PhD Thesis

Prognostic investigations in chronic myeloid leukemia

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List of abbreviations

ABL1 c-abl oncogene 1, receptor tyrosine kinase
AP acceleration phase
ATM ataxia teleangiectasia mutated
BC blastic crisis
BCR breakpoint cluster region
BCR-ABL DF dual fusion BCR-ABL1 FISH probe
BCR-ABL SF simple fusion BCR-ABL1 FISH probe
CCyR complete cytogenetic response (0/20 metaphase Ph+)
CEP8 FISH probe specific for the centromeric region of chromosome 8
CHR complete hematologic response
CML chronic myeloid leukemia
CMR complete molecular response (≤ 0,01 or 0,0032% BCR-ABL1/ABL1 ratio)
CP chronic phase
DAPI 4’,6-diamidino-2-fenilindol
ELN European Leukemia Net
e13a2 BCR-ABL1 breakpoint corresponding to M-Bcr (b2a2)
e14a2 BCR-ABL1 breakpoint corresponding to M-Bcr (b3a2)
e19a2 BCR-ABL1 breakpoint corresponding to µ-Bcr, producing p230 protein
e1a2 BCR-ABL1 breakpoint corresponding to m-Bcr, producing p190 protein
FISH fluorescence in situ hybridisation
MCyR major cytogenetic response (0-7/20 metaphase Ph+)
mCyR minor cytogenetic response (8-13/20 metaphase Ph+)
minCyR minimal cytogenetic response (14-19/20 metaphase Ph+)
MMR major molecular response (≤ 0,1% BCR-ABL1 on the international scale)
NCyR no cytogenetic response (20/20 metaphase Ph+)
OCT1 organic cation transporter 1
PCR polimerase chain reaction
PCyR partial cytogenetic response (1-7/20 metaphase Ph+)
Ph1 Philadelphia-chromosome
RB1 retinoblastoma 1
ROC-analysis „receiver operating characteristic” analysis
RQ-PCR real time, quantitative PCR
RT-PCR reverse transcription PCR
SD-1 a cell line derived of a patient with Ph+ ALL (B-lymphoblastic cell line)
XPB xeroderma pigmentosum B
Introduction

Chronic myeloid leukemia (CML) may be regarded as a paradigm of modern oncology. CML was described as the first leukemia 165 years ago. The first cytogenetic abnormality associated with a malignant disease – the Philadelphia-chromosome (Ph-chromosome) – is related to CML. Gene fusion resulting in the BCR-ABL1 gene producing a protein with abnormal tyrosine kinase function was recognized as a vital pathogenetic factor in malignant disease for the first time in CML patients. The discovery of the BCR-ABL1 protein opened a new chapter in oncotherapy, CML was the first disease where molecularly targeted therapy produced groundbreaking results. Also, CML was the first disease that required a complex molecular and cytogenetic treatment response monitoring strategy.

At the turn of the millenium the first tyrosine kinase inhibitor, imatinib appeared and brought never before seen results in the treatment of CML; 85% 8-year overall survival, and only 8% progression that in the majority of cases occurs in the first three years of therapy.

Not all patients benefit from imatinib therapy. 20-25% of patients do not show the desired therapeutic response, 8-10% is intolerant to the drug. For them the favorable survival is not guaranteed, which defines a need for more effective tyrosine kinase inhibition. Today two second generation tyrosine kinase inhibitors are already available for CML patients. Both provide more rapid and deeper therapeutic responses than imatinib and studies regarding their long-term survival benefits are underway.

Essential questions remain regarding imatinib treatment of CML. Which method leads to the earliest recognition of patients for whom imatinib doesn’t appear to be potent enough? Presently, the method of choice is the assessment of the depth of therapeutic response at specific timepoints of treatment. This assessment is performed by bone marrow karyotyping, real time quantitative polymerase chainreaction (RQ-PCR), or fluorescence in situ hybridization (FISH).

It is important to identify the factors leading to resistance. The recognition of these may help designing effective treatment protocols so the survival of imatinib resistant patients may be improved.

It is still unclear how the different translocation breakpoints of BCR-ABL1 influence the observable phenotype of the disease. The breakpoints result in different transcripts, and thus different proteins, and although these differ in their in vitro transformation potential, the prognostic significance of this difference is currently unknown.
It is widely accepted that the \textit{BCR-ABL1} translocation is necessary and sufficient for the development of CML, however, a few observations indicate that the translocation may be a secondary cytogenetic event appearing due to previous genetic instability. This possibility bears huge significance since current treatment strategies in CML focus on the selective destruction of only the \textit{BCR-ABL1} positive cells.

\textbf{Aims}

1. Determining the role of FISH in monitoring CML patients.

2. Determining the benefits of automated FISH analysis compared to manual FISH investigation regarding monitoring treatment in CML patients.

3. Investigation of the genetic and molecular background of imatinib resistance.

4. Investigation of the prognostic significance of the rare types of \textit{BCR-ABL1} breakpoints in CML.

5. Demonstration of the interpretation difficulties posed by additional citogenetic aberrations in CML by investigating a patient and his family.

6. Determining the significance of secondary aberrations appearing simultaneously in Philadelphia-positive and negative cells by investigating two CML patients.

\textbf{Materials and methods}

\textbf{Samples}

The thesis was based on 2900 bone marrow and peripheral blood samples of 356 CML patients our lab received between 2003 and 2011 for routine diagnostics mostly from Pécs, Szekszárd, Szombathely, Zalaegerszeg, Tatabánya and Veszprém.
Karyotyping

The chromosome banding was performed using G-banding after 24 or 48 hours of culturing bone marrow samples, or using direct bone marrow preparations without culturing. At least 20 metaphases were analyzed, whenever possible. The karyotypes were described using the International Human Cytogenetic Nomenclature.

Fluorescence in situ hibridisation

Commercially available dual-colour simple and dual fusion probes (BCR-ABL SF and BCR-ABL DF, Vysis Inc.) were used for FISH analysis. When manual investigation was performed, 200 cells were analyzed. The cutoff for positivity was determined based on samples of healthy adults (mean + 2 SD). For FISH analysis, peripheral blood or bone marrow samples were used.

The automated FISH analysis was performed using Metafer 4.0, a commercially available image capture and analysis software and a motorized, epifluorescent Zeiss Axioplan2ie MOT microscope.

Consecutive FISH investigations were performed using commercially available probes specific for the centromeric region of chromosome 8 (CCEP8, Vysis Inc.) as a first step. Then, images of cells were captured with Metafer 4.0, and after the removal of FISH probes, rehibridisation was performed using the BCR-ABL DF probe. The cells were relocated based on the images captured previously, thus, the clonal relationship of trisomy 8 and BCR-ABL1 translocation was determined.

PCR investigations

PCR investigations were carried out using primer combinations recommended by the Europe Against Cancer program after reverse transcription. Nested PCR was used for the qualitative assessment of BCR-ABL1 expression, the quality of RNA was controlled with PCR reactions using ABL1 exon 2 and 3 primers.

Quantitative BCR-ABL1 investigations were performed using a commercially available kit (BCR-ABL FusionQuant, Ipsogen, CT, USA) according to the protocol recommended by the manufacturer, following reverse transcription. Our lab took part in a national standardisation program that provided an International Conversion Factor of 0.9253.
ABL1 kinase domain mutation analysis

Analysis of the ABL1 kinase domain for point mutations was performed by bidirectional direct sequencing using BigDye 1.1 cycle sequencing kit and ABI PRISM 310 (Applied Biosystems, CA, USA) genetic analyzer. The labeling occurred after reverse transcription and a heminested selective PCR amplification of the ABL1 kinase domain corresponding to BCR-ABL1.

Results and discussion

1. Determining the role of FISH in monitoring CML patients

A potential role of FISH in monitoring CML patients may be to complement the established prognostic information provided by bone marrow karyotyping. To reveal this possible role, FISH results of 150 peripheral blood samples of chronic phase CML patients were compared to the results of contemporaneous bone marrow karyotyping. In cases of complete cytogenetic response (CCyR, 0 Ph+ metaphases out of 20) FISH showed BCR-ABL1 positivity in 0 – 13.0% of cells (mean: 5.0%). Based on receiver operating characteristic (ROC) analysis, the optimal cutoff value of FISH for determining CCyR was 7%. Using this cutoff FISH predicted the presence of CCyR with 81% sensitivity and 84% specificity.

Since CCyR and partial cytogenetic response (PCyR, 1 – 7 Ph+ metaphases out of 20) are overlapping categories, the relationship of FISH and major cytogenetic response (MCyR), i.e. CCyR or PCyR was also investigated. Using a cutoff value of 15%, FISH performed on peripheral blood samples predicted the presence of MCyR with 95% sensitivity and 96% specificity, when BCR-ABL SF probe was used.

120 peripheral blood samples were used as above when the performance of FISH with BCR-ABL DF probe was investigated. The optimal cutoff value was 1% for CCyR, which provided 91% sensitivity and 96% specificity. MCyR was predicted using 15% cutoff value with sensitivity and specificity of 99% and 90%, respectively.

Results of karotyping of RQ-PCR also showed strong correlation. 1% BCR-ABL1/ABL1 cutoff predicted the presence of CCyR with 98% specificity, but with only 82%
sensitivity. The relationship of MCyR and 10% RQ-PCR was characterized with 95% specificity and 90% sensitivity.

The gold standard of monitoring treatment of CML patients is bone marrow karyotyping. This method requires bone marrow aspiration, and has a relatively high limit of detection; 14% of Ph-positivity can be detected with 95% confidence. Complete and partial cytogenetic responses should be considered combined, since in cases of low level Ph-positivity amounting to a few percent, there is a considerable overlap between the two cathegories.

The role of the sensitive real-time quantitative (RQ-PCR) is rapidly expanding as a tool of monitoring CML patients. RQ-PCR is capable of demonstrating residual leukemia as low as $10^{-4-5}$. Several reports show strong correlation with karyotyping, however, the results are based on different biological backgrounds. The correlation with karyotyping becomes strong only with the advancement of treatment; cytogenetic cathegories cannot be determined with confidence when CCyR is not reached.

FISH is a cell-based method that can be performed even using peripheral blood samples. Its sensitivity is lower than that of RQ-PCR with several orders of magnitude, it cannot supplant the latter, the limit of detection is approximately 2 – 9%; the exact value depends on the type of probe used. The exact role of FISH in treatment monitoring of CML patients is not clarified in international recommendations.

Our results indicate that FISH may be used to determine the presence of MCyR and CCyR in CML patients. According to the recommendation of the European Leukemia Net, treatment response is suboptimal, if MCyR is not reached by 6 months, or CCyR is not reached by 12 months of treatment. Treatment failure is asserted when MCyR is not reached by 12 months, or CCyR is not achieved by 18 months. The loss of MCyR or CCyR is an adverse prognostic information regardless of the timepoint it occurs. The above represent the situations when in the case of unsuccessful or unavailable karyotyping FISH may be used to determine the level of cytogenetic response. The presence of MCyR and CCyR may be asserted with higher sensitivity based on FISH results compared with RQ-PCR, thus a more prudent declaration of treatment failure potentially leading to a more expensive, or more dangerous therapies may be possible.
2. Comparing automated and manual FISH analysis

We have set an automated microscope system for the analysis of FISH using simple fusion \textit{BCR-ABL1} probe, and compared the results of automated analysis with the results of manual investigation. First, we trained the automated system using positive and negative samples to detect cells and FISH spots of red and green fluorescence. Cell detection was hampered mostly by clumped cells that were recognized as single cells. The rejection of these cells from further analysis was easily achieved based on geometrical parameters, after rejection, cell detection was achieved with 99.9% specificity and 88.7% sensitivity.

The detection of red and green FISH spots was performed with 81% and 85% precision, respectively. Incorrect spot detection led to unexpected number of spots within cells. After rejecting cells with an incorrect number of spots the precision of spot detection was 99.6%.

After the above mentioned rejections, the discrimination of positive and negative cells was based on the shortest distance between red and green FISH spots. The false positivity and negativity of automated analysis amounted to 6.7% and 5.5%, respectively. The same values of manual analysis were 5.8% and 2.7%, respectively.

Next, manual and automated FISH analysis performed on peripheral blood samples of 18 CML patients were compared. Samples of patients with a low residual disease approximating the limit of detection were chosen. Manual analysis was performed by three investigators independently.

Automated and manual results showed strong correlation ($R^2 = 0.99$), the mean of difference was only 3.7%. Reproducability of the automated analysis proved to be superior to manual analysis. The results of the three investigators differed in a range of approximately 20%, in the case of twelve out of 18 samples this difference led to disagreement regarding whether the sample showed positivity surpassing the cutoff value of false positivity or not. Automated analysis performed on the same number of cells showed differences only in a range of 8%, which corresponds to sampling error. Out of the 18 samples investigated, only 7 demonstrated ambiguity regarding positivity.

Based on our results it can be concluded that automated FISH analysis is possible with adequate precision. Although false positivity and negativity are not improved compared to manual analysis, interobserver variability is eliminated, thus, precision of the analysis is improved even when analysing only 200 cells. Automation makes the increase of analysed cells easily possible, the limit of which is set by the hardware capabilities alone. Furthermore,
digital images of cells are captured with automated analysis, accomplishing documentation as well. Relocation of analysed cells is possible using the coordinates of cells, leading to the possibility of combining the analysis with immunophenotyping or additional FISH investigations.

3. Factors of imatinib-resistance in CML

The cytogenetic and molecular factors of imatinib resistance were investigated in 48 CML patients. Bone marrow karyotyping, FISH, qualitative and quantitative PCR were performed. The parameters observed were compared with similar parameters of 98 patients without signs of resistance.

The type of the Ph-translocation, interstitial deletions neighboring the breakpoints and the type of the BCR-ABL1 transcript (b2a2 or b3a2 breakpoints) did not show different frequencies in resistant and non-resistant cases, so they do not seem to influence treatment responses.

ABL1 kinase domain point mutations were detected in 29% of resistant patients. A broad range of incidence – 21 - 90% – is reported in the literature that is influenced by the phase of the disease and the level (i.e. cytogenetic or hematologic) of resistance. In only 13% of patients (6/48) could the observed point mutation be held responsible for imatinib resistance, since in the other cases, the mutation was detected after resistance commenced. It is possible that point mutations were present before the first observation of resistance below our limit of detection. However, resistance is defined as the accumulation of a residual leukemic clone; a point mutation can hardly be the cause of the lack of treatment response if it is present in only a small subset of residual leukemic cells.

The finding described above is surprising, since ABL1 kinase domain mutations are considered to be the most frequent causes of tyrosine kinase inhibitor resistance, although several publications suggest that mutations are not directly responsible for resistance, but rather are indicators of genetic instability leading to resistance.

46% (22/48) of patients with resistance showed additional cytogenetic abnormalities besides the Ph-translocation. 14/48 cases showed duplication of the Ph-chromosome, 1/48 case presented with BCR-ABL1 amplification. These frequencies are similar to the ones reported in the literature: 7/32 and 2/32, respectively.
In cases with imatinib resistance, clonal evolution and ABL1 kinase domain point mutations were observed most frequently, but their causality is ambiguous. In 14 cases no cytogenetic or molecular abnormality was detected. Presumably, inadequate treatment responses are often invoked by pharmacokinetic factors or lack of adherence to the drug that frequently causes mild side effects and needs to be taken every day.

4. Prognostic significance of rare BCR-ABL1 breakpoints in CML

Three CML cases were investigated where BCR-ABL1 transcripts of exclusively the m-bcr (e1a2) breakpoint were found. Monocytosis was not seen in either case. One of the patients received imatinib as second-line treatment after having developed accelerated phase under interferon treatment, the other two patients were initiated on first-line imatinib treatment. All three demonstrated complete hematologic response, however, cytogenetic response did not develop in one of them, although progression did not occur during 5 years of follow-up. One of the other two patients relapsed after 19 months of treatment, developed myeloblastic crisis and succumbed to the disease at 39 months. The third patient shows complete cytogenetic response after 3 years of follow-up.

An additional patient with chronic phase CML was investigated, who was presented with BCR-ABL1 transcripts of the µ-bcr (e19a2) breakpoint. Initially, interferon treatment was started, later, due to inadequate treatment response, imatinib with increased dose was administered. Finally, allogeneic bone marrow transplantation was performed because of the occurrence of blastic crisis. Complete cytogenetic reponse developed after transplantation, however blastic crisis reoccurred 5 months later. Although complete cytogenetic reponse was achieved with chemotherapy, the patient died due to septic shock.

The majority of CML patients harbor translocation breakpoints involving the major breakpoint cluster region (M-bcr) of the BCR gene leading to the formation of a 210 kDa protein. Rarely, in 1% of patients the breakpoint is located in a different region. Alternative breakpoints are the m-bcr (e1a2) that is frequent in acute lymphoblastic leukemia and results in a 190 kDa protein, and the even more rare µ-bcr (e19a2) that yields a 230 kDa protein. The different BCR-ABL1 proteins have divergent transformation potentials in vitro. The exclusive presence of m-bcr BCR-ABL1 transcripts in CML is associated with monocytosis and adverse
prognosis based on various reports, while the \( \mu \)-bcr breakpoint is related to an excess of neutrophils similar to chronic neutrophilic leukemia and a better prognosis.

The four cases investigated do not support the above associations between the observable phenotype and the rare BCR-ABL1 transcripts. The exclusive presence of the m-bcr transcript was indeed associated with poor prognosis, since two out of three such patients did not achieve CCyR, however, neither patients showed monocytosis. The patient harboring the \( \mu \)-bcr breakpoint died of his disease after suffering multiple blast crises, which does not indicate a good prognosis.

How the same genetic abnormality may lead to different phenotypes is still an unresolved question, just like the relationship between the diverse in vitro transformation potential of BCR-ABL1 proteins and the difference of phenotypes related to them.

5. Interpretation difficulties of cytogenetic aberrations besides the Ph-translocation during the follow-up of a CML patient

Bone marrow karyotyping revealed cytogenetic aberrations involving six different chromosomes besides Ph-positivity at diagnosis of a 44-year-old man with CML. The accumulation of additional cytogenetic abnormalities suggested an aggressive course of the disease. After the commencement of imatinib treatment, neutropenia and thrombocytopenia occurred leading to the reduction of the dose. Only minor cytogenetic response was observed at 8 months of treatment which is considered as suboptimal treatment response. However, complete cytogenetic response was attained at 20 months, major molecular response at 60 months.

The first follow-up cytogenetic investigation raised the possibility of the constitutional nature of the additional cytogenetic aberrations, thus, karyotyping of phorbol acetate (TPA) stimulated peripheral blood lymphocytes was performed. The investigation showed Ph-negativity in all of the cells, however, the formerly observed additional aberrations were present in every cell analysed. These aberrations were present in every cell at every subsequent cytogenetic analysis as well.

The karyotyping of peripheral blood lymphocytes of seven family members involving three generations of the patient was performed. The three constitutional translocations involving the six chromosomes were seen in six family members, two abnormalities were seen in two. The presence of all three abnormalities was restricted to the patient.
The above findings demonstrate clearly that while FISH and the PCR-based methods are targeted tests aimed at the \textit{BCR-ABL1} translocation, karyotyping yields information concerning the entire genome. The rarely occurring constitutional aberrations may confound the prognostic interpretation of secondary cytogenetic alterations. Currently, there is no conclusive evidence that constitutional cytogenetic aberrations increase the risk of malignant hematological diseases; however, our case illuminates the possibility that genetic instability leading to the accumulation of constitutional abnormalities may provide the background for the development of the Philadelphia-translocation as well. Fortunately, neither the patient nor his relatives demonstrate any sign of escalating constitutional genetic instability, and CML or any other malignant disease was not observed in family members.

6. The significance of secondary aberrations appearing simultaneously in Philadelphia-positive and negative cells in two CML patients

Samples of a 50-year-old male and a 40-year-old female patient were investigated. Both patients received first line imatinib treatment due to chronic phase CML. The dose was reduced temporarily due to cytopenias in both cases. Cytogenetic studies revealed trisomy 8 in both Ph-positive and Ph-negative cells at 6 and 16 months in the two cases, respectively.

Only minor cytogenetic response was reached with imatinib treatment in both cases, so dasatinib was initiated. Neither patient demonstrated the presence of any \textit{ABL1} kinase domain mutation during their follow-up. Significant residual disease was seen in both cases after temporary cytogenetic improvement at 23 and 27 months of dasatinib treatment, respectively. At last follow-up, all three abnormal clones – Ph-positive, Ph-positive and trisomy 8, and Ph-negative and trisomy 8 – were present in both cases.

We investigated the clonal relationship of 8 trisomy and the \textit{BCR-ABL1} translocation in the samples of the two patients with interphase FISH. The three abnormal clones were detectable in both cases starting from a very early point during follow-up. The clones did not demonstrate any obvious growth advantage; no trends were observed in their ratios as time progressed.

Both patients were resistant to both imatinib and dasatinib. There is a possibility that trisomy 8 developed due to a genetic instability predating the Ph-translocation in both cases. This possibility bears major significance: if the genetic instability characteristic of CML cells frequently leading to tyrosine kinase inhibitor resistance is not the consequence of the
abnormal tyrosine kinase function of BCR-ABL1, but rather develops before the Ph-translocation, than rapid and selective inhibition of BCR-ABL1 will not achieve protection against treatment resistance, not even using more advanced tyrosine kinase inhibitors. The identification and targeted therapy of the abnormality predating the Ph-translocation would be more beneficial.

Summary

1. We determined the potential role of FISH in monitoring the treatment response of CML patients. The method may lead to assessment of major or complete cytogenetic response even using peripheral blood samples. At the early phase of treatment, suboptimal response or treatment failure may be determined with greater sensitivity using FISH than RQ-PCR.

2. We demonstrated that the sensitivity and the accuracy of FISH investigation may be improved by automated analysis since it provides the significant increase of the numbers of investigated cells without increasing manual working hours. Additionally, automated analysis removes interobserver variability. We characterized the specifications of the automated FISH analysis of BCR-ABL1 translocation, which may provide the basis of similar specifications of detecting different genetic abnormalities.

3. We investigated the molecular and genetic factors of imatinib resistance. The most frequent cause proved to be clonal cytogenetic evolution, ABL1 kinase domain mutations were observed with slightly lower incidence compared with what is reported in the literature. In several cases we observed that the ABL1 kinase domain mutation is not the cause of the imatinib resistance, but rather is the indicator of genetic instability leading to the resistance.

4. We determined that the rare BCR-ABL1 breakpoints are not associated with an obvious, specific prognosis or phenotype. The relationship between the different BCR-ABL1 transcripts and the phenotype of the disease is still unclear.

5. We highlighted the difficulties of interpreting constitutional cytogenetic abnormalities in the case of a CML patient and his family, and we pointed at potential
differential diagnostic difficulties. Our findings also raised the possibility of an underlying genetic instability causing the Ph-translocation.

6. With thorough investigation of two CML cases, we have found support of the theory that the Ph-translocation may be a secondary abnormality in CML.
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Publications related to the thesis

Original articles
1) **Kajtár B.** A BCR-ABL1 transzlokáció molekuláris biológiája. Hemat Transzf. 2007;40:124-34.


Citable abstracts


Lectures held in the topic of the thesis:


**Other publications - original articles**


**Other publications – citable abstracts**

1) Alpár D, Nagy Zs, **Kajtár B**, Feller D, Jáksó P, Dávid M, Kosztolányi Sz, Hermesz J, Kereskai L, Pajor L. Initiating role of recurrent IgH translocations during the oncogenesis of plasma cell myeloma is questionable in a not negligible subset of cases. Haematologica 2011;S2(96):116. IF (2010).: 6,532


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**Cumulative impact factor of citable abstracts: 65,75**