GENETIC ANOMALIES INVOLVED IN THE PROGNOSIS OF MYELOPROLIFERATIVE NEOPLASMS

ABSTRACT OF PhD THESIS

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The thesis is illustrated with 70 figures and 43 tables. The summary includes a limited number of figures, retaining their numbering from thesis.
INTRODUCTION

The myeloproliferative neoplasms (MPNs) are diseases characterised by clone proliferation, the distinction between various types of MPNs being based on myelodysplasia (Vardiman et al. 2009). The management of chronic myeloid leukemia (CML) in Romania is problematic because of the defective alignment to the international standards for molecular monitoring with consequences upon the modification of treatment at the optimal time and the adequate use of the new tyrosine kinase inhibitors (TKI). Although TKI induce remission in an important number of patients, resistance and incomplete response to these agents occur frequently leading to relapse and disease progression, this condition being considered incurable. The choice of the doctoral research subject was determined by the following arguments: the present research directions are oriented both in the clinical field (a more efficient and standardized monitoring of the disease evolution and the level of minimal residual disease, the early relapse diagnosis) and in the fundamental one (the identification of genetic and epigenetic alterations, the evaluation of their involvement in the pathogeny of the disease, the clarification of cellular communication inter-relations between stem leucemic cells and the cellular micro-environment); the therapy efficiency depends on the measure in which it identifies and controls the molecular targets, both the optimal change of the therapy with new generations of TKI and the personalization of treatment being compulsory.

The general objective aimed at consisted of the identification and validation of a molecular markers panel (clone anomalies, mutations of the kinase domain of the ABL gene, mutations of the JAK2 gene, anomalies in the number of copies of other genes or epigenetic anomalies) with a potential role in the progression or relapse of CML and the evaluation of their involvement in the regulation of the expression of the BCR-ABL fusion gene. The doctoral thesis follows the contemporary advanced research directions by promoting useful tools that define personalized medicine. Besides aspects related to the synthesis of existing data, our personal research was targeted at studying the progressive particularities of CML patients, identifying the genetic anomalies involved in the evolution of the disease as well as their potential use as markers in the identification of patients with a risk of CML progression. The outcomes were possible due to the collaboration with the Oncology-Haematology Clinic and the Laboratory of Molecular Biology of the Regional Institute of Oncology and the unconditioned support and
expertise of the teams led by head of department Dr. Cătălin Dănăilă and Professor Eugen Carasevici, PhD.

II. PERSONAL CONTRIBUTIONS

3. THE EVALUATION OF TREATMENT RESPONSE AND RATE OF SURVIVAL FOR CML PATIENTS

3.1. Introduction
CML patients are usually diagnosed in CF (chronic phase) (90%), the clinical diagnosis being based on a characteristic complete blood count where the differential blood count is left shifted (Melo et al. 1993, Assouline, Lipton 2011). The introduction of the TKI treatment turned this type of leukaemia from a reduced life expectancy disease (approximately 4-6 years) into a chronic disease, with an increasing global surviving rate (Deininger 2007). Classic prognosis indicators, such as Sokal and Hasford scores, were used in order to estimate the relative risk of evolution in CML-CP (Hochhaus et al. 2008). Due to the prognosis value of the early response to treatment and the level of response obtained, cytogenetic and molecular testing for the monitoring of the therapeutical response and the minimal residual disease level have become essential elements as far as decisions regarding CML patients are concerned (Assouline, Lipton 2011).

3.2. Purpose and objectives of the study
The purpose of this study is to identify evolution particularities concerning the therapeutic response, tolerance and survival rate in patients treated with second-generation TKI in order to identify the most useful markers for establishing the prognosis and optimizing treatment changes.

3.3. Material and methods
The study batch (batch A) was formed of 87 patients diagnosed with CML, belonging to Romanian Institute of Oncology IRO, Iași. The selection of the patients was performed by including all incidental cases recorded in the period 2000-2012, their evolution being monitored in successive stages up to the year 2014. The data analysis and interpretation was performed for the cases that meet the conditions of molecular and cytogenetic monitoring of minimum 18 months. For monitoring and treatment response we performed the cytogenetic analysis and molecular evaluation of BCR-ABL transcript and evaluated the type, frequency and duration of treatment response.

Standard chromosomal analysis was performed on hematogenous marrow culture, without stimulation (minimum 2 cultures). In order to quantify the genetic expression of BCR-ABL we used the Translocation
Kit t(9;22)/M-BCR-RQ (Experteam, Italy), based on the RealTimePCR technique with hydrolysis probe (of TaqMan type). The reference gene was ABL.

3.4. Results

Characteristics of the patients batch. The statistic descriptive analysis of the batch indicated a relatively equal distribution between sexes, the average age was 41 years, people under 40 years of age being more frequently affected by the disease (55%). The blood count is suggestive, indicating hyperleukocytosis with a median of 143.2x10^6/µL in 62.1% of the cases over 100x10^6/µL. Thrombocytosis was signalled in 47.1% of the total number of patients, with a severe form (>700x10^6/µL) in 13.6% of the patients.

Evaluation of prognosis scores. The evaluation of the Sokal score revealed that 36 patients belonged to the high risk category (41.9%), while only 17 patients (19.5%) were included in the same prognosis category when applying the European Hasford score. Statistical influence was evaluated with the Kaplan Meyer survival curves, depending on the prognosis scores. The survival rate in the batch of patient with Hasford low risk was similar with that of the patients with intermediate and low Sokal risk and of those with EUTOS low risk.

Treatment. At diagnosis, in equal proportions of approximately 25%, the patients of the studied batch received treatment with Glivec (Imatinib) or Hidroxiuree (HUR) or HUR and cytarabine (C-ARA) while a very small percentage received other treatments. The Imatinib dose was increased in 66 patients, 17 patients displaying an improvement of the therapeutic response. The treatment was interrupted in 19 patients because of severe cytopenia, the evolution of the disease leading to acceleration (N=14) and CB (N=4), lack of response or loss of response. The Glivec treatment represented the second and respectively the third therapeutic option in 66 patients (75.9%), the treatment being initiated in less than 6 months from diagnosis in 54% of the cases (47 patients). At the last evaluation, 11 patients were taken out of the batch, 51 patients received the Glivec treatment while 18 (20.7%) received second-generation TKI.

Survival rate according to treatment. For the studied batch the survival rate for the patients under treatment Imatinib was, on average, of 56 months (3-126 months). The general survival rate was, on average, 76 months (18-180 months). The time until the initiation of treatment significantly influenced the survival rate, patients receiving Glivec in less than 24 months from diagnosis responding much better than the ones who received therapy later (p = 0.024).
Treatment response monitoring was performed at a haematological, cytogenetic and molecular level. All the patients received TKI treatment throughout the evolution of the disease. The therapeutic response to the Imatinib treatment varied, 59 patients (67.8%) obtaining CHR in 12 months and respectively 93.1% in 18 months from the beginning of the treatment. The major cytogenetic response (partial and complete) was obtained in only 64.5% of the patients in 12 months of treatment. At the end of the study only 49 patients presented MCyR (56.3%) while 31% had MCyR or ES. By the end of the study molecular examination was performed in 62 patients (71.3%), while after the beginning of the Glivec treatment an equal number of 22 patients CMR and MMR respectively. The evaluation of the impact of prognosis scores upon the therapeutic response to Imatinib revealed a significant statistic impact of the Sokal score both on the cytogenetic response (p = 0.001) and the molecular response (p = 0.025). The administration of Glivec as prime line therapy greatly influenced both the cytogenetic (p = 0.005) and the molecular response (p = 0.015), early administration (in less than 24 months) determining a significantly better response (p = 0.026) as compared to the late administration (third line of treatment).

3.5. Discussions

Despite the fact that initial treatment was heterogeneous, throughout the evolution of the disease all the patients received TKI treatment. In our study, CHR rate was 93.1% comparable with the one obtained in the IRIS study, of respectively 98% (Deininger M et al, 2009), as well as with the response rate of 95% obtained in the phase II study (Kantarjian H et al, 2002).

The cytogenetic response rate for the patients included in our study is lower than the one reported by the IRIS study, namely 64.5% for the patients included in this comparative study as compared to 83%, yet it is higher than the 13% rate reported in the phase I study – a study with patients recruited after showing resistance at the αInterferon (Druker et al. 2001), being comparable with the results of the phase II study in which, for a batch of 454 patients diagnosed with CML-CP, the CCyR rate was of 60% (Kantarjian et al. 2002). This difference might be motivated by the fact that our batch of patients was not homogeneous, since 54 patients (62%) received the first line of treatment with Imatinib and only 38 patients began treatment within 12 months from the diagnosis. In our study, only 74 patients were evaluated molecularly, MMR being obtained in 73% of the cases. At the end of the study only 50% of the patients maintained MMR or CMR. Although it is difficult to compare these outcomes with the ones described in literature because of the differences
in expressing the level of the BCR-ABL transcript, the MMR rate obtained in our study was similar with that reported by Lavallade et al.

In our study, the time until the initiation of treatment with Imatinib, namely less than 12 months from diagnosis, had a positive impact on the survival prognosis, as opposed to the data obtained Kantarjian et al. in a study including 368 CML patients treated with Imatinib, after the failure of the interferon treatment (Kantarjian et al. 2002).

The persistence of the prognosis Sokal score influence upon the therapeutic response and the survival rate of the CML patients after the apparition of Imatinib was discussed in several studies (Trask et al. 2012, Hoffmann et al. 2013, Uz et al. 2013). Sokal maintained the impact and the prognostic value upon the therapeutic response and survival rate even in the Imatinib era. In our study the Sokal score at diagnosis had the highest impact upon the cytogenetic molecular and survival responses. The Hasford prognostic score had a less significant influence upon the cytogenetic response, yet had a statistically more significant influence upon the molecular response.

4. IDENTIFICATION OF THE MUTATIONS IN THE TYROSINE KINASE DOMAIN OF THE BCR-ABL GENE

4.1. Introduction

The mutations in the tyrosine kinase situ (the kinase domain) of BCR-ABL are the main mechanism associated with the resistance to the TKI treatment in CML patients. More than 90 types of mutations were reported, in patients with resistance to Imatinib (Jabbour et al. 2006, O’Hare et al. 2007, Grant et al. 2010). The mutations in the kinase domain are mainly grouped into nine amino-acids positions, including T315I, Y253H/F, M351T, G250E, E255K/V, F359V and H396R, which determine different levels of sensitivity to Imatinib. The in vitro evaluation of their sensitivity to various generations of TKI offered the adequate treatment options depending on the mutational statute of BCR-ABL (eg. mutations Y253H, E255K/V or F359V/C/I – sensitivity to Dasatinib and Ponatinib). Some mutations, such as T315I, are resistant to all generations of TKI.

4.2. Purpose and objectives of the study

The purpose of the study consists in the identification of mutations in the kinase domain of the ABL gene in the BCR-ABL fusion gene which induce resistance to the 1st generation TKI treatment, in order to achieve controlled treatment alterations or transplant indication in patients who do not respond to treatment.
4.3. Material and methods

The study batch (batch B) selected for the analysis of mutations in the kinase domain of BCR-ABL was formed of 15 patients diagnosed with CML and treated with Imatinib, who did not obtain an adequate molecular and cytogenetic response according to the ELN criteria (without CHR, Ph+-> 95% at 3 months; Ph+-> 35%, or BCR-ABL> 10% at 6 months and Ph+ ≥0% and/or BCR-ABL> 1% at 12 months from the beginning of the treatment) or lost the response to treatment.

Sanger sequencing. The Sanger sequencing method with terminal fluorescence was used in the study (Dye Terminator Sequencing). For the sequencing of the kinase domain of the ABL gene involved in the translocation we performed overlapped amplifications (nested PCR) for BCR-ABL and for ABL. The primers sequences used in the amplification of the kinase domain of the BCR-ABL (Branford et al. 2002) gene were: first amplification - the forward primer – 5’ TGACCAACTCGTGTGGTGTGAAACCTC 3’, the reverse primer – 5’ TCCACTTCGTCTGAGATGACTGGATT 3’; second amplification (nested) – Forward primer – 5’ CGCAACAAGCCCACCTGCT 3’, reverse primer – 5’ TCCACTTCGTCTGAGATGACTGGATT 3’. Following the calculation of the PCR reaction parameters, a mix and a start amplification programme were set, from which any other subsequent optimizations derived. Sequencing is achieved with the GenomeLab™ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Fullerton, CA). Capilar electrophoretic migration was performed in the Beckman Coulter CEQ8000 sequencer. The migration protocol was LFR-a. The data obtained was initially interpreted with the CEQ8000 soft. The data folders were afterwards exported in the .scf format, being interpreted with the ChromasLite visualisation programe.

4.4. Results

4.4.1. The optimization process of the Sanger sequencing process of the tyrosine kinase domain of the BCR-ABL gene

1. In the first stage it was decided to modify the PCR reaction parameters in order to identify the optimal working conditions.

Concentration gradient of MgCl₂ In order to verify the optimal concentration of Mg, the following quantities were selected: 2; 3; 4; 5; 6 and 7μl MgCl₂, corresponding to th final concentrations of 1.1; 1.6; 2.2; 2.7; 3.3; 3.8 mM MgCl₂. The 2.7 mM MgCl₂ concentration was considered the accurate one.

Primers hybridization temperature gradient. Starting from the hybridization temperature set at 64°C, an amplification in temperature gradient from 60 to70°C was performed, as described below: 60; 61;
61.9; 62.9; 63.8; 64.6; 65.4; 66.3; 67.1; 68.1; 69; 70°C (Figure 40). The value of 66.5°C was chosen as the hybridization temperature.

Concentration gradient for primers. For concentrations below 0.5 μl (final concentration 0.2 μM), no unspecific amplification signal was obtained. A concentration of 0.2 μM was chosen.

Concentration gradient for template. The template concentration introduced in the PCR reaction was calculated theoretically (the technique is performed on cDNA). Following the ARN extraction a dilution up to 500 ng/μl was performed, using in the ReversTranscription reaction a quantity of 2μg. The final concentration of cDNA will be equivalent of of 100 ng/μl of RNA. The PCR amplifications were performed with 5μl undiluted cDNA and in successive dilutions in scale 1/2. A dilution of 1/8 was selected.

2. In the second stage, in order to optimize the sequencing reaction the reaction parameters were modified. The amplification programme was optimized starting from the hybridization temperature of the primers used in the PCR (66.5°C) amplification with an elongation of 72°C, and eventually a two step programme was used with a hybridization temperature and elongation of 60°C (the optimal temperature for the amplification of the enzyme in the sequencing kit, respectively).

3. In the third stage we aimed at optimizing the electrophoretic migration programme – the voltage and the migration time were modified from 2.2 kV – 110 min to 3 kV – 180 min.

4.4.2. Identified mutations

A batch of 15 patients was investigated after concluding the optimization process for the sequencing procedure. The level of the BCR-ABL/ABL ratio was over 30% in the evaluated patients. Mutations were identified only in two patients (13%).

A patient displayed two mutations at two different moments of the disease: the c.T107G mutation corresponding to the F359V phenotype and the c.C951A mutation corresponding to the p.F317L phenotype (Figure 45 A and B); one patient displayed the cG756T mutation, corresponding to the Q252H phenotype (Figure 45 C).
4.5. Discussions

4.5.1. Technical approaches used in the identification of mutations in the tyrosine-kinase domain of the ABL gene in the BCR-ABL fusion gene

The techniques aimed at target the most frequent mutations, with the most significant pathogenouos impact, or which ensure a decisive attitude in the transfer from one line of treatment to the other. Of these, the most frequently used are: allele-specific oligonucleotide PCR, alele probe-specific RealTime PCR (Iqbal et al. 2013) and RFLP (Chien et al. 2008). The general techniques are represented by procedures that ensure the possibility to identify any genetic anomaly. Contemporary approaches include: pyro-sequencing (Khorashad et al. 2006), SEQUENOM MassARRAY (Vivante et al. 2007) or specific amplification of the mutant clone (Nardi et al. 2008). The Sanger direct sequencing is a frequently used method as it allows the identification of all the mutations in the kinase domain of ABL, although it displays a relatively low sensitivity (over 10%-20% leukemic clone). In our study we have chosen the use of a technique that would facilitate the identification of all types of mutations in the kinase domain of BCR-ABL, namely the direct Sanger sequencing. The reasoning behind the choice of the technique was determined by the multitude of mutations reported in literature for the kinase domain of the ABL gene, as well as by their relatively low incidence, since the use of a targeted technique might present the risk of a negatively false result.

4.5.2. The significance of mutations in the tyrosine kinase domain of the BCR-ABL gene

The mutations of the tyrosine kinase domain of the ABL gene were reported with a global prevalence of 30-60% in patients with resistance to Imatinib (Jabbour et al. 2006, O’Hare et al. 2007, Grant et al. 2010). In our study we identified mutations in 2/15 patients (13%) in LMC-BC, their low frequency being explained by the existence of a reduced batch.
of patients and the possible existence of certain reduced mutant clones, below the method detection limit.

One of the mutations identified in our study was Q252H, on a patient displaying the loss of the molecular and haematological response, after 60 months of favourable evolution under treatment with Imatinib. The Q252H mutation, together with other mutations of the coupling loop with the ATP, were considered as having a great degree of resistance to Imatinib and a very reserved prognosis (Branford et al. 2003). This aspect was also noticed in the case of the patient included in our batch for which no response was obtained during the treatment with Dasatinib, as the patient deceased 4 months after the identification of the mutation.

Two mutations were identified in the ligation site of Imatinib, F359V and F317L, respectively, in two successive samples (from different moments in the evolution of the disease) at the same patient. The c.1075T>G (F359V) mutation confers a reduction of response to treatment with Imatinib and Nilotinib and partially retains the response to the therapy with Dasatinib (Soverini et al. 2011). In the case of the female patient included in our study this treatment was initiated, with a favourable evolution (CCyR and MMR were obtained after three months, the response being valid for 10 months). The c.951C>G (F317L) mutation determines a reduced/moderate sensitivity to the treatment with Imatinib (Shah et al. 2007) and Dasatinib (Laneuville et al. 2010) and preserves high sensitivity to the therapy with Nilotinib (O’Hare et al. 2007, Soverini et al. 2011). In the case of the female patient included in our study the identification of the mutation was achieved too late, as the patient died before the result was obtained. The high incidence of the F317L mutation following the treatment with Dasatinib (50% of all detected mutations) support the in vitro studies that demonstrated the potential occurrence of mutations (F317L included) under treatment with Dasatinib (Bradeen et al. 2006). In a manner similar to the data reported by Bradeen et al., in the case of the female patient included in our study, the F317F mutation occurred during the treatment with Dasatinib.

5. EVALUATION OF GENETIC ANOMALIES RESPONSIBLE FOR THE EVOLUTION TOWARDS BLASTIC CRISIS OF CML

5.1. Introduction

Between 15% and 20% of the patients can progress towards CML-BC (Fabarius et al. 2011). The acquisition of secondary chromosomal aberrations (CSA), such as + 8, the duplication of the Ph chromosome, i 17q, +19 or translocations and associate inversions LAM, was interpreted
as a sign of evolution being associated with a high progression risk towards the accelerated phase or the blastic crisis (Fabarius et al. 2011). The evolution towards the BC limits therapeutic options, which seriously reduces the survival rate (Cortes et al. 2008). Early recognition of the patients with a risk of evolution towards BC is thus very important. Deletions in IKZF1, PAX5, and/or CDKN2A were frequently reported in lymphoid CML-BC (Mullighan et al. 2008, Alpar et al. 2012). Multiple genes were identified as being methylate in the bone marrow in patients with CML or AML, including CDH1, CDKN2A/CDKN2B, DAP kinase, RUNX3, WT1 (Hess et al. 2008, Homig-Holzel, Savola 2012).

5.2. Purpose and objectives of the study

The purpose of our study is to identify genetic and epigenetic anomalies in patients who either are resistant or lost their response to the TKI treatment, in order to evaluate the overlapping events that could explain the unfavourable evolution, as well as to identify new markers that could discriminate between the respondent and non-respondent patients as far as this therapy is concerned.

5.3. Material and methods

For this study, the batch (batch C) was formed of 92 patients diagnosed with CML, for whom molecular and cytogenetic investigations were performed. The selection of the patients was made by including the cases recorded in the period 2005-2013, their evolution being monitored until the year 2014 (cytogenetic or molecular monitoring of minimum 6 months). For all the patients we evaluated the frequency and types of SCA identified through the standard karyotype. Chromosomal anomalies were regarded as clonal evolution if at least two cells were discovered to have the same chromosomal arrangement/supplementary chromosome(s) or at least three cells were identified to have the same monosomy.

From the study batch we selected the sub-batch D, made of 30 patients: 15 patients who showed no response to the treatment or relapsed (the level of the BCR-ABL transcript went up to minimum 25%) and 15 control patients diagnosed with CML-CP, with a favourable evolution. All the patients in sub-batch D were investigated using the MLPA method and the P335 set (aimed at detecting anomalies in the number of copies of the IKZF1, CDKN2A, PAX5, EBF1, ETV6, BTG1, RB1 genes and in the PAR1 area: CRLF2, CSF2RA, IL3RA) and the ME002 set, which evaluates the methylation stages of thye promontory regions of a set of suppressing genes for tumours frequently involved in the carcinogenesis process. The number of DNA copies was estimated using the Coffalyser.net programme.
5.4. Results

5.4.1. The characterization of batch C as far as cytogenetic anomalies and evolution are concerned

Conventional cytogenetic analysis revealed the presence of the Ph chromosome in 92.4% (85/92) of the patients, as well as the presence of other associated anomalies in 18% (17/92) of the patients. Fifteen of the patients with SCA (15/17 – 88%) had an unfavourable evolution (acceleration or accutization phases). The most frequent major SCA were trisomy 8 (8 cases – 53%) and the duplication of the Ph chromosome (7 cases - 46%), 4 patients presenting the four anomalies simultaneously. The identified chromosomal anomalies are presented in Table 38.

<table>
<thead>
<tr>
<th>Nr</th>
<th>Sex</th>
<th>Age</th>
<th>Months to the SCA</th>
<th>Cytogenetic aberrations additional to t(9; 22)(q34;q11) or the t(v,22) version, at diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>46</td>
<td>60</td>
<td>47,XX,t(9,22)(q34;q11.2),+8[7]/46,XX,t(9,22)(q34;q11.2)[4]</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>55</td>
<td>96</td>
<td>46,XX[13]/47,XX,t(9,22)(q34;q11.2),+8[17]</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>41</td>
<td>6</td>
<td>46,XX,t(9,22)(q34;q11.2),inv(16)(p13;q22)[17]/46,XX,t(9,22)(q34;q11.2)[2]/46,XX[1]</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>40</td>
<td>0</td>
<td>47,XX,t(9,22)(q34;q11.2)[21]</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>55</td>
<td>6</td>
<td>46,XX[23]/46,XX,t(9,22)(q34;q11.2)[4]/46,XX,t(16;17)[3]</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>34</td>
<td>12</td>
<td>46,XX,t(9,22)(q34;q11.2)[16]/47,XX,t(9,22)(q34;q11.2),+8[10]</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>56</td>
<td>12</td>
<td>46,XX[4]/47,XX,t(9,22)(q34;q11.2)X2[8]/48,XX,t(9,22)(q34;q11.2)X2, +6[4]</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>63</td>
<td>6</td>
<td>46,XX[14]/46,XX,t(9,22)(q34;q11.2)[3]/48,XX,t(9,22)(q34;q11.2)x2,+8[1]</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>24</td>
<td>38</td>
<td>46,XY,t(9,22)(q34;q11.2)[15]/47,XY,t(9,22)(q34;q11.2)x2[5]/47,XY,t(9,22)(q34;q11.2),+8[6]</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>30</td>
<td>12</td>
<td>46,XY,t(9,22)(q34;q11.2)[10]/47,XY,t(9,22)(q34;q11.2),+17[6]/47,XY,t(9,22)(q34;q11.2),+19[8]</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>24</td>
<td>0</td>
<td>46,XX,t(9,22)(q34;q11.2)[8]/47,XX,t(9,22)(q34;q11.2)x2[4]</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>55</td>
<td>6</td>
<td>46,XX,t(9,22)(q34;q11.2)[16]/48,XX,t(9,22)(q34;q11.2)X2, +8[15]</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>20</td>
<td>6</td>
<td>46,XY,t(1;3)(p22;q29),t(9,22)(q34;q11.2)[20]</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>59</td>
<td>0</td>
<td>46,XY,t(17)(qter→q10::q10→qter)[10]</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>55</td>
<td>0</td>
<td>46,XY,der(2)?,der(2)?,t(9;22)(q34;q11.2)[20]</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>36</td>
<td>12</td>
<td>48,XY,t(9,22)(q34;q11.2)x2,+8[20]</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>28</td>
<td>0</td>
<td>46,XY,t(9,22;11)(q34;q11;13)[30]</td>
</tr>
</tbody>
</table>

F - female, M - male; *age in years at the time of diagnosis; SCA- supplementary chromosomal anomaly

5.4.2. Molecular and clinical characterization of a particular case of CML which coexpresses the CBFβ-MYH11 and BCR-ABL fusion genes

In the case of patient 3 we identified, at the moment of accutization, the presence of a inv(16), while further on it was proved that it was present even since the diagnosis. The evaluation of the CBFβ-MYH11
fusion gene revealed the E type transcript. Two mutations were identified in the kinase domain of ABL F359V and F317L, respectively. The patient deceased because of the progression of the disease and lack of response to TKI and chemotherapy, 18 month after diagnostication.

5.4.3. Evaluation of the variations in the number of genic copies through the MLPA technique, using the P335-B1 set

In the sub-batch D there were identified 3 cases (10%) that associated anomalies of the number of genic copies, in the group with resistance to the TKI treatment (Figure 60, Figure 61, Figure 62 – partial images).

![Figura 60](image1)
![Figura 61](image2)
![Figura 62](image3)

Figure 1. Heterozygous deletion at the level of exon 1 of the IL3RA gene or polymorphism mutation at this level (patient A1); Figure 61. Heterozygous deletion at the level of the entire IKZF1 gene (patient A2); Figure 62. Heterozygous deletion of the exons 4-7 of the IKZF1 gene and of the CSF2RA, P2RY8 and IL3RA genes, in the PAR1 region (patient A3)

5.4.4. The evaluation of the methylation status of a set of tumour-suppressor genes through MS-MLPA using the ME002 kit

With the help of the MS-MLPA technique there was identified abnormal methylation of the following tumour-suppressor genes: WT1, CDH13, GATA5, CD44, ESR; they were methylated in 23% of the patients (7/30). Out of the patients with abnormal methylation, 6 patients were among the ones who did not respond to the TKI and evolved towards BC (Table 40).

Table 40. Characteristics of patients who displayed abnormal methylation through MS-MLPA

<table>
<thead>
<tr>
<th>Age*/Sex</th>
<th>BCR-ABL*</th>
<th>Genes with abnormal methylation</th>
<th>Treatment*</th>
<th>Evolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 65 years, M 45%</td>
<td>CDH13</td>
<td>Dasatinib</td>
<td>LMC-CP</td>
<td></td>
</tr>
<tr>
<td>A2 58 years, F 35%</td>
<td>ESR1, CDH13; (del CDK6, CFTR)</td>
<td>Imatinib 400</td>
<td>Myeloid BC - death</td>
<td></td>
</tr>
<tr>
<td>A3 58 years, M 79%</td>
<td>CDH13;</td>
<td>Dasatinib</td>
<td>Lymphoid BC - death</td>
<td></td>
</tr>
<tr>
<td>A4 24 years, F 28%</td>
<td>WT1</td>
<td>Dasatinib</td>
<td>Myeloid BC—</td>
<td></td>
</tr>
<tr>
<td>A5 38 ani.M 68%</td>
<td>CD44</td>
<td>Imatinib 400</td>
<td>LMC-CP</td>
<td></td>
</tr>
<tr>
<td>A6 58 years, M 100%</td>
<td>WT1,CDH13, GATA5</td>
<td>-</td>
<td>Lymphoid BC</td>
<td></td>
</tr>
</tbody>
</table>
In patient A2 (presented in the sub-chapter 5.4.3), the MS-MLPA revealed a heterozygous deletion of genes CDK6 and CFTR, situated on 7q21-q22 and 7q31.2, respectively, as well as methylation anomalies at the level of genes ESR and CDH13 (Figure 63). The frequency of methylated genes was 13% (4/30) for the CDH13 gene, 6.6% (2/30) for genes WT1 and CD44 and 3.3% (1/30) for genes ESR and GATA5.

Figure 63. MS-MLPA aspect in patient A2. Superior panel: heterozygous deletion at the level of genes CDK6 and CFTR. Inferior panel: abnormal methylation of genes ESR and CDH13

5.5. Discussions

5.5.1. Secondary chromosomal anomalies

Types and frequency of secondary chromosomal anomalies

In our study 18% of the cases associated clonal SCA, their incidence in the accelerated or blastic phase being of 75%. The type and frequency of identified SCA are similar to those reported in previous studies (Farag et al. 2004, Fabarius et al. 2011), the most frequent being major SCA (11/17 - 64%), respectively trisomy 8 (53%) and duplication of the Ph chromosome (46%), 4 patients (23%) displaying a complex karyotype with the association of both anomalies. In the study batch one patient displayed t(v;22), with the further involvement of a single chromosome, namely chromosome 11: 46,XY,t(9;22;11)(q34;q11;q13) [30]. The patient’s evolution was favourable (CCyR, MMR), similar to the one reported in previous studies (El-Zimaity et al. 2004, Marzocchi et al. 2011). Rare SCA were identified (as far as we know, they have not been reported) in three patients: t(16;17), t(1;3)(p22;q29) and respectively two derivative chromosomes 2, possible by means of a mutual translocation between homologous chromosomes.

Prognosis impact of secondary anomalies in CML
Correspondingly to the data reported by Fabarius et al., who evaluated 1151 patients with the same diagnosis and treatment, in our study, major SCA had an unfavourable prognosis, with an average survival rate of 41 months. Out of these, complex chromosomal anomalies, with more than three abnormal cellular lines (two cases) were associated with the patients death. Major chromosomal anomalies developed during treatment were confirmed to be signals of acceleration/accutization. Death occurred in two patients with rare chromosomal anomalies usually detected in acute leukemias: inv(16) and t(1;3). The co-existence of t(9; 22) and inv(16) in CML seems to indicate an unfavourable prognosis, as well as resistance to both chemotherapy and TKI (Merzianu et al. 2005, Roth et al. 2011).

5.5.2. Anomalies in the number of genic copies

Anomalies in the number of copies were detected in only three cases (3/30 -10%). The affected genes were similar to the ones reported in literature (Mullighan et al. 2007, Mullighan et al. 2008, Kuiper et al. 2010, Calderon-Cabrera et al. 2013, van der Sligte et al. 2014). In the BC cases there were identified deletions of the IKZF gene. Patient A3, with lymphoid CML-BC limfoidă associated the most frequent type of deletion, namely the deletion of exons 4-7 which was associated with the deletion of genes in the pseudoautosomal region CSF2RA, P2RY8 and IL3RA. In the case of the patient A2, we identified a rarely reported deletion, namely the heterozygous deletion of the entire IKZF1 gene (exons 1-8) which was phenotypically associated with myeloid CML-BC. Thorough investigations through the use of MS-MLPA revealed supplementary methylation anomalies and a heterozygous deletion of genes CDK6 and CFTR, situated on 7q21-q22. The presence of the deletion at the level of chromosome 7 on both arms can indicate the absence of the entire chromosome 7 (monosomy 7). No cytogenetic examination was performed for this case. Monosomy 7 was described in approximately 5% of the supplementary chromosomal anomalies in CML, being more frequently associated with lymphoid CML-BC (Johansson et al. 2002). Deletions in the IKZF1 gene were associated with a reserved prognosis, similar to the data reported in literature (Mullighan et al. 2008, Kuiper et al. 2010). We have not been able to establish a marker that could discriminate between patients with CML-CP who are resistant to the therapy with Imatinib and those with an optimal response to the treatment.

5.5.3. The methylation profile of tumour suppressor genes in CML
In our study, the genes identified with abnormal methylation and the frequency of these alterations are similar to the results reported in literature (Nguyen et al. 2000, Deutsch et al. 2003, Roman-Gomez et al. 2003, Janssen et al. 2010). In positive patients, methylation was heterogenous, each individual having a different combination of affected genes, an aspect which might be connected to an increased expression of di-methyltransferases, as it was proven DNMT13A and 3B in CML-BC (Mizuno et al. 2001). Abnormal methylation was significantly more frequent in patients who did not respond to the TKI treatment (6 of 7 patients), 5 of them evolving towards BC. The increased number of methylated genes in BC suggests that abnormal methylation is a non-specific process which characterizes the acute stage of the disease, at least for a sub-group of patients.

The CDH13 gene had the most frequent abnormal methylation, being identified in both BC patients (3 cases) and in CP (1 case). These results were similar to outcomes of previous studies (Roman-Gomez et al. 2003, Janssen et al. 2010). The methylation of the CDH13 gene was not correlated with a certain evolutive phenotype. The presence of abnormal methylation in CP, as well in the progressive stages of the disease, suggests that the aberrant methylation of the promoter takes place in an early stage during the CML pathogenetics and it probably influences the clinical behaviour of the disease. The abnormal methylation of the CD44 gene occurred in isolation in two patients, one with CML-CP who had a favourable response to the Imatinib treatment and one with myeloid BC, who displayed SCA, namely inv(16). In this context, the aberrant methylation of the CD44 gene promoter takes place in an early stage in the CML pathogenesis and probably influences the progression of the disease by losing the control of the micro-environment upon the tumoral cells. The hyper-methylation of the WT1 gene was identified in two patients with progressive evolution. The alterations in the WT1 expression seem to be involved in the defective progenitor cells and the progressive evolution of the disease (Rampal et al. 2014).

6. EVALUATION OF CLINICAL AND EVOLUTIVE IMPLICATIONS OF THE CONCOMITANT PRESENCE OF THE JAK2V617F MUTATION AND THE BCR-ABL FUSION GENE IN PATIENTS WITH MYELOPROLIFERATIVE NEOPLASMS

6.1. Introduction

The Ph chromosome is associated with CML and it occurs as the effect of the balanced reciprocal translocation between chromosomes 9 and 22 (t(9;22)(q34;q11)), resulting in the production of the BCR-ABL
fused gene. In patients with negative MPN for BCR-ABL a punctiform mutation was discovered that leads to the replacement of valine with phenylalanine in codone 617 of the JAK2 gene. This mutation leads to the constitutive activation of the JAK2 kinase and consequently to cellular proliferation and resistance to apoptosis. It was initially believed that the two anomalies are mutually exclusive (Jelinek et al. 2005), yet in the past few years some rare cases were described about patients in which both anomalies co-exist, the BCR-ABL fusion gene and the JAK2 V617F mutation (Hussein et al. 2007, Inami et al. 2007), raising questions regarding the phenotypical and prognosis relevance of the co-existance of the two specific markers.

6.2. Purpose and objectives of the study

This study aims at investigating the incidence of this double mutant phenotype in patients with MPN at diagnosis, in order to evaluate the clinical characteristics of the differences in the responses to treatment for patients testing positive for the JAK2V617F mutation and BCR-ABL, as compared to patients who do not display the JAK2V617F mutation, but are positive for BCR-ABL. A comparison with the outcomes described in literature was also aimed at.

6.3. Material and methods

This study was conducted in the period between January 2012 and February 2014 and included 190 MPN cases selected from the Regional Institute of Oncology Iasi, which formed batch E. For all the patients, we evaluated both the JAK2V617F mutation, and the presence of the BCR-ABL gene. The evaluation of the p210 BCR-ABL (b2a2, b3a2) transcript was achieved by means of the Real Time PCR quantitative method, with Taqman hydrolysis probe, using Translocation Kit t(9;22)/M-BCR-RQ (Expertteam, Italy). The evaluation of the JAK2V617F mutation was performed with Real Time PCR Taqman hydrolysis probe.

6.4. Results

Out of the 190 patients for which we evaluated both the BCR-ABL fusion gene and the JAK2V617F mutation, 94 (84) patients (49.4%) displayed the JAK2V617F mutation (79 heterozygotes, 15 mutants) without the presence of the BCR-ABL gene, 68 (79) patients (35.8%) displayed JAK2 genotype wild type and tested negative for BCR-ABL while 26 patients (13.7%) tested negative for the JAK2 mutation (wild type genotype) and positive for the BCR-ABL fusion gene. Only two patients simultaneously displayed both the JAK2V617F mutation and the BCR-ABL transcript, namely 1.05%. The two patients’ phenotypes were essential thrombocythemia and polycythemia vera, respectively.
Case 1 is a 62 year old female patient in which the presence of the b2a2 transcript (p210) was identified with a ratio of BCR-ABL/ABL of 99.54% at diagnosis. The patient also tested positive for the JAK2V617F mutation in heterozygous state. In these circumstances, the diagnosis was CML with associated JAK2V617F mutation. The therapeutic response was favourable at Glivec 400mg/day, obtaining CHR and MMR after 3 months. The JAK2V617F mutation remains present in heterozygous state in successive determinations. Case 2 is a 45 year old male patient in which the JAK2V617F mutation was identified in heterozygous state. The analysis of the p210 transcript of the BCR-ABL fusion gene proved the existence of the latter in reduced quantity at the time of diagnosis, with a ratio BCR-ABL/ABL of 2.6%. the patient was diagnosed with polycythemia vera, which associates the presence of the BCR-ABL major transcript.

6.5. Discussions

The cases we presented contribute, along with the ones described in literature in the last years, to a better understanding of the rare situation in which the JAK2V617F mutation and the BCR-ABL fusion gene co-exist in patients with MPN. In our study, the cases incidence that display the BCR-ABL fusion gene at the same time with the JAK2V617F mutation was of 1.05%, in a batch of 190 evaluated patients (2/190). A similar result was reported by Cappetta et al. who analyzed 1320 cases with a suspicion of MPN and identified 5 (0.37%), patients with non-typical forms of MPN.

The two patients whose cases were detailed in this study associate both anomalies concomitantly at diagnosis, having, however, different phenotypes. Patient 1 displayed a non-typical phenotype for CML, associating elements specific to essential thrombocythemia (ET). The cases associating CML and ET phenotype are extremely rare (Veronese et al. 2010, Lee et al. 2013, Pastore et al. 2013). Case 2 was diagnosed with PV without phenotypical modifications specific to CML. Only two cases described in literature display the BCR-ABL fusion gene (with reduced levels of expression) without clinical signs of CML, throughout the evolution of MPN BCR-ABL negative (Bornhauser et al. 2007, Park et al. 2013). As far as response to treatment is concerned, in most reported patients the suppression of the BCR-ABL positive clone was obtained under TKI treatment (CCyR or MMR/CMR), yet with a partial haematological response, or the progression of marrow fibrosis. In the case of patient 1 described in this study, the response to the Imatinib 400 mg/day treatment was very good, leading to CHR and CMR in 3 months.
Two hypotheses were proposed to explain the presence of the JAK2V617F mutation and the BCR-ABL fusion gene at the same patient. According to the first hypothesis negative MPN BCR-ABL and CML are supposedly two distinctive diseases which develop in different clones of the cellular progenitors, the phenotype being determined by the dominant clone (Hussein et al. 2008, Bee et al. 2010, Veronese et al. 2010, Pastore et al. 2013, Xu et al. 2014). The second hypothesis posposes the existence of a single subclone of progenitors of the hematopoietic cells that acquire the two anomalies in the same cell, at different moments (Bocchia et al. 2007, Bornhauser et al. 2007, Inami et al. 2007, Kramer et al. 2007, Hussein et al. 2008, Jallades et al. 2008). The particularities of the cases described in our study support the second hypothesis, namely the co-existence of the two mutations within the same clone, with the occurrence of BCR-ABL in a sub-clone of positive JAK2V617F cells, the latter gaining proliferative advantage. However, we cannot totally exclude the possibility of two different mutant clones co-existing. The CML evolution does not seem to be influenced by the presence of the JAK2V617F mutation, yet it can constitute a rare cause explaining the lack of haematological response to the TKI treatment and the unfavourable prognosis for these patients.

7. FINAL CONCLUSIONS

1. Thorough monitoring of the cytogenetic and molecular response to TKI allows for the accurate appreciation of the CML patients’ evolution, the detection of patients with high risk of resistance and the identification of candidates to the second-generation TKI treatment or transplant. Rates of haematological, cytogenetical and molecular responses are similar to the ones reported in literature.

2. Analysis at diagnosis of different parameters indicated that the response to Imatinib therapy is influenced by the Sokal score and the early initiation of treatment, in the first 12 month from diagnosis. The Sokal score had the highest impact on the cytogenetic, molecular and survival responses, the prognosis Hasford score having, in turn, a statistically significant influence upon the molecular response. The time to the initiation of the Imatinib treatment, less than 12 month from diagnosis, had a positive impact upon the survival prognosis.

3. Molecular monitoring is essential for identifying patients with a high risk of resistance and can optimally identify candidates for testing the mutations in the kinase domain of ABL. The sequencing technique has the advantage of identifying all mutations in the tyrosine kinase domain of BCR-ABL.
4. The mutations identified in the study batch Q252H, F359V and F317L are frequently reported in the cases of resistance to the Imatinib treatment, being involved in the progression of the disease towards the BC. Both the mutations in the P loop and those in the connection situ of Imatinib were associated with an unfavourable prognosis, as none of the patients survived.

5. Mutations sensitivity to the TKI treatment was similar to the data reported in literature for the F359V mutation (induces sensitivity to the treatment with Dasatinib) and F317L (generally reduced sensitivity to Dasatinib), yet different for the Q252H mutation, that was resistant to the Dasatinib treatment.

6. Systematic performance of the standard karyotype in CML Ph+ patients is necessary both for appreciating the response to treatment (attaining and maintaining CCyR) and for identifying the clonal evolution in patients losing response to the TKI treatment, whose disease progresses. Classic cytogenesis is irreplaceable in the detection of supplementary chromosomal anomalies, leading to additional molecular testing.

7. Numerical chromosomal anomalies are the most frequent form of clonal evolution, the most frequent being major SCA, trisomy 8 and the duplication of the Ph chromosome. Patients displaying this anomaly associate a significantly poorer prognosis as compared with the all other patients, needing early monitoring and intensive therapeutical intervention.

8. Identified minor SCA, associated with an unfavourable prognosis, included derivative 2 chromosomes, t(1;3)(p22;q29), trisomy 19 and mosaic trisomy 17 and inv(16). Inv(16) is a rare event in CML patients being associated with the rapid progression of the disease. Particularly, the case identified in our batch associated two successive mutations in the kinase domain of BCR-ABL, the F359V mutation followed by the F317L mutation, which worsened the clinical prognosis. Taking optimal therapeutic decisions in such cases is very difficult, because of the limited knowledge regarding the evolution of these patients.

9. The MLPA technique allowed the identification of clonal anomalies in the number of copies of certain genes involved in the control and regulation of the haemato-lymphoid system, which are rare in CML-CP, yet are common to the progression stages of the disease. The deletion of the IKZF1 gene was most commonly identified in patients with lymphoid BC.

10. MS-MLPA allowed the detection of abnormal methylation in the samples taken from CML patients at the level of genes CDH13, CD44,
WT1, GATA5 and ESR with an increased frequency in CML-BC, as compared with the CP, in which methylation was almost absent. Our results sustain the hypothesis according to which the progression of the disease is frequently accompanied of methylation alterations. The identification of certain clinically relevant methylation models in CML can contribute to a better understanding of the disease progression mechanisms. Generating a MS-MLPA set specific for CML by including some specific targets could render this test more efficient in appreciating the disease progression risks and optimal altering of the treatment in the case of these patients.

11. The incidence of cases displaying the BCR-ABL fusion gene concomitantly with the JAK2V617F mutation was low (1.05%, in the batch of 190 evaluated patients), the identified cases, along with those described in literature, supporting the possibility of the co-existence of the two anomalies in the same patient, the most frequent phenotype being the CML one.

12. The screening for the presence of the JAK2V617F mutation and the BCR-ABL transcript is recommended at diagnosis in case of MPN suspicion, as well in the case of patients diagnosed with CML who develop myeloproliferations, despite achieving MMR

8. PERSPECTIVES OPENED BY THE DISSERTATION

In the context of molecular medicine development and molecularly targeted medication, the aim of this paper was to broaden the perspective by identifying genetic and epigenetic anomalies involved in the CML progression, taking into account the evolutive particularities of patients in North-Eastern Romania.

Applying the MLPA and MS-MLPA techniques in CML represents a new approach for Romania; gathering information regarding the genetic/epigenetic defects auxiliary in CML might lead to the generation, in the near future, of a specific set for this condition.

Optimizing the sequencing technique for the mutations in the kinase domain of BCR-ABL will allow its routine use, which will result in a controlled change of treatment, thus saving critical time in the attempt to conserve the state of disease remission. The sequencing technique has the advantage of identifying new mutations, besides the ones already described in literature, for which the gathering of data will allow a stronger correlation with the response to various classes of inhibitors.

Although it is a frequently studied disease, CML remains a high interest field due to the constant need of identifying alternative variants for both systemic and niche level treatments. In this respect, future
research focus on the relationship between leukemic cells and marrow stroma, especially the possibilities of artificial introduction of the immune system cellular response in order to identify neoplastic entities for each patient, creating thus the premises for the application of personalized medicine. Another research direction is represented by following the consecutive alterations or even those preceding the fusional alteration that can generate the activation of genomic reconfiguration factors, that did not undergo any cellular and/or micro-environment control system, involving genic alterations recognition molecules genic alterations repair molecules and control mechanisms for the repairs performed at the genomic level. Another field of interest is represented by the study at the marrow niche level of the communication between the leukemic cell and the immune system cells responsible with the identification and correlation of the defects, which eventually lead to the transformation of the latter to the toleration of the leukemic cell (cancelling the anti-tumoral response) or even offering a protection zone of the organism against the tumour.

**PUBLICATIONS DURING THE DOCTORAL PROGRAMME**

**Main author**


**Co-author**

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