GENETIC POLYMORPHISMS OF ALCOHOL INDUCIBLE *CYP2E1* IN TURKISH POPULATION

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ABSTRACT

GENETIC POLYMORPHISMS OF ALCOHOL INDUCIBLE CYP2E1 IN TURKISH POPULATION

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Cytochrome P4502E1 (CYP2E1), the ethanol-inducible isoform of cytochrome P450 superfamily, catalyzes many low molecular weight endogenous and exogenous compounds, including ethanol, acetone, drugs like acetaminophen and chlorzoxazone, and industrial solvents like benzene and styrene, most of which are carcinogenic. Besides, it has a high capacity to produce reactive oxygen species. CYP2E1 is induced by ethanol and isoniazid, as well by some pathophysiological conditions like diabetes and starvation. *CYP2E1* gene shows genetic polymorphisms which are thought to play a major role in interindividual variability in drug response and in susceptibility to chemical-induced diseases, like several types of cancers.

It is well established that *CYP2E1* polymorphisms vary markedly in frequency among different ethnic and racial groups. Therefore, in this study, the frequency of two important *CYP2E1* polymorphisms; the single nucleotide polymorphisms C-1019T / G-1259C in 5'-flanking region and T7678A poymorphism in intron 6, in Turkish population was investigated. For this purpose, whole blood samples were collected from 132 healthy volunteers representing Turkish population

and genomic DNA for each subject was isolated in intact form. The genotypes were determined by PCR amplification of corresponding regions followed by restriction endonuclease *Rsa*I, *Pst*I (for C-1019T / G-1259C SNPs) and *Dra*I (for T7678A SNP) digestions.

The genotype frequencies, for C-1019T / G-1259C SNPs, which are in complete linkage disequilibrium, were investigated on 116 DNA samples, and determined as 97.4% for homozygous wild type (c1/c1), 2.6% for heterozygotes (c1/c2) and 0.0% for homozygous mutants (c2c2). The allele frequency of wild type allele (c1) was calculated as 98.7% and that of mutated allele (c2) as 1.3%. The genotype frequencies for T7678A SNP, investigated in 108 DNA samples were determined as 80.6% for homozygous wild type (DD), 19.4% for heterozygotes (CD) and 0.0% for homozygous mutants (CC). The corresponding allele frequencies were 90.3% for wild type allele (D), and 9.7% for mutated allele (C). Genotype frequencies of both polymorphisms fit Hardy-Weinberg equation and showed no significant difference with respect to gender.

The genotype distributions of both polymorphisms showed similarity when compared to other Caucasian populations like French, Swedish, German, and Italian populations, while both polymorphisms studied differed significantly from Chilean, Japanese, Taiwanese and Chinese populations, as compared with Chi-Square test (χ^2-test).

Key words: Cytochrome P4502E1, genetic polymorphism, Turkish population, C-1019T / G-1259C single nucleotide polymorphism, T7678 single nucleotide polymorphism, *Rsa*I/*Pst*I RFLP, *Dra*I RFLP.

ÖZ

TÜRKİYE POPÜLASYONUNDA ALKOL İLE İNDÜKLENEBİLEN CYP2E1 GENETİK POLİMORFİZMİ

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Sitokrom P450 süperailesinin etanol ile indüklenebilen izoformu olan sitokrom P4502E1 (CYP2E1); etanol, aseton, asetaminofen ve klorzokzazon gibi ilaçlar, pek çoğu kanserojen olan endüstriyel çözücüler (benzen ve stiren gibi) de dahil olmak üzere düşük moleküler ağırlıklı pek çok endojen ve egzojen maddeleri katalize eder. Bunun yanısıra CYP2E1, reaktif oksijen türlerinin oluşmasında da etkindir. Bu izoform, etanol ve izoniazid gibi maddeler ve diyabet ve açlık gibi bazı patofizyolojik durumlarda indüklenir. *CYP2E1* geni, kimyasallarla indüklenen kanser gibi pek çok hastalığa maruz kalma riskinde gözlemlenen bireyler arası değişkenlikte etkili olduğu düşünülen genetik polimorfizmler göstermektedir.

CYP2E1 polimorfizm frekanslarının çeşitli etnik ve ırksal gruplar arasında değişkenlik gösterdiği kanıtlanmıştır. Bu çalışmada da, önemli iki *CYP2E1* polimorfizminin; genin 5'- ucundaki C-1019T / G-1259C ve intron 6'daki T7678A tek bazlık polimorfizmlerinin, Türkiye popülasyonundaki frekansı belirlenmiştir. Bu amaç doğrultusunda, Türkiye popülasyonunu temsil eden 132 sağlıklı gönüllüden kan örnekleri toplanmış ve bu örneklerden genomik DNA parçalanmamış halde izole

edilmiştir. Genotipler, bahsedilen bölgelerin polimeraz zincir reaksiyonu ile çoğaltılmasıyla elde edilen gen fragmanlarının, restriksiyon endonükleazlar *Rsa*I, *Pst*I (C-1019T / G-1259C polimorfizmleri için) ve *Dra*I (T7678A polimorfizmi için) ile kesilmesiyle belirlenmiştir.

C-1019T / G-1259C tek bazlık polimorfizmleri yeni jenerasyonlara birbirlerine bağlı olarak aktarılırlar. Bu polimorfizmlerin genotipi 116 DNA örneği için belirlenmiştir ve frekans dağılımı homozigot yabanıl (c1/c1) genotip için %97,4, heterozigot (c1/c2) genotipi için %2,6 ve homozigot mutant (c2/c2) genotipi için %0,0 olarak bulunmuştur. Buna denk düşen alel frekansları yabanıl alel (c1) için %98,7 ve mutant alel (c2) için %1,3 olarak hesaplanmıştır. T7678A tek bazlık polimorfizmin genotipi 108 DNA örneği için belirlenmiştir ve genotip frekansları homozigot yabanıl (DD) için %80,6, heterozigot (CD) için %19,4 ve homozgiot mutant (CC) için %0,0 olarak bulunmuştur. Yabanıl (D) alel frekansı %90,3, mutant (C) alel frekansı da %9,7 olarak hesaplanmıştır. Her iki polimorfik bölgenin genotip frekans dağılımının Hardy-Weinberg denklemine uygun olduğu, ve cinsiyete bağımlı bir farklılık göstermediği bulunmuştur.

Bu çalışmada belirlenen her iki polimorfizmin genotipik dağılımı, Fransız, İsveç, Alman ve İtalyan gibi diğer Kafkas popülasyonlarıyla Ki-kare testi (χ^2-testi) uygulanarak karşılaştırıldığında önemli bir farklılık göstermezken, Şili, Japon, Tayvan ve Çin popülasyonlarından önemli derecede farklılık göstermiştir.

Anahtar kelimeler: Sitokrom P4502E1, genetik polimorfizm, Türkiye popülasyonu, C-1019T / G-1259C, T7678 tek bazlık polimorfizmler, *Rsa*I/*Pst*I RFLP, *Dra*I RFLP.

dedicated to my parents,

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ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
BSA	Bovine serum albumin
COMT	Catechol O-methyltransferase
DPD	Dihydropyrimidine dehydrogenase
EDTA	Ethylene diamine tetra acetic acid
EMs	Extensive metabolizers
FAD	Flavin adenine dinucleotide
FMN	Flavin adenine mononucleotide
GIT	Gastrointestinal tract
GST	Glutathione S-transferase
HMT	Histamine methyl-transferase
HNF1α	Hepatic Nuclear Factor1α
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NAT	N-acetyltransferase
NDMA	N-nitrosodimethylamine
NQO1	NADPH:quinone oxidoreductase
PAHs	Polyaromatic hydrocarbons
PCR	Polymerase chain reaction
PMs	Poor metabolizers
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism

SRSs	Substrate-recognition sites
STs	Sulfotransferases
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TNE	Tris-NaCl-EDTA
TPMT	Thiopurine methyltransferase
UGTs	Uridine 5’-triphosphate glucuronosyltransferases
UMs	Ultra-rapid metabolizers

CHAPTER 1

INTRODUCTION

As every organism has to interact with its environment, it is subject to environmental insults from which it should protect itself, like an insect must protect itself from the toxins of plants that it feeds on. For the highest organisms, humans, these environmental insults involve environmental pollutants, food additives, industrial chemicals, drugs, herbicides and pesticides, which can be totally called as xenobiotics.

Most organisms have developed complex mechanisms by which they protect themselves from xenobiotics. A number of drug metabolizing enzyme superfamilies, including cytochrome P450 dependent monooxygenases, are thought to have evolved as adaptive responses to environmental insult.

Drug metabolizing enzymes are responsible for the processing of xenobiotics to inert derivatives that can be easily eliminated from the body but they also sometimes mediate the toxicity or carcinogenicity caused by chemicals through metabolic activation of protoxins and procarcinogens. Most of the drug metabolizing enzymes show genetic polymorphisms which create interindividual variability in xenobiotic metabolism and hence in the susceptibility of chemical-related diseases or carcinogenesis.

1.1 Polymorphism

By definition, polymorphism is a monogenic trait occuring at least in one population that is caused by the presence of more than one allele at the same gene which yields more than one phenotype in the organism. The frequency of less common allele is usually more than 1%. Polymorphisms generally do not cause any sickness or other problems that would decrease reproductive efficiency. That's why a polymorphism can be found in a population at such high frequency. In contrast, a genetic defect or mutation that causes a serious disease such as cystic fibrosis are found at extremely low frequencies (less than 1%) in humans because sick people do not efficiently reproduce and transmit the trait (Gonzalez, 1999).

1.1.1 Mechanisms of Polymorphism

Types of polymorphisms can be divided into two as functional and nonfunctional polymorphism. 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 non-coding regions of the gene. The coding region of a gene stipulates the structure of the encoded enzyme and variability here has the potential to alter enzyme activity or protein function. The non-coding regions of a gene is 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 non-coding regions is associated with altered levels of protein rather than changes in the protein itself (McKinnon and Evans, 2000).

Polymorphism involves nucleotide or gene insertions and/or deletions, as well as single nucleotide substitutions. The most widely observed polymorphism in human genome, particularly in drug metabolizing enzymes is nucleotide substitutions, or in other word, single nucleotide polymorphisms (SNPs).

1.1.2 Single Nucleotide Polymorphism (SNP)

SNP is a stable substitution of a single base with a frequency of more than 1% in at least one population (Evans and Johnson, 2001). SNPs are distributed throughout the human genome at an estimated overall frequency of one in every 1900 bp, with 1 SNP per approximately 1080 bases in exons (Int. SNP Map Work. Group, 2001). For many genes encoding drug metabolizing enzymes, the frequency of SNPs appear to be more common than the frequency for the whole genome which can be explained by the situation that these enzymes are not essential for survival from an evolutionary perspective (Evans and Johnson, 2001). The widespreadly occuring SNPs in drug metabolizing enzymes may result in (a) 'silent' polymorphisms in coding sequences, which does not cause a change in amino acids, hence in protein properties; (b) formation of a variant protein, which might have altered properties as a consequence of change in structure; (c) polymorphic sequences within both exon and intron regions, which can also result in differential splicing, protein truncation and additional functional anomalies; and (d) polymorphisms in regulatory regions that can alter gene expression, mRNA levels and stability, and consequently protein expression levels (Williams *et al.,* 2001. A scheme showing the significance of SNPs in drug/xenobiotic metabolism is depicted in Figure 1.1.

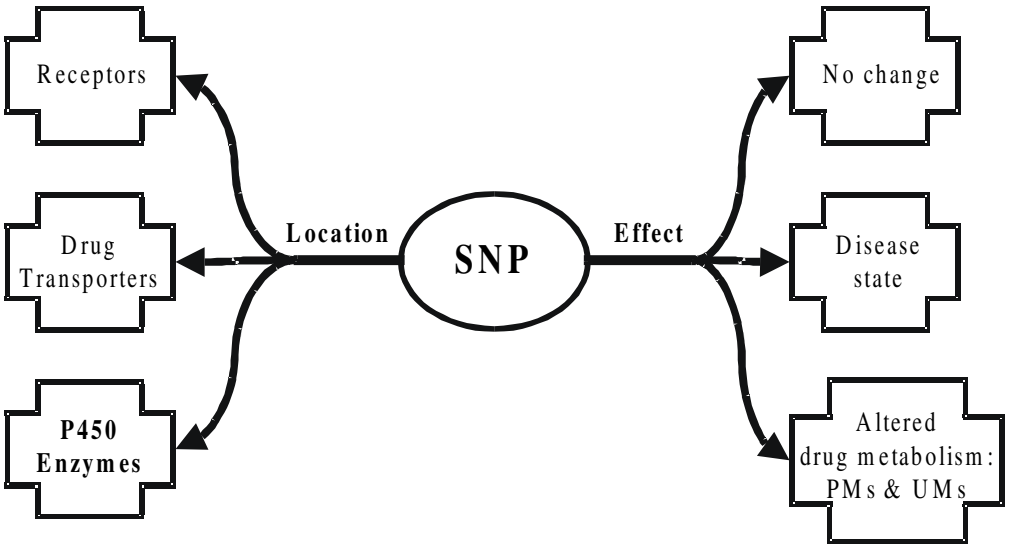


Figure 1.1 Single nucleotide polymorphisms and their significance in drug/ xenobiotic metabolism (taken from Agarwal *et al.,* 2002).

As a result of recent advances in genome technology, it is now easier to identify and study the polymorphisms and their phenotypic consequences in drug metabolizing enzymes. The highly occurring polymorphisms in these enzymes would result in an altered susceptibility to specific chemical-induced diseases. Studies on the association between the drug metabolizing enzyme polymorphisms and the incidence of related diseases are the subjects of pharmacogenetics and pharmacogenomics.

1.2 Pharmacogenetics and Pharmacogenomics

Pharmacogenetics is the study of the linkage between an individual's genotype and that individual's ability to metabolize a foreign compound, and it has a relatively narrower spectrum. Pharmacogenomics has its roots in pharmacogenetics, and is quite broad in scope, uses a genome-wide approach to identify the network of

genes that govern an individual's response to drug therapy (Evans and Johnson, 2001). Anyway, the distinction between these two terms are arbitrary and they are now commonly used interchangebly (Evans and Relling, 1999).

The first observation in this field was done by Pythagorous in 510 BC on some people developing hemolytic anemia after consumption of fava bean (Agarwal *et al.,* 2002). In early 1900's, scientists connected drug related disorders with Mendelian genetics. In 1950's, an enzyme variation in glucose 6-phosphate dehydrogenase, and a deficiency of N-acetyltransferase using the drug isoniazid were discovered. Vogel, in Germany, started the use of the term "Pharmacogenetics" in 1957 (Eichelbaum, 1999). The elucidation of the molecular genetic basis for inherited differences in drug metabolism began in the late 1980s, with the initial cloning of a polymorphic human gene encoding the drug metabolizing enzyme debrisoquin hydroxylase (CYP2D6) (Gonzalez *et al.,* 1988). Pharmacogenomics was born in early 2001 with all other –omics world during the near completion of draft human genome sequence.

1.2.1 Assessing Genetic Variability of Xenobiotic Metabolizing Enzymes

There are often large differences among individuals in the way they respond to drug or xenobiotic exposure. Potential causes for variability in xenobiotic effects include the individual's age, race, gender, nutritional states, smoking status, drug interactions, and concominant illnesses. Although these factors are often important, genetic polymorphisms in the metabolism and diposition of drugs and xenobiotics – which are called pharmacogenetic polymorphisms-, can have an even greater influence.

The variation in drug metabolism gives rise to three phenotypes as poor metabolizers (PMs), extensive metabolizers (EMs) and ultra-rapid metabolizers (UMs). Extensive metabolizers are most widely seen in the population. Poor metabolizers have lower levels of drug or xenobiotic metabolism resulting in higher drug concentrations compared to EMs, which may lead to toxicity. On the other

hand, ultra-rapid metabolizers have increased enzymatic activity which does not allow the drug to reach therapeutic concentrations upon administration in standard doses. Such individuals fail to respond to drug therapy. When the parent compound administered is a prodrug, which requires the bioactivation by the enzyme to the active drug, opposite effects can be observed in PMs and UMs (Weide and Steijns, 1999). Within a population, the appearance of bimodal or trimodal phenotype is strongly suggestive of the presence of genetic polymorphism in drug metabolizing enzymes (McKinnon and Evans, 2000). Variability in drug metabolism may be assessed at the level of phenotype -by investigating the enzyme activity, or genotype -by determining which alleles are present in the gene under question.

Phenotyping is accomplished by administration of a probe drug, known to be selectively metabolized by the enzyme under study (for example, chlorzoxazone for CYP2E1), followed by measurement of the metabolic ratio -the ratio of unchanged drug to its metabolite in serum and/or urine (Weide and Steijns, 1999). The population studies of phenotyping is followed by biochemical and eventually molecular elucidation of the genetic defect responsible for the phenotypic outliners. Such studies, however, have inherent limitations such that they do not distinguish drug-drug interactions; the effect of external factors, like alcohol consumption or cigarette smoking; disease states like impaired renal and liver functions; and malnutritional states. So phenotyping studies provides an indication of altered enzyme activity but does not identify if variability is due to genetic or other causes (McKinnon and Evans, 2000).

Recent advances in genetic, especially sequencing technology, gave rise to the genotypic assessment of drug metabolism variability. Genotyping is usually performed by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) techniques, which can be carried out easily and quickly in a minimally invasive procedure by the use of peripheral leukocytes. The genotyping studies are followed by biochemical studies to assess whether these genetic polymorphisms have phenotypic consequences. The genotyping studies are not affected from external factors, underlying diseases or concominant drug therapy. When the presence of high

number of alleles in drug metabolizing enzymes are considered, new technologies like microarray systems are now introduced to detect all these polymorphisms at once. One example of such technology is the P450 gene chips, introduced by Affymetrix in the United States. These gene chips feature synthetic copies of identified P450 alleles immobilized onto a solid support using photolithographic technology. Such chips offer the potential to accurately genotype large populations with respect to all clinically relevant P450 genes (McKinnon and Evans, 2000).

So in opposite to phenotyping studies, which starts from the phenotypic variation to reach to the genetic polymorphism, genotyping studies first identifies the polymorphisms in the gene and then seeks for their phenotypic outcomes. This latter framework may permit the elucidation of polymorphisms in drug metabolizing enzymes that have more subtle, yet important, consequences for interindividual variability in human drug response and may allow straightforward relationship of a particular genotype to disease incidence such as cancer (Evans and Johnson, 2001).

1.3 Xenobiotic Metabolizing Enzymes

The xenobiotic-metabolizing enzymes are responsible for protecting the organism by rapidly processing chemicals to inert derivatives that can be easily eliminated from the body through urine or bile. Besides detoxification, they also quite often mediate the toxicity of chemicals through metabolic activation of pro-toxins and pro-carcinogens, so they are also thought to have a role in individual susceptibility to chemical induced diseases and cancer. Liver is the main organ for xenobiotic metabolism and transformation reactions. These occur in two distinct stages; namely phase I and phase II reactions.

Major phase I enzyme is the superfamily of cytochrome P450-dependent monooxygenases. P450s exist as a large superfamily of proteins and are the principal enzymes involved in oxidation of foreign compounds including therapeutically used drugs and the metabolic activation of carcinogens and toxins. The common chemical reactions involved in phase I are aromatic hydroxylations, aliphatic hydroxylations,

oxidative N-dealkylations, oxidative O-dealkylations, S-oxidations, reductions and hydrolysis (Schenkman, 1991). Most often this simple functionalization could be sufficient to make a drug more soluble, facilitating the elimination through urine or bile.

Conjugation of the drug or xenobiotic occurs in phase II reactions. Phase II enzymes have the role of conjugating either the parent compound or the metabolite from phase I by glucuronidation, sulfation, acetylation, methylation or glutathione conjugation to facilitate elimination (Gunaratna, 2000).

Almost all enzymes in drug metabolism show differences in expression among species and they are highly polymorphic, contributing to interindividual variability in drug response (Gonzalez, 2001). Drug metabolizing enzymes that are known to exhibit genetic polymorphisms with obvious phenotypical consequences are shown in Figure 1.2.

Cytochrome P450s are the most important components of the drug metabolizing system, as most of the drugs or xenobiotics first encounter with these enzymes in the liver.

1.4 Cytochrome P450s

The cytochrome P450s are hemoproteins that play critical roles in the bioactivation and detoxification of a wide variety of xenobiotic substances. Besides, they have also roles in the metabolism and synthesis of endogenous compounds. These enzymes are encoded by a superfamily of genes and in mammalian cells, they are localized predominantly in the smooth endoplasmic reticulum of hepatocytes. The term “cytochrome P450” originates from the observation that the reduced state of the protein has an absorption band with absorption maxima at 450 nm after binding to carbon monoxide (Omura and Sato, 1964).

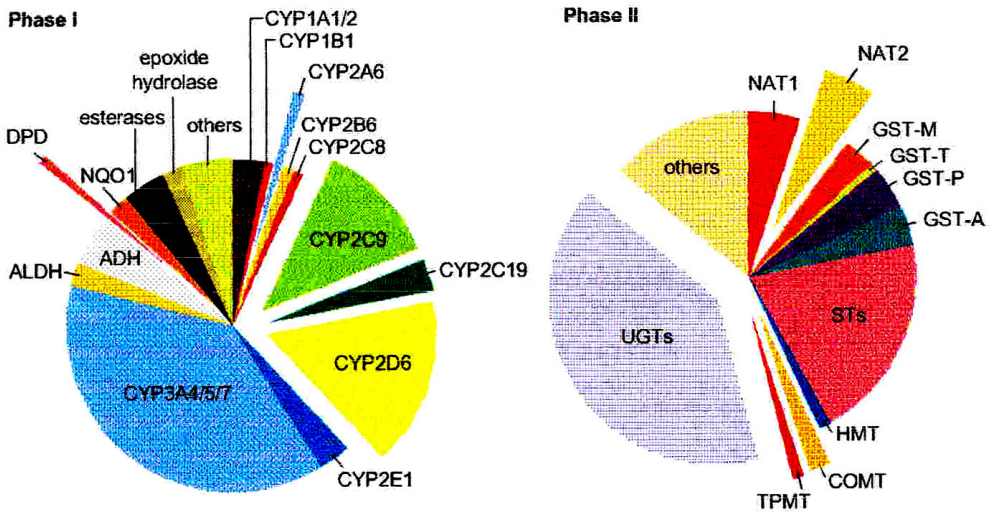


Figure 1.2 Phase I and phase II xenobiotic metabolizing enzymes that exhibit relevant genetic polymorphisms. Enzyme polymorphisms that have already been associated with changes in drug effects are separated from the corresponding pie charts. The percentage of phase I and phase II metabolism of drugs that each enzyme contributes is estimated by the relative size of each section of the corresponding chart (taken from Evans and Relling, 1999).

As shown for the first time by Lu and Coon (1968), in the microsomal system, cytochrome P450 monooxygenase system has three components: cytochrome P450, which catalyzes the monooxygenation reaction; FAD and FMN containing NADPH dependent cytochrome P450 reductase, which catalyzes the electron transfer from NADPH to cytochrome P450; and lipid, which is proposed to facilitate the transfer of electrons from NADPH-cytochrome P450 reductase to cytochrome P450 (Lu and Levin, 1974). 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 and Coon, 1968; Lu and Levin, 1974; Schenkman and Johnson, 1975; Arınç and Philpot, 1976; Black and Coon, 1986; Adalı and Arınç, 1990; Arınç, 1993).

The general reaction catalyzed by cytochrome P450 can be written as follows:

$$NADPH,H^+ + O_2 + RH \longrightarrow NADP^+ + H_2O + ROH$$

where R represent the substrate that has a site for oxygenation, such as an alkane, alkene, aromatic ring or heterocyclic substituents. This is a monooxygenation reaction as one of the two oxygen is incorporated into substrate while the other is reduced to water. Depending on the particular reaction and the nature of various unstable intermediates, different reactions can occur. These include oxidative and reductive dehalogenation; N-hydroxylation and N-oxidation; oxidative deamination; S-, N-, and O-dealkylation; and aliphatic and aromatic hydroxylation (Schenkman, 1991; Guengerich, 1993).

It has been estimated that the total number of P450 substrates may exceed 200,000 which would include the majority of drugs and other xenobiotics, together with several types of endogenous compounds. The endogenous substrates of P450s include saturated and unsaturated fatty acids, eicosanoids, sterols and steroids, bile acids, vitamin D_3 derivatives, retinoids, and uroporphyrinogens. Also, many cytochrome P450 enzymes can metabolize various xenobiotics including drugs, plant- or fungal-derived secondary metabolites consumed with food, and thousands of environmental pollutants- e.g. halogenated hydrocarbons, polycyclic aromatic hydrocarbons, arylamines, ingredients of combustion, industrial complex mixtures, herbicides, and pesticides, resulting in their detoxification (Gonzalez, 1989). Besides, the actions of P450 enzymes can also generate toxic metabolites that contribute to increased risks of cancer, birth defects, and other toxic effects (Nebert and Russell, 2002). Such P450 substrates include polycyclic aromatic hydrocarbons, nitrosamines, hydrazines, and arylamines.

It must be also noted that expression of many P450 enzymes is often induced by the accumulation of the substrate (Schuetz, 2001) and the overlapping substrate specificities of P450 isoforms can result in drug-drug interactions.

1.4.1 Characteristic Structural Features of Cytochrome P450s

Cytochrome P450s have both structurally conserved and variable regions, which maintains its catalytic monooxygenation activity and the great substrate variability, respectively.

Highest structural conservation is found in the core of the protein around the heme and reflects a common mechanism of electron and proton transfer and oxygen activation. The conserved core comprises first, the heme-binding loop with the absolutely conserved cysteine that serves as fifth ligand to the heme iron, which is considered as a P450 signature; second, the absolutely conserved motif on the proximal side of heme, probably stabilizing the core structure; and finally, the proton transfer groove on the distal side of the heme (Werck-Reichart and Feyereisen, 2000).

The heme group of cytochrome P450 is the iron protoporphyrin IX, and the fifth ligand of the heme is the thiolate anion provided by the highly conserved cysteine residue of the cytochrome P450. This stucture contributes to the unusual spectral and catalytic properties of P450. The sixth coordination position of the heme may be occupied by exchangeable water molecule, or by O_2 when iron is reduced.

The most variable regions of cytochrome P450s are associated with either amino terminal anchoring or targetting of membrane-bound proteins, or substrate binding and recognition; the latter regions are located near the substrate-access channel and catalytic site, and are often referred to as substrate-recognition sites or SRSs, in short. These sites are flexible and move upon binding of substrate so as to favor the catalytic reaction. Other variations reflect differences in electron donors, reaction catalyzed or membrane localization (Gotoh, 1992).

1.4.2 Classification and Nomenclature of Cytochrome P450s

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 McKinnon, 1994; Şen and Arınç, 1997,1998;). As of January, 19, 2004, a total of 3043 P450s were identified from various organisms, so that 1277 animal, 1098 plant, 207 lower eukaryotic and 461 bacterial P450s were counted (http://drnelson.utmem. edu/CytochromeP450. html).

Presence of so many P450s in various organisms created a need for systemic nomenclature for cytochrome P450s, and it has been devised in 1987 by Nebert and coworkers which is now being used worldwide. This systemic nomenclature is based on the structural homology, that is, on amino acid sequence similarities between P450 proteins.

According to this system, the cytochrome P450 superfamily is categorized into respective families and subfamilies. P450 proteins exhibiting more than 40% amino acid sequence similarity are classified within the same family, while proteins exhibitng more than 55% sequence similarity are grouped into the same subfamily (Nebert and McKinnon, 1994; Nelson *et al.,* 1996).

In naming of the individual cytochrome P450 enzymes, the abbreviation 'CYP' (Cyp for Drosophila and mouse) -meaning for CYtochrome P450-, is used as a preface to designate the protein under question is a cytochrome P450-dependent monooxygenase. Proteins belonging to the same family are designated with an Arabic numeral, and those belonging to the same subfamily with a capital letter. Lastly, the individual isoform is shown by an Arabic numeral following the capital letter. So, for instance, the ethanol-inducible isoform of cytochrome P450 superfamily is designated as CYP2E1, shortly. The gene encoding the cytochrome P450 isoforms are denoted similarly, but with italics, like *CYP2E1*.

As noted above, there are enormous numbers of P450 in the organisms, a major portion of them belonging to the plants and animals. Also, it is well established that mammals like rats, mice, rabbits, monkeys and -in highest degree- human exhibit genetic polymorphisms in *P450* genes (Gonzalez, 1999). An explanation for the high number of P450 isoforms in organisms and the presence of high degree of polymorphisms in human *P450* genes can be found in the evolution of P450s.

1.4.3 Evolution of Cytochrome P450s

As the sequence comparisons indicated extensive similarity between cytochrome P450 identified in man and bacteria, it is suggested that the P450 superfamily originated from a common ancestral gene about 3 billion years ago (Nebert and Russell, 2002).

P450s are encountered in aerobic organisms, which is expected, as both prokaryotic and eukaryotic P450s utilize oxygen to metabolize chemicals via monooxygenation (Wickramasinghe and Villee, 1975; Cloud, 1976). So the dramatic rise in atmospheric oxygen levels over the last 2 billion years (Cloud, 1976) probably directed the functions of P450 towards utilization of dioxygen for the metabolism of, firstly, endogenous compounds and, subsequently, xenobiotics. Besides the steady rise in atmospheric oxygen levels, the radiation of animal phyla, which is thought to have commenced 543-533 million years ago, corresponds with the appearance of new P450 isoforms (Lewis *et al.,* 1998).

As mentioned before, conserved regions of P450s which are necessary for catalytic activity exhibit substantially lower mutation rates than those related to substrate specificity variations, such as the substrate recognition sites (SRSs) (Gotoh, 1992). Such alterations in substrate specificities of xenobiotic-metabolizing P450s may have resulted from changing dietary requirements during the course of evolution, and coevolution of plants and animals following land colonization (Gonzalez and Nebert, 1990).

The "Plant-Animal Warfare" hypothesis was developed to account for evolution of cytochrome P450s. According to this thesis, the evolution of cytochrome P450s, as well as other xenobiotic-metabolizing enzymes, could be driven by plants producing new chemicals that are toxic to animals, and animals developing new enzymes that can metabolize these toxins (Gonzalez, 1999). This situation has been well documented with the Monarch butterfly where strains having a P450 that detoxifies a furanocoumarin toxin found in a dietary plant source (Cohen *et al.,* 1994; Berenbaum, 1995). As a result, over the course of time, individual populations and different species had their own unique "battery" of enzymes that are reflective of their history of dietary exposure to plants. Accordingly, evolution gave rise to species differences and polymorphisms (Gonzalez, 1999).

During the course of human evolution, the requirement for detoxification of nitrogenous plant toxins became unnecessary due to dietary changes; thus, allelic variants –polymorphisms- which would have arisen spontaneously, became established in the human genome in higher frequencies due to the lack of coevolutionary pressures to supress them (Gonzalez, 1999). This explains why the degree of polymorphism that have phenotypic consequences are higher in human compared to the other organisms.

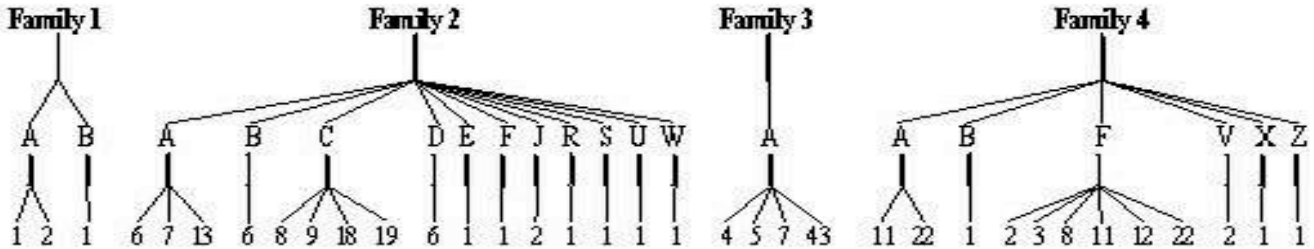
1.4.4 Human Cytochrome P450s

Although the existence of P450 enzymes in human tissues has been known for many years, Kaschnitz and Coon for the first time, were able to partially separate P450, NADPH-P450 reductase and phospholipid from human liver microsomes in 1975; and demonstrated the need for all three fractions in the reconstitution of catalytic activity (Kaschnitz and Coon, 1975). In the late 1970s efforts in several laboratories led to the purification of human P450s to a high degree of purity. It is now possible to express individual human P450 cDNAs in yeast and mammalian cells, and a number of P450s have been prepared in such a way (Guengerich *et al.*, 1991).

The completion of the human genome sequence enabled scientists to determine the human P450 genes, although identification of some genes, especially the pseudogenes creates some difficulty. As of December 5, 2003, 18 P450 families, with a total of 57 P450 genes are determined in humans (http://drnelson.utmem.edu /hum.html). Figure 1.3 represents the human cytochrome P450 genes identified so far (according to the last update on December, 5, 2003). Besides, there are 58 pseudogenes, most of them belonging to the families 2, 3 and 4. A pseudogene is a defective gene that does not produce a functional protein. They are relics of gene duplications where one of the copies has been degenerated and lost its function. They are represented by the letter "P" coming after the individual enzyme name, like CYP2F1P. More detailed information on human cytochrome P450 pseudogenes can be obtained from http://drnelson.utmem. edu/58human.pseudo.html.

The majority of P450s are expressed in human liver, but they are also expressed in extrahepatic tissues –like lung, kidney, brain, gastrointestinal tract, skin, heart and placenta- on a smaller scale (Raunio *et al.* 1995). A few CYP isoforms participating in the metabolism of foreign compounds are found only in extrahepatic tissues, for example CYP2F is located in lung, CYP4A11/F2 and F3 in kidney, CYP4B1 in lung and placenta, and CYP2S1 in lung and gastrointestinal tract (Rylander *et al.,* 2001; Anzenbacher and Anzenbacherova, 2001).

Table 1.1 lists the human cytochrome P450 families and their functions. The enzymes in the families 1-3 are mostly active in the metabolism of xenobiotics- thus called xenobiotic metabolizing P450s-, whereas the families 5-51 have important endogenous functions (see Table 1.1). Isoforms in family 4 have also roles in xenobiotic metabolism besides fatty acid hydroxylation. However, some isoforms in xenobiotic metabolizing P450s also metabolize endogenous compounds, such as steroid hormones and arachidonic acid (Gonzalez 1992, Capdevila *et al.* 2000). There is one enzyme in these families, CYP2J2, which has not been shown to metabolize foreign compounds (Wu *et al.* 1996).



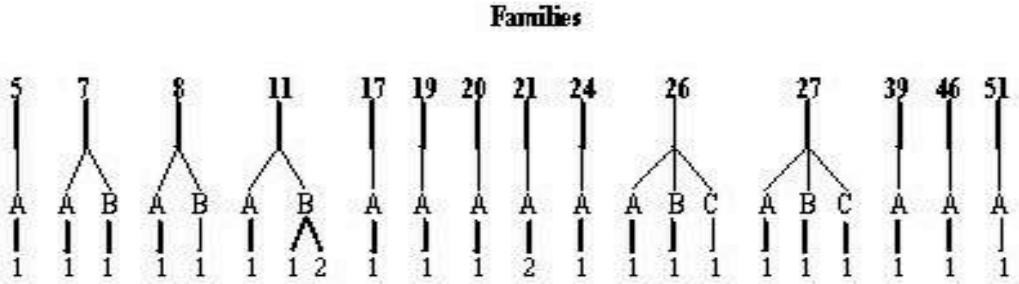


Figure 1.3 Human cytochrome *P450* genes (based on Stoilov *et al.*, 2001; and updated from drnelson.utmem.edu/human P450 table.html; Dec ,5,2003).

Table 1.1 Human cytochrome P450 families and their main functions (adapted from Hukkanen, 2000; Nebert and Russel, 2002).

CYP Family	Main Functions
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic metabolism Arachidonic acid metabolism
CYP3	Xenobiotic and steroid metabolism
CYP4	Fatty acid hydroxylation Xenobiotic metabolism
CYP5	Thromboxane synthesis
CYP7	Cholesterol 7α-hydroxylation
CYP8	Prostacyclin synthesis
CYP11	Cholesterol side-chain cleavage Steroid 11β-hydroxylation Aldosterone synthesis
CYP17	Steroid 17α-hydroxylation
CYP19	Androgen aromatization
CYP20	Unknown
CYP21	Steroid 21-hydroxylation
CYP24	Steroid 24-hydroxylation
CYP26	Retinoic acid hydroxylation
CYP27	Steroid 27-hydroxylation
CYP39	24-hydroxycholesterol 7α-hydroxylation
CYP46	Cholesterol 24-hydroxylation
CYP51	Sterol biosynthesis

There are differences between the characteristics of genes encoding xenobiotic metabolism (CYP1-3 and 4), and those of importance for the metabolism of endogenous compounds (CYP5-51). First, the greatest variability in number of members of the subfamilies lies in families 2, 3 and 4, as can be seen in Figure 1.3. The same families also contain the highest number of pseudogenes (52 of total 58 pseudeogenes belongs to families 2, 3 and 4; the rest 6 pseudogenes belong to families 1, 21, 46, and 51). In addition, it is evident that the number of polymorphisms is much greater among the *P450* genes coding for drug metabolizing enzymes, so that all *P450* genes belonging to families 1-3 show polymorphisms (Ingelman-Sundberg, 2002). By contrast, so far, only 3 isoforms, namely CYP5A1, 8A1 and 20A1 are known to show polymorphisms in families 5-51 (http://www.imm.ki.se/CYPalleles). As a result, it can be stated that the human *P450* genes coding for xenobiotic metabolizing enzymes are susceptible to changes or "disturbances", while the ones involved in endogenous metabolism are highly conserved. The reason for this situation may be the lack of evolutionary pressures which would maintain the structure of the gene undisturbed, as discussed in section 1.4.3 (Gonzalez, 1999).

Some general properties of drug metabolizing human P450s are given in Table 1.2. The major isoforms important for drug metabolism are CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, whereas CYP1A1, CYP1A2, CYP1B1, and CYP2E1 are the most important isoforms responsible for metabolic activation of procarcinogens (Ingelman-Sundberg, 2002). These xenobiotic-metabolizing P450s compose approximately 80% of total liver P450 content, and they can be ordered according to their relative content as CYP3A4/3A5 (~35%) > CYP2 (20%, major component being 2C9) > CYP1A2 (~12%) > CYP2E1 (~7%) > CYP2A6 (~4%) > CYP2D6 (~2%) > CYP2B6 = CYP1A1 (~1%) (Tanaka, 2001). When the drugs metabolized by P450s are concerned, the majority of them are metabolized by CYP3A4/3A5, CYP2D6 and CYP2C family, estimated fractions being 52%, 30% and 11%, respectively (Anzenbacher and Anzenbacherova, 2001).

Table 1.2 General properties of human xenobiotic metabolizing P450s (based on Watanabe, 1998; Anzenbacher and Anzenbacherova, 2001; Ingelman-Sundberg, 2002).

CYP Isoform	Localization	Substrates (examples)	Polymorphism frequencies	Functional Effects of Polymorphisms
1A1	Lung, liver, brain, gastrointestinal tract (GIT), lymphocytes, heart	Carcinogens (polyaromatic hydrocarbons-PAHs)	Relatively high	Lung, breast, colorectal cancers
1A2	Liver	Drugs, carcinogens (caffeine, aromatic amines, PAHs)	High	Colorectal cancer, myotonic dystrophy
1B1	Skin, brain, heart, lung, placenta, liver, kidney, GIT, spleen	Carcinogens, estrogens (PAHs)	Rare null alleles	Clinical consequence in glaucoma, drug toxicity
2A6	Liver	Drugs, carcinogens, steroids (coumarin, nicotine)	High in Orientals, less in Caucasians	Liver cirrhosis
2B6	Liver, heart	Drugs, nicotine	Relatively high	Reduced drug metabolism
2C8	Liver, kidney	Drugs, retinoids (taxol)	High	Taxol metabolism
2C9	Liver	Drugs (tolbutamide, diclofenac)	Relatively low	Drug toxicity
2C19	Liver, heart	Drugs (S-mephenytoin, omeprazole, diazepam)	High	Drug toxicity
2D6	Liver, brain, heart	Drugs (antidepressives, β-blockers)	High	Lung, liver, gastric cancers, Parkinson disease
2E1	Liver, lung, kidney, brain, endothelium, heart, bone marrow, nasopharyngeal tissues	Carcinogens, solvents, some drugs(ethanol, nitrosamines, acetaminophen)	High	Lung cancer, liver disease
3A4/5	Liver, GIT, kidney, lung, brain, endothelium, placenta, lymphocytes	Drugs, carcinogens, steroids (Ca^{2+}-channel blockers, cyclosporin, taxol)	High in 3A5	Drug toxicity

As mentioned above, all *P450* genes belonging to families 1-3 show genetically functional polymorphisms, resulting in pharmacogenetic heterogenity between individuals. However, although CYP2E1 isoform is polymorphic, it is relatively well conserved, for example, none of the CYP2E1 polymorphisms result in a significant change in protein structure (Ingelman-Sundberg, 2002).

Opposite to the inactivating mutations in the P450s with physiological functions leading to serious diseases, similar mutations in xenobiotic-metabolizing P450s are rarely the direct cause of diseases. However, they affect individuals drug metabolism and susceptibility to some diseases, without directly causing disease (Nelson 1999). Genetic polymorphisms in P450s that contribute to chemical toxicity is likely to be a significant factor in determining susceptibility to diseases related to exposure of these compounds (Daly *et al.*, 1998). For this reason, CYP2E1 polymorphisms drive considerable attention on them, as they may be the causes for interindividual differences in susceptibility to especially occupational diseases caused by chemicals or chemical-induced carcinogenesis.

1.5 Cytochrome P4502E1 (CYP2E1)

Perhaps the most striking feature of CYP2E1 is its role in ethanol metabolism, at least it is the reason for its discovery. Therefore it is meaningful to mention the microsomal ethanol oxidizing system, firstly.

Ethanol is found in mammals in trace amounts, but it is primarily an exogenous compound that is readily absorbed from the gastrointestinal tract and metabolized principally in liver. In 1960s, it was believed that there was only one significant pathway for ethanol metabolism, involving multiple forms of alcohol dehydrogenase (ADH). However, some features of ethanol metabolism, such as the adaptive increase after chronic consumption could not be explained on the basis of ADH, therefore raised the suspicion of the existence of another pathway (Lieber, 1997). The morphological observations that, in rats and humans, ethanol feeding resulted in a proliferation of the smooth endoplasmic reticulum (Iseri *et al.,* 1966;

Lane and Lieber, 1966), which resembled the situation observed after P450 induction, raised the idea that ethanol might also be metabolized by a similar process.

Such a cytochrome P450 involving system was then demonstrated in liver microsomes *in vitro* and found to be inducible by chronic ethanol feeding *in vivo* (Lieber and DeCarli, 1970). Ethanol-inducible cytochrome P450 was first purified from rabbits and named as LM3a (Koop *et al.,* 1982; Ingelman-Sundberg and Johansson, 1984) and later in rats, named as P450j (Ryan *et al.,* 1985; Patten *et al.,* 1986) and human (Wrighton *et al.,* 1986, 1987). Acording to the new nomenclature system for *CYP* gene superfamily, it was proposed that the ethanol-inducible form be designated as CYP2E1.

The cDNA of CYP2E1 was cloned from the rat and human liver in 1986 (Song *et al.,* 1986). The deduced amino acid sequences of the rat and human CYP2E1 both contained 493 amino acids with calculated molecular weights of 56,634 and 56,916, respectively. Figure 1.4 and Figure 1.5 give the amino acid sequences of bacterial CYP102, human, rabbit, rat and mouse CYP2E1; and model structure of CYP2E1, respectively. Human 2E1 shared 75% nucleotide and 78% amino acid similarities to rat 2E1. Amino acid alignment revealed that 2E1 was 48% similar to 2B1 and 2B2 and 54% similar to 2C6 and 2C7, but had lower similarities to other P450s (Umeno *et al.,* 1988).

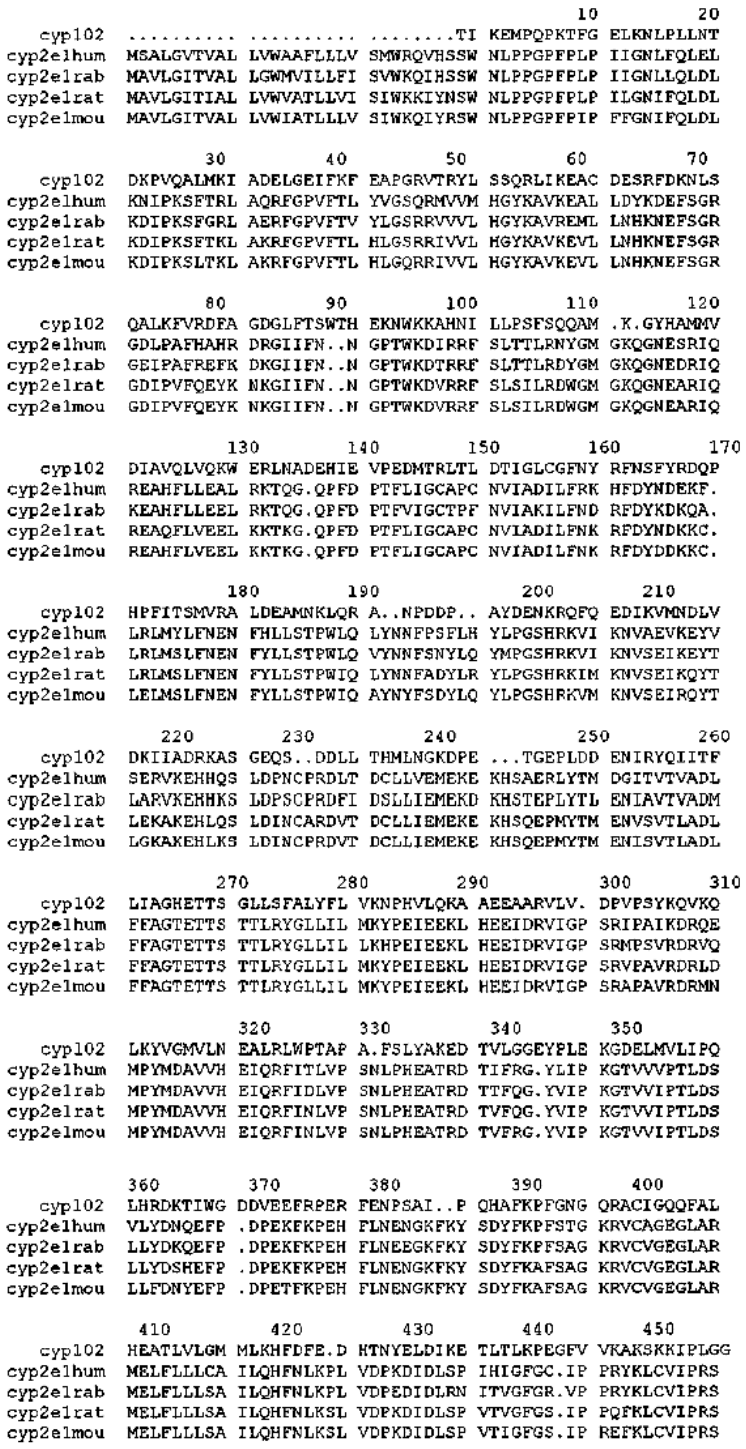


Figure 1.4 Amino acid sequence alignments of bacterial CYP102, and human, rabbit, rat and mouse CYP2E1 proteins (taken from Lewis *et al.,*2000).

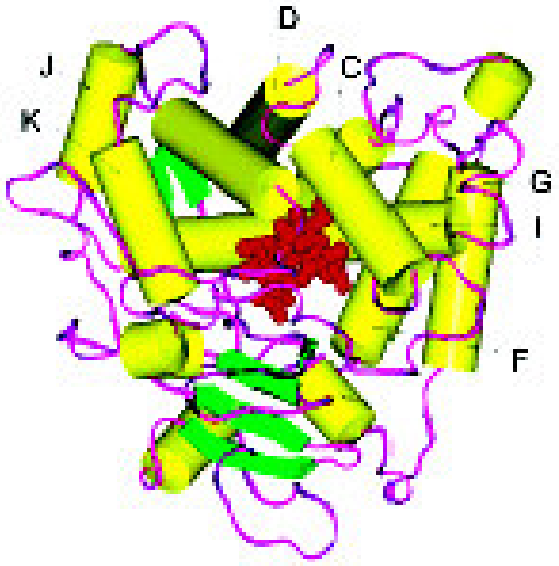


Figure 1.5 Model structure of CYP2E1. Cylinders, green ribbons and magenta ribbons represent helices, β-sheets and coils, respectively (taken from Park and Harris, 2003).

CYP2E1 is mainly found in liver, but significant amounts are also found in extrahepatic tissues including lung, kidney, brain, endothelium of large blood vessels, heart, bone marrow and nasopharyngeal tissues (Ding *et al.,* 1986; Ingelman-Sundberg *et al.,* 1993). In liver, the enzyme is not homogenously distributed: the expression of enzyme, both constitutively and after induction, is restricted to the centrilobular region of the liver and, in particular, to the three to four layers of hepatocytes most proximal to the central vein (Ingelman-Sundberg *et al.*, 1988). Thus, hepatic damage caused by CYP2E1 activity is mainly observed in these regions of the liver.

1.5.1 Substrates of CYP2E1

A number of different chemicals of diverse structures have been found to be metabolized selectively by CYP2E1. Besides ethanol, CYP2E1 metabolizes some other endogenous compounds but most of its substrates are exogenous, including industrial solvents, procarcinogens and a few pharmaceutical drugs. It is difficult to find a common structure among all these substrates, but it appears that small and hydrophobic compounds in general provide efficient targets for CYP2E1-dependent catalysis (Ingelman-Sundberg *et al.,* 1993). Examples for the substrates, inducers and inhibitors of CYP2E1 is listed in Table 1.3.

CYP2E1 is among the most conserved forms in the CYP2 family and the catalytic activities of CYP2E1 across species are quite similar, suggesting that it has a physiological importance. Acetone, a ketone body, is metabolized by CYP2E1 to acetal and then to methylglyoxal (Koop and Casazza, 1985) which can be used for the synthesis of glucose (Landau and Brunengraber, 1987). Its activity toward acetone and acetal oxidation suggests that CYP2E1 is involved in the pathway of gluconeogenesis during the fasting state and diabetes (Gonzalez, 1989).

Among the substrates of CYP2E1, the 6-hydroxylation activity on muscle-relaxant chlorzoxazone is being used as a probe substrate to measure CYP2E1 activity *in vivo* (Peter *et al.,* 1990). However, some studies have shown that recombinant human CYP1A1 and 1A2 were also involved in the metabolism of chlorzoxazone (Carriere *et al.,* 1993; Ono *et al.,* 1995). Also, N-demethylation of nitrosodimethylamine, aniline 4-hydroxylase and *p*-nitrophenol hydroxylase activities of CYP2E1 are used to measure the in vitro activity of this enzyme (Lieber, 1997; Arınç *et al.,* 2000a,b; Arslan *et al.,* 2003).

Table 1.3 Substrates, inducers and inhibitors of CYP2E1.

Substrates		
Endogenous	**Exogenous**	
	Therapeutic drugs/ anaesthetic gases	**Solvents and other chemicals**
Ethanol Acetone Acetoacetate Acetol Acetaldeyde Fatty acids (arachidonic acid, lauric acid) Glycerol	Acetaminophen Chlorzoxazone Dapsone Enflurane, sevoflurane, isoflurane Halothane Disulfiram Isoniazid *p*-nitrophenol Phenacetin	Acrylonitrile, methacrylonitrile Alcohols, ethers, alkanes Acetone Benzene (and derivatives) Styrene Chloroform Carbontetrachloride Pyrazole Phenol Pyridine Acrylamide Nitrosamines (e.g. NDMA) Ethyl carbamate, vinyl carbamate Vinyl chloride, vinyl bromide Diethylether Hexane Butadiene Ethylene dibromide Ethylene dichloride Methyl chloride Methylene chloride 1,1,1-trichloropropanol 1,2-dichloropropanol

Inducers	**Inhibitors**
Ethanol Acetone Benzene Isoniazid Isopropanol Pyrazole Pyridine Starvation Diabetes	Diallylsulfide, diallylsulfone Chlormethiazole Diethyldithiocarbamate Isothiocyanates 4-methyl-pyrazole Disulfiram

Although the drugs metabolized by CYP2E1 are relatively low in number, it is shown to be the major enzyme responsible for the acetaminophen toxicity, as clearly established in the studies with CYP2E1-null mice protection against acetaminophen toxicity (Gonzalez, 2001). CYP2E1 takes a role in the formation of a reactive quinoneimine in acetaminophen metabolism that can bind to hepatic and renal proteins (Guengerich and Shimada, 1991; Manyike *et al.,* 2000).

Also, as can be seen on the Table 1.3, an important portion of CYP2E1 substrates involves industrial chemicals and procarcinogens/carcinogens. Among them are butadiene (Melnick and Kohn, 1995), small molecular weight hydrocarbons, such as benzene and styrene (Guengerich, 1995), certain chloroalkanes and chloroalkenes, like chloroform, tetrachloromethane, trichloroethane and vinyl chloride (Guengerich *et al.,* 1991; Gonzalez and Gelboin, 1994), and nitrosamines including NDMA (Garro *et al.,* 1981; Arınç *et al.*, 2000a,b). CYP2E1 activates these chemicals into more toxic or carcinogenic forms, therefore receives a great deal of attention in terms of occupational liver-diseases and cancer.

Another important and peculiar feature of CYP2E1 is its capability to reduce molecular oxygen, resulting in the formation of H_2O_2 and $O_2^{\bullet-}$ radicals (Gorsky *et al.,* 1984; Elkstrom and Ingelman-Sundberg, 1989; Persson *et al.,* 1990). It is known that unlike most other P450s, CYP2E1 contains a proportion of the hemoprotein that is present naturally in the high-spin state even in the absence of substrate (Koop *et al.,* 1982, Guengerich and Johnson, 1997), and this feature of CYP2E1 would let it be reduced by an electron provided by NADPH-cytochrome P450 reductase (in the absence of substrate), resulting in the generation of reactive oxygen species (Elkstrom *et al.,* 1986; Cederbaum, 1987; Kukielka and Cederbaum, 1994).

It appears that oxygen radicals generated by CYP2E1 have the capability to initiate membranous lipid peroxidation. Thus, CYP2E1 turned out to be the most efficient isozyme in the initiation of NADPH-dependent lipid peroxidation. Its importance is also verified with studies showing almost complete inhibition of NADPH-dependent lipid peroxidation in microsomes using CYP2E1 antibodies

(Elkstrom and Ingelman-Sundberg, 1989). So, it is clear that CYP2E1 plays a key role in the pathogenesis of liver injury.

Membranous lipids are not the sole target of free radical attack. The increased microsomal generation of reactive oxygen derivatives could also contribute to ethanol toxicity through radical-mediated inactivation of metabolic enzymes (Dicker and Cederbaum, 1988), including CYP2E1 itself (Koop and Tierney, 1990).

Besides all, reactive oxygen species can cause DNA damage (Halliwell and Gutteridge, 1990; Wiseman and Halliwell, 1996) and can, therefore, represent factors in the overall carcinogenicity of some CYP2E1 substrates and inducers (Parke, 1987, 1994; Terelius *et al.,* 1993; Ioannides *et al.,* 1995).

As a summary, CYP2E1 receives attention as it is thought to be related to diabetes and fasting due to its capacity to metabolize acetone, as well, it is of great concern especially in terms of chemical-induced occupational diseases, and carcinogenicity, because of its spectum of substrates and capacity to create reactive oxygen species. But the scene becomes more complicated when the regulation of CYP2E1 is also involved, as it comprises of complex mechanisms of transcriptional, posttranscriptional and posttranslational events.

1.5.2 Regulation of CYP2E1

Most CYP2E1 substrates are shown to be also inducers of the enzyme, like ethanol, isoniazid, acetone, benzene and pyridine. As well, some pathophysiological conditions such as starvation, diabetes, obesity, and high fat feeding are also found to induce CYP2E1 (Koop and Casazza, 1985; Hong *et al.,* 1987, Song *et al.,* 1987; Schenkman *et al.,* 1989; Kim *et al.,* 1990; Arınç *et al.,* 1991; Yoo *et al.,* 1991; Arınç *et al.,* 2000a,b; Arslan, 2003). Inhibition of CYP2E1 is observed upon treatment with diallylsulfide, disulfiram, diethlydithiocarbamate, isothiocyanates, and 4-methyl pyrazole (Lieber, 1997; Rendic and Di-Carlo, 1997).

As mentioned above, the regulation of CYP2E1 expression is complex, involving transcriptional, posttranscriptional, and posttranslational events with polymorphism playing a role (Song, 1995). Transcriptional regulation seems to have a minor role, in contrast to many other xenobiotic-metabolizing P450 forms. A summary of the modes of CYP2E1 regulation is given in Figure 1.6.

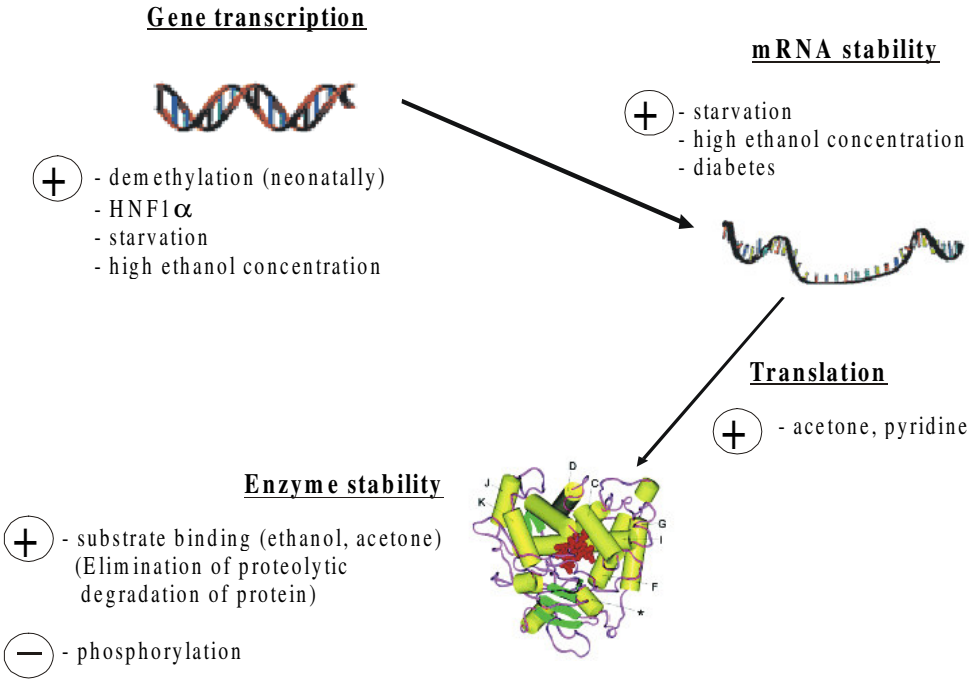


Figure 1.6 Mechanisms of regulation of CYP2E1 expression (adapted from Ingelman-Sundberg *et al.,* 1993).

1.5.2.1 Transcriptional Regulation of CYP2E1

Transcriptional activation of CYP2E1 was demonstrated in rats right after birth (Song *et al.,* 1986) which is accompanied by demethylation of cytosine residues located within the 5'-flanking region of the gene (Lieber, 1997).

It is also reported that hepatic nuclear factor-1α (HNF1α) - a homodomain-containing transcription factor that is expressed in liver, kidney, intestine, stomach and pancreas (Lee *et al.,* 1998; Blumenfeld *et al.,* 1991), can bind to *CYP2E1* gene between –113 bp to –7 bp in 5'-flanking region and probably controls the transcription of this gene (Liu and Gonzalez, 1995). In a recent study, it is established that *CYP2E1* gene is found out to be expressed at markedly lower levels in the livers of HNF1α-deficient mice, with a corresponding decrease in the protein level, verifying that HNF1α may act as a positive regulator of *CYP2E1* gene (Cheung *et al.,* 2003). However, the role of this transcription factor in the regulation of *CYP2E1* gene needs further investigation.

Transcriptional activation of *CYP2E1* gene is also seen after extensive starvation (Albano *et al.,* 1993), and at high ethanol concentrations (Badger *et al.,* 1993; Ronis *et al.,* 1993). Acute ethanol treatment does not influence the CYP2E1 mRNA levels, indicating that no specific ethanol responsive element is present within the *CYP2E1* gene (Ingelman-Sundberg *et al.,* 1993).

1.5.2.2 Posttranscriptional and Posttranslational Regulation of CYP2E1

One of the most profound examples of posttranscriptional regulation is seen with the CYP2E1 enzyme. Administration of small organic compounds such as acetone, pyrazole, and ethanol to rats caused a rapid induction of CYP2E1 protein without affecting the levels of CYP2E1 mRNA (Khani *et al.,* 1987). Furthermore, in a HepG2 cell line, which stably and constitutively expresses the coding sequences of human CYP2E1, addition of ethanol (2-100 mM) for 2 days resulted in an increased

CYP2E1 content without any raise in mRNA levels. (Carrocio *et al.,* 1994). These results indicate a posttranscriptional regulation of CYP2E1 at either the translational level or by stabilization of the protein against degradation. The stabilization of enzyme was proposed to be achieved by binding of substrates to CYP2E1, switching its degradation from an endoplasmic reticulum proteolytic pathway (with half-life of 7 hours) to a lysosomal proteolytic pathway (with half-life of 37 hours) (Eliasson *et al.,* 1992).

Controversially, other studies on hamsters (Kubota *et al.,* 1988), rats (Diehl *et al.,*1991; Kim and Novak, 1994) and humans (Takashi *et al.,* 1993), showed that administration of low molecular weight substrates of CYP2E1 like ethanol results with an increase in CYP2E1 mRNA levels in liver. According to these results, it appears that dose of inducer triggers different modes of induction, such that CYP2E1 induction with ethanol occur in two steps: 1) posttranslational mechanism at low ethanol concentrations and 2) an additional transcriptional one at high ethanol levels (Ronis *et al.,* 1993; Badger *et al.,* 1993).

In spontaneously diabetic rats or in rats made diabetic by treatment with streptozotocin or alloxan, CYP2E1 protein was induced up to 6-fold above the level seen in untreated rats (Song *et al.,* 1986; Favreau *et al.,* 1987; Bellward *et al.,* 1988; Dong *et al.,* 1988). This increase was accompanied by up to a 10-fold increase in CYP2E1 mRNA in the absence of an increase in transcription of the *CYP2E1* gene (Song *et al.,* 1986). Fasting also caused an increase in CYP2E1 mRNA and protein (Hong *et al.,* 1987), suggesting that the rate of mRNA degradation may have been retarded under these conditions (Hong *et al.,* 1987; Song *et al.,* 1987). Recently, studies carried out using rabbits made diabetic experimentally with alloxan treatment have shown that CYP2E1 protein is induced and CYP2E1-associated enzyme activities are stimulated compared to untreated control rabbits (Arslan, 2003; Arslan *et al.,* 2003). Several studies have also shown that fasting and diabetes results in elevated levels of ketone bodies (Miller and Yang, 1984), which are also the substrates of CYP2E1, so they are thought to be involved in the induction response either directly, or indirectly (Bellward *et al.,* 1988).

1.5.2.3 Inhibition of CYP2E1

Some examples for the inhibitors of CYP2E1 are listed in Table 1.3. Among them, disulfiram is a clinically used inhibitor (Guengerich & Shimada 1991). It is also well established that CYP2E1 activities may be decreased by some food components, such as diallyl sulfide present in garlic and onion and after consumption of cruciferous vegetables (Ioannides, 1999). Brady and coworkers have demonstrated that diallyl sulfide and disulfiram were protective aginst NDMA-induced hepatotoxicity (Brady *et al.,* 1991a,b).

The mechanism-based inactivation of CYP2E1 was demonstrated *in vitro* with diallyl sulfone, an oxidative metabolite of diallyl sulfide (Brady *et al.,* 1991b). It is believed that diallyl sulfone is converted by CYP2E1 to a reactive intermediate which modifies the heme moiety of this cytochrome and causes inactivation (Brady *et al.,* 1991b). It was also demonstrated that diallyl sulfone has a protective action against acetaminophen toxicity. Therefore, it may have the potential for practical application in the prevention of acetaminophen toxicity, especially in alcoholics whose CYP2E1 levels are usually elevated (Yang and Smith, 1995). Phenethyl isothiocyanate also inactivates CYP2E1 by a suicide mechanism (Ishizaki *et al.,* 1990).

The wide variety of substrates and complex mechanisms of regulation makes this enzyme important for occupational and environmental medicine. Associated polymorphisms in *CYP2E1* gene receives a great deal of attention especially after the development of genetic techniques and completion of the human genome project, as polymorphisms in xenobiotic- metabolizing enzymes, including CYP2E1, is one of the major reasons for interindividual variability in susceptibility to related diseases and chemical-induced carcinogenesis.

1.5.3 Polymorphisms of *CYP2E1*

The human *CYP2E1* gene is located in 10q24.3-qter region of chromosome 10, and spans 11,413 base pairs with nine exons and a typical TATA box. (Umeno *et al.,* 1988). The gene contains several polymorphisms, some of which seem to effect the expression of the protein. Figure 1.7 shows a schematic representation of the gene and associated polymorphisms.

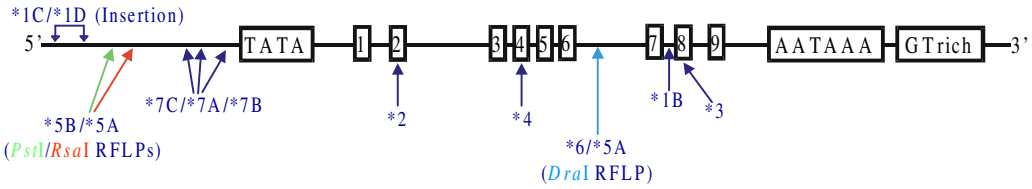


Figure 1.7 Schematic representation of human *CYP2E1* gene with associated polymorphisms. The numbered boxes represent the exons; polymorphisms occuring throughout the gene are indicated by dark blue arrows; the polymorphisms investigated in this study are indicated in green, red and light blue arrows. Alleles are designated according to the CYP Allele Nomenlature Committee assigments (http://www.imm.ki.se /CYPalleles) (see also Table 1.4 for nomenclature of CYP2E1 alleles).

The blast of studies on polymorphisms resulted in inconsistencies in numberings of SNPs and different nomenclatures of alleles, which created a need for an official source to present a systemic nomenclature. The Human Cytochrome P450 Allele Nomenclature Committee is developed to solve this problem and establishes genetic variants of CYP isozymes under http://www.imm.ki.se/CYPalleles. According to this committee, to be assigned as a unique allele, it should contain nucleotide changes that have been shown to affect transcription, splicing, translation, posttranscriptional or posttranslational modifications or result in at least one amino

acid change. To name new P450 alleles, the gene name and allele are separated by an asterix followed by Arabic numerals. The normal version of each gene, designated wild type, is always given the number 1. Thus *CYP2E1*1* is the wild-type *CYP2E1* allele while *CYP2E1*4* is the third identified variant. Alleles which display only minor differences thought to be of no functional significance are given the same numerical designate but differentiated by a letter (e.g. *CYP2E1*5A*). Table 1.4 shows the polymorphisms in *CYP2E1* gene based on the assignments of the Human Cytochrome P450 Allele Nomenclature Committee.

1.5.3.1 *CYP2E1*5A*, *CYP2E1*5B* and *CYP2E1*6* Polymorphisms

The most frequently studied genetic polymorphisms in *CYP2E1* are the RFLPs *Pst*I/*Rsa*I (mutant allele: *CYP2E1*5B*) located in the 5'-flanking region of the gene, which have been related to altered enzyme expression *in vitro* (Hayashi *et al.* 1991), as well as the *Dra*I polymorphism (mutant allele: *CYP2E1*6*) located in intron 6 (Uematsu *et al.* 1991). These polymorphisms are partly related, and they form the common allele designated *CYP2E1*5A*.

Recently, Haufroid and co-workers described a trend to lower 'chlorzoxazone metabolic ratios' for individuals possessing at least one mutant *CYP2E1*6* allele, compared to the homozygous wild type (Haufroid *et al.*, 2002). The relationship of *CYP2E1*5A* allele with CYP2E1 enzyme expression and the associated consequences in the metabolism of chemicals remain to be definitely established, as available studies are conflicting in this respect (Hayashi *et al.* 1991; Kim *et al.* 1996).

Vodicka *et al.* (2001) have shown that polymorphisms in 5'-flanking region and in *Dra*I site in intron 6 are significantly correlated with single strand breaks in DNA. The *Dra*I polymorphism was found to be associated with an increased risk for lung cancer in several studies (Uematsu *et al.,* 1992; Wu *et al.,* 1998; Le Marchand *et al.,* 1998), for breast cancer among premenapausal smokers only (Shields *et al.,* 1996), and for renal carcinoma among Caucasian women (Farker *et al.,* 1998). The *Rsa*I c2 mutated allele in 5'-flanking region was found to be

Table 1.4 Nomenclature of *CYP2E1* alleles (taken from http://www.imm.ki.se/ CYPalleles).

Allele	Protein	Nucleotide Changes	RFLP	Effect	Enzyme Activity		References
					in vivo	in vitro	
*CYP2E1*1A*	CYP2E1.1	None			Normal	Normal	Umeno *et al.,* 1988
*CYP2E1*1B*	CYP2E1.1	9896C>G	*Taq*I-				McBride *et al.,* 1987; Brockmöller *et al.,* 1996
*CYP2E1*1C*	CYP2E1.1	6 repeats in the 5'-flanking region					Hu *et al.,* 1999
*CYP2E1*1D*	CYP2E1.1	8 repeats in the 5'-flanking region	*Dra*I and *Xba*I		Incr. activity after alcohol exposure and in obese		McCarver *et al.,* 1998; Hu *et al.,* 1999
*CYP2E1*2*	CYP2E1.2	1132G>A		R76H		Reduced	Hu *et al.,* 1997
*CYP2E1*3*	CYP2E1.3	10023G>A		V389I		Normal	Hu *et al.,* 1997
*CYP2E1*4*	CYP2E1.4	4768G>A		V179I		Normal	Fairbrother *et al.,* 1998
*CYP2E1*5A*	CYP2E1.1	-1293G>C; -1053C>T; 7632T>A	*Pst*I+ *Rsa*I- *Dra*I-				Watanabe *et al.,* 1990; Hayashi *et al.,* 1991; Persson *et al.,* 1993;
*CYP2E1*5B*	CYP2E1.1	-1293G>C; -1053C>T	*Pst*I+ *Rsa*I-				Watanabe *et al.,* 1990; Hayashi *et al.,* 1991
*CYP2E1*6*	CYP2E1.1	7632T>A	*Dra*I-				Persson *et al.,* 1993
*CYP2E1*7A*	CYP2E1.1	-333T>A					Fairbrother *et al.,* 1998
*CYP2E1*7B*	CYP2E1.1	-71G>T; -333T>A					Fairbrother *et al.,* 1998
*CYP2E1*7C*	CYP2E1.1	-333T>A; -352A>G					Fairbrother *et al.,* 1998

associated with adenocarcinoma (El Zein *et al.,* 1997), esophagus cancers in Chinese population (Lin *et al.,* 1998), nasopharyngeal carcinoma (Kongruttanachok *et al.,* 2001), as well as oral cancer cases (Liu *et al.,* 2001). It was also reported that the percentage of *Rsa*I c2 allele was significantly higher in alcoholics than non-alcoholics, suggesting that this polymorphism may be associated with greater alcohol consumption (Sun *et al.,* 2002; Konishi *et al.,* 2003).

1.5.3.2 Other *CYP2E1* Polymorphisms

Hu and Fairbrother have described three single nucleotide polymorphisms (SNP) that lead to associated amino acid changes in the CYP2E1 protein (*CYP2E1*2, CYP2E1*3,* and *CYP2E1*4*), but showing very low frequencies (Hu *et al*., 1997; Fairbrother *et al.,* 1998).

McCarver (1998), for the first time, described an approximately 100 bp of insertion in the promoter region of *CYP2E1*, and tentatively localized it to a region between the nucleotide positions -2270 and -1672. A phenotyping study by using chlorzoxazone as a probe was also accompanied and the presence of the insertion appeared to be associated with greater metabolic activity, but only among individuals who either were obese or had recently consumed alcohol. These data were found to be consistent with the idea that the insertion is associated with altered induction behaviour of CYP2E1, generally pointing to more complex gene–environment interactions (McCarver *et al.,* 1998). Later, Hu and coworkers determined two major alleles associated with the insertion, carrying either six or eight sequence repeats, which they designated *CYP2E1*1C* (common allele) and *CYP2E1*1D* (rare allele). The wild type, *CYP2E1*1A*, contained only five of these repeats (Hu *et al.,* 1999).

Fairbrother and co-workers (1998) have identified a polymorphism (allocated as -297T>A, now being addressed as -333T>A; *CYP2E1*7A*) that (in combination with the -71G>T detected by Hu et al., 1997; classified as *CYP2E1*7B*) exhibited a higher *in vitro* activity in a promoter construct than the wild type. This led to

increased interest in possible metabolic consequences of the *CYP2E1*7* group of polymorphisms. In 2000, Thier and coworkers have reported that *CYP2E1*7C* allele was associated with the production of higher levels of acrylonitrile-specific adducts upon acrylonitrile exposure (Thier *et al*. 2000).

1.6 The Aim of This Study

CYP2E1 is a special xenobiotic-metabolizing P450 with its substrate specificity, including many industrial chemicals most of which are at the same time procarcinogens, as well as some widely used therapeutic drugs, like acetaminophen. Besides it has a unique property to reduce molecular oxygen and create reactive oxygen species which can cause liver pathology and DNA adducts, hence chemical-induced carcinogenesis. Its ability to metabolize ethanol depicts its importance in ethanol metabolism, especially upon induction while it is also proposed to have roles in disease states like starvation and diabetes due to its ability in metaboizing ketone bodies, like acetone. Besides having a complex mechanism of regulation, *CYP2E1* also possess several polymorphisms throughout its gene.

Polymorphisms associated with the gene drives much attention in recent years because genetic polymorphisms are proposed as being the major reasons for interindividual differences in xenobiotic response and in susceptibility to related diseases, including several types of cancer. The most widely studied polymorphisms of *CYP2E1* gene are the *Pst*I / *Rsa*I polymorphisms in 5'-flanking region and *Dra*I polymorphism in intron 6. Association of these polymorphisms with several cancer types and alcoholic liver diseases is presented in Section 1.5.3.1.

It is also well established that *CYP2E1* polymorphisms differ markedly in frequency among ethnic and racial groups, like most other xenobiotic-metabolizing enzymes (Evans and Relling, 1999). That is why identification of *CYP2E1* polymorphisms in different populations, as well Turkish population, becomes important.

The purpose of this study is to investigate the two important *CYP2E1* polmorphisms- the C-1019T/G-1259C (*Pst*I/*Rsa*I RFLPs) polymorphisms in 5'-flanking region and T7678A (*Dra*I RFLP) polymorphism in intron 6, in a sample of healthy Turkish individuals that represents the population, and to identify the genotype distributions and allele frequencies of these polymorphisms. To accomplish this purpose, this study is designed to comprise:

- isolation of genomic DNA in intact form from blood samples,

- amplification of two regions in *CYP2E1* gene; one in 5'-flanking region and one in intron 6 by PCR,

- digestion of the amplified fragments with *Rsa*I/*Pst*I and *Dra*I restriction enzymes to determine the genotype of each individual for C-1019T / G-1259C and T7678A single nucleotide polymorphisms, respectively,

- determination of the genotype and allele frequencies of C-1019T / G-1259C and T7678A single nucleotide polymorphisms for Turkish population, and comparison of these results with other studies performed on different ethnicities.

This study is also planned as a prior work for further studies on correlating *CYP2E1* genotypes with incidence of related diseases and chemical-induced cancers.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1 Subjects and Blood Sample Collection

A total of 132 blood samples were obtained from healthy volunteers with the collaboration of METU Health Center, Biochemistry Laboratory. For each subject, informed consents were taken. The informed consent form also included questions regarding their age, birth place of the volunteer and his/her parents, and any diseases they had had. A copy of the informed consent form is given in Appendix 1, with permission of the volunteer. Subjects having diseases –like cancer, diabetes etc.- that are supposed to be related with CYP2E1 were excluded from the study.

4-5 mL of blood samples from subjects was taken in EDTA-containing vacuumed tubes and stored at -20°C till use. Blood samples were kept in 4°C while they were in active use.

2.1.2 Enzymes and Chemicals

Agarose (A-9539), bromophenol blue (B-5525), chloroform (C-2432), ethidium bromide (E-7637), ethylene diamine tetra acetic acid disodium salt (EDTA; E-5134), ficoll (F-2637), phenol (P-4557), sodium chloride (NaCl; S-3014), sodium

dodecyl sulfate (SDS; L-4390), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T-1503), xylene cyanoll FF (X-4126) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Borate (11607), and absolute ethanol (32221) were the products of Riedel de Haën, Seelze. Isoamyl alcohol (A2610) and Proteinase-K (A3830) were purchased from AppliChem, Ottoweg, Darmstadt.

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 enzymes *Pst*I (#ER0611), *Rsa*I (#ER1121), *Dra*I (#ER0221) –which were supplied with their buffers O^+, $Y^{+/}$TANGO^{TM}, B^+, respectively- were purchased from MBI Fermentas, USA.

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

Two sets of oligonucleotide primers were used for the amplification of polymorphic regions of CYP2E1 gene from genomic DNA. They were derived from known sequences of human *CYP2E1* gene (Hayashi *et al.,* 1991; Wu *et al.,* 1998).

C-1019T / G-1259C Single Nucleotide Polymorphisms was analyzed using:

Sense strand primer:	5'-CCA GTC GAG TCT ACA TTG TCA-3'
Antisense strand primer;	5'-TTC ATT CTG TCT TCT AAC TGG -3'

T7678A Single Nucleotide Polymorphism was analyzed using:

Sense strand primer:	5'-TCG TCA GTT CCT GAA AGC AGG-3'
Antisense strand primer;	5'-GAG CTC TGA TGC AAG TAT CGC A-3'

Primer pairs purified by HPSF® (High Performance Salt Free) technology were purchased from MWG (MWG Biotech, Ebersberg, Germany). Primers were stored at -20°C. Some properties of these primers are presented in Table 2.1.

Table 2.1 Properties of primers used throughout the study.

SNPs	C-1019T / G-1259C		T7678A	
Forward and reverse primers	5'CCAGTCGAGTCTACATTGTCA3' 5'TTCATTCTGTCTTCTAACTGG 3'		5'TCGTCAGTTCCTGAAAGCAGG3' 5'GA GCTCTGATGCAAGTATCGCA3'	
	Forward	Reverse	Forward	Reverse
Length	21 base	21 base	21 base	22 base
GC Content	47.6%	38.1%	52.4%	50%
Melting Temp. (Tm)	57.9°C	54.0°C	59.8°C	60.3°C
Tm difference	3.9°C		0.5°C	
Annealing Temp.	55°C		61°C	
Fragment lenght	412 bp		997 bp	

2.2. Methods

2.2.1 Isolation of Genomic DNA From Human Whole Blood Samples

For the isolation of genomic DNA from human whole blood samples, 100 µL of whole blood was transferred into an eppendorf tube after complete thawing and 300 µL of TNE (Tris-NaCl-EDTA) buffer (pH 8.0), 30 µL of 100 mM Tris-HCl (pH 8.0), 5 µL of Proteinase-K and 10 µL of 20% SDS were added onto the whole blood (for the preparation of reagents, see Appendix 2). The tubes were mixed by inversions and incubated at 50-55°C overnight with continuous shaking in an incubator.

An equal volume of (445 µL) phenol was added on the above lysis mixture and the organic and aqueous phases were mixed gently by inversions for 10 minutes. The two phases were separated by centrifugation at 3,000x*g* (6,000 rpm) for 10 minutes at 4°C using Sigma 1-15 benchtop microfuge (Sigma, Postfach 1713-D-37507, Osterode). After centrifugation, two layers were formed in the tube; top layer was the aqueous phase and contained genomic DNA, the bottom layer was the organic phenol phase which contained the rest of macromolecules while the proteins precipitated at the interface of two layers. The top aqueous phase was transferred into a new eppendorf tube with the help of a micropipette carefully not to suck the proteins at the interface. These extraction steps were repeated two more times with phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1) successively. All the extraction steps were carried out under the hood as phenol and chloroform may show toxic effects upon inhalation.

Two volumes of ice-cold absolute ethanol was added onto the aqueous phase obtained after chloroform:isoamyl alcohol (24:1) extraction and the mixture was kept at -20°C for 2 hours to improve the precipitation of DNA. At this point, the DNA could be seen as a small precipitate in the solution. Then the DNA was precipitated by centrifugation of the mixture at 10,000x*g* (10,500 rpm) for 10 minutes at 4°C. The supernatant was discarded immediately after centrifugation and the pellet was washed with ice-cold 70% ethanol and centrifuged again at 10,000x*g* (10,500 rpm) for 10 minutes at 4°C. The supernatant was discarded immediately as soon as the centrifugation was completed and the pellet was air-dried until no drops of ethanol remained in the tube. The pellet was resuspended in 100 µL of TE (Tris-EDTA) buffer (pH 8.0) (see Appendix 2) and kept at 4°C overnight. The DNA samples were stored at 4°C while they were in active use and kept at -20°C for long-term storage.

2.2.2 Spectrophotometric Quantification of Genomic DNA

For the determination of the concentration of DNA in the sample, absorbance values at 260 nm and 280 nm were measured in quartz cuvettes 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 50 µg/mL for double-stranded DNA, the concentration of DNA in the sample was 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) was used to estimate the purity of the nucleic acid. Pure DNA preparations gave the ratio of 1.8 while the higher or lower values showed either RNA or protein (or phenol) contaminations, respectively.

2.2.3 Qualification of Genomic DNA by Agarose Gel Electrophoresis

Determination of the intactness of DNA samples was performed by 0.5% agarose gel electrophorsesis using Biogen horizontal agarose gel electrophoresis unit having a gel tray in 8 cm x 9 cm dimensions.

0.5% agarose gel was prepared by adding 0.15 g agarose to 30 mL of 0.5x TBE (Tris-Borate-EDTA) buffer, pH 8.3 (see Appendix 2) in an erlenmeyer flask so that the buffer did not occupy more than half of the volume of the flask, and the open end of the flask was closed with a piece of aliminium foil. The slurry in the erlenmeyer flask was heated on a magnetic stirrer, and continuously stirred during heating with a magnet, until all of the grains of agarose dissolved.

The solution was cooled to approximately 60°C, but not directly on the bench top to prevent heterogenous cooling. When cooled enough, ethidium bromide was added from a stock solution of 10 mg/mL (see Appendix 2) in water to a final concentration of 0.5 µg/mL and the solution was mixed throughly.

While the agarose gel is being heated, the mold, plastic tray and comb was cleaned with 70% ethanol. The plastic tray was settled in the mold on a horizontal section of bench and the comb was placed 0.5-1.0 mm above the plate so that a complete well should be formed when the agarose was poured. The warm agarose solution was poured into the mold and any air bubbles-if present-, especially under or between the teeth of the comb were removed with the help of a pipette tip. The gel was allowed to solidify completely for approximately 20-40 minutes at room temperature.

The gel tank was filled with approximately 300 mL of 0.5x TBE buffer. The comb was carefully removed from the gel and the gel in the plastic tray was mounted in the electrophoresis tank so that the slots of the gel faced towards the negative pole-cathode. 0.5x TBE buffer was added to the tank until it covered the gel to a depth of about 1 mm. Any air bubbles in the wells, if present, were removed with the help of a pipette tip.

5 µL (0.25-0.5 ng) of DNA sample was mixed with 1 µL of gel loading buffer (see Appendix 2) by sucking in and out of a micropipette and the mixture was slowly loaded into the slots of the gel. The lid of the tank was closed and the electrical leads were attached to the power supply. The power supply was set to the constant voltage of 100 volts so that a voltage of 5 V/cm (measured as the distance between the electrodes) was applied. The gel was run for 45 minutes and then examined under UV light and the photograph was taken by using Vilber Lourmat Gel Imaging System (Marre La Vallee, Cedex, France) and Bio-Capture (Version 99.03) computer software.

2.2.4 Polymerase Chain Reaction (PCR)

Two regions of *CYP2E1* gene; one in 5'-flanking region and one in intron 6 was amplified to study the C-1019T / G-1259C and T7678A single nucleotide polymorphisms, respectively. Thermocycler used in PCR was purchased from Techne Progene (Techne (Cambridge) Ltd., Duxford, Cambridge).

2.2.4.1 C-1019T / G-1259C Single Nucleotide Polymorphisms

In order to obtain a single band belonging to the region in 5'-flanking region of *CYP2E1* gene, devoid of non-specific bands, different parameters were tested in PCR. These parameters included the change in concentration of $MgCl_2$ as 1.0, 1.25, 1.5, 1.75, 2.0 and 3.0 mM; change in concentration of primer pairs as 20, 40 and 100 pmol; and change in concentration of template DNA as 100, 200, 300 and 500 ng. Besides, addition of 10 µg BSA into reaction medium was tested to check whether an improvement in the efficiency of PCR was obtained or not. In the amplification programme, an initial denaturation at 94°C for 5 minutes was also tried. As a result, optimized PCR conditions given in Table 2.2 were used in this study.

Table 2.2 Components of PCR mixture for C-1019T / G-1259C SNPs.

Constituent	Stock Concentration	Volume to be added	Final Concentration in 50 µl reaction mixture
Amplification buffer*	10x	5 µL	1x
$MgCl_2$*	25 mM	3 µL	1.5 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 2 for these reagents			

Amplification programme used was as follows:

Denaturation	94°C	1 min	35 cycles
Annealing	55°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	6 min	

PCR products were analysed on 2% agarose gel (0.6 g of agarose dissolved in 30 mL 0.5x TBE buffer) as described in section 2.2.3. 5µL of PCR product was mixed by 1 µL of gel loading buffer and applied to the wells of gel. 3 µL of DNA ladder (50-1000 bp) (see Appendix 2) was applied to the first well of the gel. The gel was run for 1 hour at 100V.

2.2.4.2 T7678A Single Nucleotide Polymorphism

In order to obtain a single band belonging to the region in intron 6 of *CYP2E1* gene, devoid of non-specific bands, different parameters were tested in PCR. These parameters included the changes in concentration of $MgCl_2$ as 1.0, 1.5, and 2.0 mM; change in concentration of primers as 10, 20 and 40 pmol; and change in concentration of template DNA as 100, 200 and 500 ng. Besides, addition of 10 µg BSA into reaction medium was tested to check whether an improvement in the efficiency of PCR was obtained or not. In the amplification programme, an initial denaturation at 94°C for 5 minutes was also tried. As a result, optimized PCR conditions given in Table 2.3 were used in this study.

Table 2.3 Components of PCR mixture for T7678A SNP.

Constituent	Stock Concentration	Volume to be added	Final Concentration in 50 µl reaction mixture
Amplification buffer*	10x	5 µL	1x
$MgCl_2$*	25 mM	3 µL	1.5 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 2 for these reagents			

Amplification programme used was as follows:

Denaturation	94°C	3 min	
Annealing	61°C	1 min	35 cycles
Extension	72°C	1 min	
Final extension	72°C	6 min	

PCR products were analysed on 1.5% agarose gel (0.45 g agarose dissolved in 30 mL 0.5x TBE buffer) as described in section 2.2.3. 5µL of PCR product was mixed by 1 µL of gel loading buffer and applied to the wells of gel. 3 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100V.

2.2.5 Restriction Endonuclease Digestion of PCR Products

*Rsa*I/ *Pst*I and *Dra*I restriction endonucleases were used in RFLP analysis of CYP2E1 polymorphisms; C-1910T/ G-1259C and T7678A substitutions, respectively.

2.2.5.1 C-1019T Single Nucleotide Polymorphism

PCR products were digested with *Rsa*I restriction enzyme in a reaction mixture as described in Table 2.4. Digestions with *Rsa*I were carried out at 37°C for 12, 18 and 24 hours.

Table 2.4 Components of digestion mixture for C-1019T SNP.

Constituent	Stock Concentration	Volume to be added	Final Concentration in 30 µl reaction mixture
Buffer Y^+/TANGO™ *	10x	3 µL	1x
PCR Product		10 µL	
***Rsa*I**	10 U/µL	1 µL	10 U
Sterile apyrogen H_2O		16 µL	
* see Appendix 2 for the components of this buffer			

The mixture was incubated at 37°C for 18 hours for complete digestion and then analysed on 2.5% agarose gel (1.25 g agarose dissolved in 50 mL 0.5x TBE buffer). 20 µL of digestion product was mixed with 5 µL of gel loading buffer and applied to the wells of the gel. 10 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1.5 hour at 100V.

2.2.5.2 G-1259C Single Nucleotide Polymorphism

PCR products were digested with *Pst*I restriction enzyme in a reaction mixture as described in Table 2.5. Digestions with *Pst*I were carried out at 37°C for 12, 18 and 24 hours.

Table 2.5 Components of digestion mixture for G-1259C SNP.

Constituent	Stock Concentration	Volume to be added	Final Concentration in 30 µl reaction mixture
Buffer O^+ *	10x	3 µL	1x
PCR Product		10 µL	
***Pst*I**	10 U/µL	1 µL	10 U
Sterile apyrogen H_2O		16 µL	
* see Appendix 2 for the components of this buffer			

The mixture was incubated at 37°C for 18 hours for complete digestion and then analysed on 2.5% agarose gel (1.25 g agarose dissolved in 50 mL 0.5x TBE buffer). 20 µL of digestion product was mixed with 5 µL of gel loading buffer and applied to the wells of the gel. 10 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100V.

2.2.5.3 T7678A Single Nucleotide Polymorphism

PCR products were digested with *Dra*I restriction enzyme in a reaction mixture as described in Table 2.6. Digestions with *Dra*I were carried out at 37°C for 12, 18 and 24 hours.

Table 2.6 Components of digestion mixture for T7678A SNP.

Constituent	Stock Concentration	Volume to be added	Final Concentration in 30 µl reaction mixture
Buffer B^+ *	10x	3 µL	1x
PCR Product		20 µL	
***Dra*I**	10 U/µL	0.6 µL	6 U
Sterile apyrogen H_2O		6.4 µL	
* see Appendix 2 for the components of this buffer			

The mixture was incubated at 37°C for 24 hours for complete digestion and then analysed on 1.8% agarose gel. 20 µL of digestion product was mixed with 5 µL of gel loading buffer and applied to the wells of the gel. 3 µL of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour at 100V.

2.2.6 Statistical Analysis

In this study, non-parametric Chi-Square Test was used to check if the calculated frequencies for both C-1910T/ G-1259C and T7678A SNPs fit to the Hardy-Wienberg equilibrium. The same test was also used for other purposes such as the comparison of genotype distributions of Turkish population determined in this study with other populations.

Whenever any of the expected frequencies was less than 5, "Yates' correction for continuity" was made for Chi-Square Test. The formula of Chi-Square Test is given below:

$$\chi^2 = \sum \frac{(|F_o - F_e| - 0.5)^2}{F_e}$$

Yates' correction factor

where F_o is the observed frequency and F_e is the expected frequency.

CHAPTER 3

RESULTS

This study comprised a total of 132 DNA samples obtained from blood of healthy volunteers representing Turkish population. The two important single nucleotide polymorphisms in *CYP2E1* gene was determined on these samples by PCR-RFLP techniques, using the corresponding restriction enzymes. Genotyping of *Pst*I/*Rsa*I polymorphism in 5'-flanking region included a total of 116 individuals while that of *Dra*I polymorphism in intron 6 included 108. Genotypes of each individual are listed in Appendix 3.

Among 132 subjects studied, 78 of them were females and 54 were males. The mean age for all samples was 32.78 ± 12.86, for females was 31.0 ± 12.41, and for males was 35.35 ± 13.18. For C-1019T / G-1259C SNPs (*Pst*I/*Rsa*I RFLP), 116 subjects (mean age: 32.00 ± 12.35) were included comprising of 71 females and 45 males (mean ages: 30.82 ± 12.29 and 33.96 ± 12.47, respectively). T7678A SNP (*Dra*I RFLP) was investigated for 108 subjects (mean age: 32.51 ± 12.58) in total, among them 62 were female and 46 were male (mean ages: 31.10 ± 12.19 and 34.41 ± 12.96, respectively). The number of subjects involved in this study and correspondig means for ages is represented in Table 3.1.

Table 3.1 Number of subjects involved in the study and means of ages.

Study sample	Whole subjects (Females & Males)		Females		Males	
	Number	Mean of Age	Number	Mean of Age	Number	Mean of Age
Total	**132**	**32.78 ±12.86**	**78**	**31.0 ± 12.41**	**54**	**35.35 ± 13.18**
C-1019T / G-1259C	**116**	**32.00 ± 12.35**	**71**	**30.82 ± 12.29**	**45**	**33.96 ± 12.47**
T7678A	**108**	**32.51 ± 12.58**	**62**	**31.10 ± 12.19**	**46**	**34.41 ± 12.96**

3.1 Genomic DNA Isolation from Human Whole Blood and Analysis

DNA isolation was a critical step of this study as contamination of DNA with protein or phenol interferes with the subsequent PCR steps. The purity of DNA samples were determined as described in section 2.2.2. DNA samples having OD_{260}/OD_{280} ratio below 1.6 or above 2.0, were discarded and the isolation procedure was repeated for those samples.

Another important point to be considered in DNA isolation is to obtain the DNA in intact form. The intactness of DNA was determined by agarose gel electrophoresis for each sample. Intact genomic DNA should be observed as an intense single band at the wells. A smear on the gel implies that DNA is degraded and can not be used in further studies. As an example, a photograph of genomic DNA samples after isolation is represented in Figure 3.1.

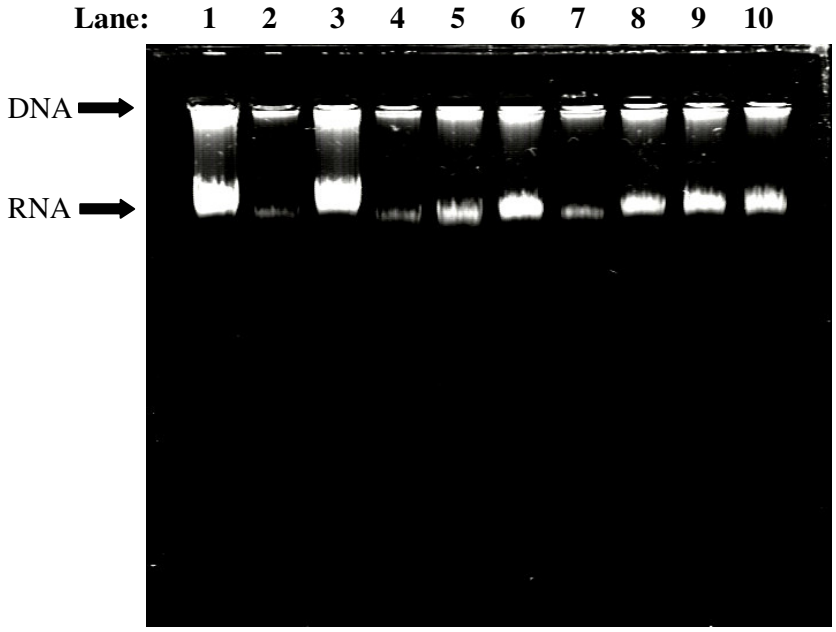


Figure 3.1 Determination of intactness of isolated genomic DNA on 0.5 % agarose gel electrophoresis. 5 µL of DNA sample was loaded in each well and run for 45 minutes at 100 V. Lanes 1-10 contained genomic DNA of samples 162, 163, 164, 8, 13, 21, 22, 31, 32, 38, 39, 43, in order. The bands on the upper position were genomic DNA while the lower bands were total RNA, as RNase treatment was not performed during DNA isolation.

3.2 Amplification of Polymorphic Regions of Human *CYP2E1* Gene by Polymerase Chain Reaction

Two regions of CYP2E1 gene; one in 5'-flanking region and one in intron 6 were amplified to study the C-1019T / G-1259C (*Rsa*I/*Pst*I) and T7678A (*Dra*I) single nucleotide polymorphisms, respectively.

3.2.1 C-1019T / G-1259C Single Nucleotide Polymorphisms in 5'-Flanking Region of Human *CYP2E1* Gene

In order to obtain a single band belonging to the region in 5'-flanking region of *CYP2E1* gene, devoid of non-specific bands, different parameters were tested in PCR. These parameters included the change in concentration of $MgCl_2$ as 1.0, 1.25, 1.5, 1.75, 2.0 and 3.0 mM; change in concentration of primer pairs as 20, 40 and 100 pmol; and change in concentration of template DNA as 100, 200, 300 and 500 ng. Besides, addition of 10 µg BSA into reaction medium was tested to check whether an improvement in the efficiency of PCR was obtained or not. In the amplification programme, an initial denaturation at 94°C for 5 minutes was also tried. Agarose gel results of the optimization experiments is presented in Appendix 3. The optimized reaction medium contained 1.5 mM $MgCl_2$, 200 µM of each dNTP, 20 pmol of forward and reverse primers, ~200 ng of genomic DNA and 2.5 U of Taq DNA polymerase as described under Materials and Methods. A single band of ~400 bp was obtained as a result of PCR which was visualized by 2% agarose gel electrophoresis; an example is presented in Figure 3.2.

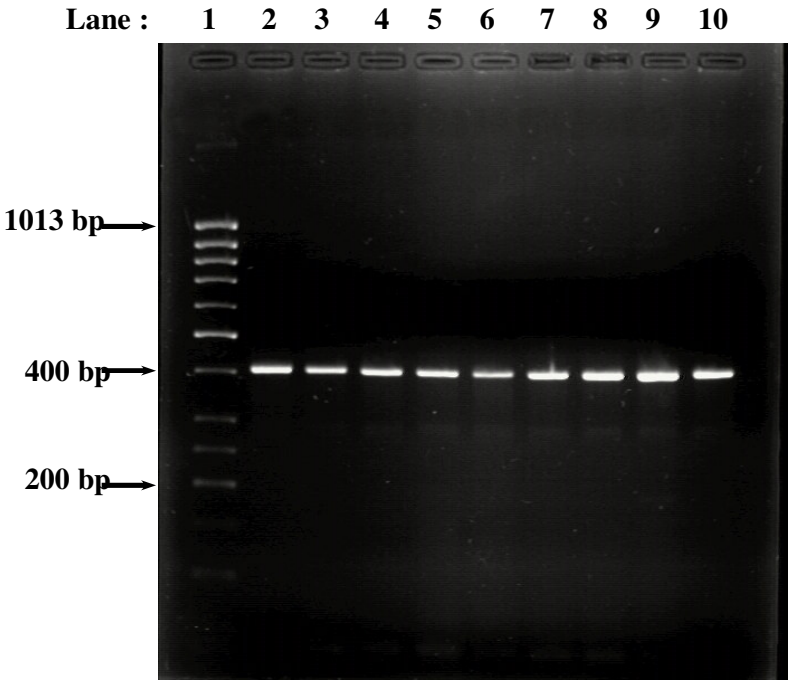


Figure 3.2 Agarose gel electrophoresis showing PCR products of amplified ~400 bp fragment in 5'-flanking region of *CYP2E1* gene. 5 µL of PCR product was loaded on 2% agarose gel and run for 1 hour at 100 V. As expected, a single band of ~400 bp was obtained. Lane 1 was DNA Ladder (50-1000 bp), Lanes 2-10 contained PCR products of samples 1, 2, 4, 5, 6, 7, 10, 11, 12, in order.

3.2.2 T7678A Single Nucleotide Polymorphism in Intron 6 of Human *CYP2E1* Gene

The region amplified in intron 6 of *CYP2E1* gene, that contains the SNP T7678A and corresponding restriction endonuclease *Dra*I recognition site, was comprised of 997 nucleotides. In order to obtain a single band belonging to the region in intron 6 of *CYP2E1* gene, devoid of non-specific bands, different parameters were tested in PCR. These parameters included the changes in concentration of $MgCl_2$ as 1.0, 1.5, and 2.0 mM; change in concentration of primers as 10, 20 and 40 pmol; and change in concentration of template DNA as 100, 200

and 500 ng. Besides, addition of 10 µg BSA into reaction medium was tested to check whether an improvement in the efficiency of PCR was obtained or not. In the amplification programme, an initial denaturation at 94°C for 5 minutes was also tried. Agarose gel results of the optimization experiments is presented in Appendix 3. The optimized reaction medium contained 1.5 mM $MgCl_2$, 200 µM of each dNTP, 20 pmol of forward and reverse primers, ~200 ng of genomic DNA and 2.5 U of Taq DNA polymerase and PCR was carried out as described in Materials and Methods. The PCR products were run and visualized on 1.5% agarose gel. The PCR results showed that the reaction yielded a single band of ~1000 bp, as expected. A photo of the PCR products is represented in Figure 3.3.

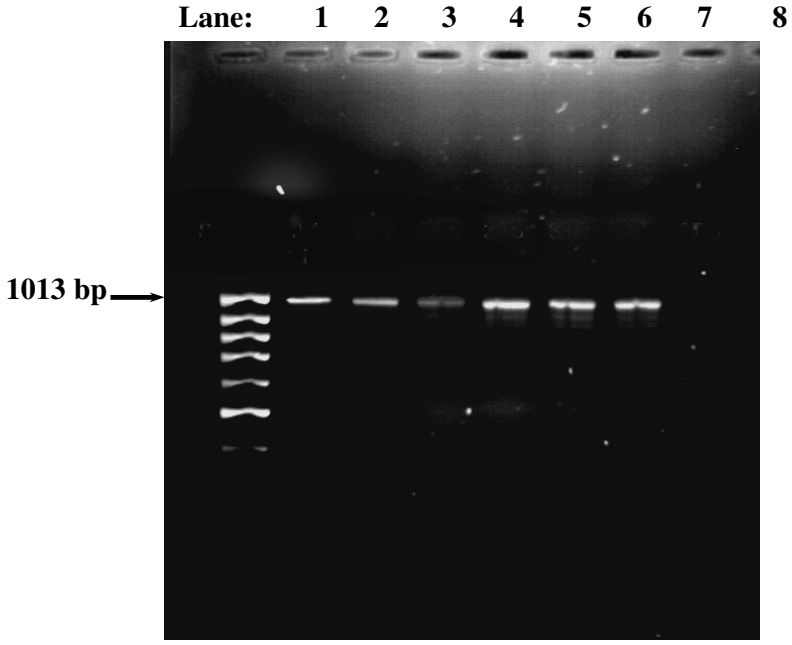


Figure 3.3 Agarose gel electrophoresis after amplification of ~1000 fragment in intron 6 of *CYP2E1* gene. 5 µL of PCR product was loaded on 1.5% agarose gel and run for 1 hour at 100 V. As expected, a single band of ~1000 bp was obtained. Lane 1 contained DNA Ladder (50-1000 bp), Lanes 1-7 contained samples 3, 13, 21, 32, 33 and 35, in order. Lane 8 is empty.

3.3 Digestion of PCR Products of Human *CYP2E1* Gene with Restriction Enzymes

PCR products of both intron 6 and 5'-flanking regions contained the single nucleotide polymorphisms. It is possible to detect these polymorphisms by RFLP technique if the polymorphic nucleotide is a part of a restriction endonuclease recognition site, so that with the base substitution from wild type to mutated form, either a restriction site is lost or created. As a result, digestion with the corresponding restriction enzyme reveals the genotype of the individual. Both situations are observed in studied human *CYP2E1* single nucleotide polymorphisms.

The restriction endonucleases *Rsa*I/*Pst*I and *Dra*I were used for genotyping of C-1019T / G-1259C and T7678A single nuclotide polymorphisms, respectively. The recognition sites of these restriction enzymes and the corresponding SNPs are given in Table 3.2.

Table 3.2 The single nucleotide polymorphisms of *CYP2E1* gene and corresponding restriction endonucleases with recognition sequences.

Region of SNP	SNP	Restriction Endonuclease	Recognition Site of the Restriction Enzyme
5'-Flanking	C-1019T	*Rsa*I	5'..G T ↓A C..3' 3'..C A ↑T G..5'
	G-1259C	*Pst*I	5'..C T G C A ↓G..3' 3'..G ↑A C G T C..5'
Intron 6	T7678A	*Dra*I	5'..T T T ↓A A A..3' 3'..A A A ↑T T T..5'

3.3.1 C-1019T / G-1259C Single Nucleotide Polymorphisms in 5'-Flanking Region of Human *CYP2E1* Gene

The amplified 412 bp region in the 5'-flanking region of the CYP2E1 gene contains two SNP sites: C-1019T and G-1259C, which are located in the recognition sites of *Rsa*I and *Pst*I, respectively. Figure 3.4 represents the sequence of amplified region in 5'-flanking region, showing the location of primers, both SNPs and corresponding restriction endonuclease recognition sites. For genotyping, the PCR product in 5'-flanking region was separately digested with *Rsa*I and *Pst*I.

3.3.1.2 *Rsa*I Restriction Endonuclease Digestion for C-1019T SNP

In C-1019T single nucleotide polymorphism, -1019 nucleotide of CYP2E1 gene is occupied with C in wild type allele, while the same location is T in mutated allele. In wild type allele, with C in position –1019, the PCR product bears a recognition site for restriction endonuclease *Rsa*I (recognition site: 5'-GTAC-3'), as can be seen in Figure 3.4 and Figure 3.5. Digestion with *Rsa*I at 37°C for 18 hours, efficiently cuts the PCR product, producing two fragments of approximately 350 bp and 50 bp. However, in the mutated allele, -1019 position is occupied with T, so there is no suitable sequence that *Rsa*I can recognize and cut the PCR product. In this case, digestion of PCR products with *Rsa*I yields one fragment of 412 bp and that is the undigested PCR product itself.

So, if the individual is homozygous wild type, the presence of recognition site for *Rsa*I would cause effective digestion of the PCR product and two bands of 350 bp and 50 bp would be obtained, while in a homozygous mutated individual, restriction enzyme would fail to cut the PCR product and a single 400 bp fragment would be the result. In heterozygotes, as expected, both undigested 400 bp band and digested fragments of 350 bp and 50 bp would be observed. The digestion pattern of *Rsa*I in wild type and mutated alleles is presented schematically in Figure 3.5.

-1382 CACCCGTGAG CCAGTCGAGT CTACATTGTC AGTTCTCACC TCGAGGGGTG CCAAAAACCA

-1322 GAGGGAAGCA AAGGCCCCTG AAGCCTCTGC CAGAGGCCAA CGCCCCTTCT TGGTTCAGGA

-1262 TGCAGT GTTAGGTGCA GCACAACCAA TGACTTGCTT ATGTGGCTAA TAAATTGTCA

-1202 AGAGAAAAAC TGGGTTAGAA TGCAATATAT AGTATGTAGT CTCATTTTTG TATAAATACA

-1142 AGTATAGAAT GGCATAACTC AAAATCCACA AGTGATTTGG CTGGATTGTA AATGACTTTT

-1082 ATTTTCTTCA TTTCTCATCA TATTTTCTAT TATACATAAA GATTCATTGT TAATATAAAA

-1022 GTACAAAATT GCAACCTATG AATTAAGAAC TTCTATATAT TGCCAGTTAG AAGACAGAAT

-962 GAAAAAACATT CTCTTCATTC TAA……..

58

Figure 3.4 Sequence of amplified fragment in 5'-flanking region of *CYP2E1* gene that includes C-1019T / G-1259C single nucleotide polymorphisms. The blue highlighted sequences are forward and reverse primers, red highlighted nucleotides show the location of SNPs, and the yellow underlined and highlighted sequences show the recognition sites for restriction enzymes *Pst*I and *Rsa*I, as indicated (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

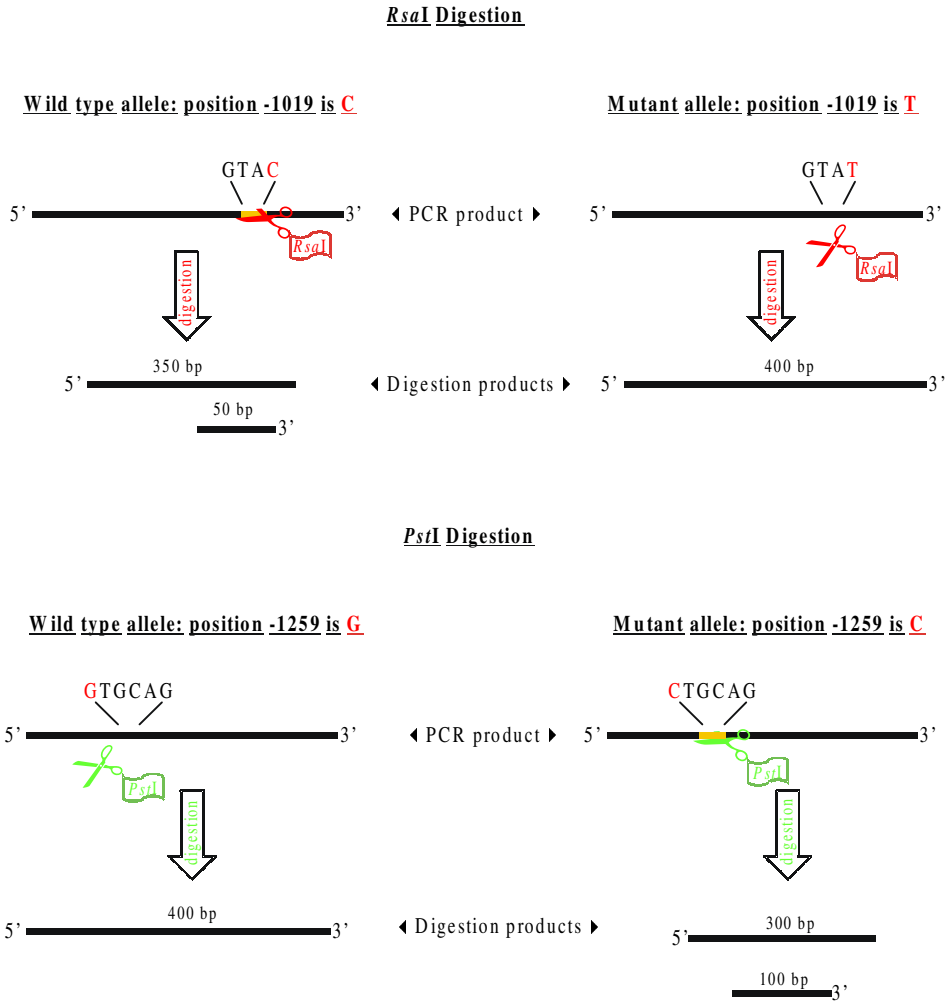


Figure 3.5 Schematic representation of the banding patterns of the amplified 5'-flanking region of *CYP2E1* gene upon digestion with *Rsa*I and *Pst*I. The upper part shows the *Rsa*I digestion pattern of wild type and mutated allele (indicated in red) while the lower part shows those of *Pst*I digestion (indicated in green). The yellow signs on the genes indicates the presence of a suitable recognition sequence for the enzyme, the scissors are representatives of restriction enzymes.

In this study, 10 µL of amplified PCR product of *CYP2E1* gene was digested with 10 units of *Rsa*I in a buffer recommended by manufacturer. The digestion was carried out at 37°C for 18 hours to assure complete digestion of fragment by *Rsa*I. Digestion of all possible fragments is important as any undigested product would cause a conflict in determination of genotype. The digestion products were analyzed with 2.5% agarose gels, and each individual's genotype was determined according to the band pattern after digestion: two bands of 350 and 50 bp indicated homozygous wild type individuals, three bands of 400, 350 and 50 bp revealed the genotype of a heterozygous individual. Throughout the study, a homozygous mutated individual, with a single band of 400 bp was not encountered. As an example to *Rsa*I digestion, a photograph is represented in Figure 3.6.

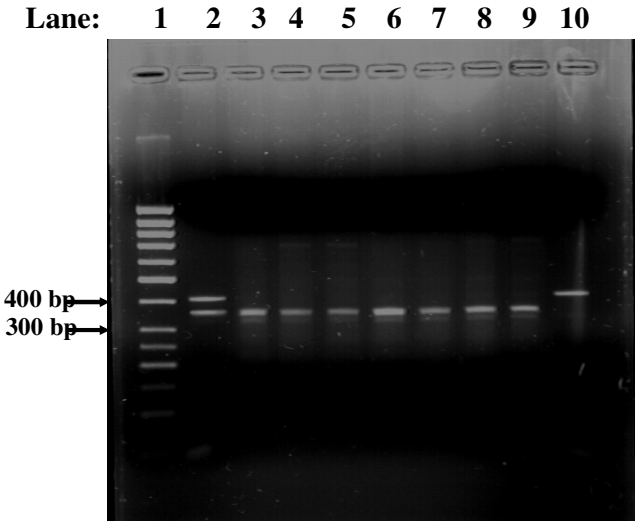


Figure 3.6 Agarose gel electrophoresis of *Rsa*I digestion of amplified fragment in 5'-flanking region of *CYP2E1*. 20 µL of digestion product was loaded in 2.5% agarose gel and electrophoresed for 1.5 hour at 100 V. Lane 1 is DNA Ladder (50-1000 bp). Lanes 3-9 represents homozygous wild individuals (sample no's: 67, 68, 69, 70, 71, 72, 73). Lane 2 is a heterozygous individual (sample no: 66), as both 400 bp and 350 bp bands are observed. Lane 10 is the PCR product, which was not subjected to digestion. All lanes (except 10) also contain a 50 bp band which is not observable in the photo.

3.3.1.3 *Pst*I Restriction Endonuclease Digestion for G-1259C SNP

-1259 position of *CYP2E1* gene is occuppied by G in wild type alleles, while it is substituted to C in the mutated alleles. In wild types, with G at position –1259, there is no recognition site for restriction endonuclease *Pst*I in the amplified fragment of 5'-flanking region. (see Figure 3.4 and Figure 3.5; recognition site for *Pst*I is 5'-CTGCAG-3'). However, in the mutated allele, that bears C at position –1259, there is a recognition site for *Pst*I (underlined with yellow in Figure 3.4). So, situation in this case is opposite to that in *Rsa*I digestion; *Pst*I restriction endonuclease can not cut the PCR product in wild types as its recognition site is not present; but an appopriate recognition sequence is present in mutated alleles, therefore *Pst*I cuts the 400 bp PCR product into two fragments of 300 and 100 bp.

As a result, when analyzing the *Pst*I digestion results in agarose gel, a homozygous wild type individual would yield a sigle band of 400 bp, as *Pst*I fails to cut the PCR product; while a homozygous mutated individual would yield two bands of 300 and 100 bp, as the enzyme manages to cut the PCR product. Obviously, heterozygotes are expected to yield three bands with 400, 300 and 100 bp. The digestion pattern of *Pst*I is represented schematically in Figure 3.5.

In this study, 10 µL of PCR product of human *CYP2E1* gene was digested with 10 units of restriction endonuclease *Pst*I at 37°C for 18 hours, again to assure complete digestion of every possible fragment. The digestion products were analyzed on 2.5% agarose gels. Genotypes were determined according to the band pattern observed on the gel, so that a single band of 400 bp showed homozygous wild type individuals, while the presence of all three bands (400, 300 and 100 bp) indicated the genotype of a heterozygote. Throughout this study, a homozygous mutated individual (300 and 100 bd bands) was not encountered. Agarose gel results of the samples digested with *Pst*I is presented in Figure 3.7. The DNA samples obtained from the same subjects were also used for *Rsa*I digestion results presented in Figure 3.6.

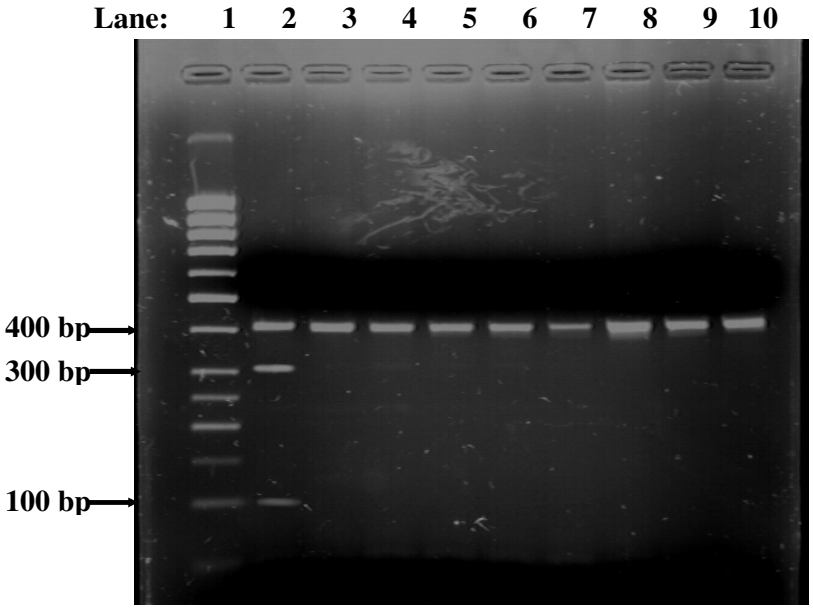


Figure 3.7 Agarose gel electrophoresis of *Pst*I digestion products. 20 µL of digestion product was loaded into 2.5% gel and run for 1 hour at 100 V. DNA Ladder (50-1000 bp) was loaded in Lane 1. Lane 3-9 represents homozygous wild type individuals having a single band of 400 bp (sample no's: 67, 68, 69, 70, 71, 72, 73). Lane 2 shows a heterozygous individual having three bands of 400, 300 and 100 bp (sample no: 66). Lane 10 is a PCR product which was not subject to digestion.

3.3.2 *Dra*I Restriction Endonuclease Digestion for T7678A SNP in Intron 6 of Human *CYP2E1* Gene

The 1000 bp amplified region in the intron 6 of *CYP2E1* gene included a single nucleotide polymorphism at nucleotide position of 7678. This position is occupied by either T or A, in wild type alleles and mutants, respectively. Figure 3.8 represents the partial nucleotide sequence of the amplified region, showing the location of primers, the single nucleotide polymorphism and the recognition sites of the restriction enzyme *Dra*I. The amplified fragment contained two recognition sites as shown in Figure 3.8, one of which includes the SNP in its sequence.

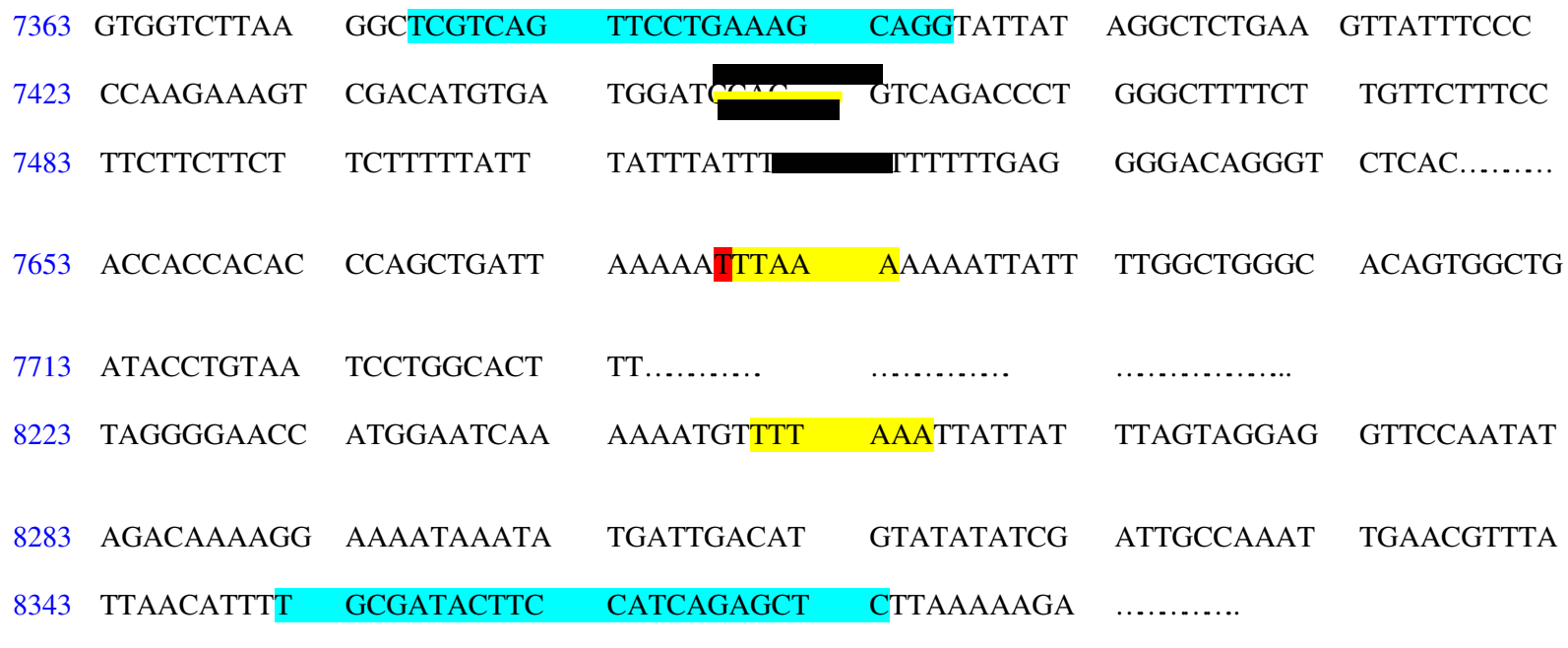


Figure 3.8 Sequence of amplified fragment in intron 6 of *CYP2E1* gene that includes T7678A single nucleotide polymorphism. The amplified region contains two recoginition sites for *Dra*I restriction enzyme, and T7678A SNP is located in one of them. The blue highlighted sequences are forward and reverse primers, red highlighted nucleotide shows location of SNP, and the yellow highlighted sequences show the recognition sites for restriction enzyme *Dra*I, as indicated. The dots indicates that there are many nucleotides there, which are not presented for convenience (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov)

The wild type alleles that contain a T at position 7678, also bear a recognition site around that nucleotide, enabling *Dra*I restriction endonuclease to cut the PCR product from that position. But in the mutant alleles with A in postion 7678, there is not a recognition site around the SNP, preventing *Dra*I to cut the fragment (Figure 3.8 and Figure 3.9). Therefore, as the PCR product contains an additional recognition sequence for the restriction enzyme, independently from the presence or absence of SNP, the 1000 bp PCR product is cut into two; yielding 900 bp and 100 bp fragments upon *Dra*I digestion. In the wild type alleles, the 900 bp fragment is further cut into 600 bp and 300 bp fragments, as a second recognition site in the 900 bp fragment is present. In mutated allele, however, due to base substitution T to A is present, the corresponding recognition site is absent, and further digestion does not occur, resulting two fragments of 900 and 100 bp.

Accordingly, the expected banding patterns upon digestion of amplified region in intron 6 of *CYP2E1* gene with *Dra*I restriction enzyme is as follows: In homozygous wild types, presence of two recognition sequences would yield three bands of 600, 300 and 100 bp, while in homozygous mutants, as one recognition sequence around SNP is lost due to base substitution, only two bands of 900 and 100 bp, are expected. The heterozygotes would contain in total four bands in lenghts of 900, 600, 300 and 100 bp. The band pattern of *Dra*I digestion is shown schematically in Figure 3.9.

In this study, 20 µL of PCR product from intron 6 of *CYP2E1* gene was digested with 6 units of restriction endonuclease *Dra*I and the mixture was incubated at 37°C for 24 hours for complete digestion. As expected, three bands of 600, 300 and 100 bp were observed for homozygous wild type individuals and four bands of 900, 600, 300 and 100 bp were observed for heterozygotes. However, none of the individuals investigated was homozygous mutated. Figure 3.10 is an agarose gel showing the results of *Dra*I digestion of PCR products from intron 6 of human *CYP2E1* gene.

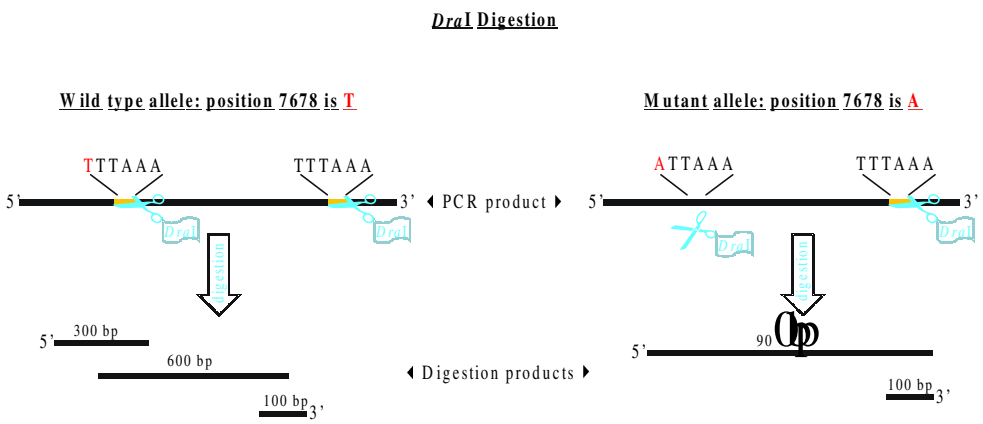


Figure 3.9 Schematic representation of the banding patterns of amplified fragment in intron 6 of *CYP2E1* gene upon digestion with *Dra*I. The yellow sings on the gene indicate presence of a suitable recognition site for *Dra*I. The scissors in blue represents the restriction enzyme *Dra*I.

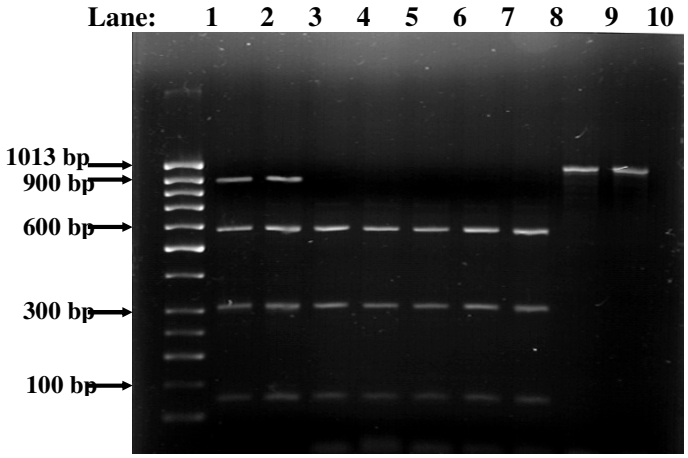


Figure 3.10 Agarose gel electrophoresis of *Dra*I digestion of PCR products from intron 6 of *CYP2E1* gene. 20 µL of digestion product was loaded on 1.8% agarose gel and the gel was run for 1 hour at 100 V. Lane 1 is DNA ladder. In lane 2 and 3, four bands of 900, 600, 300, and 100 bp indicates heterozygotes (Sample no's: 13, 14). Lanes 4-8 with 600, 300 and 100 bp bands belong to homozygous wild type individuals (Sample no's: 15, 16, 17, 18, 19). Lanes 9 and 10, with single 1000 bp bands, are PCR products that were not subject to *Dra*I digestion.

3.4 Genotyping for C-1019T/G-1259C Single Nucleotide Polymorphisms in 5'-Flanking Region of Human *CYP2E1* Gene

The two single nucleotide polymorphisms, C-1019T and G-1259C are located in the 5'-flanking region of the *CYP2E1* gene with close proximity to eachother. It is well established that these two polymorphisms are in complete linkage disequilibrium (Watanabe *et al.,* 1990; Hayashi *et al.,* 1991), and the genotypes are named as c1/c1 for homozygous wild type, c2/c2 for homozygous mutated, and c1/c2 for heterozygotes, accordingly c1 depicts wild type allele for C-1019T and G-1259C, while c2 depicts mutated allele for both SNP sites. Complete linkage disequilibrium indicates that the two SNPs are associated and always inherited together, for example, if –1019 is occupied with C, -1259 is always occupied with G, but never C. Table 3.3 summarizes the possible genotypes in 5'-flanking region of *CYP2E1* gene.

Table 3.3 Genotyping of 5'-flanking region of *CYP2E1* gene.

Genotype of 5'-Flanking Region	**Genotype of C-1019T SNP (*Rsa*I polymorphism)**	**Genotype of G-1259C SNP (*Pst*I polymorphism)**
c1c1	Homozygous wild type	Homozygous wild type
c1c2	Heterozygous	Heterozygous
c2c2	Homozygous mutated	Homozygous mutated

In this study, *Rsa*I / *Pst*I polymorphisms are identified for 116 individuals. 113 of them were homozygous wild type for both polymorphic sites (C-1019Tand G-1259C), while 3 individuals were heterozygous for both sites. A homozygous mutated individual was not identified in this study. In other words, of 116 individuals studied, 113 of them had the genotype of c1/c1 and 3 had c1/c2 genotype, while

c2/c2 genotype was not observed. The corresponding genotype frequencies were 97.4% for c1/c1, 2.6% for c1/c2 and 0.0% for c2c2. The allele frequency of c1 was calculated as 98.7% and that of c2 as 1.3%. The genotype and allele frequencies are presented in Table 3.4. The complete documentation with respect to each individual is given in Appendix 4.

Table 3.4 Genotype and allele frequencies of C-1019T/G-1259C SNPs.

Genotype	N	Frequency (%)
c1/c1	113	97.4
c1/c2	3	2.6
c2/c2	0	0
Total	116	100

Allele	Frequency (%)
c1	98.7
c2	1.3

3.5 Genotyping for T7678A Single Nucleotide Polymorphism in Intron 6 of Human *CYP2E1* Gene

As mentioned before, T7678A SNP is located in intron 6 of *CYP2E1* gene and RFLP analysis of this polymorphism was done with the restriction enzyme *Dra*I. The wild type allele for this polymorphism is denoted as D, while the mutated allele is denoted as C. Accordingly, homozygous wild type individuals are designated as DD, homozygous mutated individuals as CC and the heterozygous individuals as CD.

In this study, a total of 108 individuals were investigated for *Dra*I polymorphism. Among them, 87 were homozygous wild type (DD), while 21 of them were heterozygotes (CD). Homozygous mutated individuals (CC) were not encountered throughout the study. The corresponding genotype frequencies were

80.6% for DD and 19.4% for CD (0.0% for CC). The allele frequencies was found to be 90.3% for D and 9.7% for C. The genotype and allele frequencies are summarized in Table 3.5. The complete documentation for each individual is given in Appendix 4.

Table 3.5 Genotype and allele frequencies of T7678A SNP.

Genotype	N	Frequency (%)
DD	87	80.6
CD	21	19.4
CC	0	0.0
Total	108	100

Allele	Frequency (%)
D	90.3
C	9.7

CHAPTER 4

DISCUSSION

There are often large differences among individuals in the way they respond to drug or xenobiotic exposure. Potential causes for variability in xenobiotic effects include the individual's age, race and gender, nutritional states, smoking status, drug interactions, and as a major factor, pharmacogenetic polymorphisms in the drug metabolizing and related genes.

As a result of recent advances in genome technology, it is now easier to identify and study the polymorphisms and their phenotypic consequences in drug metabolizing enzymes. Genotyping approach in assessing the variability in xenobiotic response has an advantage over phenotyping approach, in the sense that it is not affected from external factors, underlying diseases or drug-drug interactions, which are usually obstacles in relating phenotyping studies with genetic polymorphisms. However, although genotyping provides direct information on which xenobiotic-metabolizing alleles are present, accurate interpretation requires an understanding of the relationship between any given genotype and the resultant phenotype. So genotyping studies should be accompanied with biochemical studies to accomplish the relation of polymorphisms with phenotypic outcomes.

On the other hand, initially, inherited differences in drug metabolism were discovered following clinical observations of marked interindividual differences in drug response, and this clinically based approach made it likely that pharmacogenetic polymorphisms would have clinical consequences for drug effect as their discovery was based on a clinical phenotype. However, genotyping studies showed that there are many pharmacogenetic polymorphisms which do not have phenotypic outcomes, but an important portion of these polymorphisms are thought to have more subtle, yet important, consequences for interindividual variability in human xenobiotic response, and can be risk factors for the susceptibility to related disease incidence such as cancer (Evans and Johnson, 2001). In this sense, genetic polymorphisms in xenobiotic-metabolizing enzymes, including cytochrome P450s that contribute to chemical toxicity, is likely to be a significant factor in determining susceptibility to diseases related to exposure of xenobiotics (Daly, 1998).

CYP2E1 is a special isoform among xenobiotic-metabolizing P450s with its substrate specificity; including many industrial chemicals most of which are at the same time procarcinogens or carcinogens, as well as some widely used therapeutic drugs, like acetaminophen. Besides, it has a high capacity to reduce molecular oxygen and form reactive oxygen species which can cause liver pathology and DNA adducts, hence chemical-induced carcinogenesis. Its ability to metabolize ethanol depicts its importance in ethanol metabolism, especially upon induction while it is also proposed to have roles in patophysiological states like starvation and diabetes due to its ability in metabolizing ketone bodies, like acetone. Besides having a complex mechanism of regulation, CYP2E1 also possess several polymorphisms throughout its gene.

CYP2E1 polymorphisms drive considerable attention on them, as they may be the causes for interindividual differences in susceptibility to especially occupational diseases caused by chemicals or chemical-induced carcinogenesis.

The most frequently studied genetic polymorphisms in *CYP2E1* are the single nucleotide polymorphisms C-1019T/G-1259C, located in the 5'-flanking region of the gene, which can be detected by RFLPs *Rsa*I/*Pst*I (Hayashi *et al.,* 1991), as well as the T7678A single nucleotide polymorphism located in intron 6, which can be detected by *Dra*I RFLP (Uematsu *et al.* 1991).

It is also well established that *CYP2E1* polymorphisms differ markedly in frequency among ethnic and racial groups, like most other xenobiotic-metabolizing enzymes (Evans and Relling, 1999). That's why identification of *CYP2E1* polmyorphisms in different populations become important.

For this purpose, determination of *CYP2E1* polymorphisms in Turkish population receives attention, and in this study, the two important *CYP2E1* polymorphisms, C-1019T / G-1259C SNPs in 5'-flanking region, and T7678A SNP in intron 6 were investigated in a sample of healthy volunteers representing Turkish population.

The genotypes of C-1019T / G-1259C SNPs in 5'-flanking region are designated as c1/c1 for homozygous wild types, c1/c2 for heterozygotes and c2/c2 for homozygous mutated individuals while the T7678A single nucleotide polymorphism is designated as DD for homozygous wild types, CD for heterozygotes and CC for homozygous mutated individuals.

The allele frequencies and genotype frequency distributions of both C-1019T/G-1259C and T7678A polymorphisms are given in Table 4.1.

Table 4.1 Allele frequencies and genotype distributions of C-1019T / G-1259C and T7678A polymorphisms.

SNP	Allele frequencies			Genotype distribution			
	Allele	N	Frequency	Genotype	N	Frequency	Expected N *
C-1019T/G-1259C (*Rsa*I/*Pst*I RFLP)	**c1**	229	0.987	**c1/c1**	113	0.974	113.01
	c2	3	0.013	**c1/c2**	3	0.026	2.97
				c2/c2	0	0.000	0.02
	$\chi^2 = 0.00$; df = 2; $p<0.005$						
T7678A (*Dra*I RFLP)	**D**	195	0.903	**DD**	87	0.806	88.02
	C	21	0.097	**CD**	21	0.194	18.96
				CC	0	0.000	1.02
	$\chi^2 = 0.0031$; df = 2; $p<0.005$						

***** Expected N were determined by the Hardy-Weinberg equilibrium.

The two single nucleotide polymorphisms: C-1019T and G-1259C base substitutions, which are in close proximity to each other, were found to be in complete linkage disequilibrium (Watanabe *et al.,* 1990; Hayashi *et al.,* 1991). As described previously in section 3.4.1, as a result of complete linkage disequilibrium, if a base substitution is present in –1019 position, it is always accompanied by a base substitution in –1259 position, meaning that an individual cannot have the variant nucleotide 'T' at position –1019 while having the wild type nucleotide 'G' at position –1259, or vice versa. This situtation was also confirmed in this study, so that all individuals heterozygous for C-1019T SNP were also heterozygous for G-1259 SNP as revealed by *Rsa*I and *Pst* restriction enzyme digestions.

The genotype frequencies of C-1019T / G-1259C SNPs in 5'-flanking region were found out to be 0.974 for c1/c1, 0.026 for c1/c2 and 0.00 for c2/c2. The allele frequency of c1 was calculated as 0.987 and that of c2 as 0.013. The genotype frequencies were found to fit Hardy-Weinberg equilibrium as analyzed by χ^2-test (χ^2= 0.00; df= 2; p<0.005) which was in accordance with other studies (Hayashi *et al.,* 1991; Wu *et al.,* 1997; Garte *et al.,* 2001; Sikdar *et al.,* 2003; Silvestri *et al.,* 2003; Ogawa *et al.,* 2003).

The Human Cytochrome P450 Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles) defines *CYP2E1*5B* allele as *Pst*I+*Rsa*I- (see Table 1.4), meaning for the situation *Pst*I restriction endonuclease can cut the fragment while *Rsa*I cannot cut. *Pst*I cuts the fragment if -1259 position is mutated, and *Rsa*I cannot cut if the position –1019 position is mutated (see Figure 3.5). This allele definition, in short, meaning mutated for both sites, corresponds to the c2 allele presented here. So it should also be stated that in this study, *CYP2E1*5B* allele frequency was calculated as 1.3%.

The genotype frequencies for T7678A single nucleotide polymorphism were found to be 0.806 for DD and 0.194 for CD (0.00 for CC). Accordingly, the allele frequencies were calculated as 0.903 for D and 0.097 for C. The genotype frequencies were found to fit Hardy-Weinberg equilibrium as analyzed by χ^2-test (p<0.005; χ^2= 0.40; df= 2), which was in accordance with other studies (Hayashi *et al.,* 1991; Wu *et al.,* 1997; Garte *et al.,* 2001; Sikdar *et al.,* 2003; Silvestri *et al.,* 2003; Ogawa *et al.,* 2003).

The Human Cytochrome P450 Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles) defines *CYP2E1*6* allele as *Dra*I- (see Table 1.4), which demonstrates that 7678 position on *CYP2E1* gene is occupied by A (instead of T), and the restriction enzyme *Dra*I can not cut the fragment (see Figure 3.9). In this study, the C allele corresponds to the *CYP2E1*6* allele, so it can be stated that in this study, the frequency of *CYP2E1*6* allele was 9.7%.

The two single nucleotide polmyorphisms C-1019T/G-1259C and T7678A were also found to be related with each other and another allele representing the situation where both C-1019T/G-1259C and T7678A sites are mutated (c2 and D alleles together) was defined as *CYP2E1*5A* by the Human Cytochrome P450 Allele Nomenclature Committee (see Table 1.4). However, none of the subjects genotyped in this study possessed such a mutated allele for both regions.

The genotype distribution comparisons of both C-1019T / G-1259C and T7678A SNPs with respect to gender is summarized in Table 4.2.

Table 4.2 Genotype distribution comparisons of C-1019T/G-1259C and T7678A polymorphisms with respect to gender.

SNP	Genotype	Females			Males			Total	
		N	Freq.	Exp. N*	N	Freq.	Exp. N*	N	Freq.
C-1019T/G-1259C (*RsaI*/*Pst*I RFLP)	**Total N**	71		71	45		45	116	
	c1/c1	69	0.972	69.16	44	0.978	43.84	113	0.974
	c1/c2	2	0.028	1.86	1	0.022	1.16	3	0.026
	c2/c2	0	0.000	0.00	0	0.000	0.00	0	0.000
	$\chi^2 = 0.0016$; df = 1; $p<0.005$								
T7678 (*Dra*I RFLP)	**Total N**	62		62	46		46	108	
	DD	51	0.823	49.94	36	0.783	37.06	87	0.806
	CD	11	0.177	12.06	10	0.217	8.94	21	0.194
	CC	0	0.000	0.00	0	0.00	0.00	0	0.000
	$\chi^2 = 0.268$; df = 1; $p<0.005$								

* Expected N were calculated with respect to the total N distribution.

When the genotype distribution of C-1019T / G-1259C SNPs (*Rsa*I/*Pst*I RFLP) was examined with respect to gender, it was found that among 71 females investigated, 69 of them showed c1/c1 genotype and 2 of them had c1/c2 genotype (corresponding frequencies: 0.972 and 0.028, respectively); while among 45 males investigated, 44 had c1/c1 genotype and 1 had c1/c2 genotype (corresponding frequencies: 0.978 and 0.022, respectively). When the genotype distributions in two genders are compared by using χ^2-test, there was no significant difference between two genders in terms of genotype frequency distribution ($p<0.005$; $\chi^2= 0.032$; df= 1). This result was in accordance with other studies (Garte *et al.,*2001).

In examination of genotype distribution of T7678A SNP (*Dra*I RFLP) with respect to gender, it was found that in total 62 females, 51 had DD genotype and 11 had CD genotype (corresponding frequencies: 0.823 and 0.177, respectively); while in total 46 males, 36 had DD genotype and 10 had CD genotype (corresponding frequencies: 0.783 and 0.217, respectively). When the genotype distributions in two genders are compared by using χ^2-test, there was no significant difference between two genders in terms of genotype frequency distribution ($p<0.005$; $\chi^2= 0.268$; df= 1). This result was in accordance with other studies (Garte *et al.,*2001).

As mentioned before, polymorphisms in drug-metabolizing enzymes, including CYP2E1, are thought to be associated with interindividual differences in susceptibility to related diseases, including cancer. Although many studies using case-control approaches have examined the association of polymorphisms with cancer risk, many of them produced conflicting results, in part because of the low penetrance of this category of susceptibility, resulting in insufficient statistical power. To clarify the role of individual and composite genotypes at the most interesting and/or highly studied loci in cancer susceptibility, since 1996, in scope of the International Project on Genetic Susceptibility to Environmental Carcinogens (GSEC), data from all around the world on the frequencies of genetic polymorphisms of genes associated with carcinogen metabolism has been being gathered (Taioli, 1999).

In 2001, Garte and co-workers have determined the allele and genotype frequencies of more commonly studied metabolic genes –including *CYP2E1*- in human population using the GSEC database containing information on over 15 000 control (non-cancer) subjects. In this study, major and significant differences were observed in gentoype frequencies of metabolic genes between Caucasians (n= 12 525); Asians (n= 2 136); and Africans and African-Americans (n= 996), and some, but much less, heterogeneity was observed within Caucasian populations from different countries (like Canada, Denmark, Finland, France, Germany, Italy, Netherlands, Norway, Portugal, Saudi Arabia, Slovakia, Slovenia, Spain, Sweden, UK and US). Besides, no differences in allele frequencies were seen by age and sex (Garte *et al.,* 2001).

The genotype frequencies for Caucasian and Asian populations, (data for African populations were not available) determined by Garte and co-workers is represented in Table 4.3, together with the results of this study for both *CYP2E1 Rsa*I/*Pst*I RFLP (C-1019T/G-1259C SNPs) *Dra*I RFLP (T7678A SNP). When the genotype frequency distributions were compared with χ^2-test, it was found that the genotype frequency distribution of both *Rsa*I/*Pst*I and *Dra*I RFLPs of Turkish population sample differed significantly from Asian populations ($p<0.001$, χ^2-test, df=2), but there was no significant difference from Caucasian populations ($p<0.005$, χ^2-test with Yates' correction, df=2) (Table 4.3).

Table 4.3 Comparison of *CYP2E1* genotype frequencies determined in this study with Caucasian and Asian populations.

Population	C-1019T/G-1259C SNPs (*Rsa*I/*Pst*I RFLP)				T7678A SNP (*Dra*I RFLP)			
	N	c1/c1	c1/c2	c2/c2	N	DD	CD	CC
Turkish	**116**	**0.974**	**0.026**	**0.000**	**108**	**0.806**	**0.194**	**0.000**
Caucasian[†]	1454	0.924	0.075	0.001	1360	0.854	0.138	0.008
Asian*[†]	719	0.595	0.359	0.046	286	0.483	0.423	0.094

* statistically significant difference ($p<0.001$, χ^2-test, df=2).
[†] Garte *et al.,* 2001.

Table 4.4 summarizes the genotype frequency distribution of this study and of different populations from other studies with regard to C-1019T/G-1259C SNPs (*Rsa*I/*Pst*I RFLP).

Table 4.4 *CYP2E1* gene C-1019T/G-1259C SNPs (*Rsa*I/*Pst*I RFLPs) genotype frequency distributions from various populations.

Population (N)	Genotype Frequency for C-1019T/G-1259C SNPs (*Rsa*I/*Pst*I RFLP)			Reference
	c1/c1	c1/c2	c2/c2	
Turkish (116)	**0.974**	**0.026**	**0.000**	**Present study**
Brazilian (191)	0.900	0.090	0.010	Nishimoto *et al.*, 2000
Caucosoid (155)	0.968	0.032	0.000	Yang *et al.*, 2001
Chilean (148)*	0.709	0.270	0.021	Quinones *et al.*, 2001
Egyptian (235)	0.983	0.017	0.000	Hamdy *et al.*, 2002
French (172)	0.916	0.047	0.000	Bouchardy *et al.,*2000
German (304)	0.951	0.049	0.000	Farker *et al.*, 1998
German (373)	0.943	0.057	0.000	Brockmöller *et al.*, 1996
Indian (227)	0.980	0.020	0.000	Sikdar *et al.*, 2003
Italian (114)	0.910	0.090	0.000	Ingelman-Sundberg *et al.*, 1993
Mexican-American (92)*	0.707	0.283	0.110	Wu *et al.*, 1997
Spanish (200)	0.900	0.100	0.000	Gonzales *et al.*, 1998
Swedish (148)	0.900	0.090	<0.01	Persson *et al.*, 1993
Turkish (153)	0.961	0.039	0.000	Ömer *et al.*, 2001
US-Caucasian (459)	0.922	0.078	0.000	London *et al.*, 1996
African-American (114)	0.868	0.123	0.090	Wu *et al.*, 1997
African-American (US) (247)	0.980	0.020	0.000	London *et al.*, 1996
Chinese (122) *	0.516	0.435	0.049	Persson *et al.*, 1999
Chinese (150) *	0.440	0.513	0.047	Tan *et al.*, 2000
Japanese (196) *	0.612	0.347	0.041	Ogawa *et al.*, 2003
Japanese (200) *	0.545	0.415	0.040	Murata *et al.*, 2001
Japanese (612)*	0.640	0.320	0.040	Oyama *et al.*, 1997
Taiwanese (231) *	0.580	0.351	0.069	Wang *et al.*, 1999
Taiwanese (251) *	0.574	0.386	0.040	Wu *et al.*, 2002
Taiwanese-Chinese (297) *	0.636	0.347	0.017	Kongruttanachok *et al.*, 2001

* statistically significant difference ($p<0.005$; χ^2-test, with Yates' correction where necessary; df=2).

When the genotype distributions of this study was compared with other populations by using χ^2-test, it was found that there was no significant difference between French, Swedish, Indian, Egyptian, Italian, German, Spanish, US, Caucosoid, and Brazilian populations ($p<0.005$; χ^2-test with Yates' correction; df=2), confirming the comparison of the results of this study and those of Garte *et al.* (2001) (see Table 4.2). The genotype frequency distributions of Turkish population sample were significantly different from those of Chilean ($p<0.005$; $\chi^2= 37.28$, with Yates' correction; df=2) and Mexican-American ($p<0.005$; $\chi^2= 38.00$, with Yates' correction; df= 2) populations. In both populations, the c1/c2 genotype frequencies were observed to be significantly higher than those in this study and other Caucasian populations.

When studies carried out on African-American populations (Wu *et al.,* 1997; London *et al.,* 1996) were compared with this study, it was found that there was no significant difference with regard to C-1019T/G-1259C SNPs ($p<0.005$; χ^2-test with Yates' correction, df=2).

When the genotype distributions between this study and studies on Asian populations (Japanese, Taiwanese and Chinese populations) were compared, there found to be statistically significant difference ($p<0.005$; χ^2-test with Yates' correction where necessary, df=2). These comparison results are in acordance with the comparison between this study and Asian populations determined in the study of Garte *et al.* (2001). The c1/c2 genotype frequencies in all Japanese, Taiwanese and Chinese populations investigated were significantly higher than those in this study and other studies conducted on Caucasian and African-American populations (see Table 4.4). It also worths to note that the homozgous mutated allele c2/c2, which is encountered very rarely in Caucasian and African-American populations, are observed more frequently in these Asian populations.

The genotype frequency distribution of this study and of different populations with regard to T7678A SNP (Dr*a*I RFLP) is summarized in Table 4.5.

Table 4.5 *CYP2E1* gene T7678A SNP (*Dra*I RFLP) genotype frequency distributions from various populations.

Population (N)	Genotype Frequency for T7678A SNP (*Dra*I RFLP)			Reference
	DD	DC	CC	
Turkish (108)	**0.806**	**0.194**	**0.000**	**Present study**
Caucosoid (155)	0.832	0.161	<0.006	Yang *et al.*, 2001
Chilean (129) *	0.636	0.310	0.054	Quinones *et al.*, 2001
Finnish (121)	0.790	0.200	0.010	Hirvonen *et al.*, 1993
French (172)	0.878	0.116	0.006	Bouchardy *et al.*, 2000
German (304)	0.862	0.131	0.007	Farker *et al.*, 1998
German (373)	0.873	0.124	0.003	Brockmöller *et al.*, 1996
Indian (227) *	0.648	0.321	0.031	Sikdar *et al.*, 2003
Italian (114)	0.830	0.170	0.000	Ingelman-Sundberg *et al.*, 1993
Mexican-American (104)	0.721	0.240	0.039	Konishi *et al.*, 2000
Swedish (152)	0.810	0.180	<0.01	Persson *et al.*, 1993
Turkish (153)	0.843	0.150	0.007	Ömer *et al.*, 2001
Chinese (122) *	0.484	0.467	0.049	Persson *et al.*, 1999
Japanese (76) *	0.566	0.289	0.145	Uematsu *et al.*, 1994
Taiwanese (231) *	0.537	0.377	0.086	Wang *et al.*, 1999

* statistically significant difference ($p<0.005$; χ^2-test, with Yates' correction where necessary; df=2).

When the genotype distribution of T7678A SNP of this study and other studies were compared, no significant difference were found in French, Swedish, German, Italian, Finnish and Caucosoid populations ($p<0.005$; χ^2-test with Yates' correction where necessray, df=2), which is an accordance with the comparison of this study with Caucasion populations' genotype distribution determined by Garte *et al.* (2001) (see Table 4.3).

The genotype distribution differed significantly from Indian ($p<0.005$; $\chi^2= 11.37$, with Yates' correction; df= 2) and Chilean ($p<0.005$; $\chi^2= 15.42$; df= 2) populations. In both populations, the frequencies of both DC and CC genotypes were higher than those determined in this study and in other Caucasian populations

investigated. There was also a significant difference between Turkish and Chilean populations in terms of C-1019T/G-1259C SNPs genotype distribution (see Table 4.4). However, it is interesting to note that no significant difference was observed between Indian and Turkish populations with respect to C-1019T/G-1259C SNPs (see Table 4.4) in opposite to the presence of significant difference with regard to T7678A polymorphism (see Table 4.5). Besides, it was found that there was no significant difference between Mexican-Americans and Turkish population sample in this study in terms of T7678A SNP ($p<0.005$; χ^2-test with Yates' correction; df= 2), while there was a significant difference with regard to C-1019T/G-1259C SNPs (see Table 4.4).

When the previous studies on Asian populations (Taiwanese, Chinese and Japanese) were compared with the Turkish population sample of this study, there was a significant difference with respect to genotype distributions of T7678A SNP ($p<0.005$; χ^2-test; df= 2) (see Table 4.6), the results confirming the comparison between this study and Asian populations determined by Garte *et al.* (2001). In all three Asian populations, the heterozygous CD genotypes were observed in higher frequencies than either this study or other Caucasian populations. As well, the rare homozygous mutated genotype was also encountered in considerably higher frequencies among these Asian populations compared to Caucasian populations (see Table 4.5).

A CYP2E1 DraI RFLP study on African ethnicity was not encountered in the literature to perform a comparison test with the Turkish population sample of this study.

To summarize, the genotype distribution for both C-1019T/G-1259C and T7678A SNPs showed no significant difference between Turkish population sample of this study and other Caucasian populations including French, Swedish, German, Italian and Caucosoid populations; besides there was also no significant difference between Egyptian, Spanish, US, and Brazilian populations with respect to

C-1019T/G-1259C SNPs (see Tables 4.4 and 4.5). These results were in accordance with the comparison of Caucasian populations determined by Garte *et al.*(2001) and Turkish population sample of this study (see Table 4.3).

There was a significant difference in terms of both RFLP regions between this study and Chilean population (see Table 4.4 and 4.5). When Indian population study was compared with this study, there was no significant difference between two populations with respect to C-1019T/G-1259C SNP, while a significant difference was present for T7678A SNP. The reverse situation holds true for the comparison of Turkish population sample of this study and Mexican-American populations so that a significant difference was observed with respect to C-1019T/G-1259C SNPs, while no difference was observed with regard to T7678A SNP. It must also be noted that in both studies on Mexican-American population (Wu *et al.,*1997; Konishi *et al.,* 2000), the study sample size is relatively lower compared to other studies.

Confirming the comparison results of Turkish population sample on this study with the Asian populations determined by Garte *et al.* (2001) (see Table 4.3), both RFLP sites investigated in this study showed a significant difference compared to Asian –Japanese, Taiwanese and Chinese- populations (see Table 4.4 and 4.5).

CHAPTER 5

CONCLUSION

In this study, from the whole blood of 132 healty individuals representing Turkish population (mean age being 32.78± 12.86 for the whole sample, 31.0 ± 12.41 for females and 35.35 ± 13.18 for males), genomic DNA was isolated in intact form and three SNPs in *CYP2E1* gne; C-1019T / G-1259C SNPs in 5'-flanking region, and T7678A SNP in intron 6 was investigated by PCR-RFLP techniques, using *Rsa*I/*Pst*I and *Dra*I restriction enzymes, respectively.

The genotype frequencies of C-1019T / G-1259C SNPs in 5'-flanking region, were found out to be 0.974 for c1/c1, 0.026 for c1/c2 and 0.00 for c2c2. The allele frequency of c1 was calculated as 0.987 and that of c2 as 0.013. The genotype frequencies for T7678A SNP in intron 6 were found as 0.806 for DD, 0.194 for CD, and 0.00 for CC. The allele frequencies was found to be 0.903 for D and 0.097 for C. The genotype frequencies are found to fit Hardy-Weinberg equilibrium for both polymorphisms as analyzed by χ^2-test ($p<0.005$; df= 2). Also there was no difference in the genotype distributions of both polymorphisms with respect to gender as analyzed by χ^2-test ($p<0.005$; df= 2).

When the genotype distribution of Turkish population sample of this study was compared with other studies, it was found that for both polymorphisms, there was no significant difference between this study and other studies of Caucasian

populations, including French, Swedish, Italian, German, and Caucosoid populations. As well, the genotype frequency distribution for both polymorphic sites showed no significant difference between this study and a previous study on Turkish population (Ömer *et al.,* 2001) ($p<0.005$; χ^2-test with Yates' corection; df=2). The genotype and allele frequencies for both polymorphism sites were close to eachother in both studies, so it can be stated that these two studies on Turkish population, confirm eachother.

There was a significant difference between Turkish population sample of this study and Asian populations –Japanese, Taiwanese and Chinese populations- for both polymorphism sites ($p<0.005$; χ^2-test with Yates' correction where necessary; df= 2). Also a significant diference was observed with respect to both polymorphic sites between this study and Chilean populations (χ^2-test; df= 2; $p<0.005$).

The comparison results of individual Caucasian and Asian populations with the Turkish population sample of this study were found to be in accordance with the comparison of results of Caucasian and Asian populations determined by Garte *et al.*(2001), using the database of GSEC international project.

It was found that there was no significant difference between Indian and Turkish populations with respect to genotype frequency distribution of C-1019T/G-1259C SNP, but a significant difference was present with respect to T7678A SNP, while there was a significant difference between Turkish and Mexican-American populations for C-1019T/G-1259C SNPs, but no difference was present for T7678A SNP.

As can be noticed from Tables 4.4 and 4.5, the homozoygous mutated genotype frequencies for both polymorphism sites (c2/c2 and CC) are found to be very low (both frequencies were determined as 0.00 in this study), especially in Caucasian populations, which may create conflicting results in studies correlating

these polymorphisms with disease states like cancer. That's why, sample size in such studies becomes very important to obtain high statistical power.

CYP2E1 is thought to be of importance in terms of susceptibility to related disease states. Accordingly, this study was designed as a prior study to investigate the effect of genetic polymorphisms of *CYP2E1* gene in susceptibility for the related disease states, like diabetes, occupational diseases or chemically-induced cancers. It should be also noted that another genetic polymorpism in *CYP2E1* gene is also planned to be involved in further studies.

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APPENDIX 1

INFORMED CONSENT

with permisson of the volunteer to print the consent form in this thesis.

ONAY BELGESİ

Benden alınan 3-5 mL kan örneğinin, O.D.T.Ü. Biyoloji Bölümü'nde Araştırma Görevlisi Gülen Ulusoy'un "Türkiye popülasyonunda insan CYP2E1 enziminin polimorfizmi" konulu Yüksek Lisans Tez çalışması için kullanılacağını biliyorum ve onay veriyorum.

Kan verenin Adı, Soyadı:
Haydar ŞELİK

İmza

ANKET

1. Kadın ☐ Erkek ☒

2. Yaş: 25

3. Doğum yeri ve yılı:

4. Annenizin doğum yeri: KONYA
Babanızın doğum yeri: KONYA

5. Sağlığınızla ilgili gönüllü olarak vermek istediğiniz bilgiler (geçirdiğiniz hastalıklar, örneğin diyabet, kanser vb.) YOK

Bu belgenin Gülen Ulusoy'un "Alkol ile İndüklenebilen İnsan CYP2E1 Polimorfizmi" başlıklı tezinde yayınlanmasına izin veriyorum.

Haydar Şelik

APPENDIX 2

BUFFERS AND SOLUTIONS

All the plasticware and glassware used throughout this study were sterilized by autoclaving and ultra-pure water was used for 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.

NaCl (5M);

29.22 g of NaCl was dissolved in 100 mL dH_2O. Solution was autoclaved for sterilization and stored at 4°C.

TEN (Tris-EDTA-NaCl) Buffer, pH 8.0;

10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1 M NaCl.
Solution was autoclaved for sterilization and stored at 4°C.

TE (Tris-EDTA) Buffer, pH 8.0;

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

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

Proteinase-K;

10 mg/mL in dH_2O. Solution was stored at –20°C.

20% SDS;

20 g molecular grade SDS was dissolved in 70 mL dH_2O. Solution was heated to 68°C to assist dissolution. pH was adjusted to 7.2 by the addition of few drops of concentrated HCl. Volume was completed to 100 mL with dH_2O. This solution did not require sterilization. Solution was stored at room temperature.

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 necessary 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 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 was completely dissolved. As this solution is light sensitive, the bottle was covered with alimium foil and stored at room temperature.

Gel Loading Buffer;

0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll.

Solution was stored at room temperature.

PCR Amplification Buffer (10x) (MBI 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 (MBI Fermentas);

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

Buffer O^+ (digestion buffer of *Pst*I) (MBI Fermentas);

50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/mL BSA.

This buffer was supplied together with the restriction enzyme *Pst*I. The restriction enzyme and buffer were stored at -20°C.

Buffer Y^+/TANGO^{TM} (digestion buffer of *Rsa*I) (MBI Fermentas);

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

This buffer was supplied together with the restriction enzyme *Rsa*I. The restriction enzyme and buffer were stored at -20°C.

Buffer B^+ (digestion buffer of *Dra*I) (MBI Fermentas);

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

This buffer was supplied together with the restriction enzyme *Dra*I. The restriction enzyme and buffer were stored at -20°C.

Gene Ruler 50 bp DNA Ladder (0.5 mg DNA/mL) (MBI 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):

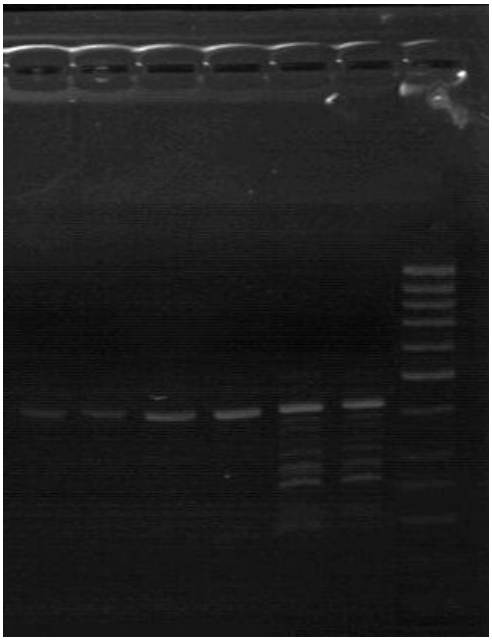
1031 900 800 700 600 500 400 300 250 200 150 100 50

APPENDIX 3

EXAMPLES FOR PCR OPTIMIZATION

Optimization for Amplification of Fragment in 5'-Flanking Region of *CYP2E1* Gene for C-1019T/G-1259C SNPs

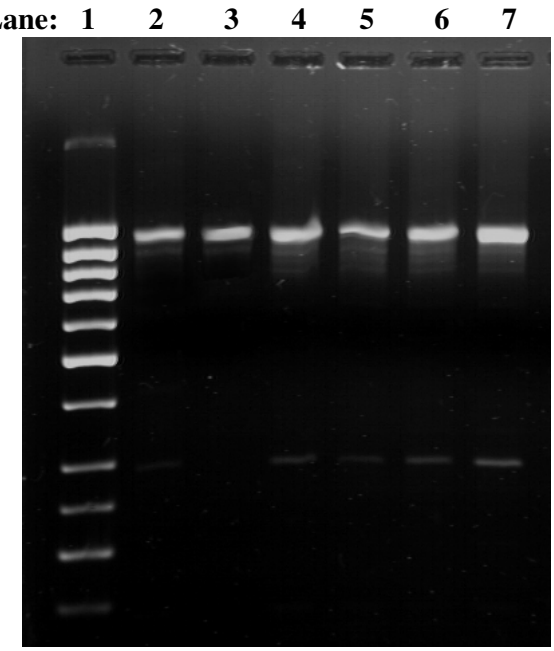
Lane: 1 2 3 4 5 6 7



Lanes 1-6: 200 ng of template DNA; 20 pmol of each primer; in each lane; $MgCl_2$ concentration: 1.0, 1.25, 1.5, 1.75, 2.0 and 3.0 mM in order. 1.5 mM of $MgCl_2$ was chosen for the rest PCR amplification.

Lane 7: DNA Ladder (50-1000 bp)

Optimization for Amplification of Fragment in Intron 6 of *CYP2E1* Gene for T7678A SNP



Lane 1: DNA Ladder (50-1000 bp)

Lanes 2-7: 200 ng of template DNA and 1.5 mM of Mg Cl_2 in all lanes; Lanes 2 &3 contain 10 pmol primer, Lanes 4 & 5 contain 20 pmol of primer, Lanes 6 & 7 contain 40 pmol of primer; Lanes 3, 5 and 7 contain 10 µg BSA. For the rest of PCR amplification, 20 pmol of primer was chosen and BSA was not added into the reaction medium as it did not increase the efficiency of the reaction significantly.

APPENDIX 4

LIST OF SUBJECTS

With Demographic Information and Genotypes

Sample no	Gender	Age	Place of birth			Genotype	
			Individual	**Mother**	**Father**	**C-1019T/G-1259C (*Rsa*I/*Pst*I) SNPs ***	**T7678A (*Dra*I) SNP ****
1	♂	24	Denizli	Denizli	Denizli	c1/c1	DD
2	♀	23	Afyon	Afyon	Afyon	c1/c1	DD
3	♂	23	Doğanhisar	Doğanhisar	Doğanhisar	c1/c1	DD
4	♀	40	Ankara	Ankara	Ankara	c1/c1	CD
5	♀	39	Kırşehir	Kırşehir	Kırşehir	c1/c1	DD
6	♀	21	Edirne	Edirne	Edirne	c1/c1	CD
7	♀	40	Niğde	Niğde	Niğde	c1/c1	DD
8	♂	58	Ankara	Ankara	Ankara	c1/c1	-
9	♂	22	Bozüyük	Bozüyük	Bozüyük	c1/c1	DD
10	♂	20	Kırklareli	Kırklareli	Kırklareli	c1/c1	DD
11	♀	54	Ankara	Ankara	İstanbul	c1/c1	DD
12	♂	26	İstanbul	Antalya	Denizli	c1/c1	DD

List of Subjects (Continued)

Sample no	Gender	Age	Place of birth			Genotype	
			Individual	Mother	Father	C-1019T/G-1259C (*Rsa*I/*Pst*I) SNPs *	T7678A (*Dra*I) SNP **
13	♀	42	Çankırı	Şabanözü	Çankırı	c1/c1	CD
14	♂	24	Tuzluca	Kağızman	Varto	c1/c1	CD
15	♀	37	Ankara	Erzincan	Erzincan	c1/c1	DD
16	♀	23	Ankara	Samsun	Samsun	c1/c1	DD
17	♂	19	Adana	Adana	Adana	c1/c1	DD
18	♀	40	Ankara	Ankara	Ankara	c1/c1	DD
19	♀	23	Ankara	Kırıkkale	Gümüşhane	c1/c1	DD
20	♀	40	Btilis	Bitlis	Bitlis	c1/c1	DD
21	♂	22	Malatya	Malatya	Malatya	c1/c1	CD
22	♂	20	Ankara	Ankara	Ankara	-	CD
23	♂	20	Afyon	Afyon	Afyon	c1/c1	DD
24	♂	50	Çorum	Çorum	Çorum	c1/c1	CD
25	♂	28	Konya	Konya	Konya	c1/c1	DD
26	♀	19	Antalya	Antalya	Antalya	c1/c1	DD
27	♀	29	Nevşehir	Kayseri	Nevşehir	c1/c1	DD
28	♀	26	Ankara	İstanbul	İstanbul	c1/c1	DD
29	♀	20	Denizli	Nevşehir	Nevşehir	c1/c1	DD
30	♂	47	Trabzon	Giresun	İzmir	c1/c1	DD
31	♂	49	Polatlı	Eskişehir	Polatlı	c1/c1	DD
32	♀	20	İstanbul	İzmit	İzmit	c1/c1	DD
33	♂	43	Yozgat	Yozgat	Yozgat	c1/c1	CD

List of Subjects (Continued)

Sample no	Gender	Age	Place of birth			Genotype	
			Individual	Mother	Father	C-1019T/G-1259C (*Rsa*I/*Pst*I) SNPs *	T7678A (*Dra*I) SNP **
34	♀	20	Sivas	Sivas	Sivas	c1/c1	DD
35	♂	62	İstanbul	İstanbul	İstanbul	c1/c1	DD
36	♀	30	Ardahan	Ardahan	Ardahan	c1/c1	DD
37	♀	22	İzmir	İzmir	İzmir	c1/c1	DD
38	♀	21	Kırcaali	Kırcaali	Kırcaali	-	DD
40	♀	16	Ankara	Ilgaz	Ankara	c1/c1	-
41	♀	21	Antalya	Eskişehir	Siirt	c1/c1	DD
42	♀	40	Ilgaz	Kars	Kars	c1/c1	DD
43	♂	39	Denizli	Denizli	Denizli	c1/c1	DD
44	♀	19	Kastamonu	Erzurum	Kastamonu	c1/c1	-
45	♀	48	Bozüyük	Sungurlu	Sungurlu	c1/c1	DD
46	♀	46	Niğde	Niğde	Niğde	c1/c1	DD
47	♂	43	Göle	Göle	Göle	c1/c1	-
48	♀	19	Rize	Ankara	Rize	c1/c1	DD
49	♂	34	İzmir	İzmir	İzmir	c1/c1	-
50	♂	55	Ankara	Samsun	Kandiye	c1/c1	-
51	♀	25	Sivas	Sivas	Sivas	c1/c1	CD
52	♂	48	Ankara	Varna	Ankara	c1/c1	DD
53	♀	23	Turgutlu	Manisa	Manisa	c1/c1	DD
54	♀	57	Burdur	Burdur	Semirkent	c1/c1	DD
55	♀	20	İstanbul	Kırklareli	İstanbul	c1/c1	DD

List of Subjects (Continued)

Sample no	Gender	Age	Place of birth			Genotype	
			Individual	Mother	Father	C-1019T/G-1259C (*Rsa*I/*Pst*I) SNPs *	T7678A (*Dra*I) SNP **
56	♀	55	İstanbul	Niğde	İstanbul	c1/c1	-
57	♂	23	Aydın	Aydın	İzmir	c1/c1	DD
58	♀	23	Kırcali	Kırcali	Kırcali	c1/c1	CD
59	♀	22	Uşak	Uşak	Uşak	c1/c1	-
60	♀	40	Ankara	Ankara	Ankara	c1/c1	CD
61	♂	18	Bornova	Posuf	Ankara	c1/c1	DD
62	♀	45	Van	Sivas	Van	c1/c1	DD
63	♀	38	Tarsus	Tarsus	Tarsus	c1/c1	CD
64	♀	21	İstanbul	İstanbul	İstanbul	c1/c1	DD
65	♂	42	Boyabat	Boyabat	Boyabat	c1/c1	DD
66	♀	19	Ankara	Ankara	Ankara	c1/c2	DD
67	♀	23	Eskişehir	Ankara	Ankara	c1/c1	DD
68	♂	38	Zonguldak	İstanbul	Bartın	c1/c1	-
69	♀	23	Ankara	Kayseri	Maraş	c1/c1	-
70	♀	22	Iskenderun	Adana	Antakya	c1/c1	DD
71	♀	26	Eskişehir	Amasya	Trabzon	c1/c1	-
72	♀	24	Bursa	Bursa	Bursa	c1/c1	-
73	♂	22	Zonguldak	Zonguldak	Zonguldak	c1/c1	DD
74	♀	24	Afyon	Dinar	Dinar	c1/c1	-
75	♂	31	İstanbul	Balıkesir	Balıkesir	c1/c1	DD
76	♀	46	Arhavi	Arhavi	Arhavi	c1/c1	-

List of Subjects (Continued)

Sample no	Gender	Age	Place of birth			Genotype	
			Individual	Mother	Father	C-1019T/G-1259C (*Rsa*I/*Pst*I) SNPs *	T7678A (*Dra*I) SNP **
77	♂	22	İzmit	Kütahya	Ayvalık	c1/c1	-
78	♂	23	Mersin	Mersin	Mersin	c1/c1	-
79	♂	42	Edirne	Edirne	Edirne	c1/c1	DD
80	♂	53	Şavşat	Şavşat	Şavşat	c1/c1	-
81	♀	40	Ankara	Ankara	Ankara	c1/c1	-
82	♀	19	Ankara	Yozgat	Yozgat	c1/c1	-
83	♀	38	Ankara	Ankara	Ankara	c1/c1	-
84	♂	41	Kırşehir	Kırşehir	Kırşehir	c1/c1	DD
85	♂	38	Balıkesir	Elazığ	Elazığ	c1/c1	DD
86	♀	39	Ankara	Ankara	Ankara	c1/c1	CD
87	♀	23	Ankara	Adana	Adana	c1/c1	DD
88	♀	42	Ankara	Amasya	İstanbul	c1/c1	DD
89	♀	26	Konya	Konya	Konya	c1/c1	DD
90	♂	36	Ankara	Urfa	Ufra	c1/c1	DD
91	♀	22	Nevşehir	Kayseri	Nevşehir	c1/c1	-
92	♀	57	Ankara	İzmir	Sinop	c1/c1	CD
93	♀	28	Adana	Ankara	Adana	-	DD
94	♂	27	Ankara	Ankara	Bilecik	c1/c1	DD
95	♂	47	Ankara	Ankara	Ankara	-	CD
96	♀	23	Ankara	Adana	Şebinkarahisar	c1/c1	DD
97	♀	25	İzmir	Manisa	Manisa	c1/c1	DD

List of Subjects (Continued)

Sample no	Gender	Age	Place of birth			Genotype	
			Individual	Mother	Father	C-1019T/G-1259C (*Rsa*I/*Pst*I) SNPs *	T7678A (*Dra*I) SNP **
98	♂	50	Samsun	Mardin	Mardin	c1/c1	CD
99	♀	47	samsun	Samsun	Trabzon	c1/c1	DD
100	♀	24	Mersin	Adana	Adana	c1/c1	DD
101	♀	24	Kırkağaç	Kırkağaç	Kırkağaç	c1/c1	DD
102	♂	35	Hakkari	Hakkari	Hakkari	c1/c1	CD
103	♀	17	Balıkesir	Ankara	Balıkesir	c1/c1	DD
104	♀	24	Ankara	Zonguldak	İstanbul	c1/c1	CD
105	♀	42	Amasya	Amasya	Amasya	c1/c1	DD
106	♂	19	Kayseri	Kayseri	Kayseri	c1/c1	DD
107	♀	22	Aksaray	Niğde	Niğde	c1/c1	DD
108	♂	23	Zonguldak	Karabük	Gümüşhane	c1/c1	DD
109	♂	27	Konya	Çankırı	Konya	c1/c1	DD
110	♂	48	Nevşehir	İstanbul	Nevşehir	c1/c1	DD
111	♂	43	Çankırı	Erzincan	Çankırı	c1/c1	DD
112	♂	24	Erzurum	Tokat	Tokat	c1/c2	DD
113	♂	24	Ankara	Karabük	Ankara	c1/c1	DD
114	♀	23	Bartın	Bartın	Bartın	c1/c2	-
115	♀	14	Ankara	Akşehir	Balıkesir	c1/c1	DD
116	♀	24	Zonguldak	Edremit	Van	c1/c1	-
117	♀	63	Sinop	Kastamonu	Selanik	c1/c1	-
118	♀	62	Kırıkkale	Kırıkkale	Kırıkkale	c1/c1	DD

List of Subjects (Continued)

Sample no	Gender	Age	Place of birth			Genotype	
			Individual	Mother	Father	C-1019T/G-1259C (*Rsa*I/*Pst*I) SNPs *	T7678A (*Dra*I) SNP **
119	♂	24	Ankara	Ankara	Ankara	-	DD
120	♀	27	Lefkoşa	Kıbrıs	Kıbrıs	-	DD
121	♀	25	Ankara	Bolu	Ankara	-	DD
122	♀	22	Adapazarı	İstanbul	Sivas	-	DD
123	♂	65	Ürgüp	Ürgüp	Ürgüp	-	DD
126	♂	26	Ankara	Ankara	Ankara	-	DD
127	♀	50	İstanbul	İstanbul	İzmir	-	DD
128	♂	49	Kırıkkale	Kırıkkale	Kırıkkale	-	CD
133	♂	48	Isparta	İstanbul	Karaman	-	CD
134	♂	57	Tekirdağ	Tekirdağ	Tekirdağ	-	DD
135	♂	43	Ankara	İstanbul	İstanbul	-	DD
136	♀	57	Ankara	Ankara	Maraş	-	CD
162	♀	31	Bursa	Bursa	Bursa	c1/c1	DD
163	♂	25	Konya	Konya	Konya	c1/c1	DD
164	♀	24	Ankara	Manisa	Ankara	c1/c1	DD

***** Heterozygote subjects for this polymorphism is shown in red.

****** Heterozygote subjects for this polymorphism is shown in blue.