RNASEL G1385A VARIANT AND BREAST CANCER SUSCEPTIBILITY

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BY

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AUGUST, 2003

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ABSTRACT

RNASEL G1385A VARIANT AND BREAST CANCER SUSCEPTIBILITY

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RNASEL (MIM# 180435) encodes for the ubiquitously expressed ribonuclease L (RNase L), which mediates the antiviral and pro-apoptotic activities of the 2-5A system. Recently, *RNASEL* Arg462Gln (G1385A) variant is shown to be implicated in up to 13% of prostate cancer cases. Furthermore, *RNASEL* mutations segregating with disease within hereditary prostate cancer (HPC) families, and loss of heterozygosity (LOH) in tumor tissues have been reported. RNase L has been proposed to suppress the development of cancer through its ability to degrade RNA and initiate a cellular stress that leads to apoptosis. Analysis for allelic losses at the long arm of the chromosome 1 suggested that inactivation of a gene(s) on 1q23-32, which encompasses the *RNASEL* locus, might contribute to the genesis of breast cancer. Based on chromosomal location and function of *RNASEL*, and pleitropic effects of cancer associated mutations, we south to investigate the hypothesis that Arg462Gln variant of *RNASEL* is associated with breast cancer risk. The homozygote mutant (odds ratio (OR) = 0.75, 95% CI= 0.49-1.14), heterozygote (OR=1.02, 95% CI= 0.76-1.37), or the genotype having at least one mutant allele (OR= 0.94, 95% CI=0.72-1.24) was found to be not associated with the breast cancer risk. The adjustment of the data with age, menopausal, smoking status, body-mass-index, age at menarche, age of 1st pregnancy, number of children, and family history of breast cancer did not change the results (homozygote mutant (OR= 0.72, 95% CI= 0.46-1.12), heterozygote (OR= 0.95, 95% CI= 0.70-1.29), or genotype having at least one mutant allele (OR= 0.89, 95% CI= 0.66-1.18)). In conclusion, our study reports no association between the *RNASEL* G1385A variant and breast cancer risk.

ÖZET

RNASEL G1385A MUTASYONUNUN MEME KANSERİ İLE İLİŞKİSİ

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RNASEL (MIM# 180435), tüm dokularımizda sentezlenen ve 2-5A sisteminin antivirütik ve pro-apoptotik aktivitelerini gerçekleştiren, "ribonükleaz L" (RNase L) enzimini kodlar. *RNASEL* Arg462Gln (G1385A) varyantının, prostat kanserli hastaların %13' ünde etkili olduğu saptanmıştır. Ayrıca, *RNASEL* mutasyonlarının prostat kanserli ailelerde hastalık ile kalıtıldığı ve tümör dokularında heterozigotluğun kaybı (Loss of heterozygosity, LOH) rapor edilmiştir. RNase L'in, hücresel stress yaratarak programlanmış hücre ölümünü başlattığı, ve kanser oluşumunu engellediği önerilmiştir. 1. kromozomun kısa kolundaki allel kayıplarının incelenmesi ile, *RNASEL* lokusunu da içeren 1q23-32 bölgesindeki gen veya genlerin işlevlerini yitirebilecekleri ve meme kanserinin oluşumunda etkili olabilecekleri gösterilmiştir. *RNASEL*'in kromozomal lokalizasyonu ve fonksiyonu, ve kanserle ilişkili genlerin "pleiotropik" etkilerini göz önüne alarak, *RNASEL* Arg462Gln varyantının meme kanseri ile ilişkisinin olduğu hipotezini kurduk. Çalışmamız, homozigot mütant (odds ratio (OR) = 0.75, 95% CI= 0.49-1.14), heterozigot (OR=1.02, 95% CI= 0.76-1.37), veya en az bir mutant allele sahip genotiplerin (OR= 0.94, 95% CI=0.72-1.24) meme kanseri oluşumuna bir etkisinin olmadığını göstermiştir. Verilerin yaş, menaposal, sigara içme durumları, vücud-kütle endeksleri, *menarche* ve ilk hamilelik yaşları, çocuk sayıları, ve ailelerindeki kanser hikayelerine göre ayarlanmaları da sonucu değiştirmedi (homozigot mütantlarda (OR= 0.72, 95% CI= 0.46-1.12), heterozigotlarda (OR= 0.95, 95% CI= 0.70-1.29), veya en az bir mütant allele sahip genotiplerde (OR= 0.89, 95% CI= 0.66-1.18)). Sonuç olarak, çalışmamız *RNASEL* G1385A varyantı ile meme kanseri arasında bir ilişki olmadığını gösterdi.

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ABBREVATIONS

2-5A	2’,5’-linked oligoadenylates
A	Adenine nucleotide
APC	Adenopolyposis Coli
Arg	Arginine
ARMS	Amplification Refractory Mutation System
ASPCR	Allele Specific Polymerase Chain Reaction
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
BMI	Body Mass Index
Bp	Base pairs
BRCA1	Breast Cancer Susceptibility Gene 1
BRCA2	Breast Cancer Susceptibility Gene 2
Cdk	Cyclin dependent kinase
CHEK2	Cell cycle checkpoint kinase 2
CI	Confidence interval
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
E	Expected (in statistical calculations)
EDTA	Ethylene diamine tetra acetic acid
F	Forward primer
G	Guanine nucleotide
G6PD	Glucose-6-phosphate dehydrogenase
Gln	Glutamine
GTP	Guanosine Triphosphate
HPC	Hereditary Prostate Cancer
IBC	Inflammatory Breast Cancer
IFN	Interferons
IL	Interleukin
kb	Kilobase(s)
kDa	Kilo Dalton(s)

LOH	Loss of Heterozygosity
M	Molar
mg	Milligram
min	Minutes
ml	Milliliter
MLH1	Mut H Homolog 1
MSH2	Mut S Homolog 2
mM	Millimolar
µl	Microliter
µg	Microgram
O	Observed (in statistical calculations)
OAS	2-5A synthetases
OR	Odds Ratio
PASA	Polymerase chain reaction amplification of specific alleles
PC	Prostate Cancer
PCR	Polymerase Chain Reaction
PIN	Prostate intraepithelial neoplasia
pmol	Picomol
R	Reverse primer
RB	Retinoblastoma
RNA	Ribonucleic Acids
rpm	Revolutions per minute
s	second(s)
T	Tymine
TBE	Tris Borate EDTA
TP53	Tumor Protein p53
UV	Ultraviolet light
w/v	Weight per volume
WHO	World Health Organization
χ^2	Chi-square

1. Introduction

1.1. Introduction to cancer

"Cancer" is accepted as a group of diseases characterized by uncontrolled cellular growth and the spread of abnormal cells, which is believed to be dictated by a series of genetic alterations.

It is now well recognized that, cancer is one of the most common and severe problems of human population. According to World Health Organization (WHO), more than 1.2 million people worldwide are diagnosed with breast cancer annually. An estimated 211,300 new cases of invasive breast cancer are expected to occur among women in the United States during 2003, being the most commonly diagnosed non-skin cancer in women. In addition, 1,300 cases of male breast cancer are also predicted. An estimated 40,200 deaths (39,800 women and 400 men) are anticipated from breast cancer this year in U.S. only (American Cancer Society, "http://www.cancer.org"). In order to completely understand the concept of cancer, we must also know the history of today's problem.

1.1.1. History of cancer

Although the ancient origin of the word "cancer" is uncertain, it is believed to be derived from Latin for "crab", presumably because the cancer "adheres to any part that it seizes upon in an obstinate manner like the crab".

Incidents of breast cancer have been documented back to the early Egyptians, when the popular treatment was "cautery" of the diseased tissue. Surgery was practiced, but it was an extremely radical treatment since anesthesia or antisepsis was not available. The reason for the disease was suggested to be melancholia (by the Greek physician Caudius Galen, 130-200 AD), where the suggested treatments were special diets. The next suggested treatment to control bleeding was mastectomy (by Andreas

Vesalius, a Flemish anatomist of Renaissance). Due to lack of detailed records, the level of success associated with these archaic treatments is not known.

After the mid 1800's, surgeons first began to keep detailed records, which provides us the information that the patients treated with mastectomy had a high rate of recurrence within eight years – especially when the glands or lymph nodes were affected. Nevertheless, the common therapy was removal of the breast and the surrounding glands in an effort to stave off any further tumor development, which shows us the belief, that breast cancer is a systemic disease and could spread and affect other parts of the body. The cure was based only on a "three-year survival rate". Although it is hardly for today, such a survival rate was acceptable at those times.

The treatment improvements were noticeable between the 1930's and 1950's, because of a better classification of the stage and progression of the tumors. Therefore, the survival rates increased dramatically during the 1900's (ten year survival rate, 10% in the 1920's to roughly 50% in the 1950's).

It was not before 1975, that the role of the accumulation of the genetic variations was shown in the development of cancer. Following that discovery, scientists identified approximately 70 genes that can spur cancerous growths and at least a dozen genes that should deter such growth but do not (Breast Cancer Society of Canada, "http://www.bcsc.ca").

1.1.2. Epidemiology of cancer

Knudson's "two-hit" hypothesis and its molecular confirmation in retinoblastoma focused attention in certain rare cancers (Knudson, 1971), and the contribution of "genetic susceptibility" (Macleod, 2000).

Before 1980s, the origins of common cancers were dominantly viewed as "environmental". This was because of the studies performed in 1960s and 1970s. The varying frequencies of cancer types observed in different populations and the convergence towards local cancer rates among immigrants also strengthened the

"environmental view" (Peto, 2001). The transition of the cancer pattern of an immigrant population from their original to the pattern of their new country was another supporting evidence (Balmain *et al.*, 2003). The transition of cancer pattern was also verified among the Turks residing in Germany (Zeeb *et al.*, 2002). The results of these studies led scientists to conclude that most cancers are in principle preventable and many could be avoided by a suitable choice of life style and environment.

By the early 1980s, many important clues about the causes of cancer were identified and this increased the emphasis on the role of genetic predisposition in the common cancers (Peto, 2001, and Balmain *et al*., 2003).

After a quarter century of rapid advances, cancer research has generated a rich and complex body of knowledge, underlining the involvement of dynamic changes in the genome (Hanahan *et al.*, 2000). Besides the genetic susceptibility, many other factors have been identified. The most important ones being;

1. Oncogenic viruses: Identification of the carcinogenic effects of infectious pathogens was one of the most important discoveries of the past two decades (Peto, 2001).
2. Smoking: The identification of the effect of tobacco in cancer development was one of the most important hallmarks in history of cancer epidemiology (Peto, 2001). Now it is well understood that incidences of many cancer types are increased by tobacco use, i.e. lung cancer, esophageal cancer, stomach cancer, liver cancer. Tobacco use cause 13% (and will probably cause 33%) of deaths in men (Liu *et al.*, 1998).
3. Reproductive and hormonal factors: The impact of reproductive and hormonal factors was first verified on breast and ovarian cancer (Peto, 2001, and Baselga *et al.*, 2003).
4. Obesity: Up to a third of cancers of breast, colon, kidney, and digestive tract were shown to be due to obesity (Josefson *et al.*, 2001). Although the impact of obesity is subject to change among populations, it is clearly stated for the post-menopausal breast cancer and cancer of the endometrium, gall-bladder and kidney (Bergstorm *et al.*, 2001).

1.1.3. Conceptualizing cancer

The word “cancer” does not refer to a single disease. Actually it is used to name a great variety of diseases characterized by masses of growth in an uncontrolled manner. The growth of the mass of the cells is autonomous, uncontrollable, increasingly malignant, and if untreated, invariably fatal. A tumor is formed by a parenchyma of proliferating cells, with a stroma of connective tissue and blood vessels (Thompson, 1991, p365). There are three main forms of tumors,

1. Sarcomas, in which the tumor has arisen in mesenchymal tissue,
2. Carcinomas, which originate in epithelial tissue,
3. Hematopoetic and lymphoid malignancies, such as leukemias and lymphomas.

Within the major groups, tumors are classified by site, tissue type and degree of malignancy (Thompson, 1991, p365).

The presence of “uncontrolled growth” is gained through the accumulated variations in the genetic materials of the cells. It was suggested that, the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan *et al.,* 2000). These alterations are summarized in Figure 1.1, where the crab is the cancer, and the six legs are the acquired capabilities of cancer.

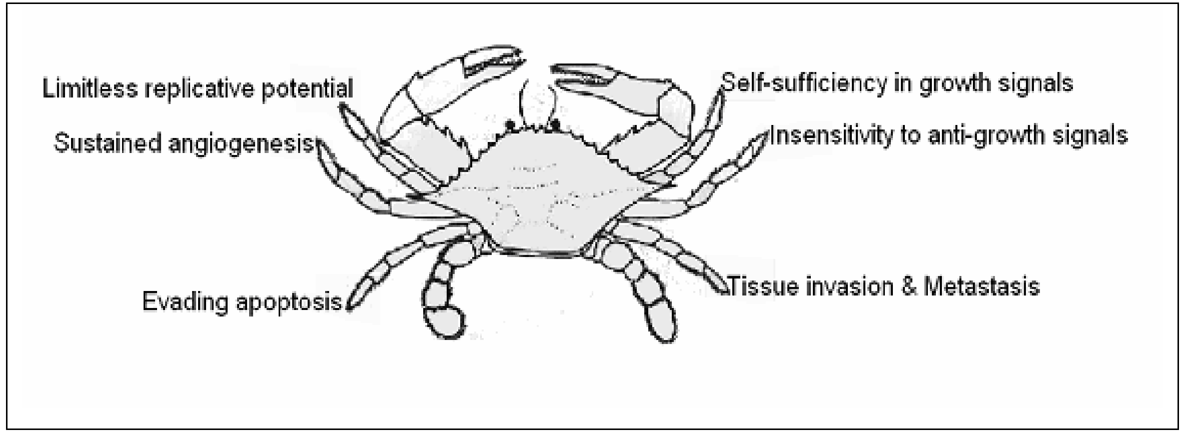


Figure 1.1. Hallmarks of cancer.

Genomic integrity may be disrupted in many ways. It may be sporadic, because of environmental factors (i.e. ionizing radiation), lifestyle (i.e. smoking, diet), or hereditary (i.e. germ line tumor-suppressor gene mutations).

Up to now, the only environmental exposure proven to induce breast cancer is ionizing radiation (Grover *et al.,* 2002). The reactive oxygen species (ROS) produced upon radiation exposure causes the genomic damage.

Documentation of family history in different types of cancers has shown that some individuals are more susceptible to cancer because of their genomic heritage. More than a century ago, Paul Broca described four generations of breast cancer in his wife's family which underlined, probably for the first time, contribution of the hereditary factors in tumorigenesis (Lynch *et al.,* 1994).

Population-based epidemiological studies showed the familial pattern of some cancers. This supported the implementation of genetic models rather than the environmental ones. Furthermore, it was also shown that, genetic alterations might account for a substantial fraction of cancer incidence without necessarily causing evident familial clustering. The demonstration of genetic linkage in breast cancer (Hall *et al*., 1990) by the use of DNA sequence polymorphisms dispelled the contribution of genetic susceptibility (Balmain *et al.,* 2003).

1.1.4. Cancer and related genes

All cancers are found to be the result of abnormalities in DNA sequence. During its life, genetic material is subject to changes, and these changes are repaired by the sophisticated genome maintenance mechanisms. If these changes can not be repaired, they may result in the stable alteration of a critical gene, possibly providing a growth advantage to the cell in which it has occurred and result in the emergence of an expanded clone, derived from this cell (Figure 1.2) (Futreal *et al.,* 2001).

With few exceptions, cancers are derived from single somatic cells and their progeny (Ponder, 2001). This clonal nature of cancer is supported by many evidences. The original evidence came from the study of tumors in women heterozygous for the X-linked enzyme glucose-6-phophate dehydrogenase (G6PD). Due to the process of X-inactivation, only one pair of a pair of X-linked allele in a female heterozygote is expressed in a somatic cell. Cell lines derived from tumors in these women expressed one or the other G6PD allele, but not both, indicating that each tumor had grown from a single cell. Some other chromosomal deformations also occur in the same way. All of the evidences indicate that these malignancies are of single-cell origin (Thompson, 1991, p366).

Genetic instability has long been hypothesized to be a cardinal feature of cancer. A huge body of evidence also strengthened the proposal that mutational alterations conferring instability occur early during tumor formation. The ensuing genetic instability drives tumor progression by generating mutations in oncogenes and tumor-suppressor genes. These mutant genes provide the cancer cells the selective advantages (Cahill *et al.,* 1999).

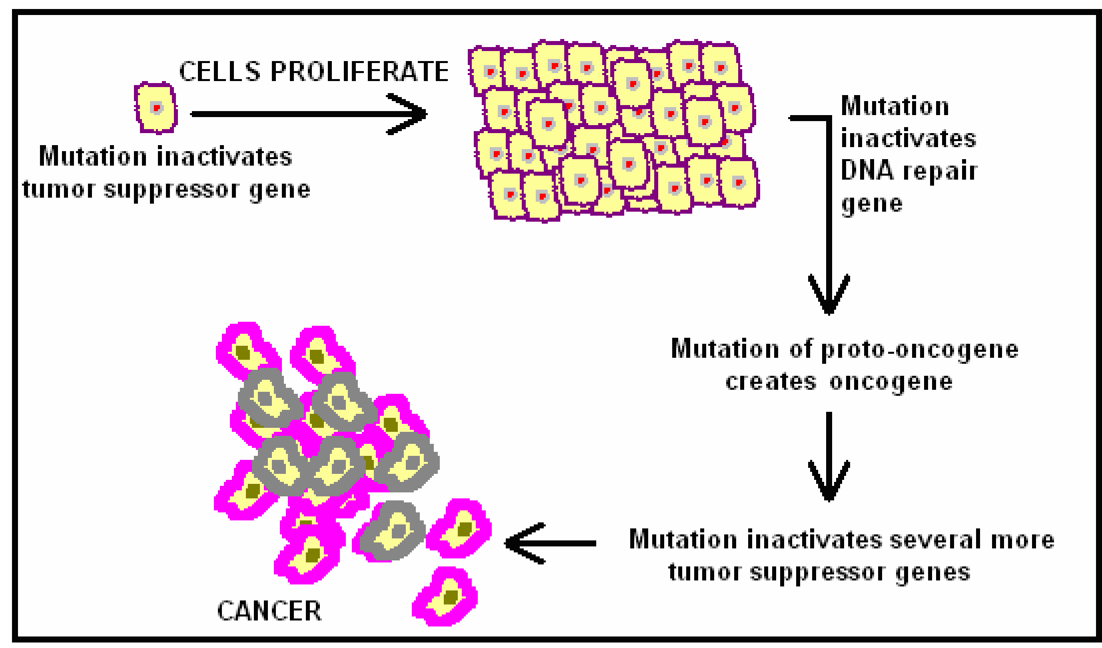


Figure 1.2. Genetic alterations in progression of cancer.

Studies of inherited and sporadic colorectal cancer have demonstrated that in the overwhelming number of cases the primary mutation targets a single signal transduction pathway (Bienz *et al*., 2000).

After the initial promoting mutation in the primary cell of the tumor clone, additional mutations in the relevant target genes, and consequent waves of clonal expansion, produce cells that invade surrounding tissues and metastasize (Futreal *et al.,* 2001). It is obvious that, any alterations in any gene will show its effect through the protein product of this gene. Mostly, this altered protein product is found to be involved in important cellular processes. Some critical ones are,

- Transcription factors in breast cancer development (reviewed in, Benz ,1998),
- Telomerase in breast cancer (reviewed in, Herbert *et al.,* 2001),
- Centrosome abnormalities in carcinogenic progression (reviewed in, Duensing *et al.,* 2001),

While considering the genes in the progression of cancer, we may classify them into two broad groups, tumor-suppressor genes and oncogenes. The two classes have opposite effects on tumor development in their activated forms. Tumor-suppressor genes block tumor development, and oncogenes facilitate malignant transformation. So, cell proliferation and cell death are essential yet opposing cellular processes. Crosstalk between these processes promotes a balance between proliferation and death, and it limits the growth and survival of cells with oncogenic mutations (Guo *et al*., 1999).

1.1.4.1. Tumor suppressor genes

Tumor suppressor genes encode for the proteins that block the abnormal growth and malignant transformation. These proteins are generally involved in the growth regulatory or differentiation pathways. They generally contribute to malignancy when both alleles are lost. So, the mutations in these genes are told to be "recessive" at the cellular level.

The identification of cancer-susceptibility genes has revolutionized our understanding of cancer. Most of these genes were originally thought to control cellular proliferation directly, acting as "gatekeepers". But afterwards it became clear that genes that maintain the integrity of the genome ("caretakers") may be even more frequent causes of inherited predisposition to cancer (Kinzler *et al.,* 1997). So the tumor suppressor genes are divided into two categories: gatekeepers and caretakers. By definition, the genes whose mutation or altered expression disrupts the cell-cycle control and cell division, death or life-span, promoting the outgrowth of cancer cells are termed "gatekeepers" (e.g. *Rb*). And those, which cause genomic instability, increase the frequency of alteration in gatekeeper genes are defined as "caretakers" (i.e. *MLH1*, *BRCA1*) (Figure 1.3).

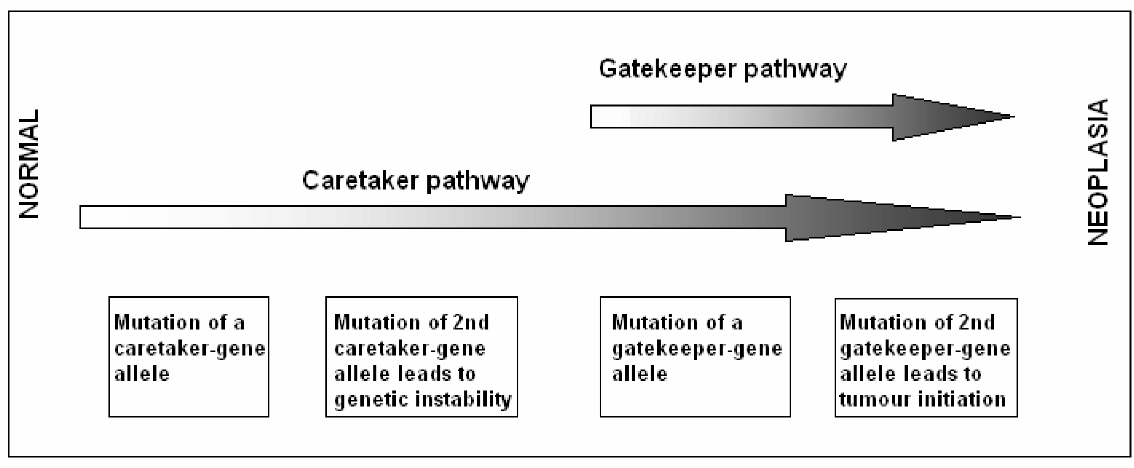


Figure 1.3. Caretakers and gatekeepers (Adapted from Kinzler *et al.*, 1997).

1.1.4.2. Oncogenes

Oncogenes encode for the proteins that dictate cell growth and development. “Proto-oncogene” is the name used for the unaltered form of these genes. The protein products of these genes are generally involved in the regulation of cell cycle, cell division, and differentiation. If a proto-oncogene is altered or over expressed (that is, become an oncogene), the cell undergoes uncontrolled growth, and eventually become malignant.

Oncogenes exhibit a “dominant” phenotype at the cellular level, and activation of one copy of oncogenes is enough to result in gain-of-function. The activation is gained through several different ways; a point mutation due to a small change, partial deletions and chromosomal translocations as large scale changes. These changes may occur in the exons of the gene (protein coding sequences) or in the sequences controlling the expression levels of the gene. Another way to achieve high expression levels may be the presence of extra copies of the gene, due to gene amplification events. Oncogenes may be transmitted from generation to generation when the mutation is present in the germ-line.

1.1.4.3. Genomic variations at a glance

It is widely accepted that cancer results from the accumulation of mutations in the genes that directly control cell birth or cell death. But the way a cell acquires these changes is a subject of continuing debate. It is suggested that an underlying “mutator phenotype” is required to create the rest of the mutations (Lengauger *et al.,* 1998). However, the opposite argument claims that normal rates of mutation along with the clonal nature of the cancer are enough to dictate malignant transformation (Heoijmakers, 2001).

Cells must guard the integrity of their genome to avoid both the inheritance of deleterious mutations and the accumulation of mutations in genes that control cell proliferation. Although cells employ many safeguards to protect their genomic integrity, cellular DNA is constantly bombarded by mutagens from endogenous and exogenous sources. DNA repair and cell-cycle checkpoints must all interlink to promote cell survival following DNA damage and preserve the integrity of chromosomes (Levitt *et al.,* 2002).

There are three main types of causes leading to the formation of DNA lesions that may lead to mutations if they are left unrepaired (Figure 1.4).

First type is the environmental agents such as ultraviolet (U.V) component of the sunlight, ionizing radiation, and numerous genotoxic chemicals.

Second type is the (by) products of normal cellular metabolism. These include the reactive oxygen species (superoxide anions, hydroxyl radicals and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation.

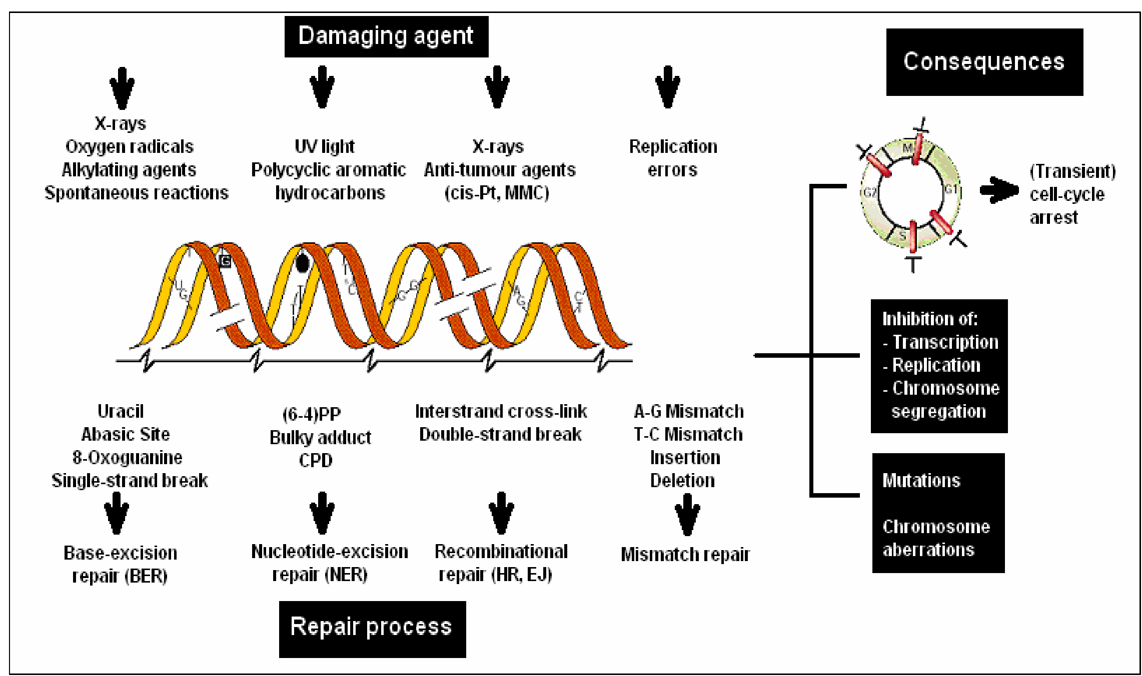


Figure 1.4. DNA damage, repair and consequences
(Adapted from Heoijemakers, 2001).

Finally, some chemical bonds in DNA tend to spontaneously disintegrate under physiological conditions. For example, hydrolysis of nucleotide residues leaves non-instructive abasic sites. Spontaneous or induced deamination of cytosine, adenine, guanine or 5-methylcytosine converts these bases to the miscoding uracil, hypohxantine, xanthine and thymine, respectively. Figure 1.4 summarizes some of the most common types of DNA damage and their sources (Heoijmakers, 2001).

1.1.5. Molecular profiling of cancer

Categorization of the tumors has been performed on the basis of histology. But, it is now clearly known that the staining patterns of cells viewed under the microscope is not sufficient to reflect the underlying molecular events that drive the neoplastic process. But, using today's technology, reading the molecular signature of an individual's tumor by surveying thousands of genes at once –using DNA arrays– is possible (Liotta *et al.,* 2000). So the variations in the gene expression profiles will be beneficial to fully understand different cancers. It is generally accepted that four to seven rate limiting genetic events are required for the development of the common epithelial cancers (Rennan *et al.,* 1993). It is noteworthy that the patterns of genetic alterations differ between cancers of different types and even of the same type. But fortunately, the patterns are not random (Liotta *et al.,* 2000 and Suzuki *et al.,* 2000).

The main aim of the recent use of DNA arrays (also protein arrays) is to be able to understand the sophisticated disease mechanisms and treatment targets (Liotta *et al.,* 2000). So, the identification of the molecular signatures of the tumors in genomic alterations or expression profiles will enable us to understand the possible mechanisms involved in tumor development, which may also enable us to obtain valuable information about clinics (Suzuki *et al.,* 2000).

1.1.6. Inherited predisposition

Family based studies led scientists to recognize the inherited predisposition to cancer. Since, cancer is a common disease; some families may contain several cases only due to chance. But there is a spectrum of probability that a given family history reflects inherited predisposition from near-certainty of strong predisposition in the rare inherited cancer syndromes, to the possibility of weak effects in familial clusters (Table 1.1) (Ponder, 2001).

Contribution of genetic factors to the development of cancer phenotype can be in varying degrees. Some genes may confer a high cancer risk to the individual but some not.

So, the concept of "inherited predisposition" must be investigated under two sections of "strong predisposition" and "weak predisposition". For example, germ-line mutations in *BRCA1* and *BRCA2* genes confer a high risk of breast cancer (Bertwistle *et al*., 1998; Ozdag *et al.*, 2000, and Manguoğlu *et al.*, 2003), whereas mutations in other cancers such as GSTM1 do not confer a high breast cancer risk.

Ironically, the frequencies of these two types of mutations are inversely related to their penetrances. The mutations, conferring a high risk are generally rare in the populations, whereas the mutations conferring a low risk are generally more frequent.

Table 1.1. Inherited predisposition to cancer.

	Contribution to overall cancer incidence	*Clinical feature*	*Frequency of predisposing alleles*	*Effect on individual risk*
Inherited cancer syndromes	1-2% at most	Rare/unusual cancers or combinations of cancers. Sometimes with associated developmental defects or non-neoplastic phenotype. Mendelian dominant inheritance.	Rare (nearly 1:1,000 or less)	Strong: lifetime risks of cancer up to 50-80%
Familial cancers	Up to 10% depending on definition	Families with several cases of common cancers that fall into a recognized pattern of cancer types. Spectrum from families with multiple cases at young age to two or three cases at older ages: many of the latter will be due to chance or to combinations of weaker genes. Generally show pattern consistent with dominant inheritance.	Uncommon to common	Moderate to weak
Predisposition without evident familial clustering	No precise figure possible. Distribution of risk within population may result in substantial fraction of cancer incidence within predisposed minority.	Single cases of cancer at any site, some with one or two affected relatives. The distribution of these cases in the population is probably determined by the combined effects of multiple genetic and non-genetic risk factors.	Multiple common alleles	Weak

1.1.6.1. Strong predisposition

A number of relatively rare, high-risk genes have been identified which predispose to common cancers such as breast, colon, and melanoma (Goldgar, 2002). The human inherited cancer syndromes and their transgenic mouse counterparts have been extensively studied. List of familial cancers and related genes are summarized in Table 1.2. As a result of these studies it was clearly seen that the strong predisposition to cancer results either through inheritance of one of the events on the cancer "pathway", or through effects on DNA repair of genome stability (Ponder, 2001).

The tissue specificity and variability of expression are two important features of strong predisposition. All inherited predisposition to cancer seems to show a considerable degree of tissue specificity, even in the case of defective DNA repair. The mechanism governing tissue specificity is still unknown. There may also be considerable variation in the age at onset of cancer and in the specific types of cancer that predominate not only within a given syndrome, but also within a single family. Some of this variation is due to different germ-line alleles of the main predisposing gene, and some is environmental or chance. But much of the within-family variation is probably attributable to the effects of genetic modifiers (Ponder, 2001).

Some other characteristics of strong predisposition are the vertical and not sex-specific transmission of the cancer predisposition, specific clinical characteristics (early age of diagnosis, presence of two or more primary cancers) (Ponder, 2001).

The first predisposing genes were identified as rare, mutated alleles. These mutated genes result in multiple cases of the disease in families. They were identified using genetic linkage and positional cloning. The prototypic gene associated with familial cancer syndromes is the retinoblastoma gene (*RB1*), which has turned out to be one of the most important hubs of cellular signaling. Other key signaling molecules such as p53 (encoded by *TP53*) were initially identified as important targets of viruses or somatic mutations in tumors and were subsequently found to function as germline-inherited tumor predisposition genes (Balmain *et al.,* 2003).

High penetrance alleles have provided many fundamental and unexpected insight into various aspects of cancer biology, including identification of the adenomatosis polyposis coli (*APC*), β-catenin and Tcf-4 pathway, and the phosphatase *PTEN*, which is implicated in Cowden syndrome and in the development of a variety of tumor types (Balmain *et al.,* 2003).

It is important to consider that, most of the genes whose altered forms are found to be involved in "strong predisposition", encode for the proteins of DNA damage repair or related pathways (i.e. *BRCA1* and *BRCA2*) (Heojimakers *et al.*, 2002). This is obviously due to the high number of studies investigating the impact of DNA damage or related pathway genes. But, considering the variety of the pathways in the cellular metabolism, other pathways and genes must also be studied.

The explanation provided by the investigations on the high penetrance genes for how cancers develop is very incomplete. For example, we still have no mechanisms for the tissue specificity of many of the inherited cancer syndromes (Balmain *et al.,* 2003).

Table 1.2. List of familial cancer genes and syndromes (Adapted from National Cancer Institute web site; "http://www.cancer.gov").

Gene	Cancer syndrome	Location	Discovery
APC	Familial polyposis of colon	5q21	1991
BRCA1	Hereditary Breast/Ovarian cancer	17q21	1994
BRCA2	Hereditary Breast/Ovarian cancer	13q12.3	1995
CDH1	Familial gastric sarcoma	16q22.1	1998
CDK4	Hereditary Melanoma 2	11q14	1996
CDKN2A	Cutaneous malignant melanoma	9p21	1994
CDKN1C	Beckwith-Weideman syndrome	11p15.5	1995
CYLD	Familial cylindramotosis	16q12-q13	2000
EXT1	Multiple exostoses type 1	8q24.1	1995
EXT2	Multiple exostoses type 2	11p12	1996
MADH4	Juvenile polyposis	18q21.1	1996
MEN1	Multiple endocrine neoplasia type I	11q13	1997
MET	Hereditary Papillary Renal Carcinoma	7q31	1997
MLH1	Hereditary non-polyposis colon cancer	3p21.3	1994
MSH2	Hereditary non-polyposis colon cancer	2p21	1993
NF1	Neurofibromatosis type 1	17q11.2	1990
NF2	Neurofibromatosis type 2	22q12.2	1993
PMS1	Hereditary Non-polyposis Colon Cancer3	2q32	1994
PMS2	Hereditary Non-polyposis Colon Cancer4	7p22	1994
PRKAR1A	Cancer complex	17q23-q24	1996
PTCH	Nevoid basal cell carcinoma	9q22.3	1996
PTEN	Cowden's syndrome	10q23.3	1997
RB1	Familial retinoblastoma	13q14.1	1986
RET	Multiple endocrine neoplasia MEN2A, MEN2B and	10p11.2	1993
SDHD	Familial paraganlioma	11q23	2000
SMARCB1	Rhabdoid predisposition syndrome	22q11	1996
TP53	Li-Fraumeni syndrome	17p13.1	1990
TSC1	Tuberous sclerosis 1	9q34	1996
TSC2	Tuberous sclerosis 1	16p13.3	1993
STK11	Peutz-Jegers syndrome	19p13.3	1997
VHL	Von Hipple-Lindau syndrome	3p25	1993
WT1	Familial Wilms' tumor	11p13	1990

1.1.6.2. Weak predispositon

Weak predisposition to cancer may in principle result from weak alleles of the pathway or caretaker genes. The study of weak predisposition is of interest both for its possible public-health implications and because just as the study of inherited cancer syndromes identified "pathway" genes, so weak predisposition may point to a wider range of processes that are relevant to cancer development, and to interactions between them.

The risk attributable to the effect of pathway genes and the low penetrance genes is more than the risk due to subtle sequence variants or polymorphisms (which can be associated with small to moderately risk for cancer). Low penetrance gene candidates are found in many pathways such as environmental carcinogen detoxification, steroid hormone metabolism and DNA damage repair. There is no doubt about the future of genetic medicine will allow us to identify more genes in several other pathways (Chakravarti, 2001).

Such factors modifying the probability are of extreme importance in sporadic cancers. They may be strongly associated either with disease susceptibility or with some other aspects of the disease phenotype, such as the treatment response of survival (Cardon *et al.*, 2001).

Predisposition by combinations of weak genetic variants may be of much greater significance to public health than the marked individual risks seen in inherited cancer syndromes (Pharoah *et al.,* 2002). Population based epidemiological studies have shown that only 15-20% of the observed familial clustering of breast cancer occurs in families that carry a strongly predisposing *BRCA1* or *BRCA2* mutation (Figure 1.5) (Balmain *et al.,* 2003). In principle, the remaining 80-85% of familial risk might have a genetic or environmental origin, but evidence from studies of breast cancer in twins (Peto, 2001) and the pattern of inheritance in families suggests that genetic factors predominate (Balmain *et al.,* 2003).

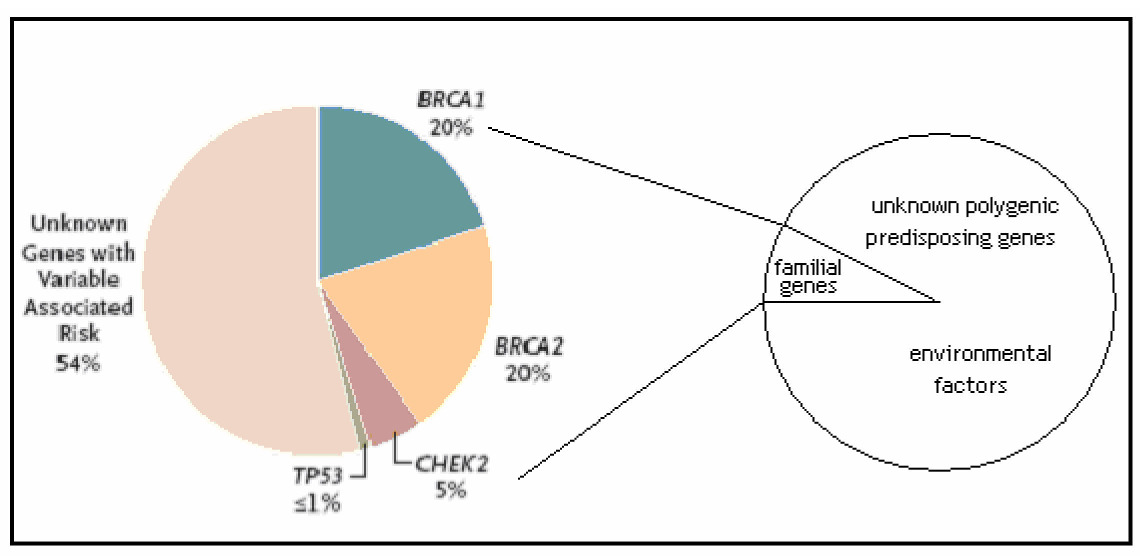


Figure 1.5. Breast cancer susceptibility genes (Adapted from Balmain *et al*., 2003).

1.2. Breast cancer

Breast cancer is the most commonly diagnosed cancer among women, after nonmelanoma skin cancer. It is the second leading cause of cancer deaths after lung cancer.

An estimated 211,300 new cases are expected to occur among women in United States during 2003. About 1,300 new male breast cancer cases are also expected. This year, 40,200 deaths from breast cancer will occur in United States only. It becomes the first leading cause of death among women with age between 15-54 (Atlanta, 2002).

In recent years, improved diagnostic tools have made it possible to detect breast cancers at early, even pre-invasive stages leading to a significant decrease in breast cancer mortality rates over the past decades. Despite all these improvements in the diagnostic tools of breast cancer, approximately a quarter of breast cancer patients die of their disease (Polyak, 2001).

1.2.1. Setting the stage

The mammary gland is a remarkable organ with respect to its development and functional differentiation. Unlike most mammalian organs, development of the mammary gland is primarily postpubertal. Mammary glands start to develop during the 4th week of gestation in mammals. Mammary epithelium is derived from epidermis, but the initiation is dependent on the presence of a specialized mesenchyma, called fat pad. Signals from mammary fat pads underlying the epidermis direct epidermal cells to a mammary differentiation pathway and induce their migration into the mammary fat pad (Figure 1.6, part A) (Polyak, 2001).

Although, the genes governing the mammary gland development are not clearly identified, the homeobox genes are suggested as the possible candidates based on their roles in the development of other organs. Recent genetic and expression analyses

clarified the functions of homeobex genes at specific transition points in the mammary gland development (Lewis, 2000).

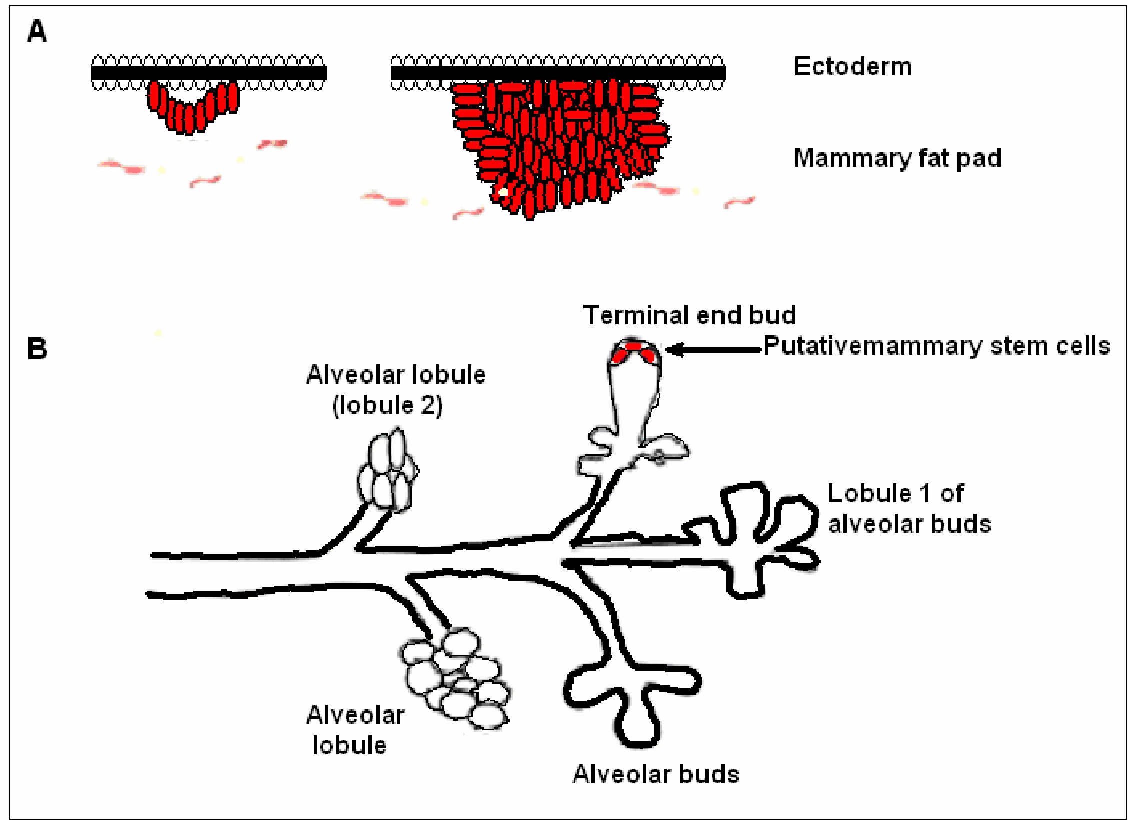


Figure 1.6. Mammary gland and its development (Adapted from Polyak, 2001).

Only few, poorly branched mammary ducts are formed during embryogenesis. The mammary gland remains in this rudimentary form until puberty. During puberty, hormones, particularly estrogen and progesterone, induce further elongation, branching and extension of the already existing ducts. This leads to the generation of lobules that contain a terminal duct splitting into alveoli. These lobules are relatively simple and not branched in nulliparous women (lobule 1 in Figure 1.6 part B). Fully mature gland (extensive branching, alvoelogenesis, and terminal differentiation) only occur during "full-term pregnancy" (Polyak, 2001).

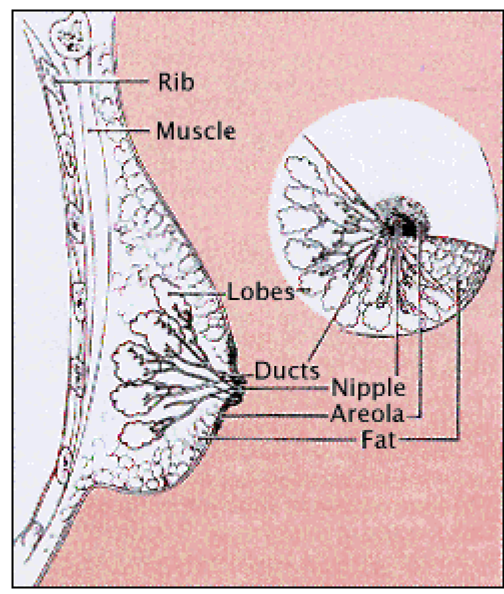


Figure 1.7. Main anatomic structures of breast (http://www.imaginis.com).

The smallest structure in breast is the tiny bulbs that can produce milk. These tiny bulbs form the lobules, and lobules forms the lobes. Ducts are the tiny tubes connecting the bulbs, lobules, and lobes. These ducts lead to the nipple in the center of a dark area of skin called areola.

The remaining part of the breast is filled with fat and vessels carrying the colorless fluid named "lymph". Muscle tissue is present only under the breast and covers the ribs.

The main origin of the breast carcinoma is the epithelial tissue of the mammary gland, including the milk-producing lobules and the ducts that carry milk to the nipple. The stromal, vascular, or fatty components of the breast are not generally included in the transformation process, excluding some very rare cases. The progression profile somehow reflects the clinics of the disease. For example, inflammatory breast cancer (IBC) is an aggressive form of locally advanced breast cancer that affects approximately 5% of women with breast cancer (Kleer *et al.,* 2000).

Breast cancer results from a combination of many factors including inherited mutations or polymorphisms of cancer susceptibility genes, environmental agents that influence the acquisition of somatic genetic changes and several other systemic and local factors (Figure 1.8) (Polyak, 2001). These factors may be grouped under sections of, *behavioral* (i.e. parity, life-style), *environmental* (i.e. chemicals, radiation), *systemic* (hormones, growth pressure, immune system), *local factors* (surrounding cells, autocrine factors, paracrine factors), and lastly *genetic* factors which is accepted as the major factor on the disease, since all the other factors may regulate and/or supplement the contribution of genetic factors.

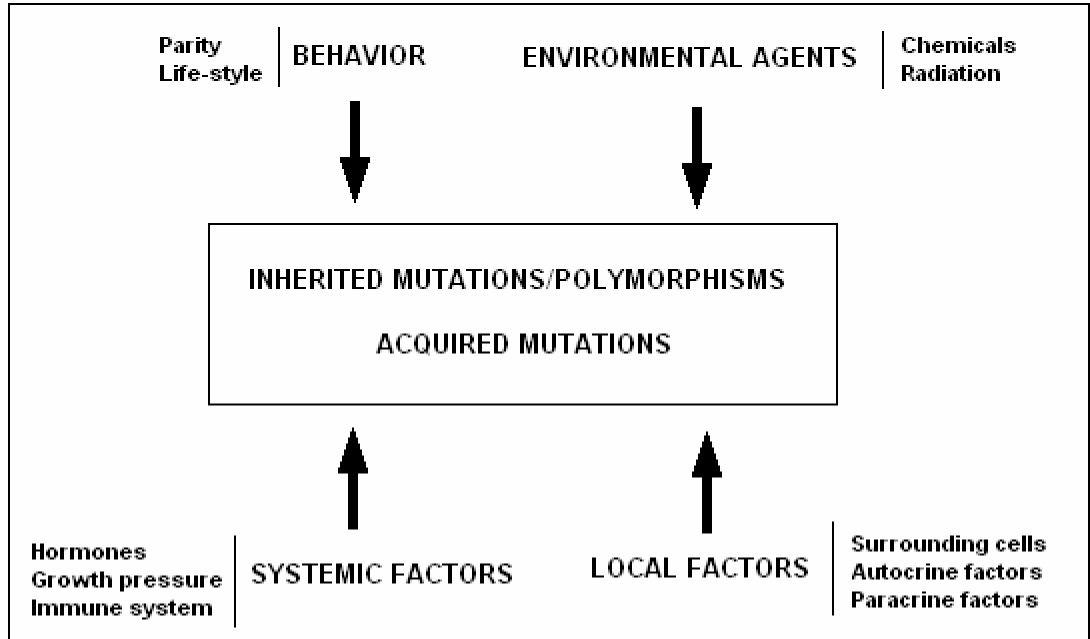


Figure 1.8. Summary of factors influencing breast carcinogenesis (Adapted from Polyak, 2001).

The factors listed in Figure 1.8 actually act on the development of breast cancer in various combinations. For example, when we consider, parity, we must also mention the effect of hormones. So, the factors mentioned in Figure 1.8 is summarized below.

The frequency of breast cancer is clearly shown to be associated with the body-mass-index (bmi) of the patient. Although the relationship between the bmi and the development of breast cancer is complex, the underlying factor is supposed to be the elevated levels of estrogen due to the production in adipose tissue (DeVita *et al*., 2001).

The development of breast cancer in many women appear to be related to the exposure of female reproductive hormones. Early age at menarche, nulliparity, late age at first full term pregnancy, late age at menopause increase the risk of breast cancer due to the hormonal exposure levels (DeVita *et al*., 2001).

The natural history of breast cancer involves a sequential progression through defined clinical and pathologic stages starting with atypical hyperproliferation, progression to in situ then invasive carcinomas, and culminating in matestatic disease (Figure 1.9 and Table 1.3) (Polyak, 2001).

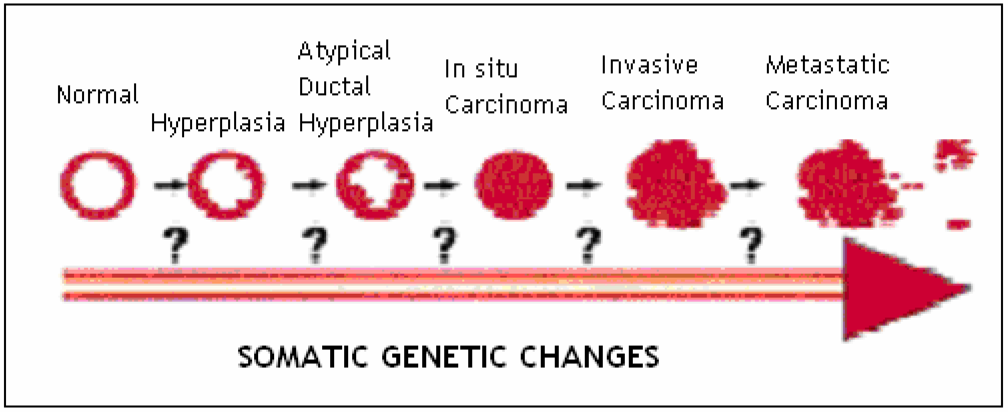


Figure1.9. A hypothetical multi stage model of breast carcinogenesis (Adapted from Polyak, 2001).

The stage at the time of diagnosis is very important in determining the treatment modalities and prognosis. So the staging of breast cancer is very important. Although many staging systems have been proposed, the most commonly used system is the one adopted by both the American Joint Committee (AJC) and the International Union Against Cancer (UICC). The staging system is a detailed TNM (tumor, nodes, metastasis) (Table 1.3).

Table 1.3. TNM Staging.

Stage 0	Carcinoma in situ
Stage I	Tumor 2 cm, axillary nodes not involved
Stage II	Tumor between 2 and 5 cm and/or involved but mobile axillary lymph nodes
Stage III	Tumor larger than 5 cm and/or fixed axillary lymph nodes; includes inflammatory breast cancer
Stage IV	Distant metastases beyond ipsilateral axillary lymph nodes

1.2.2. Genetics of breast cancer

In breast cancer, the risk to close relatives of a case, averaged across all ages, is about two-fold (Ponder, 2001). 5-10% of the cases have a first- or second-degree relative with the disease. The remaining nearly 90% of cases are sporadic (non-inherited) (Figure 1.5) (Wooster, 2003).

The hereditary breast and ovarian cancer syndromes are shown to involve genetic alterations in various susceptibility genes such as *BRCA1, BRCA2, p53, ATM, PTEN* or *MSH2, MLH1, PMS1, MSH3,* and *MSH6* (Palevic, 2001). Two of these are regarded as the major susceptibility genes, breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*) (Venkitaraman, 2002). However, mutations in these genes account for only 2 to 3 percent of all breast cancers, which indicates the presence of other susceptibility genes (Wooster, 2003).

Recently, the structure and expression of *CHEK2* was analyzed in breast cancer. *CHEK2* was found to be implicated in a significant proportion of sporadic breast cancers, but unlikely to represent a susceptibility gene for a high proportion of hereditary breast cancer (Sullivan *et al.*, 2002). In conclusion, *CHEK2* 1100DelC variant is a low penetrance, and low frequency predisposing allele (Offit *et al*., 2003). More recent experiments stated the association of this variant and prostate cancer risk (Meijers-Heijboers *et al*., 2003, and Dong *et al.*, 2003).

1.2.2.1. Somatic mutations in breast cancer

Studies of sporadic breast cancers led scientists to understand the pathogenetic mechanisms underlying the development of breast cancer. Approximately 90% of all breast cancer cases are sporadic. The genes coding for growth factors and receptors, intracellular signaling molecules, regulators of cell cycle, genome maintenance mechanisms, adhesion molecules and proteases are the first targets of the somatic mutations. Some examples are:

- The tumor suppressor protein p53 plays a central role in regulating progression through cell cycle and the genome maintenance. p53 mutations have been detected in 15-45% of human breast cancer specimens in several studies.
- Cyclin proteins are regarded as the central regulators of cell cycle progression, which are also shown to be over expressed in breast cancer (Evan, 2001).
- The proto-oncogene bcl-2 and c-myc which suppress apoptosis over expressed in 30-45% of breast cancer cases (Evan, 2001).
- Frequent alterations of the FHIT locus in breast cancer, suggest its role in the pathogenesis of breast tumors. FHIT protein was shown to be directly involved in the control of cell growth and/or proliferation. (Ingvarsson, 2001).

1.2.2.2. Germline mutations in breast cancer

Clinical investigations of familial aggregation of breast cancer have identified several genetic syndromes with an autosomal dominant pattern of inheritance that features breast cancer (Tonin, 2000). Breast cancer cases due to germline mutations have several distinctive clinical features. For example, age-of-onset is relatively low than sporadic breast cancer, the prevalence of bilateral breast cancer is higher, and in the presence of associated tumors in affected individuals is noted in some families. Associated tumors may include ovarian, colon, prostate, and endometrial cancers and sarcomas. However, inherited breast cancer does not appear to be distinguished by histologic type, metastatic pattern, or survival characteristics (Vogelstein *et al.*, 1998).

The syndromes in which genes are known or are suggested to cause inherited breast cancer and other cancers are shown in Table 1.4.

Table 1.4. Hereditary cancer syndromes that feature breast cancer (Tonin, 2000).

Sydrome	***Gene***	***Manifestations***
Breast-ovarian cancer (MIM # 113705)	BRCA1	Breast (female and male), ovarian and pancreas cancers
	BRCA1 & BRCA2	Breast cancer (female and male)
Li-Fraumeni syndrome (MIM # 151623)	TP53	Sarcoma, leukemia, breast, brain and adrenal cancers
Cowden disease (MIM # 158350)	PTEN	Breast and thyroid cancers, multiple hamartomas of skin and gastrointestinal tract
Ataxia telangiectasia (MIM # 208900)	ATM	Leukemia, lymphoma, breast cancers

BRCA1 and BRCA2

The existence of the *BRCA1* gene, which predispose to breast cancer, was demonstrated by linkage analysis in 1990 (Hall *et al*., 1990). Using polymorphic markers, which would distinguish the parental origins of alleles and are representative of different chromosomal regions, linkage was established to the long arm of chromosome 17 at region q21. Families with early age of onset (pre-menopausal) of breast cancer were more likely to be linked to the *BRCA1* locus. Through an intense cloning effort, the identity of *BRCA1* was discovered in 1994 (Miki *et al*., 1994, and, Brown MA, 1995). In the following year, a human *BRCA1* gene knockout (Boyd, 1995) and the aberrant subcellular localization was identified (Chen *et al*., 1995).

In addition, linkage analyses provided sufficient evidence for the presence of another susceptibility gene (Wooster *et al.*, 1994), which was identified about a year later (Wooster *et al.*, 1995, and, Tavtigian *et al.*, 1996). Germ-line mutations in *BRCA1* and *BRCA2* have been reported in at least two syndromes that feature breast-cancer: site-specific breast cancer and breast-ovarian cancer syndrome (Table 1.14). The striking feature common to families of both syndromes is the young age of onset of breast cancer (Tonin, 2000).

Population-based studies have reported lower risks of breast and ovarian cancer in mutation carriers. It has been suggested that other factors may modulate the risk in mutation carriers, and may account for the reduced penetrance. Recent studies have shown that lifestyle choices such as smoking may modulate the risk of breast cancer in mutation carriers. More than 100 mutations in each gene have been described to date, and the majority of the mutations is private and reported in only one family (Please refer to the Breast Information Core Data Base).

BRCA1 is comprised of 5.592 nucleotide pairs with 24 exons. *BRCA2* is comprised of 10,254 nucleotide pairs and 27 exons. The coding sequences of both genes are spread across large tracts of DNA, comprising more than 1,000,000 nucleotides. The large size and complexity of each gene, and the absence of "hot-spots" for mutations, have made sequence analysis an ardous and costly endeavor.

TP53

Li-Fraumeni syndrome (LFS), now known to be associated with germ line mutations in TP53, was first identified as a syndrome in 1969 in a description of four kindreds in which cousins or siblings had childhood soft-tissue sarcomas and other relatives had excessive cancer occurrence (Vogelstein *et al.*, 1998). Underlying genetic defect in the Li-Fraumeni syndrome is a germline mutation in the *TP53* gene (MIM# 191170) as first described by Malkin *et al.,* in 1990. But now, there are nearly 250 independent germ-line *TP53* mutations in numerous publications.

Li-fraumeni syndrome is associated with a variety of different tumor types occurring over a wide age range, including childhood. The definition of LFS originated from Li and Fraumeni's work as a proband with a sarcoma aged under 45 years with a first-degree relative aged under 45 years with any cancer, plus an additional first- or second-degree relative in the same lineage with any cancer aged under 45 years or a sarcoma at any age (Li *et al*., 1988). Now, LFS is defined as a proband with any childhood tumor, or a sarcoma, brain tumor, or adrenocortical tumor aged under 45 years plus a first- or second-degree relative in the same lineage with a typical LFS tumor at any age, and an additional first- or second-degree relative in the same lineage with any cancer under the age of 60 years (Varley, 2003).

Bone and soft-tissue sarcomas, premenopausal breast carcinoma, brain tumors, adrenocortical carcinomas and leukemias are the first identified tumors of LFs. Subsequent studies reported wider range of tumors such as melanoma, Wilm's tumor, and lung, gastric, and pancreatic carcinoma (Varley, 2003).

The cellular role of p53 is well characterized. p53 is a sequence specific DNA binding protein, that functions as a transcription factor. The sequence specific transcription factor activity appears to be essential for its role as a tumor suppressor (Picksley *et al.*, 1994). The impact of p53 on multiple cellular functions such as gene transcription, DNA synthesis and repair, cell cycle arrest, senescence, and apoptosis is well documented (Hussain *et al*., 2001). The phosphorylation status of the protein is found to be regulating its function (Prives *et al.*, 2001).

ATM

ATM (ataxia telangiectasia mutated) is one of the key proteins involved in the cellular response to DNA damage. In the autosomal recessive disorder ataxia telangiectasia (A-T) ATM protein is defective. The heterozygous *A-T* gene carrier frequency in the population is ~1% and the disease incidence is ~1/40000. Affected individuals develop progressive cerebellar ataxia (loss of balance and coordination) such that most are wheelchair bound by their early teenage years. Telangiectasias are tortuous dilated blood vessels that develop in the eyes and sun-exposed skin. A-T is associated with a 30–40% lifetime risk of developing a malignancy, usually of lymphoid origin and occurring in childhood. And relevant studies showed that women with *ATM* mutation have an elevated risk of developing breast cancer. A-T individuals are also more susceptible to infections, and aspiration pneumonia is a common cause of death. Life expectancy is reduced, with a median age at death of ~30 years (Levitt *et al.,* 2002).

PTEN

Cowden disease is best characterized by multiple hamartomatous lesions, especially of the skin, mucus membranes, colon, breast, and thyroid, and multiple facial trichilemmomas. Hamartomatous polyps of the colon also occur, and there are neoplasms of the thyroid and breast. Family-based analysis suggested an autosomal dominant mode of inheritance with high penetrance in both sexes, and a high frequency of breast cancer (up to 30%) in females. Linkage analysis of Cowden disease families revealed a locus on chromosome 10q22-23. *PTEN* was the strongest candidate gene that mapped to this interval on chromosome 10, and was previously shown to harbor somatic mutations in a number of tumor types, particularly breast cancer, that feature in Cowden disease. Therefore, a combination of linkage analysis and candidate gene approaches led to the discovery that individuals with Cowden disease harbored germline mutations in *PTEN*. Although the reported mutations are dispersed throughout the gene, there is a tendency of mutations to cluster in exon 5 (Tonin *et al.,* 2000).

1.3. RNASEL

RNASEL (MIM# 180435) encodes for the ubiquitously expressed ribonuclease L (RNase L). The *RNASEL* gene maps to the hereditary prostate cancer (HPC) predisposition locus at 1q24-q25 (*HPC1*).

1.3.1. Prostate cancer

Prostate cancer (PC) is the second leading cause of cancer deaths in men >50 years of age and the most frequent visceral cancer in males (Silverman, 2003). Prostate cancer is a significant international public health problem, with a world-wide estimate of 239,000 deaths resulting from this disease annually, in the U.S. only (Xu *et al.*, 2000).

The prostate is a walnut-sized gland of the male reproductive system located beneath the bladder and in front of the rectum that produces and stores the seminal fluid (Silverman, 2003). Precursor lesions known as prostate intraepithelial neoplasia (PIN) can progress after many years of overt carcinoma and finally to metastatic cancer (Figure 1.12) (Abate-Shen *et al.*, 2002). The most common sites for metastasis are lymph nodes and bones (pelvis and axial skeleton) (Silverman, 2003).

Aging, hormonal, environmental, and genetic factors are all believed to play roles in the pathogenesis of prostate cancer.

This cancer type usually appears after the sixth decade, and so it is generally considered as a disease of aging. Prostate cancer is diagnosed in very few people younger than 50 years (<0.1% of all patients). The mean age of patients with this disorder is 72-74 years, and about 85% of patients are diagnosed after age of 65 years (Grönberg *et al.*, 2003).

Prostate cancer is rare in males castrated before puberty and the tumor growth is inhibited by orciectomy or chemical hormone-ablation theraphy. Also, there is a large body of evidences indicating the role of “androgen signaling system” in the development of prostate cancer (Grossman *et al.*, 2001).

Environmental causes are found to be implicated in prostate cancer development by the geographic data on prostate cancer incidence and observations that relative risk of developing prostate cancer is associated with migrations between low and high incidence regions of the world (Siverman, 2003).

It has been recognized for some time that prostate cancer tends to cluster in families (Wang *et al.*, 2002). Remarkably, men with three or more first degree relatives with prostate cancer have a 100-fold increased risk compared with men that have no family history of prostate cancer (Silverman, 2003). Segregation analysis suggests that this familial clustering can best be explained by at least one rare dominant susceptibility gene (Wang *et al.*, 2002). And this dominant susceptibility gene must be rare, autosomal, highly penetrant for the hereditary prostate cancer with early onset (Silverman, 2003). However, there is also a considerable evidence on the presence of a complex genetic basis, involving multiple susceptibility genes and variable phenotypic expression (Simard *et al.*, 2002). On the basis of linkage studies of families with high risk of PC, six PC-susceptibility loci were identified (Eeles *et al.*, 1998, and, Wang *et al.*, 2002).

1. *HPC1* (1q24-25),
2. *HPCX* (Xq27-q28),
3. *PCAP* (1q42),
4. *CAPB* (1p36),
5. *HPC20* (20q13), and
6. *HPC2* (17p11) (reviewed in Ostrander *et al.,* 2000).

HCP1 was the first such prostate cancer locus, mapped in 1996 to chromosome 1q24-25 (Smith *et al*., 1996). Initial gene mapping studies placed *RNASEL* and several other genes in the critical *HPC1* region in chromosome 1q25 (Carpten *et al*., 2000). *HPC2* was mapped to 17p11 (Tavtigian *et al.*2001, and, Suarez *et al.*, 2001).

To overcome limitations due to genetic heterogeneity and a low frequency of mutations in any particular susceptibility gene, the International Consortium for Prostate Cancer Genetics (ICPCG) performed a joint analysis from 722 families. They have confirmed linkage of hereditary prostate cancer to the *HPC1* locus (Xu, 2000, and, Xu *et al.*, 2001). A second important study was performed with 2410 individuals, including 662 men with prostate cancer compared several potential prostate cancer susceptibility loci (*HPC1*, *PCAP*, *HPCX*, and *CAPB*). They have demonstrated that only *HPC1* commonly segregated within families with the most severe cases of prostate cancer (Goode *et al.*, 2001).

The linkage of *HPC1* to *RNASEL* suggests that RNase L directly or indirectly suppresses one or more steps is prostate tumorigenesis and/or metastasis (Figure 1.12).

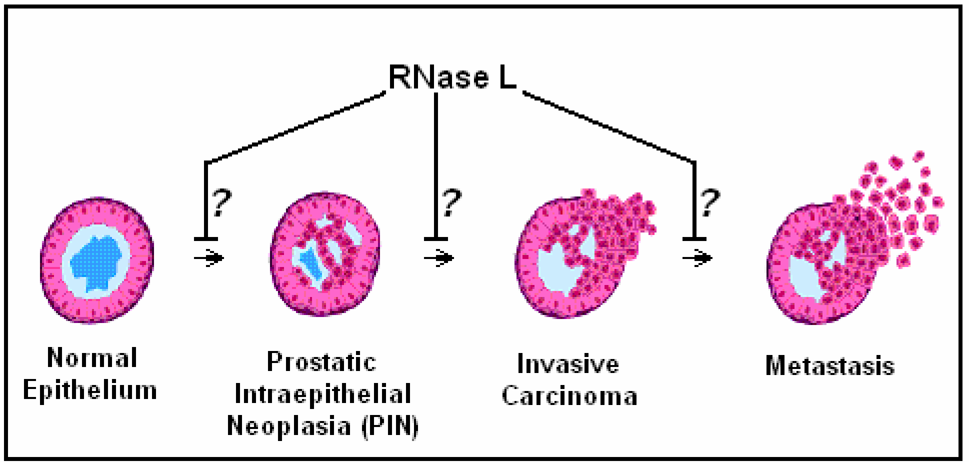


Figure 1.10. Possible role of RNase L in prostate carcinogenesis

(Adapted from Silverman, 2003).

1.3.2. RNase L

RNase L is a fascinating tightly regulated endoribonuclease of higher vertebrates that plays essential roles in mediating diverse types of cellular responses (Zhou, 1993). The activation of RNase L requires the production of unusual effector molecules, 2',5'-linked oligoadenylates, $p_{1\text{-}3}A(2'p5'A)_{>=2}$ (2-5A) (Dong *et al.*, 2001). 2-5As are produced from ATP by 2-5A-synthetases (OAS enzymes). The genes coding for OAS enzymes are activated upon interferon treatment of mammalian cells (Dong *et al.*, 1997).

OAS enzymes were discovered in the mid-1970s by I.M.Kerr and colleagues. They are found to be activated by double stranded RNA (dsRNA). They convert ATP to PP_i and a series of short 2' to 5' linked oligoadenylates, collectively referred to as 2-5As (Figure 1.11) (Silverman, 2003).

IFN treatment of the cells activates the JAK-STAT pathway which also activates the expression of OAS genes (Stark *et al.*, 1998). In humans, there are four related genes (*OAS1*, *OAS2*, *OAS3*, and *OASL*) encoding eight or more isoforms as a result of alternative splicing (Silverman, 2003).

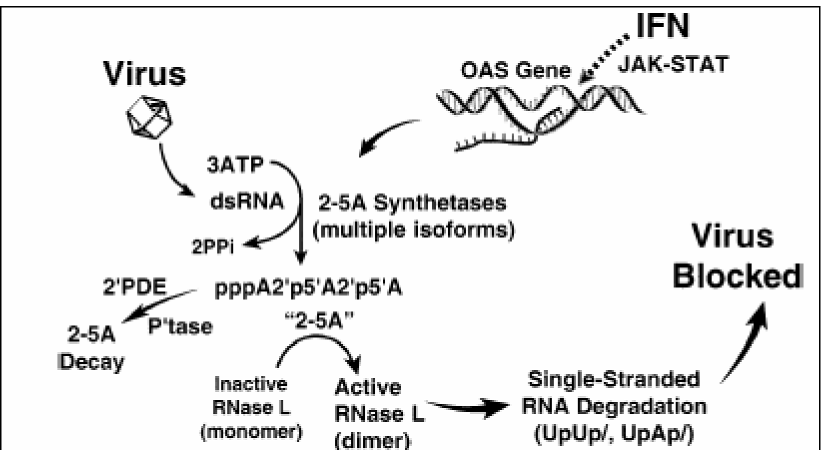


Figure 1.11. Role of RNase L in the antiviral activity of IFNs (Silverman, 2003).

Up to date, the only well-established biochemical function of 2-5A is the activation of RNase L (Zhou *et al.*, 1997). The significance of the dsRNA requirement for 2-5A synthetase activity is that it is a common intermediate or by product of viral infections.

RNaseL protein consists of 741 amino acids. RNase L has been detected only in reptiles, birds and mammals. Only mouse and human RNase L sequences are available. The presence of RNase L was shown for many mammalian tissues.

C-terminal region contains the RNase domain and the kinase-like domain. N-terminal domain represses the ribonuclease domain in the C-terminal region in the absence of 2-5A. The N-terminal region of the protein is called the repressor part. It contains 9 ankyrin repeats. Anykrin repeats are common protein/protein interaction domains. The presence of two P-loop motifs (GTK) is the characteristic property of RNase L. Because the Lysine residues in these regions are the sites of 2-5A binding (Figure 1.12) (Silverman, 2003).

A kinase domain was assigned to the C-terminal region of the protein based on the sequence comparisons. But this assignment lacks experimental evidence. On the contrary, experimental evidence suggests that the kinase like motif is implicated in enzyme dimerization (Dong *et al.,* 1999), which is the crucial step in enzyme activation (Figure 1.12) (Silverman, 2003).

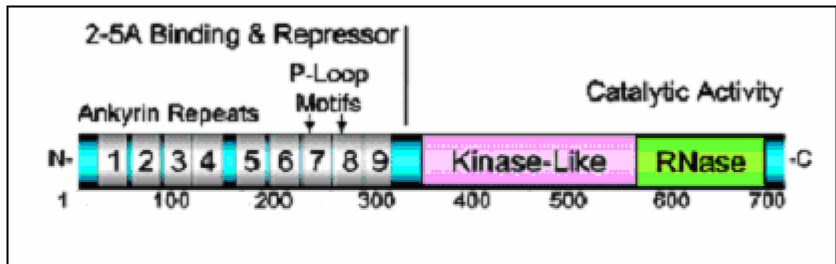


Figure 1.12. RNase L protein structure (Adapted from Silverman, 2003).

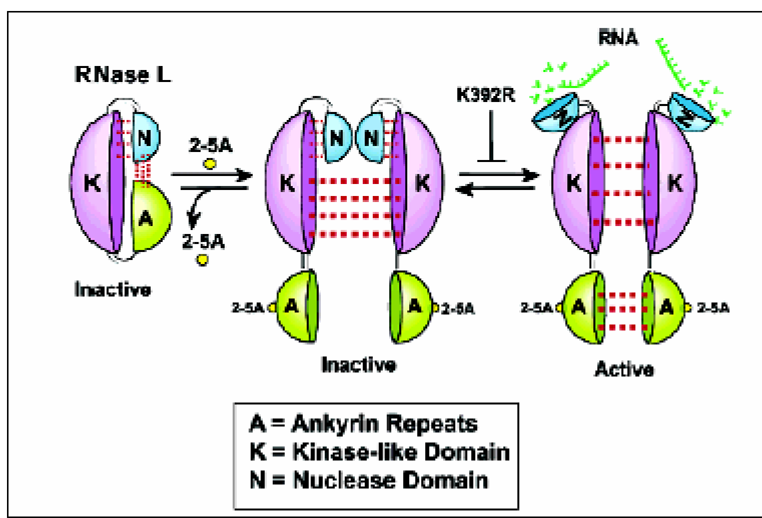


Figure 1.13. Functional model for the activation of RNase L by 2-5A (Adapted from Silverman, 2003).

The binding of 2-5As leads to the formation of a potent dimeric endoribonuclease (Dong *et al.,* 1995). 2-5A binding to the P-loops relieves binding of repressor domain. This conformational change ceases the inhibition by the ankyrin repeats on the dimerization and ribonuclease domains. Accessible dimerization domains enables the dimerization of the enzyme, which enables formation of active enzyme (Dong *et al.,* 2001).

Cleavage sites for the RNase L enzyme are UpNp dinucleotide sequences (primarily UU and UA) (Silverman, 2003).

Considering the production of 2-5As and the 2-5A dependent activation of the enzyme, it may be concluded that the RNase L action is located in the vicinity of the dsRNA. This enables specificity to the system to degrade only the viral RNA. The experimental evidence is the preferential degradation of viral RNA in comparison to cellular RNA in EMCV-infected cells (Li *et al.,* 1998). Along with PKR, RNase L constitutes the antiviral arm a group of mammalian stress response proteins (Williams, 1999).

The other important cellular role of RNase L is the initiation of apoptosis. Presumably, this function of the enzyme is also atributable to RNA degradation activity of the enzyme.

The degradation of 28S and 18S rRNA by RNase L in intact ribosomes has been long known as a hallmark of IFN and viral infections. Cleavage of 28S rRNA by RNase L maps to the L1 protuberance implicated in the formation of the exit of E site of the ribosome, possibly interfering with the release of deacetylated tRNA (Iordanov *et al.,* 2000).

The possible sequence specific gene silencing activity of RNase L was also investigated. Suggested mechanism involves the endoribonucleolytic activity of RNase L directed towards a specific mRNA molecule. Antisense oligonucleotides conjugated with 2-5A sequences are the mediators of the mRNA degradation. Where, the antisense oligonucleotide provides the mRNA specificity, and the 2-5A molecule activates the enzyme (Torrence *et al*.,1993).

The involvement of possible RNA decay pathways in the repression of tumor development is not a new idea. The ribonuclease, onconase, the N-glucosidase ricin A chain that attacks ribosomal RNA, and the anti-FLT-1 (VEGF receptor) ribozyme, angiozyme, have been explored as cancer therapeutics in clinical trials with varying success (Weng *et al.,* 2001, Mikulski *et al.,* 2002, and Schnell *et al.,* 2002).

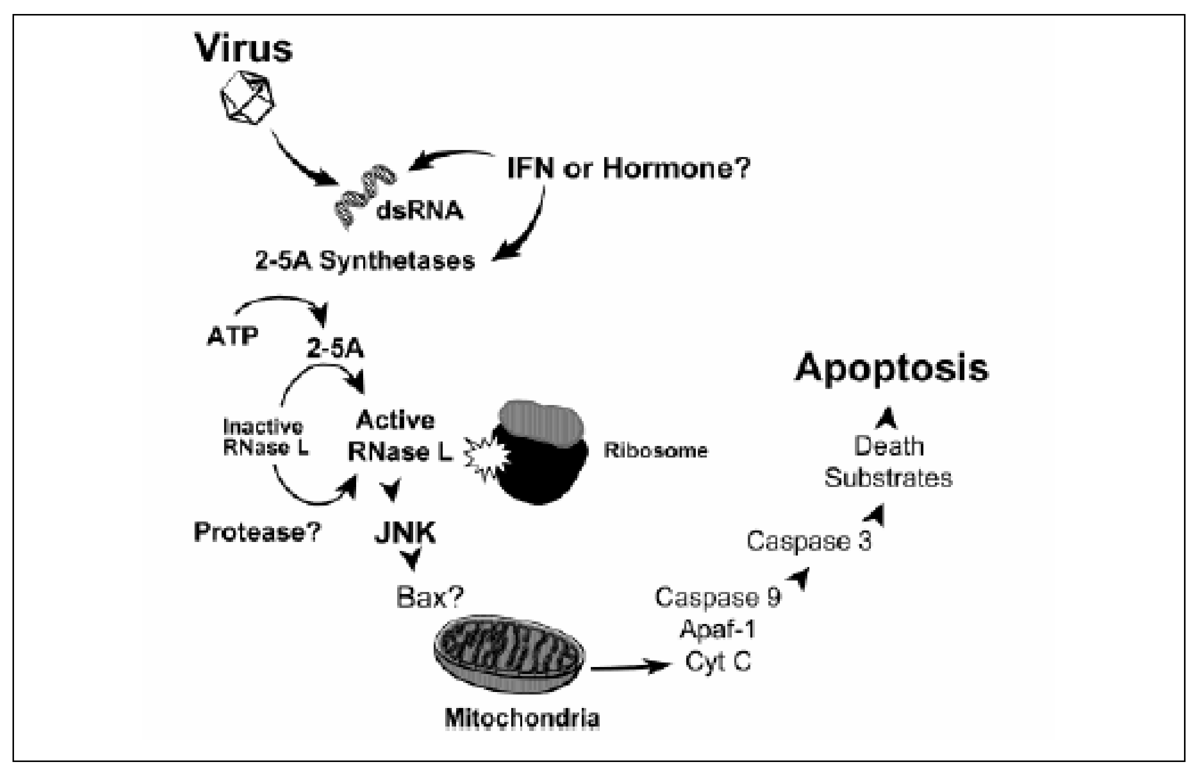


Figure 1.14. The pro-apoptotic role of RNase L (Silverman, 2003).

An RNaseL-based approach might have certain advantages in the treatment of cancers. RNase L is a candidate tumor suppressor that is normally dormant but whose antitumor activity can be activated by a small molecule, 2-5A. It is also possible to target RNase L to particular cancer associated RNAs, such as telomerase RNA, by linking 2-5A to antisense (Kondo *et al.,* 1998). In cancers where RNase L is present, including many prostate tumors, its activation by a 2-5A analogue might produce an antitumor response as was demonstrated in a mouse model of human prostate cancer (Kondo *et al.,* 2000).

1.3.3. *RNASEL*

Ubiquitously expressed *RNASEL* consists of eight exons. Northern blot analysis showed that there are two mRNA species of 5 kb and 9.5 kb in the spleen, thymus, prostate, testis, uterus, small intestine, colon and peripheral blood leukocytes. Expression level varies according to the tissue, with the highest expression in the spleen and thymus.

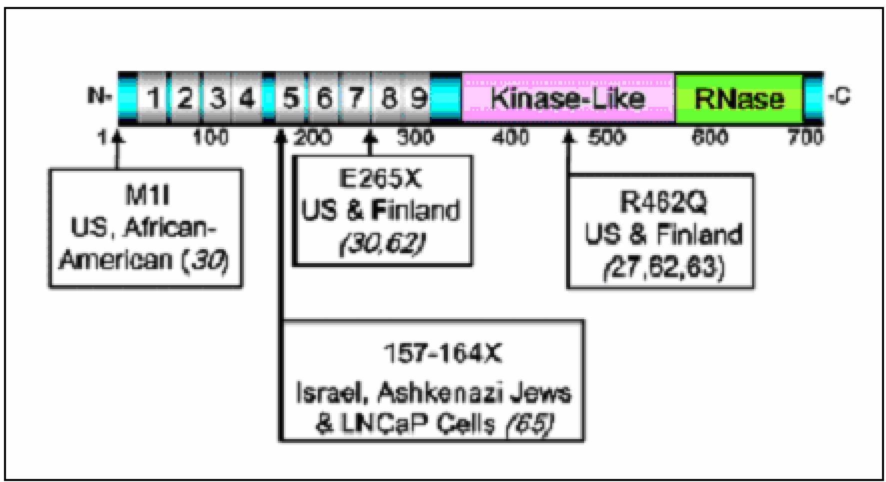


Figure 1.15. *RNASEL* (Silverman, 2003).

RNASEL in cancer genetics

RNASEL has been proposed as a candidate tumor suppressor after the involvement of RNase L in the antiproliferative activity of interferons was represented. The location of the *RNASEL* in 1q25, a region found to be deleted or rearranged in some breast cancers was the second evidence (Hassel *et al.,* 1993, Lengyel, 1993, and Squire *et al.,* 1994). Furthermore, RNase L was shown to be deficient in human leptoma cell line HEPG2 (Tnani *et al.,* 1998). The important studies regarding the impact of *RNASEL* in cancer genetics are summarized below.

The first in vivo evidence of RNase L as a tumor suppressor was the identification as the candidate for *HPC1*. *RNASEL* was identified by using a combination of recombination mapping and candidate gene analysis. Nonsense mutations and mutation in initiation codon were shown to segregate independently in two HPC1-linked families (Carpten *et al*., 2002).

The second important study was performed in 116 Finnish families with HPC, 492 patients with PRCA, 223 patients with benign prostatic hyperplasia (BPH), and 566 controls. In addition to 4 previously identified variations in *RNASEL* (Carpten *et al*., 2002), they have identified 3 more variants in 66 patients with HPC. Neither E265X nor R462Q was found to be sufficient for the familial clustering of the disease. But, both mutations were shown to effect age of onset in HPC patients (Rökman *et al.,* 2002).

Third study concerns the effects of 1385G→A (resulting in R462Q variant), and 1623T→G (resulting in D541E), on the enzymatic activity of RNase L and their possible association with HPC. Enzymatic activity of the D541E variant was shown to be identical to the wild-type of the enzyme. But the enzymatic activity of R462Q variant was shown to be three times lower than the wild type of the enzyme. Relying on the functional significance of the R462Q variation, the association of this variant to HPC was investigated in a family based case-control study. Each control was chosen to have an effected relative involved in the study as case. By using a standard statistical analysis of matched data (conditional logistic regression), a log additive model was found to best fit the results. This implied the significant association between the *RNASEL* 1385G→A variant and prostate cancer (P=0.011). The association was a little bit more significant among men of European descent only (P=0.007). The values of corresponding odds ratios suggest that carrying one copy of the mutated allele increases risk of prostate cancer by an approximately doubling a man's risk of developing this disease. Their study reported that approximately 13% of prostate cancer cases in population may be attributable to 1385G→A mutation (Casey *et al.,* 2002).

The conflicting conclusions about the impact of R462Q variant in two studies (Rökman *et al.,* 2002, and Casey *et al.,* 2002), may be attributable to the sampling methods of the studies. This underlines the possible impact of familial links.

Three *RNASEL* variants, I97L, R462Q, and D541E, were investigated in 499 sporadic cases and 510 controls. The variants I97L and D541E were shown not to be associated with the disease. R462Q variant was shown to be associated with familial (*P*=0.02) but not sporadic prostate cancer (*P*=0.92) incidence (Wang *et al.,* 2002).

A recent study reported a founder null mutation in Ashkenazi Jewish men, 471delAAAG. But the results of that study is not found as statistically significant and needs further support (Rennert *et al.*, 2002).

The results of these studies are summarized in Table 1.5.

Table 1.5. Summary of *RNASEL* sequence variants implicated in HPC.

Variant	Exon	Codon	Position & Change
175G→A	2	59	175; G→A (GGC→AGC / Gly→Ser)
793G→T	2	265	793; G→T (GAG→TAG / Glu→Stop)
1179G→A	2	393	1179; G→A
1217C→T	2	406	1217; C→T (TCT→TTT / Ser→Phe)
1385G→A	2	462	1385; G→A (CGA→CAA / Arg→Gln)
1623T→G	4	541	1623; T→G (GAT→GAG / Asp→Glu)
2172G→A	7	724	2127; G→A

The complete role of RNase L in the cellular metabolism is not fully understood yet. But the dependence of the critical balance between hormonally regulated cell growth and cell death on the pathways of this enzyme appears as a stong evidence for the impact of this gene in the tumor development. It is rational to speculate that the absence of the full-capacity of this enzyme may shift the balance towards the growth of the cells, which will lead to a favorable environment for the development of cancer. In conclusion, *RNASEL* is suggested as a candidate tumor-suppressor gene due to the known cellular functions of RNase L, and the cancer association studies.

1.4. Our Aim

We sought to investigate the hypothesis that, Arg462Gln variant of *RNASEL* is associated with breast cancer risk based on the following observations and considerations:

1. The chromosomal location of *RNASEL* (1q25) in a region (1q23-q32) that was found to be implicated in breast cancer (Chen *et al.*, 1989),
2. The cellular function of *RNASEL*, and its involvement in pro-apoptotic and antiviral activities (Silverman, 2003),
3. The pleitopic effects of cancer associated mutations, as exemplified by *BRCA1* in both breast and ovarian cancers or *CHEK2* in both breast and prostate cancers (Dong *et al.*, 2003),

For this purpose, genotyping analysis of 835 individuals (453 affected and 382 controls) representing two Eastern Mediterranean populations, Turkish and Greek, was performed and the possible association between this variant and breast cancer risk was investigated.

2. Materials and Methods

2.1. Materials

2.1.1. DNA samples and collaborators

DNA samples (453 affected and 382 controls, total 835) genotyped in the study were kindly provided by our collaborators from Turkey and Greece.

List of participants:

1. Bilkent University – Department of Molecular Biology and Genetics, 06800, Ankara, Turkey; Işık G. Yuluğ, Gülsen Çolakoğlu.
2. Gazi University –Faculty of Pharmacy, 06330, Ankara, Turkey; Ali Esat Karakaya, Semra Sardaş, and Neslihan Aygün Kocabaş.
3. Akdeniz University – Departments of Medical Biology and Genetics, and Surgery, Faculty of Medicide, 07070, Antalya, Turkey; Esra Manguoğlu, Güven Lüleci, Taner Çolak.
4. Ankara Numune Research and Teaching Hospital – Department of Surgery, 06100, Ankara, Turkey; Betül Bozkurt and Ömer Cengiz.
5. Molecular Diagnostic Laboratory, I/R-RP, National Center for Scientific Research Demokritos, Ag. Paraskevi Attikis, 15310, Athens, Greece; Drakoulis Yannoukakos, Irene Konstantopoulou.
6. Institute of Biology, National Center for Scientific Research Demokritos, 15310, Athens, Greece; Voutsinas Gerassimos, George Nasioulas.
7. Molecular Biology Research Center "HYGEIA" – "Antonis Papayiannis", 15123, Athens, Greece; Eirene Papadopoulou.
8. Alfalab, Molecular Biology and Cytogenetics Center, 11524, Athens, Greece; Lina Florentin.
9. IVF & Genetics, 15232, Athens, Greece; Elena Kontogianni.

2.1.2. Study population

Our study population consisted of 835 females (mean age: 49.23, standard deviation: 13.37, age range: 15-88); divided into two groups of effected individuals previously diagnosed with breast cancer (n=453) and control individuals with no history of breast cancer (n=382). 301 affected and 218 control individuals were from Turkey, and, 152 affected and 164 control individuals were from Greece (Figure 2.1). The details of the groups are presented in the following sections.

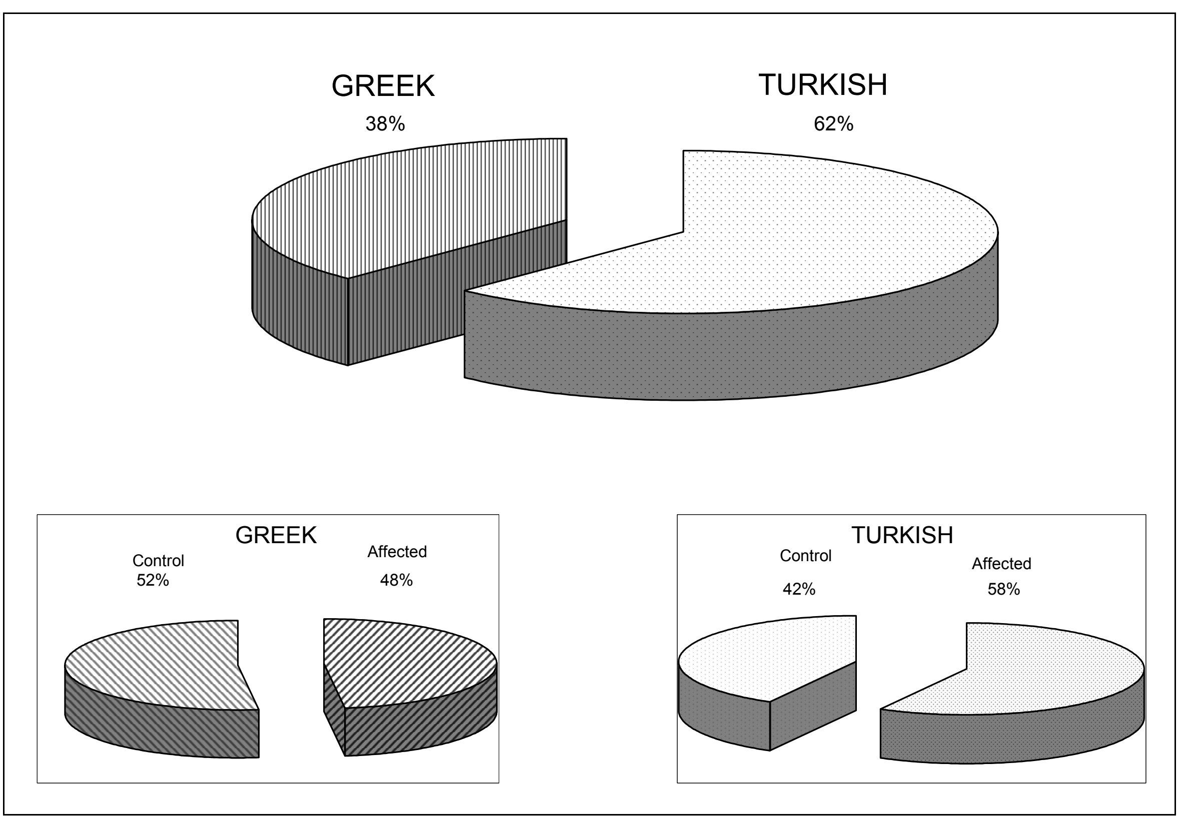


Figure 2.1. Cohort facts.

Informed consent, blood samples and personal information was obtained from all Turkish participants. At the time of blood donation, each individual completed a standardized questionnaire (Figure 2.2) including data on age, weight, height, menstrual and reproductive histories, family history of breast and other cancers (first degree relatives; only mother, sister or daughters) and smoking status. Information about the histopathology of the tumors, estrogen receptor status, and progesterone receptor status were obtained from the medical records. The clinical information of the Greek participants were not available except for the age and menopausal status, which were kindly provided by our collaborators.

Selected characteristics of our study population are summarized in Table 2.1.

2.1.2.1. Patients

Our patient group consisted of 453 individuals previously diagnosed with breast cancer (invasive breast carcinoma, mean age: 49.65, standard deviation: 12.95, age range: 20-86). The individuals were from two countries, Greece and Turkey. Greek patients (n = 152, mean age: 50.04, standard deviation: 15.02, age range: 20-86) were diagnosed with breast cancer at the institutions 5-9 listed in Section 2.1.1. Turkish patients (n = 301, mean age: 49.55, standard deviation: 12.50, age range: 20-80) were diagnosed with breast cancer at the institutions 1-4 listed in Section 2.1.1.

The patient group was divided into two groups of premenopausal (n = 203, mean age: 40.29, standard deviation: 7.82, age range: 20-58) and postmenopausal (n = 250, mean age: 57.40, standard deviation: 11.15, age range 31-86).

2.1.2.2. Controls

Our control group consisted of 382 individuals, with no history of breast cancer (mean age: 48.84, standard deviation: 13.68, age range: 15 - 88). The individuals were from two countries, Greece and Turkey. Greek control individuals (n = 152, mean age: 50.83, standard deviation: 13.29, age range: 24-89) were from institutions 5-9 listed in Section 2.1.1. Turkish control individuals (n = 218, mean age: 47.45, standard deviation: 13.84, age range: 15-83) were from the institutions 1.4 listed in Section 2.1.1.

Control group was also divided into two groups of premenopausal (n=180, mean age: 37.91, standard deviation: 8.05, age-range: 15-52), and post menopausal (n=202, mean age: 58.55, standard deviation: 9.77, age range: 30-88).

Table 2.1. Selected characteristics of our study population.

	Case (n=453)		**Control (n=382)**	
	Turkey (n=301)	**Greece (n=152)**	**Turkey (n=218)**	**Greece (n=164)**
Age mean (standard deviation)	49.55 (12.50)	50.04 (15.02)	47.45 (13.84)	50.83 (13.29)
Age range	20-80	20-86	15-83	(24-88)
Age at first birth, mean (standard deviation)	22.52 (5.17)	24.60 (4.90)	20.79 (3.99)	28 (3.74)
Age at menarche, mean (standard deviation)	13.61 (1.39)	12.73 (1.21)	13.87 (1.43)	12.25 (1.28)
Number of children, mean (standard deviation)	2.71 (1.95)	1.57 (2.40)	3.06 (2.08)	1.64 (1.08)
Body mass index (kg/m^2), mean (standard deviation)	27.44 (4.89)	n/a*	27.10 (5.17)	n/a*
Menopausal status at the time of blood donation				
Premenopausal	121	82	107	73
Postmenopausal	180	70	111	91

n/a* : calculation was not performed due to the absence of the data.

HASTA ANKET FORMU

1. Adı Soyadı:
2. Yaşı:
3. Medeni hali:
4. Yaşadığı şehir ve süresi:
5. Ağırlığı:
6. Boyu (cm):
7. Mesleği:
8. İlk menstürasyon periyodunun başlama yaşı:
9. Menapozal durumu:

Premenapozal ise; son menstürasyon periyodunun kaç gün önce olduğu:
Postmenapozal ise; son menstürasyon periyodunun kaç gün önce olduğu:

10. Tanı konulduğu zamanki menapozal durumu:
11. Tanının ne zaman konulduğu:
12. Uygulanan tedavi:
13. Daha önce hormon tedavisi gördü mü? Ne tip?
14. Oral kontraseptif kullandimı? Nedir?
15. Kaç çocuğu var?

a. İlk doğumunu yaptığı yaş?
b. Son doğumunu yaptığı yaş?

16. Daha önce meme ile ilgili operasyon geçirdi mi?
17. Ooferektomi (yumutalıkların alınması) yapıldı mı? Yapıldı ise kaç yıl önce?
18. Sigara içme alışkanlığı:

Hiç içmedim ( ) Eskiden içerdim ( )
1-10 sigara/gün ( ) 11-20 sigara/gun ( ) 20 ve daha fazla/gün ( )
1 yıldır içiyorum ( ) 2-5 yıldır içiyorum ( ) 5-10 yıldır içiyorum ( )
10-15 yıldır içiyorum ( ) 15-20 yıldır içiyorum ( ) 20 ve daha fazla yıldır içiyorum ( )

17. Sigara içilen ortamda sıkça bulunuyor musunuz?
    (a) Evet (b) Hayır
18. Alkol kullanıyor musunuz?
    (a) Evet (b) Hayır
    Nadiren Haftada 1 kez Haftada 2-3 kez Haftada 4-5 kez Haftada 6-7 kez
19. Beslenme alışkanlığınızda size en fazla uyan tanım aşağıdakilerden hangisidir?

a.Kızartma ağırlıklı yağlı diyet
b. Sebze ağırlıklı yağsız diyet
c.Dengeli beslenme

20. Radyasyona maruz kaldınız mı? Hangi sıklıkla?
    a) Evet (b) Hayır
21. Tiroid ile ilgili bir rahatsılığınız var mı?
    (a) Evet (b) Hayır
    Hipertiroidizm ( ) Hipotiroidizm ( )
22. Aile bireylerinde ve sizde genetik bir rahatsızlık var mı? Tipi.
    (a) Evet (b) Hayır
23. Ailenizde meme kanserli başka bireyler var mı? (Anne, kardeş, anneanne,vb)
    (a) Evet (b) Hayır
24. Tümörün histopatolojisi
25. Tümör grade
26. Tümör stage
27. Östrojen reseptör durumu (+) veya (-)
28. Progesteron reseptör durumu (+) veya (-)

Figure 2.2. “Hasta Anket Formu”.

2.1.3. Primers

Two pairs of primers (common reverse primer) for *RNASEL* and one pair of primer for the amplification of a control gene were used in the study. The sequences of the primers used in the study (Casey et al., 2002) are listed below (Table 2.2).

Table 2.2 List of primers for the amplification reactions.

Primer	**Sequence of the Primer**	**Target Gene**
1385G	5'-GTGGAAAATGAGGAAGATGAATTTGCCAG-3'	***RNASEL***
1385A	5'-GTGGAAAATGAGGAAGATGAATTTGCCAG-3'	
1385R	5'-ATTGGGGACTCACCTATTAAGATGTTTTG-3'	
ARMS-A	5'-CCCACCTTCCCCTCTCTCCAGGCAAATGGG-3'	**Control gene***
ARMS-B	5'-GGGCCTCAGTCCCAACATAGGCTAAGAGGTG-3'	

* The control amplicon is a 393-bp fragment of *GKC* (available in NCBI, Blast, AV690557)

2.1.4. Chemicals and reagents

Table 2.3. Chemicals, reagents and their producers.

Chemical Used	Producer
Agarose	Basica LE, EU
Boric Acid	Sigma, St. Louis, MO, USA
Bromophenol blue	Sigma, St. Louis, MO, USA
EDTA	Boehringer Mannheim, Mannheim, Germany
Ethidium Bromide	Sigma, St. Louis, MO, USA
Ficoll Type 400	Sigma, St. Louis, MO, USA
TrisHCl	Merck, Schuchardt, Germany
Xylene cyanol	Sigma, St. Louis, MO, USA

2.1.5. PCR materials

The amplification was carried out using;

- Taq polymerase (5 U/µl) enzyme, MBI Fermentas Inc. , NY, USA.
- 10X PCR reaction buffer (100 mM Tris-HCl, pH 8. 8 at 25°C, 500 mM KCl, 0. 8% Nonidet P40), MBI Fermentas Inc., NY, USA.
- 25 mM $MgCl_2$, MBI Fermentas Inc., NY, USA.
- 10 mM dNTP mix, MBI Fermentas Inc., NY, USA.
- Primers (listed in Table 2.2), Iontek Co, Bursa, TURKEY.

The amplification reactions were carried out in "Gene Amp PCR system 9600", Perkin Elmer, Foster City, CA, USA.

2.1.6. Standard solutions and buffers

- Agarose gel loading buffer (6X) (Maniatis *et al*., 1989, page B.24).

  15 % ficoll

  0.05 % bromophenol blue

  0.05 % xylene cyanol

- Ethidium Bromide (10 mg/ml) (Maniatis *et al*., 1989, page B.11).

  1 g of ethidium bromide

  100 ml H_2O

- Tris-boric-acid-EDTA (TBE) (10X) (1L) (Maniatis *et al*., 1989, page B.23).

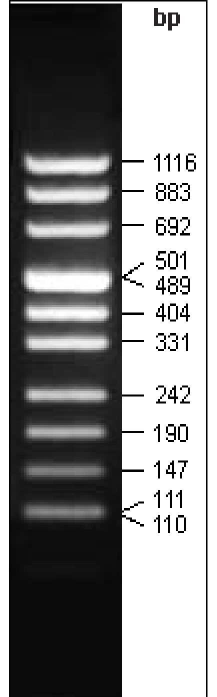
  108 g Tris HCl

  55 g boric acid

  20 m 0.5 M EDTA

  Add ddH_2O to a final volume of 1 L.

• Standard DNA size markers;



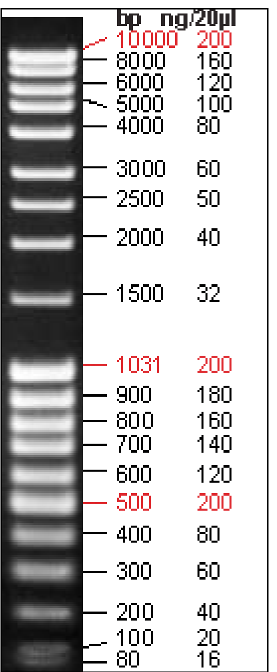


Figure 2.3. Standard DNA size markers. **pUC** Mix Marker, 8* (*on the left*) and Mass Ruler^{TM} DNA Ladder, Mix, ready-to-use** (*on the right*).

* *1.7% agarose, 0.5µg/lane, 8cm length gel, 1X TBE, 12V/cm* (MBI, Fermentas).

** *1% agarose, 20µl/lane, 8cm length gel, 1X TAE, 17V/cm* (MBI, Fermentas).

2.2. Methods

2.2.1. Amplification Refractory Mutation System (ARMS)

The genotyping of the individuals were carried out using the Amplification Refractory Mutation System (ARMS). ARMS, also known as Allele Specific PCR (ASPCR) or PCR Amplification of Specific Alleles (PASA), is used in detection of known single-base substitutions or micodeletions/insertions.

DNA polymerases are the enzymes responsible for the synthesis of a new DNA strand on a template strand. During this process, these enzymes can add the new nucleotide to the growing strand only if the previously added nucleotide is completely matched with the previous nucleotide on the template strand. This is also valid for the last nucleotide of the oligonucleotide primers, for the addition of the next nucleotide of the strand to be synthesized. This is the basis of the “Refractory Mutation System” (Lewin, 1997, pp472-7).

In Amplification Refractory Mutation System (ARMS) two complementary reactions are performed. One reaction contains a specific primer for the normal allele and the other for the mutant allele. They are included in the PCR reaction in addition to the other common components of the PCR reaction (with one common primer) (Stratchan *et al*., 1999, pp431-2).

Each of the primers perfectly matches one of the alleles, one for the wild-type and one of the mutant. Genotyping is based on whether there is amplification in the reaction. For example, if the amplification with the wild-type specific primer occurs, this means the individual contains that allele, and the just in the same manner if the amplification with the mutant-specific primer occurs, this means the individual contains the allele. If a sample leads to only one band with the one type of reaction, this means that, the individual is homozygote for that allele. And if a sample leads to two bands in each lane, this means that the individual is heterozygote (Amplification Refractory Mutation System, “www.ich.ucl.ac.uk/cmgs/arms98.htm”).

2.2.1.1. Polymerase Chain Reaction (PCR) for ARMS

One of the most notable developments in the field of applied molecular genetics in the last decade was the invention of a simple DNA amplification strategy called the polymerase chain reaction (PCR). The idea for PCR is credited to Karry Mulis, who was a research scientist in the 1980s at a California biotechnology company called Cetus. Karry Mullis showed that the amplification of defined segments of DNA (or cDNA) could be performed with oligonucleotide primers. Mullis was awarded the Nobel Prize in Chemistry in 1993 for his recognized contribution to the development of PCR (Miesfeld, 1999, p143).

For the time being, PCR is a frequently used method for oligonucleotide primer directed enzymatic amplification of a specific DNA sequence of interest, with two important benefits, first the enormous amplification achieved, and the high specificity of amplification. PCR amplification occurs under the dictation of two oligonucleotide primers that flank the DNA fragment which is to be amplified. The amplification begins with an initial denaturation of the template DNA. Following cycles includes denaturation, annealing of the primers to their complementary sequences, and extension of the annealed primers with heat stable Thermus aquaticus DNA polymerase (Old *et al.,* 1998, pp178-83).

This is just a simple copy of the naturally occurring event in vivo, under the experimental conditions. Provided enzyme works on the template using the provided substances (primers, dNTPs…) obeying to the physicochemical rules governed by the conditions that the researcher adjusts. At the end of each cycle the number of the amplicons grows exponentially, resulting in nearly 2^n of amplicons at the end of n cycles. And a final elongation step at the end of the cycles.

PCR reactions were carried out in a total of 15 µl of volume. 50-100 ng of genomic DNA was amplified by using 5 pmol of primers of matching forward and reverse. 3 pmol of control primers were used. 1 U Taq Polymerase, 1x Polymerase Buffer, 1 µl of $MgCl_2$, 0.3 µl of dNTP mix was used for each reaction. PCR conditions are as follows; Initial denaturation is at 95°C for 5 minutes. Denaturation of 30 seconds

at 94°C, annealing 30 seconds at 56°C, polymerization for 40 seconds at 72°C is repeated for 30 cycles. Final extension was 10 minutes at 72°C, which is followed by 4°C soak. This reaction amplifies a 123-bp fragment of our interest and 393–bp fragment of internal control.

2.2.2. Agarose Gel Electrophoresis

The separation of nucleic acids by electrophoretic mobility is used for both analytical and preparative purposes. DNA and RNA molecules are negatively charged due to the phosphate backbone. The polymer structure leads to a constant charge to mass ratio. Therefore, in a uniform electric field, nucleic acid molecules move through a solid support matrix from the negatively charged cathode towards the positively charged anode at a rate that is inversely proportional to the $\log_{10}$ of the molecular weight. There are two types of matrices with different ranges of molecular weights of nucleic acids that can be analyzed. Agarose gels are generally used to separate nucleic acid molecules of 0.2 – 10 kb, and acrylamide gels are best suited for resolving nucleic acids less than 500 kb nucleotides long.

Agarose is a linear polysaccharide polymer derived from a red seaweed. The agarose powder is mixed with buffer to make the gel. This gel may also contain the staining agents like Ethidium Bromide (EtBr). EtBr embeds between the nucleotides, and due to the luminescence property of EtBr, the DNA molecules can be easily viewed under UV light (Miesfeld, 1999, p18-9).

2% (w/v) agarose gels were prepared in 1xTBE buffer and 1µl of Ethidium Bromide solution from 10 mg/ml was added to the buffer. 3 µl 6x loading buffer was added to the PCR tube containing 15 µl of amplicon. And the mix was loaded onto the gel. The products were run at 90 volts for nearly 45 minutes. The gel was then analyzed under the transilluminator and photographs were taken. pUC mix8 or Mass Ruler^{TM} DNA Ladder was used as the DNA size marker.

2.2.3. Genotyping of Individuals

The *RNASEL* G1385A mutation analysis was performed by using Amplification Refractory Mutation System (ARMS). The genotypes of each individual were scored by two independent researchers. In order to provide further reliability, some selected samples are subjected to genotyping again.

Two different forms of the sequence were analyzed by the presence of the bands in the corresponding reaction products. The presence of the control bands in both reactions was expected to assure that the reaction has worked properly. The presence or the absence of the bands in the corresponding region indicated the presence of the genotype.

Both of the reactions were performed in same conditions for the two tubes. The amplified fragments were electrophoresed in 2% agarose (Figure 2.5).

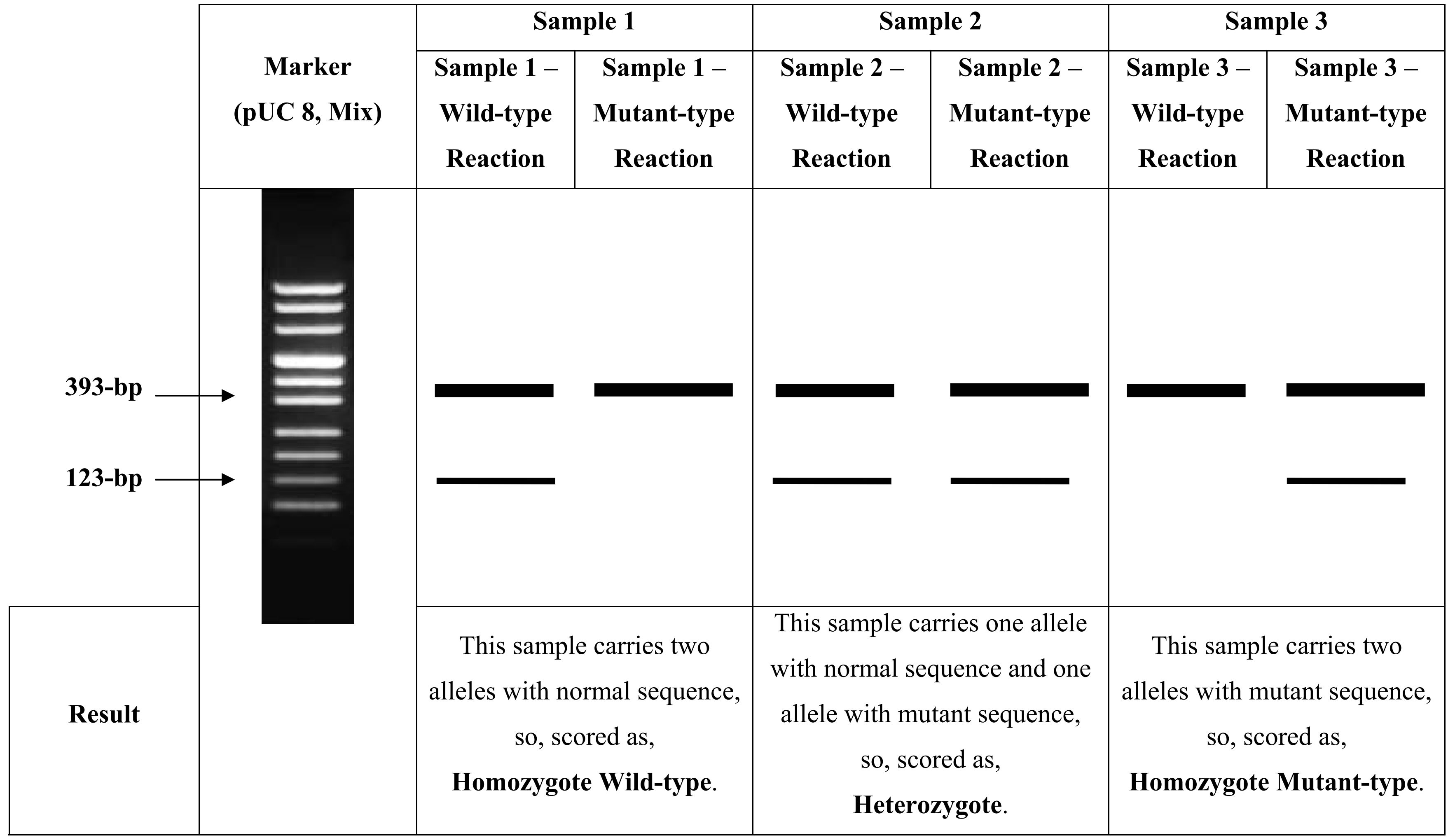


Figure 2.5. Schematic representation of *RNASEL* genotyping.

2.2.4. Statistical analyses

Statistical analyses (*P*-value, Crude Odds Ratio and Adjusted Odds Ratio with 95% Confidence Interval calculations), were carried out with the SPSS 9.0.0 software program. The power calculation of the study was done with the EpiCacl 2000 v1.02.

We sought to investigate the hypothesis that the Arg462Gln variant of *RNASEL* is implicated in the development of breast cancer. This was our null hypothesis. For the statistical calculations, null hypothesis is the hypothesis to be tested. The opposite statement is the alternative hypothesis, which holds true if our data rejects our null hypothesis. The statistical analysis to proof the null hypothesis is called hypothesis testing. For this purpose, appropriate statistical tests will be performed, and if the results are in accordance with the acceptance of the null hypothesis, than we will conclude that our hypothesis is true.

2.2.4.1. Chi-square (χ^2) test for *P*-value calculation

In studies ending with count or frequency data, the chi-square distribution is the most frequently used technique to understand if any relationship between the two variables exists. Two types of variables yielding two types of data (numerical or categorical) are generated from the randomly drawn populations. Numerical data is in the format of continuous numbers (i.e. age; 1,2,3,…), but the categorical data is in the format of two or more values representing the categories of data (i.e. smoking status; “1” for smokers and “0” for nonsmokers).

The quantity chi-square is a measure of the agreement of the observed and expected frequencies. The value of the chi-square is small when there is a close agreement between the observed and expected frequencies, but the value is large, when the agreement is poor. This can be easily understood regarding the formula of the chi-square.

$$X^2 = \Sigma [(O_i - E_i)^2 / E_i]$$

Where, the O_i is the observed frequency for the ith category of the variable of interest. Just in a similar manner, E_i is the expected frequency for the ith category of the variable of interest.

When the distributions of the two variables are similar, the term "$(O_i - E_i)$" will be close to zero, which will lead to a χ^2 value smaller, or close to zero.

And in the case of small values for χ^2, we are unable to reject the H_0. When we have a large χ^2 value, we reject the H_0, which means that, the distributions of the two groups of data for the observed and expected are similar. And this tells us that the association between the two variables is strong. In another words, the results are close to the ones that we expect.

2.2.4.2. P-value calculation

P-value is generally used in the evaluation of the statistical significance of the results. Chi-square test is used for the calculation of the P-value. P-value indicates a probability ranging from zero to one. P-value operates in the manner of accepting or rejecting a null hypothesis. The previously defined null hypothesis is tested by the P-value.

If the P-value calculated from the study is smaller than the previously set threshold value, then we may conclude that the results are significant. If the P-value is larger than the previously set threshold value, then we may conclude that the results are not significant. The threshold value for the P-value is traditionally chosen as 0.05.

The interval of results that are regarded as possible alternatives due to the size and variability of the results are called confidence interval (CI). The intervals are calculated for any desired degree of confidence. But the traditionally used confidence is 95%. The interval indicates that, if you continue the research, you will obtain data within the interval in 95% of the trials.

2.2.4.3. Odds Ratio calculation

Observational study

An observational study is a scientific investigation, in which neither the subjects nor any of the variables of interest are manipulated (For example, in our study, we do not attempt to change the *genotype* of any individual).

We have two types of variables. First type is the dependent variable, which is also named as the *outcome*. This is the breast cancer phenotype in our study. The other type is the independent variable, which is also named the *risk factor*. The genotypes of the individuals are the risk factors in our study, or in the calculation of the adjusted odds ratio, when we defined more than one risk factor such as; the age or menopausal status, they are the risk factors.

Two types of observational studies

Observational studies may be divided into two groups of prospective and retrospective studies according to their sampling schemes. In prospective studies two random samples of subjects are selected, one sample consisting of subjects possessing the risk factor and the other sample consisting of subjects who do not possess the risk factor. The classification of the subjects into the two sample groups of outcome variable are recorded in the time interval that is they are followed prospectively. But in a retrospective study, the samples are selected from those belonging to the two or more categories of the outcome variable. Determination of the presence or the absence of the risk factor in those groups is performed to evaluate the possible association of the risk factor, and the outcome. So, we are setting two categories upon the outcome, the study group of affected individuals and the control group of the individuals with no history of outcome. By using the calculated frequencies of the risk factor in each group, we perform statistical analysis to evaluate the association.

The data resulting from those studies involving two dichotomous variables are generally displayed in a 2x2 contingency table. This table provides information about the numbers of individuals with and without the risk factor, in the groups of exposed and not exposed (Table 2.4).

Two ways of expressing the risk for two types of observational studies

The estimation of the risk of the outcome in a population can be done in two different ways in two different types of observational studies. "Relative Risk" (RR) is more commonly used in the risk estimation in prospective studies and the Ratio of the Odds (Odds Ratio; OR) is more commonly used in the risk estimation in retrospective studies. Both are actually the ratio of the risk of developing a disease among subject with the risk factor to the risk of developing the disease among subjects without the risk factor.

Odds ratio (OR) is an expression of the risk. It can have a value between zero and infinity. If the OR has a value of zero, than we may conclude that the risk of developing the disease does not have any association with the presence risk factor. The OR smaller than 1 indicates the reduced odds of disease among the subjects with the risk factor, than the subjects without the risk factors. But the OR bigger than 1 indicates the increased odds of disease among the subjects in whom the risk factor is present.

As described before, we have also calculated a confidence interval (with 95% confidence) for our OR. To assign an odds ratio as statistically significant, the confidence interval must exclude the value of "1". Only in such a condition we may say with 95% confidence that the further trials will end with results within the interval.

Table 2.4. Sample 2x2 Table for Odds Ratio Analysis.

Risk Factor	Control	Case
Present	a	c
Absent	b	d

a: number of controls with the risk factor

b: number of controls without the risk factor

c: number of cases with the risk factor

d: number of cases without the risk factor

then;

$$\mathbf{OR = ad / bc}$$

and,

$$\mathbf{95\%\ CI = e^{\ln[OR] \pm 1.96 \text{ times square root of } (1/a + 1/b + 1/c + 1/d)}}$$

2.2.4.4. Multivariate adjusted odds ratio calculation

The association of the disease status and the risk factor may also be investigated also considering other independent variables (risk factors). In other words, we also calculated the association of the *RNASEL* genotype and the breast cancer status considering the other possible risk factors for the breast cancer, such as the menopausal status, age, reproductive and hormonal histories, smoking status, body-mass-index, family histories of cancer, number of children of the individuals. The basis of the selection of additional independent variables is a strong association with the outcome but no association with other independent variables. Multiple variant descriptions on the

binary logistic regression analysis is performed to calculate the adjusted odds ratio for these independent variables.

2.2.4.5. Calculation of the power of our study

In order to evaluate the reliability of the statistical analyses that we have performed, we also calculated the "Statistical power" of our study. Statistical power of a study depends on the following variables;

- The size of the study group (actually the ratio of the cases to controls is important),
- The significance level must be set (traditionally the significance level is set as $\alpha=0.05$),
- The allele frequencies (the frequency of the controls that are exposed to the risk factor is required),
- A specific odds ratio.

The ratio of the cases to controls must be as low as possible. That is the number of controls must be high enough. Significance level is generally accepted as $\alpha = 0.05$.

Apart from other variables, the involvement of a specific odds ratio is, somehow unusual. The power may be calculated for a specific odds ratio of any value. That is, knowing all the other variables, a researcher may calculate the power value for a specific odds ratio of 2, or a specific odds ratio of 1. But as the specific odds ratio is decreased, the power of the study also decreases. So, a balanced level must be set between these two variables. A study must have adequate power for an acceptable specific odds ratio. The traditionally acceptable power for such studies is 80% (or higher). So, a specific odds ratio is chosen that study has a suitable power.

3. Results

We investigated the association for *RNASEL* G1385A variant and breast cancer risk. The genotyping of the individuals was done using Amplification Refractory Mutation System (ARMS). The results were scored by two independent researchers and some selected samples were subjected to second genotyping in order to check the accuracy of our results.

3.1. Cohort information

We have also performed statistical analyses of the genotyping data. In order to perform statistical calculations, we had to further analyze the characteristics of our study population. For example, the percentage of individuals having a BMI value higher than the mean BMI of the control individuals was calculated. These values are presented in Table 3.1.

Table 3.1 also states the association between the cancer occurrence and the established risk factors. The Odds Ratios (OR) were calculated for the risk factors and the breast cancer, and the results are in accordance with the previously stated importances of these factors.

The possible effect of menopausal status on the breast cancer risk well documented. 40.20% of the Turkish and 53.95% of the Greek patients are premenopausal, and 59.80% of the Turkish and 46.05% of the Greek patients are post menopausal. 49.08% of the Turkish and 44.51% of the Greek controls were premenopausal, and 50.92% of the Turkish and 55.49% of the Greek controls are post menopausal. The mean age of menarche is 13.61 for the Turkish and 12.73 for the Greek patients, and, 13.87 for the Turkish and 12.25 for the Greek patients.

Owing to the stated importance of the body mass index (BMI) on the breast cancer risk, the percentage of individuals having BMI higher than a threshold value is calculated. Since the BMI data was missing for the Greek samples, we could perform the

calculations for only the Turkish patients and controls. The mean BMI of the control individuals were used as the threshold value. The 47.09% of the patients and 52.91% of the control individuals were having a BMI value higher than the mean of controls. Of the patients, 5.59% is premenopausal, and 41.50% is postmenopausal. Of the controls, 6.94% is premenopausal and 45.97% is post menopausal.

3.2. Genotyping of *RNASEL* and genotype distributions

We performed genotyping of *RNASEL* G1385A by using Amplification Refractory Mutation System (ARMS). The samples were subjected to PCR amplification due to the instructions of ARMS. The reaction produces a 123-bp fragment of our interest if the allele (that the primer is specific for) is present. And upon the presence or the absence of the bands the genotyping of the individuals were performed (An example of the PCR result is shown in Figure 3.1). There were three possible outcomes, G/G, G/A, and A/A. The wild-type form is G/G, and the homozygote mutant form is A/A. The G/A genotype is the heterozygote. The G→A conversion in the *RNASEL* sequence results in Arg→ Gln conversion in the protein product.

The number of patients and controls genotyped for *RNASEL* G1385A variant were, 453 and 382, respectively. There were 301 Turkish and 152 Greek patients, and 218 Turkish and 164 Greek control individuals in our study.

The paired PCR amplification for the ARMS procedure with the specific primers gives two separate bands of different length. First one is the control band (393-bp), which indicates the success of the amplification. And the second band (123-bp), if present, indicates the presence of the allele. Sample result of ARMS procedure is shown in Figure 3.1 (Also refer to Figure 2.5 for schematic representation).

The genotype distribution in the whole group, the Turkish and the Greek population is summarized in Table 3.2. Further sub-grouping of the results on the menopausal status is presented in Table 3.3.

Table 3.1. Characteristics of participants in our study.

	Cases n=453		Controls n=382		OR (95% CI)
	Tr[1] n=301	Gr[1] n=152	Tr[1] n=218	Gr[1] n=164	
Age (yr), mean	49.55	50.04	47.45	50.83	1.0045 (0.9938 – 1.0153)
1st degree relative with breast cancer	5.65%	n/a[2]	0	n/a[2]	
Body mass index, mean	27.44	n/a[2]	27.10	n/a[2]	
Age at first live birth, mean	22.52	24.60[3]	20.79	28.00[3]	1.0670 (1.0243 – 1.1115)
Number of children, mean	2.71	1.57[3]	3.06	1.64[3]	0.9702 (0.8953 – 1.0513)
Age at menarche, mean	13.61	12.73[3]	13.87	12.25[3]	1.0104 (0.9028 – 1.1308)
Smoking Status					0.6775 (0.4251 – 1.0798)
Menopausal Status					1.0974 (0.8350 – 1.4422)
Premenopausal	40.20%	53.95%	49.08%	44.51%	
Postmenopausal	59.80%	46.05%	50.92%	55.49%	
BMI ≥ 27.10[3] (n=189)	47.09%	n/a[2]	52.91%	n/a[2]	1.0134 (0.9733 – 1.0551)
Premenopausal	5.59%	n/a[2]	6.94%	n/a[2]	
Postmenopausal	41.50%	n/a[2]	45.97%	n/a[2]	
Age at menarche ≤ 13.87[3]	62.13%	n/a[2]	86.70%	n/a[2]	

[1] Tr; Turkish, Gr; Greek

[2]This calculation was performed with Turkish data. Greek data excluded due to the high number or whole missing data.

[3]This value was calculated using the data for the known samples. The unknown samples are excluded.

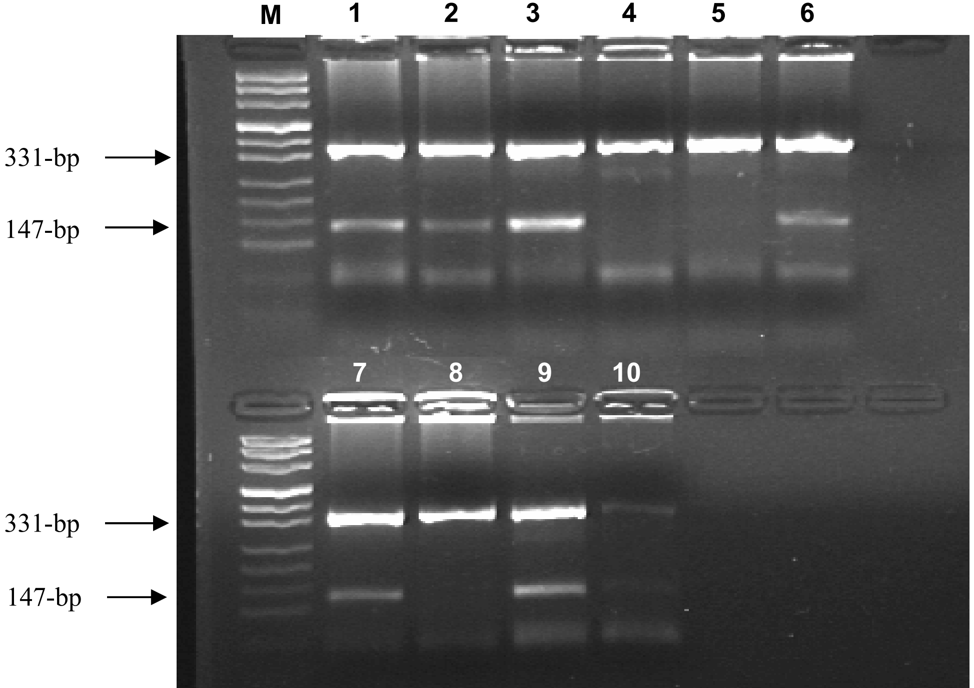


Figure 3.1. Genotyping of *RNASEL* G1385A variant in breast cancer patients. A 123-bp *RNASEL* specific, and a 393-bp *GKC* (control) specific amplicon is observed. The *RNASEL* G allele specific primers were used in lanes 1, 3, 5, 7, and 9; and *RNASEL* A allele specific primers were used in lanes 2, 4, 6, 8, and 10. The breast cancer patients genotyped in this experiment are BC-211 (lanes 1 and 2), BC-303 (lanes 3 and 4), BC-305 (lanes 5 and 6), BC-306 (lanes 7 and 8), and BC-309 (lanes 9 and 10). Based on the results BC-211 and BC-309 are heterozygous for the *RNASEL* G1385A variant. BC-303 and BC-306 are homozygous wild-type, and BC-305 is homozygous mutant. M: DNA size marker (pUC mix 8).

Distribution of the genotypes and the alleles

In the whole study group, which consists of 453 affected and 382 control individuals, genotype distributions are as follows; G/G 45.48% (n=206), G/A 42.16% (n=191) and A/A 12.36% (n=56) in the patients and G/G 43.98% (n=168), G/A 40.05% (n=153), and A/A 15.97% (n=61) in the control group, and G/G 50.00% (n=109), G/A 40.37% (n=88), and A/A 9.63% (n=21) for the controls. The combined frequency of G/A and A/A genotypes, which represent the frequency of the presence of at least one allele is 54.52% (n=247) for the patients and 56.02% (n=214) for the controls (Table 3.2).

In the Turkish population, the genotype distributions for the patients are G/G 48.50% (n=146), G/A 41.20% (n=124), and A/A 10.30% (n=31), and G/G 50.00% (n=109), G/A 40.37% (n=88), and A/A 9.63% (n=21) for the controls. The combined frequency of G/A and A/A genotypes is 51.50% (n=155) for the patients and 50.00% (n=109) for the controls (Table 3.2).

In the Greek population, G/G genotype was observed in 39.47% (n=60) of the patients and 35.98% (n=59) of the controls, G/A genotype in 44.08% (n=67) of the patients and 39.63% (n=65) of the controls, and A/A genotype in 10.30% (n=25) of the patients and 9.63% (n=40) of the controls. The frequency of the individuals having at least one mutant allele was 60.53% (n=92) in the patients and 64.02% (n=105) in the controls (Table 3.2).

Table 3.2. Distribution of *RNASEL* G1385A genotypes and breast cancer risk in the age matched controls and breast cancer patients.

Population	Genotype	Case n=453 (%)	Control n=382 (%	OR (95% CI) Crude	OR (95% CI) Adjusted[a,b]
Whole group	**G/G**	206 (45.48)	168 (43.98)	1.00	1.00
	G/A	191 (42.16)	153 (40.05)	1.02 (0.76- 1.37)	0.95 (0.70- 1.29)
	A/A	56 (12.36)	61 (15.97)	0.75 (0.49- 1.14)	0.72 (0.46- 1.12)
	G/A or A/A	247 (54.52)	214 (56.02)	0.94 (0.72- 1.24)	0.89 (0.66-1.18)
Greek group	**G/G**	60 (39.47)	59 (35.98)	1.00	1.00
	G/A	67 (44.08)	65 (39.63)	1.01 (0.62- 1.66)	0.78 (0.42- 1.46)
	A/A	25 (16.45)	40 (24.39)	0.62 (0.33- 1.14)	0.67 (0.32- 1.42)
	G/A or A/A	92 (60.53)	105 (64.02)	0.86 (0.55- 1.36)	0.74 (0.42- 1.31)
Turkish group	**G/G**	146 (48.50)	109 (50.00)	1.00	1.00
	G/A	124 (41.20)	88 (40.37)	1.05 (0.73- 1.52)	0.77 (0.46- 1.28)
	A/A	31 (10.30)	21 (9.63)	1.10 (0.60- 2.02)	1.07 (0.48- 2.39)
	G/A or A/A	155 (51.50)	109 (50.00)	1.06 (0.75- 1.50)	0.82 (0.51-1.33)

Gr: Greek, *Tr*: Turkish populations.

ORs and 95% CIs were calculated using binary logistic regression. Adjusted for;

[a]age and menopausal status (*Gr*, *Gr*+*Tr*) and

[b]smoking status, body-mass-index, age at menarche, age of 1st pregnancy, number of children, family history of breast cancer (*Tr*).

Table 3.3. The allele frequencies and sample odds ratios in subgroups according to menopausal status.

Menopause	Population	Genotype	Case n=453 (%)	Control n=382 (%)	Crude OR (95% CI)
All	Whole group	G/G	206 (45.48)	168 (43.98)	1.00
		G/A	191 (42.16)	153 (40.05)	1.02 (0.76- 1.37)
		A/A	56 (12.36)	61 (15.97)	0.75 (0.49- 1.14)
		G/A or A/A	247 (54.52)	214 (56.02)	0.94 (0.72- 1.24)
	Greek group	G/G	60 (39.47)	59 (35.98)	1.00
		G/A	67 (44.08)	65 (39.63)	1.01 (0.62- 1.66)
		A/A	25 (16.45)	40 (24.39)	0.62 (0.33- 1.14)
		G/A or A/A	92 (60.53)	105 (64.02)	0.86 (0.55- 1.36)
	Turkish group	G/G	146 (48.50)	109 (50.00)	1.00
		G/A	124 (41.20)	88 (40.37)	1.05 (0.73-1.52)
		A/A	31 (10.30)	21 (9.63)	1.10 (0.60- 2.02)
		G/A or A/A	155 (51.50)	109 (50.00)	1.06 (0.75- 1.50)
Premenopausal	Whole group	G/G	92 (45.32)	75 (41.67)	1.00
		G/A	86 (42.36)	77 (42.78)	0.91 (0.59- 1.40)
		A/A	25 (12.32)	28 (15.56)	0.73 (0.39- 1.35)
		G/A or A/A	111 (54.68)	105 (58.33)	0.86 (0.57- 1.29)
	Greek group	G/G	29 (35.37)	22 (30.14)	1.00
		G/A	39 (47.56)	31 (42.47)	1.88 (0.78- 4.54)
		A/A	14 (17.07)	20 (27.39)	1.80 (0.78- 4.12)
		G/A or A/A	53 (64.63)	51 (69.86)	0.79 (0.40- 1.55)
	Turkish group	G/G	63 (52.07)	53 (49.53)	1.00
		G/A	47 (38.84)	46 (42.99)	0.86 (0.50- 1.49)
		A/A	11 (9.09)	8 (7.48)	1.16 (0.43- 3.09)
		G/A or A/A	58 (47.93)	54 (50.47)	0.90 (0.54- 1.52)
Postmenopausal	Whole group	G/G	114 (45.60)	93 (46.04)	1.00
		G/A	105 (42.00)	76 (37.62)	1.31 (0.74- 2.29)
		A/A	31 (12.40)	33 (16.34)	1.47 (0.83- 2.61)
		G/A or A/A	136 (54.40)	109 (53.96)	1.02 (0.70- 1.48)
	Greek group	G/G	31 (44.29)	37 (40.66)	1.00
		G/A	28 (40.00)	34 (37.36)	1.52 (0.64- 3.66)
		A/A	11 (15.71)	20 (21.98)	1.50 (0.62- 3.65)
		G/A or A/A	39 (55.71)	54 (59.34)	0.86 (0.46- 1.62)
	Turkish group	G/G	83 (46.11)	56 (50.45)	1.00
		G/A	77 (42.78)	42 (37.84)	0.96 (0.44- 2.09)
		A/A	20 (11.11)	13 (11.71)	1.19 (0.54- 2.63)
		G/A or A/A	97 (53.89)	55 (49.55)	1.19 (0.74- 1.91)

Genotype distribution in different menopausal groups

The genotype distribution in our study population was also investigated in different menopausal groups. The genotype distributions in premenopausal and post menopausal individuals were calculated in whole group and the two countries separately (Table 3.3).

The grouping of the individuals upon their menopausal status (regardless of their country) did not reveal any statistically significant results. G/G genotype was detected in 45.32% (n=92) and 41.67% (n=75) premenopausal patients and controls, respectively. The same genotype was observed in 45.60% (n=114) and 46.04% (n=93) postmenopausal patients and controls, respectively. 42.36% (n=86) and 42.78% (n=77) premenopausal patients and controls, respectively, and 42.00% (n=105) and 37.62% (n=76) postmenopausal patients and controls, respectively are found to be G/A genotype. A/A genotype was observed in 12.32% (n=25) and 15.56% (n=28) premenopausal patients and controls, and 12.40% (n=31) and 16.34% (n=33) postmenopausal patients and controls, respectively. Finally, as mentioned in the previous sections, in order to calculate the risk contribution correctly, the percentage of the individuals having at least one mutant allele is also calculated. At least one mutant allele was present in 54.68% (n=111) and 58.33% (n=105) premenopausal patients and controls, and 54.40% (n=136) and 53.96% (n=109) postmenopausal patients and controls, respectively.

Genotype distribution in different menopausal groups of Turkish individuals

In the Turkish population, consisting of 301 affected and 218 control individuals, the genotype frequencies in different menopausal groups were as follows; G/G 52.07% (n=63) and 49.53% (n=53) in premenopausal Turkish patients and controls, 46.11% (n=83) and 50.45% (n=56) in postmenopausal Turkish patients and controls, respectively; G/A genotype in 38.84% (n=47) and 42.99% (n=46) premenopausal

Turkish patients and controls, 42.78% (n=77) and 37.84% (n=34) postmenopausal Turkish patients and controls, respectively; A/A genotype in 9.09% (n=11) and 7.48% (n=8) in premenopausal Turkish patients and controls, and 11.11% (n=20) and 11.71 (n=13) in postmenopausal Turkish patients and controls, respectively. Finally, the individuals having at least one mutant allele is 47.93% (n=58) and 50.47% (n=54) in premenopausal Turkish patients and controls, and 53.89% (n=97) and 49.55% (n=55) in postmenopausal Turkish patients and controls, respectively.

Genotype distribution in different menopausal groups of Greek individuals

In the Greek populations, consisting of 152 affected and 164 control individuals, the genotype distributions in different menopausal groups were as follows; G/G 35.37% (n=29) and 30.14% (n=22) in premenopausal Greek patients and controls, and 44.29% (n=31) and 40.66% (n=37) in postmenopausal Greek patients and controls, respectively. G/A genotype in 47.56% (n=39) and 42.47% (n=31) in premenopausal Greek patients and controls, and 40.00% (n=28) and 37.36% (n=34) in postmenopausal Greek patients and controls, respectively. A/A genotype in 17.07% (n=14) and 27.39% (n=20) in premenopausal Greek patients and controls, and 15.71% (n=11) and 21.98% (n=20) in postmenopausal Greek patients and controls, respectively. Finally, the percentage of individuals having at least one mutant allele is 64.63% (n=53) and 69.86% (n=51) in premenopausal Greek patients and controls, and 53.89% (n=97) and 49.55% (n=55) in postmenopausal Greek patients and controls, respectively.

3.3. Statistical analysis

We performed statistical analysis of the genotyping data. The statistical analyses were performed by using SPSS 9.0.0 software program and EpiCalc 2000 v1.02 for the power calculation of our study.

3.3.1. *P*-value calculation

In order to evaluate the association between the breast cancer risk and the risk genotype in the cases and controls we have performed *t*-test to calculate *p*-values.

The *P* value for the whole group was $P = 0.665$. Since, the value is higher than the threshold value of 0.05, it indicates the absence of association between the breast cancer risk and *RNASEL* G1385A variant. The calculated *P* values for either populations or either menopausal groups were similarly higher than the threshold value, which again indicated the absence of association (Table 3.4).

Table 3.4. *P*-values.

Manopausal Status	Population	P-value
All	Whole group	0.665
	Greek group	0.521
	Turkish group	0.737
Premenopausal	Whole group	0.472
	Greek group	0.489
	Turkish group	0.703
Postmenopausal	Whole group	0.926
	Greek group	0.644
	Turkish group	0.472

3.3.2. Results of Odds-Ratio calculation (Crude)

The possible association between the genotype and the development of breast cancer risk was investigated using the odds ratio. The odds ratios calculated from these data are summarized in Table 3.2.

1385G→A conversion is suggested to be a risk factor for the development of breast cancer. So, the individuals with G/G genotype were assigned as the control group. So, the G/A and A/A genotypes are assigned as the risk genotypes. Their possible impact on the breast cancer risk was investigated either for each and both. In order to investigate the impact of having one mutant and one wild-type allele, we assigned the G/G genotype as the control and the G/A genotype as the risk factor. In order to investigate the impact of having two mutant alleles, we assigned the G/G group as the control and the A/A as the risk genotype. We also investigated the impact of having at least one mutant allele. For this calculation, we assigned the individuals with G/G as the control group, and the individuals with G/A or A/A genotypes as the risk group. The results of the crude odds ratio calculation are summarized in Table 3.2.

Considering both the G/A and A/A genotypes as the risk factors, the crude odds ratio for the study population is 0.94 (95% CI, 0.72-1.24). This value ia 1.02 (95% CI, 0.76-1.36) when the G/A genotype alone was assigned as the risk factor and 0.75 (95% CI, 0.49-1.14) when the A/A genotype alone was assigned as the risk factor.

The crude odds ratio for the Greek group is 0.86 (95% CI, 0.55- 1.36) for the G/A and A/A genotypes combined. This value is 1.01 (95% CI, 0.62- 1.66) for the G/A genotype alone and 0.62 (95% CI, 0.33- 1.14) for the A/A genotype alone.

Considering the Turkish group, the crude odds is 1.06 (95% CI, 0.75-1.50) for the G/A and A/A genotypes combined. This value is 1.05 (95% CI, 0.73-1.52) for the G/A genotype alone and 1.10 (95% CI, 0.60-2.02) for the A/A genotype alone.

The crude odds ratio values for the menopausal groups are summarized in Table 3.3. The crude odds ratio values were 0.86 (95% CI, 0.57-1.29) for the premenopausal group and 1.02 (95% CI, 0.70-1.48) for the postmenopausal group of our study population. These values were 0.90 (95% CI, 0.54-1.52) and 1.19 (95% CI, 0.74-1.91)

for the premenopausal and postmenopausal Turkish groups, respectively. Considering the Greek population, 0.79 (95% CI, 0.40-1.55) and 0.86 (95%CI, 0.46-1.62) were the crude odds ratio values for the premenopausal and postmenopausal groups, respectively.

Although these results cannot be regarded as statistically significant results, the crude odds ratio values are higher in post menopausal groups. They are also smaller than 1, but with higher values. This may reflect a possible effect of menopausal status in addition the *RNASEL* 1385G→A variant in our study group.

3.3.3. Results of Odds-Ratio calculation (Adjusted)

In order to provide further level of confidence to our statistical analyses, we have also performed the calculation of adjusted odds ratio. For the calculation of the adjusted odds ratio, all the variables that will be used in the adjustment of the data must be available for every individual. But the only data available for all the Greek individuals were the age and the menopausal status. So, the adjustment of the data in the Greek group and the whole group was only done for the age and menopausal status. But the adjusted odds ratio for the Turkish population was calculated for all the variables (age, menopause status, smoking status, body-mass-index (BMI), age at menarche, age of first pregnancy, number of children, and family history of breast cancer). Appendix A includes the variables for all the individuals, as well as the genotypes.

In the whole study group, the adjusted odds ratios for the risk genotypes were as follows; 0.89 (95% CI, 0.66-1.18) for the presence of at least one mutant allele, 0.95 (95%, CI 0.46-1.12) for the G/A genotype, and 0.72 (95% CI, 0.46-1.12) for the A/A genotype.

The adjusted odds ratios in the Turkish population for the risk genotypes are 0.82 (95% CI, 0.51-1.33), 0.77 (95% CI, 0.46-1.28), and 1.07 (95% CI, 0.48-2.39) for the presence of at least one mutant allele, G/A genotype and A/A genotype, respectively.

For the given risk genotypes, the adjusted odds ration in the Greek population were as follows; 0.74 (95%, CI, 0.42-1.31) for the presence of at least one mutant allele,

0.78 (95% CI, 0.42-1.46) for the G/A genotype, and 0.67 (95% CI, 0.32-1.42) for the A/A genotype.

3.3.4. Results of power calculation for our study

We investigated the statistical power of our study by performing power calculation. We calculated the power of our study using EpiCacl v1.02 developed by Joe Gilman & Mark Myatt 1998, Brixton Books.

We calculated that, given the sample size and the allele frequencies of the study population, we had 90% of power to detect an odds ratio as low as O.R.=1.8 on a significance level of $\alpha = 0.05$. And to the best of our knowledge on the powers of calculations a power of 90% is reliable (actually, a study having a power higher than 80% is accepted as a statistically strong study).

3.3.5. Further possible stratification of the Turkish group data

3.3.5.1. Stratification according to Body-Mass-Index

Considering the possible risk attributable by the high BMI, odds ratio for the high BMI individuals and low BMI individuals was calculated. The results are summarized in Table 3.5.

BMI data was available for 397 of the 519 individuals (192 patients and 195 controls). 89 of 192 patients had low-BMI, and 103 of them had high-BMI. 103 of 195 controls had low-BMI, and 92 of them had high-BMI.

Table 3.5 summarizes the odds ratio values in low and high BMI groups. Although the odds ratios are slightly higher in high-BMI group, these odds ratios cannot be regarded as statistically significant results.

Table 3.5. Odds ratios in low and high BMI of Turkish group.

Genotypes	Low BMI (< 27.10)				High BMI (≥ 27.10)			
	Frequency in;		Odds Ratio (95% CI)	Odds Ratio[a] (95% CI)	Frequency in;		Odds Ratio (95% CI)	Odds Ratio[a] (95% CI)
	Cases (n=89)	Control (n=103)			Case (n=103)	Control (n=92)		
G/G	50.56%	46.60%	1.00	1.00	51.46%	51.09%	1.00	1.00
G/A	39.33%	42.72%	0.85 (0.50-1.55)	0.75 (0.36-1.56)	34.95%	39.13%	0.89 (0.48-1.64)	0.76 (0.37-1.55)
A/A	10.11%	10.68%	0.86 (0.31-2.36)	0.72 (0.20-2.57)	13.59%	9.78%	1.44 (0.55-3.78)	1.30 (0.45-3.73)
G/A or A/A	49.44%	53.40%	0.85 (0.48-1.51)	0.74 (0.37-1.50)	48.54%	48.91%	1.01 (0.57-1.79)	0.87 (0.45-1.68)

Odds ratios were calculated using “binary logistic regression”.

[a] Adjusted odds ratio were calculated by adjusting the data with age, menopausal status, smoking status, age at menarche, age of 1st pregnancy, number of children, family history of breast cancer.

4. Discussion

The past decade has seen great strides in our understanding of the genetic basis of cancer (Hanahan *et al.*, 2000). Although the same conclusion is valid for the genetics of breast cancer, it still remains as an important cause of death in women (Wooster *et al.*, 2003). The incidence rate is 111 and the mortality rate is 24 cases per 100,000 woman-years in the United States (U.S.) (Baselga *et al.*, 2002). In Turkey, breast cancer is among the leading causes of death among women (Özsari *et al.*, 1997). Therefore, the improvements in the molecular based new therapies are of utmost importance. The key to the improvement of such approaches lies in the identification of genes and pathways involved in breast tumorigenesis (Polyak, 2001).

In the U.S., 10-20% of patients with breast cancer have a first- or second-degree relative with the disease. Two major genes associated with susceptibility to breast cancer –breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*)– have been identified. Population-based epidemiological studies have shown that only 15-20% of the patients with familial clustering have alterations in the two major breast cancer susceptibility genes, *BRCA1* and *BRCA2*. Mutations in either of these genes confer 60-85% lifetime risk of breast cancer. But their incidence is less than 5% in all cancer cases, and susceptibility alleles in other genes, such as *TP53*, *PTEN*, and *ATM*, are even less common causes of breast cancer (Wooster *et al.,* 2003). The recent discovery of the common 1100delC mutation in the cell-cycle-checkpoint kinase gene (*CHEK2*) validates the prediction that DNA-sequence variants confer a small but appreciable enhanced risk of cancer. Although the penetrance is low, the genetic analysis on other susceptibility genes needs further research. Therefore, we sought to investigate *RNASEL* as a candidate for the development of breast cancer.

RNASEL encodes for the ubiquitously expressed ribonuclease L (RNase L). RNase L is an endoribonuclease that is activated by 2'-5' adenylated oligonucleotides (2-5As). The 2-5As are produced upon interferon stimulation (Player *et al.,* 1998), and the activation of RNase L leads to RNA degradation. Consistent with its activity,

RNASEL has been shown to be mediating the pro-apoptotic and pro-inflammatory responses of interferon.

IFN treatment of cells induces a family of OAS genes through the JAK-STAT signal transduction pathway. To date, the only well established biochemical function of 2-5A is activation of RNase L (Silverman, 2003). The significance of the dsRNA requirement for 2-5A synthetase activity is a common intermediate or byproduct of viral infections. Thus, the 2-5A synthetases may be regarded as receptors/sensors for the presence of viral dsRNA that allow the cell to respond by initiating a host-defense mechanism resulting in activation of RNase L (Silverman, 2003). These evidences underline the antiviral function of RNase L in the immune system.

RNase L is shown to be mediating the pro-apoptotic activities via the same biochemical function. The activation and RNA degradation activity of RNase L creates a cellular stress, which leads to the activation of programmed cell death (Castelli *et al.,* 1998). The involvement of RNA decay pathways in cancer development is not a new idea. Several other candidate enzymes and genes have been investigated and some of them received varying degrees of success in experimental investigations.

Furthermore, involvement of RNase L in sequence specific gene silencing pathways was also suggested. Proposed mechanism involves 2-5A oligonucleotides fused to antisense sequences. The sequence specificity is provided by the antisense sequences, and 2-5A oligonucleotides activates the enzyme (Torrence *et al.*, 1993).

The proapoptotic activity of the enzyme is another important phenomenon to consider. The molecular machinery at the nexus of apoptosis and inflammation includes caspase-1, an activator of IL-1 beta, and IL-18, as well as the double-stranded-RNA-dependent protein kinase pathway and RNase L pathway, which are key regulators of antiviral immunity (Restifo, 2000). Recent studies also increase the number of candidate proteins. Along with the sequence homology with RNase L, down-regulation in high-grade prostate cancers, and unaltered level of expression in low grade prostate cancers, many new proteins are suggested as potential prognostic markers for the prostate cancer (i.e. SAPC) (Yu *et al.*, 2001).

Identification of germline mutations in *RNASEL* segregating in hereditary prostate cancer families that show linkage to the *HPC1* (hereditary prostate cancer 1) region at 1q24-25, was the first *in vivo* evidence for the impact of this gene in the development of prostate cancer. Furthermore, loss of heterozygosity (LOH) with markers flanking the *RNASEL* locus was observed in tumor tissues obtained from prostate cancer patients (Smith *et al.*, 1996, and Carpten *et al.*, 2002).

Consequently, several variants of *RNASEL* were identified and the association of these variants with the risk of prostate cancer was also investigated. Most notably, Arg462Gln variant was reported to be implicated in up to 13% of the prostate cancer cases (Casey *et al.*, 2002).

RNASEL was initially proposed as a candidate tumor suppressor on the basis of its involvement in the antiproliferative activity of IFs (Lengyel, 1993). We believe, the chromosomal location of this gene, which is 1q25 may also give important clues about the involvement of *RNASEL* in cancer development. For example, HAS 1q25 is a region found to be implicated in the genesis of breast cancer (Chen *et al.*, 1989). Considering the chromosomal localization and function of *RNASEL*, and the pleiotropic effects of cancer associated mutations, as exemplified by *CHEK2* in both breast and prostate cancers (Dang *et al.*, 2003), we sought to investigate the hypothesis that Arg462Gln variant of this gene is associated with breast cancer risk.

In our study, 835 individuals (453 breast cancer patients and 382 controls) from two countries (Turkey and Greece) were subjected to genotype analysis. The genotype analysis of the individuals was performed using Amplification Refractory Mutation System (ARMS). The genotype results were subjected to statistical analysis in order to evaluate the association between *RNASEL* Arg462Gln variant and incidence of breast cancer. Odds ratio calculation was performed using binary logistic regression procedure of SPSS 9.0.0 software program. Finally, statistical power of our study was calculated using EpiCalc 2000 v1.02.

The combined Greek and Turkish population allele frequencies of the *A* allele is 0.334 and 0.359 for cases and controls, respectively. Although the *A* allele frequencies were slightly different in two populations (Greek cases: 0.385, and controls: 0.442;

Turkish cases: 0.309 and controls: 0.298), the genotype distributions in the control groups were in Hardy-Weinberg equilibrium in both populations. The frequencies of the G/G, G/A and A/A genotypes in the whole study group were, 45.48%, 42.16% and 12.36% in the cases and 43.98%, 40.05% and 15.97% in the controls, respectively. The genotype frequencies in Greek population were, G/G: 39.47% and 35.98%, G/A: 44.08% and 39.63%, A/A: 16.45% and 24.39% in cases and controls, respectively. These values were G/G: 48.50% and 50.00%, G/A: 41.20% and 40.37%, and A/A: 10.30% and 9.63% in cases and controls of the Turkish population.

Based on these results, the odds ratio calculations showed no significant association between the *RNASEL* Arg462Gln variant and breast cancer risk (Table 3.3). Calculated odds ratios were generally below the threshold value of "1", indicating the lack of association between the Arg462Gln variant and breast cancer risk. Although, several odds ratio values were higher than the threshold value of "1" (i.e. postmenopausal individuals in the whole group or Turkish population), they were not regarded as statistically significant since their confidence intervals included "1".

Adjusting the odds ratio for age and menopausal status in the Greek population; age, menopausal status, smoking status, body-mass-index, age at menarche, age of first pregnancy, number of children, family history of breast cancer in the Turkish population; or age and menopausal status in both populations combined did not change the results (Table 3.2).

The association of the *RNASEL* Arg462Gln variant with breast cancer risk was also investigated in the Turkish patients and controls stratified according to body mass index (BMI). The threshold BMI value was selected as the mean of the controls. Individuals with BMI values higher than the threshold were grouped as "high-risk" and individuals with lower values were grouped as "low-risk". The crude odds ratio was 0.85 (95% CI, 0.48-1.51) for the low-risk group and 1.01 (95% CI, 0.57-1.79) for the high-risk group. Neither value is statistically significant. Adjusted odds ratios according to age, menopausal status, smoking status, age at menarche, age of 1st pregnancy, number of children, family history of breast cancer, were not statistically significant (Table 3.5).

Our cohort was also analyzed for established risk factors (Table 3.1). Since the data for most of the variables are not available for the Greek population, most of the calculations were performed with the Turkish population data;

- Age of menarche was not found to be significantly associated with the risk of developing breast cancer (1.0104, 95% CI 0.9028 – 1.1308).
- High body mass index was also not found to be associated with breast cancer (1.0134, 95% CI 0.9733 – 1.0551).
- Age of first pregnancy was found to be truly associated with the risk of developing breast cancer (1.0670, 95% CI 1.0243 – 1.1115). This is also consistent with the previous studies.

Given the sample size and allele frequencies, our study had a power of 90% to confirm an odds ratio as low as OR =1.6 on a significance level of α =0.05. Such a power value indicates the statistical strength of the study.

To the best of or knowledge this is the first genetic study that investigates the association between *RNASEL* Arg462Gln variant with breast cancer. Inclusion of two different Eastern Mediterranean populations and a fairly large number of cases and controls makes our study relatively strong.

5. Conclusions and future perspectives

To the best of our knowledge, we performed the first study on the possible association between the *RNASEL* G1385A variant and the risk of breast cancer following the identification of this variant as the candidate susceptibility allele in prostate cancer.

Our study has provided the following data;

- It appears there is no significant association between RNASEL G1385A variant and breast cancer risk in the Turkish and Greek populations.
- Adjusting the odds ratio with the available variants did not change the results (Turkish data could be adjusted for the age, menopausal status, smoking status, BMI, age at menarche, age of 1st pregnancy, number of children, family history of breast cancer, and the lack of significance remained unchanged. Greek data could be adjusted only for age and menopausal status, and similarly the lack of significance remained unchanged. The whole group of both populations could be adjusted for age and menopausal status).
- The odds ratio analysis in low- and high-BMI groups did not reveal any significant results in the Turkish population.
- Power calculation showed that our study had 90% of power to confirm an odds ratio as low as OR =1.6 on a significance level of $\alpha = 0.05$.

As next step, polymorphisms in other genes, which may have regulatory roles on the action of *RNASEL*, can be studied. The possible interaction of these genes with our variant can explain the further impact of our variant on breast cancer.

In conclusion, our study suggests no significant association between *RNASEL* G1385A variant and breast cancer risk in the Greek and Turkish populations. These results may need to be further corroborated by other investigations and in different populations since this is the first study reporting on the association of *RNASEL* G1385A variant and incidence of breast cancer.

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Appendix A. The Results.

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	001	**MCK-001**	GG	1	58	0	38	1,54	16,02	15	17	4	0
1	1	002	**MCK-002**	GA	1	58	0	84	1,51	36,84	15	17	6	0
1	1	003	**MCK-003**	GA	1	63	1	60	1,58	24,03		24	3	0
1	1	004	**MCK-004**	GG	1	52	0	95	1,64	35,32		17	6	0
1	1	005	**MCK-005**	GA	1	47								
1	1	006	**MCK-006**	GG	1	49								
1	1	007	**MCK-007**	GG	1	48	1	80	1,68	28,34	13	27	2	
1	1	008	**MCK-008**	GA	1	47								
1	1	009	**MCK-009**	GG	0	34	1	58	1,63	21,83	14	22	2	0
1	1	010	**MCK-010**	GG	0	44	0	68	1,67	24,38	12	20	3	0
1	1	011	**MCK-011**	GG	1	49	1	50	1,55	20,81	13	34	2	0
1	1	012	**MCK-012**	GG	0	41	0	65	1,55	27,06	13	21	1	0
1	1	013	**MCK-013**	GA	1	62	1	47	1,63	17,69	13	26	1	0
1	1	014	**MCK-014**	GA	0	38	1	76	1,7	26,30	13		3	0
1	1	015	**MCK-015**	GG	0	29	0	65	1,55	27,06	12	23	1	0
1	1	016	**MCK-016**	GG	0	50	0	66	1,67	23,67	12	29	2	0
1	1	017	**MCK-017**	GA	1	74	0	60	1,6	23,44	15	22	2	0
1	1	018	**MCK-018**	GG	1	58	0	65	1,55	27,06	14	20	4	0
1	1	019	**MCK-019**	GG	1	64	0	65	1,7	22,49	14	19	3	0
1	1	020	**MCK-020**	AA	1	61	0	72	1,52	31,16	15	21	5	0
1	1	021	**MCK-021**	GG	1	63	0	72	1,5	32,00	15	19	5	0
1	1	022	**MCK-022**	GG	1	60	0	85	1,65	31,22	14	20	6	0
1	1	023	**MCK-023**	GG	0	24	1	64	1,58	25,64	11	19	2	0
1	1	024	**MCK-024**	GG	1	47	0	70	1,5	31,11	15	21	2	0
1	1	025	**MCK-025**	GG	0	45	0	77	1,57	31,24	12	18	3	0
1	1	026	**MCK-026**	GG	0	43	0	62	1,53	26,49	14	20	4	0
1	1	027	**MCK-027**	GA	1	45	0	65	1,6	25,39	13	29	2	0
1	1	028	**MCK-028**	GA	1	65	0				13	16	8	0
1	1	029	**MCK-029**	GA	1	72	0	77	1,56	31,64	14	18	4	0
1	1	030	**MCK-030**	GG	1	55	0	82	1,62	31,25	15	21	2	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	031	**MCK-031**	AA	1	53	0	80	1,62	30,48	13	28	2	0
1	1	032	**MCK-032**	GA	1	65	0	70	1,55	29,14	16	19	5	0
1	1	033	**MCK-033**	GA	1	49	1	73	1,56	30,00	12	22	2	0
1	1	034	**MCK-034**	GA	0	50	0	80	1,55	33,30		21	3	0
1	1	035	**MCK-035**	GG	1	52	0	68	1,55	28,3	14	18	4	0
1	1	036	**MCK-036**	GG	0	42	0	72	1,6	28,12	17	21	2	0
1	1	037	**MCK-037**	GA	0	51	0	87	1,64	32,35	12	38	1	0
1	1	038	**MCK-038**	GG	1	58	0	75	1,63	28,23	14	19	4	0
1	1	039	**MCK-039**	AA	0	36	0	80	1,5	35,56	13	21	2	0
1	1	040	**MCK-040**	GA	1	45	0	91	1,63	34,25	13	26	2	0
1	1	041	**MCK-041**	GA	0	40	0	87	1,6	33,98	12	22	3	0
1	1	042	**MCK-042**	GA	1	48	1	47	1,5	20,89	14	33	2	0
1	1	043	**MCK-043**	GG	1	53	0	75	1,55	31,22	14	17	4	0
1	1	044	**MCK-044**	GG	1	68	0	70	1,65	25,71	14	20	6	0
1	1	045	**MCK-045**	GG	1	51	0	50	1,5	22,22	13	19	3	0
1	1	046	**MCK-046**	GA	1	69	0	70	1,58	28,04	12	18	2	0
1	1	047	**MCK-047**	GA	1	61	0	78	1,58	31,24	15		0	0
1	1	048	**MCK-048**	GA	1	66	0	62	1,58	24,84	14	20	3	0
1	1	049	**MCK-049**	AA	1	46	1	60	1,55	24,97	13	26	1	0
1	1	050	**MCK-050**	AA	0	42	1	95	1,57	38,54	15	20	2	0
1	1	051	**MCK-051**	GA	1	60	0	80	1,55	33,3	12	35	2	0
1	1	052	**MCK-052**	GA	1	54	0	57	1,56	23,42	13	22	6	0
1	1	053	**MCK-053**	GG	1									0
1	1	054	**MCK-054**	GA	1	48	1	67	1,57	27,18	12	19	2	0
1	1	055	**MCK-055**	GG	1	49	1	55	1,6	21,48	14	18	2	0
1	1	056	**MCK-057**	GG	1	55	0	75	1,6	29,3	13	23	5	0
1	1	057	**MCK-058**	GG	0	35	0	90	1,68	31,89	12	23	2	0
1	1	058	**MCK-059**	GA	1	42	1	60	1,58	24,03	12	27	2	0
1	1	059	**MCK-060**	GG	1	41	0	58	1,62	22,1	13	18	4	0
1	1	060	**MCK-061**	AA	1	50	1	84	1,65	30,85	11	27	1	0
1	1	061	**MCK-062**	GA	1	40	1	87	1,65	31,96	13	16	3	0
1	1	062	**MCK-063**	GA	0	48	0	75	1,6	29,3	13		0	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	063	**MCK-064**	AA	0		0	61	1,65	22,41	14		0	0
1	1	064	**MCK-065**	GG	0	48	0	68	1,54	28,67	14	17	8	0
1	1	065	**MCK-066**	AA	1	47	1	60	1,62	22,86	13	20	4	0
1	1	066	**MCK-067**	GG	1	56	0	70	1,55	29,14	14	16	4	0
1	1	067	**MCK-068**	GG	1	76	0	75	1,6	29,3	14	21	2	0
1	1	068	**MCK-070**	GA	0	35	1	63	1,66	22,86	11		0	0
1	1	069	**MCK-071**	GA	1	78	0	76	1,56	31,23	13	18	3	0
1	1	070	**MCK-072**	GG	1	43	0	72	1,62	27,43	12	23	2	0
1	1	071	**MCK-073**	GA	1	79	0	70	1,71	23,94	13	18	2	0
1	1	072	**MCK-074**	GG	1	70	0	63	1,59	24,92	13	17	4	0
1	1	073	**MCK-075**	GG	1	58	0	75	1,65	27,55	14		0	0
1	1	074	**MCK-076**	GA	0	33	1	67	1,62	25,53	13	21	2	0
1	1	075	**MCK-077**	GG	1	61	1	80	1,57	32,46	12		0	0
1	1	076	**MCK-078**	GG	1	56	0	78	1,67	27,97	12	21	5	0
1	1	077	**MCK-079**	GG	0	41	0	70	1,7	24,22	13		4	0
1	1	078	**MCK-080**	GG	1	48	1	65	1,6	25,39	12		0	0
1	1	079	**MCK-081**	GG	0	33	0	50	1,63	18,82	14	20	3	0
1	1	080	**MCK-082**	GA	1	51	0	66	1,56	27,12	14		0	0
1	1	081	**MCK-083**	AA	1	51	1	60	1,6	23,44	12	30	2	0
1	1	082	**MCK-084**	AA	1	80	0				11	20	3	0
1	1	083	**MCK-085**	GG	0	30	0		1,6		14	20	2	0
1	1	084	**MCK-086**	GA	1	56	0		1,6		14	17	4	0
1	1	085	**MCK-087**	GG	1	73	0		1,5		14			
1	1	086	**MCK-088**	GG	1	50	0		1,7		14			
1	1	087	**MCK-089**	AA	1	62	1		1,8				0	0
1	1	088	**MCK-091**	GA	1	68	0		1,6		13			0
1	1	089	**MCK-092**	GG	1	49	0		1,6					
1	1	090	**MCK-093**	GG	1	51	0		1,7		14	18	3	0
1	1	091	**MCK-094**	AA	0	34	0		1,5		14	17	2	0
1	1	092	**MCK-096**	AA	1	51	0		1,6		14	29	1	0
1	1	093	**MCK-097**	GA	0	44	0		1,5		15		2	0
1	1	094	**MCK-098**	GA	1	58	0		1,7		13	17	2	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	095	**MCK-099**	GA	1	64	0		1,6		15	16	5	0
1	1	096	**MCK-100**	GG	0	34	0	60	1,57	24,34	13	26	2	0
1	1	097	**MCK-101**	GA	0	33	0	70	1,6	27,34	13		0	0
1	1	098	**MCK-102**	GG	0	20	0	59	1,65	21,67	14		0	0
1	1	099	**MCK-103**	GA	0	33	1	51	1,47	23,6			0	
1	1	100	**MCK-105**	GG	0	49	1	73	1,65	26,81	13		0	
1	1	101	**MCK-106**	GG	1	48	0	84	1,66	30,48			5	0
1	1	102	**MCK-107**	GA	1	50	0	85	1,6	33,2	13	18	8	0
1	1	103	**MCK-108**	GG	0	36	0	78	1,6	30,47	15	22	2	0
1	1	104	**MCK-109**	GG	1	79	0	80	1,5	35,56	13		0	0
1	1	105	**MCK-110**	GG	1	70	0	80	1,57	32,46		20	3	0
1	1	106	**MCK-111**	GG	1	43	0	78	1,6	30,47	12	18	2	0
1	1	107	**MCK-112**	AA	1	60	0	80	1,53	34,17	13	30	2	0
1	1	108	**MCK-114**	GG	1	43	0	60	1,57	24,34	13	19	2	0
1	1	109	**MCK-115**	GG	1	54	1				13	27	2	0
1	1	110	**MCK-118**	GA	0	37	0	66	1,63	24,84	13	25	2	0
1	1	111	**MCK-119**	GG	1	64	1	62	1,55	25,81	14	20	3	0
1	1	112	**MCK-121**	GA	0	38	1	61	1,55	25,39	13	18	2	0
1	1	113	**MCK-122**	GG	0		0	75	1,65	27,55	13	22	2	0
1	1	114	**MCK-125**	GA	0	33	1	48	1,65	17,63		23	1	0
1	1	115	**MCK-126**	GA	1	63	0	74	1,65	27,18	13	17	12	0
1	1	116	**MCK-127**	GG	1	43	0	63	1,47	29,15			0	0
1	1	117	**MCK-128**	GG	1	60	0	78	1,55	32,47	15	17	6	0
1	1	118	**MCK-129**	GA	1	77	0	62	1,48	28,31	14		0	0
1	1	119	**MCK-130**	AA	1	65	0	93	1,63	35	14	17	5	0
1	1	120	**MCK-131**	GG	0	30	0	70	1,6	27,34	13	18	2	0
1	1	121	**MCK-132**	AA	1	55	0	64	1,56	26,3	13	17	3	0
1	1	122	**MCK-133**	GA	1	62	0	87	1,52	37,66	12	16	3	0
1	1	123	**MCK-135**	GG	0	30	0	62	1,7	21,45	14	23	2	0
1	1	124	**MCK-136**	GG	0	44	0	76			14	24	2	0
1	1	125	**MCK-138**	GA	0	28	0	67	1,68	23,74	12	26	1	0
1	1	126	**MCK-139**	GA	1	74	1	50	1,58	20,03	20	21	5	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	127	**MCK-140**	GA	0	32	1	59	1,64	21,94	14	23	2	0
1	1	128	**MCK-141**	GG	1	74	0	74	1,57	30,02	12		0	0
1	1	129	**MCK-142**	GA	0	38	0	74	1,6	28,91			0	0
1	1	130	**MCK-143**	GG	0	45	0	57	1,65	20,94	13	19	2	0
1	1	131	**MCK-144**	AA	0	50	0	63	1,6	24,61	15			0
1	1	132	**MCK-145**	GG	1	64								
1	1	133	**MCK-148**	GA	0	28								
1	1	134	**MCK-149**	GG	0	33								
1	1	135	**MCK-150**	GA	0	49								
1	1	136	**MCK-152**	AA	0	38								
1	1	137	**MCK-155**	GA	1	51								
1	1	138	**MCK-156**	GG	1	70								
1	1	139	**MCK-159**	GA	0	44								
1	1	140	**GB-001**	AA	1	62	0	91	1,6	35,55	18	21	4	0
1	1	141	**GB-003**	GG	1	70	0	40	1,53	17,09	14	15	4	0
1	1	142	**GB-004**	GA	1	49	0	60	1,56	24,65	15	20	4	0
1	1	143	**GB-006**	GG	1	40	0	50	1,53	21,36	15	30	1	0
1	1	144	**GB-007**	GA	1	39	0	63	1,63	23,71	15		0	0
1	1	145	**GB-008**	GA	0	35	0	52	1,53	22,21	13		0	0
1	1	146	**GB-010**	GA	1	44	1	66	1,75	21,55	13	23	2	0
1	1	147	**GB-011**	GG	1	51	0	58	1,55	24,14	14	21	3	0
1	1	148	**GB-012**	GA	1	41	0	83	1,55	34,55	15	17	4	0
1	1	149	**GB-013**	GG	0	32	0	52	1,6	20,31	14	21	4	0
1	1	150	**GB-016**	GA	1	50	1	57	1,61	21,99	13	22	2	0
1	1	151	**GB-017**	GA	0	49								
1	1	152	**GB-018**	GG	1	48	1	73	1,5	32,44	14	17	2	0
1	1	153	**GB-019**	GG	1	72	0	75	1,58	30,04	11		0	0
1	1	154	**GB-020**	GG	0	47	0	107	1,6	41,80	14		0	0
1	1	155	**GB-021**	GA	0	49								
1	1	156	**GB-022**	GG	0	51	1	75	1,6	29,30	14	26	2	0
1	1	157	**GB-023**	GG	0	43	0	70	1,6	27,34	17	33	2	0
1	1	158	**GB-024**	GG	1	48	0	60	1,55	24,97	15	25	3	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	159	**GB-026**	GG	0	40	1	58	1,6	22,66	18	33	3	0
1	1	160	**GB-027**	GG	0	36	0	64	1,55	26,64	14	20	4	0
1	1	161	**GB-028**	GG	1	58	0	75	1,55	31,22	13	21	10	0
1	1	162	**GB-029**	GG	1	31	0	47	1,52	20,34	15	23	2	0
1	1	163	**GB-030**	GG	1	73	1	70	1,57	28,40	14	16	3	0
1	1	164	**GB-032**	AA	1	45	0	82	1,65	30,12	13	19	5	0
1	1	165	**GB-033**	GG	1	55	0	60	1,7	20,76	14	20	3	0
1	1	166	**GB-034**	AA	1	46	0	72	1,6	28,12	14		0	0
1	1	167	**GB-036**	GG	1	54	0	51	1,52	22,07	16	33	5	0
1	1	168	**GB-037**	GA	1	47	0	55	1,55	22,89	18	20	8	0
1	1	169	**GB-038**	GG	1	64	0	55	1,55	22,89	14	27	6	0
1	1	170	**GB-039**	AA	1	43	0	84	1,5	37,33	13	23	3	0
1	1	171	**GB-040**	GG	1	40	0	76	1,58	30,44	12	24	4	0
1	1	172	**GB-041**	GG	1	52	0	84	1,7	29,07	16	18	5	0
1	1	173	**GB-042**	GG	0	40	0	80	1,65	29,38	14	30	3	0
1	1	174	**GB-043**	AA	1	45	0	70	1,6	27,34	13	22	4	0
1	1	175	**GB-044**	GA	1	66	0	60	1,63	22,58	15	26	7	0
1	1	176	**GB-045**	GG	0	40	0	83	1,55	34,55	13	22	2	0
1	1	177	**GB-046**	GA	1	38	0	55	1,52	23,81	15	21	3	0
1	1	178	**GB-047**	GA	1	49	0	63	1,6	24,61	14		0	0
1	1	179	**GB-048**	GG	1	54		70	1,6	27,34	16	20	3	0
1	1	180	**GB-049**	GA	1	40	0	85	1,5	37,78	13	22	1	0
1	1	181	**GB-050**	GG	0	34	0	63	1,55	26,22	13	23	3	0
1	1	182	**GB-051**	GA	1	54	0	75	1,56	30,82	15	24	6	0
1	1	183	**GB-052**	GG	1	71	0	90	1,62	34,29	12	21	1	0
1	1	184	**GB-053**	GA	0	32	0	70	1,52	30,30	13	19	3	0
1	1	185	**GB-054**	GA	0	46	0	51	1,55	21,23	14	26	1	0
1	1	186	**GB-055**	GA	1	46	0	71	1,53	30,33	13	16	7	0
1	1	187	**GB-056**	GA	0	44	0	80	1,65	29,38	15	30	3	0
1	1	188	**GB-057**	AA	1	77	1	54	1,6	21,09	12	21	2	0
1	1	189	**GB-058**	GG	0	42	0	75	1,65	27,55	14	18	4	0
1	1	190	**GB-059**	GG	1	66	0	73	1,55	30,39	15	19	3	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	191	**GB-060**	AA	1	36	1	52	1,52	22,51	13	25	1	0
1	1	192	**GB-062**	GA	1	46	0	70	1,6	27,34	14	18	3	0
1	1	193	**GB-063**	GA	0	41	0	75	1,64	27,89	12		0	0
1	1	194	**GB-064**	GA	1	68	0	78	1,56	32,05	14	19	10	0
1	1	195	**GB-065**	GG	1	45	0	80	1,52	34,63	14	24	4	0
1	1	196	**GB-066**	GG	0	38	0	60	1,5	26,67	15	20	4	0
1	1	197	**GB-067**	GG	0	38	0	64	1,68	22,68	16	20	5	0
1	1	198	**GB-068**	GG	0	40	0	83	1,55	34,55	14	22	5	0
1	1	199	**GB-069**	GA	1	44	0	58	1,5	25,78	15	32	3	0
1	1	200	**GB-070**	GA	1	70	0	78	1,57	31,64	14	16	3	0
1	1	201	**GB-071**	GA	0	35	0	63	1,58	25,24	14	18	6	0
1	1	202	**GB-072**	GG	0	34	0	70	1,6	27,34	13	25	2	0
1	1	203	**GB-073**	GA	1	78	0	58	1,62	22,10	15	22	2	0
1	1	204	**GB-074**	GG	0	33	0	56	1,5	24,89	14	22	5	0
1	1	205	**GB-075**	GA	0	30	0	55	1,7	19,03	13	22	3	0
1	1	206	**GB-076**	GG	1	76	0	55	1,65	20,20	13	19	2	0
1	1	207	**GB-077**	GG	1	52	0	56	1,62	21,34	14	20	6	0
1	1	208	**GB-080**	GA	0	32	0	52	1,6	20,31	13	19	3	0
1	1	209	**GB-081**	GA	1	42	1	80	1,62	30,48	16	34	1	0
1	1	210	**GB-082**	GA	0	44	0	82	1,6	32,03	12	18	2	0
1	1	211	**GB-083**	GG	1	53	0	100	1,55	41,62	14	19	6	0
1	1	212	**GB-084**	GA	1	76	0	80	1,6	31,25	15	33	1	0
1	1	213	**GB-088**	GG	1	53	0	80	1,55	33,30		23	3	0
1	1	214	**GB-089**	AA	0	48	0	75	1,6	29,30	15	17	4	0
1	1	215	**GB-090**	GG	1	44	1	65	1,65	23,88	12	23	2	0
1	1	216	**GB-091**	GG	0	37	0	75	1,6	29,30	13	22	3	0
1	1	217	**GB-092**	GG	1	74	0	70	1,55	29,14	13	18	7	0
1	1	218	**BC-001**	GG	0	47					13		0	0
1	1	219	**BC-002**	GA	0	45		67	1.6		13	20	2	0
1	1	220	**BC-003**	GG	1	59		110	1.65		14.5	17	9	0
1	1	221	**BC-004**	GA	1	57		50	1.5		12	21	2	0
1	1	222	**BC-005**	GA	1	55					14		2	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	223	**BC-007**	GA	1	62		98	1.6		14	18	4	0
1	1	224	**BC-008**	GA	1	54		103	1.64		16	23	3	0
1	1	225	**BC-009**	GA	1	53		70	1.6		11	33	2	0
1	1	226	**BC-010**	GA	1	66					12.5	22	2	0
1	1	227	**BC-011**	GG	0	44		59	1.43		16.5	35	3	0
1	1	228	**BC-012**	GA	1	46		76	1.68		16	18	2	0
1	1	229	**BC-013**	GA	0	34		60	1.58		12	29	2	0
1	1	230	**BC-014**	GG	1	40		70	1.58		17.5	20	2	1
1	1	231	**BC-015**	GA	1	50		61,5	1.55		13	23	2	1
1	1	232	**BC-016**	GA	1	48		58	1.51		14	38	2	1
1	1	233	**BC-017**	GG	0	35						25	2	0
1	1	234	**BC-018**	GG	0	44		70	1.62		15	39	0	0
1	1	235	**BC-019**	GA	1	58		92	1.66		12	15	2	1
1	1	236	**BC-020**	GA	1	52		60	1.61		13.5	25	2	0
1	1	237	**BC-021**	GG	1	52							0	0
1	1	238	**BC-022**	GG	1	65		70	1.61		12	22	3	0
1	1	239	**BC-023**	GA	0	39		70	1.60		13	19	3	1
1	1	240	**BC-024**	GG	0	43		77	1.72		12	21	3	0
1	1	241	**BC-028**	GA	1	73		69	1.55		14	21	3	1
1	1	242	**BC-029**	GA	1	55		80	1.57		15	19	3	1
1	1	243	**BC-030**	GA	1	62					14	19	3	0
1	1	244	**BC-031**	GA	0	48		87			14	22	3	0
1	1	245	**BC-036**	GG	0	29		105	1.7		13	22	2	0
1	1	246	**BC-038**	GA	0	40		65	1.6		12	21	3	0
1	1	247	**BC-039**	GG	1	75		45	1.5		14	15	10	0
1	1	248	**BC-040**	GA	1	52		70	1.6		16	42	3	0
1	1	249	**BC-041**	GA	1	50		75	1.64		12.5		0	0
1	1	250	**BC-042**	GG	0	47		66	1.68		13	17	2	0
1	1	251	**BC-043**	GA	0	44		70	1.75			37	1	0
1	1	252	**BC-044**	GA	0	43		58	1.7		13	22	3	0
1	1	253	**BC-045**	GG	0	42		60	1.5				0	0
1	1	254	**BC-046**	GG	0	40		50	1.51		12	23	4	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	255	**BC-047**	GA	1	55						37	2	0
1	1	256	**BC-048**	GA	1									
1	1	257	**BC-049**	GG	1	57		57	1.62			30	2	1
1	1	258	**BC-050**	GG	0	44		57	1.55			29	4	0
1	1	259	**BC-051**	GA	0	31		60	1.6		12	25	2	0
1	1	260	**BC-052**	AA	0	43		75	1.67		11	21	2	0
1	1	261	**BC-053**	GG	0	30		68	1.64			20	1	0
1	1	262	**BC-055**	GA	0	43		46	1.6		14	26	2	0
1	1	263	**BC-060**	GG	0	54		80	1.65			25	2	0
1	1	264	**BC-061**	GA	1	51		48	1.55		13	26	3	0
1	1	265	**BC-062**	GA	0	40		85	1.75		14	36	2	0
1	1	266	**BC-063**	AA	1	72						29	1	
1	1	267	**BC-064**	GG	0	37		70	1.65		13	23	2	0
1	1	268	**BC-065**	GG	1	46		86	1.67		11	28	0	1
1	1	269	**BC-068**	GG	0	43						22	2	0
1	1	270	**BC-069**	GA	1	65		64	1.57		13	24	2	0
1	1	271	**BC-070**	GG	0	43		65	1.58		13	23	2	0
1	1	272	**BC-071**	GG	1	54		87	1.67		13		0	1
1	1	273	**BC-072**	GG	1	44		87	1.67		13	18	4	1
1	1	274	**BC-073**	GG	1	51		60	1.55		16	30	2	0
1	1	275	**BC-074**	GG	0	34		94			12.5	30	1	1
1	1	276	**BC-075**	GA	1	62		50	1.61		12	25	4	0
1	1	277	**BC-076**	GA	0	45								1
1	1	278	**BC-077**	GG	0	48		75	1.57		16	20	3	0
1	1	279	**BC-078**	AA	0	48		96	1.7		13	23	4	0
1	1	280	**BC-080**	GG	0	40						25	2	0
1	1	281	**BC-081**	GG	1	78		86	1.55		12	18	3	0
1	1	282	**BC-082**	GG	0	37		60	1.55		12.5	27	2	0
1	1	283	**BC-085**	GG	0	42		59	1.5		12	24	1	0
1	1	284	**BC-086**	GA	1	55		70	1.6		13	26	2	0
1	1	285	**BC-087**	GG	1	53						28	2	0
1	1	286	**BC-088**	GG	0	37		59	1.68		13	25	2	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	1	287	**BC-089**	GG	1	70						34	3	0
1	1	288	**BC-090**	GA	0	30						20	2	0
1	1	289	**BC-091**	GG	0	40					12	20	1	0
1	1	290	**BC-202**	GA	1	54		78	1.58		13	20	2	0
1	1	291	**BC-203**	GA	0	40		60	1.6		13	25	2	0
1	1	292	**BC-204**	GA	0	34		62	1.5		13.5	16	2	0
1	1	293	**BC-207**	GG	0	43		62	1.56			27	3	1
1	1	294	**BC-208**	GA	1	61						15	2	0
1	1	295	**BC-209**	GA	0	35		85	1.74		13	26	2	1
1	1	296	**BC-210**	AA	0	51		77	1.69		13		0	0
1	1	297	**BC-211**	GA	0	38		56	1.6		18	24	3	0
1	1	298	**BC-303**	GG	1	51		66	1.65		14	23	3	1
1	1	299	**BC-305**	AA	0	47		66	1.72		14	24	2	0
1	1	300	**BC-306**	GG	1	53		63	.153		13	18	2	1
1	1	301	**BC-309**	GA	0	27		73	1.62		13	20	2	0
2	1	302	**62**	GG	0	54					12	28		1
2	1	303	**63**	AA	0	50					11	24	2	1
2	1	304	**64**	GA	0	48		62.5			13	25	2	1
2	1	305	**65**	GG	1	74					14	26		1
2	1	306	**66**	GG	0	52					12	23		1
2	1	307	**67**	AA	1	59					13	19		1
2	1	308	**68**	GA	0	48					12	23	2	1
2	1	309	**70**	GG	0	45					12	21	2	1
2	1	310	**71**	GG	0	51					13	21	2	1
2	1	311	**72**	GG	1									1
2	1	312	**73**	GG	1	81					13	23		1
2	1	313	**74**	GG	0						12	23		1
2	1	314	**75**	GA	0	50					13		0	1
2	1	315	**76**	GG	1						13	25		1
2	1	316	**77**	AA	1	82					14	26		1
2	1	317	**78**	AA	0	51					13	31		1
2	1	318	**79**	GG	1	72					11	36		1

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	1	319	**80**	GG	1	72					13			1
2	1	320	**81**	GG	1	80							0	1
2	1	321	**83**	GG	1						11	18		1
2	1	322	**85**	AA	0	42					13	35	1	1
2	1	323	**86**	AA	1	48					14	29	2	1
2	1	324	**87**	GA	0									1
2	1	325	**88**	AA	1	60					12	20	2	1
2	1	326	**90**	GG	0	56					13	22		1
2	1	327	**92**	GG	1	50								1
2	1	328	**94**	GA	1	52					13	24		1
2	1	329	**98**	GA	0	56		81	1.67		13	18	3	1
2	1	330	**99**	GG	0			60	1.68		11	21	2	1
2	1	331	**120**	GA	1									1
2	1	332	**123**	GA	1	65					13		0	1
2	1	333	**124**	GG	0	38					13		0	1
2	1	334	**125**	GA	0	56					14		2	1
2	1	335	**126**	GG	0	43					13		2	1
2	1	336	**127**	GA	1	66					13	18	3	1
2	1	337	**128**	GA	0	30					15		1	1
2	1	338	**129**	GA	0	32					12	21	2	1
2	1	339	**130**	AA	0	32					14		0	1
2	1	340	**131**	GA	0	37					12	25	2	1
2	1	341	**132**	GA	0	28					10		0	1
2	1	342	**133**	GA	0	28							0	1
2	1	343	**134**	GG	0	53							2	1
2	1	344	**137**	GA	0	42					14	20		1
2	1	345	**138**	GA	1	86					12	22	2	1
2	1	346	**139**	GG	1	49					14	24	1	1
2	1	347	**140**	GA	1	73					12			1
2	1	348	**141**	GA	0	58						21		1
2	1	349	**143**	AA	0									1
2	1	350	**145**	GA	1									1

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	1	351	**146**	AA	0									1
2	1	352	**150**	GG	1									1
2	1	353	**151**	GA	0									1
2	1	354	**152**	GG	1									1
2	1	355	**153**	GA	0									1
2	1	356	**154**	AA	0									1
2	1	357	**155**	GA	1									1
2	1	358	**156**	GA	0									1
2	1	359	**157**	GA	0									1
2	1	360	**158**	GA	0									1
2	1	361	**159**	GG	1									1
2	1	362	**160**	GG	0									1
2	1	363	**162**	GG	1									1
2	1	364	**163**	GA	1									1
2	1	365	**165**	GG	1									1
2	1	366	**167**	GG	1									1
2	1	367	**169**	AA	1						14		0	1
2	1	368	**171**	GA	0	40					11	23	2	1
2	1	369	**174**	GA	1									1
2	1	370	**175**	AA	0	48								1
2	1	371	**176**	GA	0	51								1
2	1	372	**180**	AA	0									1
2	1	373	**182**	AA	1									1
2	1	374	**186**	GA	0									1
2	1	375	**197**	GA	1									1
2	1	376	**198**	GG	0									1
2	1	377	**199**	GA	1									1
2	1	378	**200**	GA	1									1
2	1	379	**201**	GG	1									1
2	1	380	**206**	AA	0									1
2	1	381	**207**	AA	1									1
2	1	382	**208**	GG	0	39					13	29	1	1

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	1	383	**209**	GA	0									1
2	1	384	**211**	GA	1									1
2	1	385	**219**	GG	1	40								1
2	1	386	**221**	GG	0	50								1
2	1	387	**222**	GG	0	25								1
2	1	388	**224**	AA	1	54								1
2	1	389	**227**	GA	0	48								1
2	1	390	**228**	GG	0	49								1
2	1	391	**229**	GA	0									1
2	1	392	**230**	GA	0									1
2	1	393	**231**	GA	0	49								1
2	1	394	**232**	GG	1	63								1
2	1	395	**233**	GA	0									1
2	1	396	**234**	GA	1	53								1
2	1	397	**235**	GA	1									1
2	1	398	**239**	GA	0	47					14		0	1
2	1	399	**240**	GA	0	36					12		0	1
2	1	400	**244**	GA	0	49					15	30	1	1
2	1	401	**245**	GA	1									1
2	1	402	**246**	GA	0									1
2	1	403	**247**	GG	0									1
2	1	404	**248**	GA	1									1
2	1	405	**249**	GG	0	20								1
2	1	406	**250**	AA	0	42								1
2	1	407	**251**	AA	0	31								1
2	1	408	**252**	GG	0	28								1
2	1	409	**253**	AA	0	32								1
2	1	410	**254**	GA	0	45					11	22	2	1
2	1	411	**255**	GA	0									1
2	1	412	**256**	GG	1	63					12		0	1
2	1	413	**257**	AA	1	69					15	30	1	1
2	1	414	**258**	GG	0	39					16		0	1

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	1	415	**259**	GG	1								2	1
2	1	416	**260**	GA	1									1
2	1	417	**261**	AA	1	59		63	1.58			33	2	1
2	1	418	**262**	GG	0	38					13	26	2	1
2	1	419	**266**	GG	1									1
2	1	420	**267**	GG	1									1
2	1	421	**270**	GA	0									1
2	1	422	**271**	GA	0	45								1
2	1	423	**273**	GA	0	38					14	32	1	1
2	1	424	**274**	GG	1									1
2	1	425	**275**	GA	0									1
2	1	426	**276**	GA	0	29								1
2	1	427	**277**	AA	0									1
2	1	428	**279**	GG	0	21								1
2	1	429	**281**	GA	1									1
2	1	430	**282**	GA	1									1
2	1	431	**283**	GG	0	39					12		0	1
2	1	432	**284**	GA	1						11		2	1
2	1	433	**286**	AA	1	62					12		0	1
2	1	434	**287**	GG	0	72					13	35	2	1
2	1	435	**288**	GA	0	29					12		0	1
2	1	436	**289**	GA	1									1
2	1	437	**291**	GG	1									1
2	1	438	**292**	GG	0									1
2	1	439	**293**	GA	1						15		0	1
2	1	440	**294**	GA	1	74					12		2	1
2	1	441	**295**	GG	1	54					12		17	1
2	1	442	**296**	GA	1	58					13			1
2	1	443	**297**	GA	1									1
2	1	444	**303**	GG	0									1
2	1	445	**304**	GG	1	71								1
2	1	446	**305**	GG	1									1

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	1	447	**306**	GG	1	72								1
2	1	448	**307**	GG	0									1
2	1	449	**308**	GA	1	49								1
2	1	450	**310**	GG	0	36					12	20	2	1
2	1	451	**311**	GG	1	60					12	21	2	1
2	1	452	**312**	GG	0	38					11	20	2	1
2	1	453	**317**	GG	1									1
1	0	454	**NK-001**	GA	0	38	1	55	1,6	21,48	13	23	3	0
1	0	455	**NK-002**	GA	1	50	0	75	1,57	30,43	14	23	5	0
1	0	456	**NK-003**	GA	0	43	1	73	1,65	26,81	13	19	3	0
1	0	457	**NK-004**	AA	1	51	1	55	1,57	22,31	15	15	4	0
1	0	458	**NK-005**	GG	1	69	0	85	1,7	29,41		29	7	0
1	0	459	**NK-006**	GG	1	63								
1	0	460	**NK-007**	AA	1	72	0	48	1,47	22,21	15	20	5	0
1	0	461	**NK-008**	GG	1	75	0	75	1,52	32,46	13	20	4	0
1	0	462	**NK-009**	GG	0	41								
1	0	463	**NK-010**	AA	1	57	0	84	1,63	31,62	13	20	3	0
1	0	464	**NK-011**	GA	1	51								
1	0	465	**NK-012**	GG	0	52	0	53	1,63	19,95	14	18	3	0
1	0	466	**NK-013**	GA	1	56	0	48	1,34	26,73	14	16	7	0
1	0	467	**NK-014**	GA	1	73	0	65	1,47	30,08	14	22	3	0
1	0	468	**NK-015**	GA	0	42	0	81	1,66	29,39	13	19	3	0
1	0	469	**NK-016**	AA	1	62	0	55	1,56	22,60	18	22	2	0
1	0	470	**NK-017**	GG	0	40	0	58	1,6	22,66	15	20	3	0
1	0	471	**NK-018**	GG	0	34	1	50	1,67	17,93	15		0	0
1	0	472	**NK-019**	GG	1	50	0	58	1,57	23,53	15	20	2	0
1	0	473	**NK-020**	GA	1	41	0	92	1,6	35,94	10	20	2	0
1	0	474	**NK-021**	GA	0	42								
1	0	475	**NK-022**	GA	0	37	1	57	1,55	23,73	16	33	1	0
1	0	476	**NK-025**	AA	0	46	1	71	1,61	27,39	15	23	2	0
1	0	477	**NK-026**	GG	0	15	0	70	1,57	28,40	13			0
1	0	478	**NK-027**	GG	0	18	0	50	1,55	20,81	15			0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	0	479	**NK-029**	GG	1	64	0	61	1,52	26,40	16	22	2	0
1	0	480	**NK-030**	GG	1	64								
1	0	481	**NK-031**	GG	0	34	0	85	1,64	31,60	14	22	1	0
1	0	482	**NK-032**	GA	0	18	1	55	1,68	19,49	12			0
1	0	483	**NK-034**	GA	0	27	0	63	1,6	24,61	14	18	2	0
1	0	484	**NK-035**	GA	1	53	0	80	1,6	31,25	14	21	3	0
1	0	485	**NK-036**	GA	0	41	0	89	1,64	33,09	13	23	2	0
1	0	486	**NK-037**	GA	1	30	1	116	1,68	41,10	12		0	0
1	0	487	**NK-038**	GA	0	32	1	60	1,58	24,03	14	16	2	0
1	0	488	**NK-039**	GA	0	47	0	84	1,58	33,65	14	16	5	0
1	0	489	**NK-040**	GG	0	34	0	80	1,6	31,25	14	21	4	0
1	0	490	**NK-043**	GA	0	47	0	65	1,62	24,77	12	19	5	0
1	0	491	**NK-045**	GA	0	46	0	63	1,57	25,56	14	18	2	0
1	0	492	**NK-046**	GG	1	70	0	72	1,6	28,12	12		0	0
1	0	493	**NK-047**	GG	1	70								
1	0	494	**NK-048**	GG	0	47	0	61	1,62	23,24	14		0	0
1	0	495	**NK-049**	GA	1	74	0	55	1,6	21,48	14		0	0
1	0	496	**NK-050**	GA	1	67	0	55	1,51	24,12	17	20	5	0
1	0	497	**NK-051**	GG	1	66	0	77	1,5	34,22	14	16	6	0
1	0	498	**NK-052**	GA	0	37	1	45	1,6	17,58	17	25	2	0
1	0	499	**NK-053**	GA	0	46	1	84	1,53	35,88	13	16	2	0
1	0	500	**NK-054**	GA	1	53	0	63	1,51	27,63	14	19	7	0
1	0	501	**NK-055**	GG	0	34	1	58	1,63	21,83	15	21	2	0
1	0	502	**NK-057**	GG	1	53								
1	0	503	**NK-058**	GG	0	47	0	80			15	30	1	0
1	0	504	**NK-059**	GA	1	71	1	55	1,5	24,44	13	24	6	0
1	0	505	**NK-061**	GA	0	45								
1	0	506	**NK-062**	GG	1	65	0	70	1,5	31,11	12	15	6	0
1	0	507	**NK-063**	GG	1	58	0	65	1,57	26,37	14	21	4	0
1	0	508	**NK-065**	GG	0	34	0	65	1,59	25,71	14	24	3	0
1	0	509	**NK-066**	GA	1	63	0	80	1,6	31,25	13	20	3	0
1	0	510	**NK-067**	GG	0	41	0	63	1,5	28	12	30	2	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	0	511	**NK-068**	GG	1	51	0				12	22	3	0
1	0	512	**NK-069**	GG	0	20	0	55	1,57	22,31	12	18	1	0
1	0	513	**NK-072**	GA	0	46	0	72	1,6	28,12	16	18	5	0
1	0	514	**NK-073**	GA	1	56	0	65	1,56	26,71	12	18	6	0
1	0	515	**NK-074**	GA	1	54	0	74	1,56	30,41	16	17	4	0
1	0	516	**NK-075**	GG	0	49	0	89	1,51	39,03	13	15	7	0
1	0	517	**NK-076**	GA	1	66	0	50	1,52	21,64	12	20	5	0
1	0	518	**NK-077**	GA	0	17	0	59	1,54	24,88	14		0	0
1	0	519	**NK-078**	GG	0	34	0	70	1,58	28,04	15	20	3	0
1	0	520	**NK-079**	AA	1	57	0	80	1,54	33,73	17	19	4	0
1	0	521	**NK-083**	GA	0	45	1	63	1,67	22,59	11	15	2	0
1	0	522	**NK-084**	GG	0	45	0				13	17	3	0
1	0	523	**NK-086**	GG	0	20	0	61	1,68	21,61	14		0	0
1	0	524	**NK-091**	GA	0	33	1	57	1,65	20,94	13	20	1	0
1	0	525	**NK-092**	GA	0	35	0	52	1,6	20,31	14		0	0
1	0	526	**NK-094**	GG	1	56	1	59	1,6	23,05	13	15	4	0
1	0	527	**NK-095**	GG	0	24	0	45	1,55	18,73	13		0	0
1	0	528	**NK-096**	GA	0	26	0	65	1,6	25,39	15		0	0
1	0	529	**NK-097**	GG	0	28	0				14	18	1	0
1	0	530	**NK-098**	GG	0	41	1	66	1,55	27,47		24	2	0
1	0	531	**NK-100**	GA	1	55	0	85	1,55	35,38	12	20	4	0
1	0	532	**NK-101**	GA	0	37	1	68	1,69	23,81	13		0	0
1	0	533	**NK-102**	GA	0	48	0	70			19	20	2	0
1	0	534	**NK-103**	GA	1	75	0	70	1,6	27,34	14	27	2	0
1	0	535	**NK-104**	GA	1	55	0	66	1,6	25,78	17	21	1	0
1	0	536	**NK-105**	GA	0	19	0	60	1,55	24,97	13		0	0
1	0	537	**NK-106**	GG	1	59	0	86	1,66	31,21	13	19	4	0
1	0	538	**NK-107**	GA	1	63	0	92	1,7	31,83				0
1	0	539	**NK-108**	GA	1	83	0	76	1,55	31,63			3	0
1	0	540	**NK-109**	GG	1	50	0	80	1,6	31,25	13	17	3	0
1	0	541	**NK-110**	GG	1	64	1	75	1,65	27,55	12	16	5	0
1	0	542	**NK-111**	GA	0	32	1	70	1,56	28,76	13	20	3	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	0	543	**NK-112**	GA	0	36	0	58	1,5	25,78	12	24	2	0
1	0	544	**NK-113**	GA	1	50	0	70	1,59	27,69	15	19	8	0
1	0	545	**NK-114**	GG	1	55	0	44	1,62	16,77	15	16	5	0
1	0	546	**NK-116**	GG	1	53	0	70	1,5	31,11	14	17	10	0
1	0	547	**NK-117**	GG	0	33	1	40	1,65	14,69	14	24	3	0
1	0	548	**NK-118**	GA	0	43	0	80	1,6	31,25	13	19	3	0
1	0	549	**NK-119**	GG	1	75	0	95	1,63	35,76	13	23	8	0
1	0	550	**NK-122**	GA	1	50	0	73	1,6	28,52	16	21	5	0
1	0	551	**NK-127**	GG	0	31	0	74	1,64	27,51	12	19	1	0
1	0	552	**NK-128**	AA	1	52	0	60	1,58	24,03	14	21	5	0
1	0	553	**NK-131**	AA	0	27	1	41	1,52	17,75	13	19	2	0
1	0	554	**NK-133**	GA	0	38	0	63	1,56	25,89	17		0	0
1	0	555	**NK-134**	GG	1	61	0	83	1,61	32,02	15	18	6	0
1	0	556	**NK-135**	GA	0	43	0	64	1,58	25,64	15	17	4	0
1	0	557	**NK-137**	GG	1	54	0	61	1,55	25,39	14	29	2	0
1	0	558	**NK-138**	GG	0	45	1	60	1,5	26,67	14	16	6	0
1	0	559	**NK-139**	GA	1	52	1	92	1,6	35,94	14	18	5	0
1	0	560	**NK-140**	GG	1	45	0	83	1,58	33,25	13	18	5	0
1	0	561	**NK-142**	GG	0	27	0	50	1,68	17,72	13	20	1	0
1	0	562	**NK-143**	GG	0	46	0	110	1,58	44,06	16	16	4	0
1	0	563	**NK-147**	GG	0	39	1	84	1,7	29,07	14	22	2	0
1	0	564	**NK-148**	GG	1	51	0	78	1,61	30,09	16	20	3	0
1	0	565	**NK-149**	AA	0	40	1	60	1,59	23,73	15	19	3	0
1	0	566	**NK-151**	GA	0	47	0	67	1,6	26,17	17	22	5	0
1	0	567	**NK-153**	GA	1	77								
1	0	568	**NK-154**	GA	1	70								
1	0	569	**NK-155**	AA	0	26								
1	0	570	**NK-156**	AA	1	52								
1	0	571	**NK-159**	GA	0	33								
1	0	572	**NK-163**	GA	1	64								
1	0	573	**NK-164**	GG	1	55								
1	0	574	**NK-165**	GG	0	38								

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	0	575	**NK-169**	GG	0	45								
1	0	576	**NK-170**	GA	1	74								
1	0	577	**GK-001**	GG	1	69	0	62	1,59	24,52		25	3	0
1	0	578	**GK-002**	GG	0	36	1	55	1,56	22,60	14	22	2	0
1	0	579	**GK-003**	GG	1	52	1	52	1,52	22,51	15		2	0
1	0	580	**GK-004**	GG	0	34	0	75	1,64	27,89	13	21	2	0
1	0	581	**GK-005**	GA	1	49	1	90	1,65	33,06	13	17	4	0
1	0	582	**GK-007**	GG	1	56	0	60	1,55	24,97	12	17	4	0
1	0	583	**GK-008**	GG	0	52	0	66	1,61	25,46	13	21	2	0
1	0	584	**GK-009**	AA	0	43	0	73	1,56	30,00	13	20	1	0
1	0	585	**GK-010**	GG	1	65	0	58	1,5	25,78	13	17	5	0
1	0	586	**GK-011**	GG	1	53	1	72	1,65	26,45	14	22	3	0
1	0	587	**GK-012**	GG	1	48	0	68	1,68	24,09	13	27	2	0
1	0	588	**GK-013**	GG	1	50	0	60	1,6	23,44	14	22	1	0
1	0	589	**GK-014**	GG	1	44	0	81	1,62	30,86	16			0
1	0	590	**GK-015**	GA	1	42	0	103	1,61	39,74		20	4	0
1	0	591	**GK-016**	GG	1	69								
1	0	592	**GK-017**	GG	0									
1	0	593	**GK-018**	GG	1	69								
1	0	594	**GK-019**	GA	1	69								
1	0	595	**GK-020**	GG	1	55	0	70	1,56	28,76	11	25	5	0
1	0	596	**GK-021**	GG	0	37	0	89	1,5	39,56	13	17	5	0
1	0	597	**GK-022**	AA	1	46	0	60	1,52	25,97		19	2	0
1	0	598	**GK-023**	GG	1	39	0	70	1,58	28,04	13	14	10	0
1	0	599	**GK-024**	GA	1	55	0	80	1,5	35,56	14	17	3	0
1	0	600	**GK-025**	AA	1	56	1	75	1,56	30,82	13	19	3	0
1	0	601	**GK-026**	GG	1	42	0	80	1,63	30,11		22	3	0
1	0	602	**GK-027**	GG	0	39	1	61	1,56	25,07	14	24	1	0
1	0	603	**GK-028**	GG	0	47	0	67	1,6	26,17	13	17	2	0
1	0	604	**GK-029**	GG	1	67	0	80	1,55	33,30		27	5	0
1	0	605	**GK-030**	GG	1	65	0	65	1,65	23,88	13	24	4	0
1	0	606	**GK-031**	GG	1	62	0	66	1,6	25,78	15	17	3	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	0	607	**GK-032**	GG	1	32	0	63	1,56	25,89		20	2	0
1	0	608	**GK-033**	GG	0	39	0	80	1,62	30,48	15	16	3	0
1	0	609	**GK-034**	GG	1	55	0	80	1,62	30,48	15	20	5	0
1	0	610	**GK-035**	GA	0	45	0	63	1,58	25,24	13	17	2	0
1	0	611	**GK-036**	GG	0	38	0	90	1,64	33,46	13	18	2	0
1	0	612	**GK-037**	GG	0	45	1	75	1,59	29,67	11	27	2	0
1	0	613	**GK-038**	GA	0	52	0	65	1,52	28,13	12	20	3	0
1	0	614	**GK-039**	GA	1	60	1	85	1,66	30,85	15	21	4	0
1	0	615	**GK-040**	GG	0	31	1	67	1,68	23,74	13	19	3	0
1	0	616	**GK-041**	GG	1	69	0	87	1,52	37,66	14	18	3	0
1	0	617	**GK-042**	GA	1	55	0	75	1,64	27,89	15	19	4	0
1	0	618	**GK-043**	GA	0	40	0	72	1,6	28,12	15	26	1	0
1	0	619	**GK-044**	GA	1	56	0	65	1,54	27,41	15	27	4	0
1	0	620	**GK-045**	GG	0	45	1	61	1,6	23,83	14	27	2	0
1	0	621	**GK-046**	GA	0	47	0	72	1,5	32,00	13	21	2	0
1	0	622	**GK-047**	AA	0	29	1	60	1,65	22,04	14	29	1	0
1	0	623	**GK-048**	GG	1	44	1	59	1,56	24,24	14	24	2	0
1	0	624	**GK-049**	GA	1	49	0	60	1,6	23,44	15	23	4	0
1	0	625	**GK-051**	GG	1	50								
1	0	626	**GK-052**	GG	0	33	1	54	1,64	20,08	15		0	0
1	0	627	**GK-053**	GG	1	50	0	75	1,53	32,04	14	24	6	0
1	0	628	**GK-054**	GA	1	67	0	66	1,62	25,15	16	20	5	0
1	0	629	**GK-055**	GA	1	49	1	55	1,55	22,89	14	23	2	0
1	0	630	**GK-056**	GA	1	45	0	65	1,65	23,88	13	22	4	0
1	0	631	**GK-057**	GA	0	39	1	69	1,6	26,95	12	27		0
1	0	632	**GK-058**	GA	0	41	0	54	1,7	18,69	13		0	0
1	0	633	**GK-059**	GA	1	53	0	66	1,65	24,24	12	26	2	0
1	0	634	**GK-060**	GG	0	46	0	97	1,73	32,41	14	21	1	0
1	0	635	**GK-061**	GG	0	34	0	59	1,55	24,56	14	16	4	0
1	0	636	**GK-062**	GG	0	36	0	54	1,64	20,08	15	22	4	0
1	0	637	**GK-063**	GG	0	46	0	97	1,73	32,41	14	21	1	0
1	0	638	**GK-064**	GG	0	37	0	80	1,5	35,56	15	25	3	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	0	639	**GK-066**	GG	0	48	1	48	1,52	20,78	15	23	2	0
1	0	640	**GK-067**	GG	0	48	0	80	1,63	30,11	14	18	3	0
1	0	641	**GK-068**	GG	1	43	0	77	1,68	27,28	15	16	4	0
1	0	642	**GK-069**	GG	1	62	0	72	1,53	30,76	14	22	6	0
1	0	643	**GK-070**	GG	1	38	0	75	1,65	27,55	12	27	2	0
1	0	644	**GK-071**	GG	0	34	0	72	1,53	30,76	14	23	3	0
1	0	645	**GK-072**	GG	1	40	0	58	1,5	25,78	13			0
1	0	646	**GK-073**	GG	0	25	0	48	1,62	18,29	16		0	0
1	0	647	**GK-075**	AA	1	70								
1	0	648	**GK-076**	GA	0	42	0	72	1,58	28,84	14	23	4	0
1	0	649	**GK-077**	AA	0	20	0	58	1,65	21,30	13		0	0
1	0	650	**GK-079**	GA	1	69	0	76	1,54	32,05	14	19	5	0
1	0	651	**GK-080**	GG	0	43	1	65	1,58	26,04	12	37	1	0
1	0	652	**GK-081**	GA	0	38	0	41	1,55	17,07	14		0	0
1	0	653	**GK-082**	GA	0	31	0	66	1,59	26,11	17	17	1	0
1	0	654	**GK-083**	GA	0	27	1	55	1,67	19,72	14	15	3	0
1	0	655	**GK-084**	GA	0	29	1	48	1,6	18,75	15	26	1	0
1	0	656	**GK-085**	AA	1	55	0	80	1,53	34,17	16	18	7	0
1	0	657	**GK-086**	GA	0	34	1	57	1,65	20,94	13	19	2	0
1	0	658	**GK-087**	GA	0	33	1	66	1,65	24,24	13	25	2	0
1	0	659	**GK-088**	GG	1	75	0	60	1,5	26,67	14	17	8	0
1	0	660	**GK-089**	AA	0	47	1	86	1,58	34,45	13	20	4	0
1	0	661	**GK-090**	GG	0	28	1	55	1,67	19,72	12	19	2	0
1	0	662	**GK-091**	GG	1	54	0	79	1,65	29,02	13	19	4	0
1	0	663	**GK-092**	GA	1	45	1	67	1,6	26,17	14	32	1	0
1	0	664	**GK-093**	GA	0	40	0	80	1,54	33,73	13	26	2	0
1	0	665	**GK-094**	AA	1	43	1	61	1,6	23,83	15	26	2	0
1	0	666	**GK-095**	GG	1	42	1	50	1,59	19,78	16	21	2	0
1	0	667	**GK-096**	GA	1	51	1	56	1,55	23,31	13	30	2	0
1	0	668	**GK-097**	AA	1	51	0	77	1,54	32,47	12	20	2	0
1	0	669	**GK-098**	GG	1	73	0	63	1,54	26,56		19	12	0
1	0	670	**GK-099**	GA	0	43	0	70	1,54	29,52	14	16	4	0

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
1	0	671	**GK-100**	GG	1	43	0	83	1,56	34,11	11	16	4	0
2	0	672	**7264**	GA	1	58								
2	0	673	**8215**	GG	1	63								
2	0	674	**7861**	AA	1	54								
2	0	675	**7224**	AA	1	55								
2	0	676	**8495**	GA	0	29								
2	0	677	**8268**	GA	0	32								
2	0	678	**4423**	GA	0	34								
2	0	679	**5932**	GA	0	39								
2	0	680	**1493**	GA	1	71								
2	0	681	**1828**	GA	0	47								
2	0	682	**7462**	GG	1	59								
2	0	683	**8209**	GA	0	49								
2	0	684	**8428**	GA	1	52								
2	0	685	**7977**	GG	0									
2	0	686	**8313**	GA	0	40								
2	0	687	**4396**	GG	0	40								
2	0	688	**5887**	AA	0	35								
2	0	689	**4718**	GG	1	57								
2	0	690	**1891**	GA	0	41								
2	0	691	**7311**	GA	0	46								
2	0	692	**7956**	GA	1	54								
2	0	693	**7726**	GG	1	58								
2	0	694	**8126**	GG	1									
2	0	695	**8500**	GA	1	65								
2	0	696	**8259**	GA	0	33								
2	0	697	**4606**	GA	0	45								
2	0	698	**8359**	AA	0	39								
2	0	699	**7164**	GG	1	60								
2	0	700	**5713**	GG	1	55								
2	0	701	**1717**	GG	0	38								
2	0	702	**4720**	GA	1	55								

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	0	703	**1944**	GG	0	45								
2	0	704	**5846**	AA	0	48								
2	0	705	**2290**	GA	1	58								
2	0	706	**1751**	GA	0	44								
2	0	707	**2195**	GA	0	36								
2	0	708	**8100**	GG	1	64								
2	0	709	**7363**	GA	1	50								
2	0	710	**8150**	GG	1	59								
2	0	711	**7688**	AA	0	47								
2	0	712	**8408**	GG	0	37								
2	0	713	**7693**	GA	0	32								
2	0	714	**2540**	GG	0	43								
2	0	715	**2718**	AA	1	56								
2	0	716	**6096**	AA	0									
2	0	717	**1883**	GA	1									
2	0	718	**7127**	GG	0	44								
2	0	719	**8097**	GG	0	45								
2	0	720	**7715**	GG	1	57								
2	0	721	**8293**	AA	0	34								
2	0	722	**7000**	GA	0	31								
2	0	723	**4610**	GG	0	35								
2	0	724	**2670**	AA	0	41								
2	0	725	**6355**	AA	1	56								
2	0	726	**1889**	GA	1	60								
2	0	727	**7342**	GA	0	49								
2	0	728	**8053**	AA	0	38								
2	0	729	**7685**	AA	0	42								
2	0	730	**5948**	GG	1	62								
2	0	731	**6536**	GG	0	49								
2	0	732	**2504**	GG	1	55								
2	0	733	**2910**	GG	1	62								
2	0	734	**6391**	AA	0	41								

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	0	735	**2852**	GG	1	56								
2	0	736	**7355**	AA	0	43								
2	0	737	**8408**	GG	0	37								
2	0	738	**7273**	GA	0	45								
2	0	739	**6648**	AA	0									
2	0	740	**4509**	GG	0	43								
2	0	741	**2884**	AA	1	58								
2	0	742	**4729**	GA	0	35								
2	0	743	**1727**	GG	1	58								
2	0	744	**8281**	GA	1	50								
2	0	745	**7940**	GG	1	60								
2	0	746	**8013**	GG	1	61								
2	0	747	**5820**	GG	0	36								
2	0	748	**6559**	GG	1	55								
2	0	749	**3081**	GA	1	56								
2	0	750	**6316**	GA	0	40								
2	0	751	**1499**	GA	0	49								
2	0	752	**7931**	GA	1	51								
2	0	753	**8431**	AA	0	49								
2	0	754	**6584**	GA	0	48								
2	0	755	**8530**	AA	0	46								
2	0	756	**8125**	GG	0	35								
2	0	757	**3001**	GA	1									
2	0	758	**4918**	GG	1	58								
2	0	759	**1452**	GG	0									
2	0	760	**A1**	AA	1	59					12		3	
2	0	761	**A2**	GG	1	70					13		2	
2	0	762	**A3**	GA	1	68					12		2	
2	0	763	**A4**	GA	1	70					12		3	
2	0	764	**A5**	GA	1	60					11		2	
2	0	765	**A6**	AA	1	58					10		2	
2	0	766	**A7**	GA	1	50					12		2	

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	0	767	**A8**	GG	1	79					12		4	
2	0	768	**A10**	GG	1	62					12		2	
2	0	769	**A11**	AA	1	53					11		1	
2	0	770	**A12**	GG	1	66					14		2	
2	0	771	**A13**	GG	1	52					12		0	
2	0	772	**A14**	AA	1	88					11		4	
2	0	773	**A15**	AA	1	65					14		4	
2	0	774	**A16**	AA	1	78					12		2	
2	0	775	**A17**	GA	1	72					13		2	
2	0	776	**A18**	GA	1	54					13		2	
2	0	777	**A19**	GG	1	52					12		2	
2	0	778	**A20**	GA	1	70					12		2	
2	0	779	**A21**	GA	1	51					13		1	
2	0	780	**A22**	GA	1	64					11		2	
2	0	781	**A23**	GA	1	61					12		2	
2	0	782	**A24**	GG	1	60					14		1	
2	0	783	**A25**	GG	0	49					13		2	
2	0	784	**A26**	GG	1	77					15		1	
2	0	785	**A27**	AA	1	71					13		0	
2	0	786	**A28**	GA	1	71					12		0	
2	0	787	**A29**	GG	1	52					13		2	
2	0	788	**A30**	AA	1	52					10		2	
2	0	789	**A31**	GA	1	53					11		2	
2	0	790	**A32**	GA	1	74					13		1	
2	0	791	**A34**	GA	1	72					12		2	
2	0	792	**A35**	GG	1	56					13		2	
2	0	793	**A36**	AA	1	55					13		0	
2	0	794	**A38**	GG	1	68					11		2	
2	0	795	**A39**	GG	1	69					12		2	
2	0	796	**A40**	AA	1	58					10		1	
2	0	797	**A42**	GA	1	60					13		2	
2	0	798	**A44**	GA	1	56					12		3	

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	0	799	**A46**	GG	1	62			56		14		3	
2	0	800	**A48**	AA	1	51					11		2	
2	0	801	**A49**	GG	1	63					14		3	
2	0	802	**A50**	AA	1	51					13		2	
2	0	803	**A51**	GG	1	80					15		5	
2	0	804	**A53**	AA	1	58					11		2	
2	0	805	**A54**	AA	1	70					12		2	
2	0	806	**A55**	GG	1	68					14		1	
2	0	807	**A56**	GG	1	68					11		1	
2	0	808	**A57**	GA	1	50					13		2	
2	0	809	**A59**	GA	1						13			
2	0	810	**A60**	GG	0						11			
2	0	811	**A62**	AA	1						13			
2	0	812	**O1**	AA	0	37		68	1.67		12	25	2	
2	0	813	**O2**	AA	0	35		62	1.68		14	30	2	
2	0	814	**O3**	AA	0	32		58	1.72		14	27	1	
2	0	815	**O4**	AA	0	24		62	1.64		13	21	2	
2	0	816	**O10**	GG	0	42		58	1.70		14	23	2	
2	0	817	**O12**	GA	0	41		54	1.60		12	26	2	
2	0	818	**O13**	GA	0	34		105	1.68		12	30	2	
2	0	819	**O14**	AA	0	41		59	1.59		14	30	2	
2	0	820	**O15**	AA	0	35		52	1.60		13		0	
2	0	821	**O16**	GA	1	52		50	1.59		11	28	2	
2	0	822	**O17**	GA	1	52		68	1.67		15		0	
2	0	823	**O18**	GG	1	51		75	1.60		12	35	1	
2	0	824	**O19**	GA	0	46		64	1.65		12	26	2	
2	0	825	**O20**	GA	0	30		59	1.60		11		0	
2	0	826	**O21**	GG	0	31		50	1.69		12		0	
2	0	827	**O22**	GG	0	26		55	1.70		11		0	
2	0	828	**O23**	GA	0	26		60	1.60		10		0	
2	0	829	**O24**	GG	0	47		67	1.60		10	33	2	
2	0	830	**O25**	GG	0	27		50	1.65		10		0	

C	S	ID	ID	Genotype	Men. Stat.	Age	Smoking	Weight	Height	BMI	Age at Men.	1st Preg.	# of Children	Fam. Hist.
2	0	831	**O27**	GA	0	30		60	1.58		13	30	1	
2	0	832	**O28**	GA	0	31		65	1.65		12	28	1	
2	0	833	**O30**	GA	0	24		64	1.54		10		0	
2	0	834	**O31**	GA	0	40		55	1.55		12		0	
2	0	835	**O32**	GA	0	26		88	1.62		11		0	

"**C**", **Country** (1: Turkey, 2: Greece)
"**S**", **Status** of the individual (1: Patient, 0: Control)
"**ID**", ID number assigned just to order.
"**S. ID**", Sample ID written on the sample DNA.
"**Men. Stat.**", Menopausal Status (0: Premenopausal, 1: Post menopausal)
"**Smoking**", Smoking Status (1: Smoker, 0: Never Smoke)
"**BMI**", Body-mass-index
"**Age at Men.**", Age at menarche
"**1st Preg.**", Age of first pregnancy
"**# of Children**", Number of healthy born children
"**Fam. Hist.**", Family history of breast cancer (0: No, 1:Yes)