VIA MEDICA

CASE REPORT

Katarzyna Osmańska[1], Barbara Mucha[1], Katarzyna Skonieczka[1], Alicja Bartoszewska-Kubiak[1], Dominik Chraniuk[2], Małgorzata Całbecka[2]

[1]Department of Clinical Genetics, Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz, Poland
[2]Department of Haematology, Municipal Hospital, Toruń, Poland

Primary myelofibrosis with normal karyotype and cryptic aberrations detected by FISH: case report

Corresponding author:
Katarzyna Osmańska
Department of Clinical Genetics, Collegium Medicum
Nicolaus Copernicus University
Skłodowskiej-Curie Street No. 9
85–094 Bydgoszcz, Poland
Phone: +48 52 585 36 81
Fax: +48 52 585 35 68
e-mail: kosmanska@wp.pl

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ABSTRACT

Introduction. Myeloproliferative neoplasms (MPNs) are the result of clonal haematopoietic stem cell disorders. The most common cytogenetic aberrations are: partial trisomy 1q, 13q–, 20q–, trisomy 8 and abnormalities of chromosomes 1, 7 and 9. Conventional karyotyping is a routinely used method. Fluorescent *in situ* hybridisation (FISH) analysis however may also be an integral component of the diagnostic evaluation, especially when the abnormality is cryptic.

Subject and methods. A 70-year-old woman was admitted to the Department of Haematology in September 2013 with suspected acute myeloid leukemia (AML). The final diagnosis was primary myelofibrosis and NYHA class III heart failure. Bone marrow (BM) was used for karyotyping and FISH. Peripheral blood (PB) was used for PCR.

Results. Cytogenetic GTG analysis revealed normal female karyotype — 46,XX [22]. The result of analysis of *JAK2* V617F mutation was negative. Analysis using LSI BCR/ABL Dual Fusion Probe, JAK2 Break Probe and RB1 Deletion Probe showed abnormal cells, of which the numbers were beyond the normal cutoffs. FISH examinations using p53 Deletion Probe and LSI CDKN2A/CEP 9 showed normal cells.

Conclusion. The diagnosis of primary myelofibrosis may pose a problem. We still do not know the specific abnormalities (i.e. genomic and chromosomal aberrations or gene mutations), the occurrence of which may help to diagnose and assess a probable time of survival of patients with PMF. Further examinations are needed (e.g. using aCGH) to find out more about myeloproliferative neoplasms.

Key words: myeloproliferative neoplasms (MPNs), primary myelofibrosis (PMF), chromosome aberrations, FISH, trisomy 13, *JAK2*

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Introduction

Myeloproliferative neoplasms (MPNs) are the result of clonal haematopoietic stem cell disorders characterised by the proliferation of one or more of the myeloid lineages (granulocytic, erythroid, megakaryocytic or mast cell) of the bone marrow (BM). MPNs are characterised by hypercellularity of the bone marrow. The haematopoietic maturation is still effective and the numbers of granulocytes, red blood cells and/or platelets increase in the peripheral blood (PB). Splenomegaly and hepatomegaly are common and are caused by the sequestration of excess blood cells or the proliferation of abnormal haematopoetic cells. MPNs have the potential to progress (i.e. marrow failure, ineffective haematopoiesis, transformation to acute leukemia) [1, 2]. In 1951, William Dameshek used the term 'myeloproliferative disorders' (MPDs) to describe polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and chronic myeloid leukemia (CML) — four different diseases with clinical and biological similarities. Other rare chronic haemopathies were subsequently included [3]. In 2008, the World Health Organisation (WHO) presented a new classification of chronic myeloproliferative disorders (CMPDs) and recommended altering the term 'myeloproliferative disorders' to 'myeloproliferative neoplasms' (the new term better reflects the clinical pattern and course of the diseases) [1, 3]. MPNs involve CML, three major non-CML diseases: PV, ET, PMF and several less common entities: systemic

mastocytosis (SM), chronic eosinophilic leukemia/ /hypereosinophilic syndrome (CEL/HES), not otherwise classified chronic neutrophilic leukemia (CNL), and unclassified MPNs. Increased production of granulocytes is the most typical for CML. An increased number of platelets is typical of ET, and an increased number of red cells is a major feature of PV. Bone marrow fibrosis and cytopenias are characteristic of PMF [1, 2]. The reason for the changes in the classification was a somatic mutation of *JAK2* (located at the short arm of chromosome 9 — 9p24), which was shown to play a major role in the pathogenesis of many cases of *BCR-ABL1* negative MPNs. This discovery was made in 2005. *JAK2* V617F is the most common mutation and it results in a constitutively active cytoplasmic JAK2, which plays a central role in the signalling from myeloid cytokine receptors. The frequency of *JAK2* mutation is different in each MPN: 95% in PV but 50–55% in ET and PMF [1–4]. Primary myelofibrosis is defined as a clonal MPN characterised by the proliferation of predominantly megakaryocytes and granulocytes in the bone marrow that in the fully developed disease is associated with reactive deposition of fibrous connective tissue and with extramedullary haematopoiesis. PMF has a poor prognosis, with median survival of patients of about five years. The prognosis depends on: haemoglobin concentration at diagnosis, age, low or high leukocyte counts, cytogenetic abnormalities and number of circulating $CD34^+$ blasts. The frequency of PMF is about 0.5––1.5/100,000 cases per year. It occurs most commonly in the sixth and seventh decades of life and males and females are nearly equally affected. The most common cytogenetic aberrations are: partial trisomy 1q, 13q–, 20q–, trisomy 8 and abnormalities of chromosomes 1, 7 and 9. Chromosomal aberrations are found in about 10–15% of MPN patients and 30–56% of PMF patients [1, 2]. Conventional karyotyping is a routinely used method. However, fluorescent *in situ* hybridisation (FISH) analysis may also be an integral component of the diagnostic evaluation, especially when the abnormality is 'cryptic' (i.e. not evident after using conventional karyotyping) [5]. In this report, we describe a case of *JAK2*-negative primary myelofibrosis with normal karyotype and cryptic aberrations detected by FISH.

Subject and methods

Case report

A 70-year-old woman was admitted to the Department of Haematology, Municipal Hospital in Toruń in September 2013 due to the suspicion of acute myeloid leukemia (AML). Medical history excluded chronic diseases. The patient was taking only analgesic medicines. On the day of admission, the patient complained of strength reduction, periodic diarrhoea, and constantly increasing massive oedema of both lower extremities. There were no episodes of bleeding disorders, fever or infections. Abnormal findings in physical examination included obesity. Ultrasonography (USG) showed hepatosplenomegaly and the presence of fluid in the abdominal cavity. Routine blood examination revealed white blood cell (WBC) count at 100 G/L and platelets at $28 \times 10^3/\mu$L. Myelogram showed 4% blasts and the presence of micromegakaryocytes as well as dysplasia in erythrocyte and leukocyte lineages. A peripheral blood smear showed a left shift (with cells at all stages of maturation) and the presence of erythroblasts. Immunophenotyping showed 2.5% of cells expressing CD34 ($CD34^+$ cells). All examinations excluded AML and suggested primary myelofibrosis. Four units of leukoreduced red blood cells (LRBC) and five units of platelet concentrate were transfused to the patient. Drug treatment reduced the oedema of the lower extremities. Peripheral blood and bone marrow samples were collected for the next examinations: karyotyping, FISH and polymerase chain reaction (PCR). The patient was discharged from the hospital with the final diagnosis of primary myelofibrosis and NYHA class III heart failure. The patient did not come to the medical check-up and was lost to follow-up.

Chromosome preparation

A G-banded chromosome study was performed using the standard cytogenetic protocol. Unstimulated cultures of bone marrow aspirate were set up in RPMI-1640 medium supplemented with 10% fetal bovine serum. The cells were cultured for 24 and 48 h at 37°C and 5% CO_2. At the end of the culture, following 35 minutes of incubation with colcemide at a final concentration of 9 μl/mL, the cultures were exposed to hypotonic solution (0.075 M KCl) and fixed with a mixture of three parts methanol to one part acetic acid. Drops of fixed cell suspension were placed onto glass slides and the fixative was allowed to evaporate. As the slides dried completely, they were stained with GTG banding. Twenty two metaphases were analysed and archived with Applied Spectral Imaging software (Applied Spectral Imaging, Edingen, Neckerhausen, Germany). The karyotype was described according to the International System for Human Cytogenetic Nomenclature 2013 [6].

Allele-specific PCR for the detection of *JAK2* V617F mutation

Genomic DNA was extracted from peripheral blood using the QIAamp DNA BloodMini Kit (Qiagen). The *JAK2* V617F mutation was detected according to the modified protocol of Baxter et al. [7]. 100 ng of patient DNA was amplified in a 35-cycle PCR reaction at the

annealing temperature of 58ºC. The primers used for multiplex PCR were R1-Reverse: 5'CTGAATAGTCCTACAGTGTTTTCAGTTTCA3', F1-forward (specific): 5'AGCATTTGGTTTTAAATTATGGAGTATATT3' and F2-forward (internal control): 5'ATCTATAGTCATGCTGAAAGTAGGAGAAAG3'. The PCR products were analysed on 2% agarose gel. The primers R1 and F2 amplify wild-type product (364 bp), while the primers R1 and F1 amplify a 203 bp product when the *JAK2* V617F mutation is present.

Fluorescent *in situ* hybridisation

FISH was performed on BM interphase and metaphase cells following the manufacturer's guidelines. Five FISH probes were used: LSI BCR/ABL Dual Fusion Probe (Abbott Molecular, USA), JAK2 Break Probe (Kreatech, the Netherlands), RB1 Deletion Probe (Cytocell, Great Britain), p53 Deletion Probe (Cytocell, Great Britain) and LSI CDKN2A/CEP9 Probe (Abbott Molecular, USA). All probes were used to detect deletion or rearrangement of appropriate genes. Each analysis covered 200 interphase cells and some metaphase cells altogether. The normal cutoff for each analysis was the following: 6.0% (*BCR*/*ABL*); 3.49% (*JAK2*); 4.0% (*RB1*); 5.1% (*TP53*); and 2.0% (*CDKN2A*).

The study was approved by the Bioethics Committee of the Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland (No. KB 358/2013).

Results

Cytogenetic GTG analysis revealed normal female karyotype – 46,XX [22] (Fig. 1). The result of the analysis of *JAK2* V617F mutation was negative (the mutation was absent). The analysis using LSI BCR/ABL Dual Fusion Probe showed 10% of abnormal cells — 19 cells with additional *BCR* signal (3G2R pattern) and one cell with deletion of *ABL* signal (2G1R pattern) (Fig. 2). FISH examination using JAK2 Break Probe showed 6.5% of abnormal cells — four cells with rearrangement of *JAK2* (1Y1G1R pattern), seven cells with the deletion of distal region of *JAK2* (1Y1R pattern), and two cells with the deletion of proximal region of *JAK2* (1Y1G pattern) (Fig. 3). The analysis using RB1 Deletion Probe showed 18.5% of abnormal cells — 37 cells with three red signals (*RB1*) and three green signals (13q subtelomeric region), which might suggest trisomy of chromosome 13 (3G3R pattern) (Fig. 4). FISH examinations using p53 Deletion Probe and LSI CDKN2A/CEP 9 showed no abnormality (numbers of abnormal cells were under the normal cutoff) (Tab.1).

Discussion

In this study, we reported the results of karyotyping, *JAK2* mutation analysis by allele-specific PCR, and *BCR*/*ABL*, *JAK2*, *RB1*, *CDKN2A* and *TP53* gene rearrangements analysis by FISH in patient with primary myelofibrosis and tried to understand their role in the pathophysiology, prognosis and monitoring of the patient.

Primary myelofibrosis is a rare myeloproliferative neoplasm and there is no identified specific genetic defect for this illness. Although 50% of patients have *JAK2* V617F mutation, the same mutation can be found in PV and ET. About 5% of patients with PMF have *MPL* mutation (*MPL* W515K/L), but this is also reported in occasional cases of ET. About 30–56% of PMF patients have cytogenetic aberrations. The presence of the

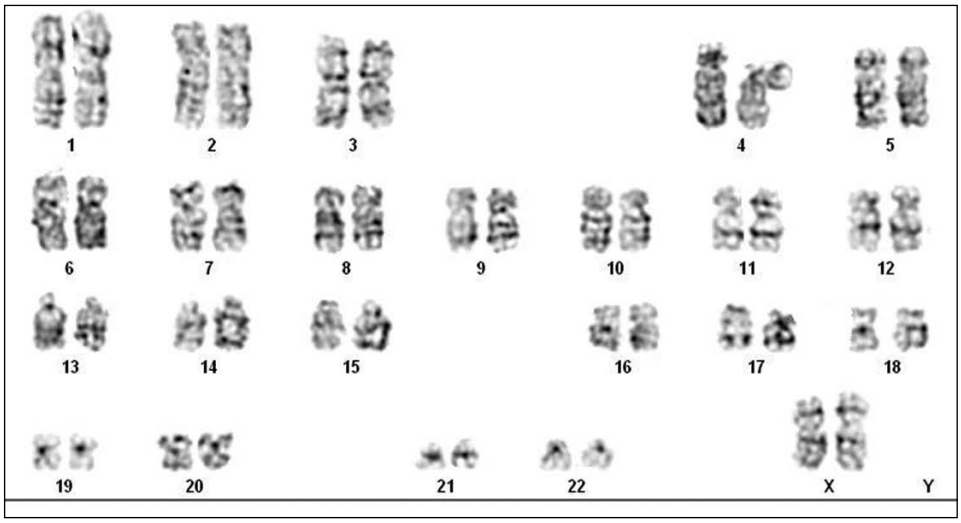

Figure 1. Representative GTG-banded normal karyotype — 46,XX

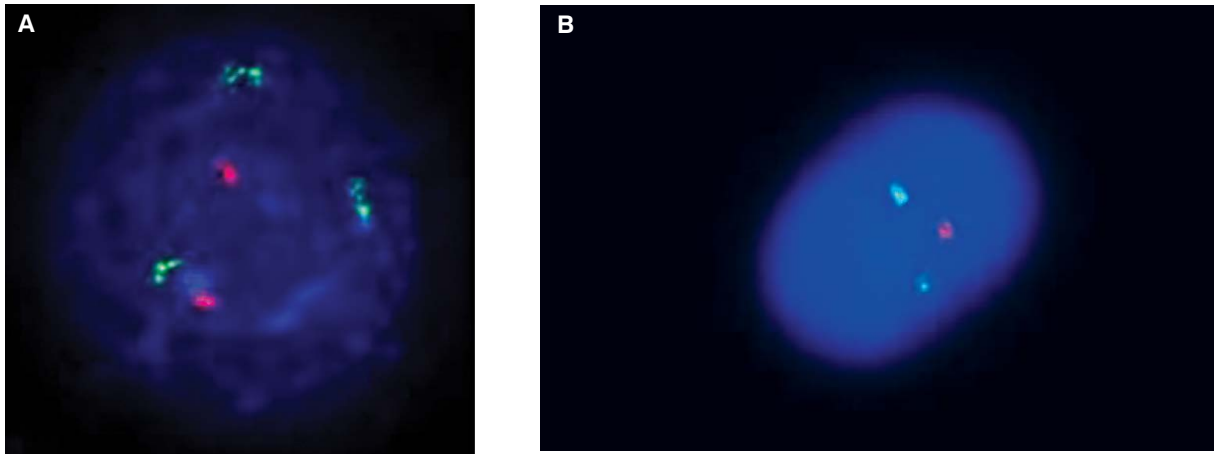

Figure 2. Different interphase FISH patterns found with the LSI *BCR*/*ABL* Dual Fusion Probe: green signal (G) — *BCR*, red signal (R) — *ABL.* **A.** 3G2R pattern — additional *BCR* signal. **B.** 2G1R pattern — deletion of *ABL* signal

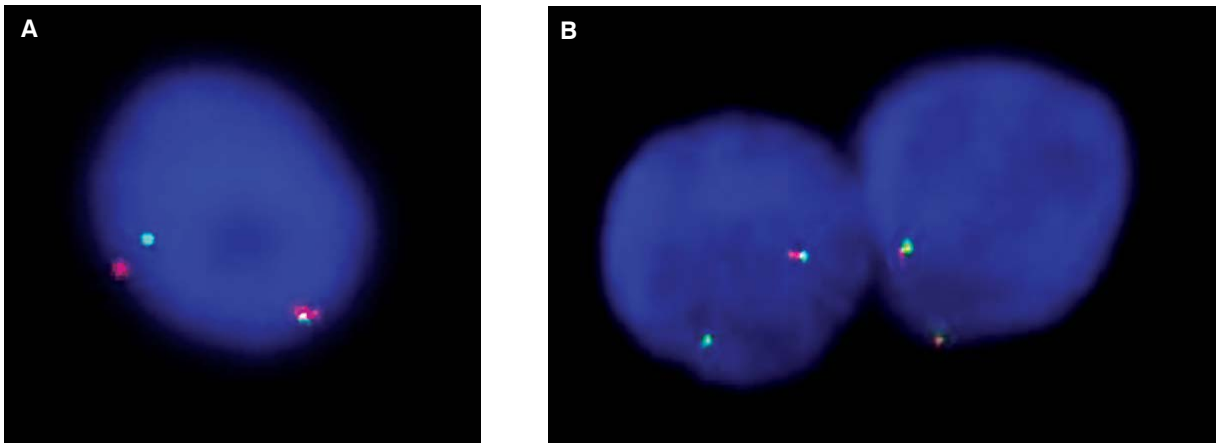

Figure 3. Different interphase FISH patterns found with the *JAK2* Break Probe: green signal (G) — distal region of *JAK2*, red signal (R) — proximal region of *JAK2*, yellow signal (Y) — normal gene. **A.** 1Y1G1R pattern — rearrangement of *JAK2*. **B.** 1Y1G pattern (left nucleus) — deletion of proximal region of *JAK2*; 2Y pattern (right nucleus) — normal nucleus

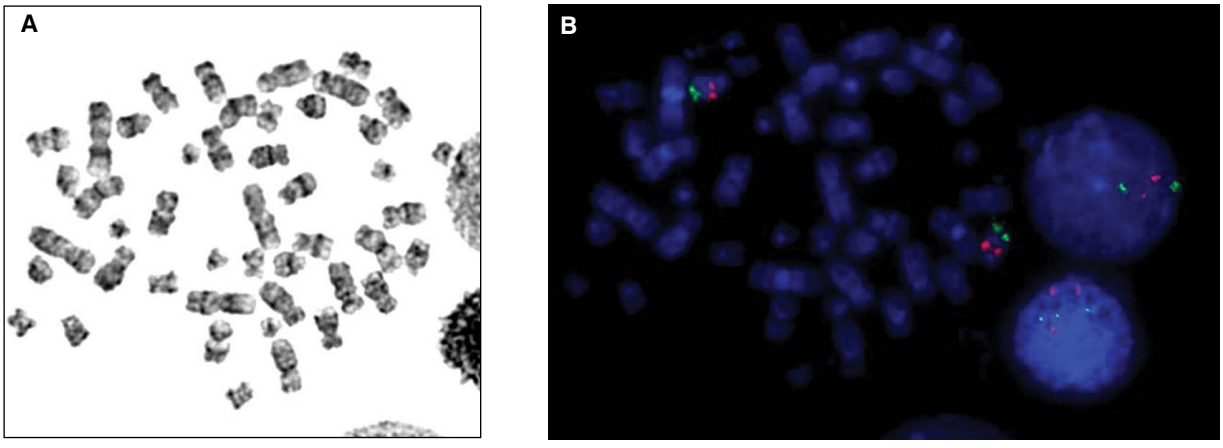

Figure 4A. GTG-banded metaphase used to FISH preparation. **B.** Fluorescence *in situ* hybridisation using RB1 Deletion Probe: red signal (R) — *RB1*, green signal (G) — 13q subtelomeric region; normal metaphase (2G2R pattern) and interphase nucleus (2G2R pattern) and nucleus with trisomy 13 (3G3R pattern)

Table 1. Results of FISH investigations in the described patient with primary myelofibrosis

Type of probe	LSI BCR/ABL Dual Fusion Probe		JAK2 Break Probe		RB1 Deletion Probe		p53 Deletion Probe		LSI CDKN2A/ /CEP9 Probe	
Normal cutoff (%)	6.0		3.49		4.0		5.1		2.0	
Type of signal	**Normal**	**Abnormal**	**Normal**	**Abnormal**	**Normal**	**Abnormal**	**Normal**	**Abnormal**	**Normal**	**Abnormal**
Number of signals	180	20[a]	187	13[b]	163	37[c]	197	3[d]	198	2[e]
Percentage of abnormal signals	10.0		6.5		18.5		1.5		1.0	

[a]19 cells — additional signal of *BCR*, one cell — deletion of *ABL*; [b]four cells — rearrangement of *JAK2*, seven cells — deletion of distal region of *JAK2*, two cells — deletion of proximal region of *JAK2*; [c]37 cells — three signals of *RB1* and three signals of 13q subtelomeric region; [d]three cells — deletion of *TP53*; [e]one cell — deletion of *CDKN2A*, one cell — chromosome 9 monosomy

disease is strongly associated with del(20q), partial trisomy 1q, trisomy 9, trisomy 8, del(13)(q12-22) and der(6)t(1;6), but these abnormalities are not sufficient to diagnose PMF [1, 2]. Clinical trials are needed to assess the prognostic significance of recurring chromosome abnormalities in primary myelofibrosis. The identification of risk groups based on specific chromosomal findings will have major clinical implications. In addition, the finding of new recurring chromosome abnormalities will provide insight into the gene region(s) that may be important in the malignant transformation of PMF [8, 9].

PMF is a Ph-negative MPN. That is why we did not find *BCR-ABL* fusion gene. However, Taştemir et al. showed that atypical patterns also may have diagnostic and prognostic implications. Atypical patterns were seen as a result of additional numerical changes (most often gains or losses of chromosomes 9 or 22) [10]. Rice et al., using SNP arrays in 12 MPN patients (in group of 87 MPN patients), described the gain in region 9q34.13 — locus of *ABL* [11]. We had such atypical patterns in two cases in our analysis.

PMF and other Philadelphia chromosome-negative myeloproliferative neoplasms were recognised as clonal haematopoietic stem cell disorders about 40 years ago, but until 2005 little was known about their genetic basis [12]. Najfeld et al. revealed with FISH two types of *JAK2* rearrangements: gain and/or structural rearrangements (patients with Ph-negative MPNs and non-MPNs). Gain and amplification of *JAK2* was primarily observed in patients who were *JAK2* V617F-positive. Additionally, *JAK2* attracts multiple gene partners (e.g. *TEL*/*ETV6*) and its translocations may contribute to disease progression in patients with MDS and B-cell malignancies, while the *JAK2* copy number appears to be important in the pathogenesis of Ph-negative MPNs [13]. Rice et al., using SNP arrays in six MPN patients (in a group of 87 MPN patients), described the gain in region 9p24.3–9p23 (9p24.1 — locus of *JAK2*) [11]. Three different rearrangements of *JAK2* were observed in our FISH examinations.

Deregulated function of *RB1* (13q14) may be involved in the transformation of undifferentiated myeloid cells [9]. The deletion of *RB1* is the most frequent aberration of chromosome 13 associated with MPNs and has been identified in 19% of PMF patients [2, 3]. Our results showed trisomy of chromosome 13. Trisomy 13 (as a sole cytogenetic abnormality) was first described in 1990 by Döhner et al. This numerical aberration was identified in eight of 621 consecutive successfully karyotyped adults with de novo acute leukemia. Döhner et al. concluded that trisomy 13 in acute leukemia is associated with a poor prognosis, with survival ranging from 0.5 to 14.7 months [14]. Trisomy 13 is a rare cytogenetic abnormality associated with a low complete remission rate and a short remission duration. Moreover, trisomy 13 clusters overwhelmingly with high-risk myeloid malignancy [15, 16]. One case of primary myelofibrosis with trisomy 13 was described by Mesa et al. (the research involved 27 patients with an isolated trisomy; any clinical data about the PMF patient was added) [16].

The product of *TP53*, p53 protein, is involved in various biological activities such as control of DNA repair, control of cell-cycle checkpoints, and apoptosis. p53 is frequently inactivated in human cancers — e.g. mutations in *TP53* occur in approximately 10% of AML samples. Mutations of *TP53* are not connected with the chronic phase of MPNs. However, *TP53* mutations have been identified in 20% of post-MPN-AML patients, which may suggest that they play a prominent role in the transformation process [3, 17]. Aberrations of *TP53* (e.g. large deletions at region 17p which harbors *TP53*) play a significant role in the pathogenesis of leukemias [18].

The biological functions of *CDKN2A* are similar to *TP53*. This gene is frequently mutated or deleted in a wide variety of tumours. Congenital mutations in *CDKN2A* are associated especially with melanoma, pancreatic cancer, colorectal cancer, breast cancer and lung cancer. The acquired ones are associated with e.g. leuke-

mias. Rice et al., using SNP arrays in seven MPN patients (in a group of 87 MPN patients), described the gain in region 9p23–9p13.3 (9p21.3 – locus of *CDKN2A*) [11]. We did not observe any aberration of *TP53* and *CDKN2A* in our examinations.

Conclusion

The diagnosis of primary myelofibrosis may pose a problem. We still do not know the specific abnormalities (i.e. genomic and chromosomal aberrations or gene mutations), the occurrence of which may help to diagnose and assess a probable time of survival of patients with PMF. Further examinations are needed (e.g. using aCGH) to find out more about myeloproliferative neoplasms.

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Conflict of interest statement

The authors declare no conflict of interest.

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