ABSTRACT

Background

Chronic Myelogenous Leukemia (CML) is a clonal stem cell disease with deregulated tyrosine kinase activity of BCR-ABL. DNA methylation in gene promoters influences disease development, therapeutic response, and clinical outcome in various diseases.

Objectives

This study aimed to investigate the methylation pattern in promoter regions of the runt-related transcription factor 3 (RUNX3) gene and its effect on the response to imatinib therapy, among CML patients.

Patients and Methods

Blood samples were collected from 100 participants from November 2020 to February 2022, in two groups 75 samples of CML patients and 25 samples of healthy controls, genomic DNA was extracted from the samples by using the salting-out method, then Isolated DNA was treated with sodium bisulfite. By Methylation-Sensitive High-Resolution Melting (MS-HRM), the samples were analyzed.

Results

The promoter sequence methylation level is significantly higher (P<0.05) in DNA samples from CML patients than in the control group, the methylation level in most of the healthy control samples 96% was detected between 0-24% while in CML patients was 16%, in 25-49%, RUNX3 methylation levels in Health individuals were 4%, moreover in CML patients were 24% also, in 50-100% of methylation level, health control was 0%, in CML patient showed 60%. According to methylation detection of RUNX3 among Imatinib resistance and good response, the results of IM resistance were detected between 0-40% was 15.21%, Furthermore, in good response was 79.31%. In other hand at 41-100% RUNX3 methylation levels in IM resistance was 84.78%, while a good response was recorded at 20.68%.

Conclusion

The study concluded that RUNX3 gene hypermethylation often occurs in CML patients. Additionally, there is a strong correlation between RUNX3 hypermethylation and poor imatinib respondents. We found that both IM resistance and CML patients have higher levels of RUNX3 gene hypermethylation. Moreover, research on a larger sample size is required to validate these findings, which may serve as possible indicators of disease progression and imatinib resistance.

Keywords: Chronic Myeloid Leukemia, RUNX3, Imatinib, Epigenetics, Promoter hypermethylation

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INTRODUCTION

Chronic myeloid leukaemia (CML) is a well-known malignant condition of hematopoietic stem cells (HSCs), which leads to a marked proliferation of granulocytes in the blood (1). CML is caused by a reciprocal chromosomal translocation between chromosomes 9 and 22: t(9;22)(q34;q11), which leads to the formation of the Philadelphia (Ph) chromosome, generating BCR/ABL1 fusion (2). The product of the hybrid gene BCR-ABL is a chimeric protein with constitutive tyrosine kinase activity that phosphorylates target proteins to encourage the proliferation of hematopoietic stem and progenitor cells and a hematopoietic stem cell (HSC) is transformed into a leukemic stem cell (LSC) (3, 4). CML is a tri-phase disease with a chronic (CP), accelerated (AP, and lymphoid or myeloid blast phase (BP). CML often appears in the chronic phase (CP), which is defined by clonal proliferation of mature myeloid cells. All untreated patients will eventually move to a deadly blast phase (BP), which may be preceded by an expedited phase (AP) (4, 5).

In CML, the BCR-ABL1 mutation not only transforms the HSC to an LSC but also causes epigenetic reprogramming (6). The bulk of epigenetic changes happen during cell differentiation and are carried through several cell cycles (6). Transcription, replication, DNA alterations, histone variations and modifications, DNA repair, chromatin structure, and genome stability all rely on epigenetic processes (7). The most common event implicated in the pathogenesis of some human malignancies is DNA methylation of cytosine to 5-methylcytocine, primarily at CpG dinucleotides in gene promoter regions. Methylation, in particular, inactivates genes related to DNA repair, cell cycle regulation, and tumour suppressor genes in haematological malignancies, resulting in the accumulation of additional secondary mutations, a specific feature of disease progression (8, 9).

The Runt-Related Transcription Factor gene family (RUNX) consists of RUNX1, RUNX2, and RUNX3, each of which expresses differently in different tissues, their roles are cell context-dependent and connected to major developmental pathways (10). The RUNX3 gene located on chromosome lp36.11 encodes a transcription factor that is highly expressed in all hematopoietic stem cells (11). RUNX3 promoter hypermethylation caused transcriptional suppression of RUNX3, which was found in myeloid leukaemia cells, confirming RUNX3’s tumour-suppressive action and reductions in expression of RUNX3 may confer the fitness of normal and malignant stem cells in bone marrow with ageing (12).

Imatinib (Gleevec/STI-571, Novartis, Switzerland), a tyrosine kinase inhibitor, IM, Glivec, is the main treatment for CML patients and has significantly improved the therapy of CML. It is particularly effective for treating CP-CML but has inferior results in those with accelerated phase CML disease (13, 14).

Variability in disease development and therapy response (Imatinib) might be attributed to molecular events such as secondary mutations or/and any other chromosomal abnormalities, as well as CpG methylation patterns or histone modifications that occur following BCR/ABL1 fusion (15). Therefore, this study aims to investigate the methylation pattern in promoter regions of the RUNX3 gene and its effect on the response to imatinib therapy, among CML patients.

MATERIALS AND METHODS

Study population

Blood samples were collected from 100 participants from November 2020 to February 2022, in two groups 75 samples of CML patients and 25 samples of healthy controls, the samples were provided from the private clinic in Sulaymaniyah City/Iraq and Pasteur Institute in Tehran/Iran. All pertinent clinical and epidemiological data were recorded for both groups, and EDTA vacutainers were used to collect 5 ml of blood from each research participant.

DNA Extraction

Genomic DNA was extracted from EDTA peripheral blood using the salting-out method, and the nontoxic DNA extraction method extracts high-quality DNA from whole blood according to Miller, Polesky method (16). The concentration and purity of the extracted DNA were measured using a UV spectrophotometer (NanoDropTM 1000, Thermo Fisher Scientific, Waltham, MA, USA) to assess its quality. By measuring the absorbance at 260 nm (A260) and 280 nm (A280), the absorbance A260/A280 ratios were used to evaluate the DNA’s purity.
**Methylation Pattern of the RUNX3 Gene and its Impact on Response to...**

**Primer Design**

The MethPrimer Software free online tool was used for primer design. The Primer design for MS-HRM is essential and critical, so primers were designed based on criteria stated by Wojdacz (17) with some additional changes; the primer design was performed in gene promoter and exon 1. Primers are listed in (Table 1).

![Table 1. Primer sequences of RUNX3 used for MS-HRM amplification.]

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Annealing temperature</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUNX3 - MF</td>
<td>GACGAGGGAGGTGAGTAATTTTTTG</td>
<td>62° C</td>
<td>130</td>
</tr>
<tr>
<td>RUNX3 - MR</td>
<td>ACCGAACTCCCCAACTTACTAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M: Methylated, F: Forward, R: Reverse

**Bisulfite Conversion of DNA**

After extraction of genomic DNA, between 500 ng-2μg of the DNA (1000 ng) was subjected to bisulfite treatment. Bisulfite conversion was performed according to the respective kit protocols applied by EZ DNA Methylation Gold Kit (Catalog No: D5001, ZYMO Research, USA) following the manufacturer's recommendation. Besides the patient samples, universal methylated DNA and unmethylated DNA were converted with bisulfite, applying the same kit. Unmethylated cytosines (C) are deaminated to uracil (U) by the bisulfite reaction. Since methylated cytosines can resist the deamination process, they are protected during the conversion of bisulfite (18).

**Standards**

To mimic DNA samples with specific degrees of DNA methylation, Universal methylated DNA and unmethylated DNA (Qiagen, Germany) were used as 100% and 0% methylation DNA control, respectively, were combined in varied ratios (0%, 5%, and 10%, 25%, 50%, 75%, 100% methylated DNA). These standards were used to assess the assay's sensitivity and to estimate gene promoter methylation in clinical samples in a semi-quantitative manner.

**MS-HRM methylation analysis**

PCR amplification and MS-HRM analysis were performed according to Wojdacz et al., (17) using Step One plus a Real-Time PCR Detection System (Applied Biosystem, USA). The PCR conditions and annealing temperature were optimized, and after each run, immediate HRM analysis was performed. The PCR was performed in a volume total of 10μl containing 2μl of 5X master mix Evagreen (Solis body company), 0.4μl from each primer, 1μl bisulfite-treated DNA template and 6.2μl DNase-free water. Then the mixture was placed in Real real-time PCR machine and the amplification programs were configured as shown in (Table 2).

![Table 2. PCR Amplification and melting stage with HRM analysis steps for RUNX3 gene.]

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles number</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Amplification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>95° C</td>
<td>12 minutes</td>
<td>-</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95° C</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>62° C</td>
<td>60 seconds</td>
<td>40</td>
</tr>
<tr>
<td>Extension</td>
<td>72° C</td>
<td>35 seconds</td>
<td>-</td>
</tr>
<tr>
<td>Mutated PCR Amplification</td>
<td>95° C</td>
<td>10 seconds</td>
<td>-</td>
</tr>
<tr>
<td>Melting stage (HRM Analysis)</td>
<td>Heteroduplex formation</td>
<td>60°C – 95°C (In 0.3°C increments)</td>
<td>15 seconds</td>
</tr>
</tbody>
</table>

**STATISTICAL ANALYSIS**

The distributions of baseline features were compared for qualitative variables using χ 2 and Fisher exact test and for age as a quantitative variable using a t-test. ROC curve was generated from the numerical data to determine the optimal cut-off point of RUNX3 methylation levels in discriminating the IM good response and resistance CML patients. The numerical
data of RUNX3 methylation levels were divided into two groups according to the cut-off point calculated. A simple logistic regression test was performed to evaluate the relationship between RUNX3 promoter methylation levels and IM resistance and disease progression risk in CML patients by computation of Odds ratios (ORs) and 95% confidence intervals (CIs). Statistical analysis was performed using the R software (http://www.R-project.org). P values of < 0.05 were considered statistically significant.

RESULTS

Study Demographic

In the current study, 100 samples are included, of which 75 CML patient samples, 40 (53.33%) male and 35 (46.67%) female in addition, 25 samples from healthy controls 13 (52%) male and 12 (48%) female were included. CML patients comprised both the IM good response (n = 29) and IM resistant (n = 46) groups were recruited.

MS-HRM analysis of RUNX3 gene

The methylation level of each sample from the CML patient and control groups was determined by comparing the melting profiles of the PCR product and standards to a known ratio of methylated and unmethylated templates. Step one plus a Real-Time PCR Detection System was used for PCR amplification and MS-HRM analysis (Applied biosystem, USA). Step one plus Melt Analysis was used to examine the HRM data. Figure 1 shows the melting curves and difference plot of the RUNX3 gene obtained from standards and representative samples. According to the results of the MSHRM analysis for the RUNX3 gene, the promoter sequence methylation level is significantly higher (P<0.0001) in DNA samples from CML patients than in the control group, as shown in Table 3.

According to methylation detection of RUNX3 among CML patients and healthy controls, the results of healthy control were detected between 0-24% was 96% (24), while in CML patients was 16% (12). In 25-49%, RUNX3 methylation levels in healthy individuals were 4% (1), while in CML patients recorded 24% (18). Also, in 50-100% of methylation level, health control was 0% (0), in CML patients showed 60% (45). A highly significant difference was detected between the healthy control groups and the CML patients’ group (P<0.05). (Table 3) shows the methylation percentage frequencies of RUNX3 gene promoters in the healthy control group and CML patients.

RUNX3 methylation level and IM response among CML patients

Regarding to methylation detection of RUNX3 among Imatinib resistance and good response, the results of IM resistance were detected between 0-40% was 15.21%, while in good response was 79.31%. In 41-100% RUNX3 methylation levels in IM resistance were 84.78%, while a good response was recorded at 20.68%. A highly significant difference was detected between various methylation levels and response to IM therapy (P<0.05) as shown in (Table 4)

Figure 1. HRM curve for RUNX3 gene. A: comparison of Aligned melt curves Serial standard controls (0,10,25,50,75,100) with melt curve one patient sample with methylation percentage (range 50-75%). S: patient sample with methylation percent 50-75% (light blue line). B: Difference plot for RUNX3 gene in serial standard controls and one sample of patients with methylation percentage of 50-75%.
Methylation Pattern of the RUNX3 Gene and its Impact on Response to...

Table 3. Methylation percentage frequencies of RUNX3 gene in CML patients and healthy controls.

<table>
<thead>
<tr>
<th>RUNX3 methylation level (%)</th>
<th>Healthy controls (n=25)</th>
<th>CML Patients (n=75)</th>
<th>P–value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24</td>
<td>24</td>
<td>12</td>
<td>3.03e-12</td>
</tr>
<tr>
<td>25-49</td>
<td>1</td>
<td>18</td>
<td>0.0005572</td>
</tr>
<tr>
<td>50-100</td>
<td>0</td>
<td>45</td>
<td>6.031e-07</td>
</tr>
</tbody>
</table>

Table 4. Risk association RUNX3 methylation level and IM response among CML patients.

<table>
<thead>
<tr>
<th>RUNX3 methylation (%)</th>
<th>IM resistance (n=46)</th>
<th>Good response (n=29)</th>
<th>P value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-40</td>
<td>7</td>
<td>23</td>
<td>&lt;0.0001</td>
<td>21.35</td>
<td>6.39-71.33</td>
</tr>
<tr>
<td>41-100</td>
<td>39</td>
<td>6</td>
<td></td>
<td></td>
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</tbody>
</table>

IM (Imatinib), n (Number), OR (Odd ratio)

DISCUSSION

Methylation frequencies of the RUNX3 gene between healthy controls and CML patients

The runt-related transcription factor 3 gene (RUNX3), which is found on chromosome 1p36 has 2 promoters, 6 exons, and is around 67 kb in length. It is primarily a gene that regulates cell proliferation, growth, differentiation, angiogenesis, adhesion, and invasion also, has also been linked to cancer (1, 15). RUNX3 gene, a member of the transcription factor runt domain family, has recently attracted interest for its function in the development of tumors (19). Moreover, it was shown that 45% of human gastric cancer cells had hemizygous deletions that significantly decreased the expression of the human RUNX3 gene, and 60% of these cells had hypermethylation in the promoter region (13). There have also been reports of RUNX3 inactivation in other cancer types, including breast (20), lung (21, 22), pancreatic (23) and colorectal cancer (24), as a result of hypermethylation. However, the RUNX3 gene promoter has been reported to be hypermethylated in AML patients (25).

In the current study, High-resolution melting (HRM) analysis, a novel process for the investigation of promoter methylation, was employed to screen a sample with CML for promoter methylation of the RUNX3 gene, and we analyzed the methylation percentage difference between CML patients in different stages and healthy controls, and also between response groups, to find association of methylation status of RUNX3 gene with healthy control and CML patients, as well as relationship of CML patients and drug response with methylation level.

Our findings indicate that, as compared to healthy controls, CML patients have a considerable hypermethylation in the RUNX3 gene. Furthermore, compared to healthy controls, there were substantially more patients with hypermethylation of the promoter RUNX3 gene (25–49 and 50%–100% methylation level) in CML patients, our results show that by increasing methylation levels, the number of the number of patients with CML had increase.

This result is in agreement with the results of Mori et al., methylation of the studied gene was frequent in CML patients (26). However, recent findings differed from Uhm et al., results that did not find any promoter sequence methylation of the RUNX3 gene in 21 CML samples (27). We believe that this difference came from disease progression, level of methylation in CML groups, sample numbers, smoking state, and BCR-ABL transcript types.

Unfortunately, there has been little reported on the methylation status of RUNX3 in CML. Considering the relevance of this epigenetic inactivation of those genes in solid tumors and other hematological malignancies, it seems necessary to shed some light on this issue.

Methylation frequencies of RUNX3 and correlation between I.M. resistance and good response for IM

In our current research, we examined the methylation percentage difference between response and resistance groups of CML patients in order to determine the relationship between the methylation status of the RUNX3 gene and the patient’s response to treatment, significant differences were noted between the two groups.
Compared to patients who had a good response, our data indicate that CML patients have a significant hypermethylation throughout the examined gene for 41% cutoff point. Furthermore, the percentage of patients with hypermethylation of the RUNX3 promoter (41%–100% methylation level). Moreover, the risk of IM resistance in patients with hypermethylation 41-100%, 21.35-fold higher than the low category level (0-40%). Although the finding’s clinical significance is yet unknown, it could be connected to a mechanism underpinning the progression of the disease.

To the best of our knowledge, there are no prior reports on this correlation between RUNX3 promoter hypermethylation and resistance IM in CML patients. It has been shown that imatinib mesylate resistance is correlated with epigenetic changes, specifically increased gene promoter region hypermethylation (6,28, 29). Based on those reports, it is reasonable to suggest that promoter methylation of the RUNX3 gene might be a potential epigenetic mechanism in the BCR-ABL-independent pathway in promoting IM resistance among CML patients. So, the higher level means the greater resistance to IM.

In conclusion, the current study concluded that RUNX3 gene hypermethylation often occurs in CML patients. Additionally, there is a strong correlation between RUNX3 hypermethylation and poor imatinib responders. We found that both IM resistance and CML patients have higher levels of RUNX3 gene hypermethylation. Moreover, research on a larger sample size is required to validate these findings, which may serve as possible indicators of disease progression and imatinib resistance.

Abbreviations

CML, chronic myeloid leukemia; RUNX3, runt-related transcription factor 3 gene; IM, imatinib mesylate; OR, odds ratio; CI, confidence intervals; TKD, Tyrosine kinase domain; SD, standard deviation; MS-HRM, Methylation Sensitive High-Resolution Melt.

REFERENCES

Methylation Pattern of the RUNX3 Gene and its Impact on Response to...


