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Signal transduction pathway analysis in fibromatosis: receptor and nonreceptor tyrosine kinases*

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Summary

Despite reports of receptor tyrosine kinase activation in desmoid-type fibromatosis, therapeutic benefits of kinase inhibitor therapy are unpredictable. Variability in signal transduction or cellular kinases heretofore unevaluated in desmoid tumors may be responsible for these inconsistent responses. In either case, a better understanding of growth regulatory signaling pathways is necessary to assess the theoretical potential of inhibitor therapy. Immunohistochemical analysis of tyrosine kinases and activated isoforms of downstream signal transduction proteins was performed on a tissue microarray containing 27 cases of desmoid-type fibromatosis and 14 samples of scar; 6 whole sections of normal fibrous tissue were studied for comparison. Platelet-derived growth factor receptor, β type, and focal adhesion kinase 1 were expressed in all desmoid tumors and healing scars but only 80% and 50% of nonproliferative fibrous tissue samples, respectively. Hepatocyte growth factor receptor was detected in 89% of desmoids and all scars tested, but not in any of the fibrous tissue samples. Epidermal growth factor receptor was detected in only 12% of desmoids and not in scar or fibrous tissue. Mast/stem cell growth factor receptor, receptor tyrosine–protein kinase erbB-2, and phosphorylated insulin-like growth factor 1 receptor/insulin receptor were negative in all study cases. Variable levels of phosphorylated downstream signal transduction molecules RAC- $\alpha/\beta/\gamma$ serine/threonine-protein kinase, mitogenactivated protein kinase, and signal transducer and activator of transcription-3 were observed in desmoids (58%, 62%, and 67%), scar tissues (100%, 86%, and 86%), and fibrous tissue (33%, 17%, and 17%). These results indicate that tyrosine kinase signaling is active in both fibromatosis and healing scar,

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but not in most nonproliferating fibrous tissues. Although platelet-derived growth factor receptor, β type, is expressed ubiquitously in desmoids, the kinases driving cell proliferation in desmoids remain unresolved.

Keywords

Desmoid-type fibromatosis; Receptor tyrosine kinase; Nonreceptor tyrosine kinase

1. Introduction

Desmoid-type fibromatosis (desmoid tumor) is a rare myofibroblastic tumor that most commonly affects adolescents and young adults [1]. Although lacking metastatic potential, the deep fibromatoses are locally aggressive and cause significant morbidity. Repeated local recurrences sometimes result in death when the tumors involve vital structures [1]. Complete surgical excision is often combined with adjuvant radiation therapy to minimize the potential for local recurrence [2]. Other than wide surgical reexcision and perhaps adjuvant radiation therapy, there is no standard treatment of this recurrent disease [1,3].

Aberrations in the Wnt signaling pathway are well described in desmoid tumors [1,2]. Recent evidence suggests that receptor tyrosine kinases (RTKs) are also active in desmoid tumors. Activated isoforms of downstream signal transduction molecules such as phosphorylated RAC- $\alpha/\beta/\gamma$ serine/threonine-protein kinase (p-Akt), mitogen-activated protein kinase 3/1 (p-MAPK), and signal transducer and activator of transcription-3 (p-STAT3) have been detected in a limited number of desmoid tumors examined thus far [4,5]. However, prior investigations have failed to disclose correlations between the expression or activation status of RTKs such as platelet-derived growth factor (PDGF) receptor, α or β type (PDGFR α or PDGFR β), epidermal growth factor receptor (EGFR), receptor tyrosine-protein kinase erbB-2 (HER2), or mast/stem cell growth factor receptor (KIT) and clinical outcomes or responses to receptor tyrosine kinase inhibitor therapy in deep fibromatosis [2,4–11]. Consequently, it is unknown why patients generally fail to respond to these agents [12].

Specific, as-yet-undetected intralesional molecular abnormalities or differences in the functional status of target molecules may be responsible for unpredictable therapeutic benefits. Alternatively, other tyrosine kinase receptors implicated in myofibroblastic differentiation or proliferation such as insulin-like growth factor-1 receptor (IGF1R) [13,14], hepatocyte growth factor receptor (MET) [13,15,16], or focal adhesion kinase 1 (FAK) may represent the molecular targets of inhibitor therapy.

Current advances in targeted molecular therapy offer new opportunities to treat fibromatosis patients [7]. A better understanding of the signaling pathways active in fibromatosis, particularly those that may affect Wnt signaling, is necessary to determine whether there exists the theoretical potential for inhibitor therapy in this debilitating disease. Several tyrosine kinases and their downstream signal transduction proteins were evaluated in 27 cases of deep fibromatosis and compared with normal fibrous tissue and healing scars (nonneoplastic, reactive myofibroblastic proliferations). The results demonstrate that RTK

activity is detectable in the proliferating fibroblasts of desmoid-type fibromatosis and healing scars, but not in quiescent fibrous tissue. The presence of MET in desmoid tumor further suggests that this kinase may represent a promising molecular therapeutic target, offering new treatment modalities for patients with this disease.

2. Materials and methods

2.1. Tissue samples

Surgical pathology archives were searched for cases diagnosed as “fibromatosis” or “desmoid tumor” between 1990 and 2005. A representative series of healing cutaneous scars obtained from reexcision specimens for non-desmoid-related diseases ($n = 14$) and nonneoplastic fibrous tissues ($n = 6$) from reduction mammoplasty or panniculectomy specimens were collected for comparison. Clinicopathologic data (tumor location, tumor size, patient demographic data, (neo)adjuvant therapy administered, and recurrence-free and overall survival intervals) were extracted from electronic medical records. This study was approved by an institutional review board, and a waiver of consent was granted.

2.2. Tissue microarray and immunohistochemistry

The original hematoxylin and eosin-stained slides were reviewed, and diagnostic tissue was marked for construction of a tissue microarray using a manual arrayer (Beecher Instruments, Sun Prairie, WI). Two tissue cores (1.5 mm each) of representative areas from each of the selected formalin-fixed, paraffin-embedded tissue blocks were used for the array. The antisera and immunohistochemistry (IHC) protocols used are presented in Table 1. Antigen retrieval was performed for 25 minutes in a preheated steamer, followed by a 15-minute cooldown to room temperature. Sections were then incubated with primary antibodies for 1 hour at room temperature, unless otherwise noted. Appropriate positive and negative control sections were included for each antibody and assay run, respectively.

IHC stains were independently reviewed by 2 of the authors, and the expression of each marker was scored in a semiquantitative manner. Because most lesional cells stained uniformly within each tissue microarray core, only staining intensity was recorded and scored as either negative (0), weaker than the corresponding positive control (1+), similar to the corresponding positive control (2+), or stronger than the corresponding positive control (3+). There were no major discrepancies (difference >1 score category) between the 2 observers. The modal IHC intensity score for each pathologic specimen was used in subsequent statistical analyses.

2.3. Statistical analysis

Median IHC intensity scores for each diagnostic category were compared using the Kruskal-Wallis test with a conservative Bonferroni correction applied; $\alpha = .005$ was considered statistically significant. For markers showing statistical differences among the 3 diagnostic categories, post hoc analyses were performed using Dunn multiple comparison test [17]. IHC data were compared with clinicopathologic features using standard univariate methods. Kaplan-Meier recurrence-free survival curves were compared using the log-rank test.

3. Results

3.1. Clinicopathologic data

Twenty-seven cases of sporadic desmoid-type fibromatosis were available for analysis. The study group was composed of 14 female patients (median age 26 years; range, 4–73 years) and 13 male patients (median age, 23 years; range, 1–58 years). None of the study patients had clinical documentation of familial adenomatous polyposis or Gardner syndrome. The most common sites of involvement were the extremities ($n = 7$); other common sites included the thoracic ($n = 4$) or abdominal walls ($n = 3$), neck ($n = 4$), and limb girdles ($n = 4$). Two cases arose in the juxta-opharyngeal soft tissues, and the site of origin was not specified for 3 cases. Tumor size ranged from 1 to 15 cm in the greatest dimension (median, 5.5 cm). Gross tumor volume was calculable for 21 cases; the median tumor volume was 53.5 cm³ (range, 0.4–421 cm³). Resection margins were either grossly or microscopically positive in 12 of 22 cases for which margin status could be determined. Adjuvant therapy (radiation and/or chemotherapy) was administered to 3 patients with positive margins. Of the 20 patients with follow-up information, the median follow-up interval was 87 months (range, 9.3–254 months). During this interval, 11 patients had at least 1 local recurrence (55%), with a median time to recurrence of 15.6 months (range, 6.5–38.1 months). No patient has died of complications of desmoid tumor. Nuclear β -catenin was detected in 71% of desmoid tumors and 14% of hypertrophic scars but not in quiescent fibrous tissue (data not shown).

3.2. Receptor and non-RTKs

Representative IHC stains for selected markers and the distribution of IHC intensity scores for each diagnostic category are presented in Figs. 1 and 2, respectively. Strong expression (2+ or 3+) of PDGFR β was detected in almost all desmoids, scars, and quiescent fibrous tissues, with levels in desmoids significantly higher than those in fibrous tissue (Kruskal-Wallis, $P < .001$; Dunn post test, $P = .01$ –.001) but not higher than in healing scar. Low-to-intermediate levels of MET (1+ or 2+) were detected in 89% of desmoid tumors and 100% of scars but not in nonneoplastic fibrous tissue. FAK was detected in all desmoids and scars, whereas fibrous tissues variably expressed FAK; no statistically significant differences in staining intensities between desmoid or scar and fibrous tissue were detected. Similarly, the phosphorylated isoforms of FAK (p-FAK [Y397] and p-FAK [Y861]) were observed in a subset of cases from each of the diagnostic categories examined, without statistically significant differences in immunoreactivity (Fig. 2). Low-to-intermediate levels of EGFR were detected in only 11% of desmoids but not in either scar or fibrous tissue; IHC stains for phosphorylated EGFR (p-EGFR) disclosed similar findings (data not shown). The other RTKs studied (KIT, HER2, and phosphorylated IGF1R/insulin receptor [IR]) were not detected in any of the samples examined. Intralesional mast cells were positive for KIT (data not shown), which likely accounts for the reports of KIT expression in desmoid tumors detected by Western blot analysis.

3.3. Downstream markers of RTK activity

Phosphorylated Akt was detected in 56% of desmoids. Significantly higher intensity levels were observed in scar tissues, however (Kruskal-Wallis, $P = .0002$; Dunn post test, $P = .01$ –.

001). In contrast, low levels of p-Akt were detected in only 2 of 6 samples of fibrous tissue. Variable levels of p-MAPK and p-STAT3 were observed in both desmoid and scars, whereas low-intensity staining for these markers was seen in only 1 sample of fibrous tissue. These findings were not statistically significant after Bonferroni correction for multiple comparisons. Thus, it appears that RTK signaling is active in both scar tissue and desmoids but not in nonproliferating fibrous tissue.

3.4. Clinicopathologic correlations

Correlation analysis was performed using the Spearman rank test (Table 2). As expected, immunohistochemical staining indices for the 2 phosphorylated isoforms of activated FAK (Y397 and Y861) strongly correlated with each other but not with total FAK immunoreactivity. The only other correlation that maintained statistical significance after Bonferroni correction was between p-MAPK and p-STAT3, perhaps indicative of RTK activity in cases that coexpress these 2 activated signaling molecules. Nuclear β -catenin accumulation did not correlate with any of the markers studied. Neither did any of the IHC markers correlate with patient age, greatest dimension of tumor, or total tumor volume by Spearman rank test (data not shown). None of the IHC markers were associated with sex or anatomic location of the tumor, and there were no associations between any IHC marker and recurrence-free survival by log-rank tests of Kaplan-Meier survival curves (data not shown).

4. Discussion

The expression of several signaling proteins and activated downstream signal transduction molecules in desmoid-type fibromatosis was compared with reactive myofibroblasts in healing scars and quiescent fibroblasts in nonproliferative fibrous tissue to identify deregulated signal transduction pathways in desmoid-type fibromatosis. The IHC staining intensities for each of the markers studied, except for p-Akt, were not significantly different between desmoid-type fibromatosis and healing scar (p-Akt levels were significantly higher in healing scar tissue). Thus, tyrosine kinase signaling appears active in the proliferating fibroblasts/myofibroblasts of both desmoid tumors and healing scars. However, it remains unclear which kinases are driving cell proliferation in these conditions.

PDGF is a crucial cytokine involved in myofibroblastic transformation and proliferation and thus is an important factor in the wound healing response as well as fibrotic diseases [13,18]. Although PDGFR β and PDGFR α are expressed in desmoid-type fibromatosis [5–7], several points of evidence argue against these RTKs having significant pathogenetic roles in desmoid tumors. First, activating mutations have not been identified [4,19,20]. Second, our findings and those of others show that PDGFR β levels are not significantly different between desmoid tumors, healing scars, and normal fibroblasts [20,21]. Our data also corroborate that of Heinrich et al [20], who were unable to demonstrate correlations between PDGFR β expression and the phosphorylated isoforms of Akt, MAPK, or STAT3. Furthermore, Heinrich et al [4,20] and Dufresne et al [5] showed that PDGFR β is only weakly activated in desmoid tumors and that the α -type PDGFR likewise appears inactive. Nonetheless, given significant differences in plasma PDGF levels in patient with fibromatosis [20], low levels of PDGF in normal fascia and mature scar tissues [21], and

reports of phosphorylated PDGFR β in desmoid tumors (reviewed in Signoroni et al [7]), further comparisons of the levels of phosphorylated PDGFR β in neoplastic and reactive fibroblasts might demonstrate significant differences in the activation of this pathway.

Activation of the RTK MET in mesenchymal cells stimulates cell motility and proliferation [15,22], making it an apt target for inhibition therapy. However, because MET is expressed in both desmoid tumors and scars, it may not be instrumental in the pathogenesis of desmoid tumors. Instead, it appears to play a role in limiting the fibrotic response of myofibroblasts to transforming growth factor β (TGF- β) [15,16]. Given the possible role of TGF- β in promoting desmoid tumor growth [23–25], inhibition of MET may not be beneficial in fibromatosis. Contrariwise, because MET has been implicated in stimulating the Wnt pathway, decreased MET signaling may reduce aberrant β -catenin-mediated transcriptional activity in desmoid tumors [26–29]. Although none of the IHC markers in this study correlated with nuclear β -catenin accumulation, further investigations of the role of noncanonical mechanisms of β -catenin activation (particularly through MET signaling) in desmoid tumor growth seem warranted.

Cell adhesion and integrin-mediated signaling via the non-RTK FAK is also important for myofibroblast differentiation and survival [30–32]. FAK expression and activation status have not been previously evaluated in desmoid tumors. FAK and its activated isoforms phosphorylated on Y397 and Y861 were detected in all desmoids and scar tissues studied, whereas fibrous tissue variably expressed FAK. Thus, FAK may be activated in both reactive and neoplastic fibroblasts/myofibroblasts, and FAK signaling does not appear to be aberrant in desmoid-type fibromatosis.

The RTKs EGFR, KIT, and HER2 have not been detected in most desmoid tumors studied thus far and are, therefore, poor candidates for targeted therapies [8,9]. Despite reports to the contrary [6,10], desmoid cells do not express KIT [2,4,7–9,11,20,33]. KIT immunoreactivity seen in extracts of desmoid tumors on Western blots is likely derived from intralesional mast cell infiltrates instead [4,33]. Although insulin-like growth factors have been reported as important mitogenic cytokines responsible for myofibroblastic differentiation and proliferation [13] and the insulin-like growth factor pathway has been implicated in fibromatosis [14], immunohistochemical stains for the activated isoforms of the IGF1R/IR were negative in all cases studied.

Variable levels of phosphorylated downstream signal transduction molecules indicative of RTK activity (p-Akt, p-MAPK, and p-STAT3) were detected in desmoid tumors. Although Dufresne et al [5] were unable to document the presence of p-Akt (S473) in fibromatosis by IHC, Heinrich and coworkers [4] have identified it in desmoid tumors using Western blots. The difference in the IHC results reported here for p-Akt (S473) is likely due to the different antigen retrieval method used and prolonged incubation with primary antibody in our IHC protocol. In assessing the extracellular signal-regulated kinase (ERK) arm of the mitogen-activated protein kinase cascades, Dufresne et al [5] reported staining for p-MAPK in a similar percentage of desmoid tumors as we show herein. The presence of these phosphorylated downstream signal transduction molecules in both desmoids and healing scars is consistent with active RTK signaling in proliferating fibroblasts and myofibroblasts,

but not in quiescent fibrous tissue. Consequently, as with the RTKs studied, these downstream signal transduction markers fail to distinguish desmoids from healing scar tissue.

In summary, the expression profiles of selected receptor and non-RTKs, together with downstream markers of RTK activity, were examined in desmoid tumors and compared with reactive scar and quiescent fibrous tissue. Although there appears to be evidence for active RTK signaling in both fibromatosis and healing scar, it is not clear which kinases are responsible. The presence of MET in desmoid tumor represents a promising molecular therapeutic target, potentially offering new treatment modalities for patients with this disease as well as for individuals with hypertrophic scars or keloids. However, further study is necessary to determine whether the MET tyrosine kinase is activated in desmoid tumors. The question of which signaling pathways are affected in imatinib-sensitive desmoid tumors remains unanswered. One possibility is that because (a) imatinib mesylate inhibits the fibrogenic activity of TGF- β and (b) p21-activated kinase-2 and Abelson non-RTK are activated during TGF- β -induced proliferation and fibrosis, other cellular kinases are responsible for the cytostatic effects of imatinib [34,35].

Abbreviations

EGFR	epidermal growth factor receptor (erbB-1)
FAK	focal adhesion kinase 1 (protein-tyrosine kinase 2)
HER2	receptor tyrosine–protein kinase erbB-2
IGF1R	insulin-like growth factor 1 receptor
IHC	immunohistochemistry
KIT	mast/stem cell growth factor receptor (CD117)
MET	hepatocyte growth factor receptor
PDGF	platelet-derived growth factor
PDGFRβ	platelet-derived growth factor receptor β type
p-Akt	phosphorylated RAC- $\alpha/\beta/\gamma$ serine/threonine-protein kinase
p-EGFR	phosphorylated epidermal growth factor receptor
p-FAK	phosphorylated focal adhesion kinase 1
p-IGF1R/IR	phosphorylated insulin-like growth factor 1 receptor/insulin receptor
p-MAPK	phosphorylated mitogen-activated protein kinase 3/1 (ERK1/2)
p-STAT3	phosphorylated signal transducer and activator of transcription-3
RTK	receptor tyrosine kinase
TMA	tissue microarray

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Fig. 1. Representative immunohistochemical stains for PDGFR β , MET, p-Akt, and p-MAPK are demonstrated in desmoid-type fibromatosis (Desmoid), reactive myofibroblasts in healing scar (Scar), and quiescent fibrous tissue (Fibrous tissue) (magnification $\times 100$).

Fig. 2.

Scatter plots demonstrate the distribution of IHC intensity scores of various receptor and non-RTKs and activated downstream signal transduction molecules among desmoid-type fibromatosis, healing scar tissue, and quiescent fibrous tissue. Lines denote median intensity scores. P values from Kruskal-Wallis tests are given in parentheses, and results of Dunn post test comparisons are denoted on the plots as follows: *** $P < .001$ and ** $P = .001-.01$.

Table 1

Antibodies and IHC protocols used

	Clone	Dilution	Antigen retrieval	Detection	Source ^d
β-Catenin	β-Catenin-1	1:200	Dako Target Retr ^b	EnVision+ ^c	Dako
EGFR	H11	1:100	Proteinase K ^d	LSAB2 ^e	Dako
FAK	Polyclonal	1:25	EDTA ^f	EnVision+	Abgent
HER2	4D5	1:100	Proteinase K	LSAB2	Genentech
KIT	Polyclonal	1:600	Dako Target Retr ^b	EnVision+	Dako
MET	Polyclonal	1:100	Citrate ^g	EnVision+	Zymed
PDGFRβ	Polyclonal	1:200	EDTA	LSAB2	Santa Cruz
p-Akt (S473)	736E11	1:40 ^h	Citrate	LSAB2	Cell Signaling
p-EGFR (Y1173)	74	1:500	Citrate	CSA II ⁱ	Chemicon
p-FAK (Y397)	Polyclonal	1:50	Proteinase K	EnVision+	Calbiochem
p-FAK (Y861)	Polyclonal	1:500	Proteinase K	EnVision+	Novus
p-IGF1R/IR (Y1161/Y1185)	Polyclonal	1:200	Proteinase K	EnVision+	Abcam
p-MAPK (T202/Y204)	Polyclonal	1:50	Trilogy ^j	LSAB2	Cell Signaling
p-STAT3 (Y705)	Polyclonal	1:25	Citrate	EnVision+	Cell Signaling

Abbreviation: p-IGF1R/IR, phosphorylated IGF1R/IR.

^aDako North America Inc, Carpinteria, CA; Abgent, San Diego, CA; Genentech, San Francisco, CA; Zymed, Invitrogen Corporation, Carlsbad, CA; Santa Cruz Biotechnology, Santa Cruz, CA; Cell Signaling Technology, Danvers, MA; Chemicon International, Inc, Temecula, CA; Calbiochem, San Diego, CA; Novus, Littleton, CO; Abcam, Cambridge, MA.

^bDako Target Retrieval Solution (Dako North America Inc).

^cEnVision+ (Dako North America Inc).

^dProteinase K, prediluted (Dako North America Inc) incubated for 5 minutes at room temperature.

^eLSAB2 (Dako North America Inc); incubation overnight at 4 °C.

^f1× EDTA buffer, pH 8.0 (Zymed, Invitrogen Corporation).

^g1× Citra buffer, pH 6.0 (Dako North America Inc).

^hOvernight incubation.

ⁱCSA II, Biotin-Free Catalyzed Amplification System (Dako North America Inc).

^jTrilogy, pH 8.0, prediluted buffer (Cell Marque, Hot Springs, AR).

Table 2Correlation analysis between immunohistochemical markers in desmoid-type fibromatosis^a

	β -catenin	MET	FAK	p-FAK (Y397)	p-FAK (Y861)	p-STAT3	p-MAPK	p-Akt
β -Catenin	–	0.067	0.000	0.044	–0.239	0.387	0.303	0.464
MET	0.740	–	0.398	0.051	0.128	0.385	0.028	0.293
FAK	1.000	0.054	–	0.042	0.131	0.156	–0.151	0.353
p-FAK (Y397)	0.828	0.802	0.847	–	0.772	0.480	0.315	0.235
p-FAK (Y861)	0.229	0.526	0.542	0.001	–	0.252	0.095	0.103
p-STAT3	0.046	0.048	0.468	0.011	0.205	–	0.569	0.397
p-MAPK	0.133	0.893	0.491	0.118	0.644	0.002	–	0.415
p-Akt	0.017	0.147	0.091	0.247	0.616	0.045	0.039	–

^aUpper right panels, Spearman rank correlation coefficients; lower left panels, *P* values.