NAT2 SLOW ACETYLATION, GSTM1 NULL GENOTYPING AND RISK OF INVASIVENESS OF BLADDER CANCER IN TURKISH BLADDER CANCER PATIENTS

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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ABSTRACT

The effect of GSTM1, GSTT1 and NAT2 gene disorders on invasive bladder cancer was examined. People carries different risk factors depending on their genotypes.

GSTM1 and GSTT1 are the phase2 enzymes and responsible for detoxification. When a mutation or any other gene disorder occured, enzyme loses its activity partially or completeley. In this way, DNA may be caused by xenobiotics. DNA might be damaged under the xenobiotic exposure.

NAT2 enzyme functions to both activate and deactivate arylamine and hydrazine drugs and carcinogens. Polymorphisms in this gene, N-acetylation polymorphism, directly result in rapid, intermediate, and slow acetylator phenotypes. Polymorphisms in NAT2 are also associated with higher incidences of cancer and drug toxicity.

The study aims to provide new insight for understanding of the relationship between invasive bladder cancer and gene disorders. The data will facilitate the determination of association between specific gene disorders and cancer.

Keywords: Glutathione, bladder neoplasms, Glutathione transferase, cancer patients, N-acetyltransferase

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ÖZ

GSTM1, GSTT1 ve NAT2 genlerinde meydana gelen bozuklukların invazif mesane kanseri üzerine etkileri araştırıldı. Kişiler sahip oldukları genotip özelliklerine göre farklı risk grubunda bulunmaktadırlar.

GSTM1 ve GSTT1, her ikisi de faz 2 enzimlerindendir ve detoksifikasyondan sorumludurlar. Bu genlerde meydana gelen mutasyon ya da bir diğer gen bozukluğu, gen tarafından kodlanan enzimde parsiyel ya da tamamını kapsayan aktivite bozukluklarına neden olur. Bu durumda, DNA , zenobiyotikslere maruz kalabilir. Bunun sonucu olarak DNA hasarlarının olması beklenebilir.

NAT2 enzimi, arilamin ve hidrazin ilaçları ile kansorejenlerin aktive ve deaktive edilmesinden sorumlu enzimdir. Bu gende meydana gelen polimorfimler N-asetilasyon

enzimleri olarak bilinir ve fenotipte hızlı, yavaş ve orta asetilasyon olarak görülür. NAT2 enzimindeki polimorfizm, ilaç toksissitesi ve kanser gelişimi ile yüksek oranda ilişkilidir.

Bu çalışmanın kapsamı kanser gelişimi ile bu üç gen bozukluğunun ilişkisine kayda değer bir katkı yapacak şekilde yeni bir bakış açısı kazandırmaktır. Elde ettiğimiz veriler belli bir takım gen bozuklukları ile kanser gelişimi arasındaki ilişkiyi ortaya koymamıza yardımcı olacaktır.

Anahtar Kelimeler: Glütatyon, mesane neoplazmları, glütatyon transferaz, kanser hastaları, N-asetil transferaz

Dedicated to my parents.

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

APS	:	Ammonium per sulfate
EtBr	:	Ethidium Bromide
GSTM1	:	Glutathione-S-transferase-M1
GSTT1	:	Glutathione-S-transferase-T1
NAT2	:	N-acetyl-transferase-2
PCR	:	Polymerase Chain Reaction
RFLP	:	Restriction Fragment Length Polymorphism
TBE	:	Tris Boric EDTA
TCC	:	Transitional Cell Carcinoma
TEMED	:	N,N,N',N'-Tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

In the USA bladder cancer is the fourth most common cancer among men and the eighth most common in women, with Transitional Cell Carcinoma comprising nearly 90% of all primary bladder tumours. In 2004, it was estimated that 60 250 new patients were diagnosed with bladder cancer and 12,710 died from this disease. Although most patients with bladder cancer is diagnosed with superficial bladder tumours, 20–40% will either present with or subsequently develop muscle-invasive disease. Invasive bladder cancer is a lethal malignancy; if left untreated, >85% of patients will die from the disease within 2 years of diagnosis [1]. Despite an early and aggressive approach toward high-grade, invasive bladder cancer [2], nearly 25% of patients have pathological evidence of lymph-node metastases at the time of cystectomy [3].The risk of bladder cancer is increased by approximately three times more in smoker group and accounts for 50to 60 % of the bladder cancer patients among men and one – third of those in women [4]. Bladder cancer risk can be modulated by genetically based metabolic polymorphisms. Cytosolic glutathione-S-transferase [GST] are very large family of isoenzymes which play role in detoxification of many electrophilic substrates by their conjugation with reduced glutathione [5]. The inability to replicate results on many associations between common genetic polymorphisms and complex diseases has raised scepticism in this area of research [6]. One of the few exceptions could be the association between the risk of bladder cancer and polymorphisms in two carcinogen-detoxification genes—*NAT2* and *GSTM1* [7]. Tobacco smoking is an important cause of bladder cancer, [8] and previous analyses have suggested that the relative risk from smoking is stronger for *NAT2* slow acetylators than for rapid or intermediate acetylators

[9]. This interaction is biologically plausible, since aromatic amines, which are thought to be the most important class of bladder carcinogens in tobacco smoke, [10] are detoxified by *NAT2* [11].

1.1 GLUTATHIONE S-TRANSFERASE

Cytosolic and membrane-bound forms of glutathione S-transferase are encoded by two distinct supergene families. At present, eight distinct classes of the soluble cytoplasmic mammalian glutathione S-transferases have been identified: alpha, kappa, mu, omega, pi, sigma, theta and zeta. This gene encodes a glutathione S-transferase that belongs to the mu class. The mu class of enzymes functions in the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione. The genes encoding the mu class of enzymes are organized in a gene cluster on chromosome 1p13.3 and are known to be highly polymorphic. These genetic variations can change an individual's susceptibility to carcinogens and toxins as well as affect the toxicity and efficacy of certain drugs. Null mutations of this class mu gene have been linked with an increase in a number of cancers, likely due to an increased susceptibility to environmental toxins and carcinogens. Multiple protein isoforms are encoded by transcript variants of this gene. [12]

Glutathione S-transferase (GST) theta 1 (GSTT1) is a member of a superfamily of proteins that catalyze the conjugation of reduced glutathione to a variety of electrophilic and hydrophobic compounds. Human GSTs can be divided into five main classes: alpha, mu, pi, theta, and zeta. The theta class includes GSTT1 and GSTT2. The GSTT1 and GSTT2 share 55% amino acid sequence identity and both of them were claimed to have an important role in human carcinogenesis. The GSTT1 gene is located approximately 50kb away from the GSTT2 gene. The GSTT1 and GSTT2 genes have a similar structure, being composed of five exons with identical exon/intron boundaries. [13]

Glutathione S-transferase are a family of inducible enzymes that are important in carcinogen detoxification. They catalyze the conjugation of a variety of different compounds with the endogenous tripeptide glutathione [GSH]. Cytosolic GSTs can be divided into four human families [isoenzymes] with different but sometime overlapping substrate specificities. They are termed A [alpha], M [my], P [pi] and T [theta] [14].

Both the expression and the protein levels of GST isoenzymes vary between individuals, making them predisposed to the toxic effects of environmental carcinogens. Also, high levels of GSTs have been found in human cancer tumors compared with normal tissue [15]. Variation in levels of tumor GST may be associated with resistance or susceptibility to chemotherapeutic agents [16].

Not only single polymorphism within each GST class may be used as biomarkers of cancer risk. Combinations of polymorphisms within different GST classes also influence the risk. Individuals with two high risk genotypes in M1, P1 or T1 have a significantly higher risk of developmenting prostate cancer [17], and the risk of CLL is increased 2.8 fold if M1, T1 and P1 are “high risk” genotypes [18]. It is even more complicated when high-risk genotypes of other phase II enzymes are combined with GSTs. A polymorphic NAT2 combined with a GSTM1 and T1 null genotype changes the risk of development of breast cancer in women [19].

Individuals with GSTT1 null genotype show reuced ability to detoxify metabolites of 1,3 butadiene and ethylene oxide [20]. GSTM1 deficiency suspected to increased risk of bladder cancer was first reported by Bell et al., 1993 [21] a number of studies appeared wtih supporting findings [22]. In contrast to GSTM1, potential role GSTT1 genotype in individual susceptibility to bladder cancer have been reported with inconsistent results. A few studies exhibited non-significant decreased risk of bladder cancer with GSTT1 null genotype [23]. However; some other studies exhibited

increased risk of bladder cancer with GSTT1 null genotype [24]. Inconsistent results have also emerged from recent studies exploring the combined effect of GSTM1 and GSTT1 genotype in the development of malignancy [25].

1.2 SUSCEPTIBLE GENES FOR BLADDER CANCER

Reduced DNA repair capacity is thought to be a risk factor for cancer. There are four major DNA repair pathways in human cells: nucleotide excision repair [NER], base excision repair [BER], double strand break repair [DBR], and mismatch repair [MMR]. A genetic mutation that results in a deficiency at any point along one of these pathways can lead to genetic instability and an increased risk for carcinogenesis. Polymorphisms for different DNA repair genes can slightly alter the structure of DNA repair enzymes and, in turn, modulate cancer susceptibility. Shen, et al examined three such genes and their interactions with environmental exposures commonly associated with bladder cancer [26]. *XRCC1 [Arg* ^{399}*Gl]* is a component of the base excision repair pathway. It functions as a "scaffolding protein" that fixes base damage and DNA single strand breaks. The amino acid substitution is not known to have functional consequences. *XRCC3*[*Thr* ^{241}*Met*] is located at exon 7, which is not known to be a functional domain. It takes part in homologous recombination repair of DNA double strand breaks and cross- links, and interacts directly with Rad51 [a eukaryotic homolog of the bacterial RecA recombinase]. *XPD*[*Lys* ^{751} *Gln]* functions in the nucleotide excision repair pathway and works as an ATP- dependent helicase. It is necessary for normal transcription initiation and nucleotide excision repair. Mutations may cause defects in DNA repair, transcription, and apoptosis [27].

Although smoking, PAHs and aromatic amines have long been associated with increased bladder cancer risk, the results of studies that evaluate the relationship between polymorphisms of several DNA repair genes and bladder cancer risk have been

inconsistent. Recently, for example, Stern, et al found *XRCC1* codon 399 to be protective [28]. Matullo, et al found *XRCC3* codon 241 to increase bladder cancer risk [29] and Sanyal, et al found no effect of *XRCC1, XRCC13,* or *XPD* polymorphisms on bladder cancer risk [30]. While rare mutations of DNA repair genes cause lethal genetic disease, it is the more common DNA repair gene polymorphisms that are thought to modulate an individual's disease susceptibility [31].

Epidemiological studies have shown an association between low folate intake and an increased cancer risk. Major genes involved in folate metabolism include methylene-tetrahydrofolate reductase [*MTHFR*] and methionine synthase [*MS*]. Lin et al. investigated joint effects of polymorphisms of the *MTHFR* [677 C->T, 1298A->C] and *MS* genes [2756 A->G], dietary folate intake and cigarette smoking on the risk of bladder cancer in a case-control study [32].

Bladder cancer is associated with smoking, occupational exposures, and glutathione *S*-transferase [GST] M1 and *N*-acetyltransferase [NAT] 2 polymorphisms that may influence carcinogen metabolism, but somatic *p53*mutations are often CpG dinucleotide G:C-A:T transitions that can occur spontaneously. We conducted a case-control study to determine whether *p53*mutation characteristics might distinguish cases with environmental *versus* endogenous causes. *p53*exons 4–9 were amplified from 146 bladder tumors by PCR, screened by single-strand conformational polymorphism analysis, and sequenced. Thirty-one cases were *p53*-positive, and 112 were *p53*-negative [germ line or silent]. G:C-A:T transitions were also subclassified as CpG or non-CpG. Cases and 215 clinic controls were interviewed. *GSTM1, NAT1,* and *NAT2* polymorphisms were assayed from peripheral blood. Odds ratios [ORs] and 95% confidence intervals [CIs] were estimated using logistic and polytomous regression [33].

Recent molecular epidemiological studies have analyzed the relationship between various metabolic enzymes, such as N-acetyltransferases [NATs], cytochrome

P450 [CYP] and glutathione S-transferases [GSTs] in bladder cancer to determine as biomarkers [34]. In humans, hereditary differences in Nacetylation activity have lead to phenotypic classification of individual as rapid or slow acetylators. It has been reported that genetically variable NATs, CYP P450 and GSTs are involved in the metabolism of drugs, carcinogens and natural products; and therefore act as candidate genes for cancer susceptibility [35; 36].

1.3 N-ACETYL-TRANSFERASE-2

This gene encodes N-acetyltransferase 2 (arylamine N-acetyltransferase 2). This enzyme functions to both activate and deactivate arylamine and hydrazine drugs and carcinogens. Polymorphisms in this gene are reponsible for the N-acetylation polymorphism in which human populations segregate into rapid,intermediate, and slow acetylator phenotypes. Polymorphisms in NAT2 are also associated with higher incidences of cancer and drug toxicity. A second arylamine N-acetyltransferase gene (NAT1) is located near NAT2 [37].

A survey through the literature reveals that individuals with the slow NAT2 acetylator type are supposed to be at higher risk for drug side effects, such as peripheral neuropathia after isoniazid treatment [38], or certain disorders, such as drug-induced lupus erythematosus. This severe disease may be caused, in susceptible individuals, by a large number of drugs involved in acetylation metabolism. The association to the idiopathic form, however, is discussed controversially [39]. Peripheral neuropathy provoked by isoniazid overdosage may be a major problem in ethnicities with a high frequency of slow acetylators, such as those in Northern Africa. Low drug efficacy may be expected in rapid acetylators, due to fast metabolization. The kinetics of amrinone, a phosphodiesterase inhibitor for the treatment of severe heart failure, correlates with the acetylation status (unpublished observations). Because*N*-acetylamrinone may cause

thrombocytopenia [40], rapid acetylators may be at higher risk for this severe side effect.

Major substrates of arylamine *N*-acetyltransferases are xenobiotics. Therefore, many studies have focused on the distribution of slow and rapid acetylators in certain xenobiotics-induced malignancies. In particular, slow acetylation was shown to be a susceptibility factor for bladder cancer [41]. Especially, exposure to arylamines, formerly used in the aniline industry, now preferentially related to cigarette use, makes slow acetylation a hereditary risk factor, as proven by recent genotyping studies [42].

In humans, hereditary differences in N-acetylation activity have lead to phenotypic classification of individual as rapid or slow acetylators. The absence of a restriction site indicated the presence of defective alleles identified the slow acetylators [43]. NAT2 slow acetylator phenotype(s) infer a consistent and robust increase in urinary bladder cancer risk following exposures to aromatic amine carcinogens. However, identification of specific carcinogens is important as the effect of NAT2 polymorphism on urinary bladder cancer differs dramatically between monoarylamines and aryldiamines. Misclassifications of carcinogen exposure and NAT2 genotype/phenotype confound evidence for a real biological effect [44].

1.4 RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination. Most RFLP markers are co-dominant (both alleles in heterozygous sample will be detected) and highly locus-specific.

Table 1.1 Restriction enzymes and digested product size of NAT2 gene locus

NAT 2 alleles	Restriction enzyme	Size of the fragments [547 bp] for wild type	Size of the fragments presence of mutated alleles
NAT2*5	KpnI	433- bp, 114- bp	547 bp
NAT2*4	MspI	345-, 137-, and 65-bp	345-, 114-, and 65-bp
NAT2*6	TaqI	222-, 170-, and 155-bp	392- and 155-bp
NAT2*7	BamHI	490- and 57-bp	547 bp

CHAPTER 2

EXPERIMENTAL PART

2.1 MATERIALS

2.1.1 DNA Samples

Performing the experiment, two different DNA group were used.

2.1.1.1 Patient Samples

DNA samples were obtained from the outside laboratory. All DNAs were extracted from the blood sample using “Salting-out Extraction Method”. Patients are from “Bezm-i Alem Valide Sultan Vakif Gureba Educational and Research Hospital, Urology Clinique”.

2.1.1.2 Control Samples

DNA samples were extracted from the buccal swap sample. These were obtained from the people who are healthy and has no risk factor for bladder cancer. People are randomly selected from the Fatih University students.

2.1.2 Equipments

Autoclave	:	CERTO CLAW A-4050 Traun, Austria
Balance	:	Sartorius, Wender Landstrasse 94-108 D-37075 Goettingen, Germany
Camera	:	Sony Cybershot DSC T520
Centrifuges	:	Hettich, Mikro 22
Deep freezers	:	BEKO, Turkey
Electrophoretic Equipment	:	Bio-Rad Sub Cell, GT
Magnetic Stirrers	:	Chiltern Hotplate Magnetic Stirrer
Power supplies	:	Bio-Rad Power PAC-300
Refrigerator	:	Ariston, +4 ^{o}C
Thermocyclers	:	TECHNE TC-512
Transilluminator	:	Bio-Rad GelDoc 2000
Vortex	:	IKA LABORTECHNIK
Water Purification System	:	Millipore, Water Purification System, Ultra Pure Water

2.1.3 Chemicals

Many of the chemicals in this thesis are supplied from either MERCK [Germany] or SIGMA [USA]. Alcohols are purchased from RIEDEL DE-HAEN [Germany].

2.1.4 Buffers and Solutions

During DNA isolation and visualization of the PCR products, different buffers were used.

2.1.4.1 DNA Isolation

Buccal Cell Isolation kit	:	Gentra Systems, Puregene, Genomic DNA Purification Kit
Alcohol	:	RIEDEL DE-HAEN [Germany].

2.1.4.2 PCR

Taq polymerase buffer	:	Fermentas, 10x Taq Buffer + $[NH_4]_2SO_4$ – $MgCl_2$

2.1.4.3 Gel Electrophoresis

Both horizontal and vertical electrophoresis were used.

Agarose;

10 x TBE buffer	: 54 g Trsibase, 27.50 g Boric acid, 4.650 g NaEDTA dissolved in 500ml distilled water
10 x loading buffer	: 2.5mg/ml bromophenol blue, 1% SDS in 2 ml of glycerol
Ethdidum Bromide	: 10 mg/ml
2 % agarose	: 2 % agarose [MERCK] in 0.5 x TEB buffer

Polyacrylamide;

Loading buffer	: 95 % formamide, 20mM EDTA, 0.05%Xylene Cyanol, 0.05% Bromophenol blue
6% acrylamide solution	: 6% acrylamide/bisacrylamide [19:1]
APS	: 10 % Ammonium persulfate
TEMED	: N,N,N',N'-Tetramethylethylenediamine

2.1.4.4 Staining of Gels

In order to confirm the results silver staining was used in addition to EtBr staining.

EtBr Staining;

Ethidium Bromide : 10 mg/ml

Silver stainig;

Buffer A : 10 % Ethanol, 0.5 % Glacial Acetic Acid

Buffer B : 0.1 % $AgNO_2$

Buffer C : 15 g NaOH, 0.1 g $NaBH_4$, 4 ml Formaldehyde [37%], completed to 1 ml with dH_2O

Buffer D : 7.2 g Na_2CO_3 dissolved in 1 liter dH_2O

Buffer E : 5 % Glycerol

2.1.5 Oligonucleotide Primers

Oligonucleotide primers were used to amplify specific gene loci, which are called GSTM1, GSTT1, ß – globulin and NAT2.

Table 2.1 Oligonucleotide Primers

Gene	Primer Sequence	Product size
GSTT1	5' - ttc ctt act ggt cct cac atc tc - 3 '	480 bp
	5' - tca ccg gat cat ggc cag ca - 3'	
GSTM1	5' - gaa ctc cct gaa aag cta aag c - 3'	215 bp
	5' - gtt ggg ctc aaa tat acg gtg g - 3'	
ß - globulin	5’ - caa ctt cat cca cgt tca cc - 3'	268 bp
	5’ - gaa gag cca agg aca ggt ac - 3'	
NAT2	5' - gga aca aat tgg act tgg - 3'	547 bp
	5' - tct agc atg aat cac tct gc - 3'	

2.2 METHODS

2.2.1 DNA Extraction

DNA was isolated using the Genomic DNA Purification Kit which is supplied by Gentra Systems. Buccal cells are collected by using sterile nylon bristle cytology brush which is included in the kit. Brush is removed into 1.5ml microcentrifuge tube cotaining 300µl cell lysis solution. Tubes are incubated at 65 ºC for 45 minutes in the waterbath in order to complete the cell lysis activity. After removing the sample from the waterbath, brushes are take out from the microcentrifuge tube. Remaining cell lysate is cooled to room temperature by placing them on ice for 1 minutes. 100 µl Protein precipitation solution is added into cell lysate. That solution is vigorously vortexed at the highest speed for 20 seconds in order to mix the Protein precipitation solution uniformly with the cell lysate. Tubes are placed in an ice bath for 5 minutes. Centrifuge at 13,000-16,000 x g for 3 minutes. The white pellet contains precipitated proteins. İf the tight structure of the protein precipitation is not provided vortex and centrifugation is repeated. DNA containing supernatant is poured into a clean 1.5ml microfuge tube which contains 300µl 100% isopropanol [2-propanol] and 0.5µl glycogen solution[20mg/ml, Gentra catalog number R-5010]. Tubes are mixed by inverting gently 50

times and incubated at room temperature for at least 5 minutes. Centrifugation was performed at 13,000-16,000 x g for 5 minutes. The DNA may or may not to be visible as a small white pellet at bottom of the tube. Supernatant is poured off and tube is drained on a clean absorbent paper. Add 300µl of 70% ethanol and tube is inverted several times to wash DNA. Centrifugation is performed again at 13,000-16,000 x g for 1 minute. Carefully pour off the ethanol and watch pellet. Invert and drain the tube on a clean absorbent paper and allow it to dry 10-15 minutes. 20µl of DNA hydration solution is added. If the yield is 1µg DNA, concentration will reach 50ng/µl when 20 µl is added. DNA is rehydrated by incubating the sample for overnight at room temperature. The DNA was stored at -20 ºC.

2.2.2 Polymerase Chain Reaction [PCR]

All the PCR reactions is performedin a total volume of 25 µl. The master mix which is the mixture of PCR reagents contains 10x Taq Buffer + $[NH_4]_2SO_4$ – $MgCl_2$,2mM dNTP mix, 1 unit Taq polymerase and 100ng of genomic DNA. Thermocycler Techne was used for amplification reaction.

2.2.2.1 Amplification of the GSTM and GSTT and β-globulin gene loci

PCR was performed in the thermocycler TECHNE. Amplification reaction includes 5 minutes denaturation at 94 ºC for genomic DNA and three consecutive steps are repeated as a module of 35 cycles. Last step is ten minutes extension and the storage step is followed at a proper temperature. β-globulin is amplified as an internal control. By this way, amplification reaction is confirmed

94 ºC		5 minutes	[initial denaturation]	
94 ºC		30 seconds	[denaturation]	
57 ºC	▲ 5 ºC	30 seconds	[annealing]	35 cycle
72 ºC		2 minutes	[elongation]	
72 ºC		10 minutes	[final extension]	
4 ºC				

2.2.2.2 Amplification of NAT2 gene locus

PCR was carried out in the thermocycler TECHNE. A program which consist of initial denaturation at 94 ºC for 3 minutes and 35 cycle repeating module of 94 ºC 30seconds[denaturation], 60 ºC for 1 minute[annealing] and 72 ºC for 2 minutes[elongation] was performed. Finally, 72 ºC for 10 minutes is the final extension step.

94 ºC	5 minutes	[initial denaturation]	
94 ºC	30 seconds	[denaturation]	
60 ºC	30 seconds	[annealing]	35 cycle
72 ºC	2 minutes	[elongation]	
72 ºC	10 minutes	[final extension]	
4 ºC			

2.2.2.3 Optimization of PCR conditions

In order to obtain proper annealing temperature and amount of reagents for optimum PCR, optimization was carried out according to results of initial reactions. Starting to PCR, annealing temperature was adjusted to 55 ºC and 5 ºC gardient is used. 5 ºC gardient means wells on the thermocycler will be heated starting from 50ºC to 60 ºC . However, in this temperature many non-specific bands were observed. Annealing temperature is increased to 57 ºC and gradient degree still remains as 5 ºC. Temperature is increased and non-specific bands are dissappeared. Amount of $MgCl_2$ is increased from 2 µl to 2.5 µl. In the less concentration of the $MgCl_2$, low brightness in the bands was detected. So, concentration of the $MgCl_2$ is elevated. In all the reactions, double distilled water was used in order to prevent problems caused by water.

2.2.3 Agarose Gels

PCR products were resolved on 2% agarose gel. Gel is prepared adding 1.6 gram of powdered agarose gel into 80ml of 0.5 x TBE buffer solution and it is boiled until the agarose is completely dissolved in the buffer solution. Four µl of Ethidium Bromide was added when the boiled solutions began to cool down and reach approximately 55 ºC. Solutions is mixed homogenously by making hand-shaking. It directly poured into horizontal agarose gel platform and the comb including 8 or 22 wells is placed one side of the gel. Let the gel solidify for 45 minutes.

2.2.4 Page Analysıs

The resultant products of the digested PCR products were resolved on 6% polyacrylamide gels. Polyacrylamide gel was prepared using the 37.5% stock solution of acrylamide. Amount of the acrylamide is 7.5ml and 5ml of 10x TBE buffer, 700 µl Ammonium per sulfate [APS], 70 µl TEMED and 36.75ml of double distilled water is added. Tetramethylethylenediamine [TEMED] is used as initiator and catalyzer of the polymerization reaction. The gel contains 20 wells and it has 1mm thickness.

2.2.5 Restrıctıon Fragment Length Polymorphısm [RFLP]

PCR products of NAT2 amplification reaction were digested with different restriction endonucleases. These enzymes cut the DNA backbone in the specific region. BamHI, TaqI, KpnI and MspI/AluI restrintion enzymes were used in order to determine the specific mutation in the same PCR region. 10 µl of PCR products was used for restriction reaction. In addition to this, MspI/AluI, KpnI and BamHI needs 18 µl ditilled water, 2 µl reaction buffer and 2 µl enzyme whereas TaqI needs 16 µl distilled water, 2 µl reaction buffer and 2 µl restriction enzyme. All the reactions occurs in 37 ºC in water bath except TaqI. Optimum temperature for TaqI is 65 ºC.

2.2.6 Staining of Gels

In agarose gel EtBr staining was used as usual. Moreover, Silver staining was applied on Polyacrylamide gel to get more specific result.

2.2.6.1 EtBr Staining

Ethidium Bromide is substance which inter catalates the DNA backbone. This dye is visible under UV. The dye is added into directly to agarose gel solution and gel must be homogenous before pouring into platform. For acrylamide gel, 5 µl is dropped into 100ml water and the gel placed inside the solutions for staining.

2.2.6.2 Silver Staining

Silver staining is the the sensitive, easy to do and safer method compared the other radioactive and immunologic labelling methods. Silver staining technique was used to determine the bands in polyacrylamide gel.

In the staining, five different buffers were used. Buffer C must be prepared just before staining protocols.

The procedure is :

- Incubate in buffer B for 10 minutes.
- Wash briefly twice with dH_2O.
- Incubate in buffer C until bands appear.
- Incubate in buffer D for 5-10 minutes
- Seal the gel

In order to obtain more qualified view, some arrangements are necessary in view of the original method.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 GSTM1 and GSTT1 deletion analysis

Glutathione-S-transferase T-1, Glutathione-S-transferase M-1 and β-globulin genes are amplified in PCR reaction and they are visualized in 2 % agarose gel. Size of the GSTM1 gene is 215 bp, size of the GSTT1 gene is 480 bp and size of the β-globulin is 268 bp. Every separate lane refers an independent patient. In the first lane, DNA ladder is loaded in order to be able to compare the size of the PCR products with marker.

Tablo 3.1. PCR product size of GSTM1, GSTT1, β-globulin.

Gene name	PCR product size
GSTM-1	215 bp
GSTT-1	480 bp
β-globulin	268 bp

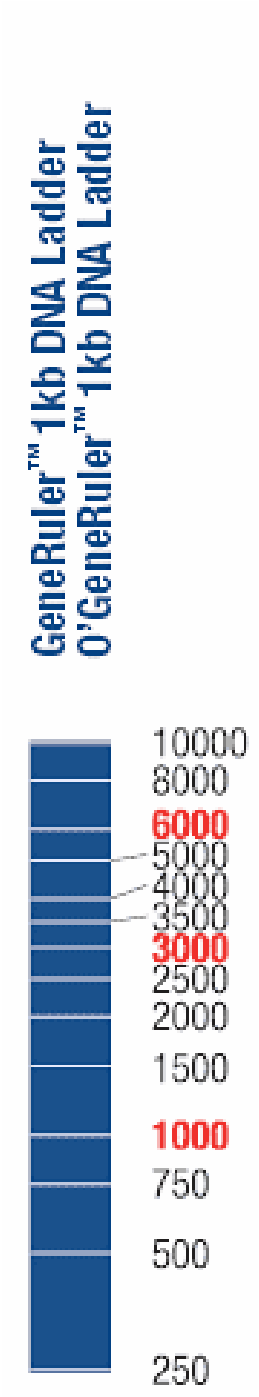

Figure 3.1 Gene ruler 1kb DNA ladder, Fermentas. In the first lane of gel picture, the mixture of 2 µl DNA ladder, 2 µl loading dye and 1 µl distilled water was loaded.

Gene ruler is a commercially available PCR products in different sizes. During the gel electrophoresis, sample bands are compared to gene ruler and determine the size of the our DNA. In this study, 1kb gene ruler was used. After the effect of the electrical current this ladder shows different bands in different sizes [figure3.1]. starting from the 250 bp until 10,000 bp it can be detected using this 1kb DNA ladder. As the Gene ruler is amplified several times, it is highly concentrated. In the agarose gel, that highly concentrated material is hard to run. So, Gene ruler is diluted by adding distilled water. In addition to this, loading dye was also added the generuler to detect the place of the ladder while it is runnig.

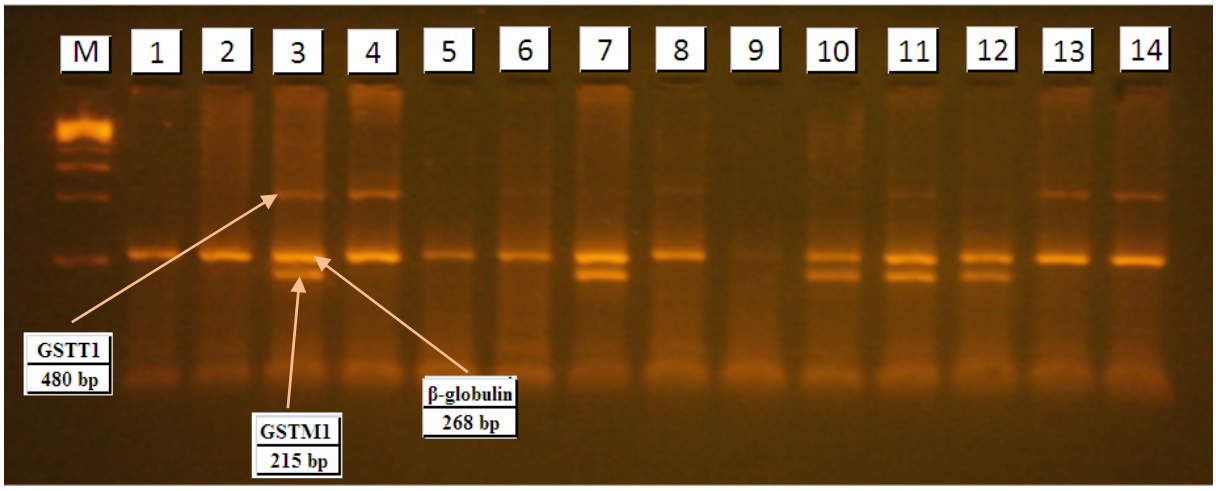

Figure 3.2 In the figure, lane 3 and lane 11 patients have all three genes, - GSTT1, GSTM1 and β-globulin – lane 4, lane 8, lane 13 and lane 14 has only GSTT1 and β-globulin, lane 1, lane 2, lane 5, lane6 has only β-globulin gene which is an internal positive control whereas lane 7, lane 10, lane 11, lane12 has only β-globulin and GSTM1. In Lane 9, there is no amplification.

Observing the bands on the gel refers to proper amplification of the targeted gene. On the other hand, GSTM-1 and GSTM-2 analysis are deletion detection

experiments. Determining bands means amplification of the gene, but, it is hard to specify whether one or both alleles are amplified. In order to reach to exact result, light intensity of the all bands are compared by using the software called MathLab. According to the brightness of the band, it will definitely give results starting from blue color until red one. In figure 3, Mathlab analysis of the figure 2, three dimensional view of the gel picture seen on figure 2. Figure 4 shows the two dimensional picture. It again belongs to figure 3.2.

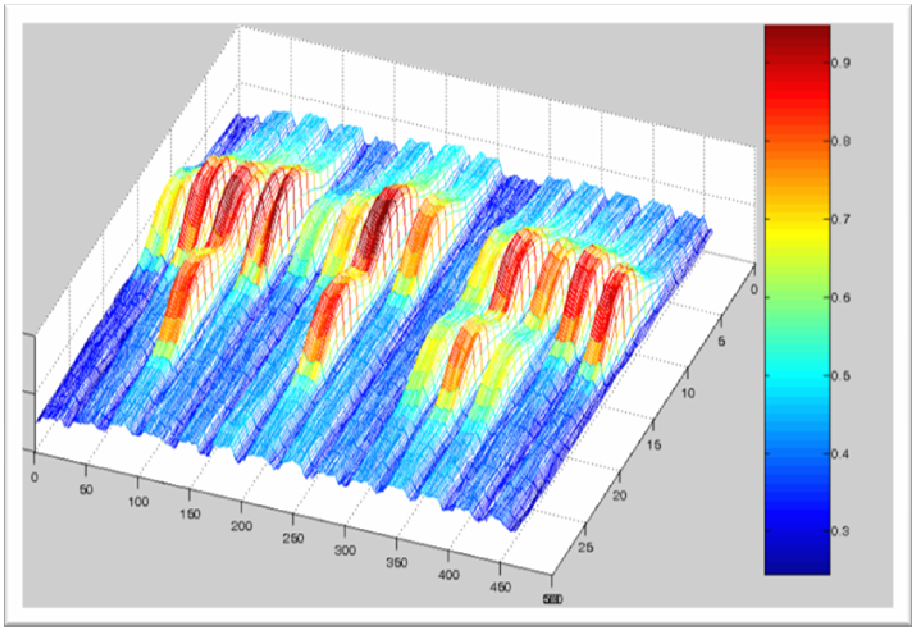

Figure 3.3 Three dimensional analysis of the Figure 3.2.

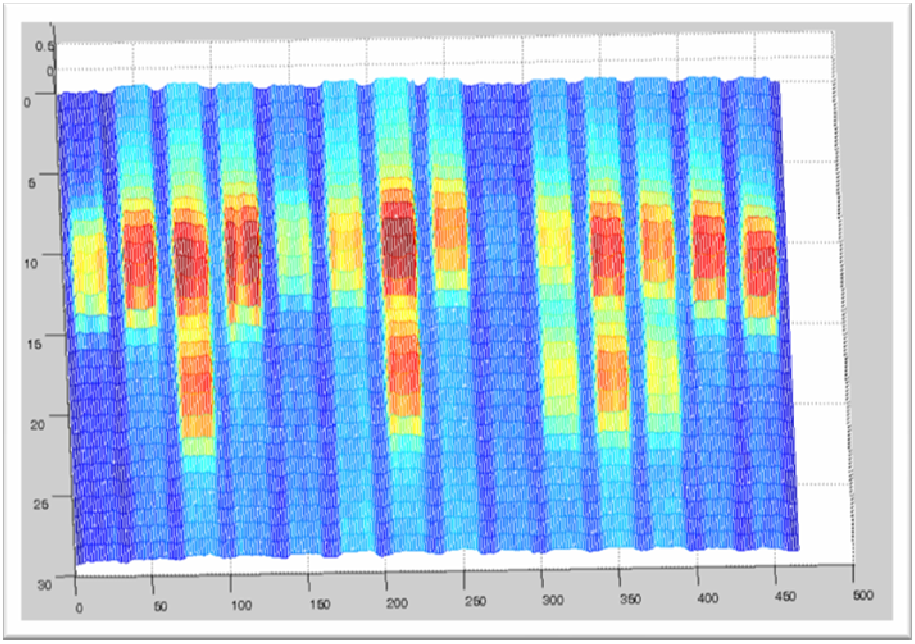

Figure 3.4 Two dimensional analysis of the Figure 3.2.

By using this Mathlab software program, “0/0” null genotype and “0/+” partial deletion patients are clearly separated from each other.

3.2 N-acetyl transferase- 2 [NAT2] gene analysis

PCR reaction was performed for NAT2 gene and a 547 bp fragment was amplified. Four different alleles of NAT2 was identified by using Restriction Fragment Lenght Polymorphism [RFLP] method. According to independent restriction sites, four different restriction endonucleases was used and four alleles were determined. Digested PCR products were identified on the 2 % agarose gel. The following restriction enzymes *Kpn*I [MBI, Fermentas], *Taq*I [MBI, Fermentas], MspI[MBI, Fermentas], and *Bam*HI [MBI, Fermentas] were used to detect the presence or absence of mutations at the positions C481T, G590A, A803G, and G857A, respectively. PCR products of mutated alleles which ish C to T transition, at position 481 [allele *NAT2*5*], were

resistant to *Kpn*I digestion [absence of restriction site], the 547bp PCR product was not digested into 433- and 114-bp fragments. The alleles with A803G mutation [allele *NAT2*4*] were determined by the presence of an additional *Msp*I restriction enzyme site; thus the normal pattern 345-, 137-, and 65-bp fragments were replaced by 345-, 114-, and 65-bp fragments. Alleles with G to A transition at position of 590 [allele *NAT2*6*] were identified by loss of *Taq*I restriction enzyme site, producing 392- and 155-bp fragment instead of 222-, 170-, and 155-bp fragments. Restriction endonuclease *Bam*HI was used to identify the alleles with G to A transition at the position of 857 [allele *NAT2*7*]. They were identified by loss of the *Bam*HI restriction enzyme site, result in 547-bp fragment, instead of 490- and 57-bp fragments. The wild-type allele *[NAT2*4]* was recognized by the occurrence of the restriction sites for the *Kpn*I, *Bam*HI, *Msp*I, and *Taq*I endonucleases.

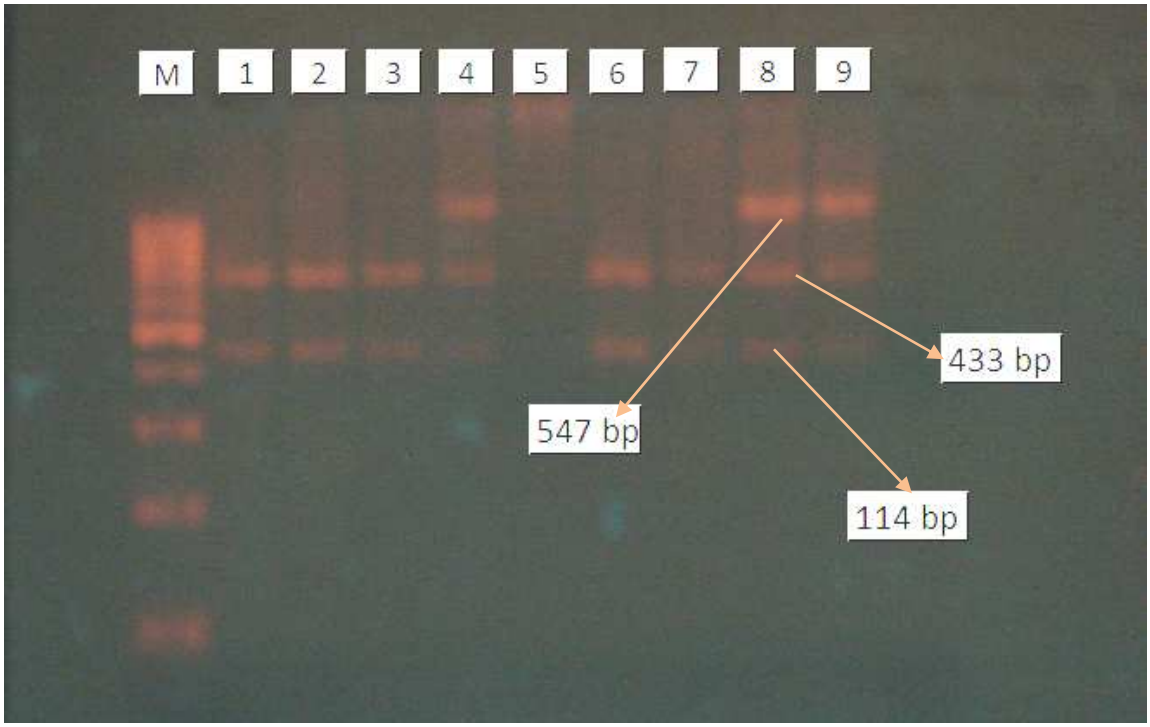

Figure 3.5 KpnI digestion of the amplified NAT2 gene locus. People in the lane 1, lane 2, lane 3, lane 6 and lane 7 are wild type. Lane 4, lane 8 and lane 9 are heterozygous for KpnI digestion. In lane 5, no amplification is observed.

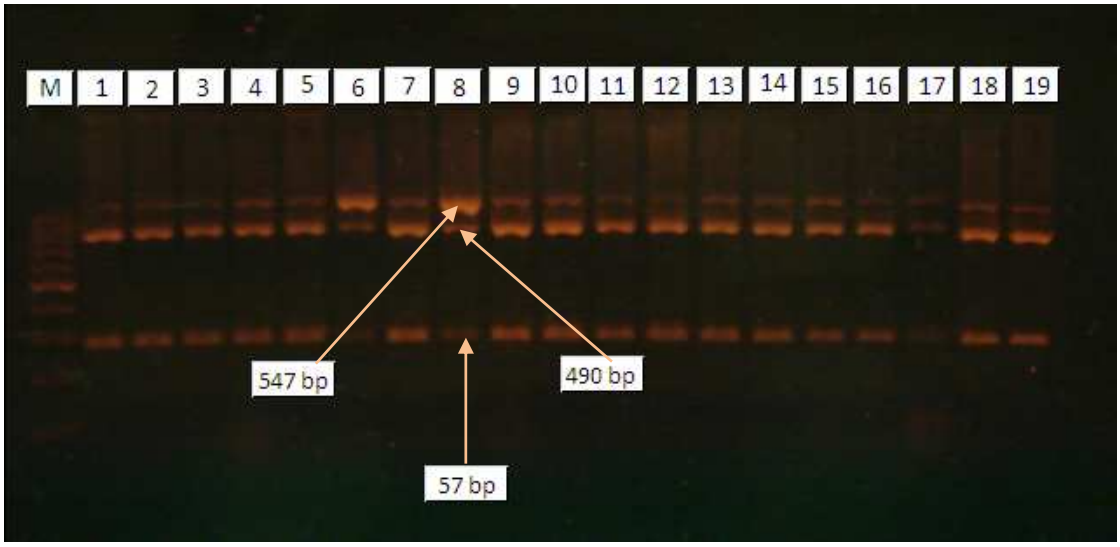

Figure 3.6 BamHI digestion of NAT2 PCR product. Lane 1, lane 2, lane 3, lane 11, lane 12 and lane 16 are wild type genotypes. Lane 4, lane 5, lane 6, lane 7, lane 8, lane 9, lane 10, lane 13, lane14, lane 15, lane 18 and lane 19 are heterozygous for BamHI restriction site. Lane 6, both alleles have mutation and result in disappearing of recognition site.

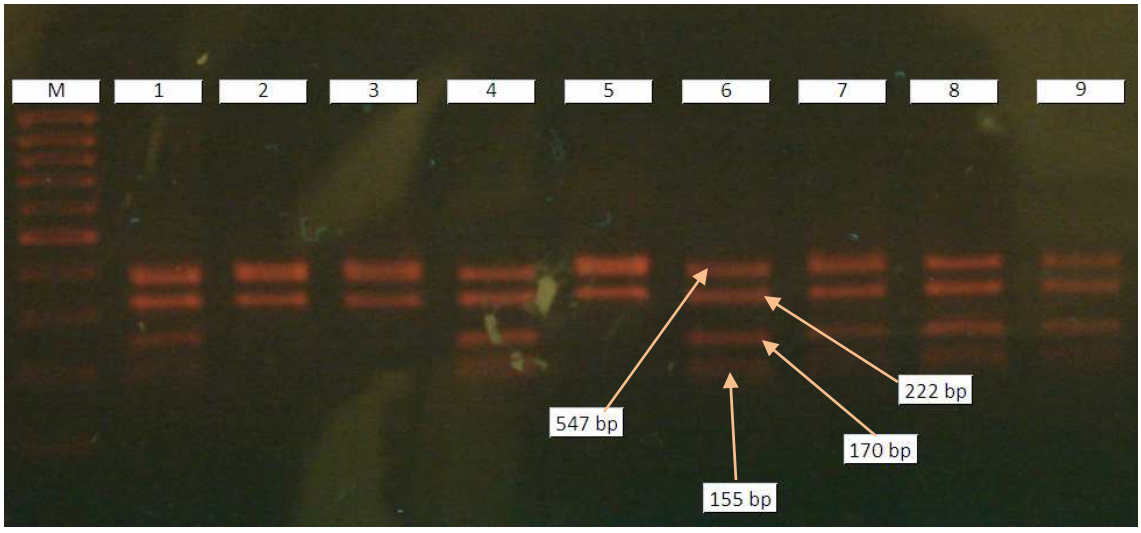

Figure 3.7 TaqI digestion of amplified NAT2 region. Lane 1, lane 4, lane 6, labe 7, lane 8, lane 9 are heterozygous for TaqI restriction site. Lane 2, lane 3 and lane 5 are homozygous mutant for Taq I.

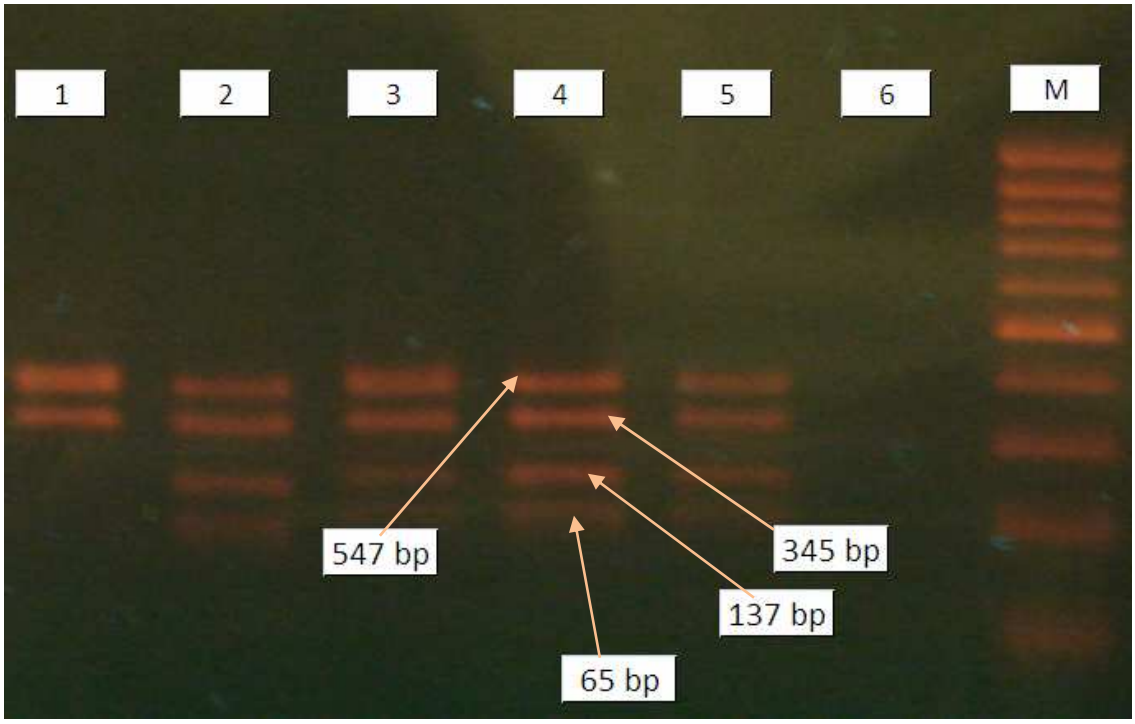

Figure 3.8 Lane 2, Lane 3, lane 4 and Lane 5 are heterozygous for MspI restriction site. Lane 1 is homozygous mutant. Lane 6 is negative control for NAT2 amplification and MspI digestion reaction.

Table 3.2: Amplification results of patient and control groups

Gene	Genotype	Patient (59)	control (81)
GSTM1			
	0/0	35	26
	0/+	2	1
	+/+	22	54
GSTT1			
	0/0	19	13
	0/+	0	2
	+/+	40	66
NAT2			
	Fast Acetylator	3	40
	Intermediate Acetylator	4	29
	Slow Acetylator	52	12

3.3 Statistical Analysis

Logistic Regression allows one to predict a discrete outcome, where the independent or predictor variables can take any form of categorical, or mix of continuous and categorical,

In binary logistic regression the dependent variable is dichotomous, that is, the dependent variable can take the value 1 with a probability of success θ, or the value 0 with probability of failure $1-\theta$.

Logistic regression have no assumption about the distribution of the independent variables. Independent variables do not have to be normally distributed, linearly related

or have equal variance within each group. The relationship between the predictor and response variables is not a linear function in logistic regression, instead, the logistic regression function is used, which is the logit transformation of θ:

$$\theta = \frac{e^z}{1+e^z} \text{ , where } z = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_k X_k$$

Where β_0 = the constant of the equation and, β_i = the coefficients of the predictor variables.
An alternative form of the logistic regression equation is:

$$\log it[\theta(x)] = \log\left[\frac{\theta(x)}{1-\theta(x)}\right] = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_k X_k$$

The goal of logistic regression is to predict the category of outcome for individual cases using the most parsimonious model. To achieve this goal, a model is created that includes all predictor variables that are useful in predicting the response variable. Since logistic regression calculates the probability or success over the probability of failure, the results of the analysis are in the form of an odds ratio. Logistic regression also provides knowledge of the relationships and strengths among the variables

Wald test is used to test the statistical significance of each coefficient (β) in the model. Agresti (1996) states that the likelihood-ratio test is more reliable for small sample sizes than the Wald test. Log transformation of the likelihood functions yields a chi-squared statistic. This is the recommended test statistic to use when building a model

Table 3.3 Frequency analysis of gene disorders

	Genotype	Freq.	Percent
Control Group	0	78	57,78
Patient Group	1	57	42,22
GSTT	0	61	45,19
	1	74	54,81
GSTM	0	31	22,96
	1	104	77,04
NAT2	0	62	45,93
	1	32	23,7
	2	41	30,37
Total		135	100

Logistic regression	Number of obs	=	135
	LR chi2(3)	=	78.37
	Prob > chi2	=	0.0000
Log likelihood = -52.749641	Pseudo R2	=	0.4262

Table 3.4 Coefficeint and Standart deviation chart of genes

	Coef. (β)	Std. Err.	z	P>|z|	[95% Conf. Interval]		Odds Ratio (e^{β})	%
Constant	2.540.534	.6605605	3.85	0.000	1.245.859	3.835.209		
GSTT	-.680811	.4992556	-1.36	0.173	-1.659.334	.297712	.5062063	-49.4
GSTM	-1.244.738	.6124112	-2.03	0.042	-2.445.042	-.0444344	.2880163	-71.2
NAT2	-234.263	.4034688	-5.81	0.000	-3.133.415	-1.551.846	.0960746	-90.4

% = percent change in odds for unit increase in X

The likelihood ratio chi-square of 78.37 with a p-value of 0.0000 tells us that our model as a whole fits significantly. In the table we see the coefficients, their standard errors, the z-statistic (sometimes called a Wald z-statistic), associated p-values, and the 95% confidence interval of the coefficients. Both **GSTM** and **NAT2** are statistically significant while **GSTT** is not. The interpretation of the coefficients can be awkward. Researchers prefer to exponentiate the coefficients and interpret them as odds-ratios

We can say that for a one unit increase in **GSTM**, the odds of being patient (vs. not being patient) increased by a factor of 0.29. Similarly, for a one unit increase in **NAT2,** the odds of being patient (vs. not being patient) increased by a factor of 0.01.

% column in the table below shows the percent change in odds for unit increase in X. For example for a unit increase in GSTM, we expect %71.2 decrease in the odds

of in cancer. One unit increase in NAT2 variable decreases the odds of cancer by 90.4%. One unit increase in GSTT variable decreases the odds of cancer by 49.4.

Even odds ratios can be hard to interpret. Instead, one can also use predicted probabilities, which are sometimes easier to understand than the coefficients or odds ratios, to interpret the results.

Although GSTT is not statistically significant, as we can see, the predicted probability of being patient is 0.25 if the GSTT is 1 and 0.40 if the GSTM is 0 while GSTM and NAT2 are held constant at their mean value. The predicted probability of being patient is 0.26 if GSTM is 1 and 0.55 if GSTM is 0. The predicted probability of being patient is 0.26 if GSTM is 1 and 0.55 if GSTM is 0. The predicted probability of being patient is 0.03 if NAT2 is 2, and 0.24 if NAT2 is 1, and 0.77 if NAT2 is 0.

Table 3.5. Predicted probabilities of positive outcome for cancer

		Prediction
GSTM		
	0	0.5471
	1	0.2581
GSTT		
	0	0.4021
	1	0.2540
NAT2		
	0	0.7700
	1	0.2434
	2	0.0300

Result of the gene statistics

GSTM = 0.77037037
GSTT = 0.54814815
NAT2 = 0.84444444

We obtained the predicted probabilities when the predictor variables are set to specific values: GSTM =1, GSTT=1, or NAT2=0, 1, 2. As you can see, when one's GSTT is 1, the predicted probability of being patient is 0.25, and 0.75 of not being patient. When GSTM is 1, the probability of being patient is0.24, 0.76 of not being patient and when one's NAT2 is 1, the predicted probability of being patient is 0.24 and not being patient is 0.76

Many reported associations between common genetic polymorphisms and complex diseases have not been confirmed in subsequent studies. An exception could be

the association between NAT2 slow acetylation, GSTM1 null genotype, and bladder-cancer risk.

We investigated polymorphisms in NAT2, GSTM1, GSTT1, in 59 patients with invasive cancer of the urinary bladder and 71 controls in Turkey.

This work to our knowledge is the first reported one for invasive bladder cancer association with the above mentioned genotypes. It provides compelling evidence of an increased bladder-cancer risk associated with the GSTM1 null and NAT2 slow-acetylation genotypes. There seems to be no correlation between GSTT1 null genotype alone and combined with NAT2 slow acetylation and GSTM1 null genotype and invasive bladder cancer risk.

Table 3.6 PCR and RFLP Results

Genotype	**GSTM1**	**GSTT1**	**NAT2**	**Patient #**	**%**	**Control #**	**%**
G1	+/+	+/+	*SA*	12	8.571	5	357.143
G2	+/+	+/+	*FA*	1	0.714	21	15
G3	+/+	+/+	*IA*	1	0.714	20	142.857
G4	+/+	*0/0*	*SA*	8	5.714	-	0
G5	+/+	*0/0*	*FA*	1	0.714	4	285.714
G6	+/+	*0/0*	*IA*	1	0.714	4	285.714
G7	*0/0*	+/+	*SA*	24	17.14	8	571.429
G8	*0/0*	+/+	*FA*	-	0	-	0
G9	*0/0*	+/+	*IA*	1	0.714	4	285.714
G10	*0/0*	*0/0*	*SA*	9	6.429	1	0.71429
G11	*0/0*	*0/0*	*FA*	-	0	3	214.286
G12	*0/0*	*0/0*	*IA*	1	0.714	1	0.71429

The relationship between *NAT2* slow acetylation and bladder-cancer risk showed that this association is robust and similar among the invasive bladder cancer patients. 24+9/59 (17.14+6.429 %) patients have the GSTM1 null and NAT2 slow acetylator

genotype whereas only 8+1/71 (5.71+ 0.714 %) controls have this highly susceptible genotype. The expected least susceptible bladder cancer genotype was 1+1/59 (0.714+0.714) in the patient group whereas 21+4/71 (15+2.85%) in the control group. This significant difference explains us the direct relationship between invasive bladder cancer risk and carrying GSTM1 null genotype and NAT2 slow acetylator simultaneously. NAT2 slow acetylator genotype alone is the most susceptible genotype on invasive bladder cancer, 45/50 (frequency). Although NAT2 fast acetylator genotype seems to be high 28/71 among controls, decreasing the susceptibility, the genotype number G8 with no individuals in both patient and control groups can be an indication for the combined effect of GSTM1 (+/+) genotype on NAT2 fast acetylator type. 12 patients have wild type GSTM1 and NAT2 slow acetylator. In the control group, 5 people have this genotype. Percentage in the patient and control group are 8,57 % and 3,57 % respectively. The number of individuals which have the GSTM1 null genotpe and wild type NAT2 is 3 in the control group and noone among patients. This data also highlightes the direct relationship between GSTM1 and NAT2 slow acetylation genotype in invasive bladder cancer patients.

Analyses by binary logistic regression suggested a strong association between risk of invasive bladder cancer and *NAT2* slow acetylator and *GSTM1* null genotypes.

This study aimed to explain the relationship between two genetic polymorphisms and cancer risk. Although the results showed the relations, the sample sizes for both controls and patient groups must be increased in order to have robust opinion about this correlation .

CHAPTER 4

CONCLUSION

GSTT1, GSTM1 and NAT2 gene region were amplified by PCR. Amplification reaction was performed to confirm the presence of the gene locus or to be able to analyze the mutation.

First two genes, GSTT1 and GSTM1 are the genes responsible for detoxification. Gene disorder that may occur in these genes are called “deletion”. Deletion is the physical process in which the gene is broken off partially or completeley. In the deletion analysis of GSTT1 and GSTM1, we amplified the these region by using oligonucleotide primers. These primers are site specific and they attach only in these genes. After amplification reaction, PCR products were visualized in agarose gel. In the gel, under the electrical effect, PCR products run and separated from each other depending on their size differences. In addition to this, every human being has two copy of a one single gene. It is hard to detect whether one or two copies of the gene is amplified by looking the bands. In order to be able to detect, we used Matlab Program. We uploaded our gel pictures and made brightness analysis. Brighter one shows two allele while the other refers to one allele. In this method, amplification is confirmed more precisely. Result of the experiments showed 35 patients carry GSTM1 mutation and 19 patient carry GSTT1 mutation.

NAT2 gene locus is also amplified with PCR. NAT2 is more polymorphic gene. In this study, we analyzed four alleles of NAT2. Every allele is determined by different mutation in different part of the gene. In order to detect this mutation, restriction endonucleases was used. These enzymes are site specific and has specific cutting site.

In the thesis, we analyzed NAT2*4, NAT2*5,NAT2*6 and NAT2*7 alleles. If the patient carries two or more mutation, it is called “Slow acetylator”. If the patient carries one mutation , it is calle “Intermediate Acetylator”. No mutation directly refers to “Fast Acetylator”. At the end of the experiments we obtained, 52 patients were slow acetylator, 4 patients were Intermediate acetylator and 3 patients were fast acetylator.

Statistical Analysis showed that both **GSTM** and **NAT2** are statistically significant while **GSTT** is not.

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