Interactions of hereditary and acquired genetic factors in *BCR-ABL1* negative myeloproliferative neoplasms

Ph.D. thesis

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Budapest
2017

1. Introduction

Myeloproliferative neoplasms (MPN) are a group of diseases, that are characterised by clonal hematopoiesis with increased production of different mature myeloid cells. The 2016 World Health Organization (WHO) classification define several MPN entities. Essential thrombocythaemia (ET), polycythaemia vera (PV) and primer myelofibrosis (PMF) belong to the *BCR-ABL1* (breakpoint cluster region – Abelson1 fusion gene) also known as Philadelphia chromosome negative classical MPN group. ET, PV and PMF are characterized mainly by megakaryocyte, erythrocytes, and both megakaryocyte-granulocyte proliferation respectively.

Mutations of Janus kinase 2 (*JAK2*), calreticulin (*CALR*) and thrombopoietin receptor (*MPL*) genes were identified in the genetic background of *BCR-ABL1* negative classical MPN. Each of them activates the JAK/STAT (STAT: signal transducer and activator of transcription) signaling pathway. First V617F somatic point mutation (substitution of valine for phenylalanine at position 617) was recognized in the exon 14 of the *JAK2* gene. Later *MPL* exon 10 mutations in V617F negative ET and PMF, and *JAK2* exon 12 mutations in V617F negative PV patients were identified. The most frequently acquired *MPL* mutations affect serine and tryptophan amino acids at positions 505 and 515 in the receptor's transmembrane

domain. *JAK2* exon 12 mutations are located within the coding region of JAK2 protein: substitutions, deletions (del), insertions (ins) and duplications in the region of 533-547 codons. Genetic alterations affecting exon 9 of *CALR* gene occur in *JAK2* and *MPL* negative ET and PMF. The most frequent frameshift mutations are type 1 (52 base pair deletion, c.1092_1143del) and type 2 mutations (5 base pair insertion, c.1154_1155insTTGTC). *JAK2*, *CALR* and *MPL* mutations are MPN-specific, they occur mutually exclusively. These mutations are infrequent in other hematologic malignancies. Since one of these three genes is affected in 85-90% of classical MPN patients, examination of their mutations has diagnostic significance. In 10-15% of the ET and PMF cases *JAK2*, *CALR* and *MPL* mutations cannot be detected, these patients represent the triple negative group.

In the majority of MPN patients additional gene mutations are present beside the oncogene mutations, which affect mainly epigenetic modifiers and tumour suppressor genes. These mutations themselves are not responsible for the development of MPN symptoms, and often occur in other myeloid malignancies. Additional mutations may indicate advanced stages of MPN, depending on the number and the order of appearance. Identification of these alterations has prognostic relevance in MPN.

Presence of the same mutations (*JAK2* V617F*, CALR, MPL*) in different MPN diseases suggests that other genetic factors may play a

role in MPN pathogenesis. Beyond acquired mutations hereditary genetic factors can also affect the predisposition to MPN, clinical features and disease course. Beside the *JAK2* 46/1 haplotype, the rs2736100_C variant in the second intron of telomerase reverse transcriptase (*TERT)* gene was recently identified as a susceptibility factor for MPN and solid tumours. The *TERT* gene encodes the catalytic subunit of telomerase, which is essential for the maintenance of telomere length, the region of repetitive nucleotide sequences at the tips of chromosomes. The "C" variant of rs2736100 increase *TERT* transcription, which may contribute to the increased susceptibility to MPN development.

2. Aims

The aim of our study was to examine the hereditary and acquired genetic factors and their impact within our classical MPN cohort. The aims were the following:

1) To establish methods in order to detect acquired oncogene mutations (*JAK2*, *CALR*, *MPL*), and to apply a complex array of molecular techniques.
2) To identify the acquired mutations and test the frequencies. To investigate their impact on clinical parameters, outcome and prognosis.
3) To compare *JAK2* V617F and *CALR* mutant allele burden, in order to analyse the effects of higher allele burden on clinical parameters.
4) To determine the allele frequency of *TERT* rs2736100 and *JAK2* rs12343867 polymorphisms, to examine their individual and combined role in predisposition to MPN.
5) To study the effect of *TERT* polymorphism on MPN phenotype, complications and overall survival.

3. Methods

3.1 Patients

The occurrence of *JAK2*, *CALR* and *MPL* mutations was investigated in *BCR-ABL1* negative classical MPN patients diagnosed between 1974 and 2013. The patient cohort consisted of 949 individuals (353 PV, 469 ET and 127 PMF). Patients were diagnosed according to WHO 2008 criteria. Laboratory parameters and clinical data were collected retrospectively, occurrence of myelofibrotic or acute leukemic transformation and coagulation complications were recorded.

3.2 Detection of *JAK2* V617F mutation

The presence of *JAK2* V617F point mutation was identified by allele-specific polymerase chain reaction (AS-PCR) in all MPN patients. The quantitative determination of the V617F allele was performed in a subset of patients (n=484) and 30 healthy bone marrow donors by real-time PCR using the fluorescent TaqMan detection system on LightCycler 480 (LC 480) device.

3.3 Detection of *JAK2* exon 12 mutations

The *JAK2* exon 12 mutations were tested by fragment analysis in *JAK2* V617F negative PV patients. In case of positive results the

nucleotide sequence of the DNA fragment was determined by Sanger sequencing.

3.4 Detection of *CALR* exon 9 mutations

The *JAK2* V617F negative ET (n=181) and PMF (n=53) patients were screened for *CALR* mutations by fragment analysis. We calculated the mutant load from the ratio of mutant and wild type peak heights, and expressed the mutant allele in the percentage of the total *CALR* alleles. In positive cases, the exact type of the mutation was identified by Sanger sequencing.

3.5 Detection of *MPL* exon 10 mutations

In *JAK2* V617F and *CALR* negative ET (n=63) and PMF (n=23) patients, we investigated the presence of *MPL* mutations by three different methods: high resolution melting analysis (HRM), sequencing and AS-PCR.

3.6 SNP analysis

TERT rs2736100_C and *JAK2* rs12343867_C polymorphisms were identified in 584 *BCR-ABL1* negative MPN patients and 400 healthy controls. Genotyping was performed by real-time PCR followed by melting curve analysis on LC 480 device.

4. Results

4.1 Establishing laboratory methods

Since 2014 sensitive real-time PCR has been performed to detect *JAK2* V617F point mutation. *JAK2* exon 12 and *CALR* exon 9 mutations were determined by fragment sizing. In mutant cases, the exact mutation type was identified by Sanger sequencing. End-point PCR followed by fragment analysis is a suitable semi-quantitative method to define *CALR* mutation load. Screening for *MPL* exon 10 mutations was performed by HRM analysis which can detect rare genetic alterations as well. In case of positive screening test, the exact missense nucleotide substitutions were determined by sequencing. The negative cases were further examined by AS-PCR separately for each mutation (S505, W515). Allelic discrimination method with hybridization probe detection format was applied to detect risk alleles of *JAK2* rs12343867 and *TERT* rs2736100 polymorphisms.

4.2 Oncogene mutations in classical MPN

A complex array of molecular genetic techniques was applied to identify oncogene mutations in the genetic background of MPN, in 353 PV, 469 ET and 127 PMF patients. *JAK2*, *CALR* and *MPL* mutations are mutually exclusive, concomitant mutations represent rarity in the literature. Therefore mutation detection was performed sequentially. In suspicion of PV, *JAK2* V617F mutation was screened

at first. In absence of V617F, the *JAK2* exon 12 mutations were investigated. Investigation was also started with *JAK2* V617F mutation in ET and PMF patients, and according to the mutation frequencies in earlier observations the V617F negative samples were further screened for *CALR*, then *MPL* mutations. In patients with PV, 98.6% *JAK2* V617F and 1.4% *JAK2* exon 12 mutation-positive cases were identified. In ET and PMF the frequency of mutations were similar: 61-58% *JAK2* V617F, 25-24% *CALR* and 2-6% *MPL* mutations were found. In the remaining 11-12% of the cases none of *JAK2*, *CALR* and *MPL* mutations could be detected.

Relationship between the presence of different oncogene mutations and clinical characteristics was also studied. We compared the clinical and laboratory parameters of the *CALR*^{mut} and *JAK2* V617F^{mut} ET patients. In the *CALR*^{mut} ET cohort we found male predominance, younger age at diagnosis, lower hemoglobin levels, lower white blood cell counts and higher platelet counts. Coagulation complications were more frequent in *JAK2*^{mut} ET patients, while myelofibrotic transformation occurred more frequently in the *CALR*^{mut} cohort. Similar analyses in the PMF group showed younger age at diagnosis, higher platelet counts and less frequent splenomegaly in the *CALR*^{mut} subgroup compared to the *JAK2*^{mut} PMF patients. Other variables were not significantly different.

Outcome parameters were analysed by Kaplan-Meier approach, in patients with ET and PMF. Survival information was not available in each cases, therefore we performed survival analysis on a subgroup of patients. In contrast to ET, the overall survivals proved to be different in PMF subgroups according to genetic alterations. *CALR*^{mut} patients had the best and the triple-negative ones the worst overall survival. This was further confirmed by pairwise comparisons. The difference was significant comparing both *CALR*^{mut} vs *JAK2*^{mut} groups (p=0.04), and *CALR*^{mut} vs triple-negative groups (p=0.01).

We determined the allele burden of driver mutations in case of *JAK2* V617F and *CALR* mutations. *JAK2* V617F allele quantities were available in 215 PV, 154 ET and 56 PMF patients, while *CALR* allele burdens were accessible in 96 ET and 25 PMF cases. Comparing the subgroups, quantitative data of samples taken at diagnosis and samples taken at a later time point were separated. Performing pairwise comparisons, we made the following observations. (i) Neither *JAK2* V617F^{mut}, nor *CALR*^{mut} ET patients' mutational loads were significantly different, while PV patients showed a tendency toward increasing allele burden comparing the samples taken at diagnosis and at a later time point. (ii) *JAK2* V617F loads increased gradually in parallel with the appearance of more advanced stages of MPN (ET<PV<post-PV MF). Same tendency was observed in case of *CALR* mutant loads (ET<post-ET MF). (iii) In contrast to *JAK2*

V617F^{mut} load, *CALR*^{mut} load only rarely exceeded 50%. (iv) Within patients with ET, the *CALR*^{mut} load was significantly higher than the *JAK2* V617F^{mut} load.

We investigated the distribution of different types of *CALR* mutations in the ET (n=96) and the PMF (n=25) cohorts. The occurrence of these variants was as follows: in the ET subgroup 50% type 1, 32% type 2 and 18% other; in the PMF subgroup: 64% type 1, 12% type 2 and 24% other *CALR* mutations were found. An increased frequency of type 2 mutations versus non-type 2 mutations was observed in ET (32%) compared to PMF (12%). Mutant *CALR* allele burden was further investigated in 31 ET patients with samples available within 1 year after diagnosis. Our study demonstrated for the first time, that hematologic laboratory parameters (hemoglobin level, white blood cell and platelet counts) were directly influenced by *CALR* mutational burden in ET patients. White blood cell counts (9 vs 11 G/L, p=0.025) and platelet counts (848 vs 1406 G/L, p=0.04) were lower among patients with low *CALR*^{mut} load (<38%), while hemoglobin concentration was higher in the same comparison (138 vs 122 g/dL, p=0.04).

4.3 *TERT* rs2736100 and *JAK2* rs12343867 polymorphisms

Among hereditary genetic factors, we investigated the role of *TERT* and *JAK2* polymorphisms as predisposing factors to MPN. Both *TERT* and *JAK2* variants showed an increased allele frequency compared to controls (*TERT* rs2736100_C: 62.7% vs 48.8%; *JAK2* rs12343867_C: 45.7% vs 29.8%). Carriership of the *TERT* variant was similarly associated either with *JAK2* V617F^{mut} or *CALR*^{mut} MPNs. In contrast, the effect of the *JAK2* rs12343867_C allele was more pronounced in the *JAK2* V617F^{mut} MPN.

In our MPN cohort, *TERT* homozygosity [Odds Ratio: OR=3.2 (2.2-4.7)] increased MPN predisposition additively compared with *TERT* heterozygosity [OR=2.2 (1.5-3.1); p=0.009]. As the presence of a single *TERT* or *JAK2* allele increased MPN susceptibility, we examined their combined effect. Heterozygous genotype counted as single, while homozygous genotype as double risk alleles. The presence of two risk alleles increased MPN risk 6-fold, while combined homozygosity close to 10-fold. These results suggest different pathomechanisms in the background of MPN susceptibility owing to *TERT* and *JAK2* variants.

As a next step, we investigated the potential effect of *TERT* polymorphism on MPN signs and symptoms. Except the elevated white blood cell counts, no remarkable effect of *TERT* rs2736100_C variant was observed on the hematologic parameters and the

frequencies of different hematologic complications. In contrast, we noticed that *TERT* variant altered long-term overall survival of carriers in PV. Detailed past medical history was available in 356 cases. Comparing the causes of death, analysis revealed that carriers of *TERT* CC genotype had a higher probability to die from solid tumours unrelated to the hematologic malignancy. Solid tumours were present in 8.2% of *TERT* wild-type (AA genotype), in 16.2% of heterozygous (AC genotype) and 23.1% of homozygous (CC genotype) patients (p=0.014). Multivariate analyses showed that *TERT* rs2736100_C genotype and cytoreductive therapy were independent risk factors for nonhematologic tumour formation [p=0.045; OR=3.08 (1.03–9.26)].

5. Conclusions

1) Molecular genetic screening and confirming methods were established in our laboratory, which are suitable to determine somatic oncogene mutations in more than 90% of PV, ET or PMF cases. To detect alterations in *JAK2*, *CALR* and *MPL* genes, a combination of qualitative and quantitative AS-PCR, fragment-sizing, HRM and Sanger sequencing was applied.
2) The frequencies of detected mutations in our MPN cohort were similar to earlier observations. The presence of different and mutually exclusive oncogene mutations is associated with distinct clinical characteristics. In our PMF subgroup $CALR^{mut}$ patients had better overall survival (OS) than $JAK2^{mut}$ ($p=0.04$) or triple-negative ($p\leq0.01$) ones.
3) We studied for the first time the effect of *CALR* mutant allele load using an alternative semi-quantitative PCR approach. Our study shows for the first time, that hematologic laboratory parameters (hemoglobin concentration, white blood cell and platelet counts) were directly influenced by *CALR* mutational burden in ET patients. Our results indicate that effect of *CALR* allele burden on disease is similar to those of *JAK2*, which increase gradually in parallel with the appearance of more advanced stages of MPN.

4) We confirmed the previously reported increased allele frequencies of both *TERT* rs2736100_C and *JAK2* rs12343867_C variants regardless of MPN disease type or molecular background. Both heterozygous and homozygous form of the *TERT* variant predispose to the development of *BCR-ABL1* negative MPN. Our results support, that *TERT* and *JAK2* risk alleles have independent and additive impact on MPN predisposition.
5) We found that *TERT* polymorphism do not have influence on blood cell parameters (except higher white blood cell count) and on the frequency of different hematologic complications in MPN. However, we observed different long-term OS according to *TERT* rs2736100 genotypes in PV patients. Investigating the reasons, we revealed that patients with homozygous *TERT* rs2736100_CC genotype had a higher probability to die from solid tumours unrelated to the hematologic malignancy, which is generally assumed to be the consequence of cytoreductive treatment. In multivariate analyses we demonstrated for the first time, that *TERT* rs2736100_C polymorphism and cytoreductive therapy were independent risk factors for subsequent nonhematologic tumour formation.

6. List of publications

6.1 Publications related to the present thesis

- **Krahling T**, Balassa K, Kiss KP, Bors A, Batai A, Halm G, Egyed M, Fekete S, Remenyi P, Masszi T, Tordai A, Andrikovics H. Co-occurrence of myeloproliferative neoplasms and solid tumors is attributed to a synergism between cytoreductive therapy and the common TERT polymorphism rs2736100. CANCER EPIDEMIOLOGY BIOMARKERS & PREVENTION 25:(1) pp. 98-104. (2016)
- **Krahling T**, Balassa K, Meggyesi N, Bors A, Csomor J, Bátai A, Halm G, Egyed M, Fekete S, Reményi P, Masszi T, Tordai A, Andrikovics H. Complex molecular genetic algorithm in the diagnosis of myeloproliferative neoplasms. ORVOSI HETILAP 155:(52) pp. 2074-2081. (2014)
- Andrikovics H, **Krahling T**, Balassa K, Halm G, Bors A, Koszarska M, Batai A, Dolgos J, Csomor J, Egyed M, Sipos A, Remenyi P, Tordai A, Masszi T. Distinct clinical characteristics of myeloproliferative neoplasms with calreticulin mutations. HAEMATOLOGICA 99:(7) pp. 1184-1190. (2014)

6.2 Publications not related to the present thesis

- Balassa K, **Krahling T**, Remenyi P, Batai A, Bors A, Kiss KP, Torbagyi E, Gopcsa L, Lengyel L, Barta A, Varga G, Tordai A, Masszi T, Andrikovics H. Recipient and donor JAK2 46/1 haplotypes are associated with acute graft-versus-host disease following allogeneic hematopoietic stem cell transplantation. LEUKEMIA & LYMPHOMA 58(2) pp. 391-398. (2017)
- Koszarska M, Meggyesi N, Bors A, Batai A, Csacsovszki O, Lehoczky E, Adam E, Kozma A, Lovas N, Sipos A, **Krahling T,** Dolgos J, Remenyi P, Fekete S, Masszi T, Tordai A, Andrikovics H. Medium-sized FLT3 internal tandem duplications confer worse prognosis than short and long duplications in a non-elderly acute myeloid leukemia cohort. LEUKEMIA & LYMPHOMA 55:(7) pp. 1510-1517. (2014)