Abstract

Purpose: To explore the molecular bases of potential new pharmacologic targets in aggressive fibromatosis (desmoid tumor).

Experimental Design: Tumor specimens from 14 patients surgically treated for aggressive fibromatosis (6 familial adenomatous polyposis and 8 sporadic cases), analyzed for adenomatous polyposis coli (APC) and CTNNB1 (β-catenin) mutations, were further investigated for β-catenin, cyclooxygenase-2 (COX-2), platelet-derived growth factor (PDGF) receptor α (PDGFRα)/PDGF receptor β (PDGFRB), their cognate ligands (PDGFA and PDGFB), and KIT using a comprehensive immunohistochemical, biochemical, molecular, and cytogenetic approach.

Results: No CTNNB1 (β-catenin) mutations were found in the familial adenomatous polyposis patients, but previously reported activating mutations were found in six of the eight sporadic patients. All of the cases carrying an altered WNT pathway showed nuclear and cytoplasmic immunoreactivity for β-catenin, whereas β-catenin expression was restricted to the cytoplasm in the sporadic patients lacking CTNNB1 mutations. COX-2 protein and mRNA overexpression was detected in all 14 cases, together with the expression and phosphorylation of PDGFRα and PDGFRB, which in turn paralleled the presence of their cognate ligands. No PDGFRB mutations were found. The results are consistent with PDGFRα and PDGFRB activation sustained by an autocrine/paracrine loop.

Conclusions: Aggressive fibromatosis is characterized by WNT/oncogene pathway alterations triggering COX-2–mediated constitutive coactivation of PDGFRα and PDGFRB, and may therefore benefit from combined nonsteroidal anti-inflammatory drug + tyrosine kinase inhibitor treatment.

Aggressive fibromatosis, also known as desmoid tumor, is a rare clonal fibroblastic proliferation that may arise in abdominal or extra-abdominal sites (deep soft tissues), characterized by infiltrative growth and a high risk of local recurrence even after complete surgical excision (1). Aggressive fibromatosis may occur in patients with familial adenomatous polyposis or as sporadic form.

Familial adenomatous polyposis patients are characterized by germ-line inactivating mutations of the adenomatous polyposis coli (APC) gene (2), whereas patients with sporadic aggressive fibromatosis usually harbor somatic β-catenin activating mutations (3). Because APC and β-catenin are members of the WNT pathway, we can conclude that the WNT pathway is altered in all aggressive fibromatoses irrespective of familial or sporadic origin (4). In normal cells, the WNT pathway leads to changes in gene expression profile; in fact, APC is able to form a multiprotein complex with glycojen synthase kinase-3β and axin, and then binds to β-catenin, which in turn is phosphorylated by glycojen synthase kinase-3β and subsequently degraded by the proteasome. In tumor cells, when either APC or β-catenin is mutated, the multiprotein complex could not be formed and, therefore, β-catenin accumulates into the cytoplasm and then translocates to the nucleus, where it activates the T-cell factor, which in turn causes transcription of target genes such as cyclooxygenase-2 (COX-2; ref. 5).

COX-2 is the inducible form of the COX enzyme family, whose members regulate the conversion of arachidonic acid to prostaglandins, which play critical roles in a large number of biological processes including immune function regulation, kidney development, reproductive biology, and gastrointestinal integrity. COX-2 is overexpressed in various cancer tissues, and it has been found that its activity contributes to tumorogenesis...
by inhibiting apoptosis, stimulating angiogenesis and invasiveness, and modulating cell proliferation by increasing the expression of growth factors such as platelet-derived growth factors (PDGF). It is also well known as a major pharmacologic target in chemoprevention, and its inhibition by nonsteroidal anti-inflammatory drugs seems to reduce the risk of developing cancer (5, 6).

Local control of aggressive fibromatosis is a challenge for oncologists. Surgery and radiotherapy are currently the principal modes of treatment, but some resections may be mutilating (and not resolutive) and radiotherapy has several drawbacks (4). Recently, there have been reports of responses to the oral kinase inhibitor imatinib (Novartis), which are mediated by the inhibition of PDGF receptor β (PDGFRB) kinase activity (7) rather than KIT (8).

Here, we studied a series of surgical tumor specimens taken from a monoinstitutional cohort of sporadic and familial adenomatous polyposis–associated patients to assess the mutational status of APC and CTNNB1 (β-catenin) as well as the expression of β-catenin, COX-2, PDGF receptor α (PDGFRα)/PDGFRB (and their cognate ligands), and KIT by means of a comprehensive immunohistochemical, biochemical, molecular, and cytogenetic approach.

The results show that aggressive fibromatosis is characterized by WNT/oncogene pathway alterations triggering the COX-2–mediated constitutive coactivation of PDGFRα and PDGFRB, and may therefore be suitably treated with a combination of nonsteroidal anti-inflammatory drugs + tyrosine kinase inhibitors.

Materials and Methods

Patients. After informed consent was obtained, we investigated a monoinstitutional cohort of 14 patients surgically treated for aggressive fibromatosis at Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. Eight of them were sporadic cases and six had a family history of familial adenomatous polyposis. The median patient age was 37 years (range, 15–70 years). Further details of clinical features are reported in Table 1.

Fifty healthy donors who gave informed consent were included. This group were disease-free on the basis of routinely serologic and instrumental analyses.

DNA and RNA extraction. DNA extraction from frozen tissues was carried out according to the QiAmp DNA Minikit protocol (Qiagen). Genomic DNA from blood was extracted by using the QiAmp DNA Blood kit (Qiagen).

Total RNA extraction was done using the TRIzol method (Life Technologies, Inc.) following the manufacturer’s instructions. One microgram of total RNA was reverse transcribed with Superscript reverse transcriptase (Life Technologies) using both oligodeoxythymidylic acid and random hexamers following the standard protocols. Thirty micro-liters of sterile 10 mmol/L Tris (pH 8)-1 mmol/L EDTA were added to 20 μL of total volume of cDNA obtained from each sample.

All the samples were tested for cDNA integrity by amplifying the β-actin housekeeping gene.

APC germ-line mutations. The APC germ-line mutations of familial adenomatous polyposis patients, detailed in Table 1, were characterized according to the protocols already published (2). Cases 2, 5, 8, and 12 have previously been reported (2).

Mutational screening of CTNNB1. CTNNB1 exon 3 mutational screening was done on genomic DNA using the protocol already reported (9).

Immunohistochemical analyses. Immunophenotyping for β-catenin, COX-2, KIT, PDGFRα, and PDGFRB was carried out as previously described (10–12) using the monoclonal mouse primary antibodies anti-β-catenin (Transduction Laboratories; 1:50 diluted) and anti–COX-2 (Cayman Chemical; 1:50 diluted) and the polyclonal rabbit antibodies anti-KIT (DAKO; 1:50 diluted), anti-PDGFRα (C-20; Santa Cruz Biotechnology; 1:200 diluted), and anti-PDGFRB (P-20; Santa Cruz Biotechnology; 1:100 diluted).

Two colorectal cancer samples previously characterized were used as positive control for β-catenin and COX-2 (10, 11). A gastrointestinal stromal tumor, molecularly characterized for KIT and PDGFRα, was used as positive control for KIT and PDGFRα staining, and a chordoma was used as positive control for PDGFRB reactivity as previously described (12).

COX-2 mRNA analysis by reverse transcription-PCR. The analysis of mRNA expression of COX-2 was done on the basis of the protocol already reported (11). Amplified products were run on 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

PDGFRα and PDGFRB real-time PCR. PDGFRα and PDGFRB cDNA was relatively quantified by real-time quantitative PCR (ABIPRISM 5700 PCR Sequence Detection Systems, Applied Biosystems) using a

### Table 1. Patients’ characteristics and results of APC and β-catenin mutational analyses

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<th>Patient ID</th>
<th>Age (y)</th>
<th>Sex</th>
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<th>APC germ-line mutation</th>
<th>β-Catenin IHC Mutations</th>
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Abbreviations: ABD, abdominal primary site; EXT, extra-abdominal primary site. FAP, familial adenomatous polyposis. IHC, immunohistochemistry; + c, cytoplasmic staining; + c/n, cytoplasmic and nuclear staining.
TaqMan-based analysis. Assays-on-Demands, consisting of a mix of unlabeled PCR primers and TaqMan probe (FAM dye-labeled) for the target genes, were purchased from Applied Biosystems. Each reaction was done with 10 μL of 1× TaqMan Universal master Mix (Applied Biosystems), 1 μL of probe/primer mix, and 1 μL of template cDNA. Cycling was started with 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

GAPDH mRNA, detected by a specific probe, was used as an internal control.

The 2^−ΔΔCt method (bulletin; ref. 13) was used to calculate the relative amount of PDGFRα and PDGFRβ mRNA by means of real-time quantitative PCR experiments: ΔΔCt = [ΔCt (unknown sample) - ΔCt (calibrator sample)] = [Ct g,GR (unknown sample) - C1,GB (calibrator sample)] - [Ct g,GR (calibrator sample) - C1,GB (calibrator sample)], where GI is the gene of interest, GR is the gene reference, and the calibrator is obtained from a pool of normal mesenchymal tissues expressing a basal level of receptors mRNAs and chosen to represent 1× expression of the gene of interest.

**Immunoprecipitation/Western blotting analyses of PDGFRα and PDGFRβ.** Protein extraction and immunoprecipitation/Western blotting experiments were done as previously described (12). In particular, specific rabbit polyclonal antibodies [PDGFRα (Upstate) and PDGFRβ (Santa Cruz Biotechnology)] were used for immunoprecipitation/Western blotting to detect PDGFR expression and anti-pY7 mouse monoclonal antibody (clone 4G, Upstate) to detect phosphorylation.

NIH-3T3 cell line (American Type Culture Collection, Manassas, VA) and 2N5A cell line (derived from the NIH-3T3 cell line and expressing the COL1-PDGFB fusion characterizing dermatofibrosarcoma protuberans, kindly provided by Dr. A. Greco, Experimental Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy) were used as positive control for the PDGFRα and PDGFRβ expression/phosphorylation experiments, respectively.

A pool of normal mesenchymal tissues (muscle, vessel wall, adipose tissue, and nerve) was used as a negative control of receptor expression levels.

**PDGFRβ mutational screening.** PCR amplification of genomic DNA for PDGFRβ (exons 10, 12, 14, and 18) was done using the following primer sequences (designed) and annealing temperatures: exon 10, primer forward, 5′-CCTCAGTTCCTCCGTCT-3′; primer reverse, 5′-AGTGGGAGATGCCATCT-3′; T annealing, 60°C; exon 12, primer forward, 5′-TCAGGGCCCTGAGACTCCCTCC-3′; primer reverse, 5′-CTCCCACACTGCGCACA-3′; T annealing, 60°C; exon 14, primer forward, 5′-GGGGAGAGATCAGAAATA-3′; primer reverse, 5′-CTGGTGCGGAGCAGCITTT-3′; T annealing, 60°C; exon 18, primer forward, 5′-GAAGGTTCCTTCCCTCCCA-3′; primer reverse, 5′-GAATCTGTCTCCGGGTAC-3′; T annealing, 60°C.

All PCR products were subjected to automated sequencing by 3100 ABI-PRISM Genetic Analyzer (Applied Biosystems) and analyzed with Sequencing Analysis and Sequence Navigator software programs by ABI-PRISM (Applied Biosystems). All mutated sequences were confirmed by repeating analysis at least twice starting from independent PCR products. In each case, the detected mutation was confirmed in the sequence as sense and antisense strands.

**PDGFRβ fluorescence in situ hybridization analysis.** Sections of 2 μm were placed on xylanized slides, air-dried, and baked at 56°C overnight in HYBrite Thermoblock (Vysis) and then deparaffinized by means of three 10-min xylene washings, rehydrated in a graded alcohol series, washed in Tris-EDTA (15 min at 96°C), and placed in double-distilled water for 2 min. After digestion with 10 mg/mL pepsin in 0.01 N HCl for 15 to 20 min at 37°C, they were dehydrated in alcohol for 3 min. BAC clones RP1-1-368O19 (PDGFRβ gene, 5q31-32) and RP1-1-52C13 (Sp11.1, as control) were used as the fluorescence in situ hybridization (FISH) probes, as previously described (12). The following probe mixtures were prepared for dual-color FISH:

- **A pool of normal mesenchymal tissues (muscle, vessel wall, adipose tissue, and nerve) was used as a negative control of receptor expression levels.**
SG-labeled PDGFRB+SO-5p. The mixture was placed on the preselected target area under microscopic control, covered with a coverslip, and sealed with rubber cement. The samples were denatured and hybridized in HYBrite Thermoblock (Vysis) at 85°C for 1 min and at 37°C overnight, after which the slides were washed in 2×SSC (pH 7) at 73°C for 2 min, air-dried, and mounted using 4',6-diamidino-2-phenylindole H antifade (Vysis). The slides were observed using a Zeiss Axioscope equipped with a 100-W mercury lamp and 4',6-diamidino-2-phenylindole, SC, and SO filters (Vysis); the signals were counted in at least 40 nuclei per slide under ×600/×1,000 magnification. The images were acquired using a cooled CHOI 4912 camera (Applied Imaging Corp.) and McProbe software (Vysis).

**Results**

**APC germ-line mutations of familial adenomatous polyposis patients**

APC germ-line mutations were detected in six of six familial adenomatous polyposis patients and are detailed in Table 1.

**CTNNB1 (β-catenin) mutational status**

The analysis of exon 3 of CTNNB1 (β-catenin), the region most frequently mutated, revealed no mutations in familial adenomatous polyposis patients, whereas activating mutations, already reported in literature, were detected in six of eight sporadic patients (Table 1). In the remaining sporadic cases, no mutations were observed (case nos. 6 and 14).

**Immunohistochemical analysis**

**β-Catenin.** Cytoplasmic and nuclear β-catenin immunoreactivity was found in all patients carrying an altered APC/β-catenin pathway. In the sporadic patients lacking exon 3 CTNNB1 (β-catenin) mutations (case nos. 6 and 14), β-catenin expression was restricted to the cytoplasm (Fig. 1).

**COX-2.** All cases carrying an altered APC/β-catenin pathway showed COX-2 immunodecoration. We also detected COX-2 immunoreactivity in the two sporadic cases without exon 3 CTNNB1 (β-catenin) mutations (Fig. 2).

**PDGFRα, PDGFRβ, and KIT.** The representative sections incubated with specific antibodies showed a cytoplasmic decoration for PDGFRβ and PDGFRα (Figs. 3 and 4) and a null immunophenotype for CD117 antibody (with positive inbuilt control).

**COX-2 reverse transcription-PCR**

mRNA analysis done by reverse transcription-PCR (RT-PCR) revealed a COX-2 overexpression in all the cases, in keeping with immunohistochemical results (Fig. 2).

**Detection and relative quantification of PDGFRα and PDGFRβ expression by real-time PCR**

PDGFRα and PDGFRβ were relatively quantified by means of real-time quantitative PCR.

We quantified receptors expression in all the 14 aggressive fibromatoses using the $2^{-ΔΔC_t}$ method. The calibrator sample, obtained from a pool of normal mesenchymal tissues expressing a basal level of receptors mRNAs, showed a $ΔC_t$ value of 6.6 for PDGFRα and 6.74 for PDGFRβ. In Table 2, the values of relative expression are reported. The median value $2^{-ΔΔC_t}$ was 3.4 for PDGFRα and 38.3 for PDGFRβ, indicating that aggressive fibromatoses express the receptors more than the pool of normal tissues. PDGFRβ were found to be more expressed than PDGFRα.

**PDGFRα and PDGFRβ biochemical analyses**

Immunoprecipitation/Western blotting experiments were done by immunoprecipitating equal amounts of total protein extracts.

**PDGFRα.** Membrane incubation with α-pTyr antibody revealed a 170-kDa band corresponding to an activated receptor in all the patients. As shown in Fig. 3, a similar phosphorylation level was present in all patients. The comparison with positive control (NIH-3T3 cell line) revealed a lower level of phosphorylation but higher than the reference.

The incubation with the anti-PDGFRα antibody showed that all the patients had similar PDGFRα expression level but lower than positive control (NIH-3T3 cell line; Fig. 3).

**PDGFRβ.** Membrane incubation with α-pTyr antibody revealed a 180-kDa band corresponding to an activated receptor in all the patients. As shown in Fig. 4, different phosphorylation levels were present and some patients were shown to have an activated receptor comparable to the one detected in the 2N5A cell line used as positive control. All aggressive fibromatoses were found to be more phosphorylated than the pool of normal mesenchymal tissues used as reference.

The incubation with the anti-PDGFRβ antibody showed that all the patients had similar PDGFRβ expression level comparable to those observed in positive control (2N5A cell line; Fig. 4).

**Fig. 2.** COX-2 protein and mRNA expression. A, COX-2 immunodecoration of tumor cells from a representative patient (ID 1); B, COX-2 mRNA expression of four representative patients (ID 1, 2, 3, and 4).
In keeping with real-time results, PDGFRB was more expressed than PDGFRA.

**PDGFRB mutational screening**

In cases 1, 2, and 9, we identified a base substitution in position 1,809 (G→A) in exon 10, which is responsible for the amino acid substitution Glu485Lys485.

This type of mutation was also found in normal tissues of the same patients and in 6 of 50 blood samples from healthy donors, thus suggesting that it is a polymorphism. \( \chi^2 \) analysis showed that the distribution was not statistically different \((P \leq 1)\). No correlation between the identified polymorphism and the expression/phosphorylation of the receptor was found.

**PDGFRB FISH analysis**

Gene copy numbers were investigated by FISH, with a complete analysis of PDGFRB. All cases showed a disomic hybridization pattern.

**PDGFRA and PDGFB expression (mRNA)**

The 14 analyzed cases resulted positive for both ligands (Figs. 3 and 4).

**Discussion**

The standard treatments of aggressive fibromatosis involve extensive surgical resection and/or radiation therapy although...
Table 2. COX-2, PDGFRA, PDGFRB, and KIT by immunohistochemistry, mRNA expression, and biochemical and ligand analyses

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<th>Patient ID</th>
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NOTE: IHC data: +, cytoplasmic staining; -, no staining. mRNA data: +, expressed. IP/WB data: +, protein expression; ++, high protein expression; P, phosphorylated. Ligand expression data: +, expressed. Abbreviation: IP/WB, immunoprecipitation/Western blotting.

tamoxifen, chemotherapy, and anti-inflammatory agents have been used with varying degrees of success in cases of unresectable, recurrent, or advanced disease (4). It has recently been shown that imatinib is clinically active in advanced aggressive fibromatosis presumably as a result of the inhibition of PDGFRB (7).

Taking advantage of the availability of cryopreserved and formalin-fixed material from a series of consecutive patients with abdominal and extra-abdominal aggressive fibromatosis in whom the mutational status of APC and β-catenin have been assessed, the aim of this study was to explore the molecular bases for potential new targets in aggressive fibromatosis lesions. Considering that COX-2 is dramatically up-regulated in the presence of WNT signaling deregulation and that COX-2 is known to increase the expression of growth factors, among which PDGFB and PDGFA (6), we assumed that COX-2 might represent an attractive target for therapeutic strategies in aggressive fibromatosis.

For this, we carried out an immunohistochemical screening to assess COX-2, PDGFRA, PDGFRB, KIT, and β-catenin expression in the above-mentioned series of aggressive fibromatoses carrying germ-line APC (six of six) or somatic β-catenin (six of eight) mutations. With the exception of KIT, which was null in all cases, COX-2, PDGFRA, PDGFRB, and β-catenin were found to be immunoreactive in all 14 cases. Remarkably, all of the cases carrying an altered APC/β-catenin pathway presented β-catenin nuclear and cytoplasmic decoration, whereas the decoration of the two wild-type β-catenin cases was restricted to the cytoplasm, thus further confirming the absence of any alteration in the APC/β-catenin pathway in these two cases.

To verify the immunohistochemical results, we analyzed the presence of COX-2 mRNA by means of RT-PCR and of PDGFRA/PDGFRB mRNA by means of real-time PCR. The analysis confirmed COX-2 overexpression in all the cases carrying APC or β-catenin gene mutations, which is in line with the idea that the COX-2 promoter has a binding site for T-cell factor (14), a nuclear factor that forms complexes with β-catenin, and therefore that APC/β-catenin pathway deregulation may drive COX-2 overexpression (15). In the two cases with wild-type APC and β-catenin, it is likely that COX-2 expression was sustained by inflammatory cytokines (16). The relative quantification of PDGFRA and PDGFRB mRNAs obtained using a pool of normal mesenchymal tissues as calibrator revealed that PDGFRB was more expressed than PDGFRA and that both were more expressed than in the pool.

The biochemical analyses further supported the immunophenotyping and mRNA molecular findings by showing that both receptors were expressed/phosphorylated, and thus activated. Interestingly, in line with the mRNA quantification data, PDGFRB was more expressed than PDGFRA. The data of activation of both PDGFRs we observed were at variance with the results of a previously reported study where only PDGFRB was involved owing to its expression in absence of a clear evidence of phosphorylation (7). This discrepancy could be due to the different methodologic approaches used for the detection of phosphorylated tyrosine and to the different antibodies used in the experiments.

Because the expression and activation status of PDGFRB was higher than that of PDGFRA, we wanted to exclude the presence of gene gain or mutations of this receptor. No mutations were found and FISH assay turned out to be negative according to Liegl et al. (17). Furthermore, we identified a polymorphism not yet described in three patients. However, the analysis of a cohort of 50 healthy donors revealed the same polymorphism in six cases, supporting the notion that this alteration is likely to be not associated with the activation status of PDGFRB.

As the lack of mutations and gene number alterations strongly suggested autocrine/paracrine activation of the PDGFRs, we used RT-PCR experiments to seek the presence/absence of the cognate PDGFA and PDGFB ligands, both of which were found to be expressed in all cases.
Taken together, our results indicate that both PDGFRs are activated, and that this activation takes place by an autocrine/paracrine loop and is mediated by COX-2 overexpression, which in turn is triggered by deregulation of the APC/\(\beta\)-catenin signaling pathway or, possibly, inflammatory cytokines. The latter needs further investigations.

Our results confirm that imatinib may be effective in treating aggressive fibromatosis as indicated by Heinrich et al. (7) and as suggested by Poon et al. (18) and recently by Gega et al. (19) in relation to chemotherapeutic regimens that aggressive fibromatosis patients may also benefit from combined anti–COX-2 nonsteroidal anti-inflammatory drug and tyrosine kinase inhibitor treatment. The latter is principally aimed to block PDGFR family (e.g., sunitinib; refs. 20, 21) or, alternatively, kinases involved in the PDGFR signaling downstream pathway, blocking phosphatidylinositol 3-kinase or mammalian target of rapamycin for which new compounds have been developed and clinically tested (22).

References

Correction: Article on Cyclooxygenase-2 and Platelet-derived Growth Factors as Targets in Treating Aggressive Fibromatosis

In the article by Signoroni and coworkers on the treatment of fibromatosis, beginning on page 5034 of the September 1, 2007, issue of Clinical Cancer Research, Fig. 4 was rendered incorrectly. The correct version of Fig. 4 appears below.
Cyclooxygenase-2 and Platelet-Derived Growth Factor Receptors as Potential Targets in Treating Aggressive Fibromatosis

Stefano Signoroni, Milo Frattini, Tiziana Negri, et al.


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