Inactivation of P16 (INK4a) Gene by Promoter Hypermethylation is Associated with Disease Progression in Chronic Myelogenous Leukaemia

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Abstract

Background: Chronic Myelogenous Leukemia (CML) has a typical progressive course from transition from a chronic phase to a terminal blast crisis phase. The mechanisms that lead to disease progression remain to be elucidated. Promoter hypermethylation is one of the putative mechanisms underlying the inactivation of negative cell-cycle regulators in haematological malignancies. Therefore, aim of our study was to examine whether the methylation status of P16 (INaK4A) gene is a useful biomarker in the development and progression of CML.

Material and Methods: The methylation status of p16INK4A gene was evaluated by Methylation Specific Polymerase Chain Reaction (MSP) in 200 CML patients among which, 81 were in CP-CML, 54 in AP-CML and 65 in BC-CML.

Results: The p16INK4A gene was hypermethylated in 84 of 200 (42%) of CML patients (P<0.0001). Among the three stages p16 (INK4A) promoter gene was methylated in 26% (CP-CML), 43% (AP-CML) and 68% (BC-CML) patient (P<0.0001). Methylation was more frequent in elastic and accelerated phase patients than in chronic phase. A significant correlation was found between p16INK4A methylation and loss of Imatinib response. Similarly higher frequency of p16INK4A methylation was reported in CML patients with haematological (P<0.02) and molecular resistances (P<0.04). Significantly higher (p<0.0001) frequency of p16INK4A promoter methylation was reported in patients with thrombocytopenia. However no correlation was found between p16INK4A hypermethylation and other clinic-pathological parameters like age, gender, BCR-ABL transcripts etc.

Conclusion: Our results suggest that p16INK4A is a primary target for inactivation by promoter methylation in the disease progression of CML patients and that its detection is useful in the follow up of patients with a high risk of developing CML and resistance to Imatinib therapy.

Introduction

Chronic Myeloid Leukemia (CML) is a clonal disorder of the hematopoietic stem cell caused by the BCR-ABL receptor tyrosinekinase. The hallmark of CML is Philadelphia (Ph) chromosome. The Philadelphia chromosome (Ph) is a shortened of chromosome 22 which is due to reciprocal translocation of chromosome 9 and 22 [1]. This translocation leads to formation of the BCR-ABL fusion oncogene gene, the protein product of which (p210, p190 and rarely p230) has constitutive tyrosine kinase activity implicated in the pathogenesis of the disease. Chronic Myelogenous Leukemia (CML) has a typical progressive course with transition from the chronic phase to the terminal blast crisis phase [2]. The mechanisms that lead to disease progression have yet to be elucidated.

Genetic alternations including mutation, deletion, and DNA amplification have been shown to play an important role in tumorigenesis [3]. Epigenetic alteration of the DNA such as methylation of CpG island in promoter region participates in the regulation of gene expression that is now recognized as an additional method to be involved in human malignancies [4-6]. Methylation in the promoter region is capable of causing gene silencing, which may provide an alternative pathway to gene inactivation, in addition to deletions or mutations. CALCA, ESR, HIC1, TFAP2A and ABL1, were found to be frequently methylated in CML [7-10]. Moreover, methylation of the ABL1 gene is associated with the progression of CML [11].

The p16INK4A gene is a tumour suppressor gene and is associated with tumourogenesis when it is inactivated [12]. The p16 gene is also known as CDKN2A is implicated in the cell cycle control. This gene is located on region 9p21, comprised of 3 exons, and codes for a 16 kDa protein. The p16INK4A protein belongs to a family of regulators of the cell cycle, called Cyclin Dependent Kinase Inhibitors (CDKI), which
bind themselves to cyclin-CDK complexes. The formation of such complexes causes, as a result, the arrest of the cell cycle in the G1 phase, a way through which the p16NK4a protein can stop the proliferation of neoplastic cells [13].

The inactivation of p16 gene has been shown to play a key role in the pathogenesis and the progression of leukemia and is a reliable prognostic factor that predicts shortened survival times [14]. Moreover, abnormality of P16 and calcitonin were shown to be associated with mixed cell blast crisis in CML [15]. Hypermethylation of the p16 promoter region has been detected in various types of human cancers [16,17]. Most studies to date evaluating CDKN2A and patient outcome have done so using methylation specific PCR [18].

The aim of our study was to determine the role of aberrant P16 methylation in the prognosis of chronic myeloid leukemia using methylation-specific polymerase chain reaction.

Material and Method

Two hundred (200) CML patients were collected from the Maulana Azad Medical College and Associated Hospitals, New Delhi and were diagnosed by clinical and haematological criteria like bone marrow hyperplasia, leukocytosis, splenomegaly, high fever, fatigue and had disease confirmation by detecting t(9;22) or BCR/ABL fusion gene (p210 BCR-ABL) by reverse transcriptase (RT)-PCR.

Study design

Sample collection: Peripheral blood samples i.e. 5ml venous blood samples were collected in EDTA vials from CML patients as well as from 25 healthy donors. Buffy coat was isolated and washed in red cells lysis buffer. All samples were stored at -80°C until the RNA and genomic DNA was extracted.

CML diagnosis

Inclusion and exclusion criteria: The study included diagnosed CML patients of all the three stages from chronic phase, accelerated phase and blast crisis. All patients were treated with Imatinib with a dose of 400mg/ day. All patients gave written informed consent to participate in the study before entry, and the study was reviewed and approved by a recognized ethics review committee. The exclusion criteria included Chronic Myelomonocytic Leukaemia’s (CMML) patients, other myeloproliferative disorder patients.

Patient evaluation: Patients were evaluated for hematologic and molecular responses and relapse at specified intervals. The patients follow up was maintained regularly and peripheral blood samples were obtained and analyzed, after every 3 months of Treatment until achieved and confirmed. The classic criteria used for Imatinib mesylate responses in chronic myeloid leukemia for hematologic and molecular responses are depicted in Tables 1A and 1B respectively.

RNA isolation: Total RNA was isolated from mononuclear cells with guanidinium isothiocyanate (Trizol LS™ - Invitrogen), according to the protocol provided by the manufacturer. The presence of RNA was confirmed by running the product on 2% agarose gel.

cDNA synthesis: The concentration of RNA was measured spectrophotometrically. cDNA was then synthesized using M-MuLV Reverse Transcriptase and other reaction components (Fermentas CAT# K1622), according to the protocol provided by the manufacturer.

Multiplex RT-PCR for BCR-ABL: BCR-ABL transcripts were detected using allele-specific primers for p210 and p190 primer sequences, as already described [19] listed in table 2. PCR was carried out in a total volume of 25 ul reaction mixture containing 1 U/μL Taq polymerase, 240 μM dNTP, 1.8 M MgCl₂, and 0.6 μM of primers. A program was employed, under the following conditions: an initial denaturation step at 95°C for 10 min., then followed by 40 cycles of denaturing at 94°C for 40 s, primer annealing at 55°C, extension at 72°C for 45 s, and a final extension step at 72°C for 5 min. The expected bands were as follows: 808bp, normal BCR; 481 bp, e1a2; 385bp, b3a2; 310bp, b2a2.

DNA extraction and Bisulphite treatment: The methylation status of the promoter CpG islands of P16 gene in all DNA samples was analyzed by MS-PCR on the sodium-bisulphite converted DNA. Genomic DNA from peripheral blood was extracted using genomic DNA extraction kit (Gene Aid CAT#GB 100). The quality and integrity

<table>
<thead>
<tr>
<th>Complete or major haematological response</th>
<th>Partial or minor haematological response</th>
<th>No or minimal haematological response</th>
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</table>

Table 1A: Hematological responses criteria in CML patients.

<table>
<thead>
<tr>
<th>Major molecular response</th>
<th>Minimal or No Molecular response</th>
</tr>
</thead>
<tbody>
<tr>
<td>It indicates nonquantifiable and nondetectable BCR-ABL gene transcript (BCR-ABL/ABL) &lt; 0.10. * Check every three months</td>
<td>If indicates quantifiable and detectable BCR-ABL gene transcript (BCR-ABL/ABL) &gt; 0.10. * Check every three months</td>
</tr>
</tbody>
</table>

*BCR-ABL to control gene ratio according to international scale (IS)

Table 1B: Molecular responses criteria in CML patients.

<table>
<thead>
<tr>
<th>BCR-ABL primers</th>
<th>Oligonucleotides used in multiplex RT-PCR for detection of BCR-ABL transcript as the target gene and BCR transcripts as the internal control.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5e 5’-ATAGGATCTTTGCAACCGGCTCTCAA-3’</td>
<td>Table 2:</td>
</tr>
</tbody>
</table>
of the DNA was determined by the A260/280 ratios. Genomic DNA (1μg) was modified with sodium bisulphite using EZ-DNA methylation kit (Zymo research, CA). Bisulphite-treated DNA was used for methylation-specific PCR by using previously published primer sets [20] to distinguish between methylated and unmethylated DNA. The PCR products were electrophoresed on a 2% agarose gel.

Methylation-specific polymerase chain reaction: MSP was performed using primers shown in table 3. With a complete chemical modification reaction, U primers amplified only unmethylated DNA, and M primers amplified only methylated DNA in the region of P16 gene promoter.

The thermo cycling conditions were 45 cycles of denaturation at 94°C for 45s, annealing at 62°C for 45s and extension at 72°C for 60s, then a final extension at 72°C for 10 minutes. The PCR products were then analyzed on a 2.5% agarose gel. The expected bands were as follows: 151bp for methylated DNA and 150 bp for unmethylated DNA.

Statistical Analysis

Statistical analysis was performed using the SPSS 16.0 software package. Chi-square analysis and Fisher exact test were carried out to compare the difference of frequencies between groups of patients. P value of ≤ 0.05 was considered statistically significant.

Results

Clinical-pathological classification

Methylation profile of p16 gene was established in 200 chronic myeloid leukemia patients (72 females and 128 males). We were able to detect p210 transcripts, such b2a2 or b3a2 transcripts in 200 patients at presentation, b3a2 transcript was detected in 62% cases, b2a2 in 29.5% and both b3a2 and b2a2 in 8.5% patients (Table 4).

MS-PCR analysis results

Out of 200 CML patients we could detect 84 (42%) patients which displayed methylated bands M, 151 bp products, unmethylated Band U, 150 bp product was visible in all patients with varied intensity (Figure 1).

Clinico-pathological correlation of p16INK4a hypermethylation with different parameters

P16 promoter methylation was slightly higher in females (44.4%) than in males (40.6%). Patients in age group >45 showed 46.5% methylation compared to 33.8% in patients having ≤ 45 age, but the results could not reach stastical significance. We did not find any significant correlations between the methylation status of P16 gene and the clinical features such as age, gender, chromosomal abnormalities like BCR-ABL and its transcripts (Table 5).

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmethylated (U)</td>
<td>TTATTAGAGGTTGGGGTGGATTGT</td>
<td>CAACCCCAAACCAACACCTAA</td>
</tr>
<tr>
<td>Methylated (M)</td>
<td>TTATTAGAGGTTGGCGGATCGC</td>
<td>GACCCGAAAGCGGATCGTAA</td>
</tr>
</tbody>
</table>

Table 3: Sequence of oligonucleotides used for methylated and unmethylated DNA.

Table 4: Demographic classification in CML patients.
P16 hypermethylation in stages of CML

P16 promoter methylation was detected in 21% (26%) in CP-CML patients. 23 (42.6%) AP-CML patients had P16 promoter methylation and 40 (61.5%) showed P16 promoter methylation. Methylation increased significantly (P < 0.0001) with the acceleration of disease from early to advanced phase in CML. Taken together, out of 84 total methylated patients, 63 patients were in advanced phase (AP, BC) of the disease, suggesting that inactivation of cell cycle control gene P16 by promoter hypermethylation plays a significant role in the progression of CML.

Correlation of P16 hypermethylation with haematological response

30 CML patients had major HR, 46 minor HR and 124 minimal haematological responses respectively. The frequency of p16 promoter methylation was reported higher in patients with minor or minimal response. In minor HR group 43.5% aberrant methylation was detected, in minimal HR 46.8% aberrant methylation was detected and 20% aberrant methylation was detected in major haematological responders group. A significant correlation (P<0.02) was reported between p16 methylation and haematological response in CML patients.

P16 hypermethylation higher in patients with loss of molecular response

Among the 200 CML patients at the time of analysis, 50 displayed major MR, 130 minimal or no molecular responses. The higher frequency of P16 promoter methylation was reported in molecular resistant cases (46%) than the good molecular responders (30%). A statistically significant difference was found between the two groups (p=0.04).

P16 hypermethylation with thrombocytopenia

The most notable side effect of Imatinib mesylate is thrombocytopenia. The risk of Imatinib mesylate induced thrombocytopenia in CML increases with disease stage. The side effects were more pronounced in blast crisis. The thrombocytopenic patients were found to have significantly (p<0.0001) higher percentage of p16 methylation (58%) than in patients with no thrombocytopenia (26%).

Discussion

The majority of recent studies have focused on the study of epigenetic changes resulting in many types of cancers. DNA methylation was the first epigenetic alteration to be observed in cancer cells. DNA methylation involves addition of a methyl group to the carbon 5 position of the cytosine ring. This reaction is catalyzed by DNA methyltransferase in the context of the sequence 5’-CG-3’, which is also referred to as a CpG dinucleotide [21,22]. The methylation of gene, particularly the methylation of CpG-rich promoters, could block transcriptional activation [21]. The potential contribution of DNA methylation to oncogenesis is mediated by one or more of mechanisms that include DNA hypomethylation, hyper-methylation of tumour suppressor gene and chromosomal instability in cancers, [23-29].

P16 tumour suppressor gene plays a monitor role in the passage of cells through the G1 to S phase of the cell cycle by binding to cyclin dependent kinase 4 and inhibiting its effect on cyclin D1.p16 is the most commonly altered gene in human malignancies [29]. Hypermethylation of the p16 tumor suppressor gene and its effect on transcriptional down regulation or silencing is one of the major mechanisms of p16/INK4a gene inactivation in various types of cancers. Methylation of cytosine residues at CpG sites in p16 gene promoter, resulting in a silenced p16 expression, has been reported in many cell lines, including CRC, and some primary carcinomas in varied origins, such as colon, brain, breast, bladder, ovary, lung, and myeloma and so on. aberrant promoter methylation correlates with lack of p16 expression and results in collapse of Retinoblastoma and P53 pathways, thus inactivation of P16 gene by promoter methylation has a growth advantage.

Several past studies have shown that p16 methylation occur frequently in both haematological as well as in solid tumours. P16 methylation was found in 12 (50%) of 24 ALL cell lines, 5 (50%) of 10 MLL cell lines, 10 AML cell lines [30], Martinez-Delgado et al. detected Hypermethylation...
Imatinib response (p=0.0001) indicating higher rate of P16 methylation haematological resistance (p=0.02), the patients which were showing significantly higher in patients which were showing minor and No associated with disease progression. We found aberrant P16 methylation age is not associated with the increase in the risk of CML with respect observations were also obtained in the present study, suggesting that inactivation of cell cycle control gene by promoter hypermethylation plays a significant role in the progression of CML.

**Conclusion**

Our results suggest that p16\(^{INK4a}\) is a primary target for inactivation by promoter methylation in the disease progression of CML patients and that its detection is useful in the follow up of patients with a high risk of developing CML and resistance to imatinib therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgement**

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**References**


**Table 6:** Frequency of P16\(^{INK4a}\) methylation in different haematological malignancies.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>% methylation</th>
</tr>
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<tbody>
<tr>
<td>B cell-Lymphoma (31)</td>
<td>20%</td>
</tr>
<tr>
<td>T cell-Lymphoma (31)</td>
<td>15%</td>
</tr>
<tr>
<td>MALT Lymphoma (31)</td>
<td>67%</td>
</tr>
<tr>
<td>AP-CML (32)</td>
<td>40%</td>
</tr>
<tr>
<td>Childhood B-ALL (33)</td>
<td>34.4%</td>
</tr>
<tr>
<td>Adult B-ALL (33)</td>
<td>26.7%</td>
</tr>
<tr>
<td>Multiple myeloma (34)</td>
<td>10%</td>
</tr>
<tr>
<td>Haematological malignancies (35)</td>
<td>10.9%</td>
</tr>
<tr>
<td>Acute Myeloid Leukemia (36)</td>
<td>0%</td>
</tr>
<tr>
<td>Acute Myeloid Leukemia (37)</td>
<td>83%</td>
</tr>
<tr>
<td>Acute Lymphoblastic leukemia (37)</td>
<td>85%</td>
</tr>
<tr>
<td>Acute Lymphoblastic leukemia (36)</td>
<td>6%</td>
</tr>
<tr>
<td>Chronic lymphoblastic leukemia (38)</td>
<td>14.3%</td>
</tr>
<tr>
<td>Present study (CML)</td>
<td>42%</td>
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