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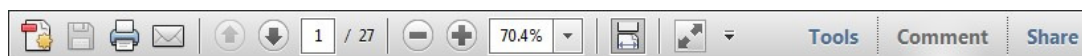## USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:

This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:
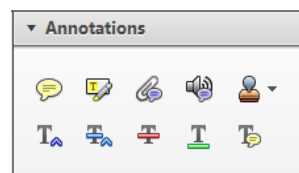
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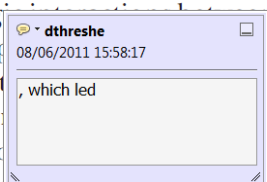### 1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

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5. Attach File Tool – for inserting large amounts of text or replacement figures.

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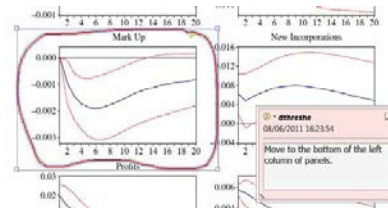
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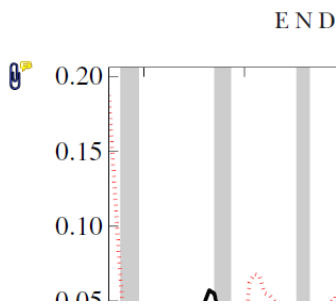
6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.
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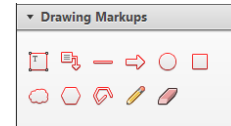
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Platelet-derived growth factor receptor β (PDGFRβ) immunohistochemistry highlights activated bone marrow stroma and is potentially predictive for fibrosis progression in prefibrotic myeloproliferative neoplasia

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Platelet-derived growth factor receptor β (PDGFRβ) immunohistochemistry highlights activated bone marrow stroma and is potentially predictive for fibrosis progression in prefibrotic myeloproliferative neoplasia

Aims: Myelofibrosis is the result of aberrant stromal activity which is determined routinely by reticulin staining in bone marrow biopsies. As matrix fibres are the product of activated fibroblasts, we analysed fibre accumulation compared to stromal cell activity during myelofibrosis progression using the fibroblast activation marker platelet-derived growth factor receptor β (PDGFRβ) by immunohistochemistry.

Methods and results: Initial and follow-up bone marrow biopsies from 84 patients with myeloproliferative neoplasia, including 55 cases with primary myelofibrosis, were evaluated from five haematopathology centres. The stromal mass was measured by conventional reticulin staining [myelofibrosis (MF) grade, 0–3] and PDGFRβ-positive cells using a novel PDGFRβ scoring system (0–3). Results were correlated for prediction of progression. The MF grade and the PDGFRβ score showed excellent correlation (Spearman's $r = 0.83$, $P < 0.0001$). Elevated PDGFRβ scores (higher than MF-grade) predicted myelofibrosis progression in total with 43% sensitivity and 57% specificity, and short-term (within 1 year) progression with 82% sensitivity and 53% specificity. Progression of prefibrotic disease to manifest myelofibrosis could be forecast with 90% sensitivity and 75% specificity.

Conclusion: PDGFRβ highlights stromal cell activation in marrow fibrosis, which is closely related to matrix accumulation, indicating a direct clinical impact especially in prefibrotic myeloproliferative disorders.

Keywords: bone marrow, immunohistochemistry, platelet-derived growth factor receptor, primary myelofibrosis

Introduction

Myelofibrosis (MF) is the result of bone marrow stromal cell activation occurring frequently in different pathological conditions of the bone marrow.[1–3] Accumulation of the non-haematopoetic stroma consists of both mesenchymal cellular components and interstitial matrix, the latter composed predominantly of reticulin and collagen fibres.[4,5] While the matrix fibre deposition is determined routinely using reticulin silver staining, much less attention is paid to the

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activation and proliferation of stromal cells. Fibroblasts responsible for the production of reticulin and collagen fibres are not prominent in the normal bone marrow[4] and become numerous only upon stimulation from the microenvironment.[6,7] Several growth factors, including megakaryocyte-derived basic fibroblastic growth factor (bFGF), transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF), have been proposed to be the most important inducers of fibroblast activation and fibre production during the process of MF.[8–11] Growth factors act by specific triggering of the appropriate surface membrane receptor pathways in the target cells.[12,13] Previous studies have demonstrated that platelet-derived growth factor receptors (PDGFR) show a differential expression in the mesenchymal cell types of the bone marrow and that the PDGFRβ subunit is expressed predominantly by activated fibroblasts in a cell type-specific manner.[6,12] Moreover, the extent of the PDGFRβ subunit demonstrated by immunohistochemistry (IHC) in bone marrow biopsy samples correlated closely with the degree of matrix fibre accumulation in MF.[6] Notably, despite the strong correlation between stromal cell activation and fibre accumulation, fibroblast-related PDGFRβ immunostaining proved to be more prominent than the amount of fibre accumulation in selected cases, suggesting a progressive potential of the stromal changes.

As PDGFRβ appeared to be useful for the characterization of MF, the question was raised as to whether the histological demonstration of activated fibroblasts might principally indicate ongoing fibrosis, and thus predict the course of MF progression. For this purpose, a statistically significant number of sequential bone marrow samples from the same patients need to be evaluated, which are rarely provided. To evaluate the utility of PDGFRβ assessment by IHC and to address its predictive potential in the long- and short-term follow-up of primary myelofibrosis (PMF), a retrospective multicentre study was initiated with the participation of the members of the European Bone Marrow Working Group. Initial and follow-up bone marrow biopsy samples featuring different degrees of MF were collected and evaluated for the fibroblast activation marker PDGFRβ using IHC and for the fibre products highlighted by conventional reticulin silver staining.

Materials and methods

Archival cases of 84 patients having had multiple bone marrow examinations were collected by the following participants: the Institute of Pathology at the University Hospital Basel, Switzerland, the Radboud University Nijmegen Medical Center, the Netherlands, the Charité – University Medicine, Berlin, Germany, the Laboratory of Tumor Pathology and Molecular Diagnostics, Szeged, Hungary and the Department of Pathology, University of Debrecen, Hungary. Clinical and histological diagnoses and basic patient data, including time of sampling, were provided in an anonymized fashion. Detailed evaluation of clinical and laboratory parameters was not the aim of this study. Histopathological evaluation of the slides was performed in accordance with the general ethical regulations and with the approval of the local ethical committees.

A total of 193 biopsy samples were obtained from 84 patients, including 55 PMF, eight essential thrombocythaemia (ET), five polycythaemia vera (PV), three chronic myeloid leukaemia (CML) and a further three unclassifiable myeloproliferative neoplasias (MPN-U) and four overlapping MPN/myelodysplastic neoplasias (MDS). Myeloproliferative neoplasia was associated with other bone marrow disorders or conditions in six additional cases [lymphoproliferative disorders, granulocyte–macrophage colony-stimulating factor (GM-CSF) therapy]. Usually two serial samples per case represented the course of the disease, although for some cases three or more biopsies were provided. The mean follow-up time was 34.4 months (range = 2–151 months).

Within the study collection, PMF represented the largest homogeneous disease group which could be evaluated separately. Altogether, 126 initial and follow-up biopsy samples from 55 PMF cases were analysed. The mean follow-up time was 30.1 months (range = 2–143 months).

Detailed evaluation of stromal fibrosis using reticulin silver staining and cellular PDGFRβ expression following immunohistochemistry was performed at the Department of Pathology, University of Debrecen, Hungary. To specifically demonstrate PDGFRβ-positive bone marrow cells, samples were incubated with the anti-PDGFRβ (ab-32570; Abcam, Cambridge, UK) primary monoclonal antibody at a dilution of 1:100, as described previously.[6] Antibody binding was visualized by the Dako EnVision FLEX/HRP and FLEX DAB3 Chromogen detection system (Dako, Glostrup, Denmark). The PDGFRβ score was determined by microscopic analysis according to a previously proposed scoring system.[6] The reticulin grade staining was determined following Gömöri's reticulin staining according to the European Consensus on grading bone marrow fibrosis.[5] To enhance reproducibility,

both stainings were evaluated in a blinded fashion using the same criteria by two histologists independently, and the results were compared. In case of disagreement a consensus decision was achieved.

Statistical evaluation and graphs were made using the GraphPad Prism software. Mean and standard deviations were calculated for each group and analysed with Student's *t*-test. *P*-values <0.05 were considered statistically significant. Correlations between data sets were obtained using linear regression analysis.

Results

RETICULIN GRADE AND PDGFRβ EXPRESSION CORRELATE STRONGLY IN MF IRRESPECTIVE OF THE TYPE OF MPN

IHC preparations were generally of high quality and optimal for microscopic analysis, irrespective of the admission site, and semiquantitative microscopic evaluations were performed in all 193 samples from 84 patients using the previously established scoring system (Figure 1). The relation of MF grade and PDGFRβ score for each sample was assessed and a strong correlation was found between the amount of accumulated fibres (MF grade) and the activated stromal cell component expressed by the PDGFRβ score (Spearman's $r = 0.83$, $P < 0.0001$) (Figure 2A). Evaluation of the 126 samples from 55 cases with PMF diagnosis gave similar results (Spearman's $r = 0.86$,

$P < 0.0001$) (Figure 2B). This correlation was statistically constant through all grades of PMF when the initial (Spearman's $r = 0.84$, $P < 0.0001$) and the follow-up samples (Spearman's $r = 0.82$, $P < 0.0001$) were evaluated separately.

PREDICTION OF PROGRESSION IN ALL MF SAMPLES USING THE PDGFRβ SCORING SYSTEM

At the time of diagnosis, 14 of 84 cases showed MF-0, 30 cases MF-1, 21 cases MF-2 and 19 cases MF-3, while the PDGFRβ score was PDGFRβ-0 in four cases, PDGFRβ-1 in 31 cases, PDGFRβ-2 in 24 cases and PDGFRβ-3 in 25 cases. The PDGFRβ score was higher than the MF grade in 29 of 84 cases (34.5%), equal to the MF grade in 51 of 84 cases (60.7%) and lower than the MF grade in only four of 84 cases (4.7%). Thirty-four of the total 84 cases showed progression in MF grade during the whole follow-up period, while 40 cases did not show any change. The remaining 10 cases showed regression/reduction of fibrosis. Of these 10 cases, in three cases anti-MF medication was applied, while in a further three cases stem cell transplantation was performed; in the four remaining cases no clinical data were available. Representative morphological changes related to fibrosis progression are presented in Figure 3. We evaluated the relation of initial PDGFRβ scores with MF progression defined by the increase of MF grade during the follow-up period. Cases with no change or a decrease in fibre content during follow-up were

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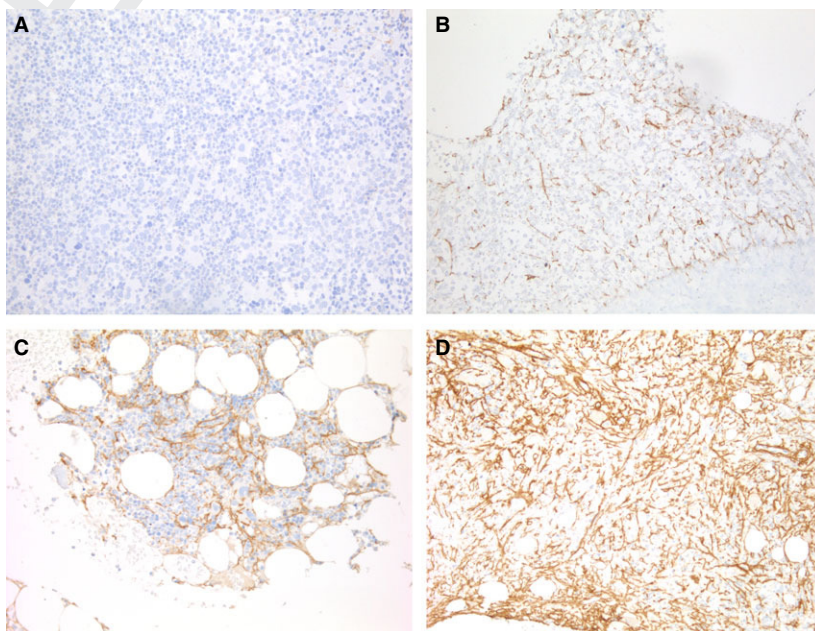
Figure 1. Platelet-derived growth factor receptor β (PDGFRβ) immunohistochemistry in bone marrow trephine biopsies highlights myelofibrosis-related stromal cell activation. Positive staining represents stromal fibroblasts in different amounts in parallel with reticulin fibre accumulation. A four-grade (0–3) scoring system was uniformly applicable for all initial and follow-up samples collected in our collaborative study (**A** = score 0, **B** = score 1, **C** = score 2, **D** = score 3).

Figure 2. Graphical presentation of the correlation between myelofibrosis (MF) grades and platelet-derived growth factor receptor β (PDGFRβ) score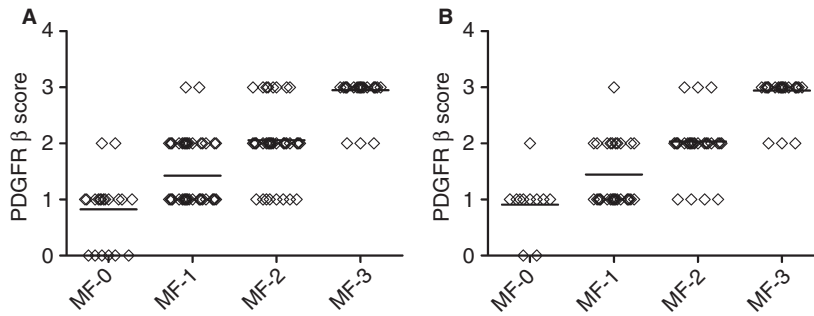s of the same bone marrow samples. The reticulin grade and the PDGFRβ immunohistochemistry (IHC) score were determined and matched in the 193 samples from all the 84 cases collected within this study (**A**) and in the 126 samples from the 55 primary myelofibrosis (PMF) cases (**B**).
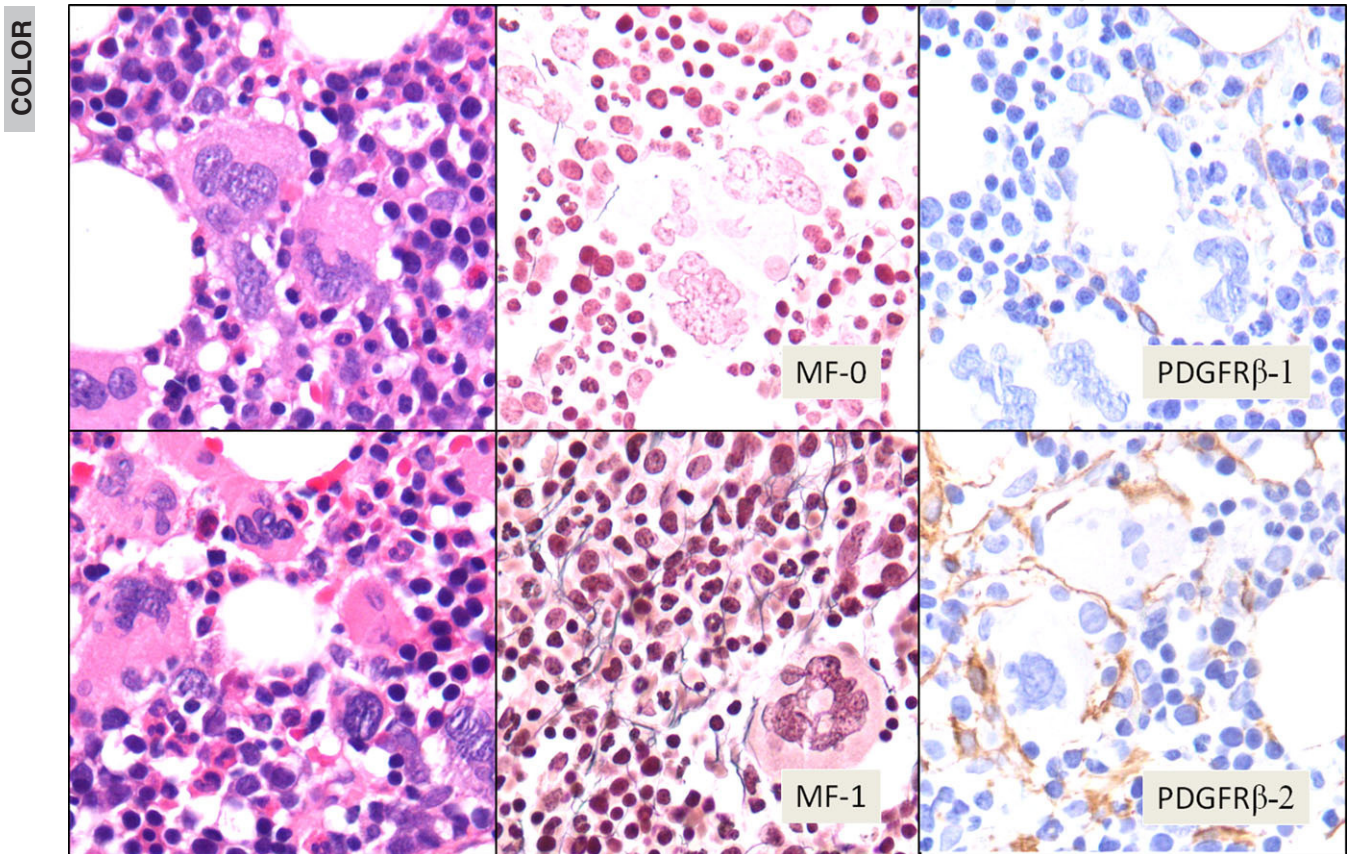
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Figure 3. Examples of the microscopic changes in the successive bone marrow samples from a case with progressive primary myelofibrosis (PMF). Hypercellularity [haematoxylin and eosin (H&E) staining], no fibre accumulation (MF-0, reticulin staining) and mild stromal cell activation [platelet-derived growth factor receptor β (PDGFRβ) score-1, immunohistochemistry] was visible in the initial biopsy sample (upper row). The same cellularity accompanied with mild increase in fibrosis (MF-1) and with extended stromal cell activation (PDGFRβ score-2) indicated progression in the follow-up sample obtained 7 months later (lower row).

considered as non-progressive. Nineteen of the 84 cases (22.6%) had already presented with fully developed myelofibrosis (MF-3) at the time of the initial diagnosis, and thus further progression was no longer possible. The statistical analysis was performed after their exclusion. Figure 4A shows the outcome of MF in cases with higher (*n* = 28), equal (*n* = 34) and lower (*n* = 3) PDGFRβ scores compared to MF grade
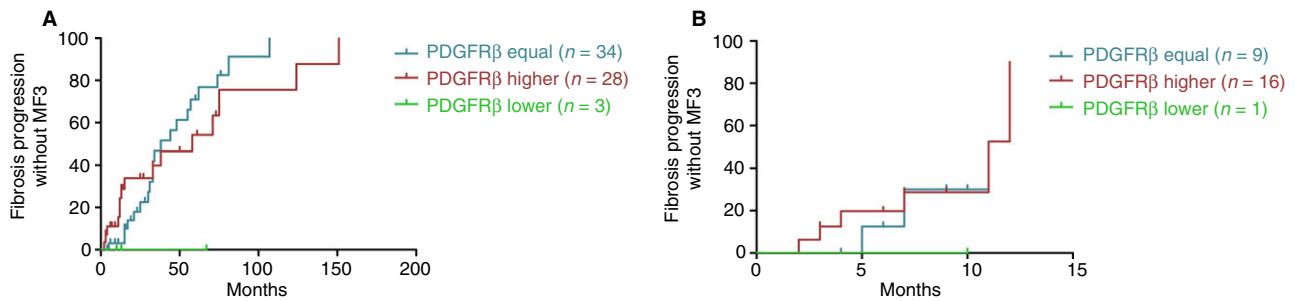
Figure 4. Probability of progression respective to the platelet-derived growth factor receptor β (PDGFRβ) score. The initially higher score did not predict fibrosis progression in the total sample collection after the removal of MF-3 cases (**A**). The same result was seen when short-term follow-up (12 months or less) was considered (**B**). Increase in myelofibrosis (MF) grade was defined as progression, while cases with decrease or no change in the MF grade were considered as non-progressive.

during the whole follow-up period. Using the log-rank test there was no significant difference in the probability of fibrosis progression in the different PDGFRβ subgroups ($P = 0.4547$). To evaluate the short-term effect of stromal cell PDGFRβ expression, the same analysis was performed for cases having follow-up samples within the initial 12 months after diagnosis (26 cases). Eleven of the 26 (42.31%) matched short-term sample pairs showed progression, while in 15 cases (57.69%) there was no progression. The log-rank test did not show any significant difference in the probability of fibrosis progression in these short-term follow-up series ($P = 0.7398$) (Figure 4B). PDGFRβ scores higher than the MF grade did not predict the progression of MF in general ($n = 65$), with appropriate sensitivity (43%) and specificity (57%) after the exclusion of MF-3 cases on a long-term basis (Table 1). In the short-term analysis, considering only the initial 12 months follow-up time ($n = 26$), the elevated PDGFRβ score proved to be predictive of progression, with 82% sensitivity and 53% specificity (Table 1).

Most interestingly, prefibrotic cases (initial MF-0) showing an elevated PDGFRβ score (ranging 1–2+) turned frequently to fibrotic cases, as measured by reticulin staining in the repeated biopsy. The progression from the prefibrotic to the fibrotic status could be predicted with 90% sensitivity and 75% specificity based on the initially higher PDGFR staining (Table 1). The prefibrotic samples evaluated here were of heterogeneous origin, including ET (two cases), PV (two cases), PMF (six cases), CML (two cases) and MPN-U (two cases). No other objective clinical (gender, age, medication) or histological (bone marrow involvement, cellularity, inflammation) parameter potentially influencing the extent and dynamics of the bone marrow stromal reaction could be identified.

Table 1. Predictive value of the platelet-derived growth factor receptor β (PDGFRβ) scoring for myelofibrosis (MF) progression

	All cases	All cases within 1 year follow-up	All prefibrotic cases
Number of cases	65	26	14
TP	15	9	9
TN	17	8	3
FP	13	7	1
FN	20	2	1
Sensitivity	43% (15/35)	82% (9/11)	90% (9/10)
Specificity	57% (17/30)	53% (8/15)	75% (3/4)

TP = true positive; TN = true negative; FP = false positive; FN = false negative.
Specificity and sensitivity was calculated considering all evaluated cases for the full length of follow-up, for the short-term (1 year) follow-up and separately for prefibrotic cases only. End-stage (MF-3) fibrosis was excluded, as these cases were not subjects of further progression.

PREDICTIVE VALUE OF PDGFRβ IN SELECTED CASES OF PRIMARY MYELOFIBROSIS

The group of 55 cases with a diagnosis of PMF was the largest and most homogeneous disease group within our multicentre study. At the time of diagnosis, six PMF cases proved to be prefibrotic (MF-0) and 20 cases showed MF-1, 11 cases MF-2 and 18 cases MF-3 reticulin grade. At the same time, the PDGFRβ score was PDGFRβ-0 in one case, PDGFRβ-1 in 18
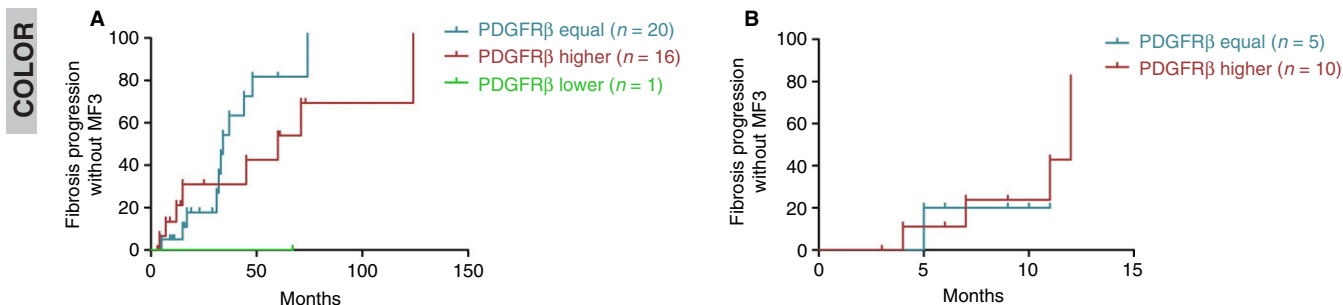
Figure 5. Probability of progression in primary myelofibrosis (PMF) reflected by the platelet-derived growth factor receptor β (PDGFRβ) score. The initially higher score did not predict progression of fibrosis during the full-length (**A**) or short-term (**B**) follow-ups. Initial MF-3 cases were excluded as these were not subjects of further progression.

cases, PDGFRβ-2 in 15 cases and PDGFRβ-3 in 21 cases. During disease follow-up, 24 of 55 cases (43.6%) with PMF remained unchanged, while 24 cases (43.6%) showed MF progression and seven cases (12.8%) regression/reduction of the MF degree. The stromal cell reaction measured by the PDGFRβ score remained unchanged in 33 of 55 cases (60.0%), 16 showed an increase (29.1%) and six cases (10.1%) a decrease during the total follow-up period.

The PDGFRβ score was higher than the MF grade in 14 of 55 cases (25.5%), equal to the MF grade in 39 of 55 cases (70.9%) and lower than the MF grade in only two of 55 cases (3.6%). Cases with a fully developed myelofibrosis (MF-3) at the time of diagnosis (18 of the 55) were excluded from further evaluation regarding progression. Figure 5A shows the progression of MF in cases with higher ($n = 16$), equal ($n = 20$) and lower ($n = 1$) PDGFRβ scores compared to the MF grade. There was no significant difference in the probability of fibrosis progression in the different subgroups ($P = 0.3905$). The dynamics of PMF on a short-term basis (within 12 months' follow-up history, a total of 15 cases) was also not associated statistically with the PDGFRβ scores ($P = 0.7915$), despite a prominent increase in selected cases with progression (Figure 5B).

In PMF, a higher PDGFRβ score than MF grade predicted the progression of MF with 40% sensitivity and 53% specificity after the exclusion of MF-3 cases ($n = 37$) (Table 2). The short-term ($n = 15$) progression prediction was effective with 83% sensitivity and 44% specificity (Table 2). The six prefibrotic PMF cases enrolled into this study did not allow a conclusion regarding the predictive utility of PDGFRβ scoring.

The potential effect of the initial PDGFRβ finding on the follow-up parameters of MF was also evaluated (Table 3). While the correlation between MF grade and PDGFRβ score was reproducibly strong within the same bone marrow samples at any time during our investigations, the initial PDGFRβ score correlated only moderately with either the MF grade or PDGFRβ score of the endpoint follow-up sample.

Discussion

Although a previous report also demonstrated PDGFR expression in thrombopoietic cells, our earlier studies and current observations revealed that PDGFRβ expression is limited to the endosteal and the perivascular stromal niche and reticular fibroblasts in the bone marrow.[14] These differences may be explained by the specificity of applied antibodies. PDGFRβ is a dynamically expressed and potentially targetable tyrosine-kinase receptor of this set of mesenchymal cell types. The role of PDGFR signalling has been studied extensively experimentally[15] and its role could be demonstrated in fibrotic disorders, such as in local and systemic sclerosis and in neoplasias of mesenchymal origin.[16–19] The increased amounts of PDGFRβ-expressing fibroblasts also proved to be a characteristic feature of the different degrees of myelofibrosis. Using PDGFRβ immunohistochemistry, the topography and dynamics of the newly generated fibroblasts can be followed in the diseased bone marrow. Although the link between receptor expression and matrix fibre synthesis is not completely clear, the total amount of the PDGFRβ immunopositivity was associated strongly with reticulin fibre deposition, which could also be reproduced by the present study. According to current opinion, the degree of MF is an important clinical parameter reflecting disease activity. The amount and distribution of reticulin and collagen fibres, however, is a result of the activity and proliferation of stromal fibroblasts. For this reason, we speculated that the cellular activation marker

Table 2. Specificity and sensitivity of the elevated platelet-derived growth factor receptor β (PDGFRβ) score to predict progression in primary myelofibrosis (PMF)

	PMF cases	PMF cases within 1 year follow-up	Prefibrotic PMF cases
Number of cases	37	15	6
TP	8	5	4
TN	9	4	0
FP	8	5	1
FN	12	1	1
Sensitivity	40% (8/20)	83% (5/6)	(4/5)
Specificity	53% (9/17)	44% (4/9)	(0/1)

TP = true positive; TN = true negative; FP = false positive; FN = false negative.
Initially, MF-3 cases were excluded, as these were not subjects of further progression. Analysis was performed for all PMF cases within the full follow-up range and also for cases having short-term follow-up. Prefibrotic PMF could not be evaluated separately due to low case number ($n = 6$).

PDGFRβ might be useful to highlight ongoing cellular processes occurring in association with or even in advance of stromal fibre deposition.

Using a microscopic scoring system, detailed evaluation of 193 bone marrow samples from five European centres clearly validated the close relationship between the conventionally determined MF grade and the fibroblast activation marker PDGFRβ. Moreover, elevated PDGFRβ scores indicated stromal activity in a significant portion of the samples, also disclosing early lesions lacking prominent reticulin fibrosis. According to our evolving concept, fibroblast activation may also reflect early phases of stromal reaction, the result of which can only be identified in subsequent bone marrow samples as clinically relevant fibrosis. In our comparison, however, no strong predictive role could be stated for the actual PDGFRβ status in myeloproliferative disease – even if the follow-up period was limited to only 1 year. Conversely, patients at risk for progressive bone marrow disease could be identified with a sensitivity of 90% and specificity of 75% among cases presenting initially with MF-0 (prefibrotic) if the PDGFRβ score was elevated (Table 1). Unfortunately, the low number of cases did not allow us to reproduce the same predictive effect

Table 3. Correlation of the initial platelet-derived growth factor receptor β (PDGFRβ) score with the follow-up status in primary myelofibrosis (PMF); PDGFRβ score correlated well with the MF grade of the same sample, but regression coefficients did not support a strong relation with the end-point bone marrow status

	Correlations with initial beta score ($n = 55$)		
	MF initial	MF endpoint	Beta endpoint
Spearman's r	0.8463	0.4002	0.4931
P-value (two-tailed)	<0.0001	0.0025	0.0001

when PMF was evaluated separately (six prefibrotic/ 55 PMF cases).

According to current opinion, MF is itself an accompanying condition appearing with variable dynamics in myeloproliferative disease. The course of myelofibrosis is influenced by many factors, and the appreciation of MF progression is difficult and somewhat limited. Early fibrosis may occur in different degrees throughout the bone marrow, frequently with only focal presentation in biopsy samples. In particular, the MF-2 grade may be associated with significant heterogeneity and a broad spectrum in parenchymal reticulin accumulation, making a single-parameter histological evaluation and statistical consideration problematic. The dynamics of fibrosis in bone marrow disease are also reliant upon the local cytokine/growth factor stimuli depending on the composition of the neoplastic microenvironment. Conversely, the fibre production may be affected significantly by the treatment applied. Due to this complexity, fibroblast activation and extracellular fibre accumulation represent two different biological aspects of the same pathological process. As a major finding of our study, we validated the generally strong linear association between bone marrow fibrosis and PDGFRβ-expressing mesenchymal cells. Further, the elevated PDGFRβ expression in initial prefibrotic bone marrow lesions indicated progression in the absence of fibre deposition. According to our concept, up-regulation of the PDGF receptor for optimal ligand binding is required very early in advance of significant stromal proliferation and matrix production, a feature that can be demonstrated uniformly in parallel with the neoplastic transformation of the bone marrow. Thus, increased PDGFRβ expression can be expected in all phases and even before manifest myelofibrosis (MF-0). Finally, the fibrotic process

may be gradually tuned down due to the loss of paracrine stimuli in end-stage fibrotic bone marrow disease (MF-3). For these reasons, the use of stromal activation markers, together with the classical disease characteristics, offers a completely new insight into the pathological processes against a background of ongoing stromal fibrosis. In clinical practice, PDGFRβ IHC provides valuable additional information when the reticulin staining is difficult to interpret or when immunostainings are preferred to classical histochemistry. Interpretation of the staining pattern requires only minimun effort and the proposed scoring is easily comparable with the reticulin-based grading.

In the current study we focused on the pathological progression of MF as an endpoint of our investigations, which covered mainly histological parameters. Clinical correlations regarding the PDGFR expression similar to the reticulin MF grade are highly difficult to measure due to the heterogeneous nature and the different, individually adapted clinical management of the myeloproliferative neoplasias. Probably for the same reasons, despite a consistent correlation within the same sample, an association between the initial and endpoint MF grade or PDGFR score could not be demonstrated (Table 3).

In summary, despite the above-mentioned biological–histological variables, PDGFRβ immunohistochemistry proved to be a robust indicator of marrow stromal reaction that could be measured reproducibly in bone marrow biopsies. PDGFRβ highlights the cellular aspects of fibrosis that are related closely to the reticulin and collagen accumulation. Its potential value to predict progression in early, prefibrotic myeloproliferative neoplasias could also be demonstrated based on a small number of retrospective follow-up cases which, however, calls for validation in a larger sample collection.

Acknowledgements

Conflicts of interest

The authors report no potential conflicts of interest.

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