STROMAL GROWTH RELATED BIOMARKER AND MATRIX PROTEIN EXPRESSION IN PRIMARY MYELOFIBROSIS IN RELATION TO GÖMÖRI'S SILVER GRADING

PhD thesis

Tamás Székely MD

Doctoral School of Pathological Sciences
Semmelweis University

Supervisor: Tibor Krenács, PharmD, DSc

Official reviewers: Béla Kajtár, MD, PhD and Deján Dobi, MD, PhD

Head of the Comprehensive Exam Committee: Miklós Kellermayer, MD, DSc

Members of the Comprehensive Exam Committee: Pál Maurovich-Horváth, MD, DSc and Miklós Kozlovszky, PhD

Budapest
2023

1. Introduction

Myeloproliferative neoplasms are a diverse collection of diseases caused by the abnormal development of myeloid cell lines. Primary myelofibrosis (PMF) is a member of the myeloproliferative neoplasms (MPN) characterized by aberrant clonal stem cell-derived myeloproliferation. Chronic myeloid leukemia (CML), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), essential thrombocythemia (ET), polycythemia vera (PV), juvenile myelomonocytic leukemia (JMML) are other conditions that fall within the MPN umbrella. While CML is a Philadelphia chromosome (*BCR::ABL1*) positive disease , PMF, ET, and PV are *BCR::ABL1*-negative MPNs. All of these neoplasms are characterized by clonal myeloid lineage growth and in PMF an increased stromal cell activation. By overproducing extracellular matrix as a result of stromal cell activation, the bone marrow space shrinks, which physically compromises hemopoiesis. Certain genetic mutations are present in PMF. Driver mutation in the Janus kinase 2 (JAK2), Calreticulin (CALR), or proto-oncogene, thrombopoietin receptor (MPL) is present in roughly 90% of PMF patients. Less commonly, mutations of additional clonal markers (such as ASXL1, EZH2, TET2, IDH1/2, SRSF2 and SR3B1) are also involved. Functional modification of the JAK binding cytokine receptor is the common final consequence of genotypical alterations. Deregulated overproduction and release of pro-inflammatory cytokines (such as IL-1, TGF-ß), growth factors (such as bFGF, PDGF, and VEGF), and extracellular matrix components (fibronectin, laminin and collagens) are caused by constitutive JAK-STAT signaling, which is triggered by abnormal receptors. These chemical factors' secondary effects on the bone marrow matrix have been directly linked to the development of fibrosis and osteosclerosis. Increased cytokine-

directed signaling, increased activation of the ERK/MAPK and PI3K/Akt pathways, as well as the action of chemicals released upon neutrophil engulfment that have dysregulated receptor activity, all contribute to increased synthesis of connective tissue fibers In fibroblast activation, increased production and release of platelet-derived growth factor (PDGF), fibroblast growth factor receptor (FGF), transforming growth factor beta (TGFβ) and vascular endothelial growth factor (VEGF) play important roles .

PMF is more aggressive condition than ET, PV, and pre-MF. It is progressive and has a dramatic impact on the patients' quality of life. Patients with ET, PV, or pre-MF run the risk of developing myelofibrosis, which may be referred to as post-ET-MF, post-PV-MF, etc. Patients with PMF have more mutations, cytogenetic abnormalities, and a higher likelihood of leukemic transformation. Up to one-third of patients are asymptomatic at the time of diagnosis, and many of these people are identified when unrelated blood tests reveal minor abnormalities, such as anemia and thrombocytopenia.

1.1 Diagnostic procedures of PMF

The diagnosis and grading of PMF are still mainly established by using microscopic evaluation of bone marrow biopsy sample. Evaluation is semi-quantitative, based on Gömöri's silver staining, and requires not only strictly regulated laboratory processing but also experienced hematopathologists. Due to a number of influencing factors such as preanalytical conditions, staining variables, a lack of a positive staining internal standard, and of course diagnostic subjectivity, standardization of the selective silver impregnation is rather difficult. Inter-rater differences might be large when it comes to grade evaluation. To objectively quantify BMF, computer-assisted image analysis is being used more often in clinical studies as an auxiliary to semi-quantitative grading in addition to human evaluation. Computer-assisted image analysis has been proven to be more accurate than

regular histology in detecting BMF level changes in MF patients receiving therapy, and it also correlates well with morphology

1.2 Biomarkers involved in PMF progression

1.2.1 Stromal cell's growth related biomarkers

L-NGFR has been involved in the regulation of neuronal survival and apoptosis but it can also act as a tyrosin kinase co-receptor for the anti-apoptotic tropomyosin receptor kinase A (TrkA) to enhance MAPK pathway activation, culminating in ERK1-2 signal transducer phosphorylation and nuclear translocation in stromal cells.

CXCL12 a chemokine, ligand of CXCR receptors, produced by stromal, endothelial cells and osteoblasts which plays an important role in maintaining stem cell quiescence by safeguarding both the perivascular and endosteal bone marrow niches.

1.2.2 Stromal cell related extracellular matrix components

Collagens are fibrillar proteins, main structural component of many tissues in the body. Regenerative fibrosis and bone marrow fibrosis are associated with increased type I and type III collagen synthesis and deposition.

Fibrillin-1 is an evolutionarily conserved extracellular matrix glycoprotein. It is known for serving as a template framework in the construction of elastic fibers network. It plays a role in regulating the activity of certain growth factors, such as TGF-β, by binding to and sequestering them in the extracellular matrix.

2. Objectives

1. Our major objective was to find potentially diagnostic biomarkers of primary myelofibrosis progression. We highlighted specific molecules in association with bone marrow stromal cell activation and examined if their expression level was in line with the conventional Gömöri's silver impregnation-based myelofibrosis grades.

2. At first the expression of bone marrow stromal cell growth related biomarkers, L-NGFR receptor, CXCL12 motif chemokine, and phospho-ERK1-2 signal transducer/transcription factor were evaluated with immunohistochemical labeling. Our goal was to identify if any of these biological markers could be used to a supplement the Gömöri' silver impregnation based histological myelofibrosis grading.

3. Major extracellular matrix proteins involved in regenerative fibrosis including type I collagen, type-III collagen and fibrillin-1 were selected for immunohistochemical labeling in the second part of the study. Our goal was also to investigate the correlation between stromal cells matrix production and histological grades classified by Gömöri's silver staining, and select the biomarker with the strongest and statistically most significant association with MF grades.

4. Our additional objective was to develop an immunoreaction-based, computer-assisted, semi-automated digital image quantification method using a machine-learning based computer algorithm. We intended to demonstrate if this approach can accurately reproduce and, perhaps in the future, even replace Gömöri's silver staining-based grading when set up using eye-control.

3. Methods

Our cohort included Jamshidi biopsies of patients diagnosed with primary myelofibrosis at the 1st Department of Pathology, Semmelweis University, between 2016 and 2022. The selection of samples was based on pathological diagnoses. It was followed by the collection of paraffin blocks and Hematoxylin Eosin (HE) stained slides from the archive. A quick review was done to make sure that the quality and number of samples in the block were good and enough for the study to begin. There was no repeated HE staining, however, Gömöri's reticular fiber staining was performed again in all cases that were selected for analysis to confirm or supervise the original grading. The study was divided into two parts. Firstly the expression of L-NGFR, phospho-ERK1-2, and CXCL12 were studied, later type I, type III, and fibrillin-1 were marked and evaluated on an extended sample collection. A total of 60 and 68 samples were studied in the first and the second part of the study, respectively. Due to the consumption of tissue within the paraffin blocks, some sample had to be replaced by new ones selected from the same grade group. MF-0 grade group samples were expanded with 5 additional cases for the second sample set. And also, an additional 5 non-myelofibrotic, non-fibrotic bone marrow samples were selected as negative controls for the second part of the study.

Gömöri's silver staining was used to determine the grade of MF. These results served as the classification basis for our samples. The procedure of diagnostic reticulin grading followed the European Consensus set up for bone marrow fibrosis.

With the help of a Pannoramic 1000 Scanner (3DHistech Ltd., Budapest), all stained slides used in this study were subjected to whole slide digitalization. Visual analysis on high resolution monitors and scoring of each staining series were then performed using the SlideViewer program (3DHistech). A similar

methodology to that used to evaluate Gömöri's reticulin staining was applied to evaluate the extracellular matrix immunoreactions. Score 0: only infrequent, fragmented fibrils with no intersections; Score 1: focal, loose networks with only perivascular intersections; Score 2: medium density of positive fibrils with regular intersections and some extrafibrillar deposition; Score 3: diffuse, densely intersecting fibril networks and more extrafibrillar antigen deposition.

Fibrillin-1 immunoreaction series was selected for digital image analysis. The QuantCenter software package's PatternQuant module, a semi-automated machine learning algorithm, was used for the image analysis of (all 3DHistech). Each slide was annotated with three to five representative areas, and each slide was then subjected to image segmentation using a template designed to highlight all brown staining, from faint but clearly distinct signals to strong intensities. Immune-positive (brown) immunoreactions were highlighted in red, immune-negative cells and tissue were highlighted in green, and cell-free regions were highlighted in yellow. The immunopositive tissue area fractions in % (red/(red+green) x100) within the chosen annotations were calculated for each case using the same template.

The study was conducted in accordance with the Helsinki Declaration. Our study was performed in line with the regulations of the WMA Declaration of Helsinki and was approved by the National Committee for Research Ethics (ETT TUKEB) under the number IV/129/2022/EKU.

4. Results

Stromal cell growth related biomarker expression in PMF progression

First, we analyzed the levels of expression of growth-related biomarkers, including L-NGFR, phospho-ERK1-2, and CXCL12. These biomarkers were primarily detected in the stromal cells of bone marrow, and they play a crucial role in promoting fibroblasts' matrix manufacturing activity. In our studies, it was found that the increasing density of L-NGFR receptor levels had the best correlations with myelofibrosis grades and the best overall agreement between different labs.

When demonstrated in parallel sections, it was found that the gradually increasing density of L-NGFR positive stromal cell processes matched with that of the reticular and collagen fibers of silver impregnation. Despite this, it was clear that there were fewer interconnections between L-NGFR positive projections than there were between silver-stained fibers.

Compared to the value of L-NGFR, the consolidated scores of both p-ERK1-2 and CXCL12 demonstrated a significance that was less prominent concerning their grade separation values. These markers were primarily found in the nuclei (p-ERK1-2) and in the cytoplasm (both p-ERK1-2 and CXCL12) of stromal cells, which projected only thin processes and had almost no interconnections between them.

Statistically significant data suggests that L-NGFR reaction exhibited the highest discriminating power of the biomarkers that were tested. This was the case regardless of the myelofibrosis grade. This was the only marker that could be identified solely in stromal cells and in all of the processes that they underwent. Furthermore, L-NGFR scores demonstrated the best interrater agreement between pairs of assessors.

Previously, we found that the Cx43 direct cell-cell communication channels were upregulated in pathological bone marrow samples 27. These samples had a higher ratio of stromal cells to hemopoietic cells. Therefore, L-NGFR, which was found to be the most effective marker in this study for highlighting the stromal network, and Cx43 protein were found simultaneously using immunofluorescence in samples representing all grades of myelofibrosis in order to investigate the possibility of a link between the two. The results of multilayer scanning showed the presence of Cx43-positive particles with a size of 1 μm throughout the 4 μm section thickness, as well as a significant co-localization between these particles and L-NGFR-positive stromal cell processes

Extracellular matrix protein expression in PMF progression

In the second part of the study, we focused on stromal cell related matrix proteins such as type I and type III collagens and fibrillin-1. All of the matrix protein immunoreactions that were tested showed close visual and statistical correlations with Gömöri's silver staining in patients with advanced MF-3 myelofibrosis, but fibrillin-1 was the one that had the strongest correlation within silver-based grade groups at all grade levels.

Immunostaining with antibodies directed against type I collagen also highlighted bone trabecules, including newly formed bone in osteosclerosis. However, insufficient preanalytics and bone damage in a few cases caused type I collagen to appear in the adjacent megakaryocytes in a non-specific manner, and these areas were neglected when scoring.

The reaction involving fibrillin-1, which aids in differentiating between adipocyte membranes and blood vessels, was the one that stood out the most. Only one of the five bone marrows with

normal morphology was given a score of MF-1 based on the reactions evaluated by type I collagen (by two assessors) and type III collagen (by one expert). But this case, along with all of the other normal and pre-fibrotic cases, was finally agreed to be MF-0 with any of the other markers.

The fibrillin-1 reactions that were uniformly strong offered the best visualization of the structural abnormalities of the stromal scaffolding. All of the matrix protein immunoreactions that were tested showed close visual and statistical correlations with Gömöri's silver staining in patients with advanced MF-3 myelofibrosis.

Fibrillin-1 staining may provide a quicker, more standardized substitute for the current silver impregnation-based grading procedure, according to statistically significant evidence. We have proved that not only is the correlation with silver staining-based grading significant, but the advantage of high optical contrast also qualifies it for computer-assisted automated image analysis.

5. Conclusions and novel published observations

1. All of the immunohistochemically detected biomarkers tested in this study showed a significant correlation with the progression of myelofibrosis as characterized by Gömöri's silver impregnation-based grading.

2. Low affinity nerve growth factor receptor (L-NGFR) demonstrated the strongest statistical correlation with the Gömöri's silver impregnation based myelofibrosis grading. Therefore, L-NGFR immunohistochemistry may valuably complement silver grading in the rutin diagnostic assessment of primer myelofibrosis progression.

3. Compared to L-NGFR, CXCL12, Phospho-ERK1-2 levels proved to be somewhat less accurate but valuable indicators of myelofibrosis progression indicators in line with Gömöri's silver impregnation based grading.

4. The glycoprotein fibrillin-1 demonstrated a very strong association with the regular silver impregnation-based grading among the extracellular matrix proteins examined in this study in primary myelofibrotic bone marrow. Fibrillin-1 immunohistochemical detection in primary myelofibrosis is the most promising of the biomarkers evaluated in this study for complementing or even replacing the low reproducibility silver-based grading method.

5. Compared to fibrillin-1, type I and type III collagens were proven to be less accurate but still very useful myelofibrosis progression indicators in line with Gömöri's silver staining based grading.

6. Using computer-assisted digital image analysis performed on fibrillin-1 immunostained digital whole slides resulted in highly reproducible quantitative results that statistically strongly correlated with the eye-control based results on the same digital slides.

6. Bibliography of the candidate's publications

Total of impact factors: 17.283

Publications related to PhD dissertation *(subtotal of impact factors: 10.652)*

Szekely T, Krenacs T, Maros ME, Bodor C, Daubner V, Csizmadia A, Vrabely B, Timar B: Correlations Between the Expression of Stromal Cell Activation Related Biomarkers, L-NGFR, Phospho-ERK1-2 and CXCL12, and Primary Myelofibrosis Progression. Pathol Oncol Res 2022, 28:1610217. *(journals's impact factor in 2022 : 2.874)*

Szekely T, Wichmann B, Maros ME, Csizmadia A, Bodor C, Timar B, Krenacs T: Myelofibrosis progression grading based on type I and type III collagen and fibrillin 1 expression boosted by whole slide image analysis. Histopathology 2022 *(journals's impact factor in 2022 : 7.778)*

Additional papers not related to the PhD thesis *(subtotal of impact factors: 6.631)*

Krenacs, T., Meggyeshazi, N., Forika, G., Kiss, E., Hamar, P., Szekely, T., & Vancsik, T. (2020). Modulated Electro-Hyperthermia-Induced Tumor Damage Mechanisms Revealed in Cancer Models. International journal of molecular sciences, 21(17), 6270. https://doi.org/10.3390/ijms21176270 *(journals's impact factor in 2020 : 5,924)*

Fónyad, L., & Székely, T. (2021). Online patológiai vizsgálatkérő felület létrehozása a Semmelweis

Egyetemen [Online pathology request platform at the Semmelweis University]. Orvosi hetilap, 162(49), 1962–1967. https://doi.org/10.1556/650.2021.32306 *(journals's impact factor in 2021 : 0.707)*

7. Acknowledgement

I am grateful to Prof. Tibor Krenács for providing the opportunity to work in his laboratory and for constantly supervising my work.

I am thankful to Prof. András Matolcsy for letting me work in his institution and for supporting my study in the Doctoral School of Pathological Sciencesof Semmelweis University.

I am thankful to Botond Timár for helping with sample collection, evaluation, and explaining me various hematopathological aspects of the topic in details.

I am grateful to all the members of the laboratory, especially Eva Mátrainé Balogh, for their excellent technical support in the laboratory work.

I am grateful to Máté Maros and Barna Wichmann for their excellent support in statistical analysis laboratory work.

I am also grateful to all members of the Department of Pathology and Experimental Cancers Research at Semmelweis University who helped me in any aspect of my studies.

Finally, but not least, I am truly grateful to my beloved family and friends, who give me a stable background and motivation throughout my work.