INTRODUCTION

Chronic Myeloid Leukemia (CML) is a disease characterized by uncontrolled proliferation in the bone marrow and accumulation of myeloid cells in blood.\(^1,2\) Breakpoint Cluster Region (BCR) gene on chromosome 22q11 and Abelson murine leukemia (ABL) gene on 9q34 fusion, t(9;22) (q34:q11) called the Philadelphia chromosome (Ph) is typical for CML and believed that it has the vital role in disease development.\(^2,3\) BCR-ABL chimeric protein encoded by fusion gene that leads to increase proliferation, resistance to apoptosis and genetic instability via deregulated tyrosine kinase activity.\(^6\) The fusion gene encodes 3 different sizes of proteins named as P190, P210, P230 depend on the fusion involve BCR exon parts and these proteins termed m-BCR, M-BCR, µ-BCR respectively. P210 protein is detected in 95% of patients.\(^7,8\) Subtypes of the P210 protein have been classified as b3a2 (55%), b2a2 (40%) and variants (5%).\(^3,9\) Standard diagnostic methods for CML included complete blood count, conventional cytogenetic, fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR).\(^4,10\)

Hematologic, cytogenetic and molecular response, evaluation of CML patients follow-up.\(^1\) In addition to standard detection of Philadelphia chromosome, so far FISH and conventional karyotyping in bone marrow have been used for definition of addition cytogenetic abnormalities that standard primers of RQ-PCR could not be determined.\(^11-13\) Philadelphia chromosome burden detected by these assays show the therapeutic responses of patients, according to these rates complete cytogenetic response and major molecular response termed respectively detected rate of 1% and 0.1%.\(^14,15\) During the treatment it is recommended to use cytogenetic studies in bone marrow, in 3rd, 6th and 12th months, then once in a 12 months when a complete cytogenetic response was reached and RQ-PCR must be used once in 3 - 6 months when a major molecular response was achieved.\(^4,12\) Additionally, molecular determination of BCR-ABL burden is important for treatment with tyrosine kinase inhibitors because the treatment will be changed if BCR-ABL burden is still over 10% and /or Ph chromosome 35% after 6 months.\(^16,17\) Nevertheless, RQ-PCR has been most sensitive molecular methods for determination of chimeric BCR-ABL mRNA transcript level in CML patients.\(^4\) However, FISH can be used to confirm diagnosis when cytogenetic in bone marrow is inappropriate and it can be used together with cytogenetic and RQ-PCR but is not preferred to use alone.\(^10,13\) There is a need to use noninvasive or at least less use of invasive methods in

ABSTRACT

Objective: To determine the use of the Quantitative Real Time PCR (RQ-PCR) assay follow-up with Chronic Myeloid Leukemia (CML) patients.

Study Design: Cross-sectional observational.

Place and Duration of Study: Izmir Ataturk Education and Research Hospital, Izmir, Turkey, from 2009 to 2013.

Methodology: Cytogenetic, FISH, RQ-PCR test results from 177 CML patients' materials selected between 2009 - 2013 years was set up for comparison analysis. Statistical analysis was performed to compare between FISH, karyotype and RQ-PCR results of the patients. Karyotyping and FISH specificity and sensitivity rates determined by ROC analysis compared with RQ-PCR results. Chi-square test was used to compare test failure rates.

Results: Sensitivity and specificity values were determined for karyotyping 17.6 - 98% (p=0.118, p > 0.05) and for FISH 22.5 - 96% (p=0.064, p > 0.05) respectively. FISH sensitivity was slightly higher than karyotyping but there was calculated a strong correlation between them (p < 0.001). RQ-PCR test failure rate did not correlate with other two tests (p > 0.05); however, karyotyping and FISH test failure rate was statistically significant (p < 0.001).

Conclusion: Besides, the situation needed for karyotype analysis, RQ-PCR assay can be used alone in the follow-up of CML disease.

Key Words: Chronic myeloid leukemia. Sensitivity. Quantitative real time PCR. Karyotyping.
follow-up CML patients because of long and exhausting process of monitoring and treatment of the disease. Besides avoidance of invasive procedures, use of right test at the right time affects the costs of diagnosis and treatment of the disease.14 The aim of the study was to investigate RQ-PCR assay to exclusively follow-up the patients by comparing with conventional cytogenetic and FISH based on the patient material at any stage of CML disease.

**METHODOLOGY**

The study design was cross-sectional and data analyzed from the patients, between 2009 - 2013. The inclusion criteria for this study were older than 18 years of age, diagnosed with CML and all results available from the researchers’ database. Samples were excluded from study if all test material was not gathered at the same time and results were not from the authors’ database. Karyotyping, FISH, RQ-PCR results of 177 CML patients’ samples were evaluated according to inclusion and exclusion criteria.

Bone marrow aspirate materials were evaluated at the genetic laboratories for conventional cytogenetic and FISH diagnosis. Conventional cytogenetic studies were carried out by scanning at least 20 metaphases. FISH assays were performed with commercial probes for t(9;22) (q34;q11.2) fusion and scanned at least 200 interphase cells. Bone marrow aspirate material and peripheral blood samples were taken at the same time and, unlike cytogenetic and FISH assays, RQ-PCR test assay were performed in the laboratory.

RQ-PCR method used for qualitative and quantitative analysis of BCR-ABL (b3a2/b2a2) fusion transcripts. RNA isolation from peripheral blood were performed with QiAamp RNA Blood Mini (Qiagen Inc. Valencia, CA.) Kit. RNA quality was analyzed by spectrophotometry in the range of 260 - 280 nm. RT-DX kit (Ipsogen- France), RT kit (Qiagen, USA) kits were used for cDNA synthesis respectively between 2009 - 2011 and 2011 - 2013 dates, BCR-ABL Mbcr Fusion Quant Kit (Ipsogen-Fransa), BCR-ABL1 Mbcr IS-MMR Kit (Qiagen-ABD) kits were used respectively for the same periods. PCR amplification and product analysis were performed on RG-6000 real-time instrument (Corbett Research-Australia). PCR conditions were performed according to the manufacturer’s recommendations. Software Rotor Gene Q series 1.7 was used for qualitative and quantitative analysis.

All statistical analyses were performed on SPSS (Statistical Package for Social Science Inc. Chicago, USA) version 10.0 statistical software. Receiver Operating Characteristic (ROC) curves were drawn to calculate the sensitivity of the three assays, and compared with each other. Conventional cytogenetic and FISH assay results of the specificity and sensitivity were calculated by comparison with the RQ-PCR results considered as the gold standard. The Pearson chi-square test was used to compare test failure rates for karyotyping, FISH and RQ-PCR test assays. The differences were considered to be not statistically significant when the p-value was less than 0.05. Approval was obtained from the Ethics Committee (Ege University Clinical Researches Ethics Committee, 2014, number: 14 - 4.2/15).

**RESULTS**

Test results were classified as positive, negative and test failure (Table I). ROC analysis was used for investigating relations between positive, negative results of the three methods. Sensitivity and specificity values of FISH and karyotyping were calculated by comparing with RQ-PCR results and karyotyping 17.6 - 98% (p=0.118, p > 0.05), FISH 22.5 - 96% (p=0.064, p > 0.05, Figure 1) calculated respectively. Sensitivity differences were not statistically significant (p > 0.05). FISH sensitivity was slightly higher than karyotyping but there was a correlation calculated between them (p < 0.001, Figure 2). Karyotyping sensitivity was determined as the lowest test in the study and compatible with all karyotyping positive samples had been determined positive in FISH and RQ-PCR results too. Moreover, nearly all RQ-PCR negative samples were determined negative by karyotyping and FISH tests, and compatible with this findings specificities were close to 100% determined for these tests.

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive n (%)</th>
<th>Negative n (%)</th>
<th>Test failure n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karyotype</td>
<td>19/177 (10.73)</td>
<td>137/177 (77.40)</td>
<td>21/177 (11.86)</td>
</tr>
<tr>
<td>FISH</td>
<td>29/177 (16.38)</td>
<td>140/177 (79.09)</td>
<td>8/177 (4.51)</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>116/177 (65.53)</td>
<td>58/177 (32.76)</td>
<td>3/177 (1.69)</td>
</tr>
</tbody>
</table>

Figure 1: ROC curve graphic of karyotype and FISH test's sensitivity and specificity compared with the reference.

Karyotype area: 0.578 (p=0.118)
FISH area: 0.593 (p=0.064)
Reference: RQ-PCR results
RQ-PCR test failure rate differences between karyotyping/FISH assays was not statistically significant (p > 0.05). However, there was a correlation between karyotyping and FISH for test failure rate differences (p < 0.001, Table II).

**DISCUSSION**

At present close monitoring of CML patients has a vital role of the disease management. So far conventional cytogenetic, FISH, and RQ-PCR tests are used in CML patients for diagnosis and follow response to treatment in the current approach but each of these tests have different sensitivity for detection and monitoring of Ph+ cells, furthermore, sensitivity was measured 1 - 5% for karyotyping, 0.1 - 3 % for FISH and 0.01% or fewer for RQ-PCR in literature.8-10 Moreover, several studies have shown that leukemia cells can be found in the circulation when complete cytogenetic response is achieved.10,11,18 Thus, PCR has become an indispensable for follow-up of minimal residual disease.14

This study has revealed that karyotyping and FISH sensitivities did not correlate with RQ-PCR. In accordance with the information above, the present study showed that RQ-PCR determined positive the patients who were determined negative by the other two tests via high sensitivity rate of RQ-PCR. Similar success could not be attained for the conventional cytogenetic outcomes. Although 75% sensitivity and 100% specificity values had been shown in literature.19 This study revealed only 17.6% sensitivity and 98% specificity. Apart from the specificity, the sensitivity value was markedly lower than reported in literature. In addition to this, there is no statistical differences between FISH and conventional karyotyping tests (p < 0.001) consistent with the literature.20 Moreover, FISH sensitivity was calculated to be 22.5% and found to be significantly lower than RQ-PCR (p > 0.05). Low in vitro mitotic activity of cancer cells, transport of materials, laboratory practitioners qualifications and laboratory conditions were possible explanations for the conventional cytogenetic and FISH test sensitivities being lower compared to literature. It was found that the calculation and analysis of identifying real negative patients were more successful compared with identifying real positive patients based on RQ-PCR values for conventional cytogenetic and FISH assays. These differences between real positive and false negative results supports standardization in conventional cytogenetic and FISH tests for laboratories is difficult and differences can be seen between laboratory observations. Apart from standardization, there were 3 - 10% false positive rate for FISH2 and 1 - 35% false negative rate for conventional cytogenetics tests21 in the literature.

The present findings suggest that FISH and karyotyping assays were insufficient compared with RQ-PCR when only aimed to determine Ph+ cells burden. FISH and karyotype methods alone are inadequate for follow-up of patients for the reasons stated above.

| Table II: The chi-square test for association between test failure rates. |
|-----------------------------|-----------------|-----------------|-----------------|------------------|
|                             | Karyotype       | FISH            | Total           | p    |
|                             | n   | %   | n   | %   | n   | %   |     |
| Ineligible                  | 0   | 0.0%| 8   | 4.6%| 8   | 4.5%| 1.00 |
| Eligible                    | 3   | 100.0%| 153 | 95.4%| 156 | 95.5%| 1.00 |
| Total                       | 3   | 1.7%| 174 | 98.3%| 177 | 100.0%|     |

Ineligible = Test failure; Eligible = Test is not failure

RQ-PCR test failure rate differences between karyotyping/FISH assays was not statistically significant (p > 0.05). However, there was a correlation between karyotyping and FISH for test failure rate differences (p < 0.001, Table II).
Furthermore, in the present study, RQ-PCR had a prominent success compared with test failure rates, statistically did not show a relation between the two tests ($p > 0.05$). On the contrary, karyotype and FISH failure rates were statistically concordant with each other ($p < 0.05$). In literature, the test failure rates were stated to be 10 - 25% for karyotyping, and 5 - 6% for FISH. The present findings were consistent with it, determining 4.5% and 11.9% respectively.

In addition to the test failure rates, karyotype and FISH assay processes distinctively take longer time and need more work hours compared with the RQ-PCR assay. Another advantage of RQ-PCR test assay other than to be obtained quickly and high precision determination of Ph+ cell rates, was that the commercial kits include International Scoring (IS) assay's made standardization fairly easy compared to the conventional cytogenetic and FISH tests for laboratories.

Even with all advantages of RQ-PCR, this method could not determine the additional cytogenetic abnormalities at diagnosis or that may occur during the treatment process that conventional cytogenetic method could detect. There were a number of conflicts about the additional cytogenetic abnormalities affect of the disease: a difference has not been shown between CML patients with classic variant $t(9;22)$ on the cytogenetic and molecular response or on prognosis. On the contrary, major route additional chromosomal aberrations (second Philadelphia chromosome, trisomy 8, isochromosome 17q or trisomy 19) at diagnosis had been found associated with decreases at survival and progression to the accelerated phase and blast crisis. Recently, Baccarani et al. showed that 17% of the Ph negative patients under imatinib treatment had developed clonal cytogenetic abnormalities, but shown the only clinical impact for chromosome seven involvement. Otero et al. showed no differences in cytogenetic response or survival between only Ph-positive patients and patients with additional chromosomal abnormalities.

Although there is existence of different literature findings about the role of additional chromosomal abnormalities in a survey of the disease, investigation of these abnormalities is part of follow-up of the patients so far. Unfortunately, changes in the qualitative RQ-PCR may indicate the development of additional cytogenetic abnormalities.

Detection and prediction of additional cytogenetic abnormalities by RQ-PCR was not a valid substitute for conventional cytogenetic examination, at least for the time being. For these reasons, conventional cytogenetic assay recommended in the early stage of imatinib treatment for additional cytogenetic abnormalities may develop during the treatment which are impossible to determine by RQ-PCR. Studies can be conducted on the possible relationship between the clonal abnormalities and changes of quantitative results or international scale values for CML patients will provide scientific literature and the related data.

**CONCLUSION**

The detection rate and sensitivity of RQ-PCR were significantly higher than karyotyping and FISH assays in this study; nevertheless, conventional cytogenetic method still seems indispensable. In the light of the above findings, the authors recommended that besides karyotype analysis, RQ-PCR assay can be used alone in the follow-up of CML disease. New and future developments in the isolated use of the RQ-PCR assay may reduce the frequency of invasive bone marrow aspiration and the cost of long-term follow-up for these patients.

**REFERENCES**


