RESEARCH ARTICLE

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Epigenetic Modulation of *DDIT3* and *MGMT* Expression Acts Synergistically with Resistance to Imatinib towards CML Disease Progression: A Hospital based Study

Gautam Hazarika[1,2], Manash Jyoti Kalita[2], Simanta Kalita[2,3], Partha Pratim Das[2,4], Kalpajit Dutta[2], Lipika Lahkar[5], Anjanjyoti Rajkonwar[1], Mohammed Ghaznavi Idris[2], Vinotsale Khamo[6], Giriraj Kusre[1], Subhash Medhi[2]*

Abstract

Introduction: *Imatinib Mesylate* is an authenticated drug that aids in the treatment of Chronic Myeloid Leukaemia and Philadelphia patients which is recognized as a BCR-ABL tyrosine kinase inhibitor. Indeed, DNA Methylation occupies a key role in the stability of chromosomes. **Objective:** Changes in the methylation status of genes may impart to the advancement of Chronic Myeloid Leukaemia. The present investigation aims to assess the role of expression analysis and methylation status of *DDIT3* and *MGMT* genes in imatinib-resistant and nonresistant cases. **Methods:** The Imatinib resistance was screened through RFLP. In this case maximum number of patients were recorded in the chronic phase belonging to the age group 40-59 and the accelerated and blast phase is more common in elderly patients showing the progressive nature of the disease with age. Hemoglobin and platelet count are found to be higher in cases where WBC count was minimal. A history of long-term alcohol consumption is found to be associated with the progression of the disease. **Results:** The maximum level of expression of the *DDIT3* gene was recorded in the chronic phase regardless of upstream (67.8%) and downstream (57.9%) regulation. The highest *MGMT* expression regulation was also observed in the case of chronic phase in both upstream (78.9%) and downstream (44%) regulation. Further, the *MGMT* gene showed the highest methylation of 6.6% and *DDIT3* showed 3.3% in CML cases. **Conclusion:** In the present study notable depletion of survivality was established in the Imatinib resistance patients manifesting genetic malfunction of BCR-ABL transcripts among the North East Indian inhabitants and advocating for the expansion of the disease.

Keywords: Chronic Myeloid Leukemia- Imatinib Resistant- DNA Methylation- *DDIT3- MGMT*

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Introduction

Chronic myeloid leukemia (CML) is a stem cell disorder resulting from BCR-ABL oncogenic transcription (Wang et al., 2010; Annamaneni et al., 2014). Imatinib mesylate(IM) is a validated exclusive treatment aid for CML and Philadelphia patients and is recognised as BCR-ABL tyrosine kinase-specific inhibitor (Fausel et al., 2007). 89% of CML-recognised patients under *Imatinib* therapy exhibit about five years of survivability (Kantarjian et al., 2007). Though *Imatinib* is a noteworthy assistance in the field of CML treatment, it unveiled resistance consoling barely effective (Hochhaus et al., 2013). BCR-ABL kinase mutation is one of the crucial tools that represent the tyrosine kinase inhibitor mechanism of resistance (Rejali et al., 2015; Kantarjian et al., 2007; Dhahi et al., 2013). BCR-ABL mutations were revealed in 40-60% of *Imatinib* mesylate-resistant patients (Hochhaus et al., 2013).

Aberration in DNA Methylation has been recognised in diverse hematopoietic malignancies including CML. A specific gene expression may be change due to promoter region Methylation (Pena et al., 2009). DNA Methylation in CpG islands is a robust phenomenon of gene silencing that may drive malignancy by inactivating crucial tumour - suppressor mechanisms (Jelinek et al., 2011). Hyper-methylation of DNA is a familiar witness in myelogenous leukemia (Kalinkova et al., 2022). Failure of the gene regulation may be accompanied with leukemogenesis and the development of CML.

Several studies has confirmed the role of DNA-damage-inducible transcript-3 (*DDIT3*) (Wang et al., 2010) and

*[1]Department of Anatomy, Assam Medical College &Hospital, Dibrugarh, (Assam) India. [2]Department of Bioengineering & Technology, Gauhati University, Guwahati, (Assam) India. [3]Multi-disciplinary Research Unit, DMC, India. [4]Multi-disciplinary Research Unit, FAAMCH, India. [5]Department. of Botany, Silapathar College, Dhemaji, (Assam) India. [6]Department of Pathology, Naga Hospital Authority, Kohima, (Nagaland) India. *For Correspondence: subhashmedhi@gauhati.ac.in*

MGMT (O6-methylguanine DNA Methyl Transferase) (Oliver et al., 2014), in the regulation of cellular growth and differentiation of CML. *DDIT3* is expressed ubiquitously and can be induced by a wide variety of treatments such as DNA lesion, hypoglycemia, radiation and cellular stress (Scapa et al., 2021; Matsumoto et al., 1996). It was recorded that in myeloid malignancies the level of *DDIT3* transcript has been down-regulated (Wang et al., 2010; Qian et al., 2005). Another transcripts *MGMT*, the DNA repair gene unfasten the methyl groups and devote the O (6) position of guanine (Zuo et al., 2004). Epigenetic silencing of the expression of this gene is customarily noticed in diverse types of malignancies as a ramification of transcriptional silencing persuade by hypermethylation of the CpG island of the promoter of the *MGMT* gene (Oliver et al., 2014; Hibi et al., 2009; Liu et al., 2006)

Despite numerous studies regarding mutations, methylation status and gene expression analysis in tyrosine kinase domain, no convenient reports were recorded from Assam about the interrelation among *Imatinib* resistance and BCR-ABL tyrosine kinase mutations. Thus, the aim in this investigation is to analyze of mutations on BCR-ABL using RFLP along with methylation and expression status of *DDIT3* and *MGMT* gene.

Materials and Methods

Sample Collection

A total of 219 numbers of blood samples were collected from CML patients which was divided into two groups based on *Imatinib* resistance status according to RFLP result. Samples were collected as per approval of the institutional ethical committee of AMCH, Dibrugarh, Assam upon obtaining proper informed consent either from guardian or from patients itself with dully-filled up proforma.

Data collection

Attempts were also made to collect patient level characteristics from the self-administered questionnaire, as well as demographic characterise and life style factors such as tobacco and alcohol use. To account for disease-related changes in smoking and alcohol consumption status, history was assessed for participants who have these habits during their lifetime. For comparison with previous studies and evaluation of effect modification, participant was categorized as "never versus ever" for smoking and alcohol consumption.

Inclusion Criteria

Patient inclusion was according to the confirmed molecular positive report of BCR-ABL transcripts. The patients included in this study having age group in the range of 11-80 years, regardless of any sex, patents having prior illness was not included. Peripheral blood was collected in 2ml EDTA vial.

To consider patients as *Imatinib* resistant, firstly, a dose of 400 mg daily had to be taken as *Imatinib* therapy. Secondly, succeeding three months of treatment in which WBC was higher than 10×10^9/L or platelet count was higher than 450×10^9/L. furthermore, peripheral blasts had to be observed in the blood smear along with immature granulocytes had to be higher than 5%. In addition, patients with transformation to AP- accelerate or BP-blast following an interval of time remaining at CP, were distinguished as *Imatinib* resistant. Moreover, patients who had been under tyrosine kinase inhibitors (TKIs) treatment for more than four years, had used second or third generation of TKI drugs or under gone *Imatinib* dose hike up to 500 mg or 600 mg daily, countered in the study.

The patients who did not attain the mentioned condition were not included in the study. Thus, we did not separately mention the exclusion criteria.

RNA extraction and cDNA Preparation:

RNA was extracted using a commercial kit (QIAamp RNA Blood Mini Kit, QIA-GEN, Germany) and 1µg of RNA was converted to cDNA using cDNA synthesis kit (Verso cDNA Synthesis Kit, Thermo-scientific, USA).

Imatinib resistant Mutation Screening

BCR-ABL positive cases were further carried out for restriction fragment length polymorphism (RFLP) analysis of four most common (Y253H, T315I, E255K and M351T) mutations. Mutation specific restriction enzymes (RsaI, DdeI, MnlI and NcoI) (Rejali et al., 2015) were applied for unveiling the proportion of mutated and un-mutated alleles.

Expression analysis of DDIT3 and MGMT

Expression analysis of *DDIT3* and *MGMT* gene was performed in ABI QuantStudio3 Real-time PCR using SYBR green (RR420A Takara) method. Relative expression level of the gene was estimated taking the expression level of housekeeping gene β-actin as reference. $2^{-\Delta\Delta Ct}$ method. Expression status was carried out in triplicate and healthy individuals was included as control.

Methylation- specific PCR analysis (MSP)

DNA was extracted from peripheral blood samples using QIAamp DNA blood mini extraction kit (QIA-GEN, Germany), its quality was evaluated by agarose gel electrophoresis and quantified in Nano-Drop. 1µg of genomic DNA was modified using the Epitech DNA bisulfide Modification Kit (QIA-GEN, Germany).

The methylation status in the CpG Island of the promoter region of *DDIT3* and *MGMT* was analysed by methylation specific PCR(MSP) (Wang et al., 2010; Hong et al., 2016). PCR amplification was carried out in Arktik Thermo-scientific using Master Mix (M7505 Promega). PCR products were analysed on 2.5% Agarose gels and visualized under Gel Documentation system (Science instruments, CLINX). Healthy individuals have been used for promoter methylation studies as control.

Statistical analysis

Statistical analysis was performed using SPSS (Version 24, Chicago, IL, USA). ANOVA was performed to analyse the significant mean difference between continuous variables i.e. different patient parameters and CML stages

among the studied population. Chi square test (χ^2) was performed to analyse the significant association between categorical variables i.e. Age, sex, Hb% etc. and different disease stages. Expression of *MGMT* and DDIT was presented as mean ±SD and ANOVA was performed to find the significant association between different patient parameters. Multinomial logistic regression analysis was performed to find the independent risk factors associated with disease stages considering chronic stage as reference. All the tests were 2-tailed and considered significant when the p value <0.05. Kaplan-Meier survival analysis was carried out using the log-rank test and univariate analysis was constructed using Cox's regression model. The nonparametric Mann-Whitney U test was selected for the association study with different lifestyle factors and clinicopathological parameters.

Results

Clinical observation and Analysis

The results of RFLP method for *Imatinib* for unveiling the proportion of mutated and unmutated alleles for (Y253H, T315I, E255K and M351T) selected mutations. Among these 122 numbers of samples did not show resistant pattern but total 40 samples had resistant and 57 samples were heterozygous that is single allele resistant.

Analysis of association of different clinic pathological and life style factor with CML Progression

CML disease progression from chronic stage to blast crisis stage was found to be significantly associated (p=0.001<0.05) with increasing age of the patients. Majority of the blast stage patients (N=21, 61.76%) belongs to age group 60-79 years with mean age 65.4±4.3. while patients diagnosed with accelerated (N=31, 88.57%) and chronic stage (N=70, 63.06%) of CML were found from the middle age group 40-59 years. The mean difference of blast cell count between chronic and accelerated stage was 4.170 (p=0.013<0.05) and that with blast crisis stage was 13.5 (p=0.001<0.05). Further laboratory diagnosis of BCR-ABL translocation type (B3A2, p=0.014 and B2A2, p=0.028) also exhibited significant association with chronic to blast crisis progression. Resistance to scheduled prescribed drug *Imatinib* also showed significant impact on disease progression (Table 1).

Among different life style factors, alcohol consumption habit was also found to be significantly (p=0.001<0.05) associated with progression from chronic CML to blast crisis stage with increasing duration. Further analysis of altered expression of *DDIT3* and *MGMT* among the stage stratified CML patients was significant. Overall fold change of *DDIT3* among chronic cases was 1.03±0.904 and that among accelerated and blast crisis cases were 0.87±0.68 and 0.38±0.49 respectively, representing a significant reduction upon progression from accelerated to blast crisis stage (p=0.001<0.05). Similar kind of results were obtained for fold change of *MGMT* (1.57±1.39, 0.705±0.68 & 0.48±0.75) (Table 1).

Association of expression of DDIT3 and MGMT with different parameters cum life style factors

DDIT3 expression was observed to be reduced significantly among age group 60-79 and above (0.35±0.49, p=0.005<0.05). A similar expression pattern was observed for *MGMT* expression with mean expression value of 0.57±0.78, p=0.05 among age group 60-79 years. Further, with advancing splenomegaly from mild to massive, a significant down-regulation of *DDIT3* and *MGMT* gene expression was observed (p=0.015<0.05, and p=0.001 respectively). With advancing disease stage the mean expression of *DDIT3* and *MGMT* reduced significantly from 1.03±0.90 in chronic to 0.38±0.49 at blast stage (p=0.008<0.05) and from 1.57±1.39 to 0.48±0.75 (p=0.001) respectively. *DDIT3* expression was down-regulated among smokers in comparison to non-smokers (695±0.83,p=0.001<0.05). Similarly, *MGMT* expression pattern was also found to be down-regulated among smokers (0.97±1.03,p=0.007<0.05). Further analysis of association between expression alteration and smoking frequency shows simultaneous reduction in mean expression of *DDIT3* (P=0.001) and *MGMT* (P=0.049) with increasing smoking frequency. Alcohol consumption and its duration also exhibited significant impact on altered expression of *MGMT* and *DDIT3*. Alteration of mean expression of *DDIT3* and *MGMT* was found to be marginally significant among different drug resistant group (Table 2).

Factors associated with CML disease progression by logistic regression analysis

Multinomial logistic regression analysis showed increasing age of detection (OR=1.323, 95% C.I. =1.15-1.52, P=0.001), splenomegaly (OR=26.85, 95% C.I.=7.423-97.18, P=0.001), Blast count(OR=1.035, 95% C.I.=0.961-1.115, P=0.059), Promyelocyte (OR=1.15, 95% C.I.=0.986-1.341, P=0.076), Myelocyte (OR=1.094, 95% C.I.=0.963-1.242, P=0.016), and Metamyelocyte count (OR=0.960, 95% C.I.=0.862-1.068, P=0.045) as independent predictors of disease progression. Further, Smoking tobacco and drinking alcohol was also found have considerable impact on disease progression in patients with chronic CML. Although the altered expression of *DDIT3* and *MGMT* exhibit a statistically non-significant association with CML disease progression, however the observed OR is suggestive of its probable impact upon disease progression (Table 3).

Promoter methylation analysis of *DDIT3* showed un-methylated CpG islands within the promoter region of 98(44.74%) cases. 75(34.24%) CML positives cases were found to possess partially methylated promoter region and in rest of the 46(21%) cases promoter regions were found to be hypermethylated. Among the chronic cases, the mean expression of *DDIT3* was found to be 1.64 ± 0.67 while promoter CpG islands were un-methylated. The expression was reduced to 0.35± 0.22 in partially methylated condition and the mean expression reduced further to 0.039 ±0.003 under hypermethylated condition of promoter CpG islands. The reduction in mean expression was found to be significant with p=0.001<0.05. Further stratification based on drug resistance status,

Table 1. Frequency of Occurrence of Different Disease Stages among the CML Positive Patients Stratified based on Different Clinicopathological Parameter and Life Style associated Factors.

Parameters	Frequency (N)	CML Stages			P value	
		Chronic	Accelerated	Blast	P1	P2
Age group						
10-30 yrs	N=39	N=36 (92.3%)	N=2 (6.06%)	N=1		
		23.55 ± 4.5	26.5 ± 2.1	30 ± 0.00	0.001	0.001
40-59 yrs	N=112	N=70 (62.5%)	N=31 (27.7%)	N=11 (9.8%)		
		46.74 ±4.24	48.77 ±4.9	47.9±9.01		
60-79 yrs	N=27	N=4 (14.81%)	N=2(7.4%)	N=21 (77.77%)		
		63.25±2.36	63.5±3.5	65.4±4.3		
80 yrs & above	N=2	N=1 (50%)	0	N=1 (50%)		
		80.00±0.00	N=0	81.00± 0.00		
Hb%	N= 219	N=138 (63.01%)	N=46 (21%)	N= 35 (15.9%)	0.53	0.608
		16.61 ±93.35	8.65± 2.37	9.38± 2.45		
WBC	N= 219	N=138 (63.01%)	N=46 (21%)	N= 35 (15.9%)	0.08	0.511
		1.9 ± 1.41	4.44 ±18.87	2.99±0.98		
Platelet	N= 218	N=137 (62.84%)	N=46 (21.1%)	N= 35 (16.05%)	0.949	0.014
		3.1±2.05	3.1±1.75	2.24±1.42		
Blast	N= 219	N=138 (63.01%)	N=46 (21%)	N= 35 (15.9%)	0.013	0.001
		6.3±6.93	10.47±7.6	19.8±17.6		
Myelocyte	N= 219	N=138 (63.01%)	N=46 (21%)	N= 35 (15.9%)	0.001	0.001
		11.68±5.4	15.65±4.51	16.22±7.02		
Promyelocyte	N= 219	N=138 (63.01%)	N=46 (21%)	N= 35 (15.9%)	0.009	0.001
		7.97±4.81	10.58±6.84	15.17±7.57		
Metamyelocyte	N= 219	N=138 (63.01%)	N=46 (21%)	N= 35 (15.9%)	0.011	0.001
		11.86±5.76	14.6±5.8	18.1±8.7		
Gender						
Male	N=142	N= 91 (64.08%)	N=30 (21.12%)	N= 21 (14.7%)	0.86	0.256
Female	N=77	N= 47 (61.03%)	N= 16 (20.77%)	N= 14 (18.18%)		
Laboratory Diagnosis						
B3A2	N=144	N= 86(59.72%)	N= 33 (22.91%)	N= 25 (17.36%)	0.014	0.028
B2A2	N=74	N= 51 (68.91%)	N= 13 (17.56%)	N= 10 (14.08%)		
Smoking category						
Yes	N=104	N=51 (49.03%)	N=27 (25.96%)	N=26 (25%)	0.347	0.003
No	N=115	N=87 (75.65%)	N=19 (16.5%)	N=9 (7.82%)		
Smoking Frequency						
1-5 times/day	N=61	N=32 (52.45%)	N=18 (29.5%)	N=11 (18.03%)	0.012	0.001
6-10 times/day	N=35	N=15 (42.8%)	N=9 (25.7%)	N=11 (31.42%)		
16-20 times/day	N=2	N=1 (50%)	N=0	N=1 (50%)		
Smoking Duration						
1-10 yrs	N=30	N=16 (53.33%)	N=12 (40%)	N= 2 (6.67%)	0.055	0.001
11-20 yrs	N=33	N=20 (60.6%)	N=6 (18.2%)	N=7 (21.2%)		
21-30 yrs	N=37	N=15 (40.54%)	N=9 (24.30%)	N=13 (35.13%)		
31-40 yrs	N=4	N=0	N=0	N=4 (100%)		
Alcohol Category						
Yes	N=103	N=46 (44.6%)	N=30 (29.12%)	N=27 (26.21%)	0.001	0.001
No	N=116	N=92 (79.3%)	N=16 (13.79%)	N=8 (6.89%)		
Alcohol Duration						
1-10 yrs	N= 38	N= 28 (73.68%)	N=8 (21.05%)	N=2 (5.26%)	0.013	0.001
11-20 yrs	N=44	N=18 (40.9%)	N=17 (38.63%)	N=9 (20.45%)		
21-30 yrs	N=25	N=4 (16%)	N=5 (20%)	N16 (64%)		

Table 1. Continued

Parameters	Frequency (N)	CML Stages			P value	
		Chronic	Accelerated	Blast	P1	P2
Giddiness						
Yes	N=55	N=38 (69.09%)	N=9 (16.3%)	N=9 (16.3%)	0.269	0.337
No	N=163	N=100 (61.3%)	N=37 (22.69%)	N=37 (22.69%)		
Drug resistance						
Non Resistant	N= 122	N= 114 (93.44%)	N= 2 (1.63%)	N= 6 (4.9%)	0.001	0.001
Carrier	N= 57	N=18 (31.57%)	N=38 (66.67%)	N= 1 (1.75%)		
Resistant	N=40	N=6 (15%)	N= 6 (15%)	N= 28 (70%)		
DDIT3 Fold Change						
Overall Fold change	N= 219	1.03 ± 0.904	0.87 ± 0.68	0.38 ±0.49	0.233	0.001
DDIT3 expression Regulation						
Up Regulation	N=112	N=76 (67.8%)	N=24 (21.4%)	N=12 (10.7%)	0.733	0.028
Down regulation	N= 107	N= 62 (57.9%)	N=22 (20.5%)	N= 23 (21.49%)		
MGMT Fold Change						
Overall Fold change	N=219	1.57 ± 1.39	0.705± 0.68	0.48 ± 0.75	0.001	0.001
MGMT expression regulation						
Up Regulation	N= 119	N=94 (78.9%)	N= 16 (13.44%)	N=9 (7.56%)	0.001	0.001
Down regulation	N=100	N= 44 (44%)	N= 30 (30%)	N=26 (26%)		

P, probability; P1, compares chronic stage with Accelerated stage; P2, compares Chronic with Blast stage; p<0.05 considered as significant.

Table 2. Association of Expression of *DDIT3* and *MGMT* (Mean ±SD) with Different Clinicopathological Cum Life Style associated Factor

	N	*DDIT3*		*MGMT*	
		mRNA expression	P-value	mRNA expression	P-value
Duration of age					
10- 39 years	78	0.96±0.82		1.3±1.17	
40- 59 years	112	0.97±0.86	0.005	1.31±1.39	0.05
60 - 79 years	27	0.35±0.49		0.57±0.78	
80 years & above	2	0.95±1.34		1.18±1.66	
Gender					
Male	142	0.942±0.90	0.269	1.29±1.32	0.234
Female	77	0.811±0.70		1.07±1.16	
Splenomegaly					
Mild	108	1.04±0.88	0.015	1.51±1.3	0.001
Moderate	72	0.83±0.81		1.11±1.24	
Massive	39	0.602±0.65		0.57±0.74	
Disease Stage					
Chronic	138	1.03±0.90	0.008	1.57±1.39	0.001
Accelerated	46	0.87±0.68		0.705±0.68	
Blast	35	0.38±0.49		0.48±0.75	
Smoking Category					
Yes	104	0.695±0.83	0.001	0.97±1.03	0.007
No	115	1.07±0.805		1.43±1.42	
Smoking Frequency					
1-5 times/day	61	0.71±0.81	0.001	0.99±0.96	0.049
6-10 times/day	35	0.58±0.82		0.903±1.08	
16-20 times/day	2	0.006±0.005		0.52±0.73	

Table 2. Continued

	N	*DDIT3*		*MGMT*	
		mRNA expression	P-value	mRNA expression	P-value
Smoking Duration					
1-10 yrs	30	0.56±0.69	0.01	0.767±0.69	0.037
11-20 yrs	33	0.72±0.92		1.24±1.12	
21-30 yrs	37	0.81±0.87		0.95±1.17	
31-40 yrs	4	0.62±0.71		0.47±0.87	
Alcohol habit					
Yes	103	0.825±0.89	0.237	1.02±0.99	0.037
No	116	0.95±0.78		1.38±1.46	
Alcohol habit					
1-10 yrs	38	0.717±0.91	0.027	1.07±0.93	0.034
11-20 yrs	44	1.03±0.93		1.21±1.08	
21-30 yrs	107	0.48±0.58		0.58±0.75	
Lab Diagnosis					
B3A2	144	0.795±0.825	0.262	1.23±1.26	0.861
B2A2	74	1.08±0.841		1.24±1.30	
Drug Resistance					
Non Resistant	122	1.15±0.90	0.064	1.74±1.3	0.051
Resistant	40	0.34±0.47		0.235±0.62	
Carrier	57	0.74±0.64		0.742±0.62	

P<0.05 was considered as statistically significant.

significant reduction in *DDIT3* expression was observed with changing promoter methylation pattern, with minimum mean expression level of 0.002±0.001 in hypermethylated drug resistant Chronic cases. A similar pattern of reduction in *DDIT3* expression with advancing promoter methylation was also observed among patients diagnosed with accelerated stage as well as with blast crisis stage. The observations were in close association with *Imatinib* resistance status of the patients apart from its significant association with disease progression (Table 4).

Promoter methylation analysis of *MGMT* showed significant reduction in mean expression with increasing promoter methylation from un-methylated to hyper methylated condition. Among chronic cases, mean expression with un-methylated promoter was 2.38±1.49 (n=68), and 0.99±0.59 with partially methylated promoter and further reduced to 0.27±0.22 (p=0.000) with highly methylated promoter. *Imatinib* resistance status was found

Table 3. Multinomial Logistic Regression Analysis of Factors associated with the Development Accelerated and Blast Stages in Patients with Chronic CML.

	Accelerated		Blast	
	OR (95% C.I)	p-value	OR (95% C.I)	p-value
Age	1.323 (1.15-1.522)	0.001	1.478 (1.139-1.918)	0.003
Gender	1.160 (0.343-3.921)	0.811	6.132 (0.506-74.39)	0.154
Hb%	0.824 (0.640 – 1.063)	0.136	1.007 (0.958-1.06)	0.774
Splenomegaly	26.85 (7.423-97.18)	0.001	127.05 (15.15-1063.8)	0.001
WBC count	1.034 (0.85-1.25)	0.73	1.115 (0.905-1.37)	0.306
Platelet	1.051 (0.782-1.413)	0.74	0.264 (0.089-0.785)	0.017
Blast	1.035 (0.961-1.115)	0.059	1.129 (0.998-1.277)	0.054
Promyelocyte	1.150 (0.986-1.341)	0.076	1.282 (1.02-1.613)	0.033
Myelocyte	1.094 (0.963-1.242)	0.016	0.916 (0.670-1.253)	0.584
Metamyelocyte	0.960 (0.862-1.068)	0.045	1.064 (0.846-1.340)	0.596
Smoking Habit	0.974 (0.304-3.124)	0.965	0.003 (0.005-0.348)	0.017
Alcohol Habit	0.235 (0.07-0.785)	0.019	0.02 (0.001-0.359)	0.008
DDIT3 expression regulation	1.022 (0.294-3.553)	0.973	2.215 (0.203-24.13)	0.514
MGMT expression regulation	3.201 (0.895-11.45)	0.074	0.468 (0.030-7.22)	0.587

OR, Odds ratio; C.I, 95% confidence interval

Table 4. Analysis of Altered Expression of *DDIT3* and *MGMT* in Different Disease Stage Considering the Imatinib Resistance Status among the CML Positive Patients.

Methylation status	*DDIT3* expression						Resistance status
	Chronic	p Value	Accelerated	p Value	Blast	p Value	
Unmethylated (UM)	1.6±0.67		1.99± 0.028		1.26± 0.09		Non resistant (homozygous)
	(N=78)	0.636	(N=2)	0.032	(N=3)	0.001	
	1.59±0.42		1.56± 0.35		0		Single allele resistant (heterozygous)
	(N=2)		(N=13)				
	0		0		0		Resistant (homozygous)
Partially methylated (PM)	0.118±0.15		0		0.28±0.16		Non resistant
	(N=30)	0.001		0.352	(N=3)	0.054	(homozygous)
	0.507±0.38		0.65± 0.42		0.027± 0.00		Single allele resistant (heterozygous)
	(N=13)		(N=19)		(N=1)		
	1.10±0.00		1.07± 0.00		0.796± 0.46		Resistant
	(N=1)		(N=1)		(N=7)		(homozygous)
Hyper methylated (HM)	0.0039± 0.001		0		0		Non resistant
	(N=6)	0.03		0.101		0.001	(homozygous)
	0.0073± 0.004		0.008±0.005 (N=6)		0		Single allele resistant (heterozygous)
	(N=3)						
	0.002± 0.001		0.585±0.262 (N=5)		0.153±0.323		Resistant
	(N=5)				(N=21)		(homozygous)
MGMT expression							
Unmethylated (UM)	2.51±1.51		1.6±0.33		0		Non resistant
	(N=61)	0.1	(N=2)	0.158		0.02	(homozygous)
	1.25±0.55		1.49±0.29		2.22±0.00		Single allele resistant (heterozygous)
	(N=6)		(N=13)		(N=1)		
	1.13±0.00		1.12±0.79		0.232±0.65		Resistant
	(N=1)		(N=2)		(N=9)		(homozygous)
Partially methylated (PM)	1.124±0.57		0		0		Non resistant
	(N=41)	0.001		0.001		0.001	(homozygous)
	0.464±0.322		0.375±0.03		0		Single allele resistant (heterozygous)
	(N=10)		(N=21)				
	0		0		0.212±0.55		Resistant
					(N=3)		(homozygous)
Hyper methylated (HM)	0.31±0.29		0		0.97± 0.71		Non resistant
	(N=12)	0.084		0.431	(N=6)	0.196	(homozygous)
	0.235±0.02		0.048± 0.08		0		Single allele resistant (heterozygous)
	(N=2)		(N=4)				
	0.0038±0.002		0.011± 0.014		0.46± 0.75		Resistant
	(N=5)		(N=4)		(N=11)		(homozygous)

P <0.05 considered significant, One-Way ANNOVA was performed

to be associated with partially methylated state of the promoter. As such, the observation is suggestive of the possible synergistic effect of altered *DDIT3* and *MGMT* expression due to epigenetic modulation and resistance to the prescribed drug *Imatinib* , towards progression of CML from chronic to blast crisis stage (Table 4).

Survival analysis

The study was carried out for a duration of 48 months by following up the patients and the Kaplan-Meier survival analysis was carried out with different factors to check their role in disease progression. The mean survival time reduced significantly with increasing age of patients at the time of CML detection. The mean survival duration was found to be <40 months for the age group of 60 years and above. With advancing splenomegaly, the mean survival time of patients decreased significantly (p=0.001<0.05). The patient survival rate drops further with massive splenomegaly (n=38) having 22 death (57.89%) with mean survival duration of 25.31± 6.5 months. With the advancement of the disease stage from chronic to blast stage the overall survival of the patients decreased significantly up to 29 months (p=0.001<0.05). Individual with alcohol consumption habit were found to have a mean survival duration of 40 months (p=0.021<0.05). Among the different drug resistance categories, *Imatinib* resistance groups exhibit an enhanced progression of the disease and a reduced survival duration of 31 months. Analysis of association of patient's survival with altered expression of *DDIT3* and *MGMT* also exhibited significant impact

Table 5. Survival Analysis for Different Clinicopathological Parameter and Life Style Factor under Consideration.

Parameter	Grouping	Frequency (N)	Mean estimate	S.E	95% C.I.		P Value
					Lower Bound	Upper bound	
	10-39	63	44.92	0.83	43.28	46.55	
Age group	40-59	106	44.12	0.75	42.64	45.6	0.001
	60-79	25	31.84	2.37	27.18	36.49	
	80 and above	2	35	9.19	16.98	44.01	
Gender	Male	127	42.78	0.78	41.25	44.309	0.515
	Female	69	42.609	1.21	40.23	44.98	
Splenomegaly	Mild	95	46.28	0.47	45.34	47.22	
	Moderate	63	42.07	1.15	39.81	44.34	0.001
	Massive	38	34.86	1.98	30.98	38..74	
Stage	Chronic	117	46.91	0.25	46.41	47.41	
	Accelerated	44	42.52	1.09	40.38	44.66	0.001
	Blast	35	28.94	1.9	25.14	32.74	
Smoking category	Yes	95	40.68	1.07	38.57	42.79	
	No	101	44.63	0.73	43.19	46.06	0.075
Smoking duration	1-10	24	41.79	1.72	38.41	45.16	
	11-20	32	43.28	1.63	40.07	46.49	0.009
	21-30	35	38.68	1.96	34.84	42.52	
	31-40	4	30.75	5.44	20.07	41.42	
Smoking frequency	1-5	53	40.81	1.41	38.03	43.58	
	6-10	35	40.65	1.83	37.05	44.25	0.019
	16-20	1	26	0	26	26	
Alcohol category	Yes	93	40.25	1.12	38.04	42.47	
	No	103	44.94	0.65	43.65	46.22	0.021
Alcohol duration	1-10	30	43.73	1.5	40.79	46.67	
	11-20	41	41.8	1.5	38.86	44.74	0.009
	21-30	23	33.3	2.63	28.13	38.47	
Giddiness	Yes	50	43.66	1.12	41.44	45.87	
	No	145	42.53	0.78	40.99	44.07	0.87
Lab diagnosis	B3A2	132	42.57	0.8	40.99	45.19	
	B2A2	63	42.93	1.15	40.67	45.19	0.58
Drug resistance	Non resistant (Homozygous)	107	46.17	0.4	45.38	46.96	0.001
	Resistant (Homozygous)	39	31.1	2.01	27.15	35.05	
	Single allele Resistant (Heterozygous)	50	44.38	0.85	42.7	46.05	
DDIT3 regulation	Up regulation	104	44.61	0.65	43.33	45.89	
	Down regulation	92	40.57	1.15	38.32	42.82	0.2
MGMT regulation	Up regulation	107	45.53	0.56	44.42	46.64	
	Down regulation	789	39.33	1.18	37.02	41.65	0.001

Kaplan Meir survival analysis was performed for individual covariates, P<0.05 was considered significant

on overall survival of the patients. Among the *DDIT3* up-regulation group of patients (n=104), mean survival was 45 months and that within the down-regulation group (n=92) was 41 months (p=0.020<0.05). CML cases with *MGMT* up-regulation (n=107) the mean survival time was 46 months and among down-regulation group (n=89) survival duration was reduced to 39 months (p=0.001) (Table 5).

Cox regression analysis and Hazard outcome:

Univariate cox regression analysis showed hazard ratio as the ratio of the hazard outcomes corresponding to the conditions represented by two groups of a variables. A hazard ratio of 1 represents that there is no survival difference between the two groups whereas a hazard ratio of less than or more than 1 represents considerable impact on survival of one of the two groups. In this study, the hazard (mortality of patients) ratio for older age group (70 years and above) patients was found to be 1.834 times higher (p=0.055). Similarly, a hazard ratio of 0.227 (p=0.001) and 0.465 (p=0.010<0.05) was obtained for patients with moderate and massive splenomegaly respectively in comparison to mild stage. Upon comparing chronic CML stage with accelerated stage, a hazard ratio

Table 6. Hazard Analysis (p<0.05, significant)

Parameter	Group1/Group 2	Frequency (Group 1/ Group 2)	P value	Hazard Ratio	95% C.I.	
					Lower Limit	Upper Limit
Age group	10-39/40-59	63/106	0.396	0.42	0.056	3.133
	10-39/60-79	63/25	0.316	0.361	0.049	2.652
	10-39/ 80 and above	63/2	0.055	1.834	0.245	13.746
Gender	Male/ Female	127/69	0.518	1.185	0.707	1.986
Splenomegaly	Moderate/Mild	36/95	0.001	0.227	0.124	0.414
	Massive/Mild	38/95	0.01	0.465	0.26	0.83
Stage	Accelerated/Chronic	44/117	0.001	1.09	0.051	0.16
	Blast/ Chronic	35/117	0.001	2.34	0.132	0.441
Smoking duration	11-20yrs/1-10yrs	32/24	0.079	0.313	0.086	1.142
	21-30yrs/1-10yrs	35/24	0.01	0.173	0.046	0.655
	31-40yrs/1-10yrs	Apr-24	0.13	0.385	0.112	1.325
Smoking frequency	6-10times/1-5times	35/53	0.055	0.136	0.018	1.044
	16-20times/1-5times	Jan-53	0.051	0.128	0.016	1.006
Smoking Habit	Yes/No	95/101	0.0079	1.54	0.951	2.494
Alcohol intake	Yes/No	93/103	0.023	1.751	1.079	2.84
Alcohol duration	11-20yrs/1-10yrs	41/30	0.008	1.32	1.167	2.845
	21-30yrs/1-10yrs	23/30	0.019	1.418	1.167	2.845
Giddiness	Yes/No	50/145	0.879	1.043	0.558	1.648
Lab diagnosis	B3A2/B2A2	132/63	0.588	0.871	0.529	1.435
Resistance	NR/R/single allele R	107/39	0.081	1.26	0.972	1.633
DDIT3 Regulation	Up regulation/down regulation	104/92	0.027	1.361	0.843	2.198
MGMT regulation	Up regulation/ down regulation	107/89	0.001	2.291	1.405	3.736

of 1.090 (p=0.001) was observed and with blast crisis stage the same was found to be 2.34 (p=0.001), which is suggestive of an increasing mortality with advancement of the CML disease stage. Similarly, a hazard ratio of 1.751 was observed upon comparing alcoholic individuals with non-alcoholic (p=0.023<0.05) and the same was found to be significantly associated with alcohol consumption duration (11-20yrs/ 1-10yrs, HR=1.32, p=0.008 and 21-30yrs/1-10yrs, HR=1.418, p=0.019). Further the hazard ratio was also found to be associated with drug resistance status of the patients. Upon comparing non-resistant with single allele resistant (heterozygous) and resistant (homozygous) patients, a hazard ratio of 1.260 was obtained which was marginally significant (p=0.081). Analysis of hazard outcome corresponding to altered promoter methylation and thereby expression of *DDIT3* and *MGMT* was found to be significant. Comparison of up-regulation and down-regulation of *DDIT3* showed a HR of 1.361 (p=0.027) and that for *MGMT* expression was found to be 2.291 (p=0.001) (Table: 6).

Discussion

The phases of CML determines based on number of immature WBC in blood or bone marrow that progress from relatively innocuous chronic phase to fatal blast phase (Granatowiz et al., 2015; Sawyers et al., 1999). In the present study we found the maximum number of patients were in between the age of 40-59 years, which is dominated by chronic phase of CML, whereas accelerated and blast phase were predominant among old age population (>60 years). Thus, the disease can become more progressive with the age, however the cause of which is difficult to accurately presume. A previous study on adolescent and children in East India also found chronic phase is the most common phase of CML with asthenia, splenomegaly and splenic discomfort were predominant presenting symptoms (Raut et al., 2013). The median haemoglobin percentage and platelet count were found higher in chronic phase while WBC count found lower in chronic phase compared to other two group. Whereas, a past study from France described no significant difference on leucocyte count, haemoglobin level and platelet count based on age and gender of the recruited patients (Millot et al., 2005). The positive correlation of duration of alcohol consumption and transition from chronic to blast stage of CML, might be due to immunomodulatory effect of alcohol which gradually impairs the immune functions of drinker and thereby lead to reduced immune response (Diaz et al., 2002). *DDIT3* plays an essential role in cell stresses, cell cycle arrest and apoptosis. The epigenetic changes in tumour suppressor gene like *DDIT3* have been recognized as a potential candidate and contributing factor for development of CML. The aberrant promoter methylation of this gene was previously reported on CML study (Wang et al., 2010). Indeed, in the present study almost 55% of CML cases possessed either partial methylation or hyper methylation in the promoter region

of *DDIT3* gene. Whereas, no significant correlation with clinic-pathological features with *DDIT3* methylation have been observed. However, the frequency of *DDIT3* promoter hypermethylation was found to be inversely correlated with the expression pattern of the *DDIT3* gene, which suggest the epigenetic modification concurrently influences gene expression. The DNA repair protein, *MGMT* is involved in cellular defence against mutagenesis and toxicity from alkylating agents. It's promoter hypermethylation was previously identified in certain tumour cell lines such as non-Hodgkin lymphoma and AML (Hong et al., 2016; Kraguljac et al., 2012). Besides, the methylation of this gene has also been found to be associated with longer survival and time to progression in glioblastoma multiforme, a tumour of the central nervous system (Abdel et al., 2018). However, in the present study, *MGMT* promoter hypermethylation significantly reduces the expression of the gene while advancing the CML from chronic to blast stage. This observation suggests the possible synergistic effect of altered *DDIT3* and *MGMT* expression due to epigenetic modulation and resistance to the prescribed drug *Imatinib* , towards progression of CML from chronic to blast crisis stage. The Tyrosine kinase inhibitors (TKIs) mediated drug, such as *Imatinib* resistance due to possible BCR-ABL mutation seems to be major challenges during the prognosis and treatment of CML. The significant reduction of survival capacity in *Imatinib* resistance patients in our study revealed the notion of prevailed characterized genetic abnormality of BCR-ABL fusions among the North East Indian population and advocate for the development of effective second and third generation tyrosine kinase inhibitor for frontline therapy of CML.

Author Contribution Statement

GH carried out the survey, samples collection and execution of experiments. AR & GK monitor the works as Lab In-Charge, MGI and SM conceived and designed the experiments. GH, MJK & LL critically analysed the data and prepared the manuscript, SK, PPD & KD helped in manuscript writing. VK guided in clinical data analysis.

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General

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Scientific Approval

This study is a part of PhD Thesis of Gautam Hazarika, Gauhati University, Assam.

Ethical Declaration

This research was conducted ethically in accordance with the AMCH and Gauhati University, institutional guidelines for human studies and was approved by the AMCH Ethics committee (AMC/EC/1594 Date 24/7/2020) and Gauhati University Ethics committee (GUIEC/2021/019 Date 29/9/2021).

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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