



PERFORMING SIMULTANEOUS DETECTION OF BCR-ABL AND JAK2V617F MUTATION REPRESENTS AN IMPROVEMENT FOR A MORE ACCURATE AND LESS TIME CONSUMING MYELOPROLIFERATIVE NEOPLASMS DIAGNOSIS

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Abstract. This article presents observations made following a more comprehensive study which analyzed the results of molecular tests performed for the diagnosis of myeloproliferative neoplasms in a molecular biology laboratory over a period of seven years (January 2009- December 2015). The laboratory is an ELN (European Leukemia Net) certified diagnostic laboratory for quantification of BCR-ABL. Two diagnosis tests (for BCL-ABL transcript and Jak2 V617F point mutation) were performed on a number of 614 patients with various chronic myeloproliferative neoplasms subtypes. 320 (52%) patients were found to be Jak2 V617F positive, 25 (4.07%) were found to be BCR-ABL positive. A "double negative" was found in 262 (42.67%) patients and 7 (1.17%) patients were positive for both Jak2V617F and BCR-ABL. The results are consistent with other similar works from the scientific literature and demonstrate the importance of molecular testing for an accurate diagnosis and a correct assessment of the clonal character of proliferations. Also, due to the particular situations, the simultaneous performance of both screening tests in the diagnosis of CMN is highly recommended.

Key words: Myeloproliferative neoplasms, Bcr-abl transcript, Jak2V617F mutation.

Background

The importance of genetic testing in the diagnosis of chronic myeloproliferative neoplasms was first discovered in the '60s, when the Philadelphia chromosome was found to be associated with Chronic Myeloid Leukemia (CML). The discovery of several mutations involved in the pathogeny of chronic myeloproliferative neoplasms has later imposed specific markers as major criteria in the diagnosis of these diseases.

Chronic myeloproliferative neoplasms are clonal proliferations of hematopoietic stem cells that involve one, two or all cell lines of myeloid precursors, by uncontrolled growth and accumulation of mature myeloid cells. In 1951 William Dameshek, described and defined for the first time these diseases as a group. The group of "myeloproliferative disorders" (MPDs) included five entities: chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and erythroleukemia.[9]

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The first genetic change linked to a disease was described by Novell in 1960 as Philadelphia chromosome present only in cases of CML. In 1972, Janet Rowley deciphered the Ph chromosome as a reciprocal translocation between chromosomes 9 and 22; t(9;22)(q34;q11). Later (between 1982 and 1990) an American scientists group defined Bcr-abl fusion transcript and established this one as being the disease-causing mutation in CML.[5]

The second important event was the historic observation made in 2005 by four independent laboratories that the majority of patients with classic BCR-ABL-negative MPDs carried a JAK2 gain-of-function mutation (JAK2V617F; a G to T somatic mutation at nucleotide 1849).[5]

JAK2V617F mutation was noticed in 95% of PV cases and in 60% of PMF and ET cases.[14]

These discoveries clarified some pathogenesis aspects, leading to changes in classifications and diagnosis criteria. The 2008 WHO's classification of hematologic malignancies is based on the employment of molecular biology screening for diagnosis. It also introduces for the first time the term of neoplasm, in order to emphasize the clonal character of these proliferations demonstrated in 1967). [5, 15]

Myeloproliferative neoplasms are currently divided into two groups: BCR-ABL positive myeloproliferations (CML cases showing a presence of BCR-ABL major oncogene) and BCR-ABL negative myeloproliferations (with 7 subtypes: polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic neutrophilic leukemia(CNL), eosinophilic leukemia and hypereosinophilic syndrome(CEL and HES), systemic mastocytosis (SM) (unclassifiable chronic myeloproliferative diseases).[13,15]

The most frequently found MPN BCR-ABL negative entities are: PV, ET and PMF

The use of molecular markers in the diagnosis of chronic myeloproliferative neoplasms has become essential also due to a new and revolutionary type of drugs: low molecular weight drugs with targeted action on tyrosine-kinases involved in oncogenesis. The discovery of Imatinib in 1996 has been considered by Tefferi "a paradigm shift in the cancer treatment" See [5] At the moment there are two types of tyrosin-kinase inhibitors: BCR-ABL inhibitors which are successfully used in the treatment of CML and JAK2 inhibitors used in PMF therapy and in PV cases which are resistant or intolerant to hydroxyurea.

Materials and method

Genomic DNA and total RNA were extracted from integral peripheral blood using Qiagen IVD Kits following protocols recommended by the manufacturers.

The concentrations of nucleic acids were determined by spectrophotometry using NanoDrop ACTgene, USA. Nucleic acids qualities were assessed by the 260:280 and 260:230 spectrophotometric ratios

V617F mutations were detected using ARMS assay (Amplification Refractory Mutation Screening) described by Jones et al. in Blood, 2005 Sept 15, 106 (6). [3]

DNA tetra-primer ARMS assay uses two pairs of primers that specifically amplify in the same reaction the normal and mutant sequences and a positive control fragment. Amplifications were performed under standard conditions (annealing temperature 56°C, and 35 cycles)using AmpliTaq Gold® DNA polymerase (Applied Biosystems, USA).

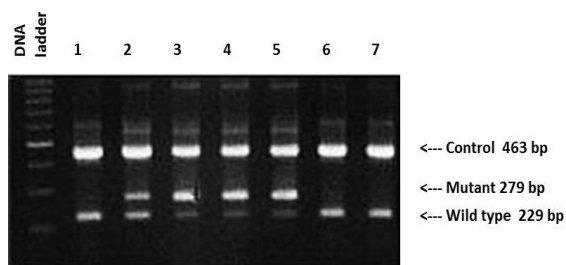


Fig.1. JAK2V617F detection by ARMS PCR:
Representative results: Tracks 1,2,3, are controls, 4 and 5 a homozygous positive patient and 6,7 a negative patient

The resulting amplicons were: a 463bp control fragment and fragments corresponding to V617F mutation (279bp) and to wild-type sequence (229bp).

The amplicons were resolved on 2% agarose gels (through electrophoresis) and visualized after staining with ethidium bromide and using UV transluminal.

Every experiment used specific controls: positive (with mutant allele) and negative (with wild-type allele). Even this method requires post-amplification manipulation ARMS PCR is considered one of the most sensitive techniques (detects low level of mutant allele:1-2%).

Detection and quantification of BCR-ABL transcription was done using real-time PCR, following EAC protocol (as described by Gabert et al., Leukemia, 2003), on StepOnePlus platform, Applied Biosystems.[1]

Ritus Biotec Laboratory is an ELN (European Leukemia Net) certified diagnostic laboratory for quantification of BCR-ABL.

Our methods are periodically validated and evaluated through external quality assessment schemes for clinical laboratories (from UK NEQAS - United Kingdom National External Quality Assessment Service).

PCR and real-time PCR assays were performed at onset of the disease and, in some particular situations, during the clinical course as well.

From 2009 to 2015, 614 suspected MPN patients were subjected to the two types of molecular biology testing in our laboratory. All patients had presumptive diagnosis of MNP in compliance with recommendations of clinical practice guidelines. See [14] The 614 patients are between 19 and 90 years old (median age:61.5years), they are both male and female(48% male;52%female) and came from different areas of Romania.

Results and discussions

The results were as follows: 320 patients (52%) were Jak2V617F positive, 25(4.07%) of the patients were BCR-ABL positive, 262 (42.67%) presented none of the two mutations and 7 patients presented both mutations (1.14%). Figure 2 describes the distribution of molecular biology test results in a series of 614 patients with a presumptive diagnosis of chronic myeloproliferative neoplasms.

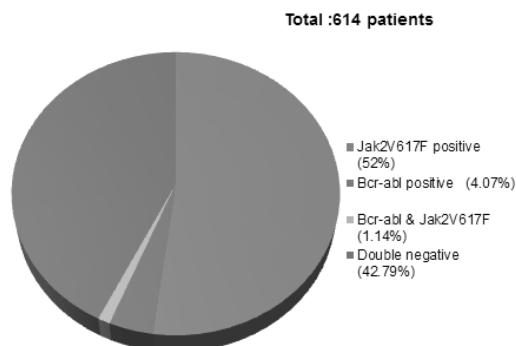


Fig.2. Test results on 614 patients

Of the 614 patients tested for both tests: 549 patients had a clinical suspected diagnosis of BCR-

ABL-negative MPN and 65 patients had a clinical suspected diagnosis of BCR-ABL-positive MPN (“CML like”).

Of the 65 patients who had a clinical suspected diagnosis of MPN-“CML like”

we noticed that about 1/3 presented Jak2 V617F(35.38%) mutation and only 9 cases (13.84%) were confirmed CML (Bcr-abl positive). 33 patients (50.79%) presented negative results in both tests. Most of the cases of MPN-CML like with JAK2V617F positive test results were proved to be a prefibrotic phase of PMF, in various stages of evolution.

The distribution of JAK2V617F mutation among the BCR-ABL negative MPN subtypes was as follows:the mutation was noticed in 66% of PV cases, in 56% of ET cases and in 54% of PMF cases. Similar data were reported in other scientific works.[8, 10, 11,15]

	No patients/%	Age(Years)	Male%	Female%
	Total(33)	56(36-91)	58%	42%
PV	Jak2V617F Positive: 22 - 66%	60(38-81)	41%	59%
	Total (63)	57(26-91)	30%	69%
ET	Jak2V617F Positive: 35 - 56%	56(26-87)	25%	75%
	Total (180)	63(28-85)	48%	52%
PMF	Jak2V617F Positive : (96) -54%	64(28-85)	59%	49%

Table 1. Results distribution of Jak2V617F positive patients according to disease, age and gender patients

We noticed the presence of BCR-ABL transcript in 16 patients with Bcr-abl negative MPN s group (14 with the presumptive diagnose of MPN unclassified and 2 with the diagnose of PMF) .

The JAK2V617F detection test alone would have lead to a wrong classification.

Interestingly was the presence of both molecular markers, as in the case of the 7 patients. It is a rare but not impossible phenomenon the 1.14% (7/614)

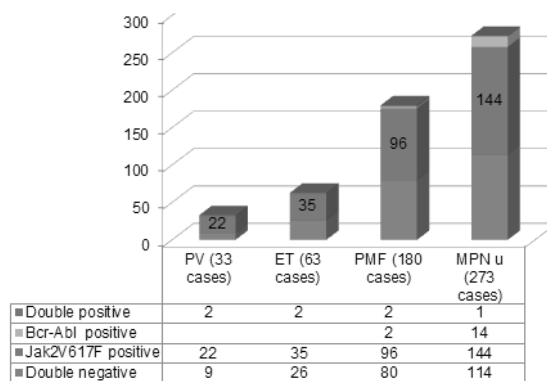


Fig.3. Result distribution according MNPs subtypes

we have obtained in our study is consistent with other scientific works: 0.37% (5/1320) in Cappetta et al., 2013; 2.25% (7/314) in Pieri et al., 2011). [2, 4, 7]

We mention that these particular cases were carefully monitored over time

These patients were integrated in various MPN entities: 2 cases of PV, 2 cases of ET, 2 cases of PMF and one unclassified CMN, according to classical diagnostic criteria.

Using clinical monitoring and laboratory data we noticed the change of the MPN phenotype, resulting in all these patients showing a CML typical aspect at various stages of the disease course, which was confirmed by the presence of BCR-ABL marker. BCR-ABL translocation gives a growth advantage comparing to JAK2V617 and thus induces the proliferation of granulocytes, resulting in CML phenotype. The JAK2V617 induced phenotype occurs only when the BCR-ABL transcript is absent/reduced due to tyrosin-kinase inhibitors therapy.

We present two of these 7 cases mentioned above as two different situations with simultaneously occurrence of two mutations that are considered mutually exclusive. The molecular diagnose and follow up testing were done in our laboratory.

First case is a Ph+ CML in treatment with Imatinib who developed after four years Jak2V617F positive PV . The second case is a PV diagnosed in 2003 based on standard clinical and biological criteria who developed after 7 years Bcr-abl positive CML[6]. The most important features are described in table nr.2

M/F	Age	Diagnostic	HLG			Bcr-abl transcript (ratio bcr-abl/abl%)	Jak2V617F	Treatment	Clinical features; Particularities
			Hg g/dl	WBCx 10 ³ /ul	PLT X10 ³ /ul				
M	51	I:CML(2009) Sept 2010 Febr 2011 Mar 2012	9.8	139	418	100%	-	Imatinib	First diagnose was CML(with low Hg level:9.8/dl,HT28%) Four years after clinical changes (pruritus, facial erythema) and important hematologic changes occurred (Hb:18gdL, Ht:54%, L-4.8x10 ⁹ /mmc,Plt-665x10 ⁹ /mmc), which raised the suspicion of polycythemia. Confirmed by bone marrow biopsy and the presence of Jak2V617F mutation.
		0.02% Undetectable Molecular Response				-	Imatinib		
		II:PV(2013) Sept 2014 Dec 2015	18	4.8	665	MR4 MR4 MR4 MR4	Positive Homozygous	Imatinib+ Phlebotomy	

M/F	Age	Diagnostic	HLG			Bcr-abl transcript (ratio bcr-abl/abl%)	Jak2V617F	Treatment	Clinical features; Particularities
			Hg g/dl	WBCx 10 ³ /ul	PLT X10 ³ /ul				
M	61	I: PV(2003)	20.8	20	750	-		Hydroxyurea	First diagnose was PV based on clinical and biological standard criteria.
		II: CML(2010))	9	108	95	100%	Negativ	Imatinib	
		Jan 2012				0.6%	Pozitive homozygous	Imatinib+ Phlebotomies	
		Oct 2012				Undetectable	Positive homozygous	Imatinib+	
		May 2013				MR4		Phlebotomies	
		Jul 2014				MR4			
Nov 2015				MR4					

(*This case was first described by Iulia Ursuleac, MD, PhD in the 2013 article The concomitant occurrence of Jak2 V617F mutation and BCR-ABL translocation with phenotypic expression - an overlapping myeloproliferative disorder or two distinct diseases? Case report.

Conclusions

The data reveals the limits of a diagnosis based mainly on clinical and standard biological criteria, showing that among the 65 MPN-“CML-like” cases, the disease could be confirmed only in 9 cases (13.8%), while in the group with BCR-ABL negative MPN suspected patients, 16 cases (2.91%) were positive for BCR-ABL.

Particular cases showing the simultaneous presence of both markers contradict the idea that these mutations would exclude each other and raise new questions over the pathogenesis of MPNs.

Nowadays an accurate diagnosis of MPN subtype (P, ET, PMF) requires more tests of molecular biology: Jak2 exon 12 mutations, MPL, newly discovered CALR mutations.[14]

Bcr-abl and Jak2V614F mutation detection maintain important as screening tests and even more, performing them simultaneously is highly recommended

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