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Gadd45g Gene Inactivation by Promoter Hypermethylation and Chronic Myeloid Leukemia Progression: Pilot Study

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Abstract
Background*: Chronic Myelogenous Leukemia (CML) is hematological cancer. Based on disease progression CML has three phases: chronic phase, accelerated phase and blast crisis phase. Most of the patients remain undiagnosed until and unless specific test for Philadelphia chromosome was performed. So if CML Patients remains untreated; progress from chronic phase to accelerated and then blast crisis which is medical emergency. So identification of progression of CML from CP to AP and AP to BC in early phase before terminal blast crisis phase is very important. The mechanism that leads to disease progression is not exactly known. There are many possible Genetic and Epigenetic Mechanism that leads to CML progression. Promoter hypermethylation is one of the possible epigenetic mechanisms that leads to inactivation of tumor suppressor gene in hematological malignancies. GADD45G is one of the tumor suppressor gene among panel of tumor suppressor genes involved in cancers especially hematological malignancy. That's why Aim of our study was: Can we use Promoter hypermethylation status of GADD45G gene as follow up prognostic biomarker for CML patients who are high risk for progression? If yes; specific targeted therapy for such epigenetic change must be developed to prevent further progression of CML.*
Materials and methods*: The study was carried out in 30 cases of CML which includes 10 patients of each phase of CML (Chronic Phase, Accelerated Phase, and Blast Crisis) patients and 30 healthy controls. Methylation status of GADD45G gene was evaluated by Methylation specific-PCR*
Results*: Frequency of promoter hypermethylation of GADD45G gene in whole blood of CML patients 40%.*
We observed statistically significant difference in methylation status of genes in whole blood DNA with cases (30 CML patients) and controls (30 healthy controls) (p-value <0.0001 by Fisher exact test), But We found that there is no significant association between methylation status of GADD45G with clinical stages of CML patients (p-value=0.03 by Fisher exact test)
Conclusions*: Promoter hypermethylation of GADD45G is an important mechanism for GADD45G gene inactivation in CML patients. GADD45G gene inactivation leads to progression of CP to AP and AP to BC in CML patients. We found that there is no significant association between methylation status of GADD45G with clinical stages of CML patients (p-value=0.03 by Fisher exact test), But we did this study as pilot study so need to be study with large sample size.*

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I. Introduction

Chronic myeloid leukemia is the commonest adult leukemia in India and the annual incidence ranges from 0.8-2.2/1,00,000 population in males and 0.6-1.6/1,00,000 population in females in India.[1] CML is a clonal disorder that is usually easily diagnosed because more than 95% of patients have a distinctive cytogenetic abnormality, the *Philadelphia chromosome* (*Ph*). The *Ph* results from a reciprocal translocation between the long arms of chromosomes 9 and 22 and is demonstrable in all hematopoietic precursors.[3] This translocation results in the transfer of the Abelson (ABL) on chromosome 9 oncogene to an area of chromosome 22 termed the breakpoint cluster region (BCR).[2] This results in a fused *BCR-ABL* gene that leads to production of an abnormal tyrosine kinase protein that causes the disordered myelopoiesis in CML. Chronic Myelogenous

Leukemia (CML) progress from a chronic phase to more severe accelerated phase and finally terminal blast crisis phase which has high mortality rate and poor prognosis. Identification of CML patient is very important in early phase especially before terminal blast crisis phase because if such patients left as such then it's going to detoriated from CP to AP and AP to BC which is a medical emergency.

The mechanisms that lead to CML progression is not exactly known. There are many cellular and genetic events play a role in CML progression is well known. Cellular events includes decreased apoptosis, differentiation block, decreased immune surveillance and drug resistance while genetic events includes mutation, deletion and polymorphism that leads to inactivation of tumor suppressor genes, activation of oncogenes and impaired DNA repair. There is also role of epigenetic alternation of the DNA such as methylation and acetyalation, ADP ribosyalation; in the regulation of gene expression and thereby causes human cancers.[3-5]Methylation in the promoter region with CpG islands is main mechanism capable of causing gene silencing. Either genetic or epigenetic mechanism are known to inactivate TSGs. Epigenetic inactivation of tumor suppressor genes (TSG) is frequently associated with tumor pathogenesis and progression.[6] The major mechanism of this epigenetic inactivation is through hypermethylation of promoter CpG islands, which leads to the binding of transcription repressors, compressed chromatin and transcription silencing.[6] Inactivation of tumor suppressor gene by DNA hypermethylation has role in cancer initiation and progression. Promoter hypermethylation is one of the possible mechanisms underlying the inactivation of tumor suppressor gene in haematological malignancies. Increasing number of TSGs has been documented with epigenetic inactivation in tumors such as *hMLH1, VHL, BRCA1* and *RASSF1*.[7] CALCA, ESR, HIC1, TFAP2A and ABL1, were found to be frequently methylated in CML.[8-11] Methylation of the ABL1 gene is associated with the progression of CML.

GADD45G (Growth Arrest and DNA Damage inducible gamma) gene is tumor suppressor gene involved in cell growth arrest and DNA damage repair, thus involved in growth regulatory and apoptotic pathway. Implicated as stress sensor that modulates the response of mammalian cell to genotoxic or physiological stress like methyl methane sulfonate, UV radiation, hydroxyurea and ionizing radiation. It is identified as IL2 induced immediate-early gene.[12]

It acts as tumor suppressor gene by activation of p38/c-Jun amino terminal kinase pathway via mitogen activated kinase1/MEKK4 in response to various environmental stresses.[13] They also inhibits the cell proliferation at different stages like G_1S and G_2M checkpoints and induced cell apoptosis.[14] *GADD45G* down-regulated in thyroid cancer[15] and pituitary adenoma.[16] Its hypermethylation seen in gastric, colorectal and pancreatic carcinoma. *GADD45G* down-regulated in 65% hepatocellular carcinoma.[17] *GADD45G* is androgen-responsive and growth inhibitory in prostate cancer cells.[18] Thus *GADD45G* is important tumor suppressor gene and its hypermethylation which has role in cancer initiation and progression. That's why Aim of our study was: Can we use Promoter hypermethylation status of *GADD45G* gene as follow up prognostic biomarker for CML patients who are high risk for progression? If yes; specific targeted therapy for such epigenetic change must be developed to prevent further progression of CML.

II. Materials And Methods

This study was carried out in the Department of Biochemistry in conjunction with the Department of Medicine and Department of Pathology of Maulana Azad Medical College and associated Lok Nayak Hospital, New Delhi. Ethical clearance was given by Institutional Ethical committee.

STUDY DESIGN: Hospital based case control study.

SAMPLE SIZE: This study was done in 30 patients of CML (which includes 10 chronic phase, 10 accelerated phase and 10 blast crisis phase patients) and 30 normal healthy controls.

SELECTION OF CASES:

Inclusion criteria

Diagnosed CML patients confirmed by qualitative PCR for BCR-ABL1.

Exclusion criteria

1. Patients having other myeloproliferative disorders.
2. Chronic myelomonocytic leukemia (CMML).
3. BCR-ABL1 positive adult ALL patients.

SELECTION OF CONTROLS: Age and sex match healthy controls was selected for study.

SAMPLE COLLECTION: 5ml of blood samples was collected in EDTA vial under aseptic conditions from cases and control by venipuncture after getting informed consent and stored at -80 ˚C until analysis.

DNA METHYLATION OF *GADD45G*

Total genomic DNA extracted from whole blood and subjected to bisulfite treatment followed by methylation specific polymerase chain reaction using specific primers and conditions mentioned below. PCR products were directly loaded onto 2% agarose gel, stained with ethdium bromide and visualized under UV illumination.

DNA EXTRACTION:DNAs from whole blood was extracted using Genomic DNA Mini Kit (Genaid, Biotec, Taiwan). Principle: Chaotropic salt was used to lyse cells and degrade protein, allowing DNA to bind to the glass fibre matrix of the spin column. Contaminants were removed using a Wash Buffer (containing Ethanol) and purifies genomic DNA was eluted by a low salt Elution Buffer.) Total genomic DNA extracted from whole blood was subjected to agarose gel electrophoresis. The DNA solutions were stored in -80 ˚C till further analysis.

BISULFITE TREATMENT OF EXTRACTED DNA: Total genomic DNA extracted from whole blood subjected to Sodium bisufite treatment by using BisulFlash™ DNA Modification Kit (Epigentek, New York).

Bisulfite modifications based on the principle that bisulfite treatment of DNA would convert unmethylated cytosine residues into uracil, whereas methylated cytosine residues would remain unmodified. Thus, methylated and unmethylated DNA sequences after bisulfite conversion would be distinguishable by sequence-specific primers.

Modified DNA was stored at –20°C for further analysis

METHYLATION SPECIFIC POLYMERASE REACTION (MSP) ANALYSIS

Promoter CpG island in genes will be examined by MSP. Forward and reverse primers will be used which correspond to the predicted sequence of methylated or unmethylated genomic DNA after sodium bisulfite treatment(Table: 1)

TABLE1: Primer sequence used in Methylation Specific PCR analysis

GENE	DIRECTION	SEQUENCE(5'-» 3')	AMPLICON(BAND) SIZE
P16$^{(INK4a)}$			
UNMETHYLATED	FORWARD	TTATTAGAGGGTGGGGTGGATTGT	150
	REVERSE	CAACCCCAAACCACAACCATAA	150
METHYLATED	FORWARD	TTATTAGAGGGTGGGGCGGATCGC	151
	REVERSE	GACCCCGAACCGCGACCGTAA	151
GADD45G			
UNMETHYLATED	FORWARD	TTAGTTTATATAAGGGTGTG	136
	REVERSE	AACCAACAACTAATTATCCACAAACA	136
METHYLATED	FORWARD	TTATTAGTTTATATAAGGGCGC	139
	REVERSE	AACCAACAACTAATTATCCACAAACG	139

Modified DNA was subjected to MSP reaction (Table: 3). Each reaction was performed in a total volume of 25 µl containing 10 µl Master Mix, a working concentration of 0.25 µl for each primer (as shown in Table 2) and 3 µl of methylated DNA.

TABLE 2: Cocktail Preparation for 1x (1x means for one reaction).

TOTAL VOLUME	25 µl
Master Mix	10 µl
Forward and Reverse Primer	0.25 µl each
Methylated DNA	3 µl
Nuclease free water	11.5 µl

TABLE 3: PCR Conditions.

PCR STEPS	TEMPERATURE AND TIME
1.Initial Denaturation	95˚C- 10 min
2.Denaturation	94˚C - 45 sec
3.Annealing	For *P16$^{(INK4a)}$* Methylated 60˚ C- 45sec For *P16$^{(INK4a)}$* Unmethylated 57˚ C- 45sec For *GADD45G* Methylated 56˚C-45 sec For *GADD45G* Unmethylated 53˚C- 45sec
4.Extension	72˚ C for 45 sec
5.Final extension	72 ˚C for 10 min

Steps 2 to 5 repeated for 40 cycles.

Then amplified products were resolved (along with molecular weight marker) using electrophoresis in 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.(Fig.)

FIGURE1: *GADD45G* METHYLATION STATUS IN CML PATIENTS.

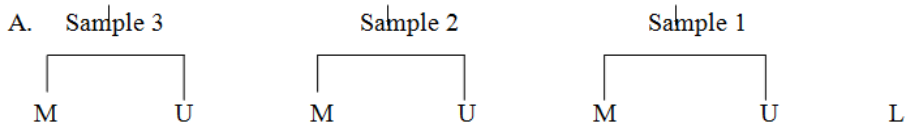


Figure A. Representative samples results showing methylation of *GADD45G* in blood samples DNA of CML patients.(L) (100bp DNA ladder).*GADD45G* methylation(product size:139bp for methylated and 136bp for unmethylated DNA).Lanes M and U,amplified products with primers recognizing methylated and unmethylated sequences, respectively.Sample 1 and 3 showing *GAAD45G* methylation

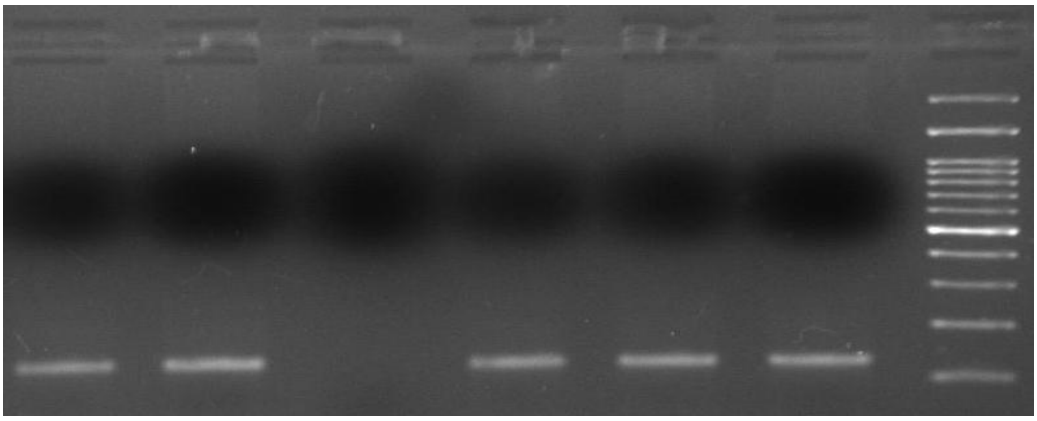
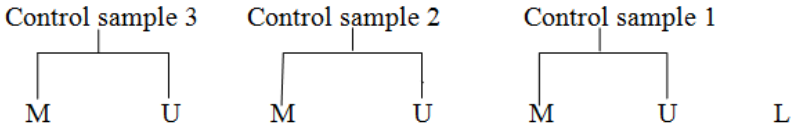


FIGURE 2: *GADD45G* METHYLATION STATUS IN HEALTHY CONTROLS.



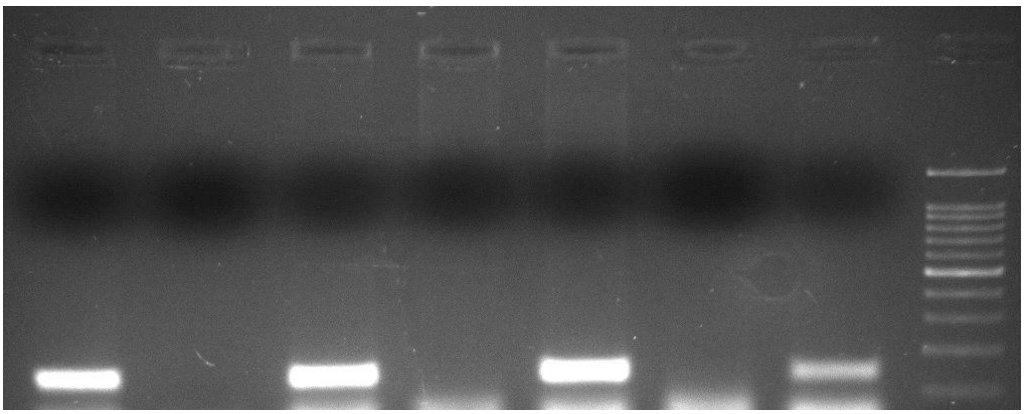


Figure (B).Results showing methylation analysis of *GADD45G* in Blood sample DNA in healthy controls. No methylation was seen in control samples.

Statistical analysis

Statistical analysis was performed using the SPSS 17.

As sample size is small, Fisher exact test was carried out to compare the difference of frequency of hypermethylation in cases and controls and compare the difference of frequencies between groups of CML patients.

p value of ≤ 0.05 is considered as statistically significant

III. Results

In our study age group of patients was ranged from 18-70 yrs and mean age of this group of patients was 37 yrs. The mean age of normal healthy control in our study was 33 yrs which match with CML patients. In our study out of 30 CML patients, 16 were males and 14 were female; whereas in controls group 15 male and 15 female were taken.

Most of the CML patients diagnosed in chronic phase. The untreated/drug resistant patients progress to accelerated phase and/or blast crisis phase.

Table 4: Frequency of promoter hypermethylation of *GADD45G* in chronic myeloid leukemia patients and healthy controls group.

STUDY GROUPS	NO OF SUBJECTS	*GADD45G* METHYLATION POSITIVE	*GADD45G* METHYLATION NEGATIVE	p-value∗
PATIENTS	30	12 (40%)	18 (60%)	<0.0001
CONTROLS	30	0(0%)	30(100%)	
MALE	16	6(37.5%)	10(62.5%)	0.52
FEMALE	14	6(42.9%)	8(57.1%)	
AGE < 45 YRS	8	4 (50%)	4 (50%)	0.39
AGE >45 YRS	22	8 (36.4%)	14 (63.6%)	

∗p-value was calculated by Fisher exact test. As shown in table 4, *GADD45G* is hypermethylated in 40% of cases which is significantly higher than that of controls (p value <0.0001).

Table 5: *GADD45G* hypermethylation status with gender of CML patients

GENDER	NO OF SUBJECTS	*GADD45G* METHYLATION POSITIVE	*GADD45G* METHYLATION NEGATIVE	p-value∗
MALE	16	6(37.5%)	10(62.5%)	0.52
FEMALE	14	6(42.9%)	8(57.1%)	

∗p-value (0.50) was calculated by Fisher exact test. We did not find any significant correlations between the methylation status of *GADD45G* gene and gender of CML patients.

Table 6: *GADD45G* hypermethylation status with age groups of CML patients.

AGE GROUPS	NO OF SUBJECTS	*GADD45G* METHYLATION POSITIVE	*GADD45G* METHYLATION NEGATIVE	p-value∗
>40 YEARS	8	4 (50%)	4 (50%)	0.39
≤40 YEARS	22	8 (36.4%)	14 (63.6%)	

∗p-value (0.50) was calculated by Fisher exact test. We did not find any significant correlations between the methylation status of *GADD45G* gene and age of CML patients.

Table 7: *GADD45G* hypermethylation status with stages of CML patients

STAGES OF CML	NO OF SUBJECTS	*GADD45G* METHYLATION POSITIVE	*GADD45G* METHYLATION NEGATIVE	p-value∗
CHRONIC PHASE	10	3 (30%)	7(70%)	0.89
ACCELERATED PHASE	10	4 (40%)	6(60%)	
BLAST CRISIS PHASE	10	5 (50%)	5(50%)	

∗p-value (0.89) was calculated by Fisher exact test.

GADD45G promoter methylation was detected in 3 (30%) in CP-CML patients, 4 (40%) AP-CML patients and 5 (50%) in BC-CML patients. Methylation increased but could not reach statistical significant level (p -value 0.89) with the acceleration of disease from Chronic phase to Accelerated phase and/Blast crisis phase in CML.

Table 8: *GADD45G* hypermethylation status in relation to early stage (Chronic phase) v/s Advanced stages (Accelerated phase and Blast crisis phase) of CML patients.

STAGES OF CML	NO OF SUBJECTS	*GADD45G* METHYLATION POSITIVE	*GADD45G* METHYLATION NEGATIVE	p-value∗
EARLY STAGE (CP)	10	3(30%)	7(70%)	0.35
ADVANCED STAGE (AP & BC)	20	9 (45%)	11(55%)	

∗p-value (0.35) was calculated by Fisher exact test

Methylation increased with the acceleration of disease from early stage(CP) to advanced stage(AP & BC), taken together, out of 12 total methylated patients, 9 patients were in advanced stage (AP, BC) of the disease but could not reach statistical significant level (p value 0.35).

This suggesting that inactivation of cell cycle control gene by *GADD45G* promoter hypermethylation has no role in the progression of CML.

IV. Discussion

Most genetic aberrations in tumor suppressor genes and oncogenes are associated with CML progression along with other cancers. Besides these, Epigenetic modification of TSGs by Promoter hypermethylation is an early events in development and progression of cancer. epigenetic changes also has role in CML progression. Hypermethylated gene represent potential biological markers for diagnosis and progression of disease, could prove to be useful tool. This is especially important in CML, where disease progress from chronic phase to accelerated and blast crisis phase which is difficult to treat as these stages patient are resistant to standard treatment with TKI. This hypermeyhlation can be reverse by demethylating agent, could useful to reverse back to chronic phase which is responsive to standard treatment by TKI.

GADD45G is most frequently methylated gene in many human cancer. Its inactivation due to hypermethylation has been detected in various cancer cell lines and primary cancer.

Study conducted by **Jianming Ying et al**. to explore frequency of *GADD45G* gene methylation and its correlation with various cancer cell lines and primary cancer reported following frequency of *GADD45G* gene methylation in various cell lines : 85% in Non-Hodgkin's lymphoma 11/13, 50% in Hodgkin's lymphoma 3/6, 33% in Leukemia 1/3, 73% in Nasopharyngeal carcinoma 8/11, 50% in Cervical carcinoma 2/4, 40% in Lung carcinoma 2/5, 29% in Esophageal carcinoma 5/17, 20% in Hepatocellular carcinoma 1/5, 20% in Colorectal carcinoma 1/5, 100% in Laryngeal carcinoma 1/1 and 0% in Breast carcinoma 0/4 and Gastric carcinoma 0/1. They also reported following frequency of *GADD45G* gene methylation in primary lymphomas : 88% in Endemic Burkitt's lymphoma 7/8, 38% in Diffuse large B-cell lymphoma 5/13, 16% in Follicular lymphoma 1/6, 33% in Post-transplant lymphoma 4/13, 63% in Nasal NK/Tcell lymphoma 5/8, 34% in Hodgkin's lymphoma 10/29, 0% in other types of lymphoma 0/11. In Primary carcinomas: 16% in Nasopharyngeal carcinoma 6/38, 11% in Esophageal carcinoma 3/27, 0% in Breast carcinoma 0/20 and Hepatocellular carcinoma 0/6 and 11% in Gastric carcinoma 2/19. Hypermethylation not reported in Immortalized normal epithelial cell lines e.g.RHEK-1, NE1, NE3, NP69(0/4) and Normal tissues like Peripheral blood mononuclear cell(0/12), Lymph node (0/3), Nasopharynx(0/10), Breast tissue (0/7), Esophageal epithelium (0/7).[19]

We investigated *GADD45G* promoter hypermethylation in both cases and controls groups. We found 40% (12/30) of *GADD45G* hypermethylation in whole blood DNA in CML patients.

No methylation was detected in plasma DNA of 30 healthy controls. *GADD45G* Promoter hypermethylation in whole blood DNA were significantly higher in CML patients compared with controls(p value <0.001).

Correlation of methylation status of *GADD45G* with age, gender and clinical stages in plasma sample of CML patients

We found that there was no statistically significant difference between different age group of (> 40yrs and ≤ 40 yrs) CML patients with regards to methylation status of *GADD45G* (p value 0.39).

We observed no significant difference between detection of frequency of hypermethylation of *GADD45G* in different gender (male and female)(p value 0.52).

We observed no significant difference between detection of frequency of hypermethylation of *GADD45G* with clinical staging(CP,AP and BC)(p value-0.89 by Fisher exact test). We observed that there is no statistically significant differenace in $P16^{INK4a}$ hypermethylation status in early stage(Chronic phase) v/s Advanced stages (Accelerated phase and Blast crisis phase) of CML patients(p value 0.35)**.**

V. Conclusion

Our results suggest that GADD45G is a primary target for inactivation by promoter methylation in the disease progression of CML patients and its detection is useful in the follow up of patients with a high risk of developing CML.

No potential conflicts of interest were disclosed.

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