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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 with 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 (INK4a) gene is a useful biomarker in the development and progression of CML.

Material and Methods: The methylation status of $p16^{INK4A}$ 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 $p16^{INK4A}$ 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 blastic and accelerated phase patients than in chronic phase. A significant correlation was found between $p16^{INK4A}$ methylation and loss of Imatinib response. Similarly higher frequency of $p16^{INK4A}$ methylation was reported in CML patients with haematological (P<0.02) and molecular resistances (P<0.04). Significantly higher (p<0.0001) frequency of $p16^{INK4A}$ promoter methylation was reported in patients with thrombocytopenia. However no correlation was found between $p16^{INK4a}$ hypermethylation and other clinic-pathological parameters like age, gender, BCR-ABL transcripts etc.

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.

Abbreviations: CML: Chronic Myelogenous Leukemia; CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A,p16(INK4a) Gene; MMR: Major Molecular Response; MHR: Major Haematological Response; mHR: Minor Haematological Response

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 alternation 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 $p16^{INK4a}$ 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 $p16^{INK4a}$ protein belongs to a family of regulators of the cell cycle, called Cyclin Dependent Kinase Inhibitors (CDKI), which

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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 $p16^{INK4a}$ 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 $MgCl_2$, 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

Complete or major haematological response	Partial or minor haematological response	No or minimal haematological response
Platelet count >150×109/L. WBC count <10×109/L. Basophils :<5% . Absence of blasts and Promyelocytes in peripheral blood. Spleen : nonpalpable spleen .	Platelet count <450×109/L. WBC count >10×109/. Basophils:>10%. Presence of blasts and Promyelocytes in peripheral blood. Spleen : Palpable spleen.	Platelet count <450×109/L. WBC count >20×109/L. Basophils:>15%. Presence of blasts and promyelocytes in peripheral blood. Spleen : Palpable spleen.

Table 1A: Hematologic responses criteria in CML patients.

Major molecular response	Minimal or No Molecular response
It indicates nonquantifiable and nondetectable BCR-ABL gene transcript (BCR-ABL/ABL) ≤ 0.10.* Check every three months	It indicates quantifiable and detectable BCR-ABL gene transcript (BCR-ABL/ABL)> 0.10.* Check every three months

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

Table 1B: Molecular responses criteria in CML patients.

BCR-ABL primers
C5e 5'-ATAGGATCCTTTGCAACCGGGTCTGAA-3'
B2B 5'-ACAGAATTCCGCTGACCATCAATAAG-3'
BCR-C 5'- ACCGCATGTTCCGGGACAAAAG-3'
CA3 5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3'

Table 2: Sequence of oligonucleotides used in multiplex RT-PCR for detection of BCR-ABL transcript as the target gene and BCR transcripts as the internal control.

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 $p16^{INK4a}$ 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).

Name	Forward primer	Reverse primer
Unmethylated (U)	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA
Methylated(M)	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA

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

Clinical feature	no.	%
Patients	200	
Healthy Controls	25	
Gender		
Males	128	64.0%
Female	72	36.0%
Age		
Age >45	71	35.5%
Age ≤45	129	64.5%
BCR-ABL transcripts		
a2b2	59	29.5%
a2b3	124	62.0%
a2b2/ a2b3	17	8.5%
Staging of the disease		
Chronic phase-CML	81	40.5%
Accelerated phase-CML	54	27.0%
Blast crisis-CML	65	32.5%
Molecular Response		
Major molecular response(MMR)	50	25.0%
No molecular response	150	75.0%
Haematological responses		
Major haematological response(MHR)	30	15.0%
Minor haematological response(mHR)	46	23.0%
No haematological response	124	77.0%
Type of therapy		
Imatinib treated	200	100%
Dasatinib treated (Imatinib failure)	10	5.0%
Thrombocytopenia		
Thrombocytopenia	100	50.0%
No Thrombocytopenia	100	50.0%

Table4: Demographic classification in CML patients.

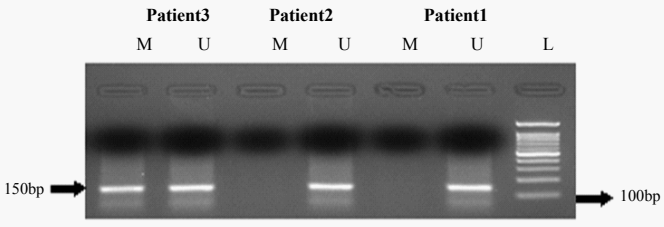

Figure 1: Showing the methylation status of P16 gene, U-unmethylated DNA, M-Methylated DNA, L-100 bp DNA ladder.

P16 ^{INK4a} 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 ^{INK4a} 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, 150 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 stastically significant difference was found between the two groups $p=0.04$.

P16 ^{INK4a} 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 AML cell lines [30]. Martinez-Delgado et al. detected Hypermethylation

Clinical feature	No of cases	P16 Unmethylated	%	P16 Methylated	%	P value
Patients	200	116	58%	84	42%	0.0001
Controls	25	23	92%	2	8%	
P16 promoter methylation Gender						
Males	128	76	59.4%	52	40.6%	0.7
Female	72	40	55.6%	32	44.4%	
P16 promoter methylation with stage						
CP-CML	81	60	74%	21	26%	0.0001
AP-CML	54	31	47.4%	23	42.6%	
BC-CML	65	25	38.5%	40	61.5%	
P16 promoter methylation with BCR-ABL transcripts						
a2b2	59	31	52.5%	28	47.4%	0.5
a2b3	124	64	51.6%	60	48.3%	
a2b2/ a2b3	17	11	64.7%	6	35.2%	
P16 promoter methylation with type of therapy						
Imatinib	200	116	58%	84	42%	0.0001
Dasatinib	10	00	00	10	100%	
P16 promoter methylation and age						
Age >45	71	47	66.1%	24	33.8%	0.08
Age <45	129	69	53.4%	60	46.5%	
P16 promoter methylation and molecular responses						
MMR	50	35	70%	15	30%	0.04
No MR	150	81	59.4%	69	46%	
P16 promoter methylation and haematological responses.						
MHR	30	24	80%	6	20%	0.02
Minor HR	46	26	56.5%	20	43.5%	
No HR	124	66	53.2%	58	46.8%	
P16 promoter methylation and Thrombocytopenia						
Thrombocytopenia	100	42	42%	58	58%	<0.0001
No Thrombocytopenia	100	74	74%	26	26%	

Table 5: Association of P16 promoter methylation with the clinicopathological features.

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

Table 6: Frequency of $P16^{INK4a}$ methylation in different haematological malignancies.

of P16 gene in approximately 20% of B cell lymphomas of both low and high grade, 15% of T cell non-Hodgkin's lymphoma and 67% in MALT (mucosa-associated lymphoid tissue) lymphomas [31].

Nagy et al. have shown that P16 methylation is a frequent event in accelerated phase of CML with more than half of their accelerated phase patients (40%) were hypermethylated suggesting that inactivation of P16 by aberrant methylation is the primary target for the acceleration of CML [32]. The incidence of P16 promoter aberrant methylation was 34.4% in childhood B-ALL and 26.7% in adult B-ALL patients [33]. In Multiple myeloma 10% methylation was detected by Wang et al. [34]. Chen et al. showed 10.9% P16 promoter methylation in different haematological malignancies [35]. No methylation of p16 was found in AML and only 6% was found in ALL in China region [36]. However, Pei-Ching Hsiao et al. showed 83% methylation in AML and 85% in ALL patients [37]. Chim et al. have shown that aberrant methylation of p16 gene was detected in 14.3% of chronic lymphocytic leukemia and there was no association between age, sex and overall survival in Chinese patients [38].

In the present study, we used MS-PCR for analysis of the methylation status of p16 gene. This method provided significant advantages over previous ones used for assaying methylation. MSP is much more sensitive than Southern analysis, facilitating the detection of low numbers of methylated alleles and the study of DNA from small samples. MS-PCR allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes.

In our study, p16 methylation in CML was found in a frequency as high as 42% with an extremely significant p value 0.0001. We didn't find any correlation between p16 methylation and various clinicopathologic factors including age, gender or BCR-ABL type. A recent study also showed that hypermethylation of the $p16^{INK4a}$ gene promoter to be unchanged according to the patient age [36]. Similar observations were also obtained in the present study, suggesting that age is not associated with the increase in the risk of CML with respect to p16 hypermethylation.

Our results indicate that p16 methylation was significantly associated with disease progression. We found aberrant P16 methylation significantly higher in patients which were showing minor and No haematological resistance (p=0.02), the patients which were showing minimum molecular response p=0.04, thrombocytopenic patients (p<0.0001) and methylation rate were higher in patients with loss of Imatinib response (p=0.0001) indicating higher rate of P16 methylation with the acceleration of disease. P16 promoter methylation was found of higher frequency in advanced stage of disease than in chronic phase p value 0.0001; hence a feature of blast crisis, accelerated phases of disease than chronic phase, 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.

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