Leukemia Research 39 (2015) 236–241

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

PDGFB hypomethylation is a favourable prognostic biomarker in primary myelofibrosis

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ARTICLE INFO

Article history:
Received 11 August 2014
Received in revised form
13 November 2014
Accepted 21 November 2014
Available online 2 December 2014

Keywords:
Primary myelofibrosis
DNA methylation
PDGFB
FGF2
LINE-1

ABSTRACT

Primary myelofibrosis (PMF) is a myeloproliferative neoplasm characterised by the clonal proliferation of the haematopoietic precursors together with the progressive development of bone marrow fibrosis. This stromal alteration is an important clinical issue and specific prognostic markers are not currently available. In bone marrow biopsies from 58 PMF patients, we explored the methylation pattern of genes encoding cytokines involved in the stromal reaction, namely platelet-derived growth factor-beta (*PDGFB*), transforming growth factor-beta (*TGFB*) and basic fibroblast growth factor (*FGF2*). We also evaluated the methylation profile of the Long Interspersed Nucleotide Element 1 (LINE-1). *PDGFB*, *FGF2* and LINE-1, but not *TGFB*, were significantly differently methylated in PMF compared to controls. Significantly, *PDGFB* hypomethylation (<16%) was correlated with a favourable PMF prognosis (grade of marrow fibrosis, $p = 0.03$; International Prognostic Scoring Systems $p = 0.01$ and Dynamic International Prognostic Scoring Systems, $p = 0.02$). Although the basis of the association of *PDGFB* hypomethylation with favourable prognosis remains to be clarified, we speculate that hypomethylation in PMF could represent the effect of acquired somatic mutations in genes involved in epigenetic regulation of the genome.

1. Introduction

Primary myelofibrosis (PMF) is a myeloproliferative neoplasm characterised by the clonal proliferation of the haematopoietic precursors, particularly of the granulopoietic and megakaryopoietic series. It is associated with the development and progression of bone marrow fibrosis, osteosclerosis and angiogenesis [1]. Although stable for a period that varies among individuals, patients develop fibrosis, which is the result of the accumulation of the extracellular matrix in the bone marrow spaces [2]. Progression to fibrosis is an important clinical and prognostic issue [3,4]; however, specific predictive markers are not currently available. Among the candidate markers there are proteins involved in the stromal reaction, in particular platelet-derived growth factor (PDGF), basic fibroblast growth factor 2 (FGF2) and transforming growth factor-beta (TGFB), cytokines that stimulate the stromal cells and particularly the fibroblasts to produce extracellular matrix components [5].

The PDGF family comprises four different polypeptides, PDGFA, PDGFB, PDGFC and PDGFD, existing as homodimers and as an AB

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http://dx.doi.org/10.1016/j.leukres.2014.11.012
0145-2126/

heterodimer [6,7]. The *PDGFB* gene is mainly expressed in vascular endothelial cells, megakaryocytes and neurons. From megakaryocytes, PDGFB is carried to the circulation by the released platelets and acts as a mediator of tissue repair. PDGFB-driven pathogenic processes are involved in various diseases, mainly related to recruitment of stromal cells, increased mesenchymal cell proliferation and deposition of extracellular matrix leading to progressive loss of organ function [8]. Elevated levels of PDGF proteins have been observed in the serum and platelets of PMF patients and it was suggested that enhanced PDGF expression could be a marker of marrow fibrosis [9]. A significant overexpression of PDGFA, PDGFB, PDGFC and PDGF receptor (PDGFR) mRNAs was documented in the bone marrow of PMF cases, particularly in those with fibrosis [10]. These data strongly suggest that growth factors of the PDGF family are implicated in the genesis of myelofibrosis via PDGF/PDGFR interaction, which promotes chemotactic activity and induces proliferation of medullary fibroblasts [11].

FGF2 protein may also contribute to the pathogenesis of PMF; as hypothesised by Bock et al. [12] FGF2 could be involved in the enhanced proliferation of megakaryocytes, rather than in the direct induction of fibroblast proliferation. It cannot be excluded that enhanced turnover of megakaryocytes could cause release of FGF2 from the disintegrating cells, which in turn could lead to a paracrine stimulation of fibroblasts [13].

TGFB stimulates the activity of genes involved in the production of the extracellular matrix [9] and it also acts by suppressing the expression of proteases that physiologically control the turnover of the matrix components [14]. TGFB mRNA levels are increased in the total bone marrow cells of PMF cases, with highest values in cells from the fibrotic phase as well as in isolated megakaryocytes [15].

Over the past 10 years, there has been a significant increase in the knowledge of the genetic alterations involved in the pathogenesis of myeloproliferative neoplasms. *JAK2* [16], *MPL* [17] and *CALR* [18,19] are frequently mutated and are mutually exclusive. Mutations of *TET2*, *ASXL1*, *DNMT3A* and *IDH1/2* genes are present in about 15–20% of PMF cases. It is intriguing that these loci are involved, at different levels, in the epigenetic regulation of cells [1,20] and are associated with epigenetic alterations in cancer, suggesting that epigenetic defects could be also present in PMF [20].

DNA methylation, the binding of a methyl group to cytosine to form 5-methylcytosine, is considered a master epigenetic mechanism for the orchestrated organisation of chromatin modelling [21]. It mainly occurs at CpG sites, which are typically located in the promoter regions, and influences gene expression. In the presence of methylated CpGs, gene transcription is generally inhibited, whereas unmethylated CpGs are common in chromatin regions accessible to the transcriptional machinery. Methylation at non-CpG islands has an important role in the maintenance of genomic stability by preventing the mobility of transposable elements such as Long Interspersed Nucleotide Elements 1 (LINE-1) [22]. LINE-1 constitute a substantial portion of the human genome and their methylation is considered to be an index of global DNA methylation status and of genome stability. In a number of tumour types, including promyelocytic leukaemia, LINE-1 demethylation is a negative prognostic marker [23] and is associated with activation of proto-oncogenes [24]. Conversely, LINE-1 hypermethylation is a favourable prognostic factor probably associated with genomic stability, as reported in colorectal cancer and glioma [25,26].

Based on the roles of PDGFB, FGF2 and TGFB in PMF fibrosis, and of LINE-1 in genomic stability, we investigated their epigenetic status in bone marrow biopsies (BMBs) from 58 PMF patients, stratified according to clinical classification and the presence of *JAK2* or *CALR* mutations, to explore associations between the epigenetic profiles of the candidate loci and PMF features.

2. Materials and methods

2.1. Patients and samples

Sixty-five BMBs from 58 PMF patients, who also had peripheral blood samples collected at the time of the diagnosis (required for mutation analyses) were available from the Haematology Unit of the IRCCS Fondazione Ca' Granda Ospedale Maggiore Policlinico. In seven cases, a second bone marrow biopsy was available. These patients received the following therapies: hydroxyurea (HU) plus aspirin (cases 3, 11, 27, 43 and 53) followed by ruxolitinib (case 35) and bone marrow transplantation (case 50).

All the cases were reviewed by two hematopathologists and reclassified according to the to the updated WHO classification [27]. The patients had a median age of 65 years (range, 32–91 years), 27 were males and 31 females.

BMBs were formalin-fixed, decalcified with 10% EDTA/Tris–HCl pH = 7.4 solution and paraffin-embedded. Serial BMB sections were used for the DNA extraction and methylation studies, histology and immunohistochemical analyses.

In accordance with the European Consensus on the grading of bone marrow fibrosis [28], the cases were categorised at diagnosis as cellular phase (MF0, $n = 12$) and fibrotic phases (MF1, $n = 29$; MF2, $n = 15$; and MF3, $n = 2$).

The clinical data were available for all patients (Supplementary Table 1). The follow-up period ranged from 6 to 287 months (mean 76 months). The International Prognostic Scoring System (IPSS) and the Dynamic International Prognostic Scoring System (DIPSS) values [29,30], used to determine a patient's risk group at diagnosis and over time, were also considered. Five of the 58 patients died: two from the evolution of PMF to acute myeloid leukaemia and three from other PMF-unrelated causes. Of the remaining 53 patients, 12 had progression of fibrosis, 24 patients had progressive disease based on the IPSS and 27 had progressive disease based on the DIPSS.

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2014.11.012.

Twenty cases of iliac bone fragments obtained from patients who had undergone surgery for iliac bone prosthesis were included as normal controls. These patients were 50–60 years old and had clinical and haematological parameters within the normal ranges. The bone fragments were treated as described for the BMBs and histologically evaluated. None of them showed neoplasia or other abnormalities.

All patients gave their informed consent and the Institutional Review Board approved the study.

2.2. PMF genotyping

JAK2 (V617F), *CALR* (ins/del in exon 9), *IDH1* (R132H) and *IDH2* (R172 K) mutation analyses were performed on DNA purified from the patient's peripheral blood granulocytes. In all patients *JAK2* (V617F) genotyping was performed as previously reported [31]. *JAK2* and *CALR* mutations are mutually exclusive and therefore *CALR* exon 9 mutations were evaluated only in the cases with wild-type *JAK2* (20 out of 58 PMF patients), according to the protocol recently reported by Klampfl et al. [18]. *IDH1* and *IDH2* were genotyped as previously reported [32].

2.3. Quantitative DNA methylation analyses

Analysis of the DNA methylation status of the *PDGFB*, *FGF2* and *TGFB* promoters and of LINE-1, was carried as follows: genomic DNA was extracted from BMBs and control specimens using QIAamp DNA FFPE Tissue Kit (Qiagen). For each sample, DNA (30–100 ng) was bisulphite-converted using EZ DNA Methylation-Direct Kit (Zymo Research Corporation) and eluted in 15 μl of elution buffer. For promoter analyses, PCR was carried out in a reaction volume of 50 μl, containing 10 pmol of each primer (detailed in Table 1) and 20–100 ng of bisulphite-treated DNA. Methylation at the analysed CpG sites was quantified by pyrosequencing using primers described in Table 1 and PyroGold SQA Reagent Kit (Qiagen).

The methylation percentage of at each CpG site was quantitatively analysed by PyroMark ID instrument (Qiagen) and software Q-CpG v.1.0.11 (Qiagen) as previously reported [33]. LINE-1 methylation analysis was assessed as previously reported [34].

2.4. Immunohistochemical analysis

Immunohistochemical analyses were performed in 58 PMF cases and in five of the control iliac bone samples.

Immunohistochemistry for PDGFB (rabbit polyclonal antibody diluted 1:800; clone N-30, sc-127; Santa Cruz Biotechnology, Santa Cruz, CA) and FGF2 (rabbit polyclonal antibody diluted 1:2000; clone (147), sc-79; Santa Cruz Biotechnology, Santa Cruz, CA) was performed using an automated staining system (BenchMark ULTRA, Ventana, Roche, Tucson, USA). Heat-induced antigen retrieval in 0.05 mol/L EDTA solution, pH 8.0 at 95 °C for 30 min. Reactions were revealed using an ultraView Universal DAB Detection Kit (Ventana, Roche, Tucson, USA) in accordance with the manufacturer's instructions. The negative control slides for these procedures were incubated with normal goat serum instead of the primary antibody.

The immunohistochemistry reactivity was evaluated by the so-called "hot spots method", as previously described [35]. Briefly, first each specimen was scanned at