PHARMACOGENETICS OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA: INVESTIGATION OF FREQUENCY OF *TPMT* RISK ALLELES FOR THIOPURINE TOXICITY AND THE ROLE OF *SULT1A1*, *EPHX1* POLYMORPHISMS AS RISK FACTORS FOR DEVELOPMENT OF THE DISEASE

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ABSTRACT

PHARMACOGENETICS OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA: INVESTIGATION OF FREQUENCY OF *TPMT* RISK ALLELES FOR THIOPURINE TOXICITY AND THE ROLE OF *SULT1A1*, *EPHX1* POLYMORPHISMS AS RISK FACTORS FOR DEVELOPMENT OF THE DISEASE

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Thiopurine methyltransferase (*TPMT*) risk alleles (mainly **2,*3B, *3C* and **3A*) are the major determinants of interindividual differences in the severe toxicity or efficacy of 6-mercaptopurine (6MP) during the treatment of childhood acute lymphoblastic leukemia (ALL). The frequencies of these risk alleles, known to functionally impair TPMT activity, were investigated among 167children with ALL and 206 healthy adult controls in Turkish population by using allele specific PCR and PCR-RFLP methods. *TPMT*3A* and *TPMT*3C* were the only deficiency alleles detected in Turkish population with an allele frequency of 0.5% for both. The total frequency of mutant *TPMT* alleles in Turkish population (1.0%) was found to be significantly lower than those of other Caucasian populations (5.3-7.0%), but it was found to be very similar to Kazak population (1.2%) which is also Caucasian in ethnic origin.

In the patient group, two individuals were found to be heterozygote for **3C* and **3A* allele. One individual was homozygous mutant *(*3B/*3C*). In this study, the clinical histories of the patients with TPMT defects were examined retrospectively from hospital records. The patients with heterozygous or homozygous mutant genotypes had systematically developed severe neutropenia, infection and some other specific conditions (like lesions around mouth, oral herpes and high fever) when they were administered with 6MP during the therapy. This study provides the first data on the frequency of common *TPMT* risk alleles in the Turkish population, based on analysis of pediatric patients with ALL. The results would contribute valuable information to the public health, as more clinicians and patients become aware of the importance of *TPMT* polymorphisms, less patients will suffer from 6MP related adverse effects.

In addition, in this study two genes *EPHX1*-microsomal epoxide hydrolase (exon 3 and exon 4 polymorphisms) and *SULT1A1*2* variant – sulfotransferase 1A1, either alone or in combination were investigated as risk modifiers in the development of childhood acute lymphoblastic leukemia due to their dual role (activation/detoxification) in the metabolism of various carcinogens. Also interactions of these polymorphisms with non-genetic risk factors (parental smoking exposure and parental age at conception) were investigated. The conclusion inferred from results was that only genetically reduced EPHX1 activity (homozygous mutant genotype for EPHX1 exon 3 polymorphism and some specific genotype combinations with exon 4 polymorphism) was found to be significantly associated with the risk of childhood ALL.

Key words: TPMT risk alleles; 6MP drug response; SULT1A1, EPHX1; childhood ALL; pharmacogenetics.

ÖZ

ÇOCUKLUK DÖNEMİ AKUT LENFOBLASTİK LÖSEMİ FARMAKOGENETİĞİ: THIOPURINE TOKSİSİTESİNDE YER ALAN TPMT RİSK ALELLERİNİN FREKANSININ ARAŞTIRILMASI VE SULT1A1, EPXH1 POLİMORFİZMLERİNİN HASTALIĞIN GELİŞİMİNDEKİ ROLÜ

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Tiyopurin metil transferaz (TPMT) risk alelleri (başlıca, **2,*3B, *3C* ve **3A*) çocukluk dönemi akut lenfoblastik löseminin (ALL) tedavisinde yaygın olarak kullanılan 6-merkaptopurine (6MP) karşı bireyler arasında görülen farklı efikasite ve toksisitelerin en önemli nedenidir. Çok düşük yada noksan TPMT aktivitesine neden olan bu risk alellerinin frekansları, Türk populasyonunda 167 ALL hastası çocuk ve 206 sağlıklı yetişkinde alel spesifik PCR ve PCR-RFLP metotları kullanılarak araştırıldı. *TPMT*3C* (%0.5) ve *TPMT*3A* (%0.5) risk alelleri Türk populasyonunda görülen tek *TPMT* mutasyonlarıdır. Türk populasyonunda *TPMT* mutasyonlarının toplam frekansı (%1.0), diğer beyaz popülasyonlarınkinden (5.3-7.0%) düşük ancak yine bir beyaz popülasyon olan Kazaklarınkine (%1.2) çok yakın bulunmuştur.

Hasta popülasyonunda, iki kişi **3A* ve **3C* mutasyonlarını heterozigot olarak taşırken, bir hastada bu iki mutasyonun her ikisi birden

(*3C/*3A) tespit edilmiştir. Bu hastaların dosyaları geriye dönük olarak incelendiğinde, hepsinde 6MP kullanımı sırasında, çeşitli ters (adverse) etkilerin ortaya çıktığı gözlemlendi. Bu etkiler nötropeni, enfeksiyon, ağız kenarında yaralar, uçuk ve yüksek ateş olarak sıralanabilir. Bu çalışma ile ilk kez, ALL hastası Türk çocukları için tüm yaygın olarak görülen TPMT risk alellerinin frekansları ortaya konulmuş ve yaygın TPMT polimorfizmlerini taşıyan hastalarda 6MP bağlı yan etkilerin ortaya çıktığı geriye dönük olarak, hasta dosyaları incelenerek gösterilmiştir. Bu çalışmanın sonuçları halk sağlığı açısından önemlidir. Daha fazla hasta ve doktor TPMT polimorfizmlerinin tedavideki öneminin farkına vardıkça daha az hasta 6MP'e bağlı yan etkileri yaşayacaktır.

Bu çalışmada, ayrıca, *EPHX1* ekzon 3 ve ekzon 4 ve *SULT1A1*2* genetik polimorfizmlerinin, tek başlarına veya kombinasyonlar halinde, çocukluk dönemi akut lenfoblastik lösemi hastalığı riskini modifiye edici rolleri araştırılmıştır. Buna ek olarak, adı geçen genetik polimorfizmlerin diğer genetik olmayan risk faktörleriyle ilişkisi de incelenmiştir. Sadece düşük EPHX1 enzim aktivitesiyle alakalı genetik polimorfizlerin (ekzon 3 polimorfizmi için homozigot mutant genotip'e sahip olanlar ve bunun bazı ekzon 4 genotipleriyle kombinasyonları) çoçukluk dönemi ALL riskini önemli derecede arttırdığı bulunmuştur.

Anahtar kelimeler: TPMT risk alelleri; 6MP ilaç cevabı; SULT1A1, EPHX1; çocukluk dönemi ALL; farmakogenetik.

Dedicated to my mother

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χ^2	Chi-Square
ALL	Acute Lymphoblastic Leukemia
AZA	Azathiopurines
BaP	Benzo(a)pyrene
COR	Case-only Odds Ratio
CYP	Cytochrome P450
DMEs	Drug Metabolizing Enzymes
GST	Glutathione S-tranferase
MALDI- TOF	Matrix Assisted Laser Desorption/Ionization-Time-of Flight
EPHX1	Microsomal Epoxide Hydrolase
6MP	6-mercaptopurine (Purinethol)
MPO	Myeloperoxidase
NAT	N-Acetly Transferase
NQO1	NAD(P)H Quinone Oxidoreductase 1
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PAHs	Polyaromatic Hydrocarbons
PAPs	3'- Phosphoadenosine 5'- Phosphosulfate
RFLP	Restriction Fragment Lenght Polymorphism
SAM	S-Adenosyl-L-Methionine
SNP	Single Nucleotide Polymorphism
SULTs	Sulfotransferases
6TG	6-Thioguanine
TGNs	Thioguanine Nucleotides
TMPT	Thiopurine methyl-transferase
UGT	UDP-glucuronosyl-transferase

CHAPTER 1

INTRODUCTION

Cancer is a serious health problem-second leading cause of death across the world. It is estimated that 7.6 million people in the world died of cancer in 2007 (American Cancer Society, 2007). However, during the past 40 years many remarkable advances have been accomplished in cancer therapies and cure rate have increased considerably with the currently available treatment regimens. During the period 1974-1976, the 5-year survival rate among adults for all cancers was approximately 50%, in 2000s, this rate increased to approximately 65% (www.cancer.gov).

The improvement in survival rate of childhood acute lymphoblastic leukemia (ALL), being the most common fatal childhood cancer (25% of all childhood cancer cases), over the past 35 years is one of the great success stories of cancer treatment. In the 1960s, while less than 5 percent of children with ALL survived for more than five years, today, about 85 percent of children with ALL live five years or more (www.cancer.gov). According to the pediatric clinicians, a 90% of cure rate for ALL is quite possible in the near future with the successful implementation of the pharmacogenetics into routine clinical practices.

1.1 Pharmacogenetics

Patients who are treated with standard doses of a given drug compound may represent large interindividual variations in respect to efficacy and toxicity of the drug. Individual's sex, age, race, organ function, nature and severity of the disease being treated, drug interactions and concomitant illness are potential reasons for individual variations in response to drug treatment, however, inherited differences in the drug transporters, drug metabolism and drug targets have a greater impact on both the efficacy of a drug and the likelihood of an adverse drug reactions (Evans and Johnson, 2001).

Regarding cancer treatment, there is a wide variability in response of individuals to standard doses of chemotherapeutic agents. This variability mostly can be attributable to genetic variations in the genes coding for drug transporters, drug metabolizing enzymes and drug targets which may result in decreased clinical response to anticancer agents or increased incidence of adverse drug reactions (therapy related side effects). In this point, pharmacogenetics takes its role and it aims to identify the individuals who are at greater risk of adverse drug effects due to these genetic variations.

Pharmacogenetics can be defined as the study of biological consequences of hereditary variations "Genetic Polymorphisms" in the genes that is responsible from drug disposition and drug response. "Genetic polymorphism is a specific change in DNA sequence that occur in more than 1% of the population" (Meyer, 2000). Polymorphisms caused by the presence of more than one allele for the same gene which result in more than one phenotype in the organisms. "Their frequencies (more than 1%) in a population are relatively high comparing to any genetic defect or mutation (less than 1%) since polymorphisms generally do not cause any

sickness or other problems that would decrease the reproductive efficiency". (Gonzales, 1999)

1.2 Types of Genetic Polymorphisms

Types of genetic polymorphisms can be examined in two aspects: functionality and structure. According to functionality, polymorphisms can be categorized as functional and non-functional polymorphisms. A functional polymorphism is a change in the DNA sequence of a gene that results in different levels of expression of protein or enzyme, or in alteration of the activity, while a non-functional polymorphism results in neither of them (Gonzalez, 1999). The functional polymorphisms can be either in the coding or in the noncoding regions of the gene. Variations in the coding region of a gene have the potential to alter enzyme activity or protein function. The non-coding regions of a gene are comprised of introns –which are spliced of posttranscriptionally; and regulatory regions, which are not transcribed but regulate the level of expression of the protein. Genetic variability in these noncoding regions is associated with altered levels of protein rather than changes in the protein itself (McKinnon and Evans, 2000).

According to structure, polymorphisms can be listed as deletions/insertions (InDel), varying number of tandem repeats (VNTR), copy number variations (CNVs), epigenetic variations of the human genome such as DNA methylations, microRNA regulations and single nucleotide polymorphisms (SNPs).

By definition InDel polymorphisms is the simply deletion or insertion of DNA sequences that are 1 to 1000 nucleotides long with the one or three base pair deletions being more frequent. At the present time,

there are probably more than 100,000 InDel polymorphisms in the human genome.

VNTRs are tandem repeats of two to ten nucleotides (microsatellites or short tandem repeat-STR), 10-100 nucleotides (minisatellites) and of relatively larger tandem repeat units -100-1000bp (satellites). Their predicted occurrence of the human genome is more than 500,000.

In respect to pharmacogenetics most important human genome variations seen in the drug disposition or drug response elements are CNVs, epigenetic variations and SNPs, for this reason, they will be handled in detail in the next section.

1.2.1 Copy Number Variations, Epigenetic Variations and SNPs

Copy number variations (CNVs) are DNA segments at 1 kb or larger with a variable number of copies in comparison with a reference genome (Ingelman-Sundberg et al., 2007). CNVs can have dramatic phenotypic consequences as a result of altering gene dosage, disrupting coding sequences, or perturbing long-range gene regulation (Stranger *et al*., 2007). Initially CNVs were thought to be very rare events however by the invention of new techniques that rapidly allow the analysis of full genomic occurrence of CNVs, it was recently found that this is a very common phenomenon in the human genome and at the present time, a total number of 1447 CNV had been identified in the human genome, covering 360 Mb (12% of the genome) (Ingelman-Sundberg *et al*., 2007). There are several well known examples of CNVs in pharmacogenetics, especially in drug metabolizing enzymes such as in CYP2D6, GSTM1/T1, and SULT1A1.

Epigenetic variations of the human genome such as variations in DNA methylation and variation in micro RNA control significantly change the pattern of gene expression from one individual to another. Such changes can significantly modify drug efficacy or initiate adverse drug reactions and so they have to be taken into consideration in clinical pharmacology.

DNA methylation occurs predominantly at CpG sites in the mammalian genome (Jones and Takai, 2001) by the DNA methyltransferase (DNMT) enzyme (Bestor, 2000; Rountree *et al*., 2001). Initially variations in the pattern of DNA methylation were also ignored like CNV however it has been recently identified that at least 20% of all genes are differentially methylated in the promoter region or in the coding regions (Brockmöller and Tzvetkov, 2008) and their importance were also validated with clinical studies. For example, O6-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme, show interindividual variation in its activity due to differential methylation pattern and this result in increased sensitivity of cancer patients for alkylating anti-cancer drugs such as cyclophosphamide or carmustine (Esteller *et al.,* 2000). Another example is a drug metabolizing enzyme- CYP1B1 which metabolizes tamoxifen and estradiol used in the treatment of breast cancer. Expression of CYP1B1 is epigenetically regulated and the extent of CYP1B1 methylation could predict survival in tamoxifen treated and non-treated patients thus predict the response of the patients to these anticancer drugs (Widschwendter *et al*., 2004).

miRNAs are a family of non-coding RNAs that base pair to target mRNA and typically decrease their expression (Massirer and Pasquinelli, 2006). At present, about 0.5–1.5% of the total genes in sequenced animal species are known miRNA genes (Carthew, 2006). At least 474 miRNA genes have been identified in human (see http://microrna.sanger.ac.uk/

cgi-bin/sequences/browse.pl). Studies on miRNA regulated genes that are pharmacogenetically important are very scarce. For example, despite the fact that, CYP1B1 is the only P450 dependent drug metabolizing enzyme that is known to be regulated with miRNA (Tsuchiya *et al*., 2006), there is not yet any evidence that this posttranscriptional regulation could be responsible for unexplained interindividual variability in CYP1B1 expression and activity.

Finally, SNP is the simplest form (presence of two different nucleotides at one defined position) and most common source of genetic variations. According to the last release of SNP database (http://www.ncbi.nlm.nih.gov/SNP/Notes/build127_announce.txt), there are almost 12 million SNPs have been found in the human genome and this makes about 90% of all human DNA polymorphisms. Regarding the interindividual differences in drug response, SNPs take much of the attentions since they are the most common inherited sequence variations occurring in the genes encoding drug-metabolizing enzymes, transporters, and target molecules. The presence of functional SNPs in these genes may affect both pharmacokinetics and pharmacodynamics of the respective drug (Yamayoshi *et al*., 2005).

1.3 Pharmacokinetic vs Pharmacodynamics

Once a drug administered, it is absorbed and distributed to its site of action where it interacts with target molecules in the body (such as receptors, ion channels, enzymes and immune system), undergoes metabolism (by drug metabolizing enzymes; DMEs), and is then excreted (Weinshilboum, 2003). Pharmacokinetics and pharmacodynamics are two important terms in the pharmacology involving each of these processes. There is a clear cut distinction between the pharmacokinetics which refers

to action of the body on drug and pharmacodynamics which refers to action of the drug on body.

Pharmacokinetic involves the processes of Absorption, Distribution, Metabolism and Excretion (ADME in short). Pharmacodynamics involves the interaction of the drug with its targets in the body. As demonstrated in Figure 1.1 each processes in pharmacokinetics and pharmacodynamics has clinically significant genetic variations and those genetic variations in turn affect the fate of drug compound (affecting the way of interaction with its target and also the way of its metabolism etc.). In relation to efficacy of a drug and adverse drug reactions, most interest has centered on the involvement of pharmacokinetic processes.

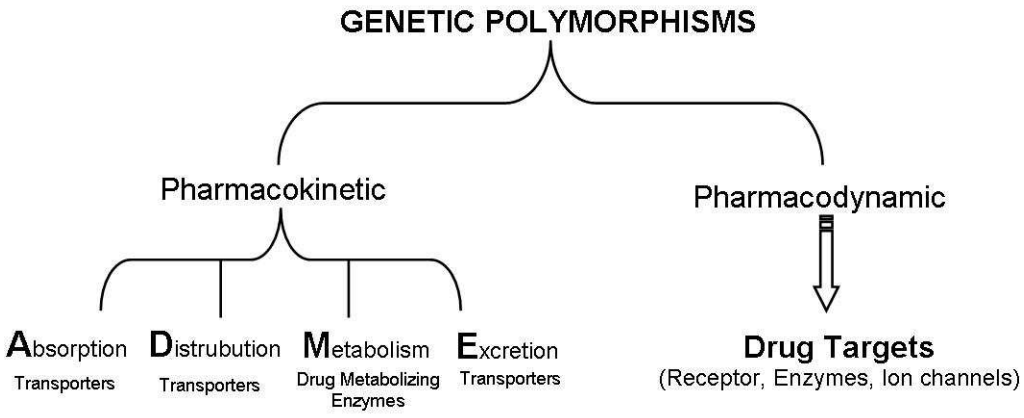


Figure 1.1 Genetic polymorphisms affect both pharmacokinetic and pharmacodynamic of the drug (Modified from Primohamed and Park, 2001).

In pharmacogenetics perspective, most of the attentions are given to the genetic variations in drug metabolizing enzymes relevant to efficacy and adverse drug reactions. In fact, pharmacogenetics originated as a result of the observation that there are clinically important inherited variations, in drug metabolism. Regarding to this information, in the first part of this thesis, main consideration will be given to the pharmacogenetics of drug metabolizing enzymes with a special emphasis on thiopurine methyl transferase polymorphisms due to its critical role in the metabolism of an anticancer drug: 6-mercaptopurine essentially used in the treatment of childhood acute lymphoblastic leukemia. Besides, today, there is increasing realization that genetic variations in drug targets, and transporters might also clinically important for the efficacy and toxicity of a drug therapy. Although research into this area is in its infancy, firstly herein a brief summary will be made in pharmacogenetics impacts of the drug transporters and drug targets.

1.4. Genetic variations in Drug Targets

Research studies on pharmacogenetics of drug targets began in late 1990 and in recent years there has been growing interest in genetic polymorphisms of drug targets in relation to therapy outcome- safety, efficacy and adverse drug reactions. There are many examples of drug targets-drug response variability and relevant drug/drug classes. This lengthy subject will not be covered here in detail. However, a specific example of tyrosine kinase inhibitors is worth to mentioning for better understanding of relationship between genetic variation in drug target and drug response.

In chronic myelogenous leukemia, the Philadelphia chromosome leads to a fusion protein of *abl* with *bcr*. The resulting fusion protein, *bcr-abl* is an active tyrosine kinase with an anti-apoptopic function (Druker *et*

al., 2001). Imatinib (Gleevec®, Novartis, Basel, Switzerland) is a specific inhibitor of bcr-abl tyrosine kinase and preventing its anti-apoptopic function (Druker *et al*., 2001; Kantarjian et al., 2002). A SNP- rs2290573 (C/T transition) have been reported to be present in the tyrosine kinase gene. A significant association between the response rate of individuals to drug and rs2290573 polymorphisms was observed in imatinib-treated patients. Patients with the CT or TT genotype showed a higher major cytogenetic response rate as compared to patients with the CC genotype and the time to progression has also been reported to differ significantly among these genotypes (Dressman *et al*., 2004). The distribution of CC:CT/TT has been reported to be 10:67 in Caucasians, 10:1 in African-Americans, and 2:1 in Asians (Dressman *et al*., 2004). Therefore, response rate to imatinib show wide interethnic variations.

1.5. Genetic Variations in Drug Transporters

It is well established that drug transport across the biomembranes is subject to active and facilitated transport processes and membrane transporters play an important role in the absorption, distribution and excretion of drugs (see Figure 1.1), (Ambudkar *et al*., 1999; Cascorbi, 2006). With recent progress in the area of pharmacogenetics, it has been well recognized that the genetic variations occurring in the drug transporters will become a marker to predict the response to drug (Okamura *et al*., 2004).

Most of the transporters belong to the ATP-binding cassette (ABC) superfamily of membrane proteins, which share many physicochemical characteristics. The human ABC transporter superfamily consists of 49 members which are divided in 7 subfamilies, from A to G, based on similarity in gene structure, order of the domains and sequence homology. These transporters play a major role in defense mechanism against

penetration of xenobiotics or transmembrane transport of various endogenous compounds. The energy necessary for the substrate translocation across biomembranes is generated from the hydrolysis of ATP and intermediate phosphorylation of the transporter, enabling active transport of substrates against steep concentration gradients (Cascorbi, 2006). Among ABC transporters, ABCB1 (P-gp, MDR1) and multidrug resistance associated proteins-ABCC (MRPs), have been investigated mostly since they are expressed in many cancer cell. They are conferring drug resistance in cancer cells and in many normal tissues and they play important roles in drug absorption and excretion.

P-glycoprotein- mostly known as MDR1 (P-gp, ABCB1) is the best characterized and most widely studied human drug transporter. Fifteen different polymorphisms in MDR1 were detected. Two SNPs: a silent mutation C3435T in exon 26 (rs1045642) and a missense polymorphism (Ala893Ser/Thr) within exon 21, G2677T/A (rs2032582) generated the greatest interest regarding the variation in drug response (*Hoffmeyer et al*., 2000). For example in one study, it has been found that in respect to C3435T polymorphism, individuals homozygous for this polymorphisms (T/T genotype) had the lowest Pgp expression and the highest digoxin (a drug used in the treatment of various heart conditions) plasma concentration compared with individuals with the wild type genotype (C/C) (Hoffmeyer *et al.,* 2000) In another clinical study comprised from 90 patients with chronic myeloid leukemia, for the G2677T/A polymorphism, the presence of G allele was found to be associated with worse response to imatinib (77.8%, TT/TA; vs 47.1%, GG/GA/GT; P = .018) (Dulucg *et al*, 2008). A large range of anticancer drugs are substrates of MDR1 and studies evaluating the relationship between genetic variations in MDR1 and response to chemotherapy are in progress.

MRPs –ABCC subfamily has thirteen isoform through ABCC1 to ABCC13. Each of them has its own group of substrate specificity. Several in vitro studies have been conducted for the identification of relationship between genetic variation in different ABCC gene and transport activity. For example, in a study using transfected human embryonic kidney cells, Condrad *et al*., (2001) demonstrated that MRP1 C1303A (Arg433Ser) polymorphisms result in less transport activity for leukotriene C4 and estrone-3-sulfate (Conrad *et al*., 2001). A number of experiments like that performed in vitro suggest that drug response could be altered by genetic polymorphisms found in the MRPs. However, further evaluations of them in clinical populations should be needed to confirm the impact on drug response.

1.6. Genetic variation in Drug Metabolism

50 years ago, pharmacogenetics originated as a result of the observation that there are clinically important inherited variations, in drug metabolism. Today also most of the attentions regarding the clinical relevance of pharmacogenetics are oriented to the effects and outcomes of genetic variations observed in drug metabolism.

Drug metabolism simply is the process that converts drug molecules to more water soluble thus easily excreted metabolites. It can also convert prodrugs (inactive form of the drug-metabolically activated) into therapeutically active metabolites, and it may even result in the formation of toxic metabolites (Conney and Burns, 1972; Weinshilboum, 2003). Metabolism is usually processing through specialized enzymatic systems that catalyze specific reactions. In classical pharmacology, these reactions are classified as “Phase I” and “Phase II” reactions. While phase I reactions occurs by hydrolysis, oxidation and reduction and phase II involves conjugation reactions i.e. acetylation, glucuronidation, sulfation

and methylation (Schenkman, 1991). Although in the metabolism of the many drug compounds phase I reactions precedes phase II, the numeric used in the names is historical, meaning that phase II reactions also preceeds phase I and even occur without prior hydroxylation, oxidation or reduction. In both type of reactions, the general purpose is the introduction or unmasking of polar molecules to the lipophilic drug compounds thus more polar metabolites of the original molecule can be easily excreted from the body.

Almost all enzymes functioning in phase I and phase II pathways exhibit genetic polymorphisms that result in mostly a phenotypic consequence, and thus contributes to interindividual variations in drug response (Eichelbaum, 1999). Generally these phenotypic consequences can be classified into three groups: Individuals with two alleles coding for “normal” enzyme function are termed as efficient metabolizers (EM or “wild-type”), whereas those with two variant alleles resulting in inactive or absent enzyme are “poor metabolizers” (PM). The ultrarapid metabolizers (UM), with more than 2 active genes (inherit multiple copies of wild-type alleles) have increased enzyme activity which does not allow the drug to reach the therapeutically effective concentrations and these patients when treated with standard doses of the drug fail to respond drug therapy. However, in case of prodrug- when the parent compound is not active and require metabolic activation for therapeutic efficacy- opposite effects can be observed in PMs and EMs (Weide and Stejins, 1999). In addition to this general classification, there may also be an intermediate metabolizer (IM) phenotype with reduced function, which usually results from the presence of one variant and one normal allele (heterozygous EM). In most of the reports, intermediate and efficient metabolizers are often collectively referred to as “efficient metabolizers”, especially in studies in which metabolizer status is assigned using phenotype (Gardiner, 2006).

Metabolizer status can be assigned to individuals by phenotypic or genotypic assessment. Phenotypic assessment *in vivo* is generally based on the metabolic ratio which is measured by the administration of a probe drug (known to be selectively metabolized by the enzyme under study, i.e., dextromethorphan for CYP2D6, chlorzoxazone for CYP2E1). This is followed by the measurement of the ratio of unchanged drug to its metabolite in serum and/or urine which simply gives the metabolic ratio (Gonzales, 1999; Weinshilboum, 2003). Genotypic assessment is mostly based on polymerase chain reaction (PCR) technology, in which the DNA containing the polymorphic area of interest is amplified. Then, the polymorphism can be detected by the restriction fragment length polymorphism (RFLP) technique or other band pattern analysis techniques (like Single Strand Conformation Polymorphisms-SSCP, heteroduplex analysis) on agorose or SDS gel, by annealing temperature, MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of Flight) analysis or direct sequences. Although phenotypic assessment based on the metabolic ratio gives the direct measure of the real condition in the serum, it doesn't necessarily mean that observed variation in the activity is due to genetic variation. There may exist many factors result in this variation such as drug-drug interaction, induction or inhibition of the enzyme, and physiological conditions of the patient. However, application of molecular genetic techniques elucidates the molecular mechanism underlying the variation. Today, the recent and very rapid progress in the field of molecular biology has allowed the identification of genetic polymorphisms in the major Phase I and Phase II drug metabolizing enzymes. These studies have been followed by the identification of the phenotypic assessment of variations and lastly their clinical relevance has been studied in large patients groups in respect to drug response and adverse drug reactions. By this approach, a wide range of clinically important genetic variations have been elucidated in phase I and phase II drug metabolizing enzymes.

1.6.1. Pharmacogenetics of Phase I Drug Metabolizing Enzymes

As mentioned above, phase I reactions involve hydrolysis, oxidation and reduction. Major phase I enzyme is the superfamily of cytochrome P450s which are the principal enzymes involved in the oxidation of the 80% of all clinically used drug compounds (Eichelbaum *et al*., 2006). The polymorphic forms of P450s are responsible for the development of a significant number of adverse drug reactions. According to Phillips *et al*. (2001), 56% of drugs that are cited in adverse drug reaction studies are metabolized by polymorphic phase I enzymes, of which 86% are cytochrome P450s (Phillips *et al*., 2001).

1.6.1.1 Cytochrome P450s

Cytochrome P450 monooxygenase system, also known as NADPH dependent cytochrome P450 mixed function oxidase, is a microsomal system. It functions as a multicomponent electron transport system, which undergoes a cyclic series of reaction. The liver microsomal cytochrome P450 dependent monooxgenase system contains 2 protein components (Lu and Coon, 1968); cytochrome P450 and cytochrome P450 reductase, and a heat stable factor lipid (Lu *et al*., 1969); which was later identified as phosphotidylcholine dilauroyl (Lu *et al*., 1970). All three components of cytochrome P450 dependent monooxygenase system (NADPH-cytochrome P450 reductase, cytochrome P450 and lipid) are required to reconstitute the full hydroxylation activity (Lu *et al*., 1969; Lu and Levin, 1974; Schenkman and Jhonsons, 1975; Arınç and Philpot, 1976; Black and Coon, 1986; Adalı and Arınç, 1990; Arınç, 1993; Şen and Arınç, 1997, 1998; Bozcaarmutlu and Arınç, 2008).

In this enzyme system, cytochrome P450 consists of a superfamily of enzymes which are categorized into respective families and

subfamilies. P450 proteins exhibiting more than 40% amino acid sequence similarity are classified within the same family, while proteins exhibiting more than 55% sequence similarity are grouped into the same subfamily (Nebert and McKinnon, 1994; Nelson *et al.,* 1996) and those that are more than 97% identical are represent alleles unless there is evidence to the contrary Nebert *et al*., 1991).

The great genetic multiplicity of P450 superfamily has been arisen a need for a standard nomenclature system that name and assign individual genes into families and subfamilies and thus P450 Nomenclature Committee was established (http://drnelson.utmem.edu/ CytochromeP450.html). According to this committee, nomenclature of P450s is based on the level of amino acid sequence identity, phylogenetic association and gene organization. In naming of individual cytochrome P450 enzyme, the abbreviation “CYP” (Cyp for Drosophila and mouse) standing for the **CY**tochrome **P**450 is followed by an Arabic number for designating individual family and this is followed by a capital letter to show the subfamily name. Following the capital letter, lastly, individual isoform is shown by an Arabic number. In accordance with this nomenclature system, the first officially named cytochrome P450 was CYP1A1. The gene encoding the cytochrome P450 isoforms are denote similarly, but with italics, like *CYP1A1.*

P450s have been characterized in many species of organisms, including bacteria, fungi, plants, fish, birds, reptiles, insects and mammalian systems (Lu and Levin, 1974; Philpot *et al.*, 1975; Arınç *et al*., 1976; Arınç and Philpot, 1976; Coon *et al*., 1978; Nebert and Mc Kinnon, 1994; Şen and Arınç, 1997, 1998; Bozcaarmutlu and Arınç, 2008). By the February, 29, 2008, a total of 8128 P450s (excluding variants, including pseudogenes) were identified from various organisms, so that 2872

animal, 2867 plant, 912 bacterial, 239 protist and 1238 fungal P450s were counted (http://drnelson.utmem.edu/CytochromeP450.html).

In humans, as of May 17, 2007, 18 families with 58 different active genes encoding P450 enzymes (http://drnelson.utmem.edu/ CytochromeP450.html) and a same number (58) of pseudogenes have been identified (http://drnelson.utmem.edu/CytochromeP450.html). These CYP families catalyze in total more than 200,000 different substrates which include mainly drug compounds, other xenobiotics, and several types of endogeneous compounds. Human cytochrome P450s that metabolize drug compounds are almost exclusively in the CYP1, CYP2 and CYP3 families. All genes encoding P450 enzymes in these CYP families (1–3) are polymorphic. At present, more than 350 functionally different CYP alleles, i.e., gene variants that affect the function and/or activity of the gene products are presented at the CYP allele Web site (http://www.imm.ki.se/cypalleles). In this web site the highest number of variant alleles are described for CYP2D6 (63 alleles), CYP2B6 (28 alleles), CYP1B1 (26 alleles) and CYP2A6 (22 alleles). (http://www.imm.ki.se/cypalleles). Approximately 40% of human P450-dependent drug metabolism is carried out by polymorphic enzymes in particular CYP2C9, CYP2C19 and CYP2D6, which can cause abolished, quantitatively or qualitatively altered drug metabolism and this makes drug dosing problematic. The major polymorphic P450 forms, their major variants alleles and ethnic group differences were given in Table 1.1 Some of the examples that have strong clinical implication in terms of given variant alleles were also listed in Table 1.1 to be able to clarify the effects of polymorphisms on drug response.

Table 1.1 Some of the clinically important polymorphic P450 forms, their major alleles, allele frequencies and clinical examples.

Enzyme-Some of the Major variant allele	**Mutation**	**Consequence for enzyme function**	**Allele frequencies (%)**	**Representative Drug Metabolized & Clinical Example**
CYP2D6				
CYP2D6*2XN	Gene duplication or multiplication	Increased enzyme activity	[a]Turkish, 6[#] [b]Caucasian, 1-5 [b]Asians, 0-2	Codeine (prodrug) is converted to morphine (active metabolite) by CYP2D6. **An infant death** was caused by a breast feeding mother of the **UM phenotype (with multiple copies of CYP2D6)** who took high doses of codeine with the resulting formation of morphine at levels being lethal to the infant (Koren *et al*., 2006).
CYP2D6*4	Defective splicing	Inactive enzyme	[a,d]Turkish, 11-21 [b]Caucasian,12-21 [b]Asians, 1	Tamoxifen is converted to endoxifen (100 times more potent than tamoxifen) by CYP2D6. Tamoxifen treated breast cancer women who are **homozygous for CYP2D6*4** allele tend to have a **higher risk of disease relapse** due to not able to produce active metabolite (endoxifen) relative to women heterozygous or homozygous wild type (Goetz *et al*., 2005).

Table 1. 1 (Continued)

Enzyme-Some of the Major variant allele	**Mutation**	**Consequence for enzyme function**	**Allele frequencies (%)**	**Representative Drug Metabolized & Clinical Example**
CYP2C9				
CYP2C9*2	Arg144Cys	Reduced affinity for P450 reductase	[c]Turkish, 10 [b]Caucasian,8-13 [b]Asians, 0	S-warfarin is an anticoagulant metabolized by CYP2C9. Patients with CYP2C9*2 allele showed **decreased level of S-warfarin clearance** which is associated with a **higher rate of major bleeding complications** during the initiation of warfarin therapy and with **longer hospitalization times** (Higashi *et al*., 2002).
CYP2C9*3	Ile359Leu	Altered substrate specificty	[c]Turkish, 9-10 [b]Caucasian,6-9 [b]Asians, 2-3	For most of the drug compound metabolized by CYP2C9, CYP2C9*3 **heterozygous individuals** had approximately **50% of the wild type total oral clearance** and **CYP2C9*3 homozygous individuals** had a **5- to 10-fold reduction** (Takahashi et al., 1998; Thijssen & Ritzen, 2003). Homozygous mutant individuals exhibited a much higher risk for major bleeding complications (Steward *et al*., 1997).

Table 1.1 (Continued)

Enzyme- Some of the Major variant allele	**Mutation**	**Consequence for enzyme function**	**Allele frequencies (%)**	**Representative Drug Metabolized & Clinical Example**
CYP2C19				
CYP2C19*2	Abberant splice site	Inactive enzyme	[a]Turkish, 12 [b]Caucasian, 13 [b]Asians, 23-32	Antiulcer drugs: proton pump inhibitors **PM phenotype (homozygous mutant)** is **beneficial** in the treatment of patient with gastrointestinal disorders using proton pump inhibitors **due to the reduced metabolism of these drugs by inactive CYP2C19*2** in such individuals, thus leading to increased drug plasma levels (Furuta *et al*., 2002)
CYP2C19*3	Premature stop codon	Inactive enzyme	[a]Turkish, 0.4 [b]Caucasian, 0 [b]Asians, 6-10	Clopidogrel (prodrug): antithrombotic agent activated by CYP2C19. **PM phenotype** does **not response to clopidogrel** since clopidogrel is a pro-drug, it can not be activated by PM phenotype thus patients suffer from an increased risk of recurrent thromboses (Hulot *et al*., 2006)

[a] Data from Aynacioglu *et al*, (1999a). [#] Data given for **MxN* represent any kind of gene duplication.
[b] Data from Ingelman-Sundberg *et al*, (1999).
[c] Data from Aynacioglu *et al*, (1999b).
[d] Data from Koseler *et. al*, (2007).

1.6.2 Pharmacogenetics of Phase II Drug Metabolizing Enzymes

As mentioned before, Phase II reactions involve acetylation, glucoronidation, sulfation, glutathione and amino acid conjugation and methylation, generally known as conjugation reactions. They are usually detoxification reactions in nature however sometimes activation of the compound can be possible. Major phase II enzymes: Glutathione S-transferases (GSTs), N-acetyltransferases (NATs), UDP-glucuronosyltransferases (UGT), methyltransferases and sulfotransferases (SULTs). All these enzymes are polymorphic in nature. The polymorphisms of some of the phase II enzymes particularly GSTs, NATs and SULTs are generally associated with the disease susceptibility which is the subject of second part of this chapter. Among the phase II enzymes, polymorphisms of UGT1A1 (a UDP-glucuronosyltransferase) and thiopurine methyltransferase –TPMT (a methyltransferases) constitute precedent for the value of pharmacogenetics in cancer treatment. They have great impact in terms of their wide range approved clinical applications in the field of cancer pharmacogenetics (Evans *et al*., 1998; Mc Leod and Shiva 2002; Innocenti and Ratain, 2002; Jong *et al*., 2006).

The clinical application of the pharmacogenetics in cancer treatment which can be designated by name “Cancer Pharmacogenetics” is especially important as many anti-cancer drugs given to cancer patients have a narrow therapeutic index. By definition therapeutic index refers to the safe concentration of a drug in the blood that yields therapeutically effective dose. Drug with a narrow therapeutic index is meaning that there is a very fine difference between the toxic and therapeutically effective dose of a given drug. In addition to narrow therapeutic index, many of the anti-cancer drugs are acting on body through toxic action. For all these reasons, any variation in the metabolism of anticancer drugs can result in

very dramatic consequences, for example, low rate of drug metabolism cause an anticancer drug to reach a toxic threshold.

There are impressive mass of data in the literature for the effects of phase I and phase II DMEs polymorphisms on cancer treatment. The general feature of all these studies is that the knowledge obtained from pharmacogenetic researches is not commonly used for the benefit of patients in the clinics (Becquemont, 2003). This is one of the drawbacks of the research studies. However, the clinical importances of two phase II DME polymorphisms: UGT1A1 and TPMT have led to calls by many researchers for prospective assessment of these polymorphisms in patients prior to initiation of therapy (Jackson *et al*., 1997; Relling *et al*., 1999; Ando *et al*., 2000; Iyer *et al*., 2002). Later on, this is approved and strongly recommended by FDA (Federal drug Administration of USA). In the next sections, pharmacogenetics of UDPGT1A1 and particularly pharmacogentics of TPMT will be discussed in detail.

1.7 UGT1A1 Polymorphisms and Irinotecan Toxicity

Irinotecan is an important anticancer agent commonly used in the treatment of colorectal cancer. It is a prodrug and it is activated to major active metabolite SN-38 through a complex pathway to which CYP3A4/3A5 and several carboxyesterases are involved. SN-38 is a topoisomerase I inhibitors and cause cell death as a result of DNA strand breaks due to the formation of cleavable complexes (Chabot, 1997). Thus, it exerts anticancer activity through cytotoxic action. UGT1A1 is the major phase II enzyme responsible for the inactivation of active metabolite SN-38 through glucoronidation. By this pathway, the concentration of active metabolite decreases in the systemic circulation. Therefore the activity of UGT1A1 is extremely important in terms of both efficacy and toxicity of the irinotecan. As irinotecan has a very narrow therapeutic

index and it is also acting on the body through cytotoxic action, it has very important dose-limiting life threatening toxicities like myelosuppression and diarrhea (Nagar and Blanchard, 2006). For this reason any decrease in the UGT1A1 enzyme activity causes high doses of SN38 active metabolites in the blood which may cause severe myelosupression. Myelosuppression caused by high doses of SN38 metabolite manifests as grade 3 to 4 neutropenia, leukopenia, severe anemia, or severe thrombocytopenia (Cersosimo, 1998).

The UGT superfamily of enzymes catalyzes the conjugation of a wide variety of endobiotics and xenobiotics. UGTs proteins are bound to the endoplasmic reticulum (ER) membrane, with the catalytic domain proximal to the lumen of the ER. The superfamily is divided into families and subfamilies based on protein sequence identity such that gene products in the UGT1 and UGT2 families exhibit less than 50% identities (Burchell, 2003). In UGT1 family, UGT1A1 is the major enzyme responsible for the glucoronidation of SN38. This gene contains many SNPs. However, by far the most well-known polymorphism studied with regard to irinotecan toxicity is an insertion/deletion polymorphism of TA-nucleotides designated UGT1A1*28 (Jong *et al*., 2006). This genetic variant is located in the promoter region of UGT1A1 that controls transcription of the UGT1A1 gene. The number of TA-repeats in the UGT1A1 gene promoter may vary between 5 and 8, and each variant is designated by a separate code: TA5-*36, TA6-*1, TA7-*28 and TA8-*37. In individuals carrying the TA7 allele (UGT1A1*28-especially those being homozygous for this variant) the UGT1A1 expression is reduced up to 70% (Nagar and Blanchard 2006; Jong *et al*., 2006). Initial preliminary reports indicated that the low-activity UGT1A1 *28 variant was associated with increased irinotecan toxicity (Ando *et al*., 2000; 2002; Iyer *et al*., 2002). Recently, another low activity UGT1A1 variant-UGT1A1*6 which is more prevelant than UGT1A1*28 in Asian populations, has been found to

be associated with irinotecan related hematologic toxic effects (Ichikawa *et al*., 2008). However this study is based on a few patients, further studies are needed to clarify this issue. As mentioned above, the general idea behind the low activity allele for UGT1A1-irinotecan toxicity is that the decreased inactivation of active metabolite SN38 causes high concentration of this cytotoxic agent in the systemic circulation. As a result, as well as cancer cells, it also causes cell death in the normal blood cells, the extreme cases of which is known as myelosuppression.

Various studies have examined UGT1A1 *28 and its association with irinotecan-related toxicity. A retrospective study, conducted by Ando *et al*., (2000) in 26 Japanese cancer patients with severe toxicity, indicated that 15% (4 of 26) of the patients with severe toxicity (leukopenia and/or diarrhea) were homozygous for the UGT1A1 *28 variant, compared to 3% (3 of 92) of patients with no toxicity (Ando et al., 2000). Another study, conducted by Iyer *et al*. (2002) prospectively in 20 solid tumor patients, revealed that 3 patients suffered from severe toxicity, and all 3 carried at least one copy of UGT1A1 *28 (Iyer et al., 2002). In the study of Innocenti et al., (2004) sixty-six cancer patients were subsequently enrolled for a large prospective study with irinotecan monotherapy and were genotyped for UGT1A1 *28 polymorphism (Innocenti *et al*., 2004). Six patients developed severe neutropenia; of these, 3 were heterozygous for UGT1A1 *28, while 3 were homozygous for UGT1A1*28. All these retrospective and prospective clinical studies and many others revealed that there is a clear and striking relationship between UGT1A1*28 polymorphisms and irinotecan related toxicities which can be life threatening. For this reason in 2005, the USA **FDA approved** the inclusion of UGT1A1 genotype information in the irinotecan package insert, with dosing guidelines based on UGT1A1 genotype and later on the **FDA approved** a clinical test for the UGT1A1*28 allele (FDA 2005).

The incorporation of genetic information linked to dosage in package inserts by the USA **FDA** made the integration of pharmacogenetics into clinical practices for cancer treatment a reality. UGT1A1 is the one of two important examples of this reality however it is not the first one. The earliest and best known example of approved DME polymorphism is TPMT polymorphisms. In 2002, FDA has added a recommendation for TPMT genetic test before the prescription of 6MP into package insert of the thiopurine drugs. (http://www.fda.gov/ medwatchSAFETY/2003/03DEC_PI /Tabloid _PI.pdf).

1.8 TPMT pharmacogenetics and Thiopurine drugs

Thiopurine drugs; azathiopurines-AZA, 6-thioguanines-6TG and 6-mercaptourine-6MP (see Figure 1.2 for their molecular structures) have been in common use over the last 45 years (Coulthard and Hogarth 2005). AZA is widely used as an immunosuppressant in the treatment of autoimmune disorders, inflammatory bowel disease and following the organ transplantation. 6TG mostly used in the treatment of acute myeloid leukemia, organ transplantation and rarely some of the acute lymphoblastic leukemia (ALL) protocols. 6MP (the generic name of the drug compound is Purinethol, throughout the text, 6MP was used for simplicity) is used almost all protocols of childhood ALL treatment. It is administered both in the remission induction and in the maintenance phase of the therapy. During remission induction, 6MP are given to the patients with a combination of various anti-cancer drugs to induce complete remission. Once a remission achieved in the patients, a maintenance period starts. During this period which last approximately 2-3 years, 6MP, alone, is given to the patients every night orally (Chessells, 1997; Evans *et al*., 1998). Therefore, the intensive use of 6MP contributes to the high cure rate (85%) achieved in the treatment of this life threatening disorder.

It is well established that most of the patients get high benefit from the use of thiopurine drugs. However, there are some groups of patients that undergo severe and even fatal toxicities. The reason for these interindividual differences in response to thiopurine therapy lies under the metabolism of these drug compounds. Thiopurines are partly metabolized by thiopurine methyl transferase enzyme. TPMT is a phase II drug metabolizing enzymes, and its activity, in all cells and tissues, is regulated by common **genetic polymorphisms** occurring throughout the gene. Genetic polymorphisms are the most important factors responsible for interindividual differences observed in the level of TPMT activity which in turn responsible for the interindividual differences observed in the metabolism, toxicity and therapeutic efficacy of thiopurines (Lennard *et al*., 1990; Mc Leod *et al*., 1993).

TPMT polymorphisms show trimodal variation in respect to enzyme activity. Population studies showed that one individual in 300 (0.3%) in white populations (Caucasians) has low or no detectable TPMT activity, 89% of population have high enzyme activity, and 11% have intermediate activity (Lennard *et. al.,* 1990; Evans *et. al.,* 1991). It is well known that patients with inherited low levels of TPMT activity experience severe or fatal hematological toxicity when administered with standard doses of the thiopurines (Mc Leod, *et al.,* 1993). The reason for developing this kind of severe toxicities can be better explained by the action mechanisms and metabolism of thiopurines.

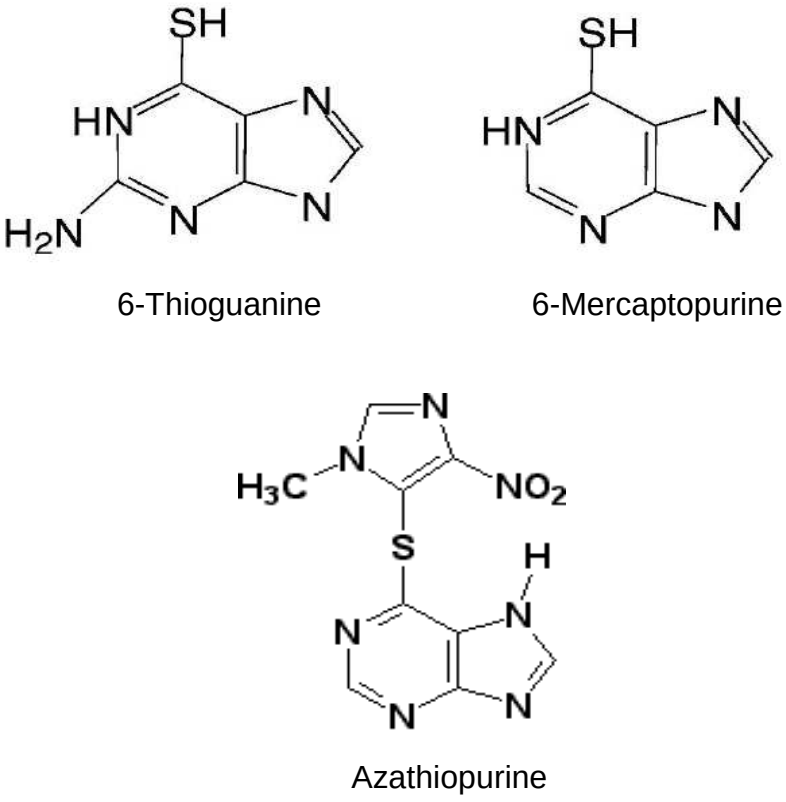


Figure 1. 2 Structure of thiopurine drugs

1.8.1 Metabolism and Action Mechanism of Thiopurine Drugs

The thiopurines are prodrugs and have to be metabolized in order to exert their anticancer action (immunosuppressive action in case of azathiopurines) (Lennard, 1992). Azathiopurine is non-enzymatically reduced to 6MP (see Figure 1.3). The main enzymes competing for the initial metabolism of 6MP are hypoxanthine guanine phosphoribosyltransferase (HGPRT), xanthine oxidase (XO) and TPMT. 6MP is metabolized by HGPRT to produce an intermediate metabolite which is then further metabolized by series of kinases and reductases to

produce cytotoxic thioguanine nucleotides (TGNs). These molecules are very similar in structure to DNA nucleotides. For this reason, they can easily incorporate into the structure of DNA (Swan *et al*., 1996).

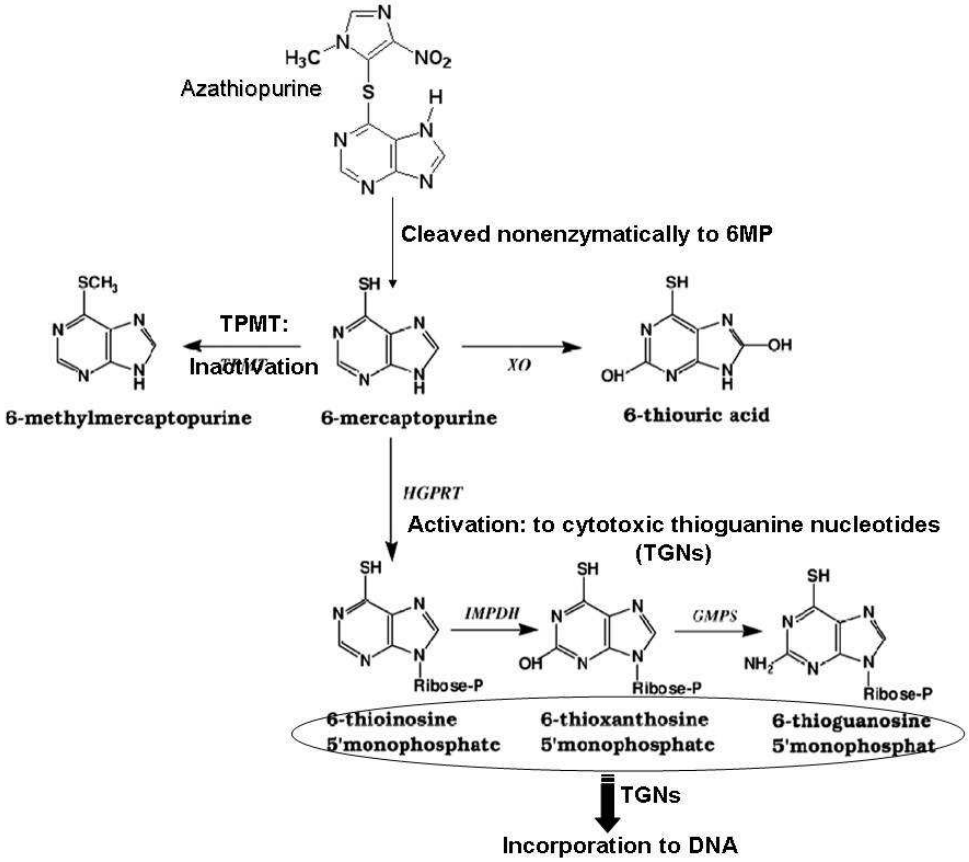


Figure 1.3 Metabolism of AZA and 6MP. Taken from Anglicheau *et al*, 2002.

The incorporation of TGNs into DNA causes DNA-protein cross-links, single strand breaks, interstrand cross-links and sister chromatid exchanges (Christie *et al,* 1984; Pan *et al,* 1990). Those changes in the structure of DNA result in activation of mismatch repair pathway, this phenomena causes the cells to undergo apoptosis. Apoptosis (programmed cell death) is a physiological process which selectively deletes cells whose function is no longer required or whose continued presence may have deleterious consequences in the host tissue as in case of tumor cells (Ellis *et al*., 1991). Generally when cells are treated with antitumor agents, apoptotic cell death has been recognized after G2 arrest in the cycling cell. However, in case of 6MP it is found that apoptotic cell death has been recognized after G0 or long G1 phases when cells are treated with active TGNs (Inamochi *et al*.,1999). By the apoptotic process, proliferation of various types of lymphocyte lines is blocked. By this way the uncontrolled proliferation of tumor cells is prevented. This simply accounts the action mechanism of 6MP via active metabolites. As well as leukomogenic lymphocytes, normal blood cells are also affected from the cytotoxic poperties of TGNs. In those cells also apoptosis can be triggered by mismatch repair pathway, and this accounts the immunosupressive properties of 6MP.

As told before, the initial metabolism of 6MP involve two additional enzymatic reaction catalyzed by Xanthine Oxidase (XO) and TPMT. XO catalyze the conversion of 6MP to thiouric acid which is an inactive metabolite. XO is a constitutively expressed enzyme and it is not polymorphic. However, the rate of reaction mediated by TPMT shows interindividual variations due to genetic polymorphisms occurring in the TPMT gene. TPMT catalyzes the conversion of 6MP into an inactive metabolite 6-methylmercaptopurine which has no cytotoxic properties, through S methylation. The inherited interindividual differences in the level of TPMT activity are mostly responsible for the variation in the therapeutic

efficacy and toxicity of 6MP. It can be accounted as follows: the cellular accumulation of TGNs is inversely proportional to TPMT activity, since high TPMT activity in the first pathway shunts more drugs down the methylation pathway, leaving less for activation to cytotoxic TGNs which result in low therapeutic efficacy of drug and may cause relapse of therapy. Conversely, TPMT-deficient patients accumulate very high cytotoxic TGNs concentrations in tissues which results in development of life threatening hematopoietic toxicities (Evans *et al.,* 1991; Mc Leod *et al.,* 1993).

1.8.2 Dose Limiting Toxicity Profile of 6MP

There are three main dose limiting and life threatening toxicities associated with 6MP: Bone marrow toxicity, immunosupression and hepatotoxicity. Bone marrow suppression which is the most consistent dose related toxicity of 6MP is usually manifested by anemia (low count of RBC), leucopenia (low count of WBC), thrombocytopenia (low count of platelet), or any combination of these. Immunosuppression is another dose associated toxicity of 6MP. In the case of immunosupression, which is generally mediated by severe neutropenia (abnormally large fall of WBC count), induction of immunity to infectious agents or vaccines is subnormal in the patients. For this reason they become more susceptible to life threatening infections. Although rarely observed, the high doses 6MP can become hepatotoxic in animals and humans. Nausea, vomiting, mouth ulcers (sores around the mouth), diarrhea, skin rashes and darkening of the skin are another rarely observed dose associated toxicity of 6MP (This information was partly taken from prospectus of 6MP-Purinethol®).

All these side effects of 6MP are related with the cytotoxic properties of active TGNs. As previously emphasized, the concentration

of active TGNs is directly related with the TPMT level of a person which shows interindividual variation due to the genetic polymorphisms. The association of 6MP dose related toxicity with TPMT polymorphisms is an important fact and cannot be ignored for the safe treatment. For this reason, since 2002, by the FDA approval, this relationship has been mentioned in the prospectus of the drug under many subsections. Under “Bone Marrow Toxicity” subsection, it includes a warning for substantial dose reductions generally required for homozygous-TPMT deficient patients. In the “Adverse Reactions” part, under “Hematological” subsection, it includes particular susceptibility of TPMT-deficient patients to hematological toxicity. In addition, it includes a subsection “TPMT Testing” informing on the availability of the tests and the variant alleles associated with low TPMT activity. Moreover, in the “Dosage and Administration” part it includes a specific subsection on “Dosage in TPMT-deficient Patients” informing about severe toxicity with conventional doses of 6MP and reemphasizes that substantial dose reduction is generally required for homozygous carriers of TPMT polymorphisms (See prescribing information for 6MP which is available at the web page: http://patient.cancerconsultants.com/druginserts/Mercaptopurine.pdf)

1.8.3 TPMT Gene and the Enzyme

Thiopurine methyltransferase (TPMT) is a cytoplasmic, phase II drug metabolizing enzyme (in the class of methyltransferases). It is present in both prokaryotes and eukaryotes. Originally found in the kidney and liver of rats and mice, than it was subsequently shown to be present in most human tissues, for example, heart, blood cells, placenta, pancreas, and intestine (Krynetski and Evans 2003).

TPMT gene has been localized to chromosome 6p22.3. It spans approximately 34 Kb in length made up of ten exons and nine introns

(Szumlanski *et al*., 1996). *TPMT* gene encodes a 245 aminoacid protein with a predicted molecular mass of 35 kD. TPMT enzyme catalyses the S-methylation of aromatic and heterocyclic sulphydryl compounds, including the thiopurine drugs (Ames *et al*., 1986). TPMT belongs to the subfamily of small molecule S-adenosyl –L-methonine (SAM)-dependent methyltransferase. It uses SAM as methyl donor. Figure 1.4 shows the representative reaction catalyzed by the TPMT. However, this enzyme does not share any recognizable sequence similarity to other SAM-dependent methyltransferases. It also lacks any of the known SAM-binding consensus motifs.

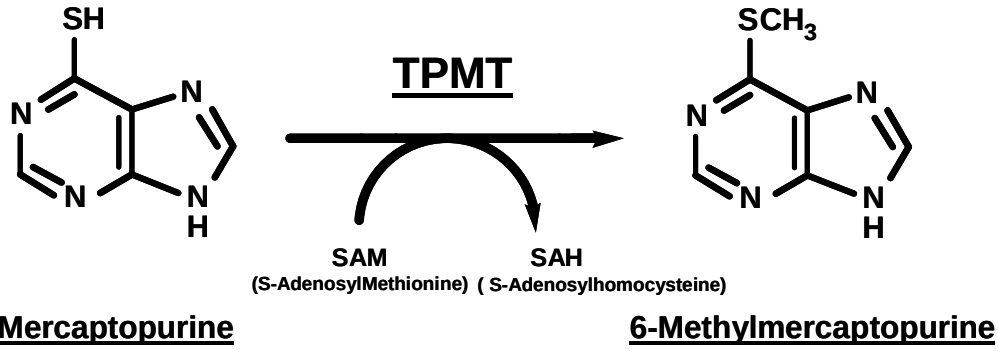


Figure 1.4 Methylation of 6MP by TPMT.

TPMT has no endogeneous substrate and its biological role remains to be unanswered. Phenotypically, TPMT-deficient individuals appear to be indistinguishable from wild type people, unless administered with thiopurines. This fact also supports that TPMT has no vital function in the body. However, efforts to identify an endogenous substrate for TPMT

still continue. Comparative genomics is a potential tool to identify the biological role of this enzyme. Integration of the comparative genomics data suggests that the biological role of TPMT is probably methylation of sulfur-, selenium-, or tellurium-containing organic and inorganic compounds, with SAM being a source of the methyl group. Formation of dimethyl selenide constitutes a detoxification pathway in rats; therefore, a hypothetical role for human TPMT is detoxification of sulfur, selenium, or tellurium via conversion to relatively nontoxic methylation products (Hassoun *et al*., 1995; Krynetski *et al*., 1995).

TPMT enzyme is a monomeric, single-domain protein consisting of a nine-stranded core β-sheet sandwiched between two sets of α-helices (Rutherford and Daggett, 2008). Figure 1.5 shows the structure of TPMT protein.

The genetic polymorphisms especially SNPs found in the *TPMT* gene causes aminoacid changes. These aminoacid changes result in distortion in active site, fattened and distorted protein structure and rapid degradation /aggregation (Rutherford and Daggett, 2008).

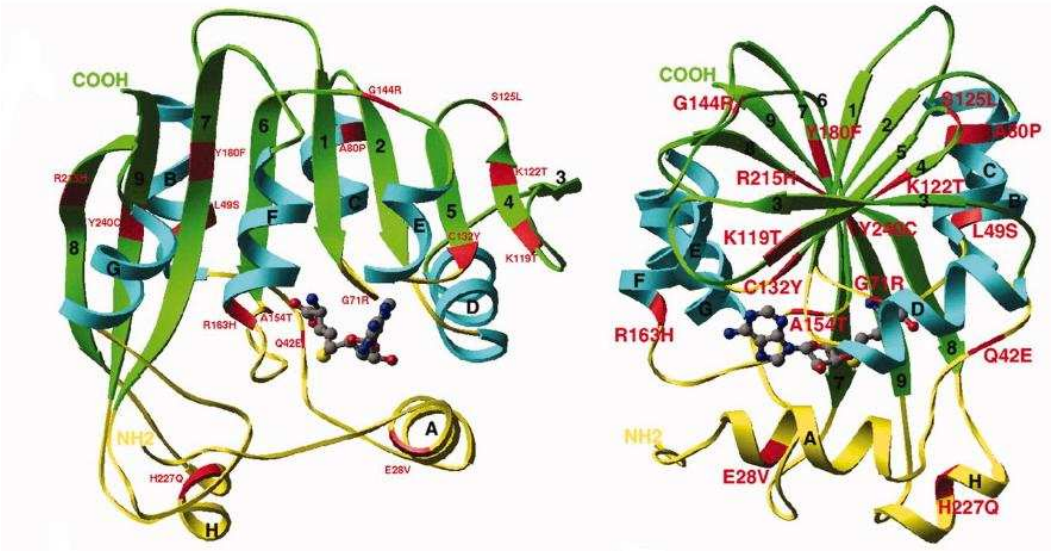


Figure 1.5 Structure of human TPMT. Two views of TPMT in ribbon model. Strands are colored green and helices cyan. The additional N-terminal helix (αA) and the insertion helix αH between strands β8 and β9 are in yellow. Naturally occurring point mutations are shown in red. The bound SAH is in ball-and-stick model with conventional coloration (carbon atoms grey; nitrogen blue; oxygen red; sulfur yellow) Taken from Wu *et al*., 2007.

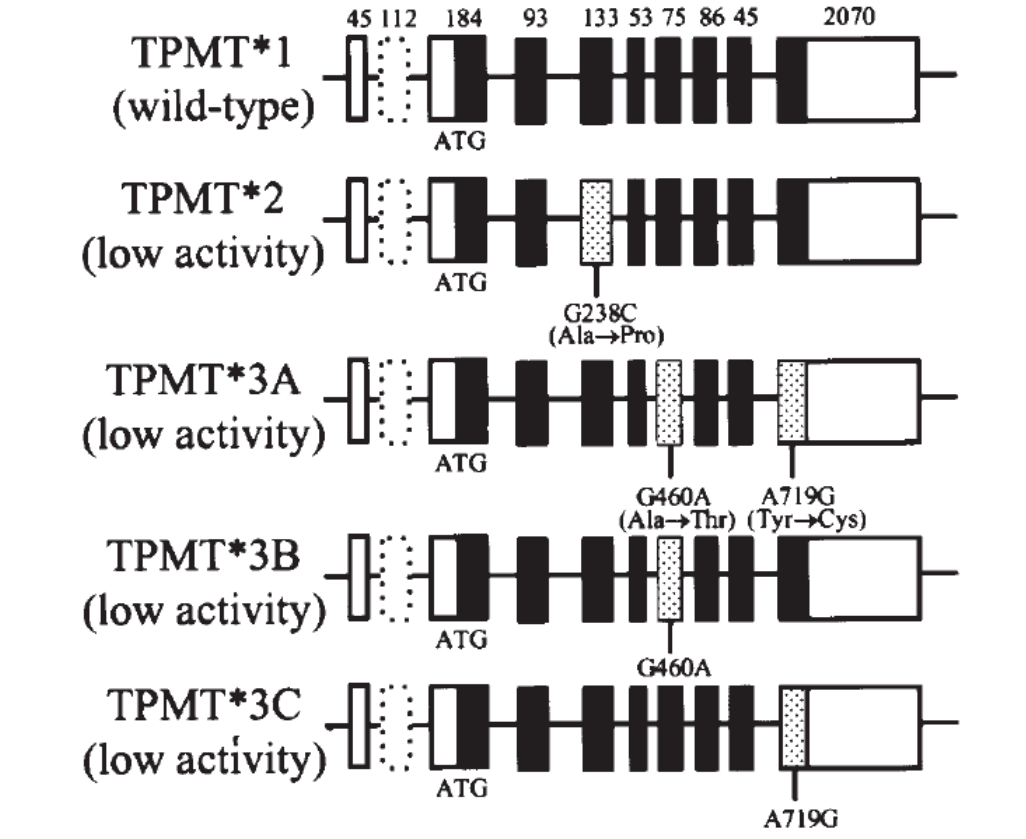
1.8.4 Polymorphisms of *TPMT* Gene in Human

Altered TPMT activity predominantly results from SNPs found throughout the gene. Various *TPMT* alleles carrying a single nucleotide change (point mutation) or a combination of mutations have been associated with deficient, intermediate, or low activity. Generally, individuals with high activity are homozygous wild type, bearing no mutations in *TPMT* gene. *TPMT* deficient individuals are homozygous for

mutant allele, while individuals with intermediate activity are heterozygous, bear one mutant and one wild type allele.

To date, 23 SNPs in the *TPMT* gene associated with deficient enzyme activity have been identified (Teml *et al*., 2007). Generally, SNPs in the open reading frame of the gene alter the encoded amino acid sequence and finally lead to enhanced protein degradation thus result in enzyme deficiency. Among the defective alleles, more than 80% of all low activity cases are attributed to three inactivating mutations (G238C, G460A, and A719G) that characterize the four defective alleles namely *TPMT*2, *3B, *3C*, and *3A* (Yates *et al*., 1997). Among them, *TPMT*2* has G238C transition in exon 5 that causes an amino Ala80Pro amino acid substitution in the encoded protein (Krynetski *et al*, 1995). *TPMT *3B* has G460A point mutation in exon 7 leading to Ala154Thr alteration in the encoded protein. *TPMT *3C* includes A719G SNPs in exon 10 resulted in Tyr240Cys alteration (Loennechen *et al*, 1998). *TPMT*3A* has A719G and G460A SNPs together on the same gene (Tai *et al*., 1997). Figure 1.6 (A) shows the schematic representation of common defective *TPMT* alleles. A study by Wang and coworkers showed that A719G and G460A SNPs, either alone or, together are misfolded and, therefore, aggregate and are targeted for rapid degradation (Wang *et al*, 2005).

(A)



(B)

Figure 1.6 (A) Four important allele variants at the human *TPMT* gene. Pointed boxes shows the single nucleotide changes that result in amino acid changes. White boxes are untranslated regions. Black boxes are exons. Taken from McLeod and Evans., 2001.

(B) Shows VNTR in the promoter region of the *TPMT* gene. Taken from Yan *et al*., 2000

Besides SNPs, *TPMT* gene has VNTRs in the promoter region that affects the level of TPMT acivity (Vayron de la Moureyre *et al.*, 1999). The repeat elements were either 17 or 18 base pairs (bp) in length, and five alleles were reported that varied from four to eight repeats in length. The most common alleles were those with either four or five repeats, **V4* or **V5*. Among the 191 subjects studied, the level of red blood cell TPMT activity was inversely related to the sum of the number of repeat elements on the two alleles in each DNA sample (ie, the larger the total number of repeats, the lower the activity), (Vayron de la Moureyre *et al.*, 1999). However, It should be emphasized that, when compared with the striking differences in level of TPMT activity associated with open reading frame-based SNPs, these VNTR-related effects were quantitatively relatively small (Yan *et al*., 2000).A schematic representation of the location of the VNTR within the human *TPMT* gene is shown in Figure 1.6 (B).

1.8.5 TPMT Phenotype- Genotype Correlation

There is an excellent correlation between TPMT genotype and phenotype. According to the large scale study of German population by Schaeffeler *et al*., (2004), the overall concordance rate between TPMT genotypes and phenotypes was reported as 98.4% with estimates of ≥90% for sensitivity, specificity, and positive and negative predictive values using *TPMT* genetics to predict the correct TPMT phenotype (Schaeffeler *et al*., 2004).

The relationship between TPMT genotype and phenotype has been most clearly defined for *TPMT*2*, *TPMT*3A, TPMT*3B,* and *TPMT*3C* alleles and in patients with leukemia and normal volunteers (Yates *et al.,* 1997). The presence of *TPMT*2*, *TPMT*3A, TPMT*3B* or *TPMT*3C* is predictive for phenotype, in that patients heterozygous for these alleles all have intermediate activity and subjects homozygous for

these alleles are TPMT deficient. In addition, compound heterozygotes (*TPMT*2/*3A*, *TPMT*3A/*3C*) are also TPMT deficient, as would be expected (Yates *et al.,* 1997).

TPMT activity for phenotype determination has been firstly measured by a radiochemical assay (Weinshilboum *et al*., 1978). Later on, HPLC methods have been published (Lennard and Singleton, 1994; Jacqz-Aaigrain *et al*., 1994). All these methods are based on *in vitro* conversion of 6MP to 6-methylmercaptopurine, using SAM as the methyl donor (see Figure 1.4 for clarity). RBC isolates are generally used as convenient source for measuring TPMT activity, because it is easy to obtain and also it reflects TPMT levels in other tissues including lymphoblasts (Mc Leod *et al*., 1995). Phenotypic measurements are usually carried out on whole blood from patients **at diagnosis** since RBC TPMT activity increased by a median of 33% within 3 months after the start of 6MP therapy in children with ALL (Lennard, 1987; 1990; Mc Leod *et al*., 1995,). However, many children with ALL require RBC transfusion at presentation, often prior to referral to the treatment center (Mc Leod *et al*., 1995). TPMT activity can not be determined accurately in a patient who received RBC transfusion because of the presence of donor's RBC. In view of those facts, in most of the cases phenotyping is not a convenient way to determine TPMT status of the patients. Considering the more than 98% correlation between genotype and phenotype, for the prediction of TPMT status genotype analysis can be an alternative way.

Today, genotype test to identify most common defective *TPMT* alleles (**2,*3A, *3C* and **3B*) were done for the patients by the PCR based methods. However, although their frequencies are very rare, there are 19 additional *TPMT* variants associated with low enzyme activity. The newer genomic approaches such as DNA chip technology or recently developed multiplex matrix assissted laser desorption ionization-time of

flight (MALDI-TOF) mass spectrum assay (Schaeffeler *et al*., 2008) allows efficient genotyping for all currently known functional *TPMT* variants. This approach provides 100% predictive ability and allows genotype guided dosage strategy for 6MP to soon become reality (Mc Leod *et al*., 2000).

1.8.6 Pharmacogenetically Guided Dosing of 6MP

As the adverse effects of 6MP in TPMT deficient patients are very severe, detecting *TPMT* polymorphism before starting the therapy is extremely important. Proper dose adjustment is crucial in “slow (heterozygous)” or “deficient (homozygous mutant)” metabolizers for avoiding life threatening toxicities. Today, two general approaches are used over the world for the use of 6MP. The first approach which is widely accepted in UK and USA, is that TPMT genotype and/or phenotype testing should be done for dose optimization in children with ALL before 6-MP therapy. The second approach is the administration of the standard dose of 6MP to the patients at the beginning and then monitoring the side effects. For example, if there is an abnormally large fall of blood counts (reported as neutropenia, anemia or thrombocytopenia), clinicians generally stop the therapy and wait for a while for the return of normal blood count levels. Then, they try to titrate dosage of 6MP until the right dose has been adjusted. During this period, patients may undergo severe toxicities for several times and in each time the therapy have to be stopped.

Today, it is very well known that patients with homozygous mutant genotype or heterozygous patient cannot tolerate full dose of 6MP at the high percentages of the therapy comparing to the wild type patients. In the study of Relling *et al*., (1999a), it was reported that while wild type patient tolerated standard doses of 6MP during 84% of the scheduled therapy, heterozygous and TPMT deficient patients tolerated full dose of 6MP only

65% and 7% of the scheduled therapy, respectively (Relling *et al*., 1999a). Accordingly, both heterozygous and deficient patient have to be withheld from all the components of the chemotherapy at great percentages of the total treatment period which makes the patients susceptible to relapse.

Therefore it is very clear that for the sake of the treatment the best way is to determine TPMT status of the patient before starting 6MP therapy and making appropriate dose adjustment. According to the many study found in the literature, patients with a 'deficient methylator' status (homozygous mutant) can be started on 6–10% of the standard dose of thiopurine therapy, while heterozygous patients (intermediate methylators) can start at 65% of the standard dosage (Evans *et al*., 1991; Lennard *et al*., 1993; Mc Leod *et al*., 1993; Andersen *et al*.,1998).

Dose reduction in TPMT deficient patients is not only important in terms of developing toxicities, but also, in terms of developing secondary malignancies. Several researchers had demonstrated the relationship between secondary malignancies and low TPMT levels. Relling *et al*., (1999b) showed that low TPMT levels tended to be associated with increased risk and shorter onset of secondary acute myeloid leukaemia among ALL patients (Relling *et al*., 1999b). Again Relling *et al.,* (1999c) demonstrated that a higher incidence of brain tumors was observed in children with ALL who had received radiotherapy treatment (six out of 52 children compared with none of 101 who did not receive radiotherapy). Four out of the six with brain tumors had TGN levels above the 70th percentile for the entire cohort of 52 patients studied and of these, three were heterozygous for TPMT, which is a greater percentage of heterozygous than in the normal population (Relling *et al*., 1999c). In another study, Thomsen *et al.*(1999), also speculated on the potential carcinogenic effect of 6-MP on bone marrow stem cells and low TPMT levels with a risk of secondary AML. In this study patients with the highest

risk of developing secondary AML had low TPMT activity and high methylated 6-MP metabolite and/or TGN levels (Thomsen *et al*., 1999).

1.8.7 The role of TPMT Pharmacogenetics in the Treatment of Other Diseses

As mentioned before besides 6MP**,** TPMT also catalyzes other thiopurine drugs: AZA which has wide use in the treatment of autoimmune disorders, inflammatory bowel disease, Chorn's disease and following the organ transplantation and 6TG which is mostly used in the treatment of acute myeloid leukemia, organ transplantation and rarely some of the ALL protocols. They are both metabolized like 6MP by the same activation and deactivation pathways. Actually AZA is non-enzymatically cleaved to 6MP in the body and the rest of the metabolism is the same with 6MP. 6TG is also activated to TGNs and inactivated by TPMT. In case of AZA and 6TG, the reported toxic events in the TPMT deficient patients are as severe as 6MP case. For example, in the study of Gummert *et al*., (1995) a TPMT deficient heart transplanted patient developed severe leucopenia after administered with standard dose of the AZA. TPMT activity was then measured in this patient and found to be below the detection limit. Subsequently the patient died from multiorgan failure due to septicaemia. As a result of this experience, author started to screen all patients for TPMT deficiency before the AZA therapy and suggesting that if TPMT deficiency is present in the patients, the AZA dosage should be adjusted or alternative immunosuppressive regimens should be considered (Gummert *et al*.,1995). As the severity of the reported cases, in 2002, together with 6MP, the FDA has also added TPMT genetic information to AZA and 6TG package insert.

Although the use of 6TG and 6MP is overlapping to some extent in the treatment of childhood ALL, actually, each thiopurine drug is

specifically addressed to different group of patients. For this reason, TPMT polymorphisms have wide range of clinical impact in different patients groups from children to adult, and from cancer patients to patients of autoimmune disorders, inflammatory bowel disease and organ transplantations. Affecting highly diverse population, the frequency and distribution of defective *TPMT* polymorphisms have been extensively studied in different races and ethnic groups.

1.8.8 Ethnic Distribution of *TPMT* Polymorphisms

Like many other drug metabolizing enzymes, the frequencies of common *TPMT* alleles show wide variations among different ethnic groups. Generally, mutant alleles are less frequent in Asian populations than in Caucasian populations. *TPMT*3A* is the major mutant allele in Caucasian populations with allele frequency of 3.2–5.7%, whereas it has not been detected in any Asian populations studied to date (Ameyaw *et al*., 1999; Hon *et al*., 1999; Hiratsuka *et al*., 2000; Rossi *et al*., 2001; Chang *et al*., 2002; Mc Leod and Siva, 2002). Actually, *TPMT*3C* is almost the only mutant allele detected in Asian populations with allele frequency of 0.6– 0.8% (Hiratsuka *et al*., 2000; Chang *et al*., 2002; Kam *et al*., 2002). *TPMT*3C* is also seen in Caucasian population with allele frequency of 0.2–1.0% (Hon *et al*., 1999; Ameyaw *et al*., 1999; Rossi *et al*., 2001). *TPMT*2* is one of the three common mutant alleles which are generally confined to Caucasians and Latin American populations, with allele frequency of 0.2–0.7% (Hon *et al*., 1999; Ameyaw *et al*., 1999). All other *TPMT* variants have been identified worldwide only in single cases.

Besides the interethnic variability, studies indicated that populations within the same ethnic origin can demonstrate significant differences in the distribution of common defective alleles (Zhang *et al*., 2004 and Wei *et al*., 2005). For example, Wei *et al*., (2005) reported that

Kazak population, which is Caucasian in ethnic origin, has a very distinct pattern and lower frequency distribution for common *TPMT* mutant alleles compared to other Caucasians (Wei *et al*., 2005). While the frequencies of main *TPMT* polymorphisms have been studied extensively in many different populations all over the world, so far, no information is available for the Turkish population which is Caucasian in ethnic origin.

1.8.9 The Future Prospect: The Role of Transporter Polymorphisms in 6MP Related Toxicities

6MP and its metabolites are substrates for several transporter proteins including multidrug resistance proteins, MRP4 and MRP5 (Wijnholds *et al*., 2000; Chen *et al*., 2001). These transporters, which are efflux transporters, are genetically polymorphic. Regarding the role of transport proteins in the intracellular level of drug compound and its metabolites, polymorphisms in these genes may be as important as *TPMT* polymorphisms since they together determines the intracellular level of drug and its active metabolites. If the variants of MRP4 and MRP5 genes have increased the activity of exports proteins, 6MP and its active TGNs metabolites would be more avidly exported out of cells; therefore intracellular level of cytotoxic metabolites decrease thus the fate of therapy completely change depending on the TPMT status of the patients. In view of this fact, there is strong evidence that in addition to TPMT pharmacogenetics, polymorphisms in drug transporters will play a critical role in altered 6MP pharmacology. In the future, this subject will take the great attention in the evaluation of 6MP related toxicities.

1.9 The Additional Area of Cancer Pharmacogenetics: The Role of Gene-Environment interactions in Cancer Susceptibility

The primary concerns of pharmacogenetics is the hereditary interindividual differences in response to drug therapy particularly variation in therapeutic efficacy and adverse drug reactions. However, soon after the field of pharmacogenetics emerged, this scope was broadened and the genetic polymorphisms were extensively studied, not only in relation to known variation in drug response differ but also as susceptibility factors for diseases in general (Brockmöller and Tzvetkov, 2008). In this aspects, evaluation of genetic risk factors within the frame of gene-environment interactions is one of the important subject in cancer pharmacogenetics.

1.9.1 Gene-Environment Interactions in Cancer Development

The vast majority of cancers arise as a consequence of exposure to environmental agents that are toxic or mutagenic. In fact, today, it is very well known that up to 90% of all cancers are possibly caused by environmental factors, such as tobacco smoke, diet and occupational exposures (Ames *et al*., 1995; Raunio *et al*., 1995).

The majorities of chemical carcinogens require metabolic activation before they interact with cellular macromolecules and can cause cancer initiation. As mentioned before, the metabolism of drugs and carcinogens is carried out by phase I (oxidation, reduction and hydrolysis) and phase II (conjugation) reactions catalyzed by DMEs. Among the phase I enzymes, cytochromes P450 comprise about 80% which introduce a functional moiety, usually one or more hydroxyl groups, into endogenous and exogenous substrates (Schenkman and Jhonson, 1975; Arınç and Philpot, 1976; Black and Coon, 1986). Phase II DMEs such as glutathione

S- transferases, *N*-acetyltransferases, and sulfotransferases use these functional groups for conjugation with the moieties of glutathione, acetate and sulfate respectively, to yield a very hydrophilic product that can be excreted easily. Although the combined effect of phase I and phase II DME activities is generally the detoxification of the drug or environmental chemicals, numerous phase I DME-mediated metabolic intermediates are highly active to form protein and DNA adducts and reactive oxygen species which cause oxidative stress. These reactive intermediates are acting as strong toxicants, teratogens, mutagens, and carcinogens (Nebert *et al*., 1999).

It can be postulated that individuals having a modified ability to metabolize carcinogens are at increased susceptibility to cancer. Therefore, polymorphisms in genes encoding DMEs have strong relevance in determining susceptibility to cancer. Accordingly, an individual carrying the more active form of an enzyme involved in the activation of carcinogen, or deficient alleles of detoxifying enzymes, will be at greater risk of developing cancer (Krajinovic *et al*., 1999). Enzymes which are particularly involved in the activation and deactivation of environmental carcinogens such as CYP1A1/1A2, CYP1B1, CYP2E1, epoxide hydrolase (EPHX1), GSTs, NATs and SULTs have many genetic polymorphisms resulting in a broad range of activities between a complete lack of activity and extremely high activity depending on the enzyme and the individual genotype. The following two examples: CYP1A1 and GSTM1 polymorphisms and exposure to some chemicals such as PAHs, dioxins etc. in relation to lung cancer may serve to illustrate the relationship between such DMEs polymorphisms, chemical exposure and cancer susceptibility.

It is estimated that 80–90% of lung cancer incidence can be attributed to cigarette smoking (Hammond and Seidman, 1980; Shopland, 1995; Dockery and Trichopoulos, 1997). Tobacco smoke contains more than 4,000 chemicals including at least 60 known carcinogens such as nitrosamines, benzene and polycyclic aromatic hydrocarbons. Some of these compounds become carcinogenic only after they are activated by specific drug metabolizing enzymes. These activated compounds can then become part of deoxyribonucleic acid (DNA) molecules and possibly interfere with the normal growth of cells. For example, benzo(a)pyrene (BaP) which is the major PAH compound found in the tobacco smoke, requires metabolic activation to exert its carcinogenic effects.

As represented in Figure 1.7, BaP is first converted to a epoxides molecule- BaP 7,8 epoxide by the action of several CYP enzymes (CYP1A1, 1A2, 2B, and 3A4). This epoxide molecule is then hydrolyzed to BaP 7,8 dihydrodiol by microsomal epoxides hydrolase (EPHX1) enzyme. BaP 7,8 dihydrodiol is converted to ultimate carcinogen BaP 7,8 diydrodiol-9,10-epoxide (BPDE) which is one of the most carcinogenic compound ever known by CYP1A1. BPDE can bind to DNA and forms DNA- adducts (Parke *et al*.,1991). CYP1A1 is induced by B(a)P and other PAHs found in the tobacco smoke at the mRNA level. The exposure to PAHs leads to greater CYP1A1 induction which in turn may result in high levels of activated carcinogens and consequently to higher degree of persistent DNA-adduct formation or to an enhanced oxidative damage (Stegeman, 1995; Arınç *et al.,* 2000). About 10% of the Caucasian population has a highly inducible form of CYP1A1 enzyme. The CYP1A1 Ile462Val (m2) mutation in the heme binding region results in a 2-fold increase in microsomal enzyme activity and in Caucasians it is in complete linkage disequilibrium with the CYP1A1 MspI (m1) mutation, which has also been associated experimentally with increased catalytic activity. In the study of Hung *et al*., (2003), the presence of the *Ile462Val*

polymorphism (*Ile*/*Val*, *Val*/*Val*) in the CYP1A1 gene was increased the risk of lung cancer 2.99 times (OR: 2.99, 95% CI: 1.51–5.91) this effect was stronger on lung adenocarcinoma (OR 4.85, 95%CI 2.03–11.6) (Hung *et al*., 2003).

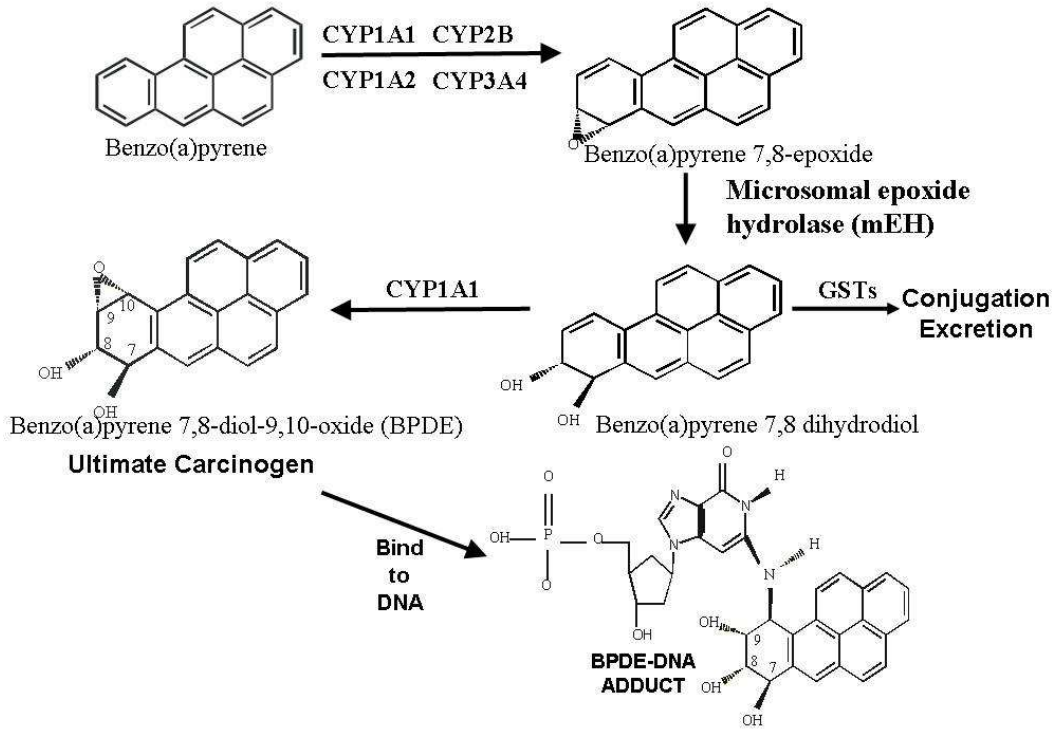


Figure 1.7 Metabolism of benzo(a)pyrene.

While CYP1A1 involve in the formation of BPDE from B(a)P, GSTM1, a phase II DME, detoxifies BPDE and many other hydrophobic electrophiles derived from the metabolism of xenobiotics, including PAHs, by catalyzing their conjugation to glutathione (see Figure 1.7), (Strange and Fryer, 1999). GSTM1 exhibit deletion polymorphisms. Homozygous deletions of this gene, called GSTM1 null genotype, result in deficient

enzyme activity. 40 to 60% of most ethnic group has a null genotype. In most of the study, the *GSTM1* null variant was associated with an increased risk of lung cancer (Carlsten *et al*., 2008).

The CYP1A1 polymorphisms and GSTM1 null polymorphisms together result in a formation of increased amount of BDPE and also other PAH derived carcinogenic metabolites. For this reason the combined effect of these two polymorphisms on the lung cancer susceptibility is very striking. For example in the study of Hung *et al*., (2003) the combination of the CYP1A1 Ile462Val variant and GSTM1 null genotype was increased the risk of lung cancer 4.67 times (OR: 4,67, 95%CI 2.00±10.9), (Hung *et al*., 2003).

The association of tobacco smoking/variation in tobacco derived carcinogen metabolism and individual's susceptibility to lung cancer is one of the model examples for genetic susceptibility to chemical carcinogenesis. As stated before, most of the environmental carcinogens require metabolic activation to exert their carcinogenic effect. The polymorphisms of DMEs involved in the activation and deactivation pathways of these carcinogens can modulate individual's susceptibility to cancer.

In the scope of the presented work individual's susceptibility to childhood acute lymphoblastic leukemia will be handled in regard of genetic risk factors, particularly genetic polymorphisms of some drug metabolizing enzymes.

1.10. Childhood ALL: Incidence and nature of the disease

Acute lymphoblastic leukemia (**ALL**) is the most common form of leukemia. It is uncontrolled growth of infection-fighting white blood cells (lymphocytes) in the blood. Without treatment, these cells will crowd out other blood cells and cause death from anemia, infection or bleeding. ALL primarily affects children, and it is the most frequent form of cancer affecting the children. It accounts for approximately 30% of all cancer diagnosed in children younger than 15 years (Linet *et al*., 1999). Throughout the world each year 2000-2500 new cases of childhood ALL are diagnosed. According to records of LÖSEV, in Turkey every year, approximately 1200-1500 children are diagnosed with ALL. In Turkey, leukemia is also the most frequently observed childhood cancer accounting for 23% of all childhood cancers, and among leukemia cases, ALL has the highest incidence rate with 81% (personal communication with Kutluk, Tezer, 2008)

Although the primary causes (molecular etiology) of childhood ALL have not been known yet, a multistage model is proposed to elucidate the mechanism by which the disease arises. This model requires minimum two steps for the development of disease- a primary event that typically occur prenatally followed by postnatal second events (Greaves 2003; Greaves *et al*., 2003; Greaves 2005; 2006). Gross chromosomal changes, particularly translocations which produce fusion genes (i.e. t(12,21) translocations produce TEL-AML1 fusion gene), are found in the majority of ALL cases (Look, 1997; Greaves and Wiemels, 2003; Catalina *et al*., 2008; Zuna *et al*., 2009). Generally translocations are initiated by double-strand DNA breaks, followed by non-homologous end joining (Kim *et al*., 2006). These chromosome translocations are often the first initiating events (first mutations) by which a preleukaemic stem cell clone is generated *in utero* during fetal development. However, the expression of

the fusion gene is a common event and alone it is not sufficient to induce ALL (Andreasson *et al*., 2001). Indeed, the preleukaemic stem cell can remain silent in most cases. In a minority of children this preleukaemic stem cell progress to leukemia after receiving further postnatal genetic hits (mutations) such as deletions of a part of fusion gene (Maia *et al*., 2001).

Although numerous studies support the multistage model of leukemia formation, factors causing the ALL-inducing mutations (such as DNA double strand breaks or deletion of a part of fusion genes) in children are not exactly known. However, it is well established that there are some exposure dependent factors known to influence the disease risk and are likely to be associated with the ALL inducing events (Kim *et al*., 2006). These are environmental agents (e.g., carcinogens, mutagens) and genetic factors (e.g., genetic polymorphisms of DMEs). Identifying these risk factors for childhood leukemia is an important step in the reduction of overall burden of the disease.

1.10.1 Environmental Risk Factors

Numerous environmental agents have been found to be associated with ALL such as inhaled particulate hydrocarbons, cabon tetra chloride, pesticides, herbicides, fungicides and benzene (Ross *et al*.,1994; Buffler *et al*., 2005, Kinney *et al*., 1991; Kinney *et al*., 2003).

In relation to multistage model of leukomogenesis, the potential environmental agent can affect the development of the disease in three critical period: preconceptional-before pregnancy, prenatal-during pregnancy and postnatal (Kim *et al*., 2006). Environmental agents that father and mother are exposed before conception can be linked to first **initiating prenatal event of multistage model**. In this aspect, father and

mother occupational exposure, household exposure, cigarette smoking, alcohol use and dietary habits can be important factors. Actually in terms preconceptional transmission, exposures occurring during an individual's father's life may be more important than exposures occurring during their mother's life. This is because spermatogenesis continues from puberty to old age and hence there is more opportunity for preconceptional mutant gene accumulation in men than women (Anderson *et al*., 2000). These genetic mutations accumulated in the father's sperm may transmit cancer susceptibility to the child. Another important factor for the occurrence of **prenatal event** is the exposure during pregnancy (prenatal period). The prenatal exposure mostly related with mother exposure since fetus expose to the environmental agents via placental transmission. Risk factor associated with breastfeeding mothers and children after birth are potentially linked to the postnatal events (Kim *et al*., 2006).

According to three critical window of exposure, Table 1.2 lists environmental exposure agents that have been associated with childhood ALL. As stated before, cigarette smoke contains many well-established carcinogens, and both active and passive smoking have been implicated in the development of several cancers during adulthood (Boffetta *et al*., 2002). Cigarette smoke constituents can readily cross the placental barrier, and have been linked to an increased frequency of chromosomal abnormalities (Pluth *et al*., 2000) oxidative damage (Fraga *et al*., 1996), and aneuploidy of sperm (Shi *et al*., 2001). However, it is unclear that whether maternal and paternal cigarette smoking before and during pregnancy is a risk factor for the developing childhood leukemia because of inconsistency of the results. For example, Mac Arthur *et al*., (2008) reported that although it is not statistically significant there was an association between maternal preconceptional and prenatal use of cigarette smoking and childhood ALL risk (see Table 1.2) (Mac Arthur *et al*., 2008). Shu *et al*., (1996) reported that there was a positive association

between paternal preconceptinal cigarette use and childhood leukemia (see Table 1.2) (Shu *et al*., 1996). However, Brondum *et al*., (1999) demonstrated that the risk of ALL was not associated with the father's ever having smoked (odds ratio [OR], 1.04, 95% confidence interval [CI] 0.90 –1.20) or the mother's ever having smoked (OR, 1.0, 95% CI 0.9–1.2) (Brondum *et al*., 1999). In terms of postnatal exposure of child to cigarette smoke, the findings again were inconsistent. However, one study (Chang *et al*., 2006) demonstrated a very strong association (OR, 3,9; 95% CI 1.3–12.4) interestingly when combined effect of paternal preconceptional smoking and maternal postnatal smoking was considered. Because of inconclusive results, the role of parental smoking at any critical window of exposure and also postnatal exposure of child should be investigated in more studies. Besides, relating cigarette smoke exposure with genetic polymorphisms of DMEs that is involved in the metabolism of tobacco smoke carcinogens can give more meaningful and reliable results.

Table 1.2 Environmental exposure agents and childhood ALL risk.

Exposure Period	Exposure Type	Childhood Leukemia	Odd Ratio (95%CI)	Reference	Exposure Place
Preconceptional Father	Cigarette Smoking	ALL	1.6 (1.0-2.4)	Shu *et al*., 1996	
	Inhaled Particulate Hydrocarbons	ALL	1.4 (1.1-1.8)	Kinney *et al*., 2003	Occupational
	Timber & Wooden Furniture	[a]Childhood Leukemia	2.7 (1.0-6.9)	Kinney *et al*., 1991	Occupational
	Carbon Tetrachloride	[a]Childhood Leukemia	2.9 (1.1-7.4)	Kinney *et al*., 1991	Occupational
	Benzene	[a]Childhood Leukemia	5.8 (1.7-26.0)	Kinney *et al*., 1991	Occupational
Preconceptional Mother	Cigarette Smoking	ALL	1.2 (0.9-1.7)	Mac Arthur *et al*., 2008	
	Paint & Thinners	ALL	1.6 (1.2-2.2)	Shu *et al*., 1999	Occupational
	Solvents	ALL	1.8 (1.3-2.5)	Shu *et al*., 1999	Occupational
	Dermal Exposure to Hydrocarbons	ALL	2.6 (1.2-4.0)	Kinney *et al*., 2003	Occupational
	Pesticide	ALL	3.5 (1.1-11.2)	Shu *et al*., 1999	Occupational
	Benzene	[a]Childhood Leukemia	4.0 (0.3-117.9)	Kinney *et al*., 1991	Occupational

[a]The overall distribution of diagnosed patients was ALL (75%), the rest was other leukemias.

Table 1.2 (Continued)

Exposure Period	Exposure Type	Childhood Leukemia	Odd Ratio (95%CI)	Reference	Exposure Place
Prenatal Mother	Cigarette Smoking	ALL	1.3 (0.9-1.8)	Mac Arthur *et al*., 2008	
	Paint & Thinners	ALL	3.2 (1.3-13.9)	Shu *et al*., 1999	Occupational
	Pesticides	ALL	6.5 (1.5-59.0)	Infante Rivard *et al*,1999	Garden
Posnatal Children	Pesticides	ALL	2.3 (1.9-2.8)	Menegaux *et al*., 2006	Household
	Insecticides	ALL	2.4 (1.3-4.3)	Menegaux *et al*., 2006	Garden
	Funcicides	ALL	2.5 (1.1-5.1)	Menegaux *et al*., 2006	Garden
	Professional Pest Control Services	ALL	3.3 (1.4-7.7)	Menegaux *et al*., 2006	Household
	Exposure to Artwork	ALL	4.1(1.1-5.1)	Freadman *et al*., 2001	Household
	Neighboring repair garages /petrol gas stations	ALL	4.0 (1.5-10.3)	Freadman *et al*., 2001	Environmental

Among the chemicals, pesticides and hydrocarbons are most commonly associated chemical classes with childhood ALL (Belson *et al*., 2007). Many studies have suggested a strong association between pesticide exposure and childhood ALL. Infante-Rivard *et al*., (1999) demonstrated that father occupational exposure to pesticides specifically to fungicides increased the risk of childhood ALL 5.11 times (see Table 1.2) (Infante Rivard *et al*., 1999). Mother exposure to pesticides in both preconceptional and prenatal period was increased the risk of disease at significantly high levels (3.5 and 6.5 times respectively see Table 1.2) (Infante Rivard *et al*., 1999; Shu *et al*., 1999). Moreover, postnatal exposure of child to pesticides and specifically insecticides, fungicides, and professional pest control services have been found to be greatly affect the risk of childhood ALL (see Table 1.2) (Menegaux *et al*., 2006).

Hydrocarbons are organic compounds made up primarily of carbon and hydrogen atoms. They are found in many household and industrial products including plastics, paraffin, waxes, paints, paint removers, thinners and solvents. An occupational study conducted by Kinney *et al*., (1991) found a strong association (see Table 1.2) between childhood leukemia and preconceptional exposure of father to inhaled particulate hydrocarbons, carbon tetra chloride and timber and wooden furniture works in which they expose to paints, paint removers, thinners, etc (Kinney *et al*., 1991). Similarly, preconceptional exposure of mother to hydrocarbons: specifically dermal exposure to hydrocarbons, solvents and paints/thinners (see Table 1.2) have been found to be increased the risk of childhood ALL (Shu *et al*., 1999; Kinney *et al*., 2003). Besides, prenatal exposure to mother to paints and thinners have been reported to be greatly increased (3.2 times, see Table 1.2) the risk of childhood ALL (Shu *et al*., 1999).

The most widely recognized hydrocarbon is benzene. It is a ubiquitous chemical used in the manufacture of paints and plastics and as constituents in motor fuels, petroleum products and hobby glues. It is also formed whenever carbon-rich materials undergo incomplete combustion. It is produced in combustion of fossil fuels like petroleum products and coal, volcanoes and forest fires. It is also a component of cigarette smoke. Benzene is one of the most widely known human carcinogens and also it has a strong association with the development of leukemia.

1.10.1.1 Benzene and Leukemia Risk

Benzene is widespread in the environment. Outdoor air may contain benzene from tobacco smoke, automobile service stations (repair garages), the transfer of gasoline, exhaust from motor vehicles, and industrial emissions. Vapors from products that contain benzene, such as glues, paints, furniture wax, and detergents, can also be a source of exposure. Air around hazardous waste sites or petrol gas stations may contain higher levels of benzene.

Carcinogenic, particularly, leukomogenic effects of benzene reported first as a single clinical case report in 1887 (Aksoy, 1989). Up to 1974, some case reports or patient series were published in the medical literature (Yarış *et al*., 2004). However, first large-scale epidemiological study that established a link between leukemia and occupational exposure to benzene comes from a Turkish scientist Prof Dr Muzaffer Aksoy in 1974. Dr. Aksoy observed that a large group of leukemic patients were among the shoemakers, or they worked in leather manufacturing. These people chronically expose to high level of benzene since it is used as a solvent in leather manufacturing and also it is the major components of glue commonly used in the shoe making process. In the study of Aksoy *et al*., (1976), from 1967 to 1974, in Istanbul, Turkey, 31 patients with

leukemia were diagnosed among 28,500 shoe, slipper, and handbag workers who are chronically exposed to benzene (Aksoy *et al*., 1976). The incidence of leukemia among this occupational group was 13.59/100,000. At that time the incidence of leukemia in general population of Istanbul was 4.00/100,000 (Yarış *et al*., 2004). According to the study of Aksoy *et al*. (1976), the incidence of leukemia was more than 3 times higher among shoe workers comparing to general population of İstanbul. This higher incidence rate clearly established a link between occupational exposure to benzene and leukemia. From now on many studies demonstrated the strong association between acute leukemia and benzene exposure (Rhuston and Romaniuk 1997; Glass 2003).

When evaluated in three critical windows of exposure, it can be seen that benzene exposure is strongly associated with childhood leukemia risk (see Table 1.2 gray-painted rows). For example, father occupational exposure to benzene before pregnancy (preconception period) increase the risk of childhood leukemia 5.8 times (see Table 1.2), (Kinney *et al*., 1991). Similarly mother exposure to benzene at the preconception period increase the disease risk 4.00 times (see Table 1.2), (Kinney *et al*., 1991). Besides direct exposure, there is lots of way to indirectly expose to benzene. Paints and thinners, pesticides, solvents, paints and glues used in the artworks contain considerable amount of benzene. Moreover, air around automobile repair garages and gas stations also contains high level of benzene. As it is clearly seen in Table 1.2 both preconceptional and prenatal exposure of mother to this chemical directly or indirectly increase the risk of childhood ALL significantly.

In regard of postnatal exposure of child to benzene indirectly (by household artwork and neighboring repair garages and petrol/gas stations) the risk of disease increased greatly (4.1 and 4 times,

respectively; see Table 1.2), (Freadman *et al*., 2001). Considering the examples represented in Table 1.2, it is clear that benzene is one of the best established environmental causative agent for childhood acute lymphoblastic leukemia.

As described in the previous section like benzene many environmental chemicals mentioned above, require metabolic activation by drug metabolizing enzymes to initiate leukemia. Most of the drug metabolizing enzymes show genetic polymorphisms which create interindividual variability in the metabolism of these environmental agents and thus in the susceptibility of chemical related disease and carcinogenesis. For this reason genetic polymorphism of DME can be considered as one of the risk factor for the development of childhood ALL. In the following section genetic polymorphisms of some of the DMEs in relation to their role in susceptibility to childhood ALL will be given.

1.10.2 Genetic Polymorphisms of DMEs and Risk of Childhood ALL

It is well established that several metabolic steps and several DMEs are required to produce toxic metabolites of benzene which in turn cause several DNA anomalies like strand breaks, stable structural chromosome aberrations, chromosome aneuploidy and chromosome translocations. These are the main genetic abnormalities found in leukemias. The first step in benzene metabolism is the formation of benzene oxide, an epoxide, by CYP2E1 enzyme (see Figure 1.8). There are at least three routes in the pathway proceeding from this intermediate (demonstrated by red colored arrows in Figure 1.8). The first involves hydroxylation of the epoxide to phenol (spontaneous event), which is then converted to hydroquinone by **CYP2E1**. Both phenol and hydroquinone are substrates for phase II enzymes: **SULTs** and **UGTs**. (Seaton *et al*., 1975). Free hydroquinone may leave the liver and reach the bone

marrow, in which it can be further metabolized to the genotoxic 1,4 benzoquinone metabolite by myeloperoxidases (**MPO**) (Snyder and Hedli, 1996). Benzoquinones are potent genotoxic and haematotoxic compounds which can be reconverted to less toxic hydroxy metabolites by NAD(P)H:quinone oxidoreductase (**NQO1**) (Smith *et al*, 2001). In the second route, by ring opening reactions, benzene oxide is converted to open ring muconate derivatives of benzene. Trans-trans muconic acid is often detected as the urinary metabolite of benzene. *Trans-trans*-muconaldehyde (MA) which is a direct acting alkylating agent is a precursor to trans-trans muconic acid and MA is proposed to be the most toxic species resulting from benzene metabolism. The third route involves conversion of benzene oxide to benzene dihydrodiol through the activity of microsomal epoxide hydrolase (**EPHX1**) then it can be further metabolized to catechol and 1,2 benzoquinone (Snyder and Hedli, 1996). The latter compound is able to bind to proteins and possibly to DNA (McDonald *et al*, 1994). In the third route, benzene oxide can also deactivated to phenyl mercaptopuric acid by glutathione S-transferases (**GSTs**) enzyme.

In this pathway, CYP2E1 plays a critical role as it is the key enzyme bioactivating benzene into several potentially carcinogenic metabolites. The other two important enzymes in the pathway are MPO and NQO1. In the bone marrow by MPO enzyme, hydroquinones and catechols are converted to 1,2 and 1,4 benzoquinones (see Figure 1.8) which are genotoxic, carcinogenic metabolites of benzene. In the reverse directions of MPO catalyzed reactions, NQO1 detoxifies (see Figure 1.8) benzoquinone species back into catechols and hydroquinones which are then conjugated by phase II enzymes and excreted from the body.

In fact, CYP2E1 is the most studied enzyme in relation to childhood ALL risk due to its role in the metabolism of various carcinogens. For

example, CYP2E1 catalyzes the activation of numerous low molecular weight compounds including ethanol, acetone, drugs like acetaminophen, isoniazid and many procarcinogens such as vinyl chloride and vinyl bromide, dimethylnitrosamine and diethylnitrosamine, acrylonitrile, urethane, styrene, acrylamide, carbon tetrachloride, and chloroform. CYP2E1 is also induced by its substrates like ethanol, benzene and acrylamide (Koop and Casazza 1985; Johansson and Ingelman-Sundberg 1988; Arınç *et al*, 2000a; Arınç *et al*., 2000b; Nuyan, 2008), as well as some patophysiological conditions like obesity, diabetes and starvation (Koop and Casazza 1985; Hong *et al*., 1987; Song *et al*., 1987; Arınç *et al*., 2005; Arınç *et al*., 2007). For this reason, long-term exposure to CYP2E1 substrates like benzene and acrylamide, and some of the conditions like diabetes may increase the amount of carcinogenic metabolites by CYP2E1 induction thus increase the individual's susceptibility to cancer.

In view of the role played by CYP2E1 in the metabolism of various procarcinogenic compounds, several studies have examined the relationship between CYP2E1 poymorphisms and risk of childhood ALL. Recently Ulusoy *et al*., (2007) investigated the possible association of *CYP2E1*5B, *6* and *7* alleles, alone or in combination, with the risk of incidence of childhodd ALL in Turkish population. Accordingly, when both *CYP2E1 *5B* and *6* alleles were considered together, the risk of childhood ALL have been found to be significantly increase to 2.9 fold (95% CI 1.0–8.5). (Ulusoy *et al*., 2006, 2007).

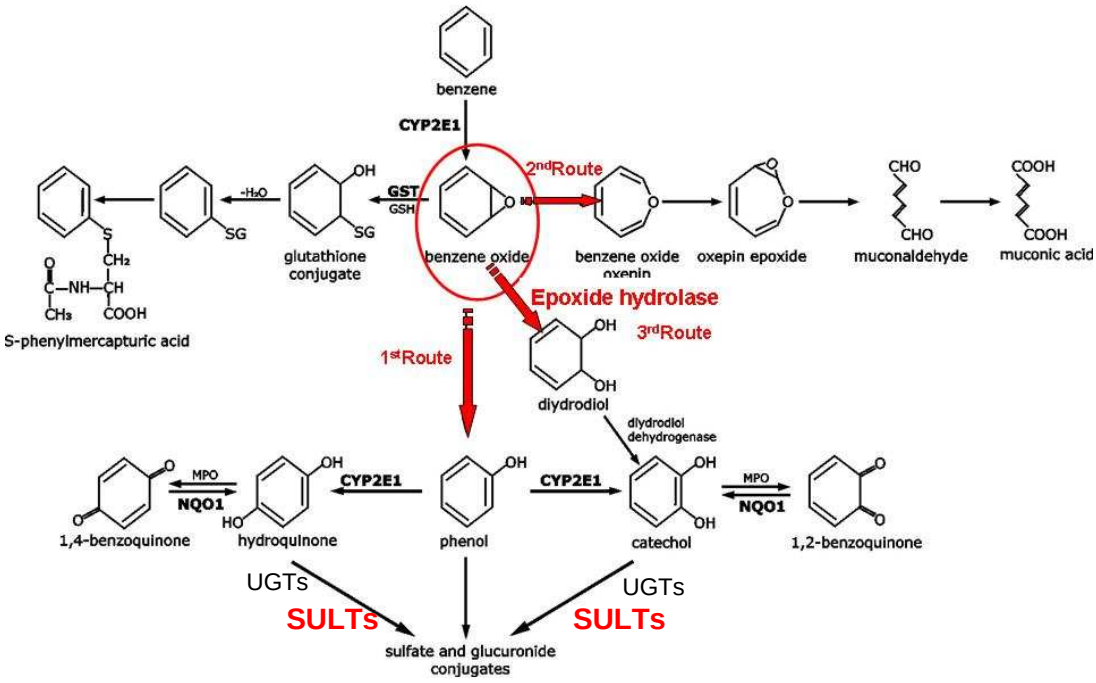


Figure 1.8 Benzene metabolism

In the benzene pathway there are two other important enzymes- EPHX1 and SULT1A1. As mentioned above, EPHX1 converts benzene oxide to benzene dihydrodiol which is then converted to catechol and 1,2 benzoquinones by further reactions. The latter compound can bind to DNA and proteins and causes carcinogenic effects. SULT1A1 is responsible for the detoxification of phenol and hydroquinones. Besides benzene metabolism, both SULT1A1 and EPHX1 participate in the metabolism of various chemical compounds including environmental and tobacco carcinogens. In this aspect, genetic polymorphisms altering the activity of SULT1A1 and EPHX1 may modify the individual's susceptibility for the childhood ALL. Polymorphisms of both SULT1A1 and EPHX1 have been widely studied in relation to various cancer types as risk modifiers.

However there are no reports evaluating the clinical significance of EPHX1 and SULT1A1 genetic polymorphisms for the risk of developing childhood ALL. In the scope of presented work these two enzymes were evaluated in terms of their risk modifier role in childhood ALL. For this reason, the polymorphisms of these enzymes will be focused on in the next sections.

1.10.2.1 SULT1A1 Polymorphisms and Childhood ALL Risk

Sulfotransferases (SULTs), a superfamily of multifunctional enzymes, catalyze O-sulfonation (sulfate conjugation) reactions which is an important pathway in the phase II metabolism of a large number of diverse drug compounds, xenobiotics and as well as endogenous compounds (Radominska *et al*.,1990; Falany, 1997).

The general mechanism of reaction catalyzed by SULTs was represented in Figure 1.9. SULTs catalyze the transfer of a sulfuryl group from endogenous sulfate donor 3' phosphoadenosine 5' phosphosulfate (PAPs) to hydroxyl or amino groups of acceptor molecules therefore forming sulfuric acid esters and sulfamates, respetively (Mulder, 1981; Banaoğlu, 2000). SULT1A1 enzyme is one of the most important members of this family playing role in the metabolism of various carcinogenic compound/metabolites. Sulfonation is generally considered as a detoxification mechanism that generates more water-soluble metabolites on the other hand, in some cases, it can lead to bioactivation of promutagens and procarcinogens including environmental and tobacco smoke carcinogens (Glatt *et al*. 2000; Wang *et al*. 2002; Zheng *et al*. 2003). For instance, SULT1A1 catalyzes the sulfonation of N-hydroxy derivatives of arylamines and heterocyclic amines both are presented in tobacco smoke, to form more reactive DNA damaging electrophiles (Ozawa *et al*., 1994; Liang *et al*., 1994; Chou *et al*., 1995).

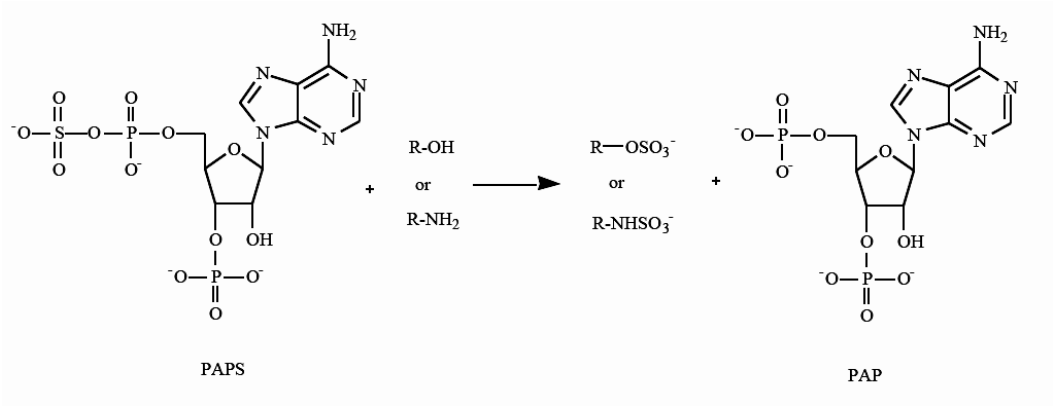


Figure 1.9 The general sulfation reaction catalyzed by the SULTs.

The human SULT1A1 gene is located on chroosome 16p12.1–p11.2 and consists of 8 exon. SULT1A1 enzyme activity shows interindividual variability due to genetic polymorphisms occuring throughout the gene. Up to now, seven allelic variants have been described for SULT1A1 enzyme. The most important polymorphism identified for SULT1A1 is G638A SNP in exon 7 region of the gene. This SNP (designated as SULT1A1*2 alelle) causes Arg213His replacement at the encoded protein. In terms of funcional consequences, enzyme coded by SULT1A1*2 allele (213His) appears to be less thermostable and exhibits decreased substrate affinity compared to the wild type SULT1A1*1 enzyme (Arg213) (Ozawa *et al*., 1999). Subjects homozygous for the SULT1A1*1 allele have approximately eight-fold higher platelet sulfotransferase activity towards *p*-nitrophenol than those with the SULT1A1*2 allele (Raftogianis *et al*., 1997).

Regarding the role of SULT1A1 in the metabolisms of various carcinogens and strong reduction of SULT1A1 activity due to SULT1A1*2 variant, several studies have examined the relationship between this polymorphism and risk of certain cancers. However the results from these previous studies are conflicting. For this reason, additional studies to address the role of SULT1A1*2 polymorphism in human carcinogenesis are needed. In case of childhod ALL, so far, there has been no study evaluating the role of SULT1A1 *2 polymorphism as risk modifier. In this study, the role of SULT1A1*2 polymorphism in the development of childhood ALL was investigated.

1.10.2.2 EPHX1 Polymorphisms and Childhood ALL Risk

Microsomal epoxide hydrolase is a critical phase I enzyme catalyzes the conversion of a broad array of xenobiotic epoxide substrates to more polar diol metabolites. Epoxides are generally electrophilic compounds and often formed by the action of another phase I enzymes; cytochrome P450s (CYPs). Hydrolysis of epoxides through the action of epoxide hydrolase enzyme generally leads to detoxification after conjugation by phase II enzymes. However, reactions of diol metabolites by CYPs generate highly toxic and carcinogenic compounds from epoxide molecules. In the metabolism of benzo(a)pyrene (BaP) which is mentioned in section 1.9.1 in detail both activation and detoxification of diol metabolites are exhibited. As represented in Figure 1.7 (under section 1.9.1), BaP is first converted to a epoxides molecule- BaP 7,8 epoxide by the action of several CYP enzymes (CYP1A1, 1A2, 2B, and 3A4). This epoxide molecule is then hydrolyzed to BaP 7,8 dihydrodiol by the EPHX1 enzyme. In the further reactions, BaP 7,8 dihydrodiol can be convertd to ultimate carcinogen BaP 7,8 diydrodiol-9,10-epoxide (BPDE) which is the most carcinogenic compound ever known by the CYP1A1. On the other hand, it can be also conjugated by GSTs and eliminated from the body.

As represented in the example of BaP, whether EPHX1 causes detoxification or activation of the epoxides is not very clear. It could be concluded that EPHX1 enzyme plays a dual role in the detoxification and activation of the epoxide molecules. Because EPHX1 is involved in the bioactivation of several carcinogens, and detoxification of toxic epoxides (Oesch, 1973), there is significant interest in genetic polymorphisms that may alter the activity of EPHX1.

EPHX1 activity demonstrates large interindividual variations due to SNPs occuring throughout the gene. Two major aminoacid-altering polymorphisms have been described for EPXH1 gene, one in exon 3 and one in exon 4. Exon 3 polymorphisms is characterized by Tyr113His replacement at the encoded protein and results in a 40-50% decrease in enzyme activity (slow activity) (Hasett *et al*., 1994). Exon 4 polymorphisms is characterized by His139Arg replacement and enhances enzyme activity by approximately 25% (fast activity) (Hasett *et al*., 1994).

In view of the dual role played by EPHX1 in the activation and detoxification of various epoxide molecules, two allelic version of EPHX1 protein (fast and slow activity enzymes) have been widely studied in relation to various cancer types as risk modifier. However the results are conflicting. In addition, in case of childhod ALL, so far, there has been no study evaluating the role of EPHX1 polymorphisms as risk modifier. In this study, the role of EPHX1 polymorphisms (exon 3 and 4) in the development of childhood ALL was investigated.

1.11. Aim of the Study

Several lines of evidence suggest that “environmental exposure” together with “environmental susceptibility genes” play major role in the development of childhood ALL. “Drug metabolizing enzymes” are regarded as one class of environmental susceptibility genes. It is well established that individuals having a modified ability to metabolize carcinogens are at increased susceptibility to cancer. Therefore, polymorphisms in genes encoding DMEs have strong relevance in determining susceptibility to cancer. Accordingly, an individual carrying the more active form of an enzyme involved in the activation of carcinogen, or deficient alleles of detoxifying enzymes, will be at greater risk of developing cancer (Krajinovic et al., 1999).

As described in more detail previously, microsomal epoxide hydrolase (EPHX1) and sulfotransferase 1A1 (SULT1A1) are two susceptible enzymes due to their dual roles in both activation and detoxification of many procarcinogens and environmental toxicants including tobacco smoke. Genetic polymorphisms altering the activity of SULT1A1 and EPHX1 may modify the individual’s susceptibility for the childhood ALL. Polymorphisms of both *SULT1A1* and *EPHX1* have been widely studied in relation to various cancer types as risk modifiers. However, so far, there have been no reports evaluating the clinical significance of *EPHX1* and *SULT1A1* genetic polymorphisms for the risk of developing childhood ALL. Therefore, a part of current work focused on the effects of *SULT1A1* and *EPHX1* genetic polymorphisms, alone or in combination, as risk modifier for the development of childhood ALL and aims related with this part of the present study included;

- Investigation of two genetic polymorphism on *EPHX1* gene, namely exon 3 and exon 4 polymorphisms, on the pediatric patients with ALL and healthy adult controls and comparison of the frequencies in case and control groups to elucidate the possible risk modifier effects of polymorphisms on the development of childhood ALL. So far, there has been no study evaluating the role of *EPHX1* polymorphisms as risk modifier. Therefore this study, investigated the association of EPHX1 polymorphisms with the risk of childhood ALL for the first time.
- Investigation of *SULT1A1*2* polymorphism in control and case group, and determination of the effects of this polymorphisms on the risk of development of childhood ALL. So far, *SULT1A1*2* polymorphism has not been studied in relation to any kind leukemia including the childhood ALL. Therefore this study, investigated the association of *SULT1A1*2* polymorphism with the risk of childhood ALL for the first time.
- The multi-locus analysis of two *EPHX1* polymorphisms together with *SULT1A1*2* polymorphism.
- Investigation of the interaction between *EPHX1* and *SULT1A1* genetic polymorphisms with non-genetic factors such as parental age at conception, smoking status of the parents, postnatal exposure of the children, in the risk of development of childhood ALL, based on case-only model.
- Lastly for this part, investigation of the frequencies of *EPHX1* polymorphisms and *SULT1A1 *2* polymorphism in control samples, which would reflect the Turkish population frequencies for these polymorphisms, and compare the frequencies of these polymorphisms in Turkish population with those in other population of different ethnic origins. To our knowledge, by the current work, the frequencies of *SULT1A1*2* polymorphism has been identified for Turkish population for the first time.

The major focus of the current study was the investigation of TPMT enzyme polymorphisms in Turkish population comprised pediatric ALL patients and healthy adult controls. In the scope this work, the clinical histories of the pediatric patients with *TPMT* defects were investigated retrospectively in relation to 6MP related side effects during the treatment of childhood ALL. As mentioned earlier in detailed, 6MP is essential drug compound used in the routine treatment of childhood ALL. S-methylation catalyzed by TPMT enzyme is the prominent route for the inactivation of this agent. TPMT exhibits interindividual variation in activity, partly due the presence of genetic polymorphisms with *TPMT*2, *3A, *3B,*and *3C* being the most frequent mutant alleles associated with reduced activity. These genetic variants have extremely important clinical implications since patients carrying the defective mutations in the *TPMT* gene suffer severe toxicities when administered with standard doses of 6-MP. On the other hand, this patients can be safely and effectively treated with the lower doses of 6MP (Evans *et al*, 1991; Lennard *et al*, 1993).

The distribution of common defective *TPMT* alleles have been studied extensively in many different populations all over the world due to its significant inter- and intraethnic variation and as well as due to its strong clinical implications. The knowledge of main defective *TPMT* frequencies in a population is essential to estimate the proportions of risk groups under 6-mercaptopurine therapy. High proportions of risk groups (homozygous mutant and heterozygous individuals) in a population may put the genetic test for *TPMT* into routine clinical practices before the prescription of the 6MP for safe dosing. On the other hand, if the proportion of risk groups in a population is very low, then the right strategy may be the genotyping of the patient who develop 6MP related side effects in the first application of the drug, rather than genotyping of every patient before the prescription of 6MP. The most important consideration

about this subject should be as more patients and clinicians become aware of the importance of *TPMT* pharmacogenetics in safe dosing of 6MP, the number of children suffering from the 6MP related side effects will decrease.

Regarding the main points related with the TPMT polymorphisms-6MP related side effects, the aims of the current study included,

- Investigation of the genotype distribution and allele frequencies of common defective *TPMT*2*, **3A*, **3B* and **3C* polymorphisms, which accounts more than 80% of all deficient or low activity cases, in Turkish population, based on the analysis of the pediatric patients with ALL and healthy controls.
- Comparison of the allele frequencies of common defective *TPMT* polymorphisms in Turkish population with those in other population of different ethnic origins.
- Retrospective investigation of the patients' clinical records who carry defective TPMT alleles (either heterozygous or homozygous mutant) in order to evaluate the relationship between 6MP related adverse reaction and genotype.
- Phenotyping of patients (analysis of TPMT activity for ALL patients by HPLC based enzyme assay)
- Determination of HPLC conditions (parameters) for phenotyping of the patients.

In order to achieve all the goals listed above, first of all, blood samples collected from children with acute lymphoblastic leukemia and healthy adult samples stored under safe conditions. Genomic DNA was isolated for all the samples and used for PCR-based reactions to determine all the genetic polymorphisms (*TPMT* polymorphisms, *EPHX1* polymorphisms and *SULT1A1*2* polymorphism) studied within the frame

of current work. For phenotyping of the patients enzyme assay and HPLC colon conditions were studied. Chi-square analysis was performed on the case and control polymorphism frequencies in order to determine the significance of the differences, as well as to compare different control population. The risk assessment was based on the odd ratio, with 95% confidence intervals, for both single gene polymorphisms and combination analyses. Interaction of non-genetic risk factors (smoking status of parents, postnatal exposure to cigarette smoke, etc.) and genetic polymorphisms in patient population as risk factors was done by determination of case-only odds ratio with 95% confidence intervals.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Subjects and Blood Sampling

Control group comprised of 206 subjects and blood samples were obtained from healthy volunteers with the collaboration of METU Health Center, Biochemistry Laboratory. Written informed consents were taken from each participant. The consent form also included questions regarding their age, birth place of the volunteer and his/her parents, and any diseases they had had. Subjects having diseases –like any type of cancer, diabetes etc.- were excluded from the study.

A total of 167 subjects were included in the ALL-patient group. The blood samples for patient group were obtained by the collaboration of Sami Ulus Children's Hospital and Ankara University, Faculty of Medicine, Department of Pediatric Haematology between Hematology between June 2005 and November 2007. Ethical approval was obtained from research ethics committee of Medical Faculty, Ankara University (The approval from ethical committee was given in Appendix A). Seventeen patients were from Ankara University and 150 patients were from Sami Ulus Children's Hospital. The information on demographic data, clinical diagnosis, stage of therapy, patient's risk group, treatment protocol, other diseases (if any), familial cancer cases (if present) were obtained with a

questionnaire. Information on subtype of ALL, age at diagnosis, white blood cell count at diagnosis, relapse conditions were obtained from the medical patient's log. Written informed consents were taken from the parents together with the questionairre, an example is given in Appendix A.

Questionnaire on the smoking status of mother and father was also obtained for 106 of the patients (an example is given in Appendix A). The questionnaire served information on the age of mother, father and child, from which the age of mother and father at the time of conception can be calculated; smoking status of mother and father, and the duration of exposure (passive or active smoking) were obtained. The questionnaire also included information on the smoking status of mother during pregnancy, and postnatal exposure of child to cigarette smoke.

4-5 mL of blood samples from contol and ALL-patient group subjects were taken in EDTA-containing vacuumed tubes and stored at -20°C till use for DNA isolation.

2.1.2 Enzymes and Chemicals Used in the Genotyping Studies

Agarose (A-9539), bromophenol blue (B-5525), ethidium bromide (E-7637), ethylene diamine tetra acetic acid disodium salt (EDTA; E-5134), sodium chloride (NaCl; S-3014), sodium dodecyl sulfate (SDS; L-4390), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T-5941) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Borate (11607), and absolute ethanol (32221) were the products of Riedel de Haën, Seelze. Magnesium chloride (A4425) and potassium chloride (A2939) were purchased from AppliChem, Ottoweg, Darmstadt.

Sucrose (7653) and Triton X-100 (11869) were the products of Merck & Co., Inc., Whitehouse Station, NJ, USA.

Taq DNA Polymerase –supplied together with $MgCl_2$ and amplification buffer- (#EP0407), dNTP mix (#R0191), Gene Ruler^{TM} 50 bp DNA Ladder (#SM0371) and restriction enzyme *Hha*I (#ER1851), *Rsa*I (#ER1121), *Mwo*I (#ER1731) which were supplied with their buffers Tango^{TM}, and *Tth111*I (#ER1331), *Acc*I (#ER1482) which were supplied with their buffers, Buffer B- were purchased from Fermentas, Int., Inc., Ontario, Canada. The componenets of amplification buffer, Tango^{TM} buffer and Buffer B were given in Appendix B.

All chemicals used in this study were of molecular grade and were obtained from commercial sources at the highest grade of purity.

2.1.3 Primers

Primers used throughout the study were selected by literature search and were derived from known sequences of human. The primer pairs were purchased from MWG (MWG Biotech, Ebersberg, Germany) or Metabion (Metabion International AG, Martinsried, Deutschland) and were all purified by HPLC. Primer stocks were brought to 100 pmol/µL concentration and stored at -20°C. Aliquots of 10 pmol/µL concentration were prepared and used for PCR.

2.1.4 Chemicals Used in the Determination of TPMT Activity by HPLC

All reagent used were of analytical grade except the chloride salt of S-adenosyl-L-methionine (SAM, 70%). SAM, 6MP, 6MMP were purchased from Sigma in highest available purity (Sigma Chemical Company, Saint Louis, Missouri, USA). Ultra-pure water was used for the

preaparation of all solutions. Phosphate buffer (pH 7.4) was prepared accrding to Sörensen with 0.067 M Na_2HPO_4/KH_2PO_4 (81.8:18.2). Thiopurines are not soluble in water or buffer, therefore, the stock solutions of 6MP (15mM) and 6MMP (6.02 mM) were prepared in 0.1 N NaOH. SAM (3.0 mM, corrected for purity) was dissolved in phosphate buffer (0.067M, pH 7.4). These stock solution were aliquated in eppendorf tubes and kept frozen (-20 ℃) until use.

2.2 Methods

2.2.1 Isolation of Genomic DNA from Human Whole Blood Samples

2.2.1.1 Manual Isolation of Genomic DNA

Genomic DNA was isolated by using a salting-out method described by Lahiri and Schnabel (Lahiri and Schnabel, 1993), with some modifications. 500 μL of whole blood which was taken into EDTA-containing vacuumed tubes is treated with an equal volume of low-salt buffer containing 10 mM Tris-HCl pH 7.6, 10 mM KCl, 2 mM EDTA, 4 mM $MgCl_2$ (TKME buffer), and 2.5% Triton X-100. The cells were lysed by inversions and the suspension is centrifuged at 1000 g for 10 min at room temperature using Sigma 1-15 benchtop microfuge (Sigma, Postfach 1713-D-37507, Osterode). The pellet was washed three more times with TKME buffer by same centrifugation condition. The final pellet was suspended in 0.2 mL of TKME buffer containing 1.0% SDS. The suspension is mixed vigorously and incubated for 20 min at 55˚C. Then 75 μL saturated NaCl (6M) is added, the tube is mixed well and centrifuged at 14000 g for 7 min, at 4˚C using cooled centrifuge. The supernatant, which contained the DNA is precipitated using absolute ethanol and collected at 10000g for 10 min at 4˚C and DNA pellet is washed once with 70% ethanol, then air dried and final DNA pellet is resuspended in 0.2 mL of 10 mM Tris-HCl pH 8.0, and 1 mM EDTA pH 8.0. (TE) and incubated overnight at 37˚C. The DNA samples were stored

at 4°C while they were in active use and kept at -20°C for long-term storage.

2.2.1.2 Genomic DNA Isolation by Nucleospin Blood Kit

Nucleospin Blood Kit (Machery-Nagel, Germany) was used for isolation of DNA from old blood samples or from the ALL patients that have reduced number of white blood cells, which manual isolation did not give effective PCR results.

After complete thawing, 200 µL of whole blood was mixed with 25 µL of proteinase-K in an 1.5 mL eppendorf tube, and vortexed immediately. Then 200 µL of lysis buffer “B3” was added, vortexed vigorously for 10-20 seconds and the tube was incubated at 70°C in a heater block for 30 minutes. In every 10 minutes of incubation, tube was vortexed for 10-20 seconds. The lysate turns into a greenish-brownish color at the end of the incubation period.

At the end of the incubation period, 210 µL of absolute ethanol was added to the mixture, vortexed and the sample in the tube was transferred to a spin column. The sample in the column was centrifuged at 14000 g for 2 minutes at room temperature using a Thermo Microlite RF (Waltham, MA, USA) refrigerated micro centrifuge. The collecting tube with flow-through was discarded. The spin column was placed in a new collecting tube and 500 µL of buffer “BW” was added to the spin column. Buffer BW serves to wash the DNA sample in the spin column. The sample in the spin column was centrifuged at 14000 g for 2 minutes at room temperature and the flow through was discarded. 600 µL of buffer “B5” was added to the column, centrifuged again as described above, and the flow through was discarded. Then the spin column was centifuged once

more as described above, without adding anything to the column, in order to get rid of the ethanol that was present in buffer “B5” completely.

In order to elute the DNA in the column, the spin column was placed in a new 1.5 mL eppendorf tube, 100 µL of preheated (to 70°C) buffer “BE” was dispensed directly onto the membrane, the tube was incubated at room temperature for 5 minutes and then centrifuged at 14000 g for 2 minutes at room temperature. The eluent was loaded onto the same spin column again, incubated for 5 minutes and centrifuged as described above, to increase the yield of DNA.

2.2.2 Spectrophotometric Quantification of Genomic DNA

The concentrations of the genomic DNA isolated from each sample were determined spectrophotometrically using Schimadzu UV-1201 Spectrophotometer (Schimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). As the DNA molecule gave maximum absorption at 260 nm, reading at this wavelength was used to calculate the concentration of nucleic acid in the sample. Based on the knowledge that an optical density of 1.0 corresponded to approximately 50µg/ml for double stranded DNA, the concentration of DNA in the sample will be calculated according to the formula:

“Concentration (µg/mL) = OD_{260nm} x 50 (µg/mL) x Dilution Factor”

The ratio between OD values at 260 nm and 280 nm (OD_{260}/OD_{280} ratio) will used to estimate the purity of the nucleic acid. Pure DNA preparations gave the ratio of 1.8 while the higher and lower values showed either RNA or protein contamination, respectively. However the ratio between 1.6 and 2.0 can be acceptable.

2.2.3 Qualification of Genomic DNA by Agarose Gel Electrophoresis

Intacness of DNA samples was determined by horizontal agarose gel electrophoresis .Agarose gel was prepared in 0.5% concentration by 0.5x TBE buffer (450 mM Tris, 450 mM Boate, 10 mM EDTA, pH 8.3) using a microvawe oven. When the gel solution was cooled to approximately 60°C, ethidium bromide was added from a stock solution of 10 mg/mL so as to obtain a final concentration of 0.5 µg/mL and the solution was mixed throughly.

The warm agarose solution was poured into the pre-settled mold and any air bubbles –if present-, especially under or between the teeth of the comb were removed by the help of a pipette tip. The gel was allowed to solidify completely for approximately 20-40 minutes at room temperature. After the agarose gel is solidified, it was mounted in the electrophoresis tank which was filled with 0.5x TBE buffer so that the slots of the gel faced the negative pole-cathode. 5µL of DNA sample was mixed with 1 µL of gel loading dye by the use of a micropipette, and the mixture was slowly added to the wells of the gel. After loading of the DNA samples were completed, the lid of the tank was closed and the electrical leads were attached to the power supply. The power supply was set to a constant voltage so that not more than a voltage of 5V/cm (measured as the distance between the electrodes) was applied (corresponds to a maximum of 150 volts for Scie-Plas HU13W horizontal gel electrophoresis unit).

The gel was run until the bromophenol blue reached to the bottom of the gel, and then examined under UV light and the photograph was taken by Vilber Lourmat Gel Imaging System (Marne La Vallee, Cedex, France) and InfinityCapt (version 12.9) computer software. Pure DNA prepartions give a single band in agarose gel electrophoresis, while RNA

contaminated preparations yield two band. A smear indicated the degredation of DNA.

2.2.4 Genotyping of Single Nucleotide Polymorphisms

In this study the genes of three drug metabolizing enzymes: *TPMT*, *SULT1A1* and *EPHX1* were genotyped for their most common single nucleotide polymorphisms. The two *TPMT* point mutations namely G460A and A719G polymorphisms, *SULT1A1*2* polymorphisms and *EPHX1* exon 3 and exon 4 polymorphisms were identified by amplification of SNP containing regions by PCR followed by appropiate restriction enzyme digestions. G238C point mutation in the *TPMT* gene was identified by allele specific PCR. The details of these methods were decsribed below. Techne Progene (Cambridge, UK) and Eppendorf Mastercycler (Hamburg, Germany) thermocyclers were used for PCR.

2.2.4.1 Genotyping for SNPs of TPMT gene

To date, 23 SNPs in the *TPMT* gene associated with deficient enzyme activity have been identified (Teml *et al*., 2007). Among the defective alleles, more than 80% of all low activity cases are attributed to three inactivating mutations (G238C, G460A, and A719G) that characterize the four defective alleles namely *TPMT*2*, **3B*, **3C*, and **3A* (Yates *et al*., 1997). In this study the frequencies of those three inactivating mutations thus four different defective *TPMT* alleles were investigated in 167 children with ALL and 206 healthy adult subjects.

For the identification of the three inactivating *TPMT* mutations, the amplified region, corresponding primer sequences, the size of PCR products, restriction enzymes used and annealing temperatures used in the PCR protocols for all three polymorphic region of the *TPMT* gene were given in Table 2.1.

Table 2.1 Regions of Amplification, Primer sequences (Yates *et al*., 1997), PCR Product Sizes, Restriction Enzymes and Annealing Temperatures for *TPMT* Genotyping.

Mutation	Primer	Sequence of primer	PCR Product Size	Restriction Enzyme Used	Annealing temperatures (℃)
G238C Exon 5	P2W P2M P2R	5'-GTATGATTTTATGCAGGTTTG-3' 5'-GTATGATTTTATGCAGGTTTC 5'-TAAATAGGAACCATCGGACAC-3'	254 bp		57
G460A Exon 7	P3F P3R	5'-AGGCTCCTAAAACCATGAGGG-3' 5'-GTATACTAAAAAATTAAGACAGC-3'	317 bp	*Mwo*I	53
A719G Exon 10	P3CF P3CR	5'-CAGGCTTTAGCATAATTTTCAATTCCT C-3' 5'-TGTTGGGATTACAGGTGTGAGCCA C-3'	293 bp	*Acc*I	55

2.2.4.1.1 Genotyping for *TPMT*2* Allele-G238C Polymorphisms

Allele specific PCR method was used to detect G238C (*TPMT*2* variant allele) SNPs. This mutation located in exon 5 region which is selectively amplified by the primers in Table 2.1. In allele specific PCR, wild type or mutant DNA fragments was amplified selectively resulting in fragments length of 254 bp. The sequence of the amplified fragment and location of G238C SNP were given in Figure 2.1. A DNA fragment was amplified with P2W (forward primer) and P2R (reverse primer) primers when G238 (wild type) is present whereas a DNA fragment will be amplified with P2M (forward primer) and P2R primers when C238 (mutant allele) is present. Accordingly, for each sample, two PCR reactions were carried out. One contains forward primer designed for detection of wild type allele and common reverse primer. The other one contains forward primer designed for detection of mutant allele and common reverse primer.

Several optimizations regarding the $MgCl_2$ (1.0, 1.25, 1.5, 2.0, 3.0, 4.0 mM) and primer concentration (20 and 40 pmol) as well as amplification program (optimization of the annealing temperature and cycle number) were carried out in order to obtain a single band for this region. Components of the optimized PCR mixture for the amplifiction of exon 5 region (containing G238C point mutation) were given in Table 2.2. Briefly, PCR reaction was carried out in 50 µl of a solution containing amplification buffer (supplied commercially with the Taq polymerase enzyme : 20 mM Tris HCl, 50 mM KCl; pH 8.5), 1 mM $MgCl_2$, 1mM dNTPs, 0.4 µM (20 pmol in 50 µl action mixture) of forward and reverse primers, 200 µM dNTP approximately 200 ng of genomic DNA, and 2.5 U of Taq polymerase.

Figure 2.1 Sequence of amplified fragment in exon 5 region of *TPMT* gene that includes G238C SNP. The yellow highlighted sequences are forward and reverse primers for amplifying wild type allele. Red marked sequences shows the forward primer for amplifying mutant allele. Turquoise highlighted nucleotide shows the location of G238C SNP (The nucleotide sequence was taken from http://www.ncbi.nlm.nih.gov).

Table 2.2 Components of PCR mixture for G238C SNPs (*TPMT*2* allele).

Constituent	**Stock Conc.**	**Volume added**	**Final Conc. in 50 µl reaction mixture**
Amplification buffer	10x	5 µL	1x
$MgCl_2$	25 mM	2.5 µL	1.0 mM
dNTP mixture*	10 mM	1 µL	200 µM
Reverse Primer	10 pmol/µL	2 µL	20 pmol
Forward Primer	10 pmol/µL	2 µL	20 pmol
Template DNA	varies	varies	~200 ng
Taq DNA Polymerase*	5 U/µL	0.5 µL	2.5 U
Sterile apyrogen H_2O		To 50 µL	
***** see Appendix B for these reagents.			

The standard amplification program used for the amplification of exon 5 region consist of an initial denaturation step at 95 °C for 5 min followed by 30 cycles of denaturating at 94 °C fo 1 min, annealing at 57°C for 2 min and extension at 72°C for 1 min. The final extension will be performed at 72 °C for 7min (Brouwer *et al.,*2005).

Amplification programme used was as follows:

Initial denaturation	95 °C	5 min	
Denaturation	94°C	1 min	30 cycles
Annealing	57°C	2 min	
Extension	72°C	1 min	
Final extension	72°C	7 min	

PCR products were analyzed on 2% agarose gel as described in section 2.2.3. 10 µL of PCR product was mixed by 2 µL of gel loading dye and applied to the wells of the gel. 5 µL of DNA ladder (50-1000bp, see Appendix B) was applied to one well. The gel was run until the bromophenol blue reached to the bottom of the gel, visualized under UV and photographed.

2.2.4.1.2 Genotyping for *TPMT*3B* allele-G460A Polymorphisms

For genotyping of *TPMT*3B* allele, first, a part of exon 7 region (a 317 bp fragment) on the TPMT gene was amplified by PCR using the primer pairs given in Table 2.1. Amplified region then was digested by restriction enzyme (*Mwo*I) for the detection of G460A point mutation. The sequence of the amplified fragment, location of the G460A SNP and sequence of the recognition site for the restriction enzyme were given in Figure 2.2.

2.2.4.1.2.1 Polymerase Chain Reaction for G460A Polymorphisms

Several optimizations regarding the $MgCl_2$ (1.0, 1.25, 1.5, 1.75, 2.0 mM) and primer concentrations (20 and 40 pmol) as well as amplification program (optimization of the annealing temperature and cycle number) were carried out in order to obtain a single band for this region. Components of the optimized PCR mixture for the amplifiction of exon 7 region (containing G460A point mutation) were given in Table 2.3. Briefly, PCR reaction was carried out in 50 µl of a solution containing amplification buffer (supplied commercially with the Taq polymerase enzyme: 20 mM Tris HCl, 50 mM KCl; pH 8.5), 1.75 mM $MgCl_2$, 1mM dNTPs, 0.4 µM (20 pmol in 50 µl action mixture) of forward and reverse primers, 200 µM dNTP approximately 200 ng of genomic DNA, and 2.5 U of Taq polymerase.

Table 2.3 Components of PCR mixture for G460A SNPs (*TPMT*3B*).

Constituent	Stock Conc.	Volume added	Final Conc. in 50 µl reaction mixture
Amplification buffer	10x	5 µL	1x
$MgCl_2$	25 mM	3.5 µL	1.75 mM
dNTP mixture*	10 mM	1 µL	200 µM
Reverse Primer	10 pmol/µL	2 µL	20 pmol
Forward Primer	10 pmol/µL	2 µL	20 pmol
Template DNA	varies	varies	~200 ng
Taq DNA Polymerase*	5 U/µL	0.5 µL	2.5 U
Sterile apyrogen H_2O		To 50 µL	
***** see Appendix B for these reagents.			

The standard amplification program used for the amplification of exon 7 region consist of an initial denaturation step at 95 °C for 5 min followed by 30 cycles of denaturating at 94 °C fo 1 min, annealing at 53°C for 2 min and extension at 72°C for 1 min. The final extension will be performed at 72 °C for 7min (Brouwer *et al.,*2005).

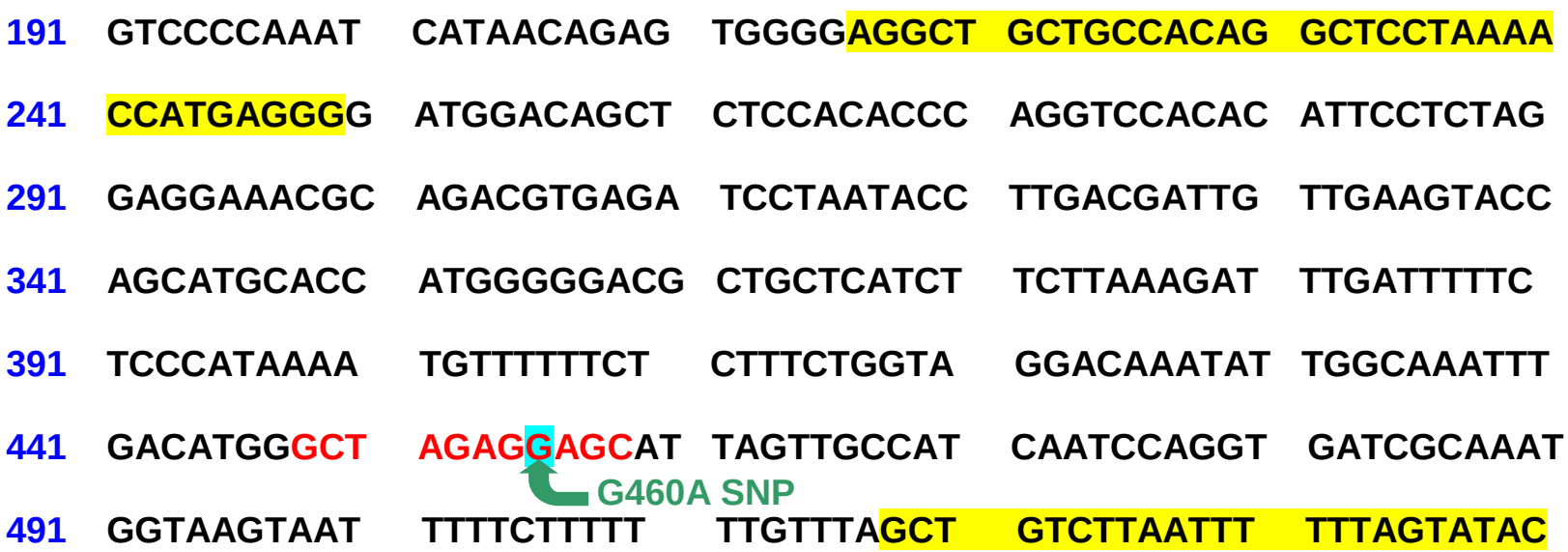


Figure 2.2 Sequence of amplified fragment in exon 7 region of *TPMT* gene that includes G460A SNP. The yellow highlighted sequences are forward and reverse primers. Red marked sequences shows the recognition site for the restriction enzyme *Mwo*I. Turquoise highlighted nucleotide shows the location of G460A SNP (The nucleotide sequence was taken from http://www.ncbi.nlm.nih.gov).

Amplification programme used was as follows:

Initial denaturation	95 °C	5 min	
Denaturation	94°C	1 min	30 cycles
Annealing	53°C	2 min	
Extension	72°C	1 min	
Final extension	72°C	7 min	

The bands were visualized as described in section 2.2.4.1.1.

2.2.4.1.2.2 Restriction Endonuclease Digestion of PCR Products for G460A Polymorphisms

Genotyping for G460A point mutation was carried out by digesting 20 µl of PCR product with 2 U of *Mwo*I enzyme in a final volume of 30 µl reaction mixture. The componenets of the reaction mixture were given in Table 2.4.

Table 2.4 Components of digestion mixture for G460A SNPs (*TPMT*3B*).

Constituent	**Stock Conc.**	**Volume added**	**Final Conc. in 30 µl reaction mixture**
Buffer*	10x	3 µL	1x
PCR Product		10 µL	
Restriction enzyme**	10 U/µL	0.2 µL	2 U
Sterile apyrogen H_2O		16.8 µL	
* Tango™ Buffer (see Appendix B)			

The reaction mixture was incubated overnight at 37℃ for 18 hours for complete digestion. The wild type alleles that contain G at position 460 bear a recognition sites around that nucleotide for *Mwo*I restriction enzyme thus providing *Mwo*I to cut the product from that position. Therefore *Mwo*I digestion results in 2 bands of 240 and 77 bp for the wild type (G460). However in mutant alleles with A in position 460, there is not a recognition site around the SNP, thus *MwoI* can not cut the amplified fragment. Figure 2.3 shows the basis of *Mwo*I digestion and represents the resulting fragments upon *Mwo*I digestion in case of different genotypes. Accordingly, the expected banding patterns upon digestion of amplified region in exon 7 of *TPMT* gene with *Mwo*I restriction enzyme were as follows: In homozygous wild types, presence of recognition sequences would yield two bands of 240 and 77 bp, while in homozygous mutants, as recognition sequence around SNP is lost due to base substitution (G at 460. nucleotide position was substituted by A in mutant allele), single band is expected. The heterozygotes would contain in total 3 bands in lenghts of 317, 240, and 77 bp.

Restriction products were analyzed on 2.5% agarose gel. 30 µL of digestion product was mixed with 5 µL of gel loading dye and applied to the wells of the gel. 5 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run until the bromophenol blue reached to the bottom of the gel, visualized under UV and photographed.

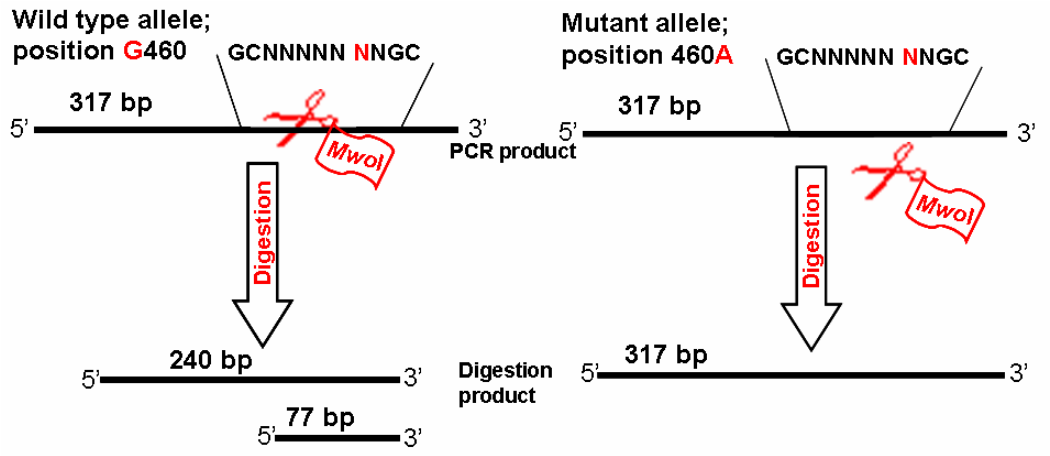


Figure 2.3 Schematic representation of the banding patterns of the amplified exon 7 region of *TPMT* gene upon digestion with *Mwo*I restriction enzyme. The left handside shows the banding pattern for wild type allele and right handside shows the banding pattern for mutated allele.

2.2.4.1.3 Genotyping for *TPMT*3C* allele-A719G Polymorphisms

For genotyping of *TPMT*3C* allele, first, a part of exon 8 region (a 293 bp fragment) on the *TPMT* gene was amplified by PCR using the primer pairs given in Table 2.1. Amplified region then was digested by restriction enzyme (*Acc*I) for the detection of A719G point mutation. The sequence of the amplified fragment, location of the A719G SNP and sequence of the recognition site for the restriction enzyme were given in Figure 2.4.

2.2.4.1.3.1 Polymerase Chain Reaction for A719G Polymorphisms

Several optimizations regarding the $MgCl_2$ (1.0, 1.25, 1.5) and primer concentrations (20 and 40 pmol) as well as amplification program (optimization of the annealing temperature and cycle number) were carried out in order to obtain a single band for this region. Components of the optimized PCR mixture for the amplification of exon 8 region (containing A719G point mutation) were given in Table 2.5. Briefly, PCR reaction was carried out in 50 µl of a solution containing amplification buffer (supplied commercially with the Taq polymerase enzyme: 20 mM Tris HCl, 50 mM KCl; pH 8.5), 1.25 mM $MgCl_2$, 1mM dNTPs, 0.4 µM (20 pmol in 50 µl action mixture) of forward and reverse primers, 200 µM dNTP approximately 200 ng of genomic DNA, and 2.5 U of Taq polymerase.

Table 2.5 Components of PCR mixture for A719G SNPs (*TPMT*3C*).

Constituent	**Stock Conc.**	**Volume added**	**Final Conc. in 50 µl reaction mixture**
Amplification buffer	10x	5 µL	1x
$MgCl_2$	25 mM	2.5 µL	1.25 mM
dNTP mixture*	10 mM	1 µL	200 µM
Reverse Primer	10 pmol/µL	2 µL	20 pmol
Forward Primer	10 pmol/µL	2 µL	20 pmol
Template DNA	varies	varies	~200 ng
Taq DNA Polymerase*	5 U/µL	0.5 µL	2.5 U
Sterile apyrogen H_2O		To 50 µL	
***** see Appendix B for these reagents.			

The standard amplification program used for the amplification of exon 8 region consist of an initial denaturation step at 95 °C for 5 min followed by 30 cycles of denaturating at 94 °C fo 1 min, annealing at 55°C for 2 min and extension at 72°C for 1 min. The final extension will be performed at 72 °C for 7min (Brouwer *et al.,*2005).

Amplification programme used was as follows:

Initial denaturation	95 °C	5 min	
Denaturation	94°C	1 min	30 cycles
Annealing	55°C	2 min	
Extension	72°C	1 min	
Final extension	72°C	7 min	

The bands were visualized as described in section 2.2.4.1.1.

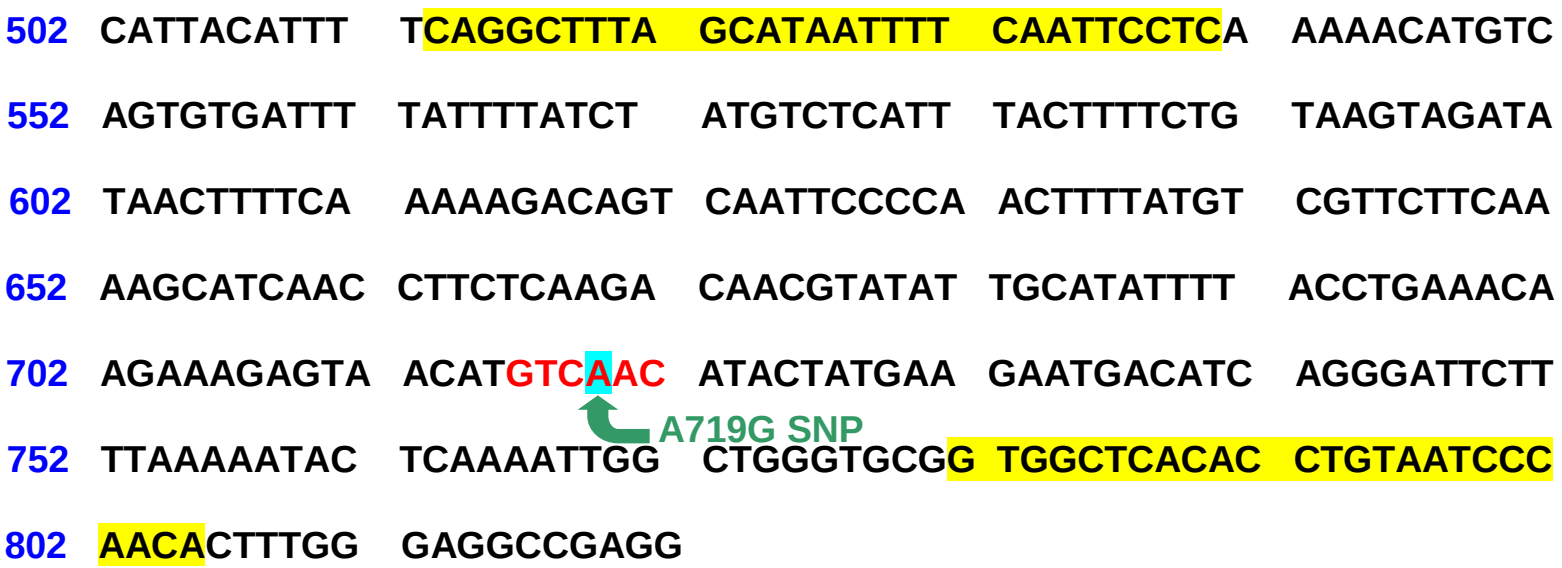


Figure 2.4 Sequence of amplified fragment in exon 8 region of *TPMT* gene that includes A719G SNP. The yellow highlighted sequences are forward and reverse primers. Red marked sequences shows the recognition site for the restriction enzyme *Mwo*I. Turquoise highlighted nucleotide shows the location of A719G SNP (The nucleotide sequence was taken from http://www.ncbi.nlm.nih.gov).

2.2.4.1.3.2 Restriction Endonuclease Digestion of PCR Products for A719G Polymorphisms

Genotyping for A719G point mutation was carried out by digesting 20 µl of PCR product with 3U of *Acc*I enzyme in a final volume of 30 µl reaction mixture. The componenets of the reaction mixture were given in Table 2.6.

Table 2.6 Components of digestion mixture for A719G SNPs (*TPMT*3C*).

Constituent	**Stock Conc.**	**Volume added**	**Final Conc. in 30 µl reaction mixture**
Buffer*	10x	3 µL	1x
PCR Product		20 µL	
Restriction enzyme	10 U/µL	0.3 µL	3 U
Sterile apyrogen H_2O		6.7 µL	
***** Buffer B (see Appendix B)			

The reaction mixture was incubated overnight at 55℃ for 18 hours for complete digestion. The mutant type allele which contains G at position 719 bears a recognition sites around that nucleotide for *Acc*I restriction enzyme. Therefore mutated allele provides *Acc*I to cut the product from that position so *Acc*I digestion results in 2 bands of 207 and 86 bp for the mutant alleles (A719G mutation). However in wild type alleles with A in position 719 there is not a recognition site around the SNP, thus *Acc*I can not cut the amplified fragment. Figure 2.5 shows the basis of *Acc*I digestion and represents the resulting fragments upon digestion in case of different genotypes. Accordingly, the expected banding patterns upon digestion of amplified region in exon 8 of *TPMT*

gene with *Acc*I restriction enzyme were as follows: In homozygous mutant types, presence of recognition sequences would yield two bands of 207 and 86 bp, while in homozygous wild type, as recognition sequence around SNP is lost due to base substitution (A at 719. nucleotide position was substituted by G in mutant allele), single band is expected. The heterozygotes would contain in total 3 bands in lenghts of 293, 207, and 86 bp.

The bands were visualized as described in section 2.2.4.1.2.2.

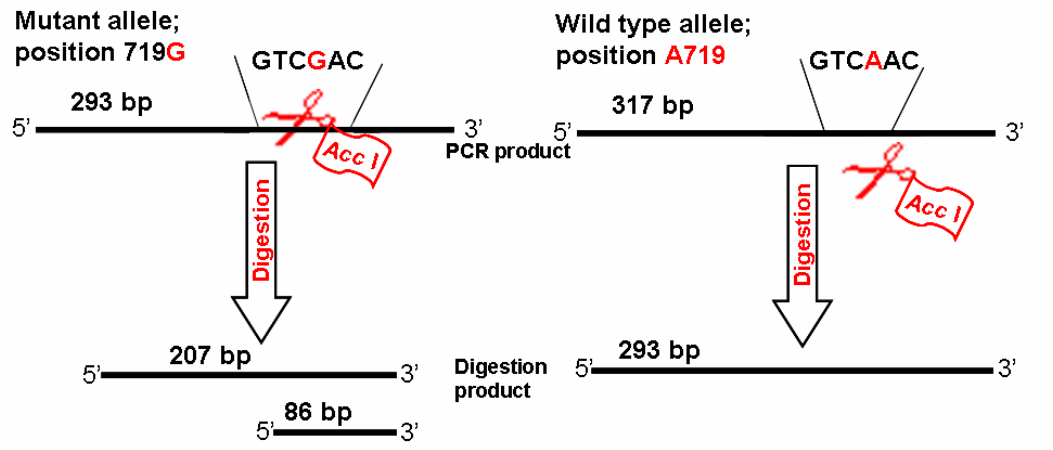


Figure 2.5 Schematic representation of the banding patterns of the amplified exon 8 region of *TPMT* gene upon digestion with *Acc*I restriction enzyme. The left handside shows the banding pattern for mutated allele and right handside shows the banding pattern for wild type allele.

2.2.4.2 Genotyping for SULT1A1 Gene-(G638A Polymorphisms)

In this study, *SULT1A1* gene was genotyped by PCR-RFLP method for the detection of *SULT1A1*2* allele which is characterized by G638A point mutation. The sequence of the amplified fragment, location of the G638A SNP and sequence of the recognition site for the restriction enzyme were given in Figure 2.6.

2.2.4.2.1 Polymerase Chain Reaction for SULT1A1*2 Variant

G638A SNP (*SULT1A1*2*) is located on exon 7 region of the gene. For this reason determination of the *SULT1A1*2* polymorphisms requires amplification of exon 7 region of the gene. A 281 bp fragment covering G638C point mutation was amplified with forward primer PSF: 5'-GGTTGAGGAGTTGGCTCTGC-3' and reverse primer PSR: 5'-ATGAACTCCTGGGGGACGGT-3' (primer sequences were taken from Ozawa et al., 1998). In order to obtain a single band devoid of non-specific bands, different primer (10 and 20 pmols) and $MgCl_2$ (1.25, 1.5, 2.0, 2.5 mM) concentrations were tested. The optimized PCR conditions as given in Table 2.7. Briefly, PCR reaction was carried out in 50 µl of a solution containing amplification buffer (supplied commercially with the Taq polymerase enzyme: 20 mM Tris HCl, 50 mM KCl; pH 8.5), 1.25 mM $MgCl_2$, 1mM dNTPs, 0.2 µM (10 pmol in 50 µl reaction mixture) of forward and reverse primers, 200 µM dNTP approximately 200 ng of genomic DNA, and 2.5 U of Taq polymerase.

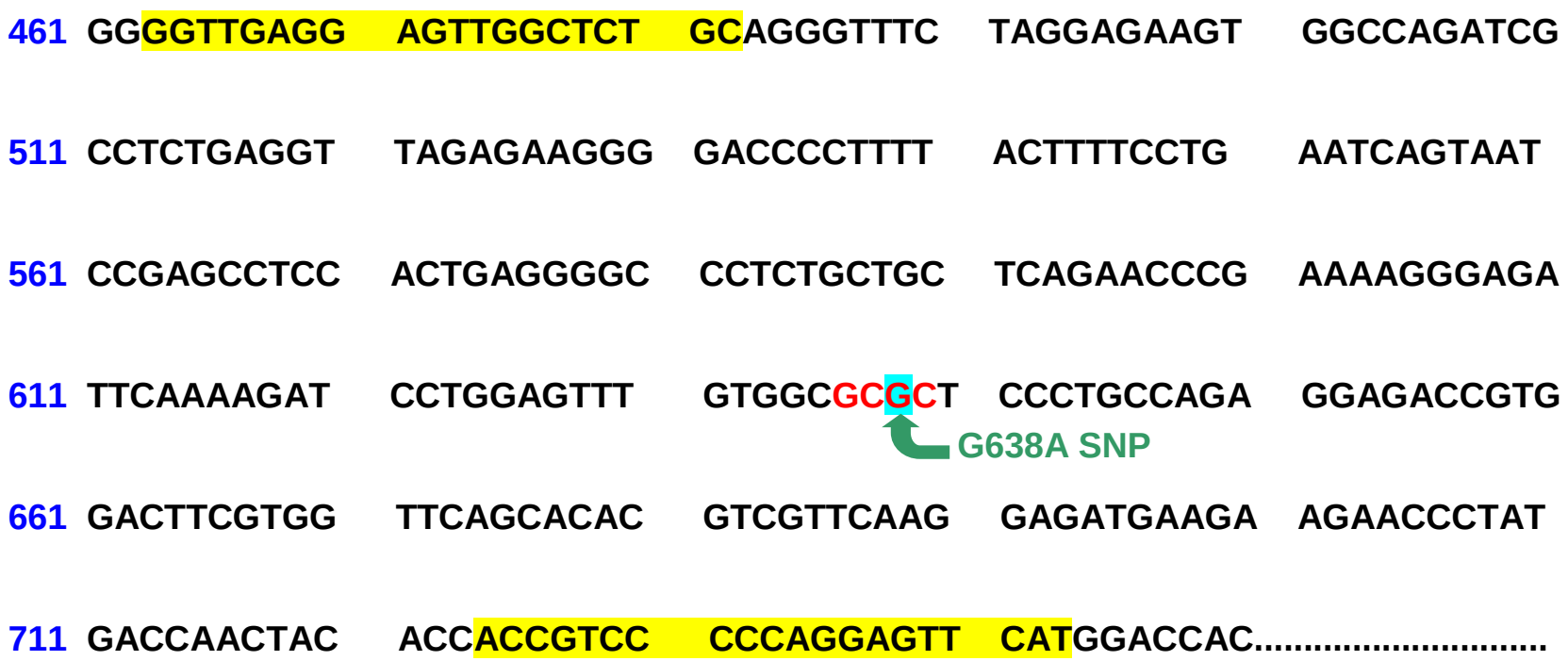


Figure 2.6 Sequence of amplified fragment in exon 7 region of *SULT1A1* gene that includes G638A SNP. The yellow highlighted sequences are forward and reverse primers. Red marked sequences shows the recognition site for the restriction enzyme *Hha*I. Turquoise highlighted nucleotide shows the location of G638A SNP (The nucleotide sequence was taken from http://www.ncbi.nlm.nih.gov).

Table 2.7 Components of PCR mixture for G638A SNPs (*SULT1A1*2*).

Constituent	Stock Conc.	Volume added	Final Conc. in 50 µl reaction mixture
Amplification buffer	10x	5 µL	1x
$MgCl_2$	25 mM	2.5 µL	1.25 mM
dNTP mixture*	10 mM	1 µL	200 µM
Reverse Primer	10 pmol/µL	1 µL	10 pmol
Forward Primer	10 pmol/µL	1 µL	10 pmol
Template DNA	varies	varies	~200 ng
Taq DNA Polymerase*	5 U/µL	0.5 µL	2.5 U
Sterile apyrogen H_2O		To 50 µL	
***** see Appendix B for these reagents.			

The PCR program used for the amplification of exon 7 region consist of an initial denaturation step at 94 °C for 5 min followed by 35 cycles of denaturating at 94 °C fo 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min. (Ozawa *et al.,*1998).

Amplification programme used was as follows:

Initial denaturation	94 °C	5 min	
Denaturation	94°C	1 min	35 cycles
Annealing	60°C	1 min	
Extension	72°C	2 min	

The bands were visualized as described in section 2.2.4.1.1.

2.2.4.2.2 Restriction Endonuclease Digestion of PCR Products for G638A Polymorphisms

Genotyping for G638A point mutation was carried out by digesting 20 µl of PCR product with 3U of *Hha*I enzyme in a final volume of 30 µl reaction mixture. The componenets of the reaction mixture were given in Table 2.8.

Table 2.8 Components of digestion mixture for *SULT1A1*2* variant

Constituent	**Stock Conc.**	**Volume added**	**Final Conc. in 30 µl reaction mixture**
Buffer*	10x	3 µL	1x
PCR Product		20 µL	
Restriction enzyme	10 U/µL	0.3 µL	3 U
Sterile apyrogen H_2O		6.7 µL	
* Tango™ Buffer (see Appendix B)			

The reaction mixture was incubated overnight at 37℃ for 18 hours for complete digestion. The wild type alleles that contain G at position 638 bear a recognition sites around that nucleotide for *Hha*I restriction enzyme thus providing *Hha*I to cut the product from that position. Therefore *Hha*I digestion results in 2 bands of 176 and 105 bp for the wild type (G638). However in mutant alleles with A in position 638, there is not a recognition site around the SNP, thus *Hha*I can not cut the amplified fragment. Figure 2.7 shows the basis of *Hha*I digestion and represents the resulting fragments upon digestion in case of different genotypes.

Accordingly, the expected banding patterns upon digestion of amplified region in exon 7 of *SULT1A1* gene with *Hha*I *restriction* enzyme were as follows: In homozygous wild types, presence of recognition sequences would yield two bands of 176 and 105 bp, while in homozygous mutants, as recognition sequence around SNP is lost due to base substitution (G at 638. nucleotide position was substituted by A in mutant allele), single band is expected. The heterozygotes would contain in total 3 bands in lenghts of 281, 176, and 105 bp.

The bands were visualized as described in section 2.2.4.1.2.2.

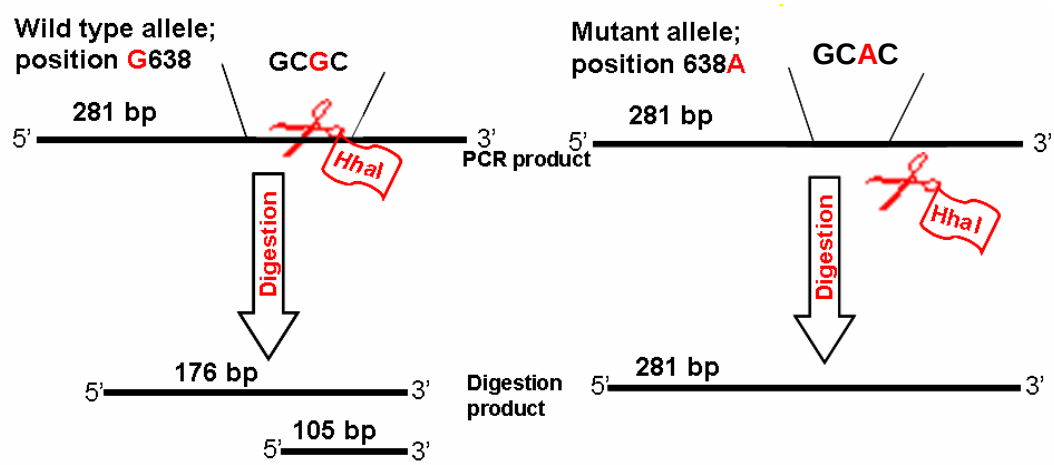


Figure 2.7 Schematic representation of the banding patterns of the amplified exon 7 region of *SULT1A1* gene upon digestion with *Hha*I restriction enzyme. The left handside shows the banding pattern for wild type allele and right handside shows the banding pattern for mutant allele.

2.2.4.3 Genotyping for EPHX1 Gene (exon 3 and exon4 polymorphisms)

In this study, *EPHX1* gene was genotyped by PCR-RFLP method for the detection of exon 3 polymorphisms that changes tyrosine residue 113 (Tyr 113) to histidine (His 113) and exon 4 polymorphisms that changes the histidine residue 139 (His 139) to arginine (Ar139).

2.2.4.3.1 Genotyping for exon 3 (Tyr113His) Polymorphism

2.2.4.3.1.1 Polymerase Chain Reaction for Exon 3 Variant

A 198 bp fragment in the exon 3 region of the *EPHX1* gene that contains a T/C SNP (causing a Tyr113His replacement in the encoded protein) was amplified with forward primer EH3 F: 5'-GGGGTCCTGAATTTTGCTCC-3' and reverse primer EH3 R: 5'-CAATCT TAGTCTTGAAGTGACGGT-3' (primer sequences were taken from Korhonen et al., 2003). The sequence of the amplified fragment, location of T/C SNP and sequence of the recognition site for the restriction enzyme were given in Figure 2.8. In order to obtain a single band devoid of non-specific bands, different primer (20 and 40 pmols) and $MgCl_2$ (1.00, 1.25, 1.50, 1.75, 2.00) concentrations were tested. The optimized PCR conditions as given in Table 2.9. Briefly, PCR reaction was carried out in 50 µl of a solution containing amplification buffer (supplied commercially with the Taq polymerase enzyme: 20 mM Tris HCl, 50 mM KCl; pH 8.5), 1.25 mM $MgCl_2$, 1mM dNTPs, 0.8 µM (40 pmol in 50 µl reaction mixture) of forward and reverse primers, 200 µM dNTP approximately 200 ng of genomic DNA, and 2.5 U of Taq polymerase.

Table 2.9 Components of PCR mixture for exon 3 polymorphisms of *EPHX1* gene (Tyr113His).

Constituent	Stock Conc.	Volume added	Final Conc. in 50 µl reaction mixture
Amplification buffer	10x	5 µL	1x
$MgCl_2$	25 mM	2.5 µL	1.25 mM
dNTP mixture*	10 mM	1 µL	200 µM
Reverse Primer	10 pmol/µL	4 µL	40 pmol
Forward Primer	10 pmol/µL	4 µL	40 pmol
Template DNA	varies	varies	~200 ng
Taq DNA Polymerase*	5 U/µL	0.5 µL	2.5 U
Sterile apyrogen H_2O		To 50 µL	
***** see Appendix B for these reagents.			

The PCR program used for the amplification of exon 3 region consist of an initial denaturation step at 95 °C for 5 min followed by 35 cycles of denaturating at 95 °C fo 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min (Korhonen *et al*., 2003).

Amplification programme used was as follows:

Initial denaturation	95°C	5 min	
Denaturation	95°C	1 min	35 cycles
Annealing	55°C	1 min	
Extension	72°C	1 min	

The bands were visualized as described in section 2.2.4.1.1.

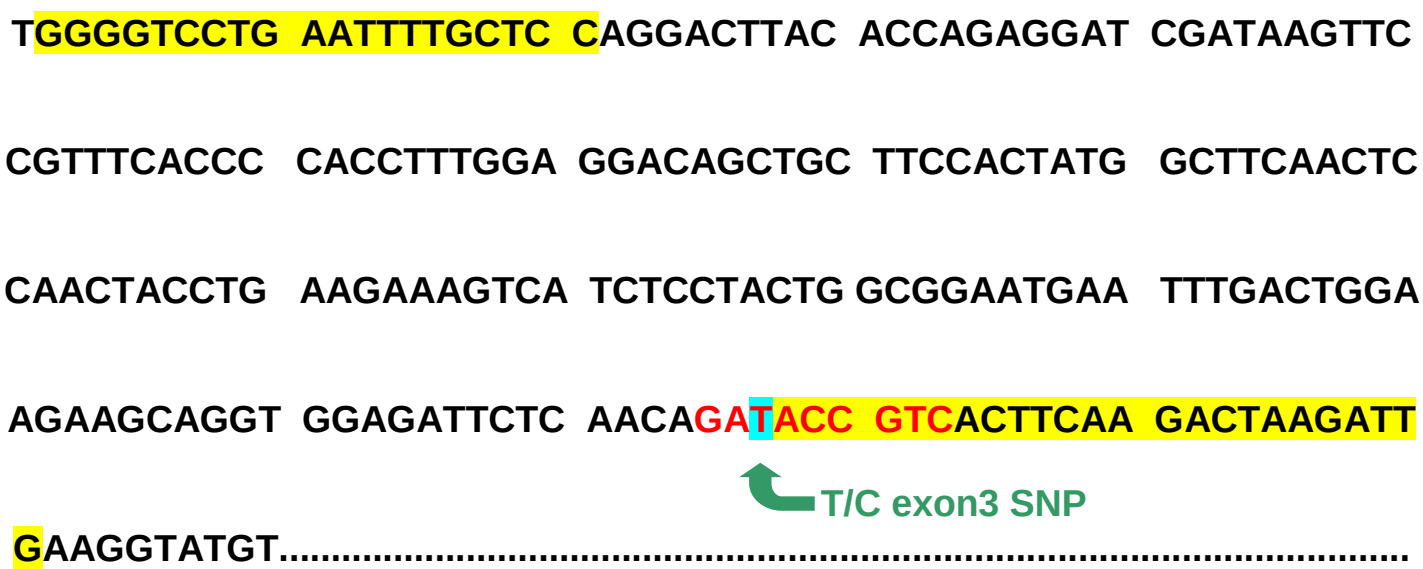


Figure 2.8 Sequence of amplified fragment in exon 3 region of *EPHX1* gene that includes T/C SNP. The yellow highlighted sequences are forward and reverse primers. Red marked sequences shows the recognition site for the restriction enzyme *Tth111*I. Note that the sequences of enzyme's recognition site and reverse primer are overlapping. Turquoise highlighted nucleotide shows the location of T/C SNP (The nucleotide sequence was taken from http://www.ncbi.nlm.nih.gov).

2.2.4.3.1.2 Restriction Endonuclease Digestion of PCR Products for Exon 3 Polymorphisms

Genotyping for exon 3 polymorphisms of EPHX1 gene was carried out by digesting 20 µl of PCR product with 3U of *Tth111*I (*Psy*I) enzyme in a final volume of 30 µl reaction mixture. The componenets of the reaction mixture were given in Table 2.10.

Table 2.10 Components of digestion mixture for exon 3 polymorphisms of *EPHX1* gene.

Constituent	**Stock Conc.**	**Volume added**	**Final Conc. in 30 µl reaction mixture**
Buffer*	10x	3 µL	1x
PCR Product		20 µL	
Restriction enzyme	10 U/µL	0.3 µL	3 U
Sterile apyrogen H_2O		6.7 µL	
***** Buffer B (see Appendix B)			

The reaction mixture was incubated overnight at 37℃ for 18 hours for complete digestion. The mutant type allele which contains C instead of T bears a recognition sites around that nucleotide for *Tth111*I restriction enzyme. Therefore mutated allele provides restriction enzyme to cut the product from that position so *Tth111*I digestion results in 2 bands of 175 and 23bp for the mutant alleles (T/C mutation). However in wild type alleles with T, the recognition site of the restriction enzyme is lost, thus *Tth111*I can not cut the amplified fragment. Figure 2.9 shows the basis of

*Tth111*I digestion and represents the resulting fragments upon digestion in case of different genotypes. Accordingly, the expected banding patterns upon digestion of amplified region in exon 3 of *EPHX1* gene with *Tth111*I restriction enzyme were as follows: In homozygous mutant types, presence of recognition sequences would yield two bands of 175 and 23 bp, while in homozygous wild type, as recognition sequence around SNP is lost due to base substitution (T/C), single band (198bp) is expected. The heterozygotes would contain in total 3 bands in lenghts of 193, 175, and 23 bp.

The bands were visualized as described in section 2.2.4.1.2.2.

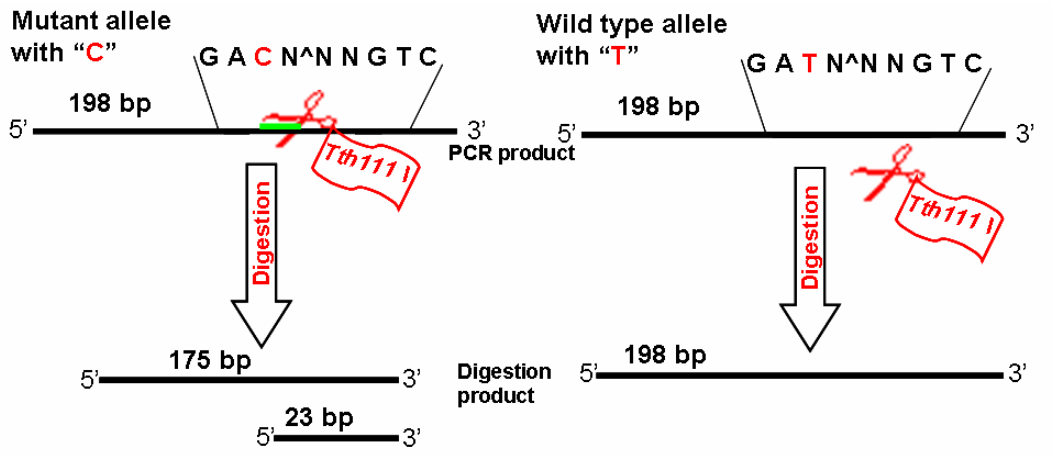


Figure 2.9 Schematic representation of the banding patterns of the amplified exon 3 region of *EPHX1* gene upon digestion with *Tth111*I restriction enzyme. The left handside shows the banding pattern for mutant allele and right handside shows the banding pattern for wild type allele.

2.2.4.3.2 Genotyping for Exon 4 (His139Arg) Polymorphism

2.2.4.3.2.1 Polymerase Chain Reaction for Exon 4 Variant

A 210 bp fragment in the exon 4 region of the *EPHX1* gene that contains A/G SNP (causing a His139Arg replacement in the encoded protein) was amplified with forward primer EH4 F: 5'-GCCACTTCCAGAGGGCAGT ATCT-3' and reverse primer EH4 R: 5'-ATGCCTCTGAGAAGCCAT-3' (primer sequences were taken from Smith and Harrison, 1997). The sequence of the amplified fragment, location of the exon 4 A/G SNP and sequence of the recognition site for the restriction enzyme were given in Figure 2.10. In order to obtain a single band devoid of non-specific bands, different primer (10 and 20 pmols) and $MgCl_2$ (1.00, 1.25, 1.50, 1.75, 2.00) concentrations were tested. The optimized PCR conditions as given in Table 2.11. Briefly, PCR reaction was carried out in 50 µl of a solution containing amplification buffer (supplied commercially with the Taq polymerase enzyme: 20 mM Tris HCl, 50 mM KCl; pH 8.5), 1.25 mM $MgCl_2$, 1mM dNTPs, 0.4 µM (20 pmol in 50 µl reaction mixture) of forward and reverse primers, 200 µM dNTP approximately 200 ng of genomic DNA, and 2.5 U of Taq polymerase.

The PCR program used for the amplification of exon 3 region consist of an initial denaturation step at 95 °C for 5 min followed by 35 cycles of denaturating at 95 °C fo 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min (Korhonen *et al*., 2003).

Amplification programme used was as follows:

Initial denaturation	95°C	5 min	
Denaturation	95°C	1 min	35 cycles
Annealing	55°C	1 min	
Extension	72°C	1 min	

The bands were visualized as described in section 2.2.4.1.1.

Table 2.11 Components of PCR mixture for exon 4 polymorphisms of *EPHX1* gene (Tyr113His).

Constituent	**Stock Conc.**	**Volume added**	**Final Conc. in 50 µl reaction mixture**
Amplification buffer	10x	5 µL	1x
$MgCl_2$	25 mM	2.5 µL	1.25 mM
dNTP mixture*	10 mM	1 µL	200 µM
Reverse Primer	10 pmol/µL	2 µL	20 pmol
Forward Primer	10 pmol/µL	2 µL	20 pmol
Template DNA	varies	varies	~200 ng
Taq DNA Polymerase*	5 U/µL	0.5 µL	2.5 U
Sterile apyrogen H_2O		To 50 µL	
***** see Appendix B for these reagents.			

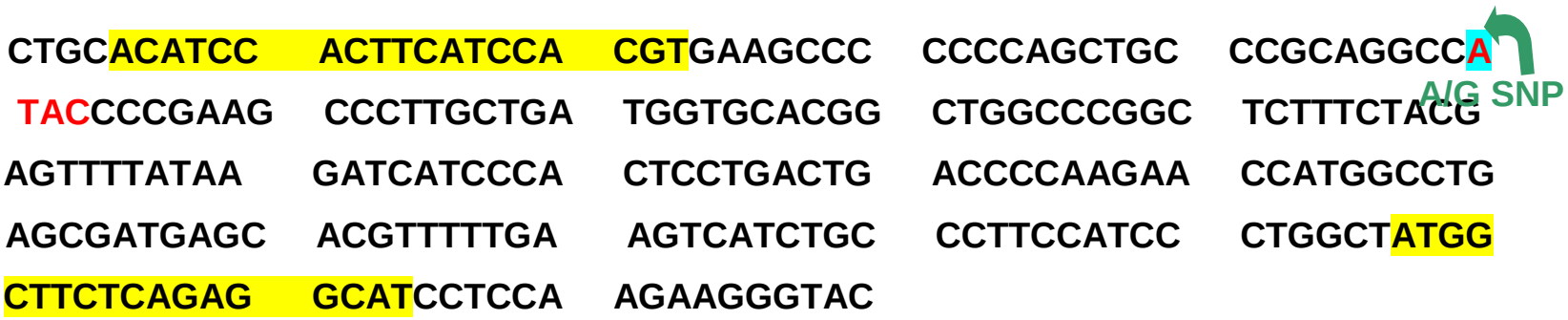


Figure 2.10 Sequence of amplified fragment in exon 4 region of *EPHX1* gene that includes A/G SNP. The yellow highlighted sequences are forward and reverse primers. Red marked sequences shows the recognition site for the restriction enzyme *Rsa*I. Turquoise highlighted nucleotide shows the location of A/G SNP (The nucleotide sequence was taken from http://www.ncbi.nlm.nih.gov).

2.2.4.3.2.2 Restriction Endonuclease Digestion of PCR Products for Exon 4 Polymorphisms

Genotyping for exon 3 polymorphisms of *EPHX1* gene was carried out by digesting 20 µl of PCR product with 3U of *Rsa*I enzyme in a final volume of 30 µl reaction mixture. The componenets of the reaction mixture were given in Table 2.12.

Table 2.12 Components of digestion mixture for exon 4 polymorphisms of *EPHX1* gene.

Constituent	**Stock Conc.**	**Volume added**	**Final Conc. in 30 µl reaction mixture**
Buffer*	10x	3 µL	1x
PCR Product		20 µL	
Restriction enzyme	10 U/µL	0.3 µL	3 U
Sterile apyrogen H_2O		6.7 µL	
***** Tango Buffer ™ (see Appendix B)			

The reaction mixture was incubated overnight at 37℃ for 18 hours for complete digestion. The mutant type allele which contains G instead of A bears a recognition sites around that nucleotide for *Rsa*I restriction enzyme. Therefore mutant allele provides restriction enzyme to cut the product from that position so *Rsa*I digestion results in 2 bands of 164 and 46 bp for the mutant alleles (A/G mutation). However in wild type alleles with A, the recognition site of the restriction enzyme is lost, thus *Rsa*I can not cut the amplified fragment. Figure 2.11 shows the basis of *Rsa*I

digestion and represents the resulting fragments upon digestion in case of different genotypes. Accordingly, the expected banding patterns upon digestion of amplified region in exon 4 of *EPHX1* gene with *Rsa*I restriction enzyme were as follows: In homozygous mutant types, presence of recognition sequences would yield two bands of 164 and 46 bp, while in homozygous wild type, as recognition sequence around SNP is lost due to base substitution (A/G), single band (210 bp) is expected. The heterozygotes would contain in total 3 bands in lenghts of 210, 164, and 46 bp.

The bands were visualized as described in section 2.2.4.1.2.2.

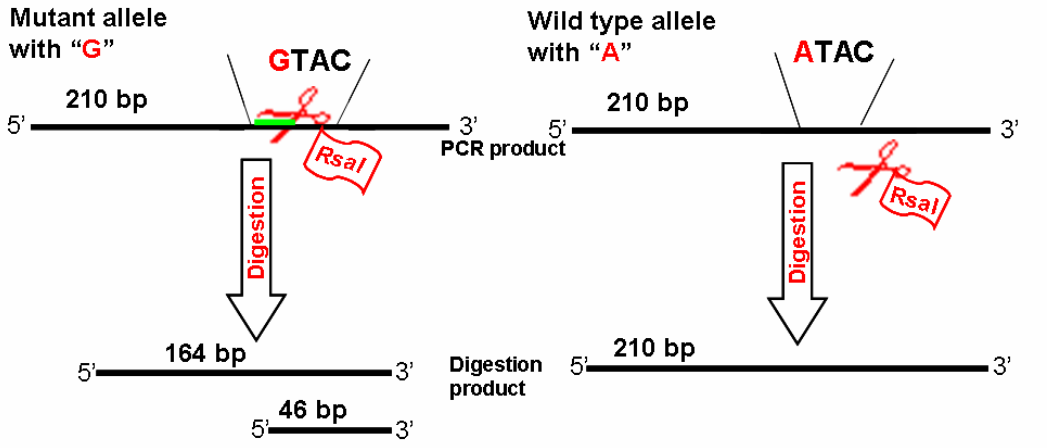


Figure 2.11 Schematic representation of the banding patterns of the amplified exon 4 region of *EPHX1* gene upon digestion with *Rsa*I restriction enzyme. The left handside shows the banding pattern for mutant allele and right handside shows the banding pattern for wild type allele.

2.2.5 Determination of Thiopurine methyltransferase Activity

2.2.5.1 Preparation of Red Blood Cell Hemolysate

Two milliliter whole blood, collected to EDTA containing tube, was centrifuged at 1400 g for 5 minutes at room temperature by using Sorvall RC-5C Plus high speed centrifuge using SS30 rotor with glass tube adapters. After centrifugation, supernatant (plasma portion) and buffy coat (leukocytes) were discarded. The pellet, red blood cell containing suspension, was washed two times in five volumes of 0.9% NaCl solution and centrifuged at 1400 g for 5 minutes in order to remove leukocytes. After each centrifugation the supernatant containing the remaining plasma and buffy caot was discarded. Then one drop of pellet was taken and put into a microscope slide. By the help of other slide, a blood smear was prepared and observed under light microscope. If no leukocyte were visible by light microscopy after the second saline washing, the erythrocytes were lysed by incubating the pellet with an equal volume of cold distilled water for one hour at 4 ℃. If leuko cytes observed under the light microscopy, then washing steps were repeated until get rid of all the leukocytes. The hemolysate was then dialysed overnight against 20 mM phosphate buffer, pH 7.4, with 2mM EDTA. Figure 2.12 demonstrates the preparation of human red blood cell hemolysate.

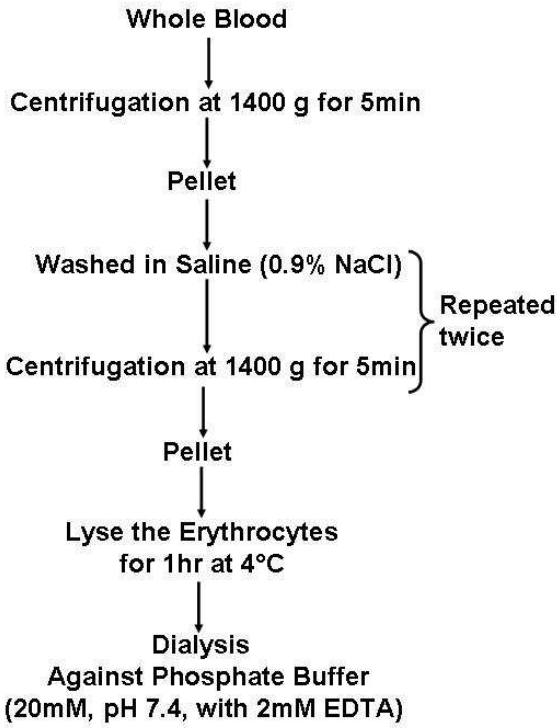


Figure 2.12 The preparation of human red blood cell hemolysate.

2.2.5.2 Determination of Haemoglobin Content

The haemoglobin content of the hemolysate was determined by using total Haemoglobin Kit (Spinreact, Spain). The Haemoglobin determination kit was commercially supplied with a concentrated working reagent (Reference No: 1001230) and ready to use standart solution (Reference No: 1001232). Before using, the working reagent was diluted by mixing 0.1 concentrated reagent with 4.9 ml of distilled water. 10 µl of hemolysate were mixed by 2.5 ml of diluted working reagent. Similarly, 10 µl of standard solution (ready to use) was mixed by 2.5 ml of diluted working reagent. Both standard and sample mixtures allowed standing 3 minutes at room temperature and absorbances were recorded at 540 nm

against blank reagent which only contained 2.5 ml of diluted working reagent. Calculation of the haemoglobin content of the red blood cell lysate was done according to the formula given below.

g/dL Haemoglobin = (OD_{540} for Sample/OD_{540} for Standard) x 15

2.2.5.3 Measurement of the TPMT Activity

Red blood cell TPMT activity was measured by a non-radiochemical modification (modified by Khalil *et al*., 2005) of the Weinshilboum radiochemical methods (Weinshilboum *et al*., 1978). The assay is based on TPMT-catalyzed conversion of 6-mercaptopurine (6MP) to 6-methylmercaptopurine (6MMP) using SAM as methyl donor and direct measurement of product (6MMP) by HPLC. In Figure 2.13, the TPMT catalyzed reaction was given.

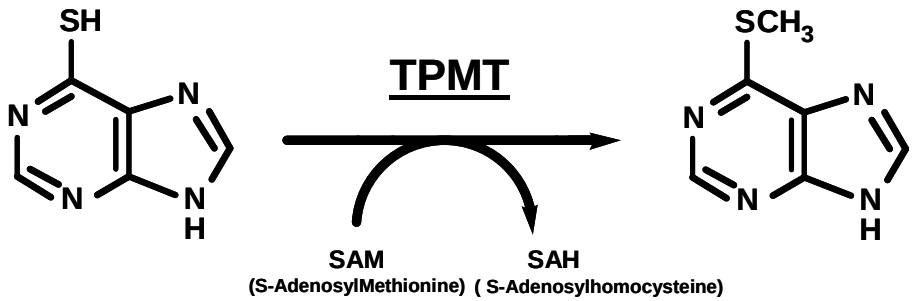


Figure 2.13 The conversion of 6MP to 6MMP in the presence of cofactor SAM by TPMT catalyzed reaction.

A typical assay mixture used for the determination of TPMT activity was given in Table 2.13. The final volume of the assay mixture was 500 µl. Twenty µl 0.1 M of HCl were diluted with 315 µl of 0.067M phosphate buffer (pH 7.4) in a 1.5 ml eppendorf tube and then 20 µl of the stock solution of 6-MP and SAM were added (resulting in final concentrations of 600 µM for 6MP and 120 µM for SAM). The enzymatic reaction was started by adding 100 µl of the prepared RBC lysate. The reaction mixture was incubated at 37 ℃ for 1 hour. Following incuba tion the reaction was terminated by adding 25 µl 60% $HClO_4$. The remnant was centrifuged for 4 ℃ min at 13,000 ×*g* after a protein precipitation of 3 min. Finally the clear supernatant was analysed by HPLC without further pretreatment.

Table 2.13 The constituents of TPMT assay mixture.

Constituents	**Stock Solutions**	**Volume to be added (µl)**	**Final concentration in 500 µl incubation mixture**
Phosphate Buffer pH 7.4	0.067 M	315 µl	0.042 M
HCl	0.1 M	20 µl	0.004 M
6MP*	15 mM	200 µl	0.60 mM
SAM**	3.0 mM	20 µl	0.12 mM

* prepared in 0.1N NaOH
** prepared in 0.067 M phophate buffer pH 7.4.

2.2.5.4 HPLC Analyses

In the current study, some important parameters for HPLC analysis to determine TPMT activity were investigated. Briefly, the retention times for substrate and product were determined. Standard calibration curve was constructed. Optimization studies were carried out by using two samples. HPLC analyses were performed at Central Research Lab, Molecular Biology and Biotechnology Research Center of Middle East Technical University. The injections and software recordings were carried out by a technical expert. The HPLC sytem (VARIAN, Prostar HPLC, USA), is consisted of a pump (VARIAN Prostar 240 Model Pump), an autosampler (Prostar 410 autosampler) with microvials (25 µl), and a PDA detector (Prostar 330 PDA). The separation was performed at room temperature on a column using Microsorb-MV 100, C18, particle size 5 µM (VARIAN, USA). The mobile phase and flow rate of the column were arranged according to Khalil *et al*., (2005). Accordingly, the flow rate was 0.5ml/min. The mobile phase consisted of 0.01 M sodium phosphate buffer adjusted to pH 2.7 with phosphoric acid (85%). For the elution of product (6MMP) 7% acetonitrile was added to the mobile phase. The 6MMP was detected at 290 nm where it gives maximum absorption and 6MP was detected at 322 nm where it gives maximum absorption.

2.2.5.5 Determination of Retention Times for 6MP and 6MMP

In this analyses, first the retention time for 6MMP and 6MP have to be determined since the column material that was used in the present study was very different from the column material used in the other reports found in the literature. For the determination of retention time for 6MMP, a solution that contains 3mM 6MMP (sufficiently high amount for clear detection) were injected to the column. The absorbance was measured at 290 nm. The solution also supplemented with phosphate

buffer (pH 7.4), $HClO_4$ and NaOH (solvent for 6MMP) at the same final concentrations found in the enzyme assay mixture. As represented in Figure 2.14 the 6MMP gave a peak at 27.2 min. In addition, 6MMP was trying to dissolve in water. Although it is not dissolved completely, the solution was filtrated to get rid of undissolved 6MMP particles and injected into the column. As represented in Figure 2.15, this injection also gave a peak around 27. min. Therefore it was determined that the retention time for 6MMP in the column condition used in the present study was around 27 min. To see the effects of solution the same concentration of blanks for each component (only $HCLO_4$, only NaOH and only phosphate buffer, pH 7.4) were prepared and injected into the column. As represented in Figure 2.16, each component also gave some small peaks around 27. min. The retention time for 6MMP was also confirmed by injecting the solution of 6MMP which was dissolved in mobile phase (see Figure 2.16).

The retention time for 6MP was also determined. As demonstrated in Figure 2.17, 3mM 6MP solution which is supplemented by phosphate buffer (pH 7.4), $HClO_4$ and NaOH (solvent for 6MMP) at the same final concentrations found in the enzyme assay mixture, injected into the column. The absorbance of 6MP was measured at both 290 and 322 nm. However, 6MP gave maximum absorption at 322 nm as can be seen in the Figure 2.17. The retention time for 6MP was determined as 18.3 min

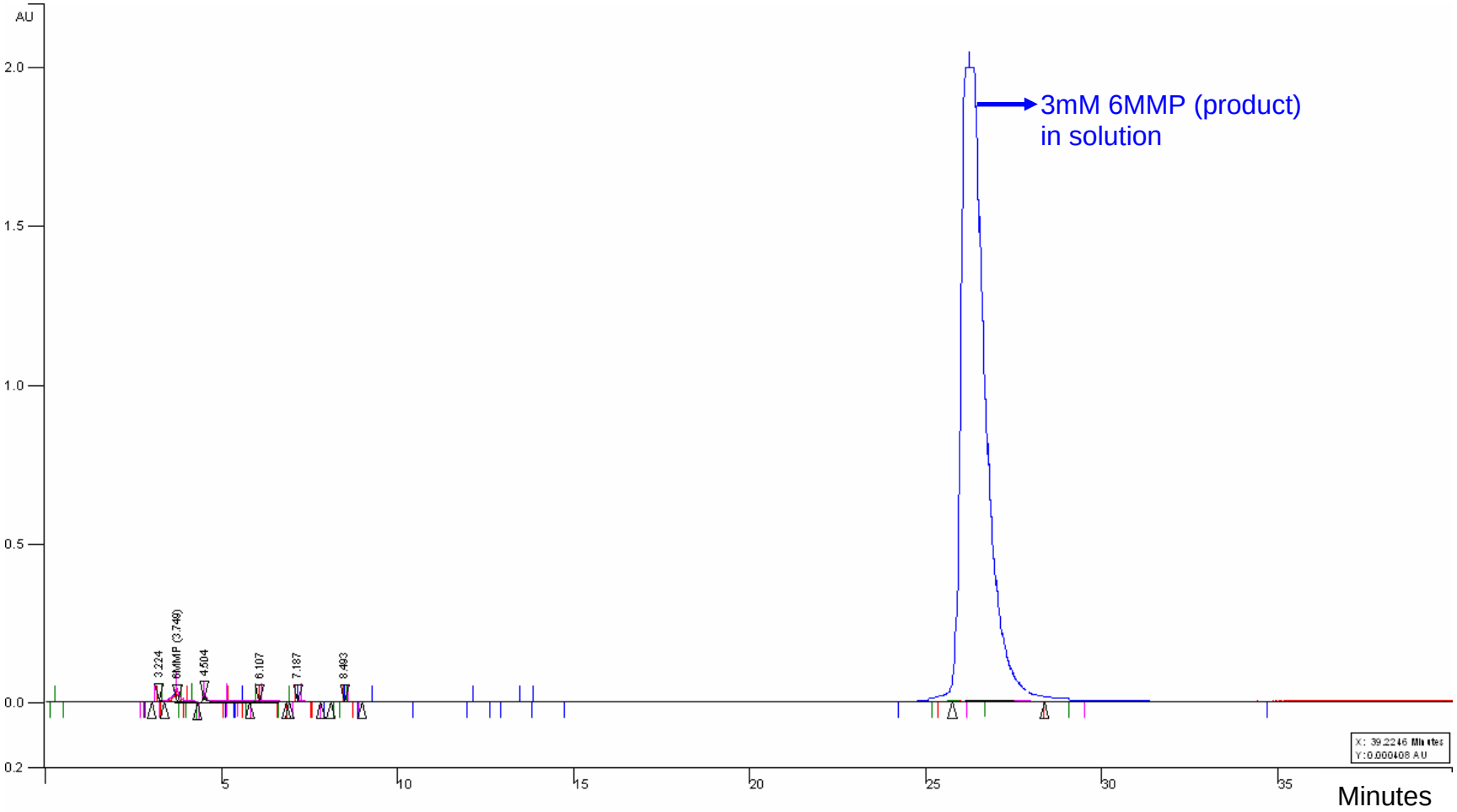


Figure 2.14 Identification of the retention time for 6MMP (1).

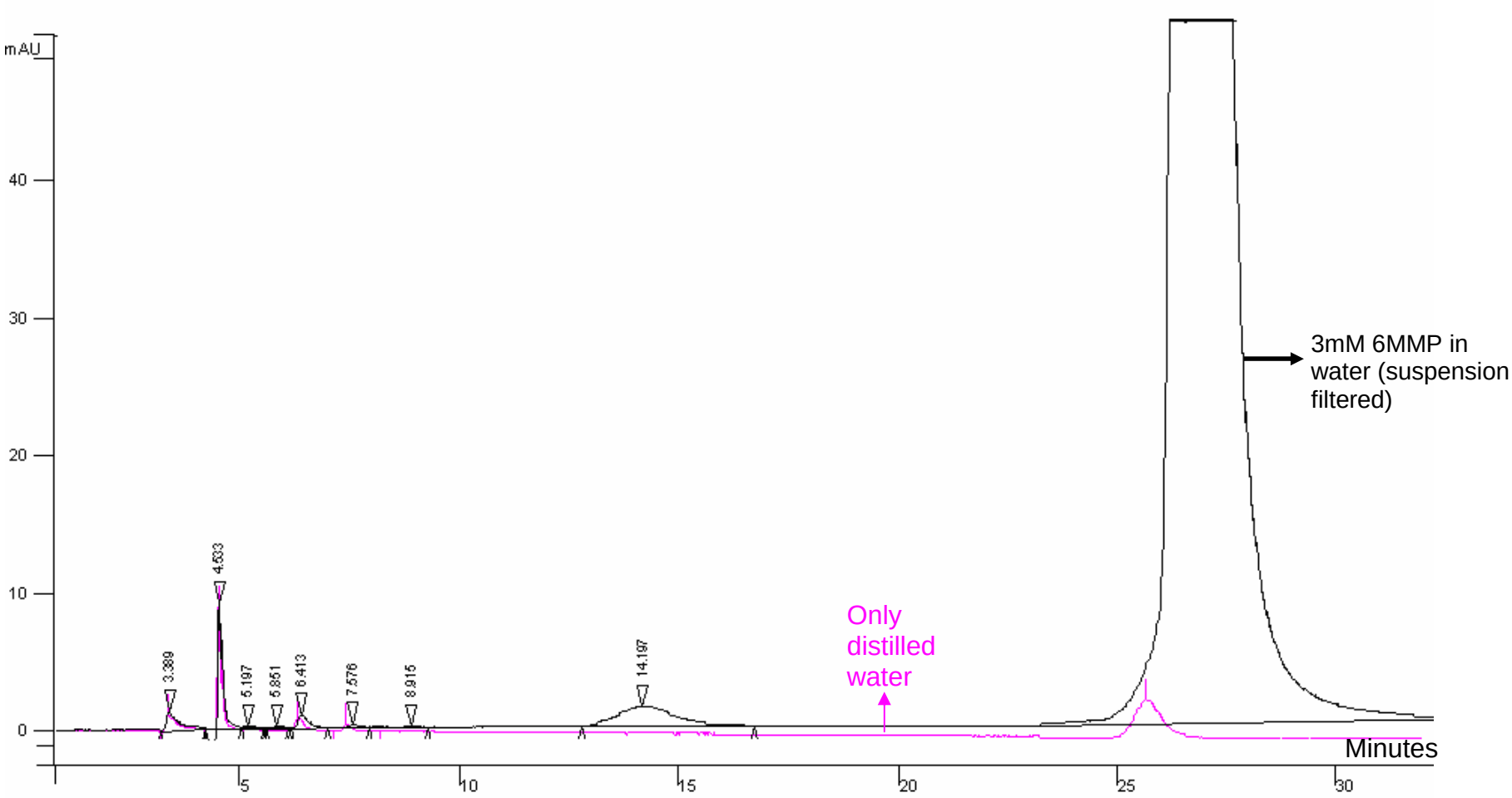


Figure 2.15 Confirmation of retention time for 6MMP.

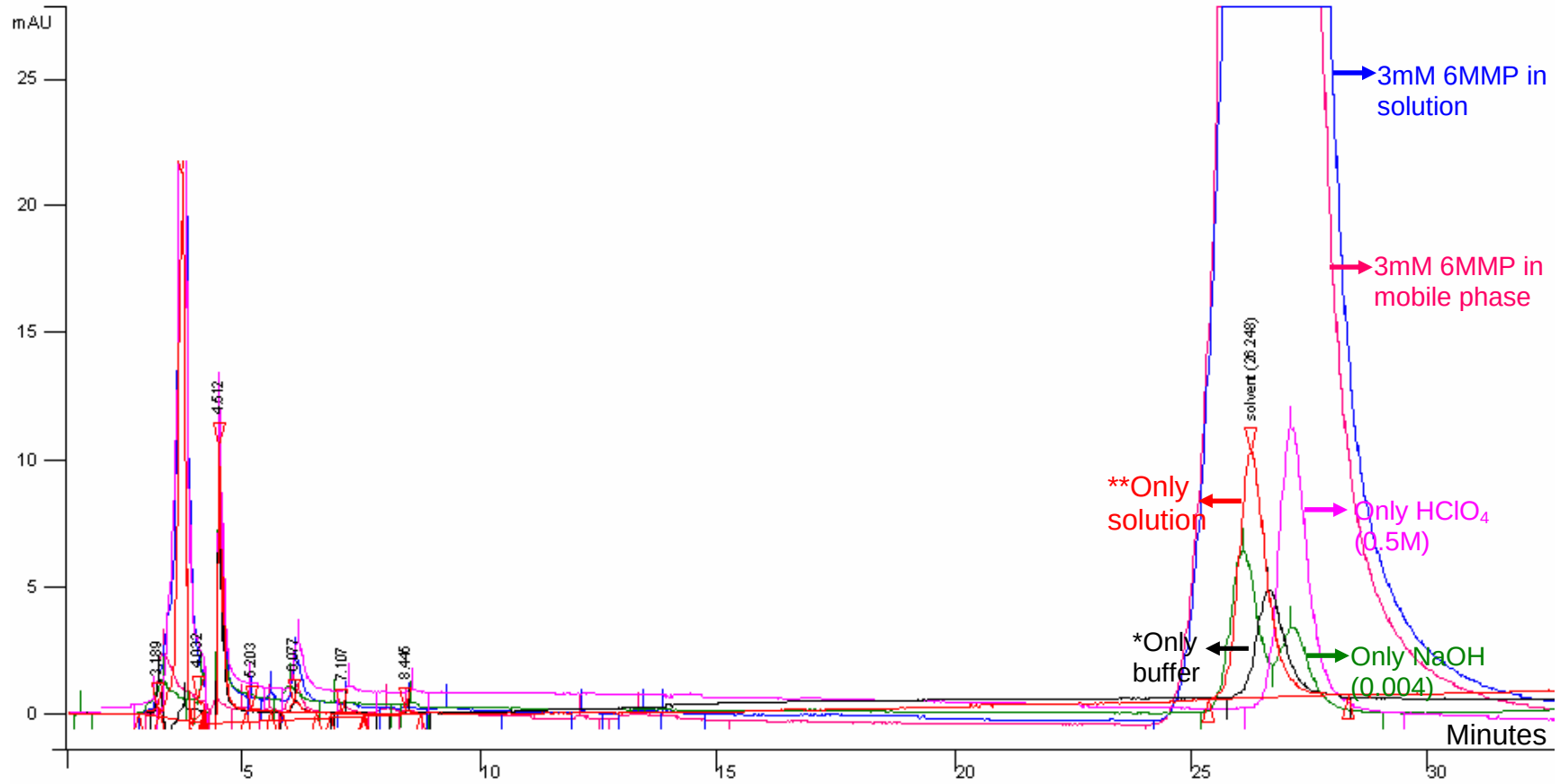


Figure 2.16 Identification of retention time for 6MMP (2). *Only buffer: 0.042 M potassium phosphate buffer pH 7.2. **Only solution: contains, 0.004 N NaOH, 0.5 M $HClO_4$, 0.042 M potassium phosphate buffer pH 7.2.

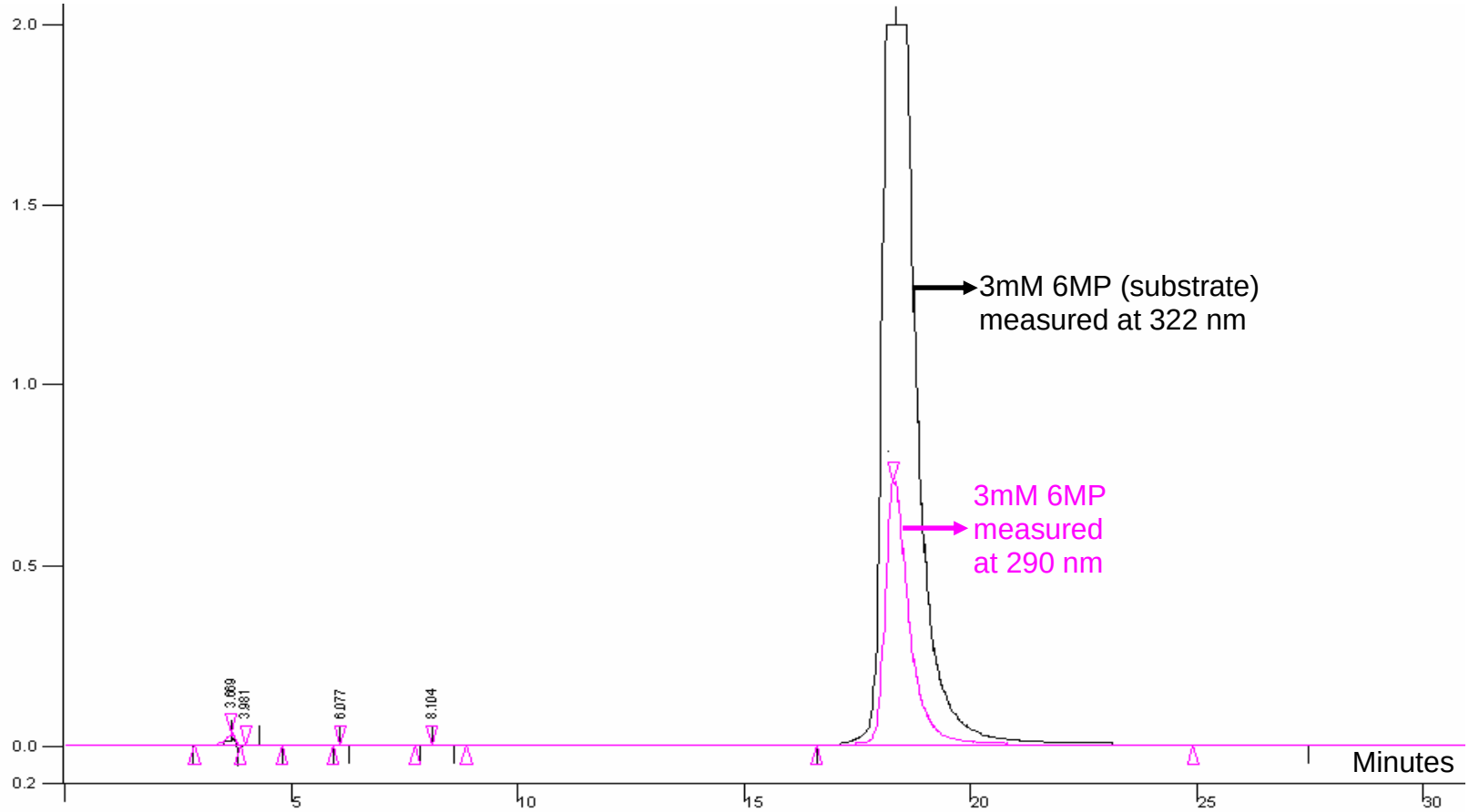


Figure 2.17 Identification of retention time for 6MP.

2.2.5.6 Construction of Standard Calibration Curve

6MMP was used as standard. Different concentrations of 6MMP solution were prepared from 0.5mg/ml stock 6MMP solution (in 0.1 N NaOH) by serial dilutions (dilutions were made by dH_2O). Seven different concentrations (25, 50, 70, 100, 140, 200 and 500 ng /ml) of 6MMP were tried. The retention time and area under curve (peak area) for each concentration of standards were given in Table 2.14. The standard calibration curve of 6MMP was given in Figure 2.18. As can be seen from figure, a linear relationship between the peak areas and 6MMP concentration was obtained over the range of 25 to 500 ng/ml.

Table 2.14 The retention time and peak areas for different standard concentration of 6MMP.

Final Concentration of 6-MMP (ng/ml)	Retention Time	Peak Areas
25	27.6	16133
50	27.5	45434
70	27.9	78034
100	27.2	136115
140	27.0	180973
200	27.0	283108
500	27.7	616700

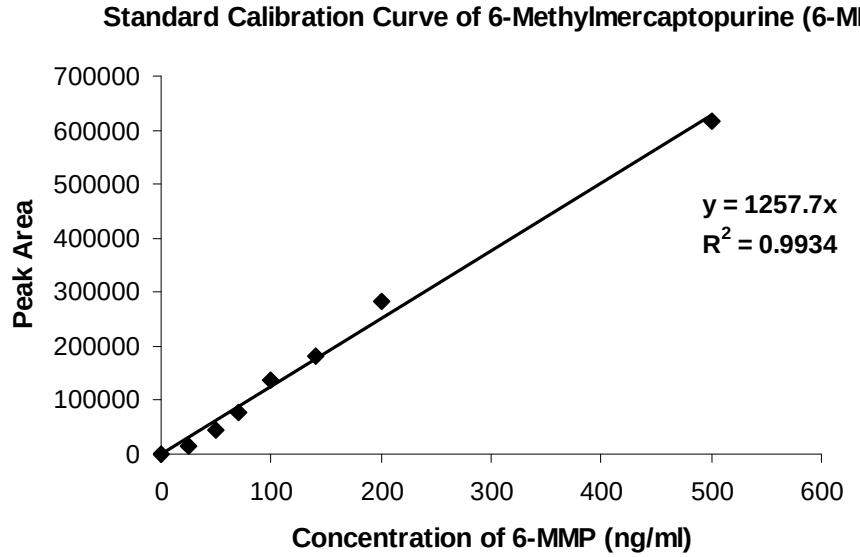


Figure 2.18 Standard calibration curve for TPMT enzyme activity determination

2.2.5.7 Determination of Enzyme Activity

TPMT enzyme activity assay was performed as described in section 2.2.5.3. The 25 µl of enzymatic assay mixture after extraction by $HCL0_4$ was injected into the column. The mobile phase contained 0.01 M sodium phosphate buffer adjusted to pH 2.7 with phosphoric acid (85%) and 7% acetonitrile. The flow rate was 0.5ml/min. A blank solution was prepared and extracted with the same manner as enzymatic assay mixture to see the effects of some components found in the assay mixture. Blank solution contains everything in the assay mixture except 6MP and SAM (RBC incubated without substrate and co-substrate) to eliminate the effects of solutions found in the assay mixture (NaOH, potassium phosphate buffer and HCl and $HClO_4$). As represented in Figure 2.16 each of these components gave small peaks around the retention time 6MMP for this reason effects coming from this solutions should be eliminated. Figure 2.19 demonstrated the peaks for blank,

enzymatic assay mixture and the standard 6MMP (single concentration was used to verify the peak coming from 6MMP). The same concentration of standard was prepared twice and injected into the coloumn twice. As can be seen from the Figure 2.19, the enzymatic assay mixture also gave a product peak around the standard (6MMP-product) peak. Quantitation was carried out from the 6MMP (product) peak area relative to the standard calibration curve represented at Figure 2.18.

At the begining of this study phenotyping of the patients had been planned. With this aim the enzyme assay and colon condition for HPLC were studied extensively and the major conditions (the identification of the each compound found in the enzymatic assay mixture based on retention time, construction of standard calibration curve) were optimized as described above. However, assessment of TPMT activity in patient group has significant limitations due to several reason. One major problem is that measurement of TPMT activity in RBC isolates can be spurious if patients have received RBC transfusion within the previous 60-90 days. This is not uncommon for ALL patients during treatment with chemotherapetic agents, even at the time of diagnosis and represents a serious limitation of assessing TPMT phenotype on the basis of TPMT activity (Relling *et al*., 1999a). Another problem related with phenotyping of the patients is that TPMT activity can be induced by 6MP within 3 months after the start of therapy in children with ALL (Mc Leod *et al*., 1995b). Besides, TPMT activity can be inhibited by common medications such as salicylic acid derivatives, furosemide and sulfasalazine (Lennard, 1998). Regarding these facts, phenotyping of the patients can only give accurate results in newly diagnosed patients who should not take RBC transfusion within 2 months. It is understood that enzyme activities of the patients can only be determined for the newly diagnosed patients.

Optimization of the HPLC conditions for the measurement of TPMT enzyme activity took about six months. However, during the six months, we did not able to get any blood samples from the newly diagnosed patients. For this reason we were not able to continue TPMT activity measurement.

In the context of current study, the main parameters for measuring TPMT activity were set for the HPLC coloumn conditions. In the future studies, these optimized conditions can be used for determining TPMT enzyme activity.

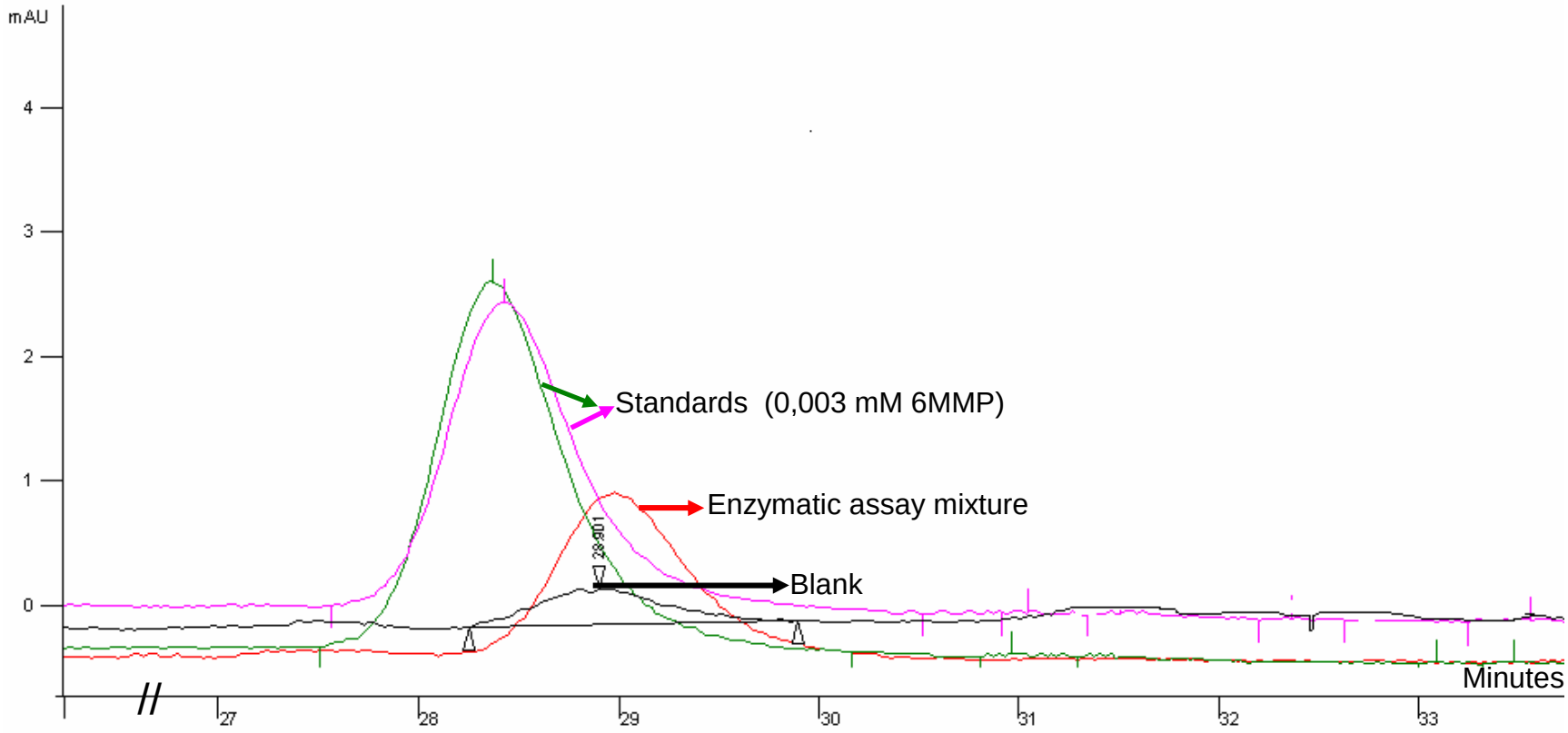


Figure 2.19 Enzymatic assay mixture, standard and blank for TPMT activity.

2.2.6 Statistical Analyses

The frequency of each variant allele was evaluated by gene counting. The allele and genotype frequencies of patients were given together with the 95% confidence interval (CI). Statistical analyses were conducted using SPSS 16.0 software or by Vassar Stats. The genotype distributions of polymorphisms were compared by χ^2-test (Pearson), Yate's correction factor was used if necessary, or Fisher's exact probability test was applied when the sample number is small so that Pearson's or Yate's χ^2 analysis could not be performed. Differences in allele frequency between Turkish population and other ethnic populations were analyzed by using Chi-square test. A *p* value <0.05 was considered to be statistically significant throughout the population comparison.

Comparison of genotypes in control and patient groups was done by calculating odds ratio (OR). The significance of association was determined by χ^2-test. For case-only studies, interaction of non-genetic factors and genetic polymorphisms for the risk of development of childhood ALL was analyzed by determining case-only odds ratio (COR) values and the significance was determined by χ^2-test.

CHAPTER 3

RESULTS

3.1 Study Populations

3.1.1 Control Population

Control population comprised of 206 healthy Turkish volunteers between the ages of 12 and 65 and mean age of 31.3 ± 12.1. Among the control subjects, 82 were male (mean age: 33.0 ± 12.3; range: 14-65 years) and 124 were female (mean age: 30.3 ± 11.4; range: 12-63 years). Subjects having diseases –like any type of cancer, diabetes etc.- were excluded from the study. The blood samples were collected from Middle East Technical Universtiy (METU) Health Center, Biochemistry Laboratory. According to the information obtained from the birth place of volunteer and his/her parents, all the regions of Turkey were equally represented by the study population. It could be concluded that control population is a sample of Turkish population. The distribution of control subjects according to their birth place was given in Figure 3.1.

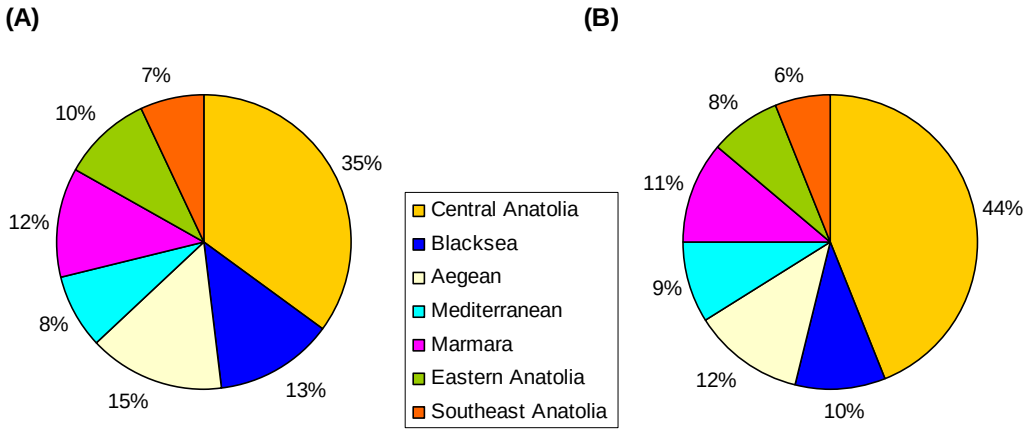


Figure 3.1 Distribution of control subjects to seven regions of Turkey, according to A) the birth place of parents of the subjects B) the birth place of subject

3.1.2 Patient Population

A total of 167 children (65 female and 102 male with the mean age at diagnosis being 7.0 ± 3.8 years, range 0.6-16 years) who were treated in Ankara University, Faculty of Medicine, Department of Pediatric Hematology and Sami Ulus Children's Hospital were included in this study. These two hospitals are one of the most crowded health centers of the Turkey and receive patients all over the country. For this reason it could be said that study population represents Turkish population. This information was also proven by the demographic data of the samples. The distribution of patient subjects according to their birth place was given in Figure 3.2.

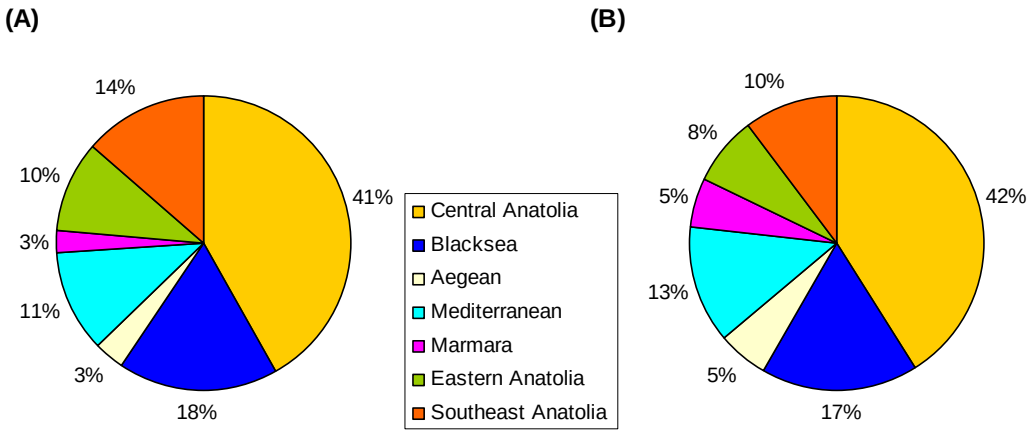


Figure 3.2 Distribution of patient subjects to seven regions of Turkey, according to A) the birth place of parents of the subjects B) the birth place of subject.

The characteristics of the patient population, derived from the informed consent, questionnaire for parent's smoking status (see Apendix A) and the patients' medical log were summarized in Table 3.1 and below within the text. However, data was not available for all patients. Besides, questionnaires on the cigarette smoking status of the parents were not filled out by all parents.

The risk group information (low, standard or high risk), which was assigned at the time of diagnosis to determine the intensity of the therapy, was available for 159 patients (information on 8 patients were missing). In total, 17 (10.7%) patients were assigned to low-risk group, 62 (40.0%) patients were assigned to standard risk group and 80 (50.3%) were assigned to high risk group.

Table 3.1 (A) Characteristics of the patient population (B) Information obtained from cigarette smoking questionnairre

(A)

Characteristic	Total Patients
N	167
Age at diagnosis	7.0 ± 3.8 (range: 0.6-16)
Risk Group	159 (8 mising)
Low Risk	17 (10.7%)
Standard Risk	62 (40.0%)
High Risk	80 (50.3%)
Treatment protocols	152 (15 missing)
Saint Jude XIII	128 (84.2%)
Saint Jude XI	10 (6.6%)
CCG1952	7 (4.6%)
CCG1961	7 (4.6%)

(B)

Information obtained from cigarette smoking questionnairre		
Maternal age at conception	**Total Patients**	
	105 (62 missing)	
Mean age	26.8 ± 6.9	
<20 years of age	10 (9.5%)	
20-35 years of age	78 (74.3%)	
>35 years of age	17 (16.2%)	
Paternal age at conception	**Total Patients**	
	N=102 (65 missing)	
Mean age	30.6 ± 9.0	
<40 years of age	87 (85.3%)	
>40 years of age	15 (14.7%)	
Parental Smoking Status	**Maternal**	**Paternal**
N	106	106
	(61 missing)	(61 missing)
Non-smoker	27 (25.5%)	19 (17.9%)
Passive smoker	56 (52.8%)	5 (4.7%)
Active smoker	23 (21.7%)	82 (77.4%)
Maternal smoking during pregnancy	**Total Patients**	
	106 (61 missing)	
Smoked	12 (11.0%)	
Not smoked	94 (89.0%)	
Postnatal exposure of child to cigarette smoke	**Total Patients**	
	105 (62 missing)	
Exposed	73 (69.5%)	
Not exposed	32 (30.5%)	

The 152 chidren were treated on the following treatment protocols: Saint Jude XIII, 128 (84.2%); Saint Jude XI, 10 (6.6%); CCG1952, 7 (4.6%); CCG1961, 7 (4.6%). The treatment protocols for 15 children were missing. All of the treatment protocols included a maintenance phase of the therapy with daily oral 6MP intake.

The information related with the age at conception was available for 105 mothers and 102 fathers. The mother's mean age at conception was 26.8 ± 6.9 and father's mean age at conception was 30.6±9. Among the mothers, 17 (16.2%) of them were older than 35 years of age, 78 (74.3%) of them were at the ages between 20 and 35 at the time of conception, and 10 (9.5%) of them were younger than 20 years of age. Among the fathers, while 87 (85.3%) of them were younger than 40 years of age at conception, 15 (14.7%) of them were older than 40 years of age at conception.

Smoking status information was available for 106 parents. Among 106 mothers, 27 (25.5%) of them were non smokers, 56 (52.8%) of them were passive smokers and 23 (21.7%) of them were active smokers. Among 106 fathers, 19 (17.9%) of them were non smokers, 5 (4.7%) of them were passive smokers and 82 (77.4%) of them were active smokers. 94 (89%) of mothers did not smoke and 12 (11%) of them had smoked during pregnancy. Information related with the postnatal exposure of child to cigarette smoke was available for 105 children, 32 (30.5%) children were not exposed and 73 (69.5%) were exposed to cigarette smoke postnatally.

As stated before, the major focus of the present work was the investigation of TPMT enzyme polymorphisms in Turkish population comprised pediatric ALL patients and healthy adult controls and also retrospective investigation of patients with TPMT defects from hospital

records in relation to 6MP related side effects. Additionally, in the scope of present study, the genetic polymorphisms of two drug metabolizing enzymes-SULT1A1 and EPHX1- which have dual role in both activation and detoxification of various environmental carcinogens, were investigated as risk modifier for the development of childhood ALL. Related with this part of the present study (risk assessment part), some non genetic risk factors -parental age at conception and cigarette smoking exposure- were investigated to evaluate their interactions with genetic polymorphisms of SULT1A1 and EPHX1 genes. These analyses were carried out by using case-only approach as the information related with non-genetic factors was only available for patient group. However, the genotyping of single nucleotide polymorphisms for all DMEs (TPMT, SULT1A1 and EPHX1) included in this study were done for all 206 control and 167 patient sample.

3.2 *TPMT* Genotyping

In the scope of this part, a total of 167 children with ALL and 206 healthy control were genotyped for the most common defective *TPMT* alleles which were *TPMT*2, *3B, *3C, *3A.*

3.2.1 *TPMT* Genotyping in the Patient Group

Allele specific PCR was performed to detect *TPMT*2* (G238C) genotypes. As represented in Figure 3.3 (lanes 1, 2), PCR products yielded homozygous wild type genotype for all individuals genotyped however, no heterozygous and homozygous mutated genotypes were observed in the sample population.

In order to determine *TPMT*3B* (G460A), *TPMT*3C* (A719G) and *TPMT*3A* (G460A and A719G together) genotypes, PCR-RFLP method

was performed. The PCR products covering G460A point mutation were digested with M*wo*I restriction enzyme and yielded homozygous wild type (lane 4) and heterozygous (lanes 5, 6) genotype as shown in Figure 3.3. The PCR product involving A719G point mutation were digested with *Acc*I restriction enzyme and resulted in homozygous wild type (lane 8), heterozygous (lanes 9, 10) and homozygous mutated genotypes (lane 11) as seen in Figure 3.3.

Table 3.2 summarizes the genotypes detected in the study sample. Accordingly, two people were found to be heterozygous in terms *TPMT*3C* and *TPMT*3A* alleles with genotype of *TPMT*1/*3C* (0.6%), (Patient's ID: 62) and *TPMT*1/*3A* (0.6%), (Patient's ID: 74). One patient was found to be compound heterozygous mutant (0.6%), (Patient's ID: 81) carrying both **3A and *3C* defective alleles (*TPMT*3A/*3C*). In the scope of this work, the clinical histories of these patients (patients with defective *TPMT* alleles) were investigated from patient's medical logs in order to examine the relationship between *TPMT* genotypes and 6MPrelated side effects. The results of these observations will be handled in detailed in the discussion part.

Overall, mutant *TPMT* alleles were found in 1.8% of the evaluated patients subjects. 164 patients (97.3%) were apparently homozygous for the wild type allele (*TPMT*1/*1*) i.e., they did not carry any of the tested polymorphisms (*TPMT*2, *3B,*3C, *3A*). In the patient group **2* allele was not detected in any of the sample.

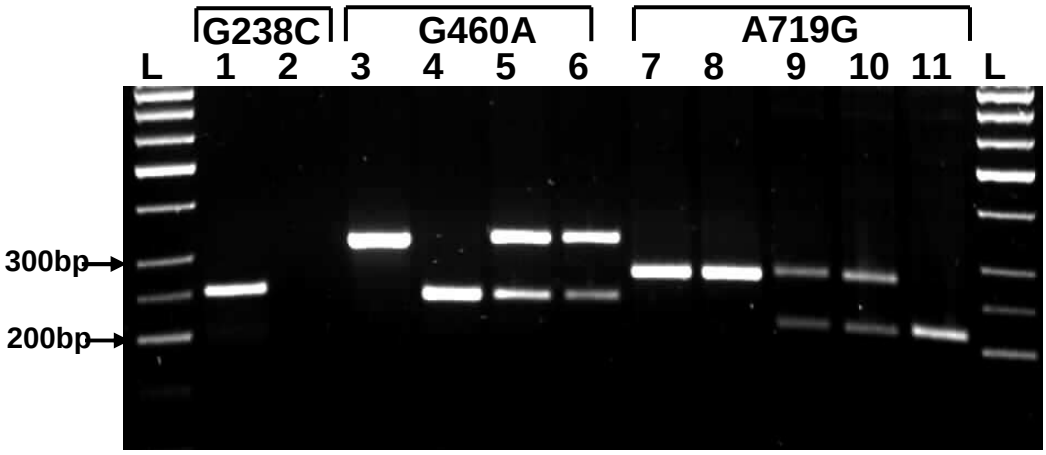


Figure 3.3 Electrophoresis patterns for *TPMT* alleles analyzed by PCR based methods. L: 50 bp DNA ladder. **Lanes 1-2:** Electrophoretic pattern of G238C point mutation analyzed by allele specific PCR with wild type primer pair (P2FW+P2R), (lane 1) and mutated primer pair (P2FM+P2R), (lane 2). Patient analyzed in lane 1 and 2 was homozygous wild type as amplification occurred only with wild type primer pair. **Lanes 3-6:** PCR-RFLP analyses of G460 point mutation; the amplified region (317 bp) bears a *Mwo*I recognition site in case of wild type allele and yielded fragments of 240 and 77 bp upon digestion. Lane 3, uncut PCR product, patient analyzed in lane 4 was homozygous wild type (Patient's ID: 62) and patients in lane 5 and 6 were heterozygous (Patient's ID: 73 and Patient's ID: 81, respectively). **Lanes 7-11:** PCR-RFLP analysis of A719G point mutation; the amplified region (293 bp) bears an *Acc*I recognition site in case of mutant allele and yielded fragments of 207 and 86 bp upon digestion. Lane 7, uncut PCR product, patient analyzed in lane 8 was homozygous wild type, patients in lane 9 and 10 were heterozygous (Patient's ID: 62 and Patient's ID: 73 respectively) and patient in lane 11 was homozygous mutant (Patient's ID: 81). These patients' ID match with those on Table 3.2.

Table 3.2 Genotype frequencies of *TPMT* variants in the patient group.

***TPMT* Genotype**	**Number of Subjects**	**%Genotype Frequency (95 % CI)**
**1/*1*	164	98.2 (94.84-99.63)
**1/*2*	0	0.0 (0.00-0.02)
**1/*3C*	1 (Patient's ID: 62)	0.6 (0.20-3.30)
**1/*3A*	1 (Patient's ID: 73)	0.6 (0.20-3.30)
**3C/*3A*	1 (Patient's ID: 81)	0.6 (0.20-3.30)
Mut/mut †	0	0.0 (0.00-0.02)

Patients' ID match with those on Figure 3.3.
†Represents homozygous mutant genotypes like *3C/*3C, *3A /*3A and *2/*2.

The allele frequencies of *TPMT* variants in the patient group were represented in Table 3.3. The *TPMT*3C* and **3A* were the only mutant alleles observed in patient group. The *TPMT*2* and **3B* alleles were not detected in any individual among the sample population. The allele frequencies for **3C* and **3A* in the patient group were determined as 0.6% thus the total frequency of mutant alleles in Turkish children with ALL was found to be 1.2%. In addition the wild type allele frequency was 98.8%.

Table 3.3 Frequencies of *TPMT* alleles in patient group.

Alleles	SNPs	No. of alleles	Frequency % (95% CI)
Total of Allele		334	
*TPMT*1*	Wild-Type	330	98.8 (96.96-99.67)
*TPMT*2*	G238C	0	0.0 (0.00-1.10)
*TPMT*3B*	G460A	0	0.0 (0.00-1.10)
*TPMT*3C*	A719G	2	0.6 (0.70-2.19)
*TPMT*3A*	G460A, A719G	2	0.6 (0.70-2.19)
Total mutant alleles		4	1.2 (0.33-3.04)

3.2.2 *TPMT* Genotyping in the Control Group

206 healthy controls were genotyped for *TPMT*2, *3B, *3C* and *3A* allele. Table 3.4 summarizes the genotypes detected in the study sample. Accordingly, two individuals were found to be heterozygous in terms of *TPMT*3C* allele with genotype of *TPMT*1/*3C* (1.0%). Two individuals were found to be heterozygous for *3A allele with genotype of *TPMT*1/*3A* (1.0%). Overall, mutant *TPMT* alleles were found in 1.9% of the evaluated subjects. 202 subjects (98.1%) were apparently homozygous for the wild type allele (*TPMT*1/*1*) i.e., they did not carry any of the tested polymorphisms (*TPMT*2, *3B,*3C, *3A*).

Table 3.4 Genotype frequencies of *TPMT* variants in the control group.

***TPMT* Genotype**	**Number of Subjects**	**%Genotype Frequency (95 % CI)**
**1/*1*	202	98.1 (95.10-99.47)
**1/*2*	0	0.0 (0.00-1.77)
**1/*3C*	2	1.0 (0.12-3.46)
**1/*3A*	2	1.0 (0.12-3.46)
Mut/mut †	0	0.0 (0.00-1.77)

†Represents mutant genotypes like *3C/*3C, *3A /*3A, *2/*2 or compound mutant like *3C/*2, *3A/*3C etc.

The allele frequencies of *TPMT* variants in the control group were represented in Table 3.5. Likewise the patient group, the *TPMT*3C* and **3A* were the only mutant alleles observed in control group. The *TPMT*2* and **3B* alleles were not detected in any of the healthy individual. The allele frequencies for **3C* and **3A* in the control population were determined as 0.5% thus the total frequency of mutant alleles was found to be 1.0%. In addition, the wild type allele frequency was 99.0%.

Table 3.5 Frequencies of *TPMT* alleles in the control group.

Alleles	**SNPs**	**No. of alleles**	**Frequency % (95% CI)**
Total of Allele		412	
*TPMT*1*	Wild-Type	408	99.0 (97.53-99.73)
*TPMT*2*	G238C	0	0.0 (0.00-0.89)
*TPMT*3B*	G460A	0	0.0 (0.00-0.89)
*TPMT*3C*	A719G	2	0.5 (0.06-1.74)
*TPMT*3A*	G460A, A719G	2	0.5 (0.06-1.74)
Total mutant alleles		4	1.0 (0.27-2.47)

3.2.3 *TPMT* Genotyping in Turkish Population

As represented in Table 3.6, when the allele frequencies of patient and control populations were compared by using two tailed Fischer's exact test, it was found that there was no significant difference between these two sample populations. Actually they were very similar. For this reason, to evaluate the allele frequencies of defective *TPMT* alleles for a sufficiently large Turkish population, control and patient populations were pooled. Accordingly, allele frequency for both **3A* and **3C* allele were found to be 0.5%. Neither **2* nor **3B* allele was detected in Turkish population. Overall, the total frequencies of defective TPMT alleles were found to be 1.0% in Turkish population. These data were summarized in Table 3.7.

Table 3.6 Comparison of *TPMT* allele frequencies between control and patient groups.

TPMT Alleles	**ALL patients (%)** [95%CI]	**Controls (%)** [95%CI]	***p*** †
**1 (Wild type)*	98.8 [96.96-99,67]	99.0 [97.53-99.73]	
**2*	0.0 [0.00-1.10]	0.0 [0.00-0.89]	
**3B*	0.0 [0.00-1.10]	0.0 [0.00-0.89]	
**3C*	0.6 [0.70-2.19]	0.5 [0.06-1.74]	*p=1.00*
**3A*	0.6 [0.70-2.19]	0.5 [0.06-1.74]	*p=1.00*
Total mutant Allele	1.2 [0.33-3.04]	1.0 [0.27-2.47]	*p=1.00*

† According to the two tailed Fischer's exact test.

Table 3.7 The frequencies of defective *TPMT* alleles in Turkish population.

Alleles	SNPs	No. of alleles	Frequency % (95% CI)
Total of Allele		746	
*TPMT*1*	Wild-Type	738	98.93 (97.90-99.54)
*TPMT*2*	G238C	0	0.0 (0.00-0.62)
*TPMT*3B*	G460A	0	0.0 (0.00-0.62)
*TPMT*3C*	A719G	4	0.5 (0.15-1.37)
*TPMT*3A*	G460A, A719G	4	0.5 (0.15-1.37)
Total mutant alleles		8	1.0 (0.46-2.10)

Considering the genotype distribution, among 373 individual, one individual were found to be homozygous mutant (actually it was compound heterozygous mutant as it has two different mutant allele: TPMT*3C/*3A) thus overall frequency of complete defective genotype was found to be 0.3% (95%CI: 0.01-1.48). Three individual were heterozygous in terms of **3C* allele (*TPMT*1/*3C*) and three individual were heterozygous interms of **3A* allele (*TPMT*1/*3A*). The overall frequency of heterozygosity among Turkish population was found to be 1.6% (95%CI: 0.59-3.47). These data were summarized in Table 3.8.

Table 3.8 The overall frequencies of *TPMT* wild type, heterozygous and homozygous mutant individuals in Turkish population.

TPMT*1*1 (Wild type) (%)[95%CI]	***TPMT*1*3C* or **3A*** (Heterozygous) (%)[95%CI]	**TPMT*3C/*3A** (Homozygous mutant) (%)[95%CI]
98.1 [96.2-99.2]	1.6 [0.6-3.5]	0.3 [0.0-1.5]

3.2.4 The Comparison of *TPMT* Genotype Distrubutions and Allele Frequencies in Turkish Population with Different Ethnic Groups

As said above, in the present study, the frequencies of **3C* and **3A* alleles between patient and control groups were found to be nearly same (0.5% vs. 0.6%, p=1.00, see Table 3.6) and the data for control and patient groups were pooled in order to obtain sufficiently large sample population. The allele frequency data obtained for overall Turkish population (373 individual) were compared with the various control adult populations and it was represented below in Table 3.9. The comparision of allele frequencies in Turkish population with other populations will be handled in detailed in the discussion part. Briefly, **3C* allele frequency in Turkish population (0.5%) was significantly lower ($p<0.05$) than the Africans like Kenyans (5.4%) and Ghanaian (7.6%), but it was similar to other Caucasians (0.2-0.9%) and Asians (0.8-2.3%) (Ameway *et al*., 1999; Hon *et al*., 1999; Hiratsuka *et al*., 2000; McLeod and Siva, 2002; Wei et al., 2005). The **3A* allele frequency (0.5%) was found to be significantly low ($p<0.05$) in Turkish population compared to British, French and American whites (McLeod and Siva, 2002; Ameway *et al*., 1999; Rossi *et al*., 2001). Both frequency of **3A* and **3C* allele in Turkish population were found to be very similar to Kazak population (0.3% and

0.9%, respectively; Wei *et al*., 2005) which was also Caucasian and Turkic in ethnic origin.

In terms of genotype distrubution, generally the wild type and homozygous mutant genotype frequencies do not differed so much among populations. It was also true for the Turkish population (see Table 3.9). But, considering the heterozygous genotype distrubution (see gray painted column in Table 3.9), it was significantly lower ($p<0.05$) in Turkish population compared to other Caucasian populations (like British, French and Americans) and African populations (like Kenyan and Ghanaian).

Table 3.9 Ethnic variation in *TPMT* geneotype distrubutions and allele frequencies.

Populations	**n for Individuals**	**Genotype Distrubution**			***TPMT* Allele Frequencies**			**n for Alleles**	**References**
		WW%	**WM %**	**MM%**	***2%**	***3A%**	***3C%**		
Turkish	**373**	**98.1**	**1.6**	**0.3**	**0.0**	**0.5**	**0.5**	**746**	**This study**
Kazak	327	97.5	2.5	0.0	0.0	0.3	0.9	654	Wei et al., 2005
French	191	85.9	13.6#	0.5	0.5	5.7#	0.5	382	Mc Leod and Siva, 2002
British	199	89.9	9.6#	0.5	0.5	4.5#	0.3	398	Ameway et al., 1999
American	282	92.5	7.4#	0.1	0.2	3.2#	0.2	564	Hon et al., 1999
Kenyan	101	89.1	10.9#	0.0	0.0	0.0	5.4	202	McLeod et al., 1999
Ghanaian	217	85.3	14.4#	0.5	0.0	0.0	7.6	434	Ameway et al., 1999
Japanese	192	98.4	1.6	0.0	0.0	0.0	0.8	384	Hiratsuka et al., 2000
Chinese	192	95.3	4.7	0.0	0.0	0.0	2.3	384	Collie-Duguid et al., 1999

#Demonstrates statistically significant differences $p<0.05$.

3.3 Genetic Risk Factors for the Development of Childhood ALL

In this part of the study, the genetic polymorphisms of two drug metabolizing enzymes SULT1A1 and EPHX1 were investigated as risk modifiers for the development of childhood ALL. G638A SNP for *SULT1A1* gene (the variant is designated as *SULT1A1*2*) was investigated in patient and control population. T/C SNP in exon 3 and A/G SNP in exon 4, in the EPHX1 gene were investigated in both patient and control population.

In the risk assessment studies, the general approach is the comparison of a group of individuals that are disease positive (the "case" group or patient group) with a group of disease negative individuals (the "control" group), with respect to the risk elevating susceptibility allele for a given gene. The statistic generated to measure association is the “odds ratio (OR)”. In order to calculate the odds ratio, the individuals carrying the risk elevating allele is proportioned to those who do not carry the risk elevating allele for both case and control groups (odds of risk elevating allele for case and control groups), separately. The odds ratio was calculated by the formula:

$$OR=(Case_{Risk}/ Case_{No\ Risk}) / (Control_{Risk}/ Control_{No\ Risk})$$

The proportion of case’s odds to control’s odds gives the ratio of the risk- the odds ratio- in terms of folds increase. An odds ratio significantly greater than 1 denotes the association of the risk elevating genotype with the disease, while a ratio far less than 1 indicates a protective role of the genotype for the disease. Odds ratio values near to 1 means there is no association between the genotype under question and disease. Odds ratio is an important term for genetic epidemiologic

studies showing both the presence or absence of association and the magnitude of it, in terms of times–fold of risk (Green *et al.,* 2000).

In case-control analyses, if more than one gene has been studied, the risk elevating properties of the gene can be studied separately or in combination. While evaluating the effect of single gene –single polymorphism - the heterozygous and homozygous mutated genotypes for a given polymorphism were stated as “risk” group, and analyzed against homozygous wild type genotype, which was placed in “no-risk” group. However, while evaluating the effect of more than one gene (or more than one polymorphisms), heterozygous and homozygous mutated genotypes for all given polymorphisms were combined as “risk” group, and analyzed against homozygous wild type genotype, which was placed in “no-risk” group. For the present study, first the possible risk modifier role of genetic polymorphisms were tested separately then their combined effects were analyzed.

In addition, for the current study, there are also non-genetic factors which could be examined for their interaction with the genetic factors (DMEs polymorphisms) in the risk of development of childhood ALL, like smoking status of parents and age of parents at conception. As information on these factors was not present for control population, case-only approach was applied to examine the interaction.

3.3.1 Case-Control Analyses

3.3.1.1 *SULT1A1* (G638C SNP) as Genetic Risk Factor

SULT1A1 gene has a single nucleotide polymorphism in the exon 7 coding region- G to A base substitution at position 638. nucleotide which causes an Arg213His replacement at the encoded protein. Wild type and mutated alleles are designated as **1* (G638) and **2* (638A) for *SULT1A1* polymorphism.

In order to determine genotypes for *SULT1A1*, PCR-RFLP method was performed. The PCR products covering G638A point mutation were digested with *Hha*I restriction enzyme. The amplified PCR product in exon 7 bears a recognition sites for the restriction enzyme *Hha*I (recognition sequence: 5'-GCG↓C -3'), in case of wild type allele (638. nucleotide occupied by G). So, up on digestion of wild type allele, PCR product was cut into 176 and 105 bp fragments (Figure 3.4, lanes 5-7). On the other hand, in mutated individuals, the nucleotide at position 638 is A, which abolish recognition site for *Hha*I, therefore upon digestion with the restriction enzyme, the 281 bp PCR product was not cut and resulted into single fragment on the gel (Figure 3.4, lane 9). Digestion of PCR fragment with *Hha*I in heterozygous individuals resulted into three fragment with 281, 176 and 105 bp (Figure 3.4, lanes 2-4, 8 and 10).

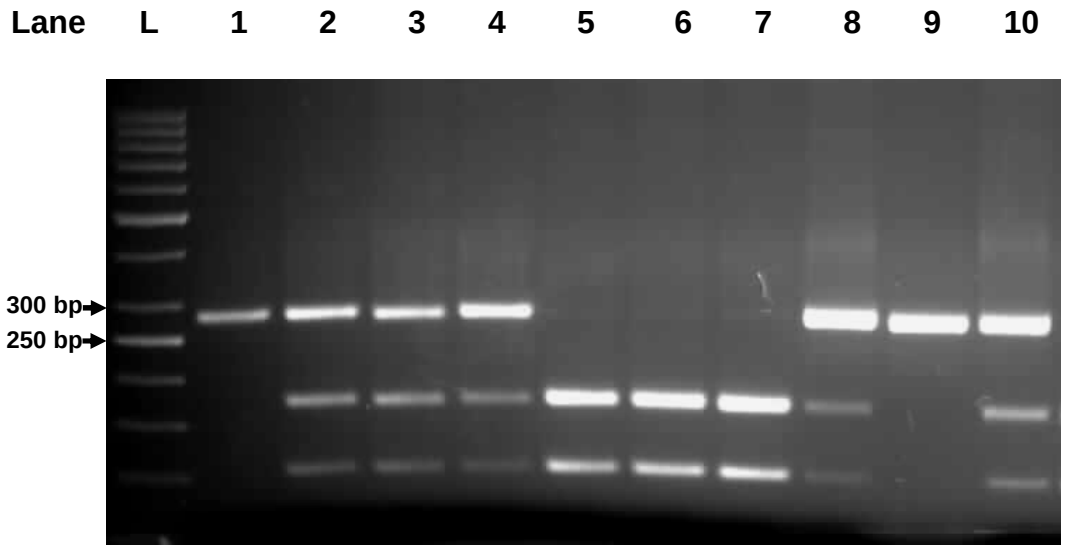


Figure 3.4 Agarose gel photo for *Hha*I digestion of exon 7 amplified region of the *SULT1A1* gene. Lane labeled as "L" is DNA ladder (50-1000 bp). Lane 1 is PCR product which was not subjected to digestion. Lane 2-4, 8 and 10 show the band patterns for heterozygous individuals with 281, 176 and 105 bp bands up on *Hha*I digestion. Lane 5-7 show the band patterns for wild type individuals with 176 and 105 bp bands. Lane 9 shows the band pattern for homozygous mutant individuals with a band of 281 bp.

The genotype distributions and allele frequencies of *SULT1A1*2* polymorphism were presented in Table 3.10 for patient and control groups. Among 206 control samples, 126 subjects, 61.2% of the control population, were homozygous wild type. The frequency is very similar in patient group- among 167 patients, 103 subjects, 61.7% of the patient samples, were homozygous wild type. In case of heterozygosity, while 31.1% of the patient samples were heterozygous, 34% of the control samples were heterozygous. The frequency distribution of the heterozygous individuals did not differ significantly between patient and

control population (OR: 0.9; 95%CI: 0.6-1.4; p=0.67). The frequency of homozygous mutant genotype was slightly higher in patient population (7.2%) compared to control population (4.9%). However, this difference was not statistically significant (OR: 1.5; 95%CI: 0.6-3.5; p=0.40). When heterozygous and homozygous mutant individuals were combined, the frequency distribution was found to be nearly same for both patient (38.3%) and control populations (38.8%). Considering the mutated allele frequency, it was found to be 23% in patient population which was very similar to control population (22%; OR: 1.1, 95%CI: 0.8-1.5; p=0.92). Therefore, considering the mutated allele frequencies and genotype distributions in control and patient groups, **2* polymorphism was not a risk factor for childhood ALL.

As the data represented below in Table 3.10 for *SULT1A1*2* polymorphisms, also include healthy control subjects, it provides information for the genotype distribution and allele frequency of *SULT1A1*2* polymorphisms in Turkish populations which is not determined yet to our knowledge. Therefore, 61.2% of the Turkish population was homozygous wild type, 34% was heterozygous and 4.9% was homozygous mutant. The mutant allele frequency (**2* allele) was 0.22 and wild type allele frequency was 0.78 for Turkish population.

Table 3.10 Genotype and allele distribution of *SULT1A1*2* polymorphism in control and patient groups.

SULT1A1 Genotypes	**Patients (n=167)**		**Controls (n=206)**		**OR (95% CI)**	***p***
	n	**%**	**n**	**%**		
*SULT1A1 *1*1*	103	**61.7**	126	**61.2**	1.0 (Referent)	
*SULT1A1 *1*2*	52	**31.1**	70	**34.0**	0.9 (0.6-1.4)	0.67
*SULT1A1 *2*2*	12	**7.2**	10	**4.9**	1.5 (0.6-3.5)	0.40
*SULT1A1 (*1*2+2*2)*	64	**38.3**	80	**38.8**	1.0 (0.6-1.5)	0.92
Allele frequency						
Arg213	258	**77.0**	322	**78.0**	1.0 (Referent)	
His213	76	**23.0**	90	**22.0**	1.1 (0.8-1.5)	0.92

The comparison of *SULT1A1**2 allele frequency in Turkish population (obtained from control population) with various control population was represented in Table 3.11. Accordingly, for the Turkish population the frequency of the mutant allele (**2*) was found to be very similar to that observed in other Caucasian populations like British (UK), German, USA and Australians. It was also found to be similar to African and Japanese population. However, the frequency of *2 allele was found to be significantly different from Chinese population ($p<0.05$).

Table 3.11 The *SULT1A1*2* allele frequency in different ethnic groups.

Populations	Allele Frequency *1	Allele Frequency *2	N	References
Turkish	**0.78**	**0.22**	**412**	**This Study**
Australia	0.68	0.32	804	Wong *et al*.,2002
British	0.68	0.32	586	Coughtrie *et al*., 1999
German	0.63	0.37	600	Raftogianis *et al*., 1999
USA	0.67	0.33	490	Raftogianis *et al*., 1996
Africans	0.74	0.26	80	Wong *et al*., 2002
Chinese	0.91	0.08[#]	580	Wong *et al*., 2002
Japanese	0.83	0.17	286	Ozawa *et al*., 1999

[#]Statistically significant difference $p<0.05$

3.3.1.2 EPHX1 T/C SNP in Exon 3 Region as Genetic Risk Factor

EPHX1 gene has two functionally important single nucleotide polymorphisms. One of them is T/C substitution in the exon 3 coding region of the gene which causes Tyr113His replacement at the encoded protein. Wild type allele is designated as *Tyr113* and wild type genotype is demonstrated as Tyr/Tyr. Mutant allele is designated as *His113* and homozygous mutant genotype is shown as His/His. Heterozygous genotype is represented as Tyr/His.

In order to determine genotypes for *EPHX1* gene, PCR-RFLP method was performed. The PCR products covering T/C point mutation in exon 3 region were digested with *Tth111*I restriction enzyme. The amplified PCR product in exon 3 bears a recognition sites for the restriction enzyme *Tth111*I (recognition sequence: 5'-GACN ↓NNGTC -3'), in case of mutant allele (when T replaced by C). So, up on digestion of mutant allele, PCR product was cut into 175 and 23 bp fragments (Figure 3.5, lanes 1 and 2). On the other hand, in wild type individuals, the

presence of “T” abolishes recognition site for *Tth111*I, therefore upon digestion with the restriction enzyme, the 198 bp PCR product was not cut and resulted into single fragment on the gel (Figure 3.5, lane 7). Digestion of PCR fragment with *Tth111*I in heterozygous individuals resulted into three fragment with 198, 175 and 23 bp (Figure 3.5, lanes 3, 5, 6 and 8).

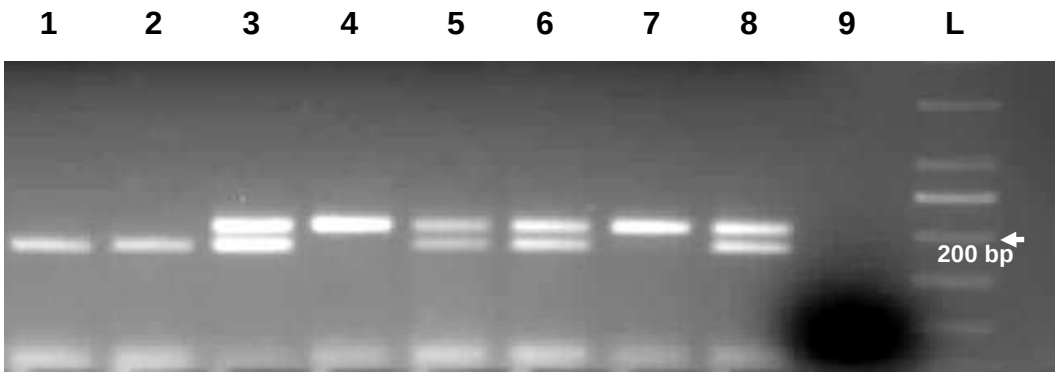


Figure 3.5 Agarose gel photo for *Tth111*I digestion of exon 3 amplified region of the *EPHX1* gene. Lane labeled as “L” is DNA ladder (50-1000 bp). Lane 1 and 2 show the band patterns for homozygous mutant individuals with the bands at 175 and 23 bp. Lane 3,5,6,8 show the band patterns for heterozygous individuals with the bands at 198, 175 and 23 bp. Lane 4 is PCR product which was not subjected to digestion. Lane 7 shows the band pattern for wild type individuals with a 198 bp band.

The genotype distribution and allele frequencies of *EPHX1* exon 3 (T/C substitution in nucleotide; Tyr113His replacement at the encoded protein) polymorphism were presented in Table 3.12 for patients and control groups. Among 206 control samples, 118 subjects, 57.3% of the

control population, were homozygous wild type. The frequency is very similar in patient group- among 167 patients, 89 subjects, 53.3% of the patient population, were homozygous wild type. In case of heterozygosity, while 28.1% of the patient population was heterozygous, 32.5% of the control population was heterozygous. The frequency distribution of the heterozygous individuals did not differ significantly between patient and control population (OR: 0.9; 95%CI: 0.6-1.5; p=0.76). The frequency of homozygous mutant genotype was significantly higher in patient population (18.6%) compared to the control population (10.2%) with an OR of 2.0 (1.1-3.6; p=0.01). When heterozygous and homozygous mutant individuals were combined the frequency distribution did not differ significantly between control (42.7%) and patient (46.7%) population (OR: 1.2; 95%CI: 0.8-1.8; p=0.44). In regard of mutated allele frequency, it was considerably higher in patient population (32.6%) compared to the control population (26.5%) with an OR of 1.4 (1.0-1.8; p=0.01). Therefore, when exon 3 polymorphism (Tyr113His) was investigated as a risk factor for childhood ALL, it was observed that the homozygous mutant genotype (His/His) increased the risk of ALL 2.0-fold, which was statistically significant (p=0.01).

As the data represented below in Table 3.12 for *EPHX1* exon 3 polymorphism (*His113*), also include healthy control subjects, it provides information for the genotype distribution and allele frequency of EPHX1 exon 3 polymorphism in Turkish populations. Therefore, 57.3% of the Turkish population was homozygous wild type, 32.5% was heterozygous and 10.2% was homozygous mutant. The mutant allele frequency (*His113*) was 0.26 and wild type allele frequency was 0.74 for Turkish population.

Table 3.12 Genotype and allele distribution of *EPHX1* Tyr113His polymorphism in control and patients groups.

EPHX1 exon 3 Genotypes	**Patients (n=167)**		**Controls (n=206)**		**OR (95% CI)**	***p***
	n	**%**	**n**	**%**		
Tyr/Tyr	89	**53.3**	118	**57.3**	1.0 (Referent)	
Tyr/His	47	**28.1**	67	**32.5**	0.9 (0.6-1.5)	0.76
His/His	31	**18.6**	21	**10.2**	**2.0 (1.1-3.6)**	**0.01***
Tyr/His+ His/His	78	**46.7**	88	**42.7**	1.2 (0.8-1.8)	0.44
Allele frequency						
Tyr113	225	**67.4**	303	**73.5**	1.0 (Referent)	
His113	109	**32.6**	109	**26.5**	**1.4 (1.0-1.8)**	**0.01***

***Statisiticaly significant difference p<0.05

The comparison of *EPHX1* exon 3 polymorphism (His113 mutant allele) in Turkish population (obtained from control population) with various control population was represented in Table 3.13. Accordingly, the mutant allele frequency (His113) found in the present study for Turkish population was highly similar to that were found in other Turkish studies (Ada *et al*.,2007; Pınarbaşı *et al*., 2007). In addition, mutant allele frequency found in present study for Turkish population was very similar to other Caucasian population like German, Bitish and France (Smith and Harrison, 1997; Harms *et al*., 2004; Clavel *et al*., 2005).

The mutant allele frequency in Asian population such as Japanese, Chinese and Taiwanese were slightly higher than that of observed for Turkish population in the present study (Yoshikawa *et al*., 2000; Cheng *et al*., 2004; Xiao *et al*., 2004).

Table 3.13 The *EPHX1*, *His133* allele frequency in different ethnic groups.

Populations	Tyr113 Wild Type	His113 Mutant Allele	N	References
Turkish	**0.74**	**0.26**	**412**	**This study**
Turkish	0.72	0.28	266	Ada *et al*., 2007
Turkish	0.65	0.35	310	Pınarbaşı *et al*., 2007
German	0.74	0.26	238	Harms *et al*., 2004
British	0.69	0.31	406	Smith, Harrison, 1997
France	0.65	0.35	210	Clavel *et al*., 2005
Taiwan	0.52	0.48	424	Cheng *et al*., 2004
Chinese	0.55	0.45	200	Xiao *et al.*, 2004
Japanese	0.56	0.44	716	Yoshikawa *et al*., 2000

3.3.1.3 *EPHX1* A/G SNP in Exon 4 Region as Genetic Risk Factor

EPHX1 gene has a A/G substitution in the exon 4 coding region of the gene which causes His139Arg replacement at the encoded protein. Wild type allele is designated as *His139* and wild type genotype is demonstrated as His/His. Mutant allele is designated as *Arg139* and homozygous mutant genotype is shown as Arg/Arg. Heterozygous genotype is represented as His/Arg.

In order to determine genotypes for *EPHX1* gene, PCR-RFLP method was performed. The PCR products covering A/G point mutation in exon 4 region were digested with *Rsa*I restriction enzyme. The amplified PCR product in exon 4 bears a recognition sites for the restriction enzyme *Rsa*I (recognition sequence: 5'-GT ↓AC -3'), in case of mutant allele (when A replaced by G). So, up on digestion of mutant allele, PCR product was cut into 164 and 46 bp fragments (Figure 3.6, lane 6). On the other hand, in wild type individuals, the presence of "A" abolishes recognition site for *Rsa*I, therefore upon digestion with the restriction enzyme, the 210 bp PCR product was not cut and resulted into single fragment on the gel (Figure 3.6, lanes 5, 7-9). Digestion of PCR fragment with *Rsa*I in heterozygous individuals resulted into three fragment with 210, 164 and 46 bp (Figure 3.6, lanes 2-4).

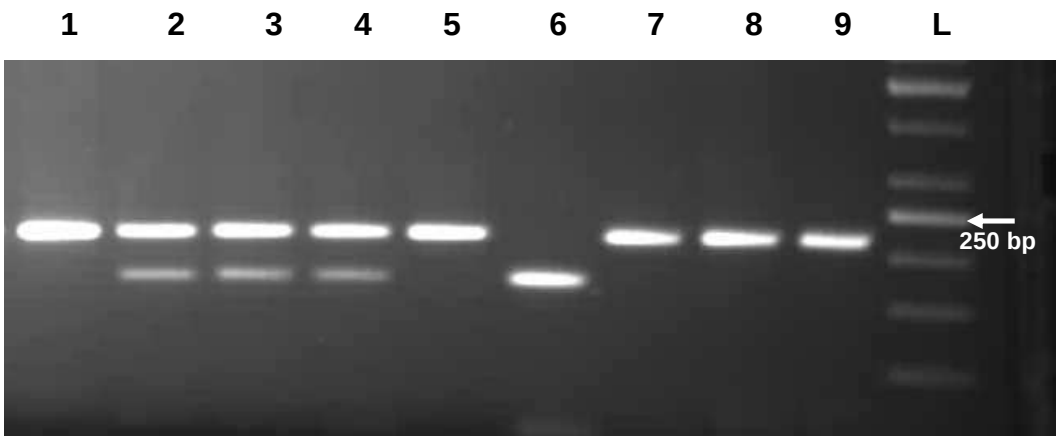


Figure 3.6 Agarose gel photo for *Rsa*I digestion of exon 4 amplified region of the *EPHX1* gene. Lane labeled as "L" is DNA ladder (50-1000 bp). Lane 1 is PCR product which was not subjected to digestion. Lane 2-4 show the band patterns for heterozygous individuals with the bands at 210, 164 and 46 bp (46 bp long band was not seen on the gel). Lane 5, 7-9 show the band patterns for wild type individuals with a 210 bp band. Lane 6 shows the band pattern for homozygous mutant individual with the bands at 164 and 46 bp.

The genotype distribution and allele frequencies of *EPHX1* exon 4 polymorphism were presented in Table 3.14 for patients and control groups. Among 206 control samples, 142 subjects, 68.9% of the control population, were homozygous wild type. The frequency is very similar in patient population- among 167 patients, 120 subjects, 71.9% of the patient population, were homozygous wild type. In case of heterozygosity, while 25.7% of the patient population was heterozygous, 29.1% of the control population was heterozygous. The frequency distribution of the heterozygous individuals did not differ significantly between patient and control population (OR: 0.9; 95%CI: 0.5-1.3; p=0.48). The frequency of homozygous mutant genotype in patient population was 2.4% which is very similar to control population (1.9%). When heterozygous and homozygous mutant individuals were combined the frequency distribution did not differ significantly between control (31.1%) and patient (28.1%) population (OR: 0.9; 95%CI: 0.6-1.4; p=0.54). Considering the mutated allele frequency, it was found to be 15.3% in patient population which was very similar to control population (16.5%; OR: 0.9, 95%CI: 0.6-1.4; p=0.65). Therefore, in regard of mutated allele frequency and genotype distribution in control and patient groups, His139Arg polymorphism was not a risk factor for childhood ALL.

As the data represented below in Table 3.14 for *EPHX1* exon 4 polymorphism (*Arg139*), also include healthy control subjects, it provides information for the genotype distribution and allele frequency of EPHX1 exon 4 polymorphism in Turkish populations. Therefore, 68.9% of the Turkish population was homozygous wild type, 29.1% was heterozygous and 1.9% was homozygous mutant. The mutant allele frequency (*His113*) was 0.17 and wild type allele frequency was 0.83 for Turkish population.

Table 3.14 Genotype and allele distribution of *EPHX1* His139Arg polymorphism in control and patients groups.

EPHX1 exon 4 Genotypes	**Patients (n=167)**		**Controls (n=206)**		**OR (95% CI)**	***p***
	n	**%**	**n**	**%**		
His/His	120	**71.9**	142	**68.9**	1.0 (Referent)	
His/Arg	43	**25.7**	60	**29.1**	0.9 (0.5-1.3)	0.48
Arg/Arg	4	**2.4**	4	**1.9**	1.2 (0.3-4.8)	0.81
His/Arg + Arg/Arg	47	**28.1**	64	**31.1**	0.9 (0.6-1.4)	0.54
Allele frequency						
His139	283	**84.7**	344	**83.5**	1.0 (Referent)	
Arg139	51	**15.3**	68	**16.5**	0.9 (0.6-1.4)	0.65

The comparison of *EPHX1* exon 4 polymorphism (*Arg139* mutant allele) in Turkish population (obtained from control population) with various control population was represented in Table 3.15. Accordingly, the mutant allele frequency (*Arg139*) found in the present study for Turkish population was highly similar to that were found in other Turkish studies (Ada *et al*.,2007; Pınarbaşı *et al*., 2007). In addition, mutant allele frequency found in present study for Turkish population was very similar to other Caucasian population like German, Bitish and France (Smith and Harrison, 1997; Harms *et al*., 2004; Clavel *et al*., 2005) and also Asian populations like Japanese, Chinise and Taiwanese (Yoshikawa *et al*., 2000; Cheng *et al*., 2004; Xiao *et al*., 2004).

Table 3.15 The *EPHX1*, *Arg139* allele frequency in different ethnic groups.

Populations	His139 Wild Type	Arg139 Mutant Allele	N	Reference
Turkish	**0.84**	**0.16**	**412**	**This study**
Turkish	0.84	0.16	266	Ada *et al*., 2007
Turkish	0.87	0.13	310	Pınarbaşı *et al*., 2007
German	0.82	0.18	238	Harms *et al*., 2004
British	0.86	0.14	406	Smith, Harrison, 1997
France	0.79	0.21	210	Clavel *et al*., 2005
Taiwan	0.81	0.19	424	Cheng *et al*., 2004
Chinese	0.91	0.09	200	Xiao *et al*., 2004
Japanese	0.86	0.14	716	Yoshikawa *et al*., 2000

3.3.1.4 Combination of *EPHX1* Polymorphisms as Genetic Risk Factors

For exon 3 polymorphism of *EPHX1* gene, Tyr113His replacement at the encoded protein results in 40–50% decrease in the enzyme activity thus the allele has been called the “slow allele”. For the second polymorphism (exon 4), His139Arg replacement at the encoded protein causes 25% increase of the enzyme activity and this allele are designated as “fast allele” (Hassett *et al*, 1994). *In vitro* expression studies demonstrated that different combinations of amino acid residues at positions 113 and 139 in EPHX1 proteins directly influence enzymatic activity, possibly by affecting protein stability. Therefore, it is reasonable to postulate that individuals with specific allelic combinations may be at differential risk for the ability to metabolize reactive epoxides efficiently (Hassett *et al*, 1994). Many authors proposed predictive enzyme activities (imputed phenotypes) for different combinatios of exon 3 and 4

polymorphisms. Pursuant to Smith and Harrison (1997), in the present study, imputed phenotypes were classified as normal, fast, slow and very slow as indicated in Table 3.16. Accordingly, normal phenotype means that no mutation in gene, or heterozygote for both exon 3 and exon 4 mutations. Fast phenotype is represented as at least one fast mutation (exon 4) and no exon 3 mutations. Slow phenotype has one slow (exon 3) allele (heterozygous for exon 3 polymorphism), and very slow phenotype is represented by the presence of two slow alleles (homozygous mutated for exon 3 polymorphism).

Table 3.16 Imputed phenotypes (predicted EPHX1 enzyme activity) based on the classification of Smith and Harrison, 1997.

Imputed phenotypes	**Combined genotypes***
Normal	W_3/W_4, H_3/H_4
Fast	W_3/H_4, W_3/M_4
Slow	H_3/W_4, H_3/M_4
Very Slow	M_3/W_4, M_3/H_4, M_3/M_4

***W_3 and W_4: Homozygous wild type genotype for exon 3 and 4 polymorphisms, respectively. H_3 and H_4: Heterozygous genotype for exon 3 and 4 polymorphisms, respectively. M_3 and M_4:Homozygous mutant genotype for exon 3 and 4 polymorphisms, respectively.

When exon 3 and exon 4 polymorphisms were considered together, nine different genotype combinations can be seen in the population. The percentages of each combination in patients and control groups were given in Table 3.17. Individuals who are homozygous wild type for both exon 3 and exon 4 polymorphisms were taken as referent.

Accordingly, individuals homozygous wild type for exon 4 polymorphism, and heterozygote for exon 3 polymorphism (H_3/W_4 genotype) did not confer a risk factor for the development of childhood ALL. The percentage of this genotype was found to be very similar between controls and cases (OR: 1.0; 95% CI, 0.6-1.7). However, the homozygous presence of exon 3 polymorphism with wild type genotype for exon 4 (M_3/W_4 genotype) was found to increase the risk of ALL 1.7 fold (95% CI: 0.9-3.5, p=0.05). These findings were found to be in accordance with the single polymorphism analysis. The homozygous presence of exon 4 polymorphism with wild type genotype for exon 3 (W_3/M_4 genotype) was found to increase the risk of ALL 2.6 fold (95% CI: 0.2-28.8). However this association was not statistically significant. The reason for such high but insignificant odds ratio for W_3/M_4 genotype could be related with the low numbers (1.2% in patients and 0.5% in controls). Individuals homozygous wild type for exon 3 polymorphism, and heterozygote for exon 4 (W_3/H_4 genotype) polymorphism did not confer a risk factor for the development of childhood ALL. The percentage of this genotype was found to be very similar between control and cases (OR: 0.9; 95% CI, 0.5-1.5). These results were also found to be in accordance with the single polymorphism analysis in which neither homozygous nor heterozygous genotype for exon 4 polymorphism were found to be a risk factor for the risk of ALL. When homozygous presence of two polymorphisms was considered together (M_3/M_4 genotype), the number was too low to compute an association. However, when heterozygous presence of two polymorphisms was considered (H_3/H_4 genotype), the numbers were found to be very similar between control and cases (OR: 0.8, 95%CI 0.3-1.8). From this result, it can be inferred that the predictive enzyme activities (imputed phenotypes) rather than genotype combinations could be more informative for the associations of combined effects of the polymorphisms with the childhood ALL risk.

Table 3.17 Combined genotypes for exon 3 and exon 4 polymorphisms and risk of childhood ALL.

Imputed Phe.	Combined Genotype	Control n=206		Patient n=167		OR (95%CI)	*P*
		n	%	n	%		
Normal	W_3/W_4	79	**38.4**	62	**37.1**	1.0 Referent	-
Normal	H_3/H_4	18	**8.7**	11	**6.6**	0.8 (0.3-1.8)	0.55
Fast	W_3/H_4	38	**18.5**	25	**15.0**	0.8 (0.5-1.5)	0.57
Fast	W_3/M_4	1	**0.5**	2	**1.2**	2.6 (0.2-28.8)	0.43
Slow	H_3/W_4	46	**22.3**	35	**21.0**	1.0 (0.6-1.7)	0.91
Slow	H_3/M_4	3	**1.5**	1	**0.6**	0.4 (0.1-4.2)	0.45
V. Slow	M_3/W_4	17	**8.3**	23	**13.8**	**1.7 (0.9-3.5)**	**0.05***
V. Slow	M_3/H_4	4	**1.9**	7	**4.2**	2.2 (0.6-8.0)	0.11
V. Slow	M_3/M_4	0	**0.0**	1	**0.6**	-	-

Predictive enzyme activities (imputed phenotypes) were attended to the genotype combinations of exon 3 and exon 4 polymorphisms as demonstrated in Table 3.16 which was also described in detail within text (section 3.3.1.2.1). *Statisitically significant difference p<0.0.5.

When individuals were grouped according to the predictive enzyme activities as normal, fast, slow and very slow, there were no significant differences in the distribution of slow and fast EPHX1 enzyme activities between the control and patient groups as indicated in Table 3.18. However, the very slow activity phenotype (represented by M_3 genotype with any combination of exon 4 polymorphism) significantly increased the risk of ALL 2.0 fold (p=0.04).

Table 3.18 Imputed phenotypes (predictive enzyme activities) for combinations of exon 3 and exon 4 polymorphisms and risk of childhood ALL.

Imputed Phenotypes	Control n=206		Patient n=167		OR(95%CI)	*P*
	n	%	n	%		
Normal	97	**47.1**	73	**43.7**	1.0 (Referent)	-
Fast	39	**18.9**	27	**16.2**	0.9 (0.5-1.6)	0.77
Slow	49	**23.8**	36	**21.6**	0.9 (0.6-1.7)	0.92
Very Slow	21	**10.2**	31	**18.6**	**2.0 (1.1- 3.7)**	**0.04***

***** Statisitically significant difference p<0.0.5.

3.3.1.5 Combination of *EPHX1* and *SULT1A1*2* polymorphisms as Genetic Risk Factors

As mentioned before, *SULT1A1*2* polymorphism causes arginine to histidine replacement at the position of 213 in the encoded protein. It has been reported that *His 213* allele is associated with low enzyme activity and low thermal stability (Raftogianis *et al*., 1997) In the single gene analysis, *SULT1A1*2* did not confer a risk factor for the development of childhood ALL. In combination analysis, *SULT1A1*2* polymorphism were combined with *EPHX1* exon 3 polymorphism (slow allele), *EPHX1* exon 4 polymorphism (fast allele), and also with the imputed phenotypes (predictive enzme activities) for *EPHX1* gene. Table 3.19 represented the combination analyses for *SULT1A1*2* polymorphism and *EPHX1* exon 3 polymorphism.

For these analyses, homozygous wild type individuals for both polymorphisms were taken as no risk group. "*SULT1A1*2* bearing genotypes" represents either homozygous mutant or heterozygous individuals for *SULT1A1*2* polymorphism. Risk case was defined in each row. Accordingly the co-presence of heterozygous genotype for *SULT1A1*2* polymorphism (*SULT1A1*1*2*) and homozygous mutant genotype for *EPHX1* exon 3 polymorphism (M_3) in the same individual considerably increased the risk of childhood ALL to 3.0 fold (95%CI: 0.9-8.1, p=0.05). Similarly, other combinations such as the co-presence of *SULT1A1*2*2* genotype and heterozygous genotype for exon 3 polymorphism of *EPHX1* gene (H_3) and co-presence of *SULT1A1*2* bearing genotype and M_3 genotype in the same individual increased the risk for ALL to 2.6 and 2.1 fold respectively, but insignificantly (95% CI: 0.5-14.8, *p*=0.26 and 95% CI: 0.8-5.7, *p*=0.07 respectivey).

Other combinations of *SULT1A1*2* polymorphism and *EPHX1* exon 3 polymorphism were not associated with the risk of development of childhood ALL, as can be seen from Table 3.19.

Table 3.19 Combination analyses for *SULT1A1*2* polymorphism and exon 3 polymorphism of *EPHX1* gene as risk factors for the development of childhood ALL.

Analysis	Control	Patients	OR (95%CI)	P
NO Risk: Wild type	72	55	1.0 (Referent)	
Risk: *SULT1A1*2*2* + M_3	2	1	0.7 (0.1-7.4)	0.73
Risk: *SULT1A1*2*2* + H_3	2	4	2.6 (0.5-14.8)	0.26
Risk: *SULT1A1*2*2* + either M_3 or H_3	4	5	1.6 (0.4-6.3)	0.48
Risk: *SULT1A1*1*2* + M_3	5	10	**3.0 (0.9-8.1)**	**0.05**
Risk: *SULT1A1*1*2* + H_3	25	15	0.8 (0.4-1.6)	0.52
Risk: *SULT1A1*1*2* + either M_3 or H_3	30	25	1.1 (0.6-2.1)	0.80
Risk: *SULT1A1*2* bearing genotypes + M_3	7	11	2.1 (0.8-5.7)	0.07
Risk: *SULT1A1*2* bearing genotypes + H_3	27	19	0.9 (0.5-1.8)	0.82
Risk: *SULT1A1*2* bearing genotypes + either M_3 or H_3	34	30	1.2 (0.6-2.1)	0.64

*M_3: Homozygous mutant individuals for exon 3 polymorphisms of EPHX1 gene.
*H_3: Heterozygous individuals for exon 3 polymorphisms of EPHX1 gene.

Table 3.20 represented the combination analyses for *SULT1A1*2* polymorphism and *EPHX1* exon 4 polymorphism. For these analyses, homozygous wild type individuals for both polymorphisms were taken as no risk group. Risk case was defined in each row. Accordingly, the co-presence of *SULT1A1*2* bearing genotype with M_4 genotype and also co-presence of *SULT1A1*1*2* genotype with M_4 genotype in the same individuals increased the risk for ALL to 3.5 fold for each case. However, the numbers of "risk" genotypes were very low in these two combinations (the number of individual 1 for control and 3 for cases) so that the association was not significant (95% CI: 0.4-34.2, *p*=0.26). Same problem existed for the combinations of *SULT1A1*2*2* genotype with either M4 or H_4 genotypes and with H_4 genotype. These two combinations increased the risk of childhood ALL 2.3 fold however the association was insignificant because of low numbers 95% CI: 0.2-26.1, *p*=0.48).

Other combinations of *SULT1A1*2* polymorphism and *EPHX1* exon 4 polymorphism were not associated with the risk of development of childhood ALL, as can be seen from Table 3.20.

Table 3.20 Combination analyses for *SULT1A1*2* polymorphism and exon 4 polymorphism of *EPHX1* gene as risk factors for the development of childhood ALL

Analyses	Control	Patients	OR (95%CI)	P
NO Risk: Wild type	86	74	1.0 (Referent)	
Risk: *SULT1A1*2*2* + M_4	0	0	-	-
Risk: *SULT1A1*2*2* + H_4	1	2	2.3 (0.2-26.1)	0.48
Risk: *SULT1A1*2*2* + either M_4 or H_4	1	2	2.3 (0.2-26.1)	0.48
Risk: *SULT1A1*1*2* + M_4	1	3	3.5 (0.4-34.3)	0.26
Risk: *SULT1A1*1*2* + H_4	22	13	0.7 (0.3-1.5)	0.33
Risk: *SULT1A1*1*2* + either M_4 or H_4	23	16	0.8 (0.4-1.6)	0.56
Risk: *SULT1A1*2* bearing genotypes + M_4 genotypes	1	3	3.5 (0.4-34.3)	0.26
Risk: *SULT1A1*2* bearing genotypes + H_4 genotypes	23	15	0.8 (0.4-1.7)	0.45
Risk: *SULT1A1*2* bearing genotypes + either M_4 or H_4	24	18	0.9 (0.4-1.7)	0.69

*M_3: Homozygous mutant individuals for exon 3 polymorphisms of EPHX1 gene.
*H_3: Heterozygous individuals for exon 3 polymorphisms of EPHX1 gene.

Table 3.21 represented the combination analysis for different genotypes of *SULT1A1*2* polymorphism with predictive enzyme activities for *EPHX1* gene. For this analysis, individuals who are homozygous mutant, heterozygous and either homozygous mutant or heterozygous (*2 bearing individuals) for *SULT1A1*2* polymorphism were combined with fast activity, slow activity and very slow activity phenotypes of *EPHX1* gene separately.

Individuals who are homozygous wild type for *SULT1A1*2* polymorphism and have normal enzyme activity phenotype for *EPHX1* gene were grouped as no risk. In case of slow activity phenotype, for example, co-presence of *SULT1A1*2* bearing genotype and slow activity phenotype was identified as risk group (the risk cases were identified in the rows of Table 3.21). Accordingly, for fast activity phenotype of *EPHX1* gene, only, the co-presence of *SULT1A1*2*2* genotype demonstrate an association. The co-presence of fast activity phenotype for *EPHX1* gene with *SULT1A1*2*2* genotype increased the risk of childhood ALL 2.2 fold but insignificantly (95% CI: 0.2-24.7, p=0.52). For slow activity phenotype of *EPHX1* gene, only the co-presence of *SULT1A1*2*2* genotype represents a risk factor (increased the risk to 2.2 fold) however it is not statistically significant (95% CI: 0.4-12.4, p=0.37). For very slow activity phenotype of *EPHX1* gene the co-presence of *SULT1A1*1*2* genotype and co-presence of *SULT1A1*2* bearing genotype increased the risk for ALL to 2.2 and 1.7 fold respectively, but these associations were not statistically significant (95% CI: 0.7-6.7, p=0.09 and 95% CI: 0.6-4.8, p=0.12). Other combination of different *SULT1A1*2* genotypes with slow, fast and very slow activity phenotype for *EPHX1* gene were not associated with the risk of development of childhood ALL, as can be seen from Table 3.21.

Table 3.21 Combination analyses for *SULT1A1*2* different genotypes and predictive enzyme activities for *EPHX1* gene as risk factors for the development of childhood ALL.

Analyses	Control	Patients	OR (95%CI)	*P*
NO Risk: Wild type	52	48	1.0 (Referent)	
Risk: *SULT1A1*2*2* + **fast** activity	1	2	2.2 (0.2-24.7)	0.52
Risk: *SULT1A1*2*2* + **slow** activity	2	4	2.2 (0.4-12.4)	0.37
Risk: *SULT1A1*2*2* + **very** slow activity	2	1	0.5 (0.05-6.2)	0.62
Risk: *SULT1A1*1*2* + **fast** activity	10	10	1.1 (0.4-2.8)	0.87
Risk: *SULT1A1*1*2* + **slow** activity	15	12	0.9 (0.4-2.0)	0.74
Risk: *SULT1A1*1*2* + **very slow** activity	5	10	2.2 (0.7-6.3)	0.09
Risk: *SULT1A1*2* bearing genotypes + **fast** activity	11	12	1.2 (0.5-2.9)	0.72
Risk: *SULT1A1*2* bearing genotypes + **slow** activity	17	16	1.0 (0.5-2.2)	0.96
Risk: *SULT1A1*2* bearing genotypes + **very slow** activity	7	11	1.7 (0.6-4.8)	0.12

3.3.2 Effect of Non-genetic Factors on the Risk of Genetic Factors for the Development of Childhood ALL: Case-Only Analyses

In case control analyses, the individuals carrying the risk elevating allele is proportioned to those who do not carry the risk elevating allele for both case and control groups separately. The proportion of cases to controls gives the ratio of the risk- the odds ratio- in terms of folds increase. Up to this section the polymorphisms of two susceptible gene *EPHX1* and *SULT1A1* were investigated in controls and cases and the odd ratios were calculated for defined “risk genotypes”. In the context of current study, there are some non-genetic factors which could be examined for their interaction with the genetic factors (DMEs polymorphisms) in the risk of development of childhood ALL, like smoking status of parents and age of parents at conception. However, information on these factors was not present for control population for this reason case-only approach was applied to examine the interaction.

In case-only analyses, the general approach is to assess the magnitude of the association (interaction) between the exposure of interest (for example, smoking status of parents) and the susceptibility genotype among case subjects. The magnitude of association was denoted by “Case Only Odd Ratio (COR)” which was calculated from only case population. The basic setup for analysis is a new 2 by 2 table, from which COR can be calculated. A model for calculating COR is represented in Table 3.22.

Table 3.22 Model for gene-environment interaction analysis in the context of a case-only study (Khoury *et al.,* 1996).

Environmental Factor	Susceptibility Genotype	
	-	+
-	a	b
+	c	d

Case-only Odds Ratio (COR) can be calculated as:
COR= (d/c) / (b/a)

COR values calculated from only case population were shown to be similar to OR values calculated for the case-control population, where the case population was the same for both analyses (Khoury *et al.,* 1996).

It should also be noted that, the case-only approach does not allow the investigators to evaluate the independent effects of the exposure alone or the genotype alone, merely their interaction (Khoury *et al.,* 1996; Goodman and Flanders, 2007).

For the present study, the age of mother and father at conception and cigarette smoking exposure information (both for parents and child) were investigated to evaluate their interactions with the genetic polymorphisms for risk of childhood ALL.

In case of parental age at conception, it is a well known fact that maternal age is younger than 20 and older than 35, besides, paternal age older than 40 at the conception is a risk factor for the development of childhood ALL (Kaye *et al.,* 1991; Dockerty *et al.,* 2001). In the current study, the ages of mothers at conception were grouped into three categories: younger than 20, between 20 and 35 and older than 35. The ages of father at conception were grouped into two categories younger

than 40 and older than 40. The numbers of mothers and fathers in each age group were represented prevously in Table 3.1. The case only analysis of mothers was done for ages younger than 20 and older than 35 against ages between 20 and 35 with different genotypes of *SULT1A1*2* polymorphism, exon 3 and 4 polymorphisms of *EPHX1* gene and imputed phenotypes for *EPHX1* gene, but, no significant associations were observed (data not shown). It means that, neither ages younger than 20 nor ages older than 35 at conception for the mother, together with genetic polymorphisms was a risk factor for childhood ALL. Another analysis for the investigation of the mother age at conception was done by grouping the ages younger than 20 and older than 35 into single group as “risky ages” however, for this analysis also no significant association was observed.

Father age younger than 40 was accepted as referent and case only analyses were carried out for age older than 40 with different genotypes of *SULT1A1*2* polymorphism, exon 3 and 4 polymorphisms of *EPHX1* gene and imputed phenotypes for *EPHX1* gene, but, no significant association was observed (data not shown).

3.3.2.1 Interaction of Cigarette Smoking Status of Parents with the Genetic Polymorphisms in Risk of Childhood ALL

Cigarette smoking questionnaires were answered by 106 parents. The questionnaire gave information on the smoking status of parents, smoking status of mother during pregnancy and postnatal exposure of the children. According to smoking status, parents were grouped as “non smoker”, “passive smoker” and “active smoker”. The numbers of individuals in each group were given previously in Table 3.1.

As can be seen from Table 3.1, among 106 mothers, 27 of them were non smokers, 56 of them were passive smokers and 23 of them were active smokers. Case-only odds ratio analysis for mothers was carried out for both passive against non-smokers and active against non-smokers groups with different genotypes of *SULT1A1*2* polymorphism, exon 3 and 4 polymorphisms of *EPHX1* gene and imputed phenotypes for *EPHX1* gene. In these analyses, genotypes and imputed enzyme activities did not demonstrate significant associations (data were not shown) for both passive and active smoker groups. Another analysis for mothers was carried out by grouping the active and passive smokers as exposed individuals. In this analysis, case only odd ratio was calculated for exposed individuals (either passive or active smokers) against not exposed individuals (non-smokers). The results of this analysis were given in Table 3.23, but as can be seen from table, no significant association was present for neither of the genotype and imputed phenotypes.

Table 3.23 Analysis of interaction of single genetic polymorphisms and imputed phenotypes for *EPHX1* gene and maternal smoking exposure as risk factors for development of childhood ALL.

Analyses	Not Exposed n= 27	Exposed n=79	COR 95%CI	p
***EPHX1* exon 3 polymorphism**				
W_3	12	40	1.0 (Referent)	
H_3	7	22	0.9 (0.3-2.7)	0.91
M_3	8	17	0.6 (0.2-1.8)	0.40
Either M_3 *or* H_3	15	39	0.8 (0.3-1.8)	0.58
***EPHX1* exon 4 polymorphism**				
W_4	19	60	1.0 (Referent)	
H_4	8	17	0.7 (0.3-1.8)	0.43
M_4	0	2	-	-
Either M_4 *or* H_4	8	19	0.7 (0.3-1.9)	0.57
***SULT1A1*2* polymorphism**				
*SULT1A1 *1*1*	17	52	1.0 (Referent)	
*SULT1A1 *1*2*	7	23	1.1 (0.4-2.9)	0.90
*SULT1A1 *2*2*	3	4	0.4 (0.1-2.1)	0.30
*Either *1*2 or *2*2*	10	27	0.9 (0.4-2.2)	0.79
Imputed phenotypes for EPHX1				
Normal	11	30	1.0 (Referent)	
Fast	4	13	1.2 (0.3-4.4)	0.79
Slow	4	19	1.7 (0.5-6.2)	0.39
Very Slow	8	17	0.8 (0.3-2.3)	0.65

Among 106 fathers, 19 of them were non smokers, 5 of them were passive smokers and 82 of them were active smokers. Case-only odds ratio analysis for fathers was carried out for both passive against non-smokers and active against non-smokers groups with different genotypes of *SULT1A1*2* polymorphism, exon 3 and 4 polymorphisms of *EPHX1* gene and imputed phenotypes for *EPHX1* gene. In these analyses, genotypes and imputed enzyme activities did not demonstrate significant associations (data were not shown) for both passive and active smoker groups. The results of analysis for grouping passive and active smokers as exposed individuals were represented in Table 3.24. Interestingly, exposure of father either passively or by active smoking together with slow activity phenotype for *EPHX1* gene increased the risk of ALL 6.2 fold. (95%CI: 0.7-52.4, p=0.05). As can be seen from table, no significant association was present for the other genotypes and imputed phenotypes. When the analysis of paternal exposure to cigarette smoke (active or passive) and co-presence of two risk genotypes were carried out, again no significant associations was found for the risk of development of childhood ALL (data not shown).

Table 3.24 Analysis of interaction of single genetic polymorphisms and imputed phenotypes for *EPHX1* gene and paternal smoking exposure as risk factors for development of childhood ALL.

Analyses	Not Exposed n= 19	Exposed n=87	COR 95%CI	p
***EPHX1* exon 3 polymorphism**				
W_3	8	44	1.0 (Referent)	
H_3	4	25	1.1 (0.3-4.2)	0.85
M_3	7	18	0.5 (0.2-1.4)	0.19
Either M_3 or H_3	11	43	0.7 (0.3-1.9)	0.50
***EPHX1* exon 4 polymorphism**				
W_4	12	67	1.0 (Referent)	
H_4	7	18	0.5 (0.1-1.4)	0.08
M_4	0	2	-	-
Either M_4 or H_4	7	20	0.5 (0.2-1.4)	0.21
***SULT1A1*2* polymorphism**				
*SULT1A1 *1*1*	13	56	1.0 (Referent)	
*SULT1A1 *1*2*	5	25	1.2 (0.4-3.6)	0.79
*SULT1A1 *2*2*	1	6	1.4 (0.2-12.6)	0.77
*Either *1*2 or *2*2*	6	31	1.2 (0.4-3.4)	0.74
Imputed phenotypes for EPHX1				
Normal	9	32	1.0 (Referent)	
Fast	2	15	2.1 (0.4-3.4)	0.74
Slow	**1**	**22**	**6.2 (0.7-52.4)**	**0.05**
Very Slow	7	18	0.7 (0.2-2.2)	0.58

The cigarette smoke questionnaire also includes the information of whether mothers smoked during pregnancy or not. Among 106 mothers, 94 of them did not not-smoked and 12 of them had smoked during pregnancy. Analyses were carried out for the interaction of genetic polymorphisms and maternal smoking during pregnancy as risk factor for the development of childhood ALL. The results of these analyses were represented in Table 3.25. The smoking of mother during pregnancy together with homozygous mutant genotype for exon 4 polymorphism of *EPHX1* gene increased the risk of childhood ALL 6.9 fold however this association was not statistically significant (95%CI: .4-119.3, p=0.12). As can be seen from table, no significant association was present for the other genotypes and imputed phenotypes. When the analysis of maternal smoking during pregnancy and co-presence of two risk genotypes (combination of the genotypes) were carried out, again no significant associations was found for the risk of development of childhood ALL (data not shown).

Table 3.25 Analysis of interaction of single genetic polymorphisms and imputed phenotypes for *EPHX1* gene and maternal smoking during pregnancy as risk factors for development of childhood ALL.

Analyses	Not Smoked n= 94	Smoked n=12	COR 95%CI	p
***EPHX1* exon 3 polymorphism**				
W_3	45	7	1.0 (Referent)	
H_3	28	1	0.2 (0.-1.9)	0.15
M_3	21	4	1.2 (0.3-4.6)	0.77
Either M_3 or H_3	49	5	0.7 (0.2-2.2)	0.49
***EPHX1* exon 4 polymorphism**				
W_4	69	10	1.0 (Referent)	
H_4	24	1	0.3 (0.0-2.4)	0.22
M_4	1	1	6.9 (0.4-119.3)	0.12
Either M_4 or H_4	25	2	0.6 (0.1-2.7)	0.46
***SULT1A1*2* polymorphism**				
*SULT1A1 *1*1*	59	10	1.0 (Referent)	
*SULT1A1 *1*2*	28	2	0.4 (0.1-2.1)	0.27
*SULT1A1 *2*2*	7	0	-	-
*Either *1*2 or *2*2*	35	2	0.4 (0.1-1.6)	0.16
Imputed phenotypes for EPHX1				
Normal	35	6	1.0 (Referent)	
Fast	16	1	0.4 (0.0-3.3)	0.35
Slow	22	1	0.3 (0.0-2.4)	0.20
Very Slow	21	4	1.1 (0.3-4.4)	0.90

Information related with the postnatal exposure of child to cigarette smoke was available for 105 children. Among them, 32 children were not exposed and 73 were exposed to cigarette smoke postnatally. The results of the analysis for association of postnatal exposure of child to cigarette smoke with genetic polymorphisms were represented in Table 3.26. Slow activity phenotype for the *EPHX1* gene and postnatal exposure to cigarette smoke was increased the risk of ALL 2.0 fold but insignificantly (95%CI: 0.6-7.3, p= 0.27). As can be seen from table, no significant association was present for the other genotypes and imputed phenotypes. When the analysis of postnatal exposure to cigarette smoke and co-presence of two risk genotypes (combination of the genotypes) were carried out, again no significant associations was found for the risk of development of childhood ALL (data not shown).

Table 3.26 Analysis of interaction of single genetic polymorphisms and imputed phenotypes for *EPHX1* gene and postnatal exposure to cigarette smoke as risk factors for development of childhood ALL.

Analyses	Not Exposed n= 32	Exposed n=73	COR 95%CI	p
***EPHX1* exon 3 polymorphism**				
W_3	14	37	1.0 (Referent)	
H_3	7	22	1.2 (0.4-3.4)	0.75
M_3	11	14	0.5 (0.2-1.3)	0.15
Either M_3 or H_3	18	36	0.8 (0.3-1.7)	0.51
***EPHX1* exon 4 polymorphism**				
W_4	22	56	1.0 (Referent)	
H_4	10	15	0.6 (0.2-1.5)	0.27
M_4	0	2	-	-
Either M_4 or H_4	10	17	0.7 (0.2-1.7)	0.39
***SULT1A1*2* polymorphism**				
*SULT1A1 *1*1*	19	49	1.0 (Referent)	
*SULT1A1 *1*2*	11	19	0.7 (0.2-1.7)	0.39
*SULT1A1 *2*2*	2	5	1.0 (0.2-5.4)	0.97
*Either *1*2 or *2*2*	13	24	0.7 (0.3-1.7)	0.44
Imputed phenotypes for EPHX1				
Normal	12	28	1.0 (Referent)	
Fast	5	12	1.0 (0.3-3.6)	0.97
Slow	4	19	2.0 (0.6-7.3)	0.27
Very Slow	11	14	0.6 (0.2-1.5)	0.25

CHAPTER 4

DISCUSSION

The major concern of the current work was the investigation of Thiopurine methyltransferase (TPMT) enzyme polymorphisms in Turkish population comprised pediatric acute lymphoblastic leukemia (ALL) patients and healthy adult controls. Interindividual variations in TPMT enzyme activity due to genetic polymorphisms have strong clinical implications during the treatment of childhood ALL. Polymorphic nature of the enzyme affects the response of patient to drug (6MP) resulting serious adverse reactions even life threatening toxicities. Regarding this, in the current work, the clinical histories of the pediatric patients with *TPMT* defects were investigated retrospectively from hospital records in relation to 6MP related side effects during the treatment of childhood ALL.

Additionally, in the scope of present study, the genetic polymorphisms of two drug metabolizing enzymes which have dual role in both activation and detoxification of various environmental carcinogens were investigated as risk modifier for the development of childhood ALL. Throughout the course of discussion, first the results related with risk assessment part will be handled than the major part related with the *TPMT* polymorphisms in Turkish population will be discussed.

Acute lymphoblastic leukemia is the most common childhood cancer representing 30% of all cancer cases occurring in children. (Cheok *et al*., 2006). There are only a few well established risk factors for childhood leukemia (such as, sex, age, race and certain congenital diseases such as Down syndrome, and neurofibromatosis), which account for only 10% of the childhood leukemia cases (Chang, 2009). Several lines of evidence suggest that “environmental exposure” together with “environmental susceptibility genes” play major role in the development of childhood ALL. “Drug metabolizing enzymes” are regarded as one class of environmental susceptibility genes. It is well established that individuals having a modified ability to metabolize carcinogens are at increased susceptibility to cancer. Therefore, polymorphisms in genes encoding DMEs have strong relevance in determining susceptibility to cancer. Accordingly, an individual carrying the more active form of an enzyme involved in the activation of carcinogen, or deficient alleles of detoxifying enzymes, will be at greater risk of developing cancer (Krajinovic et al., 1999).

As described in more detail previously, microsomal epoxides hydrolase (EPHX1) and sulfotransferase 1A1 (SULT1A1) are two susceptible enzymes due to their dual roles in both activation and detoxification of many procarcinogens and environmental toxicants including tobacco smoke. Genetic polymorphisms altering the activity of SULT1A1 and EPHX1 may modify the individual’s susceptibility for the childhood ALL. Polymorphisms of both *SULT1A1* and *EPHX1* have been widely studied in relation to various cancer types as risk modifiers. However, so far, there have been no reports evaluating the clinical significance of *EPHX1* and *SULT1A1* genetic polymorphisms for the risk of developing childhood ALL. Therefore, a part of current work focused on the effects of *SULT1A1* and *EPHX1* genetic polymorphisms, alone or in combination, as a risk modifier for the development of childhood ALL.

Case-control analyses were done on 206 adult control and 167 pediatric patients with ALL for total of three polymorphisms (two for *EPHX1* gene and one for *SULT1A1* gene) either alone or in combination. The results were represented in Tables 3.10 through 3.21, but the situations that were found to be important for the discussion were summarized below, in Table 4.1.

Considering single locus polymorphisms as risk factor for the development of childhood ALL, only *EPHX1* exon 3 polymorphism (Tyr113His-slow activity allele), alone, represent a significant risk factor for the development of childhood ALL. Neither *SULT1A1*2* (Arg238His-low activity allele) nor *EPHX1* exon 4 (His139Arg-fast activity allele) polymorphisms represented a significant association for childhood ALL. For *EPHX1* exon 3 polymorphism, the homozygous presence of the mutant allele (His/His genotype) was significantly increased the risk of childhood ALL two fold (OR: 2.0, p=0.01, see Table 4.1).

EPHX1 exon 3 polymorphism, alone, represents a risk factor for different types of cancer. For example, the study of Srivastava *et al*., 2008 comprised 160 controls and 106 patients with bladder cancer, demonstrated that *EPHX1* exon 3 polymorphism (His/His genotype) alone, were significantly associated (OR: 2.67 p=0.001) with bladder cancer (Srivastava *et al*., 2008). The study of Khedhaier *et al*., (2008) comprised 246 controls and 314 patients with breast cancer, demonstrated that *EPHX1* exon 3 polymorphism (His/His genotype) alone, were significantly associated (OR: 2.8 p=0.02) with breast cancer (Khedhaier *et al*., 2008).

Table 4.1 Analysis of genetic polymorphisms, alone, or in combination, as risk factors for the development of childhood ALL. The data for this table were drawn from Tables 3.10 to 3.21.

Genotypes	OR (95%CI)	p
SULT1A1		
*SULT1A1*1*2*	0.9 (0.6-1.4)	0.67
*SULT1A1*2*2*	1.5 (0.6-3.5)	0.40
*SULT1A1*2 bearing*	1.0 (0.6-1.5)	0.92
EPHX1 exon 3 (Tyr113His)		
Tyr/His	0.9 (0.6-1.5)	0.76
His/His	**2.0 (1.1-3.6)**	**0.01**
His bearing genotypes	1.2 (0.8-1.8)	0.44
EPHX1 exon 4 (His139Arg)		
His/Arg	0.9 (0.5-1.3)	0.48
Arg/Arg	1.2 (0.3-4.8)	0.81
Arg bearing genotypes	0.9 (0.6-1.4)	0.54
Combined genotypes		
M_3W_4[a]	**1.7 (0.9-3.5)**	**0.05**
M_3H_4[b]	2.2 (0.6-8.0)	0.11
Very slow phenotype	**2.0 (1.1-3.7)**	**0.04**
*SULT1A1*1*2+*M_3	**3.0 (0.9-8.1)**	**0.05**
*SULT1A1*2 bearing+*M_3	2.1 (0.8-5.7)	0.07

[a] M_3W_4: The co-presence of homozygous mutant genotype for exon 3 polymorphism and wild type genotype for exon 4.

[b] M_3H_4: The co-presence of homozygous mutant genotype for exon 3 polymorphism and heterozygous genotype for exon 4.

In the study of Jain *et al*., 2008; the *EPHX1* exon 3 polymorphism was investigated for the risk of squamous cell esophageal cancer (ESCC) in 107 patients and 320 controls. Accordingly, exon 3 polymorphism, particularly, homozygous mutant genotype was significantly increased (OR: 2.3, p=0.03) the risk of ESCC cancer. In this study, (Jain *et al*., 2008) also, haplotype analyses which includes different combinations of two *EPHX1* polymorphisms, were also done, however, none of the haplotype combinations of exon 3 (Tyr113His) and exon 4 (His139Arg) polymorphisms showed modulation of risk for ESCC (Jain *et al*., 2008).

Haplotype analysis for *EPHX1* polymorphisms is very meaningful in terms of functional relevance (phenotype) as both of the variants affect the enzyme activity in different ways. While exon 3 polymorphism (Tyr113His) causes 50% decrease in the enzyme activity, exon 4 polymorphism (His139Arg) causes 25% increase in the enzyme activity. Therefore, it is reasonable to postulate that individuals with specific allelic combinations may be at differential risk for the ability to metabolize reactive epoxides efficiently (Hassett et al., 1994). When exon 3 and exon 4 polymorphisms were considered together, nine different genotype combinations can be seen in the population. In the current study, among nine different genotype combinations (haplotypes), only combinations with the homozygous mutant genotype for exon 3 polymorphism (slow allele) demonstrated an association with the childhood ALL (see Table 4.1). The homozygous presence of slow allele, with wild type genotype for fast allele significantly increased the risk of childhood ALL 1.7 fold (p=0.05). Similarly the homozygous presence of slow allele with heterozygote genotype for fast allele increased the risk for all 2.2 fold (p=0.11). These results being in accordance with the single locus analysis demonstrated the importance of *EPHX1* exon 3 polymorphism in the risk of development of childhood ALL.

Many authors proposed predictive enzyme activity for different combinations of exon 3 and exon 4 polymorphisms. In the present study, according to Smith and Harrison (1997), predictive enzyme activities (imputed phenotypes) were classified as normal, fast, slow and very slow. For example, while fast phenotype is represented by the combination of at least one fast mutation (exon 4) and no mutation in exon 3, very slow phenotype represented by the any combination of exon 4 polymorphism with homozygous mutant genotype for exon 3 polymorphism. In the present study, when individuals were grouped according to predictive enzyme activities only very slow activity phenotype (homozygous mutant genotype for exon 3 polymorphism with any combinations of exon 4 genotype) significantly increased the risk of childhood ALL (OR: 2.0 p=0.04). This result also demonstrated the importance of *EPHX1* exon 3 polymorphism in the risk of development of childhood ALL. Up to this point, the results of all analyses for *EPHX1* gene including single locus, genotype combinations and predictive enzyme activities were in accordance with each other and they all emphasized the role of *EPHX1* exon 3 polymorphism in the risk of development of childhood ALL.

What lies behind the molecular etiology of *EPHX1* slow activity polymorphism being risk factor for childhood ALL? As described in detail previously, the role of EPHX1 enzyme in the detoxification or activation of chemical carcinogens is not very clear. Hydration of reactive epoxides to diol-intermediates by EPHX1 catalyzed reactions seems to be detoxification. However, as described in the metabolism of benzo(a)pyrene in detail (in section 1.9.1 and section 1.10.2.2), further reactions of diol metabolites by CYPs generate highly toxic and carcinogenic compounds (depicted in the figure that shows benzo(a)pyrene metabolism-Figure 1.7). As diol metabolites can undergo reactions with CYPs, alternatively, they can be also conjugated by phase II enzymes like GSTs (see again Figure 1.7) and eliminated from the

body. Therefore, it can be concluded that EPHX1 enzyme with dual function, is associated with both activation and detoxification process. However, several studies conducted by using transgenic mice pointed out the detoxifying role of EPHX1 enzyme in the metabolism of carcinogens and demonstrated the real *in vivo* situation (Jackson *et al*., 2000; Wickliffe *et al*., 2003; 2006). One specific example is worth to mention in this aspect: Wickliffe *et al*., (2006) demonstrated that EPHX1 deficient mice (EPHX1-null) were significantly more sensitive to mutagenic effects of 1,3-butadine which is well established mutagen and carcinogen and supported the critical role that EPHX1 plays in detoxification of reactive epoxides (Wickliffe *et al*., 2006). Therefore, it is reasonable that slow activity polymorphism of EPHX1 enzyme could be a risk factor for the development of cancer as the detoxification of carcinogens decreases due to slow activity. This is also verified by many case-control studies as described above. To our knowledge, the present work is the first one that demonstrates *EPHX1* exon 3-slow activity polymorphism as risk factor for the development of childhood ALL.

It is important to note that the role of EPHX1 in the development of carcinogenesis mostly associated with the role played by various CYP enzymes and also conjugation enzymes (see Figure 1.7 for carcinogenesis caused by benzo(a)pyrene and Figure 1.8 for the carcinogenesis caused by benzene) which in turn greatly affected by genetic polymorphisms. Therefore, investigation of the role of *EPHX1* polymorphisms as risk factor for the development of carcinogenesis could be very informative when considered together with polymorphisms of other drug metabolizing enzymes particularly CYPs and conjugation enzymes such as GSTs. Recently in the study of Ulusoy *et al*., (2007), it was demonstrated that when both *CYP2E1*5B* and **6* alleles were considered together, the risk of childhood ALL significantly increased (2.9-fold; OR = 2.9, 95% CI 1.0–8.5).

In the present study, as said before, *SULT1A1*2* polymorphism, alone, did not confer a risk factor for the childhood ALL (see Table 4.1). The major role of SULT1A1 in the metabolism of carcinogens is detoxification by generating more water soluble and often less toxic metabolites. However, for a minority of the compounds this conjugation reaction may produce electrophiles that can bind to cellular macromolecules like DNA and RNA to form carcinogenic adducts. In view of the role played by SULT1A1 in the metabolism of carcinogens and strong reduction of SULT1A1 activity resulted from *SULT1A1*2* polymorphism, there are many different studies in the literature examining the role of polymorphisms in the development of certain cancers (Steiner *et al*., 2000; Seth et al., 2000; Bamber et al., 2001; Ozawa et al., 2002). However the results from these previous studies are conflicting and some findings appear to the contrary to the prior hypothesis, therefore warranting the additional studies to address the role of this gene in human carcinogenesis. So far, no studies have been found in the literature for the investigation of this polymorphism as risk factor for the childhood ALL. However, this enzyme represents a front line of chemical defense in the human fetus. Today it is very well known that while most of the DMEs such as CYPs and UGTs etc. are not expressed at significant levels until after birth (Coughtrie et al., 1988; Hakkola et al., 1998), SULTs are abundantly expressed in the human fetus (Barker et al., 1994; Hume et al., 1996; Richard et al., 2001). For this reason, it is very reasonable to hypothesize that *SULT1A1*2* polymorphism can be a risk modifier in the development of childhood ALL. Regarding all the points discussed above, in the current study the lack of association between *SULT1A1*2* polymorphism, alone, and risk for childhood ALL seems to be puzzling. The one possibility for the explanation of this result could be as follow: the effects of *SULT1A1*2* polymorphisms as risk modifier in the development of childhood ALL could be polyallelic. The contribution of SULT1A1 to childhood ALL could be strongly influenced by one or more additional

polymorphisms within or close proximity of SULT1A1 gene. Depending on the combination of polymorphic variants, the effects of the **2* variants may be masked due to the presence of other unidentified risk alleles. This may also account for the discrepancy in the role of SULT1A1 polymorphisms in different cancer sites and in different ethnic population. Therefore further studies are needed to elucidate this possibility.

In addition, as it is said above genetic polymorphism of a single DME on its own may not be enough to significantly increase the risk for the disease. But, the combinations of two or more risk genotypes can significantly increase the risk of disease. Infact, in the present study, when analysis were carried out according to combination of the genes for the risk of childhood ALL, the results showed that the co-presence of *EPHX1* exon 3 polymorphism and *SULT1A1*2* polymorphism had a combined effect on the risk of development of childhood ALL. The co-presence of heterozygous genotype for *SULT1A1*2* and homozygous mutant genotype for exon 3 polymorphism of *EPHX1* gene (slow activity genotype) increasing the risk from 0.9 (OR for *SULT1A1*1*2*-heterozygous genotype, see Table 4.1) to 3.0 (p=0.05, see Table 4.1) fold for the risk of childhood ALL. When analysis was carried out for the combination of *SULT1A1*2* bearing genotypes (either homozygous mutant or heterozygous) with homozygous mutant genotype for exon 3 polymorphism the risk for childhood ALL increased 2.1 fold (p=0.07, see Table 4.1). Actually, EPHX1 and SULT1A1 enzymes together involve in the metabolism of various substances like polyaromatic hydrocarbons and benzene (see Figure 1.6). In these pathways, SULT1A1 generally detoxify metabolites which are produced by EPHX1 catalyzed reactions, through sulphate conjugation. Therefore, the combined effect of *SULT1A1*2* with *EPHX1* exon 3 polymorphism both of which causes low enzymatic activity, on the risk of development of childhood ALL supported the fact that genetic polymorphism of one single gene on it is own may not

enough to significantly increase risk for the disease. However, the accumulation of multiple high risk genotypes within a metabolic pathway of well-established carcinogens can have striking effects.

Related with the risk assessment part of the present study, some non genetic risk factors -parental age at conception and cigarette smoking exposure of parents and children- were investigated to evaluate their interactions with genetic polymorphisms of SULT1A1 and EPHX1 genes. These analyses were carried out by using case-only approach as the information related with non-genetic factors was only available for patient group. The way of categorization of the patients according to non-genetic risk factors and the number of patients in each category were previously represented in Table 3.1.

Parental age at conception is one of the non-genetic risk factor for the development of childhood ALL that was analyzed for the interaction with genetic polymorphism in the scope of present work. It is well established that maternal age is younger than 20 and older than 35, besides, paternal age older than 40 at the conception are risk factors for the development of childhood ALL (Kaye *et al*., 1991; Dockerty *et al*., 2001). For example, it has been shown that risk of childhood ALL was significantly increased 2.1 fold among the children of mothers older than 35 years of age and 1.6 fold among the children of older fathers (Kaye et al., 1991). In this study as represented in Table 3.1 and previously mentioned in the results part, the case only analysis of mothers was done for ages younger than 20 and older than 35 (risky ages) against ages between 20-35 (no-risk) with different genotypes of *SULT1A1*2* polymorphisms and exon 3 and exon 4 polymorphism of *EPHX1* gene and predictive enzyme activities for *EPHX1* gene. However, neither ages younger than 20 nor ages older than 35 for the mother together with genetic polymorphisms represents a risk factor for the development of

childhood ALL. In addition, same analysis was carried out for father age older than 40, but again, no significant association has been found.

The other case only analyses were done on the interaction of genetic polymorphisms and smoking exposure of the mother, father and postnatal exposure of child. Tobacco smoke contains at least 60 known carcinogens, with the major classes being volatile hydrocarbons, aldehydes, aromatic amines, polycyclic aromatic hydrocarbons (such as benzo(a)pyrene), nitrosamines and benzene. All these chemicals in tobacco smoke could interact with each other synergistically in a complex way, collectively, to gain a significant carcinogenic effect on the development of leukemia. It is well established that tobacco smoke is a risk factor for the development of adult acute myeloid leukemia (Chang, 2009). However, studies that investigate the association between parental smoking and childhood leukemia have produced inconsistent results (Shu *et al*., 1996; Brondum *et al*., 1999; Chang *et al*., 2006; see section 1.10.1 for detailed examples).

In this study, analyses were carried out by grouping the active and passive smokers as exposed individuals and case-only odd ratio was calculated for exposed individuals (either passive or active) against non-exposed individuals. Among the analyses only paternal exposure demonstrated a significant association. Maternal exposure to cigarette smoke, smoking of mother during pregnancy and postnatal exposure of child did not demonstrate an interactive effect with the genetic polymorphisms, either, alone or in combination on the development of childhood ALL. In terms of paternal exposure, only the slow activity phenotype (predictive enzyme activity) for *EPHX1* gene in children whose father exposed to cigarette smoke (either passively or actively), associated with a significant risk of 6.2 fold (p=0.05).

Why paternal exposure demonstrated a considerably high positive interactive effect with the polymorphism rather than maternal exposure on the development of childhood ALL? The majority of the studies on maternal smoking and childhood leukemia did not find a significant positive associations and some even reported an inverse associations (Chang *et al*., 2006; Chang, 2009). In contrast to studies of maternal smoking, studies of paternal smoking and childhood leukemia reported more positive associations (Chang et al., 2006; 2009). Actually, in terms of carcinogenesis, exposures occurring during an individual's father's life may be more important than exposures occurring during their mother's life. This is because spermatogenesis continues from puberty to old age and hence, there is more opportunity for mutant gene accumulation in men than women (Anderson *et al*., 2000). These genetic mutations accumulated in the father's sperm may transmit cancer susceptibility to the child. The biological mechanism for more positive association of paternal cigarette smoking with childhood ALL risk could be explained by the accumulation of cigarette smoke-induced oxidative DNA damage in human semen, which may cause chromosome breaks that ultimately lead to translocations in utero and childhood leukemia development.

The last important point to be discussed related with the risk assessment part is the frequencies of the *EPHX1* and *SULT1A1* polymorphisms in Turkish population. As risk assessment studies also include healthy control subjects, the results of the present study would also represented valuable information for the allele frequency of the studied polymorphisms in Turkish population. It is well established that the genetic polymorphisms of drug metabolizing enzymes demonstrate significant interethnic variation. In this aspect, it is very important to determine the distribution of genetic polymorphisms in different population and ethnic groups. The control samples of the current study consisted of

206 unrelated healthy individuals. As stated in section 3.1.1, the study sample represented the Turkish population quite well.

To our knowledge, in the current work, *SULT1A1*2* polymorphism was studied for the first time in Turkish population. Accordingly, the frequency of **2*-mutated allele for *SULT1A1* gene in Turkish population was found 0.22. The comparison of *SULT1A1*2* allele frequency in Turkish population (obtained from control population) with various control population was previously represented in Table 3.11. Accordingly, for the Turkish population the frequency of the mutant allele (**2*) was found to be very similar to that observed in other Caucasian populations.

The frequency of exon 3 and exon 4 polymorphisms of *EPHX1* gene were found to be 0.26 and 0.17, respectively. The comparisons of exon 3 and exon 4 polymorphisms (obtained from control population) with various control population were previously represented in Table 3.13 and 3.15, respectively. These polymorphisms have been studied in Turkish population before by other groups (Ada *et al*., 2007; Pınarbaşı *et al*., 2007). Accordingly, the allele frequencies for both polymorphisms found in the present study for Turkish population was highly similar to that were found in other Turkish studies (Ada *et al*., 2007; Pınarbaşı *et al*., 2007). In addition, allele frequencies for both polymorphisms in Turkish population were found to be very similar to other Caucasian population.

In summary, the risk assessment part of the current study provided information on the frequencies of *SULT1A1* and *EPHX1* polymorphisms in Turkish population, to the pool of data found in the literature for many populations with different ethnic origin. Also, case-control analysis revealed that *EPHX1* exon 3 (slow activity allele) polymorphism, alone, has significant associations with the development of childhood ALL, whereas exon 4 polymorphism and *SULT1A1*2* variant has not. The

haplotype analysis for *EPHX1* polymorphisms demonstrated that some haplotype combinations especially ones with homozygous mutant genotype for exon 3 polymorphism represent a significant associations with the risk of development of childhood ALL. Case-only analysis revealed that slow activity phenotype of child in terms of *EPHX1* gene together with paternal smoking exposure has considerably positive interactive effect on the development childhood ALL.

As it is pointed out at the beginning, the major focus of the current study was the investigation of thiopurine methyl transferase enzyme polymorphisms in Turkish population comprised pediatric ALL patients and healthy adult controls. In addition, in the present study, a retrospective investigation of the patients with TPMT defects were also carried out in relation to 6-mercaptopurine related adverse reactions during the treatment of childhood ALL. The drug compound “6MP” is widely used in the standard ALL treatment protocols together with many other chemotherapeutic agents. The cure rate for the childhood ALL improved considerably over the 40 years with those currently marketed medications. In fact, today, about 85% of children with ALL survived more than five years or more (Cheok *et al*., 2006).

Chemotherapeutic agents used in the treatment of childhood ALL has very narrow therapeutic index (Relling, 2003). This means the dosage that is required to achieve anticancer efficacy is not very much lower than the dosage that may result in life-threatening toxicity or adverse effects. Therefore, any variation in drug metabolism can disrupt the balance between efficacy and toxicity of a drug and lead to a drug induced adverse effects including relapse of the therapy and even death from toxicity or secondary tumors. For this reason, childhood ALL represents a disease that can greatly benefit from individualizing dosages. According to the pediatric clinicians, a 90% of cure rate for ALL is quite possible in the

near future with the successful implementation of the pharmacogenetics into clinical practices. The relation between *TPMT* polymorphisms and 6MP related side effects in the treatment of childhood ALL exhibits best example for the clinical implications of pharmacogenetics.

6MP is one of the essential chemotherapeutic agent used in the routine treatment of childhood ALL. In standard protocols, it is administered as **daily oral dose** throughout the 2-3 years during the maintenance phase of the chemotherapy (Chessells *et al*., 1997; Evans *et al*., 1998). 6MP is partly metabolized by TPMT enzyme. TPMT activity, in all cells and tissues, is regulated by common genetic polymorphisms occurring throughout the gene, which are partly responsible for interindividual differences observed in enzyme activity. Altered TPMT activity predominantly results from SNPs located in the open reading frame of the gene. Up to date, 23 SNPs (**2-*18* and **20-*23*) in the *TPMT* gene associated with low or deficient enzyme activity have been identified (Teml *et al*., 2007). Among the defective alleles, more than 80% of all low activity cases can be attributed to four *TPMT* alleles: *TPMT*2, *3A, *3B* and **3C* (Yates *et al*., 1997). The remaining 19 *TPMT* variants have been named as rare alleles as they are seen rarely in the populations. TPMT activity is inherited as an autosomal codominant trait. Accordingly inherited deficient or low TPMT activity associated with homozygous mutant genotype whereas intermediate activity is associated with heterozygote genotype.

It is well known that patients with inherited low levels of TPMT activity are prone to develop severe or even hematological toxicity when administered with standard doses of 6MP (Relling *et al*., 1999a; Mcleod and Siva, 2002). However, these individuals can safely be treated with the 6 -11% of conventional doses (Mc Leod *et al*., 1999; Relling *et al*., 1999a; McLeod and Siva, 2002). Furthermore, heterozygote individuals with

intermediate TPMT activity are highly sensitive to myelosuppressive effects of 6MP. Thus, in most of the cases, heterozygote individuals, comprising 11% of the Caucasian population, require significant dose reduction (60-70 % of standard doses) for safe treatment (Mc Leod *et al*., 1999; Relling *et al*., 1999a; McLeod and Siva, 2002). As the problem related with the toxicity of this drug is very dramatic, TPMT is the one of the first drug metabolizing enzyme for which genetic test is widely requested in many countries during the therapy with 6MP. Moreover, it is one of the first DMEs that FDA has added a recommendation for TPMT genetic test before the prescription of 6MP into package insert.

The distribution of common defective *TPMT* alleles have been studied extensively in many different populations all over the world due to its significant inter- and intraethnic variation and as well as due to its strong clinical implications. However, up to 2007, no information was available in the literature for the Turkish population. The knowledge of main defective *TPMT* frequencies in a population is essential to estimate the proportions of risk groups under 6-mercaptopurine therapy. High proportions of risk groups (homozygous mutant and heterozygous individuals) in a population may put the genetic test for *TPMT* into routine clinical practices before the prescription of the 6MP for safe dosing. On the other hand, if the proportion of risk groups in a population is very low, then the right strategy should be the genotyping of the patient who develop 6MP related side effects in the first application of the drug, rather than genotyping of every patient before the prescription of 6MP. First and foremost, as more patients and clinicians become aware of the importance of *TPMT* pharmacogenetics in safe dosing of 6MP, less children will suffer from the 6MP related side effects. With this in mind, in 2007, the preliminary results of the present study were published in order to provide clinically useful information (Tumer *et al*., 2007). The preliminary results were based on the analysis of common defective

TPMT alleles in 106 pediatric patients with ALL. In addition, in order to evaluate the relationship between genotype and 6MP related adverse reactions, clinical histories of the patients with defective TPMT were analyzed retrospectively. To our knowledge, this report (Tumer *et al*., 2007) was the first one that presents data for *TPMT* polymorphism in Turkish children with ALL. According to this preliminary data, of 106 children with ALL, two patients were found to be heterozygous in terms of *TPMT*3C* and *TPMT*3A* alleles with genotype of *TPMT*1/*3C* (patient's ID: 62) and *TPMT*1/*3A* (patient's ID: 74). One patients was found to be compound heterozygous mutant carrying both **3A* and **3C* defective alleles. The remaining 103 patients were apparently homozygous for the wild type allele (*TPMT*1/*1*) i.e. they did not carry any of the tested polymorphism (*TPMT*2*, **3B*, **3C* and **3A*). Considering this report, *TPMT*3A* and **3C* were the only deficiency alleles detected in the sample population with allele frequencies of 0.9% for both (Tumer *et al*., 2007; data represented at Table 4.2). In the further study which was carried out in the context of the current work, we increased the number of patients from 106 to 167. In addition, 206 healthy adult volunteers were genotyped for most common defective *TPMT* alleles. In accordance with the preliminary data, it was found that **3A* and **3C* were the only deficiency alleles detected in both patient (consisted of 167 individual) and control group (206 individual). As represented in Table 4.2, neither **2* nor **3B* was detected in patient and control groups. In patient group, the number of individuals with defective *TPMT* alleles did not change. However, the frequency of **3A* and **3C* decreased to 0.6% for both allele which was not significantly different from the previous finding (0.9% for both), as the number of individuals genotyped increased from 106 to 167. In control population, the frequency of **3A* and **3C* allele were found to be 0.5% for both.

In the literature, there has been no difference in the frequencies of mutant *TPMT* alleles between adult control populations and pediatric patient groups (McLeod *et al*., 1999; Ameway *et al*., 1999; Hon *et al*., 1999; Hiratsuka *et al*., 2000; Rossi *et al*., 2001; Chang *et al*., 2002; Kham *et al*., 2002). In accordance with these reports, in the present study also the frequencies of **3C* and **3A* alleles between patient and control groups did not differed so much. Actually they were found to be nearly same (0.5% vs. 0.6%, p=1.00, see Table 4.2). In view of this fact, the data for both patient and control groups were pooled in order to obtain sufficiently large sample population representing Turkish population well (overall 373 individuals). Accordingly, in Turkish population, the frequencies of **3C* and **3A* were found 0.5% for both deficiency allele (see Table 4.2).

Table 4.2 TPMT allele frequency for preliminary results (Tumer et al., 2007), for patient group (167 patient), control group (206 individual) and for patient and control group (overall; 373 individual).

	No. of Alleles	**Frequency of TPMT alleles (%)**				
		1	***2***	***3B***	***3C***	***3A***
[a]**Tumer *et al*., 2007**	212	98.2	0.0	0.0	0.9	0.9
Patient group	334	98.8	0.0	0.0	0.6	0.6
Controls	412	99.0	0.0	0.0	0.5	0.5
Patients & Controls	746	99.0	0.0	0.0	0.5	0.5

[a]Based on preliminary results of 106 pediatric patient with ALL.

The allele frequency data obtained for overall Turkish population (373 individual) were compared with the various control adult populations and it is represented in Table 4.3. Accordingly, **3C* allele frequency in Turkish population (0.5%) was significantly lower ($p<0.05$) than the Africans like Kenyans (5.4%) and Ghanaian (7.6%) but it was similar to other Caucasians (0.2-1.0%) and Asians (0.6-0.8%) (Ameway *et al*., 1999; Hon *et al*., 1999; Hiratsuka *et al*., 2000; Rossi *et al*., 2001; Chang *et al*., 2002; McLeod and Siva, 2002; Wei et al., 2005; Alves et al., 2004). In case of **3A* defective allele, the frequency in Turkish population (0.5%) was found to be significantly ($p<0.05$) lower than the American Caucasians (3.2%) and Latin American populations (3.1-3.6%) (Hon *et al*., 1999; Isaza *et al*., 2003; Larovere *et al*., 2003). Besides, the comparison of **3A* allele frequency in Turkish population with the European Caucasian populations also showed significant differences.

As seen in Table 4.3, the **3A* allele frequency (0.5%) was found to be significantly low ($p<0.05$) in Turkish population compared to British, French and Italian whites (McLeod and Siva, 2002; Ameway *et al*., 1999; Rossi *et al*., 2001). In addition the frequency of total defective allele in the current study (1.0%) was also significantly lower ($p<0.05$) than British (5.3%); French (7.0%) and Italian (4.9%) populations.

Table 4.3 Allele frequencies (%) of common TPMT variants in different populations.

	Japan	Taiwanese	Indian	Kenyan	Ghanaian	Argentina	Colombian	American	British	French	Italian	Kazak	**Turkish**
*TPMT*2*	0	0	0	0	0	0.7	0.4	0.2	0.5	0.5	0	0	**0**
*TPMT*3A*	0	0	0.5	0	0	3.1[#]	3.6[#]	3.2[#]	4.5[#]	5.7[#]	3.9[#]	0.3	**0.5**
*TPMT*3C*	0.8	0.6	0.8	5.4[#]	7.6[#]	0	0	0.2	0.3	0.8	1.0	0.9	**0.5**
No. of Alleles	384	498	400	202	434	294	280	564	398	382	412	654	**746**
References	a	b	c	d	e	f	g	h	i	j	k	l	**This study**

[#]statistically significant differences ($p<0.05$)

a: Hiratsuka *et al*., 2000; b: Chang *et al*., 2002; c: Kham *et al*., 2002; d: Alves *et al*., 2004; e: Ameway *et al*., 1999; f: Larovere *et al*., 2003; g: Isaza *et al*., 2003; h: Hon *et al*., 1999; i: Ameway *et al*., 1999; j: Mc Leod and Siva, 2002; k: Rossi *et al*., 2001; l: Wei *et al*., 2005

As described above, *TPMT*3A* and *TPMT*3C* were the only deficiency alleles detected in our sample population. Considering their percent distributions, in Turkish population it was found that both of the deficiency alleles contribute equally to the overall frequency i.e. while **3A* comprises 50% of all defective alleles **3C* accounts for the remaining 50%. However in Caucasian populations **3A* comprises 60-89% of all deficiency alleles whereas *3C accounts for only 5-15% of mutant alleles (Alves *et al*., 2004). Up to date studies showed that the genetic polymorphisms of many drug metabolizing enzymes like *CYP1A1, CYP2E1*, *CYP2C9, and GSTM1/T1* in Turkish population are similar to Caucasian population (Aynacıoğlu *et al*., 1999; Balta *et al*., 2003; Ada *et al*., 2004; Ulusoy *et al.*, 2007a, 2007b). However data represented in the scope of present work showed that distributions of *TPMT* polymorphisms are different compared to other Caucasians except Kazak population which is also Caucasian in ethnic origin. Kazaks were the only Turkic people whose *TPMT* polymorphisms were studied (Wei *et al*., 2005).

As presented in Table 4.3, the frequencies of defective *TPMT* alleles in Turkish population were found to be very similar to those found in Kazak population. Therefore, these data showed that the frequency of mutations and the allelic distributions of *TPMT* gene in Turkish and Kazak populations are different from the genetic profile found among Caucasian populations. This may be related with the migration pattern of Turkic people from Central Asia into Anatolia, Middle East and Europe. In this respect, further studies are needed to analyze *TPMT* gene polymorphism in different Turkic countries like: Azerbaijan, Kyrgyzstan, Turkmenistan, and Uzbekistan.

As stated earlier, in the context of the present study, the clinical histories of the patients with defective *TPMT* alleles were examined from hospital records retrospectively. Those patients had been received curative therapy for ALL and during their treatment period their TPMT status had not been known. Retrospective examination of their clinical histories was done with the aim of displaying a relationship between genotype and reaction to treatment involving 6MP. As a result of these examinations highly remarkable associations were observed. Before explaining those observations, briefly reviewing the action mechanism and dose limiting toxicities of 6MP make it easy to understand the association between TPMT deficiency and adverse reaction to 6MP.

As represented in Figure 4.1 (A), 6MP is activated to cytotoxic thioguanine nucleotides (TGNs) which are incorporated into the DNA and trigger cell death (Lennard and Maddocks, 1983). Therefore, 6MP exerts its anticancer effects through cytotoxicity created by active TGNs. In hematopoietic tissues, besides activation, 6MP is also inactivated through S-methylation catalyzed by TPMT enzyme (see Figure 4.1-A). By this pathway, the concentration of 6MP which is available for the activation pathway is decreased. In fact, clinical studies well established that the concentration of active TGNs was inversely proportional with the TPMT activity (Coulthard and Hogarth, 2005). In the treatment of ALL, the standard dose of 6MP is often adjusted to cause a moderate “target” degree of **myelosupression-**supressing the production of blood cells (RBC, white blood cells and platelets)- to overcome proliferation of leukemic cells (Relling, 2003). The target degree of myelosuppression can be tolerated by the patients (see Figure 4.1-A).

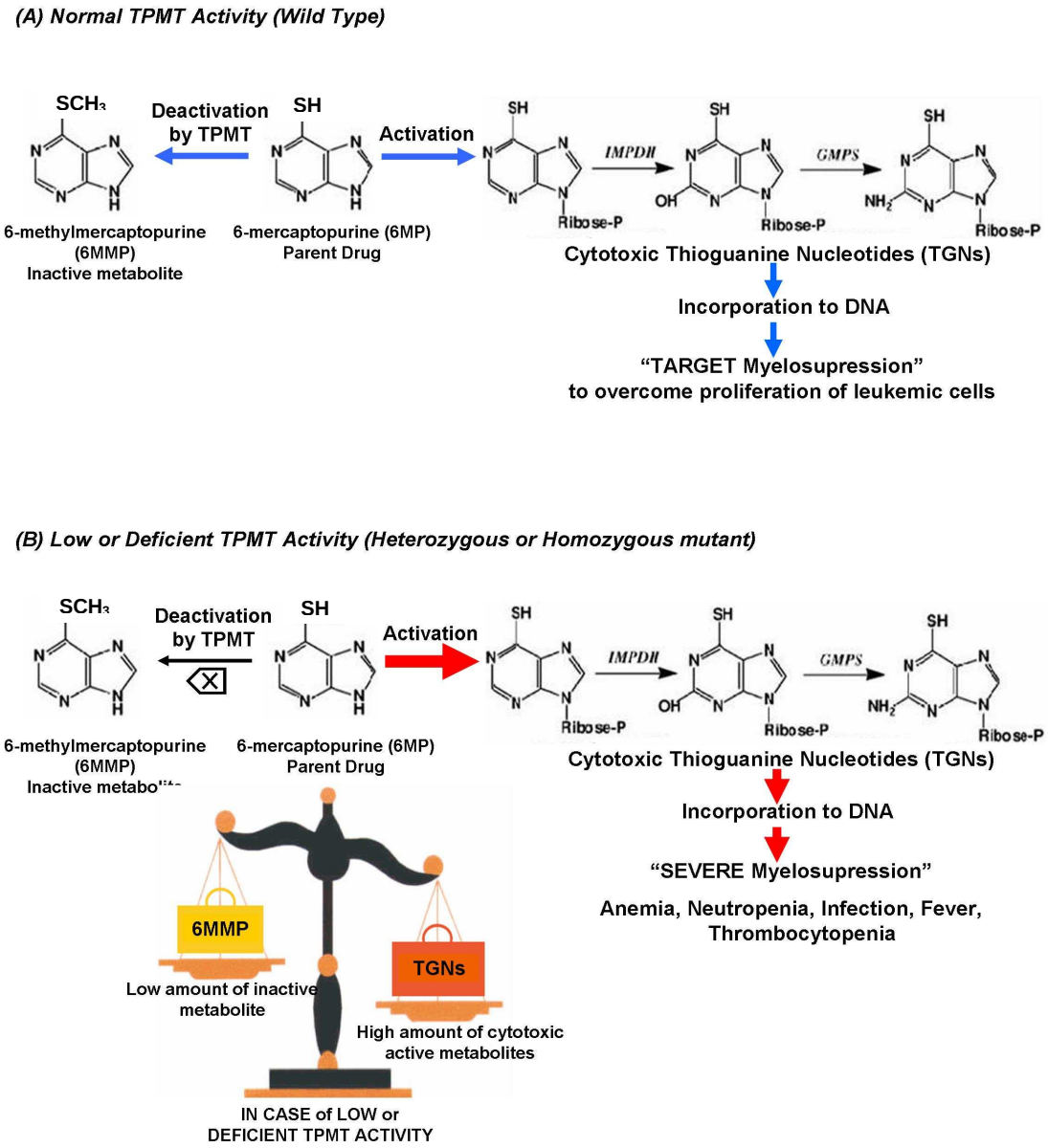


Figure 4.1 Activation and deactivation of 6MP (A) in TPMT wild type individuals (B) in TPMT heterozygous or homozygous mutant individuals. For simplicity xhanthine oxidase pathway was omitted.

However, as it is demonstrated in Figure 4.1(B) if an individual have low or deficient TPMT activity due to genetic polymorphisms, the greater amount of standard drug dose will be converted to cytotoxic TGNs which cause severe myelosupression. This condition can rapidly lead to life-threatening infection, as the body cannot produce white blood cells in response to invading bacteria and viruses, as well as leading to anemia due to lack of red blood cells and spontaneous severe bleeding due to deficiency of platelets. When patient undergo severe myelosupression, generally no therapy could be administered until the blood counts returns to the normal values. Otherwise, this could result in sepsis- a whole body inflammatory state-, and theoretically, lead to death from a serious infection (Relling, 2003).

In the current study, detailed examinations of the hospital records of the patients with defective *TPMT* alleles revealed that these patients experience severe neutropenia-abnormally large fall of neutrophil in the blood- and infections systematically after the administration of 6MP. As demonstrated in Table 4.4, it was observed that patient with compound heterozygous mutant genotype (**3C/*3A*) (Patient's ID: 81) had to be withheld from the therapy during the 42% of the maintenance period due to febrile neutropenia and infections. This observation is in agreement with the study of McLeod *et al*, (1999), who reported that one patient with two defective *TPMT* alleles could not receive the chemotherapy during the 53% of the maintenance period (McLeod *et al*., 1999). In case of heterozygote patient with **3A* defective *TPMT* allele (**1/*3A*) (Patient's ID: 73), the treatment had to be withheld about 15% of maintenance period again because of febrile neutropenia and infections. In that patient, thrombocytopenia (low platelets counts) and anemia were also reported often together with neutropenia. For this patient, it had been specifically

reported that lesions around mouth together with herpes simplex virus developed whenever 6-mercaptopurine was administered. The other heterozygote patient with **3C* defective allele (**1/*3C*) (Patient's ID: 62) had not been able to receive therapy about 28% of the maintenance period because of infections and low blood counts.

Table 4.4 6MP related side effects observed retrospectively in the patients with TPMT defects.

Genotypes of Patients	Clinical Histories
TPMT*3C/*3A Compound Heterozygous Mutant	Treatment was withheld nearly 42% of total period of therapy due to severe neutropenia, infection and anemia.
TPMT*1/*3A Heterozygous	Treatment was witheld 15% of therapy because of febrile neutropenia and anemia. Hospitalization oftenly for fever and infections, specifically lessions around the mouth together with herpes simplex virus.
TPMT*1/*3C Heterozygous	Treatment was withheld nearly 28% of total period of therapy because of neutropenia and infection.

In summary, when standard doses of 6MP are prescribed to patients with undiagnosed TPMT deficiency or heterozygosity, this can cause severe myelosupression (manifested by neutropenia, fever and infections), necessitating withholding from all the components of chemotherapy including 6MP until the blood counts recover. The interruption of the therapy for a while, adversely affects the chance for cure as the proliferation of leukemia cells may restart during this lag period (Relling *et al*., 1999a). However, as told before homozygous mutant and heterozygous individuals can be safely treated with the lower doses of 6MP. Today, it is well established that patients with a 'deficient methylator' status (homozygous mutant) can be started on 6–10% of the standard dose of 6MP therapy, while heterozygous patients (intermediate methylators) can start at 65% of the standard dosage (Evans *et al*., 1991; Lennard *et al*., 1993; Mc Leod *et al*., 1993; Andersen *et al*., 1998).

Severe myelosupression is an acute adverse effect of 6MP which occurs in patients with TPMT defects (either homozygous mutant or heterozygous) when they are administered with standard drug dose. However, in the long term period, the TPMT-deficient genotype has also been associated with a risk of a secondary malignancy, including secondary leukemia and brain tumors, in patients treated with antimetabolites and cranial irradiation (Relling *et al*., 1999b; 1999c). In view of this fact, three patients with defective *TPMT* alleles in our patient population -especially the ones with complete *TPMT* deficiency (TPMT*3C/*3A-patient's ID: 81) should be followed carefully in the years to come.

It is also important to note that in our sample population while the genotypes of all defective allele carriers were in accordance with their

clinical histories; in our patient group there were two cases with similar histories that did not have any of the tested common *TPMT* risk alleles (**2,*3A, *3B* and **3C*). However, they had not been able to tolerate 6MP for 27% and 36% of the maintenance therapy. Therefore, it is thought that these two cases may have any of the untested rare alleles (remaining 19 rare alleles: **4-*18*, **20-*23*) which were found to be associated with low or defecient enzyme activities by the various studies found in the literature (Schaeffeler et al., 2006; 2008; Lindqvist *et al*., 2004; Ujii *et al.,* 2008). This possibility should be questioned and needed further analysis.

Recently a highthroughput multiplex MALDI-TOF mass spectrum (MS) assay that allows efficient genotyping for all currently known functional *TPMT* variants: 23 *TPMT* alleles (*2 to 18 and *20 to 23) was developed by Professor Mathias Schwab' group at the Margaret Fischer-Bosch Institute of Clinical Pharmacology in Stuttgart, Germany. We contacted by Prof Schwab and asked for the analysis of two cases described above in terms of all currently known functional *TPMT* alleles (23 TPMT alleles). In his laboratory, genotyping of 156 patients including two cases described above in our patient group were kindly carried out in terms 23 *TPMT* alleles by multiplex MALDI-TOF MS assay. For this analysis, we isolated a new batch of high quality DNA samples for 156 patients and sent them to the laboratory in Germany. The details of the multiplex MALDI-TOF MS method for detecting all TPMT variants were given in Appendix C.

The MALDI-TOF MS analysis of rare alleles in our patient group detected a single case with **14* allele. None of the other rare *TPMT* alleles were detected in our sample population. The genotype of this patient (patient' ID: 57) were determined as TPMT**1*14* (heterozygous for *14 allele). *TPMT*14* allele was characterized by A to G transition in the start codon of exon 3 region. This transition occurring in highly conserved

nucleotide position results in a substitution of Methionine with Valine in the encoded protein. Up to date, *TPMT*14* allele was identified in one patient with inflammatory bowel disease and in her father in Swedish population. Both of this individuals demonstrated low TPMT activity (Lindqvist *et al*., 2004). In a recent *in vitro* study conducted by Ujii *et al*., (2008), TPMT*14 was heterologously expressed in COS-7 cells and expression level was found to be considerably lower than that of wild type allele $p<0.005$ (Ujii *et al*., 2008). For patient with **14* allele (patient' ID: 57) in our patient group, self communication with the physician who attended to treatment of this patient revealed that she successfully completed the therapy with no considerable 6MP related side effect. Now she was followed without therapy by the physician.

As said before MALDI-TOF MS analysis allows the identification of all currently known defective *TPMT* alleles including the common ones (*2 and *3). In terms of common *TPMT* mutations, the results of MALDI-TOF analysis were perfectly matched with our results. It is important to emphasize that the patient's ID and genotypes determined by MALDI-TOF MS assay were one to one matched with those determined by PCR based assays in our laboratory. Therefore, the PCR-RFLP (for detection of *TPMT*3B*, **3C* and **3A*) and allele specific PCR (for detection of *TPMT*2*) assays which were optimized in our laboratory resulted in 100% agreement with the highly robust and sensitive MALDI-TOF MS assay.

Relating the results of MALDI-TOF analysis to the clinical histories of the patients, in summary, there were three discordant cases in our patient groups. These are two cases that have not any of the known defective TPMT alleles but experiencing 6MP related side effects during therapy, and patient with **14* rare allele who did not experience any side effects during 6MP therapy. One possible explanation for these discordant cases may be related with the polymorphisms occurring in transporters.

As was the case for polymorphisms in DMEs, potential polymorphisms in genes encoding membrane transporters that are responsible for the efflux of the drug/metabolite may also influence the accumulation of drug or its metabolites in tumor and host tissue. (Wall and Rubnitz *et al*., 2003) Some ATP-binding cassette (ABC) transporters especially MRP4 and MRP5 preferentially promote the efflux of 6MP and its active/cytotoxic TGN nucleotides from tumor cells; thus, it is possible that polymorphisms that affect these transporters' function could alter 6MP/TGNs efflux. For example, any deficiency in either MRP4 or MRP5 due to genetic polymorphisms may cause accumulation of TGNs to toxic levels. This could be one possible explanation for the 6MP related adverse reactions that is observed in *TPMT* wild type individuals. Conversely, if the variants of MRP4 and MRP5 genes have increased the activity of exports proteins, 6MP and its active TGNs metabolites would be more avidly exported out of cells; therefore intracellular level of cytotoxic metabolites decrease. The latter example could be the case for individual with *TPMT*1*14* genotype in our patient population. The identification of polymorphisms in MRP4/5 still continues. In the view of all the discordant cases in our study, there is strong evidence that in addition to TPMT pharmacogenetics, polymorphisms in drug transporters possibly play a critical role in altered 6MP pharmacology. In the future, this subject will take the great attention in the evaluation of 6MP related toxicities.

Another important point with TPMT enzyme, besides 6MP, it also catalyzes other thiopurine drugs: AZA, which has wide use in the treatment of autoimmune disorders, inflammatory bowel disease, Crohn's disease and following the organ transplantation and 6TG, which is mostly used in the treatment of acute myeloid leukemia, organ transplantation and rarely some of the ALL protocols. They are both metabolized like 6MP by the same activation and deactivation pathways. Actually AZA is

non-enzymatically cleaved to 6MP in the body and the rest of the metabolism is the same with 6MP (as depicted in Figure 1.3). TG is also activated to TGNs and inactivated by TPMT. In case of AZA and TG, the reported toxic events in the TPMT deficient patients are as severe as 6MP case. Because of the severity of the reported cases, in 2002, together with 6MP, the FDA has also added recommendation for TPMT genetic test before the prescription of AZA and 6TG into package insert.

Although the use of 6TG and 6MP is overlapping to some extent in the treatment of childhood ALL, actually, each thiopurine drug is specifically addressed to different group of patients. For this reason, TPMT polymorphisms have wide range of clinical impact in different patients groups from children to adult, and from cancer patients to patients of autoimmune disorders, inflammatory bowel disease and organ transplantations. Therefore, TPMT polymorphisms have extremely important clinical implications for highly diverse and wide range of population.

Affecting such a diverse and wide range of patient population, pharmacogenetics of TPMT is one of the most active research areas in clinical pharmacogenetics. Today, TPMT testing (genotype, phenotype) have been already implemented into practical medical exercise in many countries especially in USA, UK, Germany and Netherlands (Hopkins *et al*., 2006). However, it has been still controversial that should all patients be tested for TPMT status before they receive first dose of thiopurine? Some propose that such tests should be implemented into **routine** medical practices for every patient before the prescription of 6MP to arrange safe drug dose. Some suggest that only those patients who experiencing severe acute myelosupression when administered with standard dose of 6MP should be tested for TPMT status. Nonetheless, the most important fact is that, genetic variations of TPMT have striking

impacts on the dose of thiopurines that patients can tolerate. In view of this fact, as more patients and clinicians become aware of the importance of *TPMT* pharmacogenetics in safe dosing of 6MP, genetic test will work its way into the treatment of related diseases.

In conclusion, this work, for the first time, identified the distribution and frequency of all common and rare *TPMT* alleles in Turkish population. In terms of common defective *TPMT* alleles, *TPMT*3A* and *TPMT*3C* were the only common deficiency alleles detected in Turkish population with an allele frequency of 0.5% for both. Neither *2 nor *3B were detected in any of the sample. These results also confirmed by highly robust and sensitive MALDI-TOF MS technique for 156 pediatric patients with ALL. Patient's ID and genotypes determined by MALDI-TOF MS assay were one to one matched with those determined by PCR based assays in our laboratory and demonstrated 100% consistency. In terms of rare alleles, *TPMT*14* variant was the only rare allele detected in a single case in Turkish population. By this study, for the first time, an association was represented retrospectively between 6MP related adverse effects and TPMT defects during the therapy of Turkish children with ALL. It is important to recognize that patients with defective TPMT alleles had developed neutropenia, infection and some other specific conditions (like lesions around mouth, oral herpes caused by herpes simplex virus and high fever) systematically when they were administered with 6MP. There were two cases demonstrated 6MP related side effects which could not be explained by their genotypes. The one of the possible explanation for these discordant cases could be the transporter polymorphisms. Further studies are warranted to elucidate the role of transporter polymorphisms in 6MP related adverse reactions.

Lastly, today, the importance of TPMT pharmacogenetics has been well recognized in many countries all over the world. TPMT determination

before the therapy with thiopurines or in case of acute hematotoxicity during thiopurine therapy is widely recommended for pharmacogenetically guided safe dosing. The findings of the present study also demonstrated the clinical importance of TPMT defects in Turkish population. In Turkey, as many clinicians become aware the importance of TPMT pharmacogenetics, the number of patients who experience side effects during the thiopurine therapy will decrease.

CHAPTER 5

CONCLUSION

The relation between *TPMT* genetic polymorphisms and 6MP related adverse reactions in the treatment of childhood ALL represents best example for the clinical implications of pharmacogenetics. In view of this fact, the distributions of common *TPMT* polymorphisms have been extensively studied all over the world and the allele frequencies have been established for many different populations. Therefore, in the context of the present study, our primary focus was the investigation of common defective *TPMT* variants in 167 pediatric patients with ALL and 206 healthy adult controls. In addition, we also retrospectively examined the clinical histories of the patients with *TPMT* defects in order to evaluate the relation between *TPMT* deficiency and 6MP related adverse reactions.

In the present study, besides *TPMT* polymorphisms and drug response-genotype relationship in the treatment of childhood ALL, the polymorphisms of two drug metabolizing enzymes were also investigated as risk modifiers in the development of childhood ALL. In this respect, EPHX1 and SULT1A1 drug metabolizing enzymes were selected due to their dual role in the metabolism of various carcinogens. Up to date the genetic polymorphisms of various drug metabolizing enzymes like CYP1A1, CYP2E1, GSTs, NQO1 etc., were extensively studied in association with childhood ALL. However, the roles played by EPHX1 and

SULT1A1 as risk modifiers in the development of this disease was not studied so far. To the best of our knowledge, in the scope of this study, the polymorphisms of *EPHX1* and *SULT1A1* have been studied for the first time in relation to development of childhood ALL. We investigated two polymorphisms of *EPHX1* gene (exon 3- slow allele, exon 4- fast allele) and one polymorphism of *SULT1A1* gene (*SULT1A1*2*- slow activity allele) in 167 pediatric patients with ALL and 206 healthy adult controls.

The risk modifier role of exon 3 and exon 4 polymorphisms of *EPHX1* gene and *SULT1A1*2* polymorphisms were investigated either alone or in combination in the development of childhood ALL. When investigated alone, only *EPHX1* exon 3 polymorphism, particularly homozygous mutant genotype for this polymorphisms, was found to be significantly associated with the development of disease (OR=2.0, p=0.01). In haplotype analysis for EPHX1 enzyme variants, the homozygous presence of slow allele, with wild type genotype for fast allele significantly increased the risk of childhood ALL 1.7 fold (p=0.05). Regarding the predictive enzyme activities, only very slow activity phenotype (homozygous mutant genotype for exon 3 polymorphism with any combinations of exon 4 genotype) significantly increased the risk of childhood ALL (OR: 2.0 p=0.04). All the results of case-control analyses for *EPHX1* gene, including single locus, genotype combinations and predictive enzyme activities were in accordance with each other and they all emphasized the role of *EPHX1* exon 3 polymorphism in the risk of development of childhood ALL.

Although *SULT1A1*2* polymorphism alone did not confer a risk factor for the development of childhood ALL, the co-presence of *EPHX1* exon 3 and *SULT1A1*2* polymorphisms were found to have a combined effect on the risk of development of childhood ALL. The co-presence of heterozygous genotype for *SULT1A1*2* and homozygous mutant

genotype for exon 3 polymorphism of *EPHX1* showed an increased risk of 3.0 fold (p= 0.05) for the childhood ALL. Therefore, the combined effect of *SULT1A1*2* with *EPHX1* exon 3 polymorphism on the risk of development of childhood ALL supported the fact that genetic polymorphism of one single gene on it is own may not be enough to significantly increase risk for the disease in some cases. However, the accumulation of multiple high risk genotypes can have striking effects.

In case only analysis, some non genetic risk factors -parental age at conception and cigarette smoking exposure of parents and children- were investigated to evaluate their interactions with genetic polymorphisms of *SULT1A1* and *EPHX1* genes. Accordingly, we could not find an interactive association with the paternal age at conception and studied genetic polymorphism on the risk of childhood ALL. In terms of cigarette smoking exposure, maternal exposure to cigarette smoke, smoking of mother during pregnancy and postnatal exposure of child did not demonstrate an interactive effect with the genetic polymorphisms, either, alone or in combination on the development of childhood ALL. In terms of paternal exposure, only the slow activity phenotype (predictive enzyme activity) for *EPHX1* gene in children whose father exposed to cigarette smoke (either passively or actively), associated with a significant risk of 6.2 fold (p=0.05).

As stated at the beginning, the major focus of the current study was the investigation of *TPMT* enzyme polymorphisms in Turkish population comprised pediatric ALL patients and healthy adult controls and retrospective investigation of the patients with *TPMT* defects in relation to 6-mercaptopurine related adverse reactions during the treatment of childhood ALL. Accordingly, among 373 individual (overall) *TPMT*3C* and **3A* were the only common deficiency allele with frequency of 0.5% for both. Neither **3B* nor **2* was detected in Turkish population.

Up to date, studies showed that the genetic polymorphisms of many DME in Turkish population are similar to Caucasian population. On the other hand, in the current study it was found that the distribution of *TPMT* polymorphisms in Turkish population was different compared to other Caucasians but found to be very similar to Kazak population which is also Caucasians in ethnic origin.

In patient population, among 167 individuals, two patients were found to be heterozygous in terms of *TPMT*3C* and *TPMT*3A* alleles with genotype of *TPMT*1/*3C* (patient's ID: 62) and *TPMT*1/*3A* (patient's ID: 74). One patients was found to be compound heterozygous mutant carrying both **3A* and **3C* defective alleles. In terms of common *TPMT* alleles, the genotypes of patients were confirmed for 156 individuals by highly sensitive multiplex MALDI-TOF MS analysis. This analysis was performed at the Margaret Fischer-Bosch Institute of Clinical Pharmacology in Stuttgart, Germany by a technical expert. It is important to emphasize that the patient's ID and genotypes determined by MALDI-TOF MS assay were one to one matched with those determined by PCR based assays in our laboratory and resulted in 100% agreement with the highly robust and sensitive MALDI-TOF MS assay. In terms of rare alleles, MALDI-TOF MS assay detected a single case who has **14* rare alleles (genotype-*TPMT*1*14*).

In the current study, detailed examinations of the hospital records of the patients with defective *TPMT* alleles revealed that these patients experience severe neutropenia and infections systematically after the administration of 6MP.

It is also important to note that in our sample population while the genotypes of all common defective allele carriers were in accordance with their clinical histories; there were also three cases with unexpected

histories that could not be explained by their genotypes. One of them is patient with **14* rare allele (heterozygote individual). This patient successfully completed the therapy with no considerable 6MP related side effect (this information obtained from self communatication with the physician who attended to the treatment of patient). There were also two cases that were apparently wild type for all currently known *TPMT* polymorphisms (including rare ones) however they had not been able to tolerate 6MP for 27% and 36% of the maintenance therapy. The one of the possible explanation for these discordant cases could be the transporter polymorphisms. Further studies are warranted to elucidate the role of transporter polymorphisms in 6MP related adverse reactions.

Today, the importance of *TPMT* pharmacogenetics has been well recognized in many countries all over the world. The findings of the present study also demonstrated the clinical importance of TPMT defects in Turkish population. In Turkey, as many clinicians and patients become aware the importance of TPMT pharmacogenetics, the number of patients who experience side effects during the thiopurine therapy will decrease.

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APPENDIX A

APPROVAL FROM ETHICAL COMITTEE, INFORMED CONSENT FORM AND QUESTIONAIRRE

A1. Aproval From Ethical Committee

ANKARA ÜNİVERSİTESİ TIP FAKÜLTESİ ETİK KURULU
RESEARCH ETHICS COMMITTEE OF MEDICAL FACULTY, ANKARA UNIVERSITY
ANKARA-TÜRKİYE
ARAŞTIRMA BAŞVURUSU ONAYI

BAŞVURU BİLGİLERİ	PROTOKOL KODU	
	PROTOKOL ADI	Akut Lösemi Riski ve Akut Lösemili Hastaların Tedavisinde Kullanılan İlaçların Metabolizmasında Rol Oynayan Bazı Faz I ve Faz II Enzimlerinin Polimorfizmlerinin Araştırılması
	SORUMLUARAŞTIRICI ÜNVANI / ADI	Prof.Dr.Sevgi Gözdaşoğlu Prof.Dr.Emel Arınç
	ARAŞTIRMA MERKEZİ	Ankara Üniversitesi Çocuk Sağlığı ve Hastalıkları Anabilim Dalı ODTÜ Biyoloji Bölümü
	DESTEKLEYİCİ FİRMA	

DEĞERLENDİRİLEN İLGİLİ BİLGİLER	Belge Adı	Değişiklik No. / Tarihi	Dili
	PROTOKOL		
	ARAŞTIRICI BROŞÜRÜ		
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLURU		
	OLGU RAPOR FORMU		

ÇALIŞMA ESASI	İYİ KLİNİK UYGULAMALARI KLAVUZU

KARAR BİLGİLERİ	Karar No: 81-2125	Tarih: 21 Kasım 2005
	Araştırma protokolüne tamamen uyulmak, Ankara Üniversitesi Tıp Fakültesi Etik Kurul Yönergesinde belirtilen hususlar yerine getirilmek ve Yönergenin 11/h maddesi gereği sorumluluk araştırıcılara ait olmak üzere bütçesi temin edildiği taktirde laboratuvar çalışmasının "yürütülmesinde etik sakınca bulunmadığına toplantıya katılan öğretim üyelerinin oybirliği ile karar verildi.	

ETİK KURUL ÜYELERİ				
Ünvanı / Adı / Soyadı	Uzmanlık Dalı	Kurumu	Cinsiyeti	İmza
Prof. Dr. İsmail Hakkı Ayhan Başkan	Farmakoloji	AnkaraTıp Fakültesi	E	
Prof. Dr. Efser Kerimoğlu Başkan Yardımcısı	Çocuk Psikiyatrisi	AnkaraTıp Fakültesi	K	
Prof. Dr. Özden Palaoğlu Sekreter	Farmakoloji	AnkaraTıp Fakültesi	K	

13 Aralık 2005

ASİL GİBİDİR

Syf.1

A1. Approval From Ethical Committee (Continue)

Prof. Dr. Işık Sayıl Üye	Psikiyatri	Ankara Tıp Fakültesi	K	
Prof. Dr. Sevim D.Cengiz Üye	Kadın Doğum	Ankara Tıp Fakültesi	K	
Prof.Dr. Nermin Mutluer Üye	Nöroloji	Ankara Tıp Fakültesi	K	
Prof.Dr. Sumru Beder Üye	Göğüs Hastalıkları	Ankara Tıp Fakültesi	K	
Prof. Dr. Nurten Girgin Üye	Çocuk Sağlığı ve Hastalıkları	Ankara Tıp Fakültesi	K	İstanbul'da
Prof. Dr. Ragıp Çam Üye	Genel Cerrahi	Ankara Tıp Fakültesi	E	
Prof. Dr. Ali Rıza Uysal Üye	Endokrinoloji	Ankara Tıp Fakültesi	E	
Prof. Dr. Birsel Erdem Üye	Mikrobiyoloji	Ankara Tıp Fakültesi	K	Sınavda
Prof. Dr. Ahmet Demirkazık Üye	Tıbbi Onkoloji	Ankara Tıp Fakültesi	E	
Prof. Dr. Günhan Gürman Üye	Hematoloji	Ankara Tıp Fakültesi	E	
Prof. Dr. Ajlan Tükün Üye	Tıbbi Genetik	Ankara Tıp Fakültesi	K	
Prof. Dr. Işınsu Kuzu Üye	Patoloji	Ankara Tıp Fakültesi	K	
Prof. Dr. Özer Kendi Üye	Adli Tıp	Ankara Tıp Fakültesi	E	Rahatsız
Prof.Dr. Erdal Onar Üye	Hukuk	Ankara Üniv. Hukuk Fakültesi	E	
Prof.Dr.Yasemin Oğuz Üye	Deontoloji	Ankara Tıp Fakültesi	K	Derste
Prof. Dr.Serenay Elgün Ülkar Üye	Biyokimya	Ankara Tıp Fakültesi	K	
Ecz. Funda Aytun Üye	Eczacılık	Ankara Tıp Fakültesi	K	

T.C. ANKARA ÜNİVERSİTESİ TIP FAKÜLTESİ DEKANLIĞI

13 Aralık 2005

ASLI GİBİDİR

A2. Informed Consent

ONAY BELGESİ

Hastanın tedaviye cevabını gösterecek olan ve O.D.T.Ü Biyoloji Bölümünde yütütülecek 'Türkiye'de Akut Lösemi Hastası Çocuklarda İlaç Metabolizmasında Rol Oynayan Enzimlerin Genetik Polimorfizmlerinin Araştırılması' için, çocuğumdan alınan 2 – 3 mL kan örneğinin kullanılacağını biliyor ve onay veriyorum.

Anne veya Babanın adı soyadı: Tarih:
İmza:

HASTA BİLGİLERİ

1. Hastanın adı soyadı:
2. Hastanın takip edildiği hastane ve protokol numarası:
3. Kız Erkek
4. Yaş:
5. Doğum yeri ve yılı:
6. Eş akrabalığı var mı?:
7. Anne doğum yeri: Baba doğum yeri:
8. Hastanın klinik tanısı:
9. Tedavinin aşaması:
10. Hastanın risk grubu:
11. Tedavi şekli (eğer sabit bir tedavi protokolü varsa belirtilmeli, böyle bir protokol yoksa kullanılan ilaçların adı ve dozajı belirtilmelidir):
12. Hasta son 3 aylık dönemde kan transfüzyonu aldı mı? (evet ise en son kan transfüzyonun yapıldığı tarihi belirtiniz):
13. Çocuğun daha önce geçirmiş olduğu ya da halen var olan (lösemi dışındaki) önemli hastalıklar varsa belirtiniz:
14. Ailede başka kanser hastası var mı? Varsa kanserin çeşidini ve bu kişinin hasta ile akrabalık derecesini belirtiniz:
15. Tam kan sayımı sonuçları:
    Not: Sonuçlar bilgisayar çıktısı halinde ise onay belgesinin arkasına zımbalanmalıdır.
16. Karaciğer fonkisyon testleri sonuçları:

A.3 Cigatte Smoking Status Questionnaire

MALİGNNİTE VE SİGARA İLİŞKİSİ FORMU

ADI SOYADI:

YAŞI:

MALİGNNİTE TİPİ:

EVRESİ:

REMİSYON EVET HAYIR

ANNE YAŞI:

SİGARA İÇME: AKTİF PASİF( )SÜRESİ:

BAŞLAMA YAŞI:

KAÇ YILDIR İÇİYOR:

HAMİLELİKTE İÇME: EVET HAYIR

MİKTARI: TANE/GÜN

BABA YAŞI:

SİGARA İÇME: AKTİF PASİF

BAŞLAMA YAŞI:

KAÇ YILDIR İÇİYOR:

MİKTARI: TANE/GÜN

POSTNATAL SİGARAYA MARUZİYET: EVET HAYIR

EVETSE SÜRESİ(AY OLARAK):

AKRABALIK DÜZEYİ:

AYLIK EKONOMİK NET GELİR(YTL):

APPENDIX B

BUFFERS and SOLUTIONS

All the glassware used for DNA isolation, genotyping and lymphocyte isolation procedures were sterilized by autoclaving and sterilized distilled water was used for the preparation of solutions.

Tris-HCl, pH 8.0 (100 mM);

12.1 g Tris was weighed and dissolved in 700 mL of dH_2O. pH was adjusted to 8.0 with concentrated HCl and volume was completed to 1 L. Solution was autoclaved for sterilization and stored at 4°C.

EDTA, pH 8.0 (500 mM);

186.1 g $Na_2EDTA.2H_2O$ was weighed and dissolved in 700 mL dH_2O. Dissolution of EDTA was achieved by adjusting the pH to 8.0 with NaOH. Volume was completed to 1 L. Solution was autoclaved for sterilization and stored at 4°C.

TKME (Tris-KCl-$MgCl_2$-EDTA) Buffer, pH 7.6;

10mM Tris-HCl (pH 7.6), 10 mM KCl, 4 mM $MgCl_2$, 2 mM EDTA.

Solution was autoclaved for sterilization and stored at 4°C.

Saturated NaCl (6M)

3.5064 g NaCl was weighed and dissolved in 10 mL of sterilized dH_2O.

Solution was autoclaved for sterilization and stored at 4°C.

TE (Tris-EDTA) Buffer, pH 8.0;

10 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0).

Solution was autoclaved for sterilization and stored at 4°C.

TBE (Tris-Borate-EDTA) Buffer, pH 8.3;

5x stock solution: 54 g Trizma-base and 27.5 g boric acid were weighed and dissolved in appropriate amount of water. 20 mL of 500 mM EDTA (pH 8.0) was added. pH was set to 8.3. Volume was completed to 1 L. Solution was autoclaved for sterilization and stored at room temperature to prevent precipitation.

0.5x solution: The stock solution was diluted 10 times with sterilized dH_2O prior to use to achieve 45 mM Tris-borate, 1 mM EDTA.

Ethidium Bromide (10 mg/mL);

0.1 g ethidium bromide was dissolved in 10 mL dH_2O. Solution was stirred on magnetic stirrer for several hours to ensure that dye had completely dissolved. As this solution is light sensitive, the bottle was covered with aluminum foil and stored at room temperature.

Gel loading dye

0.25% bromophenol blue, 40% sucrose in sterilized dH_2O.

Solution is stored at 4°C.

PCR Amplification Buffer (10x) (Fermentas);

100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% Nonidet P40.

This buffer and 25 mM $MgCl_2$ solution were supplied together with Taq DNA Polymerase. Taq Polymerase, amplification buffer and $MgCl_2$ solutions were stored at -20°C.

dNTP Mixture (Fermentas);

10 mM of each dATP, dCTP, dGTP and dTTP in aqueous solution. The solution was stored at -20°C.

TANGO™ Buffer (digestion buffers) (Fermentas);

33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA.

Buffer B^+ (digestion buffer) (Fermentas);

10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mg/mL BSA.

Gene Ruler 50 bp DNA Ladder (0.5 mg DNA/mL) (Fermentas);

This commercial DNA ladder was prepared from a specially designed plasmid pEJ3 DNA, containing pUC, λ phage and yeast genome sequences. pEJ3 DNA was completely digested with *Eco*147I and *Pvu*I. The ladder was dissolved in storage buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA).

6x Loading dye solution: 0.09% bromophenol blue, 0.09% xylene cyanol FF, 60% glycerol, 60 mM EDTA.

The ladder was prepared by mixing DNA ladder: 6x loading dye solution: dH_2O in 1:1:4 ratio, mixed well and applied to the gel.

The DNA ladder contained the following discrete fragments (in base pairs): 1031 900 800 700 600 500 400 300 250 200 150 100 50.

APPENDIX C

METHOD FOR MULTIPLEX MALDI-TOF MS ANALYSIS

A new batch of Genomic DNA for 156 patients was isolated to the highest purity by Nuclospin Blood Kit (Macherey-Nagel, Germany) from whole blood as described in section 2.2.1., in METU biology department. DNA sample from each patient was put into a special safe lock eppendorf tube and accurately labeled according to patient' ID. The special safe-lock eppendorf tubes to put the DNA samples were sent from Germany to us. DNA samples were sent to Dr Elke Schaeffeler in the laboratory of Prof Mathias Schwab at the Institute of Dr. Margarete Fischer-Bosch, Clinical Pharmacology in Germany. Genotyping of the 23 TPMT SNPs (genotyped for *2, 3B, 3C, 3A, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23) by MALDI-TOF analysis was carried by technical expert for 156 patients. This technique simply is based on mass spectrometry (MS) of allele specific primer extension products. Briefly, 5 ng of genomic DNA was amplified by PCR in a final volume of 8 µl containing allele specific primers (designed for each SNP) at 167 nM final concentrations and 0.1 unit HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). PCR conditions were 95℃ for 15 min for hot start, followed by 44 cycles of denaturation at 95℃ for 30 sec, annealing at 56℃ for 30 sec, and extension for 1 min at 72℃, finally followed by incubation at 72℃ for 10 min. PCR products were treated with shrimp alkaline phosphatase (SAP, Amersham, Freiburg, Germany) for 20 min at 37℃ to remove excess dNTPs followed by 10 min at 85℃ to inactivate SAP. Base extension reactions in a final volume of 10 µl contained the extension primers at a final concentration of 0.54 µM and 0.6 units TERMIPol (SolisBioDyne,

Tartu, Estonia). Base extension reaction conditions were 94℃ for 2 min, followed by 40 cycles of 94℃ for 10 sec, 52℃ for 10 sec, and 72℃ for 10 sec. All reactions including PCR amplification, SAP treatment and base extension were carried out in a Tetrad PCR thermal cycler (MJ Research, Watertown, USA). The final base extension products, were treated with the cationic exchange resin AG50W-X8 (Bio-Rad, Munich, Germany) to remove salts from the reaction buffer. For a final volume of 26 µl, 10 µl of resin-water suspension were added into each base extension reaction. Following a quick centrifugation (2,000 rpm/440 x g; 5 min), 0.5µl of reaction solution was dispensed onto a 384 format MTP Anchor ChipTM 400/384 TF (Bruker Daltonik, Leipzig, Germany) prespotted with 1µl matrix solution of 3-hydroxypicolinic acid (3-HPA) by using a Puredisk robot (CyBio, Jena, Germany). A Bruker UltraflexTM MALDI-TOF MS was used for automated data acquisition. Genotyping calls were made using GENOTools software from Bruker (Bruker Daltonik, Leipzig, Germany). All pipetting steps were carried out with the Puredisk robot (CyBio, Jena, Germany).

VITAE

PERSONAL INFORMATION

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Education

2001-2004 Middle East Technical University, Natural and Applied Sciences, Graduate School of Biochemistry, Master of Science (C.GPA: 3.93/4.00).

1996-2001 Middle East Technical University, Faculty of Education, Biology Education and Faculty of Arts and Sciences, Biology, Double Major Program, BS (C.GPA:3.10).

1993-1996 Yalova High School, Yalova

WORKING EXPERIENCES

2002-2008 Middle East Technical University, Faculty of Arts and Sciences, Department of Biological Sciences, Ankara, TURKEY
Research Assistant

2001-Fall Sem. METU College, Ankara, TURKEY
Faculty of Education, Science Education Internship

2000-2001	Turkish Doping Control Center, Hacettepe University, Ankara, TUKEY Literature Search-Part Time Job
Ağustos 2000	Bhosphorus University, Department of Molecular Biology and Genetics, İstanbul, TURKEY Internship
Haziran 2000	Turkish Doping Control Center, Hacettepe Üniversitesi, Ankara, TURKEY Internship
1998-1999	METU Education Society Voluntary Teacher

Teaching experience:

- BIO/BCH 543, Experimental Enzymology
- BIO424, Clinical Biochemistry

PUBLICATIONS

Master of Science Thesis

Boyuneğmez T. (February 2004) Biochemical Monitoring of Toxic and Carcinogenic Organic Pollutants Along the İzmir Bay After the Great Canal Project and Possible Health Effects *MS. Thesis.* 151 pages, Middle East Technical University, Ankara. Supervisor: Prof Dr Emel Arınç

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1**. Boyunegmez Tumer T**., Ulusoy G., Adali O., Sahin G., Gozdasoglu S., Arınç E. (2007) The Low frequency of defective TPMT alleles in Turkish population: A study on Pediatric Patients with Acute Lymphoblastic Leukemia. Am J Hematol. 82(10):906–10.

2. Ulusoy G., Adali O., **Boyunegmez Tumer T**., Sahin G., Gozdasoglu S., Arınç E. (2007) Significance of Genetic Polymorphisms at Multiple Loci of CYP2E1 in The Risk of Development of Childhood Acute Lymphoblastic Leukemia. Oncology. 72 (1–2):125–31.

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Publication: Ulusoy G, Adali O, **Boyunegmez Tumer T**, Sahin G, Gozdasoglu S, Arınç E (2007) Significance of Genetic Polymorphisms at Multiple Loci of CYP2E1 in The Risk of Development of Childhood Acute Lymphoblastic Leukemia. Oncology. 72(1-2):125-31.

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PRESENTATIONS
Presentations in International Congresses

1. **Boyunegmez Tumer T**., Ulusoy G., Adali O., Sahin G., Arınç E., (2008) The Role Of SULT1A1 and EPHX1 Polymorphisms as Risk Modifiers In Childhood Acute Lymphoblastic Leukemia in Turkish Population: A Case-Control Study. Drug Metabolism Reviews- Abstracts from the 10 th European ISSX Regional Meeting, May 22-28, Vienna, AUSTRIA Vol:40, Supplement 1, pp: 141-142.

2. Ulusoy G., **Boyunegmez Tumer T**., Adali O., Sahin G., Arınç E., (2008) Significance of CYP2E1 and NQO1 Polymorphisms in the risk development of childhood acute lymphoblastic leukemia. Drug Metabolism Reviews- Abstracts from the 10 th European ISSX Regional Meeting, May 22-28, Vienna, AUSTRIA Vol: 40, Supplement 1, pp: 134-135.

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4. **Boyunegmez Tumer T**., Ulusoy G., Adali O., Sahin G., Gozdasoglu S., Arınç E. (2007) The Low Frequency Of Major TPMT Variants In Turkish Population: A Study Based On Pediatric Patients With Acute Lymphoblastic Leukemia And Healthy Subjects. ESF-UB Conference in Biomedicine Pharmacogenetics and Pharmacogenomics, June 15-20, Sant Feliu de Guixols, SPAIN. Book of Abstract pp: 6.

5. Ulusoy G., **Boyunegmez Tumer T**., Adali O., Sahin G., Cevik E., Arınç E. (2007) Possible risk factors for childhood acute lymphoblastic leukemia: CYP2E1 and NQO1 polymoprhisms. ESF-UB Conference in Biomedicine Pharmacogenetics and Pharmacogenomics, June 15-20, Sant Feliu de Guixols, SPAIN. Book of Abstract pp: 27.

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9. **Boyunegmez T.,** Arınç E. (2005) Biotransformation Enzyme Activities of Feral Annular Seabream along the Pollution Gradient of Izmir Bay: EROD Activities Correlate with MROD but not with Other Monooxygenase Activities. Marine Environmetal Research-Abstract from 13th PRIMO (Polluant response in marine organisms) Meeting, June 19-22, Alessandria, ITALY. Vol:62, Supplement 1, pp: 52.

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11. **Boyuneğmez, T.**, Bozcaarmutlu, A. and Arınç, E., (2003), Biochemical monitoring of toxic and carcinogenic organic pollutants along the İzmir Bay after great canal project and possible health effects. Turkish Journal of Biochemistry-Abstract from 13th Balkan Biochemistry Biophysical Days and Meeting on Metabolic Disorders, October 12-15, Kuşadası, TURKEY. Vol: 28, pp: 178. **Won Mention Price**.

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1. Ulusoy, G., **Boyuneğmez, T.**, Bozcaarmutlu, A., and Arınç, E., (2002), Kinetic and biochemical evidence suggest that at least two isoforms of P450 participates in N-demethylation of cocaine in rabbit. 17th Biochemistry Congress, June 24-27, Ankara, TURKEY. Book of Abstract pp:534.

CONGRESSES ATTENDED

May 18-22, 2008. 10 th European Regional Meeting, Vienna, AUSTRIA. **Poster Presentation**.

June 15-20, 2007. ESF-UB Conference in Biomedicine, Pharmacogenetics and Pharmacogenomics, Sant Feliu de Guixols, SPAIN. **Poster Presentation**.

October 22-26, 2006. 14 th North American ISSX Meeting, Rio Grande, Puerto Rico, USA. **Oral Presentation**.

June 24-29, 2006. 31st FEBS Congress, İstanbul, TURKEY. **Poster Presentation**.

June 19-22, 2005. 13th PRIMO Meeting, Alessandria, ITALY. **Poster Presentation**.

October 03-08, 2004. 19th Eurepean Workshop on Drug Metabolism, Kemer-Antalya, TURKEY. **Poster Presentation**.

October 12-15, 2003. 13th Balkan Biochemical Biophysical Days and Meeting on Metabolic Disorders, Kuşadasi, TURKEY. **Poster Presentation**.

June 24-27 2002. 17th National Biochemistry Congress, Ankara, Turkey, METU, Ankara, TURKEY. **Poster Presentation**.

SOCIETY MEMBERSHIP

International Society for the Study of Xenobiotics (ISSX)