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CORRELATION BETWEEN JAK2V617F MUTATIONAL BURDEN AND THE DEGREE OF ANGIOGENESIS IN THE BONE MARROW OF BCR-ABL NEGATIVE MYELOPROLIFERATIVE NEOPLASMS.

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SUMMARY

JAK2V617F mutation is a well-recognized feature in most Ph-negative myeloproliferative neoplasms (MPNs). An activated bone marrow (BM) angiogenesis has been established in these disorders as well. Consequently, the rational question is to ascertain a possible relation among JAK2 mutation, morphological features and angiogenesis in MPNs bone marrow.

Aim: To assess bone marrow microvessel density (MVD), bone marrow cellularity and fibrosis in newly diagnosed patients with BCR-ABL-negative MPNs, and define a correlation between the degree of angiogenesis in the bone marrow and JAK2V617F mutant allele burden.

Methods: JAK2 mutational burden was determined by RT-PCR, BM angiogenesis was defined by MVDassessment using anti-CD34 for BM endothelium staining. The BM fibrosis was evaluated according to the Hanover system. The statistical analysis was performed with SPSS 17.0 software. 52 patients with newly diagnosed MPN were included in the study.

Results: The distribution of the mutational burden was as follows: 26 patients with polycythaemia vera (PV), 16 patients with primary myelofibrosis (PMF) and 10 patients with essential thrombocythaemia (ET). In patients with PV the homozygosity was found prevalent in frequency whereas in PMF and ET the heterozygous variants were dominant. In all patients a significant positive correlations between JAK2V617F and BM MVD (r=0.306, p<0.002) and between MVD and fibrosis, (r=0.523, p<0.0001), was found.JAK2 correlated positively but borderline with fibrosis. The MVD and JAK2 burden were found in significant negative correlation with the BM cellularity (r=-0.405; p<0.002 and r=-0.431, p<0.0001, resp.).

Conclusions: The significant correlation between JAK2V617F, BM angiogenic activity and the fibrosis marks out the JAK2 allele burden as a feasible parameter with prognostic significance for evolution and progression of MPN.

Key words: JAK2V617F mutation, angiogenesis, myeloproliferative neoplasm, polycythaemiavera (PV), essential thrombocythaemia (ET), primary myelofibrosis (PMF),

In 2005 four separate groups using different models and measurement techniques (Baxer et al., James et al., Kralovics et al. and Levin et al.), reported the presence of a novel somatic point mutation in the conserved autoinhibitorypseudokinase domain of the gene coding for the Janus kinase 2 (JAK2) protein in patients with classic MPNs. The mutation is located on exon 14 of the gene and was noted to cause a valine-to-phenylalanine substitution at amino acid position 617. The loss of JAK2 autoinhibition caused by JAK2V617Fmutation results in constitutive activation of the kinase and in recruitment and phosphorylation of substrate molecules including downstream signaling pathways JAK-STAT, PI3K/AKT, MAPK/ERK [1, 2]. In humans JAK2V617F mutation presents at the stem cells level and is present in hematopoietic stem cell progenitor [3].

The angiogenesis (new blood vessel formation) is a key requirement for tumor growth and spread (Folkman 1995). Vascular endothelial growth factor (VEGF) has a rate-limiting role in promoting tumor angiogenesis and exerts its effects by binding to its tyrosin kinase receptors: VEGF-R1 and VEGF-R2 [4]. Several studies suggestthe role of angiogenesis in hematological malignancies such as acute and chronic leukemia, lymphoma and multiple myeloma. The available data on angiogenesis and VEGF expression in the bone marrow of patients with BCR-ABL-negative myeloproliferative neoplasms (MPN) reveal that micro vessel density (MVD) is increased, especially in primary myelofibrosis (PMF), and that increased angiogenesis might inversely correlate with survival [5]. Our previous data from the analysis of bone marrow pathology and angiogenesis in patients with Ph-negative MPN shows increased angiogenic activity, especially in PMF [6].

The correlation between JAK2V617F mutational status and angiogenesis in the bone marrow in patients with MPNs has been addressed in few publications with contradictory results [7, 8, 9]. Since, the increased activity of the VEGF-pathway can serve as a treatment target, additional studies on the relation between mutational status and degree of angiogenesis are further required.

The aim of our study was to assess bone marrow MVD, bone marrow cellularity and fibrosis in newly diagnosed patients with BCR-ABL-negative MPNs and to define a correlation between the degree of angiogenesis in the bone marrow and JAK2V617F mutant allele burden.

PATIENTS AND METHODS

Aprospectivestudy was performed according to the regulations of the local ethics committee. We included52 patients with newly diagnosed and previously untreated MPNs. Bone marrow biopsies were analyzed for the extent of angiogenesis. Bone marrow samples were fixed in buffered neutral formalin for 12 - 48 hours. Slides were stained with Hematoxyline and Eosin and immunohistochemically for the evaluation of endothelial cells with FLEX Monoclonal Mouse Anti-Human CD34 Class II, Clone QBend 10 (DAKO). Visualization system was Envision High pH (Link) (Code K8000). Sections were examined for bone marrow MVD atx400 magnification. Five hot spot areas (areas of highest neovascularization) in CD34 stained sections were selected and the mean number of microvessels was measured. The degree of bone marrow fibrosis was established according to the Hannover system.

Analysis of JAK2 V617F mutation was performed by Polymerase Chain Reaction (PCR). Peripheral blood mononuclear cells were separated after red blood cells destruction with a lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) (Silva et al., 2002) [10]. Genomic DNA (gDNA) and/or total cellular RNA was isolated using Trizol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. JAK2 V617F mutation status was determined by two different approached using allele-specific (AS)-PCR [1] and PCR restriction fragment length polymorphism (RFLP) analysis [2] using either the genomic DNA or complementary DNA (cDNA), synthesized via reverse transcription of RNA with 200 U MMLV reverse transcriptase (USB Products, Affimetrix, Cleveland, Ohio, USA) according to the previously reported method (van Dongen et al., 1999) [11].

gDNA AS-PCR: Reaction was performed using 100 ngg DNA as a template in a total volume of 25 µl containing 1x PCR buffer, 1.5 mM $MgCl_2$, 40 pmol of each primer (control forward primer: 52 -atctatagtcatgctgaaagtaggagaaag-32; mutation specific forward primer: 5'-agcatttggttttaaattatggagtatatt-3'; common reverse primer: 52 -ctgaatagtcctacagtgtttt-cagtttca-32) (Baxter et al., 2005) [12], 200 µM each of deoxynucleoside-52 -triphosphates (dNTPs), and 1 U of Taq polymerase (Promega, USA). Forty cycles of amplification were performed with an annealing temperature of 57°C on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

gDNA PCR-RFLP: Reaction was performed using 200 nggDNA as a template in a total volume of 32 µl containing 1x PCR buffer, 2.0 mM $MgCl_2$, 40 pmol of each primer: JAK2 forward primer 5'-gggtttcctcagaacgttga-3' and JAK2 reverse primer 5'-tcattgctttcctttttcacaa-3' (Verstovsek et al., 2008)[13], 200 µM each of dNTPs, and 1 U of Taq polymerase (Promega). Forty cycles of amplification were performed with an annealing temperature of 57°C on a Thermocycler Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia). The amplified fragment was digested overnight at 37°C with 2 µl BsaXI endonuclease (New England BioLabs, Ipswich, MA).

cDNA AS-PCR: Reaction was performed using 2 µl of cDNA as a template in a total volume of 25 µl containing 1x PCR buffer, 1.5 mM $MgCl_2$, 25 pmol mutation specific forward primer: 5'-agcatttggttttaaattatggagtaggtt-3'; 25 pmol control forward primer: 5'-gaagatttgatatttaatgaaagccttg-3' and 50 pmol common reverse primer: 52 -ctgaatagtcctacagt-gttttcagtttca-32 , 200 µM dNTPs, and 1 U of Taq polymerase (Promega). Thirty eight cycles of amplification were performed with an annealing temperature of 58°C on instructions on a Thermocycler Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia).

cDNA PCR-RFLP: PCR amplication was carried out using 4 µl of cDNA as a template in a total volume of 32 µl containing 1x PCR buffer, 1.5 mM $MgCl_2$, 25 pmol of each primer 5'-taaaggcg-tacgaagagaagtaggagact-3' and 5'-ggcccatgccaactgtttagc-3' (Jamieson et al., 2006)[14], 200 µM dNTPs, and 1 U of Taq polymerase (Promega). Thirty eight cycles of amplification were performed with an annealing temperature of 58°C on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Following amplification, JAK2 amplicons were digested overnight at 37°C with 2 ìLBsaXI endonuclease (New England BioLabs, Ipswich, MA, USA).

All amplification and digestion products were run in a conventional 2% (allele-specific PCR) or 3% (PCR RFLP) agarose gel stained with Sybr Safe (Invitrogen, Karlsruhe, Germany) and visualized after UV irradiation. Appropriate controls comprising wild-type, heterozygous and homozygous JAK2 V617F mutants were included in all experiments.

The statistical analysis was performed with SPSS 17.0 software.

RESULTS

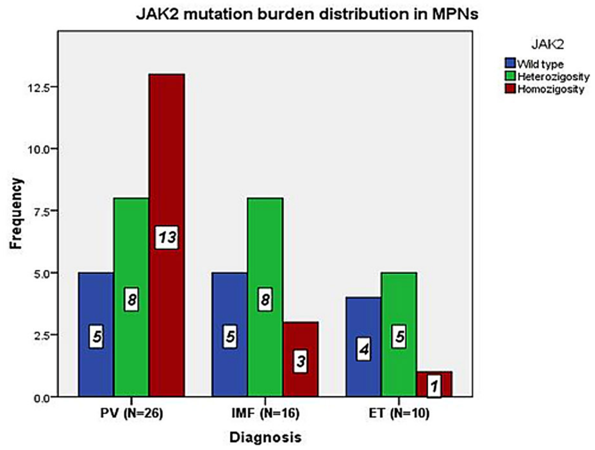
Demographic data:

The patients with Polycythemia vera (PV) were 26with average age of 55.5 years, between 23 and 75 years. A small prevalence of the female sex was established (women: men 54%: 46%).Patients with primary mielofibrozis (PMF) were 16 with an average age of 64 years (44-74), female: male ratio 40%: 60%.10 patients with essential *trombocitemia* (ET) had an average age of 61 years (37-72), with prevalence of the female sex (women: men –60%: 40%).

JAK2 mutation burden

In the group of patients with PV (26 patients) JAK2V617F mutation was registered in 81% of cases, 62% of them were homozigotes and 38% heterozygotes. In the group of patients with PMF (16 patients) 69% were carriers of the mutation (27% homozigotes and 73% heterozygotes). Among the patients with ET (10 patients) mutation carriers were 60%, as only one patient was homozygote. The distribution according to MPN type is presented on Fig.1.

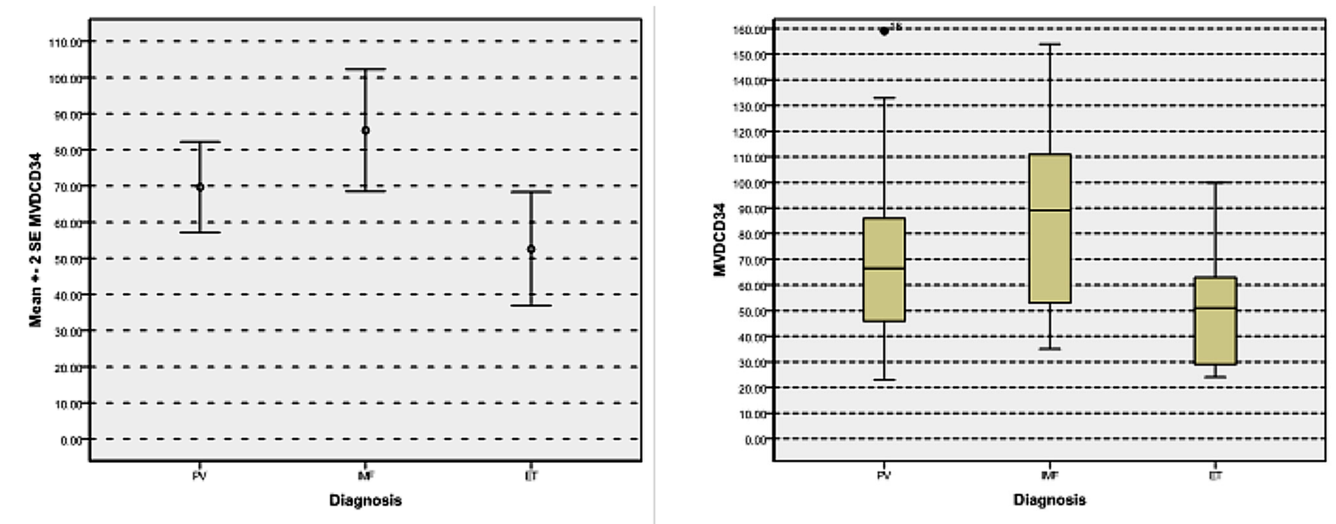
Fig. 1. Myeloproliferative neoplasms (MPNs) and JAK2V617F mutational burden distribution of the tumor load in three entities.



Microvessel density (MVD)

The most significant grade of MVDwas found in the group of patients with PMF (average MVD 85), followed by PV (average MVD 70) and ET (average MVD 52). Kruskal-Wallis Test showed a statistically significant difference between the studied groups ($p < 0.05$), the most distinct between PMF and PV and PMF and ET ($p < 0.01$) (fig. 2).

Fig. 2. Bone marrow microvessel density, determined in three groups of malignancies (PV, PLF and ET)



Significant positive correlation was found between MVD and the degree of fibrosis in the bone marrow ($r = 0.503$, $p < 0.0001$) as well as between the degree of MVD and the presence of JAK2 mutation ($r = < 0.306$, $p< 0.002$) in all patient groups (table 1). There was a pronounced significant inverse correlation between MVD and bone marrow cellularity ($r =-0.44$; $p < 0.002$). A negative correlation was found between the presence of JAK2 mutation and the cellularity in the bone marrow ($r =-0.508$; $p < 0.0001$) (table 1).

Table 1. Correlations

			MVDCD34	Fibrosis	Cellularity	JAK2	Age
Spearman's rho	MVDCD34	Correlation Coefficient	1.000	**.503****	**-.440****	**.306***	-.105
		Sig. (2-tailed)	.	**.000**	**.002**	**.027**	.457
		N	52	**48**	**48**	**52**	52
	Fibrosis	Correlation Coefficient	.503**	1.000	-.387**	**.222**	.204
		Sig. (2-tailed)	.000	.	.007	**.130**	.164
		N	48	48	48	**48**	48
	cellularity	Correlation Coefficient	-.440**	-.387**	1.000	**-.508****	-.097
		Sig. (2-tailed)	.002	.007	.	**.000**	.514
		N	48	48	48	**48**	48
	JAK2	Correlation Coefficient	.306*	.222	**-.508****	1.000	.124
		Sig. (2-tailed)	.027	.130	**.000**	.	.380
		N	52	48	**48**	52	52
	Age	Correlation Coefficient	-.105	.204	-.097	.124	1.000
		Sig. (2-tailed)	.457	.164	.514	.380	.
		N	52	48	48	52	52

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

The constitutive activation of the JAK2/STAT3 signaling pathway has been implicated in events involved in tumor-host interactions, such as angiogenesis [15]. JAK2/STAT3 signaling pathway activation mediates tumor angiogenesis by up-regulation of VEGF and basic fibroblast growth factor (bFGF) in different carcinoma types [16, 17]. There are only few publications on the relationship between the existence and the extent of a carrier of JAK2 mutation and the magnitude of the angiogenesis in the bone marrow in MPNs. In a study of Tretinski J et al. [18] a significant increase of angiogenesis in patients with PV and ET was established, but there was no correlation with the carrier of JAK2V617F mutation. In a previous study we have found that increased angiogenesis is present in all Ph negative MPNs, in highest degree in PMF [19]. Recently, new genetic mutations were detected in JAK2V617F negative patients with MPNs (ASXL1, TET2, DNMT3A, SUZ12, etc.) [20, 21]. However, the role of these mutations in the pathogenesis of Ph negative MPNs and especially in the initiation and permanent stimulation of angiogenesis still remains to be established.

CONCLUSIONS

The significant correlation between JAK2V617F, BM angiogenic activity and the fibrosis marks out the JAK2 allele burden as a feasible parameter with prognostic significance for evolution and progression of MPN.

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