PDGF Receptor Alpha Is an Alternative Mediator of Rapamycin-Induced Akt Activation: Implications for Combination Targeted Therapy of Synovial Sarcoma

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Abstract
Akt activation by the IGF-1 receptor (IGF-1R) has been posited to be a mechanism of intrinsic resistance to mTORC1 inhibitors (rapalogues) for sarcomas. Here we show that rapamycin-induced phosphorylation of Akt can occur in an IGF-1R-independent manner. Analysis of synovial sarcoma cell lines showed that either IGF-1R or the PDGF receptor alpha (PDGFRα) can mediate intrinsic resistance to rapamycin. Repressing expression of PDGFRα or inhibiting its kinase activity in synovial sarcoma cells blocked rapamycin-induced phosphorylation of Akt and decreased tumor cell viability. Expression profiling of clinical tumor samples revealed that PDGFRα was the most highly expressed kinase gene among several sarcoma disease subtypes, suggesting that PDGFRα may be uniquely significant for synovial sarcomas. Tumor biopsy analyses from a synovial sarcoma patient treated with the mTORC1 inhibitor everolimus and PDGFRα inhibitor imatinib mesylate confirmed that this drug combination can impact both mTORC1 and Akt signals in vivo. Together, our findings define mechanistic variations in the intrinsic resistance of synovial sarcomas to rapamycin and suggest therapeutic strategies to address them. Cancer Res; 72(17); 4515-25. ©2012 AACR.

Introduction
Evidence for PI3K/Akt/mTOR phosphatidylinositol 3-phosphate kinase (PI3K)/protein kinase B (PKB)/mTOR (PI3K/Akt/mTOR) pathway activation in sarcomas has made this a pathway of interest for therapeutic development. Signaling through the pathway begins with ligand activation of receptor tyrosine kinases [RTK; such as the insulin-like growth factor 1 receptor (IGF-1R) and the platelet-derived growth factor receptors (PDGFRα and PDGFRβ)] at the plasma membrane, leading to the recruitment of PI3K directly to the receptor. PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3), which then recruits Akt to the membrane where it is activated by phosphorylation.

Mouse models with activated Akt have shown that oncogenesis through this pathway is dependent upon downstream activation of mTOR (1–3). mTOR regulates protein translation through rapamycin- and nutrient-sensitive mTOR complex 1 (mTORC1), which phosphorylates S6K 1/2 (ribosomal S6 kinase 1/2) and 4E-BP1 [eukaryotic initiation factor-4E (eIF-4E)-binding protein] to promote mRNA translation. mTOR also associates with a second rapamycin-resistant complex known as mTORC2. mTORC2 phosphorylates Akt at serine 473 (4, 5), which together with threonine 308 phosphorylation results in full Akt activation (6).

The PI3K/Akt/mTOR pathway is activated in sarcomas via several mechanisms, including genetic mutations within the pathway [e.g., KIT and PDGFRα mutations in gastrointestinal stromal tumors (GIST); PIK3CA mutations in myxoid/round-cell liposarcomas; ref. 7] or other pathognomonic alterations that promote reliance upon the pathway [e.g., EWS-FLI-1 gene fusion-driven oncogenesis is dependent upon IGF-1R in Ewing sarcoma (EWS; ref. 8)]. These observations led to clinical trials testing mTORC1 allosteric inhibitors in sarcoma patients. Rapamycin (sirolimus) was the first mTORC1 inhibitor identified; several rapamycin analogs (known as rapalogues) have since been developed. Mechanistically, rapalogues bound to FK506-binding protein 12 (FKBP12) destabilize the multimeric mTORC1 protein complex, resulting in inhibition of its activity. The overall clinical activity of these agents is modest, as no more than 5% of sarcoma patients on these trials had meaningful reductions in tumor size (9, 10).

A mechanism of intrinsic mTORC1 inhibitor resistance identified in several cancers, including sarcoma, is the induction of IGF-1R–dependent Akt activation due to a release of negative feedback inhibition (11–14). Biopsies from rapalogue-treated patients confirmed that Akt activation occurs clinically...
(11, 15) and in one study portended a poorer prognosis (15). The observation that combining rapalogues with IGF-1R inhibitors results in suppression of Akt activation and enhancement of drug-mediated antiproliferative effects in preclinical models (14) led to efforts to clinically develop this combination for sarcoma patients. However, combined rapamycin and IGF-1R inhibition may not be universally applicable to all sarcoma subtypes (16–18) and IGF-1R–independent mechanisms of rapamycin-induced Akt activation may also be important. To investigate this question, we examined the IGF-1R dependency of rapamycin-induced Akt activation in a sarcoma cell line panel using the IGF-1R–targeting antibody R1507 (Roche). R1507 (Roche) is a fully human monoclonal antibody (IgG1) that binds the extracellular domain of IGF-1R with high affinity, causing displacement of IGF-1 and IGF-2 from the receptor as well as downregulation of receptor levels. R1507 does not cross react with human or mouse insulin receptor. We discovered that rapamycin-induced Akt phosphorylation in sarcoma cell lines can be either IGF-1R dependent or IGF-1R independent. In synovial sarcomas, PDGFRA is an alternate RTK that can mediate this biologic process, and hence, is an attractive therapeutic target to inhibit in combination with mTORC1.

Materials and Methods

Chemicals and drugs

R1507, the humanized monoclonal anti-IGF-1R antibody, was provided by Roche. Rapamycin was purchased from EMD Chemicals. Imatinib was purchased from LC Laboratories. R1507 was stored in a buffered solution of 250 mmol/L trehalose, 20 mmol/L C-histidine, 0.1% Tween 20, and stored at −20°C. IGF-1, EGF, stem cell factor (SCF), PDGF, and cycloheximide were purchased from Sigma-Aldrich.

Cell lines

SYO-1 (19) and HS-SY-II (20) synovial sarcoma cell lines were provided by Dr. Marc Ladanyi (Memorial Sloan-Kettering Cancer Center, New York, NY). These cell lines were authenticated by confirming expression of the pathognomonic SYT-SSX fusion gene by reverse transcriptase PCR in March 2011. EWS (TC71, CHP100, A673) and desmoplastic round cell tumor (JN-DSRCT-1; ref. 21) cell lines were provided by Dr. Samuel Singer (Memorial Sloan-Kettering Cancer Center, New York, NY). Osteosarcoma SAOS-2 cells were obtained from the American Type Culture Collection.

Cell viability assays

Cell viability assays were carried out with the Dojindo Molecular Technologies Kit per manufacturer’s instructions. Briefly, 2,000 to 5,000 cells were plated in 96-well plates and then treated with the indicated drugs. Media was replaced with 100 μL of media with 10% serum and 10% CCK-8 solution (Dojindo Molecular Technologies Kit). After 1 hour, the optical density was read at 450 nm using a Spectra Max 340 PC (Molecular Devices Corp.) to determine viability. Background values from negative control wells without cells were subtracted for final sample quantification.

Western blots

Western blots were carried out as previously described (22). Briefly, cell lysates were prepared with radioimmunoprecipitation assay (RIPA) lysis buffer. Equal amounts of protein were electrophoresed on 4% to 12% gradient gels (Invitrogen) and transferred to 0.45-micron nitrocellulose membranes (Thermo Scientific). After blocking with 5% milk, membranes were probed with primary antibodies. Bound antibodies were detected with horseradish peroxidase secondary antibodies (GE Healthcare) and visualized by Enhanced chemiluminescence reagent (GE Healthcare). All primary antibodies were obtained from Cell Signaling.

RTK assays

RTK assays were conducted with the Proteome Profiler Array Kit (R&D) per manufacturer’s instructions.

Immunoprecipitation-Western blot analysis

Immunoprecipitation was carried out using 500 μg of total protein. The cell lysates were incubated with 1:50 dilutions of PDGFRA antibody or rabbit IgG control antibody overnight at 4°C. The following day 50 μL of protein A/G agarose beads were added for an overnight incubation at 4°C. Immune complexes were then washed with RIPA buffer 5 times and suspended in 25 μL of 4X loading buffer and analyzed by Western blot.

Gene silencing

Experiments with siRNA were conducted as previously described (22). Pooled siRNA constructs targeting PDGFRA (L-003162) and nontargeting, control siRNA (D-001810) were purchased from Dharmacon (ON-TARGET plus SMART pool). The SYT-SSX siRNA was previously published and validated (23); the sense and antisense sequences 5′-CCAGAU-CAUGCCCAAGAAGdTdT-3′ and 5′-UUCCUCUGGCCAUGAU-CUGdTdT-3′ target the breakpoint of both type 1 and type 2 fusions.

Quantitative real-time PCR

Real-time PCR was carried out as previously described (24). Gene-specific probe and primer sets from TaqMan gene expression assays (Applied Biosystems) were used to detect PDGFRA, SYT-SSX (23), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. The relative expression of PDGFRA was calculated by normalizing to GAPDH.

PDGFRA overexpression

The PDGFRA expression plasmid was provided by Dr. Marc Ladanyi (Memorial Sloan-Kettering Cancer Center, New York, NY). Cells were transiently transfected with empty vector and PDGFRA expression plasmids as previously described (25).

Protein stability experiments

Synovial sarcoma cells were transfected with control or SYT-SSX siRNA (Dharmacon) as described above. After transfection,
Expression microarray analysis
RNA was isolated from 139 sarcoma patient tumor samples and 17 cell lines or xenografts representing 5 different sarcoma histologic subtypes. RNA was hybridized to Affymetrix U133A arrays. Microarray data were normalized with the robust multiarray average method. These expression microarray data are previously published (26) and are freely available at: http://cbio.mskcc.org/Public/sarcoma_array_data/.

We identified 739 probe sets on a U133A chip corresponding to 432 genes with kinase domains based on searches of the Affymetrix annotation database, published data, and genome databases. This represented 83% of the 518 known protein kinase genes. To identify differentially expressed kinase genes, we used 2-tailed t tests with a restrictive Bonferroni correction method with a cut-off P value 0.01 for multiple comparisons. Differential expression of kinase genes in synovial sarcoma was defined relative to the other 4 groups as a whole.

Immunohistochemistry analysis of clinical cases
For 57 synovial sarcoma cases, immunohistochemistry (IHC) was carried out on 4-micron formalin-fixed paraffin-embedded slides using PDGFRA and IGF-1R antibodies (Ventana, prediluted) following standard protocols. The scoring for both antibodies was quantitated on a 0 to 2+ scale: 0 = no or faint/weak staining, 1+ = moderate staining, and 2+ = strong staining. Staining of 2+ was considered positive for PDGFRA. 1+ and 2+ were considered positive for IGF-1R. Photomicrographs were taken with our Leica DM5500 microscope and Openlab 5.0 imaging software.

Western blot of clinical tumor samples
Pretreatment (within 2 weeks of drug therapy) and on treatment (between weeks 2 and 3 of therapy) biopsies were carried out on a patient participating in a National Cancer Institute (NCI) study entitled, "A Phase 1b/2 Study of Imatinib in Combination with Everolimus in Synovial Sarcoma" (NCI# 8603). This protocol was reviewed and approved by the Memorial Sloan-Kettering Cancer Center Institutional Review Board and has been registered to www.ClinicalTrials.gov (Trial registration ID: NCT01281865). Informed consent was obtained to participate in the therapeutic study as well as the research biopsies. For Western blot analysis of frozen tumor samples, 250 µL of cold RIPA buffer was added to the sample grinding kit tube (GE Healthcare), followed by a flash frozen tumor sample (1-2 mm). The tumor was disintegrated by grinding using a pestle. The samples were centrifuged and the supernatant was transferred to a fresh tube. Protein concentrations were measured and analyzed by Western blot.

Statistical analysis
All in vitro experiments were carried out at least 2 to 3 times. SD was calculated where indicated.

Results
Evaluating combined mTORC1 and IGF-1R inhibition in a panel of sarcoma cell lines reveals both IGF-1R dependence and independence
The impact of mTORC1 and IGF-1R inhibition with rapamycin and R1507, respectively, was evaluated in a panel of 12 non-GIST soft-tissue and bone sarcoma cell lines. Histologies represented in the panel included synovial sarcoma (HS-SY-II, SYO-1), EWS (TC71, CHP100, A673), embryonal rhabdomyosarcoma (RD), dedifferentiated liposarcoma (LS141, DDLS), malignant peripheral nerve sheath tumor (MPNST, ST8814), osteosarcoma (SAOS-2), and desmoplastic small round cell tumor (JN-DSRCT-1). In all the cells, rapamycin inhibited S6 ribosomal protein (S6 RP) phosphorylation (at serines 235/236), consistent with mTORC1 pathway inhibition (Fig. 1).

Rapamycin also increased Akt phosphorylation at serine 473 in 11 of the 12 cell lines (JN-DSRCT-1 was the exception; Fig. 1). In all the cell lines with high IGF-1R protein, R1507 reduced IGF-1R protein levels, indicative of receptor internalization/degradation, a marker of antibody-mediated IGF-1R targeting (27). The impact of R1507-mediated IGF-1R inhibition was grouped into 2 categories. For 7 cell lines (designated Class I), R1507 partially or completely suppressed rapamycin-induced Akt phosphorylation (Fig. 1). In almost all these cells, R1507 alone also suppressed Akt phosphorylation, suggesting a degree of generalized IGF-1R dependence in Class I cells. In the 5 remaining cell lines (designated Class II), R1507 failed to suppress Akt phosphorylation alone or in combination with rapamycin (Fig. 1). The impact upon Akt phosphorylation correlated to tumor cell viability: the R1507 and rapamycin combination cooperatively suppressed sarcoma cell viability in only the Class I, but not Class II, cells (Supplementary Fig. S1).

We concluded that Akt activation in Class II cell lines was mediated through IGF-1R-independent mechanisms, rendering them resistant to strategies directed at IGF-1R inhibition, either alone or in combination with rapamycin.

Exogenous growth factors are necessary for rapamycin-induced Akt activation in both Class I and Class II synovial sarcoma cell lines
Although susceptibility to IGF-1R targeting in rhabdomyosarcoma cell lines has been directly correlated to receptor expression levels (28), IGF-1R independence in this panel was observed both in cells with detectable IGF-1R protein (SYO-1, JN-DSRCT-1) and those with marginal receptor expression (MPNST, ST8814, and DDLS; Fig. 1). In fact, IGF-1R expression in the R1507-resistant synovial sarcoma cell line SYO-1 was comparable with that in the Class I synovial sarcoma cell line HS-SY-II (Fig. 1), showing that alternate mechanism(s) of rapamycin-induced Akt phosphorylation may be relevant even in the context of intact IGF-1R expression. We next focused upon delineating the basis for this differential susceptibility to IGF-1R targeting observed in the synovial sarcoma cell lines.

To determine whether Akt activation in these cells is dependent upon growth factor signaling, serum-starved HS-SY-II and SYO-1 cells were treated with either vehicle or rapamycin in the presence or absence of serum (Fig. 2A). In both cell lines, rapamycin-induced Akt phosphorylation was observed only in...
the context of serum, establishing that the exogenous addition of one or more growth factor ligand(s) is required for both classes of cells. Consistent with our original classification, purified IGF-1 was sufficient to restore rapamycin-induced Akt phosphorylation in serum-starved HS-SY-II cells (Class I; Fig. 2A). IGF-1 addition to serum-starved SYO-1 cells only resulted in increased basal Akt phosphorylation, but not rapamycin-induced Akt phosphorylation. Hence, although SYO-1 cells possess a functional IGF-1R signaling apparatus that can be stimulated to transduce signals to Akt, this pathway is not activated in response to mTORC1 inhibition.

PDGFRA mediates rapamycin-induced Akt phosphorylation in SYO-1 cells

To identify the potential RTK(s) responsible for the induction of Akt phosphorylation in rapamycin-treated SYO-1 cells, the phosphorylation status of 42 RTKs in both HS-SY-II and SYO-1 cells were profiled using an RTK antibody array (Proteome Profiler; R&D Systems) in the presence of serum (Fig. 2B). A strong phosphorylated IGF-1R (and insulin receptor) signal was observed in HS-SY-II cells but not in SYO-1 cells, suggesting baseline IGF-1R activation in the Class I cell line but not the Class II. Conversely, a strong phosphorylated PDGFR alpha (PDGFRA) signal was present in SYO-1 cells, whereas only a modest one was detected in HS-SY-II (Fig. 2B). This correlated to higher total PDGFRA receptor levels in SYO-1 cells compared with HS-SY-II (Fig. 2C). No signal was detected for phosphorylated PDGFR beta (PDGFRB) in the RTK antibody array. Other RTKs activated at baseline included EGFR receptor in both cell lines and Erbb2 (HS-SY-II only).

To determine whether activation of a singular RTK pathway is sufficient to facilitate rapamycin-induced Akt phosphorylation, the impact of rapamycin upon Akt was tested in SYO-1 cells exposed in isolation to purified growth factor ligands that activate each of the RTKs identified in the antibody arrays (Fig. 2D). Each growth factor with the exception of the c-kit ligand (SCF) stimulated Akt phosphorylation, but only the addition of PDGF AB ligand recapitulated rapamycin-induced Akt phosphorylation observed with serum addition. Because commercial Western blot antibodies directed against specific phosphorylated sites on RTKs, including PDGFRA, were not sufficiently sensitive or specific for serum-exposed sarcoma cells, we evaluated PDGFRA phosphorylation status by immunoprecipitating the receptor and analyzing the immune complexes for the presence of phosphorylated tyrosines by Western blot. In the presence of serum, rapamycin increased PDGFRA tyrosine phosphorylation, consistent with receptor activation upon mTORC1 inhibition (Fig. 2E). RTK arrays carried out on vehicle- and rapamycin-treated cells in the presence of serum confirmed that none of the other assayed RTKs were activated in response to rapamycin (Supplementary Fig. S2).

To assess whether PDGFRA is required for rapamycin-induced Akt phosphorylation, pooled PDGFRA-targeting siRNA constructs (Dharmacon; ON-TARGET plus SMART pool) were transfected into SYO-1 and HS-SY-II cells to suppress receptor expression. Decreasing PDGFRA levels abrogated rapamycin-induced Akt phosphorylation in only SYO-1 cells, not HS-SY-II (Fig. 3A). We next used the small molecule inhibitor imatinib mesylate to assess the impact of pharmacologically inhibiting PDGFRA kinase activity. Imatinib alone and in combination with rapamycin inhibited PDGFRA phosphorylation in both SYO-1 and HS-SY-II cells, but suppressed rapamycin-induced Akt phosphorylation only in SYO-1 cells (Fig. 3B). Although imatinib can inhibit the activity of other
PDGFRA-related type III RTKs such as PDGFRB and c-kit/CD117, each of these receptors were not detectable by Western blot in either SYO-1 or HS-SY-II cells [Fig. 2C; or in the phosphorylated RTK array after rapamycin (Supplementary Fig. S2)]. Furthermore, imatinib inhibited Akt phosphorylation induced by rapamycin in serum-starved SYO-1 cells exposed to PDGF AB ligand in isolation, affording that imatinib can specifically modulate PDGF-mediated Akt phosphorylation (Fig. 3C). As was observed with R1507, the differential impact upon Akt phosphorylation correlated to cellular viability as the combination cooperatively suppressed tumor cell proliferation in only the SYO-1 cells, and not HS-SY-II, in the context of serum (Fig. 3D). Taken together, these data showed that in SYO-1 cells, PDGFRA induces Akt phosphorylation in response to mTORC1 inhibition, suggesting that targeting alternate RTKs other than IGF-1R may be critical for overcoming intrinsic sarcoma cell resistance to mTORC1 inhibition.

**High PDGFRA expression is a critical determinant of imatinib sensitivity and can be regulated by the synovial sarcoma gene fusion SYT-SSX**

Because SYO-1 cells express higher total PDGFRA protein levels than HS-SY-II cells (Fig. 2C), we hypothesized that the differential susceptibility to PDGFRA targeting in the synovial sarcoma cell lines may be related to this discrepancy in PDGFRA. To test whether higher PDGFRA expression could confer greater susceptibility to imatinib-mediated regulation of Akt phosphorylation, HS-SY-II cells were transfected with either a PDGFRA expression plasmid or empty vector and then subjected to drug treatment. Transfection of the PDGFRA expression plasmid resulted in 2.6-fold higher PDGFRA expression relative to empty vector—transfected cells (calculated by densitometry). High PDGFRA expression conferred a new susceptibility to imatinib-mediated inhibition of rapamycin-induced Akt phosphorylation (Fig. 4A), suggesting that the level of PDGFRA expression may be a critical determinant of RTK dependence.

We next investigated the potential mechanism by which PDGFRA is differentially expressed in SYO-1 and HS-SY-II cells. Essentially all synovial sarcomas possess the pathognomonic t(X;18)(p11;q11) translocation that produces the fusion gene SYT-SSX. Variability in the Xp11 breakpoint produces a fusion of the SYT gene with either the SSX1 or SSX2 (and rarely SSX4) genes. The ratio of SYT-SSX1 (type 1 fusion) to SYT-SSX2 (type 2 fusion) tumors is close to 2:1 (29, 30), and each fusion impacts tumor biology in clinically distinct ways (30).
The HS-SY-II cell line possesses the type 1 fusion (20), and the SYO-1 cell line the type 2 (19). To investigate how each fusion type may contribute to PDGFRA expression levels, cells were transfected with an siRNA duplex that can suppress the expression of both SYT-SSX fusion types by targeting the breakpoint region of the translocation (the design and validation of this siRNA construct has been previously published (ref. 23; see Materials and Methods for sequence). Whereas decreasing type 2 gene fusion expression failed to alter IGF-1R or PDGFRA expression in SYO-1 cells, decreasing type 1 fusion expression in HS-SY-II cells increased PDGFRA protein levels (Fig. 4B), implying that the type 1 fusion gene can negatively regulate PDGFRA expression. Both PDGFRA transcript levels and protein half-life were examined to better understand the mechanism by which reducing fusion gene expression could impact PDGFRA levels. Quantitative real-time PCR revealed only a modest increase in PDGFRA mRNA levels in HS-SY-II cells after suppression of SYT-SSX1 expression, although no change was detected in SYO-1 cells (Fig. 4C). Using cycloheximide to block mRNA translation revealed that at baseline, the PDGFRA protein half-life in SYO-1 cells (t1/2 > 2 hours) is more than 2-fold longer than in HS-SY-II cells (t1/2 < 0.5 hours; Fig. 4D).

Figure 3. PDGFRA is required for rapamycin-induced Akt phosphorylation in SYO-1 cells. A, Western blot analysis of SYO-1 and HS-SY-II cells treated with rapamycin after transfection of pooled siRNA constructs. Cells were transfected with either control (CTRL) or PDGFRA pooled siRNA constructs (Dharmacon; ON-TARGET plus SMART pool) for 48 hours and then treated with either vehicle or 1 nmol/L rapamycin for 6 hours. Cell lysates were created and analyzed by immunoblot. B, Immunoprecipitation Western blot and Western blot analysis of SYO-1 and HS-SY-II cells treated with rapamycin and imatinib alone or in combination. Cells were treated with vehicle, 1 nmol/L rapamycin, 1 μmol/L imatinib, or both for 6 hours. Immunoprecipitation Western blot analysis was carried out as described in Fig. 2E to assess PDGFRA tyrosine phosphorylation status (a nontargeting IgG antibody was used as a control for the immunoprecipitation); “input” blots indicate the total PDGFRA protein levels detected in the whole-cell lysates before immunoprecipitation. Western blot on whole-cell lysates was also used to assess the phosphorylation status of Akt and S6 RP. IP ab, immunoprecipitation antibody; W, Western blot antibody. C, Western blot analysis of SYO-1 cells treated with rapamycin and imatinib alone or in combination in the context of PDGF AB ligand alone. Cells were serum starved for 24 hours and then treated with drugs as described in B for 72 hours and viability assays were carried out. % Viability = (Viability(Drug treatment))/Viability(Vehicle) × 100. Results are the mean of 3 replicates. Error bars represent SDs.
PDGFRA Can Mediate Rapamycin Resistance in Synovial Sarcoma

Fig. 4D. siRNA-mediated downregulation of SYT-SSX1 expression in HS-SY-II cells increased PDGFRA protein stability with a longer half-life approximating that observed in SYO-1 cells (Fig. 4D). Alternatively, no changes in PDGFRA protein stability were observed in SYO-1 cells with SYT-SSX2 downregulation. These data suggest that the SYT-SSX1 gene product can downregulate PDGFRA expression in synovial sarcoma cells via multiple mechanisms.

**PDGFRA transcripts are highly overexpressed in synovial sarcomas relative to other sarcoma subtypes**

To assess the clinical relevance of PDGFRA for synovial sarcoma, Affymetrix U133 arrays were used to quantify the transcript levels of kinase genes from 5 different sarcoma subtypes derived from 139 primary patient tumor specimens and 17 cell lines and xenografts: synovial sarcoma (46 patient cases and 2 cell lines), desmoplastic round cell tumor (28 patient cases, 2 xenografts, and 1 cell line), alveolar rhabdomyosarcoma (23 patient cases), alveolar soft part sarcoma (14 patient cases and 2 xenografts), and EWS (28 cases and 10 cell lines). The 739 probe sets on the U133A chip corresponded to 432 genes with kinase domains, representing 83% of the 518 known protein kinase genes (Note: this microarray expression analysis was used in a previous publication as a comparator data set for a study of myxoid chondrosarcoma; ref. 26).

Among all the kinase genes evaluated in these 5 sarcoma subtypes, PDGFRA in synovial sarcomas was the most overexpressed: synovial sarcoma cases had 14.5-fold higher levels of PDGFRA than the other 4 tumor types (Fig. 5A), representing the highest disparity in gene expression discovered in this analysis. In 25 synovial tumor cases and the 2 cell lines, no PDGFRA kinase (exons 12–16) or transmembrane
exons 18–21) domain mutations were found. IHC conducted on another set of 57 synovial sarcomas showed that 70.2% (40 of 57) of the tumors expressed PDGFRA protein, whereas only 35.1% (20 of 57) were positive for IGF-1R (Fig. 5B). This synovial sarcoma tissue analysis establishes that wild-type PDGFRA is uniquely, highly expressed in synovial sarcomas.

Preliminary clinical evidence of inhibiting Akt phosphorylation with imatinib in combination with mTORC1 inhibition

We are currently conducting a NCI phase Ib/II clinical trial testing the combination of the mTORC1 inhibitor everolimus (a rapamycin analog or rapalogue) and imatinib in patients with PDGFRA-positive, recurrent, metastatic synovial sarcoma (NCI# 8603; trial registration ID: NCT01281865). Pretherapy (within 2 weeks of starting drugs) and ontherapy (between weeks 2 and 3 of therapy) tumor biopsies were carried out on a patient who experienced a reduction in the size of lung metastases with everolimus (5 mg daily) and imatinib (400 mg oral daily; Fig. 5C). Western blot analysis with protein normalized by both tubulin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels revealed a substantial loss of phosphorylated and total S6 RP and Akt with everolimus/imatinib therapy (Fig. 5C). IHC of these same tissues revealed a corresponding loss of tumor cellularity, decreased Ki-67 staining, and loss of phosphorylated S6 RP in the tumor cells (Fig. 5C). These data showed that combined S6 RP and Akt inhibition can be achieved clinically with rapamycin and imatinib in synovial sarcoma.

Discussion

The hypothesis that induction of IGF-1R–dependent Akt activation is a critical mechanism of intrinsic resistance to rapalogue therapy has led to the development of several early phase clinical trials evaluating combined mTORC1 and IGF-1R inhibition (31–34). Early results have suggested that this approach may have greater clinical efficacy than treatment with agents that inhibit mTORC1 or IGF-1R alone (33). Nonetheless, it is also apparent that IGF-1R inhibition will likely not
PDGFRA Can Mediate Rapamycin Resistance in Synovial Sarcoma

be effective for reversing intrinsic rapamycin resistance in all sarcomas. A recent phase 1 study reported that 10 of 17 sarcoma patients treated with mTORC1 and IGF-1R inhibitors in combination still experienced increased tumor growth (34). Recent drug screens in xenograft mouse models have also suggested that this combinatorial approach may have limitations (16–18). The R1507+/− rapamycin sarcoma cell line screen presented here confirms that IGF-1R inhibition will not be an universally effective approach for overcoming intrinsic rapalogue resistance.

The identification of IGF-1R dependence and independence in 2 synovial sarcoma cell lines provided the opportunity to study an alternate mechanism of intrinsic rapamycin resistance in tumor cells of a shared histology, driven by similar genetic alterations (the SYT-SSX fusion genes). Previous studies investigating IGF-1R targeting in synovial sarcoma cell lines with small molecule inhibitors produced conflicting conclusions about IGF-1R dependence. One study argued that the IGF-1R kinase inhibitor NVP-AEW541 can inhibit the proliferation of several synovial sarcoma cell lines (35), whereas another observed that these antiproliferative effects could only be achieved with micromolar NVP-AEW541 concentrations 10- to 50-fold higher than those required for IGF-1R inhibition (36), implicating off-target drug effects rather than IGF-1R dependence. The impact of IGF-1R inhibition upon Akt phosphorylation has only been explored in the narrow biologic context of serum-starved cells exposed to purified IGF ligands (35, 37), rather than in the context of serum with multiple ligand-stimulated RTK pathways. Taking a more selective approach to receptor targeting with the monoclonal antibody R1507 and conducting these experiments in the presence of serum, we evaluated the contribution of IGF-1R and alternate RTK pathways to the induction of Akt phosphorylation following rapamycin exposure.

In SYO-1 cells, the inability to abrogate rapamycin-induced Akt phosphorylation with IGF-1R inhibition was not due to a lack of receptor expression, as it was expressed at levels comparable with those in the IGF-1R-dependent cell line HS-SY-II. The phosphorylated RTK antibody arrays of serum-exposed SYO-1 cells revealed a lack of phosphorylated IGF-1R, implying the receptor is not activated at baseline despite intact expression. Instead, we found that high PDGFRA expression mediates Akt phosphorylation in response to rapamycin in SYO-1 cells. Data from our expression microarray analysis showed that PDGFRA is uniquely overexpressed in synovial sarcomas relative to other sarcoma subtypes (also indicated in a smaller study of 16 synovial sarcomas; ref. 38), corresponding to a higher rate of PDGFRA protein immunohistochemical positivity than IGF-1R in patient tissue samples (Fig. 5B). Notably, high expression of a PDGFR pathway component has been shown to be sufficient to drive oncogenesis in other sarcoma subtypes. The COL1A1-PDGFBR fusion gene in dermatofibrosarcoma protuberans results in PDGFB ligand overexpression, hyperactivation of the PDGFR pathway, and susceptibility to imatinib (39). Recent studies have reported that high levels of phosphorylated PDGFRA in rhabdomyosarcoma cell lines correlated to susceptibility to PDGFRA inhibition achieved with sunitinib (40). For synovial sarcoma, high PDGFRA is potentially sufficient for establishing it as the dominant RTK responsible for the induction of Akt phosphorylation in response to rapalogue treatment.

Importantly, the sarcoma microarray data presented here also revealed a degree of heterogeneity with regard to how highly abundant PDGFRA transcripts may be among synovial sarcomas (Fig. 5A). In the synovial sarcoma cell lines, differences in PDGFRA expression translated to differences in PDGFRA dependence for rapamycin-induced Akt activation: resistance to PDGFRA targeting was observed in the lower PDGFRA-expressing HS-SY-II cells, whereas susceptibility to receptor targeting was found in the higher PDGFRA-expressing SYO-1 cells. Furthermore, exogenous overexpression of PDGFRA in HS-SY-II cells conferred a new susceptibility to imatinib suppression of rapamycin-induced Akt phosphorylation (Fig. 4A). A recently published phosphoproteomic analysis of sarcoma cell lines confirmed that baseline RTK activation and dependence can vary in different sarcomas (41), suggesting that the specific RTK responsible for mediating intrinsic rapamycin resistance may vary depending upon sarcoma histologic subtype or the genetic context. This phenomenon has also been observed in other cancers, for instance in pleural mesothelioma (42). Although PDGFRA does not play a role in rapamycin-induced Akt phosphorylation for HS-SY-II cells, the receptor still may contribute to pathway signaling in other contexts. PDGFRA knockdown alone in HS-SY-II did result in decreased 86RP phosphorylation and increased Akt phosphorylation (Fig. 3A), whereas PDGFRA overexpression resulted in the opposite changes (Fig. 4A). These observations suggest that at baseline PDGFRA may regulate mTOR activity in these cells and manipulating receptor expression can trigger changes to feedback pathways leading to Akt.

What remains to be better defined is how RTK expression is differentially regulated among synovial sarcomas. We found that the specific SYT-SSX fusion type expressed by the tumor can differentially impact PDGFRA expression. The type 1 fusion in HS-SY-II cells negatively regulates PDGFRA expression, whereas the type 2 fusion in SYO-1 cells does not seem to regulate PDGFRA expression at all. The SYT-SSX gene fusions are generally thought to promote oncogenesis in synovial sarcomas by altering chromatin remodeling and dysregulating the expression of several target genes (43). The impact of the type 1 fusion upon PDGFRA expression does not seem to be limited to regulating PDGFRA transcript abundance, which was only modestly altered by fusion gene downregulation, but also extended to regulating PDGFRA protein half-life. Whether fusion type can reliably predict meaningful differences in PDGFRA expression in synovial sarcomas is not clear. Although a modest trend toward higher PDGFRA transcript levels in type 2 over type 1 fusions was observed in the microarray analysis (data not shown), without the ability to assess differences in PDGFRA posttranscriptional stability and/or reliably quantify PDGFRA protein levels in clinical samples, an adequate analysis of this question is not yet possible. In the ongoing phase II study evaluating the everolimus and imatinib combination in synovial sarcoma patients, clinical activity will be correlated back to fusion status to explore how fusion type may influence susceptibility to PDGFRA targeting.
As for the mechanism by which rapamycin activates PDGFRA, mTORC1 inhibition has been shown to increase PDGFRA, tr anslocation levels in hepatocellular tumor cells [44] and in mTOR activated TSC1/−/− or TSC2/−/− mouse cells [45]. In SYO-1 cells, rapamycin increased PDGFRA phosphorylation, but did not upregulate PDGFRA expression (Figs. 2E). Furthermore, the molecular events required for PDGFRA activation seem to be different from those critical for rapamycin-induced IGF-1R activation: although rapamycin resulted in decreased phosphorylation of insulin receptor substrate-1 (IRS-1, an IGF-1R adaptor protein; ref. 11) and decreased expression of growth factor receptor-bound protein 10 (Grb10, a negative regulator of IGF-1R; refs. 46, 47) in HS-SY-II cells, these IGF-1R activating changes were not observed in SYO-1 cells (Supplementary Fig. S3A). Differences in the baseline expression or response to rapamycin of these molecules also did not seem to correlate to classifications of IGF-1R dependency (Class I versus Class II; Supplementary Fig. S3B).

In summary, intrinsic resistance to rapalogue therapy mediated by the induction ofAkt activation can occur through different mechanisms. In synovial sarcoma, uniquely high levels of PDGFRA expression can translate to the usage of this RTK for rapalogue-induced Akt activation, providing a new disease-specific mode of resistance that may be exploited therapeutically.

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

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References


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