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RESEARCH ARTICLE

The Importance of Identification of M-BCR-ABL Oncogene and JAK2^{V617F} Mutation in Myeloproliferative Neoplasms

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Background: The elucidation of the genetic background of the myeloproliferative neoplasms completely changed the management of these disorders: the presence of the Philadelphia chromosome and/or the BCR-ABL oncogene is pathognomonic for chronic myeloid leukemia and identification of JAK2 gene mutations are useful in polycytemia vera (PV), essential thrombocytemia (ET) and myelofibrosis (PMF). The aim of this study was to investigate the role of molecular biology tests in the management of myeloproliferative neoplasms.
Materials and methods: We tested the blood samples of 117 patients between April 2008 and February 2013 at the Molecular Biology of UMF Târgu Mureş using RQ-PCR (for M-BCR-ABL oncogene) and/or allele-specific PCR (for JAK2^{V617F} mutation).
Results: Thirty-two patients presented the M-BCR-ABL oncogene, 16 of them were regularly tested as a follow-up of the administered therapy: the majority of chronic phase patients presented decreasing or stable values, while in case of accelerated phase and blast phase the M-BCR-ABL values increased or remained at the same level. Twenty patients were identified with the JAK2^{V617F} mutation: 8 patients with PV, 4 with ET, 3 with PMF, 4 with unclassifiable chronic myeloproliferative disease and 1 patient with chronic myelomonocytic leukemia. There was no case of concomitant occurance of both molecular markers.
Conclusions: Molecular biology testing plays an important role in the management of myeloproliferative neoplasms: identification of the molecular markers confirms the final diagnosis, excluding secondary causes of abnormal blood count parameters. Regular monitoring of M-BCR-ABL expression level is useful in the follow-up of therapeutic efficiency.

Keywords: myeloproliferative neoplasms, JAK2^{V617F} mutation, M-BCR-ABL oncogene

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Introduction

Myeloproliferative neoplasms are clonal hematopoietic stem cell disorders characterized by the proliferation of one or more of the granulocytic, erythroid, megakaryocytic or mastocytic lineages. These disorders form a heterogeneous group, with similar symptomatology and laboratory findings, leading to a difficult differential diagnosis.

The first classification of these disorders was elaborated by William Dameshek in 1951: chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and erythroleukemia were the original components of the myeloproliferative disorders' (MPD) group [1]. Further investigations refined this classification, erythroleukemia was excluded and the remaining conditions were referred to as the classic MPDs. In 2001 the WHO classification included the classic MPDs in the chronic myeloproliferative disease (CMD) category, along with chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia/ hypereosinophilic syndrome (CEL/ HES) and unclassifiable CMPD [1].

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Due to clarification of the genetic background of these disorders and identification of specific molecular markers (BCR-ABL oncogene, JAK2 mutation, KIT mutation) for myeloid neoplasms, the WHO elaborated a new classification (2008) and included the classic MPDs, CNL, HES, CEL not otherwise categorized, unclassifiable CMPD and the newly-introduced mast cell disease (MCD) in the myeloproliferative neoplasms (MPN) category. Diagnostic criteria were updated to include the identification of these genetic abnormalities, besides clinical signs, symptoms, blood count changes and bone marrow histology [2,3].

The diagnosis of CML is based on the detection of the Philadelphia chromosome — t(9;22)(q34;q11) — by conventional cytogenetic analysis and/or the resulting BCR-ABL fusion gene detected mainly by qualitative and/or quantitative reverse transcriptase polymerase chain reaction (RQ-PCR). Depending on the breakpoint's location on the BCR gene, three main variants of this fusion gene have been described, each of them encoding a different molecular weight protein (the M-BCR-ABL gene the p190 protein, the m-BCR-ABL gene the p210 protein and the μBCR-ABL gene the p230 protein) with abnormal tyrosine kinase activity. The development of specific tyrosine

kinase inhibitors (TKIs) — imatinib, nilotinib, dasatinib — completely changed the management of CML patients and became the gold standard therapy in chronic phase patients [4].

Identification of the Janus-2 kinase (JAK2) point mutations had a great impact on the diagnostic approach of PV, ET and PMF as those can be identified in 95% of PV cases and in approximately 50% of ET and PMF patients. The most common JAK2 mutation is the V617F resulting from the substitution of valine for phenylalanine at the 617 position. The encoded mutant protein is able to activate its signaling pathways in the absence of any stimulus contributing to uncontrolled proliferation of the myeloid lineages [5]. The therapeutic perspective involves the application of specific JAK inhibitors, as several drugs (ruxolitinib, lestaurtinib, CYT387) have been developed after the discovery of JAK2 mutations and the clinical trials are being performed [3].

The aim of this study was to investigate the role of molecular biology testing in the management of myeloproliferative neoplasms with emphasis on the M-BCR-ABL oncogene and JAK2^{V617F} mutation.

Material and methods

We tested the blood samples of 117 patients at the Molecular Biology Laboratory of the Anatomy Department of the University of Medicine and Pharmacy of Tîrgu Mureş. The tests were carried out between April 2008 and February 2013 and the patients were all treated at the Hematology and Transplant Center of Tîrgu Mureş.

For the identification of M-BCR-ABL oncogene we performed RQ-PCR. One ml peripheral blood sample collected in EDTA tube was used for RNA extraction with QIAmp RNA Blood Mini Kit 50 (QIAGEN, Cat. No. 52304). RNA concentration and purity was measured using a NanoDrop spectrophotometer (ND-1000 Spectrophotometer V3.5). This was followed by DNA transcription with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat. No. 4374966) according to supplier's instructions. To quantify the M-BCR-ABL expression level, we performed RQ-PCR analysis using an ABI 7500 Real Time PCR instrument (Applied Biosystem) with 25 µl final volume: 5 µl cDNA and 20 µl TaqMan Universal PCR Master Mix (Applied Biosystem) using the primers and protocols recommended by the Europe Against Cancer Program [6]. We performed relative quantification with the ABL gene as endogenous control [7]. All reactions were made in triplicate and we also utilized known positive and negative control samples.

For the detection of the JAK2^{V617F} mutation allele-specific polymerase chain reaction was used. DNA was extracted from 200 µl peripheral blood and subjected to PCR using GeneAmp PCR buffer, dNTP, reverse primer, forward primer and AmpliTaq Gold DNA Polymerase (Applied Biosystems) in a final volume of 20 µl. After the amplification, electrophoresis was performed on 1.5% agarose gel with pUC19 (SM 0223, Fermentas) as a DNA ladder [8].

All the investigations were in accordance with the Declaration of Helsinki.

The statistical analysis was carried out with GraphPad Instat Version 3.06 software using Fischer's test and Mann-Whitney U test.

Results

Peripheral blood samples of 117 patients were tested during the mentioned period: in case of 44 patients we performed concomitant M-BCR-ABL and JAK2^{V617F} determination, 53 patients were tested only for M-BCR-ABL, and in case of 20 patients only JAK2^{V617F} analysis was performed. The results are shown in Table I.

The M-BCR-ABL positive group contains 16 patients evaluated at one occasion and 16 patients whose expression level was measured at regular intervals to monitor the therapeutic response. From the regularly monitored group two patients underwent hematopoietic stem cell transplantation (HSCT): one patient is currently in complete hematologic and molecular remission (undetectable M-BCR-ABL expression level) without any therapy after four years from the intervention, while the other presented early relapse and despite second-generation tyrosine kinase inhibitor therapy, he died at 13 months post-HSCT because of septic complications. The majority of the 14 medication treated and monitored patients receive first generation TKI (imatinib) therapy, the expression level and clinical phase changes are shown in Table II.

The case of two CP phase patients should be mentioned: after decreasing M-BCR-ABL values, they presented increasing expression levels without significant blood count changes, as shown in Table III. In case of patient number 2 we only selected two moments from the follow-up: diagnosis and significant increase of M-BCR-ABL value.

Table I. The results of the molecular analysis

CONCOMITANT analysis 44 patients	2 patients M-BCR-ABL positive + JAK2^{V617F} negative 15 patients M-BCR-ABL negative + JAK2^{V617F} positive 27 patients M-BCR-ABL negative + JAK2^{V617F} negative No patient M-BCR-ABL positive + JAK2^{V617F} positive
Only JAK2^{V617F} 20 patients	5 patients positive 15 patients negative
Only M-BCR-ABL 53 patients	30 patients positive 16 regularly monitored 14 one examination 23 negative

Table II. The relationship between M-BCR-ABL expression level and clinical phase

Clinical phase	M-BCR-ABL mRNA expression level			p value
	Decreasing	Stable	Increasing	
Chronic phase	6	2	3	0.066
Accelerated phase	0	0	1	
Blast phase	0	2	0	

Table III. Comparison between blood count parameters and M-BCR-ABL values in case of two CML patients

Patient	M-BCR-ABL	Determination date	WBC (/µl)	Hgb (g/dl)	Htc (%)	PLT (/µl)
1.	0.8%	July 2012	5780	13.1	37.5	207,000
	2.24%	Febr 2013	8470	12.9	39.7	228,000
2.	0.013%	Oct 2008	5730	11.9	38.2	197,000
	2.53%	Apr 2011	7590	13.9	43.4	274,000

Table IV. Clinical characteristics of PV and ET patients according to JAK2^{V617F} mutational status

	PV		
	JAK2^{V617F} positive	JAK2^{V617F} negative	p value
Sex: male/female	5/3	1/2	0.545
Mean age (years)	53.25 (38–73)	60.6 (49–79)	0.497
Mean WBC (/µl)	16,987.5 (9950–32,800)	11,960 (9770–15,010)	0.375
Mean Hgb (g/dl)	18.48 (16.4–20.8)	15.5 (12–17.6)	0.048
Mean Htc (%)	58.97 (53.4–63.8)	49.13 (45.1–52.7)	0.012
Mean PLT (/µl x 10^3)	413.625 (108–647)	654.3 (395–854)	0.193
	ET		
	JAK2^{V617F} positive	JAK2^{V617F} negative	p value
Sex: male/female	1/3	3/8	1
Mean age (years)	62 (58–66)	61.18 (44–78)	0.941
Mean WBC (/µl)	17,962.5 (34,290–10,240)	10,385 (4890–24,080)	0.137
Mean Hgb (g/dl)	13.075 (6.5–17)	12.85 (8.9–15.7)	0.601
Mean Htc (%)	40.6 (21.7–53.4)	38.49 (30.2–44)	0.753
Mean PLT (/µl x 10^3)	1323.25 (1091–1832)	657.3 (436–1076)	0.001

Between the two mentioned period we obtained very low (0.0055%) and even undetectable M-BCR-ABL expression level.

The JAK2^{V617F} point mutation was identified in case of 20 patients (15 patients from the concomitant group and 5 patients investigated only for JAK2^{V617F}). The majority (8 patients) were diagnosed with PV, 4 patients with ET, 3 patients with PMF, 4 patients with unclassifiable CMPD and 1 patient with myelomonocytic leukemia.

In case of 42 patients we could not identify the presence of JAK2^{V617F} mutation. In almost half of them (20 patients) a diagnosis of any hematologic disease was excluded (the abnormal blood counts were interpreted in the context of autoimmune disorders, secondary to neoplasms or idiopathic). The remaining 22 patients maintained a hematologic diagnosis: the majority (17) were diagnosed with a form of CMPD (2 CML, 11 ET, 3 PV, 1 unclassifiable CMPD) based on bone marrow analysis and 5 patients were diagnosed with other hematologic diseases (Hodgkin, non-Hodgkin lymphoma, chronic lymphoid leukemia).

The comparison between clinical characteristics of PV and ET patients according to JAK2 mutational status is shown in Table IV.

Discussion

The understanding of the pathophysiology of myeloid neoplasms at the molecular level represented a huge breakthrough in the management of these disorders from the diagnostic approach to the elaboration of targeted therapies and follow-up.

CML represents a particular CMPD, it is a real success story; the discovery of molecular pathways lead to radical changes in the management of these patients: the diagnosis is based on the identification of the Philadelphia chromosome and/or BCR-ABL fusion gene, as gold standard treatment targeted TKI therapy (imatinib 400 mg) is administered and cytogenetic and/or molecular analysis is recommended for regular follow-up [9]. In our study, we measured the M-BCR-ABL oncogene by RQ-PCR and monitored at regular intervals the changes of the expression levels after HSCT and under TKI therapy. The majority of chronic phase patients presented decreasing or stable values, while in case of accelerated phase and blast phase patients the M-BCR-ABL values increased or remained the same as we previously reported [10]. This confirmed the reduced efficacy of imatinib in these latter phases, which is concordant with international guidelines recommendations [11].

In case of two chronic phase patients we observed increasing M-BCR-ABL values, and upon comparing this result with the blood count findings, we noticed that hematologic parameters were not elevated, even when the transcript levels were much higher than previously. Consequently, monitoring patients by RQ-PCR testing, a very sensitive method, allows the timely identification of resistant cases, therefore the therapeutic changes (dose increasing, switching to second generation TKI) can be accomplished in time.

The discovery of JAK2 mutations and their contribution in Ph-negative myeloproliferative neoplasms repre-

sented an extremely important change in the management of these disorders.

Numerous studies were carried out regarding the occurrence of mutations which reported different occurrence ratios; nevertheless, it is widely accepted that the JAK2^{V617F} mutation can be identified most frequently in PV (60–95%) cases and approximately in half of ET and PMF patients. In our study, despite the small number of positive cases, we can notice the same distribution: as we detected the JAK2^{V617F} mostly in PV patients (8 out of 20 positive cases), while other disorders — ET, PMF — presented the mutation less frequently (4/3 cases). Upon analyzing the final diagnosis of JAK2 negative patients, we noted that there were significantly more ET cases than PV cases. JAK2^{V617F} is not specific for any single MPN, but because of the high incidence rate in PV it became the preferred initial testing according to WHO criteria, as it is an useful resource to exclude secondary causes of PV [12]. However, JAK2^{V617F} negative PVs were described, in such cases further investigations are needed: commonly other than V617F mutations of the JAK2 gene are involved, especially those affecting exon 12 [13,14]. Unfortunately, as the specificity of JAK mutations in ET and PMF is reduced, we can not exclude these disorders in case of absence of JAK2^{V617F}.

Comparing the blood count parameters according to the presence or absence of JAK2^{V617F} mutation in case of PV, we found significant differences in the hemoglobin and hematocrit values, those carrying the mutations presenting higher values, and JAK2^{V617F} positive ET patient's platelet counts were significantly greater than those lacking the mutation; the other variables showed statistically not significant differences. However, due to the restricted number of investigated patients and the diversity of reported data we should interpret this result cautiously. A great number of studies investigated the differences between clinical phenotype and blood count parameters according to mutational status with large variation of the results. A German study could not find any correlation between the presence of mutation and clinical parameters of PV and ET patients, however Chinese and Korean scientists reported significant correlation between the presence of mutation and blood count parameters, but the results are divergent: while in one study JAK2^{V617F} positive ET patients showed higher neutrophil and leukocyte counts, the other study found that ET patients carrying the mutations were older, with higher neutrophil counts, with greater risk for myelofibrosis and thrombotic events [15–17]. The clinical picture of MPDs is very diverse and there is no blood count parameter available that could predict the presence or absence of mutations and further research is needed to estabilish the characteristics of mutation carriers.

An interesting aspect is the role of JAK2 mutation in other myeloid malignancies, as it was described in acute myeloid leukemia and myeloproliferative/myelodisplastic syndromes (chronic myelomonocyitc leukemia-CMML, atypical Ph-CML) as well. A study conducted on 374 patients with hematologic neoplasms identified the presence of JAK2^{V617F} point mutation in 3 out of 16 cases of atypical CML and in 7 of 53 patients with CMML [18]. Regarding the presence of JAK2^{V617F} in myeloid malignancies other than PV, ET and PMF we have one CMML patient in our database with splenomegaly, anemia and high neutrophil count at presentation. This observation partially matches the results of a study performed on 78 CMML patients, which identified this mutation in 8 cases, all patients presenting splenomegaly and significantly higher hemoglobin levels and neutrophil counts than those lacking this genetic aberration [19].

We did not find any patient with concomitant occurrence of JAK2^{V617F} and BCR-ABL, but lately such cases were reported. In these case presentations there is no specific pattern: some cases presented both genetic abnormalities at the moment of diagnosis, others during the course of the disease. The first diagnosis is also variable: it was either CML, either PV, but in the majority of cases BCR-ABL subclones were the dominant ones, JAK2^{V617F} subclones becoming manifest only after the suppression of BCR-ABL with targeted TKI therapy. In such cases the patients had abnormal blood counts (leukocytosis, trombocytosis), rising the suspicion of TKI therapy failure or resistance, but molecular testing clarifies the situation [20–22].

Compared to the 2001 WHO criteria, one of the innovations of the 2008 system was the classification based on the presence of a genetic marker, but the classification does not refer to situations with more than one genetic abnormality, therefore this aspect needs to be clarified.

Conclusions

As more and more molecular pathways are understood, genetic investigations acquire an important role in the management of myeloproliferative neoplasms.

In case of CML patients, the presence of the BCR-ABL oncogene confirms the diagnosis, and monitoring the expression levels by RQ-PCR, a highly sensitive method, allows early identification of resistant cases, enabling timely therapeutic changes.

The detection of the JAK2^{V617F} point mutation is an important part of the diagnosis of myeloproliferative neoplasms as it is present in most of the PV cases, and almost half of the ET and PMF patients, helping the physician to exclude secondary causes of elevated blood count parameters. There is a perspective of introducing targeted JAK2 inhibitor therapy for mutation carrier patients, as undergoing clinical trials show promising results.

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