Epigenetic Silencing of DAPK1 Gene is Associated with Faster Disease Progression in India Populations with Chronic Myeloid Leukemia

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Abstract

Background: One of the major epigenetic changes in human cancer is DNA methylation of tumour suppressor genes which leads to silencing of gene leading to disease progression. Therefore, DNA methylation status of such genes may serve as the epigenetic biomarker for prognosis of human Chronic Myeloid Leukemia.

Material and methods: We used MSP-PCR technique for the analysis of aberrant promoter DAPK1 methylation on 200 CML venous blood samples. Stastical analysis was done for evaluating differences between different parameters using SPSS 16.0 version.

Results: We could detect 91/200 promoter methylation (45.5%) in CML patients. Percentage of methylation detected was seen higher in blast phase (63.07%) and in accelerated phase (48.1%) than in chronic phase (29.6%). A significant correlation was seen between CML stages and DAPK1 aberrant methylation. We also found a significant association of DAPK1 methylation in gender and in haematological resistance CML patients. However no correlation was found between DAPK1 promoter methylation and other clinical parameters like age, BCR-ABL type and Thrombocytopenia.

Conclusion: In summary we concluded that methylation status of DAPK1 gene is associated with advanced phase of CML and may be related to disease progression in chronic myeloid leukemia. Further study on a more number of patients is needed to explore the role of DAPK1 methylation in the prognosis of CML.

Keywords: DAPK1 Gene; Chronic myeloid leukemia; MSP-PCR technique; Methylation

Introduction

Chronic Myelogenous Leukemia (CML) is a malignant cancer of the bone marrow. It causes rapid growth of the blood-forming cells, known as myeloid precursors in the bone marrow, peripheral blood, and body tissues. CML represents about 14% of all occurrences of leukemia. CML can be divided into three phases, the chronic phase, the accelerated phase and the blast phase. The transition of the disease is not well characterized.

Diagnosis of chronic myeloid leukemia is based on the detection of BCR-ABL gene is a product derived from translocation of chromosome 22 to chromosome 9. This reciprocal translocation gives rise to BCR-ABL fusion oncogene, results in fusion messenger RNA molecules (e1a2, b2a2, b3a2, and e19a2) of different lengths that are translated into different chimeric protein products (p190, p210 and p230BCR-ABL respectively) that is characterized by constitutive activation of its tyrosine kinase activity. The presence of BCR/ABL rearrangement is the hallmark of CML [1].

The role of aberrant DNA methylation in the development of cancer is well recognized and documented. Addition of the methyl group at the fifth carbon of cytosine of CpGs is the only covalent DNA modification in vertebrate [2]. Gene hypomethylation and gene-specific hypermethylation are the two frequently observed forms of methylation changes which take place in cancer. Global hypomethylation is largely observed in gene poor regions and repetitive DNA and gene-specific hypermethylation of CpG islands [3]. Tumour suppressor gene (TSG) inactivation by promoter region CpG island hypermethylation occurs in almost all cancer types and is an important mechanism of gene silencing in cancer. To date, there are few reports of hypomethylation of human oncogenes associated with activation by over expression [4]. However, it is well-established that aberrant hypermethylation of the promoter region of tumour suppressor genes is associated with transcriptional silencing and that hypermethylation is an alternative mechanism of functional inactivation [5].

DAPK1 is a 160-kD serine/threonine, microfilament-bound kinase, a candidate tumour suppressor gene which has recently been shown to be involved in gamma interferon-induced apoptosis [6]. In several malignant cell lines, studies have shown that expression of DAPK1 is decreased or absent [7]. Additionally, decreased expression of this gene imparts resistance to interferon-induced apoptosis in cells, and a link between the loss of DAPK1 expression and cellular apoptosis has been shown to facilitate metastasis, at least in one experimental system [8]. Promoter methylation of DAPK1 gene has been observed in multiple tumor types and may increase cellular resistance to programmed cell death [9].

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Unlike genetic changes in cancer, epigenetic changes are potentially reversible. Epigenetic therapy is a rapidly expanding field and a number of drugs that alter the epigenetic profiles of cancer cells are already in clinical trials. Two hypomethylation agents, 5-azacitidine (Vidaza) and 5-aza-2′-deoxycytidine (Decitabine) are currently in use and are approved therapies for myelodysplastic syndrome [2,10]. A significant response to decitabine 5-aza-2′-deoxycytidine treatment in chronic myelogenous leukemia and myelodysplastic syndrome has been observed [11,12].

The aim of the current study was to find out aberrant promoter DNA methylation status of DAPK1 gene in chronic myeloid leukemia patients. We used methylation specific PCR (MSP-PCR) for this purpose. The data suggests that its inactivation may play an important role in disease progression of chronic myeloid leukemia.

Material and Methods

Material and method

The study was conducted on 200 CML patients collected from Maulana Azad Medical College and Associated Hospitals, New Delhi. Patients were diagnosed clinically by bone marrow hyperplasia, leukocytosis, splenomegaly, high fever and fatigue. The diagnosis was also confirmed by detecting t(9;22) or BCR/ABL fusion gene (p210 bcr-abl) which is further categorized into b3a2 or b2a2 subtypes on the basis of the BCR breakpoint by Reverse transcription polymerase chain reaction in the molecular oncology lab.

Molecular diagnosis

Selection criteria of patients:

- **Inclusion Criteria:** The study included newly diagnosed CML patients treated with imatinib with a dose of 400 mg to 800 mg/day. All three stages of cases were included. Chronic phase (CP), Accelerated phase (AP), Blast crisis (BC).

- **Exclusion criteria:** The exclusion criteria included chronic myelomonocytic leukemia’s (CML) 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 A and B.

Molecular diagnosis of CML by RT-PCR for BCR-ABL:

- **Multiplex RT-PCR:** From each CML patient venous blood (5ml) or 1ml bone marrow aspirate was collected in EDTA vials. 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. Total RNA was extracted from about 10^6 white blood cells by Trizol assay (TRI® Reagent Sigma CAT# T9424). The cDNA was synthesized using M-MuLV Reverse Transcriptase and other reaction components (Fermentas CAT® K1622). The cDNA product was amplified with 1 µl Taq polymerase, 240 µM dNTP, 1.8 M MgCl₂, and 0.6 µM of the four primers (CA3, C5e, BCR-C, and B2B). Multiplex RT-PCR assay was performed on a PCR machine with the program of 10 sec at 96°C, one min at 96°C, three min at 60°C, two min at 72°C, 10 sec at 100°C, 20 sec at 97°C, 25 sec at 58°C, 25 sec at 60°C, 10 sec at 78°C, 50 sec at 73°C, and 31 times to step five and 10 min at 73°C. The sequence of oligonucleotide primers [13] used in multiplex RT-PCR for BCR-ABL fusion transcripts as the target gene and BCR transcripts as internal control are shown in table 1. The primer combinations in multiplex RT-PCR allowed simultaneous detection of all known types of BCR-ABL and BCR transcripts in one reaction. The expected bands were as follows: 808bp, normal BCR; 481 bp, e1a2; 385bp; b3a2; 310bp, b2a2. The quality of RNA and efficiency of cDNA synthesis were analyzed by amplification of BCR gene as an internal control.

- **Methyl specific PCR analysis:** Genomic DNA was extracted from peripheral blood of CML patients using universal Genomic DNA Extraction Kit (HIMEDIA CAT# MB504). The quality and integrity of the DNA was determined by the A260/280 ratios. DNA quality was also checked by Ethidium Bromide stained of agarose gel electrophoresis (2%).

- **Bisulphite modification of genomic DNA:** Genomic DNA (1µg) was modified with sodium bisulphite using EZ-DNA methylation kit (Zymo research,CAT# D5001). Bisulphite-treated DNA was used for methylation-specific PCR by using previously published primer sets [14], (Table 2) to distinguish between methylated and unmethylated DNA. The PCR products were electrophoresed on a 2% agarose gel. After bisulphite processing, all unmethylated cytosine residues were converted to uracil, whereas the methylated cytosine residues remained unchanged. Bisulphite-modified DNA samples were stored at -80°C until use.

- **Methyl specific PCR:** Modified DNA was PCR amplified with specific primers. Each sample was amplified with two

| Table A Hematological responses Complete or major hematological response | Partial or minor hematological response | Loss or minimal hematological response |
|-------------------------------|--------------------------------------|-------------------------------------|
| Platelet count ≥150 x 10⁹/L | Platelet count <150 x 10⁹/L | Platelet count <450 x 10⁹/L |
| WBC count < 10 x 10⁹/L | Basophils: % 0-5 | WBC count >20 x 10⁹/L |
| Basophils: % >5 | Basophils: % >0-5 | Basophils: % 15 |
| Differential without immature granulocytes. | Presence of blasts | Presence of blasts and promyelocytes in peripheral blood |
| Absence of blasts and promyelocytes in peripheral blood | Spleen: Nonpalpable spleen | Spleen: Palpable spleen |

| Table B Molecular responses Major molecular response | Minimal or No Molecular response |
|---------------------------------|---------------------------------|
| It indicates nonquantifiable and nondetectable bcr-abl gene transcript (BCR-ABL/ABL) ≥0.10 | It indicates quantifiable and detectable bcr-abl gene transcript (BCR-ABL/ABL) ≥0.10 |
| No 3 log reduction of BCR-ABL/ABL | No 3 log reduction of BCR-ABL/ABL |

| C5e | 5′ATAGGATCCTTTGCAACCGGGTCTGAA3′ |
| B2B | 5′ACAAGATTCCGGCTGACCATCAATAAG3′ |
| BCR-C | 5′ACCGCATGTTCCGAGCAGAAG3′ |
| CA3 | 5′TGTTGACTGCGGTTGATGATGTTGCTTG3′ |
| Transcripts | primers used | Size (bp) |
| Control BCR | B2B and C5e | 808bp |
| b2a2 | B2B and CA3 | 310bp |
| b3a2 | B2B and CA3 | 385bp |
| e1a2 | BCR-C and CA3 | 481bp |

Table 1: Sequence of oligonucleotides used in multiplex RT-PCR for detection of BCR-ABLtranscript as the target gene and BCR transcripts as the internal control.
sets of primers, one set for methylated DNA and one set for unmethylated DNA.

The PCR amplification process was carried out after optimization of the reaction conditions. PCR products were resolved in agarose gel, and the bands were visualized by Ethidium bromide staining. MS-PCR was performed under the following cycling conditions: predenaturation at 95°C for 10 min, 45 cycles at 94°C for 40 s, 60°C for 50 s, and 72°C for 45 s, followed by final extension at 72°C for 10 min.

Statistical analysis
The data were processed by the SPSS16 Statistical software. Analysis was performed using the SPSS 16.0 software package. The association between DAPK1 and clinicopathological parameters were statistically analysed using Chi-square and Fisher exact test. P value of < 0.05 was considered statistically significant.

Results
Patient characteristics
Clinical-pathological features including the age and gender of patients, BCR-ABL type, thrombocytopenia, molecular response, haematological response and age group are shown in Table 3. We could reliably detect both a2b2, a2b3 transcripts in CML patients using multiplex PCR. 29.50% patients had a2b2 transcript, 62% patients a2b3 transcript and 8.50% patients had both transcripts.

Frequency of aberrant DAPK1 methylation in CML
The expected product size of 103bp and 98bp were for unmethylated (U) and methylated (M) DNA of DAPK1 promoter methylation. Only those cases were defined as methylation positive if their sample showed a visual band amplified with Methylated specific primers. PCR products were analyzed on 3.5% agarose gel and visualized under UV illumination. Representative example is presented in figure 1. Total frequency of aberrant methylation of DAPK1 gene was 91 out of 200 CML patients. The frequencies of aberrant methylation of DAPK1 were different according to the stages of CML.

Association between DAPK1 aberrant methylation and clinicopathological parameters
A correlation between DAPK1 promoter methylation and the clinical characteristics of CML patients is shown in Table 4. No correlation was found between aberrant DAPK1 methylation and age, BCR-ABL type, molecular response and thrombocytopenia.

DAPK1 aberrant methylation in relation to gender
DAPK1 aberrant methylation was found higher in males than in females. 66 (51.5%) out of 128 males were DAPK1 methylated compared to 25 (34.7%) out of 72 females. Methylation frequencies for DAPK1 showed a significant difference in relation to gender (p value 0.02). Relation of DAPK1 methylation with respect to gender in three stages of CML is represented in Table 5. 28.5% males and 31.25% females were in chronic phase. A significant correlation was found between DAPK1 methylation in chronic phase and in blast phase 51.5% males and 34.7% females were showing DAPK1 methylation. A significant correlation was seen in DAPK1 methylation in stages with respect to gender (p 0.003).

Methylation frequencies during CML progression
DAPK1 promoter methylation was found higher in advanced stages i.e. blast phase and accelerated phase of CML than in patients in chronic phase. Out of 200 CML patients, 81, 54 and 65 patients were in chronic, accelerated and blast phase respectively. DAPK1 aberrant methylation was detected in 91 patients (p > 0.0001). Forty one (63%) patients who showed DAPK1 promoter methylation were in blast phase, 26 (48.1%) in accelerated phase and 24 (29.6%) patients were in chronic phase. A significant correlation was found between methylation frequencies for DAPK1 and stages of CML (p > 0.0001) (Figure 2).

DAPK1 aberrant methylation frequency in haematological resistant patients
DAPK1 aberrant methylation frequency was found higher in patients showing haematological resistance. Among 200 CML patients,
DAPK1 aberrant methylation induced faster disease progression

Follow-up of patients regarding disease progression in terms of survival was performed with median duration of 60 months. A total of 91 patients suffered progression with mean follow up time of 5 years. It was observed that the DAPK1 hypermethylated cases were significantly associated with faster disease progression p < 0.004 (Figure 3).

Discussion

Chronic myeloid leukemia (CML) is a stem cell pluripotent myeloproliferative disorder and consistently associated with the BCR-ABL1 fusion gene. CML most commonly manifests in chronic phase of the disease those progresses to advanced stage disease (blast crisis) that is resistance to tyrosine kinase inhibitor therapy. Hence it is important to understand biological events involved in CML disease progression. Promoter regions methylation of Cpg islands is observed in various types of tumours. Numerous studies have reported the inactivation of tumour suppressor genes by point mutation or chromosomal deletions in the development of cancers. However epigenetic silencing of tumour suppressor genes by promoter region is also common in deletion in the development of cancers. However epigenetic silencing of tumour suppressor genes by point mutation or chromosomal deletion is resistance to tyrosine kinase inhibitor therapy. Hence it is important to understand biological events involved in CML disease progression.

As a consequence, hypermethylation can lead to the abolishing transcriptional activity of specific tumour suppressor genes and can play an important role in the induction of the cancer genesis process. It has been claimed by some authors that 65% of cases of neoplastic diseases may be linked to these epigenetic changes [17]. DAPK kinase is involved in the p53-dependent apoptosis pathway and was first identified as a mediator of IFN-γ-induced apoptosis after IFN-γ treatment [18-20]. DAPK-1 is frequently silenced in human cancers by methylation and demonstrates tumour and metastasis suppressor properties [21]. The frequency of DAPK1 methylation in different malignancies in the world is depicted in figure 4.

Katzenellenbogen et al. found a correlation between the loss of DAPK1 expression and promoter hypermethylation [22]. A scenario that may explain frequent targeting of the DAPK1 promoter by aberrant DNA methylation is the aberrant recruitment of DNA methyltransferase activity to the DAPK1 promoter by oncogenic proteins [23]. The frequency of DAPK1 methylation was found to be 28.1% in urothelial carcinoma in Korean population by Hee Jung et al. [24]. Gonzalez et al. showed 14% DAPK1 aberrant methylation in neuroblastic tumours [25]. Kim et al. detected 60.9% promoter DAPK1 methylation in B-cell lymphoma in Korean population [26]. A study by Ekmeke et al. observed the frequency of DAPK1 methylation in pediatric AML and adult AML by 70% and 55% respectively [27]. 67% methylation was detected in multiple myeloma by Margaret H.L et al. [28] Also 23.20% promoter DAPK1 methylation was detected in childhood acute lymphoblastic leukemia [29]. In South Korean population DAPK1 promoter had 94.3% methylation in Ocular adnexal lymphoma [30]. 62.7% DAPK1 methylation was shown by J Qian et al. in MDS patients [31] where as it was 23.8% in Head and Neck cancer [32]. In different haematological malignancies aberrant 28% DAPK1 methylation was reported [33]. In CML blast crisis 6%

| Clinical feature  | No. of patients | DAPK1 unmethylated | %       | DAPK1 Methylated | %       | P value |
|-------------------|-----------------|-------------------|---------|------------------|---------|---------|
| Patients          | 200             | 109               | 54.5    | 91               | 45.5    | <0.0001 |
| Males             | 128             | 62                | 48.4%   | 66               | 51.5%   | 0.02    |
| Female            | 72              | 47                | 65.2%   | 25               | 34.7%   |         |
| CP-CML            | 81              | 57                | 70.3%   | 24               | 29.6%   |         |
| AP-CML            | 54              | 28                | 51.8%   | 26               | 48.1%   | <0.0001 |
| BC-CML            | 65              | 24                | 36.9%   | 41               | 63.07%  |         |
| A2b2              | 59              | 35                | 59.3%   | 24               | 40.6%   |         |
| A2b3              | 124             | 64                | 51.6%   | 60               | 48.3%   | 0.5     |
| A2b2/ A2b3        | 17              | 10                | 58.8%   | 7                | 41.1%   |         |
| Imatinib          | 200             | 109               | 54.5%   | 91               | 45.5%   |         |
| Age >45           | 71              | 39                | 54.9%   | 32               | 45.07%  | 0.9     |
| Age <45           | 129             | 70                | 54.2%   | 59               | 45.7%   |         |
| MMR               | 50              | 31                | 62%     | 19               | 38%     | 0.4     |
| No MR             | 150             | 78                | 52%     | 72               | 48%     |         |
| MHR               | 30              | 20                | 66.6%   | 10               | 33.3%   |         |
| Minor HR          | 46              | 29                | 63.04%  | 17               | 36.9%   | 0.02    |
| Loss HR           | 124             | 60                | 48.3%   | 64               | 51.6%   |         |
| Thrombocytopenia  | 100             | 52                | 52%     | 48               | 48%     | 0.5     |

Table 4: Association of DAPK1 aberrant methylation with clinicopathological parameters.

| Stage             | No. of patients | No. of Males | DAPK1 methylation | No. of females | DAPK1 methylation | p value |
|-------------------|-----------------|--------------|-------------------|----------------|-------------------|---------|
| Chronic phase     | 81              | 49           | 14 (28.5%)        | 35 (71.5%)     | 32 (31.25%)       | 22/68.7%|
| Accelerated phase | 54              | 35           | 20 (57.1%)        | 15 (42.9%)     | 19 (31.5%)        | 13/68.5%|
| Blast phase       | 65              | 44           | 32 (72.7%)        | 12 (27.3%)     | 21 (42.8%)        | 13/47.2%|
| Total             | 200             | 128          | 66 (51.5%)        | 82 (48.5%)     | 72 (34.7%)        | 47/64.3%|

Table 5: DAPK1 methylation with respect to gender in stages of CML.

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DAPK1 methylation was reported [34] and by Qian J et al showed 51% DAPK1 aberrant methylation in chronic myeloid leukemia patients [35]. Seog et al showed DAPK1 methylation profiles in major types of human cancers as 70% in cervix carcinoma, 71% in stomach cancer, 57% in colon cancer, 13% in liver cancer, 87% in larynx carcinoma, 26% in breast carcinoma and 70% in lung cancer [36]. Review of DAPK1 methylation in different malignancies is shown in table 6.

We investigated the methylation status of the DAPK1 gene in CML chronic phase, CML accelerated phase and CML blast crisis samples. We could not find any correlation between DAPK1 methylation and various clinic pathologic factors including age, chromosomal abnormality, thrombocytopenia and BCR-ABL type.

In this study, our results indicate that DAPK1 methylation is associated with the progression of CML. Among total 91 (45.5%) DAPK1 methylation positive patients, percentage of methylation was found higher in blast phase, accelerated phase than in chronic phase with a significant correlation (p > 0.0001). We also observed a significant correlation between methylation frequencies for DAPK1 gene with respect to haematological resistance in CML (p > 0.02). The frequency of methylation was higher in patients showing minor or minimal haematological response than in patients showing major haematological resistance. Our results showed a significant association of gender with DAPK1 promoter methylation in CML patients (P < 0.02), Males were showing disease progression more than females in relation to DAPK1 methylation. We observed a significant correlation of DAPK1 methylation in three stages of CML with respect to gender (P < 0.003). Hence the assessment of the changes in DAPK1 methylation can be a prognostic factor for CML.

Thus, DAPK1 gene hypermethylation might be a valuable marker for evaluation of treatment outcome in chronic myeloid leukemia.

Table 6: Review of DAPK1 methylation in different malignancies.

| Cancer type                           | Author                | % methylation. |
|---------------------------------------|-----------------------|----------------|
| Multiple Myeloma (26)                 | Margaret et al.       | 67%            |
| Paediatric AML (25)                   | Ekmeck et al.         | 70%            |
| Adult AML (25)                        | Ekmeck et al.         | 55%            |
| Childhood ALL (27)                    | Gutierrez et al.      | 23.2%          |
| B cell lymphoma (24)                  | Sung Kim et al.       | 60.9%          |
| Ocular adenoidal lymphoma (28)        | Ho-Kyung et al.       | 94.3%          |
| Head and neck cancer (30)             | Masayuki et al.       | 23.8%          |
| Myelodysplastic syndrome (29)         | Qian et al.           | 62.7%          |
| Hematological malignancies. (31)      | Tamer et al.          | 28%            |
| CML (32)                              | Eisuke et al.         | 6%             |
| CML (33)                              | Qian et al.           | 51%            |
| Our study                             |                       | 45.5%          |

Figure 2: Frequency of DAPK1 methylation in CML patients with respect to stage.

Figure 3: DAPK1 aberrant methylation induced faster disease progression.

Figure 4: DAPK1 methylation in different malignancies in the world.

Conclusion

The data suggests that disease progression in CML might be closely associated with DAPK1 promoter methylation. Hence, detecting DAPK1 epigenetic changes in DNA may be a promising target for molecular analysis in chronic myeloid leukemia. However, further study will be needed to determine the role of DAPK1 methylation in the prognosis of CML.

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