REPUBLIC OF TURKEY
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF MEDICAL BIOLOGY

THE ASSOCIATION OF *CYP1A2, CYP2D6, GSTM1, GSTP1* AND *GSTT1* GENES POLYMORPHISMS IN IDIOPATHIC MALE INFERTILITY

MASTER THESIS

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ONDOKUZ MAYIS ÜNİVERSİTESİ
SAĞLIK BİLİMLERİ ENSTİTÜSÜ

Mohamed Ali GURE tarafından, Doç. Dr. Sezgin Guneş Danışmanlığında hazırlanan `**The Association Of *CYP1A2, CYP2D6, GSTM1, GSTP1* and *GSTT1* Genes Polymorphisms In Idiopathic Male Infertility**` başlıklı bu çalışma jürimiz tarafından 19/00/2017 tarihinde yapılan sınav ile Tıbbi Biyoloji Anabilim Dalında YÜKSEK LİSANS Tezi olarak kabul edilmiştir.

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ONAY

Bu tez, Enstitü Yönetim Kurulunca belirlenen ve yukarıda adları yazılı jüri üyeleri tarafından uygun görülmüştür.

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ABSTRACT

THE ASSOCIATION OF *CYP1A2, CYP2D6, GSTM1, GSTP1* AND *GSTT1* GENES POLYMORPHISMS IN IDIOPATHIC MALE INFERTILITY

Aim: Genetic variations in the xenobiotics metabolism have been suggested as modifiers of individual susceptibility to male infertility. This study aims to investigate the association among *GSTT1* (null polymorphism), *GSTM1* (null polymorphism), *GSTP1* (Ile105Val), *CYP1A2* (A/C), *CYP2D6* (A/G) genes polymorphisms and idiopathic infertility in Central Black Sea Region in Turkey.

Materials and Methods: In this study, 114 infertile male patients and 50 fertile controls were recruited. Peripheral blood sample was collected from azoospermic and oligozoospermic patients and fertile/normozoospermic men. Genomic DNA was isolated from the blood using salting out procedure. The genotyping was performed by polymerase chain reaction and restriction fragment length polymorphism.

Results: The sperm count of infertile men and the control group was statistically different than those infertile men (*P*=0.001). We found no significant link between *GSTM1* (*P*=0.565) and *GSTP1* (*P*=0.662) polymorphism and idiopathic male infertility. On the other hand, we found a statistically significant association between idiopathic male infertility and polymorphisms of *GSTT1* (*P*=0.005), *CYP2D6* (*P*=0.005), and *CYP1A2* (*P*=0.0001) genes.

Conclusion: Our results have suggested that genetic polymorphisms of *GSTT1, CYP2D6* and *CYP1A2* genes could play a role in idiopathic male infertility in our population.

Key words: *CYP1A2; CYP2D6*; *GSTM1; GSTP1; GSTT1*; Idiopathic male infertility

Mohamed Ali GURE, Master Thesis

Ondokuz Mayis University - Samsun, June-2017

ÖZET

CYP1A2, CYP2D6, GSTM1, GSTP1 VE *GSTT1* GENLERİ POLİMORFİZMLERİ VE İDIYOPATİK ERKEK İNFERTILİTESİ ARASINDAKİ İLIŞKİNİN ARAŞTIRILMASI

Amaç: Ksenbiyotik metabolizmasında rolü olan genlerin genetik varyasyonlarının erkek infertilitesi için risk faktörü olabileceği öngörülmüştür. Bu çalışmanın amacı Orta Karadeniz Bölgesi'nde yaşayan erkeklerde *GSTT1* (null polimorfizmi), *GSTM1* (null polimorfizmi), *GSTP1* (Ile105Val), *CYP1A2* (A/C), *CYP2D6* (A/G) genlerinin polimorfizmlerinin idiyopatik erkek infertilitesiyle ilişkisinin olup olmadığının belirlenmesidir.

Materyal ve Metot: Bu çalışmaya, 114 infertil erkek hasta ve 50 erkek hasta dahil edildi. Periferik venöz kan örnekleri azoospermik, şidetli oligozospermik ve fertil/normozoospermik erkeklerden toplandı. Genomik DNA izolasyonu, tuz çöktürme yöntemi kullanılarak yapıldı. Genotipler, polimeraz zincir reaksiyonu/restriksiyon enzimi kesimi ve multipleks polimeraz zincir reaksiyonu teknikleri kullanılarak belirlendi.

Bulgular: Sperm sayısı infertil ve fertil erkelerde istatistiksel farklılık gösterdi (P=0,001). *GSTM1* ve *GSTP1* genleri polimorfizmleri ve idiyopatik erkek infertilitesiyle anlamlı bir ilişki bulunmadı (sırasıyla, P=0,565 ve P=0,662). Diğer yandan, idiyopatik erkek infertilitesiyle *GSTT1* (P=0.005), *CYP2D6* (P=0,005) ve *CYP1A2* (P=0,0001) genlerinin polimorfizmleri arasında anlamlı ilişki bulundu.

Sonuç: Bulgularımız, çalışma populasyonumuzda *GSTT1, CYP2D6* ve *CYP1A2* genlerinin polimorfizmlerinin idiyopatik erkek infertilitesinde rolü olabileceğini göstermektedir.

Anahtar Kelimeler: *CYP1A2; CYP2D6*; *GSTM1; GSTP1; GSTT1*; İdiopathic erkek infertilitesi

Mohamed Ali GURE, Yüksek Lisans

Ondokuz Mayıs Üniversitesi - Samsun, Haziran-2017

ABBREVIATIONS EXPLANATIONS

ATP	Adenosine Triphosphate
AZF	Azoospermia Factor
CFTR	Cystic Fibrosis Transmembrane conductance
CO	Carbon monoxide
CYP	Cytochrome p450
EDTA	Ethylenediaminetetraacetic acid
FSH	Follicular Stimulating Hormone
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HO•	Hydroxyl radical
HOCl	Hypochlorous acid
ICSI	Intra-cytoplasmic sperm injection
IUI	Intrauterine insemination
IVF	*In vitro* fertilization
KS	Klinefelter syndrome
LH	Luteinizing Hormone
Mt	Mitochondrial
NO	Nitric Oxide
NOA	Non-obstructive azoospermia
OAT	Oligoasthenoteratozoospermia
OS	Oxidative Stress
PCR	Polymerase Chain reaction
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphisms
T	Testosterone
TT	Total testosterone
UV	Ultraviolet light

CONTENTS

1. INTRODUCTION

Male infertility is a worldwide problem that explained as the failure of couples to conceive pregnancy after one year of unprotected intercourse and 15% of couples are suffering to this disorder (Dehghani et al., 2012). A decrease in the male fertility may be caused by either acquired conditions or congenital like urogenital anomalies, genital tract infections, varicocele, endocrine disturbances, genetic abnormalities, immunologic problems, testicular failure, systemic diseases, cancer, alterations in the lifestyle, and exposure to gonadotoxic agents (Hamada et al., 2012).

Despite advancements in the field of genetics, the etiology of nearly half male infertility still cannot be explained and is referred to as ‘‘idiopathic infertility” (Li et al., 2013a). Genetic and epigenetic aberrations are the most frequent causes of this disease and it is currently accepted that genetic factors contributes about 60% of the idiopathic male infertility (Xu et al., 2013b) . These men have normal physical and endocrine results in laboratory examination. Routine semen analysis often presents with reduced number of spermatozoa (oligozoospermia) and decreased motility (asthenozoospermia) (Hamada et al., 2011).

Spermatozoa are susceptible to excessive quantity of reactive oxygen species (ROS) and oxidative damage may result in infertility or subfertility. Idiopathic infertile men generally show a significantly increased seminal ROS level and decreased antioxidant properties as compared to the healthy controls (Jaiswal et al., 2012a, Agarwal and Said, 2005). Therefore, excessive amounts of ROS have been linked to be one of the notable factors that cause infertility in men via oxidative DNA damages including DNA strands breaks and base damage status (Song et al., 2013).

A natural mechanism of body protection against toxic agents is detoxification. Detoxification includes a 2-stage enzymatic process for the xenobiotics neutralization, making them less harmful to the body (Jaiswal et al., 2012a). Enzymes belonging to the glutathione-S-transferase (GST) families and cytochrome p450 (CYP) are connected with two steps detoxification process of a large spectrum of xenobiotics (Salehi et al., 2012b).

The cellular detoxification of xenobiotic and physiological substances are performed by GSTs which are a super-family of phase II antioxidant enzymes. GST is primary defensive antioxidant system against oxidative stress (OS) and reduces ROS to less reactive metabolites to protect the organism. Recent studies have indicated that the

ability of protection against OS may be impaired by polymorphisms in the *GST* gene and cause to the development of a broad range of diseases (Safarinejad et al., 2010a)

In human GSTs comprises of many cytosolic (soluble), microsomal and mitochondrial enzymes. The cytosolic families have been classified into eight subclasses which are: alpha, mu, kappa, sigma, omega, pi, theta, and zeta. Functional polymorphisms of the *GSTM1*, *GSTP1*, and *GSTT1* genes coding for GSTs enzymes in the mu, theta, and pi classes have been studied (Li et al., 2013a, Agarwal and Said, 2005).

Cytochrome p450 is also an antioxidant enzyme and is involved in phase 1 metabolisms of endogenous and exogenous substance, CYP450 enzymes contain many different enzymes including *CYP1A2* and *CYP2D6.*

In our study, we aim to investigate the possible association of *CYP1A2*, *CYP2D6*, *GSTM1*, *GSTT1*, and *GSTP1* to the idiopathic male infertility.

2. GENERAL INFORMATION

Infertility is defined as the inability of a couple to achieve pregnancy following at least one year of unprotected intercourse (Han et al., 2013). The disease affects one in every five couples trying to conceive worldwide. The problem has been predicted to see an increase of about another two million cases annually according to previous trends. Epidemiological data from Poland showed that approximately 19% of couples are diagnosed with infertility-related complications. Infertility affects approximately 15% of all couples worldwide (Hwang et al., 2010), of which an estimated 45-50% is attributed to male factors. A significant percentage of this male factor-infertility remains idiopathic (Han et al., 2013). Even though large percentage of infertility cases remains completely unknown, yet some frequent etiologies of male infertility have been described, and these include: varicocele, obstruction of deferent duct, sexual dysfunction, cryptorchidism among others (Tang et al., 2016).

2.1. Origins of Male Infertility

Generally the known causes of male infertility can be grouped into two main groups: genetic and non-genetic and male infertility is associated with both (Massart et al., 2012).

2.1.1. Genetic Causes

Genetic aberrations have been showed in infertile men with azoospermia and severe oligozoospermia. Genetic factors involved in male infertility are numerical and structural chromosomal abnormalities, monogenic disorders, Y-chromosome microdeletions, mitochondrial DNA mutations, and multifactorial factors (Poongothai et al., 2009).

Chromosomal Disorders

Chromosomal aberrations account for approximately 5% of male infertility, and the frequency increases to 15% in azoospermic males. Numerical and structural chromosomal abnormalities are observed in azoospermia (the complete absence of sperm in the ejaculate) and severe oligozoospermia (less than 5 million sperm/mL in the ejaculate) (O'Brien et al., 2010).

Klinefelter Syndrome

The most frequent sex chromosome aneuploidy in males is Klinefelter syndrome (KS) occurring in approximately 0.1–0.2% in newborn males. This disorder is a primary

testicular failure with testicular hypotrophy and increased gonadotropin plasma levels. KS is common among infertile men with a very high frequency, up to 10% in azoospermic men and 5% in severe oligozoospermic men (Ferlin et al., 2006).

Y-chromosome Microdeletions

One of the most frequent pathogenetic defects identified in infertile men is Y-chromosome abnormalities. Abnormalities of the Y-chromosome, such as microdeletions are one of the main causes of azoospermia and severe case of oligozoospermia. These deletions occur spontaneously during the embryo development therefore usually fathers and brothers are not affected. Men loose genetic material from their Y-chromosome which is essential for proper sperm production. These deletions on the Y-chromosome are the cause of poor sperm quality in men with low sperm counts ($<5x10^6$ sperm/mL). Nearly half of men with Y-chromosome deletions have enough sperm to use for intra-cytoplasmic sperm injection (ICSI) but the Y-chromosome deletion will be transmitted to their male offspring and will have fertility problems (Fernando et al., 2006).

Mitochondrial DNA Mutations

Mitochondrial (mt) DNA mutations have been reported in several studies with poor semen quality. The mitochondria are involved in oxidative phosphorylation the biochemical pathway that generates ATP via the respiration chain. One-2% of the oxygen that is used is released in form of ROS, which can damage mitochondrial DNA during oxidative phosphorylation process. Higher amount of ROS can cause persistent damage and mutations of mtDNA under ROS-stressed conditions. This mitochondrial bioenergetic function is essential for proper sperm function and motility. Therefore, qualitative and/or quantitative abnormalities in mtDNA may alter the cellular functioning of the spermatozoa in three ways (Kumar and Sangeetha, 2009, Shamsi et al., 2008).

Firstly, most of the male infertility cases are related to asthenozoospermia or oligoasthenozoospermia and has been indicated in patients affected by mitochondrial DNA diseases, involving multiple deletions and point mutations of mtDNA. Secondly, spermatozoa have been demonstrated to be particularly vulnerable to developing mtDNA deletions. Some studies have demonstrated a significant association between mtDNA deletions and reduction of both fertility and motility. Finally, an association has been reported between semen quality and the functionality of the mitochondrial respiratory chain of sperm. In addition, some studies have been demonstrated that single nucleotide

polymorphisms (SNPs) of mtDNA, point mutations, mtDNA and mtDNA haplogroups can extremely effect the quality of semen (Kumar and Sangeetha, 2009, Shamsi et al., 2008).

2.1.2. Non-genetic Causes

Non-genetic causes include, hormonal disorder, reduction in sperm quality, environmental factor, exposure to chemicals, infections, life style and etc.

Hormonal Disorders

Low levels of hormones made in the pituitary gland that acts on the testes cause fertility problems about 1% of men. Low levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) can alter the testosterone levels in the testes and lead to decreased sperm production. The most prevalent hormonal abnormalities are the pituitary gland development problems leading to deficiency of FSH and LH and pituitary tumors (Agarwal, 2005).

Reduction in Sperm Quality

Decrease in human semen quality has become one of the main objects of interest in during the past ten years. Major factors for defining the semen quality impairment are reduction in sperm concentration and/or count, decline in sperm motility and an elevation in morphologically abnormal sperms. A positive association between semen parameters and the likelihood of achieving a pregnancy is known. On the other hand, the possible decrease in quality of semen parameters has not yet indicated in reports of any decrease in male fertility. The results of various studies have not shown unequivocally a decline in sperm characteristics. These results make more difficult to distinguish a cause/effect connection between declining sperm quality and exposure of pregnant women and their male offspring to endocrine hormone disrupters. Total sperm count per ejaculate (millions/ejaculate) and sperm concentration (millions/mL) are most commonly used variables (Weber et al., 2002).

Environmental Factors

Although occupational heat exposure is an important risk factor for male infertility, the controversy exists over declining human sperm concentrations worldwide. The male reproductive system is notedly defenseless to the influence of the physical and chemical environment. This may be due to dramatic events or to endemic conditions of the environment. When viewed from this aspect, industrial pollution and agricultural pesticides are important reproductive health concern. Exposure to environmental toxicants that

change the reproductive hormones, sperm function or spermatogenesis and quality may lead to idiopathic male infertility. Some studies have indicated that sperm counts have decreased in certain industrialized countries (Poongothai et al., 2009).

Exposure to Chemicals

Exposure to various chemicals can lead to adverse effects on reproductive system. Sperm quality may be affected by air pollutants present in the blood, semen and urine or other biological samples of exposed men. Various sperm function tests have demonstrated the capability of the sperm to bind to the ovulated oocyte, and also to penetrate zona pellucida and fertilize the egg is reduced by existence of high levels of lead in semen samples (Poongothai et al., 2009).

Reactive Oxygen Species

Normal cellular metabolism produces ROS during cellular metabolic activities. ROS are highly reactive molecules, containing oxygen (Aitken et al., 1998). Examples include hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), nitric oxide (NO) and hypochlorous acid (HOCl). Sources of ROS could be exogenous or endogenous. Exogenous sources of reactive oxygen species are exposure to radiation (x-rays, UV light), cigarette smoking, herbicides, alcohol abuse, chronic stress, drugs (acetaminophen) and air pollution. On the other hand, the endogenous sources are mitochondrial respiration and enzymatic systems such as xanthine oxidase and NADPH oxidase (Menezo et al., 2010). ROS induced DNA damage involves abasic sites, base modifications, single-strand and double-strand DNA breaks and DNA protein cross links. The presence of high ROS concentration results in loss of sperm motility, fertilizing potential and DNA damage (Aitken et al., 1992, Tamburrino et al., 2012, Sharma et al., 2013). Further, recent reports provide evidence to support that 20-88% of subfertile men had high presence of ROS in the semen (Agarwal et al., 2014a, Agarwal et al., 2014b).

OS is the result of an imbalance between the ROS-producing systems and the enzymatic or non-enzymatic scavengers function in the removal of ROS (Vernet et al., 2004). Virtually every human ejaculate is contaminated with leukocytes as well as by abnormal spermatozoa in semen lead to generation of potential sources of ROS and excessive generation of ROS could result in infertility. the major ROS producer is H_2O_2 in human spermatozoa (Agarwal, 2005). ROS cause infertility by two main mechanisms. First, the sperm membrane is damaged by ROS leading to reduction of sperm motility and

ability to fuse with the oocyte. Secondly, sperm DNA is damaged by ROS directly, compromising the paternal genomic contribution to the embryo. Despite the common association between compromised sperm quality and oxidative damage, men are rarely screened for this condition. Instead they are usually offered mechanical treatments such as intracytoplasmic sperm injection (IVF-ICSI) or intrauterine insemination (IUI)(Tremellen, 2008).

2.2. Cytochrome P450

Cytochrome P450 (CYP, P450 or CYP450) are a superfamily of proteins that are involved in the phase1 metabolism of both exogenous and endogenous compounds. These enzymes are also implicated in synthesis of various hormones and induce hormone-related cancers. CYP450 is characterized spectrophotometrically by an intense absorption band at 450 nm in the presence of reduced carbon monoxide (CO). These CYP450 enzymes contain an active heme iron center bound to a protein molecule through highly conserved cysteine thiolate ligand (Mittal et al., 2015b). These enzymes have a role in response and toxicity of many drugs. In CYP450 families in humans 57 gene and more than 59 pseudogenes have been recognized. On the basis of amino acid sequences all these genes are divided into 18 families and 43 subfamilies (Nelson, 2003).

These enzymes are found in all organisms from microscopic organism to plants to animals (Mittal et al., 2014) (Figure 1). In a particular, CYP450 genes in families CYP1, CYP2, and CYP3 are primarily involved in the metabolism of xenobiotics and drugs, whereas families from CYP4 to CYP51 are generally involved in the metabolism and biosynthesis of endogenous compounds and the function of about ten P450s in human tissues are not identified up to now (Polimanti et al., 2012, Seliskar and Rozman, 2007). Along with the metabolism of xenobiotics many members of the CYP450 play a key role in diverse physiological processes including steroid and cholesterol biosynthesis, fatty acid metabolism, plant toxins, drug metabolism, environmental carcinogenesis and anticancer drugs (McKinnon et al., 2008, McFadyen et al., 2004). CYP450 genes are highly polymorphic and their variants play a significant role in cancer risk and treatment. Association studies and meta-analyses have been carried out to describe the function of CYP450 polymorphisms in cancer susceptibility.

The polymorphisms of xenobiotics metabolizing CYP450 enzymes can be classified into two major classes, Class I and Class II. Class I is comprised of four

members: CYP1A1, CYP1A2, CYP2E1 and CYP2E4 which are highly conserved sequences, do not have significant functional polymorphism and are active in the metabolism of drugs and precarcinogens. However, Class II is comprised of CYP2B6, CYP2C9, CYP2C19 and CYP2D6 which have highly polymorphic and efficient in the metabolism of drugs, but not of precarcinogens (Rodriguez-Antona and Ingelman-Sundberg, 2006) .

2.2.1. Association of CYP450 enzymes with the cancer and drug metabolism

In drug metabolism the CYP450 enzymes CYP1, CYP2 and CYP3 are responsible for the metabolism of phase 1 approximately 70 to 80%. About 90% of metabolic activity depend on CYP1A2, CY2C9, CYP2C19, CY2D6, CYP2E1 and CYP3A (Wijnen et al., 2007a). A drug may be metabolized by a single site by several CYPs; multiple forms of CYPs may metabolize a single drug at different sites, however single CYP450 enzymes can often metabolize many different drugs with diverse structure. Liver is the most important site of drug metabolism mediated by CYP450 where the enzymes ubiquitously expressed (McKinnon, 2000).

In case of cancer the CYP450 isozyme system is important in this aspect. CYP450 genes are highly polymorphic and their variants play a significant role in development of cancer risk and treatment. Polymorphisms in CYP450 have been extensively studied with respect to genetic predisposition to cancer. Their clinical outcome in terms of response and to anticancer they involve the removing of carcinogenic compounds from the body. However, various studies have also shown the significance of CYP450 polymorphisms in cancer susceptibility. They can activate compounds consumed in food, converting procarcinogens to carcinogens (Mittal et al., 2015a) .

2.2.2. Tissue Distribution

CYP450 genes are associated with hepatic metabolism. CYP450 enzyme families, 1, 2, and 3 account approximately 70% of total hepatic P450 contents and are responsible for the most drug metabolism on their expression in liver. (Chang and Kam, 1999).

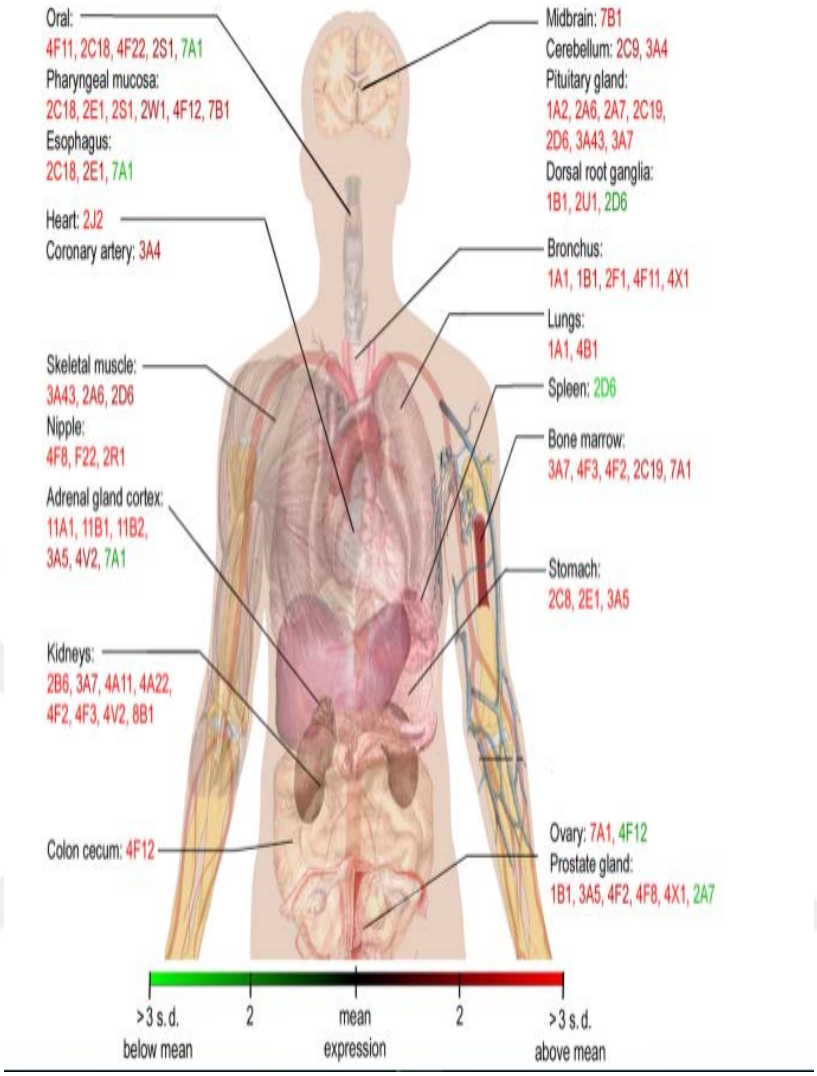

Figure 1. Cytochrome P450 enzyme expression body map. Human expression map of various CYPs in organs is demonstrated. Expression values are relative to the mean expression in all organs. Red text indiate at least three-fold higher expression in an organ. Green text indicate at least three-fold lower expression. CYPs with average expression in an organ were not indicated. A color spectrum is illustrated for the expression values in the provided scale (Preissner et al., 2013).

2.2.3. CYP2D6

CYP450C2D is one of the most important CYPs investigated and studied in relation to genetic polymorphism. The location of *CYP2D6* is on the long arm of the chromosome 22q13.1. It contains 9 exons and 8 introns and is the only functional gene in the CYP2D family. This gene consists of long chain of polypeptide that contain 497 amino acids. More than 80 allelic variants have been reported for *CYP2D6* 4,383 base pairs to

date. Several important toxins, procarcinogens, and therapeutic drugs, as well as nicotine and its metabolite, cotinine are metabolized by CYPC2D (Malaiyandi et al., 2005, Xu et al., 2002). The *CYP2D6* variants can be classified into categories, which cause null enzyme activity, poor, normal, fast or qualitatively altered catalytic activity. CYP2D6*6, CYP2D6*4, CYP2D6*5, CYP2D6*10, CYP2D6*17 and CYP2D6*41 are the most important alleles (Ingelman-Sundberg, 2005, Cascorbi, 2003). According to the four levels of activity CYP2D6 enzymes can be divided into: poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs) and ultrarapid metabolizers (UMs). PMs inherit two nonfunctional/deficient *CYP2D6* alleles and eventually metabolize drugs at very slower rate therefore this causes to an accumulation of high levels of unmetabolized drugs. EMs have two copies of normal genes and the frequency of this phenotype is highest in the population. IMs are deficient on one allele and are heterozygotes. UMs have three or more functional active gene copies (Bernard et al., 2006).

CYP2D6 is one of the most popular enzymes in cytochrome P450 families among physicians and other health professionals because of its genetic polymorphisms. CYP2D6 play an important role in the metabolism of 20 to 25 of all clinically used drugs (Niewinski et al., 2002). Although CYP2D6 represents 1–2% of the liver cytochrome p450 enzymes it is believed that high affinity/low capacity the major organ of drug matabolism for CYP2D6 is the liver however this enzyme is found in many other tissues. In the body, the activity of this enzme is not changeble with the age although CYP2D6 activity may appear to be altered because of age-associated changes in hepatic blood flow or decrease in renal elimination of metabolities. For many drugs such as psychotrotic drugs, *CYP2D6* is considered a high affinity/low capacity enzyme, which implies that CYP2D6 will preferentially metabolize drugs at lower concentration (Wijnen et al., 2007b).

Individuals deficient in functional *CYP2D6* genes metabolize substrates selectively at a lower rate, and the risk is high for adverse drug reactions. Higher drug plasma levels occur more frequently in PMs that result in adverse effects because the drug clearance is dependent on CYP2D6 (Ingelman-Sundberg, 2005).

Like other CYPs, *CYP2D6* gene may modulate cancer risk, therefore the CYP2D6 phenotype and genotype have been investigated with respect to the risk of cancers and other diseases. CYP2D6 EMs had higher risks of malignant processes as a result of increased metabolism of one or more agents in the diet or other environmental agents,

mediated by CYP2D6, forms reactive intermediaries that affect the initiation or progression of cancer in a number of tissues. It has been shown that polymorphisms at loci that encode carcinogen-metabolizing enzymes such as CYP450 catalyzes the carcinogens detoxification and may have role in cancer susceptibility. On the other hand, PMs must be exposed longer to the toxic effects of non-metabolized drugs and numerous other factors to have increased risks of cancers. Association was shown among the *CYP2D6* gene polymorphisms and oral cancer, bladder cancer and lung cancer however theses studies are still debatable (Teh and Bertilsson, 2012).

CYP2D6*10 and CYP2D6*4 are Associated with the Idiopathic Male Infertility Risk

Recent investigation have been demonstrated that various genotypes of CYP2D6*4 have a tendency to increase among infertile men whereas the wild-type allele frequency was higher in fertile normozoospermic males. Additionally, the authors have indicated elevated frequency of homozygous mutations in oligoasthenoteratozoospermia (OAT) group. Significantly lower acrosin activity index, sperm velocity, linear sperm velocity, sperm motility and sperm normal morphology were observed in men with homozygous CYP2D6*4 mutation compared with men with wild-type allele (Zalata et al., 2015).

Tamoxifen is a Trans isomer of clomiphene citrate, which is a union of two isomers that exert both estrogenic and anti-estrogenic effects simultaneously. Tamoxifen citrate enhances spermatogenesis by increasing FSH, Leydig cells sensibility to LH, and testosterone levels, which lacks an intrinsic oestrogenic effect, therefore it may be more appropriate to use in male infertility. Previous studies demonstrated that tamoxifen significantly increased sperm concentration in infertile men with oligozoospermia, but does not affect other semen values, such as volume, pH, motility, morphology, or viability because of tamoxifen's effectiveness on the seminiferous tubules during the early stages of spermatogenesis. Other studies have shown that giving tamoxifen citrate alone exerts a limited effect on sperm morphology and motility; it appears that the superior functional sperm fraction response was probably related to administration of testosterone undecanoate. CYP2D6*10 mutant genotype had a worse clinical outcome in the combined treatment of testosterone undecanoate and tamoxifen citrate in infertile men with idiopathic oligozoospermia. Analyses of CYP2D6*10 genotype may be useful for patients with idiopathic oligozoospermia, and may benefit from treatment when combining tamoxifen citrate with testosterone undecanoate (Adamopoulos et al., 2003).

2.2.4. CYP1A2

The second abundant CYP450 in adult human liver is CYP1A2. Remarkable inter individual variations in CYP1A2 activity have been reported in populations with different ethnic backgrounds. Approximately 10–200 of inter individual variability have been observed in CYP1A2 expression and activity *in vivo* or *in vitro*. Many studies have been devoted to the identification of the functional polymorphisms in the *CYP1A2* gene. The most comprehensively studied polymorphisms are CYP1A2*1C (G-3860A), CYP1A2*1F (C-163A), G-3113A, CYP1A2*1K, CYP1A2*1B (C5347A) and CYP1A2*1D (-2467delT) (Wang et al., 2013).

Susceptibility in Cancer

CYP1A2 catalyze the activation of polycyclic aromatic hydrocarbons (PAHs) and aromatic and heterocyclic amines (HAs). These compounds can arise during the broiling or frying of meat, and their metabolism via CYP1A2 lead to the formation of reactive metabolites that can bind to DNA and form DNA adducts that have the potential to induce mutations and ultimately lead to cancer. Therefore, high activity of *CYP1A2 in vivo* has been suggested to increase the susceptibility for bladder, colon and rectum cancers where exposure to compounds such as HAs and aromatic amines has been implicated in the disease etiology. This fact makes CYP1A2 one of the target enzymes for epidemiological studies on diet-related cancers such as colorectal cancer (Sachse et al., 2003).

Association with Infertility

CYP1A2 has the ability to activate and inactivate various carcinogens. Many carcinogenic compounds are activated or inactivated by complicated pathways. Estrogens play role in the development of testicular cancer and CYP1A2 catalyze the 2-hydroxylation of estradiol with formation of 2-hydroxyestrone (2-OHE1). Experimental studies have demonstrated that decreased formation of 2-OHE1 may be associated with formation of tumor and a low 2-OHE1/16a-OHE1 ratio has been found in postmenopausal breast cancer patients. Estrogens have been involved in cancer development, the complicated estrogen metabolism by CYP1A2 and other enzymes, and the possible interactions with testosterone, make it difficult to evaluate the role of CYP1A2. Therefore, studies indicate the difficulties that in the evaluation of alterations in estrogen metabolism that favors development of testicular cancer, or if it is associated with reduced detoxification of

environmental carcinogens. Therefore, CYP1A2 may have a relation with male infertility because of its association with testicular cancer (Vistisen et al., 2004).

2.3. Glutathione S Transferases

GSTs represent a superfamily of detoxication enzymes. The metabolism of various compounds usually imply well-defined stages, commonly referred to as phases I and II. Phase I metabolism catalyzes the initial oxidation of the xenobiotics by cytochrome P450 monooxygenases. This process is followed by phase II metabolism, which frequently comprises conjugation reactions catalyzed by GSTs (Hemachand, 2002) (Figure 2). Endogenous products of OS and electrophilic xenobiotics are substrates of GSTs. GSTs are the phase II enzyme families that contribute to detoxification and bioactivation by catalyzing the conjugation of glutathione to electrophilic xenobiotics such as ROS. Additionally, GSTs also have function in the biosynthesis of leukotrienes, prostaglandins, progesterone, and testosterone as well as in the tyrosine degradation (Awasthi, 2006, Hayes et al., 2005).

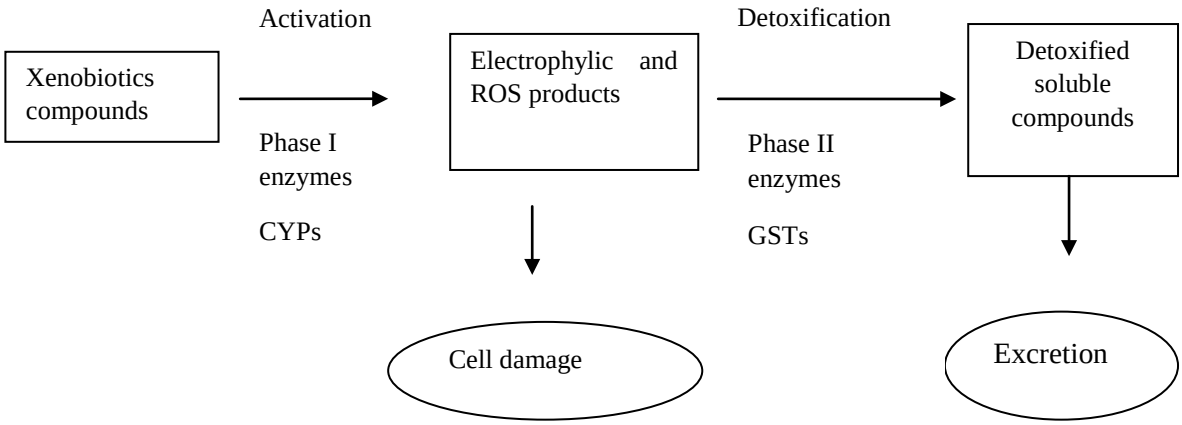

Figure 2. Metabolic process of xenobiotics

GSTs are the phase II enzyme families that contribute detoxification and bioactivation by catalyzing the conjugation of glutathione to electrophilic xenobiotics such

as ROS. *GSTM1* and *GSTT1* are responsible for detoxification of electrophilic compounds including carcinogens, therapoetic drugs, environmental toxins and OS products. Both genes are highly polymorphic and their polymorphisms are related to increasing risk of some cancer types and gaining susceptibility for toxins and carcinogens. Allele frequency of polymorphisms of *GSTT1* and *GSTM1* differs between populations.

In humans the soluble GSTs are categorized into eight classes: class alpha (α) on chromosome 6, mu (μ) on chromosome 1, theta (θ) on chromosome 22, zeta (ζ) on chromosome 14, pi (π) on chromosome11, sigma (σ) on chromosome 4, kappa (κ) chromosomal location not known , and omega (ω) on chromosome 10. These enzymes are encoded by *GSTA , GSTM , GSTT, GSTZ, GSTP, GSTS , GSTK* and *GSTO* (Safarinejad et al., 2013). Although κ enzyme is soluble it is expressed in the mitochondria not in cytoplasm. Table 1 shows GST genes, their variants and chromosomal location.

Table 1. GST genes, their variants and chromosomal location

Gene name	**Gene symbol**	**Chromosome location**	**Exon**
Glutathione s transferase alpha	*GSTA1*	6P12.2	7
	GSTA2	6P12.2	7
	GSTA3	6P12.2	7
	GSTA4	6P12.2	7
	GSTA5	6P12.2	7
Glutathione s transferase mu	*GSTM1*	1p13.3	8
	GSTM2	1p13.3	8
	GSTM3	1p13.3	8
	GSTM4	1p13.3	8
	GSTM5	1p13.3	8
Glutathione s transferase omega	*GSTO1*	10q25.1	6
	GSTO2	10q25.1	6
Glutathione s transferase pi	*GSTP1*	11q13.1	7
Glutathione s transferase sigma	*GSTS1*	4	6
Glutathione s transferase theta	*GSTT1*	22q11.23	5
	GSTT2	22q11.23	5
Glutathione s transferase zeta	*GSTZ1*	14q24.3	9

2.3.1. Structural aspects of soluble GSTs

GSTs are biologically active as subunits dimers of 23–30 kDa and with average length of 200–250 aminoacids. Each subunit consist of a helical C-terminal domain and an N-terminal domain, comprised of α helices and β strands as secondary elements (Li et al., 2013c) (Figure 3).

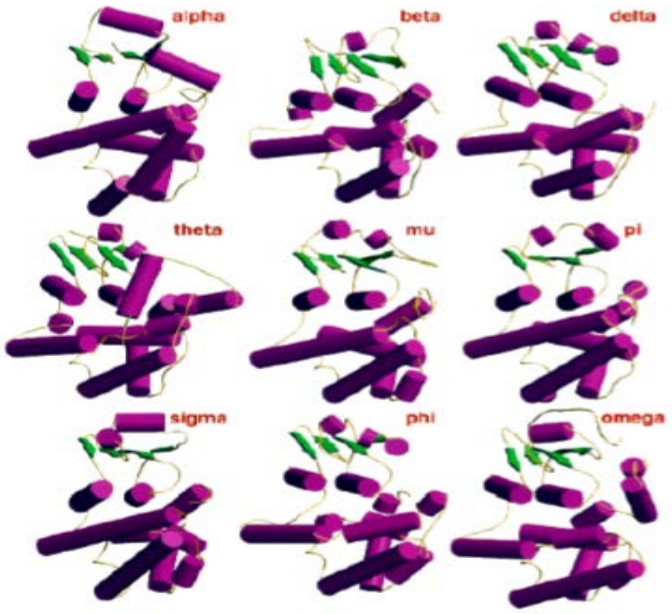

Figure 3. The common chain fold of the soluble GST family. Helices are represented as purple cylinders, and β-strands are represented as green arrows (Board et al., 2000)

2.3.2. Activity of GSTs on male germ cells

The paternal half of the genetic material is carried by male germ cells (spermatozoa) and therefore a protection from genetic damage is required for the safe reproduction of species. Spermatozoa are highly vulnerable to toxic substances that are able specifically to destroy and damage them. Germ cells are more susceptible to oxidative stresses compared with somatic cells for two reasons. Firstly, they are closely associated with the free radical-generating phagocytic sertoli cells, secondly plasma membrane of germ cells contains a eleveted level of polyunsaturated fatty acid that are sensitive to oxidation by free radicals (Rao and Shaha, 2000). GSTs isoforms are 24 kDa and are localized on the extracellular surface of the plasma membrane. Plasma membrane

of male germ cells has GSTs activity several times higher than GST activity of somatic cell plasma membrane (Rao and Shaha, 2001). GST π, α, μ and micro-somal expression is existing in and largely confined to the Sertoli and Leydig cell compartments of the normal human testis (Ritzen 1989). Sertoli cells have function in germ cell glutathione production and oxido-reductive enzyme systems (Klys et al., 1992). Human spermatozoa and seminal plasma have a variety of antioxidant systems to scavenge ROS and prevent ROS-related cellular damage. In addition, human testis GST enzymes serve for protection of germ cells from the harmful effects of reactive chemicals and other oxidative stress. In particular, fatty acid oxidations induced by leukocytes and/or defective sperm in semen, results in interuption of sperm motility and capacitation and failure of fusion oocyte and sperm (Rao and Shaha, 2001). GST are enzymatically active on sperm surface, however the precise machanism by which they detoxify and protect spermatozoa is unknown (Hemachand and Shaha, 2003).

2.3.3. Polymorphisms of *GSTM1*, *GSTT1* and *GSTP1*

2.3.3.1 GSTM1 Polymorphism

GSTM1 gene belongs to the GSTμ subfamily which consist of five genes, the four other members of the GSTμ subfamily, *GSTM2, GSTM3, GSTM4* and *GSTM5* present high levels of sequence homology and substrate specifity with *GSTM1*. *GSTM1* gene is organized in a gene cluster on chromosome 1p13.3 and has been shown to be polymorphic and it is absent in 35–60% of individual (Bhattacharjee et al., 2013) (Figure 4).

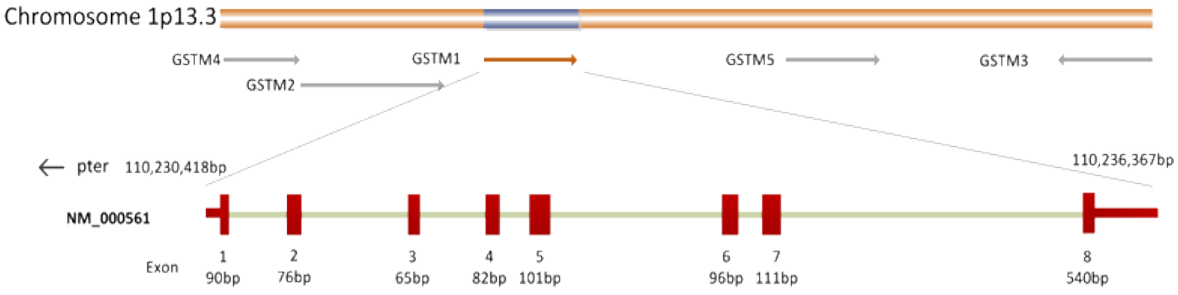

Figure 4. Localization of *GSTM1* gene on chromosome 1p13.3. The *GSTM1* gene consist of of the eights exons (red) and seven introns (green) and spans a region of 21,244 bases (http://atlasgeneticsoncology.org/Genes/GC_GSTM1.html)

GSTM1*0, GSTM1*A and GSTM1*B polymorphisms are three genetic polymorphisms of *GSTM1*. Two of these polymorphisms are enzymatically active; GSTM1*A and

GSTM1*B, and third one; GSTM*0, is a null allele. The GSTM1*A and GSTM*B alleles code for GSTM1*A and GSTM1*B proteins. These genes are identical except in a single amino acid at position number 172. The GSTM1*A protein contain lysine at this position, whereas the GSTM1*B protein contains asparagine. The products from GSTM1*A and GSTM1*B genes join and combine with each other to form two homo- and hetro-dimeric active protein. On the other hand, the GSTM1*0 (*GSTM1* null genotype) allele is a deletion and homozygotes for this allele cannot produce any functional protein. GSTM1 null genotype is thought to be related with insufficient ability to detoxify several xenobiotics, free radical mediated cellular damage, and low defense capacity against OS(Wei et al., 2012).

It estimated that about half of the human population carries polymorphic deletion of *GSTM1* gene. The genetic variants of this gene have been linked to be one of the major factors leading to susceptibility to carcinogens and toxins (such ROS and free radicals), as well as toxicity and efficacy of certain drugs. Loss of function of this gene causes many human diseases such as cancer (Wang and Leung, 2009). *GSTM1* deletion percentage is different among the population as it tends to be higher in Caucasians and Asians than Africans ranging from 58.3% in French, 43% in Chinese, 21.7% in Nigerians (Xu et al., 1998) .

2.3.3.2. GSTT1 polymorphism

The *GSTT1* gene is part of the theta-class GST gene cluster located at 22q11. The *GSTT1* gene consists of five exons, the size of exons and introns vary from 88 to 195 bp and 205 to 2363 bp, respectively. The *GSTT1* gene is embedded in a region showing more than 90% of homologies and flanked by two 18 kb regions, HA3 and HA5. A 403-bp sequence with 100% identity is settled in the central part of HA3 and HA5. During the homologous recombination of the left and right 403 bp repeats, the *GSTT1* null allele arises as a result of 54 kb deletion. Because of the sequence identity between the 403 bp repeats, the precise localization of deletion cannot be defined (Parl, 2005a) (Figure 5). Although the *GSTT1* is highly expressed in human adult liver, it is not expressed in the fetal liver. GSTT1is expressed in brain, kidney, erythrocytes, lung, skeletal muscles, heart, small intestine, the colon mucosal cytosol ,and spleen. Studies on mice, rats and humans demonstrate similar tissue expression pattern (Landi, 2000).

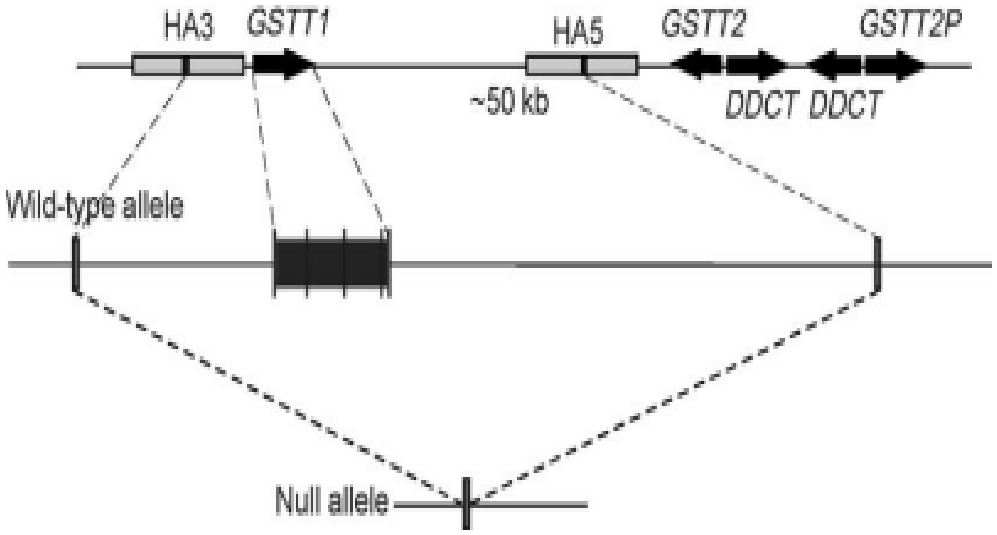

Figure 5. Structural localization of gene cluster encoding the *GSTT1* at chromosome 22q11.2. The *GSTT1*0* (*GSTT1* null allele) arises as a result of homologous recombination of the left and right 403 bp repeats, which cause to 54 kb deletion containing the entire *GSTT1* gene (Parl, 2005b)

2.3.3.3. *GSTP1* Polymorphism

GSTP1 is 2.8 kb gene located at the long arm of the chromosome 11(11q13.2) and contains seven exon SNP at the area -313 in *GSTP1*. It converts an adenine to guanine (A>G), the resulting isoluencine to valine (Val) substitution in codon 105 of exon5 (Ile105 >Val105) extensively lowers GST enzyme function (Safarinejad et al., 2012b) (Figure 6).

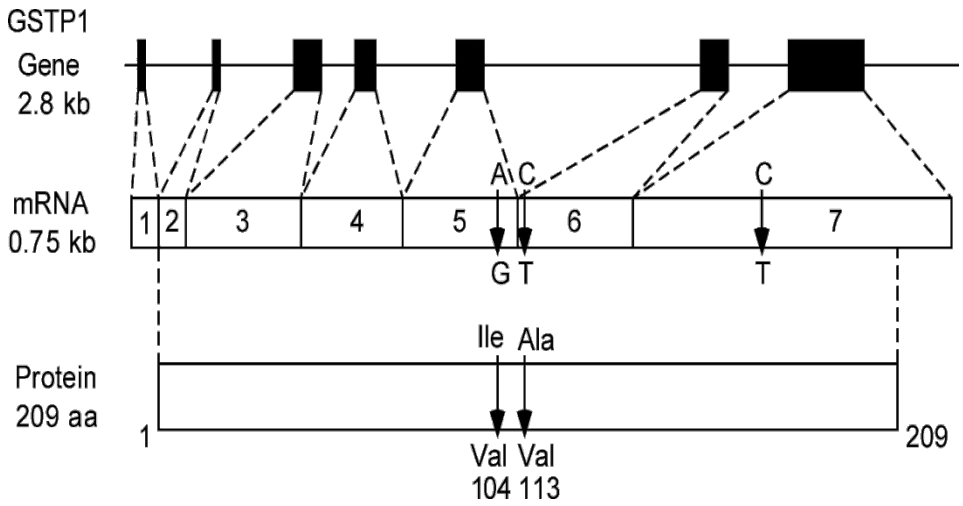

Figure 6. Schematic drawing of *GSTP1* gene, mRNA, and protein. The polymorphisms result in amino acid substitutions in codons 104 (Ile/Val) in exons 5

GSTP1 has been identified in the heart, brain, lungs and embryonic tissue, where it is predominant GSTs form, as well as in kidneys, adrenal and salivary glands, esophagus, pancreas, stomach, colon, skin and testis. This gene is not expressed in hepatocytes. This enzyme attracts much attention because of several reasons. It has long been known that *GSTP1* expression in many transformed tissues is dramatically altered in comparison to normal (Tiis et al., 2016).

2.3.4. GST Polymorphisms and Their Association with the Diseases

GST polymorphisms (*GSTM1*, *GSTP1* and *GSTT1*) have been extensively studied for their association with increased susceptibility to a variety of environmentally induced diseases including male infertility, asthma, different types of cancer and etc (Strange et al., 2001).

2.3.4.1. Association with Cancer

Several studies have indicated an association between the polymorphisms of *GSTM1, GSTT1* and *GSTP1* genes and cancers. A variety of genotypes of these genes are considered to be related to the initiation and progression of many cancers due to their roles in acute leukemia, breast cancer, and lung cancer have been presented in previous studies (Yang et al., 2013). The *GSTM1* and *GSTT1* have capability to modulate the conjugation of carcinogenic compounds to extractable hydrophilic metabolites therefore these genes are

candidate cancer susceptibility genes. Homozygous deletions genotypes that exist at both these loci are related with a lack of enzyme function. Individuals with homozygous deletions in the *GSTM1* of *GSTT1* genes may show an impaired ability to remove carcinogenic compounds and may therefore lead to elevated cancer risk.

Three critical results about the association of *GSTM1* and *GSTT1* genes with susceptibility to cancer have demonstrated by molecular epidemiological studies. First, *GSTM1* and *GSTT1* null genotype carrier frequencies are between 20-50% in most populations studies. Second, *GSTM1* gene and, possibly, *GSTT1* gene might have role in the cancer etiology at more than one site. Finally, although the risk conferred to individuals who carry *GSTM1* and *GSTT1* null genotype appears to be small in significance (OR< 2), the significance of risk is higher (OR>3-5) when considering the interactions of *GSTM1* and *GSTT1* with other factors like environmental agents, nutrients and cigarette smoking (Rebbeck, 1997).

2.3.4.2. Association with Idiopathic Male Infertility

The result of studies on the association of polymorphism in *GSTM1*, *GSTT1* and *GSTP1* genes and idiopathic male infertility are inconsistent. Male infertility has been related to multiple causes including abnormalities in sperm concentration, morphology and motility. Recent studies indicated an important decrease in quality of semen or defects of spermatogenesis over the past decades. Although the possible underlying mechanisms cannot be explained, xenobiotics have been suggested to cause adverse effects on the male reproductive system (Finotti et al., 2009). Potential sources of ROS are leukocytes and abnormal spermatozoa and excessive ROS generation in semen by these potential sources could cause infertility. Hydrogen peroxide is the major ROS producer in human spermatozoa. The major ROS producer in human spermatozoa is hydrogen peroxide (H_2O_2). ROS lead to male infertility by two leading mechanisms (Agarwal, 2005).

First, ROS wound the sperm membrane and lead to a decrease in the motility of sperm and ability to fuse with the oocyte. Second, ROS cause sperm DNA damage, compromising the paternal genomic contribution to the embryo (Tremellen, 2008). Therefore, individuals carrying the deleted *GSTM1* and *GSTT1* could be subjected to elevated levels of oxidative stress, which may be the reason of the higher risk of male infertile (Wu et al., 2013). Several epidemiological studies have demonstrated that the *GSTM1* and *GSTT1* null genotypes that cause deficiency of functional GSTM1 and GSTT1

proteins are associated with an elevated susceptibility to diseases related with OS. Although the possible role of *GSTM1* and *GSTT1* gene variants in male infertility has been already suggested, the genetic polymorphisms association studies have been shown to vary considerably among different geographical regions and ethnic groups.

An early study demonstrated that patients with a *GSTM1* null genotype have shown increased frequency of oxidative damage of sperm and seminal plasma in men with idiopathic infertility despite no significant variation were detected in the distribution of the *GSTM1* variant genotypes between idiopathic infertile men and fertile men (Aydemir et al., 2007a). In addition, Salehi and colleagues found a significant association between *GSTT1* null genotype and male infertility (Salehi et al., 2012b).

GSTP1 are the predominant members of the GST family, and deletion of the gene polymorphism Ile105Val (rs1695) of GSTP1 can impact the binding affinity of these enzymes and/or reduce or eliminate cellular GST activity. *GSTP1* plays a critical role in the biotransformation and inactivation of toxic and carcinogenetic electrophiles, especially those in cigarette smoke and also inhibits apoptosis and promotes cellular proliferation through interacting with c-Jun N-terminal kinase (JNK) pathway. The polymorphism Ile105Val in exon 5 of *GSTP1* significantly alters the activity and heat stability of the encoded enzyme, decreasing its ability to detoxify environmental mutagens and protect against oxidative damage to DNA. A number of studies have demonstrated that this polymorphism is an important risk factor for individual susceptibility to various diseases and is a biomarker to predict the efficacy of certain diseases including infertility. To date, several case-control studies have investigated the relation between the *GSTP1* polymorphism and risk of male infertility. Some scientist indicated that GSTP1 105Ile/Ile was associated with decreased risk of male infertility, whereas the genotype combination *GSTP1* 105 Ile/Val was associated with increased risk (Huang et al., 2017a).

3. MATERIALS AND METHODS

3.1. Population Sample

A total of 114 men diagnosed with idiopathic infertility and 50 fertile controls were enrolled in the study. The patients and the controls were enrolled from the Urological Department at the Ondokuz Mayis University. The OMU Clinical Research Ethics Committee approved the protocol of this study together with the consent form (OMU KAEK 2016/216). All participants signed a consent form stating their full consent to participate in the study after receiving detailed information about the study. Infertile men with sperm count of less than 5×10^6/ml at two subsequent semen analyzes and without any known reason for infertility were included in the study. The exclusion criteria for patients were karyotype abnormalities, Y-chromosome microdeletions, unilateral/bilateral vas deferens agenesis, and abnormal FSH level, epididymal obstruction due to previous surgical procedures, previous vasectomy, secondary infertility, orchidectomy, testicular malignancy, acidic semen, and radiation exposure or prescribed drug usage. Idiopathic infertile males and the normozoospermic and/or fertile controls were from the Central Black Sea Region in Turkey.

Semen analysis was done in Andrology laboratory according to World Health Organization (WHO) guidelines (WHO, 2010). After complete liquefaction of the ejaculate for 20-30 minutes at 37°C, all specimens were first assessed for volume, sperm concentration, total sperm count, sperm motility, and round cell concentration. Sperm motility and concentration were assessed using a MicroCell counting chamber (Vitrolife, San Diego, CA).

3.2. Chemicals and Equipment

3.2.1. Chemicals

- Triton X-100 (Amresco)
- Alw26I Restriction Enzyme (Thermo Fisher Scientific)
- Bsp120I Restriction Enzyme (Thermo Fisher Scientific)
- MvaI Restriction Enzyme (Thermo Fisher Scientific)
- Ethidium Bromide
- SYBR Safe - DNA Gel Stain (Thermo Fisher Scientific)
- Proteinase-K

- Taq DNA Polymerase (Thermo Fisher Scientific)
- Deoxyribonucleic Triphosphate (Larova, Germany)
- Agarose (Prona, Nu Micropor, E.U.)
- Sucrose (Bio Basic Inc.)
- Tris (Amresco)
- EDTA Sodium Salt (Merck)
- Sodium Dodecyl Sulfate
- Ethyl Alcohol (Merck)
- NaCl (Merck)
- Magnesium Chloride (Merck)
- Boric Acid (Bio Basic Inc.)
- 10 X PCR Buffer
- PCR Primers
- Enzyme Free Water
- 6 X Loading Dye (Vivantis)
- Dimethyl Sulfoxide (Sigma, Steinheim, Germany)
- Sodium Hydroxide (Merck)
- Hydrochloric Acid (Merck)

3.2.2. Equipment

- Thermocycler (GeneAmp PCR System 9700)
- Vortex (Velp Scientifica)
- pH meter (Consort C3010)
- Horizontal Electrophoresis System (Scie-Plas / Wealtec Elite 300 Plus)
- UV Transilluminator (Vilber Laurmat, France)
- Automatic Pipettes (Eppendorf, Germany)
- Multiskan GO (ThermoFisher Scientific, Finland)
- Micro-Centrifuge (Herolab, Microgen 13D)
- Centrifuge (Sanyo Centaur 2)
- Incubator (Dedeoglu Turkey)
- Magnetic Stirrer (Stuart Scientific)
- Autoclave Sterilizer (Nüve OT 4060, Turkey)

3.3. Preparation of Stock Solutions

0.5 M EDTA, pH 8.0:

- 18.61 g disodium EDTA is dissolved in 80 mL of distilled water.
- 2 g sodium hydroxide (NaOH) tablet is dissolved in the solution.
- When reaching pH 8, it is completed with distilled water to 100 mL.
- It is sterilized in autoclave and kept at the room temperature.

6 M Saturated Sodium Chloride (NaCl) Solution

- 7 g of NaCl is dissolved in 20 mL of distilled water.
- It is sterilized in autoclave and kept in the room temperature.

TRIS, pH 7.5

- 12.11 g TRIS-base is dissolved in 80 mL of distilled water.
- pH is set to 7.5 with HCl.
- It is topped up with distilled water to 100 mL.
- It is sterilized in autoclave and kept at the room temperature.

10 X TBE Stock Solution

- 108 g TRIS-base and 55 g boric acid are dissolved in 20 mL distilled water.
- Added 40 mL of 0.5 M EDTA, pH 8.0 (20 mM).
- It is topped up with distilled water to 1000 ml and kept at the room temperature.

1 X TBE Working Solution

- 100 ml 10 X TBE from stock solution and 900 ml of distilled water are mixed.

Ethidium Bromide Solution

- 1g ethidium bromide is dissolved in 10 mL of distilled water.
- Kept away from day light and stored at 4°C.
- It is prepared as 0.5 mg/mL from stock solution.

Lysis Buffer

- 320 mM Sucrose
- 10 mM TRIS, pH 7.5
- 4 mM $MgCl_2$
- 1% Triton X-100
- It is completed with distilled water to 1000 mL and sterilized after preparation.

TEN Buffer

- 10 mM TRIS pH 8
- 2 mM EDTA
- 400 mM NaCl
- It is topped up with distilled water to 50 mL and sterilized after preparation.

% SDS (Sodium Dodecyl Sulphate)

- 10 g SDS is dissolved in 100 mL distilled water.

6 M of Saturated Sodium Chloride (NaCl) Solution

- NaCl is dissolved in 20 mL distilled water.

70% Ethanol

- 70 mL %99.5 ethanol and 30 mL distilled water are mixed and stored at -20 ^{0}C.

TE Solution

- 10 mM TRIS pH 7.5
- 1 mM EDTA
- 1 ml of 500 mM TRIS pH 7.5 stock solution and 0.5 ml of 100 mM EDTA stock solution are mixed.
- It is topped up with distilled water to 50 ml.

3.3. DNA Isolation

Four mL of peripheral blood sample was collected from each participants and genomic DNA was isolated from the peripheral leucocytes by salting out method. Blood samples in EDTA containing tubes were transferred to 50 -mLpolypropylene tubes and lysis buffer (10 mM TRIS pH 7.5, 320 mM sucrose, 6 mM $MgCl_2$, 1% Triton X - 100) was added up to 45 mL. After centrifugation at 3000 rpm for 30 minutes, the supernatant was discarded. To digest the lysate, 1500 µL TEN (10mM TRIS pH=8, 2 mM EDTA, 400 mM NaCl), 100 µL %10 SDS and 25 µL proteinase K (10 mg/mL) was added onto the pellets and was incubated overnight at 37^{0}C. On the next day 500 µL 6 M NaCl were added and the samples after vortexing were centrifuged at 3000 rpm for 30 minutes. The supernatants were taken in clean propylene tubes and the centrifugation step was repeated. The supernatants were taken in another propylene tube and approximately 15 mL of 100% ethyl alcohol was added and gently mixed to allow the precipitation of the DNA in the samples. The DNA was transferred to microcentrifuge tubes containing 70% ethyl alcohol. The tubes were centrifuged at 13000 rpm for 5 minutes, incubated at 37^{0}C for about one

hour, and then 100-200 µL TE (10 mM TRIS pH 7.5, 1 mM EDTA) was added. The samples were incubated 2-3 hours at 37^0C. After incubation DNA samples were stored at -20^0C.

3.4. *GSTM1* and *GSTT1* Genotyping

GSTM1 and *GSTT1* genes were amplified simultaneously by multiplex polymerase chain reaction (multiplex PCR). Human albumin gene was used as internal control. PCR amplification was carried out in a total volume of 25 µL containing about 1xPCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.167 µM of each *GSTM1* primers, 0.1 µM of each *GSTT1* and albumin primers (Table 2), 5% DMSO, 200 ng genomic DNA and 2.0 U Taq polymerase. To check the status of contamination during PCR reactions negative controls were included in each run. Distilled water instead of DNA was added into the reaction mixture for negative controls. The reaction procedure was pre-denaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 59°C for 60 seconds, extension at 72°C for 60 seconds, and final extension at 72°C for 7 minutes. Then the PCR products were electrophoresed on a 2% agorose gel stained with ethidium bromide and visualized under UV light. The amplicon sizes of *GSTM1*, *GSTT1*, and albumin were 215 bp, 480 bp and 350 bp, respectively.

Table 2. Genes, primers sequences and restriction enzymes

Gene	Primers	Restriction Enzyme
GSTT1	(F) 5'-TTC CTT ACT GGT CCT CAG ATC TC-3'	-
	(R) 5'- TCA CCG GAT CAT GGC CAG CA-3'	
GSTM1	(F) 5'-GAA CTC CCT GAA AAG CTA AAG C-3'	-
	(R) 5'-GTT GGG CTC AAA TAT ACG GTG G-3'	
ALBUMIN	(F) 5'-GCC CTC TGC TAA CAA GTC CTA C-3'	-
	(R) 5'-GCC CTA AAA AGA AAA TCC CCA ATC-3'	
GSTP1	(F) 5'-ACC CCA GGG CTC TAT GGG AA-3'	Alw26I
	(R) 5'-TGA GGG CAC AAG AAG CCC CT-3'	
CYP2D6	(F) 5'-GCC TTC GCC AAC CAC TCC G-3'	MvaI
	(R) 5'-AAA TCC TGC TCT TCC GAG GC-3'	
CYP1A2	(F) 5'-CTA CTC CAG CCC CAG AAG TG-3'	Bsp120I
	(R) 5'-GAA GGG AAC AGA CTG GGA CA-3'	

3.5. *GSTP1* Genotyping

PCR-RFLP method was used for genotyping of *GSTP1* gene. The polymorphic sequence of *GSTP1* gene was amplified by PCR. Amplification reactions were carried out in a total volume of 25 µl containing about 1xPCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.3 µM of each primers (Table 1), 200 ng genomic DNA and 2.0 U Taq polymerase. To check the status of contamination during PCR reactions negative controls were included in each run. Distilled water instead of DNA was added into the reaction mixture for negative controls. The amplification cycles have included an initial denaturition at 94°C for 5 minutes, followed by 35 cycle of denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes. After PCR amplification, restriction fragment length polymorphism technique (RFLP) was used. 10 µL PCR amplicon was digested with 5 units of Alw26I restriction enzyme by incubating overnight at 37°C. RFLP products were electrophoresed on a 2% agorose gel stained with ethidium bromide or syber safe and visualized under UV light. Ile/Ile individuals had a single fragment of 294 bp, and Val/Val individuals had both 234 bp and 60 bp fragments. The presence of all three fragments (294 bp, 234 bp and 60 bp) corresponded to Ile/Val individuals.

3.6. *CYP2D6* Genotyping

The *CYP2D6* genotype was determined by the method of using a PCR-RFLP. The polymorphic sequence of *CYP2D6* gene was amplified by PCR. PCR amplification was carried out in a total volume of 25 µl containing about 1xPCR Buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.6 µM of each primers (Table 1), 200 ng genomic DNA and 2.0 U Taq polymerase. During PCR reactions negative controls were included in each run by using distilled water instead of DNA. The amplification cycles have included an initial denaturation at 95°C for 3 minutes, followed by 35 cycle of denaturation at 94°C for 60 seconds, annealing at 57°C for 60 seconds, extension at 72°C for 60 seconds, and final extension at 72°C for 7 minutes. After amplification, 8 µL PCR amplicon was digested with 6 units of MvaI restriction enzyme by incubating overnight at 37°C. RFLP products were electrophoresed on a 2% agorose gel stained with ethidium bromide and visualized under UV light. GG individuals had 230 bp and 104 bp fragments, heterozygous AG individuals had 334 bp, 230 bp and 104 bp fragments, AA individuals had a single fragment of 334 bp.

3.7. *CYP1A2* Genotyping

CYP1A2 genotypes were identified by using PCR-RFLP method. PCR amplification was carried out in a total volume of 25 µl containing about 1xPCR Buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.9 µM of each primers (Table 1), 200 ng genomic DNA and 2.0 U Taq polymerase. The amplification cycles included an initial denaturing at 94°C for 3 min, followed by 40 cycle of denaturation 94°C for 60 seconds, annealing step at 57°C for 60 seconds, extension step at 72°C for 60 seconds, and final extension at 72°C for 5 minutes. Then 10 µL PCR products were digested with 15 units of Bsp120I restriction enzyme by incubating overnight at 37°C. RFLP products were run on a 2% agorose gel stained with ethidium bromide and visualized under UV light. AA individuals had a single fragment of 370 bp. Hetrozygous AC individuals had 370 bp, 240 bp and 130 bp fragments and CC individuals had 240 bp and 130 bp fragments.

3.8. Agarose Gel Electrophoresis

Electrophoresis was carried out in agarose gels using the horizontal electrophoresis system (Scie-Plus, England). The electrode buffer was 1 X TBE solution (1:10 dilution of a stock buffer solution) containing TRIS buffer, 0.5 M boric acid, 0.02 M EDTA.

3.9. Statistical Analysis

Genotype and allele frequencies and their associations with infertility risk, smoking status, and diagnosis were investigated using $\chi 2$ test. Odds ratio (OR) and 95% confidence interval (95% CI) were used to assess the relative risk. Calculations were carried out using the SPSS version 15.0 (Statistical Package for the Social Sciences Inc. USA). A significance value of $P<0.05$ was specified for all analyses.

4. RESULTS

The mean age of patients, infertility duration and sperm count were compared between idiopathic infertile patients and normozoospermic controls (Table 3). The mean average age of the control group was not statistically different than those infertile men (p = 0.157). The sperm count of infertile men was statistically different than those men (p = 0.001). Idiopathic infertile group was consisting of 71 non-obstructive azoospermic men and 21 men with OAT.

Table 3. Demographic and laboratory findings of idiopathic infertile cases and control groups

Factor	**Idiopathic infertile men (n=114)**	**Normozoospermic men (n=50)**	***p*-Value**
Duration of infertility Years ±SD	4.56±3.45	NA	
Age (years) ±SD	32.04±1.6 (20-32)	38.04±5.6 (25-44)	0.157
Sperm count (10^6/ml) ±SD	2.2±2.4	44.19±1.1	0.001*

Abbreviations: NA: Not applicable; SD: standard deviation; *: statistically significant difference between groups.

The agarose gel images of *CYP1A2, CYP2D6, GSTM1, GSTT1,* and *GSTP1* polymorphisms to the idiopathic male infertility were demonstrated in Figure 7, 8, 9, and 10.

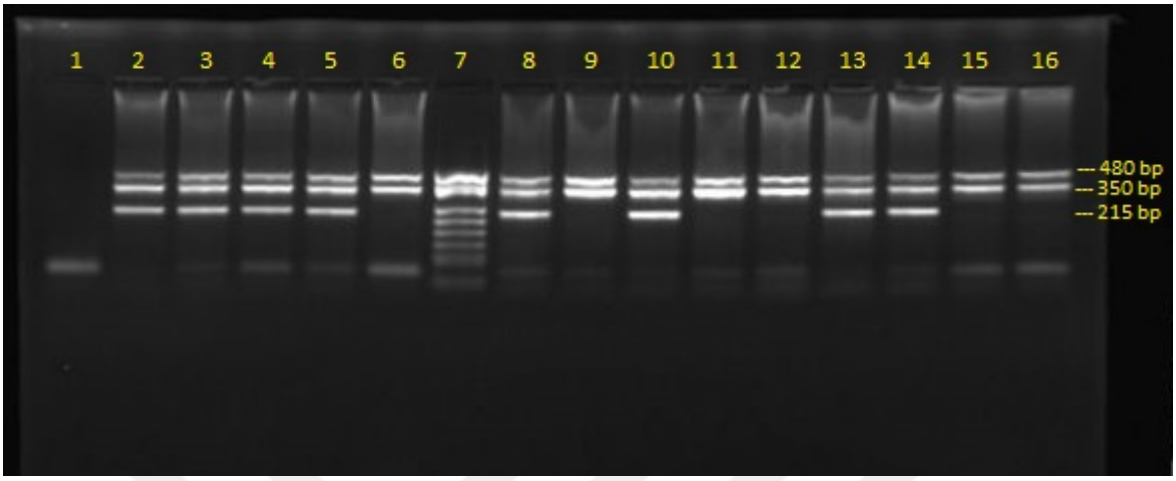

Figure 7. Multiplex polymerase chain reaction results of the *GSM1*and *GSTT1* alleles. Lanes 2, 3, 4, 5, 8, 10, 13, 14 are *GSTT1* (+)/*GSTM1* (+). Lanes 6, 9, 11, 12, 15, 16 are *GSTT1* (+)/*GSTM1* (-). Absence of 480 bp band and presence of 215 bp band was noted as *GSTT1* (-)/*GSTM1* (+) (not shown). Lane 1: negative control. Lane 7: Marker DNA (pUC19)

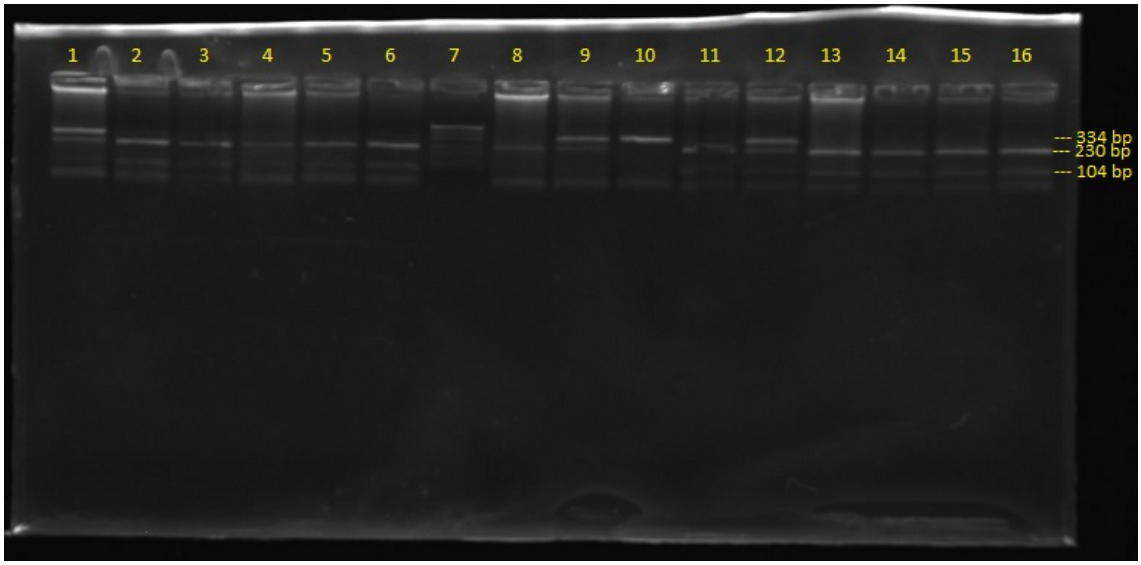

Figure 8. RFLP results of *CYP2D6* polymorphisms. Lane 10 is AA genotype. Lanes 1,9 and 12 are AG genotypes. Lanes 2,3,4,5,6,8,11,13,14,15,16 are GG genotypes. Lane 7: Marker DNA (pUC19)

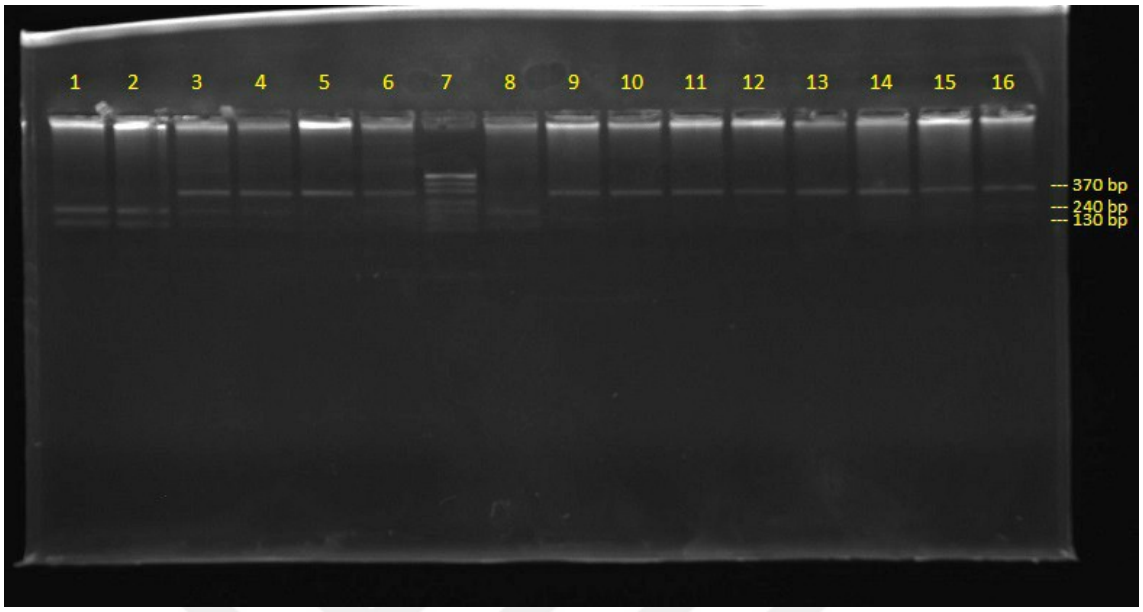

Figure 9. RFLP results of *CYP1A2* polymorphisms. Lanes 5,9,10,11,13,14,15 are AA genotypes. Lanes 3,4,6,12, and 16 are AC genotypes. Lanes 1,2,8 are CC genotypes. Lane 7: Marker DNA (pUC19)

Figure 10. RFLP results of *GSTP1* polymorphisms. Lanes 2,3,4,9,13,16 belong to Ile/Ile individuals. Lanes 1,5,6,8,10,11,14, and 15 are Val/Ile genotypes. Lane 12 belongs to Val/Val individual. Lane 7: Marker DNA (pUC19)

The frequency of the *GSTT1* null genotype was higher in infertile man (18.4%) compared with normozoospermic man (1%) (P=0.005). However, *GSTM1* null genotype was not different in both groups (49.1% compared with 54.0% in controls). Similarly, there was no association between the *GSTP1* genotypes and male infertility (P=0.662) (Table 3).

Our results have demonstrated a significant association between *CYP1A2* gene polymorphisms and male infertility. AA genotype of *CYP1A2* was more prevalent in infertile men (48.2%) compared with (22.0%) in controls (Table 4).

GG genotype of *CYP2D6* frequency was elevated in infertile men (73.7%) compared with control group (34.0%). The distribution of genotypes and alleles of infertile cases and controls are presented in Table 4.

Table 4. The distribution of *CYP1A2, CYP2D6, GSTM1, GSTT1,* and *GSTP1* polymorphisms in infertile men and controls

Genotypes	**Infertile Cases (N= 114) (%)**	**Fertile Controls (N=50) (%)**	**OR (95% CI)**	**P**
GSTT1				
Present	93 (81.6)	49 (98)	Reference	0.005*
Null	21 (18.4)	1 (2)	11.06 (1.45-84.73)	
GSTM1				
Present	58 (50.9)	23 (46.0)	Reference	0.565
Null	56 (49.1)	27 (54.0)	0.82 (0.42-1.60)	
GSTP1				
Val/Val	61 (53.5)	23 (46.0)	Reference	0.662
Val/Ile	46 (40.4)	23 (46.0)	1.33 (0.66-2.65)	
Ile/Ile	7 (6.1%)	4 (8.0%)	1.52 (0.40-5.66)	
CYP1A2				
AA	55 (48.2)	11 (22.0)	Reference	0.005*
AC	43 (37.7)	26 (52.0)	3.0 (1.34-6.79)	
CC	16 (14.0)	13 (26.0)	4.0 (1.52- 10.79)	
CYP2D6				
GG	84 (73.7)	17 (34.0)	Reference	0.0001*
AG	27 (23.7)	25 (50.0)	4.6 (2.15-9.71)	
AA	3 (2.6)	8 (16.0)	13.17 (3.16-54.82)	

5. DISCUSSION

In our investigation, we have studied to find out whether *CYP1A2, CYP2D6, GSTM1, GSTP1* and *GSTT1* genes polymorphisms were associated with idiopathic male infertility in men with defective spermatogenesis either with azoospermia or severe oligozoospermia. Our results have demonstrated a significant association between *GSTT1* and *CYP1A2* genes polymorphisms and male infertility. The sperm count of infertile men and the control group was statistically different than those infertile men ($p = 0.001$).

GSTM1 and *GSTT1* are responsible for detoxification of electrophilic compounds including carsinogens, therapoetic drugs, environmental toxins and OS products. Both genes are highly polymorphic and their polymorphisms are related to increasing risk of some cancer types and gaining susceptibility for toxins and carcinogens. Allele frequency of polymorphisms of *GSTT1* and *GSTM1* differ between populations. The present study demonstrated that the frequency of the *GSTT1* null genotype was higher in infertile man compared with normozoospermic man.

GSTP1 plays a critical role in the biotransformation and inactivation of toxic and carcinogenetic electrophiles, especially those in cigarette smoke and also inhibits apoptosis and promotes cellular proliferation through interacting with Jun N-terminal kinase (JNK) pathway. The polymorphism Ile105Val in exon 5 of *GSTP1* significantly alters the activity and heat stability of the encoded enzyme, decreasing its ability to detoxify environmental mutagens and protect against oxidative damage to DNA. A number of studies have demonstrated that this polymorphism is an important risk factor for individual susceptibility to various diseases and is a biomarker to predict the efficacy of chemotherapeutics for certain diseases including cancer (Xu et al., 2013a).

In this study we investigated the association between idiopathic male infertility and the polymorphisms of *GSTT1* and *GSTM1* in Central Black Sea Region of Turkey. Meta-analysis and systematic reviews have shown that deleted genotype of *GSTT1* and *GSTM1* might be associated with idiopathic male infertility in different populations (Li et al., 2013b, Safarinejad et al., 2012a, Tang et al., 2012). In a study conducted in a Turkish population it was found that the frequency of *GSTM1* deleted genotype was higher but *GSTT1* deleted genotype was insignificantly lower in infertile group compared the controls. In addition, the results have demonstrated no effect of these polymorphisms on semen parameters (Aydos et al., 2009). Among Iranian men an association was observed between *GSTT1* and *GSTM1* deleted genotype and idiopathic infertility (Safarinejad et al.,

2010b, Salehi et al., 2012a). On the hand, another study in Turkey, have indicated that the distribution of *GSTT1* and *GSTM1* deleted genotypes was not different in fertile and infertile groups (Aydemir et al., 2007b). This finding may be related to the number of participants in the study. Similarly, a study among Slovenian men with the participation of more subjects was showed no difference in the distribution of *GSTT1* and *GSTM1* genes polymorphisms between infertile and fertile groups (Volk et al., 2011). In another study in which patient groups consisted of men diagnosed only with non-obstructive azoospermia (NOA), the *GSTT1* deleted genotype was observed to be a protective effect for infertility (Jaiswal et al., 2012b). Similarly, an association was demonstrated between *GSTT1* positive genotype and infertility, however, the polymorphisms were not associated with smoking status among Russian men (Polonikov et al., 2010). We observed an association between *GSTT1* deleted genotype and idiopathic male infertility but not with the *GSTM1* polymorphisms. Heterogenity of the etiology of male infertility, ethnicity differences, gene-gene and gene-enviroment interactions may cause different results in populations.

Our findings indicated no difference in *GSTP1* Ile105Val polymorphism between idiopathic infertile men and normozoospermic controls. Our findings are consistent with a recent large meta analysis containing 3282 infertile cases and 3268 controls meta-analysis (Huang et al., 2017b). However, our results are inconsistent with other studies (Xiong et al., 2015, Safarinejad et al., 2012b). Similarly, Li and colleagues reported and association with *GSTP1* Ile105Val polymorphism and reduced risk of azoospermia in a Chinese Han population (Li et al., 2013c). However, the inconsistencies may be due to difference in selection criteria, exclusion and inclusion criteria, number of patient and control samples used to carry out these studies, ethnicity differences.

Our results have demonstrated a strong correlation between *CYP2D6* A/G polymorphism between azoospermia/oligozoospermia. Some alleles of CYP2D6 have been reported that they contribute the association of infertility such as CY2D6*4 and CYP2D6*10 (Zalata et al., 2015).

To the best of our knowledge this is the first study investigating the association between *CYP1A2* A/C polymorphism and male infertility. We found a strong association with male infertility and *CYP1A2* polymorphism. Estrogens have been involved in cancer development, the complicated estrogen metabolism by CYP1A2 and other enzymes, and

the possible interactions with testosterone, make it difficult to evaluate the role of CYP1A2. Therefore, *CYP1A2* may have a relation with male infertility because of its association with testicular cancer (Vistisen et al., 2004)

The conflicting results may stem from the complexity and heterogeneity of male infertility etiology, differences in the selection of population characteristics, sample size, geographic and ethnic origins, including interaction with environmental factors, and gene–gene and gene–environment interactions. Efforts to explain male infertility have faced numerous challenges, and one major obstacle has been the fact that humans are not experimental species, so researchers would inevitably rely on the next best alternative which is the use of mammalian species, classically models such as mouse and then use experimental data to explain human conditions (Li and Zhou, 2012).

6. CONCLUSIONS AND RECOMMENDATIONS

Male infertility has a multifactorial etiology that might be congenital or acquired. Diet, drug usage, and environmental factors that have an impact on the health of individuals and communities may affect to spermatogenesis and male fertility. Toxins, biological agents, radiation, excessive heat, some of drugs, exposing heavy metals, smoking and aging may increase the amount of ROS. OS reduces sperm viability and motility by causing peroxidation of sperm membrane, DNA damage and apoptosis. Elevation of ROS level may be one of the major factors in idiopathic male infertility. The underlying mechanisms of 30% of men with infertility are unexplained hence referred to as idiopathic.

The outcome of our investigation to establish the association of *CYP1A2, CYP2D6, GSTM1*, *GSTP1* and GSTT1 genes with idiopathic male infertility indicated no association among idiopathic male infertility *GSTM1* and *GSTP*. In addition, our results have suggested that genetic polymorphisms of *GSTT1*, *CYP2D6* and *CYP1A2* genes could play a role in idiopathic male infertility in our population.

Further understanding of the biological mechanism underlying these genetic variations and their relationship with environmental factors in large samples will help define the role of *CYP2D6, GSTT1* and *CYP1A2* genes with idiopathic male infertility.

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Sayın Doç. Dr. Sezgin GÜNEŞ

Etik Kurulumuza sunmuş olduğunuz ***CYP1A2, CYP2D6, GSTM1, GSTP1*** **ve** ***GSTT1*** **Genleri Polimorfizmleri ve İdiyopatik Erkek İnfertilitesi Arasındaki İlişkinin Araştırılması** başlıklı OMÜ KAEK 2016/216 Karar nolu Genetik çalışma nitelikli araştırma projeniz amaç, gerekçe, yaklaşım ve yöntemle ilgili açıklamaları, Klinik Araştırmalar Etik kurulu yönergesine göre 12.05.2016 tarihli Etik Kurulumuzda incelenmiş etik açıdan uygun bulunmuştur. Ancak araştırma bütçesinin maddi desteği henüz sağlanamadığından projeye bütçe desteği sağlanıp, tarafımıza bildirilmesinden sonra başlanmasına oy birliği ile karar verilmiştir.

Bilgilerinize arz/rica ederim.

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