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## Application of Cytogenetics for Chronic Myeloid Leukemia in Tîrgu Mureş

### Aplicarea citogeneticii pentru leucemia mieloidă cronică în Tîrgu Mureş

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#### Abstract

Chronic myeloid leukaemia (CML) is a myeloproliferative disorder characterized in about 95% of cases by the presence of the Philadelphia (Ph) chromosome, which results from a reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34;q11). Our aim was to identify and to evaluate the frequency of chromosomal abnormalities at the time of diagnosis and during drug therapy for monitoring treatment response in patients with CML in the Hematology Clinic in Tîrgu Mureş. Between January 2006 and January 2009 we received for chromosomal analysis 52 samples of bone marrow and/or peripheral blood from CML patients from the Hematology Clinics from Tîrgu Mureş. We carried out bone marrow and/or peripheral blood cell culture according to standard methods. We successfully analyzed the leukemic karyotype of 46 patients (88,5%) and identified 25 (54,3%) cases with chromosomal abnormalities. Analyses performed on the bone marrow at the moment of CML diagnosis confirmed the presence of Ph chromosome t(9;22)(q34q11), in 92% of patients. 37 cases with CML were cytogenetically analysed during drug therapy for monitoring treatment response. During therapy 20 patients were Ph negative and seven had different level of cytogenetic response.

**Keywords:** chronic myeloid leukemia, cytogenetic, Philadelphia chromosome

#### Rezumat

Leucemia mieloidă cronică (LMC) este o boală mieloproliferativă caracterizată, în 95% dintre cazuri, prin prezența cromozomului Philadelphia (Ph). Acesta apare în urma unei translocării reciproce între cromozomii 9 și 22, t(9;22)(q34;q11). Scopul nostru este să evidențiem și să stabilim frecvența anomaliilor cromozomiale în momentul diagnosticului și să monitorizăm răspunsul la tratament pe parcursul terapiei medicamentoase la pacienții cu LMC din Clinicile de Hematologie din Tîrgu Mureş.

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Au fost primite 52 de probe biologice de la pacienții cu LMC internați în Clinicile de Hematologie Tîrgu Mureș în perioada ianuarie 2006 - ianuarie 2009. S-au efectuat culturi celulare din măduva osoasă hematogenă și/sau sânge periferic, conform metodelor standard.

Analiza citogenetică a reușit în 46 dintre cazuri (88,5%), în 25 dintre ele (54,3%) evidențiind anomalii cromozomiale. Analiza citogenetică efectuată în momentul diagnosticului a confirmat prezența cromozomului Ph, t(9;22)(q34;q11) în 92% dintre cazuri. Pentru a monitoriza răspunsul la tratament, analiza citogenetică a fost efectuată în 37 dintre cazurile de LMC pe parcursul terapiei medicamentoase. Pe parcursul tratamentului 20 de cazuri au fost Ph negative, iar 7 au avut un nivel diferit al răspunsului citogenetic.

**Cuvinte - cheie:** leucemie mieloidă cronică, citogenetică, cromozom Philadelphia.

## Introduction

Chronic myeloid leukaemia (CML) is a myeloproliferative disorder characterized in about 95% of cases by the presence of the Philadelphia (Ph) chromosome (1). It is a result from a reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34;q11). The Philadelphia chromosome translocation results in an acquired somatic mutation (bcr/abl) involving the hematopoietic stem cell and fuses the abl gene from chromosome 9 with the bcr gene on chromosome 22. A chimeric mRNA (8.5 kb) is thus transcribed instead of the normal ABL transcript (6 or 7 kb) and subsequently translated to an activated bcr/abl gene product (most commonly 210 kd) instead of the normal abl gene product (145 kd). Unlike the normal ABL, which is a regulated kinase that plays a role in normal hematopoiesis, BCR/ABL localizes to the cytoskeleton and displays an up-regulated tyrosine kinase activity that leads to the recruitment of downstream effectors of cell proliferation and cell survival and consequently cell transformation (1).

The Ph chromosome is generally the only cytogenetic abnormality present in the chronic phase of disease. About 3-4% of Ph-positive patients with CML have variant translocations (2), involving another chromosome (3). These variant translocations have been classified as "simple" when another chromosome is involved and as "complex" when chromosomes 9, 22, and at least one other chromosome are involved.

The natural history of CML is one of a triphasic disease, comprising the chronic, accelerated, and the final fatal blastic phases. As the disease progresses through the accelerated phase and into the blast crisis, additional cytogenetic abnormalities become evident (4):

- **Chronic phase CML.** CML is characterised by the chromosomal translocation t(9;22)(q34;q21). The shortened derivative chromosome 22 is also known as the Philadelphia (Ph) chromosome.
- **Accelerated phase CML.** In addition to t(9;22)(q34;q21), additional karyotypic aberrations indicating clonal evolution are found. Typical additional aberrations include an extra Ph chromosome, isochromosome 17q, and trisomy 8.
- **Blastic phase CML.** Karyotypic analysis may show chromosomal aberrations additional to t(9;22)(q34;q21). The changes may be those seen during the accelerated phase. Occasionally, chromosomal changes typical for *de novo* acute leukaemias may be observed, such as t(8;21) and t(15;17).

The majority of CML patients develop secondary (i.e. additional) clonal aberrations in Ph positive cells in advanced phases of the disease. Additional abnormalities can be detected in approximately 75-80% of CML patients in blast crisis. The appearance of secondary changes is a phenomenon called cytogenetic clonal evolution. Clonal evolution is thought to reflect genetic instability of the leukemic cells and may be a sign of disease progression (5 - 7).

**Table 1. Criteria for cytogenetic response in CML**

Level of cytogenetic response	Frequency of Ph positive metaphases (%)
Complete	0.00%
Partial	1% - 35%
Minor	36%-65%
Minimal	66-95 %
No response	>95%

Secondary chromosomal aberrations are clearly non-random, the most common being the isochromosome 17q, trisomy 8, additional Ph chromosome and slightly less frequently trisomy 19. The first three changes constitute over 90% of the CML cases in whom secondary chromosomal changes are being detected. Other, less frequently seen abnormalities are for example trisomy 21 and monosomies of chromosomes 7 and 17 (8).

CML is potentially curable with allogeneic stem-cell transplantation, but fewer than 30 % of patients have suitably matched donors (9). Treatment with interferon alpha can induce a complete cytogenetic response in 5 to 20 percent of patients and result in longer survival than that achievable with chemotherapy, but it is associated with serious toxic effects. Patients in whom interferon therapy fails are usually treated with hydroxyurea or busulfan. Imatinib mesylate (Gleevec), is a potent and selective competitive inhibitor of the BCR-ABL protein tyrosine kinase. Several studies showed that daily doses of 300 mg or more of imatinib induced durable hematologic responses in nearly all patients with chronic-phase CML with minimal toxic effects (10, 11). The efficacy of

treatment can be evaluated by the rate of the cytogenetic response (Table 1) which is based on the examination of at least 16 cells in metaphase in marrow samples (11, 12).

Standard cytogenetic studies of the bone marrow show the Philadelphia chromosome, t(9;22)(q34q11), in approximately 95% of patients at diagnosis of CML. Standard cytogenetic studies should be considered at the time of CML diagnosis in all patients. During drug therapy cytogenetic evaluation is recommended at different time for monitoring treatment response. Indications of cytogenetic analysis in CML are mentioned in Table 2 (13).

Our aim is to identify and to evaluate the frequency of chromosomal abnormalities at the time of diagnosis and during drug therapy for monitoring treatment response in patients with CML in the Hematology Clinic in Tîrgu Mureş.

## Materials and methods

### Patients

Between January 2006 to January 2009, 52 samples of bone marrow and/or peripheral

**Table 2. Cytogenetic Analysis Indications in Chronic Myeloid Leukemia**

Moment of cytogenetic analysis	Sample
At diagnosis	Bone marrow
During interferon alfa or imatinib therapy	Bone marrow every 6 months until CCR, then replace with either blood FISH or RT-PCR
After hematopoietic stem cell transplantation	Bone marrow at 3-6 months, then as indicated
Disease transformation into accelerated or blast phase	Bone marrow

CCR = complete cytogenetic remission; RT-PCR = reverse transcriptase-polymerase chain reaction; FISH = fluorescence in situ hybridization;

blood from patients with chronic myeloid leukemia were sent to the Genetic Laboratory of The University of Medicine and Pharmacy in Tîrgu Mureş, Romania for cytogenetic evaluation. The study included patients with CML from Hematology Clinics from Tîrgu Mureş: 2 were in accelerated phase, 3 in blast crisis phase and 47 in chronic phase. The median age was 47 years; 41 patients (78,8%) were males and 11 females.

### Cytogenetic analysis

Heparinized bone marrow and/or peripheral blood obtained at the time of diagnosis and during drug therapy for monitoring treatment response were cultured for 1-3 days in RPMI 1640 medium supplemented with 20% fetal calf serum, 1% L-glutamine, 50 ng/ml penicillin/streptomycin with or without mitogens. After incubation, the cells were exposed to Colcemid (10 µg/ml), followed by hypotonic (75M KCl), and were fixed with a methanol and glacial acetic acid solution. Chromosomes were spread on cold, wet slides and used a special staining technique (G-banding or GTG staining). Karyotype

was interpreted according to International System for Human Cytogenetic Nomenclature (ISCN 1995) recommendation (14). A minimum of 16 metaphases were analyzed. Analysis was carried out using a BX51 Olympus microscope and images captured with an automated image analysis system (Cytovision, Applied Imaging). Cell culture failure was defined as cases where fewer than 10 analyzable metaphases were found or with poor-quality metaphases.

### Results

The 52 analyzed samples from patients with chronic myeloid leukemia included: 15 cases at the moment of diagnosis and 37 during drug therapy for monitoring treatment response. Among the 52 samples received, 6 (11,5%) were unsuitable for cytogenetic analysis due to a very low mitotic index and poor-quality metaphases obtained from the cell culture.

We successfully analyzed the karyotype of 46 leukemic patients (88,5%) and identified 25 (54,3%) cases with chromosomal abnormalities. The results were obtained from

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Figure1. Karyotype from a bone marrow metaphase showing 46,XY,t(9;22)(q34;q11). Involved chromosomes are pointed by arrows.

**Table 3. Cytogenetic analysis at the moment of diagnosis and during drug therapy**

	Cytogenetic analysis	
	At diagnosis (n)	During therapy (n)
Total	15	37
Ph +	13	7
Ph -	1	20
Ph - with other chromosomal aberration	0	5
Failed cytogenetic analysis	1	5

overnight cultures and from cultures with added growth factors, as described in the methods.

Analyses performed at the moment of diagnosis of CML from the bone marrow confirmed the presence of Ph chromosome t(9;22)(q34;q11), (Figure 1) in 92% of patients.

37 cases with CML were cytogenetically analyzed during drug therapy for monitoring treatment response. Cell culture failure was seen in 5 cases. During therapy 20 patients be-

**Table 4. The levels of cytogenetic response during Imatinib mesylate therapy**

Level of cytogenetic response	No. of cases
Complete	5
Major	1
Minor	1
Minimal	0
No response	0

came Ph negative and seven had different level of cytogenetic response (Table 3 and Table 4).

Clonal evolution with additional chromosomal aberrations was observed in 5 patients: trisomy 8 (2 patients), hyperdiploidy (1 patient), hypodiploidy (2 patients). The chromosome aberration was considered as clonal, if two or more cells with the same rearrangement or at least three cells with the same aneuploidy were found.

Trisomy of chromosome 8 was found in two cases during blastic phase: 47,XY,t(9;22)(q34;q11),+8/ 46,XY; 47,XY,t(9;22)(q34;q11)/ 47,XY,inv(9)(p12-q13),+8/ 46,XY.

Trisomy 8 (Figure 2) is considered a common additional change during transformation. The specific cytogenetic profile at the time



**Figure2. Karyotype from a bone marrow metaphase of one patient with CML showing trisomy 8 and inversion of chromosome 9.**

Table 5. The karyotypes of the Philadelphia chromosome positive (Ph+) patients

Group	No. of cases	Description	Karyotype
without additional aberrations	15	Simple translocation	46,XY,t(9;22)(q34;q11)/46,XY 46,XX,t(9;22)(q34;q11)/46,XX
with additional aberrations	2	Common additional changes	47,XY,t(9;22)(q34;q11),+8/46,XY 47,XY,t(9;22)(q34;q11)/47,XY,inv(9)(p12-q13),+8/46,XY
	3	Other additional changes	46,XX,t(9;21)(q34;q11)/54,XX,+3,+4,+8,+10,+17,+19,+mar,+mar / 46,XX 46,XY,t(9;21)(q34;q11)/45,XY,t(9;22)(q34;q11),-21/46,XY 46,XY,t(9;21)(q34;q11)/44,XY,-11,-12/46,XY

of blast transformation helps us distinguish lymphoid (chromosome 7 abnormalities) from myeloid (trisomy 8, isochromosome 17q, trisomy 19) subtype.

Seven Philadelphia chromosome positive (Ph+) chronic myeloid leukemia (CML) patients at diagnosis have been followed during Imatinib mesylate therapy. Five (71%) patients showed a complete cytogenetic response, the Ph chromosome disappeared for a period of time varying from 6 to 18 months. The levels of cytogenetic response are presented in Table 4. None of these patients have so far shown any karyotype evolution.

We also report a 27 year-old male with chronic myeloid leukemia with structural cytogenetic abnormalities. Bone marrow chromosomal study identified Philadelphia chromosome as unique abnormality, in chronic phase. Glivec (Imatinib mesylate) therapy has been started, but primary resistance was observed. Because of the resistance to Imatinib therapy and the availability of an HLA matched sibling donor the stem cell transplantation was performed. Reevaluation at 3 and 6 months after stem cell transplantation revealed that the patient achieved complete cytogenetic and hematologic remissions.

Using classic cytogenetics, the karyotype of each patient with CML was determined and Ph+ patients were divided into two main groups (Table 5):

- patients without additional chromosomal aberrations,
- patients with additional chromosomal aberrations.

Of particular interest are patients who presented two different clones in addition to the normal cell population: 46,XX,t(9;21)(q34;q11)/54,XX,+3,+4,+8,+10,+17,+19,+mar,+mar/ 46,XX; 46,XY,t(9;21)(q34;q11)/ 45,XY,t(9;22)(q34;q11),-21/ 46,XY; 46,XY,t(9;21)(q34;q11)/ 44,XY,-11,-12/ 46,XY. The presence of the additional changes may have a prognostic value. The occurrence of additional chromosome abnormalities during the course of the disease has been extensively described in the past and considered an unfavorable prognostic factor.

We found clonal chromosome aberrations in 5 Ph chromosome negative cases. In all of them chromosome abnormalities were numerical aberrations (hyperdiploidy).

On the whole, our results demonstrate that the most frequent chromosomal abnormality in CML was Ph chromosome t(9;22)(q34q11), detected in 80% of cases at diagnosis and during therapy.

## Discussion

Cytogenetic analysis is the 'gold standard' for genome-wide screening of cytogenetic abnormalities (15).

In our study, 25 (54,3%) cases showed

chromosomal abnormalities, Ph chromosome was detected in 80% of patients with CML. The Ph chromosome frequency at the moment of diagnosis in CML (92%) found in our study is within the limits reported by other authors (16). In the remaining 8%, the Philadelphia chromosome might be either masked (submicroscopic bcr/abl fusion) or part of a complex/variant chromosomal translocation (involvement of other chromosome breakpoints in addition to 9q34 and 22q11). These "Philadelphia chromosome-negative" and Philadelphia chromosome-positive cases are identified by either FISH or RT-PCR, methods with a superior sensitivity.

Therefore, standard cytogenetic studies should be considered at the time of CML diagnosis in all patients. The detection of the Philadelphia chromosome, within the context of a chronic myeloid disorder is diagnostic of CML regardless of the presentation phenotype that can sometimes mimic either essential thrombocythemia or myelofibrosis with myeloid metaplasia (17). On the other hand, the absence of the Philadelphia chromosome does not exclude the possibility of CML, and if the clinical diagnosis is suggestive, a more sensitive genetic test (eg, FISH or PCR for bcr/abl) should be performed.

Bone marrow cytogenetic studies are currently the method of choice for monitoring treatment response to both imatinib and interferon alfa. Furthermore, after treatment with these drugs, both disease-free and overall survival are best predicted by the pattern of cytogenetic response.

According to Hochhaus et al. it is often difficult to obtain wellspread metaphases with good chromosome morphology from bone marrow samples of CML patients especially on Interferon alfa therapy (18). Fugazza et al. have also observed that chemotherapeutic agents like Interferon or Imatinib provide a limited number of metaphases (10).

In our experience, bone marrow cell

cultures obtained from patients treated with Hydroxyurea and Interferon alfa provided a limited number of metaphases, which were often of poor quality. In 5 patients with CML cytogenetic analysis failed during drug therapy (Hydroxyurea and Interferon alfa) because of a very low mitotic index and poor-quality metaphases obtained from the cell culture.

Imatinib induced complete cytogenetic responses in 5 (71%) CML patients during the first 18 months of treatment as seen in other studies (13, 19, 20). According to the Fugazza et al. and Sessions J, cytogenetic evaluation is recommended in chronic myelogenous leukemia at 6 and 12 months from initiation of treatment with Imatinib, when the patient appears to be responding to treatment. If a complete cytogenetic response is reached after 6 months, it is not necessary to repeat cytogenetic evaluation at 12 months. If the patient is not in complete cytogenetic remission at 12 months, repeat cytogenetic testing is recommended at 18 months (10,21). According to the recommendations from an expert panel on behalf of the European LeukemiaNet2 cytogenetic evaluation is recommended at least every 6 months until a complete cytogenetic response has been achieved and confirmed, then every 12 months.

According to Jane Apperley, after allogenic transplant in CML, complete cytogenetic remission is the norm. This fact was proved also by one of our patients which has reached a complete cytogenetic remission after the transplant. For patients with CML, which are responding poorly or are resistant to Imatinib, allo-stem cell transplantation has the potential to be a highly effective treatment (9).

Most patients with CML will develop additional cytogenetic abnormalities (extra Philadelphia chromosome, trisomy 8, isochromosome 17q, trisomy 19) during transformation (22). Such clonal evolution is more frequent in myeloid compared to lymphoid blast crisis and may be associated with lower prognosis (22). Furthermore, the specific cytogenetic profile at

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the time of blast transformation may help distinguish lymphoid (chromosome 7 abnormalities) from myeloid [+ 8; i(17q); +19] subtype (23). On the basis of these observations, it is reasonable to perform bone marrow cytogenetics at the time of blast crisis in CML.

However, only standard cytogenetics enable the detection of additional cytogenetic abnormalities (+8, i(17q), additional Philadelphia chromosome) which, when found at the time of diagnosis, might predict a shorter duration of the chronic phase of the disease or be a component of defining an accelerated phase (24). Furthermore, baseline karyotype information is important in interpreting subsequent clonal evolution, emergence of new cytogenetically abnormal Philadelphia chromosome-negative clones, and in cytogenetic monitoring of treatment effect.

We found clonal chromosome aberrations in 5 Ph chromosome negative cases during drug therapy with Hydroxyurea or Interferon alfa. In all of them chromosome abnormalities were numerical aberrations (hyperdiploidy).

Clonal chromosome aberrations in Ph chromosome negative cells were also described in different reports (6,17). This relatively new finding was observed during cytogenetic monitoring of CML patients treated also with Imatinib. Patients have achieved cytogenetic response to the treatment, but unexpectedly other clonal chromosome aberrations were seen in Ph negative cells. The incidence of these other clonal aberrations is relatively low, being 2-15% of the Imatinib treated patients. Most frequently observed chromosome abnormalities are numerical aberrations, mainly -Y, +8 and -7. Structural changes are observed less frequently, out of which deletions of long arms of chromosomes 7 or 20 (7q-, 20q-) are more commonly seen. The mechanism of the formation of aberrant Ph negative clones is not clear. In small proportion of patients the presence of Ph negative clone has been shown in samples preceding treatment.

Conventional cytogenetics is irreplaceable in detecting clonal evolution in Ph positive cells or other clonal abnormalities in Ph negative hematopoiesis.

## Conclusion

Performing classic cytogenetics both at diagnosis and during the course of the disease is important for detecting the presence of Philadelphia chromosome and/or the development of additional chromosome changes before and during therapy, with prognostic and, consequently, therapeutic implications.

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