Phosphoproteomic Profiling Reveals ALK and MET as Novel Actionable Targets across Synovial Sarcoma Subtypes

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Abstract

Despite intensive multimodal treatment of sarcomas, a heterogeneous group of malignant tumors arising from connective tissue, survival remains poor. Candidate-based targeted treatments have demonstrated limited clinical success, urging an unbiased and comprehensive analysis of oncogenic signaling networks to reveal therapeutic targets and personalized treatment strategies. Here we applied mass spectrometry-based phosphoproteomic profiling to the largest and most heterogeneous set of sarcoma cell lines characterized to date and identified novel tyrosine phosphorylation patterns, enhanced tyrosine kinases in specific subtypes, and potential driver kinases. ALK was identified as a novel driver in the Aska-SS synovial sarcoma (SS) cell line via expression of an ALK variant with a large extracellular domain deletion (ALKΔ22–17). Functional ALK dependency was confirmed in vitro and in vivo with selective inhibitors. Importantly, ALK immunopositivity was detected in 6 of 43 (14%) of SS patient specimens, one of which exhibited an ALK rearrangement. High PDGFRα phosphorylation also characterized SS cell lines, which was accompanied by enhanced MET activation in Yamato-SS cells. Although Yamato-SS cells were sensitive to crizotinib (ALK/MET-inhibitor) but not pazopanib (VEGFR/PDGFR-inhibitor) monotherapy in vitro, both drugs were individually effective, with pazopanib efficacy likely attributable to reduced angiogenesis. MET or PDGFRα expression was detected in 58% and 84% of SS patients, respectively, with coexpression in 56%. Consequently, our integrated approach has led to the identification of ALK and MET as promising therapeutic targets in SS. Cancer Res; 77(16); 4279–92. ©2017 AACR.

Introduction

Sarcomas are a heterogeneous group of malignant tumors arising in the bone or other connective tissue. Despite multimodal treatment options comprising intensive polychemotherapy, radiotherapy, and surgery, the survival of advanced sarcoma patients, with the exception of those exhibiting gastrointestinal stromal tumors (GIST), has not improved substantially during the last decade (1, 2), and side effects may affect quality of life. This emphasizes the need for novel, targeted systemic therapies. In recent years, the specific targeting of oncogenic signaling proteins with either small-molecule tyrosine kinase inhibitors (TKI) or antibodies has been the subject of numerous preclinical and clinical studies in sarcoma. Of these, only the multikinase VEGFR/PDGFR/KIT-inhibitor pazopanib showed an increase in progression-free survival (PFS) in a phase III study, leading to its

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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registration in advanced nonadipocytic soft-tissue sarcoma (STS) after failure of standard treatment (3), and a phase I–II study with the PDGFRα antibody olaratumab led to an impressive but not yet understood overall survival improvement (4). Possible explanations for the observed limited clinical efficacy are: inclusion of a wide variety of sarcoma subtypes per study without incorporation of predictive biomarkers (2, 5), and use of single inhibitors rather than combinations, as numerous studies demonstrate that tumors often rely on multiple signaling pathways to maintain growth and progression (1, 2, 6–8). Therefore, there is a critical need to define, at a global level, the activated signaling networks that drive sarcoma progression, determine how they relate to currently defined sarcoma subtypes and whether they can provide a novel taxonomy for the disease, and identify targeted therapies and companion biomarkers for implementation of personalized treatment approaches.

So far, genetic screening underpins diagnosis of different sarcoma subtypes, yet tunable driver mutations are rarely found in non-GIST sarcomas. While disease-specific translocations such as EWS-ETS in Ewing sarcomas (ES) and SS18-SSX in synovial sarcomas (SS) have been demonstrated (9), the exact cellular functions of these fusions and their potential relationship to other oncogenic events is not fully understood, and targeting the translocation or associated activated pathways has not yet been successful. Therefore, mutation screening to select non-GIST sarcoma patients for targeted therapy has not led to clinical benefit so far (1, 10, 11). Regarding protein biomarkers, most studies to date have focused on total protein expression levels, while specific screening for phosphorylation status or other read-outs of activated kinases. The results highlight potential strategies for future personalized sarcoma treatment.

Materials and Methods

Cell culture

ES and RMS cell lines were kindly provided by Peter Houghton, SS cell lines by Kazuyuki Itoh (Yamato-SS and Aska-SS), Akira Kawai (SYO-1) and Cinzia Lanzì (CME-1); AS cell lines by Mikio Masuzawa (MO-LAS-B and ISO-HAS-B) and the GIST cell line by Jonathan Fletcher (GIST882). All cell lines were obtained between 2010 and 2015, authenticated in our laboratory upon arrival and routinely tested for mycoplasma (all negative). All translocation-associated cell lines (ES, SS, and aRMS) were authenticated in our laboratory by confirming the presence of the characteristic EWS-FLI1 (ES), SS18-SSX1/SSX2 (SS), or PAX3-FKHR (aRMS) gene fusions as listed in Table 1 by RT-PCR. Fusion-negative eRMS cell lines were additionally tested for PAX3-FKHR gene fusions, which all proved to be fusion-negative, as expected. For the GIST882 cell line, mutation analysis confirmed its known KIT mutation (K642E; c.1924A>G=p.Lys642Glu). AS and eRMS cell lines were also tested by IHC for the presence of subtype-specific protein markers, which included expression of CD31, CD34, and ERG for AS cell lines, and expression of desmin and myogenin for eRMS cell lines. Authentication methods and results of all cell lines were reviewed by an expert sarcoma pathologist (UIE Flucke, Radboud UMC). Cells were cultured according to their recommended conditions and expression of desmin and myogenin for eRMS cell lines.

Phosphoproteomic profiling

Prior to profiling studies, cells were harvested and homogenized in 8 mol/L urea lysis buffer containing phosphatase inhibitors. Lysates were sonicated and centrifuged at 14,000 × g at 4 °C for 20 minutes. For each cell line, 20 mg of protein was subjected to phosphoproteomic analysis. First, phosphopeptides were immunoprecipitated (details: Supplementary Methods). For nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS) analysis, pY-peptides were resuspended in MS-loading buffer (0.1% formic acid and 2% ACN). Peptides were separated over a 30 minutes gradient and MS was performed using a Thermo Fisher Scientific Orbitrap Plus. Up to the 10 most abundant ions (>5,000 counts) with charge states >+2 were sequentially isolated and further subjected to MS/MS fragmentation. MS data were analyzed using MaxQuant software (version 1.5.2.8.). Extracted peak lists were searched against the human UniProtKB/Swiss-Prot database (version 2010_10), concatenated with a proportionally sized decoy database for false discovery rate (FDR) as well as the reversed sequences of all entries. Protein, peptide, and site FDRs were controlled at a maximum of 1%. Raw pY-peptide spectral intensities were extracted from the "Evidence" output files generated by Maxquant. These intensities were normalized against pY-peptide intensities for the heavy-labeled spiked-in peptide standards combined with GSK3β. The label-free intensity values of EFTU, MK14, and GSK3β heavy peptides in each cell line were averaged and a subsequent normalization factor generated. MS intensity values of each pY-site quantified in the dataset were divided by the appropriate normalization factor.

Pathway and network analysis

For pathway enrichment analysis, the KEGG Orthology Based Annotation System (KOBAS) was used (16). The hypergeometric test was applied to test statistical enrichment of identified KEGG and Reactome pathways, and the P values were corrected for multiple comparisons using the Benjamini and Hochberg method (17). Physical interactions among proteins of interest were retrieved from the Protein Interaction Network Analysis (PINA)
Table 1. Sarcoma cell line characteristics

<table>
<thead>
<tr>
<th>Age group</th>
<th>Type</th>
<th>Tissue type</th>
<th>Cell line</th>
<th>Translocation</th>
<th>Translocation details</th>
<th>Subtype</th>
<th>Prognosis</th>
<th>Age at diagnosis</th>
<th>Sex</th>
<th>Primary site/specimen</th>
<th>Metastatic site</th>
<th>Ref*</th>
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<tr>
<td>Pediatric/AYA</td>
<td>ES</td>
<td>Bone</td>
<td>ES1</td>
<td>EWS-FLI1</td>
<td>Exon 7/5; Type II</td>
<td>Type II</td>
<td>45</td>
<td>F</td>
<td>Left thigh/same</td>
<td>?</td>
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<td></td>
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<td>F</td>
<td>Ilium/bone marrow</td>
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<td></td>
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<td>EWS-FLI1</td>
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<td>18</td>
<td>M</td>
<td>Right 8th rib</td>
<td>Pleural cavity</td>
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<td></td>
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<td>EWS-FLI1</td>
<td>Exon 10/5</td>
<td>15</td>
<td>M</td>
<td>Right fibula lesion</td>
<td>Bone marrow, liver, femur</td>
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<td>ES8</td>
<td>EWS-FLI1</td>
<td>Exon 7/5; Type II</td>
<td>10</td>
<td>M</td>
<td>Left proximal Femur</td>
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<tr>
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<td>EW8</td>
<td>EWS-FLI1</td>
<td>Exon 7/6; Type I</td>
<td>17</td>
<td>M</td>
<td>Abdominal mass</td>
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<td>RMS</td>
<td>STS</td>
<td>RD</td>
<td>eRMS</td>
<td>Int.</td>
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<td>F</td>
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<td>Rh18</td>
<td>PAX3-FKHR</td>
<td>aRMS</td>
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<td>16</td>
<td>M</td>
<td>Soft tissue</td>
<td>Bone marrow</td>
<td>(2)*</td>
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<tr>
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<td>Rh30</td>
<td>PAX3-FKHR</td>
<td>aRMS</td>
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<td>12</td>
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<td>Rh41</td>
<td>PAX3-FKHR</td>
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<td>Rh5</td>
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<td>SS18-SSX1</td>
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<td>DOD</td>
<td>27</td>
<td>M</td>
<td>Groin</td>
<td>Lung</td>
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<td>SS18-SSX1</td>
<td>Biphasic</td>
<td>DOD</td>
<td>30</td>
<td>M</td>
<td>Thigh</td>
<td>Lung</td>
<td>(3)*</td>
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<tr>
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<td>SS18-SSX2</td>
<td>Biphasic</td>
<td>19</td>
<td>F</td>
<td>Groin</td>
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<tr>
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<td>CME-1</td>
<td>SS18-SSX2</td>
<td>Monophasic</td>
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<td>Thigh</td>
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<tr>
<td>Adult</td>
<td>GIST</td>
<td>STS</td>
<td>GIST882</td>
<td>Meta</td>
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<td>Metastatic; site unknown</td>
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<td>AS</td>
<td>STS</td>
<td>ISO-HAS-B</td>
<td>Hemangio-sarcoma</td>
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<td>M</td>
<td>Primary parietal-scapel tumor</td>
<td>Lng, liver, spleen, adrenal gland, vertebrae, rib, pleura, diaphragm, lymph node</td>
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<td>MO-LAS-B</td>
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<td>DOD</td>
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<td>M</td>
<td>Scalp and face</td>
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Abbreviations: AS, angiosarcoma; AYA, adolescent and young adult; DOD, dead of disease; ES, Ewing sarcoma; GIST, gastrointestinal stromal tumor; Int, intermediate; Meta, metastatic; RMS, rhabdomyosarcoma (a, alveolar; e, embryonal); SS, synovial sarcoma; STS, soft-tissue sarcoma; ?, unknown.

*Provided in supplementary references list.

Personal communication with Dr. Peter Houghton/Susan Ragsdale of the Pediatric Preclinical Testing Program (PPTP; Columbus, OH); St. Jude Children’s Hospital.
Proliferation assays

The effects of small-molecule inhibitors (TAE684, crizotinib, ceritinib, and pazopanib) on cell viability was assessed by MTT proliferation assays as described previously (details: Supplementary Table S1; refs. 21, 22).

Patient cohort

Tumor samples from 43 patients diagnosed with SS (all SS18-SSX translocation positive) between 1988 and 2015, were retrieved from the clinical records. The study was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands. Patient characteristics are listed in Supplementary Table S2.

FISH

FISH was performed on 2-μm-thick formalin-fixed paraffin embedded (FFPE) tissue microarray (TMA) sections from the patient cohort described above, and an Aska-SS cytosin using an ALK (2p23) split-signal FISH DNA probe (Dako) as described previously (21).

Mutation analysis

RT-PCR and sequence analysis of the RTK domain of ALK and MET was performed on SS cell lines as described previously (21). SS cells were additionally screened with the Cancer Hotspot Panel with a focus on potential PDGFREx and EGFR mutations (23).

Sequencing library preparation and capture

Sequencing libraries were prepared from 1 μg of Aska-SS RNA using the KAPA Stranded RNA-Seq Library Preparation Kit (Roche). Capture was performed using biotinylated probes complementary to the ALK coding regions as previously described (24) with addition of the double Capture process according to manufacturer's instructions. Captured libraries were sequenced for standard depth 126-bp paired-end sequencing using the Illumina HiSeq 2500 System v4 (Illumina).

Sequence analysis

 Sequencing reads were deduplicated with Tally v15-065 and adaptor sequences removed with Cutadap v1.8.1. Postfiltering, reads were mapped to human reference genome hg38 with STAR v2.4.2a and converted to UCSC genome browser tracks using the BEDTools v2.25.0 "genomewide" command. ALK exonic coverage was calculated from uniquely mapping reads using the BEDTools coverage tool (details: Supplementary Methods).

ALK RT-PCR

One micrograms of total RNA was reverse-transcribed to DNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was performed using the primer sequences listed in the Supplementary Methods. Products were gel-purified using the Gel and PCR Clean-Up System (Promega) and were sequenced using the PCR primers.

Western blot analysis

Western blot analysis was performed as previously described (ref. 25; antibody details: Supplementary Table S3).

PathScan analysis

PathScan signaling analysis was performed on Aska-SS cells treated with TAE684 (25 nmol/L), cerizotinib (100 nmol/L), or crizotinib (750 nmol/L) and on Yamato-SS cells treated with pazopanib (10 μmol/L) and/or crizotinib (750 nmol/L), all for 24 hours, using the human PathScan RTK Signaling Antibody Array Kit (Cell Signaling Technology) according to the manufacturer's protocol.

Deglycosylation

Cells were lysed in 2% SDS and the lysates adjusted to 0.5% SDS, 40 mmol/L DTT, and centrifuged at 12,000 × g for 10 minutes at room temperature. The supernatant was then heated at 96°C for 10 minutes and 10 μg of protein treated with O-Glycosidase, Endoglycosidase H, N-glycosidase F (all from NEB), following manufacturer's protocols. Tunicamycin was obtained from Sigma. Cells were incubated with tunicamycin (2.5–20 μg/mL) for 24 hours prior to preparation of cell lysates and Western blotting.

Combination indices

To assess drug synergy between crizotinib and pazopanib, the combination index (CI) method was used (22). Cells were treated with three designated doses of crizotinib (20, 50, and 100 nmol/L) and pazopanib (100, 1,000, and 10,000 nmol/L), and effects on cell viability were assessed by MTT assays. We next identified concentrations of each monotherapy necessary to obtain a similar reduction in cell viability as observed with combined treatments. Subsequently, CI for the combination treatments was calculated using the formula CI = [Ca,x/ICa,a] + [Cb,x/ICb,b]. Ca,x and Cb,x are the concentrations of drugs A and B used in combination to achieve % drug effect, ICa,a and ICb,b are concentrations for single agents to achieve the same effects. A CI < 1 indicates synergy of the combination therapy. A CI equal to or higher than 1 indicates additivity or antagonism, respectively.

Soft-agar colony-forming assays

Soft-agar colony forming assays were performed as previously described (25).

Mouse xenografts

Animal study protocols were performed in accordance with the guidelines of the Institutional Animal Welfare Committee of the Radboud University Nijmegen and Monash University. A total of 5 × 10^6–1 × 10^7 cells of Yamato-SS or Aska-SS cells were subcutaneously inoculated into 6–7-week-old female BALB/c mice (n = 6–9 per group). Drug administration was initiated when tumors were palpable. Crizotinib, ceritinib, and pazopanib were suspended in 0.5% hydroxypropylmethylcellulose (HPMC) + 0.5% Tween-80 to the necessary concentrations. For dose–response experiments, mice bearing Yamato-SS tumors were randomized into four groups receiving either crizotinib (0 mg/kg, 12.5 mg/kg, 25 mg/kg, or 50 mg/kg) once daily or pazopanib (0 mg/kg, 10 mg/kg, 30 mg/kg or 100 mg/kg) twice daily, both orally. Mice bearing Aska-SS tumors were treated with either...
vehicle or 50 mg/kg of crizotinib or ceritinib once daily. Tumor growth was measured twice weekly with calipers. After 28 days of treatment, all mice were sacrificed and the resected tumors were fixed in formalin.

IHC

IHC of MET, PDGFRα, and ALK on SS patient TMAs and CD34 and Ki67 staining on SS tumor xenografts was performed as previously described (refs. 21, 26; antibody details: Supplementary Table S3). Patient characteristics are listed in Supplementary Table S2.

Affymetrix analysis

ALK, MET, and PDGFRα mRNA transcript levels were extracted from a publicly available Affymetrix dataset (accession number GDS2736) of 105 clinical STS specimens (including 16 SS) to compare mRNA transcript levels between sarcoma subgroups.

Results

Phosphotyrosine profiling of human sarcoma cell lines

Our cell line panel comprised 20 different sarcoma cell lines, including adult (AS and GIST) and pediatric/AYA (ES, RMS, and SS) sarcomas (Table 1). Note that even though SS can occur at all ages, all cell lines were derived from young adult patients. Tyrosine phosphorylation patterns were characterized by an immuno-affinity-coupled LC/MS-MS workflow, and after application of stringent filter criteria (see Materials and Methods), we identified 1,090 tyrosine phosphorylated peptides corresponding to 654 proteins. Of these 1,090 peptides, 334 met criteria for inclusion in subsequent hierarchical clustering analyses (Materials and Methods; Supplementary Table S4). Upon unsupervised hierarchical clustering of the different sarcoma cell lines based on their tyrosine phosphopeptide profile, the cell lines clustered into two major groups, being the adult sarcomas (GIST and AS; designated Subgroup A) and the pediatric/AYA sarcomas (SS, RMS, and ES; Fig. 1A). As the large pediatric/AYA cluster split into the ES4 cell line and two smaller clusters, we have designated the latter two pediatric/AYA clusters Subgroups B and C, respectively (Fig. 1A). Note that in this subclassification, cell lines of particular sarcoma subtypes do not always cluster together. The majority of the ES cell lines are split between subgroups B and C. In addition, examples of RMS lines were also found in these two subgroups, and this subclassification was not explained by their aRMS (translocation-positive; Table 1) or eRMS (translocation-negative) status. However, the SS cell lines clustered together in subgroup B.

To identify signaling networks that exhibited differential activity across novel subgroups A–C, an ANOVA-based approach was applied to identify subgroup-enriched phosphosites, followed by bioinformatic determination of protein–protein interaction networks or pathways associated with these sites (27). Subgroup A was characterized by hyperphosphorylation of 48 phosphosites, with significant enrichment for the pathways associated with Fc gamma receptor (FcGR)-dependent phagocytosis, EPH-Ephrin signaling, adherens junctions, and cytoskeletal organization (Supplementary Table S5). Protein–protein interaction analysis for the proteins corresponding to these phosphosites revealed a network with the key hubs PTK2 (focal adhesion kinase; FAK) and ACTB (β-actin; Fig. 1B). Other kinases present within this network were FGR, MAPK3, TYRO3, and YES1. The only phosphosite significantly enriched in subgroup B versus the other two subgroups was PKP4 Y478, whereas subgroup C was characterized by significant hypophosphorylation of 48 sites compared with
subgroups A and B. The protein–protein interaction network corresponding to these hypophosphorylated sites exhibited major hubs associated with PI3K signaling (PIK3R1, PIK3R2), and this approach also emphasized the decreased phosphorylation of certain kinases (PDGFRα, EPHA2, and JAK2) in subgroup C versus the other subgroups (Supplementary Fig. S1).

Next, tyrosine kinases (TK) were extracted from the original dataset, as these represent an important class of oncogenes and druggable targets (14). This resulted in the identification of 132 phosphotyrosine peptides belonging to 41 TKs, including 26 receptor tyrosine kinases (RTK) and 15 nonreceptor TKs (Supplementary Table S4). Unsupervised hierarchical clustering based on the tyrosine kinase–derived phosphopeptides resulted in a more complex pattern than that generated using all phosphopeptides, but served to highlight ALK and KIT as highly-phosphorylated "outliers" in the Aska-SS and GIST882 cell lines, respectively, that may act as drivers (Fig. 1C). To facilitate comparison between different (R)TKs, the total tyrosine phosphorylation count was calculated per protein (see Materials and Methods; Fig. 2A). For RTKs, ALK, EPHA2, and KIT contributed the greatest proportion of phosphopeptide counts across the panel (although this reflects major contributions from individual cell lines for ALK and KIT, Fig. 2B), while for nonreceptor TKs, FAK, LYN, SRC, and YES made the largest contributions (Fig. 2A). Considering the phosphorylation patterns across the panel, the nonreceptor TKs SRC/LYN/YES, FAK1 and the RTK EPHA2 were phosphorylated at high levels in the majority of cell lines (Fig. 2B). Some tyrosine kinases exhibited enhanced phosphorylation in subsets of cell lines without a clear preference toward a specific sarcoma subtype (e.g., EPHB3 in ES7, RH18, RH36, and Aska-SS).

ALK represents a novel driver in SS

In addition to the marked ALK tyrosine phosphorylation in Aska-SS, our MS analyses detected low ALK tyrosine phosphorylation on specific sites in ES2, EW8, RH3 and RH5. However, only Aska-SS cells demonstrated detectable phosphorylation on the ALK activation loop residues Y1278, 1282, and 1283 (Supplementary Table S4; ref. 29). Western blotting detected expression of full-length ALK (220 kDa) in several sarcoma cell lines, including ES7, EW8, RH41, and RH3 (Supplementary Fig. S2), whereas Aska-SS expressed two predominant anti-ALK immunoreactive bands of approximately 110 and 80 kDa (Fig. 2C). Importantly, these aberrant ALK proteins in Aska-SS were detected using...
ALK-antibodies directed to pY1096, Y1507 and Y1282/1283, with highest phosphorylation levels in the 110 kDa variant (Fig. 2C). The latter observation particularly applied to activation loop phosphorylation. These data indicated that Aska-SS cells express truncated, activated ALK proteins, and suggested the 110-kDa variant represents the major driver. To determine the origin of these aberrant proteins, FISH analysis was undertaken, which identified heterozygous ALK breakage in Aska-SS cells (Fig. 3A).

Figure 3. Characterization of the novel ALK driver in Aska-SS cells. A, FISH analysis demonstrating ALK breakage in the Aska-SS cell line. The arrows highlight single red signals indicating the presence of a breakpoint, whereas the other signals comprise both green and red label indicating full-length ALK. Image is at ×650 magnification. B, Discontinuous expression of ALK coding exons in Aska-SS. RNAseq was undertaken following capture of transcripts corresponding to ALK-coding regions. Note low expression of exon 1, absent expression of exons 2–17, and then high expression from exon 18 onwards. The black and blue bars represent the mean expression levels for exons 1–17 and 18–29, respectively. C, Confirmation of exon 1-exon 18 splicing by DNA sequencing. The 317-bp product generated by RT-PCR was subjected to DNA sequencing. The nature of the products in C was also confirmed by this approach. E, Schematic representation of the truncated form of ALK in Aska-SS (ALK Δ2-17). The extracellular domain of full-length ALK comprises two MAM (meprin, A5 protein and receptor protein tyrosine phosphatase mu) domains and domains (aa 264–427 and 480–626), one low-density lipoprotein class A (LDLa) motif (aa 453–477), and a glycine-rich region (aa 816–940; ref. 50). Because exons 2–17 of ALK encode the aa 223–971 region, the deletion results in a truncated form of ALK (ALK Δ2-17) lacking these four domains. TM, transmembrane; PTK, protein tyrosine kinase.

However, treatment with N-glycosidase F, which removes almost all types of N-linked (Asn-linked) glycosylation, resulted in complete conversion to a 100-kDa form consistent with the predicted size for ALK Δ2–17. This also resulted in a shift of the 80-kDa ALK band to approximately 70 kDa. The 70-kDa form is consistent with use of initiation sites within exon 18, or alternatively this may represent a cleaved version of the larger variant. Treatment of cells with tunicamycin, an inhibitor of N-linked glycosylation, confirmed the impact of this modification on mobility of ALK Δ2–17 (Supplementary Fig. S4B). ALK mutation analysis in the other SS cell lines demonstrated a previously reported ALK exon 23 deletion in Yamato-SS cells. This is known to also occur in RMS cells, and has no effect on crizotinib sensitivity (20, 30, 31).

To determine the functional role of ALK in SS, the effect of a panel of ALK inhibitors including the clinically used compounds crizotinib (ALK/MET) and ceritinib (ALK), and the tool compound TAE684 (ALK), on cell proliferation was determined (Fig. 4A; Supplementary Table S6). Aska-SS cells were extremely sensitive to all three inhibitors, with IC50 values ranging between 26 and 46 nmol/L. The other three SS cell lines exhibited markedly lower sensitivity to TAE684 and ceritinib, reflecting their lack of ALK activation. Yamato-SS cells were also sensitive to crizotinib, likely explained by high levels of MET activation, consistent with their sensitivity to the MET/VEGFR2 inhibitor foretinib (Fig. 4A; Supplementary Fig. S5). Crizotinib IC50 values were substantially higher for SYO-1 and CME-1 cells, which exhibit undetectable or
very low levels of ALK/MET tyrosine phosphorylation (Fig. 2B). Western Blot and PathScan analysis determined that the ALK inhibitors decreased ALK phosphorylation in Aska-SS cells and in general, significantly reduced activation of Erk, Akt, S6RP, STAT3, and STAT1 (Fig. 4B and C). Prolonged treatment resulted in decreased expression of cyclin A, Rb hypophosphorylation and PARP cleavage, consistent with increased cell-cycle arrest in G1, and enhanced apoptosis (Supplementary Fig. S6). Importantly, treatment of mice bearing subcutaneous Aska-SS xenografts with either crizotinib or ceritinib demonstrated a remarkable dependency on ALK for tumor growth and maintenance, with drug treatment resulting in robust and durable tumor regressions.

Figure 4.
Validation of ALK as a potential actionable target in SS. A, Effect of ALK inhibitors on SS cell line proliferation in vitro. The graphs indicate dose–response curves of SS cell lines to crizotinib, ceritinib, and TAE-684 monotherapies. Error bars, SDs. B, Effect of crizotinib on key signaling pathways in the Aska-SS cell line. Cells were treated with the indicated concentrations of crizotinib for 1 hour. Cell lysates were prepared and Western blotted with the indicated antibodies. Data are representative of duplicate experiments. C, Pathscan signaling pathway analysis for Aska-SS cells treated with ALK inhibitors. Cells were treated with TAE684 (25 nmol/L), ceritinib (100 nmol/L), or crizotinib (750 nmol/L) for 24 hours. All signals were significantly different from control ($P < 0.05$), apart from the trends observed for ceritinib on pAkt Ser473 ($P = 0.0561$), TAE684 on pAkt Thr308 ($P = 0.0728$), TAE684 on pSTAT3 Tyr705 ($P = 0.0636$), and no significant difference for crizotinib on pSTAT1 Tyr701 ($P = 0.1563$). D and E, Effect of ALK inhibitors on Aska-SS xenograft growth. Mice were treated with crizotinib (50 mg/kg; D) or ceritinib (50 mg/kg; E). Values are presented as mean relative tumor volume ± SD. $P$ values were assessed by Student $t$ test. F, Expression of ALK in human SS. Examples of patient SS sections with positive (left) and negative (right) ALK expression, as determined by IHC. Images are ×200 magnification; insets are ×400 magnification. G, IHC staining of ALK in Aska-SS (positive control for F). Image is at ×200 magnification. H, FISH analysis demonstrating ALK breakage in a SS patient. The arrows highlight single colored signals indicating a breakpoint. Image is at ×100 magnification.
Histopathology analyses demonstrated that these regressions were associated with a significant decrease in the number and size of tumor blood vessels, as well as less proliferative cells (Supplementary Fig. S7). Overall these data demonstrate that ALK A2–17 activates multiple proliferative and survival pathways, resulting in ALK addiction both in vitro and in vivo.

To confirm the clinical relevance of these findings, we first interrogated a publicly available Affymetrix dataset of 105 clinical STS specimens (including 16 SS) and compared ALK mRNA transcript levels between sarcoma subgroups. This determined that ALK mRNA was significantly more highly expressed in SS compared with other STS, suggesting a unique and specific role for ALK in SS versus other STS subtypes (Supplementary Fig. S8). In addition, we assessed protein expression of ALK in 43 primary SS patient specimens. ALK immunopositivity was observed in 6 of 43 (14%) of the patients (Fig. 4F and C; Supplementary Fig. S9). No significant correlation was found between ALK expression levels and patient outcome. The ALK-expressing samples were also evaluated by FISH. Interestingly, one of the six ALK-positive patients showed ALK rearrangement in 23% of the cells (100 cells counted; Fig. 4H). This prompted us to evaluate an additional four metastatic lesions from the same patient. All lesions were positive for ALK expression. FISH analysis reported translocations in 10%–15.3% of cells in three of the lesions (50–150 cells counted per lesion, depending on lesion size). The fourth lesion showed ALK gain of up to 6 copies. These data validate our cell line–based in vitro and in vivo studies and highlight ALK as an important therapeutic target in a subset of SS.

Kinase activation profiles and rational design of combination therapies

Although the phosphoproteomic analysis identified driver kinases in Aska-SS and GIST882 cell lines, the other sarcoma cell lines often exhibited more than one TK with high levels of activation (Figs. 1A and 2B). One way to overcome the problem of network “robustness” conferred by multiple activated kinases is implementation of combination treatments or use of appropriate multikinase inhibitors (25). Again, we focused on the SS cell lines in vitro and in vivo. Overall these data demonstrate that pazopanib and crizotinib (Fig. 5G and H). Crizotinib-treated tumors were characterized by significant reductions in blood vessel size (mean CD34+ vessel surface for vehicle, 176.8 mm²/vessel vs. crizotinib 50 mg/kg, 129.3 mm²/vessel, \( P = 0.0261 \)) and tumor cell mitotic count (vehicle, 3.1 ± 0.9 vs. crizotinib 50 mg/kg, 0.8 ± 0.3, \( P = 0.0057 \)). Therefore, Yamato-SS cells exhibit a dependency on MET for tumor growth in vivo. In pazopanib-treated tumors, there were significantly fewer CD34+ blood vessels compared with controls (vehicle, 189.7/mm² vs. pazopanib 100 mg/kg, 122.2/mm²; \( P = 0.0073 \); Fig. 5I). This effect on angiogenesis, likely mediated by VEGFR inhibition, probably underpins the contrasting sensitivity of Yamato-SS cells to pazopanib treatment in vitro and in vivo. Overall, these data demonstrate that MET represents a potential therapeutic target in a subset of SS, indicate that pazopanib may also be of benefit for specific SS patients, and highlight the potential clinical utility of crizotinib/pazopanib combination treatment.

MET and PDGFRα expression in SS patients

To interrogate MET and PDGFRα expression in SS patients, we utilized publicly available Affymetrix gene expression data and also assayed our own cohort of clinical SS specimens by IHC. There was no significant difference for MET mRNA expression in SS compared with the other sarcoma histologies, but there was a trend for higher PDGFRα mRNA expression in SS (Supplementary Fig. S8). Immunopositivity for MET or PDGFRα was detected in 58% (25/43) and 84% (36/43) of SS patients, respectively (Fig. 6A and B). In 56% (24/43) of SS patients, the two proteins were coexpressed, but the highest scores for PDGFRα expression in SS patients, and the histologic biphasic SS subtype showed a remarkable differential MET/PDGFRα coexpression pattern, with MET and PDGFRα expressed mutually exclusively in distinct areas of the tumor resembling the epithelial and spindle cell components characteristic of this subtype (Fig. 6C). These data confirm that MET and PDGFRα represent potential therapeutic targets in SS, either alone or in combination.

Discussion

In this study, we utilized mass spectrometry–based phosphoproteomics to profile the largest and most heterogeneous set of sarcoma cell lines screened to date to identify perturbed signaling networks characteristic of existing or novel subtypes, and driver kinases that may represent candidate therapeutic targets. Importantly, this has identified several potential therapeutic strategies for specific subsets of SS patients, highlighting opportunities for personalized treatment of this sarcoma subtype.

Our study identified ALK as a novel driver in the Aska-SS cell line, with exceptionally high ALK tyrosine phosphorylation levels similar to those of the driver kinase KIT in the imatinib-sensitive GIST882 cell line (28). We determined that the ALK sensitivity in Aska-SS was underpinned by expression of a novel truncated ALK variant lacking a significant proportion of the extracellular

www.aacrjournals.org Cancer Res; 77(16) August 15, 2017 4287

Published OnlineFirst June 20, 2017; DOI: 10.1158/0008-5472.CAN-16-2550

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Other activated ALK variants with extracellular domain deletions have been described in neuroblastoma and melanoma, where the deletions encompass regions encoded by exons 2–3, and all of the extracellular domain, respectively (32, 33). Consequently our data add to growing evidence that extracellular domain deletions unleash the oncogenic potential of ALK, and represent a significant mechanism for ALK deregulation in human cancers, in addition to mutation, fusion and gene amplification/overexpression (29).

Although all SS tumors harbor SS18-SSX fusion genes, there is a wide variety in clinical behavior and response to chemotherapy or targeted treatment within these tumors, emphasizing the role of
additional factors in orchestrating SS progression (34). Our detection of ALK Δ2–17 in the Aska-SS cell line, and an ALK rearrangement in an ALK-immunopositive SS patient specimen, highlight ALK alterations as one such factor. Because the ALK aberration was detected in 1 of 4 SS cell lines and 1 of 43 SS patients, yet all of our SS samples and cell lines harbored the characteristic SS18-SSX translocation, ALK is likely not associated with the characteristic SS18-SSX fusion gene. In addition, as the Aska-SS cell line harbors SS18-SSX1, while the patient specimen contained SS18-SSX2, ALK aberrations are not associated with expression of a specific SS18-SSX fusion. As ALK activation in STS has been linked to the presence of metastatic lesions (35), Aska-SS is derived from a pulmonary metastatic lesion, and the ALK breakage–positive SS patient demonstrated breakage-positive metastases. ALK aberration in SS may be required for metastatic progression. However, it is clear from our comprehensive functional analyses in Aska-SS that ALK signaling is required for cell proliferation and survival in vitro, and tumor growth and maintenance in vivo. Consequently, once ALK activation occurs, the cancer cells can become dependent on this driver, despite the presence of a SS18-SSX fusion.

We acknowledge that ALK-targeted treatment will likely not be applicable to SS tumors in general, but the dependency of a subset of these tumors on ALK signaling offers great potential for personalized treatment within this sarcoma subtype. In this regard, ALK protein expression was detected in 14% of primary SS tumors and 1 of 6 of the ALK immunopositive-tumors exhibited ALK rearrangement, with the percentage of breakage-positive cells meeting diagnostic criteria for designation as ALK aberration-positive. The presence of a small subpopulation of SS patients exhibiting these characteristics is similar to non–small cell lung cancer (NSCLC), where ALK rearrangements are found in 2%–7% of cases (36). In the latter setting, oncogenic ALK fusions are an established companion biomarker for crizotinib sensitivity. In addition, IC_{50} values for crizotinib in Aska-SS cells were similar to those of ALK-rearranged lymphoma cells, where ALK is an established clinical target (37). Consequently our work highlights ALK as an oncogenic driver and highly promising therapeutic target in a subset of SS.

ALK has also been assessed as a therapeutic target in other sarcoma subtypes. A phase I dose-escalation study of crizotinib for pediatric cancer patients, including a variety of sarcoma subtypes, reported enriched antitumor activity in patients with inflammatory myofibroblastic tumors (IMT). Here, the impressive response rate in this subtype was attributed to the presence of activating ALK aberrations. No ALK aberrations or objective responses to crizotinib were reported in other sarcoma subtypes, although no SS patients were included (38). In addition to IMT, RMS have been subject to clinical evaluation of crizotinib efficacy based on reported ALK protein expression and gene amplification (NCT01548926, NCT02034981, and NCT01742286; ref. 31). However, no objective responses have been reported in RMS...
patients (39, 40). These data may reflect a failure to detect genomic ALK rearrangements in this subtype. Furthermore, while we could detect ALK expression in certain RMS cell lines, this was not accompanied by relevant levels of ALK phosphorylation, indicating that ALK protein overexpression in RMS may not be accompanied by receptor activation.

Three other RTKs demonstrated enhanced activation in SS cell lines, MET in Yamato-SS, and PDGFRα and EGFR in all SS lines. Importantly, Yamato-SS cells were sensitive to crizotinib in vitro and in vivo, and over half of primary SS tumors demonstrated MET expression, highlighting MET as a potential therapeutic target in this sarcoma subtype. In the case of PDGFRα and EGFR, treatment of SS cells with pazopanib, gefitinib, or erlotinib was without effect in vitro, indicating that these RTKs do not act as sole drivers. However, pazopanib exhibited efficacy against Yamato-SS xenografts, likely reflecting an additional effect on VEGFRs leading to reduced angiogenesis. The latter data are consistent with pazopanib monotherapy demonstrating clinical benefit in a subset of SS patients, with 49% of the patients having stable disease at 3 months (41). Also, in the extended phase III trial, there was a trend for SS patients to have superior responses on pazopanib, though this was not significant, possibly due to the relatively small size of this subgroup (3). Clearly, our data and recent clinical studies highlight the therapeutic potential for pazopanib in SS, but further work is required to identify biomarkers of therapeutic response that allow administration in a personalized fashion.

Two additional aspects of our study are worthy of comment. First, we determined that a high proportion of clinical SS specimens coexpress both MET and PDGFRα, consistent with the high expression and activation of both MET and PDGF receptors in SS cell lines. In addition, the combination of MET and PDGF receptor targeting. Consequently, our work highlights a potential combination therapy that could be used for SS tumors that coexpress MET and PDGFRα, either in the same tumor region, or in a biphasic fashion. In this context, it is worth noting that two phase I studies in other advanced cancers are running: NCT01468922, combining the MET-inhibitor ARQ197 with pazopanib; and NCT01548144, combining pazopanib with crizotinib. A preliminary case report of the latter study reported a therapeutic response and good tolerability of the combined treatment at the lowest doses (200 mg pazopanib with 250 mg crizotinib; ref. 44), emphasizing its clinical potential.

Beyond SS, the global and unbiased nature of our phosphoproteomic profiling approach enabled us to identify potential therapeutic targets for other sarcoma subtypes, including DDR2 in ES and FGFR4 in RMS. In addition, molecular subclassification based on tyrosine phosphorylation patterns led to a novel taxonomy, with the pediatric/AYA sarcomas clustering away from adult sarcomas and subdivided into two subgroups. In this subclassification, cell line models of the ES and RMS sarcoma subtypes do not cluster together in their designated ES and RMS histologic classifications, respectively, highlighting how knowledge of histologic subtype is insufficient for assigning targeted treatments, and further molecular interrogation is required to design more personalized approaches. To this end, detailed signaling network analyses identified hyper- and hypophosphorylation signatures characteristic of each of the novel subgroups, revealing that the hyperphosphorylation pattern in subgroup A (adult sarcomas) is built around a network with PTK2 (FAK) as a key component, potentially identifying FAK as a specific, and actionable vulnerability in these tumors. Importantly, a number of small-molecule FAK tyrosine kinase inhibitors are currently undergoing preclinical and clinical testing. In particular, PF-00562271, VS-4718 and VS-6063 demonstrated promising clinical activity in patients with selected solid cancers, emphasizing their potential utility for sarcoma treatment (45, 46).

Of note, the potential of phosphoproteomic screening of sarcomas in the clinic is underlined by recent studies: in a clinical (not further specified) sarcoma sample, phosphoproteomics was capable of detecting an ALK-rearrangement (47), as we did in the Aska-SS cell line, and this technique has also enabled patient stratification in RMS (48). For clinical samples, the use of reverse-phase protein arrays (RPPA) or NanoString technology may offer a practical approach for interrogating phosphorylation status when limited amounts of tissue are available (48). The coupling of phosphoproteomic approaches with other established techniques in clinical trial design and accompanying translational studies may therefore be of great value in design of personalized sarcoma treatments (49). The potential power of a phosphoproteomics approach over genomic analyses in SS is exemplified by the study of Ishibashi and colleagues, in which pALK expression was detected in SS cases without underlying genomic aberrations (35).

In conclusion, our study has provided detailed insights into the signaling network characteristics of particular sarcoma subtypes and identified potential therapeutic targets that have been validated using both in vitro and in vivo models and patient specimens. This work has identified protein and phosphoprotein markers that, following further validation, could be incorporated into pathologic characterization of sarcomas, leading to improved patient stratification for targeted treatment approaches.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments

We thank Peter Houghton of the Pediatric Preclinical Testing Program (PPTP, Columbus, OH) for generously providing the ES and RMS cell lines, and Mikio Masuzawa (Kisato University School of Allied Sciences, Sagamihara, Japan; MO-LAS-B and ISO-HAS-B) and Jonathan Fletcher (Harvard Medical School, Boston, MA; GIST882) for donating the AS and GIST cell lines, respectively. Akira Kawai (National Cancer Center Hospital, Tokyo, Japan; SYO-1), Kazuyuki Itoh and Norifumi Naka (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan; Yamato-SS and Aska-SS), and Cinzia Lanzi (Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; CME-1) for kindly providing the SS cell lines. Luiz Zhang, Emily Humphrey, Oded Kleifeld, Jim Su and Mark del Borgo provided excellent technical advice and support. We thank the Synovial Sarcoma Research Foundation, Dutch Cancer Society, Radboud AYA Foundation, the National Health and Medical Research Council of Australia and the Netherlands Organisation for Scientific Research (NWO, Rubicon) for their financial support.

Grant Support

E.D.G. Fleuren is supported with a travel grant from the Dutch Cancer Society (KWF) and a Rubicon Fellowship from the Netherlands Organisation for Scientific Research (NWO; 019.1531.W.035). R.J. Daly is supported by a Fellowship (1058540) from the National Health and Medical Research Council of Australia. Y.M.H. Versleijen-Jonkers and W.T.A. van der Graaf are supported by the Synovial Sarcoma Research Foundation and the Radboud AYA Foundation. J. Wu is supported by SRF for ROCS, State Education Ministry (SEM), and Young Talents Program, Beijing Municipal Administration of Hospitals.

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Received September 19, 2016; revised April 21, 2017; accepted June 6, 2017.
Published OnlineFirst June 20, 2017; DOI: 10.1158/0008-5472.CAN-16-2550

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