

Monitoring M-BCR-ABL expression level in CML patients by RQ-PCR: experience of a single Center

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Abstract

Chronic myelogenous leukemia (CML) is characterized by the Philadelphia chromosome and the BCR-ABL fusion gene that encodes an abnormal tyrosine kinase. Development of specific tyrosine kinase inhibitors completely changed the management of these patients. **Materials and Methods:** Between April 2008 and July 2012, at the Molecular Biology Laboratory, University of Medicine and Pharmacy of Targu Mures, Romania, we monitored the M-BCR-ABL transcript level by real time quantitative PCR in case of 15 CML patients diagnosed at the Hematology and Transplant Center of Targu Mures. **Results:** Modification of M-BCR-ABL expression level shows statistically significant correlation ($p=0.013$) with the clinical course of these patients. **Conclusions:** Molecular biology techniques have an important role in monitoring CML patients and regular analysis is recommended.

Keywords: chronic myelogenous leukemia, real time quantitative PCR, BCR-ABL oncogene, Imatinib.

Introduction

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by three phases: begins with an indolent chronic phase (CP) that can last for years but finally progresses into a phase similar to acute leukemia (named blast phase-BP) directly or *via* an accelerated phase (AP). CML is the first human neoplasm with clarified genetic background: the Philadelphia (Ph) chromosome, result of reciprocal translocation between chromosome 9 and 22: t(9;22)(q34;q11). This translocation generates the BCR-ABL fusion gene encoding an abnormal tyrosine kinase [1, 2].

Detection of the Ph chromosome and/or the BCR-ABL oncogene has become an important diagnostic approach. The Ph chromosome can be identified by conventional cytogenetic analysis (metaphase chromosomes Giemsa banding) or fluorescence *in situ* hybridization. The molecular diagnosis of CML is based on identifying the BCR-ABL gene, most commonly by qualitative and/or quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) [3].

For many years, the treatment of CML consisted of cytarabine (Hydroxyurea, Cytarabine) or immunomodulatory (alpha-Interferon) therapy. The discovery of targeted tyrosine kinase inhibitors (TKI): Imatinib

(Gleevec/Glivec), Dasatinib, Nilotinib and Bosutinib completely changed the approach of CML patients. Currently, Imatinib mesylate represents the gold standard medicine for chronic phase CML. The newer tyrosine kinase inhibitors (Nilotinib, Dasatinib, Bosutinib) have showed improved efficacy over Imatinib and provide an effective option for patients with resistance or intolerance to Imatinib [4–6]. The only known curative therapy for CML is allogeneic bone marrow transplant. However, the difficulty in finding suitable donors, the possibility of transplant related risks and good results of tyrosine kinase inhibitor therapy limits its use [7].

For optimal coordination of CML patients' management, scientists across Europe initiated the *European LeukemiaNet* project to standardize the diagnostic and therapeutic procedures [8].

Materials and Methods

Between April 2008 and July 2012, at the Molecular Biology Laboratory, University of Medicine and Pharmacy of Targu Mures, Romania, we monitored the M-BCR-ABL transcript level by real time quantitative PCR in case of 15 CML patients from the Hematology and Transplant Center of Targu Mures.

From one mL peripheral blood sample collected in

EDTA tube, we extracted RNA using QIAmp RNA Blood Mini Kit 50 (QIAGEN, Cat. No. 52304) according to manufacturer's instructions followed by the determination of RNA concentration and purity with NanoDrop spectrophotometer (ND-1000 Spectrophotometer V3.5). DNA transcription was accomplished using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat. No. 4374966) according to supplier's instructions.

To quantify the levels of M-BCR-ABL transcripts, we performed RQ-PCR analysis on ABI 7500 Real Time PCR instrument (Applied Biosystem) with 25 μ L final volume: 5 μ L cDNA and 20 μ L TaqMan Universal PCR Master Mix (Applied Biosystem). We investigated the b3-a2 and b2-a2 subtype of M-BCR-ABL fusion gene using the primers and protocols recommended by the *Europe Against Cancer Program* [9] performing relative quantification with the ABL gene as endogenous control [10]. All reactions were made in triplicate and we also utilized known positive and negative control samples. We also performed absolute quantification using *IPSOGEN FusionQuant Standards* for both ABL (Ref. No. CGRS-01) and BCR-ABL (Ref. No. FDRS-10) genes in accordance to international guidelines [11] (Figure 1).

The statistical analysis was carried out using GraphPad Instat3 version 3.6 program. To compare the results we used a two-tailed, unpaired Fischer test. We performed two survival analyses using the Kaplan–Meier test: one with the starting point the time of diagnosis and the other the time of initiation Imatinib therapy. The assigned event (CML related death during follow-up) and end-point (overall survival) was common.

Results

Between April 2008 and July 2012, we performed 64 RQ-PCR examinations in case of 15 CML patients (eight female and seven male patients) to follow the efficacy of treatment. According to received therapy, we grouped our patients in two main categories: medication-treated group (13 patients) and hematopoietic stem cell transplantation (HSCT) group (two patients).

Medication-treated group

Among the medication-treated group, only two patients benefited of RQ-PCR analysis at the moment of diagnosis, the rest of them have already been diagnosed by cytogenetic analysis and treated before the first determination of M-BCR-ABL transcript level.

Prior to Imatinib introduction, all the patients benefited of different drug therapy (Hydroxyurea, Interferon).

Seven patients in chronic phase presented undetectable

or decreasing oncogene expression values following Imatinib therapy (one case presented in Figure 2), while two chronic phase patients transcript level increased during treatment. The two accelerated and blast phase patients' BCR-ABL levels increased or remained stable. Summarizing the relation between clinical phase and molecular evolution, we found statistically significant correlation between the decreasing values of M-BCR-ABL and chronic phase as well as the stable or increasing BCR-ABL level and accelerated/blast phase ($p=0.013$) (Table 1).

Table 1 – The relationship between the clinical phase and BCR-ABL values among medication treated patients

Clinical phase	BCR-ABL mRNA expression level			P
	Decreasing	Stable	Increasing	
Chronic phase	7	0	2	0.013
Accelerated phase	0	1	1	
Blast phase	0	2	0	

Two CP patients showed a special course:

- one patient with high-risk Sokal score at the moment of diagnosis, after a slightly successful therapy, presents increased M-BCR-ABL values in the 4th year of Imatinib 400 mg therapy due to ceasing the medicine for three months without any response to reintroduction of the same TKI therapy (Figure 3A);

- the other patient after consecutive decreasing transcript level, at the last determination presents high M-BCR-ABL value after 43 months of Imatinib therapy without any symptoms and changes in peripheral blood (Figure 3B). Further investigations regarding this case are undergoing.

HSCT group

The HSCT group showed completely opposite outcomes: while one patient has been in complete hematologic and molecular remission with no immunosuppressive therapy during four years of follow up, the other presented early relapse and despite second-generation tyrosine kinase inhibitor therapy patient dies 13 months postHSCT from septic complications (Figure 3C).

By the cutoff date of this study, the three patients have died: the one after unsuccessful HSCT and two BP patients after resistance to ITK therapy. The estimated overall survival rate at 20 months after diagnosis was 93% and after 20 months of Imatinib treatment was 93%, while after 40 months was invariable: 79% and 77% (Figure 4).

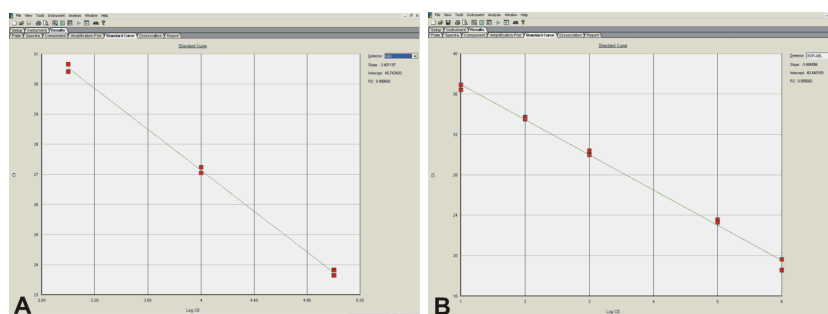


Figure 1 – Standard curves of absolute quantification: (A) ABL absolute quantification standard curve (slope -3.40; $r^2=0.998$); (B) M-BCR-ABL absolute quantification standard curve (slope -3.48; $r^2=0.995$).

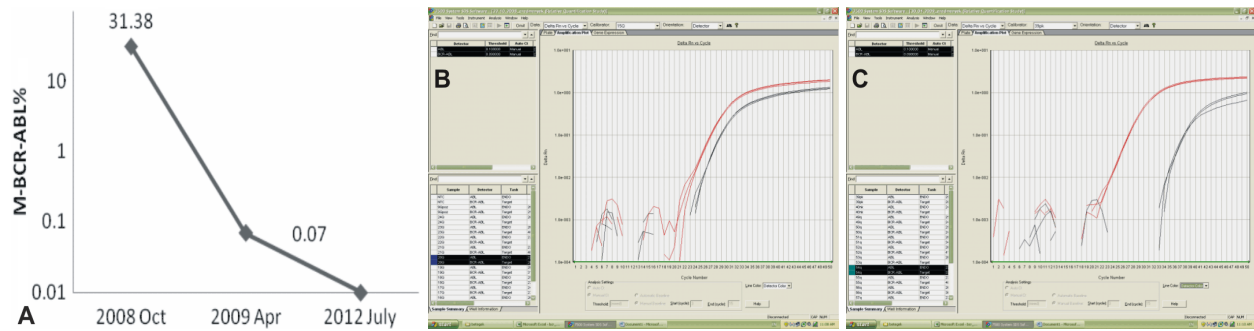


Figure 2 – Chronic phase patient's evolution under Imatinib treatment: (A) Female CP patient shows undetectable M-BCR-ABL expression level under Imatinib 400 mg therapy; relative quantification curves at three different moment of follow up: (B) 2008, October; (C) 2009, April; (D) 2012, July.



Figure 3 – Special cases: (A) Patient after ceasing Imatinib 400 mg for three months; (B) Patient's evolution under Imatinib 400 mg; (C) Patient relapse after hematopoietic stem cell transplantation (2008, September) and shows increasing values despite second generation TKI therapy.

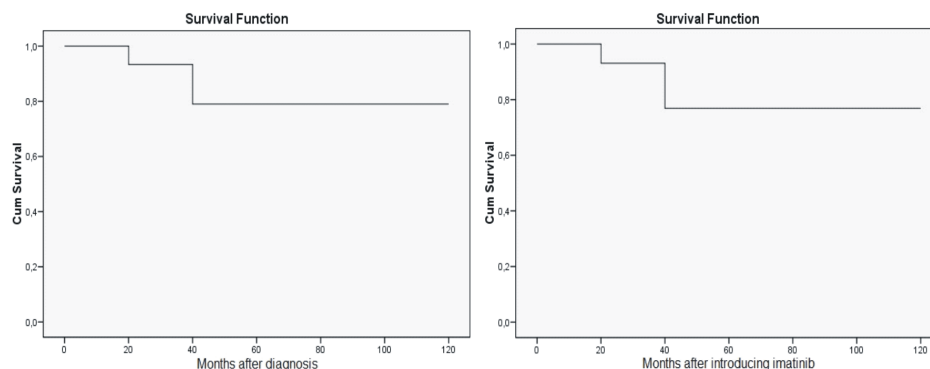


Figure 4 – Survival analysis.

Discussion

Due to the clarified genetic background and the amazing progress in the therapy of chronic myeloid leukemia, the management of CML patients has completely changed in the last years. Nowadays, there are multicenter studies based, well-developed guidelines for the supervision of these patients including the diagnostic approach, therapy recommendations and regular follow-ups.

In our Center, patient monitoring could not be conducted according to these guidelines because of multiple objective constraints: most CML patients were diagnosed before 2008, the implementation of RQ-PCR analysis at our unit, so molecular investigations could

not be carried out at the moment of diagnosis; the majority of patients did not received Imatinib as first-line therapy; cytogenetic investigations are deficient in our Center due to technical limitations; patients' residence being at significant distance from our Institute, monitoring was conducted mainly in territorial hospitals.

Due to these limitations, we cannot appreciate responses to treatment as recommended by the *European LeukemiaNet* project. We performed relative quantification for follow up these patients mainly because of these reasons (previously diagnosed, pretreated patients). Despite the fact that our Laboratory does not own a correction factor, and our results cannot be reported to the international scale, monitoring these patients is

correct and accurate, since the obtained values are reported to previous results and the analyses were performed in constant conditions. This statement is supported by the characteristics of standard curves from absolute quantification: our results (slope -3.18; $r^2=0.99$ in case of ABL; slope -3.4, $r^2=0.99$ in case of BCR-ABL) befit in quality criteria: a slope between -3.0 and -3.9 is acceptable as long as the $r^2>0.95$, but for accurate results $r^2>0.98$ is desirable [12, 13].

We have also been using this method to monitorize the evolution of M-BCR-ABL expression level in pediatric patients with CML as published previously [14].

In our study, modification of M-BCR-ABL expression level presents statistically significant correlation with the clinical course and treatment of these patients. While in patients in chronic phase treated with Imatinib we have found low levels of BCR-ABL, patients with accelerated or blastic phase treated with high-dose Imatinib (600 mg) or Dasatinib, the M-BCR-ABL values increased or remained at the same level. Effectiveness of Imatinib in these patients is lower, failing to obtain chronic phase or decreasing levels of BCR-ABL. This result is concordant with international guidelines recommendations [15].

One chronic phase patient treated with Imatinib 400 mg presents increasing BCR-ABL values after 43 months of therapy. According to IRIS (*International Randomized Study of Interferon and STI571*) study, the probability of occurrence of undesirable events is higher in the first three years of Imatinib therapy, after this period, the probability is only 0.9% [16].

Resistance to Imatinib most often is due to secondary mutations in the kinase domain (KD). More than 50 kD mutations were identified each of them providing different level of resistance to Imatinib and/or second generation tyrosine inhibitors. Identifying resistant cases early and switching to appropriate TKI constitutes an important aspect of patient monitoring. The main concern is when to perform mutational analyses: recent studies recommend the investigation in case of suboptimal response/failure to Imatinib; increase in BCR-ABL transcript levels leading to major molecular response loss or a 2.6 fold increase in mRNA level. During second generation, TKIs therapy the failure to obtain optimal hematologic or cytogenetic response, mutation analysis should be considered [17, 18]. In our follow-up, we observed this level of growth at two patients but mutation analysis will be accessible in Târgu Mureş only in the future.

A current aspect of CML patients follow-up is whether is indicated to stop Imatinib therapy when constant complete molecular response (undetectable transcript) is achieved. According to *Stop Imatinib* (STIM) study, Imatinib treatment was interrupted at patients who achieved complete molecular response and sustained for more than two years after Imatinib therapy. 60% of the patients relapsed after discontinuing the therapy within the first year, but after reintroduction of Imatinib the transcript level decreased. The only predictive aspect of

the relapse was the Sokal score: high-risk score patients relapsed more often than low risk patients [19, 20].

In our data, one patient interrupted the Imatinib therapy for three months (pregnancy planning) but the next M-BCR-ABL determination showed relapse, consequently the Imatinib therapy was reintroduced without any molecular improvement. This patient at the diagnosis was included in the high-risk group according to Sokal score [21].

Various studies (*IRIS*, *TARGET*) aim to determine survival rates in CML patients, but each of them enrolled only newly diagnosed CP-CML patients who received Imatinib as first line therapy. According to *TARGET* system data, the 639 Japanese patients after 90 months follow-up presented an overall survival rate of 95.1%, while patients included in *IRIS* study after 60 months had 89% overall survival rate, which increased to 95% after excluding the non-CML related death causes [22, 23].

Because the fact that we could only follow a small number of patients we enrolled in survival analyses all three phases CML patients: the death occurred only in case of BP patients, while CP and AP patients present 100% survival rate.

Examining the number of RQ-PCR analysis in case of each patient, we can observe absences. Whether this is caused by financial limitations, distance or simply ignorance is difficult to tell. But, proper compliance and adherence to treatment and regular monitoring are indispensable in achieving good results as shown in a study conducted in USA (patients with inadequate adherence fail to obtain major molecular response) [24].

☞ Conclusions

Effective management of patients with chronic myeloid leukemia requires a complex approach: the presence of specialists and technical background available to all patients.

Proper monitoring of CML patient is based on an interdisciplinary collaboration between hematologist/oncologist and laboratory workers.

Despite the absence of national cancer registry and the deficiency in providing prompt tyrosine kinase inhibitor therapy for newly diagnosed patients, we should strive to follow international recommendations as much as possible, and molecular biology techniques have an important role in achieving this aim.

RQ-PCR, a high sensitivity method is adequate for monitoring CML patients identifying even minimal values. Changes in oncogene's expression levels can be correlated with clinical phase (chronic phase BCR-ABL value decreases in most patients', and in other phases of evolution increases or remains stable). Because a rising transcript level is an early indication of poor response and suggests the need to reassess therapeutic strategy, regular molecular analysis is recommended.

Acknowledgments

We thank Dr. Joanne Mason, Dr. Susanna Akiki from West Midlands Regional Genetics Laboratory,

Birmingham Women's Hospital and Professor Letizia Foroni from Department of Hematology, Imperial College London, Hammersmith Hospital, for allowing to learn and practice this technique at their laboratory.

This study was partly supported by Bristol Myers Squibb Romania No Med. 20/21.04.2008, Bristol Myers Squibb Romania No Med. 127/04.06.2010, and Stichting Ondersteuning Medische Hulpverlening en Medisch Onderzoek Nederland grants.

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Received: October 18th, 2012

Accepted: January 15th, 2013