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An adaptive Src–PDGFRA–Raf axis in rhabdomyosarcoma

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ABSTRACT

Alveolar rhabdomyosarcoma (aRMS) is a very aggressive sarcoma of children and young adults. Our previous studies have shown that small molecule inhibition of Pdgfra is initially very effective in an aRMS mouse model. However, slowly evolving, acquired resistance to a narrow-spectrum kinase inhibitor (imatinib) was common. We identified Src family kinases (SFKs) to be potentiators of Pdgfra in murine aRMS primary cell cultures from mouse tumors with evolved resistance *in vivo* in comparison to untreated cultures. Treating the resistant primary cell cultures with a combination of Pdgfra and Src inhibitors had a strong additive effect on cell viability. In Pdgfra knockout tumors, however, the Src inhibitor had no effect on tumor cell viability. Sorafenib, whose targets include not only PDGFRA but also the Src downstream target Raf, was effective at inhibiting mouse and human tumor cell growth and halted progression of mouse aRMS tumors *in vivo*. These results suggest that an adaptive Src–Pdgfra–Raf–Mapk axis is relevant to PDGFRA inhibition in rhabdomyosarcoma.

1. Introduction

Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma in children [1]. This sarcoma of myogenic differentiation has two major subtypes, alveolar and embryonal. Alveolar rhabdomyosarcoma (aRMS) is frequently metastatic and even with the advent of intensified therapy, no significant improvement in outcome has occurred in nearly four decades [2,3]. In the majority of aRMS the chromosomal translocation t(2;13)(q35;q14) results in PAX3:FOX01A fusion gene [4]. This chimeric PAX3:FOX01A transcription factor has been shown to cause inappropriate activation of target genes including Pdgfra [5]. For this reason, molecularly-targeted therapies targeting Pdgfra are of great interest [5]. The conditional mouse model of aRMS expressing Pax3:Fox01A and having homozygous deleted *p53* is a useful tool our group has used for preclinical interrogation of PDGFRA as a molecular target [6].

Imatinib is a prototypic small molecule inhibitor of PDGFRA and PDGFRB [7]. Despite the tremendous success of this tyrosine kinase inhibitor therapy for CML and GIST, subsets of patients become resistant to imatinib [8,9]. Our previous studies firmly established PDGFRA as a prevalent target in aRMS, and to be functionally important by RNA interference *in vitro* and PDGFRA-specific antibodies *in vivo* [5]. Furthermore, we showed that imatinib causes tumor regression or halts the progression of tumors in our mouse model of aRMS through the inhibition of Pdgfra activity; however, nearly one-third of the mice slowly evolved resistance to imatinib therapy [5]. In the current study, we have investigated adaptive signaling mechanisms associated with narrow-spectrum PDGFRA inhibition in aRMS and explored the use of broader spectrum kinase inhibitors to overcome the molecular re-wiring and tumor progression encountered with imatinib in aRMS.

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2. Materials and methods

2.1. Western blotting

Immunoblotting was performed as described previously [10]. A summary and primary antibodies used is given in the Supplementary material.

2.2. Primary tumor cell cultures

Murine primary cell cultures were generated from fresh tumors as described previously [10].

2.3. Cell viability assays

Cell viability assays were performed as described previously [10]. A summary of the reagents (drugs and siRNAs) used is given in the Supplementary material.

2.4. Small molecule inhibitor panel

Two naïve (untreated) and two imatinib-resistant murine aRMS primary cell cultures from mice treated with 50 mg/kg/day imatinib were plated in 96-well plates at a seeding density of 4000 cells/well over graded concentrations of 66 small-molecule kinase inhibitors. A detailed summary of the drug screen is given in the Supplementary material.

2.5. In vivo studies

The mouse model for aRMS has been previously described [5,6]. Tumor-bearing mice were treated with sorafenib at the dose of 30 mg/kg/day by intraperitoneal injection for 14 days. Tumor dimensions were measured with digital calipers and volume was calculated by the formula $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. All the experiments were conducted in accordance with the institution-approved IACUC protocols. Conditional *Pdgfra* knockout mice [11] were bred to the established genetically-engineered mouse model of aRMS [5,6] to generate mice whose tumors were genetically ablated for *Pdgfra*.

3. Results

3.1. Narrow-spectrum PDGFRA inhibitor resistance is cell intrinsic

To investigate whether resistance to narrow-spectrum PDGFRA inhibitors is cell-autonomous, we treated both naïve (untreated) and mouse aRMS primary cell cultures from tumors slowly evolving imatinib resistance with varying concentrations of imatinib. Results of a 72 h cell viability assay showed the imatinib IC_{50} to be higher for primary cultures established from imatinib-resistant tumors (19 μM) than for the naïve primary cultures established from tumor bearing mice that had received no treatment (10 μM). Representative cell cultures are shown in Fig. S1A. Although imatinib effectively abrogated activation of Pdgfra in naïve (untreated) cells, imatinib did not alter activation (phosphorylation) of Pdgfra in resistant aRMS cultures (Fig. 1A).

3.2. Src is highly activated in resistant tumors

To functionally identify pathways associated with narrow-spectrum PDGFRA resistance, we screened a panel of 66 small molecule kinase inhibitors with imatinib-naïve and imatinib-resistant primary cell cultures. We identified Src family kinase (SFK) inhibitors (Table 1) as having increased activity towards the imatinib-resis-

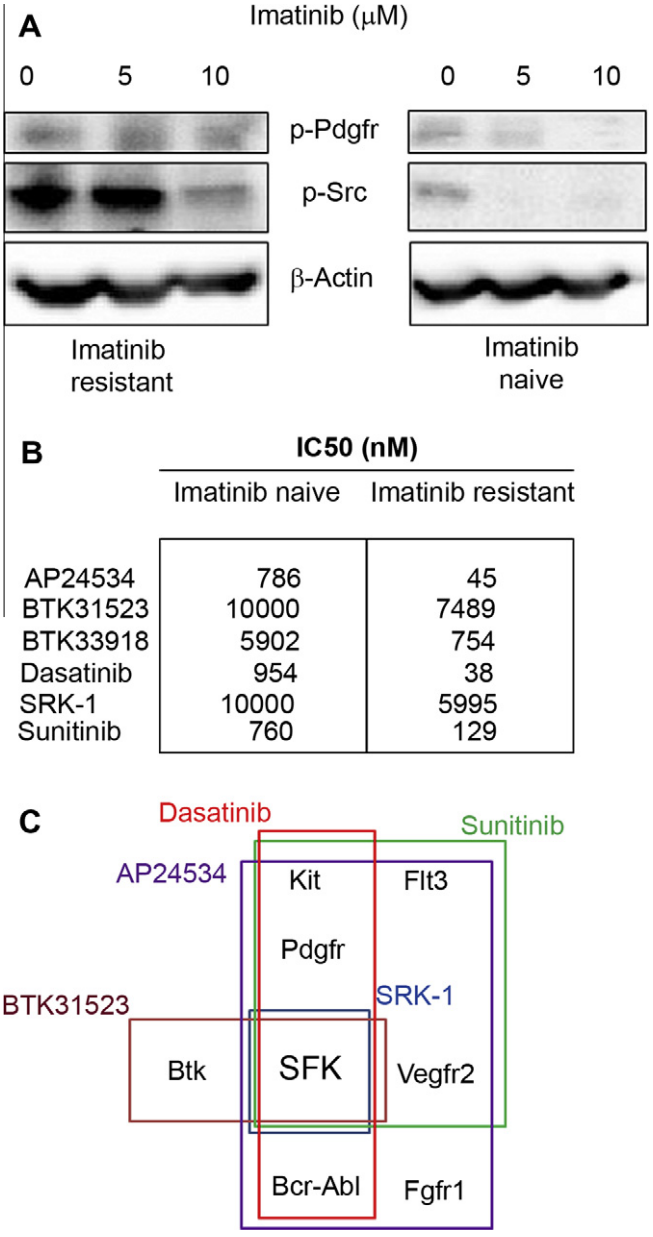

Fig. 1. Cell autonomous resistance to the prototypic PDGFRA inhibitor, imatinib. (A) Western blot analysis showing complete reduction in phospho-Src (p-Src) and phospho-Pdgfr (p-Pdgfr) levels upon imatinib treatment in imatinib-naïve primary cell cultures whereas phospho-Pdgfr and phospho-Src were still present at detectable levels upon imatinib treatment in imatinib-resistant primary cell cultures. (B) Src family kinase inhibitors that were found to be effective against imatinib-resistant primary cell cultures in a kinase inhibitor screen. (C) Venn diagram of specificities for drugs in (B) implicates Src family kinases (SFK).

Table 1
Inhibitory activity of select drugs from a screen on Src family kinases.

	C-Src	Lyn	Lck	Fyn	Hck
Src family kinases (IC_{50})					
AP24534	5.4 nM	0.24 nM	na	na	na
BTK31523	na	14 nM	97 nM	na	na
BTK33918	na	2.5 nM	20.5 nM	na	na
Dasatinib	0.21 nM	0.57 nM	0.2 nM	0.79 nM	0.35 nM
SRK-1	44 nM	na	88 nM	na	na
Sunitinib	2.1 μM	0.27 μM	0.23 μM	0.52 μM	0.88 μM

AP24534 [29]; BTK31523 and BTK33918 [30]; Dasatinib [7]; SRK-1 [31]; Sunitinib [29]; na, not available.