N-ACETYL TRANSFERASE (NAT1&NAT2) AND GLUTATHIONE-S TRANSFERASE (GSTM1&GSTT1) GENETIC POLYMORPHISMS IN BREAST CANCER

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AYÇİN ATALAY

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Prof. Dr. Canan Özgen
Director

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

Prof. Dr. Semra Kocabıyık
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Prof. Dr. Tülin Güray
Supervisor

Examining Committee Members

Prof. Dr. Mümtaz İşcan (Head of Jury)	(ANK, PHARM)	———
Prof. Dr. Tülin Güray	(METU, BIO)	———
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Ayçin Atalay:

ABSTRACT

N-ACETYL TRANSFERASE (NAT1&NAT2)
AND GLUTATHIONE-S TRANSFERASE (GSTM1&GSTT1) GENETIC
POLYMORPHISMS IN BREAST CANCER

Atalay, Ayçin

M.S, Department of Biology

Supervisor : Prof. Dr. Tülin Güray

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Breast cancer is the most frequent malignancy among women, especially in Western societies. Highly penetrant genes such as BRCA1 and BRCA2, together with the reproductive history can constitute only 30% of the cause, so there should be other common genes, which may play a role in breast carcinogenesis according to one's lifestyle. In our case, the effect of N-acetyl transferases (NAT1, NAT2) and glutathione-S transferases (GSTM1&GSTT1) were investigated, since variations in these genes may alter their enzymatic activity and therefore their capacity to biotransform xenobiotic compounds. To evaluate the potential association between NAT1, NAT2, GSTM1 and GSTT1 genotypes and development of breast cancer, a hospital based case-control study was conducted in a Turkish study population consisting of 37 histologically confirmed incident breast cancer cases and 34 control subjects with no present or previous history of cancer. The only recognizable difference between case and control groups is the percentage of GSTM1 deletion, 67.6% and 44.1% respectively (p=0.047). The frequency of rapid NAT2 acetylator genotype is 44.4% in cases and 23.5% in controls. Especially, women with NAT2 rapid acetylator and GSTM1 null genotypes were at the elevated risk (OR, 3.8; CI, 0.9-15.4). NAT1 rapid acetylator genotype showed no association with breast cancer. These results suggest that GSTM1 null genotype is a susceptibility factor for breast cancer, particularly in the presence of NAT2 rapid acetylator genotype.

Keywords:N-Acetyl Transferase, Glutathione-S Transferase, Breast Cancer

ÖZ

GÖĞÜS KANSERİNDE N-ASETİL TRANSFERAZ (NAT1&NAT2) VE GLUTATYON S-TRANSFERAZ (GSTM1&GSTT1) ENZİM POLİMORFİZMİ

Atalay, Ayçin

Yüksek Lisans, Biyoloji Bölümü

Tez Yöneticisi : Prof. Dr. Tülin Güray

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Göğüs kanseri, özellikle Batı toplumlarında kadınlar arasında en sık rastlanılan kanser tipidir. BRCA1 ve BRCA2 gibi yüksek oranda penetrant genlerin ve reprodüktif geçmişin birlikte vakaların ancak % 30'unu açıklaması, göğüs karsinogenezinde kişinin yaşam tarzına göre etki eden başka yaygın genlerin rol aldığını göstermektedir. Bu çalışmada, N-asetil transferazların (NAT1, NAT2) ve glutatyon-S transferazların (GSTM1, GSTT1) etkisi araştırılmıştır çünkü bu genlerin enzimatik aktivitesi ve dolayısıyla da ksenobiyotik bileşikleri metabolize etme kapasiteleri değişiklik göstermektedir. NAT1, NAT2, GSTM1 ve GSTT1 genotipleri ile göğüs kanseri oluşumu arasındaki potansiyel bağlantıyı değerlendirmek için, histolojik olarak tanı konmuş 37 göğüs kanseri hastası ile bugün ve geçmişte kanser bulgusu olmayan 34 kontrol hastane bazlı bir hasta-kontrol çalışması kapsamında incelenmiştir. Hasta ve kontrol grupları arasındaki tek belirgin farklılık GSTM1 delesyon oranında gözlenmiştir, %67.6 ve %44.1 ($p=0.047$). Hızlı NAT2 asetilatör genotipinin frekansı hasta grubunda %44.4, kontrol grubunda ise %23.5 olarak bulunmuştur. Özellikle, NAT2 hızlı asetilatör ve GSTM1 sıfır genotipini birlikte taşıyan kadınlar için yükselmiş risk sözkonusudur (OR, 3.8; CI, 0.9-15.4). NAT1 hızlı asetilatör genotipi göğüs kanseriyle bir bağlantı göstermemiştir. Sonuçlar, GSTM1 sıfır genotipinin, özellikle NAT2 hızlı asetilatör genotipiyle birlikte bulunduğu durumlarda göğüs kanseri için risk faktörü oluşturduğunu göstermektedir.

Anahtar Kelimeler: N-Asetil Transferaz, Glutatyon S-Transferaz, Göğüs Kanseri

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AcCoA	Acetyl Coenzyme A
BSA	Bovine serum albumin
EDTA	Ethylenediaminetetraacetic acid
gDNA	Genomic DNA
GST	Glutathione S-transferase
NAT	N-acetyl transferase
RFLP	Restriction fragment length polymorphism
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA

CHAPTER 1

1. INTRODUCTION

1.1 Breast Cancer

Breast cancer is the major cause of cancer death in women worldwide with 75.000 new cases being diagnosed annually (Kumaraguruparan *et al.* 2005). The incidence of breast cancer is quite high in different countries and the rates are increasing, for instance it is the second leading cause of cancer death in USA, Korea (Kim *et al.* 2004) and India, among Australian women it accounts for 29% percent of new cancer cases in women each year (Curran *et al.* 2000). Especially in Asia the prevelance of the disease has increased approximately 3-fold in the past 2 decades (Huang *et al.* 1999, Park *et al.* 2000). In Turkey, the rate of breast cancer incidence was found as 12.7%, in a study conducted by Ege University (Haydaroglu *et al*. 2005).

High penetrance genes, account for only 5% of cases, whereas polymorphic low penetrance genes acting in concert with lifestyle/environmental risk factors are likely to account for a much higher proportion (Williams *et al.* 2000). Many risk factors have been investigated in breast cancer susceptibility however the exact etiology of the disease remains to be fully elucidated. Xenobiotic and endogenous carcinogens have been suggested to be potential factors in sporadic breast cancer development and genetic differences in the metabolism of such carcinogens may contribute to individual variation in cancer susceptibility (Matheson *et al.* 2002). Variant forms of these genes exist in population, many of which alter enzyme activity and may thus increase or decrease exposure of humans to ultimate carcinogens. Since many of these genetic variants are relatively common, interactions between carcinogen-metabolizing enzymes and common environmental factors may have a

marked impact on the population attributable risk of cancer (Chacko *et al.* 2005).

An increased risk of breast cancer due to prolonged exposure to estrogen has been well documented by epidemiological observations showing that estrogen-related risk factors, including age at menarche, age at menopause, parity and age at first full-term pregnancy, are significantly associated with breast cancer risk (Cheng *et al.* 2005, Kocabaş *et al.* 2003). Besides reproductive history and hormonal status, diet and exposure to different carcinogens may serve as a risk factor for the etiology of the disease. The potent carcinogens implicated in breast carcinogenesis are polycyclic aromatic hydrocarbons (PAHs), aromatic and heterocyclic amines present in the diet and occupational or other environmental exposures (Krajinovic *et al.* 2001). For instance, amines produced during the cooking of meat and arylamines found in tobacco smoke are detoxified by N-acetyl transferases (NAT), whereas benzo [α] pyrene and other PAHs found in tobacco smoke and halomethanes and methyl bromide found in pesticides are detoxified or activated by glutathione S-transferases (GST).

Aromatic and heterocyclic amines require metabolic activation to electrophilic intermediates that initiate carcinogenesis. NATs (1 and 2) are important enzymes in the biotransformation of these carcinogens and exhibit genetic polymorphism. The high frequency of the NAT1 and NAT2 acetylation polymorphisms in human populations together with ubiquitous exposure to aromatic and heterocyclic amines suggest that NAT1 and NAT2 acetylator genotypes are important modifiers of human cancer susceptibility (Hein *et al.* 2002).

1.2. N-Acetyl Transferases (NATs)

The arylamine N-acetyltransferases (NATs) are found in nearly all species from bacteria to humans. They are Phase II drug-metabolizing enzymes and catalyse the acetyltransfer from acetylcoenzyme A to an aromatic amine, heterocyclic amine or hydrazine compound (Butcher *et al.* 2002). This acetyl reaction can be seen in Figure 1. NAT was first identified as responsible for the polymorphic inactivation of the antitubercular drug isoniazid, which is acetylated by human NAT2, one of the two functional human NATs (Pompeo *et al.* 2002). Both NAT isoforms have distinct but overlapping substrate specificities and they are functionally polymorphic. The NATs play an important role in the metabolic cascade, catalyzing both the N-acetylation of arylamines (usually deactivation) and, following N-oxidation, the O-acetylation (usually activation) of their N-hydroxyarylamine metabolites (Fretland *et al.* 2002). Both NAT1 and NAT2 can perform N-acetylation and O-acetylation. These two enzymes are encoded by single open reading frames of 870 base pairs that exhibit genetic polymorphism in human populations.

A phylogenetic tree for the NAT proteins is shown in Figure 2. It indicates the separate clustering of the prokaryotic and eukaryotic sequences, with the exception of *A. mediterranei* NAT which is distant from both groups (Butcher *et al.* 2002). The NAT1 and NAT2 sequences for rat, mouse and hamster cluster together suggesting that the two proteins are encoded by genes that were present before the divergence of the three rodent species. On the other hand, the two human proteins are more closely related to each other, probably because of the gene duplication later in evolution.

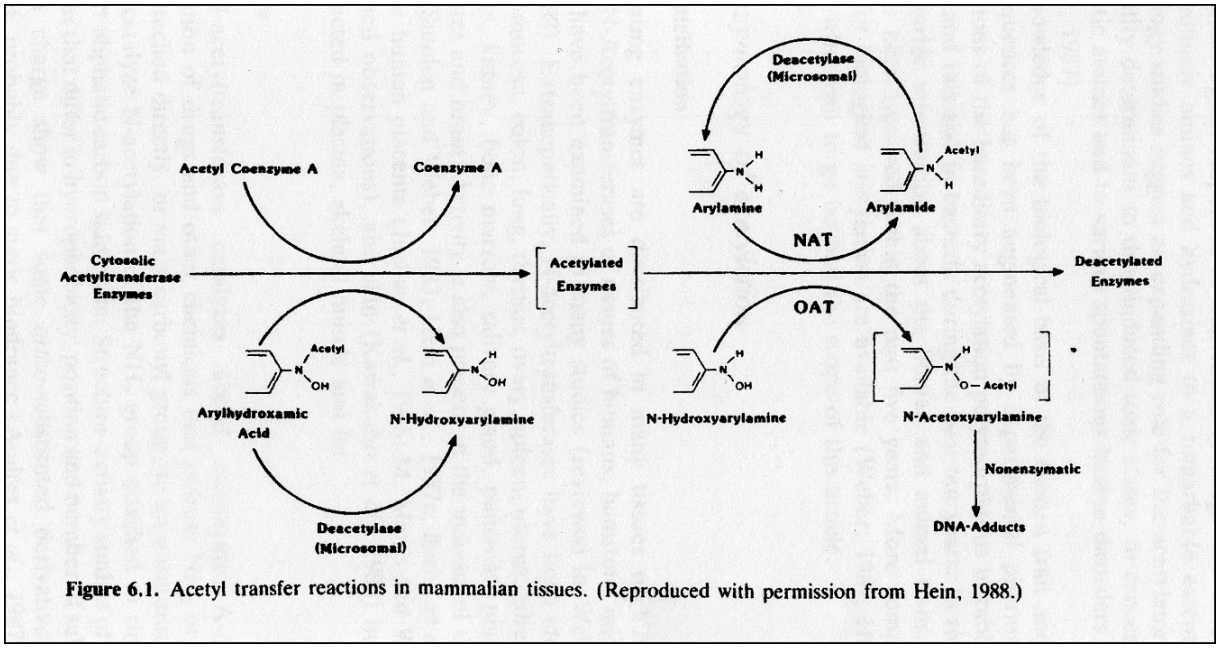


Figure 6.1. Acetyl transfer reactions in mammalian tissues. (Reproduced with permission from Hein, 1988.)

Figure 1. Acetyl transfer reactions in mammalian tissues

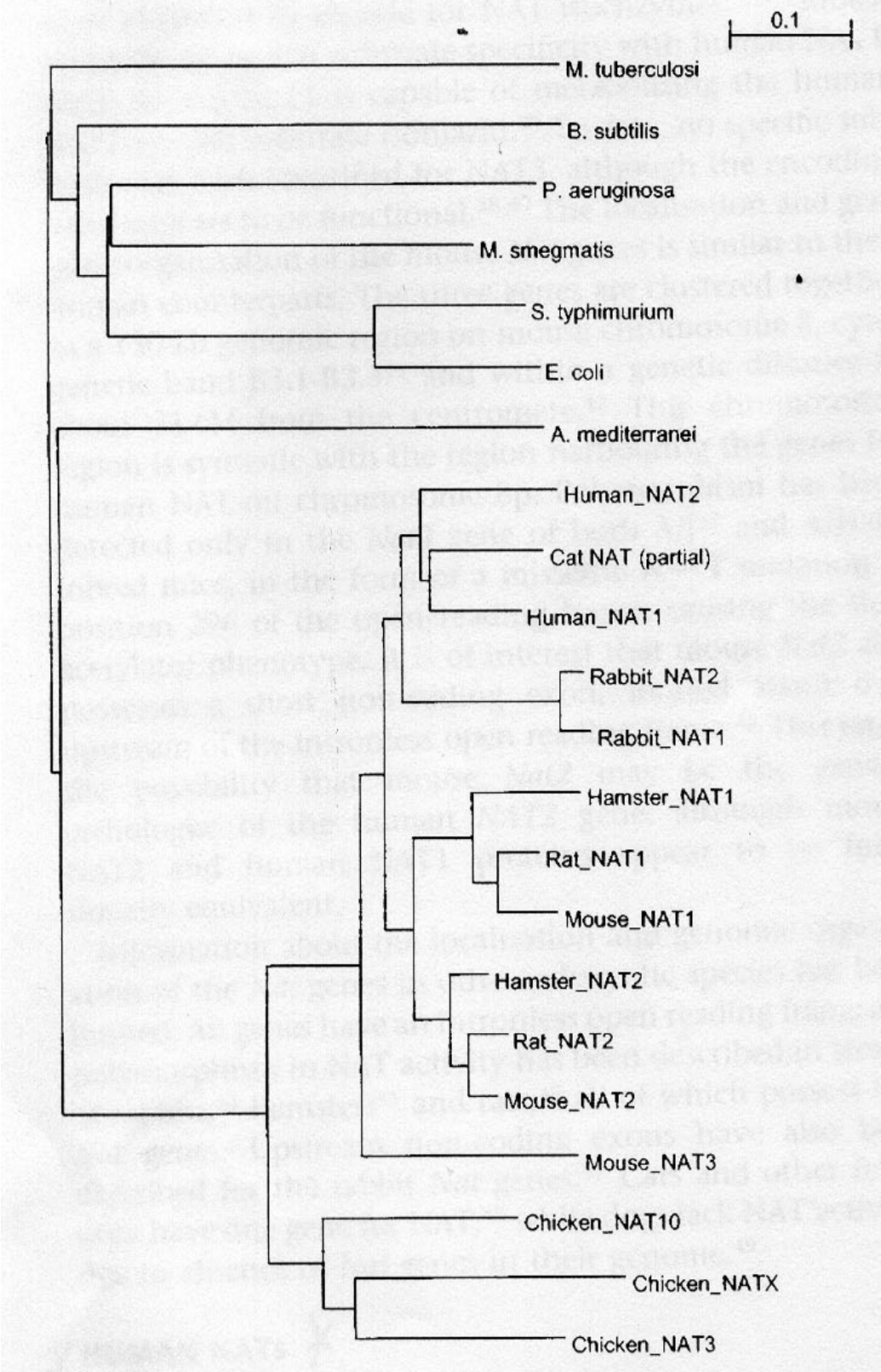


Figure 2. A phylogenetic tree for the NAT proteins

Since the first attempt to devise a consensus nomenclature for the NATs in 1995, many new alleles have been identified and thus the nomenclature was updated; the most recent information about NAT alleles can be found at **http://www.louisville.edu/medschool/pharmacology/NAT.html**.

Three NAT loci (PNAT, NAT1, NAT2) are located on human chromosome 8 at 8p22; PNAT is a pseudogene that contains premature stop codons and is not transcribed, whereas NAT1 and NAT2 both consist of an 870 base pair intronless open reading frame, encoding a functionally active 34 kDa protein (Pompeo *et al.* 2002). They share 87% nucleotide and 81% protein sequence identity but differ in tissue distribution, substrate specificity and expression levels during development. NAT1 activity is distributed to many extrahepatic tissues both during the early stages of development and during adulthood, whereas NAT2 activity is predominantly observed in the liver and intestinal epithelium. In the case of substrate specificity, NAT1 shows specificity for p-aminosalicylate and p-aminobenzoic acid, on the other hand NAT2 primarily metabolizes sulphamethazine, isoniazid and procainamide. Both enzymes take role in the metabolization of potent carcinogens; the role of NATs in predisposition to cancer depends on the type of cancer, the allelic variants and the level of exposure to carcinogens.

NATs have been identified in all mammals, including rabbit, mouse, rat, hamster and cat, but is lacking in the dog and other canids (Pompeo *et al.* 2002). Eukaryotic NAT sequences are highly homologous. Rodent NATs have 65.9-81.7% homology with human NATs and exhibit rapid and slow phenotypes, making them useful mammalian models for xenobiotic metabolism, drug disposition and toxicity studies (Vatsis *et al.* 1995)

1.2.1. N-acetyl Transferase 1 (NAT1)

Although once it was thought that NAT1 is genetically invariant, now it is well known that it has 26 different alleles and show considerable interethnic variability. NAT1*4 is the wild type allele and accepted as showing a moderate level of acetylation activity. According to the base substitution and subsequent amino acid change, the acetylation capacity may decrease, increase or not change. The allelic types of NAT1, their phenotypic properties, base substitutions and amino acid changes are summarized in Figure 3.

Allele	*Phenotype*	*Nucleotide change(s)*	*Amino acid change(s)*
*NAT1*4*	Normal	None	None
*NAT1*3*	Normal	$C^{1095}A$	None
*NAT1*5*	Normal	$G^{350,351}C$, $G^{497-499}C$, $A^{884}G$, Δ^{976}, Δ^{1105}	$Arg^{117} \rightarrow Thr$, $Arg^{166} \rightarrow Thr$, $Glu^{167} \rightarrow Gln$
*NAT1*10*	Rapid?	$T^{1088}A$, $C^{1095}A$	None
*NAT1*11A*	Normal	C^{-344T}, $A^{-40}T$, $G^{445}A$, $G^{459}A$, $T^{640}G$, $\Delta 9^{(1065-1090)}$, $C^{1095}A$	$Val^{149} \rightarrow Ile$, $Ser^{214} \rightarrow Ala$
*NAT1*11B*	Normal	C^{-344T}, $A^{-40}T$, $G^{445}A$, $G^{459}A$, $T^{640}G$, $\Delta 9^{(1065-1090)}$	$Val^{149} \rightarrow Ile$, $Ser^{214} \rightarrow Ala$
*NAT1*11C*	Normal	C^{-344T}, $A^{-40}T$, $G^{459}A$, $T^{640}G$, $T^{640}G$, $\Delta 9^{(1065-1090)}$	
*NAT1*14A*	Slow	$G^{560}A$, $T^{1088}A$, $C^{1095}A$	$Arg^{187} \rightarrow Gln$
*NAT1*14B*	Slow	$G^{560}A$	$Arg^{187} \rightarrow Gln$
*NAT1*15*	Slow	$C^{559}A$	$Arg^{187} \rightarrow Stop$
*NAT1*16*	Slow	AAA insertion after^{1091}, $C^{1095}A$	None
*NAT1*17*	Slow	$C^{190}T$	$Arg^{64} \rightarrow Trp$
*NAT1*18A*	Unknown	$\Delta 3^{(1064-1087)}$, $T^{1088}A$, $C^{1095}A$	None
*NAT1*18B*	Unknown	$\Delta 3^{(1064-1087)}$	None
*NAT1*19*	Slow	$C^{97}T$	$Arg^{33} \rightarrow Stop$
*NAT1*20*	Unknown	$T^{402}C$	None
*NAT1*21*	Rapid	$A^{613}G$	$Met^{205} \rightarrow Val$
*NAT1*22*	Slow	$A^{752}T$	$Asp^{251} \rightarrow Val$
*NAT1*23*	Unknown	$T^{777}C$	None
*NAT1*24*	Rapid	$G^{781}A$	$Glu^{261} \rightarrow Lys$
*NAT1*25*	Rapid	$A^{787}G$	$Ile^{263} \rightarrow Val$
*NAT1*26A*	Unknown	TAA insertion$^{(1066-1091)}$, $C^{1095}A$	None
*NAT1*26B*	Unknown	TAA insertion$^{(1066-1091)}$	None
*NAT1*27*	Unknown	$T^{21}G$, $T^{777}C$	None
*NAT1*28*	Unknown	TAATAA deletion$^{(1085-1090)}$	None
*NAT1*29*	Unknown	$T^{1088}A$, $C^{1095}A$, Δ^{1025}	None

Figure 3. NAT1 Polymorphism

One of the most studied allelic type of NAT1 is the NAT1*10. Generally it was associated with activity two-fold higher than that of the wild-type allele, NAT1*4. NAT1*10 has no mutations in the protein encoding region of the gene, but contains two nucleotide substitutions ($T^{1088}A$ and $C^{1095}A$) in its 3'-untranslated region. The $T^{1088}A$ base change alters the consensus polyadenylation signal (AA**T**AAA -> AA**A**AAA) leading to the suggestion that increased activity may be due to enhanced mRNA stability. However, several recent studies do not support the idea that the NAT1*10 allele is associated with elevated NAT1 activity. Therefore, the functional property of this allele remains unclear at present. Similarly, there is no aggrement about the relationship between the presence of this allele and incidence of different types of cancer. In some studies, the frequency of NAT1*10 allele was found to be higher in case groups than that of control groups (Zheng *et al.* 1999 for breast cancer, Fukutome *et al.* 1999 for prostate cancer, Boissy *et al.* 2000 for gastric cancer), whereas NAT1*10 was found to be associated with decreased risk of bladder cancer (Deguchi *et al.* 2005). On the other hand, some researchers reported that there were no significant relationship between the genotype and cancer incidence (Katoh *et al.* 2000, Zhang *et al.* 2005, Gu *et al.* 2005, Olshan *et al.* 2000).

Another investigated allelic form, NAT1*11 was shown that it do not possess a major difference in catalytic or other properties when compared to the wild type NAT1*4 protein. However, likewise to the case of NAT1*10, the results on this allele are inconsistent, some suggest that there is no significant relation between the frequency of NAT1*11 and cancer incidence (Fronhoffs *et al.* 2001 for squamous cell cancer, Cascorbi *et al.* 2001 for bladder cancer, Boissy *et al.* 2000 for gastric cancer), whereas others observed a positive correlation (Zheng *et al.* 1999 for breast cancer).

1.2.2. N-Acetyl Transferase 2 (NAT2)

In human populations, 29 different NAT2 alleles have been detected and each of these variant alleles is comprised of between one and four nucleotide substitutions (Figure 4). Thirteen base substitutions are located in the protein encoding region of the gene, nine of them lead to a change in the encoded amino acid, while the remaining four are silent. NAT2*4 is considered the wild-type allele because of its absence of any of these substitutions (Hein *et al.* 2000). Also, NAT2*4 is not the most common allele in many ethnic groups, including Caucasians and Africans. Hein *et al.* (1995) studied the acetylation capacity of 16 different NAT2 alleles in a bacterial expression system. The $T^{341}C$, $G^{590}A$, $G^{857}A$, and $G^{191}A$ substitutions being examined, produced recombinant NAT2 allozymes with reduced acetylation capacities, while the $C^{481}T$, $C^{282}T$, and $A^{803}G$ substitutions produced recombinant NAT2 allozymes with acetylation capacities similar to the wild-type NAT2*4 protein. Thus, NAT2*5, NAT2*6, NAT2*7, NAT2*14, and NAT2*17 clusters are associated with a slow acetylator phenotype.

The frequency of slow acetylator phenotypes varies considerably among ethnic groups. In Caucasian and African populations, it differs in a range between 40 and 70%, while that of Asian populations range from 10 to 30%. Besides, Caucasian and African populations possess high frequencies of NAT2*5 alleles and low frequencies of NAT2*7 alleles, whereas Asian populations exhibit an opposite distribution.

The first association between slow acetylator phenotype and urinary bladder cancer was reported 27 years ago (Lower *et al.*, 1979). Since then, several studies report a positive correlation between the incidence of cancer and these particular phenotype. In the case of colorectal cancer, a similar type of association is present with the rapid acetylator phenotype.

Several studies support the role of NAT2 acetylation polymorphism in genetic predisposition to colorectal cancer, suggesting that homozygous rapid acetylators exposed to high levels of heterocyclic amines through consumption of consistently well-done meat are at a higher risk.

In the case of breast cancer, the findings are quite inconsistent. While some studies found a positive correlation with the rapid acetylator phenotype, some suggested a positive correlation with the slow acetylator phenotype, still some found no significant relationship.

Allele	*Phenotype*	*Nucleotide change(s)*	*Amino acid change(s)*
*NAT2*4*	Rapid	None	None
*NAT2*5A*	Slow	$T^{341}C$, $C^{481}T$	$Ile^{114} \rightarrow Thr$
*NAT2*5B*	Slow	$T^{341}C$, $C^{481}T$, $A^{803}G$	$Ile^{114} \rightarrow Thr$, $Lys^{268} \rightarrow Arg$
*NAT2*5C*	Slow	$T^{341}C$, $A^{803}G$	$Ile^{114} \rightarrow Thr$, $Lys^{268} \rightarrow Arg$
*NAT2*5D*	Slow	$T^{341}C$	$Ile^{114} \rightarrow Thr$
*NAT2*5E*	Slow	$T^{341}C$, $G^{590}A$	$Ile^{114} \rightarrow Thr$, $Arg^{197} \rightarrow Gln$
*NAT2*5F*	Slow	$T^{341}C$, $C^{481}T$, $T^{759}T$, $A^{803}G$	$Ile^{114} \rightarrow Thr$, $Lys^{268} \rightarrow Arg$
*NAT2*6A*	Slow	$C^{282}T$, $G^{590}A$	$Arg^{197} \rightarrow Gln$
*NAT2*6B*	Slow	$G^{590}A$	$Arg^{197} \rightarrow Gln$
*NAT2*6C*	Slow	$C^{282}T$, $G^{590}A$, $A^{803}G$	$Arg^{197} \rightarrow Gln$, $Lys^{268} \rightarrow Arg$
*NAT2*6D*	Slow	$T^{111}C$, $C^{282}T$, $G^{590}A$	$Arg^{197} \rightarrow Gln$
*NAT2*7A*	Slow	$G^{857}A$	$Lys^{286} \rightarrow Glu$
*NAT2*7B*	Slow	$C^{282}T$, $G^{857}A$	$Lys^{286} \rightarrow Glu$
*NAT2*10*	Unknown	$G^{499}A$	$Glu^{167} \rightarrow Lys$
*NAT2*11*	Unknown	$C^{481}T$	None
*NAT2*12A*	Rapid	$A^{803}G$	$Lys^{268} \rightarrow Arg$
*NAT2*12B*	Rapid	$C^{282}T$, $A^{803}G$	$Lys^{268} \rightarrow Arg$
*NAT2*12C*	Rapid	$C^{481}T$, $A^{803}G$	$Lys^{268} \rightarrow Arg$
*NAT2*13*	Rapid	$C^{282}T$	None
*NAT2*14A*	Slow	$G^{191}A$	$Arg^{64} \rightarrow Gln$
*NAT2*14B*	Slow	$G^{191}A$, $C^{282}T$	$Arg^{64} \rightarrow Gln$
*NAT2*14C*	Slow	$G^{191}A$, $T^{341}C$, $C^{481}T$, $A^{803}G$	$Arg^{64} \rightarrow Gln$, $Ile^{114} \rightarrow Thr$, $Lys^{268} \rightarrow Arg$
*NAT2*14D*	Slow	$G^{191}A$, $C^{282}T$, $G^{590}A$	$Arg^{64} \rightarrow Gln$, $Arg^{197} \rightarrow Gln$
*NAT2*14E*	Slow	$G^{191}A$, $A^{803}G$	$Arg^{64} \rightarrow Gln$, $Lys^{268} \rightarrow Arg$
*NAT2*14F*	Slow	$G^{191}A$, $T^{341}C$, $A^{803}G$	$Arg^{64} \rightarrow Gln$, $Arg^{114} \rightarrow Thr$, $Lys^{268} \rightarrow Arg$
*NAT2*14G*	Slow	$G^{191}A$, $C^{282}T$, $A^{803}G$	$Arg^{64} \rightarrow Gln$, $Lys^{268} \rightarrow Arg$
*NAT2*17*	Slow	$A^{434}C$	$Gln^{145} \rightarrow Pro$
*NAT2*18*	Unknown	$A^{845}C$	$Lys^{282} \rightarrow Thr$
*NAT2*19*	Slow	$C^{190}T$	$Arg^{64} \rightarrow Trp$

Figure 4. NAT2 Polymorphism

1.3.Glutathione S-Transferases (GSTs)

The glutathione S-transferases (GSTs) are a family of enzymes that are important in the metabolism of a wide variety of xenobiotics, including environmental carcinogens, reactive oxygen species, and chemotherapeutic agents (Engel *et al.* 2002). They act as phase II metabolizing enzymes and catalyze GSH-dependent detoxification of electrophilic compounds. There are two distinct GST families: First, soluble GSTs, which are further grouped into 8 classes, α, κ, μ, π, σ, θ, ζ, Ω. Second, microsomal GSTs, which are structurally unrelated to soluble forms. Polymorphism has been described in many genes in these families, however most attention has focussed on allelism in the mu, theta and pi families (Strange *et al.* 2001). GSTs have been found to be expressed in most organs, however, their levels of expression vary widely between organs and between individuals (Coles *et al.* 2003).

1.3.1 GSTM1

Five mu class genes are situated in tandem in a 20 kb cluster on chromosome 1p13.3. The GSTM1 gene codes for the cytosolic enzyme GST-μ. This enzyme has received considerable attention about smoking-related cancers because of its role in the detoxification of benzo[α]pyrene and other PAHs found in tobacco smoke (Engel *et al.* 2002). Three polymorphisms of this gene have been identified. The first one is a deletion that results in a lack of functional gene product (GSTM1-0). The other two (GSTM1*a* and GSTM1*b*) differ by a C->G substitution at base position 534, resulting in a Lys->Asn substitution at amino acid 172. Because there is no evidence of functional difference between GSTM1*a* and GSTM1*b,* the two are typically categorized together as a single functional phenotype. Persons with homozygous deletion of GSTM1-null show no enzymatic activity. The frequency of null allele ranges from 23 to

41% for Africans, from 32 to 53% for Asians, from 40 to 53% for Hispanics and from 35 to 62% for Europeans (Geisler *et al.* 2001).

For the case of cancer incidence, different studies show inconsistent results, with some reporting weak-to-moderate associations and others finding no elevation in risk for the disease. GSTM1 is especially investigated for the tobacco-related cancer types. Stewart *et al.* found that the risk for lung cancer associated with GSTM1 null increased two to six-fold among heavy smokers. De Jung *et al.* (2002) suggested that GSTM1 revealed no association with colorectal adenoma or cancer. Similar inconsistent results are also prominent among studies on breast cancer. Sull *et al.* (2004) showed a slightly higher risk for women with GSTM1 deficiency, whereas Park *et al.* (2000) found that the GSM1 null genotype is significantly associated with breast cancer risk especially in premenopausal women. In a study of Curran *et al.* (2000) it is revealed that genotypes of GSTM1, GSTT1 and GSTP1 alone are not involved in breast cancer development.

1.3.2. GSTT1

The two theta genes, GSTT1 and GSTT2, are located on chromosome 22 seperated by about 50 kb (Strange *et al.* 2001). The GSTT1 locus has been mapped on chromosome 22q11.2. Two alleles have been identified at the GSTT1 locus – one functional and the other nonfunctional. Some tobacco carcinogens, such as epoxybutanes and ethylene oxide, are known substrates for GSTT1. GSTT1, like GSTM1, is known to play a role in phase II detoxification of carcinogens found in tobacco smoke as well as of other carcinogens found in pesticides, such as halomethanes and methyl bromide. Unlike the GSTM1 enzyme, however, GSTT1 has both detoxification and activation roles. For example, GSTT1 is known to

activate dihalomethanes to dichloromethane, which has been shown to cause liver and lung tumors in mice (Strange *et al.* 2001).

Unlike any other member of the GST family, GSTT1 is expressed not only in the adult liver but also in human erythrocytes and, as a result, is believed to play a more global role than GSTM1 in detoxification of carcinogens in the body (Ada AO *et al.* 2004). The frequency of nonfunctional allele ranges from 15 to 31% for Europeans, from 22 to 29% for Africans, from 10 to 12% for Hispanics. Asians have the highest reported GSTT1 deletion genotype, such as 58% for Chinese and 42-46% for Koreans (Geisler *et al.* 2001)

Matheson *et al.* (2002) studied the relation between GSTT1 null genotype and risk of premenopausal breast cancer and suggested that it may play a role in the early onset of the disease. Another study showed that among the GST genotypes the highest breast cancer risk was observed with the combination of both GSTM1 and GSTT1 null genotypes (Chacko *et al.* 2005). Again, Park *et al.* (2000) observed an especially remerkable risk of breast cancer for alcohol consuming premenopausal women lacking both the GSTM1 and GSTT1 genes.

1.4 Scope of the study

In this study, we planned to reveal the relationship between breast cancer risk and the presence of different allelic types of NAT1, NAT2, GSTM1 and GSTT1. All these Phase II enzymes play a role in the detoxification step of variable chemicals. Epidemiological studies have suggested that the environment play a significant role in the development of breast cancer. Besides, the characteristic composition of breast tissue is mostly fat and a high percentage of potent carcinogens have the ability to dissolve in lipids.

This property of breast tissue makes it an appropriate site for the storage of such compounds. Thus, activity level and acetylation capacity of these enzymes may determine the probability of the onset of a probable breast carcinoma.

By investigating all 4 enzymes in the same scope, we will be able to determine the correlation between these genes and the incidence of carcinoma in breast tissue. Up to now, evidence from certain studies revealed the presence of positive correlations, however results of other studies did not confirm such an association. These contradictions may be the result of limited data and lack of sample power in most of the studies.

Under the scope of this study, we revealed different allelic types of NAT1 and NAT2 and detect some null alleles of GSTM1 and GSTT1. Thirty seven patients with breast cancer and 34 healthy controls were screened by using Restriction fragment length polymorphism (RFLP). In addition, by the aid of a questionnaire we collected data about family history, dietary habits and reproductive and hormonal status of women being enrolled in this study.

The aim of this study is to determine whether there is an association between NAT1, NAT2, GSTM1 and GSTT1 polymorphisms and breast cancer risk and to show the possible role of environmental carcinogens and individual susceptibility to breast cancer in Turkish women.

CHAPTER 2

2. MATERIALS AND METHODS

2.1. Materials

Disodium salt (EDTA), Tris, Sodium chloride, Potassium chloride, Magnesium chloride, Sodium dodecyl sulphate (SDS), Ethanol, Ethidium bromide, Agarose, High resolution agarose, Boric acid were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

Bovine serum albumin(BSA), Restriction enzymes: TaqI, MboII, DdeI, BamHI, KpnI, HincII, BsiEI, AlwnI were obtained from Promega, Madison WI, U.S.A.

Primers, dNTPs, 10X PCR Buffer, Taq Polymerase, DNA Ladder, Loading dye were purchased from Fermentas Int. Inc., Burlington, Ontario, Canada.

Other chemicals used during research were of analytical grade and were obtained from other companies.

2.2. Methods

2.2.1. DNA Isolation from whole blood

Blood samples (5 ml) from patients with breast cancer (n=37) were obtained from Erciyes Medical School, Kayseri, whereas blood samples (5 ml) from healthy controls (n=34) were taken from people who applied to Elmadağ Hospital, Ankara. They were placed in EDTA containing tubes

(100 µl, 15% EDTA) and transferred to our laboratory in dry ice. Blood samples were stored at -80^{0}C until DNA isolation procedure was performed. In addition, by the aid of a questionnaire we collected data about family history, dietary habbits and reproductive and hormonal status of the women being enrolled in this study.

Genomic DNA is isolated from whole blood according to the method of Lahiri *et al.* (1993).

One ml blood was placed into a 15 ml falcon tube and 1 ml low salt buffer (10 mM Tris-HCl, pH 7.6 + 10 mM KCl + 2 mM EDTA + 4mM $MgCl_2$) was added. After the addition of 25 µl Triton-X-100, the tube was inverted several times. Then, samples were centrifuged at 1000xg for 10 min at 20^{0}C. The pellet (composed mostly of leukocytes) was saved and washed two more times with TKM buffer (10 mM Tris-HCl, pH 7.6 + 10 mM KCl + 4mM $MgCl_2$). Final pellet was resuspended in 0.2 ml TKM buffer and the suspension was transferred into a 1.5 ml eppendorf tube. Afterwards 20 µl of 10% SDS was added and the suspension was mixed thoroughly. Then, samples were incubated at 58^{0}C for 10 min. After the incubation step 75 µl saturated NaCl (~6M) was added and the sample was mixed well. Then the samples were centrifuged at 12000xg for 7 min at 20^{0}C and the supernatant was saved. By using ethanol, DNA was precipitated and it was transferred into an appendorf tube containing 0.2 ml TE buffer, pH 8.0. In order to sustain complete dissolvation of DNA molecule, the sample was incubated at 37^{0}C for 1.5-2 hrs. Afterwards, to determine DNA concentration in the sample and the purity of DNA, spectrometric measurement was carried out. Twenty µl from DNA sample was taken and added to 980 µl of TE. After mixing the solution, the absorbances at 260nm and 280 nm was read. The concentration of DNA in solution was determined by measuring the absorbance of the solution diluted in TE in a quartz cuvette at 260 nm (A_{260}) where for a path length of 1 cm:

A_{260}= 1⇒ 50µg/ml double stranded DNA

The purity of DNA sample was determined by looking at the ratio of OD260/OD280. Pure DNA solutions exhibit a ratio of 1.8. A value higher than 1.8 indicates a case of RNA contamination, on the other hand a value lower than 1.8 indicates a protein contamination. After completing this measurement step, all DNA samples were stored at -20^{0}C.

2.2.1. Multiplex PCR procedure to identify GSTM1 and GSTT1 +/- alleles

A multiplex PCR method was used according to the method of Abdel-Rahman *et al.* (1996) in order to detect the presence or absence of the GSTM1 and GSTT1 genes. The amplification was performed in sterile 0.5µl eppendorf tubes using approximately 1µg gDNA as template, 50 pmoles of CYP1A1 and GSTM1 and 75 pmole of GSTT1 primers, 5.0 mM dNTPs, 5 µl of 10X Thermophilic DNA Polymerase Buffer (10 mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton®X-100), 1.5 mM $MgCl_2$ and 2 U of Taq Polymerase. The reaction was made up to a total volume of 50 µl with sterile water. A negative control reaction mixture, without template DNA, was included for each PCR. reference

The PCR conditions consisted of an initial melting temperature of 95ºC for 5 minutes followed by 35 cycles of melting at 94ºC for 30 seconds, annealing at 59ºC for 45 seconds and extension at 72ºC for 90 seconds. A final extension step (72ºC) of 10 minutes terminates the process. The amplified PCR products were stored at 4ºC until analysis.

Specific primers were used to identify +/- polymorphism of GSTM1 and GSTT1 genes, and as an internal control exon 7 of the CYP1A1 gene was co-amplified in this reaction (Table 1).

Table 1. Primers used to amplify the GSTM1 and GSTT1 genes

Primers	**Sequence (5' to 3')**
GSTM1 (S)	GAA CTC CCT GAA AAG CTA AAG C
GSTM1 (AS)	GTT GGG CTC AAA TAT ACG GTG G
GSTT1 (S)	TTC CTT ACT GGT CCT CAC ATC TC
GSTT1 (AS)	TCA CCG GAT CAT GGC CAG CA
CYP1A1 (S)	GAA CTG CCA CTT CAG CTG TCT
CYP1A1 (AS)	CAG CTG CAT TTG GAA GTG CTC

Three different PCR products – 480 bp of GSTT1, 312 bp of CYP1A1, 215 bp of GSTM1 – were run on 2% agarose/0.5XTBE gel. 15 µl of each sample were loaded into wells and 90 volts, 75 milliampere were applied for 60 minutes.

2.2.2. Determination of NAT2 genotype polymorphism

Genotype analysis of the NAT2 polymorphism was performed on patients and controls using the PCR and RFLP methods. By using the genotyping method of Hickman and Sim, (1991) and Hickman *et al.* (1992) DNAs isolated from blood samples were screened for NAT2* alleles, NAT2*4 (wildtype), NAT2*5A, NAT2*5B, NAT2*5C, NAT2*6A and NAT2*7B. This

method has been shown to be accurate at predicting NAT2 phenotype in greater than 95% of individuals in a Caucasian population (Hickman *et al.* 1992). The mutant alleles differ from wildtype by up to three point mutations, which are accompanied by alterations in restriction enzyme digest polymorhisms.

Following PCR amplification of the NAT2* coding region with primers NatHu7 and NatHu8, products were digested with restriction enzymes Kpn I and Taq I. Following PCR amplification of the NAT2* coding region with primers NatHu14 and NatHu16, products were digested with restriction enzymes Bam HI and Dde I. The combination of restriction fragment patterns with the different enzymes allows determination of the alleles present in a sample (Figure 5). In order to verify that PCR amplification was specific for the NAT2* allele, the product of amplification with primers NatHu14 and NatHu16 was digested with restriction enzymes HindII and Hind III. The NAT2* coding region contains a Hind II site, but no Hind III site, whereas the NAT1 coding region contains a Hind III site, but no Hind II site (Kelly and Sim 1991, Hickman *et al.*1992).

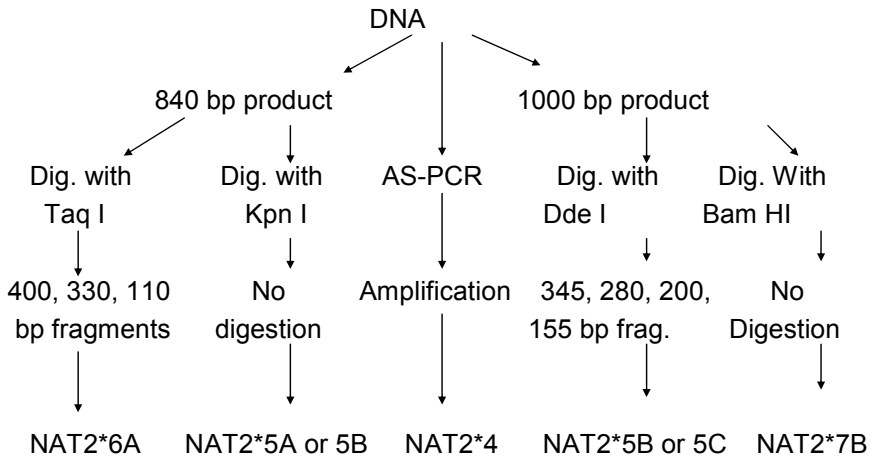


Figure 5. Allele determination of NAT2

However, the method described above is unable to distinguish between the combination of NAT2*4, 5B alleles, NAT2*5A, 5C. In order to distinguish between these combinations of alleles, an allelic specific PCR is found to be necessary (Hickman *et al.* 1992). The NAT2*5A, 5B and 5 C alleles contain a cytosine to thymine substitution at nucleotide 341 of their coding region. Genomic DNA from samples for which this ambiguity occurred were amplified with the PCR primers DH1 and NatHu16 (Figure 6).

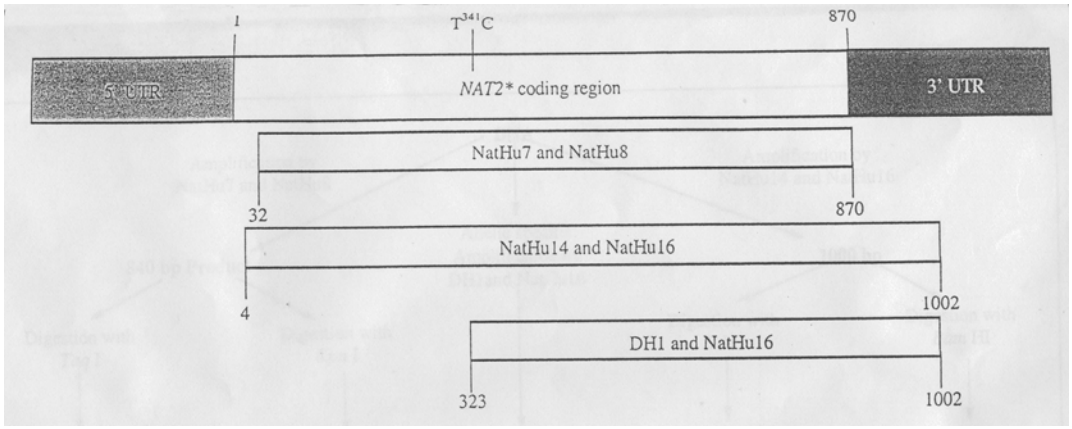


Figure 6. PCR Primers used in the amplification of the NAT2* gene to determine NAT2* genotype

Primer DH1 is specific for the wild type NAT2*4 allele and therefore any amplification occurring in these samples indicated that the sample contained a NAT2*4 allele and hence the NAT2*4, 5B combination of alleles. All primers used to amplify NAT2 gene can be seen in Table 2.

Table 2. Primers used to amplify the NAT2* gene for determination of NAT*genotype.

Primer	Sequence (5' to 3')[a]	S/AS[b]	Tm
NatHu7	$_{nt32}$GGC TAT AAG AAC TCT AGG AAC$_{nt52}$	S	58°C
NatHu8	$_{nt870}$AAT AGT AAG GGA TCC ATC ACC$_{nt850}$	AS	58°C
NatHu14	$_{nt4}$GAC ATT GAA GCA TAT TTT GAA AG$_{nt26}$	S	56°C
NatHu16	$_{nt1002}$ GAT GAA AGT ATT TGA TGT TTA GG $_{nt980}$	AS	56°C
DH1	$_{nt\ 323}$ TTC TCC TGC AGG TGA CCA $_{nt341}$	S	56°C

[a] The subscript numbers give the nucleotide of the NAT1* gene to which the primers anneal, where the adenine of the AUG start codon is labelled 1. [b] S = Sense, AS = antisense

[c] Nucleotide substitutions, necessary to create restriction enzyme digest sites, are underlined.

2.2.3. Determination of NAT1 genotype polymorphism

As seen from Figure 7, the NAT1 genotyping was detected according to the methods of Payton & Sim (1998) and Smelt (1998, 2000). Genomic DNAs isolated from blood samples of controls and patients were screened for the NAT1* alleles, NAT1*4 (wildtype), NAT1*3, NAT1*10, NAT1*11, NAT1*14A, NAT1*14B and NAT1*27. At the beginning of the procedure, genomic DNA from samples were first amplified using the primers N769 and N1113 (Figure 8).

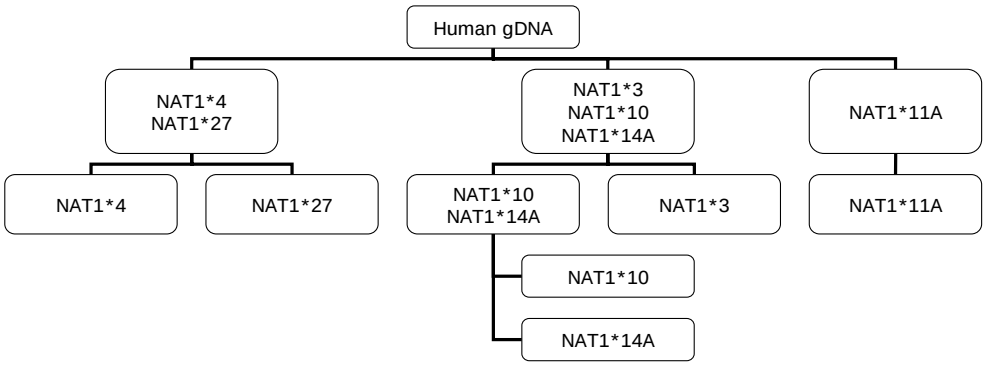


Figure 7. Stepwise genotyping procedure used in identification of the human NAT1* alleles

All primers used for the amplification of NAT1 gene can be seen in Table 3 and Figure 8.

Table 3. Primers used for the amplification of the NAT1* gene

Primer	Sequence (5' to 3')[a]	S/AS[b]	Tm
N-376	$_{nt-376}$ TAT TGC ATG ATT CTC CTG CCT A $_{nt\ -355}$	S	58°C
N1177	$_{nt1177}$ GGA ATT CAA CAA TAA ACC AAC AT $_{nt\ 1155}$	AS	58°C
N769	$_{nt769}$ ACT CGT AGT GAG GTA GAA ATA $_{nt\ 789}$	S	50°C
N1113[c]	$_{nt\ 1113}$ ACA GGC CAT CTT TAG AA $_{nt\ 1096}$	AS	50°C
N1110a	$_{nt\ 1110}$ GGC CAT CTT TAA AAT ACA TTT A $_{nt\ 1088}$	AS	54°C
N1110b	$_{nt\ 1110}$ GGC CAT CTT TAA AAT ACA TTT T $_{nt\ 1088}$	AS	54°C
N539[c]	$_{nt\ 539}$ TCC TAG AAG ACA GCA ACG ACC $_{nt\ 559}$	S	52°C
N714	$_{nt\ 714}$ GTG AAG CCC ACC AAA CAG $_{nt\ 697}$	AS	52°C

[a]The subscript numbers give the nucleotide of the NAT1* gene to which the primers anneal, where the adenine of the AUG start codon is labelled 1. [b] S = Sense, AS = antisense

[c] Nucleotide substitutions, necessary to create restriction enzyme digest sites, are underlined.

After the digestion of this initial product into a length of 344 bp (335 bp for NAT1*11A) by the aid of restriction enzyme MboII, the analysis of the resulting DNA fragments allowed the determination of the allelic type of NAT1.

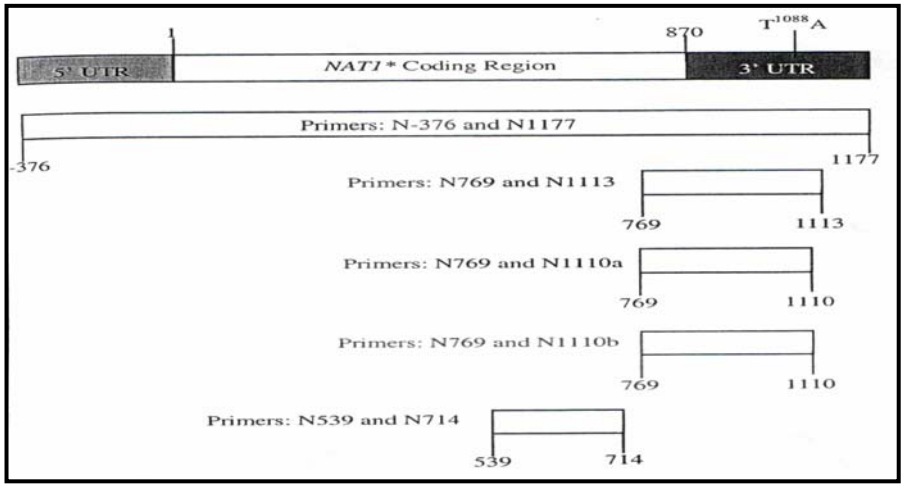


Figure 8. PCR Primers used in the amplification of the NAT1* gene to determine NAT1* genotype

According to the method, we can divide the whole procedure into 5 reaction types.

Reaction 1: A 344 bp (335 bp for NAT1*11A) PCR product was amplified as described from gDNA samples using primers N769 and N1113, 2 mM Mg Cl_2. The annealing temperature of the reaction was 50ºC, and product extension was for 45 seconds at 72ºC. Thirty five cycles were performed with a final extension at 72ºC for 5 minutes. The PCR product was digested with MboII, and analysis of the resulting restriction fragments on a 4 % High resolution agarose/0.5X TBE gel identified the possible alleles present (Table 4).

Table 4: Restriction fragments generated by MboII digestion

NAT1* Allele	MboII Restriction Fragments (bp)
NAT1*3	176, 144, 24
NAT1*4	176, 125, 24,19
NAT1*10	176, 144, 24
NAT1*11A	176, 135, 24
NAT1*14A	176, 144, 24
NAT1*27	176, 125, 24,19

Reaction 2: Two Allele Spesific-Polymerization Chain Reaction (AS-PCR) amplifications were performed as described from gDNA samples using two sets of primer pairs N769/ N1110a, and N769/ N1110b, 2.5 mM $MgCl_2$. The annealing temperature of both reactions was 54°C, and product extension was for 45 seconds at 72°C. Thirty cycles were performed with a final extension at 72°C for 5 minutes. Primer N1110a, which contains an adenine at position 1088 was designed to anneal to and amplify a 341 bp product from NAT1*3, NAT1*4, NAT1*11A and NAT1*27 allele sequences (which contain a thymine at nt 1088), but not from NAT1*10 or NAT1*14A (which contain an adenine at 1088). Primer N1110b, which contains a

thymine at position 1088 was designed to anneal to and amplify a 341 bp product from NAT1*10 and NAT1*14A allele sequences only. The PCR reactions were run on a 2% agarose/0.5X TBE gel (Table 5).

Table 5. NAT1* Alleles amplified with N769/ N1110a, and N769/ N1110b

NAT1* Allele	Product with N1110b (bp)	Product with N1110b (bp)
NAT1*3	341	-
NAT1*4	341	-
NAT1*10	-	341
NAT1*11A	341	-
NAT1*14A	-	341
NAT1*27	341	-

Reaction 3: A 175 bp was amplified as described from gDNA samples using primers N539 and N713, 2 mM $MgCl_2$. The annealing temperature of the reaction was 52°C, and product extension was for 30 seconds at 72°C. Thirty five cycles were performed with a final extension at 72°C for 5 minutes. The PCR product was digested with BsaO I, and analysis of the resulting restriction fragments on a 4% High resolution agarose/0.5X TBE gel identified the possible alleles present (Table 6).

Table 6. NAT1* alleles amplified with N539 and N713

NAT1* Allele	BsaO I Restriction Fragments (bp)
NAT1*3	155, 20
NAT1*4	155, 20
NAT1*10	155, 20
NAT1*11A	155, 20
NAT1*14A	175
NAT1*27	155, 20

The presence of mismatched cytosine and guanine at positions 555 and 556 in the N539 primer, generates a BsaO I restriction site in the amplified NAT1*3, NAT1*4, NAT1*10*, NAT1*11A and NAT1*27 allele sequences. NAT1*14A contains a guanine to adenine mutation at 560, therefore no BsaO I site is generated.

Reaction 4: A 1553 bp (1544 bp for NAT1*11A) was amplified as described from gDNA samples using primers N-376 and N 1176, 2 mM $MgCl_2$. The annealing temperature of the reaction was 52°C, and product extension was for 2 minutes at 72°C. Thirty five cycles were performed with a final extension at 72°C for 10 minutes. The PCR product was digested with AlwNI and analysis of the resulting restriction fragments on a 2% agarose/0.5X TBE gel identified the possible alleles present and provided confirmation of the NAT1*11A allele (Table 7).

Table 7. NAT1* alleles amplified with N-376 and N 1176

NAT1* Allele	AlwN I Restriction Fragments (bp)
NAT1*3	777, 776
NAT1*4	777, 776
NAT1*10	777, 776
NAT1*11A	777, 528, 239
NAT1*14A	777, 776
NAT1*27	777, 776

Reaction 5: An AS-PCR amplification was performed as described from gDNA samples using primers pairs N2 and N850, 2.5 mM $MgCl_2$. The annealing temperature of both reactions was 58°C, and product extension was for 1 minute at 72°C. Thirty five cycles were performed with a final extension at 72°C for 5 minutes. Primer N2, which contains guanine at position 21 was designed to anneal to and amplify a product from the NAT1*27 allele sequence (which contain a guanine at nt 21), but not from NAT1*3, NAT1*4, NAT1*10*, NAT1*11A or NAT1*14A (which contain a thymine at nt 21). The PCR reactions were run on a 2% agarose/0.5X TBE gel (Table 8).

Table 8. NAT1* Alleles amplified with N2 and N850

NAT1* Allele	Product with N2 (bp)
NAT1*3	-
NAT1*4	-
NAT1*10	-
NAT1*11A	-
NAT1*14A	-
NAT1*27	849

All genotyping results were interpreted by 2 independent investigators. The samples with obscure results were reexamined for quality control of the laboratory work.

2.2.5. Statistical methods

The results were expressed as the mean ± standard error (SE). Differences between the means were compared with the Student-t test, whereas differences between the medians were compared with the Mann-Whitney U test. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by binomial logistic regression. P-value of 0.05 was taken to denote significance.

NAT1 genotypes were classified into NAT1*10/any and others. Preliminary evidences suggest that the presence of one or more copies of the NAT1*10 allele is associated with increased acetylation activity, although the relationship between genotype and phenotype has not been firmly established. Individuals possessing one or two NAT2 alleles associated with high acetylation activity (NAT2*4) were classified as rapid acetylators and individuals possessing none of this allele were identified as slow acetylators.

CHAPTER 3

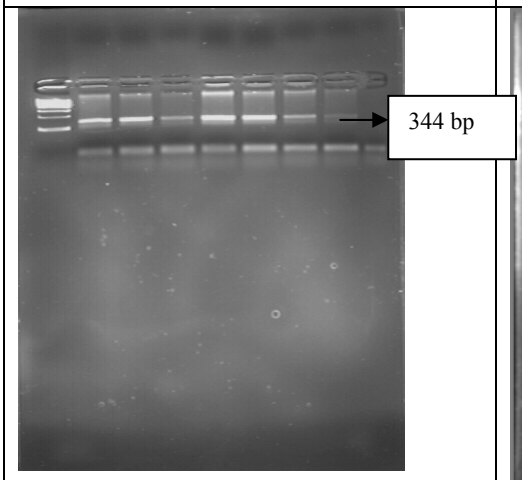
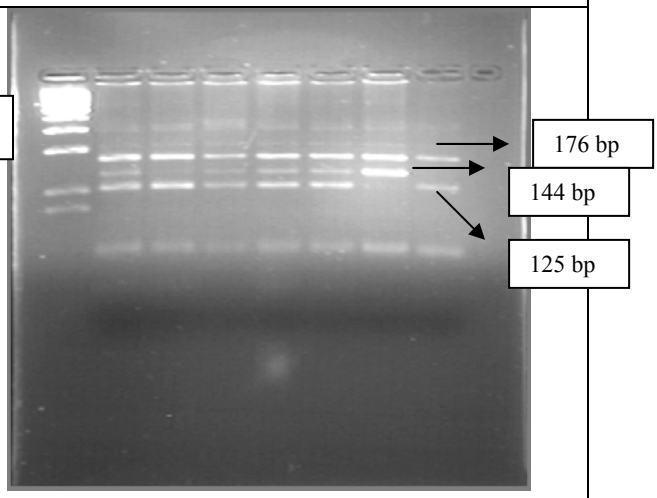
3. RESULTS AND DISCUSSION

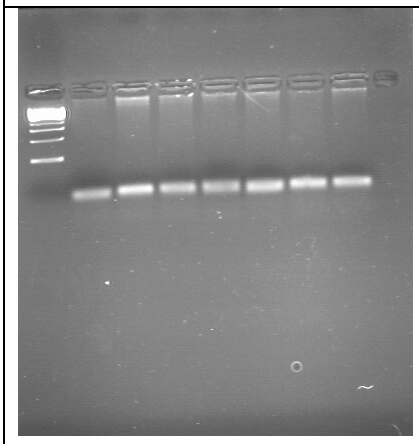
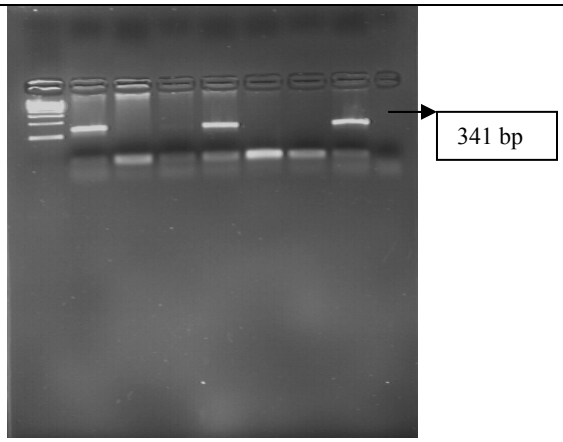
Carcinogenic heterocyclic amines are activated by N-acetyltransferase enzymes, encoded by NAT1 and NAT2 genes, to genotoxic compounds that can be accumulated in the breast pad. Genetic polymorphisms of GSTM1 and GSTT1 have also been investigated as risk modifiers in various types of cancer. We have examined the relation of polynorphisms in the genes coding for both NAT and GST enzymes to risk of breast cancer and the gene-environment interactions like dietary habbits.

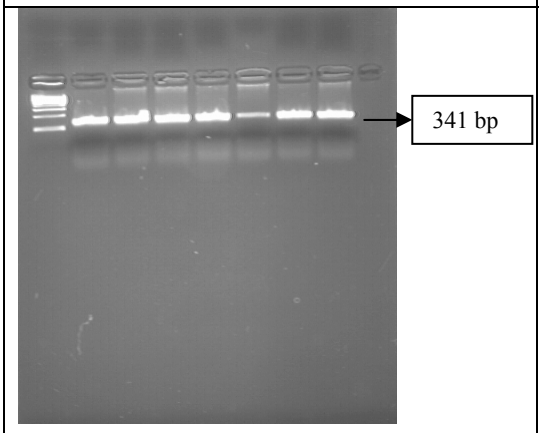
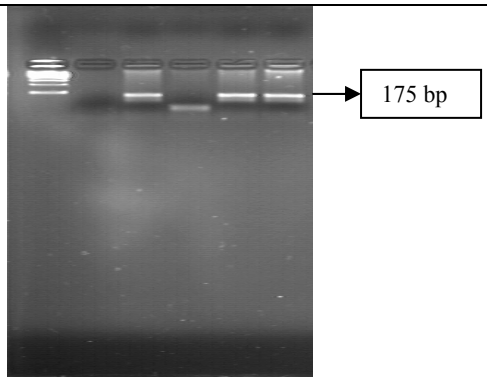
Thirty seven patients diagnosed with breast cancer and 34 healthy controls were genotyped by a Polymerization Chain Reaction (PCR)-restriction fragment length polymorphism (RFLP) method in order to determine their NAT1, NAT2 and GST genotypes. In addition, by the aid of a questionnaire we collected data about family history, dietary habbits and reproductive and hormonal status of the women being enrolled in this study. Blood samples from patients with breast cancer (n=37) were obtained from Erciyes Medical School, Kayseri, whereas blood samples from healthy controls (n=34) were taken from people who applied to Elmadağ Hospital, Ankara, for some simple reasons but were found to be in good health, not taking any medication and not having any relatives diagnosed by breast or other types of cancer.

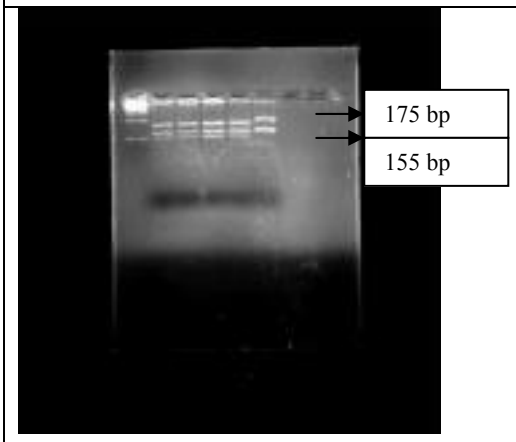
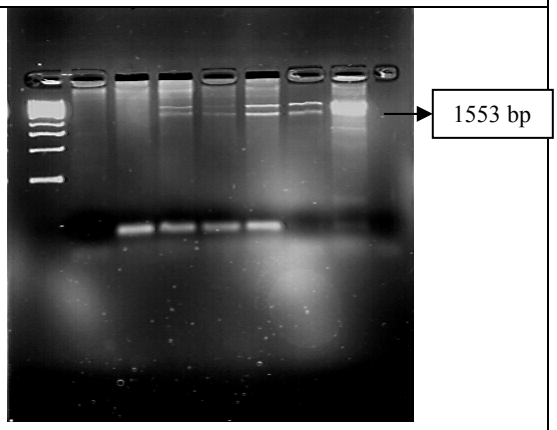
Genotype analysis of the NAT1, NAT2, GSTM1 and GSTT1 polymorphisms were performed on patients and controls using the PCR and RFLP methods. Some examples of PCR results with different primers and restriction enzyme fragments are given in Table 9.

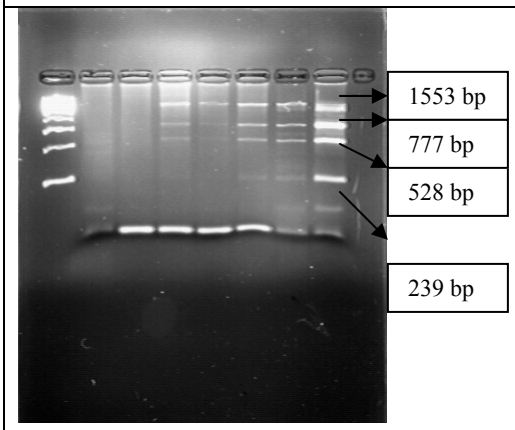
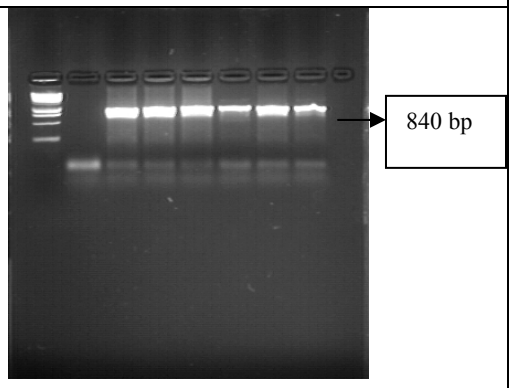
Table 9. Gel photos showing different reactions of genotyping procedures

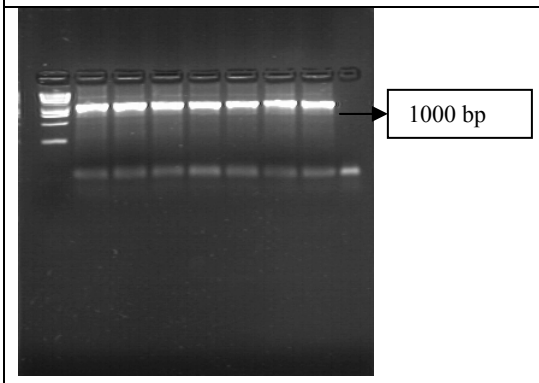
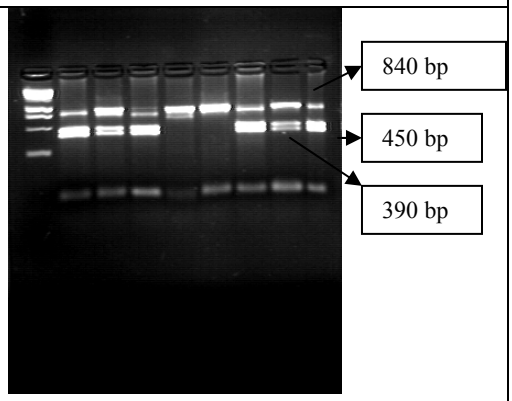
Amplification with N769 & N1110a	**Digestion with MboII**
 **M……1…2…3…4…5…6…7…8**	 **…..M…..1…..2…..3…..4…..5…..6…..7**
M: Marker **Lane 8:** Negative control **Lane 1-7:** All NAT1 alleles First amplification reaction to identify NAT1 alleles, the expected product is a 344 bp long fragment.	**M:** Marker **Lane 1, 3-5:** NAT1*3-4-10-14A-27 alleles **Lane 2, 7:** NAT1*4-27 alleles **Lane 6:** NAT1*3-10-14A alleles Digestion of this 344 bp long fragment by MboII restriction enzyme give us a pattern of smaller fragments: 176 bp: Observed in allelic forms 144 bp: Observed in 3, 10 and 14 alleles 135 bp: Observed in 11 allele (absent in this gel photo) 125 bp: Observed in 4 and 27 alleles

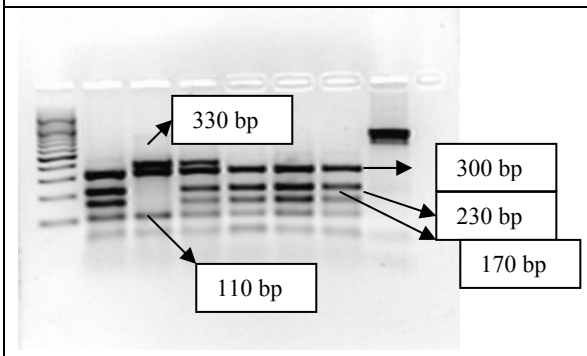
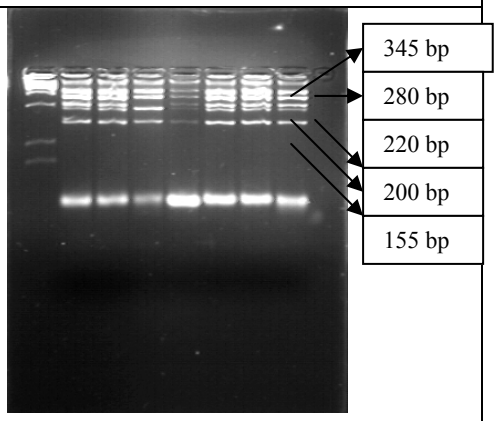
Amplification with N2&N850	**Amplification N769&N1110a**
 **M....1....2....3....4....5....6....7**	 **M.....1.....2....3....4....5....6....7....8**
M: Marker **Lane 1:** Negative control **Lane 2-7:** NAT1*27 allele (no band) Absence of the 849 bp length fragment indicates the absence of NAT1*27 allele; presence of NAT1*4 allele is confirmed.	**M:** Marker **Lane 8:** Negative control **Lane 1,4,7:** NAT1*3-4-11-27 alleles Presence of the 341 bp length band is the result of NAT1*3-4-11 or *27 alleles. Samples bearing only *10 and *14 alleles do not give any band in this reaction.

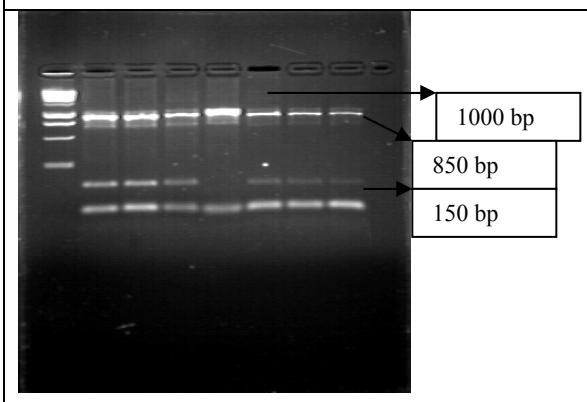
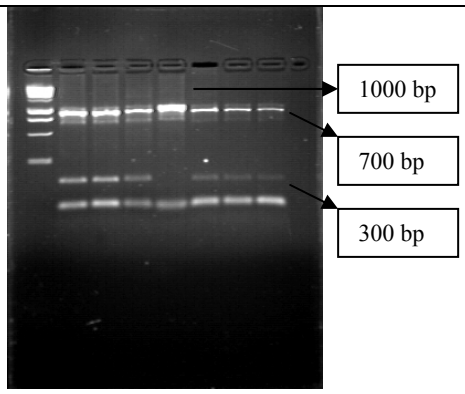
Amplification with N769&N1110b	**Amplification with N539&N713**
 **...M..1...2...3....4....5....6....7...8**	 **...M....1....2....3.....4.....5**
M: Marker **Lane 8:** Negative control **Lane 1-7:** 1 from NAT1*10-14 Presence of the 341 bp length band in this reaction indicates the presence of either NAT1*10 or *14 alleles.	**M:** Marker **Lane1:** Negative control **Lane 2,4,5:** All NAT1 alleles In order to determine whether NAT1*10 or *14 is present in our sample, an amplification step is performed with N539 and N713 primers. We should obtain a band of 175 bp length for all samples.

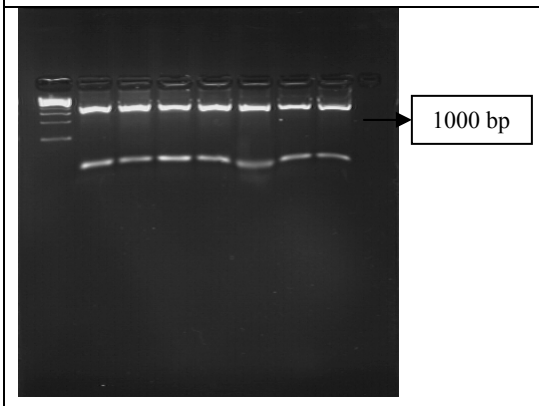
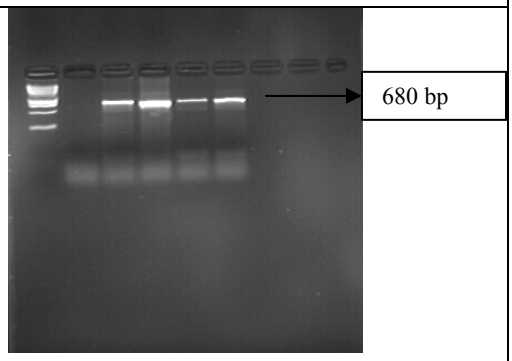
Digestion with BsiEI	**Amplification with N376&N1176**
 **.....M.1..2..3..4..5**	 **...M...1.....2....3....4...5....6....7**
M: Marker **Lane 1-4:** NAT1*3-4-10-11-27 alleles **Lane 5:** Undigested product Digested products by BsiEI are presented with a band of 155 bp length. All NAT1 alleles except *14 have a restriction site for BsiEI. So, samples bearing a *10 allele would result in a band of 155 bp length, while samples bearing *14 allele would result in a band of 175 bp length.	**M:** Marker **Lane 1:** Negative control **Lane 3-7:** All NAT1 alleles To confirm the presence of NAT1*11 allele, an amplification step is performed by using N376 and N1176 primers. All samples should give a band of 1553 bp length.

Digestion with AlwNI	Amplification with NatHu7-NatHu8
 **...M...1...2...3....4...5....6...7**	 **...M...1...2...3...4...5...6...7**
M: Marker **Lane 3-7:** NAT1*11 allele 1553 bp length PCR product is digested by AlwNI restriction enzyme. Samples containing a NAT1*11 allele have a second restriction site. Thus, samples containing an *11 allele reveal a pattern composed of 3 different bands: 777, 528 and 239 bp length, whereas other alleles would result in a single band: 777 bp length.	**M:** Marker **Lane 1:** Negative control **Lane 2-7:** All NAT2 alleles All samples would result in a 840 bp length band at the end of the amplification step with NatHu7 and NatHu8.

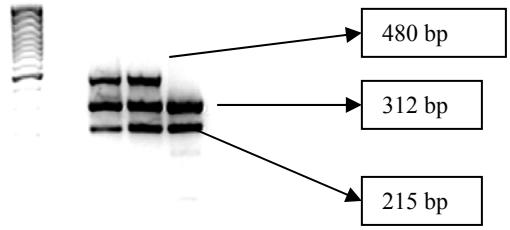
Amplification with NatHu14-NatHu16	**Digestion with KpnI**
 **...M...1...2...3...4...5...6...7...8**	 **..M...1...2...3..4..5...6...7...8**
M: Marker **Lane 8:** Negative control **Lane 1-7:** All NAT2 alleles All samples would result in a 1000 bp length band at the end of the amplification step with NatHu14 and NatHu16.	**M:** Marker **Lane 5:** Undigested **Lane 4:** NAT2* 5A-5B **Lane 1-3&6-8:** 1 from NAT2* 5A-5B, 1 from 4-5C-6A-7B Digestion of 840 bp fragment by KpnI would result in 3 different banding patterns: Undigested 840 bp band represent 5A and 5B alleles. Digested 450 and 390 bp fragments represent 4, 5C, 6A and 7B alleles. Observation of these 3 bands at the same time means one allele from 5A or 5B and one allele from the rest of possibilities.

Digestion with TaqI	Digestion with DdeI
 **....M...1.....2....3....4.....5....6.....7**	 **...M..1...2...3...4...5...6...7**
M: Marker **Lane 1,4-6:** 2 from NAT2* 4-5A-5B-5C-7B **Lanes 2:** 2 from NAT2*6A **Lane 3:** 1 from NAT2*6A, 1 from NAT2*4-5A-5B-5C-7B Digestion of 840 bp fragment by TaqI would result in 3 different banding patterns: Presence of 3 bands (110, 300 and 330 bp) represents a genotyping of NAT2*6A. Observation of 4 bands (110, 170, 230 and 300 bp) means 2 alleles coming from NAT2*4, 5A, 5B, 5C or 7B. Observation of all 5 bands represents a genotyping of one 6A allele and one from all other possibilities.	**M:** Marker **Lane1,2,5-7:** 1 from NAT2*5B-5C, 1 from 4-5A-6A-7B **Lane 3:** 2 from NAT2*5B-5C **Lane 4:** 2 from NAT2*4-5A-6A-7B Digestion of 1000 bp fragment by DdeI would result in 3 different banding patterns: Bands of 155, 280 and 345 bp length are common in all possibilities. Presence of 220 bp band represents 2 alleles from 4, 5A, 6A and 7B. Presence of 200 bp band represents 2 alleles from 5B and 5C. Observation of these two at the same time means one allele from 5B or 5C and one from 4, 5A, 6A or 7B.

Digestion with BamHI	**Digestion with Hinc II**
	
...M....1....2...3...4....5....6...7	**..M...1...2..3...4...5..6..7**
M: Marker **Lane 1-3,5-7:** 2 from NAT2*4-5A-5B-5C-6A **Lane 4:** 2 from NAT2*7B Digestion of 1000 bp fragment by BamHI would result in 3 different banding patterns: An undigested fragment represents 2 alleles from 7B. Observation of digested products, 150 and 850 bp, means 2 alleles from 4, 5A, 5B, 5C or 6A.	**M:** Marker **Lane 1-3,5-7:** 2 from NAT2*4, 5B, 5A, 5C, 6A **Lane 4:** Undigested product Digestion of 1000 bp fragment by Hinc II would end up with 3 different banding patterns. Undigested fragment means 2 alleles from 7B. Observation of digested products, 300 and 700 bp, means 2 alleles from 4, 5A, 5B, 5C or 6A.

Digestion with Hind III	**Amplification with DH1&NatHu16**
 **M.....1...2...3...4....5....6....7**	 **...M...1...2...3...4...5**
M: Marker **Lane 1-7:** 1 from NAT2*5B, 5C and 1 from NAT2*4, 5A, 6A, 7B Digestion of 1000 bp fragment by Hind III is a confirmation reaction to make sure that the amplified fragment belong to the NAT2 ORF, and not to the NAT1 ORF. Due to the lack of any restriction site within NAT2 ORF, undigested fragments should represent NAT2 gene.	**M:** Marker **Lane1:** Negative control **Lane 2-5:** Contains NAT2*4 allele This is an Allele-Spesific reaction to reveal the presence of NAT2*4 allele. Because the method described could not identify 5A*5C and 4*5B genotypes from each other, this reaction is needed and amplification of a 680 bp fragment justify the presence of *4 allele and 4*5B genotype.

Amplification of GSTM1 and GSTT1



...M...1...2...3...4

M: Marker

Lane 1: Negative control

Lane 2,3: GSTM1, GSTT1 + allele

Lane 4: GSTT1 null, GSTM1 + allele

This is a single step reaction to reveal the presence/absence of GSTM1 and GSTT1 active gene copies. The middle band of 312 bp length is an internal control, CYP1A1 gene.

The mean (± SE) age for 37 breast cancer patients was 51.5 years (± 8.9; range 37-75) and the mean (± SE) age for 34 healthy controls was 40.8 years (± 18.2; range 17-76). Case and control variables for the susceptibility of breast cancer are given in Table 10. The mean Body-mass Index (BMI) value was considerably high for both case (32.8±6.4) and controls (27.6±5.3). Whereas the mean values for age at first menarche and mean age at menopause were consistent with standards, 13.5±1.4 and 46.4±5.0 respectively for the breast cancer patients, and the mean values for age at first menarche and mean age at menopause were also consistent with the standards, 13.4±1.4 and 46.9±3.8 respectively, for controls. The mean age at first full-time pregnancy was 19.1±2.9 for cases and the mean age at first full-time pregnancy was 19.4±4.3 for controls. The age and BMI values between case and control groups show a statistically significant difference, p values of 0.003 and 0.007 respectively. However, for the variables related with age, we could not found any significant relationship. Among breast cancer patients, a higher percentage of family history (breast and ovary cancer) was observed, though statistically it was not significant (p=0.539). Nullparity was much more common among control group (p=0.014), which was totally inconsistent with the findings of Krajinovic *et al.* 2001. Estrogen intake among breast cancer patients was found to be significantly higher than that of control group (p=0.003). Literature findings (Cheng *et al.*, 2005) were consistent with this result stating that increased life-time exposure to estrogen increases the risk of developing a carcinoma of breast. Likewise to breast cancer incidence throughout the world, postmenopausal women seemed to possess a higher ratio of breast cancer (p=0.001).

The relationship between different allelic types of genes and incidence of breast cancer, investigated in our study, seemed to be not very strong. Only in the case of GSTM1 null genotype, there was a difference of border-line significance between case and control groups (p=0.047). In addition, women with NAT2 rapid acetylator and GSTM1 null genotypes

were at the elevated risk (OR, 3.8; CI, 0.9-15.4). NAT1 rapid acetylator and GSTT1 genotypes showed no association with breast cancer.

Table 10. Evaluation of variables in case and control groups

Variables	Case (n=37)	Control (n=34)	P value
Age[a]	51.5±8.9	40.8±18.2	0.003
BMI[a]	32.8±6.4	27.6±5.3	0.007
Age at first menarche[a]	13.5±1.4	13.4±1.4	0.834
Age at first full-time pregnancy[a]	19.1±2.9	19.4±4.3	0.080
Age at menopause[a]	46.4±5.0	46.9±3.8	0.762
Family history[b]	13.5	8.8	0.539
Nullparity[b]	5.4	26.5	0.014
Estrogen intake[b]	35.1	5.9	0.003
Menopausal Status:			
Premenopausal[b]	24.3	64.7	0.001
Postmenopausal[b]	75.7	35.3	0.001
GSTM1 null allele[b]	67.6	44.1	0.047
GSTT1 null allele[b]	8.1	23.5	0.073
NAT1 rapid allele[b]	51.4	55.9	0.711
NAT2 rapid allele[b]	44.4	23.5	0.065

Data denoted by a is represented as mean ± standard error and data denoted by b is represented as percentage.

Our methodology allowed to detect 7 NAT1 alleles, but only four of them (NAT1*4, NAT1*3, NAT1*10, NAT1*11) were found in our case and control group (Figure 3). Among them, NAT1*4, the wild type allele, was by far the most common allele. Then, the following most frequent one was

NAT1*10 allele and NAT1*3 and NAT1*11 alleles were found to be the least frequent ones. In the case of NAT2, NAT2*6A and NAT2*5B were the most common allelic types, here the wild type allele NAT2*4 came afterwards. NAT2*5A, NAT2*5B and NAT2*7B alleles were the less frequently observed ones in this study (Table 11).

According to our results, genotype determinations of all subjects revealed a dominant representation of slow acetylator genotypes for NAT2, while normal (wild-type-*4) acetylator genotypes were predominated for NAT1. In the literature, the role of NAT1 and NAT2 in bladder and colon carcinogenesis is well established, whereas the impact on breast cancer susceptibility is not well understood. Some studies reported an increased risk for breast cancer among slow acetylator postmenopausal women who smoked (Sillanpaa *et al.*, 2004, Alberg *et al.*, 2004). On the contrary, in some other studies, the effect of smoking on breast cancer risk was more apparent among NAT2 rapid acetylators (Krajinovic *et al.*, 2001). An association between red meat intake, modified by NAT1 functional genotypes, and the risk of breast cancer has been reported but only in relation to the NAT1*11 variant (Zheng *et al.*, 1999). Other studies failed to show any significant relationship with breast cancer risk and the presence of either NAT1 rapid or NAT2 rapid genotypes (Lee *et al.*, 2003).

Table 11. Genotype distributions of NAT1 and NAT2 among 37 breast cancer patients and 34 control subjects

NAT1 Genotypes	Case (n=35)	Control (n=34)	NAT2 Genotypes	Case (n=35)	Control (n=34)
Rapid			**Rapid**		
***4/*10	15	14	4*4	2	2
*3/*10	-	2	4*5A	-	1
*10/*10	2	3	4*5B	8	3
*10/*11	1	-	4*6A	5	2
Normal			4*7B	1	-
*4/*4	16	12	**Slow**		
*4/*3	-	1	5A*5B	1	2
*4/*11	1	2	5A*7B	-	1
			5B*5B	4	7
			5B*6A	8	9
			5C*6A	2	-
			6A*6A	3	4
			6A*7B	1	-
			5B*7B	-	2
			7B*7B	-	1

For NAT1, we detected 4 different alleles – *4, *3, *10, *11 – and there was almost no difference among case and control groups with respect to the distribution of these alleles. NAT1*4 allele was by far the most common type and subsequently came the NAT1*10 allele. Among the allelic variants of NAT1* gene, NAT1*10 allele was reported to increase the risk of breast cancer 4-fold among women who consumed well-done meat (Krajinovic *et al.*, 2001). NAT1*10 is an allele with a mutation at 3' polyadenylation site (Hein *et al.*, 2000), resulting in rapid acetylator phenotype, however, its prevalence in our population is not known. For NAT2, slow acetylator alleles, NAT2*5B and NAT2*6A were the common ones. NAT2*6A and NAT2*5B were found to be high frequency alleles among Turkish population (Kocabas *et al.*, 2003).

For the GST genes, we observed that null genotype is a much more common case for GSTM1, whereas nonfunctional allele of GSTT1 was seen rarer (Table 12).

Table 12. Genotype distributions of GSTM1 and GSTT1 among 37 breast cancer patients and 34 control subjects

	Case (n=37)	**Control (n=34)**
GSTM1		
+	12	19
Null	25	15
GSTT1		
+	34	26
Null	3	8

GSTM1 null allele was encountered much more commonly than the null genotype of GSTT1 both in case and control groups. Besides, an association of border-line significance was detected between the incidence of breast cancer and presence of nonfunctional GSTM1

genotype (p=0.047). Likewise to NAT case, there is no agreement on the effect of GSTs in breast cancer. Results from some studies did not support any involvement of the GSTM1 and GSTT1 gene polymorphisms in susceptibility to breast cancer (Curran *et al.*, 2000). On the other hand, results from other sources revealed that these genes may be modifiers of breast cancer (Sarmanova *et al.*, 2004, Sull *et al.*, 2004). Park *et al.* (2000) observed that there was an especially remarkable risk of breast cancer for alcohol-consuming premenopausal women lacking both the GSTM1 and GSTT1 genes.

In this study, case and control subjects were also compared according to data obtained from questionnaires. The mean age of case group was significantly higher than that of control group (p=0.003), however this difference was probably the result of improper sample selection. However, a significant difference was also observed in BMI value, meaning a higher BMI may increase the risk of developing a breast carcinoma. Data about the reproductive history of subjects, such as age at first menarche, age at first full-time pregnancy and age at menopause, did not reveal any relationship with cancer incidence. The most interesting finding was that the ratio of exogeneous estrogen intake was significantly higher in case group (p=0.003), suggesting that a higher level of estrogen exposure enhances the development of breast carcinoma.

In conclusion, these results indicate that there is no statistically significant relationship between breast cancer incidence and allelic type of NAT1. Previous studies of the relationship between breast cancer risk to NAT1 rapid acetylator genotypes have also not produced consistent results. In the case of NAT2, patients showed a higher percentage of rapid acetylator genotype than healthy controls, but the difference is not statistically significant. The most powerful correlation found in the scope of the study was the coexistence of GSTM1 null and NAT2 rapid acetylator genotypes. Our results suggest that GSTM1 null genotype may be a susceptibility

factor for breast cancer, particularly in the presence of NAT2 rapid acetylator genotype. In the case of hormonal status, the most important correlation was observed with the exogenous estrogen intake, either by birth control pill or hormone replacement therapy. In addition, BMI and menopausal status are other strongly related factors increasing the risk of breast cancer. Further studies with a larger and properly randomized sample size are required to confirm the presence and power of this relationships.

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5. APPENDICES

5.1.Appendix A: Questionnaire

Katılımcı takip formu:

Katılımcı takip formu					
Hasta adı:					
1. Yaş:					
2. Cinsiyet: K E					
3. Boy:					
4 Ağırlık (kg) :					
5. Meslek:					
6. Doğum yeri:					
7. Oturduğu yer:					
8. Eğitim durumu:					
	İlkokul	Lise	Üniversite	Üstü	
9. Aile geçmişi					
Yok Var	1. derece akraba	2. derece akraba			
Kanser tipi:					
10. Sigara Kullanımı					
Yok Var	1-10 tane/gün	1 paket/gün	1 paket/gun<		
Sigaraya başlama yaşı:					
11. Alkol Kullanımı					
Yok Var	1 kadeh/yıl	1 kadeh/ay	1 kadeh/gun		
12. Kırmızı et tüketimi					
Yok Var	iyi pişmiş	orta pişmiş	az pişmiş		
Tüketim sıklığı: Her /	gun	hafta	ay	yıl	
13. İlk adet görme yaşı:					
14. İlk hamilelik yaşı:					
15. Çocuk sayısı:					
16. Düşük sayısı:					
17. Erken doğum:					
18. Emzirme süresi:					
Yok Var	3 ay	6 ay	1 yıl	1 yıl<	
19 Doğum kontrol yöntemi:					
Yok Var	Doğum kontrol hapı (DKH)	Diğer			
D.K.H. Kullanım suresi:	İlaç adı:				
20. Adetten kesilme yaşı:					
21. Hormon tedavisi:					
Yok Var					
Kullanım Süresi:	İlaç adı:				
22. Kanser türü/evre/derece					
23. Kemoterapi					
Yok Var					
Kullanım Süresi:	İlaçlar:				
24. Östrojen reseptör:					
25. Progesteron reseptör:					

5.2. Appendix B: Buffer Preparations

1) 100 Mm Tris-HCl (pH 8.0):

0,121g Tris base was dissolved in about 5 mL dH_2O and pH 8.0 was adjusted with 1 M HCl. Finally, volume is completed to 10 ml.

2) 10 mM EDTA (pH 8.0):

0.0372g EDTA was dissolved in about 5 mL dH_2O and pH 8.0 was adjusted with 5M NaOH. Finally, volume is completed to 10 ml.

3) TE Buffer (pH 8.0):

10 mM Tris-HCl, pH 8.0 was mixed with 1 mM EDTA, pH 8.0 to prepare TE Buffer.

4) TBE Buffer:

54 g Tris base and 27.5 g Boric acid were dissolved in about 500 ml dH_2O, then 20mL, 0.5M EDTA was added to adjust pH at 8.0. Finally, volume is completed to 1000 ml.