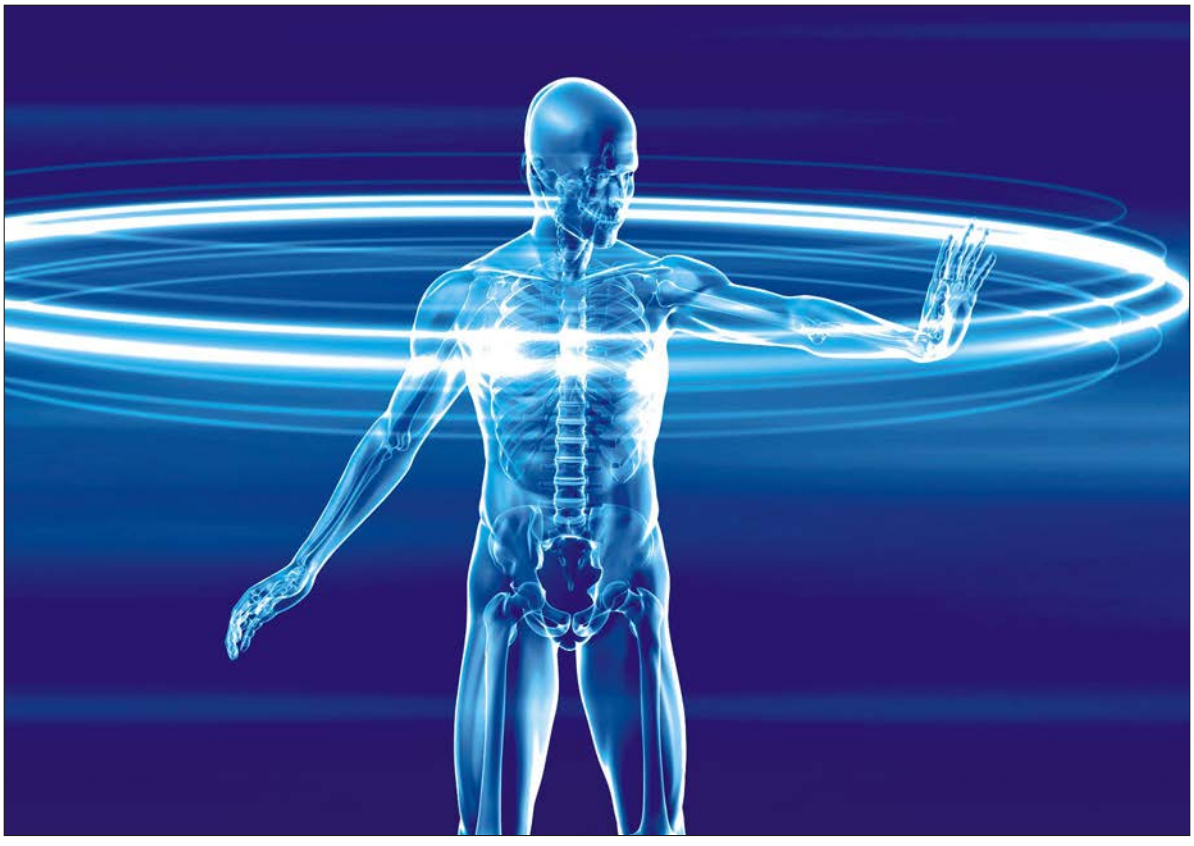

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CYP1A1, GSTM1 and GSTT1 Genetic Polymorphism in Egyptian Chronic Myeloid Leukemia Patients

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Abstract: The genetic polymorphism of xenobiotic metabolizing enzymes: phase I enzymes; cytochrome P450 (CYP1A1) and phase II enzymes; glutathione S-transferase (GSTM1 and GSTT1), were analyzed in 30 chronic myeloid leukemia patients (CML) (19 females, 11 males; age (Mean±SD) 41.7±9.5 years) and 20 age and sex matched healthy controls. The frequency of CYP1A1 alleles and of GSTT1 and GSTM1 homozygous deletions was examined by PCR-RFLP and PCR methods, respectively, using blood samples. The relationship between these genotypes and risk of CML was assessed by means of Odds Ratio (OR) with 95% confidence limits. Present results showed that the frequency of the mutant allele CYP1A1*2A was found to be 3.3% in CML patients and 45% in controls (OR = 0.042, 95% CI: 0.005-0.373; p<0.001), suggesting that this polymorphic variant may be a protective factor against CML. The frequency of individuals carrying the GSTT1 null genotype was higher among CML patients (60%) compared to controls (15%) (OR = 8.5, 95% CI: 2.038-35.458; p = 0.002). Therefore, GSTT1 null genotype may be a risk factor for CML. Although, GSTM1 null genotype frequency was slightly higher in the patient group (46.7%) than in the controls (40%), this difference was not statistically significant (OR = 1.313, 95% CI: 0.417-4.131; p = 0.642). In conclusion this data suggests that polymorphic CYP1A1 and GSTT1 genes appear to affect susceptibility to CML.

Key words: CYP1A1, GSTT1, GSTM1, polymorphisms, CML

INTRODUCTION

Adverse effects of xenobiotics, which are foreign chemical substances, are exerted via covalent interactions between intermediate metabolites and genetic materials or proteins and their related metabolites. Enzymatic reactions of xenobiotic metabolism are needed in order to avoid accumulation of lipophilic xenobiotics in cells and tissues. These reactions can be divided into two-phase activation-deactivation sequence; phase I and phase II enzymatic reactions (Guengerich, 1992; Nebert *et al*., 1996). The key enzyme systems catalyzing phase I oxidative metabolism are enzymes of the cytochrome P450 (CYP) superfamily. During these reactions, toxic metabolites are generated which might be processed by phase II enzymes (Induski and Lutz, 2000).

Cytochrome P450 enzymes are involved in the detoxification of a wide range of xenobiotics, including environmental carcinogens, chemotherapeutic agents and reactive oxygen species, they transfer electrons onto these toxicants to create highly reactive intermediates which are then coupled to glutathione or other groups, producing water-soluble compounds, or interact with DNA resulting in the formation of DNA

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adducts (Ingelman-Sundberg, 2001). CYP1A1 is one of the cytochrome P450 (CYP) superfamily. The CYP1A1 gene is a polymorphic gene that encodes this enzyme which catalyzes the bioactivation of polycyclic aromatic hydrocarbons (Induski and Lutz, 2000; Chang *et al*., 2003). CYP1A1 gene polymorhisms have been extensively studied, especially in relation to cancer susceptibility (Chang *et al*., 2003; Terry *et al*., 2003; Abbas *et al*., 2004; Lukas *et al*., 2004; Gra *et al*., 2008).

Phase II enzymes catalyze the conjugation of glutathione or glucuronide with reactive intermediates transforming them into more hydrophilic excretable metabolites and thus detoxify procarcinogens and carcinogens (Perera, 1996). Glutathione S-Transferases (GSTs) belong to the group of phase II enzymes. GSTs are polymorphic genes and involved in the metabolism of a wide range of xenobiotics. The frequencies of GSTs polymorphic alleles especially GSTT1 and GSTM1 have been reported in various cancers (Aktas *et al*., 2004; Canalle *et al*., 2004; Chan *et al*., 2005).

Numerous genetic polymorphisms have been reported for CYP and GST genes. The genetic mutations of these genes may cause lack of functional enzyme (Dufour *et al*., 2005) or lead to either increased or reduced metabolic activity (Hung *et al*., 2003). These polymorphisms may alter the ability of enzymes to metabolize the chemical carcinogens and mutagens, which may influence the susceptibility of individuals to cancer (Olshan *et al*., 2000).

Chronic Myeloid Leukemia (CML) is a malignancy of the hematopoietic stem cell (Chang *et al*., 2003). It is known that the environmental exposures to cytotoxic and genotoxic agents, particularly those derived from benzene and ionizing radiation, may be associated with increased risk of CML, otherwise there is no evidence of any significant association with either genetic or other environmental factors (Löffler *et al*., 2001).

In this study, we tried to find new evidence for the hypothesis that CML is a combined result of environmental exposure and genetic susceptibility. We used a polymerase chain reaction (PCR)-based genotyping approach to examine the relationship between genetic polymorphisms in GSTM1, GSTT1 and CYP1A1 and susceptibility to CML. We report the analysis of these loci in 30 CML patients and in 20 controls. The presence of GSTM1 allele is identified by a 230-bp fragment, while the presence of GSTT1 allele is identified by a 112-bp fragment. CYP1A1 mutations (m1), (m2) and (m4) were characterized by the PCR-RFLP approach using *MspI*, *BsrdI* and *BsaI* restriction enzymes, respectively. Mutations of m1 bands appear at 693 and 206 bp while m2 and m4 mutations are detected by loss of restriction site at 204 bp. These mutations were then used to define three distinct alleles, CYP1A1*2A (presence of m1 only), *2B (both m1 and m2) and *4 (m4 only).

MATERIALS AND METHODS

This study was conducted from September 2007 to September 2008 at Kasr Eliny hospital Cairo University.

The GSTT1, GSTM1 and CYP1A1 polymorphisms were determined using polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) method described by Krajinovic *et al.* (1999).

The present study included 30 newly diagnosed CML patients. They were diagnosed at the departments of Clinical Pathology, Faculty of Medicine, Cairo University. The diagnosis of CML was based on the standard clinico-hematological criteria and the presence of Philadelphia chromosome and/or BCR-ABL fusion gene. Twenty age and sex matched normal persons were included in this study as a control group. All the patients were subjected to careful history taking, full clinical examination and routine laboratory investigations including complete blood picture, liver and kidney functions. For each patient: 10 mL of blood were collected in a sterile heparinized vaccutainer.

Detection of GSTT1 and GSTM1 Polymorphism

Genomic DNA used was extracted from lymphocytes using the QIAamp DNA Mini isolation kit (QIAGEN, catalogue number.51304).

GSTM1 Polymorphism

The primers used for GSTM1 were as follows: P1, 5' CGCCATCTTGTGCTACATTGCCCG; P2, 5' ATCTTCTCCTCTTCTGTCTC and P3, 5' TTCTGGATTGTAGCAGATCA'. P1 and P3 amplify 230-bp product that is specific to GSTM1, whereas P1 and P2 anneal to GSTM1 and GSTM4 genes, yielding a 157-bp fragment that serves as an internal control. PCR was performed in 50 µL reaction containing 20 ng of genomic DNA, 0.5 µmol L^{-1} of each primer, 200 µmol L^{-1} of each dNTPs, 10 mmol L^{-1} Tris-HCl (pH 8.3), 50 mmol L^{-1} KCl, 1.5 mmol L^{-1} $MgCl_2$ and 0.5 U of ampliTaq DNA polymerase (Hoffman-LaRoche, Branchburg, NJ). After denaturation for 4 min at 94°C, the PCR was performed for 35 cycles of 30 sec at 94°C, 1 min at 59°C and 1 min at 72°C. The last elongation step was extended to 7 min. The presence of GSTM1 allele, identified by a 230-bp fragment, or its complete deletion (null genotype), was analyzed by electrophoresis on a 1.5% agarose gel. The absence of amplifiable GSTM1 (in the presence of the GSTM4 coamplified control) indicates a null genotype (Fig. 1).

GSTT1 Polymorphism

The primers used to amplify GSTT1 were: F46, 5' GCCCTGGCTAGTTGCTGAAG and R137, 5' GCATCTGATTTGGGGACCACA. A 268-bp fragment in the exon 1 of β-globin gene was coamplified with the primers BgloF, 5' CAACTTCATCCACGTTCACC and BgloR, 5' GAAGAGCCAAGGACAGGTAC as a control. PCR was performed in 50 µL reaction

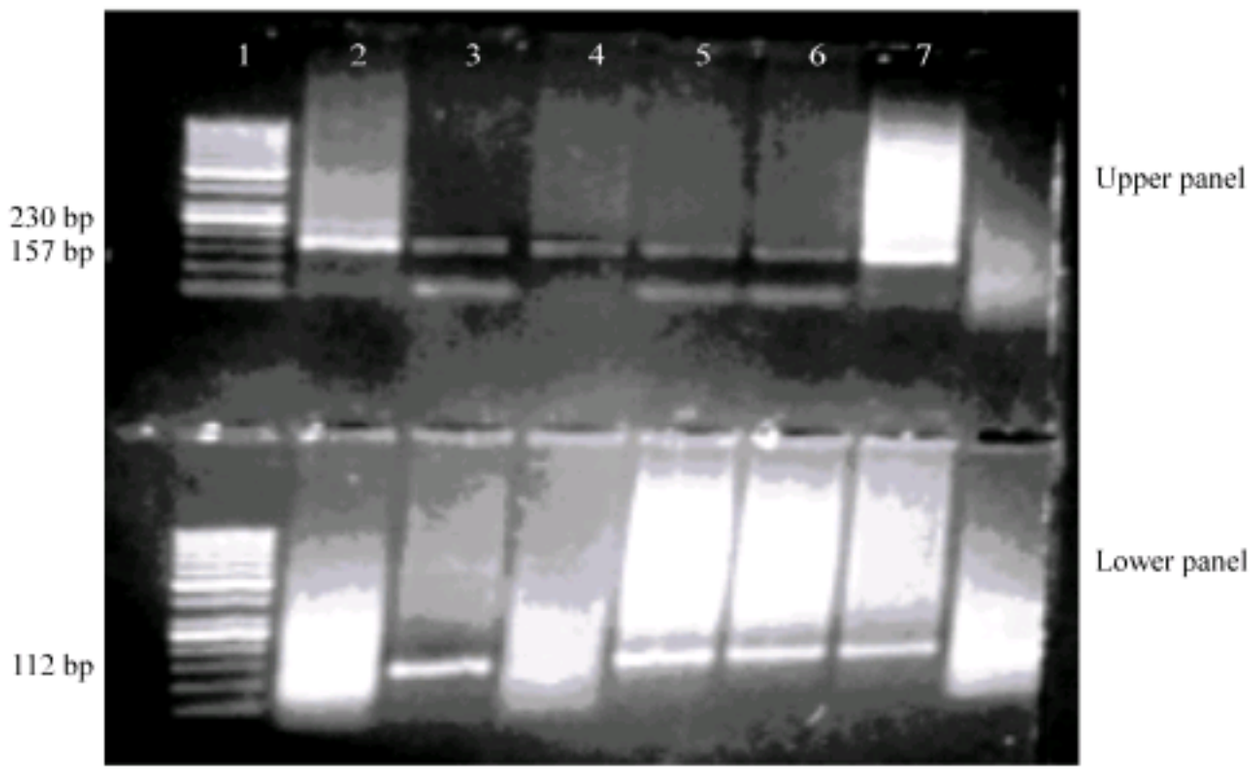

Fig. 1: Agarose gel demonstrating PCR genotyping of genomic DNA samples for detection of GSTM1 and GSTT1 polymorphism. Upper panel: Detection of GSTM1 null genotype: Presence of GSTM4 in all lanes at 157 bp as internal control. Lane 2: shows GSTM1 positive genotype, Lane 1: 50-1000 bp ladder size marker, Lanes 3, 4, 5, 6 and 7: show GSTM1 null genotype, Lower panel: Detection of GSTT1 genotype: Lane 1: 50-1000 bp ladder size marker, Lane 2 and 4: show GSTT1 null genotype, Lanes 3, 5, 6 and 7: Show GSTT1 positive genotype

containing 20 ng of genomic DNA, 0.5 µmol L^{-1} of each primer, 200 µmol L^{-1} of each dNTPs, 2.0 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} Tris-HCl (pH 8.3), 50 mmol L^{-1} KCl and 0.5 U ampliTaq DNA polymerase (Hoffman-LaRoche). After denaturation for 4 min at 94°C, PCR was performed for 35 cycles of 15 sec at 94°C, 30 sec at 60°C and 45 sec at 72°C. The last elongation step was extended to 7 min. The presence of GSTT1 alleles, identified by a 112-bp fragment, or its complete deletion (null genotype) (Fig. 1), was shown by electrophoresis on a 1.5% agarose gel. GSTT1 genotypes were scored only if the PCR signal corresponding to the β-globin internal control was evident.

Detection of CYP1A1 Polymorphism

CYP1A1 mutations T6235C (m1), A4889G (m2) and C4887A (m4) were characterized by the PCR-RFLP approach. For determination of (m1), a DNA fragment of 899 bp was amplified in 20 µL containing 20 ng of genomic DNA, 0.5 µmol L^{-1} of primers: M3F (5' GGCTGAGCAATCTGACCCTA), P80 (5' TAGGAGTCTTGTCTCATGCCT). The 200 µmol L^{-1} dNTPs, 10 mmol L^{-1} Tris-HCl (pH 8.3), 2.5 mmol L^{-1} MgCl2, 50 mmol L^{-1} KCl and 0.5 U Ampli T aq DNA polymerase. PCR was performed for 35 cycles of 30 sec at 94°C, 1 min at 63°C and 1 min at 72°C. The PCR product (5 to 10 µL) was digested with Msp1 (New England Biolabs, Schwalbach, Germany) (3 U, 37°C), resulting in smaller fragments (693 and 206 bp) in case of the mutation and subjected to electrophoresis on a 1.5% agarose gel. Mutations m2 and m4 were detected by amplifying a 204-bp fragment with primers M2F (5' CTGTCTCCCTCTGGTTACAGGAAGC)andM2R(5'TTCCACCCGTTGCAGCAGGATAGCC) as described above, followed by digestion with BsrDI (1 U, 65°C) and BsaI (2 U, 55°C), respectively. Both restriction sites were lost in case of mutation and the resulting restricted fragments were evaluated on a 2.0% agarose gel. These mutations were then used to define three distinct alleles, CYP1A1*2A (presence of m1 only) (Fig. 2), *2B (both m1 and m 2) and *4 (m4 only) (Fig. 3).

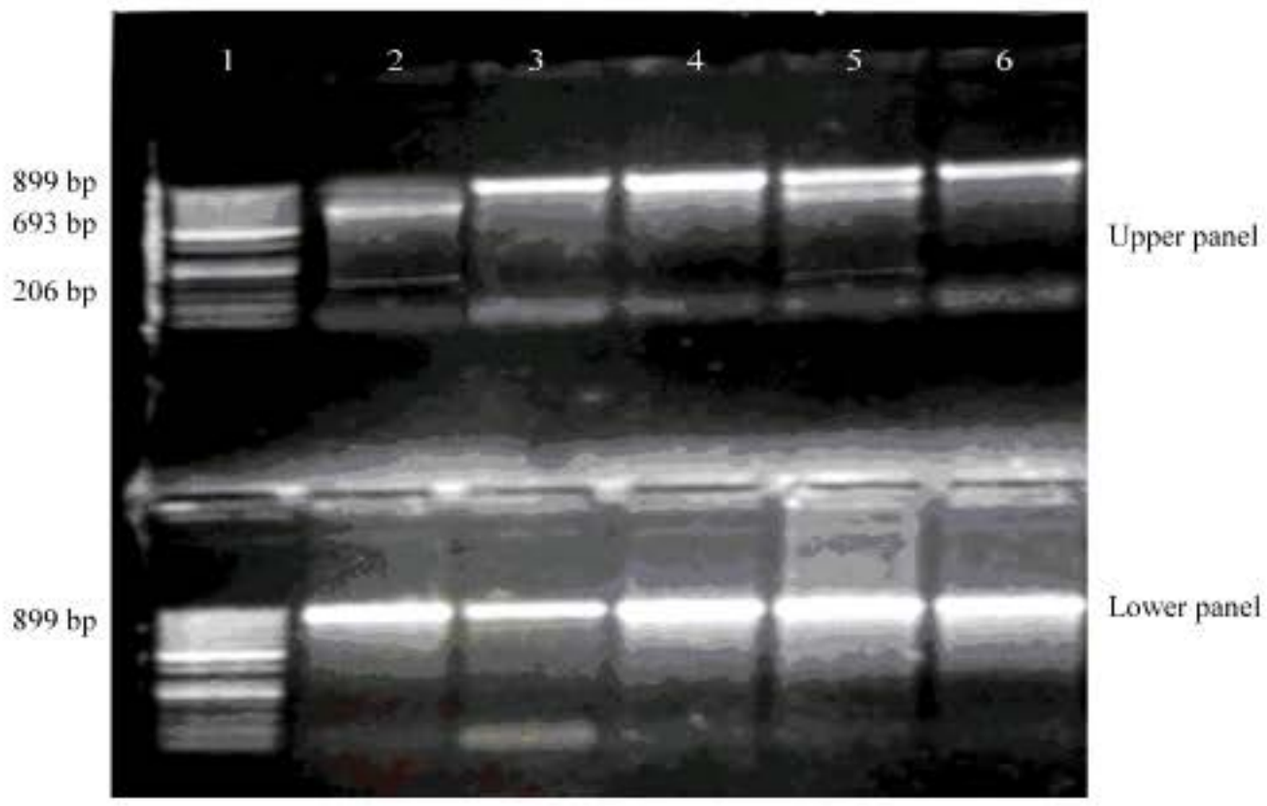

Fig. 2: CYP1A1 (m1) alleles by PCR/RFLP. Upper panel: detection of CYP1A1 allelels (m1): Lane 1: 50-1000 bp ladder size marker, Lane 2: two bands at 693 and 206 bp denoting homozygous (m1) mutation, Lanes 3, 4, 6: show only one band at 899 bp denoting no (m1) mutation, Lane 5: shows three bands at 899, 693 and 206 bp denoting heterozygous (m1) mutation. Lower panel: Lane 1: 50-1000 bp ladder size marker, Lanes 2-6: Presence of only one band at 899 bp denoting no (m1) mutation

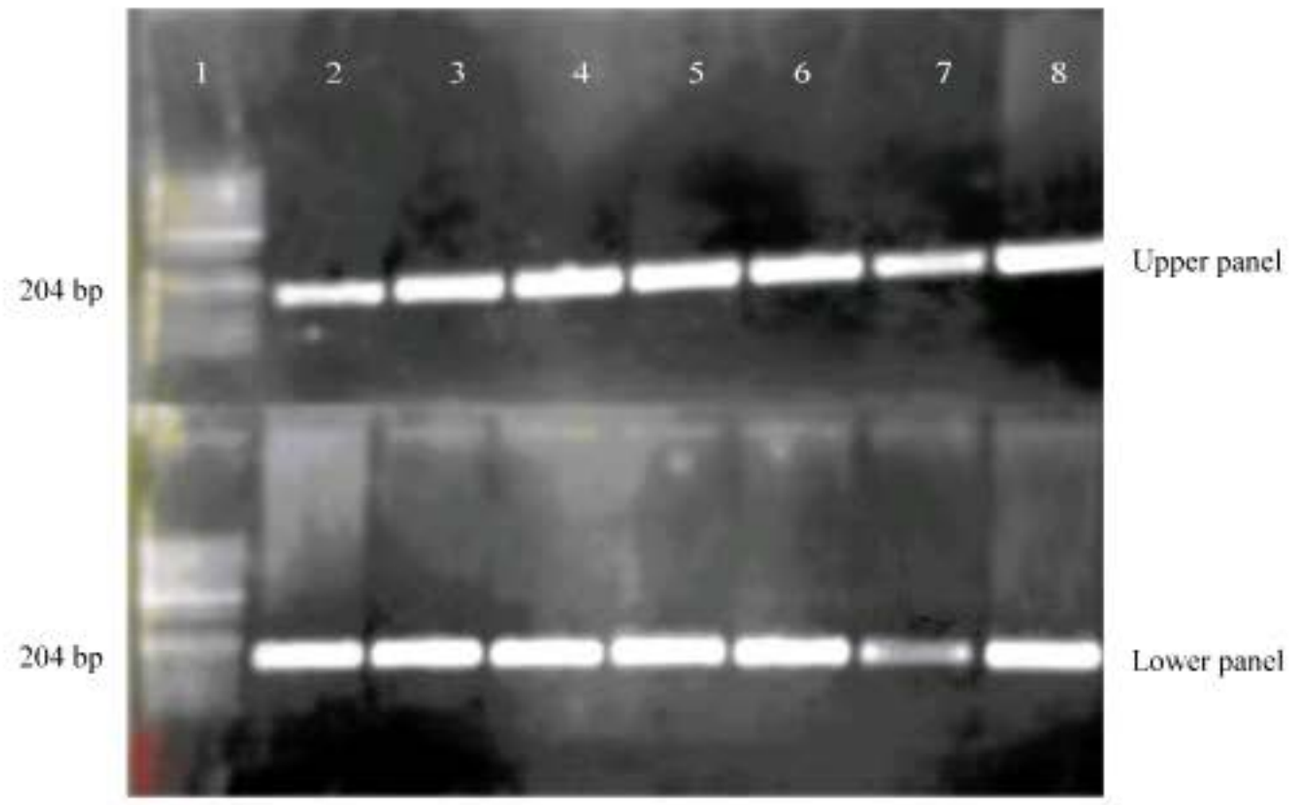

Fig. 3: CYP1A1 (m4) alleles by PCR/RFLP: Upper panel: Detection of CYP1A1 allelels (m4): Lane 1: 50-1000 bp ladder size marker, Lanes 2-8: One band at 204 bp denoting no (m4) mutation, Lower panel: Lane 1: 50-1000 bp ladder size marker, Lanes 2-8: Presence of one band at 204 bp denoting no (m4) mutation

Statistical Analysis

Data was analyzed using SPSS statistical package version 16. Numerical data were expressed as Mean±SD. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test. Risk was estimated as odds ratio with 95% confidence interval. A probability value (p-value) less than 0.05 was considered significant.

RESULTS

This study included 30 patients with CML (19 females, 11 males; age (Mean±SD) 41.7±9.5 years) and 20 normal controls. The distribution of the CYP1A1 polymorphisms, GSTT1 and GSTM1 genotypes in CML patients and controls are shown in Table 1. Whereas no significant differences between cases and controls were found in the frequencies of heterozygous or homozygous presence of the mutant alleles CYP1A1*2B and CYP1A1*4, the mutant allele CYP1A1*2A was significantly under represented among CML cases (3.3 versus 45%, $p<0.001$). As an estimate for the relative risk, the odds ratio for CYP1A1*2A was 0.042 with a 95% confidence interval of 0.005-0.373. These data indicate a reduced risk for CML in individuals carrying the mutant allele CYP1A1*2A. The frequency of individuals carrying the GSTT1 null genotype was higher among CML patients (60%) compared to controls (15%) (OR = 8.500, 95% CI: 2.038-35.458; p = 0.002). Therefore, GSTT1 null genotype may be a risk factor for CML (Table 1). Although, GSTM1 null genotype frequency was slightly higher in the patient group (46.7%) than that of the controls (40%), it was not statistically significant (Table 1).

Table 1: Comparison of CYP1A1, GSTM1 and GSTT1 genotypes in CML patients and controls

Locus	Genotype	Patients (n = 30)	Controls (n = 20)	OR	95% CI	p-value
CYP1A1	2A	1 (3.3%)	9 (45%)	0.042	0.005-0.373	<0.001 (S)
	2B	2 (6.7%)	2 (10%)	0.643	0.083-4.981	(NS)
	4	0	0	0.00	0	-
GSTM1	Present	16 (53.3%)	12 (60%)			
	Null	14 (46.7%)	8 (40%)	1.313	0.417-4.131	(NS)
GSTT1	Present	12 (40%)	17 (85%)			
	Null	18 (60%)	3 (15%)	8.50	2.038-35.458	0.002 (S)

NS: Statistically not significant, S: Statistically significant

There was no statistically significant interaction between GSTT1 and GSTM1 genotypes. This means that in this study the association between the GSTT1 (or GSTM1) genotype and CML does not depend in any way on the level of the GSTM1 (or GSTT1) genotype. The GSTT1 null genotype was not a significant risk factor among persons whose GSTM1 genotype was null or those with present GSTM1. The GSTM1 null genotype was not a significant risk factor irrespective of the GSTT1 genotype. The positions of GSTT1 and M1 genotypes towards each other were investigated by logistic regression analyses.

No correlation with established prognostic factors, including age, white blood cell counts (WBC), karyotype was detected in our study.

DISCUSSION

Little is known about the role of genetic susceptibility and environmental factors on the development of CML. Patients with CML have consistent genetic abnormality in the form of reciprocal translocation between chromosome 9 and 22, known as Philadelphia chromosome which leads to juxta-position of BCR-ABL gene. The resultant increased tyrosine kinase activity is responsible for the initiation and maintenance of leukemic process (Deininger *et al*., 2000). The only causative factor known to be associated with CML is exposure to radioactivity, however, it is likely that environmental factors such as exposure to toxins and carcinogens may be associated with the initiation of neoplastic process. It is known that the environmental exposure to cytotoxic and genotoxic agents particularly derived from benzene may be associated with increased risk of CML (Björk *et al*., 2001).

The etiology of most commonly occurring cancers in general population probably results from the complex interactions of multiple genetic and environmental factors over time. The environment-gene interaction in carcinogenesis is well reflected by metabolic enzymes involved in the activation or detoxification of environmental carcinogens (Medeiros *et al*., 2004).

This study presents combined analysis of loci encoding both phase I and II xenobiotic-metabolizing enzymes, namely; cytochrome P450 (CYP1A1) and glutathione S-transferase (GSTM1 and GSTT1) respectively. Currently, the Human Cytochrome P450 Allele Nomenclature Committee has named the polymorphic alleles of 22 CYP isoforms including more than 200 variants (Sim and Ingelman-Sundberg, 2006). Glutathione S-transferase (GSTM1 and GSTT1) constitute a superfamily of isozymes and act as the main phase II biotransformation enzymes involved in the elimination of toxic chemicals. The GSTs genes are also polymorphic genes. Polymorphism in the genes encoding carcinogen metabolizing enzymes may have relevance in determining susceptibility to cancer. Individuals carrying the more active form of an enzyme involved in the activation of carcinogens (phase-I), or less efficient alleles of detoxifying enzymes (phase-II) are at greater risk of cancer (Autrup, 2000).

Knowledge of variations in frequency of GSTM1 and GSTT1 null genotypes in different populations may help to explain differential responses to toxic chemicals (Nebert and Menon, 2001; Hishida *et al*., 2005). Homozygous deletion of the genes at the corresponding gene loci causes absence of the specific enzymatic activity (Mondal *et al*., 2005).

In this study, we found that patients with GSTT1 null genotype were more at risk to be afflicted with CML (OR = 8.5, 95% CI: 2.038-35.458; p = 0.002), therefore, the GSTT1 positive genotype is a protective factor for CML.

Metabolization of oxygen products and active carcinogens are reduced in GSTT1 null genotypes. Thus, GSTT1 null genotype may be a risk factor for cancer development. Similarly, in studies done by Mondal *et al*. (2005), Bajpai *et al*. (2007) and Taspinar *et al*. (2008); GSTT1 null genotype was associated with a significant increase in the risk of CML. Also in previous studies done by Rollinson *et al*. (2000) and Arruda *et al*. (2001), GSTT1 null genotypes were shown to be risk factors for both acute myeloid leukemia (AML) and acute Lymphoblastic Leukemia (ALL).

Also in agreement with our study, Bajpai *et al*. (2007) found statistically significant difference between GSTT1 null genotype in CML patients and controls (20 versus 8.5% in controls (OR = 2.67, 95% CI: 1.03-7.01). He also agreed with our results, reporting no difference in the frequencies of the GSTM1 null genotype and the combined GSTM1 and GSTT1 null genotypes in patients and controls.

As regard to GSTM1 null genotype alone, which was slightly higher in frequency in CML patients (46.7%) compared to controls (40%), this differences did not attain formal statistical significance.

A study from Brazil done by Loureno *et al*. (2005) on CML patients reported the frequencies of GSTM1, GSTT1 and combined null genotypes in patients and controls (43.2 versus 43.7%, p = 1.00; 18.4 versus 17.6%, p = 0.95 and 7.2 versus 7.9%, p = 0.75, respectively). They have concluded that individuals with GSTM1 (OR = 0.98; 95% CI: 0.65-1.50), GSTT1 (OR = 1.06; 95% CI: 0.62-1.80) and combined null genotypes (OR = 0.84; 95% CI: 0.36–1.96) are at similar risk of CML as compared with those without the null genotypes. Similarly, Chen *et al*. (2008) study showed no significant differences between the frequencies of GSTM1, GSTT1 and combined null genotypes in patients and controls.

The results provided by Loureno *et al*. (2005) and Chen *et al*. (2008) were different from ours regarding the role of GSTT1 null genotype as risk factor for CML, however similar results regarding the role of GSTM1 null genotype were provided.

In this study, we also investigated 3 alleles of CYP1A1 (CYP1A1*2A, CYP1A1*2B and CYP1A1*4) and found no significant differences between patients and controls regarding CYP1A1*2B and CYP1A1*4, while cytochrome P450 CYP1A1*2A allele was significantly underrepresented among CML cases compared to controls (3.3 versus 45%, p<0.001). As an estimate for the relative risk, the odds ratio for CYP1A1*2A was 0.042 with a 95% confidence interval of 0.005-0.373. These data indicate a reduced risk for CML in individuals carrying the mutant allele CYP1A1*2A. This is in accordance with the early study done by Löffler *et al*. (2001) whom study revealed that the mutant allele CYP1A1*2A was higher in frequency in control group rather than CML patients (16.0 versus 6.4%, p<0.01) and the odds ratio was 0.36 with a 95% confidence interval of 0.16-0.80. This study was further confirmed by Bajpai *et al*. (2008) study on the Indian CYP1A1 polymorphism who reported higher frequency of the CYP1A1 *2A allele among controls compared to patients (32 versus 21.8%) while the frequencies of CYP1A1 alleles *2B and *4 in cases versus controls were

18.1 versus 16% and 9 versus 4%, respectively. In contrast to our findings, Chen *et al*. (2008) found no obvious variation in frequencies of CYP1A1 genotype between healthy controls and CML patients.

This difference in results between different studies can be attributed to interindividual variability in xenobiotic metabolism. Individuals with certain genotypes that increase metabolic activation of carcinogens and decreased detoxification are inherently more susceptible to carcinogens and have higher risk of cancer. Thus these genetic polymorphisms can lead to different host phenotypes and contribute to different disease profiles. The frequencies of polymorphisms in different populations are variable due to different ethnic backgrounds, environment and even lifestyle.

In conclusion, this study showing the involvement of the mutant allele CYP1A1*2A and GSTT1 null genotype as independent protective and risk factors, respectively for CML in our population, irrespective of other prognostic factors of CML. However these findings should be considered as preliminary results, although, promising, but warrant further investigation with larger sample size. This study is important, as understanding the interplay of xenobiotic exposures, endogenous physiology and genetic variability at multiple loci will facilitate knowledge about cancer etiology and the identification of the individuals who are at increased risk of developing cancer.

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