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Association of BAX Promoter Polymorphism (-248G>A) with Chronic Myeloid Leukemia: A Case-Control Study

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ABSTRACT

Chronic Myeloid leukemia (CML) is characterized by the reciprocal translocation of 9th and 22nd chromosome, resulting in the formation of fusion oncogene, BCR-ABL (Breakpoint cluster region-Abelson). Multiple events drive the transformation of chronic phase CML into advanced stages which include accumulation of mutations, activation of downstream pathways and failure of DNA repair and apoptosis. Bax is a pro-apoptotic member of Bcl2 family involved in the mitochondrial-mediated apoptotic pathway. A -248G>A polymorphism located in the promoter region is known to alter the expression of the gene in many cancers. Hence, the study was planned to evaluate the role of this particular polymorphism in the development and progression of CML. The study was carried out on a total of 986 individuals which included 477 patients recruited from Nizams Institute of Medical Sciences, Hyderabad and 509 age and gender matched controls obtained from the local population. Genotyping was carried out through PCR-RFLP method and appropriate statistical analyses were performed. The study revealed borderline association with BAX -248 GA or AA genotype and with the variant A allele indicating reduced risk to the development of CML. More number of Chronic CML patients with GG genotype had progressed into advanced phase compared to those with GA/AA genotypes and the risk being higher for males. Further patients with GG genotype had reduced event-free and overall survival. Our study indicated the possible role of BAX -248GG genotype in CML progression and reduced survival. Hence, it can serve as a predictor marker in evaluating the progression of the CML.

INTRODUCTION

Chronic Myeloid Leukemia (CML) is characterized by the presence of Philadelphia chromosome resulting in the formation of fusion oncoprotein, BCR-ABL with deregulated tyrosine kinase activity. Targeted therapy with the primary inhibitor, Imatinib Mesylate (IM), has transformed the outcome of CML from a fatal disease into a chronic condition. IM inhibits the Bcr-Abl tyrosine kinase, thus inhibiting proliferation and inducing apoptosis of leukemic cells [1]. However, failure to completely eradicate leukemic cells might induce the CML progenitor cells to acquire a number of secondary genetic alterations leading to the transformation of the disease into an aggressive phenotype (accelerated and blast), where the cells tend to develop drug resistance [2]. The Imatinib resistance may be due to Bcr-Abl -dependent or Bcr-Abl -independent mechanisms. Bcr-Abl -dependent mechanisms include BCR-ABL acquired mutations affecting IM binding to the Bcr-Abl protein, BCR-ABL amplification or increased transcription. Independent mechanisms include genetic alterations in secondary pathways that promote survival and proliferation of Bcr-Abl +ve cells [3]. However, few studies have reported that the CML progenitors showed a normal proliferative response to growth factors, but reduced apoptosis [4]. Therefore, the onset of CML is dependent on the balance between the cell division and cell death. It was also observed that expression of Bcr-Abl inappropriately prolonged the growth factor-independent survival of CML progenitors by inhibiting apoptosis [5]. Apoptotic resistance of cells expressing the fusion protein Bcr-Abl mainly depends upon the reduced expression and/or inactivation of pro-apoptotic proteins or enhanced expression of anti-apoptotic proteins [6].

Bax (Bcl-2 associated X protein) protein is one of the pro-apoptotic members of Bcl-2 family. Studies on various cell lines and animal models had shown that it could induce apoptosis as a tumor suppressor gene through direct activation by p53. The promoter region of BAX gene contains various transcription factor binding sites (such as p53 response elements, TATA box, canonical E-boxes and NF-kappa B) which are known to regulate the expression of Bax [7]. p53 protein directly binds to the p53-binding element in the promoter of BAX gene and induces the expression of BAX [8]. -248 G>A polymorphism located in the p53 binding region of 5′-UTR region of BAX gene might cause differential binding capacity of p53 protein, thereby regulating its expression [9]. Mutations identified in the promoter region were shown to alter the function and expression of the Bax protein in many cancers. Hence, the present study was planned to evaluate the role of BAX -248 G>A polymorphism in the development and progression of CML.

MATERIALS AND METHODS

477 primary CML cases reported at Nizam Institute of Medical Sciences, Hyderabad were recruited in the present study after obtaining informed consent. 509 age and gender matched controls were obtained from the local population without family history of any cancers. Only primary Ph+ve CML cases with confirmed diagnosis who are on Imatinib therapy were included and secondary/drug induced/Ph –ve CML cases were excluded from the study.

Epidemiological information such as gender, age at the time of diagnosis, occupation, area of living, habits and diet were collected through personal interviews and the clinical information was noted down from the tumor registry with the help of medical oncologist which included: Phase of CML at the time of diagnosis, baseline clinical characteristics such as WBC count, Platelet count, Blast %, Basophils, Eosinophils and Spleen size. Based on the baseline characteristics, three different risk scores were calculated namely Sokal, Hasford and EUTOS scores, using an online calculator (http://bloodref.com/myeloid/cml/sokal-hasford). The Sokal score considers age at onset (in years), Spleen size (cm below costal margin), Platelet count (x 109/L) and Blasts %, while Hasford considers % of Eosinophils and basophils and EUTOS score considers size of spleen and % of Basophils. All the patients were followed up to assess the drug response at Hematological, Cytogenetic and Molecular levels as well as the progression into advanced phase. The response was categorized into major and poor responders (intermediate and minor) based on specific criteria [10-12]. For the purpose of calculating event free survival, event is taken as either death of the patient or progression into advanced phase [13]. The study was approved by Institutional Ethics Committee for Biomedical Research, Osmania University and Ethical committee of Nizams Institute of Medical Sciences, Hyderabad.

Genomic DNA was isolated by non-enzymatic salting out method [14] from 5 ml of blood samples collected in EDTA vacutainers from both patients and controls. Genotyping was carried out by PCR-RFLP method wherein the reaction was performed using 50 ng of DNA as template in 10 µl reaction mix comprising of 10 mM each of dNTP mix, 30 pmol each of forward and reverse primers (Fwd: 5′-CATTAGAGCTGCGATTGGACCG-3′, Rev: 5′-GCTCCCTCGGGAGGTTTGGT-3′ [15], 0.5U Taq Polymerase, 1µl of DMSO and Milli-Q water. The PCR conditions included 35 cycles of initial denaturation (94^0C for 5 minutes), denaturation (94^0C for 1 minute), annealing (58.3^0C for 45 seconds), extension (72^0C for 1 minute) and final extension (72^0C for 7 minutes). The PCR products were then digested with

2U of Msp1 enzyme (NEB) and analyzed on 4.5% agarose gel. Some of the samples were randomly selected to reconfirm the genotypes and results were found to be in 100% concordance. The data obtained was subjected to various statistical analyses like SNPSTATs (http://bioinfo.iconcologia.net/snpstats/start.htm) – for calculating the odds ratio, Hardy-Weinberg equilibrium frequencies; SPSS version 20 for calculating the event free survival (EFS) and five year overall survival. Allele frequencies and chi-square were tested using online statistical tools (http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html, http://www.quantpsy.org/chisq/chisq.htm).

RESULTS

The distribution of epidemiological variables among cases and controls were represented in Table 1. The mean age of onset was found to be 35.79 years (Median - 35 (4-80) and the incidence of CML was found to be higher in age group 20-40 years (61.22%). Twice the numbers of males (63.94%) were affected with CML compared to females (36.06%). 94.34% of patients were on non-vegetarian diet. However, other epidemiological variables did not show much variation.

Table 1: Distribution of BAX -248G>A polymorphism with Epidemiological variables

Characteristic		Controls N (%)	CML Cases N (%)	Mean age at onset ± SD
Age at onset	**<20 years**	58 (11.39%)	35 (7.34%)	15.17 + 4.15
	20-40 years	276 (54.22%)	292 (61.22%)	30.68 + 5.90
	>40 years	175 (34.38%)	150 (31.45%)	50.56 + 6.69
Gender	**Male**	305 (59.92%)	305 (63.94%)	35.97 + 12.14
	Female	204 (40.08%)	172 (36.06%)	35.48 + 12.74
Living area	**Rural**	277 (57.11%)	273 (58.33%)	35.38 + 11.74
	Urban	208 (42.89%)	195 (41.67%)	35.99 + 13.10
Occupation	**Agriculture**	23 (4.95%)	93 (19.87%)	37.84 + 11.22
	Laborers	122 (26.24%)	151 (32.26%)	35.79 + 10.85
	Others	320 (68.82%)	224 (47.86%)	34.63 + 13.59
Diet	**Veg**	78 (16.32%)	25 (5.66%)	42.84 + 13.84
	Non-veg	400 (83.68%)	417 (94.34%)	35.39 + 12.27
Habits	**Smoking, Alcoholic**	83 (17.97%)	105 (25.18%)	38.67 + 11.13
	No habits	379 (82.03%)	312 (74.82%)	35.12 + 12.76
CML cases: Mean age = 35.79 ± 12.35 ; SEM=0.75; median= 35 (4-80)				

The HapMap data of BAX -248G>A was constructed by taking the allele frequencies of various populations such as Sub-Saharan (YRI), Asian (CHB), Asian (JPT), Asian (HCB)

and European (CEU). The allele frequencies among CML patients (0.06) in the present study were comparable to those of Asian (HCB) (0.05) population while frequencies in controls (0.08) were similar to Sub-Saharan (YRI) (0.08) population (Fig. 1).

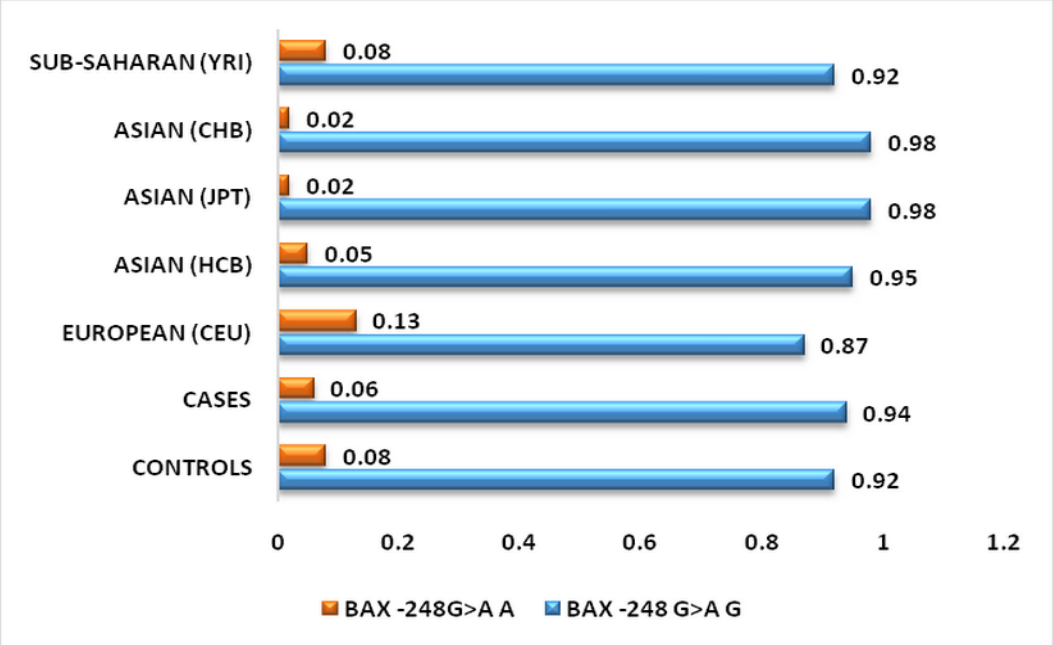

Fig. 1. HAPMAP data BAX -248G>A polymorphism

The genotype distribution of BAX -248G>A polymorphism did not show any deviation from Hardy-Weinberg Equilibrium in both cases (p-0.69) and controls p-0.16). To understand the genotypic and allelic distribution among controls and cases with respect to BAX -248G>A polymorphism, odds ratios were calculated under different models. It was observed that the frequencies of the GA and AA genotypes were reduced in CML cases (11.3% and 0.4%) while frequency of GG genotype (88.3%) was increased as compared to controls (14.5%, 1.2% and GG- 84.3%) respectively under co-dominant model. Under the dominant model, a borderline significance was observed wherein the combined frequency of GA and AA genotypes was reduced (OR-0.72; 95% CI- 0.50-1.04, p-0.08) with consequent increase in GG genotype frequency, indicating altered risk for these genotypes to develop CML. A slight decrease in the frequency of the variant allele (A) was observed in cases (6.08%) as compared to controls (8.45%) and the variant allele was found to be associated with decreased risk of CML (OR-0.70, 95% CI- 0.50-0.99, p-0.04*) (Table 2).

Table 2: Genotype distribution of BAX -248G>A polymorphism among Controls and Cases

Model	Genotype	Controls	Cases	OR (95% CI)	p-value
Codominant	**GG**	429 (84.3%)	421 (88.3%)	1.00	0.12
	GA	74 (14.5%)	54 (11.3%)	0.76 (0.52-1.10)	
	AA	6 (1.2%)	2 (0.4%)	0.32 (0.06-1.61)	
Dominant	**GG**	429 (84.3%)	421 (88.3%)	1.00	**0.08#**
	GA+AA	80 (15.7%)	56 (11.7%)	0.72 (0.50-1.04)	
Recessive	**GG+GA**	503 (98.8%)	475 (99.6%)	1.00	0.15
	AA	6 (1.2%)	2 (0.4%)	0.33 (0.07-1.66)	
Overdominant	**GG+AA**	435 (85.5%)	423 (88.7%)	1.00	0.16
	GA	74 (14.5%)	54 (11.3%)	0.76 (0.52-1.11)	
Allele Frequencies					
Wild Allele	**G**	932 (91.55%)	896 (93.92%)	1.00	
Variant Allele	**A**	86 (8.45%)	58 (6.08%)	**0.70 (0.50-0.99)**	**0.04***
Hardy Weinberg Equilibrium : Controls p=0.16; Cases p=0.69					
***p<0.05; #p<0.10**					

As the number of patients carrying the variant genotype, AA, was very less, the frequency of variant AA genotype was clubbed with that of heterozygous GA genotype for further analyses. The combined genotype frequency of GA and AA was elevated in patients who were in advanced phase (19.1%) at the time of diagnosis with corresponding increase in variant 'A' (9.57%) allele frequency (Table 3).

Table 3: Distribution of BAX -248G>A Polymorphism with respect to Phase of the disease at diagnosis

Genotype	GG	GA+AA	OR (95% CI)	p-value	G	A	OR (95% CI)	p-value
Chronic	376 (89.7%)	43 (10.3%)	1.00	0.096	794 (94.75%)	44 (5.25%)	1.00	**0.04***
Advanced	38 (80.8%)	9 (19.1%)	2.04 (0.92-4.51)		84 (89.36%)	10 (10.64%)	2.15 (1.04-4.42)	

Hence, in order to evaluate genotype association with progression of the disease, the follow-up data of patients who were in chronic phase at the time of diagnosis was stratified with

respect to BAX GG and GG + GA genotypes. It was observed that 6.38% of chronic CML patients with GG genotype have progressed into advanced phase as compared to 2.33% of the patients with GA or AA genotype (Table 4). The sex ratio among these patients who progressed into advanced stage was found to be 1.78.

Table 4: Percentage of Chronic CML patients who progressed into advanced phase

Genotype	Total no. of patients in chronic phase	Progressed	Not Progressed	OR (95% CI)	p-value
GG	376 (89.7%)	24 (6.38%)	352 (93.62%)	1.00	0.31
GA+AA	43 (10.3%)	1 (2.33%)	42 (97.67%)	2.86 (0.38-21.71)	

BAX -248G>A polymorphism did not show significant association with Imatinib response (Hematological, Cytogenetic and Molecular) as well as risk scores (Sokal, Hasford and Eutos) (Table 5, 6).

Table 5: Distribution of BAX -248G>A polymorphism with respect to Imatinib response

	GG	GA+AA	OR (95% CI)	p-value	G	A	OR (95% CI)	p-value
Hematological response								
Major	241 (89.6%)	28 (10.4%)	1.00	0.91	510 (94.8%)	28 (5.2%)	1.00	
Minor	82 (89.1%)	10 (10.9%)	1.05 (0.49-2.25)		173 (94.02%)	11 (5.98%)	1.16 (0.56-2.38)	0.69
Cytogenetic response								
Major	196 (89.1%)	24 (10.9%)	1.00	0.84	415 (94.32%)	25 (5.68%)	1.00	
Minor	113 (88.3%)	15 (11.7%)	1.07 (0.54-2.13)		241 (94.14%)	15 (5.86%)	1.03 (0.53-1.99)	0.92
Molecular response								
Complete Responders	163 (88.1%)	22 (11.9%)	1.00	0.75	348 (94.05%)	22 (5.95%)	1.00	
Non-Responders	118 (86.8%)	18 (13.2%)	1.11 (0.57-2.17)		252 (92.65%)	20 (7.35%)	1.26 (0.67-2.35)	0.48

Table 6: Distribution of BAX -248G>A polymorphism with respect to risk scores:

Genotype	GG	GA + AA	OR (95% CI)	p-value	G	A	OR (95% CI)	p-value
Sokal score								
Low risk	116 (87.2%)	17 (12.8%)	1.00	0.75	249 (93.61%)	17 (6.39%)	1.00	
High risk	285 (88.8%)	36 (11.2%)	0.90 (0.48-1.69)		604 (94.08%)	38 (5.92%)	0.92 (0.51-1.66)	0.79
Hasford score								
Low risk	146 (88%)	20 (12.1%)	1.00	0.91	312 (93.98%)	20 (6.02%)	1.00	
High risk	255 (88.5%)	33 (11.5%)	0.97 (0.53-1.76)		541 (93.92%)	35 (6.08%)	1.01 (0.57-1.78)	0.97
EUTOS score								
Low risk	259 (89%)	32 (11%)	1.00	0.66	549 (94.33%)	33 (5.67%)	1.00	
High risk	142 (87.1%)	21 (12.9%)	1.15 (0.63-2.07)		304 (93.25%)	22 (6.75%)	1.20 (0.69-2.10)	0.51

Survival analysis i.e. both event-free survival and Five year overall survival of the patients revealed that the patients with GG genotype showed lower mean EFS (41.00 ± 2.55) (p-0.02*) and reduced Five year overall survival (51.00 ± 0.86) (p-0.46) (Table 7, 8) (Fig. 2, 3).

Table 7: Kaplan-Meier Survival curve for Event Free Survival (EFS) of BAX -248G>A polymorphism in CML

Sr. No.	BAX -248 G>A	N (%)	Chronic Phase (%)	(EFS in months) Mean ± SEM	Median	p-value
1	**GG**	151 (88.82%)	134 (88.74%)	41.15 ± 2.55	36.000	0.46[a]
2	**GA + AA**	19 (11.18%)	15 (78.95%)	48.27 ± 7.11	55.000	0.25[b]
Total		170	149 (87.65%)	42.25 ± 2.38	37.000	
a) Log Rank (Mantle Cox) p value b) Breslow (Generalized Wilcoxon) p value						

Table 8: Kaplan-Meier Survival curve for Overall Survival (OS) of BAX -248G>A polymorphism in CML

Sr. No.	BAX -248 G>A	N (%)	Chronic Phase (%)	(OS in months) Mean ± SEM	Median	p-value
1	GG	402 (88.94%)	367 (91.29%)	51.29 ± 0.86	61.000	0.02*[a]
2	GA + AA	50 (11.06%)	41 (82.00%)	56.17 ± 2.01	61.000	0.02*[b]
Total		452	408 (90.27%)	51.82 ±0.79	61.000	

a) Log Rank (Mantle Cox) p value b) Breslow (Generalized Wilcoxon) p value
*p<0.05; #p<0.10

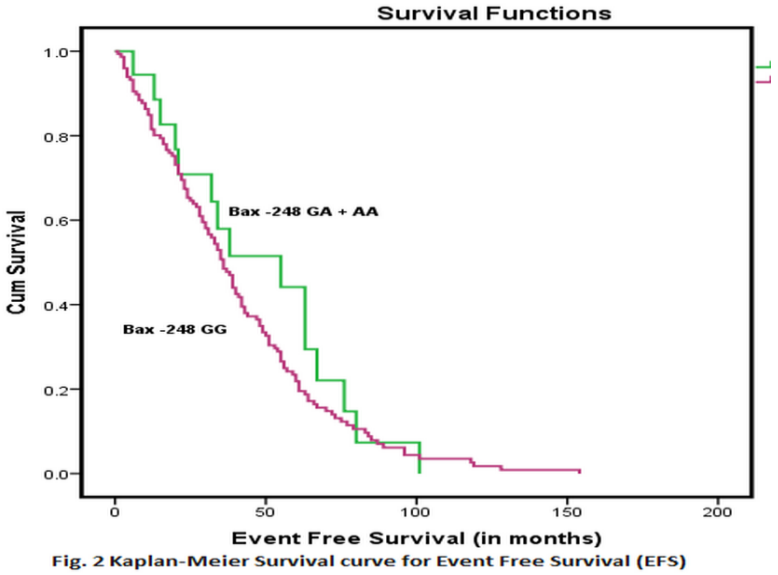

Fig. 2 Kaplan-Meier Survival curve for Event Free Survival (EFS)

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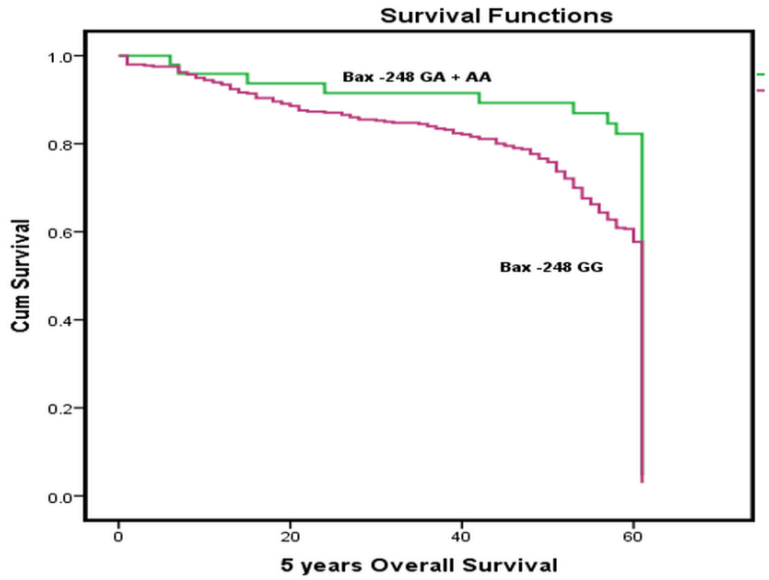

Fig. 3 Kaplan-Meier Survival curve for Overall Survival (OS)

DISCUSSION

Bax was first identified as pro-apoptotic member of Bcl2 family which was co-purified with Bcl2 in immunoprecipitation analysis [16]. Bax protein forms a heterodimer with Bcl2 and functions as an activator of apoptosis. It is known to interact with and increase the permeability of mitochondrial voltage-dependent anion channel (VDAC) resulting in loss of membrane potential and release of cytochrome c [17]. BAX gene is regulated by tumor suppressor gene, p53 and is involved in p53 mediated apoptosis. The presence of the variant 'A' allele in the 5` UTR region at -248 position were found to influence the protein expression and function [9].

The present study revealed borderline significant association between GG genotype of BAX -248G>A polymorphism and CML wherein GG was found to confer risk to CML and AA was associated with reduced risk. The results of the present study were found to be in accordance with case-control studies carried out on CLL patients from Russian population, where GG genotype was found to be associated with increased risk for the development of CLL [18] and also studies on AML from our lab [19], which reported increased risk for GG genotype. However, earlier report on CLL [20], squamous cell carcinoma of the head and neck (SCCHN) from non-Hispanic white population (Chen et al., 2007) as well as from the results of meta-analysis performed from four independent studies on CLL [21] did not report significant association with BAX -248G>A polymorphism. Nevertheless, contrasting results were observed with respect to lung cancer [22] and non-small cell lung cancer [23] where the variant AA genotype was associated with increased risk of developing cancer.

Our study indicated decreased risk for 'A' allele with increased risk for G allele which was supported by a report from Chinese population where they performed luciferase assay [24] to test the biological role of BAX -248G>A polymorphism and found that the BAX -248A allele exhibited significantly higher transcriptional activity compared with G allele, hence was associated with decreased risk. The results of our study were found to be in accordance with a case-control study on 692 CLL cases and 738 controls from Caucasian population, where 768 SNPs were genotyped from a total of 172 genes and the presence of variant A allele was found to be associated with decreased CLL risk (OR- 0.73; 95% CI, 0.58–0.92, p-0.0087) [25]. Another study reported from our lab on 221 AML patients and 305 controls also revealed the decreased risk associated with the variant 'A' allele [19].

However, Imatinib response did not show any significant association with BAX -248 G>A genotype indicating lack of influence of this SNP on drug response of CML patients. When phase of the disease was stratified, it was observed that the likelihood of chronic CML patients with GG genotype to progress into advanced phase was 3 times higher indicating that this particular genotype may tend to play a role in the progression of disease and could serve as a predictive marker. The particular risk was more pronounced for male patients.

Decreased event and overall free survival were observed among CML patients with BAX -248GG genotype. The results of the present study were found to be consistent with the results obtained on AML, where BAX -248GG genotype conferred significant risk for complete remission failure and was associated with reduced median disease-free survival (DFS) [19]. However, our results were in contrast with the reports on CLL [26], non-small cell lung cancer (NSCLC) [23,27] where the variant allele was found to be associated with shorter/poor overall survival. These results suggested that the BAX -248 GG genotype is associated with CML progression and decreased event-free/overall survival of the patients.

CONCLUSION

The promoter polymorphism (-248) in BAX gene influences mRNA transcriptional rate, thereby impacting proapoptotic ability of aberrant myeloid cells which provides survival advantage and establishment of CML clone. The variant allele enhances the mRNA transcription rate therefore associated with decreased risk. Consequently, G allele of this polymorphism with lowered transcription rate confers risk to CML development.

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critical review of manuscript; RD contributed in the collection of samples, providing clinical data.

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