Biological and Clinical Implications of Exon 8 P53 (R282W) Gene Mutation in Relation to Development and Progression of Chronic Myeloid Leukaemia Patients in India Population

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Background: TP53, located on chromosome 17p13, is one of the most mutated genes affecting many types of human cancers. To establish an association between the incidence of exon 8 p53 (R282W) gene and progression of the disease in CML and also to correlate the presence of mutation with the clinicopathological features of the disease.

Methods: p53 status was investigated by studying mutations in the p53 gene at exon 8 region after confirming the diagnosis by BCR-ABL. 100 CML samples were analyzed using the Allele-Specific Oligonucleotide PCR assay. Mutations occurred in 58% of the cases in exon 8 codon 282 region of the p53 gene. C : T transitions occurred at a high frequency with a statistically significant result (p=0.03).

Results: Of the 100 clinically confirmed specimens, 58% tested positive for the mutation. Also, the mutation was found to be higher in the progressed stages (88.2% in accelerated phase and 60.0% in blast crisis) of CML compared to the chronic stage (35.2%). A statistically significant association (p=0.001) was found between the occurrence of p53 R282W mutation and the clinical phase of CML with chronic, accelerated and blast crisis phases. The mutation was detected in a vast majority (88.2%) of patients in the accelerated and the blast crisis phase indicating that this mutation might play a critical role in predicting the progression of disease in CML. Clinicopathological correlation with TLC, platelet count and the haematological response elicited a significant association with patients harboring the mutation with (p=0.01), (p=0.001) and (p=0.01) respectively.

Conclusion: Our study suggests that p53 mutations in the exon 8 region might have a strong influence on disease progression and poor response of imatinib (Tyrosine kinase inhibitor) in CML patients.

Keywords: Chronic myeloid leukemia (CML); BCR-ABL gene; CP-CML; BC-CML; AP-CML

Introduction

Chronic Myeloid Leukemia (CML) is a malignant disease of the primitive hematologic cell, characterized by inappropriate expansion of myeloid cells [1]. BCR-ABL contributes prominently to the development of most Chronic Myeloid Leukemias (CML). BCR-ABL proteins result from chromosomal translocations, which usually involve the human chromosomes 9 and 22 [2-5]. They are found in over 90% of human chronic myeloid leukemias. Previous work has shown that the tyrosine kinase activity of BCR-ABL is greatly elevated compared to c-Abl and that this elevation directly correlates with the transformation potency of BCR-ABL [6]. The p53 gene is a multifunctional tumour suppressor that is often altered in several cancers [7-10].

The p53 gene encodes a zinc-binding protein with sequence-specific transcriptional activity and 3'-5' exonuclease activity [11-21]. p53 normally interacts with a variety of proteins involved in transcriptional regulation, DNA repair, cell-cycle progression, apoptosis, and proteasome-mediated protein degradation[22-26]. During cancer development, p53 can be altered by mutation, loss, or silencing of the p53 gene as well as by transcriptional or posttranscriptional mechanisms. Thus far, missense mutations in p53 are very common in cancer cells. Nonsense mutations, insertions, and deletions in p53 have also been observed. A missense mutation results in a single amino acid change, and this type of point mutation in the DNA-binding domain of p53 can encode a protein that is transcriptionally inactive or that displays altered transcriptional activity compared with normal wild-type p53. Although normal cells generally have a low level of p53 protein as a result of the relatively short half-life of the wild-type protein, a missense mutation in the p53 gene often encodes a protein product that is resistant to degradation, and as a result, mutant p53 protein accumulates in the nucleus [27].

Truncated forms of p53 result from an insertion, a nonsense
mutation that generates a stop codon, or a deletion in the p53 gene, and these truncation mutations encode proteins with distinct functional activity or no activity compared with wild-type p53 [28,29]. Mutant p53 proteins that are deficient in certain or all p53 functions can complex with and inactivate wild-type p53 present in the cell. This dominant negative activity can alter the behaviour and fate of the tumour cell and is thought to promote the progression of many types of cancer. Thus, the present study is aimed to study the aberration in exon 8 codon 282 DNA-binding domain as it is thought to carry high susceptibility to mutation probably due to some inherent instability or chemical predispositions to single nucleotide substitutions and may contribute to assess its role in developing CML in Indian patients.

Materials and Methods

Selection criteria of patients

Inclusion criteria: The study included newly diagnosed CML patients treated with imatinib with a dose of 400 mg/day. All three stages of cases were included, Chronic Phase (CP), Accelerated Phase (AP) , Blast Crisis (BC) . The exclusion criteria Included Chronic Myelomonocytic Leukemia’s (CMML) patients, other myeloproliferative disorder patients. The patients follow up was maintained regularly and samples were collected after every six months for imatinib response and mutation studies. The classic criteria used for imatinib mesylate responses in chronic myeloid leukemia for hematologic and molecular responses are depicted in Tables 1 and 2.

Study population

A total of 100 CML blood samples were analyzed in this study. The cohort of the 100 CML patients consisted of 60 males and 40 female patients. Out of total 100 CML samples, 68 patients were in chronic phase, 17 in accelerated phase and 15 in blast crisis phase. Corresponding data from each patient was collected and analyzed for their TLC, platelet count, their spleen size and the haematological response elicited by each patient. 5ml peripheral blood sample was collected in EDTA vials after obtaining informed consent from the patients. The study was approved by the Local Ethics Committee.

Molecular diagnosis of CML patients

RNA extraction: Total RNA was extracted from about 10⁶ white cells by Trizol (Amresco, USA) method using the manufacturer’s protocol. RNA integrity was determined by gel electrophoresis on a 2% agarose gel prior to reverse transcription as shown in Figure 1.

CDNA synthesis: For CDNA synthesis, the concentration of RNA was first measured spectrophotometrically and then the CDNA was synthesized using Omniscript Reverse Transcription Kit (Qiagen). 2μg of RNA were reverse transcribed with 200 units of M-MuLV RT in a reaction mix consisting of 10X RT buffer, 10μM random hexamer, 5 mM dNTP and 10 units/µl RNase inhibitor, in a final volume of 20 μl. Reaction conditions were 25°C for 5 minutes, 42°C for 59 minutes and 70°C for 5 minutes. The integrity of CDNA was checked by a PCR for endogenous control by using primers specific for GAPDH as shown in Table 3. The reaction conditions consisted of the following conditions: 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds. Bands corresponding to 496 bp were obtained as shown in the Figure 2.

RT-PCR for BCR-ABL: The diagnosis of CML was confirmed using primers specific for BCR-ABL, listed in Table 4. 2 μg of CDNA was used in a total reaction mixture of 20 μl. Bands corresponding to 450 bp were obtained in patients positive for BCR-ABL as shown in Figure 3. The reaction conditions for BCR-ABL are as follows: 95°C for
10 minutes, 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds followed by final extension at 72°C for 5 minutes.

**Detection of the p53 exon 8 codon 282 gene mutation:** DNA extraction from blood: DNA from CML peripheral blood samples was extracted using DNASure® Blood Mini Kit (Nucleo-pore; Genetix, India) according to the manufacturer's instructions and stored at 4°C. The ASO-PCR specific for DNA-binding domain for p53 exon8 codon282 was performed in a total reaction volume of 25 µl containing 12.5 µl each of 2 × Dream Taq Master Mix (Fermentas).0.1- 0.2 µg of DNA template was used with a working concentration of 25pm of each primer with the following sequence in Table 5 as used previously by Hainaut [8]. Bands corresponding to 207 bp were seen as shown in Figure 4.

The amplification was performed under the following conditions: 95°C for 10 minutes for initial denaturation followed by 40 cycles of denaturation at 95°C for 40 seconds, annealing at 58°C for 45 seconds, extension at 72°C for 40 seconds and completed with a final elongation step at 72°C for 10 minutes. The PCR products were visualized with ethidium bromide on a 2% agarose gel under a UV-transilluminator. The products obtained had a band-size of 207 bp.

**Statistical analysis:** The variables measured in the study were investigated for association by using the Hardy Weinberg equilibrium equation. The differences in the incidence of p53 mutations among dependent variables like age, gender, phase, TLC, platelet count, spleen size and haematological response were calculated by the Chi square test. A two-side P value of less than 0.05 was considered as statistically significant. All the analyses were carried out with the software SPSS 17.

**Results**

**Demographic characteristics of study population**

The population analysis among cases is listed in Table 6. A total of 60 males and 40 females already diagnosed with CML were included in the study. Four age groups were made; patients with age <20 years included 11 cases, between 20-30 years included 21 cases, between 30-40 years included 26 cases and >40 years included 42 cases.

**Frequency of p53 R282W mutation in CML patients with respect to gender and age**

The frequency of the p53 exon 8 codon 282 mutation was found to be higher (61.6%) in case of males as compared to the frequency of occurrence in females (52.5%), as listed in Table 7. No significant association of the mutation was observed with respect to age although a higher frequency of occurrence of the p53 R282W mutation was seen in patients <20 years of age (72.7%).

**Frequency of p53 R282W mutation in CML patients with respect to phase**

A statistically significant association (p=0.001) was found between the occurrence of p53 R282W mutation and the clinical phase of CML with chronic, accelerated and blast crisis phases. The mutation was detected in a vast majority (88.2%) of patients in the accelerated and the blast crisis phase (60.0%) indicating that this mutation might play a critical role in predicting the progression of disease in CML.

**Frequency of p53 R282W mutation in CML patients with respect to TLC**

When the association between the p53 R282W mutation was clinically correlated with the TLC, it was found that patients with TLC<20,000 had a much higher incidence of the mutation (70.0%) as compared to those who had a TLC>20,000 (42.5%) with a statistically significant (p=0.01) result. This suggests that the patients who do not respond to the Tyrosine Kinase Inhibitors (TKIs) and do not come under remission tend to harbour this mutation.

**Frequency of p53 R282W mutation in CML patients with respect to platelet count**

Patients were categorized into three groups depending on the number of platelets present per cu.mm of blood viz. normal count, with thrombocytopenia and thrombocytosis. A significant association (p=0.001) was observed when the frequency of the mutation R282W was correlated clinically, CML patients suffering from thrombocytopenia and thrombocytosis had a higher frequency

<table>
<thead>
<tr>
<th>Primer type</th>
<th>Sequence</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCR-ABL Forward</td>
<td>5'-GAAGTGTCTAGAAGCTTCC-3'</td>
<td>66°C</td>
</tr>
<tr>
<td>BCR-ABL Reverse</td>
<td>5'-CCATTGGCATTAGCCTTA-3'</td>
<td>450 bp</td>
</tr>
</tbody>
</table>

**Table 4:** Primer sequence used for BCR-ABL primers.

<table>
<thead>
<tr>
<th>Primer type</th>
<th>Sequence</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53 Forward (wild )</td>
<td>5'-CTTGGGTCTGGAGAGACCC-3'</td>
<td>58°C</td>
</tr>
<tr>
<td>P53 Forward (mutant )</td>
<td>5'-CTTGGGTCTGGAGAGACT-3'</td>
<td>207 bp</td>
</tr>
<tr>
<td>P53 Reverse (common)</td>
<td>5'-GACCTGTGTTGTGTTG-3'</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5:** Primer sequence for ASO-PCR used for p53 exon 8 codon 282 DNA-binding domains.
Table 6: Demographic characteristics of CML patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CML Patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>100(100.0)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>60(60.0)</td>
</tr>
<tr>
<td>Females</td>
<td>40(40.0)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;20 yrs</td>
<td>11(11.0)</td>
</tr>
<tr>
<td>20-30 yrs</td>
<td>21(21.0)</td>
</tr>
<tr>
<td>30-40 yrs</td>
<td>26(26.0)</td>
</tr>
<tr>
<td>&gt;40 yrs</td>
<td>42(42.0)</td>
</tr>
<tr>
<td>Phase</td>
<td></td>
</tr>
<tr>
<td>Chronic</td>
<td>68(68.0)</td>
</tr>
<tr>
<td>Accelerated</td>
<td>17(17.0)</td>
</tr>
<tr>
<td>Blast Crisis</td>
<td>15(15.0)</td>
</tr>
<tr>
<td>TLC</td>
<td></td>
</tr>
<tr>
<td>&lt;20,000</td>
<td>40(39.0)</td>
</tr>
<tr>
<td>&gt;20,000</td>
<td>60(61.0)</td>
</tr>
<tr>
<td>Platelet Count</td>
<td></td>
</tr>
<tr>
<td>Normal count</td>
<td>48(48.0)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>36(36.0)</td>
</tr>
<tr>
<td>Thrombocytosis</td>
<td>16(16.0)</td>
</tr>
<tr>
<td>Spleen size</td>
<td></td>
</tr>
<tr>
<td>Spleenomegaly absent</td>
<td>34(34.0)</td>
</tr>
<tr>
<td>Spleenomegaly present</td>
<td>66(66.0)</td>
</tr>
<tr>
<td>Haematological Response</td>
<td></td>
</tr>
<tr>
<td>Major</td>
<td>21(21.0)</td>
</tr>
<tr>
<td>Minor</td>
<td>19(19.0)</td>
</tr>
<tr>
<td>Minimal</td>
<td>60(60.0)</td>
</tr>
</tbody>
</table>

(75.0%) of the mutation as against those patients displaying a normal platelet count (39.5%).

Frequency of p53 R282W mutation in CML patients with respect to spleen size

Patients having spleenomegaly were found to carry a higher frequency (65.1%) of the mutation as compared to those patients having a normal spleen size although the results were statistically insignificant.

Frequency of p53 R282W mutation in CML patients with respect to haematological response

A statistically significant correlation (p=0.01) was found when patients carrying the mutation were assessed for major, minor and minimal haematological response. Patients exhibiting poor or minimal haematological response harbored a higher frequency (68.3%) of this mutation than patients showing minor (52.6%) and major (33.3%) haematological response.

Discussions

p53 mutations are found in a wide variety of cancers, including hematologic malignancies. These alterations apparently contribute to development of the malignant phenotype. p53 gene located on chromosome 17 at band p13.1 codes for a nuclear DNA-binding phosphoprotein with properties of a transcriptional activator [30]. We examined a cohort of 100 CML patients for alterations of p53 gene in exon 8 coding 282 mutational hotspot regions. Mutations were detected in 58% of patients carrying the Philadelphia chromosome. No specific family history was obtained to suggest inheritance of such a mutation and therefore it was apparently considered to be somatic. It has previously been reported that no significant alterations have been detected within the p53 mutational hotspot region in childhood myeloid leukemias. Our cohort consisted majority of adult population and our results explain that adults appear to have a higher frequency of p53 exon 8 codon 282 mutation as compared to that of childhood malignancies.

We limited our analysis to exon 8 as it is an evolutionarily conserved region of p53 and prior studies showed that 80% to 90% of all p53 mutations in a variety of human malignancies occur here. Besides, point mutations at codon 282 are mutational hotspots reported in other haematologic diseases [31-33] including Burkit’s lymphoma [34], myelodysplastic syndrome [35], multiple myeloma [36], T-Cell leukemia [37], acute lymphoblastic T-cell leukemia [38], lymphoid leukemia [39], chronic lymphocytic leukemia [40], acute lymphoblastic leukemia [41], adult T-Cell leukemia [42], non-Hodgkin’s lymphoma [43], acquired immunodeficiency syndrome-related lymphomas [44] and acute myelogenous leukemia [45,46]. The world scenario for the p53 mutations in some of the haematologic malignancies are shown in Figure 5.

In the present study, we evaluated the association between the appearance of p53 R282W mutation and the acquiring of chronic myeloid leukemia. A significant correlation (p=0.03) was found between the number of cases reported to be positive for the mutation and patients not carrying the mutation. The mutation was also associated with the development of progression of CML as the overall frequency of patients having the mutation in the accelerated (88.2%) and blastic phase (60.0%) was found to be greater as against those in chronic phase (35.2%) with a statistically significant result (p=0.001) as depicted in the Figure 5.

Also, it has been reported that p53 mutations may play a role in...
function of wild-type p53 providing the cells with a growth advantage.

proteins [49] and the p53 mutant protein can partially inhibit the
in this haematologic malignancy may not be induced by exogenous
conversion of cytosine into thymine. This suggests that p53 mutations
results from a spontaneous deamination at 5-methylcytosine residues
malignant phenotype The transition occurring in the exon 8 region
probably renders the patient resistant to the standard tyrosine kinase
response. This implies that the mutation in exon 8 codon 282
response in parallel to patients who showed a major haematological
poor or minimal haematological response and minor haematological
Presence of mutation was found to be higher in patients displaying a
higher percentage who presented with the mutation than the cases
thrombocytosis and a significant relation (p=0.001) was established as
development of CML than patients having a TLC of <20,000. Also
was also associated with the presence of p53 R282W mutation and a
statistically significant correlation (p=0.01) was obtained. A higher
number of patients carrying the mutation had a TLC of >20,000
which is one of the prognostic factors leading to the phenotype and
development of CML than patients having a TLC of <20,000. Also
patients with the mutation were correlated with thrombocytopenia and
thrombocytosis and a significant relation (p=0.001) was established as
the patients suffering from thrombocytopenia or thrombocytosis had a
higher percentage who presented with the mutation than the cases
having a normal platelet count. Patients were also monitored for the
kind of haemalogical response that they exhibited is shown in Figure 6.

A statistically significant (p=0.01) association was found between
the haematological response and the patients harboring the mutation.
Presence of mutation was found to be higher in patients displaying a
poor or minimal haematological response and minor haematological
response in parallel to patients who showed a major haematological
response. This implies that the mutation in exon 8 codon 282
probably renders the patient resistant to the standard tyrosine kinase
inhibitor therapy and induces the progression of the disease to a more
malignant phenotype The transition occurring in the exon 8 region
results from a spontaneous deamination at 5-methylcytosine residues
converting cytosine into thymine. This suggests that p53 mutations
in this haematologic malignancy may not be induced by exogenous
carcinogens [48]. Frameshift mutations result in short-lived, truncated proteins [49] and the p53 mutant protein can partially inhibit the
function of wild-type p53 providing the cells with a growth advantage.

To our knowledge, our study is the first to report data on the significance
of the p53 exon 8 mutation by ASO-PCR with epidemiological
correlation to CML. Using the allele-specific oligonucleotide PCR method, we have successfully examined 100 Ph positive CML patients.
Our results indicate that the ASO-PCR method is suitable for the rapid
determination of p53 R282W mutation. Thus, we concluded that the
ASO-PCR based assay is a rapid, simple, inexpensive, and accurate
method for detecting the most common p53 R282W gene mutation
among the Indian population. This method involves the selective
amplification of human p53 R282W gene with specific oligonucleotide
primers [50].

**Conclusion**

Our study suggests that R282W mutation of p53 gene might have a
strong influence on disease progression and poor response in CML
patients. The p53 mutation status being a predictive factor of response and
should be considered when following the imatinib therapy for
CML patients.

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**References**


