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ORIGINAL ARTICLE

Analysis of Expression of SIRT1 Gene in Patients with Chronic Myeloid Leukemia Resistant to Imatinib Mesylate

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ABSTRACT

Background: Chronic myeloid leukemia is a clonal myeloproliferative disease which is characterized by bcr/abl translocation. With the emergence of tyrosine kinase inhibitors such as imatinib mesylate, significant improvement has been made in treatment of this disease. However, drug resistance against this medicine is still an obstacle. SIRT1 is a gene with deacetylase activity which has been detected to have increased expression in many cancers. We aimed to determine if SIRT1 expression could play a role in the emergence of drug resistance in patients with chronic myeloid leukemia being treated with imatinib mesylate.
Methods: 48 patients with chronic myeloid leukemia referred to Dr. Shariati Hospital, Tehran, Iran, were studied. A venous blood sample of patients was collected, RNA was extracted and then cDNA were synthesized. SIRT1 gene expression was done by real-time PCR. The ratio of SIRT1 expression to ABL control gene was calculated. After calculation of CT for target gene and control gene, ΔCT was calculated. The results of SIRT1 expression levels in patients with chronic phase of CML were compared with that of the control group.
Results: 48 patients with chronic myeloid leukemia aged 15-64 years (mean: 40 years) were enrolled. 59% of the participants were men. The highest and lowest mean BCR-ABL expressions in drug-resistant patients were 1% and 57%, respectively. The results of analyzing the value of ΔCT for SIRT1 gene revealed that patients who were drug-resistant to imatinib mesylate had a lower value of ΔCT for SIRT1 than those who were not drug-resistant ($P<0.05$).
Conclusion: SIRT1 gene expression in patients resistant to imatinib mesylate was significantly higher than patients who were not drug-resistant.

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Introduction

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disease that is characterized by presence of BCR-ABL fusion gene. This oncogene itself results from chromosomal translocation between chromosomes 9 and 22, and the resulting chromosome is known as the Philadelphia chromosome.[1,2]

This disease is a myeloproliferative neoplasm which involves erythroid and myeloid cell lines. The average age at diagnosis is 53 years but all age groups could be affected. CML begins as a chronic disease which eventually may spread to accelerated phases.[3-5]

At present remarkable advances in the treatment of many cancers have been made; however, cases such as drug resistant CML and cancer progression towards advanced stages are still considered as unresolved issues.[6,7] Drug resistance and disease progression are still unsolved concerns in treatment of patients with CML. Introduction of tyrosine kinas inhibitors made a fundamental progress in treatment of CML. Over 70% of patients in the chronic phase of the disease gained complete cytogenetic response; hence, partial remission was acquired after treatment with tyrosine kinase inhibitors.[8] Despite the relative success of tyrosine kinas inhibitors in controlling

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the disease, they failed in eradication of the disease at the level of stem cells. This has led to increased threat of drug resistance and relapse.[9]

Imatinib mesylate is a tyrosine kinase inhibitor that is designed for treatment of malignant CML clones. However, primary and secondary drug resistance to the imatinib reduces the effectiveness of this drug.[10] Mechanisms of drug resistance in CML are unknown. Treatment by this drug is able to create complete cytogenetic remission in the chronic phase of the disease, but not capable of eliciting cytogenetic response in blastic crisis.[11,12]

SIRT1 functions as a deacetylase and its gene is expressed in many malignancies. The activity of the promoter of this gene in CML cells leads to survival and proliferation of these cells. This action is related to multi deacetylase characteristics of SIRT1 gene product.[13] Imatinib mesylate relatively reduces the SIRT1 expression outside the laboratory. The activity of this gene may play a role in keeping CML cells alive for chemical treatment.[14]

In hematopoietic progenitor cells whose BCR-ABL are active, SIRT1 expression can be observed actively as well. Further studies on the expression of this gene may be required to find a therapeutic target for drug resistance in cancers.[15] Excessive increase of SIRT1 product in primary tumors and hematological malignancies has been detected. Inactivation of this gene inhibits the growth of cancer cells and hence increases apoptosis in these cell lines.[16,17]

In the present study, the correlation between SIRT1 gene expression and drug resistance in patients who received imatinib mesylate will be analyzed. Furthermore, the correlation between the patients' response to this medication and pattern of gene expression will be discussed.

Patients and Methods

This study was conducted in the Research Center of Blood and Stem Cell Transplantation of Dr. Shariati Hospital in Tehran, Iran, to investigate the relationship between the expression of SIRT1 gene and drug resistance in patients with CML. 48 individuals were enrolled into the study. Moreover, 10 samples of bone marrow of healthy individuals were used as the control group. All participants in the study gave their written informed consent and the authors did their best to keep the the participants' data confidential.

Patients' RNA was extracted using TRIZOL kit (Sigma, USA). Subsequently, cDNA synthesis was performed using primers of Random Hexamer. SIRT1 gene expression analysis on patients' samples and control group was done using real time-PCR method. The results of SIRT1 expression levels in patients with chronic phase of CML were compared with that of the control group. Data were analyzed by SPSS software, version 18. The data about different phases of the disease and the patients' response to treatment were collected from the patients' medical records.

The result of this technique was reported by calculating the ratio of SIRT1 target gene expression to ABL control gene as a relative quantitative measurement. For Statistical analysis of the data in Light Cycler, the software program called "Second Derivative Maximum Method" was used. The method automatically determines cross point of each sample. In this method, the ABL gene was used as control gene. Since the result should have been relative quantitative, the ratio of SIRT1 expression to ABL control gene was calculated. After calculating CT for target gene and control gene, ΔCT was calculated.

Required primers for SIRT1 and ABL genes were designed. Real-time PCR test was conducted in 45 cycles on control group and the patients by using thermos Light cycler (Roche Diagnostics, Mannheim, Germany) and SYBR® Premix Ex Taq™ (Perfect Real Time) kit. In order to evaluate the changes in gene expression, $2^{(-\Delta\Delta Ct)}$ was calculated after estimation of CT for ABL gene expression as well as SIRT1 gene expression in samples obtained from the patients and the control group. Then, by use of formulas 1-1 to 1-4 and Excel software. $2^{(-\Delta\Delta Ct)}$was calculated for the changes of SIRT1 gene expression in two groups of drug-resistant (or Not Achieved MMR) and drug-sensitive (or Achieved MMR). The criteria for drug-resistance to imatinibmesylate was complete molecular response or (major molecular response) after 18 months of treatment with Imatinib based on the National Comprehensive Cancer network (NCCN) and European Leukemia Net (ELN) guidelines.[18] Furthermore, in order to define the criteria for complete molecular response in patients, the formula of BCR-ABL to ABL expression level was used. If this ratio is lower than 1%, the patients will be considered as appropriate responders. Likewise, this ratio must be re-calculated after 18 months of treatment.[19]

Formulas 1-1 to 1-4 were used to calculate the changes of gene expression, as follows:

Formula 1-1

$$\Delta CT_{\text{target gene in treated sample}} = CT_{\text{target gene in treated sample}} - CT_{\text{foot house in treated sample}}$$

Formula 2-1

$$\Delta CT_{\text{untreated sample}} = CT_{\text{target gene in untreated sample}} - CT_{\text{foot house gene in untreated sample}}$$

Formula 3-1

$$\Delta\Delta CT_{\text{target gene in treated sample}} = \Delta CT_{\text{target gene in treated sample}} - \Delta CT_{\text{target gene in untreated sample}}$$

Formula 4.1

$2^{(-\Delta\Delta Ct)}$=The ratio of changes in gene expression in treated sample to untreated sample

Table 1 shows the primer sequence for two genes of SIRT1 and ABL.

Results

We found that all CML patients were in the chronic phase. The patients were all treated with imatinib mesylate. They were categorized into two groups of imatinib-sensitive and imatinib-resistent based on the patterns of treatment responses.

We studied 48 patients diagnosed with CML. The patients aged from 15-46 years, with a mean age of 40 years. 28 (59%) patients were men. The minimum and maximum length of treatment for the participants in the study was 2 and 8 years, respectively. The average BCR/ABL gene expression in patients resistant to imatinib was

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Table 1: Primer sequence for two genes of SIRT1 and ABL

Gene symbol	Sequence (5' to 3')	Primer	Size
ABL-F	TGGAGATAACACTCTAAGCATAACTAAAGG	Forward	Bp124
ABL-R	GATGTAGTTGCTTGGGACCCA	Reverse	
SIRT1	AGGATAGAGCCTCACATGCAA	Forward	Bp104
SIRT1	TCGAGGATCTGTGCCAATCATA	Reverse	

14%, while the minimum and maximum level of BCR/ABL expression in patients was 1 and 57%, respectively. 10 Patients showed complete molecular response to imatinib mesylate and this group was considered as sensitive to treatment. The expression of BCR/ABL in these patients was from less than 1% to undetectable levels.

Comparing the level of SIRT1 gene expression in the two drug-sensitive and drug- resistant groups showed that the amount of ΔCT was lower in the drug-resistant group than the drug- sensitive group. This shows lower expression of SIRT1 in the drug- sensitive group and reflects that SIRT1 expression in the drug-resistant group was significantly higher ($P<0.05$).

Figure 1 shows that in order to ensure the specificity of the real-time PCR products, melting curves were plotted after each working run. In all 48 samples, there was a peak in the melting curves which indicated the specificity of the real-time PCR products. This melting curve diagram shows the performance of SIRT1 and ABL primer. As shown, the curve of all products does show one peak.

In order to draw a standard curve, dilutions of 0.1, 0.01, and 0.001 were prepared from primers of SIRT1 and ABL. After conduction of the test, amplification curves were plotted (figures 2 and 3).

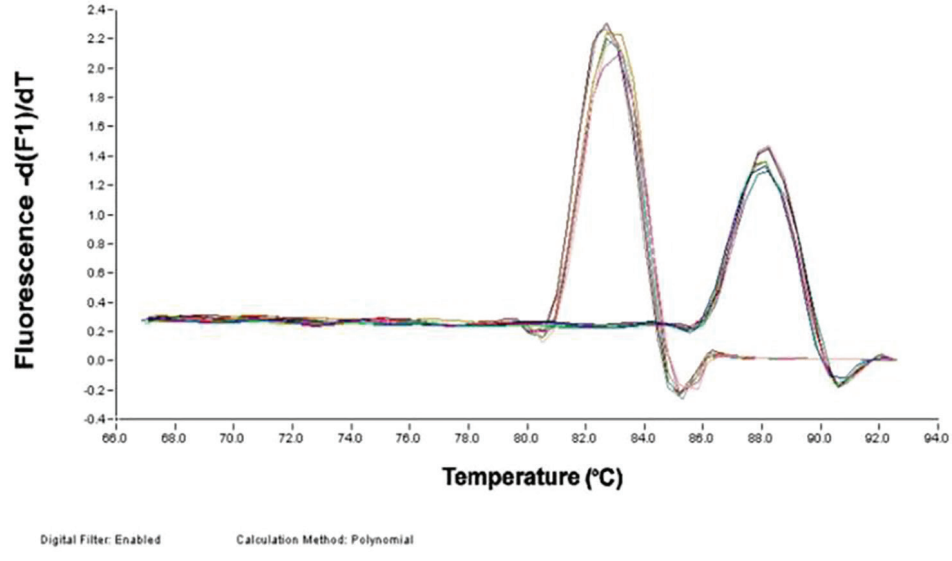


Figure 1: The melting curve of real-time PCR products

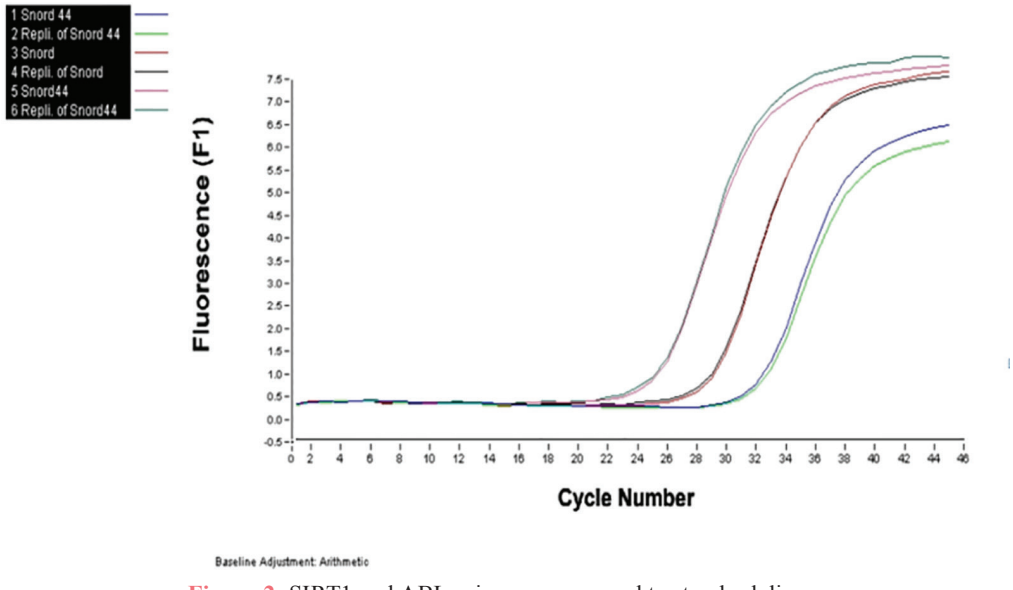


Figure 2: SIRT1 and ABL primers curve used to standard diagram

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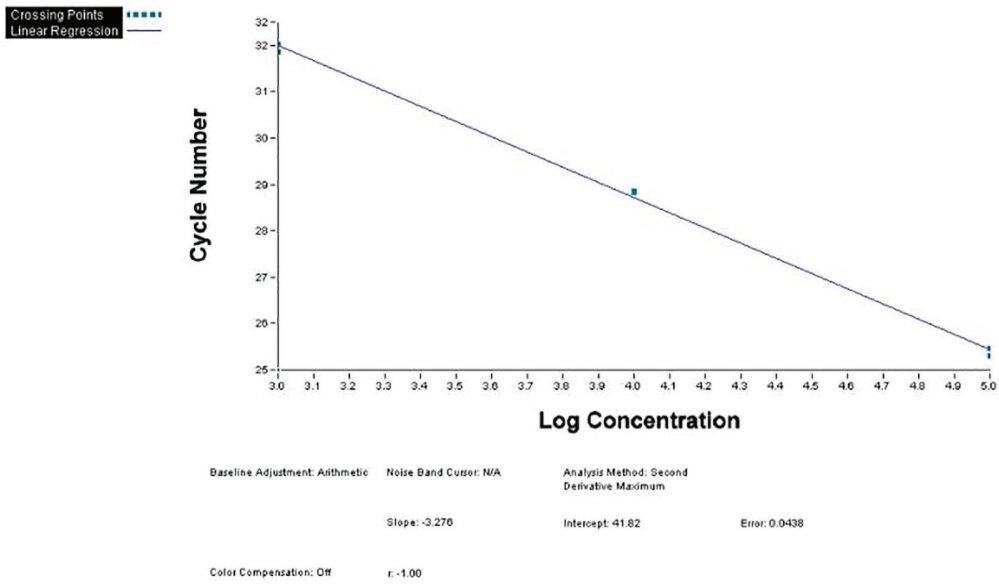


Figure 3: Standard curve for different concentrations of primers SIRT1 and ABL

For preparation of a standard diagram, the dilutions of 0.1, 0.01, and 0.001 from related cDNA to SIRT1 were prepared. The same steps were also taken for ABL primers and the curves were drawn. This step is highly important in preparation of standard diagram and optimization of the tests. After drawing the standard diagram, the slope of the diagram was calculated. Although the best range of the slope was between 3.5 and 3.6 and this slope was appropriate for calculation of the concentration of the sample, method was used in this experiment to analyze the changes in gene expression, because the slope of standard curve was equal to 3.3.

Figure 4 displays the amplification curve of real-time PCR products for primers of SIRT1 and ABL. The reactions took place in 45 cycles and the amplification curve was drawn by the machine software, based on number of cycles to intensity of fluorescence. After the end of reaction in each working run, the melting curve for aforementioned products was analyzed as well. At the end of each working run, the CT of the samples which were acquired by means of ABL and SIRT1 primers were written down.

The two groups were also compared in terms of achieving MMR based on ΔCT and the difference between the two groups was significant (figure 5).

Moreover, the two groups were compared according to ΔΔCT for SIRT1gene expression. The level of SIRT1 gene expression in the imatinib-resistant (who did not achieve MMR) group was significantly higher ($P<0.05$, figure 6).

Discussion

SIRT1 has an important role in resistance acquisition in cancers and there is an increased expression of this gene in many cancers. Previous studies showed that patients with increased expression of this gene are more likely to be resistant to many drugs, compared with patients with

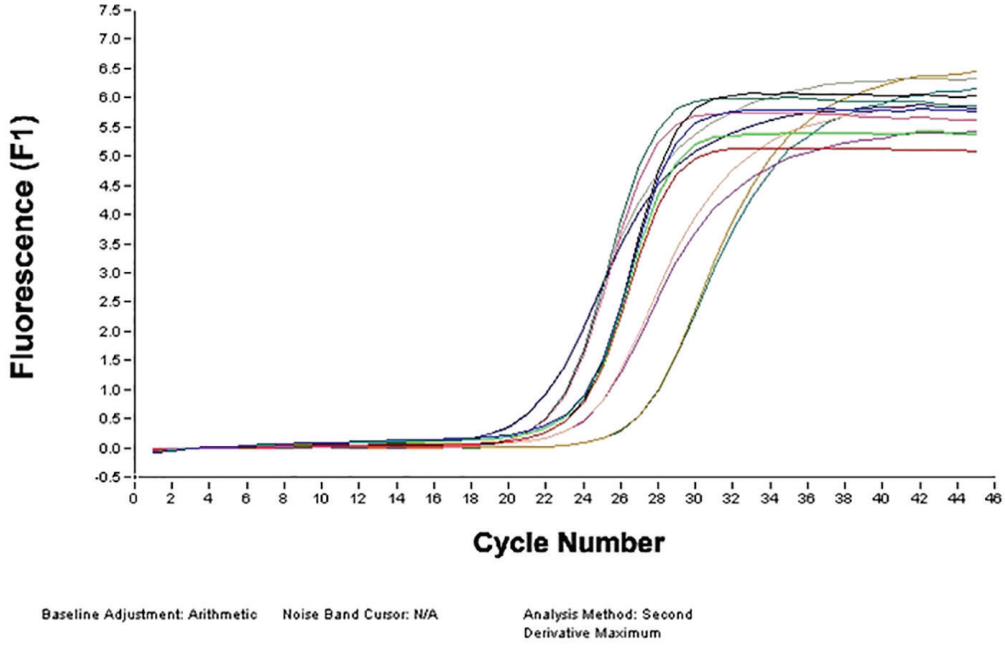


Figure 4: Amplification curve for real-time PCR products of samples obtained from the patients

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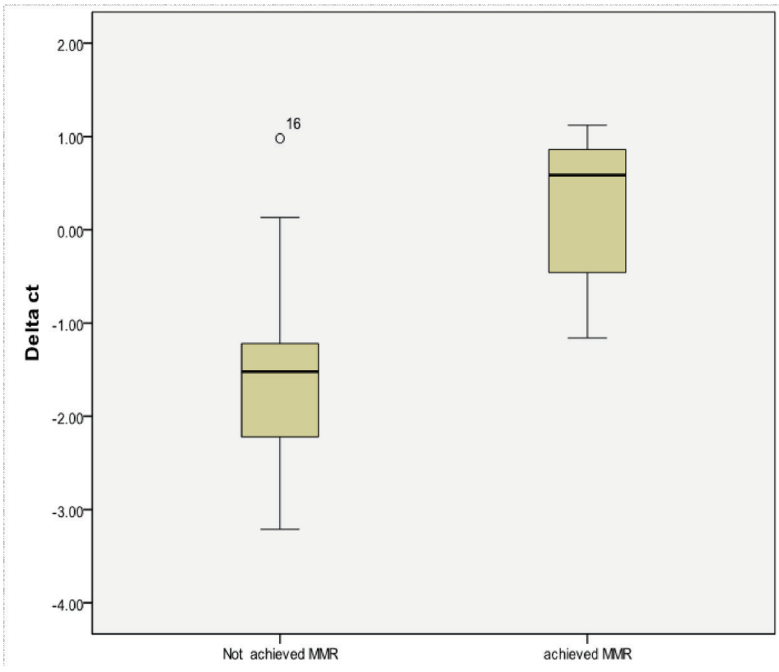


Figure 5: Comparison of two groups of patients in terms of MMR based on ΔCT (MMR= major molecular response)

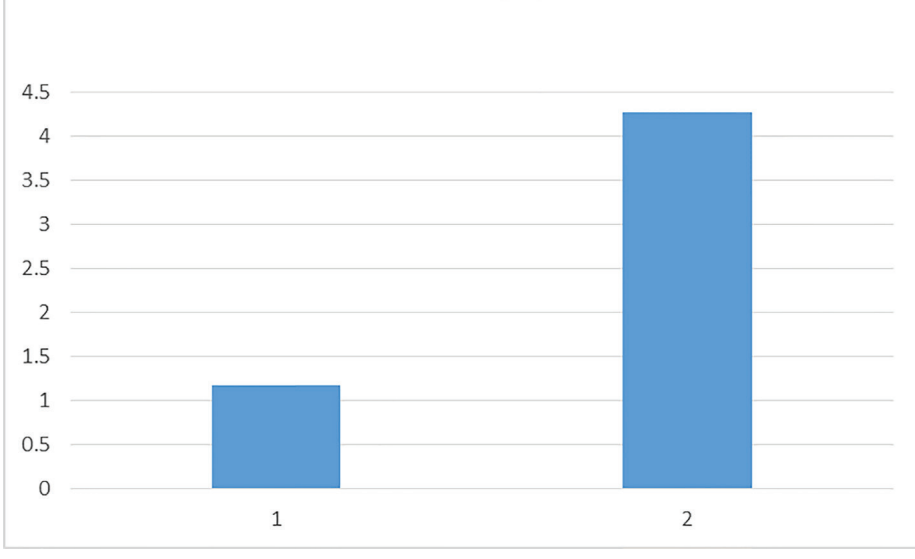


Figure 6: Comparison of the average SIRT1 gene expression in two groups of patients in terms of MMR based on ΔΔCT (1-Achived MMR; 2- Not Archived MMR)

a decreased expression of this gene.[16]

There was an increased expression of SIRT1 gene in samples from both primary chronic and blastic phases of CML cell lines. Increased expression of this gene results in acquisition of drug resistance in KCL-22 cell line.[13]

SIRT1 gene is related to different aspects of cancer such as size of the tumor and its method of development.[16] In the laboratory, blocking this gene leads to restraint of cell proliferation and cell migration. Moreover, blocking this gene leads to an increase in drug sensitivity. In animal studies the carcinogenicity and metastatic role of this gene has been confirmed. Expression of this gene has been observed in all mammalian cell lines. The product of this gene acts as a protein which plays its role in the cell nucleus. Furthermore, the expression of this gene is discussed as a factor in emergence of resistance against oxidative agents and programmed cell death. There is a controversy over the role of this gene in cancer. Increased expression of this gene has been reported in a variety of cancers, such as prostate cancer, leukemia and breast cancer. Meanwhile, there is also some role for this gene as an anti-tumor which is mentioned in some studies.[20,21]

SIRT1 gene expression is associated with advanced tumor stages and increases along with the severity of the disease. Consequently, the effects of this gene vary based on the cell type, stage of tumor progression and basic gene mutation. For example, increased expression of SIRT1 gene leads to drug resistance in liver cancers.[16]

Drug resistance in CML is in two forms; associated with the BCR-ABL and independent of BCR-ABL.[22] Role of SIRT1 in drug resistance is recently discussed in many cancers including cancer of liver, pancreas, and breast. Increased expression of this gene promotes proliferation of cancer cells and it seems this is specific to cancer cells.[23]

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Increased expression of this gene leads to up-regulation of MDR-1, a protein associated with drug resistance.[24] In some patients with CML, it was observed that this gene was responsible for deacetylation in proteins involved in DNA repair that resulted in mutations causing drug resistance. Expression of this gene not only influences over the mutation of BCR-ABL gene but also affects other genes in the carcinogenesis pathway.[25]

This study showed that imatinib-sensitive patients had a lower SIRT1 gene expression. Our results were consistent with the results of other studies on CML cell line. A distinct aspect of this study was assessment of expression of SIRT1 gene in a group of patients who were receiving imatinib mesylate for a couple of years; while previous studies were conducted on fewer patients or mostly focused on laboratory models and cell lines on culture environment.[1]

A similar study was conducted on patients afflicted with lung, liver, and prostate cancer. It was observed that patients who had an increase in SIRT1 gene expression did not respond to medications and were drug resistant.[26] Patients in our study were all in the chronic phase. SIRT1expression in a group of patients who were drug resistant had increased compared with the group who were sensitive to the drug. Likewise, in a study on KCL-22 cell line, removal of SIRT1 gene in cultivation environment led to better molecular response to treatment with imatinib mesylate.[13]

Conclusion

In our study on patients with CML, it was observed that patients with resistance to imatinib mesylate had a higher level of SIRT1 gene expression.

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Conflict of Interest: None declared.

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