The role of hereditary and acqired genetic alterations in the pathomechanism of myeloid hematopoietic stem cell disesaes

PhD thesis

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1. Introduction

Hereditary and acqired genetic alterations may simultaneously play a role in the development of myeloid hematopoietic stem cell diseases. Myeloproliferative neoplasms (MPN) are characterized by proliferation of maturated myeloid cells in the peripheral blood, while acute myeloid leukemia (AML) is characterized by proliferation of blasts in the bone marrow. Mutations of tyrosine kinases are often responsible for the uncontrolled proliferation of cells. Small molecule targeted tyrosine kinase inhibitors (TKI) can be used for inhibition of tyrosine kinase activity.

1.1 Myeloproliferative neoplasms

MPN is classified as chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primer myelofibrosis (PMF) on the basis of clinical presentation. In the genetic background of CML, a reciprocal translocation between chromosome 9 and 22 [t(9;22)(q34;q11)] was identified. The result of the translocation is the BCR-ABL gene fusion, which gene encodes the BCR-ABL fusion protein. The truncated chromosome 22 is the Philadelphia chromosome (Ph). The targeted tyrosine kinase inhibitor (TKI) imatinib is the standard first-line therapy for CML and is part of the treatment in Philadelphia-positive (Ph+) acute lymphoid leukemia

(ALL), but resistance occurs in a considerable proportion of patients. Several mechanisms have been described to cause resistance, but BCR-ABL tyrosine kinase domain (TKD) mutations and additional chromosome abnormalities beside the Ph chromosome (ACA) are probably the best characterized. The second-generation TKIs dasatinib and nilotinib are effective in the case of imatinib failure and active against several imatinib-resistant BCR-ABL mutants with the exception of T315I. Recently, alternative splicing (AS) of BCR-ABL was also proposed as a mechanism for imatinib resistance simply based on the detection of BCR-ABL splice variants in imatinib failure. Among them, the most common but less characterized BCR-ABL splice variant is exon 7 deletion.

In the background of BCR-ABL negative MPN, a common causative genetic alteration, an acquired Janus kinase 2 (JAK2) V617F (exon 12, c.1849G >T, substitution of Val617 to Phe) mutation has been identified (in >95% in PV, and in 40-60% in ET and PMF). Not only acquired, but inherited genetic alterations may also influence the susceptibility to MPN. Recent studies have demonstrated that the presence of JAK2 V617F is associated with an inherited JAK2 haplotype designated as '46/1' haplotype. Carriers of the 46/1 haplotype have higher risk of acquiring JAK2 V617F mutation, but the role of 46/1 haplotype as a predisposition factor for V617F-negative MPN is still remained controversial.

1.2 Acute myeloid leukemia

The presence of at least two different types of mutations, one that confer a proliferative advantage to cells and another that blocks the differentiation of cells, may lead to the development of AML. Several acquired genetic alterations may influence the outcome of the disease, among them the most important prognostic factor in AML is cytogenetics. Certain cytogenetic abnormalities are associated with favorable outcomes (for example, the t(15;17), t(8;21) translocations and inv(16) inversion). A number of other cytogenetic abnormalities affecting chromosome 3., 5. 7., 11., or 17 [abn(3q) del(5q), -5, del(7q)-t, -7], and complex karyotype (more than 3 cytogenetic alterations) are known to associate with a poor prognosis. About half of AML patients have cytogenetically normal karyotype (NK); they fall into an intermediate risk group. The JAK2 V617F mutation rarely occurs in de novo AML, but the prognostic impact of the mutation is unknown. The JAK2 46/1 haplotype was not investigated before in AML.

2. Aims

a) To investigate the frequency and prognostic impact of BCR-ABL TKD mutations and ACAs during first and second generation TKI therapy in TKI resistant CML patients.

b) To perform a systematic analysis of BCR-ABL exon 7 deletion on samples of CML patients and healthy controls from different time points of the disease by different PCR-based methods, and to perform bioinformatic prediction of the functionality and viability of the variant protein.

c) To test the frequency of the acquired JAK2 V617F mutation in BCR-ABL negative MPN patients and to examine associations of JAK2 V617F mutation with distinct clinical characteristics of MPN.

d) To examine the presence of the JAK2 46/1 haplotype in Hungarian V617F-positive and -negative MPN patients and to examine associations of the 46/1 haplotype with distinct clinical characteristics of MPN.

e) To examine the frequency of the inherited JAK2 46/1 hyplotype in AML patients, to compare disease characteristics between JAK2 46/1 haplotype carriers and non-carriers; and to test the prognostic impact of the 46/1 haplotype on AML outcome.

3. Methods

3.1 BCR-ABL tyrosine kinase domain mutations and ACA

To achieve exclusive amplification of BCR-ABL and to avoid the amplification of normal ABL, a 2-step (nested) PCR method was performed. During the nested PCR, the entire tyrosine kinase domain was amplified in three overlapping fragments, which were sequenced by the dideoxy chain termination method in forward and reverse directions. Karyotyping was performed using standard G-banding. Investigated patients: 71 CML and 6 Ph+ ALL patients at the time of TKI resistance.

3.2 BCR-ABL exon 7 deletion

The presence of exon 7 deletion on BCR-ABL and on ABL was investigated by four different PCR methods (direct sequencing, fragment analysis, allele-specific PCR and quantitative PCR) on CML patients (10 patients with secondary imatinib resistance and 5 patients with optimal response for imatinib) at times of diagnosis, therapeutic response and resistance, in addition to 30 healthy control samples. The functionality and viability of the variant protein was investigated by bioinformatic prediction.

3.3 JAK2 V617F mutation

The JAK2 V617F mutation was detected by allele specific multiplex PCR in the BCR-ABL negative MPN patient cohort (n=328).

3.4 JAK2 46/1 haplotype

The JAK2 rs12343867 single nucleotide polymorphism (SNP) (NT_008413.17: g.5064189T>C in intron 14) tagging the 46/1 haplotype was genotyped in 328 BCR-ABL negative MPN and 339 AML patients, and in 331 controls by LightCycler technology applying melting curve analysis with hybridization probe detection format.

4. Results

4.1 BCR-ABL TKD mutation and ACA in CML

Mutation analysis was performed at the time of imatinib resistance in 69 patients with CML and 5 patients with Ph+ ALL. Fifteen different mutations were identified (M244K/V, G250E, Y253H, E255V, D276G, E279K, T315I, M351T, E355G, F359I/V, L384M, L387M, H396R) in 27/74 (36%) imatinib resistant patients. M244V, T315I, M351T, E255V and F359I/V mutations occurred more frequently, while other mutations occurred only once. More TKD mutations were found in advanced phases of CML (early CP vs. late CP combined with AP vs. BP, p=0.026). Cytogenetic results were available in 65 cases with imatinib resistance. ACAs were detected in 30 patients (46%) at the time of imatinib failure, with chromosome 8 trisomy (+8), Ph chromosome duplication (+Ph) and isochromosome 17q [i(17q)] being the most frequent ACAs (26, 21 and 8%, respectively). No significant association between the presence of mutation and ACA status was observed. EFS and OS of 71 imatinib-resistant CML patients were analyzed according to the presence of a BCR-ABL TKD mutation and/or ACA during imatinib therapy. EFS and OS were not significantly different between the TKD mutation positive and negative groups (p = 0.884 and 0.689, respectively). The 2-year OS was 100% for ACA-negative and 76% for ACA-positive

patients (p=0.015). We did not find significant differences between the subgroups with different combinations of mutations and ACAs.

After imatinib failure, 57 patients received a second TKI (nilotinib, n = 29; dasatinib, n = 28). Subsequently, 13 of these 57 patients changed to third-line TKI after relapse (nilotinib, n = 5; dasatinib, n = 8). The following mutations disappeared on nilotinib therapy: M244V, G250E, E255V, M351T and L387M. On the other hand, Y253H, F359I and F359V persisted and 3 new mutations emerged (Y253H, T315I and F359V). In case of dasatinib therapy, Y253H and F359Vdisappeared. During dasatinib therapy, there were fewer persisting mutations (M244V and T315I) but more novel mutations (L248M, E279K and T315I). The mutation spectra for nilotinib and dasatinib did not overlap except for T315I which was the most frequent mutation during second generation TKI therapy. Out of the recurrent ACAs, i(17q) persisted in all cases during second-generation TKI therapy, whereas nilotinib and dasatinib were effective in a considerable portion of +Ph and +8 cases. In case of nilotinib, significantly more mutations (70%) were found than during imatinib (32%) and dasatinib (29%) therapies (p = 0.0483). On the other hand, T315I occurred more frequently during dasatinib therapy compared to nilotinib and imatinib (24 vs. 10 and 1%, respectively; p = 0.0016). We investigated the survival probability for second generation TKI therapy according to mutation status, ACA status and mutation status

combined with ACAs. In contrast to results with imatinib, we found significant differences between mutation positive and negative patients with regard to EFS (p = 0.006) and OS (p = 0.01). Similarly to imatinib, ACAs were also a strong prognostic factor for second-generation TKI therapy (p<0.001 for poorer EFS and OS).

4.2 BCR-ABL exon 7 deletion in CML

By sequencing, BCR-ABL$^{\Delta exon7}$ was present in 17% (12/71) of imatinib resistant patients. By the more sensitive fragment analysis, higher frequency of BCR-ABL$^{\Delta exon7}$ was found in patients at the time of imatinib resistance (70%; 7/10) compared to sequencing (p=0.001). In serial samples from imatinib-treated CML patients, BCR-ABL$^{\Delta exon7}$ was more frequent at diagnosis (80%; 12/15) and resistance (70%; 7/10) compared to samples collected at times of therapeutic response (0%; 0/9; p=0.0002). The BCR-ABL$^{\Delta exon7}$ negative samples exhibited lower BCR-ABL expression (median: 2.5%; 25–75% percentiles: 0.5–29.3%) compared to positive samples (median: 90%; 25–75% percentiles: 49–100%; p=0.001). In contrast to BCR-ABL, the frequency of ABL$^{\Delta exon7}$ was not different between samples collected at diagnosis (40%; 6/15), at resistance (70%; 7/10) and at times of therapeutic response (43%, 6/14; p=0.74). ABL$^{\Delta exon7}$ was detectable in 77% (23/30) of healthy control samples. Using allele-specific PCR, even more samples proved to be positive for BCR-ABL$^{\Delta exon7}$ [87%

(13/15) at diagnosis, 57% (8/14) at therapeutic response and 100% (10/10) at resistance]. Similarly to fragment analysis, BCR-ABL$^{\Delta exon7}$ was detected less frequently at lower BCR-ABL copy number. ABL$^{\Delta exon7}$ was present in 87% (13/15) at diagnosis, 93% (13/14) at times of therapeutic response and 90% (9/10) at resistance (p=1.0).

Bioinformatic prediction of secondary structures showed that in the presence of Δexon7, the novel protein region, which is translated from the alternative reading frame of exon 8, is short and does not affect the secondary structure of the N-terminal part of the TKD upstream from exon 6. According to a previous functional mapping of the TKD in the presence of Δexon7, ABL lacks the activation loop that prevents the truncated protein to function as a tyrosine kinase. The imatinib binding pocket of BCR-ABL$^{\Delta exon7}$ variant is also damaged (lack of 3 binding residues from the hinge region, Ala380-Phe382), so this protein is unlikely to bind imatinib. Fine structural mapping of the TKD substructure by CHASA software revealed several regions, which could preserve their structural integrity alone. The breakpoint caused by AS is located in the middle of a highly hydrophobic region. Together with the secondary structure data, the results of the above bioinformatics analyses suggest that a relatively large hydrophobic surface is exposed as a result of the exclusion of exon 7, which will trigger the UPR (unfolded protein response) mechanism to eliminate the truncated protein as a misfolded protein.

4.4 JAK2 V617F mutation in BCR-ABL negative MPN

The frequency of JAK2 V617F was 75.9% (249 of 328) in the MPN group: 87.4% (153 of 175) in PV patients, 61.1% (77 of 126) in ET patients, and 70.4% (19 of 27) in PMF patients.

Female predominance was observed in V617F-positive MPN compared with V617F-negative patients [57.4% (143 of 249) versus 41.8% (33 of 79); p=0.019]. This predominance was also present in the PV and in the PMF subgroups but not in ET. The age at presentation was significantly higher in V617F-positive CMPD (60±12 years versus 52±16 years; p<0.0001) and remained significant only in the PV subgroup. We observed higher hemoglobin (Hb) values at time of disease onset in V617F positive patients compared with V617F-negative counterparts (Hb, 165±32 g/L versus 139±30 g/L; p<0.001;), this difference remained significant in ET but not in PV and PMF subgroups. Vascular complications (thrombotic and hemorrhagic combined) were more common in V617F positive patients [p=0.039; 26.6% (64 of 241) versus 15.2% (12 of 79)].

4.4 JAK2 46/1 haplotype in BCR-ABL negative MPN and AML

Increased JAK2 46/1 haplotype frequency was found (dominant model: rs1234867 C allele carriers [CC and CT genotypes] vs. non-carriers [TT genotype]) in the entire MPN group (74.4±4.9%, p<0.0001), and in each MPN subgroups according to diagnosis (PV,

ET, PMF) compared with controls (48.0±5.5%). The 46/1 haplotype frequency was significantly higher in V617F positive MPN (77.3±5.3%), compared with V617F negative MPN (62.3±12.4%, p=0.022). The 46/1 haplotype frequency in the V617F negative MPN (ET and PMF) group was also significantly higher compared to the control group (p=0.051). The only MPN complication, which had a different frequency among 46/1 genotype groups, was myelofibrosis (PMF or post-PV/ET myelofibrosis): 32% of 'CC' homozygotes versus 12% of non-CC individuals (p=0.001).

In the entire AML group, the 'C' allele frequency (48.4±5.4%) did not show significant difference compared to controls. The V617F positive AML patients (n=4) were excluded from the study. The 46/1 haplotype frequency was significantly higher in normal karyotype (NK) AML patient group compared with the non-NK-AML group (56.6±8.7% vs. 42.0±7.2%, p=0.012). Upon comparing carrier frequencies versus non-carriers, a tendency toward increased frequency of 46/1 haplotype carriers in younger age (NK-AML <45 years vs. control p=0.036) was observed.

In those AML patients who were characterized according to the prognostic impact of JAK2 46/1 haplotype (n=176), the distributions of age, sex and AML etiology were similar between 46/1 carriers and non-carriers. There were considerably fewer cases with AML with maturation (FAB M2) morphology within the group of 46/1 carriers

than in the group of non-carriers (5.6% vs. 17.2%; p=0.018), and conversely acute myelomonocytic leukemia (FAB M4) was more frequent among 46/1 carriers [28.1% vs. 14.9%; p=0.044]. A similar distribution was observed in NK-AML (FAB M2: p=0.031, FAB M4: p=0.035). In the entire cohort, 46/1 haplotype carriers and non-carriers had similar complete remission (CR) rates, while there was a tendency towards a lower complete remission rate among 46/1 carriers than among non-carriers in NK-AML (78.7% vs. 94.1%; p=0.064). Relapse rate was similar among 46/1 carriers and non-carriers both in all AML patients and in the NK-AML subgroup. Among NK-AML cases, the rate of deaths in remission or in aplasia caused by infections established by autopsy or ante-mortem clinical signs of infections was significantly higher in 46/1 carriers than in non-carriers (46.8% vs. 23.5%; p=0.038). In the discovery cohort, univariate analysis revealed significantly longer disease-free survival and overall survival in younger patients (<45 years) and in patients with intermediate and favorable karyotypes, while sex, *FLT3* ITD and *NPM1* mutations alone were not associated with altered disease-free survival or overall survival. The 46/1 haplotype did not influence survival in the entire discovery cohort. The 46/1 haplotype was associated with shorter disease-free survival and overall survival independently of age and NPM1-FLT3 status in NK-AML (p=0.024).

5. Discussion

a) According to our knowledge, our study is the first systematic analysis of the presence and the prognostic role of the two major TKI resistance mechanisms (BCR-ABL TKD mutation and ACAs) separately and in combination in imatinib-resistant patients during imatinib therapy and also second-generation TKI treatment. The presence of ACA predicted shorter survival during first and second generation TKI therapy, while TKD mutations only influenced survival during second generation TKI therapy. Screening for BCR-ABL TKD mutations is recommended in TKI resistance before changing TKI, because the presence of different mutations may influence the selection of TKI and the therapeutic response. In case of patients carrying T315I mutation, alternative therapeutic options should be considered.

b) In case of BCR-ABL exon 7 deletion, we concluded that Δexon7 is not associated with imatinib resistance since it is abundantly detected on BCR-ABL mRNA in imatinib naive CML patients. As Δexon7 also occurs on the non-translocated (normal) ABL mRNA, the alternative splicing (AS) process is likely to be independent from BCR-ABL translocation. The detection rate of Δexon7 is highly dependent on the expression levels of BCR-ABL or ABL and the sensitivity of detection method. By bioinformatic methods, we confirmed that, the Δexon7 isoform is not associated with drug

resistance in CML, because of the significant structural changes in the truncated protein and its TKD.

c) MPN patients carrying the JAK2 V617F mutation were older ($p<0.001$) and had higher Hb levels ($p<0.001$). Vascular complications (thrombotic and hemorrhagic) were more common in V617F carrier patients (26.6% vs. 15.2%, p=0,039). We found a female predominance among V617F positive MPN compared with V617F negative patients (57.4% vs. 41.8%, p=0.019).

d) Our study confirms earlier observations that JAK2 46/1 haplotype is a susceptibility factor for JAK2 V617F positive MPN and lends further support that, it is a potential common inherited susceptibility factor for JAK2 V617F negative MPN. The 46/1 haplotype is not associated with MPN manifestations and vascular complication, except the increased risk of the development of myelofibrosis in homozygous cases.

e) In this study, we report an association of the JAK2 46/1 haplotype with disease characteristics and treatment outcome in AML patients. The JAK2 46/1 haplotype influences morphological distribution increasing a predisposition to the acute myelomonocytoid form. Our data indicate that the JAK2 germline 46/1 haplotype may be an independent adverse prognostic factor affecting the severity of infections occurring during treatment in NK-AML.

6. List of publications

6.1 Publications related to the present thesis

- M**eggyesi N**, Kalmár L, Fekete S, Masszi T, Tordai A, Andrikovics H. Characterization of ABL exon 7 deletion by molecular genetic and bioinformatic methods reveals no association with imatinib resistance in chronic myeloid leukemia. Med Oncol. 2011 Oct 30.

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6.2 Publications not related to the present thesis

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