SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

BONE MARROW STROMAL ACTIVATION IN DIFFERENT ONCOHEMATOLOGICAL DISORDERS

by
Judit Bedekovics, MD

Supervisor:
Gábor Méhes, MD, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE

DEBRECEN, 2014

BONE MARROW STROMAL ACTIVATION IN DIFFERENT ONCOHEMATOLOGICAL DISORDERS

By Judit Bedekovics, MD

Supervisor: Gábor Méhes, MD, PhD

Doctoral School of Clinical Medicine, University of Debrecen

Head of the Examination Committee: Prof. Dr. Zoltán Hernádi, MD, PhD, DSc
Members of the Examination Committee: Dr. Péter Antal-Szalmás, MD, PhD
Dr. Ilona Kovács, MD, PhD

The Examination takes place in the library of the Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Debrecen, December 15, 2014, 11:00 am.

Head of the Defense Committee: Prof. Dr. Zoltán Hernádi, MD, PhD, DSc
Reviewers: Dr. Enikő Bagdi, MD, PhD
Dr. Lajos Gergely, MD, PhD

Members of the Defense Committee: Prof. Dr. Zoltán Hernádi, MD, PhD, DSc
Dr. Péter Antal-Szalmás, MD, PhD
Dr. Enikő Bagdi, MD, PhD
Dr. Lajos Gergely, MD, PhD
Dr. Ilona Kovács, MD, PhD

The PhD Defense takes place at the Lecture Hall of Building ‘A’, Department of Internal Medicine, Faculty of Medicine, University of Debrecen; December 15, 2014, 13:00 pm.

1 INTRODUCTION

The microenvironment takes a significant part in the maintenance of bone marrow homeostasis and hemopoiesis. The stromal cells control the amount and distribution of cytokines, extracellular matrix components and ions which regulate the proliferative activity, differentiation and mobilization of hemopoietic stem cells.

In pathological conditions the activation of the microenvironment may lead to fiber accumulation called myelofibrosis. According to the composition of the extracellular matrix, myelofibrosis can be divided into two categories: reticulin and collagen fibrosis.

In the routine practice myelofibrosis can be most frequently detected in association with neoplasms such as primary lesions of the bone marrow (e.g. acute and chronic myeloid or lymphoid leukemias, myelodysplastic syndrome, myeloproliferative neoplasm, plasma cell dyscrasia, histiocytic neoplasm or mast cell leukemia). Beside these, fiber accumulation can develop due to solid tumor involvement of the bone marrow.

Matrix fiber accumulation has variable clinical significance in the different disorders. In primary myelofibrosis reticulin and collagen accumulation is one of the major features of the disease and it is an independent prognostic factor for the overall survival. In chronic myeloid leukemia fibrosis is also a negative prognostic factor which predicts the inadequate therapeutic response. Primary myelodysplastic syndrome is also frequently associated with myelofibrosis. It has been established that fibrosis is a negative prognostic factor for the overall survival in MDS. However, in the remaining large group of disorders, the effect of the mesenchymal cell expansion and the consequential myelofibrosis on the survival is less evident.

The detection of fiber accumulation is usually performed in decalcified, formalin fixed, paraffin-embedded trephine biopsies taken from the iliac crest. Microscopic semi-quantitative methods were introduced for the evaluation of myelofibrosis to make the extension of fiber accumulation comparable in clinical studies. The Gomori's silver staining is a widespread method for the detection of myelofibrosis which is based on the identification of the glycoprotein matrix surrounding the collagen fibrils. Using Gomori's silver impregnation the thin black reticulin fibers can be distinguished from the branches of thick yellowish-brown appearing collagen fibers. In the bone marrow of healthy individuals reticulin fibers can be detected in the vessel wall, and surrounding adipocytes in a scattered fashion, while collagen fibers are not present. Altogether ten semi-quantitative grading systems are known among which the Bauermeister's scale (1971) and the grading system of the European Consensus (2005) are widely accepted, both adapted to the same silver staining.

In fibrotic disorders the proliferation of fiber producing cells and the fiber production itself is under the control of tissue growth factors, such as the platelet derived growth factor (PDGF), which is responsible for the proliferation of fibroblasts.

PDGF receptors (PDGFR) are members of the membrane tyrosine-kinase receptor family, composed of the two subunits PDGFRα and PDGFRβ, which alternatively form homo- or heterodimers in a cell type specific manner. The different PDGFR dimers can activate signal pathways which show partial overlap. The most important effects of PDGF receptor activation are cell cycle promotion, actin reorganization and chemotaxis. Furthermore, PDGF receptors take part in embryo- and organogenesis.

Generally, fibroblast activation and proliferation and subsequent extracellular matrix production are the major steps in the development of fibrosis. The activation of PDGFR pathway is dominantly involved

in this first step. PDGF is secreted by macrophages and other inflammatory cells together with other cytokines which may stimulate the expression of PDGFR. In addition to reactive cell types megakaryocytes of the bone marrow show a high expression of the receptor ligand.

The PDGFR signaling pathway can be influenced in a number of ways, from which the inhibition of tyrosine-kinase activity seems to be highly effective. Currently, imatinib mesylate (imatinib, ST1571, Gleevec) is one of the most frequently used tyrosine-kinase inhibitor interacting with PDGFRα, PDGFRβ, bcr-abl fusion protein, c-kit and Flt3 activity. Imatinib and newer generation TK-inhibitors are widely used for the treatment of Philadelphia-chromosome-positive chronic myeloid leukemia, gastrointestinal stromal tumors and disorders bearing PDGFR gene involvement.

According to the current theory the cellular component of the bone marrow stroma may reflect basic information regarding the fibrotic process in addition to the assessment of fiber accumulation. Due to earlier studies it can be supposed that surface receptor compounds required for cell activity will specifically highlight fibrosis related changes. In contrast to the bone marrow fiber content, the extension of fiber producing cells may be a more dynamic parameter which may be altered faster by the surrounding factors. Based on the pathogenesis of myelofibrosis it can be hypothesized that the stromal cell proliferation may be a predictive factor for myelofibrosis progression potentially useful in disorders where the presence of fibrosis influences the survival.

Based on the PDGFR expression profile of normal bone marrow samples, positive and negative internal controls can be defined, which increase the reliability of the method. The immunohistochemical detection of PDGFRβ expression can be standardized more accurately than silver impregnation.

The features of immunohistochemical staining method let us precisely differentiate between the immunonegative and immunpositive areas offering the opportunity for digital image analysis. In contrast to the partially subjective semi-quantitative grading system, parameters provided by digital image analysis allow precise comparison of follow-up samples. It could be useful in cases where the efficiency of newer anti-fibrotic therapies is evaluated. The assessment of PDGFR expression may be at the same time the identification of a new therapeutic target in the era of tyrosine kinase inhibitors.

2 AIMS

1. The evaluation of PDGFRα and PDGFRβ expression pattern in normal, human bone marrow samples.

2. The assessment of PDGFR expression in different oncohematological disorders associated with myelofibrosis.

3. Correlation analysis between the amount of PDGFRβ positive stromal cells and the extension of fiber accumulation in myelofibrosis using a newly introduced semi-quantitative microscopic score system.

4. The evaluation of the predictive value of PDGFRβ expression on myelofibrosis progression in myeloproliferative neoplasms, where the presence of fiber accumulation influences the outcome according to the literature.

5. Development of a digital image analysis based method allowing to determine and to automatically measure the PDGFRβ immunpositive areas of the bone marrow.

6. Assessment of the correlation between the myelofibrosis grade and the image analysis based continuous parameters representing the number and extension of PDGFRβ positive cells.

3 METHODS:

3.1 Patients

3.1.1 Assessment of PDGFR expression and its correlation with MF grade

For the evaluation of PDGFRα and PDGFRβ expression, altogether 60 bone marrow biopsies were retrospectively evaluated, which had been collected for diagnostic purposes at the Hematology Department of the Institute of Internal Medicine, University of Debrecen, Hungary. The age ranged from 2 to 90 years, the number of females was 35, while the number of males was 25. We selected 47 representative bone marrow samples with diagnoses which potentially lead to MF, according to the literature. A further 13 cases with normal non-fibrotic (MF-0) bone marrow were selected as controls.

3.1.2 The predictive role of PDGFRβ expression in myelofibrosis progression

To assess the predictive role of PDGFRβ expression in myelofibrosis progression 193 initial and follow-up bone marrow biopsy samples from 84 patients with myeloproliferative neoplasia were provided by five hematopathology centers in collaboration with the European Bone Marrow Working Group. Usually two serial samples per case represented the course of the disease, although for some cases more biopsies were provided. The mean follow-up time was 34.43

months (2-151 months). Within the study collective PMF represented the largest homogenous disease group, which was evaluated separately. Altogether 126 initial and follow-up biopsy samples from 55 PMF cases were analyzed. The mean follow-up time was 30.17 (2-143 months).

3.1.3 The objective evaluation of PDGFRβ expression by digital image analysis

Altogether 79 trephine biopsy BM samples were selected with different degrees of myelofibrosis. All bone marrow specimens were obtained at the Hematology Unit of the University of Debrecen for diagnostic purpose. To evaluate the correlation between newly introduced PDGFRβ related image parameters and microscopic semi-quantitative methods (MF grade and PDGFRβ score), 10 non-pathologic and 32 pathologic samples were selected. For the validation of the algorithm 37 further pathologic BM samples were evaluated.

3.2 Processing of bone marrow biopsy samples

Bone marrow biopsies taken in or sent to the University of Debrecen were fixed in formaldehyde for 1 day which was followed by decalcification and embedding in paraffin wax.

3.3 The evaluation of MF grade

Reticulin staining and the evaluation of MF grade was performed in all part of the scientific work. The grade of reticulin

staining was determined in the microscope according to the European Consensus on grading bone marrow fibrosis. This is a widespread semi-quantitative system with four grades (MF-0, -1, -2, -3).

Gomori's silver impregnation

Reticulin silver impregnation (Gomori's staining) was done according to a standard protocol. Slides were oxidized with 0.5% potassium permanganate (60458, Sigma-Aldrich) for 5 min which was followed by rinsing with three changes of distilled water. A 1% solution of potassium metabisulfite (31628, Sigma-Aldrich) for 1 min was used for destaining. Slides were then washed with running tap water for 3 min and rinsed with four changes of distilled water. A freshly made solution of 10% silver nitrate (21572.188 VWR AnalaR Normapur) containing 2% potassium hydroxide (P5958, Sigma-Aldrich) was used for 1 min for impregnation. After impregnation the slides were rinsed with distilled water and then 10% solution of formalin (03300, Formaldehid Molar Chemicals) was used for 5 min, and slides were washed again. Exposure to a 0.2 % solution of chloride gold (520918, Sigma-Aldrich) for 30 s was followed by washes in distilled water. Finally, slides were fixed in 1% sodium thiosulphate (S7026, Sigma-Aldrich) and rinsed with two changes of distilled water before dehydration and coverage with a cover-glass.

3.4 Immunohistochemical examination

PDGFRβ immunohistochemical staining and the assessment of PDGFRβ score was performed in all part of the scientific work. The PDGFRβ scoring system is morphologically comparable to the current MF classification, it also has four scores (PDGFRβ-0, -1, -2, -3).

Besides the assessment of PDGFRβ expression, PDGFRα immunohistochemical staining was performed as well. In double immunohistochemical stainings anti-PDGFRα and anti-PDGFRβ antibodies were combined with each other and also with anti-nestin, anti-CD34 and anti-SMA primary antibodies.

PDGFR and double immunohistochemical stainings

For immunohistochemistry, samples were incubated with peroxidase-blocking reagent which was followed by antigen retrieval. To specifically demonstrate PDGFRβ and PDGFRα positive bone marrow cells, samples were incubated with the anti-PDGFRβ and anti-PDGFRα primary monoclonal antibody. Antibody binding was visualized by DAB chromogen detection system which was followed by hematoxylin counterstaining.

The intensity and the distribution of the immunoreaction was assessed by light microscope and for archiving the characteristic changes photos were taken by digital camera.

Double immunostaining was performed sequentially with the En Vision FLEX/HRP system. Following the first IHC staining terminating with the EnVision FLEX/HRP detection step, the incubation with the second primary monoclonal antibody was performed. Anti-PDGFRα, anti-PDGFRβ, anti-SMA, anti-CD34 and anti-nestin antibodies were used in different combinations. In addition to DAB (brown color) the chromogen VIP was used to highlight the second IHC reaction in a different color (dark violet). For double staining experiments methyl-green counterstaining was performed.

3.5 Imaging and digital image analysis for the objective evaluation of PDGFRβ expression

PDGFRβ IHC stained slides were captured by the Panoramic slide scanner device which consists a multicolor digital camera (Stingray F146C IRF Medical, Allied Vision Technologies, Stadtroda, Germany) supplied by 3DHistech (Budapest, Hungary). Digitalized images were decompressed to obtain image tiles 768x768 in size which were used for evaluation. Altogether 1437 tiles from 42 bone marrow biopsy samples were used for the development of the algorithm, which can be used for the objective assessment of PDGFRβ expression.

3.6 Statistical evaluation

*S*tatistical evaluation and graphs were made using the GraphPad Prism and Microsoft Excel softwares. Using semi-quantitative methods discrete values could be obtained and non-parametric statistical tests were performed, such as Spearman correlation and Wilcoxon paired test. The r value was calculated with 95% confidence interval, $p<0.05$ was considered to be a significant correlation, $r > 0.75$ was considered as strong correlation.

4 RESULTS:

4.1 PDGFR expression pattern of normal bone marrow samples

The evaluation of PDGFRα and β receptor expression profile was performed in non-fibrotic bone marrow biopsy samples. The two types of tyrosine kinase receptor subunits showed different distribution.

PDGFRα expression could be detected in endothelial and endosteal cells. In contrast, PDGFRβ subunit was expressed by perisinusoidal/pericapillary stromal cells (pericytes) and adventitial fibrocytes of the larger vessels in a pattern similar to normal reticulin fiber distribution. PDGFR expression could not be detected in adipocytes.

Double immunohistochemical stainings revealed PDGFRα/β co-expression in the single layer of bone lining endosteal cells. Significant PDGFRβ could be detected in the adventitial layer of the vessels, while these cells were negative for PDGFRα subunit, as well as for CD34 and SMA. Both the pericapillary and the perisinusoidal stromal cells (pericytes) were constantly positive for PDGFRβ and SMA, while the endothelial cells surrounded by them showed PDGFRα and CD34 expression.

Interstitial stromal cells (reticular cells) apart from the vascular system and the endosteal layer were virtually free of PDGFRβ labeling in the non-fibrotic control bone marrow group or only a minimum amount of scattered cells could be detected.

4.2 PDGFR expression pattern in myelofibrosis

Our studies on pathologic bone marrow samples showed, that only PDGFRβ expression level increased in fibrotic alterations while PDGFRα expression not. Increased PDGFRβ expression – similarly to the fiber accumulation – appeared in several different disorders. Our work focused on the myeloproliferative neoplasms and the myelodysplastic syndromes, although stromal activation could be identified in lymphoid and other myeloid neoplasms as well as in solid tumor metastases.

4.3 The correlation between MF grade and PDGFRβ score

To address the PDGFRβ subunit expression more accurately an immunohistochemistry scoring system was applied to different degrees of bone marrow fibrosis (ranging from 0 to 3 grades), which followed the quantitative approach of the well-established reticulin scoring system.

On the basis of 60 samples the correlation between the MF grade and the PDGFRβ scores proved to be strong (the Spearman r was 0.83). There was a good agreement between the PDGFRβ expression (score 0-3) and the myelofibrosis grade (grade 0-3) in the non-fibrotic control group (mean 0.0 (SD=0.0) vs. mean 0.15 (SD=0.38) respectively, p= 0.35; n=13). The MF grade was MF-0 in the control group by definition, while the PDGFRβ score was PDGFRβ-0 or

PDGFRβ-1. The stromal cell proliferation never exceed score 1 in this group.

Similarly, there was an agreement regarding MF and PDGFRβ scores in the majority of the pathologic bone marrow changes, 26 of 47. However, 21 evaluated in total cases showed a higher PDGFRβ score compared to the conventional myelofibrosis grade. In cases with pathologic bone marrow the score of PDGFR β expression was significantly higher than the myelofibrosis grade (mean 1.21 SD=0.98 vs. mean 1.66 SD=0.84 respectively, $p < 0.0001$)

Non-fibrotic cases with extended stromal cell activation raised the possible predictive role of PDGFRβ IHC staining. Unfortunately, follow-up samples from these cases were not available.

4.4 The predictive role of PDGFRβ expression in myelofibrosis progression

4.4.1 Correlation between PDGFRβ expression and MF grade

The statistical analysis of all 193 bone marrow samples from 84 cases resulted in a strong correlation between the amount of accumulated fibers (MF grade) and the activated stromal cell component (PDGFRβ score); Spearman $r=0.83$, $p<0.0001$. Statistical evaluation of the 126 samples from 55 cases with PMF diagnosis led to very similar results; Spearman $r=0.86$; $p<0.0001$. 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.

Follow-up cases were classified into two groups according to the changes in MF grade. MF progression was defined by the increase of MF grade during follow-up period (n=34). Cases without any change or

decrease in fiber content during follow-up were considered as non-progressive (n=50). The outcome of MF was assessed during the whole follow-up period in cases with higher (n=28), equal (n=52), and lower (n=4) PDGFRβ score compared to MF grade. Using the log rank test there was no significant difference in the probability of fibrosis progression in the different PDGFRβ subgroups (p=0.3028). However, 19 of 84 cases presented already at the time of the initial diagnosis with fully developed myelofibrosis (MF-3) and thus, could not progress further. The statistical analysis done after their exclusion ended with a similar result (p=0.4547).

To evaluate the short term effect of PDGFRβ activation, the same analysis was performed for cases having follow-up samples within 12 months (37 cases). The log rank test did not show significant difference in the probability of fibrosis progression in these short-term follow-up series (p=0.2084) which looked similar after the exclusion of MF-3 cases (p=0.7398).

It could be hypothesized that in pre-fibrotic cases PDGFRβ expression predicted MF progression, but this predictive feature disappeared in cases with advanced fibrosis. At the time of initial diagnosis 14 cases proved to be pre-fibrotic (grade MF-0). Difference in the probability of fibrosis progression could not be stated using the log-rank analysis (p=0.1982).

PDGFRβ scores higher than MF grade predicted the progression of MF in general (n=65) with a sensitivity of 43% and a specificity of 46% after the exclusion of MF-3 cases. In the short-term analysis considering only the initial 12 months follow-up time (n=26), elevated PDGFRβ score proved to be predictive of progression with a sensitivity of 82% and a specificity of 53%. Most interestingly, pre-fibrotic cases (initial MF-0) showing an elevated PDGFRβ score became frequently fibrotic and the progression could be predicted with a sensitivity of 90% and a specificity of 75%.

4.4.2 Correlation between PDGFRβ expression and MF grade in PMF

PMF was the largest and most homogenous disease group within our multi-center study, so all analysis were repeated in this subgroup.

The probability of MF progression was evaluated in cases with higher (n=14), equal (n=39) and lower (n=2) PDGFRβ score compared to the MF grade. There was no significant difference in the probability of fibrosis progression in the different subgroups (p=0.3905). We also performed the evaluation of PMF cases with only a short (within 12 months) follow-up history (20 cases). Although progressive cases were associated with higher PDGFRβ score, there was no statistically significant difference between the subgroups (p=0.7002).

In PMF, a bone marrow status with higher PDGFRβ score than MF grade predicted progression of MF with a sensitivity of 40% and a specificity of 53% after the exclusion of MF-3 cases (n=37). The short-term analysis resulted in 83% sensitivity but only 44% specificity for the progression prediction of elevated PDGFRβ score (n=15).

4.5 Digital image analysis for the objective evaluation of PDGFRβ expression

Based on hue value and saturation, two separated layers were created during image processing of digitalized slides. The first layer defined only the IHC related brown staining or “brown layer” while the rest of the hematoxylin stained bone marrow parenchyma was determined as the “violet layer”. The sum of purified brown objects obtained from the brown layer after background correction was called “brown component (Bc)”, while the purified immunonegative, violet

layer was called “violet component (Vc)”. Vc and Bc components together formed the intertrabecular hemopoietic parenchyma, which was called “region of interest, (ROI)”.

Using the region of interest (ROI) and the brown component (Bc) the following image parameters were evaluated. In first line the number of objects: the sum of the intertrabecular brown objects, which corresponds to the number of PDGFRβ positive cells and cell clusters. SumArea: the sum of the area (number of pixels) of all brown objects. SumPerimeter: the sum of the perimeter of all brown objects. SumSkeleton: the sum of the skeleton of all brown objects. An additional parameter used was the weighted Perimeter (wPerimeter) where the number of cross-points and end-points within the objects were considered. The highest fifty derivates of values (Top50 values) were also calculated, such as Top50Area, Top50Perimeter, Top50Skeleton. Top50 parameters can be given for each tile of a case or for the whole slide as well. Relevant parameters were also evaluated in relation to the total marrow parenchyma (/ROI), so the cellularity of the sample could be also considered.

Overall 13 parameters were defined, which were the followings SumArea, SumPerimeter/ROI, SumSkeleton/ROI, Number of objects/ROI, Top50Area, Top50Perimeter, Top50Skeleton, Number of objects/Bc, wPerimeter/ROI.

4.6 Correlation between PDGFRβ expression related, image analysis based parameters and MF grade

4.6.1 Validation of the detection of PDGFRβ expression

From the 13 parameters eight showed strong correlation with the microscopic MF grade ($r>0.75$) and with the PDGFRβ score as well

(r>0.75) in the first set of 42 samples. These eight parameters were further assessed. Image processing and statistical analyses were repeated for validation purpose in further 37 samples. Significant correlation of the eight image analysis parameters with MF grade and PDGFRβ score could be clearly validated. Spearman r ranged between 0.72 and 0.81 for MF grade and between 0.70 and 0.85 for PDGFRβ score.

4.6.2 PDGFRβ image parameters correlation with MF grade

There was no measurable difference between the normal control BM and pathologic BM lacking fibrosis (MF0). In contrast, a gradual increase in all evaluated data sets could be seen from the MF0 to the MF3 grades in the diseased bone marrow. All of the eight selected parameters showed significant difference between MF0 and MF1 grades (P values between 0.012 and 0.047); M0 and MF2 grades ($P<0.0001$ in all settings); and MF0 and MF3 grades ($P<0.0001$ in all settings). All parameters of MF1 (mild fibrosis) vs. MF2 (intermediate reticulin fibrosis) cases showed robust differences with high significance levels. None of the applied parameters resulted in statistically significant differences between pathologic MF2 and pathologic MF3 grades (p values between 0.2914 and 0.9957). ROC-analysis revealed similar differences in cases with different MF grades.

4.6.3 Comparison of PDGFRβ expression in follow-up cases using image analysis

Bone marrow heterogeneity is well known in MF. The bone marrow parenchyma is not uniformly involved by the distribution of extracellular matrix fibers. The classical MF grading is mainly based on the dominant changes occurring in the section. Besides the MF grade,

the proliferation of PDGFRβ positive stromal cell show the same heterogeneity. PDGFRβ immunohistochemistry based image measurements can reflect the mean of all events irrespective of their distribution.

Tissue heterogeneity is difficult to consider using the semi-quantitative evaluation done by microscopy and this is also true for fine differences between follow-up cases. Using selected image parameters an objective comparison of individual samples became possible.

5 DISCUSSION

Myelofibrosis is characterized by stromal activation which results in reticulin and collagen accumulation. In PMF and MDS the presence of fiber accumulation is prognostically relevant and correlates with the overall survival.

The Gomori's staining is performed as gold-standard method for the detection of myelofibrosis. To assess the extension of the fibrosis a semi-quantitative grading system is used which has four grades (0-3).

The MF grade reliably describes the BM fiber content, but does not provide information about cellular participants of stromal activation. Activation and proliferation of stromal cells were detected in several fibrotic disorders and the role of certain growth factors in the pathogenesis of fibrosis was also established. Among these growth factors, TGF-β, PDGFAA and PDGF BB have major role. The PDGF ligand binds to the PDGF receptor, which has two subtypes: PDGFRα and PDGFRβ. The PDGFR belongs to the tyrosine-kinase receptor family, so its activity can be inhibited by tyrosine-kinase inhibitors.

The identification of PDGFR expression in fibrotic disorders led to the testing of tyrosine-kinase inhibitors in these alterations. Several *in vitro* studies supported the fact that fibroblast proliferation can be blocked by tyrosine-kinase inhibitors. In animal models the tyrosine-kinase inhibitors improved the outcome of pulmonary fibrosis, cirrhosis and nephrosclerosis, even though in clinical trials the results were either controversial or not available yet.

The increased production of growth factors has been established in myelofibrosis as well, although the assessment of stromal cell

proliferation has not been studied yet. The tyrosine-kinase receptors may be potential therapeutic targets in myelofibrosis as well, however, only indirect results were reported so far.

One of the aims of the PhD work was to assess the PDGFRα and PDGFRβ expression in stromal cells of the normal and fibrotic bone marrow and to evaluate the correlation between the amount of PDGFR positive stromal cells and myelofibrosis.

In normal bone marrow, PDGFRα expression was detected in endothelial and endosteal cells together with the partial expression of interstitial fibroblasts. PDGFRβ was expressed by pericapillary and perisinusoidal pericytes, stromal cells in the adventitial layer of vessel wall, endosteal cells and by interstitial fibroblasts.

In bone marrow samples with fibrosis, increased PDGFRβ expression could be detected.

To assess the correlation between the PDGFRβ expression reflecting the amount of fiber producing fibroblasts and the MF grade reflecting the amount of fiber deposition a semi-quantitative scoring system was created. The score ranges from 0 to 3, to make the system practical and comparable with the widely used reticulin scoring system. On the basis of 60 cases strong correlation could be detected between the MF grade and the PDGFRβ score. However, in 21 of 60 cases, the PDGFRβ score exceeded the fibrosis grade. This observation raised the potential predictive role of increased PDGFR expression.

According to our hypothesis stromal cell activation and proliferation is required for fiber production, therefore, the predictive value of elevated PDGFRβ expression for myelofibrosis progression in myeloproliferative neoplasms was analyzed. In this disorder the negative prognostic effect of fiber accumulation was established, so that its early detection may have clinical relevance.

193 initial and follow-up bone marrow biopsy samples from 84 patients with myeloproliferative neoplasia were retrospectively

assessed. In general, higher PDGFRβ scores than MF grade did not increase significantly the probability of MF progression. Cases with short-term follow-up and prefibrotic initial sample showed similar result. However, elevated PDGFRβ scores predicted the MF progression in the prefibrotic group with the highest sensitivity (90%) and specificity (75%). Our data indicate to a direct prognostic impact in prefibrotic myeloproliferative disorders.

Microscopic analysis and semi-quantitative grading method do not enable us to compare follow-up samples precisely. To evaluate the therapeutic efficiency of new treatment protocols, objective and more accurate methods are needed. A digital image analysis based algorithm was developed during the PhD work which allows the objective assessment of stromal activation and precise comparison of different samples.

Our image analysis based method can be used for automated processing of such bone marrow biopsy samples, where DAB chromogen was used in the immunohistochemical staining. After that the immunopositive (Bc), immunonegative (Vc) areas and the region of interest (ROI) was determined, 13 parameters were defined from which 8 showed strong correlation ($r>0,75$) with the MF grade and also with the PDGFRβ score. All of these parameters showed significant difference between prefibrotic cases (MF-0), cases with mild (MF-1), moderate (MF-2) and advanced (MF-3) fibrosis.

Image parameters reflecting the PDGFRβ expression highlight properly the heterogeneity of BM samples, which cannot be assessed using conventional microscopic methods. Objectivity is increasingly required for the comparison of follow-up biopsy samples to measure the efficiency of newer anti-fibrosis therapies.

As a conclusion, the major statements of the PhD work are the followings.

1. Bone marrow stromal cell express PDGFRα and PDGFRβ receptor subtypes in a highly special distribution and mostly limited to vessels and bone trabecules in normal conditions.

2. PDGFRβ expression is increased in disorders with BM fiber accumulation.

3. The PDGFRβ expression reflecting the number of activated stromal cells strongly correlates with the MF grade.

4. The PDGFRβ score has a potential predictive value for myelofibrosis progression in the prefibrotic bone marrow, however, this statement requires further examinations.

5. The newly developed digital image analysis based algorithm is able to automatically detect the hematopoietic areas and immunopositive areas with high specificity.

6. The image analysis based parameters reflecting the size and complexity of PDGFRβ positive cell groups strongly correlate with the MF grade determined by microscopy. Image analysis can be reliably used for the comparison of follow-up samples.

6 SUMMARY

Reticulin and collagen accumulation, directing to stromal activation, can occur in different oncohematological conditions and even in solid tumor metastasis in the bone marrow. The occurrence of myelofibrosis (MF) has prognostic impact in myeloproliferative neoplasias – first of all in primary myelofibrosis – and in myelodysplastic syndrome. It has been established that platelet derived growth factor (PDGF) produced by megakaryocytes has a major role in the proliferation of fiber producing cells. On the other hand, the overexpression of PDGF receptor (PDGFR) can be seen in several fibrotic disorders. Currently, some studies focus on the potential therapeutic role of tyrosine-kinase inhibitors in these alterations.

The aim of the PhD work was to examine the PDGFR expression pattern and distribution in the normal bone marrow and to assess the correlation between the PDGFR expression and myelofibrosis grade. Further, the predictive potential of PDGFR overexpression in progressive myelofibrosis was evaluated. Finally, an objective image analysis method was introduced to detect the amount and distribution of the cellular component during stromal activation of the bone marrow highlighted by PDGFR immunohistochemistry.

We concluded, that different expression patterns of the PDGFRα and β isoforms characterize the bone marrow stromal cell populations. A strong correlation between MF grade and PDGFRβ expressing fibroblasts could be established. On the basis of our observations, PDGFRβ overexpression may be predictive for MF progression in pre-fibrotic cases, although further examinations are needed to prove it. Using digitalized slides with PDGFRβ immunostained bone marrow

biopsy sections and a newly developed software algorithm we proposed an automated method, which recognizes hemopoetic bone marrow parenchyma and immunpositive areas with high specificity. This method reflected significant differences in stromal activity and demonstrated intraparenchymal heterogeneity in different hematopathological conditions of the bone marrow. In summary, PDGFRβ expression proved to be a reliable and useful marker of the cellular component during stromal activation in the bone marrow.

7 ACKNOWLEDGMENTS

The work was supported by the TÁMOP- 4.2.2.A-11/1/KOV-2012- 0045 "Research network on vascular biology/medicine" project grant. Judit Bedekovics was a research fellow of the „National Excellence Program – Elaborating and operating an inland student and researcher personal support system" (TÁMOP 4.2.4. A/2- 11-1-2012-0001) subsidized by the European Union and co-financed by the European Social Fund.

UNIVERSITY OF DEBRECEN
UNIVERSITY AND NATIONAL LIBRARY
PUBLICATIONS

DEENK

Register number: DEENKÉTK/169/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Judit Bedekovics
Neptun ID: DA081R
Doctoral School: Doctoral School of Clinical Medicine
MTMT ID: 10036513

List of publications related to the dissertation

1. **Bedekovics, J.**, Szeghalmy, S., Beke, L., Fazekas, A., Méhes, G.: Image analysis of platelet derived growth factor receptor-beta (PDGFRβ) expression to determine the grade and dynamics of myelofibrosis in bone marrow biopsy samples.
   Cytom. Part B-Clin. Cytom. 86 (4), 31 p., 2014.
   IF:2.231 (2012)

2. **Bedekovics, J.**, Kiss, A., Beke, L., Károlyi, K., Méhes, G.: Platelet derived growth factor receptor-beta (PDGFRβ) expression is limited to activated stromal cells in the bone marrow and shows a strong correlation with the grade of myelofibrosis.
   Virchows Arch. 463 (1), 57-65, 2013.
   DOI: http://dx.doi.org/10.1007/s00428-013-1434-0
   IF:2.676 (2012)

List of other publications

3. **Bedekovics J.**, Méhes G.: A csontvelőfibrosis patomechanizmusa és előfordulása neoplasticus kórképekben.
   Orvosi Hetilap. 155 (10), 367-375, 2014.
   DOI: http://dx.doi.org/10.1556/OH.2014.29823

H- 4032 Debrecen, Egyetem tér 1. ¤ E-mail publikaciok@lib.unideb.hu

4. Selmeczi A., Udvardy M., Illés Á., Telek B., Kiss A., Batár P., Reményi G., Szász R., Ujj Z., Márton A., Ujfalusi A., Hevessy Z., Pinczés L., **Bedekovics J.**, Rejtő L.: Heveny myeloid leukaemiás betegeink kezelésével szerzett tapasztalataink (2007-2013).
   Orv. Hetil. 155 (17), 653-658, 2014.
   DOI: http://dx.doi.org/10.1556/OH.2014.29884

5. **Bedekovics, J.**, Rejtő, L., Telek, B., Kiss, A., Hevessy, Z., Ujfalusi, A., Méhes, G.: Identification of NPMc+ Acute Myeloid Leukemia in Bone Marrow Smears.
   Appl. Immunohistochem. Mol. Morphol. 21 (1), 73-78, 2013.
   DOI: http://dx.doi.org/10.1097/PAI.0b013e318256da37
   IF:1.828 (2012)

6. Szeghalmy, S., **Bedekovics, J.**, Méhes, G., Fazekas, A.: Digital Measurement of Myelofibrosis Associated Platelet Derived Growth Factor Receptor β (PDGFR β) Expression in Bone Marrow Biopsies.
   J. Comput. Inf. Sci. Eng. 1, 47-56, 2013.
   DOI: http://dx.doi.org/10.2498/cit.1002109
   IF:0.488 (2012)

7. **Bedekovics J.**, Hevessy Z., Kappelmayer J., Kiss C., Csáthy L.: Sejtfelszíni antigének expressziójának változása a gyermekkori akut lymphoblastos leukaemia kezelése alatt-négyszínű MRD-detektálással szerzett tapasztalataink.
   Hematológia-Transzfuziológia. 43 (3), 215-224, 2010.

8. **Bedekovics J.**, Rejtő L., Telek B., Udvardy M., Ujfalusi A., Oláh É., Hevessy Z., Kappelmayer J., Kajtár B., Méhes G.: Mutáns nucleophosmin fehérje kimutatása akut myeloid leukaemiában: Az NPMc+ AML biológiai és klinikai jellemzői.
   Orv. Hetil. 150 (22), 1031-1035, 2009.
   DOI: http://dx.doi.org/10.1556/OH.2009.28623

Total IF of journals (all publications): 7.223
Total IF of journals (publications related to the dissertation): 4.907

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

10 July, 2014

H- 4032 Debrecen, Egyetem tér 1. ¤ E-mail publikaciok@lib.unideb.hu