Co-activated PDGFRA and EGFR are potential therapeutic targets in intimal sarcoma

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Grant Support: This work is supported by research grants from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (G.0589.09, MD-R) and by a Concerted Action Grant 2006/14 from the K.U.Leuven. PV is senior clinical investigator of FWO.

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Running title: PDGFRA and EGFR as therapeutic targets in intimal sarcoma

Key words: intimal sarcoma, PDGFRA, EGFR, MDM2

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Abstract

Intimal sarcoma is a rare, malignant and aggressive tumor that shows a relentless course with a concomitant low survival rate, and for which no effective treatment is available. In this study, 21 cases of large arterial blood vessel intimal sarcomas were analyzed by immunohistochemistry and fluorescent in situ hybridization, and selectively by karyotyping, array-CGH, sequencing, phospho-kinase antibody arrays and Western immunoblotting, in search for novel diagnostic markers and potential molecular therapeutic targets. Ex vivo immunoassays were applied to test the sensitivity of intimal sarcoma primary tumor cells to the receptor tyrosine kinase (RTK) inhibitors imatinib and dasatinib. We demonstrated that amplification of the platelet-derived growth factor receptor alpha (PDGFRA) is a common finding in intimal sarcoma, which should be considered as a molecular hallmark of this entity. This amplification is consistently associated with PDGFRA activation. Furthermore, the tumors reveal persistent activation of the epidermal growth factor receptor (EGFR), concurrent to PDGFRA activation. Activated PDGFRA and EGFR frequently co-exist with amplification and overexpression of the MDM2 oncogene. Ex vivo immunoassays on primary intimal sarcoma cells from one case showed the potency of dasatinib to inhibit PDGFRA and downstream signaling pathways. Our findings provide a rationale for investigating therapies that target PDGFRA, EGFR or MDM2 in intimal sarcoma. Given the clonal heterogeneity of this tumor type and the potential crosstalk between PDGFRA and EGFR signaling pathways, targeting multiple RTKs and aberrant downstream effectors might be required to improve therapeutic outcome for patients with this disease.
Introduction

It is well established that platelet-derived growth factors (PDGFs) are involved in several pathological settings, including tumor growth \(^1\,^2\). PDGFs bind to two receptor tyrosine kinases (RTKs), platelet-derived growth factor receptor-alpha (PDGFRA) and -beta (PDGFRB), which activates the receptor kinases and initiates a number of signaling pathways, including RAS/ERK1/2, phosphoinositide-3-kinase (PI3K)/AKT, and protein kinase C (PRKC) pathways. The cellular responses to PDGF signaling involve proliferation, survival, migration and differentiation. In mice models, elevated PDGFRA activation leads to connective tissue hyperplasia, progressive chronic fibrosis in many organs, and to sarcomagenesis \(^3\). In humans, aberrant PDGFRs signaling has been associated with a number of malignancies \(^4\). In sarcomas, several types of genetic alterations, including gene amplification, translocations and activating mutations, result in ligand and/or receptor overexpression \(^5\). These aberrations may lead to disregulated activity of PDGFRA/B. The activating mutations of \(PDGFRA\) in gastrointestinal stromal tumors (GIST) and the overexpression of PDGFB due to a unique \(COL1A1-PDGFB\) fusion in dermatofibrosarcoma protuberans serve as examples \(^6\,^7\). Imatinib mesylate, the inhibitor of the tyrosine kinase activity of BCR-ABL, which also targets PDGFRA/B; proved to be very effective for the treatment of both conditions \(^8\,^9\). There are several reports on \(PDGFRA\) amplifications in solid tumors, such as glioblastomas \(^10\,^11\), anaplastic oligodendrogliomas \(^12\) and esophageal squamous cell carcinomas \(^13\).

Intimal sarcoma (IS) is a rare, lethal mesenchymal tumor, arising in the large arteries, mainly the pulmonary artery and the aorta. Presumably, these sarcomas...
develop from the subendothelial cells of the blood vessel wall located in the intima. The defining feature is the predominant intraluminal growth with obstruction of the lumen of the vessel of origin and embolic tumor dissemination. On histology, IS usually displays features of a poorly differentiated sarcoma, composed of spindle cells, with varying degrees of atypia, mitotic activity, nuclear polymorphisms and necrosis. Some tumors may show considerable heterogeneity in terms of extracellular matrix and tumor cell composition. Large myxoid regions or an epithelioid component can be observed, as well as areas of rhabdomyo-, angio- or osteosarcomatous differentiation. Angiosarcoma and leiomyosarcoma are the most important entities that have to be included in the differential diagnosis. The clinical presentation of IS is often not specific and related to tumor emboli. Pulmonary IS behaves very aggressively on site, conferring lung infiltrations in 40% of the patients. Aortic IS mostly arises in the abdominal aorta. Arterial embolic tumor dissemination might result in distant metastases involving bone, peritoneum, liver and mesenteric lymph nodes. The prognosis of IS is poor with a mean survival time of about 12 months.

To date, the understanding of the histogenesis and the molecular mechanisms leading to the development of IS remains fragmentary. In recent years recurrent molecular genetic events associated with IS development have been reported based on the analysis of few cases. By comparative genomic hybridization (CGH), recurrent amplification of the regions 4q12 and 12q13-q15 was described in a subset of IS. Amplification of the chromosomal region 12q13-q15 was reported in 75% of these tumors. Multiple genes in this region, such as the MDM2, SAS, CDK4 and GLI1, are
frequently amplified in many types of human cancer\textsuperscript{14,26}. Notably, amplification of the \textit{PDGFRA} gene, which maps to chromosome 4q12, was reported in five out of eight (62.5\%) pulmonary artery IS\textsuperscript{25}. In a more recent report, deregulated copy numbers of \textit{PDGFRA/PDGFRB, KIT} and \textit{EGFR} were described in seven examined IS cases, and the activation of the PDGFRs and EGFR has been disclosed by biochemical assays in one of those IS\textsuperscript{24}.

Due to the rareness of IS, these lesions have been a neglected area and there is a lack of specific biomarkers. The therapy is currently limited to surgery\textsuperscript{23}. In this study, we aimed to identify potential novel diagnostic markers and molecular targets for the treatment of IS. To this aim we have performed an integrated histopathological, cytogenetic, molecular and biochemical analysis of a large cohort of 21 cases with IS. In view of the clinical availability of several therapeutic agents against RTKs, we have focused on the relevance of activation of RTKs in the pathobiology of IS and on the inhibitory effect of RTK inhibitors on primary IS cells.

**Material and methods**

**Patients and histopathology**

The present study included 21 patients (11 females and 10 males; age range 27-74 years, median 52 years) (Table 1). The tumors were located in the pulmonary artery trunk (n=12), right or left pulmonary artery (n=3), heart (n=4), femoral artery (n=1), splenic artery (n=1) and one presented as the femoral embolus from the aorta primary site.
Histopathological examination was performed on formalin fixed, paraffin embedded (FFPE) tissue. Five µm sections were used for routine hematoxylin and eosin (H&E), and immunohistochemical stainings (avidin-biotin-peroxidase complex method), using the following monoclonal (mc) and polyclonal (pc) antibodies: alpha smooth muscle actin (α-SMA) (mc, 1/100; DAKO, Glostrup, Denmark), desmin (mc, 1/20; ICN, Aurora, OH, USA), CD31 (mc, 1/50; DAKO), CD34 (mc, 1/10, Becton Dickinson, San Jose, USA), MDM2 (mc, 1/100; Invitrogen, Life Technologies, Carlsbad, CA, USA), CD117 (pc, 1/250; DAKO), EGFR (EGFR PharmDx™ Kit, DAKO) and HER2 antibody (HercepTest™, DAKO). EGFR and HER2 protein expression was reported as membranous brown staining of neoplastic cells using a three-tier system ranging from 1+ (weak intensity) to 3+ (strong intensity).

**Cytogenetic analysis**

Karyotyping was performed on primary tumor cells from three cases, after seven days in culture.

**Array-CGH (aCGH) analysis**

Genomic DNA was isolated from eight frozen tumor tissues using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Basel, Switzerland). The Agilent Human Genome Microarray Kit 244A (Agilent Technologies, Santa Clara, CA) was used to perform aCGH analysis. Labeling, hybridization, washing and scanning were carried out in accordance with the protocols provided by the manufacturer. The Agilent Feature Extraction software (Version 10.5.1.1) was used to process the images obtained from...
the scanner (Agilent DNA microarray scanner, with up to 2 µm precision) and to generate a text file (FE file). Subsequently, the FE files were analyzed using the Agilent Genomic Workbench Standard Edition 5.0.14 software, based on the HG18 genome build. The ADM-2 algorithm was chosen for reporting copy number aberrations (CNA). Regions with a log₂ ratio > 2 were considered as amplified, regions with a log₂ ratio < -1 were considered as homozygously deleted. The data were submitted to GEO data base (accession # 15834535).

**Fluorescence In Situ Hybridization (FISH)**

FISH analysis was performed on all 21 cases as previously described. Pretreatment and enzyme digestion were done using the SPoT-Light Tissue Pretreatment Kit (Invitrogen). Chromosome 4 and PDGFRA copy numbers were determined by co-hybridization of SpectrumGreen(SG)-labeled centromer-specific probe CEP4 (Vysis Inc., Abbott Laboratories, Abbott Park, IL, USA) and SpectrumOrange(SO)-labeled, bacterial artificial chromosome (BAC) DNA probe RP11-231C18, which maps to PDGFRA (Research Genetics, Huntsville, AL, USA). For evaluation of the integrity of PDGFRA, BAC probes centromeric (RP11-3H20-SO) and telomeric (RP11-24O10-SG) to the PDGFRA locus (both from Research Genetics) were used. The co-amplification of PDGFRA and MDM2 or EGFR on a cellular level was determined by co-hybridization of PDGFRA/RP11-24O10-SO and MDM2/RP11-1064P9-SG or PDGFRA/RP11-24O10-SG and EGFR/RP11-231C18-SO labeled probes. The BAC DNA was isolated and labeled with fluorochrome dyes using standard techniques. Detection was performed as previously described. In addition, the MDM2, EGFR and HER2 gene copy numbers...
were investigated using double-color locus specific identifier (LSI) MDM2-SpectrumOrange(SO)/CEP12-SG (Applied Biosystems/Ambion, Life Technologies, Carlsbad, CA, USA), LSI EGFR-SO/CEP7-SG and LSI HER-2-SO/CEP17-SG probes (both from Vysis), respectively, according to the manufacturer’s recommendations. Slides were counterstained with 0.1 μM 4,6-diamino-2-phenylindole in an antifade solution and viewed under a fluorescence microscope equipped with an ISIS digital image analysis system (MetaSystems, Altlussheim, Germany). Signal copy numbers were counted from 100 non-overlapping nuclei from at least three different areas and the percentage of affected nuclei was recorded. Chromosome polysomy was defined as > 4 gene signals per nucleus paralleled by similar increases in chromosome centromere signals in at least 10% of tumor cells. A gene/CEP ratio of > 2 in at least 10% of tumor cells was defined as specific gene amplification.

Mutation analysis

Mutational analysis was performed on genomic DNA extracted from frozen tumor tissues (n=6). The whole coding sequence of the PDGFRA gene, and the sequence coding for the juxtamembrane and/or kinase domains of PDGFRB and EGFR, were amplified. Specific primers for amplified fragments are listed in Suppl. Table 1. The PCR products were purified and bi-directionally sequenced using the ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystems).

Receptor tyrosine kinases (RTKs) activation profiling
The expression and activation of RTKs and their downstream signaling pathways were analyzed using the Proteome Profiler™ Array kits (ARY001 and ARY003, R&D Systems, Minneapolis, MN, USA), according to the manufacturers' protocol and using 500 μg of protein lysate per array. The images were captured and the level of RTK activation was densitometrically quantified with AIDA software (TBC Software Inc., Topeka, KS, USA). The intensity values of the probes and the local background of the probes were log₂ transformed in order to obtain a more symmetric distribution, and the difference between these two resulted in a log₂ transformed ratio (further referred to as log₂-intensity ratios). Subsequently, the mean of the log₂-intensity ratios for each kinase within an array was calculated. Probes with a log₂-intensity ratio larger than mean plus standard deviation were considered as significantly activated.

**Western immunoblotting**

Tumor specimens (n=7) were snap-frozen at -80°C and used for Western immunoblotting. A primary GIST that harboured a PDGFRA-D842V mutation, and the human GIST882 and SQD9 squamous cell carcinoma cell lines were incorporated as controls. Cell lysis, SDS-PAGE, and immunoblotting were carried out as previously described. Membranes were immunoblotted sequentially using rabbit antibodies against phospho-EGFR(Tyr845) (R&D Systems), total EGFR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-PDGFRA(Tyr754) (Santa Cruz), total PDGFRA (Santa Cruz), phospho-ERK1/2 (Cell Signaling, Beverly, MA), total ERK1/2 (Invitrogen, Life Technologies), phospho-AKT (Cell Signaling), total AKT (Cell Signaling), and total actin (Sigma Aldrich, St. Louis, MO, USA), diluted in 5% blocking reagent. The
HRP-conjugated anti-rabbit IgGs were used to visualize with Enhanced Chemiluminescence (Thermo Scientific, Rockford, IL, USA).

Ex-vivo assay

Primary cells from a collagenase disaggregated tumor specimen from patient #1 were used for the cell cultures, as previously described. In short, cells were grown for three days in DMEM supplemented with 10% fetal bovine serum, MITO+ serum extender (according to manufactures’s recommendation) (BD Biosciences, San Jose, CA, USA), 30 µg/ml Bovine Pituitary Extract (BD Biosciences), 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. Cells were exposed to either imatinib, dasatinib or vehicle alone for 2 hours at +37°C. After a wash in ice-cold PBS, cells were lysed and used for western immunoblotting as described above.

Compounds

The inhibitors imatinib (Glivec/Gleevec, Novartis) and dasatinib (BMS-354825, Bristol-Meyers-Squibb), were provided by MedImmune Inc. (Gaithersburg, MD, USA). The inhibitors were diluted in culture medium directly prior use.

Results

Histopathology

On histology, the tumors showed a variable morphology, ranging from spindle cell proliferations with variable cellularity to islands of high anaplastic pleomorphic tumor
cells with atypical mitotic figures (Suppl. Figure 1). Low and high grade appearing areas were simultaneously present in all tumors. The matrix was usually collageneous, but on occasion very myxoid, and in one case osteoid production was observed. Immunohistochemistry revealed focal and weak expression of CD31, CD34 and desmin in 10-20% of cases (Table 1). About half of the tumors investigated were α-SMA positive. EGFR was expressed in all but one case, although the staining pattern was heterogeneous, from strongly diffuse (3+) (n=6) to intermediate (2+) (n= 7) or low and focal (1+) (n=7). MDM2 staining was positive in 52% of examined cases. CD117 (KIT) expression was rare, and occurred in only two tumors mainly as focal and weak staining.

Karyotype analysis

All three IS tumors featured a polyploid range of chromosome number and complex structural rearrangements, with the presence of homogenously staining regions within unidentified markers in two cases (#1 and #4) and double minutes in the third case (#19) (data not shown).

aCGH study

Using high-resolution oligonucleotide aCGH, we studied copy number aberrations (CNA) in eight cases for which frozen tissue was available. There was a median of 64 gains (range 36-83) and 42 losses (range 13-63) per tumor. CNA affecting six or more tumors (≥ 75% of cases) were identified on chromosomes 3q, 4q, 7p, 8q, 9p, 10q and 12q (Table 2, Figure 1). Major segments of high level amplification localized within chromosomes 1, 3, 4, 6, 8, 9, 12, 16, 21, 22 and X (Suppl. Table 2). Six out of eight
tumors showed chromosome 4q12 amplification, with the common region (54.78-55.21 Mb) containing only the PDGFRA gene (Figure 2). The region at 12q15, at 68.32–68.45 Mb, was amplified in 50% of the cases; the common region of amplification encompassed the BEST3 gene (the encoded protein is a Ca-activated Cl channel candidate in the development of vascular smooth muscle cells)\textsuperscript{32}. Three chromosomal regions were amplified in 37.5% of cases, i.e. the chromosomal regions 12q14.1 (KUB3), 12q15 (at 67.43-67.79 Mb; containing MDM2, SLC35E3 and CPM genes) and 22q11.23 (GSTT1). Four additional regions, i.e. 8p11.23-p11.22, 12q13.3-q14, 12q14.2-q14.3 and Xp21.1, were amplified in two cases each. Seventeen other amplified regions, which harbor known cancer-related genes, were observed in single cases.

Homozygous deletions were found on chromosomes 3, 5, 6, 7, 9, 10, 12, 13, 18, 20 and 22 (Suppl. Table 2). The homozygous deletions at 9p21.3-p21.2 (21.89-25.65 Mb), covering the CDKN2A/CDKN2B loci, were found in three out of eight tumors. In addition, loss of one copy of CDKN2A/CDKN2B loci was revealed in three additional cases. This finding indicates that loss of CDKN2A/CDKN2B, although not tumor-type specific, may be important for IS development and/or progression. The three chromosomal sub-bands 3p26.2 (ITPR1), 3p14.1 (FOXP1) and 18q22.3 (FBXO15) were homozygous lost in two cases each. Twelve other homozygous deletions were found in one case each, and some of those harbor known putative tumor suppressor genes.

FISH analysis
The eight tumors analyzed with 244A Agilent array and 13 additional paraffin embedded IS were evaluated by FISH (Table 1, Figure 3), using specific probes for PDGFRA, EGFR, MDM2, and HER2. The PDGFRA amplification was the most prevalent change, found in 17 out of 21 (81%) cases (Figure 3A). Notably, two tumors (#2 and #7) that showed focal PDGFRA amplification (from 12% to 18% affected cells) by FISH did not reveal this amplification by parallel aCGH analysis (Figure 2 and 3B). This finding indicates that the FISH technique is more suitable than aCGH for detecting the focal CNA in the context of cytogenetically heterogeneous and polyploid tumors. Three other IS showed high-level polysomy of PDGFRA, and only one case did not reveal any CNA of PDGFRA. Importantly, none of the examined cases demonstrated a rearrangement of PDGFRA.

The MDM2 gene was amplified in a large subset of tumors in our series (11/17 cases; 65%), and one case showed MDM2 gene polysomy.

In total, 16 (76%) tumors showed either EGFR amplification (n=2) or EGFR aneuploidy (n=14). Additionally, the status of the gene encoding HER2, a close family member and important dimerization partner of EGFR, was investigated. There was polysomy of HER2 in one case. By IHC, strong EGFR positive staining tended to correlate with EGFR gene amplification and aneuploidy, suggesting that the amplified EGFR is transcribed and translated to protein.

In the subsequent step of FISH analysis, we have explored the co-localization of PDGFRA, EGFR and MDM2 amplicons on a cellular level in three cases (1, #4 and
#19). Co-amplification of PDGFRA with MDM2 or EGFR genes was observed as partially overlapping large-clustered amplicons and/or as multiple, scattered signals (Figure 3C and 3D). Interestingly, sub-populations of tumor cells with mutual or exclusive amplification of PDGFRA and MDM2 or EGFR co-existed in each of the examined specimens.

**Mutation analysis**

No activating mutations were identified in PDGFRA/B and EGFR in the six IS cases (# 1, 2, 3, 7, 9 and 10) analyzed.

**RTKs phosphorylation profiling using phospho-RTK and phospho-kinase antibody arrays**

All seven analyzed tumors revealed strongly co-activated PDGFRA and EGFR kinases (Figure 4). Six cases concurrently showed phosphorylation of PDGFRB. Four cases concomitantly demonstrated low level phosphorylation of EPHB2, and three cases also had low level phosphorylation of AXL. Strikingly, there was no detectable activation of KIT or VEGF receptors, nor activation of the members of the SRC family proteins. By analyzing the signaling pathways (the profiles of 46 kinases and protein substrates), CREB, eNOS, TP53, JUN, STAT5B and AMPKalpha1 were the most frequently and strongest phosphorylated proteins (Suppl. Table 3, Figure 4).

The consistent protein expression and activation of PDGFRA and EGFR was confirmed by Western immunoblotting or immunohistochemistry (Suppl. Figure 2 and Table 1). By immunoblotting, both AKT and ERK1/2 downstream signaling pathways
were activated, the latter to a much stronger level, which is in line with PDGFRs/EGFR driven tumors\textsuperscript{31,33,34}.

\textit{Ex-vivo} biochemical assay using primary IS cells

We examined the consequences of PDGFRA inhibition with imatinib and dasatinib in primary IS cultures from case \# 1. Both inhibitors caused a dose responsive decrease in PDGFRA phosphorylation (Figure 5). Dasatinib completely blocked the phosphorylation of the target PDGFRA at 0.1 \(\mu\)M. In contrast, 0.5 \(\mu\)M imatinib only slightly affected PDGFRA activation, and 5.0 \(\mu\)M imatinib was needed to completely abrogate PDGFRA phosphorylation. Hence, dasatinib inhibited PDGFRA at much lower (clinically achievable) concentrations. Using p-AKT and p-ERK1/2 proteins as molecular surrogates of downstream RTKs signaling, we found that both AKT and ERK1/2 pathways were activated in untreated IS primary cells. Importantly, even though the PDGFRA activation was completely abolished at 5 \(\mu\)M imatinib, the downstream signaling pathways were still active. In contrast, complete inactivation of PDGFRA was associated with substantial inactivation of both ERK1/2 and AKT, at 0.5 \(\mu\)M dasatinib. This suggests that other dasatinib targets, next to PDGFRA, might be responsible for the inactivation of downstream ERK1/2 signaling by dasatinib.

\textbf{Discussion}
Using a variety of methodological approaches, we aimed for a comprehensive characterization of IS in search for relevant diagnostic biomarkers and therapeutic targets.

As expected, high-resolution 244A Agilent aCGH revealed complex CNA in all examined cases across the genome, including previously recognized oncogenes and tumor suppressor genes but also completely novel loci. Most importantly, high level amplifications or copy number gains frequently involved chromosomal regions that contain the \textit{PDGFRA}, \textit{EGFR} and/or \textit{MDM2} genes.

Accordingly, \textit{PDGFRA} amplification and high-level polysomy by FISH was present in 81% and 15% of tumors, respectively. Remarkably, using high-resolution array, we were able to delineate the common region of 4q12 amplification to 54.78-55.21 Mb, containing only the \textit{PDGFRA} locus. Thus, \textit{PDGFRA} is the most likely target for amplification in IS. Our findings are comparable to results reported previously on smaller IS cohorts\textsuperscript{24,25}. To the best of our knowledge, no other sarcoma reported so far presents consistent \textit{PDGFRA} amplification\textsuperscript{35}. Thus, given the specificity and high frequency of occurrence, the \textit{PDGFRA} amplification in IS might be considered as a molecular hallmark of the entity. Importantly, \textit{PDGFRA} amplification, detected by FISH, is a valuable marker for IS diagnosis, distinguishing them from the other undifferentiated sarcomas known to reside in similar anatomical sites.

The amplification of the 12q15 region, harboring \textit{MDM2} locus, was another consistent and frequent feature revealed by aCGH. Amplification and consequent up-
regulation of the *MDM2* oncogene is a recurrent molecular feature of many sarcomas, as exampled by dedifferentiated liposarcomas \(^{36}\), and it was also reported in IS \(^{14,24,25}\). The extended FISH analysis revealed 65% of tumors with *MDM2* amplification in our cohort. *MDM2* amplification was closely correlated with the protein expression of this oncogene, as evidenced by immunohistochemistry. Since the amplification or overexpression of MDM2 has been found in many cancers, inhibiting MDM2 and hereby reactivating TP53 in cancer cells is an emerging new therapeutic strategy. Nutlin-3 and MI-219 are MDM2 inhibitors with desirable pharmacological properties that recently moved to advanced preclinical development or early-phase clinical trials \(^{37}\). Interestingly, cancer cell lines with *MDM2* gene amplification were shown to be more sensitive to Nutlin-3 than cell lines lacking this amplification \(^{38}\).

The gain of chromosome 7p, encompassing the *EGFR* locus, was accounted in 6 out of 8 cases analyzed by aCGH in the present study. High level *EGFR* aneuploidy (or rarely amplification) was confirmed by FISH (76% of cases). EGFR belongs to the ERBB family of RTKs which recognizes at least eleven known ligands \(^{39,40}\). There is ample evidence of amplified and activated forms of EGFR in many human cancers, and drugs which specifically target EGFR show promise in inhibiting the growth of certain tumor types \(^{39}\). Sato *et al.* demonstrated that 168 of 281 (60%) adult soft tissue sarcomas overexpressed EGFR \(^{41}\), this overexpression significantly correlated with higher histological grade, poorer survival and chemoresistance. Noteworthy, gefitinib, an EGFR inhibitor, has shown potential antitumor effects in several sarcoma cell lines when used in combination with irinotecan \(^{42}\).
To define the compendium of co-activated RTKs in IS, we used an antibody array that allows simultaneous assessment of the phosphorylation status of 42 RTKs. At utmost importance and coherent with the crucial biological role for the IS pathogenesis, the PDGFRA and EGFR kinases were consistently activated in all seven investigated cases of our study. The PDGFRB kinase was activated in a smaller sub-group of tumors. The co-activation of multiple RTKs is not a distinctive feature of IS, because similar patterns were reported in other tumor types\textsuperscript{43-46}. Importantly, the concomitant activation of multiple RTKs serves to reduce dependence on a single RTK for the maintenance of critical downstream signaling, and thus renders such tumors refractory to single-agent RTK inhibition.

The recurrent high level amplification and strong activation of PDGFRA in absence of resistance mutations prompted us to investigate the effect of the PDGFRA inhibitors, imatinib and dasatinib, in IS. Both inhibitors were able to specifically inhibit the PDGFRA kinase activity, but dasatinib demonstrated higher potency. The stronger inhibitory capacities of dasatinib towards PDGFR in reference to imatinib were previously demonstrated in rat A10 vascular smooth muscle cells\textsuperscript{47}. Additionally, dasatinib showed a more effective inhibition of downstream ERK1/2 and AKT signaling than imatinib in primary IS tumor cells. It is likely that IS cells under PDGFRs targeted treatment are still able to maintain downstream signaling through the ERK1/2 and AKT pathways due to EGFR co-activation. By RTK array, the propagated ex vivo tumor cells lacked activation of SRC family members. Thus, the possibility that inhibition of SRC family kinases could contribute significantly to the inhibitory effect of dasatinib on the
PDGFRs downstream signaling is remote. Yet, the discrete SRC activation to levels not discernible by our technique could still exist in the tested cells, rendering them more prone to dasatinib inhibition. Interestingly, it was recently shown in lung cancer that dasatinib may reduce EGFR activation through the inhibition of SRC, which mediates the phosphorylation of the Y845 residue in the receptor.\textsuperscript{48-50}

It is essential to emphasize that the success of the therapy in IS might strongly depend on the genetic context of the tumor, which is complex, as indicated by the numerous CNA of cancer-related genes identified by aCGH. The \textit{CDKN2A/CDKN2B} losses were recurrent in IS in our cohort. This is in line with previous studies that have shown a synergistic effect on neoplasia induction when aberrant oncogenic PDGFRA signaling is combined with loss of the tumor suppressors \textit{CDKN2A/CDKN2B} \textsuperscript{3,47}. Moreover, the TP53 pathway seems to be frequently affected in IS, as exampled by common \textit{MDM2/CDK4} amplifications, pointing to confounding factors that might attenuate the response to RTK inhibition.

Secondly, an important and hitherto not reported finding in our study is the concomitant existence of cells with exclusive amplification \textit{versus} co-amplification of \textit{PDGFRA}, \textit{EGFR} or \textit{MDM2} within a single tumor, as detected by FISH. The mechanism leading to gene co-amplification remains to be clarified. In breast cancer, the co-amplification most likely arises by the selection of clones which have both foci amplified rather than as a direct result of prior fusions.\textsuperscript{51} In our study, the populations with co-amplification and single amplifications appear to be able to co-exist independently. It is
equally important to keep in mind that selection of certain populations might happen once targeted treatment is started. This provides a rationale for multi-targeted treatment of IS.

In conclusion, *PDGFRA* amplifications are common and consistently associated with activation of this gene in IS. Activation of EGFR is concurrent to PDGFRA activation and may co-exist with amplification and overexpression of MDM2. Our data provide a rationale for the targeted treatment of IS with specific PDGFRA, EGFR and MDM2 inhibitors. Dasatinib may be more efficient than imatinib for the inhibition of activated PDGFRs, but the effective therapy of these tumors may require combined regimens targeting multiple RTKs and/or downstream pathway regulatory effectors.

**Acknowledgements**

The authors would like to acknowledge Dr. Pascale Cervera for tumor tissue biopsy for this study.
References


27. Debiec-Rychter M, Lasota J, Sarlomo-Rikala M, Kordek R, Miettinen M. Chromosomal aberrations in malignant gastrointestinal stromal tumors:


Figure legends

Figure 1. Frequency of CNA detected by 244A Agilent aCGH in eight IS. The genomic positions of the most frequent imbalances are presented in Table 2.

Figure 2. (A) Representative CNA profile by 244A Agilent aCGH: case 4, case 2 and case 19. The individual array probes are arranged according to their genomic location on the X axis and their respective tumor/reference log2 ratios on the Y axis. (B) The 244A Agilent aCGH profiles of a selected region of chromosome 4q12 (54.2-56.0 Mb): case 4 and case 19 demonstrate the high level amplification of this region, with the smallest common region of amplification containing only the PDGFRA gene. The low level and focal amplification of PDGFRA in case 2, as identified by FISH (Figure 3B), is not detected by aCGH.

Figure 3. Representative examples of dual-color interphase FISH images on paraffin sections in intimal sarcomas. (A) Case 5, showing high-level amplification of PDGFRA (> 90% of nuclei), as detected by the co-hybridization of SpectrumOrange labeled BAC’s PDGFRA/RP11-24010 DNA probe (red signals) and SpectrumGreen labeled chromosome 4 CEP (green signals). (B) Using the same combination of probes, case 2 reveals low-level and focal (12% of cells) amplification of PDGFRA. Of note, this amplification is not detected by aCGH, as pictured in Figure 2B. (C) The co-hybridization of PDGFRA/RP11-24010 SpectrumOrange (red signals) and MDM2/RP11-1064P9 SpectrumGreen (green signals) labeled probes in case 19 reveals cells with exclusive amplification of PDGFRA (long arrows) or MDM2 (short arrows), intermingled with cells
showing separate amplicons for both genes. (D) The same intra-tumor heterogeneity is detected by the co-hybridizations of PDGFRA/RP11-24O10 SpectrumGreen (green signals) and EGFR/RP11-231C18 SpectrumOrange (red signals) labeled probes; cells with exclusive amplification of PDGFRA and EGFR are indicated by long and short arrows, respectively.

Figure 4. The PDGFRA and EGFR kinases and their downstream signaling intermediates are activated simultaneously in intimal sarcomas. Representative images from phospho-RTK (left panel) and phospho-kinase (right panel) arrays from whole-tumor lysates of case 4, case 2 and case 19. Each kinase is spotted in duplicate. The pairs of dots in each corner are positive controls. Each pair of the most positive kinase dots is denoted by a numeral, with the identity of the corresponding kinases listed as follows: 1) PDGFRA, 2) EGFR, 3) PDGFRB, 4) EPHB2, 5) AXL, 6) CREB(S133), 7) AMPKα1(T174), 8) TP53(S392), 9) TP53(S46), 10) STAT5b(Y699), 11) JUN(S63), 12) eNOS(S1177), 13) HSP27 (S78/S82), 14) P27 (T198).

Figure 5. Immunoblots showing the effect of the 2 hours exposure of the primary IS cells to increasing doses of imatinib or dasatinib on the phosphorylation of PDGFRA and the downstream effectors, ERK1/2 and AKT, with actin as a loading control. The lysates from the frozen tissue specimen, IS 1 (case 1) and IS 2 (case 2), and from the GISTs carrying KIT^K642E (GIST_KIT mut.) or PDGFRA^D561V (GIST_PDGFRA mut.) mutations were run in parallel as control samples.
Table 1. Clinical features of the patients, immunohistopathological data and FISH results in 21 intimal sarcomas.

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<th>N°</th>
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<td>2</td>
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</tr>
<tr>
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<td>28</td>
<td>heart</td>
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<td>16</td>
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<td>M</td>
<td>62</td>
<td>a. l.</td>
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Abbreviation: Desm., desmin; F, Female; M, Male; r., right; l., left; p. a., pulmonary artery; f. a., femoral artery; f. e., femoral artery embolism; a. l., arteria.lienalis; d, diffuse staining; f, focal staining; C, cytoplasmatic staining; M, membranous staining; a., amplification; p., polysomy; t., trisomy; n.d., not done; neg., no copy number changes.
Table 2. Recurrent copy number losses and gains in IS by aCGH.

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<th>Cytogenetic location</th>
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<th>Size (Mb)</th>
<th>Frequency</th>
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<td>0.75</td>
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<tr>
<td>4q12</td>
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*Minimal region gained or lost
Co-activated PDGFRA and EGFR are potential therapeutic targets in intimal sarcoma

Barbara M Dewaele, Giuseppe Floris, Julio Finalet-Ferreiro, et al.

*Cancer Res* Published OnlineFirst August 4, 2010.

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