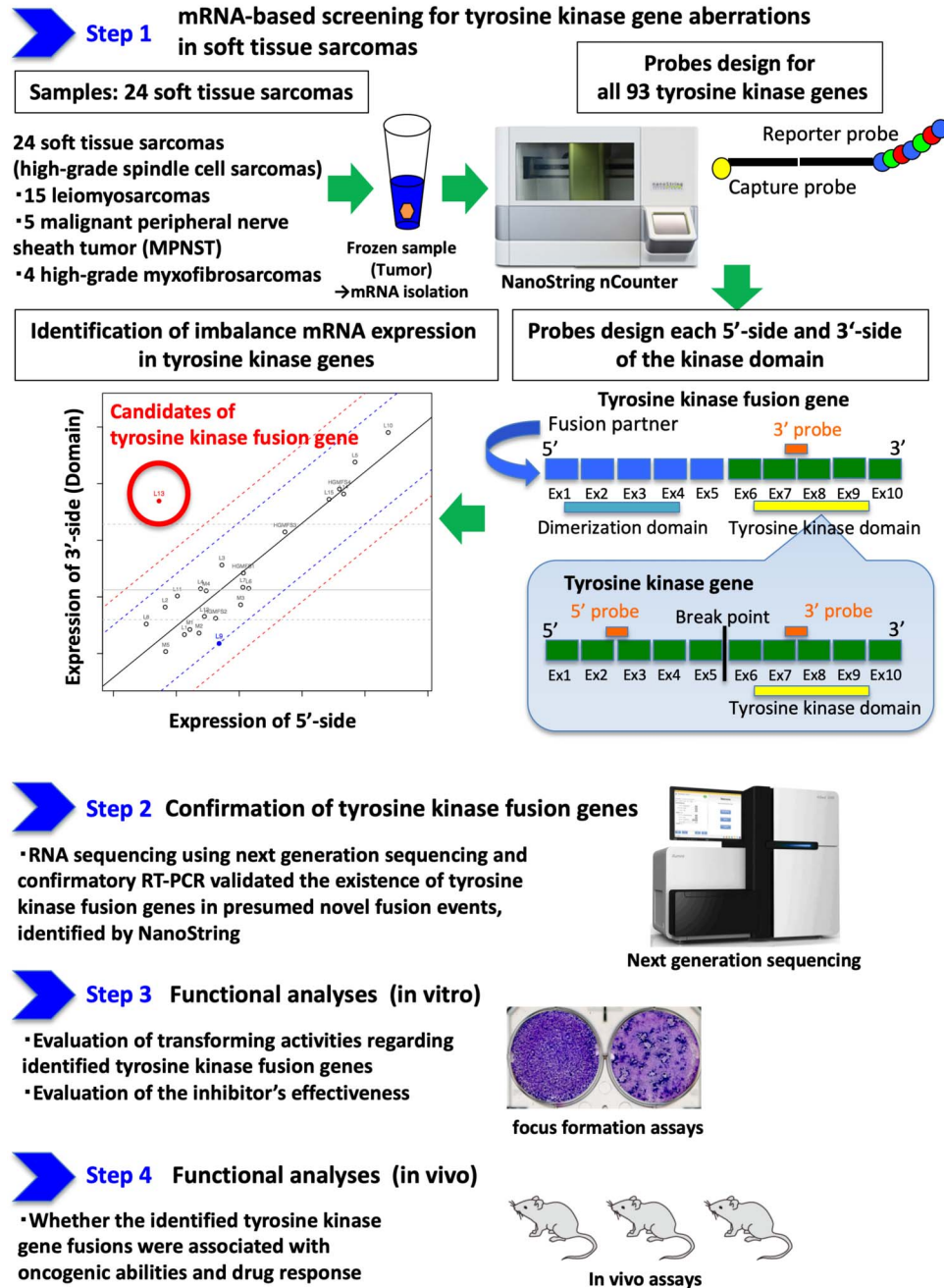
Fig. 1 Step 1: To identify tyrosine kinase fusion genes, NanoString-based transcriptome assay analyzed 24 tumor samples with STSs including 15 leiomyosarcomas, five with MPNSTs, and four with high-grade myxofibrosarcomas. This assay screened the expression balances regarding transcripts of 90 tyrosine kinases at two points: the 5' and 3' endpoints of the kinase domain. Step 2: RNA sequencing using next generation sequencing and confirmatory reverse transcription-polymerase chain reaction (RT-PCR) validated whether NanoString identified existing tyrosine kinase fusion genes in presumed novel fusion events. Step 3: Functional in vitro analyses were performed to elucidate whether the identified tyrosine kinase gene fusions were associated with oncogenic abilities. Step 4: Functional analyses consisting of in vivo assays were also performed to elucidate whether the identified tyrosine kinase gene fusions were associated with oncogenic abilities and drug responses.

classification of tumors [16]. Sample collection was conducted between 2015 and 2019, when 172 patients with STSs were treated with surgical resection at Juntendo University Hospital. To be eligible for this study, a patient needed to have high-grade spindle cell and non-translocation sarcomas, including leiomyosarcoma, MPNST, and high-grade myxofibrosarcoma; 72 patients met these criteria. In planning the analysis of leiomyosarcoma and MPNST, we found analysis size (a total of 24 samples) during the start of the study in 2016 was limited. Therefore, for leiomyosarcoma, we employed 11 leiomyosarcoma samples in the 2015 and 2016 series and excluded those (n = 2) patients who did not have sufficient frozen tumor samples and those who declined to participate, leaving a total of nine patients. We additionally collected six leiomyosarcoma samples from a sample bank at the Tokyo Medical and Dental University Hospital and analyzed a total of 15 leiomyosarcomas in this study. For MPNSTs, all five samples were collected from a sample bank at the Tokyo Medical and Dental University Hospital. To bring the sample size to 24 patients, we added four high-grade myxofibrosarcoma samples in the 2015 series from Juntendo University. These resected samples had not received any pretreatment with radiation or chemotherapy. Imbalances in the expression of mRNA in tyrosine kinase genes were screened in these 24 patients with STSs using NanoString analysis (Table 1).

RNA Extraction and cDNA Synthesis

RNA was extracted from frozen samples using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and first-strand synthesis was performed using 5 μg of RNA and the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA).

NanoString Analysis

To identify imbalances in the expression of mRNA between the 5'- and 3'-sides of 93 tyrosine kinase genes (Table 2), we used the NanoString system (NanoString Technologies Inc) using our original custom probes based on our previous study [28] (Supplemental Fig. 1; Supplemental Digital Content 1, http://links.lww.com/CORR/A465). In the NanoString system, 400 ng of RNA was hybridized to probes (a reporter probe and a capture probe) at 65°C for 18 to 24 hours using a thermal cycler. Samples were then inserted into the nCounter Prep Station (NanoString Technologies Inc) for removal of excessive probes, purification, and immobilization onto the internal surface of a sample cartridge for 3 hours. Finally, the sample cartridge was transferred to the nCounter

Table 1. Characteristics of patients in whom tumors were assessed

Sample	Age in years	Sex	Location
LMS1	37	Male	Thigh
LMS2	54	Female	Groin
LMS3	38	Female	Groin
LMS4	73	Male	Groin
LMS5	67	Female	Forearm
LMS6	47	Male	Abdominal wall
LMS7	65	Female	Forearm
LMS8	47	Male	Chest wall
LMS9	68	Female	Upper arm
LMS10	86	Male	Upper arm
LMS11	69	Male	Thigh
LMS12	83	Female	Forearm
LMS13	90	Female	Thigh
LMS14	71	Female	Buttocks
LMS15	71	Female	Thigh
MPNST1	62	Male	Shoulder
MPNST2	70	Male	Knee
MPNST3	52	Female	Thigh
MPNST4	ND	ND	ND
MPNST5	ND	ND	ND
HGMFS1	75	Female	Chest wall
HGMFS2	47	Male	Upper arm
HGMFS3	78	Male	Lower leg
HGMFS4	75	Male	Upper arm

LMS = leiomyosarcoma; MPNST = malignant peripheral nerve sheath tumor; HGMFS = high-grade myxofibrosarcoma; ND = not determined.

Digital Analyzer (NanoString Technologies Inc), where color codes were counted and tabulated for each target molecule. The expression number of the base sequence of the probes was calculated using nSolver Analysis Software (NanoString Technologies Inc) (Supplemental Table 1; Supplemental Digital Content 2, http://links.lww.com/CORR/A466). The raw data were statistically analyzed and plotted on graphs (abscissa: log2 [5'-side expression], ordinate: log2 [3'-side expression]) with 2 and 3 SD lines. If either 3'- or 5'-side expression showed higher than 10-fold SD or plotted on the area higher than 2 SDs of the graph, the abnormality was judged as having imbalanced gene expression.

Detection of Fusion Genes by RNA Sequencing

Total RNA was extracted from fresh frozen samples using RNA-Bee (Tel-Test Inc, Gainesville, FL, USA), followed by treatment with DNase I (Thermo Fisher

Table 2. Tyrosine kinase genes investigated

Number	Gene name	Number	Gene name	Number	Gene name
1	AATK	32	ERBB4	63	MST1R
2	ABL1	33	FER	64	MUSK
3	ABL2	34	FES	65	NTRK1
4	ALK	35	FGFR1	66	NTRK2
5	ARAF	36	FGFR2	67	NTRK3
6	AXL	37	FGFR3	68	PDGFRA
7	BLK	38	FGFR4	69	PDGFRB
8	BMX	39	FGR	70	PTK2
9	BRAF	40	FLT1	71	PTK2B
10	BTK	41	FLT3	72	PTK6
11	CSF1R	42	FLT4	73	PTK7
12	CSK	43	FRK	74	RAF1
13	DDR1	44	FYN	75	RET
14	DDR2	45	HCK	76	ROR1
15	EGFR	46	IGF1R	77	ROR2
16	EPHA1	47	INSR	78	ROS1
17	EPHA2	48	INSRR	79	RYK
18	EPHA3	49	ITK	80	SRC
19	EPHA4	50	JAK1	81	SRMS
20	EPHA5	51	JAK2	82	STYK1
21	EPHA6	52	JAK3	83	SYK
22	EPHA7	53	KDR	84	TEC
23	EPHA8	54	KIT	85	TEK
24	EPHA10	55	LCK	86	TIE1
25	EPHB1	56	LMTK2	87	TNK1
26	EPHB2	57	LMTK3	88	TNK2
27	EPHB3	58	LTK	89	TXK
28	EPHB4	59	LYN	90	TYK2
29	EPHB6	60	MATK	91	TYRO3

Table 2. continued

Number	Gene name	Number	Gene name	Number	Gene name
30	ERBB2	61	MERTK	92	YES1
31	ERBB3	62	MET	93	ZAP70

AATK = apoptosis associated tyrosine kinase; *ABL1* = ABL proto-oncogene 1, non-receptor tyrosine kinase; *ABL2* = ABL proto-oncogene 2, non-receptor tyrosine kinase; *ALK* = ALK receptor tyrosine kinase; *ARAF* = a-raf proto-oncogene, serine/threonine kinase; *AXL* = AXL receptor tyrosine kinase; *BLK* = BLK proto-oncogene, src family tyrosine kinase; *BMX* = BMX non-receptor tyrosine kinase; *BRAF* = b-raf proto-oncogene, serine/threonine kinase; *BTK* = bruton tyrosine kinase; *CSF1R* = colony stimulating factor 1 receptor; *CSK* = c-terminal src kinase; *DDR1* = discoidin domain receptor tyrosine kinase 1; *DDR2* = discoidin domain receptor tyrosine kinase 1; *EGFR* = epidermal growth factor receptor; *EPHA1* = erythropoietin-producing hepatoma receptor a1; *EPHA2* = erythropoietin-producing hepatoma receptor a2; *EPHA3* = erythropoietin-producing hepatoma receptor a3; *EPHA4* = erythropoietin-producing hepatoma receptor a4; *EPHA5* = erythropoietin-producing hepatoma receptor a5; *EPHA6* = erythropoietin-producing hepatoma receptor a6; *EPHA7* = erythropoietin-producing hepatoma receptor a7; *EPHA8* = erythropoietin-producing hepatoma receptor a8; *EPHA10* = erythropoietin-producing hepatoma receptor a10; *EPHB1* = EPH receptor b1; *EPHB2* = EPH receptor b2; *EPHB3* = EPH receptor b3; *EPHB4* = EPH receptor b4; *EPHB6* = EPH receptor b6; *ERBB2* = erb-b2 receptor tyrosine kinase 2; *ERBB3* = erb-b2 receptor tyrosine kinase 3; *ERBB4* = erb-b2 receptor tyrosine kinase 3; *FER* = proto-oncogene tyrosine-protein kinase FER; *FES* = FES proto-oncogene, tyrosine kinase; *FGFR1* = fibroblast growth factor receptor 1; *FGFR2* = fibroblast growth factor receptor 2; *FGFR3* = fibroblast growth factor receptor 3; *FGFR4* = fibroblast growth factor receptor 4; *FGR* = FGR proto-oncogene, src family tyrosine kinase; *FLT1* = FMS related receptor tyrosine kinase 1; *FLT3* = FMS related receptor tyrosine kinase 3; *FLT4* = FMS related receptor tyrosine kinase 4; *FRK* = fyn related src family tyrosine kinase; *FYN* = FYN proto-oncogene, src family tyrosine kinase; *HCK* = HCK proto-oncogene, src family tyrosine kinase; *IGF1R* = insulinlike growth factor 1 receptor; *INSR* = insulin receptor; *INSRR* = insulin receptor related receptor; *ITK* = il2 inducible t cell kinase, *JAK1* = janus kinase 1; *JAK2* = janus kinase 2; *JAK3* = janus kinase 3; *KDR* = kinase insert domain receptor; *KIT* = KIT proto-oncogene; receptor tyrosine kinase, *LCK* = LCK proto-oncogene, src family tyrosine kinase; *LMTK2* = lemur tyrosine kinase 2; *LMTK3* = lemur tyrosine kinase 3; *LTK* = leukocyte receptor tyrosine kinase; *LYN* = LYN proto-oncogene, src family tyrosine kinase; *MATK* = megakaryocyte-associated tyrosine kinase; *MERTK* = MER proto-oncogene, tyrosine kinase; *MET* = MET proto-oncogene, receptor tyrosine kinase; *MST1R* = macrophage-stimulating 1 receptor; *MUSK* = muscle-associated receptor tyrosine kinase; *NTRK1* = neurotrophic receptor tyrosine kinase 1; *NTRK2* = neurotrophic receptor tyrosine kinase 2; *NTRK3* = neurotrophic receptor tyrosine kinase 3; *PDGFRA* = platelet-derived growth factor receptor alpha; *PDGFRB* = platelet-derived growth factor receptor beta; *PTK2* = protein tyrosine kinase 2; *PTK2B* = protein tyrosine kinase 2 beta; *PTK6* = protein tyrosine kinase 6; *PTK7* = protein tyrosine kinase 7; *RAF1* = raf-1 proto-oncogene, serine/threonine kinase; *RET* = ret proto-oncogene; *ROR1* = receptor tyrosine kinase-like orphan receptor 1; *ROR2* = receptor tyrosine kinase-like orphan receptor 2; *ROS1* = ROS proto-oncogene 1, receptor tyrosine kinase; *RYK* = receptor-like tyrosine kinase; *SRC* = SRC proto-oncogene, non-receptor tyrosine kinase; *SRMS* = src-related kinase lacking c-terminal regulatory tyrosine and n-terminal myristylation sites; *STYK1* = serine/threonine/tyrosine kinase 1; *SYK* = spleen-associated tyrosine kinase; *TEC* = tec protein tyrosine kinase; *TEK* = TEK receptor tyrosine kinase; *TIE1* = tyrosine kinase with immunoglobulin-like and egf-like domains 1; *TNK1* = tyrosine kinase non-receptor 1; *TNK2* = tyrosine kinase non-receptor 2; *TXK* = TXK tyrosine kinase; *TYK2* = tyrosine kinase 2; *TYRO3* = TYRO3 protein tyrosine kinase; *YES1* = YES proto-oncogene 1, src family tyrosine kinase; *ZAP70* = zeta chain of t cell receptor associated protein kinase 70.

Scientific) and poly(A)-RNA selection before cDNA synthesis. The library used for the RNA sequencing was prepared with a NEBNext Ultra Directional RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) in accordance with the manufacturer's protocol. Sequencing was conducted from both ends of each cluster using the HiSeq 2500 System (Illumina, San Diego, CA, USA). RNA sequencing was aligned to hg19 using TopHat (v2.0.9; https://ccb.jhu. edu/software/tophat/index.shtml). The expression level of each gene was calculated using Cufflinks (v2.1.1; http://cole-trapnell-lab.github.io/cufflinks), and gene fusion was detected using the deFuse pipeline (https:// bitbucket.org/dranew/defuse).

Real-time Polymerase Chain Reaction

We performed real-time PCR analyses to confirm the expression of *MAN1A1-ROS1* using PCR SuperMix (Thermo Fisher Scientific). *MAN1A1-ROS1* was amplified by combining either of the following primers: *MAN1A1*: 5'-TGGTGGACTACTCTCAGCCT -3', 5'-ACGCTTTGTTGGTGGACTACT -3', 5'-GGAGAAGA AGAAGGTGGCCC-3', and 5'-CGCGAGAAAAGGGC AAAGAT-3'; and *ROS1*: 5'-TCTTCAGCTTTCTCC CACTGT-3', 5'-TCAGCTTTCTCCCACTGTATTGA-3', and 5'-GCAAACACTACTGCAGGATCC-3'. The expression of glyceraldehyde-3-phosphate dehydrogenase was used to control the RNA quality with the following primers: 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'- GAAGAT GGTGATGGGATTT-3'.

Immunohistochemistry

To diagnose leiomyosarcoma accurately, we performed an immunohistochemical analysis with the streptavidin-biotin method using the antibodies desmin (mouse monoclonal, 1:100; Roche, Basel, Switzerland), smooth muscle actin (mouse monoclonal, 1:200; Dako, Santa Clara, CA), M-actin (mouse monoclonal, 1:100; Dako), and h-Caldesmon (mouse monoclonal, 1:1; Dako). Two board-certified pathologists (TS, TH) assessed the immunostaining independently. The assessment was qualitative. When discrepancies arose, the slides were reviewed using a multiheaded microscope to reach a consensus.

Preparation of Retrovirus, Gene Transduction into 3T3 Cells, and Focus Formation Assay

Recombinant plasmids were introduced with packaging plasmids (Takara Bio Inc, Shiga, Japan) into HEK293T cells to obtain recombinant retroviral particles. For the focus formation assay, 3T3 mouse fibroblasts purchased from the American Type Culture Collection were infected with eco-tropic recombinant retroviruses using 4 μg/mL of polybrene (Sigma-Aldrich, St Louis, MO, USA) for 24 hours. With respect to focus formation assay, they were further cultured for up to 2 weeks in Dulbecco's modified eagle medium-nutrient mixture F-12 supplemented with 5% bovine serum albumin. A focus formation assay was performed using 3T3 cells infected with recombinant retroviruses expressing wild-type cDNAs (*ROS1*), mutant cDNAs (*CD74-ROS1*: [*CD74*_exon 6 / *ROS1*_exon 32] and *MAN1A1-ROS1*: [*MAN1A*_exon 5 / *ROS1*_exon 31]) and green fluorescent protein as a negative control. After 2 weeks, the cells were stained with Giemsa solution and transforming activity was measured. Cell transformation was performed quantitatively in triplicate and assessed by staining with Giemsa solution as quantitative.

Xenograft Tumor Assays

We performed in vivo animal model studies to clarify the antitumor activity of crizotinib in the *ROS1* fusion-transfected cells. All animal studies were conducted following the protocols approved by the Animal Ethics Committee of the National Cancer Research Center, Tokyo, Japan. Before injection, 3T3 cells (1.0×10^6) were mixed in phosphate-buffered saline with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) at a 1:1 ratio. The cell suspension was injected subcutaneously (200 μL/mouse) into the back of 6-week-old female BALB/c nude mice (CREA Japan, Tokyo, Japan). In accordance with our previous study, which similarly evaluated the drug efficacy using a 3T3 xenograft model, five age- and sex-matched mice of this strain were randomly assigned to each group (wild-type, *ROS1* fusions, and crizotinib treatments) [29]. The mice were inoculated subcutaneously with transfected cells with *ROS1* wild-type and *ROS1* fusion genes including *CD74-ROS1* and *MAN1A1-ROS1*. These inoculated mice were administered either crizotinib or a vehicle (dimethyl sulfoxide). Additionally, *ROS1*-transfected cells (control) with no drug administration were also analyzed. When tumors reached approximately 100 to 150 mm^3 in size, the mice were randomized into two groups. Either 50 mg/kg of crizotinib or vehicle control was administered by intraperitoneal injection. Both treatments were administered for 5 days per week over a series of weeks. Tumor volume was used as the endpoint to directly evaluate drug efficacy with regard to tumor growth inhibition. The tumor volume (mean $\pm$ SD) in each group is expressed in cubic millimeters and was calculated using the formula $\pi/6 \times$ (largest diameter) $\times$ (smallest diameter)^2. Tumor size assessment was completed by observers

(SK, SM, NH) blinded to the study groups of the animals and their treatments. The mice were euthanized when the tumor reached 1.5 cm in diameter, and the collected data were analyzed.
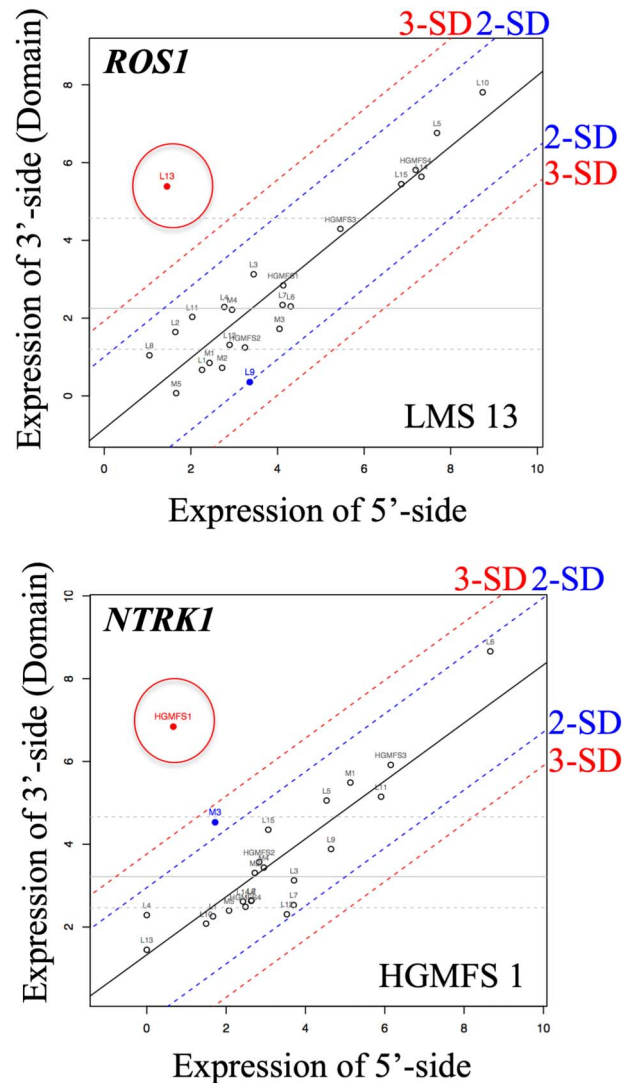
Results

Are There Novel Therapeutic Tyrosine Kinase Targets in Tumors from Patients with STSs That are Detectable Using mRNA Screening?

Using scatter plots constructed with data acquired form the NanoString system, we identified an imbalance in the expression of mRNA in tumors in two patients. These imbalances consisted of *ROS1* in a patient with a leiomyosarcoma and *NTRK1* in a patient with a high-grade myxofibrosarcoma (Fig. 2).
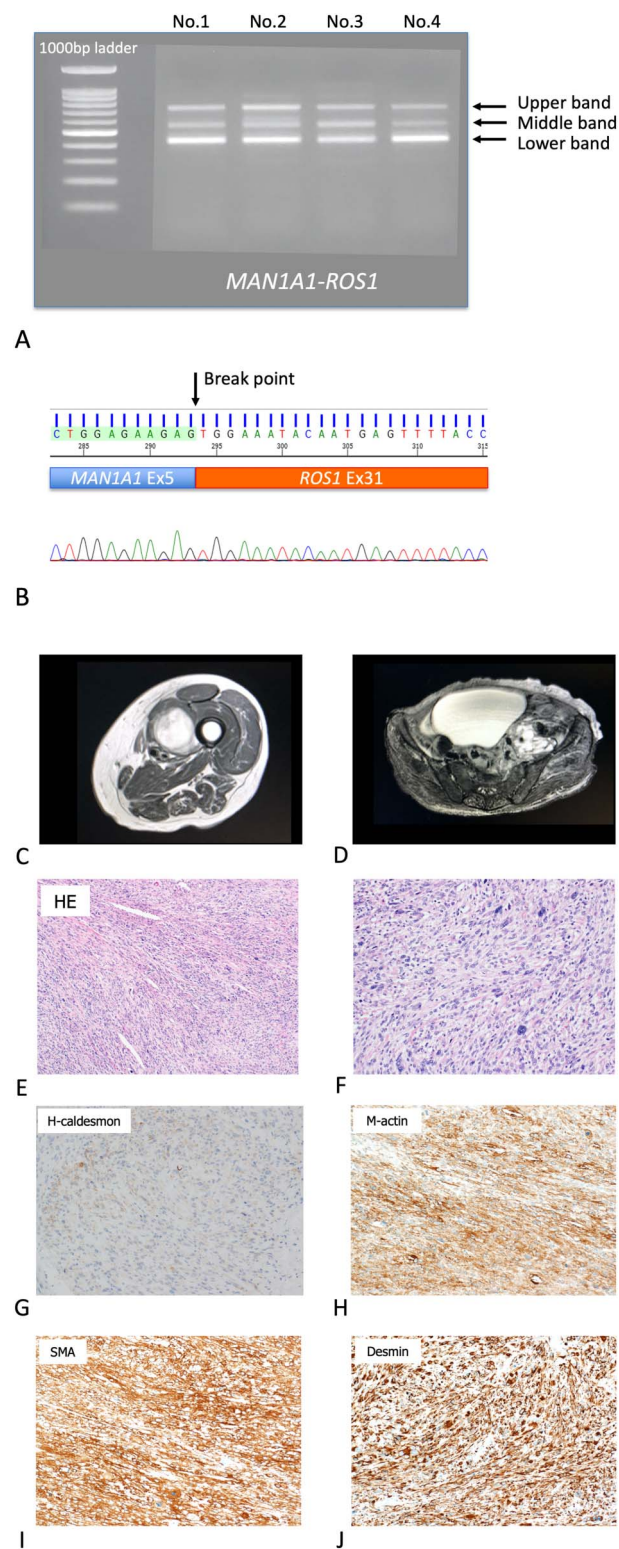
Can Potential Targets be Verified by RNA Sequencing and RT-PCR?

The results of RNA sequencing in two patients with an imbalance in *ROS1* and *NTRK1* mRNA expression identified a novel *MAN1A1-ROS1* (*MAN1A1*_exon 5 / *ROS1*_exon 31) fusion gene in the patient with a leiomyosarcoma (Supplemental Fig. 2; Supplemental Digital Content 3, http://links.lww.com/CORR/A467). Regarding the *NTRK1* gene, there was no rearrangement in the tumor in the patient with a high-grade myxofibrosarcoma based on RNA sequencing (Supplemental Fig. 3; Supplemental Digital Content 4, http://links.lww.com/CORR/A468). The in-frame fusion of *MAN1A1* exon 5 to *ROS1* exon 31 was confirmed with independent RT-PCR, and the RT-PCR product was also sequence-verified (Fig. 3A-B). This tumor was sampled from the thigh of a 90-year-old woman (Fig. 3C-D). Histologically, the tumor was composed of fascicular proliferation of spindle-shaped cells (Fig. 3E). Tumor cells with bizarre giant nuclei were also scattered throughout the lesion (Fig. 3F). Immunohistochemistry revealed that the spindle tumor cells were positive for desmin, smooth muscle actin, M-actin, and h-caldesmon (Fig. 3G-J). Additionally, these tumor cells showed an MIB-1 (Ki-67) index of approximately 60%. Based on the histologic features, immunohistochemistry findings, and imaging studies, this patient had a diagnosis of stage III pleomorphic leiomyosarcoma. The primary lesion in the thigh was resected and used for further analyses. This patient was followed with imaging studies and did not receive adjuvant chemotherapy because of her older age. Two years postoperatively, a metastasis developed in the pelvis. Based on the patient's genetic profile, the *ROS1* inhibitor crizotinib was considered to be beneficial to the

Fig. 2 NanoString assay results for *ROS1* and *NTRK1*. The *ROS1* gene showed imbalance in the expression of mRNA between the 5'- and 3'- sides in a tumor in a patient with leiomyosarcoma (patient LMS13 in Table 1; 5'-side value: 2.7, 3'-side value: 41.9, the rate of 3'/5'-side value showed over 3 SD). In patients with high-grade myxofibrosarcoma, *NTRK1* showed differences in the expression of mRNA between the 5'- and 3'- sides in an HGMFS1 tumor in a patient with high-grade myxofibrosarcoma (patient HGMFS1 in Table 1; 5'-side value: 1.6, 3'-side value: 114.7, the rate of 3'/5'-side value showed over 3 SD). These samples were subjected to RNA sequencing, and *MAN1A1-ROS1* was identified in the LMS13 tumor; LMS = leiomyosarcoma, HGMFS = high-grade myxofibrosarcoma, and the dots circled indicate candidates of tyrosine kinase fusion gene.

patient. However, because the patient was older than 90 years when the metastasis developed, we decided not to use the inhibitor against the *ROS1* fusion-positive tumor to avoid any adverse events. At 2.5 years postoperatively, the patient died.

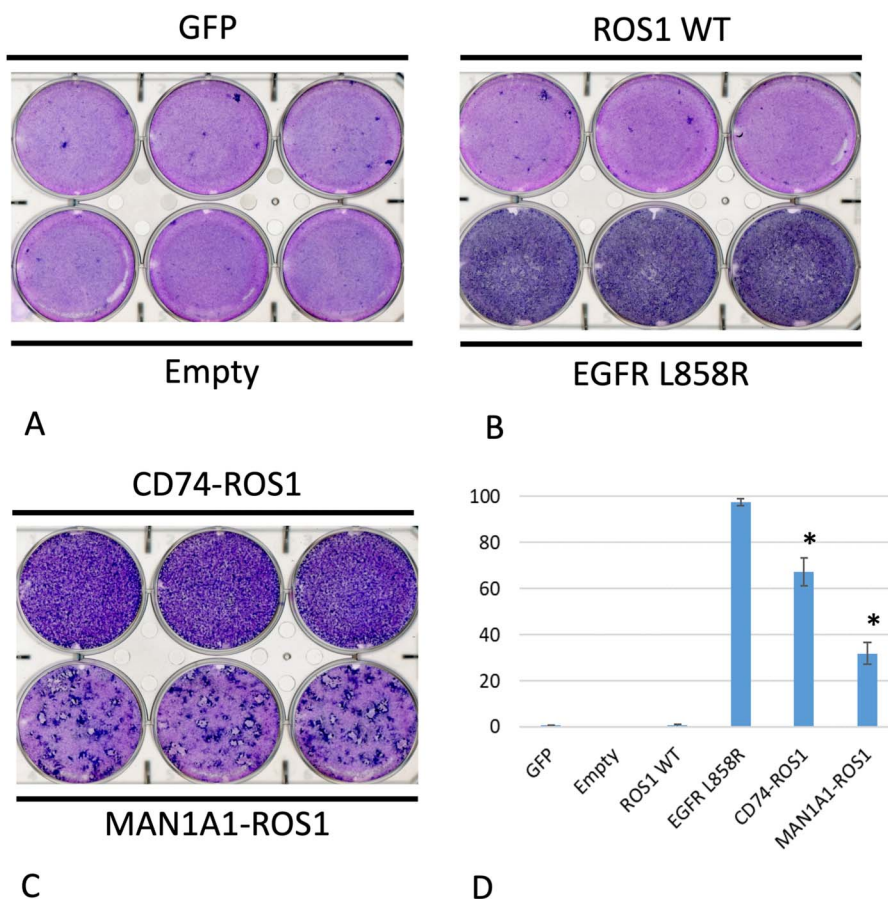## Will Potential Fusion Genes Transform Cells in In Vitro Assays?

In the focus formation assay, plate A shows the top row represent GFP (control) and the bottom row represent Empty (control) (Fig. 4A). Plate B shows the top row represent *ROS1* wild-type (control) and the bottom row represent *EGFR L858R* (positive control) (Fig. 4B). Plate C shows the top row represent *CD74-ROS1* (positive control) and the bottom row represent *MAN1A1-ROS1* (Fig. 4C). Accumulated foci were observed in the *CD74-ROS1* and *MAN1A1-ROS1* fusion-transfected cells, indicating that a strong transforming potential was associated with these rearrangements (Fig. 4D) compared with the negative control. With respect to the effectivities of the ROS1 inhibitor in vitro, in the *ROS1*-transfected cells, including *MAN1A1-ROS1*, *CD74-ROS1*, and the controls, crizotinib treatment inhibited cell viabilities in *ROS1* fusion transfected cells, including *MAN1A1-ROS1* and *CD74-ROS1* fusions (Supplemental Fig. 4; Supplemental Digital Content 5, http://links.lww.com/CORR/A469). These results demonstrated that crizotinib exerted anticell activities in *ROS1* fusion cells, including *MAN1A1-ROS1* and *CD74-ROS1* fusions in vitro.

Will Tumors in Mice That Have an Identified Fusion Gene Respond to Treatment with a Therapeutic Drug Directed Against That Target?

To detect whether tumor presence of this fusion gene would respond to a drug treatment with crizotinib, we

Fig. 3 A-J (**A**) Identification of *MAN1A1-ROS1* fusion. Real-time polymerase chain reaction showed three bands in the LMS13 tumor sample. An upper band revealed *MAN1A1* exon 5 to *ROS1* exon 31 as in-frame. A middle band revealed *MAN1A1* exon 5 to *ROS1* exon 32 as frameshift. A lower band revealed *MAN1A1* exon 5 to *ROS1* exon 33 as frameshift. We repeated gels of the same experiment four times (No.1 - 4). (**B**) Sanger sequencing using the cDNA product showed in-frame fusion of *MAN1A1* exon 5 to *ROS1* exon 31 in the tumor sample. We repeated gels of the same experiment four times (No.1 - 4). (**C**) This image shows a soft tissue mass in the left thigh of a 90-year-old woman. T2-weighted MRI showed a 5.0-cm mass in the abductor muscles. (**D**) After 2 years, a metastasis developed. T2-weighted MRI showed an 8.5-cm mass in the left retroperitoneum. (**E**) The histologic diagnosis was leiomyosarcoma (the low power microscope of HE). (**F**) A high-power microscope of HE showed several pleomorphic tumor cells. (**G**) Immunohistochemical staining was performed using h-Caldesmon, and the immunostaining test result was diffuse positive. (**H**) Immunohistochemical staining was performed using M-actin, and the immunostaining test showed positive. (**I**) Immunohistochemical staining was performed using smooth muscle actin, and the result was positive. (**J**) Immunohistochemical staining was performed using desmin, and tumor cells stained as positive.

Focus formation assay (Day 14)

Fig. 4 A-D (**A**) This image shows a functional assay of *ROS1* variants. An NIH 3T3 focus formation assay was performed in triplicate. A *MAN1A1-ROS1* mutant was transduced into a 3T3 mouse fibroblast cell line to investigate its transforming potential. Plate A shows the top row represent GFP (control) and the bottom row represent Empty (control). (**B**) Plate B shows the top row represent *ROS1* wild-type (control) and the bottom row represent *EGFR L858R* (positive control). (**C**) Plate C shows the top row represent *CD74-ROS1* (positive control) and the bottom row represent *MAN1A1-ROS1*. Focus formation after culturing for 14 days in 5% bovine serum albumin was evaluated in 3T3 cells transduced with *MAN1A1-ROS1* or control genes (*GFP, EGFR L8585R, ROS1* wild-type, or *CD74-ROS1*). (**D**) The focus number was quantified using ImageJ software (NIH, Bethesda, MD, USA) and normalized to that of the negative control. *p < 0.01 versus ROS1 WT, t-test. These assays showed the *MAN1A1-ROS1* had the highest focus number.

conducted in vivo assays, which revealed no differences in tumor growth between *ROS1* fusion genes (vehicle: *CD74-ROS1* and *MAN1A1-ROS1*) and control (*ROS1* wild-type) (Fig. 5). There was a difference in tumor growth between mice with crizotinib treatment and those with the vehicle in *ROS1* fusion genes (*CD74-ROS1* mean ± SD: $308 ± 178$ mm^3 versus $1276 ± 533$ mm^3; 253 mm^3 < the mean difference with 95% CI < 1809 mm^3; p < 0.01 and *MAN1A1-ROS1* mean ± SD: $123 ± 53$ mm^3 versus $2304 ± 854$ mm^3; 1156 mm^3 < the mean difference with 95% CI < 3206 mm^3; p < 0.01) (Fig. 5). These results indicate

that the novel *MAN1A1-ROS1* fusion gene had oncogenic abilities and crizotinib exhibited anti-tumor activity in the *MAN1A1-ROS1* fusion cells.

Discussion

STSs, including leiomyosarcoma, are malignant tumors that have poor prognoses, and novel systemic therapies are being searched for to improve patient survival. Recently, tyrosine kinase fusion genes, including *ALK, ROS1, RET,* and *NTRK,*

ROS1 fusion tumor size
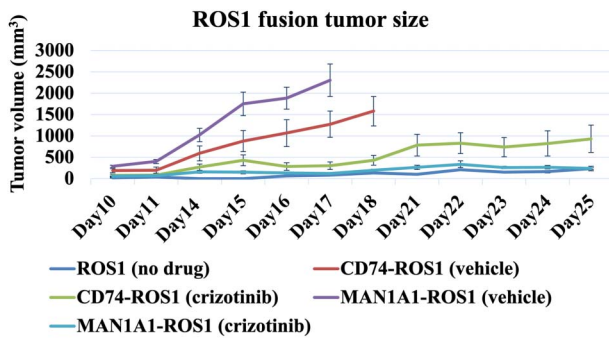
Fig. 5 Formation and crizotinib treatment in vivo. 3T3 cells with *MAN1A1-ROS1*, *CD74-ROS1*, or *ROS1* wild-type were subcutaneously injected into female BALB/c nude mice. The tumor growth with crizotinib or vehicle (dimethyl sulfoxide) treatment was evaluated. This graph shows tumor growth in size/volume versus time for the different groups (no crizotinib treatment: *MAN1A1-ROS1*, *CD74-ROS1*, or *ROS1* wild-type [control], crizotinib treatment: *MAN1A1-ROS1*, *CD74-ROS1*). With respect to comparisons including *MAN1A1-ROS1*, *CD74-ROS1*, or *ROS1* wild-type (control) with no crizotinib treatment, on day 17, the cells expressing *MAN1A1-ROS1* and *CD74-ROS1* had bigger tumor volumes than *ROS1* wild-type (control). Regarding crizotinib treatments, on day 17, the tumor volume of the cells expressing *MAN1A1-ROS1* that were treated with crizotinib were compared with corresponding cells treated with the vehicle. Error bars, SD; *p < 0.01.

have been found to be effective therapeutic targets for a variety of cancers [25, 28]. The comprehensive analysis of cancer-related genes that was innovated by Next Generation Sequencing technology made tyrosine kinase targets more accessible, having opened new avenues in the treatment modalities for various tumor types [27, 8, 14, 15]. However, the identified frequencies of tyrosine kinase targets in STSs, including leiomyosarcoma, are still low. DNA-based gene panel assays are the current main tests in clinical settings. However, there are technical issues; DNA-based gene panel assays are often inadequate for detecting tyrosine kinase fusion genes compared with RNA-based panel assays [2, 11, 12]. Therefore, in this study, we conducted a comprehensive transcriptome screening for all tyrosine kinases to identify new therapeutic targets in STSs. We successfully identified a novel *MAN1A1-ROS1* fusion gene in a leiomyosarcoma with mRNA screening, and *MAN1A1-ROS1* rearrangement revealed a strong transforming potential and was inhibited by crizotinib. These findings potentially provide a novel therapeutic option for this intractable malignancy in STSs.

Limitations

This study has several limitations. We performed tyrosine kinase screening using only 24 nontranslocation-related sarcomas, and in the 24 tumors we investigated, only one had a

"targetable" fusion product. To clarify the accuracy of the tyrosine kinase fusion rates in nontranslocation-related sarcomas, we must conduct screening using larger STS cohorts, but we view this study as a first step to encourage more investigations. As a second limitation, with respect to the malignant transformation of the *MAN1A1-ROS1* fusion gene, is that we performed a focus formation assay on *ROS1* fusions, including *CD74-ROS1* and *MAN1A1-ROS1*, and confirmed that the *MAN1A1-ROS1* fusion gene had strong transforming potential. We also confirmed that the strong transforming potential of *MAN1A1-ROS1* had the same power as that of the commonly mutated *ROS1* fusion gene (*CD74-ROS1*). Furthermore, in vivo assays successfully verified the malignant potential of *MAN1A1-ROS1* as tumor growths. Therefore, since the in vitro and in vivo assays have classified malignant potential with tumor growths, we did not conduct the other cell growth assays, including CCK-8 cell proliferation and transwell migration assays in this study. Additionally, several articles have described these assays using several partner types of *ROS1* fusion genes, including *CD74-ROS1* fusion, and confirmed their cell proliferation and invasion potentials [10, 21]. Therefore, we assumed that *MAN1A1-ROS1* fusion would have their proliferation and invasion potentials. As a third limitation, using in vivo assay, the tumor of *MAN1A-ROS1* fusion has not been verified for the differentiation of leiomyosarcomas using immunohistochemistry in this study. However, we confirmed that the tumor cells had *MAN1A1-ROS1* fusion (data not shown). As a fourth limitation, with respect to the false-positive detection of *NTRK1* fusion in the NanoString assay in this study, the 5'-side probe (*NTRK1*_Ex1/2 probe) was designed to detect the break point between exon 1 and exon 2 in *NTRK1* (Supplemental Table 1; Supplemental Digital Content 2, http://links.lww.com/CORR/A466), and our validation study with RNA sequencing revealed that exon 2 had fewer RNA reads than the other exons in *NTRK1* did (Supplemental Fig. 3; Supplemental Digital Content 4, http://links.lww.com/CORR/A468). Therefore, we supposed that the 5'-side probe might have detected a few RNA reads in exon 2 instead of the fusion gene.

Are There Novel Therapeutic Tyrosine Kinase Targets in Tumors from Patients with STSs That Are Detectable Using mRNA Screening?

We wanted to investigate whether the NanoString assay and a confirming RNA panel in a next-generation sequencing assay using RNAs may aid in identifying novel therapeutic gene fusions in STSs, especially nontranslocation-related sarcomas, and detect *MAN1A1-ROS1* fusion in a tumor from a patient with leiomyosarcoma. The NanoString nCounter assay is a single assay that can use RNA isolated from frozen and formalin-fixed paraffin-embedded samples [5]. It uses a

high-throughput hybridization technique using target-specific probes that can be customized to test for numerous fusion transcripts. A previous study on STSs using a NanoString assay targeting histologic fusion genes, including 174 fusion junctions in 25 sarcoma subtypes, successfully identified histologic fusion genes in 96 of 212 patients with STSs [4]. Furthermore, our previous assay for tyrosine kinase screening identified *KIF5B-RET* and *CAPG-ROS1* fusion genes in non-small cell lung cancers [28]. Based on these previous studies and our current findings, we suggest that mRNA screening might be able to detect novel therapeutic tyrosine kinase targets in STSs.

Can Potential Targets be Verified by RNA Sequencing and RT-PCR?

We confirmed this finding regarding a *ROS1* imbalance mRNA expression by an RNA panel in a next-generation sequencing assay. This sequencing identified novel fusion partners of *ROS1* rearrangements with *MAN1A1* in leiomyosarcoma. Additionally, we confirmed the in-frame fusion of *MAN1A1* exon 5 to *ROS1* exon 31 with independent RT-PCR. *ROS1* is a receptor tyrosine kinase and its fusion products have been observed in several types of cancers including glioblastoma, non-small cell lung cancer, cholangiocarcinoma, ovarian cancer, gastric adenocarcinoma, and colorectal cancer [10, 22, 28]. Fusion partners of *ROS1* rearrangements harboring *SLC34A2, CD74, TPM3, SDC4, FN1,* and *GOPC* have been described in detail [10, 22, 28]. *ROS1* fusion genes have been reported in STSs, including inflammatory myofibroblastic tumors [30], angiosarcomas [17, 23], epithelioid hemangioendotheliomas [10], perivascular epithelioid cell tumors [31], and synovial sarcomas [31]. With respect to sequencing, interestingly, it has been revealed that DNA-based large gene panel Next Generation Sequencing assays could not detect driver gene fusions or oncogenic isoforms perfectly compared with the alterations identified via RNA-based approaches [2, 11, 12]. Even though Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) is FDA cleared and is one of the best examples of DNA-based large gene panel Next Generation Sequencing assays [5, 30], some genes harboring *NTRK3* and *NRG1* are not included as certain important introns are too large (> 90 Kb each). Furthermore, it is extremely difficult for Next Generation Sequencing to read contained, highly repetitive elements due to their recurring presence across the genome [2, 11]. Based on these data, as current clinical cancer sequencing tests have mainly employed DNA-based large gene panel Next Generation Sequencing assays in the clinical setting, we believe that these (DNA-based) clinical sequencing tests might have been unable to identify this novel *ROS1* fusion that we identified.

Will Potential Fusion Genes Transform Cells in In Vitro Assays?

We next demonstrated the overexpression of *MAN1A1-ROS1* fusion by focus formation assay, which further substantiated our findings. This study additionally revealed that the novel *ROS1* fusion gene had good sensitivity toward the anti-ROS1 inhibitor crizotinib in vitro (Supplemental Fig. 4; Supplemental Digital Content 5, http://links.lww.com/CORR/A469). Therefore, the novel *ROS1* fusion gene had oncogenic potential. With respect to the fusion partner as *MAN1A1*, *MAN1A1* encodes Class I mammalian Golgi 1,2-mannosidase, a Type II transmembrane protein. N-glycan maturation involves Golgi mannosidase and plays a major role in cancer progression. In a previous report, ovarian cancers showed poor prognosis in the presence of high *MAN1A1* expression [19]. Recent studies showed that *MAN1A1* plays a critical role in tumorigenesis in breast and ovarian cancers [1, 20]. Although a low expression of *MAN1A1* was associated with poor prognosis in breast cancer, a high expression of *MAN1A1* conversely contributed to poor prognosis in ovarian cancer [1, 20]. Therefore, to elucidate its functions in various cancers, further studies are needed.

Will Tumors in Mice That Have an Identified Fusion Gene Respond to Treatment with a Therapeutic Drug Directed Against That Target?

Regarding in vivo analysis, we found that the novel *MAN1A1-ROS1* fusion gene had oncogenic abilities, and crizotinib exhibited antitumor activity in the *MAN1A1-ROS1* fusion cells. Crizotinib is a multiple tyrosine kinase inhibitor targeting *ALK* and *ROS1* [24]. The clinical response to crizotinib in *ROS1*-rearranged non-small cell lung cancers has been reported. The FDA recently approved crizotinib for the treatment of a few types of cancers with *ROS1* rearrangement, including non-small cell lung cancers [24]. Both RNA- and DNA-based multitarget assays could be critical in developing novel therapeutic strategies for detecting tyrosine kinase fusion genes. Additionally, NanoString, which could detect imbalances in the expression of mRNA between the 5' and 3' exons in tyrosine kinase genes, might support these detections. Several reports have demonstrated that tyrosine kinase inhibitors, including crizotinib, exert antitumor activities in STS [8, 13, 14, 15]. Therefore, tyrosine kinase fusion genes would be useful therapeutic targets for STSs.

Clinicopathogenic Futures and Our Patient Who Had a Tumor with *MAN1A1-ROS1* Fusion Gene

Leiomyosarcoma originates from the smooth muscles and frequently occurs in the uterus, retroperitoneum, and blood

vessels [13, 16]. Leiomyosarcoma is a high-grade sarcoma and may develop into a metastasis with resistance to standard cytotoxic chemotherapy [13, 16]. Recent large-scale studies using a multiplatform genome-wide dataset of patients with leiomyosarcoma revealed several gene alterations, including copy number loss involving *PTEN, RB1, CDH1,* and *TP53*; gains involving *MYOCD* and *IGF1R; PTEN* mutations; and alternative telomere lengthening in *ATRX, RBL2,* and *SP100* as frequent gene alterations [1, 3, 7]. The genomic landscape of leiomyosarcoma demonstrates that leiomyosarcoma has a genetically complex karyotype; no pathognomonic chromosomal rearrangements have been detected [3, 7]. Furthermore, there is a low incidence of actionable gene alterations and very few oncogenic kinase fusions through clinical sequencing in leiomyosarcoma [9, 13, 31]. To the best of our knowledge, *ROS1* rearrangements have not been identified in leiomyosarcoma, and this patient is the first instance of which we are aware of leiomyosarcoma with *ROS1* fusion gene. With respect to our patient, these tumors harboring novel *MAN1A1-ROS1:* fusion genes were diagnosed as pleomorphic leiomyosarcoma based on clinicopathological features. The primary lesion in the thigh was resected. This patient was followed with imaging studies and did not receive adjuvant chemotherapy because of her age. Two years postoperatively, a metastasis developed in the pelvis. Based on the patient's genetic profile, the *ROS1* inhibitor crizotinib was considered to be beneficial to the patient. However, because the patient was older than 90 years when the metastasis developed, we decided not to use the inhibitor against the *ROS1* fusion-positive tumor to avoid any adverse events. At 2.5 years postoperatively, the patient died.

Conclusion

We conducted tyrosine kinase screening using NanoString to identify novel therapeutic tyrosine kinase targets including tyrosine kinase fusions in STSs. Additionally, we identified novel *MAN1A1-ROS1* fusion in a patient with leiomyosarcoma and elucidated the malignant potential of the fusion gene via functional analyses. This NanoString-based screening system could be useful for detecting tyrosine kinase fusion genes in high-grade STSs and may provide novel therapeutic targets for treating advanced STSs.

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