Detection of *JAK2* exon 12 mutations in 15 patients with *JAK2*V617F negative polycythemia vera

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ABSTRACT

To further characterize *JAK2* exon 12 mutations, we performed molecular screening in 409 patients with polycythemia vera or unclear erythrocytosis with unmutated *JAK2*V617. The frequency of *JAK2*exon12 mutations was 10/63 (15.9%) in PV but only 5/346 (1.4%) in the erythrocytosis cases. Nine different mutations including four new types (D544-L545del, H538DK539LI540S, H538-K539del, V536-F547dup) were detected. In 2 cases we found evidence for the presence of cells homozygous for mutated *JAK2*exon12. As this was the case in only 2/15 cases with *JAK2*exon12 mutations (13%) homozygosity seemed to be less frequent than in V617F-mutated polycythemia vera (69%) ($p<0.001$). There were more females than males in the group of patients with a *JAK2*exon12 mutation (10 *vs.* 5) compared to the group with wildtype *JAK2* (132 *vs.* 262; $p=0.012$). Median age of onset was lower than in the V617Fmut controls (58.5 *vs.* 67.8 years, $p<0.001$). In conclusion, *JAK2* exon 12 mutation analysis contributes to diagnostics in polycythemia vera or erythrocytosis.

Key words: JAK2 exon 12, PV, erythrocytosis, V617.

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Introduction

The *JAK2*V617F mutation (exon 14) is a valuable marker in *BCR-ABL* negative chronic myeloproliferative diseases (CMPD),[1-5] as it is found in ~50% of patients with primary myelofibrosis (PMF), in 45% with essential thrombocytosis (ET), and in 95% of polycythemia vera (PV) cases. Stimulation of the JAK-STAT pathway leads to increased cell proliferation and cytokine independence.[1-4]

Recently, Scott *et al.* identified a novel mutation in exon 12 of *JAK2* in 10 out of 11 V617 unmutated PV patients.[6] Similar to the effects of the V617Fmut in a murine transplantation model, mutation in exon 12 resulted in a myeloproliferative subtype including erythrocytosis,[6] and *in vivo* stimulation of erythroid proliferation was demonstrated.[6]

According to some authors patients with *JAK2*exon12mut show isolated erythrocytosis[7] and are younger than those with a V617F,[6] whereas others have suggested that granulopoiesis and megakaryopoiesis might also be increased.[8] In PV patients, frequencies of *JAK2* exon 12 mut of 3-4% were described.[6-9]

To further evaluate frequency and phenotypes of mutation in exon 12, we performed analyses in 409 patients with V617 unmutated erythrocytosis and compared the results to 719 PV patients with *JAK2*V617F.

Patients

Screening for mutation in exon 12 was performed in 409 patients with *JAK2*V617 unmutated erythrocytosis. Samples were sent to the MLL Munich Leukemia Laboratory for diagnosis after obtaining the written informed consent of all patients between 01.08.2005 and 31.12.2007. The study was carried out in part consecutively and in part retrospectively. Analyses were performed on bone marrow in 130 cases and on peripheral blood in 279 cases. Sixty-three patients fulfilled the WHO criteria of PV whereas the remaining 346 cases had

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unclear erythrocytosis. There were 267 males and 142 females (15-88 years; median: 58 years). For further evaluation the data of the patients not carrying the *JAK2*V617 mutation were compared to a control cohort of 719 PV patients carrying the *JAK2*V617F mutation (376 females; 340 males; 29-88 years; median 67 years).

Design and Methods

Screening for mutation of *JAK2* exon 12 was performed by a LightCycler 2.0 based melting curve assay covering codons 535-555. Analyses were performed on cDNA from peripheral blood or bone marrow. Sequence analyses were performed on samples with an aberrant melting curve and analyzed by the Mutation Surveyor Software V3.01 (Softgenetics, PA, USA). Analyses for the *JAK2*V617F were performed as previously described.[10]

PCR reaction was performed in a 20 µL reaction volume with each 0.5 µM of forward (tgtaccaacctcaccaacat) and reverse primer (cagttgaccgtagtctcct) 0.75 µM of each Hyb-Probes (ATGGTGTTTCACAAAATCAGAAATGAAGATTTGAT-Fluorescein and LC Red 640-TTAATGAAAGCCTTGGCCAAGGCACT-phosphate), 4 mM $MgCl_2$ and 2 µL LightCycler-FastStart DNA Master Hybridization Probes (Roche Diagnostics, Mannheim, Germany). LightCycler data were analyzed using the LightCycler 4.05 software (Roche Diagnostics, Mannheim, Germany). Each 20 µL reaction contains 2 µL of cDNA. Amplification was performed with 40 cycles using 60°C annealing temperature. Final melting curve analysis was started at 40°C up to 85°C with slope of 0.2°C/sec and continuous detection with channel F2/F1. Direct sequencing of the PCR products was performed using BigDye v1.1 chemistry (ABI, Darmstadt, Germany) and a 3130 sequence detection system (ABI).

Mutation assay

A LightCycler PCR assay able to detect at least nine different mutation types in *JAK2* exon 12 was established. Different shapes of the curves allowed identification of all nine mutations. Relative mutation/wildtype ratios could be assessed, and cases with lower ratios were separated from high level mutations. A limited dilution series of cDNA of a sample with a homozygous mutation (based on its mutation to wt ratio of >1) in cDNA of an exon 12wt case showed a sensitivity of ≥10% (Figure 1).

Frequency

JAK2 exon 12 mutation were detected in 15 out of 409 patients (3.7%) with confirmed PV or erythrocytosis, all with unmutated *JAK2*V617. In detail, 10 out of 63 cases with confirmed PV were carrying the mutation of *JAK2* exon 12 (15.9%). The other 5 out of 346 cases (1.5%) had unclear erythrocytosis or were suspected to suffer from PV. Thus, *JAK2* exon 12 mutation screening may help to discriminate malignant from reactive polygobulia in a very few cases (Table 1).

Mutation types

Nine different mutations were identified in the 15 patients. Five subtypes had previously been described, but four were novel: H538-K539del, E544-L545del, H538DK539LI540S, and V536-F547dup.

Mutational ratios

The relative ratios of the *JAK2* exon 12 mutation in comparison to the wt-alleles were estimated in comparison to limited dilution assays (Figure 1). Ten cases had low ratios of 0.1-0.3. Three cases had high ratios of 0.5-0.8. A mutation/wt of >1 indicating homozygosity in at least part of the analyzed cells was detected in 2 cases only (one with a ratio >1; one with an undetectable wt-allele). Case #2 was investigated after two years of therapy with phlebotomy, acetylsalicylic acid, and hydroxyurea; case #12 showed leukemic transformation.

The total frequency of homozygosity of the *JAK2* exon 12 mutation was only 13% (2 out of 15 cases). This was significantly lower than >1 ratios in *JAK2* V617F mutation PV where it was found in 69% (496 of 719) ($p<0.001$).

Cytomorphology and cytogenetics

Bone marrow cytomorphology was available in three cases all with increased cellularity. Erythropoiesis and

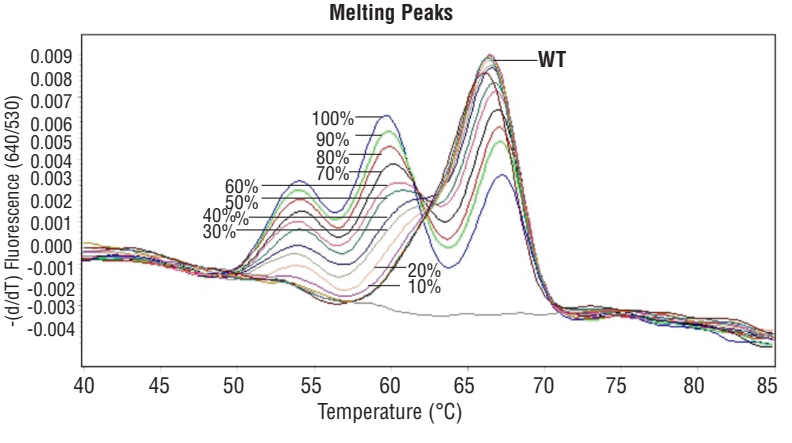

Figure 1. Limited dilution series of cDNA with an H538QK539L diluted in cDNA of a JAK2wt sample showing sensitivity of this assay of approximately 1:10.

Table 1. Biological and clinical data, laboratory parameters, and results of genetic analyses in 15 cases with CMPD carrying the *JAK2* exon 12 mutations.

Patient ID	Sex	Age at analysis (years)	Age at onset (years)	Clinical presentation	Source	Mutation	Ratio mut/wt	Karyotype	Previous treatment
1	F	54	48	PV	PB	D544-L545del	0.1	ND	HU, ASS
2	M	82	80	PV	PB	V536-F547dup	homo	ND	HU, ASS, PT
3	F	20	16	PV	PB	H538-K539del	0.2	ND	PT
4	F	77	75	PV	PB	H538DK539LI540S	0.5	46,XX	PT
5	F	75	73	Familial PV	PB	E543-D544del	0.2	ND	PT, ASS
6	F	74	74	Isolated erythrocytosis	PB	F537-K539delinsL	0.2	ND	PT, ASS
7	F	22	22	Isolated erythrocytosis	BM	H538QK539L	0.3	46,XX	PT, ASS
8	M	39	31	PV	BM	K539L	0.5	ND	PT, clopidogrel
9	M	79	79	PV	PB	E543-D544del	0.8	ND	–
10	F	63	63	PV	BM	F537-K539delinsL	0.2	46,XX	–
11	M	71	71	Isolated erythrocytosis	PB	H538QK539L	0.3	ND	–
12	F	52	52	PV (transformation)	BM	H538QK539L	homo	46,XX	–
13	M	50	47	Isolated erythrocytosis	BM	N542-E543del	0.2	46,XY	–
14	F	51	51	Isolated erythrocytosis	PB	N542-E543del	0.1	46,XX	–
15	F	85	85	PV	BM	N542-E543del	0.3	46,XX	–

F: female; M: male; y: years; PV: polycythemia vera; PB: peripheral blood; BM: bone marrow; homo: homozygous; ND: not determined; ASS: acetylsalicylic acid; PT: phlebotomy, HU: hydroxurea; wt: wildtype; mut: mutated.

megakaryopoiesis were increased in all cases, with dysplastic features in 2. Increase and left-shift of granulopoiesis were observed in 2 cases each. One patient with a high mutation/wildtype ratio and a normal karyotype showed a *classical* PV in transformation to s-AML (#12), whereas all others had no blast increase.

Seven cases carrying the *JAK2* exon 12 mutation where cytogenetics were available had normal karyotypes. In comparison, in cases carrying the V617F mutation, cytogenetic data were available in 263 cases; 51 (19.4%) had cytogenetic aberrations; 25 (9.5%) had +9, eight +8, six 20q-, and 12 showed various rare aberrations.

Clinical characteristics of patients carrying the JAK2 exon 12 mutations

In 8 patients mutations were detected at diagnosis, in 7 patients diagnostics was performed 2-8 years after initial manifestation of the PV or erythrocytosis (median four years). In these cases samples from the initial diagnosis were not available. Previous treatment was acetylsalicylic acid or clopidogrel for anticoagulation, hydroxyurea (HU), or phlebotomy in the 6 cases which were analyzed during follow-up of the disease (data were available in 14/15 cases) (Table 1). One patient (#5) had familial PV with father and sister being affected.

JAK2 exon 12 mutations were more frequent in females (n=10; 7.0%) than in males (n=5; 1.9%) compared to the patients without an exon 12 mutation (females: n=132; 93% *vs.* males: n=262, 98.1%) (p=0.012 by two-tailed Fisher's exact test). In contrast, the V617F mutated cohort had a balanced male to female ratio (340:379). Median age at detection of the *JAK2* exon 12 mutation disease was 58 years (range 16-80 years), which was in the range of the cases not carrying the *JAK2* V617 mutation (median: 58 years, range 16-88 years), but significantly lower than in patients with PV carrying the *JAK2* V617F mutation (median: 67 years; range: 29-88 years, p<0.001).

Peripheral blood values showed higher hematocrit levels in the patients carrying the *JAK2* exon 12 mutation (males: median 53%; range 49-61%; females: median 58%; range 44-71%) and lower erythropoietin levels with a median of 2.6 U/L (range 2.4-5.2). Ten out of 15 exon12mut patients (67%) showed isolated erythrocytosis. Thrombocytes were not elevated and leukocytes were not or marginally elevated. Hemoglobin was equal in *JAK2* exon 12- and V617F mutation (150 g/L *vs.* 158 g/L; p=0.950), but cases of exon 12 mutation had lower WBC and platelets than V617F mutation: WBC: 6.2×10^9/L *vs.* 11.8×10^9/L); platelets 282×10^9/L *vs.* 479×10^9/L. Marked organomegaly was present in one out of 8 patients carrying the exon 12 mutation where data were available (12%). However, some patients had received previous treatment so these results should be compared with caution.

Results and Discussion

In PV, the *JAK2*V617F mutation was included as major criterion in a revised WHO classification.[11] However, in consideration of the ≥5% of PV cases with an unmutated *JAK2*V617 codon the recent description of mutations in exon 12 of *JAK2* contributes to the genetic background of this disorder.[6] We here analyzed 409 patients with PV or erythrocytosis and not carrying the *JAK2*V617 mutation. The rate of exon 12 mutations in patients with confirmed PV but not carrying the *JAK2*V617 mutation according to the WHO[12] was

15.9%. Some previous studies had described higher rates in V617 unmutated patients of up to 83% probably due to better characterization of patients or different PCR techniques,[6-8,13,14] whereas in this study cases were sent by diverse hematologic centers and hospitals. The mutation rate in this study was similar to Williams *et al.* who detected exon 12 mutations in 9% of patients with erythrocytosis.[13] Most frequent were the N542-E543del, H538QK539L, and F537-K539delinsL subtypes. Changes of glutamin acid 543 such as the N542-E543del7 and K539L[7,9] had been previously described in cases carrying the exon 12 mutation. Our data thus confirm the exon 12 mutation cluster within the region covered by codons 537-543 as described by Scott *et al*. and Pardanani *et al*.[6,8] There was one large duplication spanning eleven codons (V536-F547dup) reminiscent of the FLT3-ITD in AML conferring constitutive activation of the FLT3 receptor tyrosine kinase. Two similar mutations – a V536-I546dup11 and a F537-I546dup10+F547L – were described by Pietra *et al*.[7] However, deletions are far more frequent in exon 12 mutation cases.

Further, we focused on the proportions of mutated cells. Here, one case showed a >1.0 mutation to wildtype ratio, and in one case the wildtype was undetectable. This rate of 2/15 (13%) was much lower than >1 ratios in V617F mutated cases (69%). In two published cases the mutant burden was not quantitated,[7,15] and in a third case the mutant allele burden was 69% in granulocytes.[16] However, most reported cases showed lower mutational ratios when compared to this study. Thus, even considering that inapparent homozygosity might be more frequent,[7] the frequency of homozygous exon 12 mutations is probably lower when compared to PV carrying the *JAK2*V617F mutation where homozygous mutations were detected in ≥27% of patients,[2,17] and where single cell homozygosity was reported in nearly all mutated patients.[16,18]

This suggests that mitotic recombination leading to loss of heterozygosity might be less frequent in patients with exon 12 mutations compared to those with V617F.[7] As one of the 2 high ratio patients of this cohort showed transformation from PV to s-AML, loss of heterozygosity of exon 12 mutated cells might be associated with progression of PV comparably to the situation in V617F[5,17,19] but more patients with a homozygous exon 12 mutation status are needed to allow definite conclusions to be drawn.

In addition to loss of heterozygosity gain of chromosome 9 was frequently shown to indicate progression in PV.[2] Cytogenetic analysis was available in only 7 cases with *JAK2* exon 12 mutations, and all revealed normal karyotypes. However, due to the few cases with cytogenetic data it is not clear whether chromosome 9 abnormalities really are absent in cases carrying the exon 12 mutation.

This study further supports the previous finding that PV carrying the *JAK2* exon 12 mutation shows a trend to female gender and younger age at onset in comparison to PV with V617F (although in a limited group of 15 patients).[6] These differences emphasize different patterns of development and effects of both *JAK2* mutational subtypes. Likewise *MPL*W515 mutated[20] cases were reported to show significant phenotype differences from cases carrying the V617 mutation e.g. by lower hemoglobin levels or higher platelet counts.[21,22]

It should be added that in this cohort of patients carrying the exon 12 mutation one case had familial PV as observed before.[6]

In conclusion, these data support previous suggestions that *JAK2* exon 12 mutation are associated to a specific PV subtype.[6-8,9] The discovery of activated tyrosine kinases in the majority of CMPD patients shows the need for further research in cases without known genetic causes[23] and to determine if *JAK2* signaling inhibition is effective. All mutations discovered so far, the *JAK2*V617F, the *MPL*W515L/K, and the *JAK2* exon 12 mutations, result in JAK-STAT activation. Small molecular *JAK2*-inhibitors have already undergone successful pre-clinical testing and proceeded to early phase human trials in PMF.[4]

Thus, the detection of the exon 12 mutations in PV patients with an unmutated *JAK2*V617 position contributes to a new perception of the CMPD increasingly based on genetic aspects.[24] Cases with PV and not carrung the *JAK2*V617 mutation should be investigated for *JAK2* exon 12 mutations in a stepwise algorithm.

Authorship and Disclosures

SS: principal investigator, molecular analysis, data evaluation, writting of paper; UB: data evaluation and writting of paper; CH: cytogenetic analysis; TG, PM, JM, PP, RS, JS, RS, HWT and JW did the patient care and provided clinical data; WK: immunophenotyping and statistics; TH: cytomorphology and final revision of the manuscript.

SS, WK, CH and TH are owners of the MLL Munich Leukemia Laboratory GmBH that is offering leukemia diagnostic services. The other authors declare no potential conflict of interest.

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