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ANALYSIS OF TUMOR SUPPRESSOR GENES IN TESTIS CANCER

**A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
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IN PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE**

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**BY
REŞAT ÜNAL
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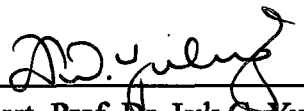
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ABSTRACT

ANALYSIS OF TUMOR SUPPRESSOR GENES IN TESTIS CANCER

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At least two classes of genes are involved directly or indirectly in the regulation of cell growth and differentiation. One group of these genes, known as tumor suppressor genes, is involved in cellular regulation by inhibiting uncontrolled proliferation. The most frequent genetic alteration in tumor suppressor genes is loss of one of their alleles and this is called “loss of heterozygosity” (LOH). Through several studies it is found that LOH in tumor suppressor genes is associated with uncontrolled cellular proliferation in many cancers.

Testis cancer is a common cancer among young men. The disease is lethal for 20-30% of the young patients and the risk of having testis cancer increases in industrialized countries. Turkish population is a young population where testis cancer might be an important threat. The relation between distinct genetic alterations and testis cancer has been studied. LOH studies in testis cancer also have been performed however the number of such studies is very low.

In this study the relation between testis cancer and the genes *BRCA1*, *BRCA2* and *PTEN* is investigated on 18 tumor samples of 10 individuals by using 8 highly polymorphic intragenic and extragenic markers with a PCR based LOH assay. LOH in *BRCA1* was observed in two tumors of two individuals, and LOH in *BRCA2* was observed in five tumors of two individuals. No LOH was found within the *PTEN* gene where only one intragenic marker was used for studying LOH status.

ÖZET

TESTİS KANSERLERİNDE TÜMÖR BASKILAYICI GENLERİN TANIMLANMASI

REŞAT ÜNAL

Yüksek Lisans Tezi, Moleküler Biyoloji ve Genetik Bölümü

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En az iki tür gen, doğrudan ya da dolaylı olarak hücre büyümesi ve farklılaşması üzerinde rol oynamaktadır. Bu genlerin bir grubu tümör baskılayıcı genler olarak bilinmektedir ve hücrel düzenleme görevini, kontrolsüz hücre bölünmesini engellemek suretiyle yerine getirmektedir. Tümör baskılayıcı genlerde en sık rastlanan genetik değişiklik ilgili genin alellerinden bir tanesinin kayba uğramasıdır ki tümör baskılayıcı genler için karakteristik olan bu olay heterozigotluğun kaybı (loss of heterozygosity) olarak tanımlanır.

Çeşitli çalışmalar sonucunda tümör baskılayıcı genlerdeki LOH ile birçok kanser türündeki kontrolsüz hücre bölünmesini arasındaki bağlantı gösterilebilmiştir. Testis kanseri genç erkekler arasında sıklıkla görülen bir kanser türüdür. Bu hastalık genç hastaların %20-30'unda ölümle sonuçlanmaktadır ve sanayileşmiş ülkelerde testis kanserine yakalanma riski artmaktadır. Türk popülasyonu testis kanserinin ciddi bir tehdit oluşturabileceği genç bir popülasyondur. Çeşitli genetik değişimler ile testis kanseri arasındaki ilişki çalışılmıştır. Testis kanserlerindeki heterozigotluğun kaybına ait bazı çalışmalar varolmasına karşın bunların sayısı oldukça azdır.

Bu çalışmada testis kanseri ile *BRCA1*, *BRCA2* ve *PTEN* genleri arasındaki ilişki 10 hastaya ait 18 tümör varyantı üzerinde 8 yüksek oranda polimorfik gen içi ve gen dışı işaretleyici kullanılarak PCR temelli bir LOH analizi yöntemi ile araştırılmıştır. İki hastaya ait iki tümörde *BRCA1* geninde ve iki hastaya ait beş tümörde *BRCA2* geninde LOH'a rastlanmıştır. Yalnızca tek bir gen içi işaretleyicinin kullanıldığı *PTEN* geninde LOH gözlenmemiştir.

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ABBREVIATIONS

APC	Adenomatous polyposis
APS	Ammonium persulphate
AFP	Alpha fetoprotein
α -P	α -phosphate radioactive labelled nucleotide
<i>BRCA1</i>	Breast-Ovarian Cancer Susceptibility Gene 1
<i>Brca1</i>	Mouse homolog of <i>BRCA1</i>
<i>BRCA2</i>	Breast Cancer Susceptibility Gene 2
<i>Brca2</i>	Mouse homolog of <i>BRCA2</i>
bp	basepair
°C	degrees celcius
Ci	Curie
CIS	Carcinoma In Situ
CR	Complete Response
cM	CentiMorgan
ddH ₂ O	double distilled water
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytosinetriphosphate
dGTP	deoxyguanosinetriphosphate

dTTP	deoxythymidinetriphosphate
DNA	Deoxyribonucleicacid
DCC	Deleted in Colon Cancer
dNTP	deoxyribonucleosidetriphosphate
EC	embryonal carcinoma
EDTA	ethylenediaminetetra-acetic acid
EX	Not alive
EtOH	Ethylalcohol
g	gram
hCGT	Human chorionic gonadotropin
kb	kilobase
kDA	kilodalton
LOH	Loss of Heterozygosity
LH	Luteinizing Hormone
LDH	Lactate dehydrogenase
mRNA	messenger ribonucleicacid
MA.TC	Mature Teratocarcinoma
mM	milimolar
ml	mililiter
MgCl₂	magnesium chloride
MCC	Mutated in Colon Cancer
MMAC1	Mutated in Multiple Advanced Cancers 1
μM	micromolar
μl	microliter
N	Normal

OD₂₆₀	Optic density at 260 nanometer
OD₂₈₀	Optic density at 280 nanometer
PBS	Phosphate Buffer Saline
PIC	Polymorphism Information Content
<i>PTEN</i>	Phosphate and Tensin Homolog
PCR	Polymerase Chain Reaction
PET	Paraffin Embedded Tissue
pH	Negative concentration of hydrogen ions
pmol	picomol
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleicacid
<i>RB (Rb)</i>	Retinoblastoma
rpm	revolution per minute
<i>SSCP</i>	Single Stranded Conformational Polymorphism
SE	Seminoma
SDS	Sodiumdodacylsulphate
T	Tumor
TC	Teratocarcinoma
TBE	Tris-boric acid EDTA Buffer
U	Unit
V	Volt
VNTR	Variable number of tandem repeat
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight

CHAPTER 1. INTRODUCTION

1.1 Tumor Suppressor Genes

Today it is known that at least two classes of genes are involved directly or indirectly in the regulation of cell growth and differentiation. Acquired or inherited alterations of these genes are crucial in the initiation and progression of human malignancies. The first group of these genes stimulates cell growth in a positive manner and is known as proto-oncogenes. The second group of these genes is tumor suppressor genes. The characteristic of tumor suppressor genes is their capability to inhibit uncontrolled growth and proliferation of cells. Cells therefore become malignant in absence or inactivation of their products.

Tumor suppressor genes often show association with the loss of one chromosome or a part of a chromosome which results in the conversion of a heterozygote genotype to a homozygote genotype through elimination of one allele of a tumor suppressor gene or surrounding marker. The function of tumor suppressor genes and their alterations that lead to tumorigenesis is summarized in Figure 1.1

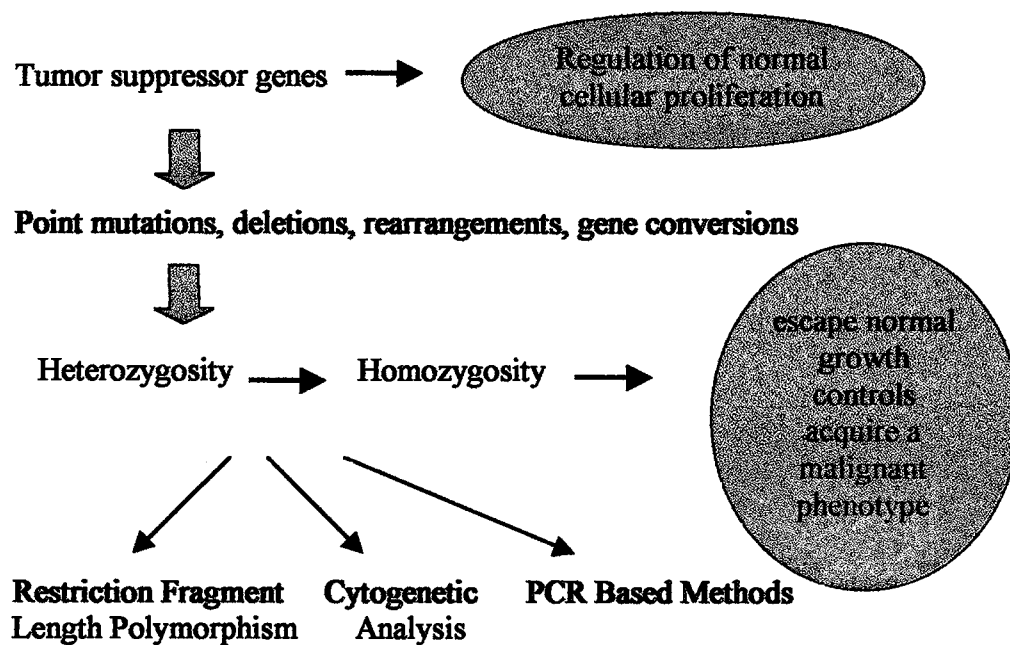


Figure 1.1: Functions of tumor suppressor genes and their role in tumorigenesis

The existence of tumour suppressor genes is revealed by malignant-nonmalignant cell fusion studies. The hybrids formed by this way did not show the malignant cell phenotype at first but after the loss of some specific chromosomes in the hybrid the malignant phenotype reappeared. Another important characteristic of several tumour suppressor genes is the loss of normal alleles, which is termed loss of heterozygosity (LOH) (Strohmeyer and Slamon, 1994).

The other property of tumor suppressor genes is their requirement of two separate mutational events in order to cause tumor development. This hypothesis is known as the two hit hypothesis. One of the mutational events may occur in the germ line and the second may then occur sporadically or both these mutational events may occur sporadically and cause tumor progression. It is more likely for a tumor to follow a

sporadic mutation in a cell that already contains an inherited mutation rather than two somatic independent mutations occurring in a single cell lineage.

The two hit hypothesis has been revealed by the studies of retinoblastoma and extended to other tumors to explain the nature of tumor suppressor genes.

1.2 Loss of Heterozygosity (LOH)

LOH is a key pointer for the existence of tumor suppressor genes. In studies where DNA polymorphisms close to the *RB-1* locus in both hereditary and non-hereditary tumors were analyzed, it was revealed that a significant difference exists between normal cells and tumor cells. The normal cells were heterozygous at many loci whereas the tumors were homorozygous at the same loci. The reason for this finding was that tumor DNA contained alleles from only one of the two homolog chromosomes of interest (Goodrich and Lee, 1993).

The mechanisms that lead to LOH were shown by retinoblastoma studies. The loss of a whole chromosome, loss of a chromosome followed by the reduplication that results in the generation of three copies of the chromosome, deletion of the wild type allele or point mutations are indicated as the possible mechanisms that result in the conversion of heterozygosity to homozygosity. The mechanisms that lead to the conversion of heterozygosity to homozygosity are summarized in Figure 1.2.

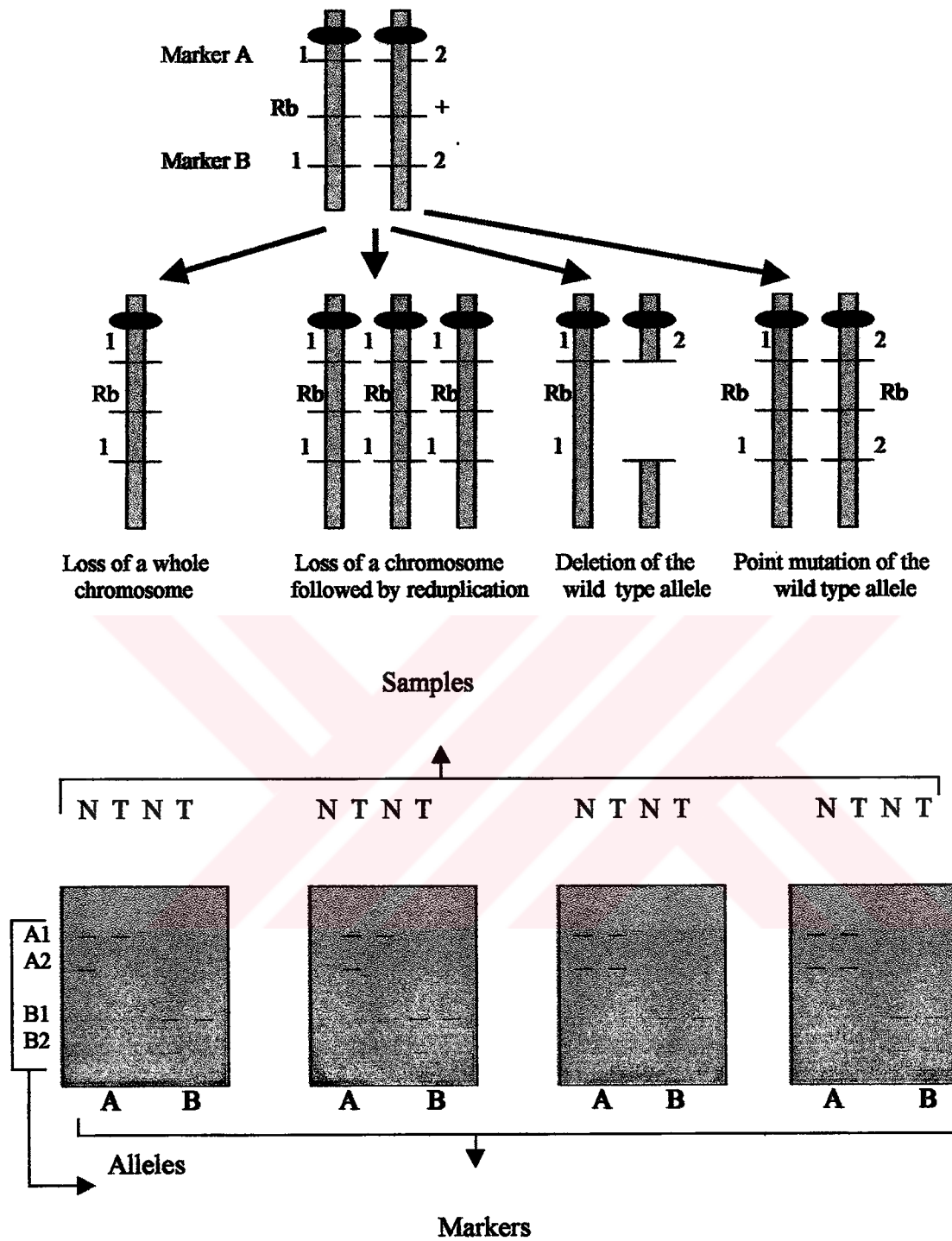


Figure 1.2: Mechanisms of loss of wild type allele

1.3 Polymorphism and Polymorphic Markers

DNA sequence variants play important roles in several aspects of genetic investigation such as evolutionary and population structure studies, forensic and the analysis and the diagnosis of genetic diseases. DNA sequence variants which are commonly known as DNA polymorphisms result from mutations and may or may not have functional consequences.

Molecular polymorphism studies were restricted to the field of immunogenetics until Smithies developed the starch gel electrophoresis in 1955. The first DNA polymorphisms observed was restriction fragment length polymorphism (RFLP) on Southern blots of genomic DNA. This kind of polymorphism is based on the restriction fragment size due to the cleavage or noncleavage of the DNA at particular sites caused by single nucleotide polymorphisms. In 1985 Jeffreys defined another class of common RFLP in which the restriction fragment length variability is due to the variable number of tandem repeats (VNTR). These repeats are named minisatellites because of their similarity to the much larger satellite DNA repeats. The conventional class of RFLP has generally only two alleles while minisatellites show hundreds of alleles per locus. Weber identified another subclass of VNTR polymorphism in which the repeat unit consists of only two base pairs. These dinucleotide repeats are called microsatellites. The advantage of using microsatellites is that they can be easily scored by the polymerase chain reaction. Variability, number and relative ease of scoring are the most important factors that make the microsatellites excellent markers for genetic studies. (Schafer and Hawkin, 1997).

The use of polymorphic markers is especially important in molecular genetic studies for several reasons. The co-inheritance of pairs of DNA markers with the genes that determine variable phenotypic effects is useful in determination of the genetic distances between them. The co-inheritance may also be used in the determination of the distance between the marker and the gene responsible for the variable phenotypic trait. The DNA probe for a marker may also be used in order to find its physical location.

The polymorphic markers are useful when they are informative. Informativeness is maximized if the marker has many alleles rather than two. The informativeness of a marker is measured by its polymorphism information content (PIC) value. PIC is a number between 0 and 1, the closer the value is to 1 the better is the marker. Above 0.7 is considered as a good value for the heterozygosity of a marker.

The polymorphic markers used in this study and their properties are summarized in Table 1.3.1.

Table 1.3.1: List of polymorphic markers used in LOH study of testis cancer.

Marker	Amplified Product Size	Allele number	Repeat type	Maximum Heterozygosity
<i>D17S776</i>	120bp	-	Dinucleotide	0.4000
<i>D17S800</i>	168-178bp	6	Dinucleotide	0.7433
<i>D17S855</i>	143-155bp	7	Dinucleotide	0.8220
<i>D17S856</i>	228-264bp	6	Tetranucleotide	0.3891
<i>D13S260</i>	158-171bp	9	Dinucleotide	0.7800
<i>D13S267</i>	148-162bp	6	Dinucleotide	0.6900
<i>D13S289</i>	260-276bp	8	Dinucleotide	0.7400
<i>PTEN</i>	154bp	-	Dinucleotide	Unknown

The alleles and allele frequencies of the markers are as listed in Tables 1.3.2, 1.3.3 and 1.3.4.

Table 1.3.2: Alleles and allele frequencies in *D17S800* and *D17S855* markers

D17S800

D17S855

Allele	Value	Frequency	Allele	Value	Frequency
1	0.168kb	0.0500	1	0.155kb	0.0500
2	0.170kb	0.1000	2	0.153kb	0.1900
3	0.172kb	0.4200	3	0.151kb	0.1800
4	0.174kb	0.0700	4	0.149kb	0.1500
5	0.176kb	0.2300	5	0.147kb	0.1200
6	0.178kb	0.1000	6	0.145kb	0.2600
			7	0.143kb	0.0500

Table 1.3.3: Alleles and allele frequencies in *D17S856* and *D13S260* markers

<u><i>D17S856</i></u>			<u><i>D13S260</i></u>		
Allele	Value	Frequency	Allele	Value	Frequency
1.	0.264kb	0.0100	1.	0.165kb	0.1071
2.	0.260kb	0.7700	2.	0.163kb	0.4107
3.	0.256kb	0.0300	3.	0.171kb	0.1071
4.	0.240kb	0.0500	4.	0.167kb	0.0536
5.	0.236kb	0.1200	5.	0.161kb	0.1250
6.	0.228kb	0.0100	6.	0.159kb	0.0357
			7.	0.169kb	0.0893
			8.	0.158kb	0.0357
			9.	0.173kb	

Table 1.3.4: Alleles and allele frequencies in *D13S267* and *D13S289* markers

<u><i>D13S267</i></u>			<u><i>D13S289</i></u>		
Allele	Value	Frequency	Allele	Value	Frequency
1.	0.154kb	0.1731	1.	0.260kb	0.4464
2.	0.148kb	0.4423	2.	0.264kb	0.1964
3.	0.160kb	0.2885	3.	0.276kb	0.0536
4.	0.156kb	0.0385	4.	0.274kb	0.0536
5.	0.150kb	0.0385	5.	0.272kb	0.1071
6.	0.162kb	0.0192	6.	0.262kb	0.0714
			7.	0.268kb	0.0179
			8.	0.270kb	0.0536

1.4 Breast-ovarian Cancer Susceptibility Gene (*BRCA1*)

The tumor suppressor gene *BRCA1* is responsible for a significant proportion of inherited breast and ovarian cancers (Lu *et al.*, 1996). It is a large gene that spreads over approximately 100 kb of genomic DNA and is localized to human chromosome 17q21.3. It contains 24 exons, 22 of which is responsible for the expression of an mRNA in abundant levels in variable tissues such as breast, ovarian, testis and thymus. The individuals that have one copy of *BRCA1* inactivated in their germ lines are predisposed to developing breast, ovarian and other malignancies (Scully *et al.*, 1997, Feunteun and Gilbert, 1996).

BRCA1 encodes a protein of 1863 aminoacids, which carries a zinc finger domain in its amino terminal region (Miki *et al.*, 1994). Today it is known that the product of *BRCA1* is required for early embryonic proliferation and development. Because of its ability to interact with Rad 51, which is a major component in double strand break repair and homologous recombination, it is thought that *BRCA1* plays an important role in the maintenance of genomic integrity (Scully *et al.*, 1997).

BRCA1 is expressed in normal breast epithelial cells and its product is intact in these tissues. In normal cells *BRCA1* is localized to nuclei. *BRCA1* is detected in the cytoplasm of almost all breast and ovarian cancer cells and is also localized to the cytoplasm in normal and tumor tissues other than breast. The expression of *BRCA1* in other tumor tissues like cervix, bladder, colon has also been shown, and the expression of *BRCA1* in these tissues is more aberrant than that in breast cancer tissues (Chen *et al.*, 1995). The expression of *BRCA1* has been shown to be five to

fifteen-folds lower in invasive cancer cells than in normal breast tissue. In adults *BRCA1* is expressed highly in the thymus and testes (Marquis *et al.*, 1995) and aberrantly in the breast, ovary, uterus, spleen and liver (Wilson *et al.*, 1997).

In order to find the biological functions of *BRCA1*, mutant mouse models were used. In a study where the fifth and sixth exons of *BRCA1* gene were deleted in mouse, it was revealed that this gene plays a crucial role in the control of the proliferative process that occurs in the early embryonic development (Hakem *et al.*, 1996). A partial deletion including aminoacids 300-361 of mouse *Brcal* exon 11 was introduced into the genome of the embryonic stem cell. In the case of heterozygosity, no effect was observed. However the introduction of the mutation to both alleles leads to severe retardation and death of the mouse in early developmental stage. This study supported that *BRCA1* plays an important role in tissue proliferation and differentiation (Liu *et al.*, 1996). Mutant mouse models were also used in order to analyze the histological alterations caused by mutations. Wild type and mutant mice were obviously distinguishable; the mutant mouse characteristically had a smaller, poorly organized abnormal phenotype. In contrast, the wild type embryos exhibited a well-organized ectoderm and endoderm. The classification of abnormal phenotype was done by dividing it into two distinct groups, one of them less severe than the other one. The less severe embryos showed epiblast, primitive streak was not detectable, and the parietal and visceral endoderms were very thin. In addition the extraembryonic region was poorly organized. The severe class did not appear to progress significantly beyond 5.5th embryonic day (Hakem *et al.*, 1996).

1.4.1 Loss of Heterozygosity on chromosome 17

LOH in a tumor is accepted as an evidence for the location of tumor suppressor genes. Through a study in which the deletions of chromosome 17 were assessed in order to measure LOH it was shown that *BRCA1* was involved in sporadic breast carcinomas. A common region on chromosome 17q of approximately 120 kb between two loci within the *BRCA1* was identified to be frequently deleted. By using 17 polymorphic markers it was revealed that *BRCA1* is involved in sporadic breast carcinomas. *THRA1*, *D17S776*, *D17S855* and *D17S856* markers were 4 out of the 17 markers used in order to find the LOH status of *BRCA1* in sporadic breast cancers. The percentage of the informative tumors that were deleted by LOH for the markers *THRA1*, *D17S776*, *D17S855* and *D17S856* was 27%, 30%, 27% and 19% respectively. It was concluded that the observed LOH affecting the wild type chromosome in tumors from affected breast/ovarian cancer patients was consistent with the hypothesis that *BRCA1* is a tumor suppressor gene (Cropp *et al.*, 1994).

In another study in which the LOH in familial breast carcinomas was studied it was revealed that *BRCA1* on 17q and *p53* on 17p might play important roles in the etiology of familial breast cancers. The LOH percentage found was 24% in the *BRCA1* locus and 31% in the *p53* locus. Chromosome 8q (33%) and 19q (27%) were also implied as chromosomes in which the LOH rates were high in sporadic breast carcinomas (Lindblom *et al.*, 1993).

Allele losses on chromosome 17 have also been studied in ovarian cancers. 16 epithelial ovarian carcinomas and 2 benign ovarian adenomas were studied for their

LOH status by using four polymorphic probes. A high frequency of allele loss on 17q (77%) and lower frequency of allele loss on chromosome 17p (69%) were detected (Eccles *et al.*, 1990).

In the existence of both sporadic and familial epithelial ovarian tumors it was revealed that a common region of deletion appeared on chromosome 17q. By using a sample size of 32 sporadic and 8 familial epithelial ovarian tumors and 21 polymorphic loci it was suggested that LOH took place in a high proportion which indicated that a distinct tumor suppressor gene from *BRCA1* localized on chromosome 17q may be involved in tumorigenesis. One of the polymorphic markers used in this study was *D17S855* and the LOH rate for this marker was 73% (Godwin *et al.*, 1994).

In order to find the involvement of *BRCA1* in prostate cancer, the LOH status was measured in 23 prostate cancer patients by using PCR based methods. Five highly polymorphic markers *D17S855*, *D17S856*, *D17S250*, *D17S579* and *D17S588* were used. The LOH percentage found was 44% in *D17S855*, 40% in *D17S856*, 10% in *D17S250*, 10% in *D17S579* and 15% in *D17S588*. According to these data it was concluded that *BRCA1* might have an important role in the pathogenesis of prostate cancer. (Gao *et al.*, 1995).

Fig.1.4.1 shows the chromosomal location of markers used in LOH studies to determine the presence of the *BRCA1* gene.

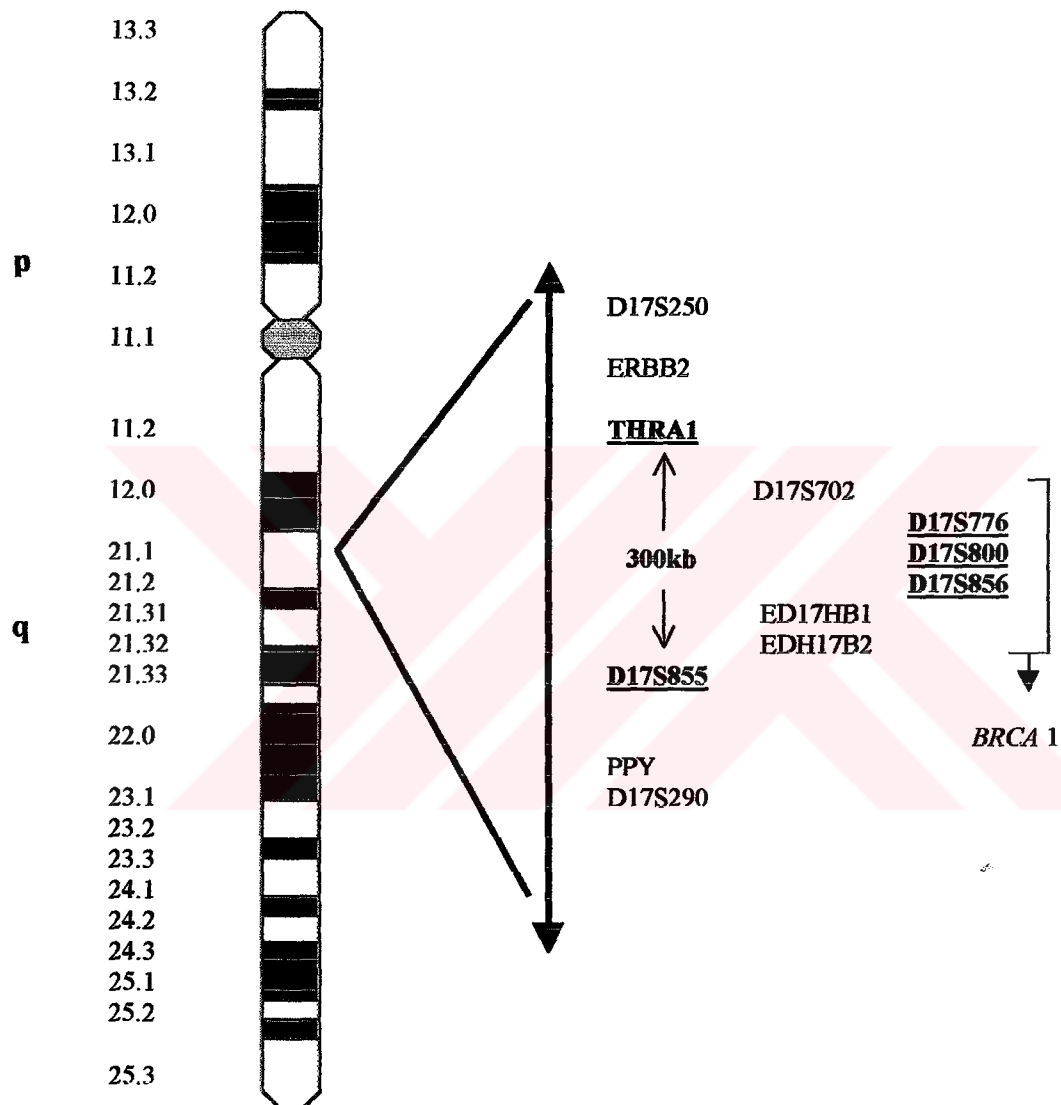


Figure 1.4.1: The chromosomal locations of chromosome 17 markers

1.5 Breast Cancer Susceptibility Gene 2 (*BRCA2*)

Another gene thought to be involved in breast cancer was identified and named as *BRCA2*. The difference between *BRCA1* and *BRCA2* is that germ line mutations in *BRCA2* predispose male carriers to an increased risk of breast cancer and female carriers to an increased risk of ovarian cancer (Rajan *et al.*, 1996). Early onset female breast cancer cases, increased risks of male breast cancers and also ovarian, prostate and pancreatic breast cancers are thought to be associated with *BRCA2* (Gayther *et al.*, 1997).

BRCA2 is composed of 10248 nucleotides encoded by 27 exons. The first exon is a noncoding exon. *BRCA2* encodes a protein of 3418 aminoacids, 383 kDA. (Connor *et al.*, 1997). Most of the exons are relatively small but exons 11 and 12 are very large and account for 60% of the entire coding region. *BRCA2* is localized on chromosome 13q12-13.

It has been shown that the pattern of expression in *BRCA2* is very similar to the pattern of *BRCA1* in multiple fetal and adult tissues and that *BRCA2* is expressed highly in proliferating cells. It is therefore thought that similar pathways as in *BRCA1* may regulate *BRCA2*. *BRCA2* is expressed in cellular compartments during fetal development. Detectable levels of expression in *BRCA2* was observed in the spleen, small intestine and placenta. High levels of expression in testis and moderate levels in the thymus and ovary were observable (Rajan *et al.*, 1997).

In order to investigate the function of *BRCA2* and its role in predisposing to breast and other cancers, targeted mutations were introduced to *BRCA2* homologs in mice. In *Brca2* (as in *Brca1*), the inactivation of both alleles resulted in developmental retardation and disorganization which lead to the death of mice in early embryological development. The onset of phenotypic abnormalities in *Brca2* was earlier than that in *Brca1*. Although the phenotypic features and time of death was highly variable in *Brca1* null homozygotes, they were much more uniform in *Brca2* homozygotes (Ludwig *et al.*, 1997).

Developmental expression of *BRCA2* in adult tissues and its colocalization with *BRCA1* were investigated by *in situ* hybridization studies. By using mouse models it was shown that *Brca2* mRNA expression in the breast was restricted to the epithelium and it was found to be highest in proliferating cellular compartments, particularly those undergoing differentiation including the testis, thymus, prostate, spleen and liver (Rajan *et al.*, 1997).

1.5.1 LOH on chromosome 13

The evidence that *BRCA2* is a tumor suppressor gene came from LOH studies that found 30-40% LOH in sporadic breast cancers and 50-60% LOH in sporadic ovarian cancers on chromosome 13q12-13 (Stratton, 1996). Another study in which 200 breast tumors were tested for LOH in order to find the involvement of *BRCA2* in sporadic breast cancer by using 11 highly polymorphic markers such as *D13S260*, *D13S267* and *D13S289* determined that 25-30% of all informative

cases showed LOH (Cleton-Jansen *et al.*, 1995). *BRCA2* was localized to a 6 centiMorgan interval on chromosome 13q12-13 by using the same polymorphic markers (Wooster *et al.*, 1995). The localization of these markers on chromosome 13 is shown in Figure 1.5.1. (Ford and Easton, 1995).

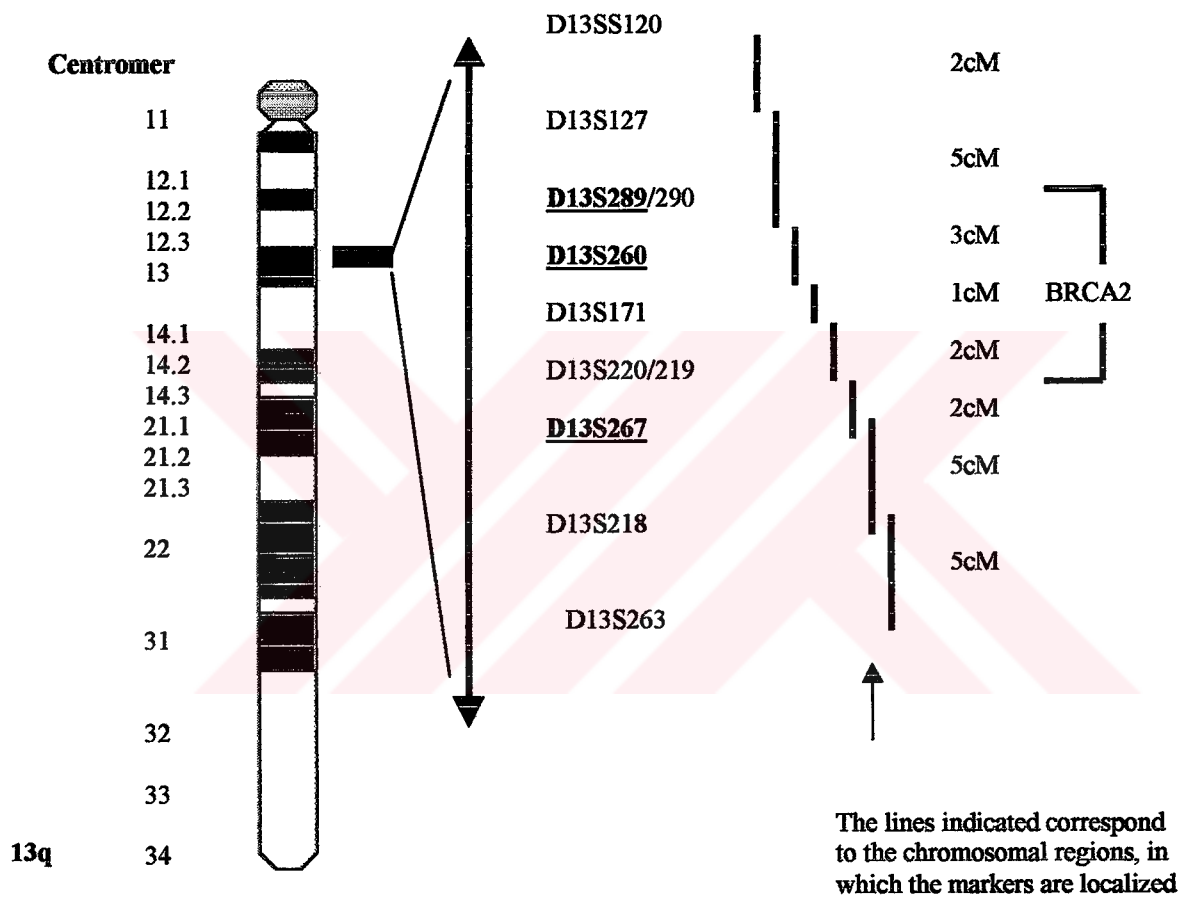


Figure 1.5.1: The chromosomal locations of chromosome 13 markers

1.6 Phosphate and Tensin Homolog (*PTEN*)

The discovery of genes involved in tumorigenesis is especially important in developing therapeutic strategies for solid tumors. Gliomas are neoplasms that account for the vast majority of brain tumors and it was thought that studies of allelic loss in gliomas could be important for localizing and cloning the gene which may be related with its progression. It was found that deletions on chromosome 10 were among the most frequent genetic alterations in glioblastomas. LOH on chromosome 10 was found to be associated with the progression of glioblastomas (Maier *et al.*, 1997).

Through the study of LOH at chromosome 10q23 it was found that 75% of all informative cases show LOH in glioblastomas and 60% of all informative cases show LOH in advanced prostate cancers. In another study in which the Cowden locus was assessed for LOH in an interval of 6cM, LOH was observed in 26% of informative follicular thyroid adenomas and 11% in Hurthle cell adenomas (Marsh *et al.* 1997).

According to these data it was thought that a tumor suppressor gene might be localized in this region. The reintroduction of the deleted part to nude mice resulted in suppression of tumorigenicity which supported the suggestion of existence of a putative tumor suppressor gene in this region. Finally a candidate tumor suppressor gene identified and named as phosphatase and tensin homologue was found to be deleted from chromosome 10 (*PTEN*) or mutated in multiple advanced cancers (*MMAC1*). The germ line mutations of *PTEN* in the three related, inheritable neoplastic disorders Cowden disease, Lhermitte-Duclos disease and Bannayan-

Zonana syndrome supported the suggestion that *PTEN* may be a putative tumor suppressor gene.

PTEN shares homology with the family of protein tyrosinases as well as with the cytoskeletal protein tensin. *PTEN* is classified within the family of dual-specificity protein phosphatases that phosphorylate serine, threonine and tyrosine residues (Myers *et al.*, 1997). The family of enzymes, which *PTEN* is included in, is characterized by the presence of a catalytic motif which is located within a conserved secondary structure consisting of seven α -helices and ten β -sheets. *PTEN* exhibits substrate specificity, and prefers extremely acidic substrates.

Many point mutations in *PTEN* result in neoplastic disorders. It is thought that this is due to the disruption of enzyme activity of *PTEN*. Germ line mutations in *PTEN* give rise to three related distinctive disorders. It was found that *PTEN* was also mutated in advanced prostate cancers and one half of endometrial cancers. Disruptions of *PTEN* in breast cancer and gynecological cancers were found to be rare (Myers *et al.*, 1997).

1.7 Testis Cancer

It is thought that the development of germ cell cancer is associated with two major factors, intrauterine prenatal effects, and postpubertal environmental or infectious effects in genetically susceptible individuals. Higher circulating *in utero* estrogen levels were associated with an increased testis cancer risk. Through segregation

studies it was confirmed that familial testis cancer was caused by a relatively high-frequency recessive gene (Oliver, 1998).

Testis cancer develops in young and middle aged men. It is most common in men between 15-34 years of age. The disease is lethal for 20-30% of the young patients (Bartkova *et al.*, 1991, Parker 1997, Moul 1997, CancerNet: National Cancer Institute). Most testis cancer cases occur sporadically and the lifetime prevalence of testis cancer in men is 4.9 per 100 000 (Peng *et al.*, 1993). The incidence of testis cancer varies from one population to another and is higher in industrialized countries (Vistisen *et al.*, 1997). It has increased two-fold in North and West Europe during the past 30 years. (Strohmeyer and Slamon., 1997). The worldwide incidence has also doubled in the last 40 years. The incidence of testis cancer presents variation according to geographic area. Scandinavia has the highest, while Asia and Africa have the lowest incidence of testis cancer. The incidence is intermediate in the United States and Germany (Bosl *et al.*, 1997).

Histological appearance of a normal testis is shown in Figure 1.7.1



Figure 1.7.1: The histological appearance of normal testis.

This is the microscopic appearance of normal testis. The seminiferous tubules have numerous germ cells. Sertoli cells are inconspicuous. Small, dark, oblong spermatozoa are seen in the center of the tubules.

Testis cancer that is highly treatable and curable is divided into two broad groups for treatment planning as seminomas where the cure rate exceeds 80% and nonseminomas. Nonseminomas are subdivided into choriocarcinomas, embryonal carcinomas, yolk sac carcinomas and mixed tumors (Strohmeyer *et al.*, 1997). Choriocarcinoma contains the highest and teratoma the lowest risk for metastasis (CancerNet: National Cancer Institute).

The serum markers play an important role in the indication of diagnosis and follow-up of testis cancer. Alpha fetoprotein (AFP), human chorionic gonadotropin (hCGT), luteinizing hormone (LH) and lactate dehydrogenase (LDH) are the most frequently used serum markers to detect tumors too small to be detected by physical examination. Although very high levels of serum markers indicate a worse prognosis, absence of serum markers does not mean the absence of tumor (CancerNet: National Cancer Institute).

1.7.1 Germ Cell Tumor of Testis

The term germ cell tumor of testis indicates that the origin of tumors is primordial germ cells (Bosl and Motzer., 1997). Germ cell tumors of men account for a biologically complex tumor system (Murty *et al.*, 1994), including the histogenesis and relationship of various histological types of testis germ cell tumors (Bartkova *et al.*, 1991). It is thought that all histological variants of testis germ cell tumors except spermatocytic seminoma are derived from the same precursor called carcinoma in

situ (CIS) (Jorgensen *et al.*, 1990, Kuczyk *et al.*, 1996). CIS of the testis is a preinvasive lesion that with time progresses into invasive germ cell tumor. The conversion of CIS into an invasive germ cell tumor takes approximately five years (Bosl and Motzer, 1997). It is thought that CIS cells are malignant cells derived from gonocytes during embryonic development. The reason of this suggestion was the morphological and immunohistochemical similarities between CIS cells and primordial germ cells, which could not be observed in comparison with spermatogonia. The observation of CIS in almost all seminiferous tubules adjacent to seminoma and nonseminoma testis tumors supports the association between CIS and germ cell tumors (Jorgensen *et al.*, 1990).

1.7.2 Causes of Testis Cancer

The most favourite hypothesis that explains the link between the declining sperm counts and increase of testis cancer is the concept that estrogen-mimicking chemicals damage the fetal germ cells. It is recognized that estrogen-mimicking chemicals act transplacentally to effect the germ cells *in utero*. However, it is thought that other mechanisms also contribute to the progress of testis cancer. Studying the comparative risks of father-to-son and sibling-to-sibling occurrence of testis cancer reveals the importance of genetics in comparison to intrauterine environmental influences. Brothers but not father and sons might share an *in utero* factor such as specific maternal hormone levels (Oliver, 1997). Patients with a history of cryptorchidism, contralateral testes, gonadal dysgenesis, infertility and undescended gonads are under higher risk of progression to testis cancer. (Jorgensen *et al.*, 1990,

Parker, 1997). Molecular genetics based studies have concluded that loss of tumor suppressor genes might be associated with testis cancer.

1.7.3 Cellular Classification and Staging in Testis Cancer

Today it is known that testis tumors arise from two origins, germ cells that account for 95% of testis neoplasms and nongermlinal sex cord stromal cells.

Although most germinal tumors are aggressive, nongermlinal tumors are usually benign. The concept that testis germ cell tumors arise from primordial germ cells is supported through studies in which the expression of *H19* gene was investigated. This gene is known to have a well-defined expression during embryonal development. It was found that the level of expression of *H19* was highly correlated with stages of maturation and differentiation in various testis tumors which supported a primordial germ cell origin of tumors (Prowse *et al.*, 1998).

Reflecting their origin in primordial germ cells and their remarkable ability to differentiate *in vivo*, testis tumors are histologically classified into two broad groups by the World Health Organization (Bosl and Motzer, 1997). Tumors showing single cell type are subdivided into seminomas, embryonal carcinomas, teratomas, choriocarcinomas and yolk sac carcinomas. Tumors showing more than one histological pattern are indicated as embryonal carcinoma and teratoma with or without seminoma, embryonal carcinoma and yolk sac carcinoma with or without seminoma, embryonal carcinoma and seminoma, yolk sac carcinoma and teratoma

with or without seminoma and choriocarcinoma and any other elements. (CancerNet: National Cancer Institute). Figure 1.7.3.1 shows the histological appearance of embryonal carcinoma.

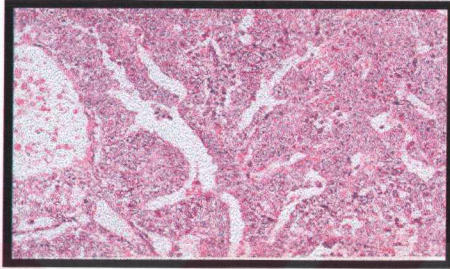


Figure 1.7.3.1: The histological appearance of embryonal carcinoma

This is the histological pattern of embryonal carcinoma sheets. The blue cells are trying to form primitive tubules.

The most common type of germinal tumors is seminomas that account for 30% of all germinal tumors. These are divided into three subgroups, as typical, anaplastic and spermatocytic carcinomas. A typical seminoma is derived from the proliferation of primary germ cells and has a uniform property. Anaplastic seminomas show greater cellular and nuclear irregularity with more frequent giant tumor cells and many mitoses. Seminomas are most frequent in fourth decade of life. Seminomas may contain high mitotic rate (anaplasty), syncytiotrophoblastic giant cells and increased human chorionic gonadotropin (hCGTP) levels.

The histological appearance of seminoma is shown in Figure 1.7.3.2

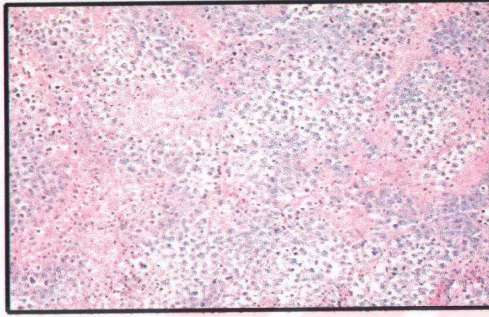


Figure 1.7.3.2: The histological appearance of seminoma

This is the microscopic appearance of the histological pattern of the seminomas. Lobules of neoplastic cells have an intervening stroma with clear infiltrates. The seminoma cells are large with vesicular nuclei, and pale watery cytoplasm.

Spermatocytic seminoma is a rare variant, which has no association with CIS. Its relation with other germ cell tumors is unknown. These tumors rarely metastasize and are found usually in elderly men.

Nonseminomatous germ cell tumors are usually found in the third decade of life. Embryonal carcinomas, teratomas, yolk sac carcinomas and choriocarcinomas are classified within this group.

The most undifferentiated cell type is embryonal carcinoma. It has totipotential

capacity to differentiate into the other nonseminomatous cell types. Embryonal carcinomas are more aggressive and lethal than seminomas. Embryonal carcinoma cells are capable of synthesizing elevated serum concentrations of hCGT and alpha fetoprotein.

Choriocarcinomas are made up of cytotrophoblasts and syncytiotrophoblasts. They are rarely pure. They with their association with metastatic diseases and high serum concentrations of hCGT are characteristic.

Yolk sac carcinoma is the most common testis tumor in infants and children. It is also known as endodermal sinus tumor and occurs frequently in combination with embryonal carcinoma in adults.

Teratomas contain somatic cell types of two or more germ-cell layers (ectoderm, mesoderm or endoderm). Mature teratomas contain a collection of differentiated cell types.

Immature teratomas are tumors with partial somatic differentiation elements where of the three germ cell layers are incompletely differentiated and not arranged in organoid fashion (Bosl and Motzer, 1997). The histogenesis of testis tumors is summarized in Figure 1.7.3.3.

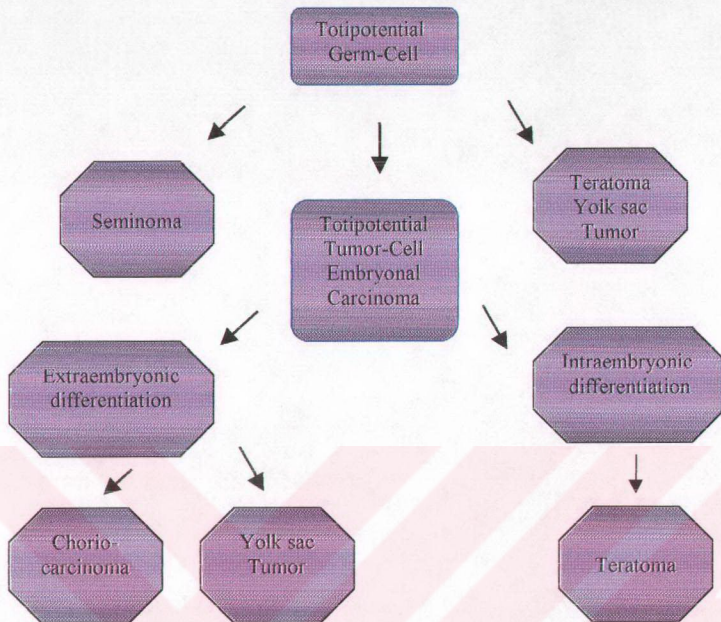


Figure 1.7.3.3: The histogenesis of testis tumors

The preinvasive state in which testis cancer is limited to the testis, epididymis, or spermatic cord is known as stage I testis cancer. The spread to the retroperitoneal or para-aortic lymph nodes in the region of kidney besides testis is defined as stage II. This stage is further subdivided as stages IIA (nodes less than 2cm in maximal diameter), IIB (nodes between 2 and 5cm in diameter) and IIC (nodes greater than 5 cm in diameter) Spread beyond the retroperitoneal nodes is implied as stage III.

1.7.4 Treatment of Testis Cancer

It is believed that all cases of CIS will progress sooner or later into a germ cell cancer. It is better to treat the disease at the CIS stage because treatment at the later cancer stages may include radiotherapy and/or chemotherapy, which have serious side effects. The surgical removal (orchietomy) of the effected testis is proposed as the treatment of choice in cases of unilateral CIS if the other gonad has a potential for fertility. In the case of patients with testis tumor or CIS of the contralateral testis the treatment method of choice is localized irradiation (Jorgensen *et al.*, 1990, CancerNet: National Cancer Institute). Chemotherapy is the method of treatment used to prevent the dissemination of germ cell tumors. Chemoterapy results in a complete response (CR) in 70%-80% of patients whereas 20%-30% still dies from this disease (Motzer *et al.*, 1990). The risk for relapses in stage I seminomas accounts for 4%, for stage IIA and stage IIB 10%. Chemotherapy cures more than 90% of patients who have a relapse after radiation therapy. (Bosl and Motzer, 1997)

1.7.5 Unanswered questions in testis cancer

There are still many problems that seem unsolved. The reason why two age peaks exist in testis cancer, and how and why undescended testes, gonadal dysgenesis, androgen insensitivity increase the risk of testis cancer is not known. It is also not answered whether chemotherapy and radiotherapy are the right treatment strategies in terms of fertility (Ginsburg, 1997). The genetic basis of testis cancer has not been enlightened enough to answer all the questions.

1.8 Molecular Biology and Genetics of Testis Cancer

Through cytological studies on testis cancer, it was revealed that isochromosome 12p (12p) presence is the most common and frequent chromosomal abnormality in testis cancer cases. This finding is present in up to 100% of all germ cell tissues studied. It is thought that isochromosome 12 appearance is an early event during oncogenesis. Other alterations such as trisomies, duplications, deletions and other rearrangements for chromosomes 1, 11, X and Y were also shown by cytological studies. It is revealed that several proto-oncogenes and growth factors are localized to the chromosomes, which are frequently altered in germ cell tumors. Using immunohistochemistry and flow cytometry, it was found that low levels of *c-myc* protein product p62 are observable in normal testicular tissue whereas high levels are present in seminoma tumor tissues. It was shown that the expression of p62 is increased due to the increase in cell differentiation in nonseminomas and the germ tumor cell line Tera-2. The incidence of *ras* mutations was found to be low (Strohmeyer *et al.*, 1994). It has been also shown that *N-ras* and *Ki-ras* mutations do exist in testis tumors and the expression of *c-myc* and *c-kit* oncogenes are high in germ cell tumors of testis (Shuin *et al.*, 1993).

It was revealed that frequent allelic losses take place in chromosomes 2p, 3p, 3q, 5p, 9p, 9q, 10q, 11p, 12q, 13q, 17p, 17q, 18p, 18q in testis tumors (Murty *et al.*, 1994).

APC, *MCC*, *DCC*, *TP53*, *RB* and *WT-1* tumor suppressor genes were also investigated for their LOH status in a high number of tumors and tumor cell lines of testis by using PCR-based methods. 28% LOH was observed at *APC*, 30% at *RB*, 23% at

MCC, 27% at *WT-1* and 22% at *TP53* (Peng *et al.*, 1995). *p53* is a tumor suppressor gene that is most frequently deleted in a variety of human tumors. Although the differential expression of *p53* in testis cancer was shown, mutations of *p53* in germ cell tumors of testis are not reported (Chou *et al.*, 1997).

In order to find the role of *p53* in testis cancer, *p53* mutations in both sporadic and familial testis cancer samples were assessed. Mutation detection was performed by using two efficient screening methods, constant denaturing gel electrophoresis and single stranded conformational polymorphism. No mutation was found in *p53*. It is thought that other tumor suppressor genes instead of *p53* may play a role in the development of testis cancer and that *p53* might mediate toxin damage to sperm count (Peng *et al.*, 1993). In contrast to most solid cancers, *p53* was found to be unmutated in testis cancers. However, Kuczyk *et al.* was able to show mutational band shifting at the *p53* gene locus in 12 out of 18 (67%) samples by using a sensitive assay called RNA-single stranded conformational polymorphism assay (RNA-SSCP) (Kuczyk *et al.* 1996. Oliver, 1997).

In 1994 Murty *et al.* reported that *DCC* gene was frequently deleted in male germ cell tumors. Using germ cell tumors and tumor cell lines of testis resulted in the study concluded that LOH at 18q21 was characteristic of seminomas as well as in all subsets of nonseminomas (Murty *et al.*, 1994).

The number of families with multiple numbers of testis cancer cases is low and those, which are reported, contain only two affected members. In a study in which 35 families with two affected sibs were typed by using 220 microsatellite markers, six

regions were found as candidates for a testis cancer susceptibility gene. The physical location of these regions are 1p36, 4p13-14, 4cen-q13, 5q12-21, 14q13-q24.3 and 18q21.1-q23 (Leahy *et al.*, 1995).

Some LOH studies performed in order to determine the LOH status in several cancers are given in Table 1.8.



Table 1.8: The results of LOH studies in several cancers

Gene	Sample Size	% LOH	Reference
<i>P53</i> <i>APC</i> <i>MCC</i> <i>DCC</i> <i>WT-1</i> <i>RB</i>	44 <u>Testis cancer</u>	22% 28% 23% 55% 27% 30%	Peng <i>et al.</i> , 1995
<i>BRCA1</i>	23 <u>Prostate cancer</u>	44% <i>D17S855</i> 40% <i>D17S856</i>	Gao <i>et al.</i> , 1995
<i>BRCA1</i>	130 <u>Sporadic breast cancer</u>	<i>THRA1</i> 27% <i>D17S776</i> 30% <i>D17S855</i> 27%	Cropp <i>et al.</i> , 1994
<i>BRCA1</i> <i>P53</i>	78 <u>Familial breast carcinomas</u>	12-19% 16-33%	Lindblom <i>et al.</i> , 1993
<i>BRCA1</i>	20 <u>Sporadic breast carcinomas</u>	<i>THRA 1</i> 79% <i>D17S250</i> 50% <i>D17S579</i> 59%	Futreal <i>et al.</i> , 1992
<i>BRCA2</i>	50 <u>Sporadic ovarian Cancer</u>	47-67%	Foster <i>et al.</i> , 1996
<i>PTEN</i>	38 <u>Sporadic thyroid Tumors</u>	26% follicular thyroid adenomas	Marsh <i>et al.</i> , 1997

1.9 AIM AND STRATEGY

This project aims to determine the status of three candidate genes on chromosome 17 (*BRCA1*), 13 (*BRCA2*) and 10 (*PTEN*) for loss of heterozygosity in order to identify potential tumor suppressor genes, which may be involved in testis tumors. We got 18 histological tumor variants of 10 testis patients. We wanted to analyse these 18 tumor with highly polymorphic dinucleotide and tetranucleotide repeat markers by using a PCR-based LOH assay.

The strategy we used was as follows;

- This study has been set up in collaboration with Hacettepe University Medical School, Department of Urology and Department of Pathology.
- The patients have been examined and operated at Department of Urology and their tumor samples were sent to pathology department for analysis. Nonseminoma IIC and IIIC patient groups were selected for molecular genetic analysis.
- The normal and tumor tissue sections of patient samples were fixed into paraffin blocks at pathology department. The sections of samples were cut and dissected under the microscope to separate normal and different variants of tumor tissues.
- These samples were send to Bilkent University, Department of Molecular Biology and Genetics for molecular analysis in PBS solution.
- The tissue samples were stored at -70°C until they were used. DNA was isolated from dissected tissue samples and used to investigate LOH status of different

genes. Peripheral blood DNA was also isolated and used to optimize the PCR conditions.

- The PCR conditions for radioactive and normal PCR were optimized to obtain the PCR products of our interest.
- The PCR products were run in nondenaturing polyacrylamide gels in order to separate the alleles.
- Depending on the method used autoradiogram or silver staining was performed to observe the alleles and decide whether allele loss had occurred or not.



CHAPTER 2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents

The reagents Tween 20, phenol, chloroform, isoamylalcohol, ethidiumbromide, boric acid, acetic acid, formaldehyde, sodium hydroxide, formaldehyde, bromophenol blue, xylene cyanol, glycerol, silver nitrate and DMSO were from Sigma Chemical Co. (St.Louis, U.S.A)

Tris-Cl and Tris-Base were from Stratagene (Heidelberg, Germany). EDTA and sodium hydroxide were from Carlo Erba (Rodano, France). Sodium acetate was from Merck (Darmstadt, Germany). Ethanol was from Delta Kim. San. (Istanbul, Turkey). Hexa Labe Labelling Kit was from MBI Fermentas Inc. (N.Y, U.S.A).

2.1.2 Nucleic Acids

100 bp DNA ladder, *Hinf*I digested Φ x174 DNA and pUC mix marker were used as DNA molecular weight standards in this study. All these molecular weight markers were from MBI Fermentas Inc. (N.Y, U.S.A). Ultrapure deoxyribonucleotides were from Pharmacia (Vienna, Austria).

2.1.3 Oligonucleotides

The oligonucleotides used in polymerase chain reaction were synthesized in the Beckmann Oligo 1000M DNA synthesizer (Beckmann Instruments Inc. CA, U.S.A) at Bilkent University, Department of Molecular Biology and Genetics (Ankara, Turkey).

2.1.4 Enzymes

Rnase A was from Sigma Chemical Co. (St.Louis, U.S.A). Proteinase K was from Appligene-Oncor (U.S.A). *Taq* DNA Polymerase and Klenow fragment of *E.coli* DNA Polymerase I large fragment were purchased from MBI Fermentas Inc. (N.Y, U.S.A).

2.1.5 Electrophoresis Materials

Agarose, acrylamide, bisacrylamide, N-N-N trimethylethylenediamine (TEMED), ammonium persulfate (APS) were obtained from Sigma Chemical Co. (St.Louis, U.S.A).

Horizontal electrophoresis apparatus was from Stratagene (Heidelberg, Germany). The power supply used was Power-PAC300 from Bio Rad Laboratories (CA, U.S.A). The vertical gel apparatuses used for polyacrylamide gel electrophoresis were Bio-Rad Proteon II xi Cell from Bio Rad Laboratories (CA, U.S.A) and EC 175 Adjustable Vertical Gel System from E.C Apparatus Co. (FL, U.S.A)

2.1.6 Photography and Autoradiography

The UV transilluminator was from Herolab. The video graphic printer UP-890CE was from Sony Corporation (Japan). Polaroid film UP 110 HD was from Sony Corporation (Japan). The films used for autoradiography were from Kodak Corporation (U.S.A). Cassettes and intensifying screens were from Amersham International (England). Photographic fixer and developer were from Kodak Corporation (Japan).

2.1.7 Radioisotopes

α -³²P- dCTP (3000 Ci/mmol) and α -³³P- dATP (3000 Ci/mmol) were from Orbital Ltd.Sti. (Istanbul, Turkey).

2.2 SOLUTIONS

2.2.1 General Solutions

EDTA:	1M, pH 8.0
1xTris-boric acid-EDTA (TBE):	45mM Tris-borate, 1mM EDTA
Ethidiumbromide:	10 mg/ml in water (stock solution) 30 ng/ml (working solution)
1x Gel loading buffer:	0.25% bromophenol blue, 0,25% xylene cyanol, 50% glycerol, 1mM EDTA
10% SDS	0.1g/ml in water (stock solution)
Tris-Cl	0.5M, pH 8.0
Proteinase K:	20mg/ml proteinase K in water (stock solution) 10µl (10mg/ml) proteinase K was used for each DNA isolation reaction.

2.2.2 Solutions for cold and radioactive polymerase chain reactions

dNTP mix (stock solution):	100mM each of four different kinds of dNTP (dATP, dTTP, dCTP, dGTP) were mixed to a final concentration of 0.2mM
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Radioactive PCR dNTP mix (stock solution):

dATP reduced dNTP mix (stock solution): 5mM dATP, 100mM dTTP
100mM dGTP, 100mM dCTP

dCTP reduced dNTP mix (stock solution): 100mM dATP, 100mM dTTP
100mM dGTP, 5mM dCTP

MgCl₂ (stock solution): 25mM MgCl₂

10x PCR Buffer: 100mM Tris-Cl (pH 8.8)
500mM KCl, 0.8% Nonidet P40

2.2.3 Solutions for DNA Isolation from blood

20x SSC: 3M NaCl, 0.3M trisodium citrate, pH 7.0

TE buffer: 10mM Tris, pH 8.0, 1mM EDTA, pH 8.0

Extraction buffer: 10mM Tris-Cl, pH 8.0
10mM EDTA, pH 8.0
0.5% SDS

Sodium acetate: 3M, pH 5.2

Phenol/chloroform/isoamylalcohol: 25:24:1 (v/v/v)

2.2.4 Solutions for DNA Isolation from paraffin embedded tissue

Digestion buffer: 50mM Tris-Cl, pH 8.0
 1mM EDTA
 1% Tween 20

2.2.5 Solutions for non-denaturing polyacrylamide gel electrophoresis

Polyacrylamide: 40% (w/v), Acrylamide/bisacrylamide,
 19:1 (w/w), (stock solution)

500ml 40% stock solution preparation: $500\text{ml} \times 40\% = 200\text{g (w/v)}$

$200 \times 19/20 = 190\text{g}$ Acrylamide was added into $200 \times 1/20 = 10\text{g}$ Bisacrylamide and dissolved in 500ml deionized water.

Ammonium persulfate: 10% APS in water

2.2.6 Solutions for silver staining

Developer solution: 1.5% NaOH
 0.1% Formaldehyde

Fixative solution: 10% EtOH
 0.5% Acetic acid

Silver-nitrate: 0.1% AgNO_3

2.2.7 Solutions for radioactive labelling

10 x Reaction buffer: 500mM Tris-Cl(pH 8.0)

dNTP mix: 10mM dATP, 10mM dTTP
10mM dGTP, 10mM dCTP

Mix C: dNTP mix minus dCTP



2.3 PATIENT INFORMATION

The patients selected for this study were nonseminoma stage IIC and IIIC patients.

18 histological tumor variants of 10 individuals were used for the study.

The detailed information about the patients is summarized in Table 2.4

The abbreviations for the Table 2.4 are as follows;

NI: No Information

SE: Seminoma histology

TC: Teratocarcinoma Histology

YS: Yolk Sac Tumor Histology

MAT.TC: Mature Teratocarcinoma Histology

EX: Not Alive

Table 2.3: Patient Information

Patient code	Age	Diagnosis date	Histology	PEI Sample	Last prognosis	STAGE	Chemotherapy	Chemotherapy before sample dissection	Chemotherapy after sample dissection
TEC91	NI	NI	NI	NI	NI	NI	NI	NI	NI
TEC93	32 (EX)	22/10/87	TC+EC	23/10/87	5/6/88	IIIC	+	+	-
TEC94	15(EX)	23/3/83	EC	25/3/83	28/12/83	IIC	+	+	-
TEC95	29	11/12/87	EC+YS	17/12/87	1/2/89	IIC	+	+	-
TEC96	44	25/11/89	TC+SE+EC	4/12/89	21/8/91	IIC	+	+	-
TEC97	54	13/7/91	SE+EC	13/7/91	29/12/95	IIC	+	+	-
TEC98	51	3/9/86	TC+YS	8/9/86	30/6/95	IIC	+	+	-
TEC99	39	26/8/89	MAT.TC	22/8/89	25/1/90	IIC	+	+	-
TEC100	30	13/1/89	TC	14/10/89	15/7/90	IIC	+	+	-
TEC101	53	4/11/81	EC	6/11/81	5/2/82	IIC	+	+	-

2.4 METHODS

2.4.1 DNA Isolation from blood

700µl blood samples were aliquoted into 1.5ml eppendorf tubes and frozen. The samples were thawed and DNA isolation was performed by phenol/chloroform/isoamylalcohol extraction. 800µl 1xSSC was added to wash the samples. The samples were then centrifuged at 13,000 rpm for one minute and the supernatants were removed and discarded. 1.4ml 1x SSC was added and after a brief vortex the samples were centrifuged for one minute. After the removal of supernatant, 800µl extraction buffer was added into the tubes. This step was followed by the addition of 10µl (10mg/ml) Proteinase K. The tubes were vortexed briefly and incubated at 56°C for one hour. 400µl phenol/chloroform/isoamylalcohol was added into the tubes and the tubes were vortexed for 60 seconds. The upper aqueous layer formed after a five-minute centrifugation was removed and transferred into new tubes. The phenol/chloroform/isoamylalcohol extraction step was repeated in order to get a clean interphase. DNA was precipitated from the aqueous solution by addition of 1/20th volume 3M sodium acetate and 2 volumes ice-cold absolute ethanol. The tubes were mixed and placed at -20°C for 30 minutes and then centrifuged at 13,000 rpm for 30 minutes at 4°C. The pellet was washed with 70% ethanol, air-dried and resuspended in approximately 25µl deionized water.

2.4.2 DNA Isolation from paraffin embedded tissue

The paraffin embedded tissue sections were placed into 1.5ml eppendorf tubes and stored at -70°C. The tubes were treated with 500µl digestion buffer. This step was followed by the addition of 15µl (10mg/ml) Proteinase K. The samples were incubated overnight at 55°C. The next day an equal volume of phenol/chloroform/isoamylalcohol was added into the tubes which were then centrifuged for 10 minutes at 5,000 rpm. The upper layers were transferred into new tubes and 2µl RNase A was added to the samples. The samples were incubated at 37°C for 30 minutes. An equal volume of phenol/chloroform/isoamylalcohol was then added into the tubes and the centrifugation step was repeated. After the centrifugation, the top layers were collected and 2.5 volume ethanol and 0.1 volume sodium acetate were added to precipitate the DNA samples. The samples were left at -20°C for one hour and centrifuged at 13,000 rpm for 30 minutes. Ethanol was air-dried and the DNA samples were resuspended in 25µl double-distilled water.

2.4.3 Agarose Gel Electrophoresis and Visualization of DNA fragments

DNA fragments were fractionated by horizontal gel electrophoresis using standard buffers and solutions (Maniatis, 1989). Agarose, the amount of which was determined according to the required percentage, was completely dissolved in 1x TBE buffer. The agarose concentration varied between 0.8% and 2% (w/v) to maximize the resolution of fragments. Ethidiumbromide was then added at a final concentration of 30µg/µl. The samples were mixed with one volume loading buffer

and loaded onto gels. The gel was run in 1x TBE at different voltages and times depending on the size and the fragment separation required. The DNA fragments were visualized under ultraviolet light.

2.4.4 DNA Size Markers

The length of the DNA fragments was determined by using commercially available DNA size markers, 100 bp DNA ladder, Φ x174/*Hinf*I or pUC mix.

Fragment sizes were as follows:

100 bp DNA ladder: (kb)	1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.08
Φ x174/ <i>Hinf</i> I: (kb)	0.726, 0.713, 0.553, 0.5, 0.427, 0.417, 0.413, 0.311, 0.249, 0.2, 0.151, 0.14, 0.118, 0.1
pUC mix: (kb)	1.116, 0.883, 0.692, 0.501, 0.489, 0.404, 0.331, 0.242, 0.19, 0.147, 0.111, 0.110
1kb DNA ladder (kb)	10.0, 8.0, 6.0, 5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.5

2.4.5 Polyacrylamide Gel Electrophoresis

Electrophoretic separation of polymorphic alleles was performed by the following method:

8%-10% polyacrylamide gels were used. 40%(w/v), 19:1(w/w) polyacrylamide stock solutions were diluted to the required percentage with 1x TBE buffer. The gel apparatus was assembled according to the manufacturer's instructions (Bio Rad Laboratories, CA, U.S.A and E.C Apparatus Co., FL, U.S.A).

In order to prepare an 8% gel, 10 μ l acrylamide:bisacrylamide (19:1,w/v), 5 μ l 10xTBE, 200 μ l TEMED and 40 μ l 10% APS were mixed and poured into the gap between two glass plates. The polymerization of the gel takes about 15-25 minutes. The gels were pre-run for 30 minutes. The samples were mixed with one volume-loading buffer and loaded into the wells. The gel was run in 1x TBE at 250V for different time periods (6-8 hours) depending on the size of the fragments at room temperature. The gel was then removed and either stained with silver nitrate or dried for autoradiography.

2.4.6 Quantification of DNA

Concentrations and purity of nucleic acids were determined by measuring absorbance at 260nm and 280nm in a spectrophotometer (Beckmann Instruments Inc., CA., U.S.A). The ratio between absorbance values at A_{260} and A_{280} was taken.

Nucleic acid samples displaying OD₂₆₀ and OD₂₈₀ values in the range of 1.8 to 2.0 are regarded as highly pure.

A value of A₂₆₀ = 1.0 corresponds to a concentration of approximately 50µg/µl double stranded DNA and 20µg/µl for oligonucleotides. (Maniatis).

The molar extinction coefficient of oligodeoxynucleotides was determined to estimate the accurate concentration. The oligonucleotide sequences were entered to the spectrophotometer and the concentration was determined.

The concentration of the oligonucleotides was determined by using the equation;

$$A_{260} = E (n \epsilon G + n \epsilon C + n \epsilon T + n \epsilon A) C l$$

A₂₆₀ is the absorbance at 260 nm.

C is the concentration in mM.

l is the path length in cm.

E is the sum of all the mM extinction coefficients of the nucleotides present

n is the number of bases.

The mM extinction coefficient (ε) values for the nucleotides are as follows:

$$\epsilon \text{ dGTP} = 12.01$$

$$\epsilon \text{ dCTP} = 7.05$$

$$\epsilon \text{ dATP} = 15.20$$

$$\epsilon \text{ dGTP} = 8.40$$

2.4.7 Polymerase Chain Reaction (PCR)

Both cold and radioactive polymerase chain reactions have been used in this study.

2.4.7.1 Optimization of PCR Conditions

PCR conditions were optimized by changing the annealing temperatures, MgCl_2 , DNA and primer concentrations. The optimum conditions required for PCR were, 1.5mM final concentration of MgCl_2 , 1pmol/ μl primers and variable annealing temperatures between 55-60°C. The DNA samples were diluted 10 times and used for PCR reactions.

2.4.7.2 Cold PCR

Polymerase chain reactions were performed using Perkin-Elmer 2400 or 9600 PCR machines (Perkin Elmer, U.S.A). Standard conditions were 1pmol/ μl of each primer, 200 μM final concentration of dNTP, 1x PCR buffer, 1.5mM final concentration of MgCl_2 and 1U *Taq* DNA Polymerase for each reaction in 25 μl final volume. The PCR conditions were as follows:

The initial denaturing was at 95°C for 5 minutes and the final extension was at 72°C for 5 minutes for all the primer pairs. 35 cycles of PCR reactions were carried out, each one beginning with an initial denaturation step at 94°C for 30 seconds and finishing with a polymerization step at 72°C for 30 seconds. The temperatures for the annealing step varied between 55-60°C. The PCR conditions are summarized in table 2.4.7.2.

Table 2.4.7.2: Oligonucleotides synthesized and their optimal PCR Conditions

GENE-MARKER	PRIMER SEQUENCE (5'-3')	ANNEALING TEMPERATURE PRODUCT SIZE
<i>D17S776F</i>	TCCATGACCCTATGGACCA	55°C
<i>D-17S776R</i>	AAACCTCTGTCTCTTTGCAG	120bp-130bp
<i>D17S800R</i>	GGTCTCATCCATCAGGTTT	55°C
<i>D17S800R</i>	ATAGACTGTGTACTGGGCATTGA	168bp-178bp
<i>D17S855F</i>	GGATGGCCTTTTAGAAAGTGG	56°C
<i>D17S855R</i>	ACACAGACTTGTCTACTGCC	143bp-155bp
<i>D17S856F</i>	AAGGCAAGACTTCGTCGAGA	58°C
<i>D17S856R</i>	CATTCCCTGGTCCTGTGC	228bp-264bp
<i>PTENF</i>	AAATGTACGGTTCATTGACTT	58°C
<i>PTENR</i>	GACTGACTACAAATGGGCA	154bp
<i>D13S260F</i>	AGATATTGTCTCCGTTCCATGA	55°C
<i>D13S260R</i>	CCCAGATATAAGGACCTGGCTA	158bp-171bp
<i>D13S267F</i>	GGCCTGAAAGGTATCCTC	55°C
<i>D13S267R</i>	TCCCACCATAAGCACAAAG	148bp-162bp
<i>D13S289F</i>	CTGGTTGAGCGGCATT	57°C
<i>D13S289R</i>	TGCAGCCTGGATGACA	260bp-276bp

2.4.7.3 Radioactive PCR

Radioactive PCR amplifications of DNA were carried out as described above with some modifications. 0.05µl ³³P-dATP (3000 Ci/mmol) or ³²P-dCTP (3000 Ci/mmol) was added into each 25µl reaction, and the concentration of the non-radioactive dATP or dCTP was reduced to 125µM final concentration (1.25mM stock solution concentrations). Other conditions were optimized as described previously.

2.4.8 Radioactive labelling of DNA

DNA was labelled by the random oligonucleotide-primed synthesis method based on the random primer method developed by Feinberg and Vogelstein.

Approximately 80-100ng DNA was used for the labelling reaction. DNA, 10µl hexanucleotide in 5x reaction buffer (dilution was made from the commercially available 10x reaction buffer) and 42µl ddH₂O were mixed in a 1.5ml eppendorf tube. The tube was then vortexed and centrifuged for 5 minutes. The mix was boiled for 10 minutes and centrifuged quickly. Then, 3µl MixC (dNTP mix minus dCTP), 4µl ³²P-dCTP and 1µl Klenow fragment (DNA polymerase I large fragment) were added to the reaction. The reaction tube was centrifuged for 5 seconds and incubated at 37°C for 10 minutes. After that, 4µl dNTP mix was added and the tube was left for final incubation at 37°C for 5 minutes. Finally the reaction was terminated by the addition of 1µl 0.5M EDTA, pH 8.0.

2.4.9 Silver Staining

Silver staining was one of the methods which was used initially in this study to investigate the loss of heterozygosity (LOH) status. The protocol was as follows:

The gel was washed two times with ddH₂O for one minute and then treated two times with 1x fixative solution for 3 minutes and washed with ddH₂O. The next step was staining for 9-10 minutes with 0.1% silver nitrate solution. The silver nitrate solution

was discarded and the gel was rapidly washed twice with deionized water. This is a critical step, which is to be done as quickly as possible. After this step the gel was treated with ice-cold developer solution. The development of the gel takes approximately 1-1.5 hours. The gel was placed into 7.5% acetic acid solution for 15-20 minutes. The acetic acid solution was then removed and the gel was stored at room temperature between two transparency sheets.

2.4.10 Treatment of radioactive gels

The gel containing radioisotope-labelled PCR products was dried for autoradiography. The gel was next placed over a layer of Whatman paper and covered with stretch film. It was then placed into the gel drier and dried for two hours at 80°C under vacuum. The stretch film was removed and the dried gel was placed into a cassette containing intensifying screens. An x-ray film was placed over the gel and left for exposure for 9-10 hours. The film was then developed.

CHAPTER 3. RESULTS

The most recent method used frequently to investigate allele losses is known to be PCR-based LOH Assay. According to this method DNAs are extracted from the source of interest and fractionated by agarose gel electrophoresis to check whether isolation has occurred or not. These samples are then used to amplify the highly polymorphic markers localized very close or within the gene selected for investigation. After the amplification the products are separated by polyacrylamide gel electrophoresis that has a high resolution and visualized by autoradiography or silver staining. Finally due to the whole allele loss or intensity reduction it will be concluded whether LOH has occurred or not.

The method used in this study was also PCR based LOH assay and the order of the experimental design was same as mentioned above.

3.1 DNA Isolation

3.1.1 DNA Isolation from Peripheral Blood

In the beginning of the study DNA was isolated from peripheral blood. It was planned to obtain the tumor tissues of the patients whose blood samples were already present. Therefore, DNA isolation of 94 samples was performed. The isolation protocol worked with high efficiency and enabled the isolation of most of the DNA samples intact and with good yield. The DNA samples isolated from peripheral blood are shown in Figure 3.1.1.



Figure 3.1.1 DNA samples isolated from Peripheral Blood

The DNA samples were run on an 0.8% agarose gel. The DNA size marker is lambda DNA digested with *Hind* III (Lanes 1 and 11). The other lanes contain the samples as follows; 2: TEC45, 3: TEC46, 4: TEC47, 5: TEC48, 6: TEC49, 7: No sample, 8: TEC50, 9: TEC1, 10: TEC5.

3.1.2 DNA Isolation from Paraffin Embedded Tissues (PET)

The tumor tissue samples used in this study were originally embedded into paraffin blocks. The isolation of DNA from paraffin embedded tissues was much more difficult in comparison with that of DNA from peripheral blood. It is thought that this might depend on the age of the paraffin embedded sample, the fixative used, or both.

The main problem in isolating DNA from paraffin-embedded tissue was the inability to obtain the genetic material intact. DNA runs as smears on the agarose gel electrophoresis and this indicates the degradation of high molecular weight genomic DNA. Therefore DNA samples isolated from paraffin-embedded tissues were diluted before they were used for further applications. In conclusion it was found that degradation of DNA was not a severe problem in order to use it for further applications. The DNA samples isolated from paraffin embedded tissue, are shown in Figure 3.1.2.

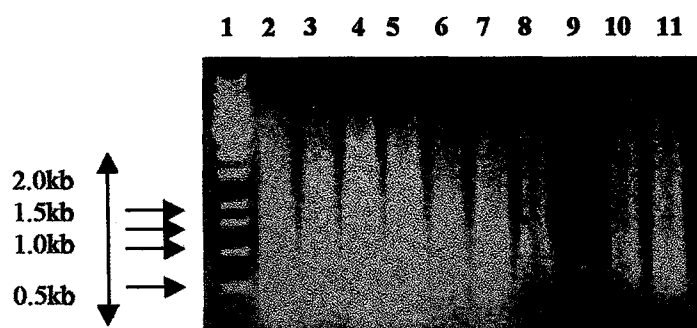


Figure 3.1.2 DNA samples isolated from Paraffin Embedded Tissue (PET)

The DNA samples were run on an 0.8% agarose gel. The DNA size marker is 1kb ladder (Lane1). The other lanes contain the samples as follows;
2: TEC93N, 3: TEC93T1, 4: TEC93T2, 5:TEC93T3, 6:TEC94N, 7: TEC94T, 8: TEC95N, 9: TEC95T1, 10:TEC95T2, 11:TEC96N. (N: Normal tissue, T: Tumor Tissue).

3.2 Polymerase Chain Reaction (PCR)

3.2.1 Optimization of Polymerase Chain Reaction Conditions

Polymerase Chain Reaction is a method to amplify a DNA fragment. It basically consists of three steps which are denaturation, annealing and extension. In the denaturation step double-stranded DNA becomes single-stranded in order to provide a template to which oligonucleotides will bind. The second step is the annealing step in which the annealing temperature plays a much important role. The annealing temperature is important for oligonucleotides to bind to template DNA and avoid the unspecific amplifications. The last step is extension in which the polymerization of DNA samples takes place. Here the primer or magnesium chloride concentrations or the amount of *Taq* DNA polymerase are the critical factors that make the amplification reaction successful. To find optimal conditions in terms providing good amplification patterns is defined as optimization of PCR conditions.

In this study DNA samples isolated from peripheral blood were used to find the optimal PCR conditions for several polymorphic markers. In the beginning appropriate annealing temperatures were investigated for specific amplifications. The annealing temperatures of the primers were first calculated according to base content and a temperature interval was determined for each primer. By trying the amplification of the DNA within this temperature interval, the annealing temperature for each primer was found.

Primer concentrations were determined to find the right primer amount that should be used for PCR reactions. PCR reactions were performed by using 20pmol and 25pmol

of primers per reaction. The PCR amplification by using 25pmol primer concentration per reaction was found to be the appropriate primer amount. Then the magnesium chloride concentration was assessed for an optimal amplification. PCR reactions were performed in which 1,5mM, 2mM and 2,5mM of magnesium chloride concentrations were used. 1,5mM of magnesium chloride was found to be sufficient for a specific amplification. The total reaction volume was determined as 25µl and 1 unit *Taq* DNA Polymerase was found to be sufficient for a good PCR reaction. 1% DMSO as a final volume was used in order to prevent the unspecific product amplifications. The PCR products which were obtained by optimized PCR conditions for the markers *D17S855* and *D17S856* are shown in figure 3.2.1.



Figure 3.2.1 PCR products of *D17S855* and *D17S856* markers

The size marker is ϕ X174 (Lane 1). Lane 2 and lane 6 are the negative controls for *D17S856* and *D17S855* respectively. Lanes 3, 4 and 5 contain the PCR amplification products when 1.5mM, 2.0mM and 2.5mM magnesium chloride concentration were used for *D17S856* respectively. Lanes 7 and 8 contain the PCR amplification products when 1.5mM and 2.0mM magnesium chloride concentration were used for *D17S855* respectively.

3.2.2 Cold Polymerase Chain Reaction

Once the optimal PCR conditions for each primer set of polymorphic markers were determined, paraffin-embedded tissue samples were used for PCR amplification.

Although DNA samples from PET were degraded, the specific PCR product bands could be observed by agarose gel electrophoresis. In conclusion successful amplification could be performed with PET. An example of PCR amplification products of normal and tumor PET samples by using primers for *D17S856* marker are shown figure 3.2.2

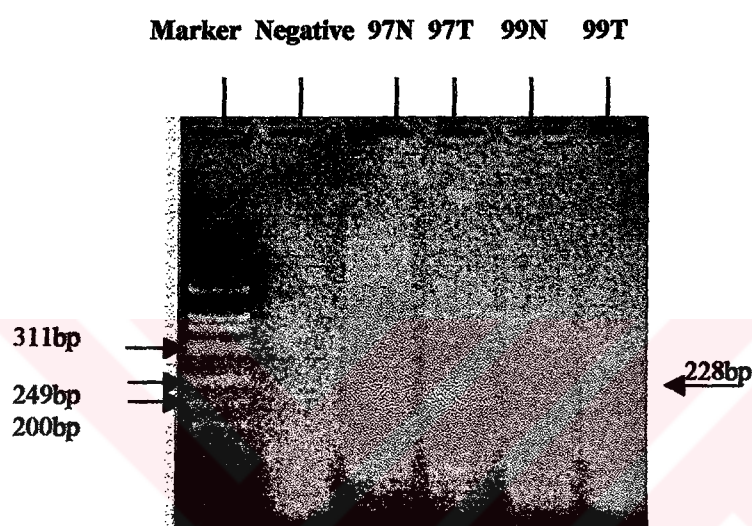


Figure 3.2.2 PCR products of normal and tumor DNA samples

The PCR products were run on an 2% agarose gel. The marker used is ϕ X174 (Lane 1). Lane 2 is the negative control of PCR reaction. The other lanes contain the PCR products of the paraffin embedded normal and tissue samples of TEC97 and TEC99 respectively. The marker used for amplification was *D17S856*.

3.2.3 Radioactive Polymerase Chain Reaction

Silver staining can be used to observe the separation of alleles with high resolution.

However autoradiography is a much more sensitive method for observing the alleles.

Autoradiography requires the radioactive labelling of PCR products by using one of the dNTPs radioactively labelled in the PCR mixture.

Although optimal conditions for radioactive PCR resemble that of cold PCR, some differences exist between two conditions. The main difference is the amount of dNTP's used in these two PCR methods. Deoxyribonucleosidetriphosphate stock solutions were prepared in which the concentrations of dCTP and dATP were reduced. The reason for preparing these stock solutions was to promote the introduction of radioactive labelled dCTPs and dATPs to the polymerization of the template DNA strand instead of unlabelled deoxyribonucleosides. This application resulted in the generation of PCR products which could be observed with autoradiography.

Figure 3.2.3 shows the first example of radioactively labelled PCR products which were run on an 8% non-denaturing polyacrylamide gel.

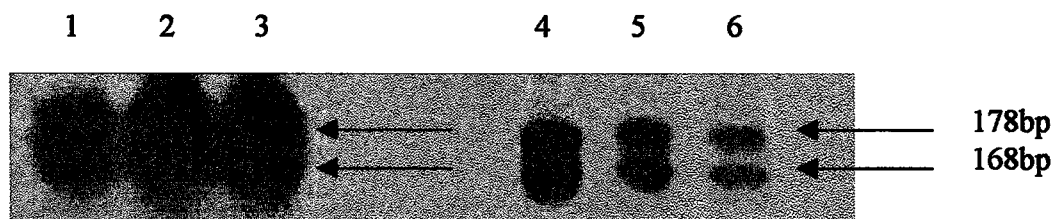


Figure 3.2.3 Autoradiography result of radioactively labelled PCR products

Lane 1 and 4 contain the PCR product of TEC49 normal DNA isolated from peripheral blood. This sample was used as a positive control. Lanes 2, 3, 5 and 6 contain PCR products of DNA samples TEC94 normal tissue (Lane 2 and 5) and TEC 94 tumor tissue (Lane 3 and 6) isolated from paraffin embedded tissues. The first three lanes contain PCR products which were labelled with α - P^{32} -dCTP. In the next three lanes the samples were labelled with α - P^{33} -dCTP. The amplified marker was *D17S800*.

3.3 Non-Denaturing Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis is a method used for different aims. It can be used in order to study proteins, to detect mutations or to dissociate alleles of DNA. The basic principal in order to fulfil all these aims is the movement of proteins or nucleic acids from the negatively charged to the positively charged pole. The advantage of using polyacrylamide gels is that it provides the high resolution of genetic or protein products.

In this study, nondenaturing polyacrylamide gel electrophoresis was used to investigate whether allele losses had occurred or not by employing several polymorphic markers. Silver staining or autoradiography enabled observation of the samples that were run on polyacrylamide gel. All markers were studied for their allele loss by silver staining or autoradiography. Polyacrylamide used in this study was prepared as 40% stock solution. Before being poured to the electrophoresis apparatus it was diluted to 8% and 200µl ammonium persulphate (APS) and 40µl triethylmethylenediamine (TEMED) were added to the working solution. The polymerization of gels takes about 15-25 minutes.

3.4 Determination of Allele Loss for Each Marker by Silver Staining and/or Autoradiography

The reason of choice of radioactivity was its high sensitivity to enable observation of alleles in comparison to silver staining. Some of markers were assessed only by silver staining some only by autoradiography and some by using both methods.

3.4.1 Assessment of *D17S776* for Loss of Heterozygosity

D17S776 is an intragenic marker, which means that it is localized within the gene *BRCA1*. Its loss is shown in several types of urogenital cancers. Although the maximum heterozygosity rate of this marker is not very high the intragenic property of this marker makes it one of the markers of choice for LOH studies. The loss of intragenic markers might result directly in loss of alleles. That is why the intragenic markers are important in studying allele losses.

There is no information about alleles and allele frequencies of this marker. The maximum amplified sequence also is unknown. It is known that the length of PCR products of marker *D17S776* varies between 120bp-130bp.

Both silver staining and autoradiography were used in this study in order to study LOH. 13 tumors of 9 individuals were studied. In three individuals the PCR amplification of five tumors could not be obtained and therefore LOH status could not be determined. By silver staining it was revealed that the alleles were amplified in these

samples studied and they were in expected sizes. Both alleles for all samples existed in all samples studied. In conclusion it was found that no loss of allele occurred in marker *D17S776*.

Figure 3.4.1 shows the silver stained polyacrylamide gel result where the alleles for marker *D17S776* were separated after PCR reaction.

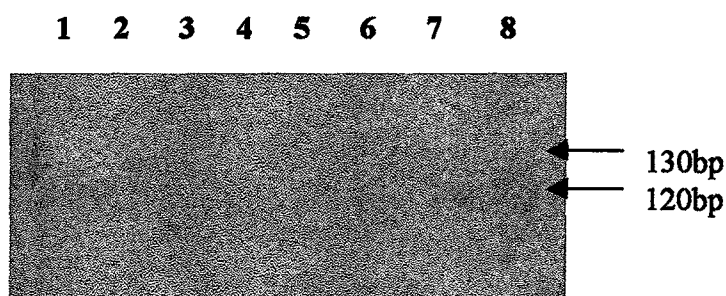


Figure 3.4.1.1 The silver stained polyacrylamide gel of *D17S776* alleles

The PCR products of marker *D17S776* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are between 120bp and 130bp. The lanes contain the samples as follows; 1: TEC 93N, 2: TEC 93T, 3: TEC 94N, 4: TEC 94T, 5: TEC 95N, 6: TEC 95T, 7: TEC 96N, 8: TEC 96T. All the samples were amplified by PCR and the PCR worked for all the samples shown in this figure. The amplification pattern was faint. Both alleles were protected for the samples TEC 93N, 93T, 94N, 94T, 95N, 95T, 96N and 96T however, the intensities of the alleles on the polyacrylamide gel were weak.

The study of allele loss for *D17S776* using autoradiography is shown in Figure 3.4.1.2

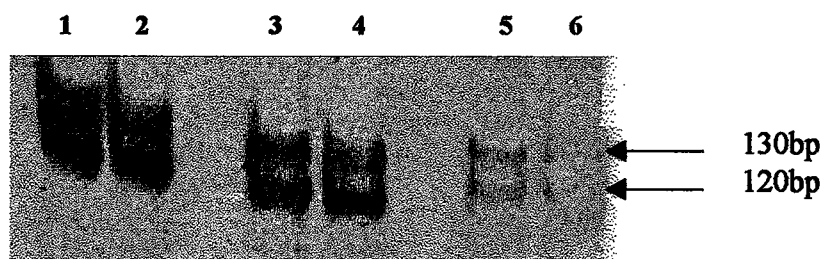


Figure 3.4.1.2: Autoradiography result of radioactively labelled PCR products of marker *D17S776*

The PCR products of marker *D17S776* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are between 120bp and 130bp. The radioisotope used was α - 32 P-dCTP. The lanes contain the samples as follows; 1: TEC 96N, 2: TEC 96T, 3: TEC 97N, 4: TEC 97T, 5: TEC 98N, 6: TEC 98T. All the samples shown in this figure contained both of the alleles and allele loss in any of the tumor samples could not be observed. No LOH was observed for any one of the samples for the marker *D17S776*.

3.4.2 Assessment of *D17S800* for Loss of Heterozygosity

D17S800 is another intragenic polymorphic marker used in order to study LOH. Its chromosomal localization is between *D17S776* and *D17S856*. It has six different alleles with different allele frequencies. The lengths of these alleles vary between 0.168kb and 0.178kb and the allele frequencies lie between 0.0500 and 0.4200. Allele1 is the allele with the lowest allele frequency and has a frequency of 0.0500. In contrast allele 3 is the allele with highest allele frequency and has an allele frequency of 0.4200. That means that the likelihood of obtaining an allele fragment with a length of 0.168kb is lowest and with a length of 0.178kb is highest for the marker *D17S800*.

By using both silver staining and autoradiography 16 tumors out of 18 of 9 individuals were studied. The appropriate lengths of PCR products were obtained. Both alleles

were observed in all the samples studied. No LOH was observed. The silver stained polyacrylamide gel of *D17S800* alleles shown in Figure 3.4.2.1.

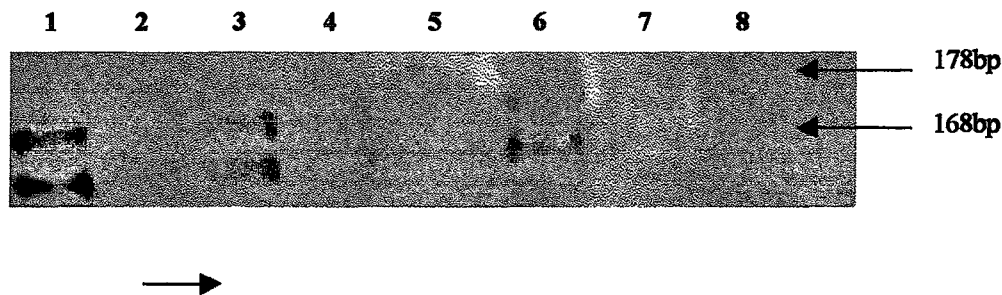


Figure 3.4.2.1: The silver stained polyacrylamide gel of *D17S800* alleles

The PCR products of marker *D17S800* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are between 168bp and 178bp. The lanes contain the samples as follows; 1: TEC 98N, 2: TEC 98T1, 3: TEC 98T2, 4: TEC 98T3, 5: TEC 100N, 6: TEC 100T, 7: TEC 101N, 8: TEC 101T. Both paternal and maternal alleles were observable in all of the samples studied. The alleles of the sample TEC 100T were observable in the polyacrylamide, however their banding patterns were very faint.

LOH study for the marker *D17S800* by using radioactive labelled dNTPs is shown in Figure 3.4.2.2.

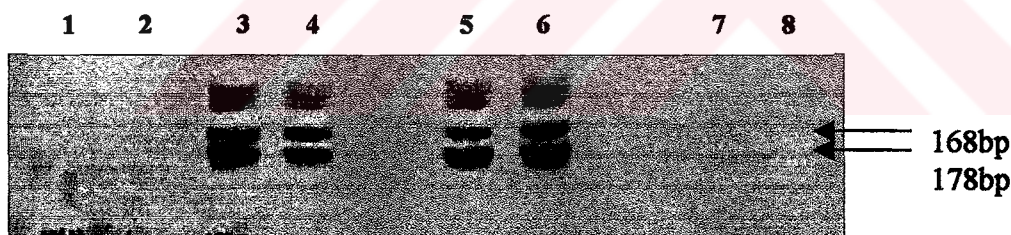


Figure 3.4.2.2: Autoradiography result of radioactively labelled PCR products of marker *D17S800*

The PCR products of marker *D17S800* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are between 168bp and 178bp. The radioisotope used is α -³²P-dCTP. The lanes contain the samples as follows; 1: TEC 101T, 2: TEC 101N, 3: TEC 100T, 4: TEC 100N, 5: TEC 99T, 6: TEC 99N, 7: TEC98T, 8: TEC 98N. All the samples shown in this figure contain both of the alleles of paternal and maternal origin. There were some problems with the PCR amplification of the samples 100N, 100T, 98N and 98T. Therefore the banding patterns of these samples on polyacrylamide gel were very faint. However the alleles of these samples obtained were informative and sufficient to conclude that no LOH exists for their tumor samples.

3.4.3 Assessment of *D17S855* for Loss of Heterozygosity

D17S855 is an extragenic marker, which is very close to *BRCA1*. Therefore the loss of this marker might be important in determining whether a putative tumor suppressor gene could be localized to this region or not. It has seven different alleles with different allele frequencies. The lengths of these alleles vary between 0.143kb and 0.155kb. The allele frequencies lie between 0.0500 and 0.2600. Allele 1 and allele 7 are the alleles with the lowest allele frequencies and have a frequency of 0.0500. In contrast allele 6 is the allele with highest allele frequency and has an allele frequency of 0.2600. That means that the likelihood of obtaining an allele fragment with a length of 0.143kb is the lowest and with a length of 0.145kb is the highest for the marker *D17S855*.

Through autoradiography and silver staining 14 out of 18 tumors in 9 out of ten individuals were investigated. The PCR amplification was performed successfully and expected banding patterns were observed in polyacrylamide gel. No LOH was observed in the samples. The silver stained polyacrylamide gel result of *D17S855* is shown in Figure 3.4.3.1.

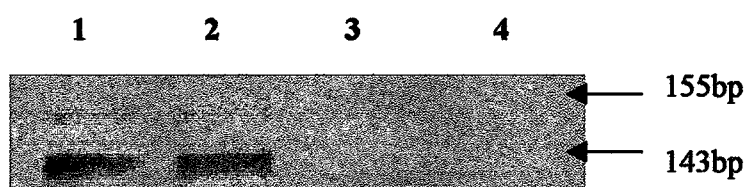


Figure 3.4.3.1: The silver stained polyacrylamide gel of *D17S855* alleles

The PCR products of marker *D17S855* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are about 143bp and 155bp. The lanes contain the samples as follows;

1: TEC 96N, 2: TEC 96T, 3: TEC 97N, 4: TEC 97T

The study of LOH for the marker *D17S855* by using radioactive labelled dNTPs is shown in Figure 3.4.3.2

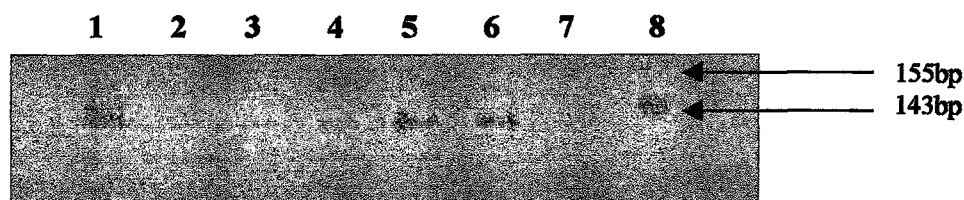


Figure 3.4.3.2: Autoradiography result of radioactively labelled PCR products of marker *D17S855*

The PCR products of marker *D17S855* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are about 143bp and 155bp. The radioisotope used is α - 32 P-dCTP. The lanes contain the samples as follows;

1: TEC 91N, 2: TEC 91T, 3: TEC 93N, 4: TEC 93T1, 5: TEC 93T2, 6: TEC 93T3, 7: TEC94N, 8: TEC 94T

3.4.4 Assessment of *D17S856* for Loss of Heterozygosity

D17S856 is another intragenic marker used for assessing LOH in *BRCA1*. It contains six alleles whose lengths differ between 0.228kb and 0.264kb. The values of allele frequencies lie between 0.0100 and 0.7700. Alleles 1 (0.001) and 6 (0.001) are the alleles with lowest frequencies. Allele 2 (0.7700) is the allele with the highest frequency.

In this study 15 out of 18 tumors in 9 out of 10 individuals were analyzed for LOH. In two samples, TEC 94 and TEC 99 LOH was found. LOH was seen both by using mutation detection enhancement (MDE) gel and routine non-denaturing polyacrylamide gel. The other samples did not show LOH. The banding patterns were in expected sizes and the loss of one allele in tumor samples were obviously observable in both gels.

The LOH detected by the MDE gel is shown in Figure 3.4.4.1

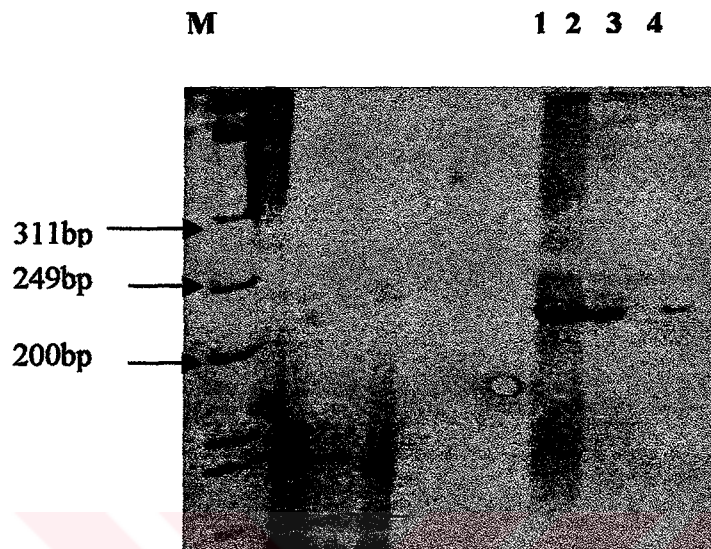


Figure 3.4.4.1: LOH in TEC 94 by Mutation Detection Enhancement (MDE) Gel

The PCR products of marker *D17S856* were run on a 12% Mutation Detection Enhancement (MDE) gel which is highly effective to detect mutations was used. The loss of one allele was observed in TEC 94T (Lane 3). The size marker used is ϕ X174 (Lane M). The allele sizes are between 228bp and 264bp. The lanes contain the samples as follows;

1: TEC 96T, 2: TEC 96N, 3: TEC 94T, 4: TEC 94N

The LOH using silver stained non-denaturing polyacrylamide gel is shown in Figure 3.4.4.2.

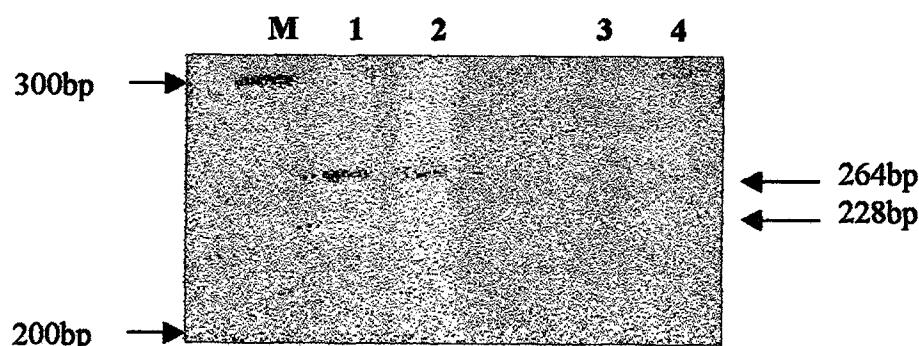


Figure 3.4.4.2: LOH in TEC 99 by non-denaturing polyacrylamide gel

The PCR products of marker *D17S856* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The loss of one allele was observed in TEC 99T (Lane 2). The size marker used is 100bp ladder (Lane M). The allele sizes are between 228bp and 264bp. The lanes contain the samples as follows; 1: TEC 99N, 2: TEC 99T, 3: TEC 100N, 4: TEC 100T. All the samples shown in this figure are informative, both paternal and maternal alleles are present in normal tissues. In the tissue sample TEC 99T the loss of one allele is observed and it is concluded that LOH has taken place for the sample TEC 99. The tumor tissue of the other sample TEC 100, contained both of the alleles of maternal and paternal origin and no LOH was observed for this sample.

3.4.5 Assessment of *D13S260* for Loss of Heterozygosity

D13S260 is one of the intragenic markers used in this study in order to find the LOH status in *BRCA2*. Its maximum heterozygosity is 0.7800 and it contains eight alleles. The existence of these two factors together makes *D13S260* an ideal marker for LOH studies. The values of allele frequencies of *D13S260* are between 0.0357 and 0.4107. Alleles 6 and 8 share the property of having the minimum allele frequency with a frequency of 0.0357 and fragment length of 0.159kb. Allele 2 contains the maximum allele frequency of 0.4107 with a fragment length of 0.163kb.

Only autoradiography was used to determine whether LOH has occurred in this marker or not. 17 out of 18 tumors in 10 individuals were studied. No LOH was observed in any of the tumors.

The assessment of LOH for *D13S260* by using autoradiography that resulted in the absence of LOH for the samples TEC 96 N, 96T1, 96 T2, TEC 97N, 97T1, 97T2 and 97 T3 are shown in Figure 3.4.5.

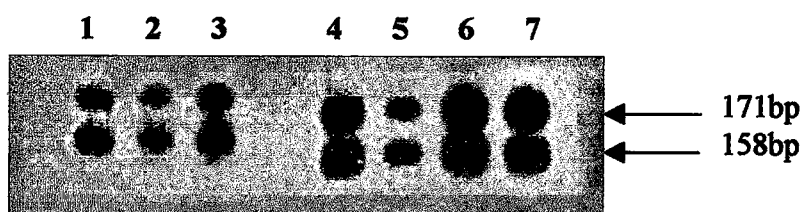


Figure 3.4.5.: Autoradiography result of radioactively labelled PCR products

TEC 96 and TEC 97 of marker *D13S260*

The PCR products of marker *D13S260* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are 158bp and 171bp. The radioisotope used is α -³²P-dCTP. The lanes contain the samples as follows;

1: TEC 96N, 2: TEC 96T1, 3: TEC 96T2, 4: TEC 97N, 5: TEC 97T1, 6: TEC 97T2, 7: TEC 97T3

3.4.6 Assessment of *D13S267* for Loss of Heterozygosity

D13S267 is an extragenic marker localized very close to the BRCA2 gene. As it contains a heterozygosity value of 0.6879 and six alleles it serves as a useful marker for LOH studies. The allele frequencies vary between 0.0192 and 0.4423 with fragment lengths lying between 0.148kb and 0.162kb. Allele 2 has the highest allele frequency, and is the most common allele with a fragment length of 0.148kb. Allele six however is the allele with the lowest frequency but biggest fragment size.

All 18 tumors of ten individuals were studied for LOH status. Only autoradiography was used to determine allele loss. The banding patterns of the tumors were as expected and no allele loss was observed in the samples studied. However in the tumors of TEC 98 it was observed that the intensities of the two alleles were not same. Although the intensities of both alleles in normal tissue of TEC 98 were same, in tumor tissues the amplification of one allele was obviously reduced. Due to these data it was suggested that LOH had occurred in TEC 98 tumor tissue samples.

The autoradiography in which allele loss was observed is shown in Figure 3.4.6.1.

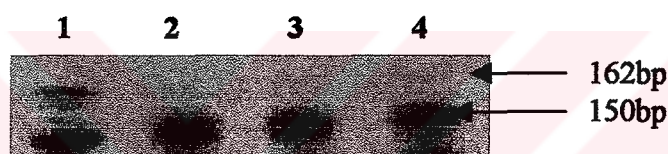


Figure 3.4.6.1: Autoradiography result of TEC 98 PCR products of marker

D13S267

The PCR products of marker *D13S267* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are about 150bp and 162bp. The radioisotope used is α - P^{32} -dCTP. The lanes contain the samples as follows;
1: TEC 98N, 2: TEC 98T1, 3: TEC 98T2, 4: TEC 98T3

The LOH study using autoradiography in order to show allele loss in *D13S260* is shown in Figure 3.4.6.2

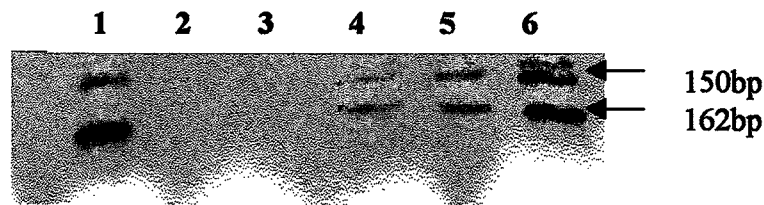


Figure 3.4.6.2: Autoradiography result of radioactively labelled PCR products of marker *D13S267*

The PCR products of marker *D13S267* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are about 150bp and 162bp. The radioisotope used is α -³²P-dCTP. The lanes contain the samples as follows;

1: TEC 96N, 2: TEC 96T1, 3: TEC 96T2, 4: TEC 97N, 5: TEC 97T1, 6: TEC 97T2. The bands representing the amplification pattern of TEC 96 T1 and 96 T2 were faint (Lanes 2 and 3).

3.4.7 Assessment of *D13S289* for Loss of Heterozygosity

D13S289 is an intragenic marker within the gene *BRCA2* with a maximum heterozygosity of 0.7400. This marker has eight alleles and the allele frequencies vary between 0.0536 and 0.4464. Allele 1 has the highest frequency of 0.4464. Alleles 3, 4 and 8 share the same lowest frequency of 0.0536.

13 out of 18 tumors in 7 out of 10 individuals were investigated for LOH. Only autoradiography was used to determine allele loss. In one of them (TEC 96) LOH was observed. The other samples did not show allele loss for the marker *D13S289*.

The LOH study using autoradiography in order to show allele loss in *D13S289* is shown in Figure 3.4.7

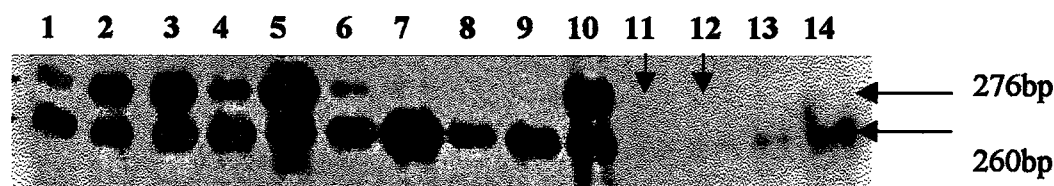


Figure 3.4.7: Autoradiography result of radioactively labelled PCR products of marker *D13S289*

The PCR products of marker *D13S289* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The allele sizes are about 260bp and 276bp. The loss of one allele was observed in TEC 96T1 and 96T2 (Lane 11 and lane 12) The radioisotope used is α -³²P-dCTP. The lanes contain the samples as follows;

1: TEC 93N, 2: TEC 93T1, 3: TEC 93T2, 4: TEC 93T3, 5: TEC 94N, 6: TEC 94T, 7: TEC 95N, 8: TEC95T1, 9: TEC 95T2, 10: TEC 96N, 11: TEC 96T1, 12: TEC 96T2, 13: TEC 100N, 14: TEC 100T

3.4.8 Assessment of *PTEN* for Loss of Heterozygosity

PTEN is a newly identified gene. Therefore no studies are available on the alleles and allele frequencies of *PTEN*. It is only known that the marker used in this study is an intragenic marker and it amplifies a fragment length of minimum 0.154kb.

All 18 tumors of 10 individuals were analysed for LOH with the silver staining method.

No LOH was observed in the samples studied.

Silver staining method was used to show allele loss in *PTEN* (Figure 3.4.8)

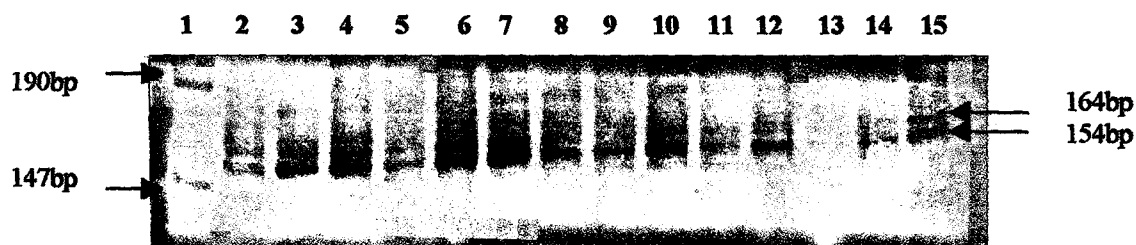


Figure 3.4.8: The silver stained polyacrylamide gel of *PTEN* alleles

The PCR products of marker *PTEN* were run on an 8% non-denaturing polyacrylamide gel to separate the alleles. The size marker used is pUC mix marker (Lane 1). The allele sizes are 154bp and 164bp. The lanes contain the samples as follows;

1: Marker, 2: TEC 91N, 3: TEC 91T, 4: TEC 93N, 5: TEC 93T1, 6: TEC 93T2, 7: TEC 93T3, 8: TEC94N, 9: TEC 94T, 10: TEC 95N, 11: TEC 95T1, 12: TEC 95T2, 13: TEC 96N, 14: TEC 96T1, 15: TEC 96T2

The results of this study for each marker are summarized in Tables 3.4.1, 3.4.2, 3.4.3, 3.4.4, 3.4.5, 3.4.6, 3.4.7 and 3.4.8.

Table 3.4.1: Results of LOH study for the marker *D17S776*

GENE	<i>BRC11</i>
MARKER	<i>D17S776</i>
POLYMORPHISM	CA DINUCLEOTIDE
CHROMOSOMAL LOCALIZATION	17pter-17qter
MAXIMUM HETEROZYGOSITY	0.4000
PRIMER NAME	TC 140/93
FORWARD PRIMER SEQUENCE	TCCATGACCCTATGGACCA
REVERSE PRIMER SEQUENCE	AAACCTCTGTCTCTTTGCAG
AMPLIFIED SEQUENCE MINIMUM LENGTH	0.120kb
AMPLIFIED SEQUENCE MAXIMUM LENGTH	0.130kb
SAMPLE	LOSS OF HETEROZYGOSITY
TEC 91T	–
TEC 93T1	ND
TEC 93T2	ND
TEC 93 T3	ND
TEC 94T	–
TEC 95T1	–
TEC 95T2	ND
TEC 96T1	–
TEC 96T2	–
TEC 97T1	–
TEC 97T2	–
TEC 97T3	–
TEC 98T1	–
TEC 98T2	–
TEC 98T3	–
TEC 99T	–
TEC 100T	–
TEC 101T	ND

– : No LOH was observed

ND: The samples in which the result was not determined

13 out of 18 tumors in 9 out of 10 individuals were studied for their LOH status. In none of them allele loss was observed.

Table 3.4.2: Results of LOH study for the marker *D17S800*

GENE	<i>BRCA1</i>
MARKER	<i>D17S800</i>
POLYMORPHISM	CA DINUCLEOTIDE
CHROMOSOMAL LOCALIZATION	17pter-17qter
MAXIMUM HETEROZYGOSITY	0.7433
PRIMER NAME	TC 155/156
FORWARD PRIMER SEQUENCE	GGTCTCATCCATCAGGTTTT
REVERSE PRIMER SEQUENCE	ATAGACTGTGTACTGGGCATTGA
AMPLIFIED SEQUENCE MINIMUM LENGTH	0.168kb
AMPLIFIED SEQUENCE MAXIMUM LENGTH	0.178kb
SAMPLE	LOSS OF HETEROZYGOSITY
TEC 91T	–
TEC 93T1	–
TEC 93T2	–
TEC93 T3	–
TEC 94T	–
TEC 95T1	–
TEC 95T2	–
TEC 96T1	ND
TEC 96T2	ND
TEC 97T1	–
TEC 97T2	–
TEC 97T3	–
TEC 98T1	–
TEC 98T2	–
TEC 98T3	–
TEC 99T	–
TEC 100T	–
TEC 101T	–

– : No LOH was observed

ND: The samples in which the result was not determined

16 out of 18 tumor tissue samples in 9 out of 10 individuals were studied for their LOH status. In none of them LOH was observed.

Table 3.4.3: Results of LOH study for the marker *D17S855*

GENE	<i>BRC41</i>
MARKER	<i>D17S855</i>
POLYMORPHISM	CA DINUCLEOTIDE
CHROMOSOMAL LOCALIZATION	17q
MAXIMUM HETEROZYGOSITY	0.8220
PRIMER NAME	TC 94/139
FORWARD PRIMER SEQUENCE	GGATGGCCTTTTGAAAAGTGG
REVERSE PRIMER SEQUENCE	ACACAGACTTGTCTACTGCC
AMPLIFIED SEQUENCE MINIMUM LENGTH	0.143kb
AMPLIFIED SEQUENCE MAXIMUM LENGTH	0.155kb
SAMPLE	LOSS OF HETEROZYGOSITY
TEC 91T	–
TEC 93T1	–
TEC 93T2	–
TEC93 T3	–
TEC 94T	–
TEC 95T1	–
TEC 95T2	–
TEC 96T1	–
TEC 96T2	ND
TEC 97T1	–
TEC 97T2	ND
TEC 97T3	ND
TEC 98T1	–
TEC 98T2	–
TEC 98T3	–
TEC 99T	–
TEC 100T	–
TEC 101T	ND

– : No LOH was observed

ND: The samples in which the result was not determined

14 out of 18 tumors of 9 out of ten patients were studied for their LOH status. In none of them LOH was observed. In TEC 101 PCR amplification did not work and no result was obtained.

Table 3.4.4: Results of LOH study for the marker *D17S856*

GENE	<i>BRC41</i>
MARKER	<i>D17S856</i>
POLYMORPHISM	TETRANUCLEOTIDE
CHROMOSOMAL LOCALIZATION	17q
MAXIMUM HETEROZYGOSITY	0.3891
PRIMER NAME	TEC 137/138
FORWARD PRIMER SEQUENCE	AAGGCAAGACTTCGTCGAGA
REVERSE PRIMER SEQUENCE	CATTCCCTGGTCCTGTGC
AMPLIFIED SEQUENCE MINIMUM LENGTH	0.228kb
AMPLIFIED SEQUENCE MAXIMUM LENGTH	0.264kb
SAMPLE	LOSS OF HETEROZYGOSITY
TEC 91T	–
TEC 93T1	–
TEC 93T2	–
TEC93 T3	–
TEC 94T	+
TEC 95T1	–
TEC 95T2	ND
TEC 96T1	–
TEC 96T2	ND
TEC 97T1	–
TEC 97T2	–
TEC 97T3	–
TEC 98T1	–
TEC 98T2	–
TEC 98T3	–
TEC 99T	+
TEC 100T	–
TEC 101T	ND

– : No LOH was observed

+ : LOH was observed

ND: The samples in which the result was not determined

15 out of 18 tumors in 9 out of 10 patients were studied for LOH in the marker *D17S856*. In two tumor samples TEC 94T and TEC 99T the loss of one allele was observed. In other tumor samples no LOH was observed. The PCR amplification and polyacrylamide gel electrophoresis did not work for the sample TEC 101.

Table 3.4.5: Results of LOH study for the marker *D13S260*

GENE	<i>BRCA2</i>
MARKER	<i>D13S260</i>
POLYMORPHISM	CA DINUCLEOTIDE
CHROMOSOMAL LOCALIZATION	13q
MAXIMUM HETEROZYGOSITY	0.7800
PRIMER NAME	TC 195/196
FORWARD PRIMER SEQUENCE	AGATATTGTCTCCGTTCCAAGA
REVERSE PRIMER SEQUENCE	CCCAGATATAAGGACCTGGTTA
AMPLIFIED SEQUENCE MINIMUM LENGTH	0.159kb
AMPLIFIED SEQUENCE MAXIMUM LENGTH	0.171kb
SAMPLE	LOSS OF HETEROZYGOSITY
TEC 91T	–
TEC 93T1	–
TEC 93T2	–
TEC93 T3	–
TEC 94T	–
TEC 95T1	–
TEC 95T2	–
TEC 96T1	–
TEC 96T2	–
TEC 97T1	–
TEC 97T2	–
TEC 97T3	–
TEC 98T1	–
TEC 98T2	–
TEC 98T3	ND
TEC 99T	–
TEC 100T	–
TEC 101T	–

– : No LOH was observed

ND: The samples in which the result was not determined

17 out of 18 tumors of all individuals were studied. In none of the tumors studied LOH was found.

Table 3.4.6: Results of LOH study for the marker *D13S267*

GENE	<i>BRC42</i>
MARKER	<i>D13S267</i>
POLYMORPHISM	CA DINUCLEOTIDE
CHROMOSOMAL LOCALIZATION	13q
MAXIMUM HETEROZYGOSITY	0.6900
PRIMER NAME	TC 191/192
FORWARD PRIMER SEQUENCE	GGCCTGAAAGGTATCCTC
REVERSE PRIMER SEQUENCE	TCCCACCATAAGCACAAG
AMPLIFIED SEQUENCE MINIMUM LENGTH	0.150kb
AMPLIFIED SEQUENCE MAXIMUM LENGTH	0.162bp
SAMPLE	LOSS OF HETEROZYGOSITY
TEC 91T	–
TEC 93T1	–
TEC 93T2	–
TEC93 T3	–
TEC 94T	–
TEC 95T1	–
TEC 95T2	–
TEC 96T1	–
TEC 96T2	–
TEC 97T1	–
TEC 97T2	–
TEC 97T3	–
TEC 98T1	+
TEC 98T2	+
TEC 98T3	+
TEC 99T	–
TEC 100T	–
TEC 101T	–

– : No LOH was observed

+ : LOH was observed

ND: The samples in which the result was not determined

All 18 tumor samples are studied for LOH. In three tumor tissue samples of one individual (TEC 98T1, T2 and T3) it was seen that the allele intensities were different although the intensities in the normal sample of the same individual was same. Therefore it was concluded that LOH had occurred in TEC 98T1, T2 and T3. In other samples no LOH for the marker *D13S267* was observed.

Table 3.4.7: Results of LOH study for the marker *D13S289*

GENE	<i>BRC42</i>
MARKER	<i>D13S289</i>
POLYMORPHISM	CA DINUCLEOTIDE
CHROMOSOMAL LOCALIZATION	13q
MAXIMUM HETEROZYGOSITY	0.7400
PRIMER NAME	TC 197/198
FORWARD PRIMER SEQUENCE	CTGGTTGAGCGGCATT
REVERSE PRIMER SEQUENCE	TGCAGCCTGGATGACA
AMPLIFIED SEQUENCE MINIMUM LENGTH	0.260kb
AMPLIFIED SEQUENCE MAXIMUM LENGTH	0.276kb
SAMPLE	LOSS OF HETEROZYGOSITY
TEC 91T	–
TEC 93T1	–
TEC 93T2	–
TEC93 T3	–
TEC 94T	–
TEC 95T1	UI
TEC 95T2	UI
TEC 96T1	+
TEC 96T2	+
TEC 97T1	–
TEC 97T2	–
TEC 97T3	–
TEC 98T1	ND
TEC 98T2	ND
TEC 98T3	ND
TEC 99T	ND
TEC 100T	–
TEC 101T	ND

– : No LOH was observed

+: LOH was observed

ND: The samples in which the result was not determined

UI: Uninformative

Seven out of ten individuals and 13 out of 18 tumors were studied for LOH. In two tumor variants of TEC 96 (TEC 96T1 and TEC 96T2) LOH were observed. The PCR amplifications and polyacrylamide gel electrophoresis of the samples TEC 98, TEC 99 and TEC 101 have been done however no results were obtained. In TEC 95 the strong amplification of one band in both normal and tumor tissue samples was observed and this is suggested as an uninformative case.

Table 3.4.8: Results of LOH study for the marker *PTEN*

GENE	<i>PTEN</i>
MARKER	<i>PTEN</i>
POLYMORPHISM	CA DINUCLEOTIDE
CHROMOSOMAL LOCALIZATION	10q
MAXIMUM HETEROZYGOSITY	Not known
PRIMER NAME	<i>PTEN</i> F/R
FORWARD PRIMER SEQUENCE	AAATGTACGGTTCATTGACTT
REVERSE PRIMER SEQUENCE	GACTGACTACAAATGGGCA
AMPLIFIED SEQUENCE MINIMUM LENGTH	0.154bp
AMPLIFIED SEQUENCE MAXIMUM LENGTH	0.164bp
SAMPLE	LOSS OF HETEROZYGOSITY
TEC 91T	–
TEC 93T1	–
TEC 93T2	–
TEC93 T3	–
TEC 94T	–
TEC 95T1	–
TEC 95T2	–
TEC 96T1	–
TEC 96T2	–
TEC 97T1	–
TEC 97T2	–
TEC 97T3	–
TEC 98T1	–
TEC 98T2	–
TEC 98T3	–
TEC 99T	–
TEC 100T	–
TEC 101T	–

– : No LOH was observed

All 18 tumor samples are studied for LOH. In none of them LOH was found.

The results of the whole study is summarized in Table 3.4.9

Table 3.4.9: Results of the LOH study for the genes *BRCA1*, *BRCA2* and *PTEN*

LOH	D17S776	D17S800	D17S855	D17S856	D13S260	D13S267	D13S289	PTEN
TEC 91T	–	–	–	–	–	–	–	–
TEC 93T1	ND	–	–	–	–	–	–	–
TEC 93T2	ND	–	–	–	–	–	–	–
TEC 93T3	ND	–	–	–	–	–	–	–
TEC 94T	–	–	–	+	–	–	–	–
TEC 95T1	–	–	–	–	–	–	UI	–
TEC 95T2	ND	ND	–	ND	–	–	UI	–
TEC 96T1	–	ND	–	–	–	–	+	–
TEC 96T2	–	–	ND	ND	–	–	+	–
TEC 97T1	–	–	–	–	–	–	–	–
TEC 97T2	–	–	ND	–	–	–	–	–
TEC 97T3	–	–	ND	–	–	–	–	–
TEC 98T1	–	–	–	–	–	+	ND	–
TEC 98T2	–	–	–	–	–	+	ND	–
TEC 98T3	–	–	–	–	ND	+	ND	–
TEC 99T	–	–	–	+	–	–	ND	–
TEC 100T	–	–	–	–	–	–	–	–
TEC 101T	ND	–	ND	–	–	–	ND	–

– : No LOH was observed

+: LOH was observed

ND: The samples in which the result was not determined

UI: Uninformative

3.5 CONCLUSION

In this study, three genes *BRCA1*, *BRCA2* and *PTEN* are studied for their LOH status in testis cancer. Eight highly polymorphic intragenic and extragenic markers are used to investigate the allele losses in these genes.

The LOH within *BRCA1* is observed in two samples TEC 93 and TEC 99 by using the marker *D17S856*. The other samples did not show LOH for any marker used to study *BRCA1*. Two out of ten individuals showed LOH for *D17S856* and this value is suggested to be considerable to study the relation between testis cancer and LOH.

The LOH within *BRCA2* is observed in three histological variants of TEC 98 (TEC 98T1, T2 and T3) by using the marker *D13S267* and two histological variants of TEC 96 (TEC 96T1 and T2) by using the marker *D13S289*. In *D13S267*, due to the difference in allele intensities between the normal and tumor tissues and in *D13S289*, due to the obvious allele loss it is suggested that LOH has been occurred. Also for *BRCA2*, the LOH values observed are suggested to be important to study the relation between *BRCA2* and LOH.

By increasing the sample number and the number of the markers used, the LOH status for *BRCA1*, *BRCA2* and *PTEN* might be further assessed and statistically more considerable results might be obtained.

CHAPTER 4. DISCUSSION

Because of its considerable death rate (20-30%), testis cancer might be accepted as a severe health problem for young men between the ages of 15-34. The significantly high lifetime prevalence of 4.9/100000 and variable incidence of testis cancer among different populations suggests that the studies of testis cancer have also a social side. Molecular studies would therefore be of great importance as they provide the necessary information to understand the causative mechanisms underlying this disease.

There is no data about the incidence of testis cancer in Turkey. Turkish population is a young population where testis cancer might be an important threat. The various status of industrialization in several regions of Turkey might be another reason to study testis cancer since it is thought that this disease also has an environmental basis. How these environmental factors act in molecular manner and which molecular mechanisms might be involved in the progression of testis cancer are questions that are waiting to be enlightened.

Worldwide, in molecular terms, testis cancer is not studied very frequently because of its high cure rate. This situation is also same for Turkey. All the factors mentioned above make the study of testis cancer desirable.

Several studies have revealed that distinct molecular alterations such as isochromosome 12 existence, trisomies, deletions, duplications, and other rearrangements of particular chromosomes, oncogene overexpressions and allele losses of some tumor suppressor genes are characteristic in testis cancer. Allele loss was known to be the key pointer in the existence of tumor suppressor genes and therefore LOH analyses were thought to be the most appropriate approach to analyse tumor suppressor genes. Some chromosomes were found to be more susceptible to allele losses in testis cancer and the tumor suppressor gene analyses studies were focused on these chromosomes. Chromosomes 10, 13 and 17 are among the chromosomes in which allele losses are very frequent (Looijenga *et al.*, 1994). This study was therefore focused on the analysis of tumor suppressor genes localized within these chromosomes. *PTEN* is a tumor suppressor gene recently identified and localized to chromosome 10. It was known that the allele loss of this gene was observable in three related diseases (Myers *et al.*, 1997). It was also shown that the allele loss of *PTEN* occurred in advanced prostate cancers (Marsh *et al.*, 1997). Therefore it was thought that this gene might also be involved in testis cancer and the allele loss of this gene was studied to analyse loss of heterozygosity. The second gene selected in this study for analysis was *BRCA1*. *BRCA1* is a gene whose LOH was shown in significant proportions in many cancers such as prostate cancer, breast cancer and ovarian cancer. The fact that the allele loss within this gene existed in many cancers and the high expression of this gene in testicular tissue made *BRCA1* a

strong candidate for this study. The last gene analysed in this study was *BRCA2*. The chromosomal localization of the gene and its relation with *BRCA1* made this gene important for this study. The most important fact for the study of these three genes was that LOH studies for those genes were not performed for testis cancer previously.

The patients selected for this study were nonseminomatous stage IIC and stage IIIC patients. Nonseminomatous histology exhibits lower differentiation however seminomatous cells are much more differentiated in comparison to nonseminomatous cells. Nonseminomatous cases are much more severe than seminomatous cases. In order to find the underlying factors of testis cancer at the beginning of the disease and to treat it as early as possible and to develop molecular methods providing this aim, it was thought that it would be more useful to analyse nonseminomatous patient samples instead of seminomatous cases.

It is known that several mechanisms might result in LOH. Through mutation analysis studies it might be investigated which mechanisms leading to LOH are much more frequent in testis cancer. Pure LOH based studies were not sufficient to decide for which mechanism causing LOH might be involved in the allele loss. The determination of the mechanism leading to LOH requires mutation detection analysis. However LOH studies are necessary in order to initiate mutation detection studies of tumor suppressor genes. It is possible to come across situations in which LOH occurs but mutation does not exist. However, there is not any case reported in which mutation in an allele has occurred without the existence of LOH. Therefore the best approach to investigate tumor suppressor genes might be to begin with LOH studies and continue with mutation detection analyses.

The informativeness of polymorphic markers is especially important in LOH studies. The distance of the polymorphic marker used to the gene of interest, allele number and frequency of the polymorphic marker are the important factors in choosing the ideal marker. Once markers are chosen it might be concluded whether a putative tumor suppressor gene exists in the area of interest or the allele loss has occurred in the tumor suppressor gene of interest. The polymorphic markers used in this study contained both low and high PIC values. Those that contain low PIC values were useful because they were intragenic markers and the loss of these markers were sufficient to determine the loss of the allele without the need of a high PIC value. The reason for using both intragenic and extragenic markers is again to determine the existence of a putative tumor suppressor gene or allele loss within the gene analysed.

This study was done on 10 individuals. The samples of these individuals were from the archives of Hacettepe University Pathology Department. The samples were paraffin embedded and old and thus the DNA could not be isolated in intact form. Another reason for this could be the fixative the samples were embedded in. Although the DNA could not be isolated intact, it could be successfully amplified by PCR. It was thought that this was due to the small fragment length of the sequences amplified.

Three genes were analysed in the existence of eight polymorphic markers in this study. Ten patients were investigated for LOH status. Both intragenic and extragenic markers were used to investigate the presence of a putative tumor suppressor gene around or LOH within *BRCA1* and *BRCA2* genes. The markers, *D17S776*, *D17S800*, *D17S855* and *D17S856* were used for investigating *BRCA1* gene. The distance between the markers selected was approximately 250-300 kilobases and an average of

one polymorphism every 60 kilobases was represented. The markers *D13S260*, *D13S267* and *D13S289* were used to determine the LOH status in *BRCA2*. *PTEN* was typed in the existence of a single intragenic marker and investigated for its LOH status.

In this study it was found that *BRCA1* gene had undergone allele loss for the marker *D17S856* in 2 out of 10 individuals. These patients were TEC 94 and TEC 99. The proportion of allele loss accounts for 20% which is a significant ratio. Loss within this intragenic marker directly indicates the loss of the allele within *BRCA1*. Patient TEC 94 showing LOH had embryonal carcinoma histology. This patient is dead probably due to the aggressive character and totipotential capacity of this histology. The other patient TEC 99 had mature teratoma histology. Since mature teratomas contain a collection of differentiated cell types the risk of having a genetic alteration is higher than in a single cell type histology. The other markers did not show any allele loss. This might be because of the loss of a very small area or a point mutation within the gene or the distance of the markers to each other. Even though the markers used were very close to each other the deletion might be so small that it does not affect the other markers used for the analysis.

BRCA2 gene was also assessed for its LOH status. Interesting results were obtained by the LOH study of *BRCA2*. In patient TEC 98, due to the difference in two allele banding patterns of tumor tissues it was suggested that one of the alleles was not amplified strongly as its homolog counterpart by using the polymorphic marker *D13S267*. However the intensity of two homolog alleles in the normal tissue of the sample did not show any difference. Since there was a quantitative change in the

existence of one allele in tumor tissues, it was thought that an allele loss had occurred in the tumor samples of patient TEC 98. Patient TEC 98 had mix germ cell histology consisting of teratocarcinomas and yolk sac tumors.

LOH was found also in patient TEC 96 for the marker *D13S289*. Here one of the two alleles was lost and therefore it was suggested that LOH had occurred in the tumors of TEC 96. Patient TEC 96 exhibited mix germ cell histology consisting of teratocarcinoma, seminoma and embryonal carcinoma histologies. The aggressive and totipotential character of embryonal carcinoma, the property of teratoma being prone to contain various histologies together might increase the risk for a genetic alteration.

PTEN is a newly identified gene. The loss of its alleles is shown in advanced cancer. The chromosome PTEN located in is prone to allele losses. These factors together make the LOH studies for *PTEN* desirable. The number of polymorphic markers found for PTEN is low. The LOH studies will extend with the increase of the numbers of polymorphic markers. *PTEN* was assessed only by using one marker. This marker was intragenic and the absence of this marker was an important indicator in the presence of LOH. However *PTEN* should be analysed by extending the number of markers and final suggestion should be done after the analyses by using additional markers.

FUTURE PERSPECTIVES

- The study was performed on 10 individuals. 18 histological variants of these 10 individuals were investigated for their LOH status. The next step would be to continue the study by increasing the number of patients studied.
- Mutation detection analyses for the samples in which LOH was found might be performed. Single stranded conformational polymorphism analysis or heteroduplex analyses might be carried out in order to determine whether a mutation has occurred within the gene or not.
- Sequencing might be performed for the samples where the intensity differences were observed to determine whether a point mutation might be the mechanism resulting in the generation of LOH.

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