DEVELOPMENT OF A NON-RADIOACTIVE DIAGNOSTIC TEST FOR THE DETECTION OF MICROSATELLITE INSTABILITY IN COLORECTAL CANCER

A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE
OF BİLKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

BY
KORKUT VATA
AUGUST 1997

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ANNEME

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a dissertation for the degree of Master of Science

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ABSTRACT

Development of a non-radioactive diagnostic test for the detection of microsatellite instability in human tumors

Korkut Vata

M.S. in Molecular Biology and Genetics
Supervisor:Assoc. Prof. Dr.Tayfun Özçelik
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Stepwise accumulation of mutations in the human genome is the initial step in carcinogenesis. Microsatellites are the regions which are first hit by the mutations resulting from mismatch repair deficiency. Until today microsatellite alterations have been shown mainly in hereditary non-polyposis colon cancer (HNPCC) and several other cancer types. Recent advances indicate that microsatellite alterations can also be detected in DNA samples (such as blood, urine, etc.), which are shed from tumors. This is an important finding for the early diagnosis of cancer since malignant cells can be detected in tissues other than the primary tumor. Therefore microsatellite analysis, when coupled with an easy, powerful screening technique could have a high diagnostic value for cancer types other than colorectal cancer. Despite its drawbacks the most common microsatellite screening method is based on the use of radioisotopes and autoradiography. However in the clinical setting non-radioactive detection methods are preferred.

The aim of this thesis is development of a non-radioactive diagnostic test for the detection of microsatellite instability in genomic DNA which can be used for the early detection of some forms of cancer. Therefore we have optimized the PCR conditions for eight microsatellite markers which are: *i.* mononucleotide repeats BAT25 and intragenic repeat region of BAX gene, *ii.* dinucleotide repeats D5S105, D6S291, D11S904, D13S175, D17S855, and *iii.* tetranucleotide repeat FGA. In addition, we have analyzed the mononucleotide repeat markers in blood, paraffin embedded and fresh tumor samples of six colorectal cancer patients with polyacrylamide gel electrophoresis and silver staining

ÖZET

İnsan tümörlerinde görülen mikrosatellit kararsızlığının incelenmesi için radyoaktif olmayan bir tanı testinin geliştirilmesi

Korkut Vata

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Tez Danışmanı: Doç. Dr. Tayfun Özçelik
Ağustos 1997, 60 sayfa

İnsan genomunda çeşitli sebeplerle mutasyonların oluşumu kanser gelişimi için atılan ilk adımdır. DNA tamir mekanizmasının bozulması veya eksik çalışmasına neden olan mutasyonlar ikincil olarak "mikrosatellit bölgeleri" olarak adlandırılan ve bir DNA dizi motifinin tekrarlandığı bölgelerde yeni mutasyonların oluşmasına yol açarlar. Bu mikrosatellit bölgelerinde bulunan tekrar dizileri aynı kişinin normal ve tümör gelişmiş dokusunda incelendiğinde farklılıklar gösterir. Bu durum "mikrosatellit kararsızlığı" olarak adlandırılmaktadır. Özellikle kolon kanserinin bir türü olan ailesel polipozsuz kolon kanseri başta olmak üzere bir çok kanser türünde tümör dokusunda "mikrosatellit kararsızlığının" varlığı gösterilmiştir.

Yapılan son araştırmalara göre mikrosatellit kararsızlığı tümör dokusundan vücut sıvılarına (kan, idrar ve bunun gibi) karışan DNA örneklerinin incelenmesi ile de tespit edilebilmektedir. Tümor dokusunun direkt olarak incelenmesini gerektirmeyen bu gelişme kanserin erken tanısında kullanılabilecek önemli bir DNA testi olma potansiyelini taşımaktadır. Henüz araştırma laboratuvarlarında yürütülen mikrosatellit analizi deneylerinde radyoaktiviteye dayanan teknikler kullanılmaktadır. Halbuki klinik tanı amaçlı DNA testlerinin radyoaktif olmayan metodlarla gerçekleştirilmesi tercih edilmektedir.

Bu tezin amacı insan tümörlerinde görülen mikrosatellit kararsızlığının incelenmesi için radyoaktif olmayan bir tanı testinin geliştirilmesidir. Bu nedenle aşağıda belirtilen mikrosatellit işaretleyicileri seçilmiş ve polimeraz zincir reaksiyonu (PCR) ile çoğaltılması için gerekli koşullar optimize edilmiştir. Bu mikrosatellit işaretleyicileri şunlardır: *i.* tek nükleotid tekrarları BAT25 ve BAX geninin bir bölgesi, *ii.* ikili nükleotid tekrarları D5S105, D6S291, D11S904, D13S175, D17S855, ve *iii.* dörtlü nükleotid tekrarı FGA. Buna ek olarak tek nükleotid tekrarları altı kolorektal kanser hastasının parafine gömülü veya taze doku örnekleri ile kan örneklerinden elde edilen DNA üzerinde poliakrilamid jel elektroforezi ve gümüş boyama teknikleri kullanılarak incelenmiştir.

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First and foremost I would like to thank my mentor Assoc. Prof. Dr. Tayfun Özçelik for the patience he showed during the period we have worked together. Eventhough the student is zealous, it is not easy to work with a young M.S. student. My mentor had helped me a lot by showing laboratory discipline and insight into the basic laboratory applications. But it must be also noted that we could have done better if we had worked in harmony at the beginning of the laboratory period.

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ABBREVIATIONS

APS	ammonium persulfate
bisacrylamide	N, N, methylene bis-acylamide
BOT	border ephithelial tumors
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
ds	double strand
EDTA	ethylene diamine tetra acetic acid
EtBr	ethidium bromide
GTBP	GT binding protein
HNPCC	hereditary non-polyposis colorectal cancer
hMLH1	human Mut L homologue 1
hMSH2	human MutS homologue 2
hPMS1	human post-meiotic segregation 1
hPMS2	human post-meiotic segregation 2
NER	nucleotide excision repair
mg	milligram
MMP	microsatellite mutator phenotype
MMR	mismatch repair
MI	microsatellite instability
min	minutes
ml	milliliter
µl	microliter
MQ	MilliQ water

MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
TBE	tris-boric acid-EDTA
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
UV	ultraviolet

1.INTRODUCTION

1.1. Cancer

In modern society cancer is the disease most feared by the majority of the people throughout the world. Roughly one person in five, in the prosperous countries of the world, will die of cancer. Actually, the term "cancer" refers to at least 100 different kinds of diseases. Almost every cell in the body can produce malignancies; some even yield several types. Even though each cancer has unique features, the basic processes that produce these diverse tumors appear to be quite similar. Trillions of cells of the normal, healthy body live in a complex, interdependent harmony, regulating one another's proliferation.(Prichopoulos *et al.*, 1996). Very occasionally, the exquisite controls that regulate cell multiplication break down and although the body has no need for further cells of its type, a cell begins to grow and divide. Ultimately, a mass called a tumor may be formed by this clone of unwanted cells. Mutation, competition, and natural selection operating within the population of somatic cells are the basic ingredients of cancer cells. Another characteristic of cancer cells is their ability to migrate to other places in the body (metastasis) (Bruce *et al.*,1994). Tumors composed of such malignant cells become more and more aggressive over time, and they become lethal when they disrupt the tissues and organs needed for the survival of the organism as a whole (Lodish *et al.*, 1996).

1.1.1. Genetic bases of cancer

Throughout the past 20 years, scientists have uncovered a set of basic principles that govern the development of cancer. It has been known for a long time that the cells in a tumor descend from a common ancestral cell that one point-usually decades before a tumor becomes palpable-initiated a program of inappropriate reproduction. Three classes of genes whose alterations lead to carcinogenesis have been defined (Bruce *et al.*,1994.):

a) Oncogenes that positively regulate cell growth i.e. when mutated they become carcinogenic and drive excessive multiplication;

b) Tumor suppressor genes that negatively regulate cell growth i.e. they contribute to carcinogenesis when they are inactivated by mutations and,

c) DNA repair genes that indirectly control proliferation by limiting the rate of mutations of growth controlling genes.

The mutations of these three kinds of genes may be caused by, basically;

i) controllable factors, including lifestyle habits such as smoking

ii) uncontrollable factors, including heredity

iii) exposure to carcinogens (such as asbestos and ultraviolet radiation)

iv) unknown factors

1.1.2. Oncogenes

Oncogenes are positive players of cell division mechanism. Activation of an oncogene is an important step towards tumorigenesis. The process of activation of these genes is termed as proto-oncogene activation. At present several mechanisms for proto-oncogene activation are known including point mutations and expanded deletions both in coding and regulatory regions of proto-oncogenes, translocations involving proto-oncogenes, and viral integration in the site of the proto-oncogene location (insertional mutagenesis) are some of them (Tabin *et al.*, 1982). DNA rearrangements caused by the above events may lead to misregulation of gene transcription resulting in the expression of the encoded protein in an inappropriate place or/and time. Mutations in the coding region may result in a protein with a transforming potential. Following translocation, the coding region of a gene may appear immediately downstream of a quite different promoter. Another possible consequence of translocation is the occurrence of a novel fusion protein that is absent in normal cells.

Oncogenes are mainly elements of cell-cycle machinery and signal transduction system The main known proto-oncogene products among others (Cantley. *et al,* 1991; Cross & Dexter,1991) include:

i) Growth factors, such as PDGF (platelet derived growth factor), FGF (fibroblast growth factor) family members;

ii) Membrane receptor proteins with tyrosine-kinase activity, such as CSF-1 receptor;

iii) Membrane associated GTP-binding/ GTPases, such as H-Ras;

iv) Cytoplasmic protein-kinases, such as c-abl;

v) Cyclins that are regulatory subunits for nuclear cell-cycle dependent protein kinases - cyclin D1;

vi) A large number of transcription factors: such as Myc, , E2F-1, etc.;

vii) Proteins encoded by the genes of the bcl family (bcl-2, bcl-x, bax, bad, etc). They are involved in the regulation of apoptosis, however their biochemical function is not clear yet.

1.1.3. Tumor suppressor genes

As opposed to oncogenes tumor-suppressor genes are negative players of cell-cycle machinery and signal transduction pathway. Thus, a functional tumor suppressor gene is a barrier on the way of uncontrolled cell division (Cordon-Cardo & Reuter VE, 1997; Greene, 1997; Lairmore TC & Norton JA, 1997). The most important distinction between tumor suppressor-genes and oncogenes is that both copies (alleles) of a tumor-suppressor gene should be inactivated for tumorigenesis to occure whereas activation of one copy (allele) of an oncogene is enough. This distinction is very important in terms of explaining the difference between familial (hereditary) and sporadic cancers (Thomson *et al.*,1995).

A possible classification of tumor-suppressor genes is according to their localization in the cell (Marshall, *et al.*, 1991) is:

i) Membrane bound receptors such as TGF-β receptor. It binds to a growth inhibitory signal agent.

ii) Cytoplasmic proteins such as NF-1 which binds to ras oncogene and somehow inactivates the ras proto-oncogene.

iii) Nucleus bound suppressors such as p53, Rb, BRCA-1. Rb is the major negative player of the cell-cycle at the G1 phase whereas p53 is at a point where cell-cycle, DNA repair and apoptosis meet. BRCA-1 is a tissue specific tumor-suppressor gene whose function is not clear yet.

1.1.4. Sporadic versus hereditary cancers

The hereditary predisposition of a cancer type was first recognized by a French neurosurgeon and anthropologist Paul Broca in 1866 when he analyzed the pedigree of his wife's family and recognized a hereditary predisposition to breast cancer. The other two malignancies recognized as hereditary were Xeroderma Pigmentosum (by James Cleaver) which is due to a defect in DNA repair genes and retinoblastoma, a malignancy resulting from a defective tumor-suppressor gene (for review see Bishop J. M. 1995). The pattern of inheritance of these familial tumors appeared to be autosomal dominant, but with exceptions. In 1971 Alfred Knudson published his first paper proposing two

paper the first mutation is carried in the germline, and the second mutation occurs later on during lifetime leading to the inactivation of the second copy of the Rb gene and resulting in tumorigenesis. This hypothesis also explains the rare sporadic cases. These cases must be due to two different mutations on the Rb alleles occurring during life-time. The two hit hypothesis also makes clear that mainly defects of tumor-suppressor genes and DNA repair genes are responsible for the familial cancer cases. The number of cancers which have a hereditary component is growing rapidly with the identification of tumor suppressor genes and better delineation of the phenotype. Some of these familial cancers, their incidences and their genes is indicated in Table 1. Conversely since only one mutation (hit) is enough for an oncogene to be activated, it can be said that their alterations are observed in sporadic cancer cases.

HEREDITARY CANCERS

NAME	INCIDENCE	GENES or LOCUS
HNPCC	*1:200*	MLH1,MSH2,PMS1,PMS2
FAMILIAL BREAST / OVARIAN CANCER	*1:300*	BRCA1, BRCA2
NEUROFIBROMATOSIS 1	*1:3000*	NF-1
WILM'S TUMOR	*1:8000*	WT-1
FAMILIAL ADENOMATOUS POLYPOSIS	*1:10000*	APC
RETINOBLASTOMA	*1:20000*	RB1
MULTIPLE ENDOCRINE NEOPLASIA 2	*1:25000*	RET
VON HIPPEL LINDAU	*1:36000*	VHL
NEUROFIBROMATOSIS 2	*1:37000*	NF-2
BASAL CELL NERVOUS SYNDROME	*1:56000*	PTC
LI FRAUMENI	*very rare*	p53
FAMILIAL MELANOMA	*rare*	p16

Table 1. *Some of the important hereditary cancers, their incidences and their genes.*

1.1.5. DNA repair

Once it was recognized that DNA is the main chemical component of all the genetic material, it was thought that this macromolecule must be extraordinarily stable in order to maintain the high degree of fidelity required for the original copy. It was suprising to learn that DNA is subject to alteration in the chemistry or sequence of individual bases. Many of these changes arise as a consequence of errors introduced during replication and recombination. Some changes are due to various environmental factors, such as chemical and physical factors (Friedberg *et al.*, 1995). If these errors were left totally uncorrected, both growing and nongrowing somatic cells might accumulate so much genetic damage that they could no longer function (Lodish *et al.*, 1995). The integrity of genome can only be explained by the presence of a repair mechanism. Several repair systems protect the genome by repairing the modified bases, DNA adducts, cross-links, and double strand breaks. A possible classification of these repair systems is as follows (Sancar A. 1995):

i) Direct repair:

Two known examples of direct repair are photoreactivation, and alkyl transfer. Photoreactivation is the reversal to monomers of pyrimidine cyclobutane dimers by a blue-light dependent enzyme. Alkyl transfer is the removal of the methyl group from O^6-MeGua in DNA to a cystein residue in the enzyme by an irreversible reaction.

ii) Base excision repair:

Base excision repair works mainly on non-bulky base adducts. In this repair system the modified, damaged base or base remnant is removed by an enzyme called DNA glycosylase. The resulting AP-deoxyribose is released by a pair of AP endonucleases that incise 3` and 5` to the AP site. The missing nucleotide is then replaced by a DNA Pol III and ligated.

iii) Nucleotide Excision Repair (NER):

The damaged base is removed by hydrolyzing phosphodiester bonds on both sides of the lesion. Two excision mechanisms could accomplish this removal; Endonuclease-exonuclease and exinuclease mechanisms (Sancar A., 1996). The first repair genes implicated in tumor predisposition were those responsible for NER and associated with Xeroderma Pigmentosum and related autosomal recessive inherited syndromes.

iv) Mismatch repair: Mismatch repair will be discussed in the following section in detail.

1.1.6. Mismatch repair in E. coli:

Both prokaryotic and eukaryotic cells are capable of repairing mismatched base pairs in their DNA. Mismatched base pairs in DNA can arise by several processes. One of the most important is replication errors. Another mechanism is the formation of a heteroduplex between two homologous DNA molecules as part of a recombinational process. If the two DNAs differ slightly in their sequence, as a consequence either of a mutation used as a genetic marker or of sequence changes acquired during evolutionary divergence, mismatches can be formed In this case, the DNA and proper correction of the mismatch contributes to the maintenance of the fidelity of the genetic information (Kolodner R., 1996).

The basic enzymology of the major mismatch repair process appears to be very similar between prokaryotic and eukaryotic organisms. The mechanism of mismatch repair has been studied most thoroughly in *E. coli.* The research groups of Mordrich, Kolodner (Kolodner R., 1996) and others have reconstituted the repair process from purified proteins. The proteins that have been identified as the initiators the repair process are MutS, MutL, and MutH (Figure 1.).

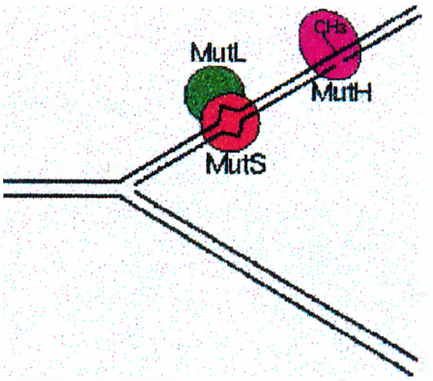


Figure 1. *Illustration of the action of the E. coli Mut HLS mismatch repair system on a mispair at a replication fork.*
Repair is initiated by binding of MutS protein to a mismatch. The subsequent binding of MutL to MutS is required to activate MutH, which then nicks the unmethylated strand of DNA at hemimethylated GATC sites. Nicking of the unmethylated strand is then followed by the excision from the nick to the mispair and resynthesis to fill in the resulting gap. These interactions result in the coupling of mismatch repair to DNA replication, so that mismatches form during DNA replication are repaired using the methylated parental strand as template, resulting in a reduction of misincorporation errors. (Adapted from Kolodner *R.*, 1996)

The fact that the old strand, but not the new, is methylated near the replication fork allows *E. coli* cells to distinguish the old (presumably correct) strand from the newly-synthesized (presumably incorrect) strand. The MutS-MutL complex activates MutH, which locates a nearby methyl group and nicks the newly synthesized strand opposite the methyl group. Excision is accomplished by cooperation between the UvrD (HelicaseII) protein, which unwinds from the nick in the direction of the mismatch, and a single stranded exonuclease of appropriate polarity (one of several in *E. coli*), followed by resynthesis (polymerase III) and ligation (DNA ligase).

It is important to note that the use of methylation to distinguish the parental strand is probably peculiar to *E. coli*. Data from yeast and mammalian *in vitro* mismatch repair experiments suggest that single-strand nicks provide a signal for strand specificity in these organisms. Note that single-strand breaks are present in nascent DNA strands,

between Okazaki fragments in the lagging strand at the 3` end of the leading strand. Although lacking homologues of MutH and uvrD, eukaryotic organisms possess numerous homologues of MutS and MutL.

E. coli protein	*S. cerevisea* protein	Human protein
MutS	MSH2	MSH2
	MSH3	MSH3
	MSH6	GTBP, p160
MutI	PMS1	PMS2
	MLH1	MLH1
	MLH2	PMS1

Table 2. *Repair gene products in E. coli and their homologues in S. cereviseae and in humans (*Adapted from Kolodner R.,1996)

The eukaryotic proteins listed in Table 2 appear to be homologues of the corresponding *E. coli* genes both in terms of amino acid sequence and in terms of functional similarities. Current evidence suggest that, whereas MutS and MutL function as monomers, the eukaryotic proteins form homo- or hetero-dimers. It appears that dimers of the MutS homologues, such as the dimer of MSH2 and MSH6/GTBP, are responsible for initial recognition of mismatches (or small insertions/deletions), and dimers of the MutL homologs (MLH1 and PMS1/PMS2) interact with the resultant complex, as in *E. Coli.* It

should be noted that human PMS2 is a better homologue of yeast PMS1 than its human PMS1.

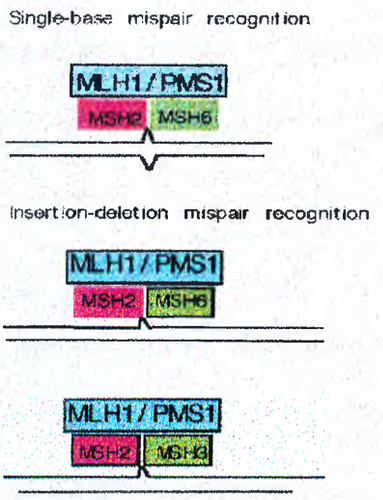


Figure 2:*Model for mismatch recognition in S. cereviseae*. The various postulated complexes between MSH2 and either MSH3 or MSH6 are illustrated interacting with either a single-base substitution mispair or an insertion/ deletion mispair, exactly which of the proteins in these complexes-MSH2, MSH3 or MSH6-actually interacts with the mispaired base is not known. Also indicated is the previously described MLH1-PMS1 complex that interacts with the mispair recognition complex. The *S. cereviseae*. protein names are given as primary names, the human protein names are the same except for PMS1, which is called PMS2 in humans, and MSH6, which has been called GTBP or p160 in humans (Adapted from Kolodner *R.*, 1996)

1.1.7. Mismatch repair and cancer

Mutations of the mismatch repair genes and their consequence, non-functional mismatch repair system illustrates the relationship between mutations in cancer susceptibility genes and mutational alteration of cancer genes (Jiricny J., 1994). A cancer gene can be defined as a gene; playing role in one of the four processes; cell growth, differentiation, senescence and survival. "Cancer susceptibility genes", on the other hand are defined as

those genes involved in any of the multiple types of DNA alterations, mutations (pre-oncogenic mutations), which influence the probability of occurrence of mutations in cancer genes (oncogenic mutations) (Perucho M., 1996a). There is a chronological difference in the involvement of two types of genes on the way to carcinogenesis. "Cancer susceptibility" genes are involved in the early phases of tumorigenesis whereas "cancer genes" are directly involved in later phases and most of the time due to the mutations of "cancer susceptibility genes".

These two kinds of genes are also referred as "caretakers" and "gatekeepers``(Kinzler K.W. & Vogelstein B., 1997). "Gatekeepers" are the early players, whereas "caretakers" play a pole in the later phases of carcinogenesis. Since all the genes involved in the repair mechanism belong to the former group, mismatch repair genes are good examples of the so called "caretakers".

The relationship between the genomic instability due to "caretaker" mutations and mutations in cancer genes such as tumor suppressor genes and oncogenes had been assumed for a long time (Loeb A. L., 1994). However no proof for a causal relationship between the deficiencies in mismatch repair and mutations in cancer genes had been found. Recently, it has been shown that the TGF-β receptor type-II gene is inactivated by a frameshift mutation in a poly(A) tract present in its coding region (Markowitz *et al.*, 1995). TGF-β receptor type-II gene codes for a membrane bound receptor which inhibits proliferation of the normal epithelial cells (Takenoshita *et al.*, 1996). This has been the first direct demonstration of the link between the mutations of the "caretakers" and the

mutations of the "gatekeepers", but also the most informative example of the relationship between the mutator and the suppressor pathways of cancer. In February 1997, frameshift mutations in intragenic (G)8 tract of BAX gene have been shown to be present in more than 50 percent of HNPCC cases. (Rampino *et al.*, 1997). BAX gene is a Bcl-2 related protein that promotes apoptosis (Yin *et al.*, 1997). Another intragenic repeat region alteration has been also reported in the insulin-like growth factor II receptor gene (Ouyang *et al.*, 1997). These three cases are good examples of inactivation of "gatekeepers" due to mutations of "caretakers".

1.2. Hereditary non-polyposis colorectal cancer (HNPCC)

Hereditary non-polyposis colorectal cancer (HNPCC), is one of several hereditary colorectal cancer syndromes. The clinical spectrum of colorectal cancers is given in Figure 3.

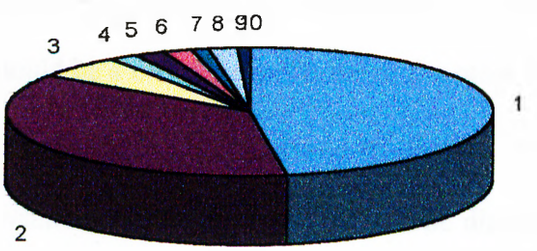


Figure 3. *Description of the extent of clinical heterogeneity in hereditary colorectal cancers:* **1.** Hereditary discrete colonic polyps, **2.** Sporadic (multifactorial polygenic), **3.** HNPCC: LynchI and II, **4.** Familial IBC, **5.** FAP, **6.** HFAS, **7.** Peutz -Jeghens syndrome, **8.** Familial juvenile polyposis,
9. Turcot`s syndrome

(Adapted from Cancer control Journal, http://www.moffit.us.edu/providers/ccj/v3n1/article1, 1997)

From the clinical point of view hereditary non-polyposis colorectal cancer can be examined in two groups; Lynch syndrome I and II. Lynch syndrome I is an autosomal dominantly inherited predisposition to colorectal cancer with right-sided predominance and an excess of multiple primary colorectal cancer. Lynch syndrome II not only shows all of the features of Lynch syndrome I, but also involves an enormous array of extra-colonic cancers, particularly endometrial carcinoma, carcinoma of the ovary, small bowel, stomach, pancreas, and transitional cell carcinoma of the urether and renal pelvis (Lothe *et al.*, 1993).

In August 1990 thirty leading experts on HNPCC from eight different countries met in Amsterdam to discuss various problems associated with the study of HNPCC. Discussions in the meeting focused upon the need to develop minimum criteria for the identification of HNPCC based on familial information (Vasen *et al.*, 1991; Aaltonen, *et al.*, 1994). The so called Amsterdam Criteria has three conditions:

1) At least three relatives should have histologically verified colorectal cancer; one of them should be a first degree relative to the other two.
2) At least two successive generations should be affected.
3) In one of the relatives colorectal cancer should be diagnosed under 50 years of age.

The lowest known estimate of HNPCC occurrence is 1%, which translates into 1500 new cases of HNPCC annually in the United States. Estimates of HNPCC incidence

range as high as 5%, or 7500 new occurrences of HNPCC in the United States each year. Either estimate indicates that HNPCC poses a major public health problem, since each new case would signify a family prone not only to colorectal cancer, but also to a variety of extra-colonic cancers. (Vasen *et al.*, 1991).

The genetic basis for HNPCC has been proven by genetic linkage between cancer occurrences and co-segregation of chromosome 2p markers in some families (Leach F.S. *et al.*, 1993), and chromosome 3p markers in others (Bronner *et al.*, 1994). Localization of a DNA mismatch repair gene in the critical region of chromosome 2p was documented with the discovery of hMSH2 mutations in this gene in several HNPCC families (Papadopoulos *et al.*,1993; Leach *et al.*, 1993). Subsequently, a second mismatch repair gene was found in the critical region of 3p, and mutations of that gene were found in HNPCC families previously linked to chromosome 3p (Papadopoulos *et al;* 1994, Liu *et al*, 1994). Inherited mutations of genes involved in mismatch repair (MMR) have been shown to be responsible for hereditary non-polyposis colorectal carcinoma (HNPCC) (Modrich *et al.*, 1994). Mutations in these genes appear to account for 90% of all known HNPCC families with a mutation within the mismatch repair genes.(Papadopoulos *et al.* 1994, Liu *et al*, 1994, Moslein *et al.*, 1996; Boyer *et al.*, 1995) and the mutations of hPMS1, hPMS2 and GTBP proteins together contribute only to10% of the cases.

1.2.1. Microsatellite instability in HNPCC

It is known that all of the protein-coding regions account for only about 3% of the human genome. However, most polymorphisms are observed in the 97% of the human genome, which does not code for proteins and called as "junk DNA". Even though these regions seem to be non-functional they may play vital roles in normal genome function. Since these regions do not code for proteins, variations therein are functionally inconsequential and hence well-tolerated during evolution This has allowed to develop tremendous genetic diversity in these regions. Much of this non-coding DNA consist of highly repetitive segments known as "DNA repeats" (Bruce et al., 1994). A classification of DNA repeats is given in Table 3. Repeated sequences can occur as tandem arrays. Such sequences, called Variable Number of Tandem Repeats (VNTRs), are unique to each person and are the basis for the precise DNA fingerprinting used in forensics. One such class of sequences in humans consists of Short Tandem Repeats (STRs), often a dinucleotide (sometimes mono-, tri-, or tetra-) repeat of CA (adenosine and cytosine) on one DNA strand and GT (guanine and thymidine) on the other. Such repeats of 2-5 nucleotide segments are known as microsatellite DNA. A single pair of PCR oligonucleotide primers that flank such sequences produce variable-sized DNA fragments depending on the number of repeats. Since mismatch repair is responsible for detecting and repairing short segments of mismatched base pairs, disorders of the mismatch repair pathway lead to errors in these polymorphic segments (Heale S.M. & Peters T.D, 1995., 1995; Jiricny *et al.*, 1994; Ionov *et al.*, 1993; Wooster *et al*, 1994).

At-Risk Sequence		Possible Intermediate		
	Occurance in			Annealed
	Human	loops	loop	Short
Type	Genome	<4nt	>30nt	Repeats
Microsatellites	>100000	+	+	+
(1-4 bp.tandem repats)				
Minisatellites	>10000	-	+	+
(30-100bp tandem repeats)				
Short (4-6bp)				
non-tandem repeats	>10000000	-	+	+
separated by 30-100 bp				

(Adapted from Kolodner, 1996)

Table 3. *Examples of at-risk sequences for detecting the presence of human mutators*

Based on the analysis of these polymorphic segments new microsatellite alleles are observed in tumor ("T") DNA when compared to non-neoplastic ("N") DNA. The addition of novel microsatellite alleles in the tumor is called **microsatellite instability (MI).** One of the mechanisms that explain microsatellite instability is slippage during DNA replication (Figure 4).

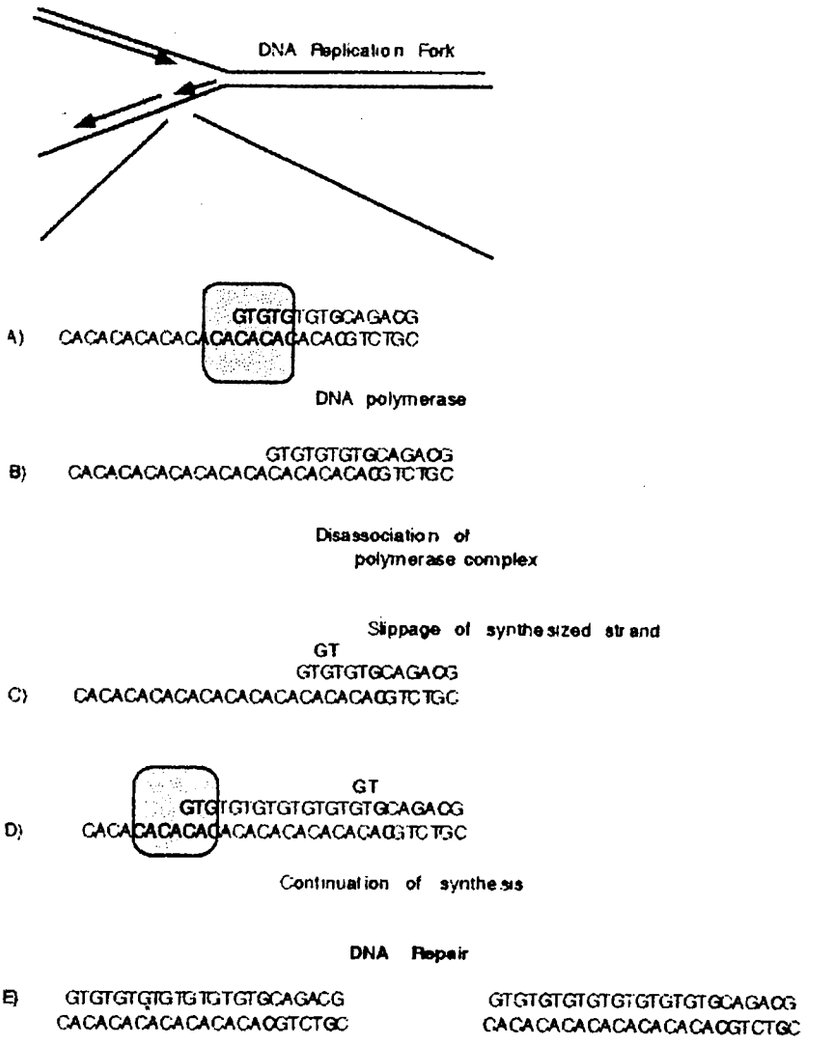


Figure 4. *Slippage mechanism during DNA replication.* A model explaining why repeated sequences are prone to accumulation of mutations in the case of mismatch repair deficiency. (Adapted from Wells R.D. 1996.)

The microsatellite instability is found to be a recessive trait by the studies on somatic cell-hybrids on MI (+) and on MI (-) cells (Casares *et al.*, 1995). Mutator cell lines lacking functional DNA mismatch repair have dinucleotide microsatellite mutation frequency

among the highest yet observed in tumors, up to 0,01 mutations per cell division. Therefore, in setting of deficient mismatch repair, microsatellite mutations can be used as indicators of tumorigenesis (Shibata *et al.*, 1996). Once this genome-wide instability was recognized, the detection of alterations in a few microsatellite loci could be explained by the existence of a deep genomic instability underlying a mutator phenotype of cancer. (Parsons *et al.*, 1995) This led to a group of papers describing "microsatellite instability" in a variety of tumors.

1.2.2. Microsatellite instability in cancer types other than HNPCC

Microsatellite instability has been studied in other types of cancer as well. Recent studies include; breast, bladder, lung, prostate, head and neck tissue, esophagus, kidney, ovary, stomach, uterus, brain, germline, mouth and skin tumors. A short summary of the recent publications are as follows;

i. 4-13% of the breast cancer cases studied were positive in terms of MI (Shaw *et al.*, 1996).
ii. Tangir and his coworkers studied 13 microsatellite markers on chromosome 3; five of the 18 BOT (Borderline Epithelial Tumors) and 2 of the 31 IEOC (invasive epithelial ovarian cancer) cases displayed MI (Tangir *et al.*, 1996).
iii. In 1995 the group led by Suzuki worked on17 loci on 9 chromosomes and they find out that 7 of the 48 cases (14.6%) displayed microsatellite instability in prostate cancer cases (Suzuki *et al.*, 1995).

iv. 15 microsatellite markers on a population composed of 20 paired normal and primary non-metastatic prostatic-tumor samples have been studied by Lacombe and his coworkers in 1996. Overall, 65% (13/20) of the cases analyzed were positive in terms of microsatellite instability. 66 patients with prostatic adenocarcinoma were screened for somatic instability (Lacombe *et al.*, 1996).

v. Microsatellite instability was examined at 36 loci, and found in 9 (43%) of the 21 prostatic cancers. (Watanabe *et al.*, 1995)

vi. In 1995 microsatellite markers D2S136, MSX2 (5q34), D5S82 (5q14-21) and TP53 (17p13.1) were studied by Shinmura and his coworkers.The prevalence of microsatellite instability in patients with multiple gastric cancer was greater (65% versus 24%) than those with solitary gastric cancer. (Shinmura *et al.*, 1995).

vii. Matsuda and his coworkers studied three loci, D2S123, D3S1067, and TP53 in 1996, genetic instability was found in 5 out of 17 patients with renal carcinoma (29%) (Matsuda *et al.*, 1996)

viii. 144 sporadic brain neoplasms are examined. These include 33 astrocytic tumors, 33 oligodendrogliomas, 6 gangliomas, 42 meningiomas, 10 vestibular schwanomas and 31 pitutary adenomas. Instability of microsatellite markers was detected in four oligodendrogliomas (17.4%), one pitutary adenoma (3.2%), one meningioma (2.4%), one astrocytic tumor (3.0%) and not at all in gangliomas and schwannomas (Rowley *et al.*, 1996).

ix. 91 oral tumors have been analysed for microsatellite instability, 6 (7%) of the cases were positive. Instability was observed at multiple loci with a range of 50-74% of loci affected (Ishwald *et al.*, 1995).

x. 26 microsatellite repeat sequences in the DNA of normal and tumor pairs from 100 head and neck, bladder, and lung cancer patients are analysed. 26% of the patients were positive in microsatellite instability. The most interesting point of this study is that the identical microsatellite alterations are detected in the corresponding urine, sputum, and surgical margines from affected patients. (Mao et al., 1996a). This paper demonstrates that microsatellite analysis on DNA samples shaded from tumors into body fluids is promising for the detection of several cancer types

1.3. Detection methods for microsatellite instability

The analysis of microsatellites relies mostly on PCR amplification of the sequences of interest and polyacrylamide gel electrophoresis (PAGE analysis) followed by different methods aiming to visualize the bands on the gels.

1.3.1. Autoradiography

Autoradiography is an efficient method for the visualization of the bands on the polyacrylamide gels, however its main drawback is the usage of radioisotopes.

There are three major problems with the radioactivity:

a) Radioactivity is hazardous.
b) Radioactive material should be delivered immediately since it has a certain half-life. For developing countries like Turkey this is a big problem since nearly all of the radioactive material is purchased from overseas. Most of the time the radioactive material is non-functional by the time it is received by the consumer.
c) Radioisotopes are expensive.

The selection of the radioisotope for a particular experiment depends mainly on the level of sensitivity and resolution required. Nucleotides with a specific activity of about 3000

Ci/mmol are most frequently chosen for the majority of applications, e.g.($^{alpha-32}$P)dCTP. Phosphorus-33 can also be used in filter hybridization. ^{33}P has the advantage of lower emission energy compared to ^{32}P, allowing increased resolution. However, this leads to longer exposure times. It is particularly suitable for microsatellite analysis and other techniques where high resolution is required. ^{35}S labeled nucleotides can be used for filter hybridization experiments but are not recommended as the low specific activity necessitates very long exposure times. Despite its drawbacks autoradiography is the most common visualization method for microsatellite analysis.

1.3.2. Fluorescence

Autoradiograhy is the direct exposure of film by beta particles or gamma rays, whereas fluorography is the exposure of the film by secondary light which was generated by the excitation of a fluor or a screen by beta particle, a gamma ray or a laser beam with a certain wavelenght. Fluorescence is the most suitable labeling method for automated analysis. One of the PCR primers is synthesized with a 5`-fluorescent label. The sample is loaded with an internal lane standard. As the DNA fragments with different lengths pass through a detector, a laser beam excites the fluorescence and the fluorescence is measured by a special camera (CCD camera most of the time). The sizes of the internal standard is known and so the sizes of the PCR products are calculated by a special software accordingly (Cawkwell *et. al.*,1995; Toh *et al.*, 1996). Multiplex analysis by using more than one fluorescence label is also possible with these kinds of systems.

1.4. Aim

Microsatellite analysis is important for the differential diagnosis of hereditary non-polyposis colorectal cancer patients since microsatellite instability is a strong determinant of germline mismatch repair deficiency in the affected individuals. Although a careful family history may also reveal involvement of germline mutations, it is rather difficult to obtain an accurate family history in the absence of a genetic counselor. Therefore microsatellite instability analysis should become an integral part of the post-operative laboratory workout in colorectal cancer patients to differentiate hereditary versus sporadic colorectal cancer cases. In addition recent advances indicate that microsatellite alterations can also be detected in DNA samples (such as in blood, and in urine), which are shed from tumors. This is an important finding for the early diagnosis of cancer since malignant cells can be detected in tissues other than the primary tumor. These include small cell lung carcinoma, head and neck cancers (Nawroz *et al.*, 1996), and bladder cancer (Steiner *et al.*, 1997, and Uchida *et al.*, 1996). Therefore microsatellite analysis, when coupled with an easy, powerful screening technique could have a high diagnostic value. However, like most of the other DNA-based diagnostic techniques microsatellite instability analysis is at present performed only in the research laboratories due to the high cost of the test, requirement of expertise, difficulty in the interpretation of the test results etc.

Autoadioactivity is the most commonly used method for microsatellite instability analysis. As stated above, autoradioactivity has two main drawbacks; it is hazardous and

therefore requires special protection for working and since it has a certain half-life one has to work with it in a limited time interval. Autoradiography necessitates also radioactively labeled primers, which is an additional step in the oligonucleotide synthesis. Moreover one has to calculate the high price of radioactivity. Eventhough fluorescence detection methods seem to be safe with respect to autoradiography, they also require an additional labeling step in the oligonucleotide synthesis and fluorescence labels are not cheap either.

Polyacrylamide-gel electrophoresis is the most useful method to separate DNA fragments with a resolution enough to conclude about microsatellite instability. A visualization method after PAGE analysis which is easy to perform, non-hazardous, and low-cost will definitely increase the applicability of microsatellite analysis in laboratories.

Based on these facts, I aimed to develop a non-radioactive diagnostic test for human tumors. Silver staining is chosen among the other non-radioactive methods due to its ease of application, high sensitivity and low cost.

2. MATERIALS and METHODS

2.1 Tissue samples

We have obtained eighty extracted DNA samples that belong to colorectal cancer patients from Dr. Tamer Yağcı, Yedigen-İstanbul. These samples have previosly been analyzed for genomic instability with several DNA markers (Yağcı et al., 1996). The samples are paired colorectal tumor and adjacent normal samples. They have been obtained from the Departments of Pathology of Istanbul Faculty of Medicine and Cerrahpaşa Faculty of Medicine. During the collection of samples no pre-selected criteria was used such as "Amsterdam criteria" for HNPCC. Of the 12 samples analyzed in this study 2 were paraffin embedded and 10 were fresh tumor samples.

2.2 Primers used for microsatellite analysis

Eight pairs of primers have been used for microstellite analysis. They have been synthesized in house using Beckman1000 M oligosynthesizer. The sequences of these primer pairs are as follows:

2.2.1. D17S855 (Gao et al., 1995)

F:	**GA97:**	GGA TGG CCT TTT AGA AAG TGG
R:	**GA98:**	ACA CAG ACT TGT CCT ACT GCC

2.2.2. D6S291 (Gyapay et al., 1994)

F:	**GA99:**	CTC AGA GGA TGC CAT GTC TAA AAT A
R:	**GA100:**	GGG GAT GAC GAA TTA TTC ACT AAC T

2.2.3. FGA (Primer pairs designed in house)

F:	**GA101:**	ACT CAC AGA TTA AAC TGT AAC CAA AA
R:	**GA102:**	GTG ATT TGT CTG TAA TTG CCA

2.2.4. D11S904 (Weissenbach et al., 1992)

F:	**GA103:**	ATG ACA AGC AAT CCT TGA GC
R:	**GA104:**	CTG TGT TAT ATC CCT AAA GTG GTG A

2.2.5. D13S175 (Weissenbach et al., 1992)

F:	**GA105:**	TAT TGG ATA CTT GAA TCT GCT G
R:	**GA106:**	TGC ATC ACC TCA CAT AGG TTA

2.2.6. D5S107 (Weissenbach et al., 1992)

F:	**GA107:**	GAT CCA CTT TAA CCC AAA TAC
R:	**GA108:**	GGC ATC AAC TTG AAC AGC AT

2.2.7. BAT25 (Parsons et al., 1995)

F:	**GA190:**	TCG CCT CCA AGA ATG TAA GT
R:	**GA191:**	TCT GCA TTT TAA CTA TGG CT

2.2.8. BAX (Rampino et al., 1997)

R:	**GA272:**	ATC CAG GAT CGA GCA GGG CG
R:	**GA273A:**	ACT CGC TCA GCT TCT TGG TG

Locus: D11S904 (Dinucleotide repeat)
Primer Name: GA103-104
Fragment Lenght: 185-210 bp

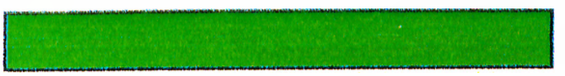
Locus: D13S175 (Dinucleotide repeat)
Primer Name: GA105-106
Fragment Lenght: 101-113 bp.

Locus: D5S107 (Dinucleotide repeat)
Primer Name: GA107-108
Fragment Lenght: 143-155 bp.

Tm: 55

Locus:BAT25 (Mononucleotide repeat)
Primer Name: GA190-191
Fragment Lenght: 105-112 bp.

Locus: BAX intragenic repeat (Mononucleotide repeat)
Primer Name: GA 272-273A
Fragment Lenght: 94 bp.



Locus: D17S855 (Dinucleotide repeat)
Primer Name: GA97-98
Fragment Lenght: 133-155 bp.

Locus: D8S291 (Dinucleotide repeat)
Primer Name: GA99-100
Fragment Lenght: 198-210 bp.

Tm:60

Locus: FGA (Tetranucleotide repeat)
Primer Name: GA101 -102
Fragment Lenght: 177 bp.

Table 4. List of the microsatellite loci and their relative fragment lengths

2.3. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR), the repetitive bi-directional DNA synthesis based on primer extension of a region of nucleic acid, is a simple design and can be used for many purposes. There are three distinct events during a PCR cycle:

1) denaturation of the template: DNA denaturation occurs when the reaction is heated to 92-96°C.
2) primer annealing: After denaturation, the oligonucleotide primers hybridize to their complementary single-stranded target sequences. The temperature of this step varies from 37°C to 65°C, depending on the homology of the primers for the target sequence as well as the base composition of the oligonucleotides.
3) DNA synthesis by the thermostable polymerase: The last step is the extension of the oligonucleotide primer by the thermostable polymerase. Traditionally this portion of the reaction is carried out at 72°C. Ussually the larger is the template the longer is the time required for a proper extension.

2.3.1. PCR Conditions

-10 X Buffer	2.5 µl
100mMTris-HCl (pH:8), 01% Gelatin, 1% TritonX, 2.0mM $MgCl_2$, 250mM KCl	
-Taq Polymerase (MBI Cat No: EP0282)	0.8 unit (0.2µl)
-dNTP mix (Sigma A4916) 10mM	1 µl
-Primers(50pmol/µl)	1 µl (0.5 F+0.5 R)
-Template DNA	100-400 ng
-Final Volume	25 µl (MQ ddH_2O upto 25 µl)

2.3.2. Thermal cycler conditions

Initial denaturation:	94°C for7min
Cycles X 35:	94 °C for 30``- (55 or 60°C)* for 30``-72°C for 30``
Final Extension	72°C for 7min

Perkin Elmer thermal cycler model 9600 was used during the experiments.

** The Tm values of primers are indicated in Table 4.*

2.4. Agarose gel electrophoresis

The progress of the first experiments on cutting and joining of DNA molecules was monitored by velocity sedimentation in sucrose gradients. However, this has been entirely superseded by gel electrophoresis. Gel electrophoresis is not only used as an analytical method, it is also used routinely for the purification of specific DNA fragments. The gel is composed of polyacrylamide or agarose. Agarose is convenient for separating DNA fragments ranging in size from a few hundred to about 20kb. Polyacrylamide is preferred for smaller DNA fragments (Primrose S.B. & Old.R.W.S., 1989.)

A gel is a complex network of polymeric molecules. DNA molecules are negatively charged, and under electric field DNA molecules migrate through the gel at rates dependent upon their sizes: a small DNA molecule can thread its way through gel easily and hence migrates faster then a larger molecule.

In any event, gel electrophoresis frequently performed with marker DNA fragments of known size which allow accurate size determination of an unknown DNA molecule by interpolation. The bands of DNA in the gel are stained with the intercalating dye ethidium-bromide, and as little as 0.01μg of DNA in one band can be detected as visible fluorescence when the gel is illuminated under ultraviolet light.

2.4.1. Procedure

In order to prepare a 2% agarose gel, 1.6 gr agarose is weight and put into 500ml erlenmeyer flask. 80ml of 1X TBE is poured on to the agarose. This erlenmeyer flask is placed in to the microwave-oven and heated in the half power for five minutes, until all of the agarose is melted. 1μl of EtBr is added on to the melted agarose and the gel solution is left to cool on a magnetic stirrer, while mixing the solution with the lowest possible speed to prevent formation of bubbles. The gel is poured into the pre-casted gel tray and left to polymerize.

2.5. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis is a method used to differentiate between DNA fragments with a very high resolution. When coupled with a detergent, like SDS, polyacrylamide gel electrophoresis can also be used for the analysis of proteins. However there are two main drawbacks of acrylamide gels; in comparison with agarose gels they are more difficult to polymerize and they are potentially more toxic.

There are two main types of polyacrylamide gels. These are denaturing and non-denaturing polyacrylamide gels.

2.5.1. Denaturing polyacrylamide gels

This types of gels contain urea or formamide to keep two strands of the DNA molecules apart. Denaturing gels allow us to differentiate between fragment lengths of single stranded DNA with a very high resolution. The resolution obtained is only depended on the molecular weight and not on the conformation. This is the main distinction between the denaturing and non-denaturing poly-acrylamide gels. Microsatellite analysis is not possible on non-denaturing gels.

2.5.2. Non-denaturing polyacrylamide gels

Buffers, similar to those used for agarose are used for the preparation of these gels (TBE, TAE). Non-denaturing gels are especially useful for detection of the conformational changes in DNA molecules (e.g. due to bends or DNA binding)

2.5.3. Solutions

♦ Sticky Solution

Bind Silane..... 50µl
Glacial Acetic Acid.....50µl
99% Ethanol.....9900µl

♦ 40% 19:1 Acrylamide-bisacrylamide stock solution

38 gr Acrylamide

2 gr Bisacrylamide

Add ddH_2O to 100ml

Solutes are dissolved on a magnetic stirrer

♦ 10% Ammonium persulfate solution

0.1 g of APS is weight and put into 1.5ml Eppendorf tube

1ml of ddH_2O (MQ) is added onto the 0.1 gr APS just prior usage.

It is recommended that APS should not be kept in Eppendorf tubes longer than 15 days.

♦ 8% Denaturing polyacrylamide gel

33.3 gr Urea

8 ml 10X TBE

16 ml of 40% 19:1 Acrylamide-Bisacrylamide stock solution

400 µl APS

20 µl + 150 µl TEMED

33.3 g of urea is dissolved in 50ml ddH_2O(MQ) on a hot magnetic stirrer

16 ml of 40% 19:1 Acrylamide-Bisacrylamide stock solution and 8ml of 10X TBE is added,

400 µl of 10% fresh APS is added,

ddH_2O(MQ) is added upto 80ml

The solution is filtered through a 22 µm filter

The solution is divided into two volumes; 20 ml and 60 ml ,

On to 20 ml, 150 µl TEMED is added and used immediately for sealing the bottom part of the gel cassette.

2.5.4. Casting the PAGE apparatus

Apparatus:

Sequi-Gen Nucleic Acid Sequencing Cell (21X50) (Bio-Rad, Cat.no: 165-3601)

Reagents:

Sigmacote (Sigma Cat.no:Sl-2)

Bind Silane (Promega Cat no: 2530-84-0)

99% Ethanol

2.5.5. Procedure

The glass plates are laid down on a smooth surface. One side of the glass plates is swept with ethanol (99%) for three times. One has to be careful at this step because the glass plates have to be ultra clean for the proper assembly. 1 ml of silicone solution (sigmacote) is dropped on to the glass plate connected to the buffer chamber and dispersed on to the entire surface of the plate by using a paper towel. 1 ml of sticky solution (silane) is dropped on to the notched plate and thoroughly dispersed on to the entire surface of the gel. It must be noted that the paper towel used for the silicone should not be used for the sticky solution. After 7-8 minutes the plates are cleaned with ethanol three times. All the time new paper towels are used. The spacers which have been cleaned with ethanol before are placed on to the plate with buffer chamber and the nothced plate is placed on to the spacers facing its sticky side inwards. Before putting on the clamps the superposition of the glass plates are controlled and they should exactly fit onto each other to prevent leakage of unpolymerized acrylamide. The clamps are put on

and tightened. The bottom part of the plates are sealed with agarose. Three 1.75 X 15cm, 3mm Whatman papers are cut and placed on to the bottom part of the PAGE apparatus. 20 ml of the prepared polyacrylamide solution is poured on to the Whatman papers and 150 µl TEMED is added. The cassette is immediately (before polymerization of the polyacrylamide) placed onto the bottom tray and the clamps are fastened so that the glass plates are forced against the tray. The polyacryamide gel is left to polymerize for 7-8 minutes. On to the bottom tray agarose is poured by using a Pasteur pipette to prevent any possible leakage of the polyacrylamide solution after pouring the gel in-between the plates. After the agarose used for sealing is polymerized the remaining 60 ml polyacrylamide solution is put into a pisette with a curtailed tip and 20 µl of TEMED is added and mixed throughly. The Page cassette is hold in a nearly vertical position (70-80 ^{O}) and the polyacrylamide solution is poured in-between the plates. One has to be careful at that step, over pressurizing of the solution may cause bubbles which definitely will interfere with the migration of the DNA fragments. The casting apparatus is slanted step by step as the level of the gel-solution between the plates increases. Finally the gel is laid down in a nearly horizontal position (10-20^{O}) and the back side of the comb is placed so that the top of the gel is smooth. The gel is left to polymerize at least for two hours.

2.5.6. Prerun of the gel

1000 ml 1X TBE is prepared from the 10X stock solution. 450 ml of the 1X TBE is poured into the buffer chamber in the base of the apparatus. The gel cassette is placed on

to the base unit and fixed by fastening the screws on the base. The remaining 550 ml 1X TBE is poured into the buffer chamber in the gel cassette. The comb is removed very carefully without damaging the gel. 25 ml syringe is used to remove the urea from the top of the gel. The electrodes of the apparatus are connected and the temperature probe is placed in the middle of the notched glass plate. The temperature is set at 48 °C and the power at 45W. The prerun is carried out under these conditions for 2 hours. At the beginning the voltage of the set up is 1850 V and it decreases gradually as the temperature increases (1650V after 90 minutes when the temperature reaches 45 °C).

2.5.7. Sample preparation and loading

Denaturing Loading Buffer

Formamide.....1350µl

EDTA 0.5M.....3µl

Bromophenol -Blue..5µl

Xylene-Cyanol.....5µl

ddH2O.....+.137µl

.....1500µl

5 µl of the 25 µl PCR reactions is used for agarose gel electrophoresis. 7 µl denaturing loading buffer is added on to the remaining 20µl of the PCR product. 10µl from the sample-buffer mixture is taken and denatured at 95°C for 2 min. The comb is gently placed on to the top of the gel so that the teeth are dipped into the gel to eliminate well to well leakage. Each well under the comb is cleaned by using a 25 ml syringe and the

samples are loaded with a 10p pipette. Well to well leakage is monitored continuously and noted immediately if observed.

2.5.8. Running the Gel

After the samples have been loaded the power supply is set to 48°C and 45W. The gel is run for 2 hours. At the end of 2 hours the dye Bromophenol-Blue should have come out of the gel.

2.6. Silver Staining

There are a variety of techniques available for staining nucleic acids in TBE polyacrylamide gels, each with their own advantages and disadvantages, depending on the desired end-result.

Ethidium-bromide staining is by far the most common method of staining nucleic acids. It is a fast technique to visualize nucleic acids. However, ethidium-bromide is a toxic mutagen and should be handled carefully.

Silver staining is a highly sensitive method for staining single and double stranded DNA and one can expect 4 times higher sensitivity of the standard ethidium-bromide technique.

2.6.1. Reagents

Silver Nitrate (Sigma Cat No:S-81-57)

Glacial Acetic acid (Carlo Erba Cat. No. 64-19-7)

Formaldehyde 37% (Sigma Cat. N0:F-8775)

Sodium Carbonate (Sigma Cat No:S-2127)

2.6.2. Solutions

Solution I: 10 % Acetic Acid solution

Solution II: Silver nitrate solution(contains 2 mg Sodium thiosulfate and 1.5ml 37 % formaldehyde)

Solution III: 3% Sodium carbonate solution (contains 1.5ml Formaldehyde/liter)

2.6.3. Procedure

Since the gel to be stained is very thin (0.4 mm), it is sticked on the notched glass plate throughout the staining procedure. After the poly-acrylamide gel electrophoresis was completed the electrophoresis apparatus is disassembled. The cassette containing the glass plates and the gel is left for cooling in the cold-room (+4°C) for 20 minutes. Afterwards the cassette is disassembled and the glass-plates are taken carefully apart. The gel remained on the notched glass-plate which has been treated with the sticky solution before. The notched plate is placed in the cuvette containing 10% glacial acetic acid (solution I) and is shaken for 30 minutes. At the end of 30 minutes the 10% acetic acid solution (solution I) is collected for further use. The cuvette and the glass plate is washed for 5 minutes with excess deionized water (MQ) to remove all of the urea remained. Water is poured off.

1.5ml formaldehyde is added for each of the1 liter of freshly prepared 0.1% silver nitrate solution (solution II) and the solution is poured in to the cuvette. The cuvette containing the glass plate is shaken vigorously for 35 minutes to make sure that the gel is stained homogeneously.

At the end of 35 minutes silver nitrate solution (solution II) is poured off and the cuvette is rinsed with deionized water. The back side of the glass plate is swiped with a paper towel to remove the remaining of the silver nitrate solution.

The sodium carbonate solution (solution III) was prepared while the glass plate was being shaked in the solution I and kept at +4°C, but 1.5 ml formaldehyde and 2 mg of sodium thiosulfate is added just before pouring the solution into the cuvette. The third solution is shaken very vigorously to make sure that all the parts of the gel develop with the same quality.

The staining reaction is stopped as soon as the expected bands are seen by pouring off the third solution and immersing the glass plate in the first solution for 15 minutes. (It must be noted that over-development of the gel in the third solution will result in an excessive background). Afterwards the glass-plate is incubated in deionized water for one hour to remove all of the acetic acid. The glass plate with the gel is left to dry out over night. The bands become sharper after the gel has been dried out

3. RESULTS:

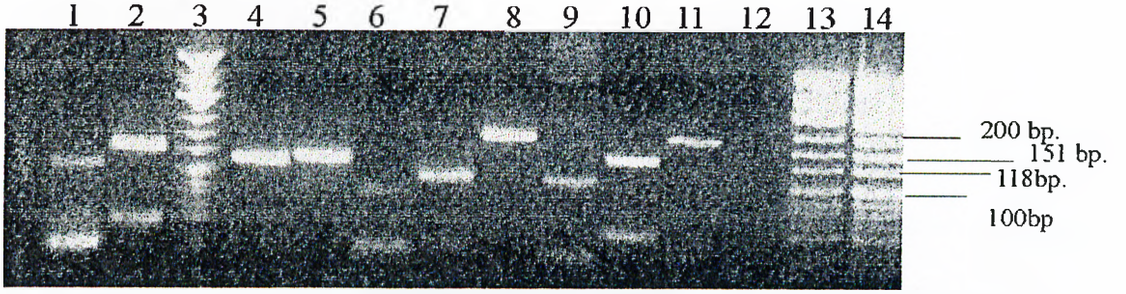


Figure 5. *Visualisation of the PCR amplification products of six microsatellite loci on 2% Agarose-gel*

1.Hilo1/15- DNA + GA97-98 (Locus: D17S855)
2.Hilo1/15-DNA + GA99-100 (Locus: D6S291))
3.Marker: ϕX174 + Hinf dIII
4.Hilo1/15-DNA + GA101-102 (Locus: FGA)
5.Hilo1/15-DNA + GA103-104 (Locus:D11S904)
6.Hilo1/15-DNA + GA105-106 (Locus: D13S175)
7.Hilo1/15-DNA + GA107-108 (Locus: D5S107)
8.Hilo1/15-DNA + GA23-24 (control primer)
9.Urine-3DNA + GA97-98 (Locus:D17S855)
10.Urine-3DNA + GA99-100 (Locus: D6S291)
11.Urine-3DNA + GA23-24
12.No DNA + GA23-24
13.Marker: ϕX174 + Hinf dIII
14.Marker: ϕX174 + Hinf dIII

3.1 Agarose gel analysis of the microsatellite loci: D17S855, D6S291, D11S904, D13S175, D5S107, and FGA

Amplification of the microsatellite loci; D17S855 (133-155bp), D6S291 (198-210 bp.), FGA (177 bp.), D11S904 (185-210bp.), D13S175 (101-113 bp.), D5S107 (143-155 bp.) have been performed by using PCR conditions described above. The bands corresponding to the loci are in the expected size region with respect to the marker (Figure 5).

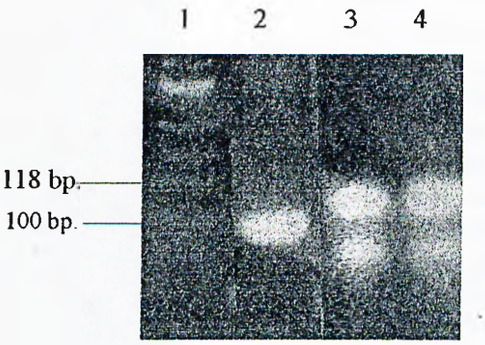


Figure 6. *Efficient amplification of the Bat25 locus and BAX intragenic repeat region*

*1.***Marker:** ϕX174 Hinf III., *2.BAX* intragenic repeat region (94 bp.), *3.* and 4.BAT 25 (105-112 bp.),

3.2 Agarose Gel Analysis of Bat25 and BAX intragenic repeat region

The bands corresponding to BAT25 and BAX intragenic repeat region are observed within the expected size range, (105-112) and 94 bp, repectively.

Figure 7. *Analysis of eight microsatellite loci on 8% PAGE gel*

1.DNA49 + GA272-273A (BAX intragenic repeat, **2.** DNA49 + GA105-106 (D13S175)), **3.**, DNA49 + GA-97-98 (D17S855), **4.** DNA49 + GA190-191(Bat25), **5.** DNA49+GA103-104 (D11S291), **6.** DNA49 + GA101-102 (FGA **7.** DNA49 + GA-99-100 (D6S291), **8.** DNA49 + GA107-108 (D5S108), **9**.DNA50 + GA272-273A (BAX intragenic repeat, **10.** DNA50 + GA105-106 (D13S175)), **11.**, DNA50 + GA-97-98 (D17S855), **12** DNA50 + GA190-191(Bat25), **13.** DNA50+GA103-104 (D11S291), **14** DNA50 + GA101-102 (FGA **15.** DNA50 + GA-99-100 (D6S291), **16** DNA50 + GA107-108 (D5S108),

(***DNA50:*** *Tumor DNA sample from a sporadic colorectal cancer patient,* ***DNA49:*** *normal DNA sample from the same sporadic colorectal cancer patient)*

3.3 Polyacrylamide gel analysis of the microsatellite loci, D17S855, D6S291, FGA, D11S904, D13S175, D5S107, Bat25, Bax intragenic repeat region

The microsatellite loci; D17S855 (133-155bp), D6S291 (198-210bp.), FGA (177bp), D11S904 (185-210bp.), D13S107 (101-113bp.), D5S107 (143-155bp.), BAT25 (105-112bp.), BAX intragenic repeat region (94bp.) are all found to be within the expected size range.

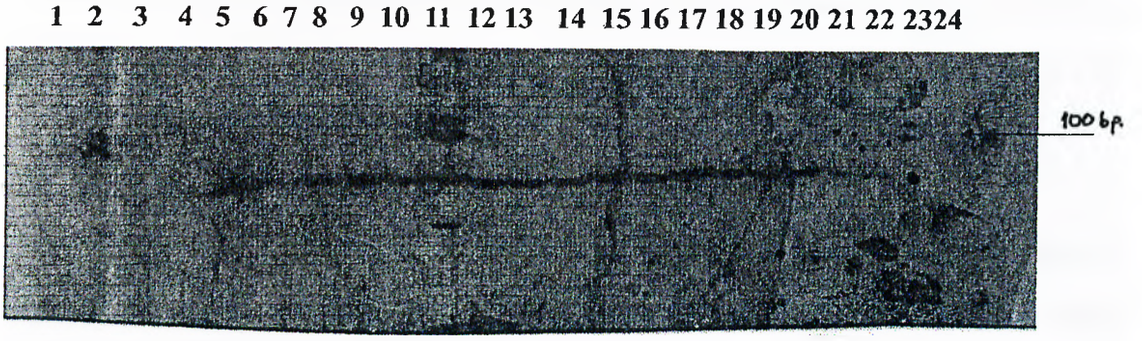


Figure 8. *Analysis of the intragenic repeat of the BAX gene in colorectal cancer patient samples on 8% PAGE gel.*

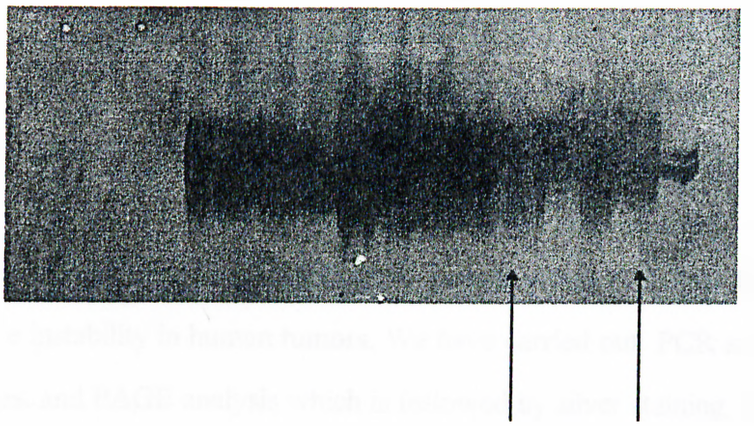
1.Marker;ϕX174+ hinfIII, **2.** Marker; ϕX174 +EcoR1+ HindIII, **3.** Sample#55F (normal), **4.** Sample#56F (tumor), **5.** Sample#71P (normal), **6.** Sample#72P (tumor), **7.** Sample#61P (normal), **8.**Samlpe#62P (tumor), **9.**Sample#71P (normal), **10.**Sample#72P(tumor), **11.** Marker; ϕX174, **12.**Sample#49F(normal), **13.**Sample#50F (tumor), **14.**Sample#51F(normal), **15.**Sample#52F(tumor), **16.**Sample#75F(normal), **17.**Sample#76F(tumor), **18.**Sample#77F(normal), **19.**Sample#78F(tumor), **20.** Sample#79F(normal), **21.** Sample#61P (normal), **22.** Samlpe#62P (tumor), **23.** Marker; ϕX174 +EcoR1+ HindIII, **24.** Marker;ϕX174+ hinfIII

3.4. Analysis of the BAX intragenic repeat region in colorectal cancer patients

All of the bands are observed in the expected size range (94bp.) with respect to the marker (Figure 8). No alteration of the BAX locus in the normal and tumor samples of the individuals is observed. BAX gene is one of the key elements of the apoptotic pathway, the locus we have studied is an intragenic repeat on this gene. Mutations of this locus, in terms of single base additions or deletions have been observed in 21 of the 41 MMP+ colorectal cancer cases (51%) (Rampino *et al*, 1997).

Here we analysed 5 normal and tumor pairs of fresh tumor samples and two pairs of paraffin embedded tumor samples. Fresh tissue samples are from patients with a distal localization of the tumor. These samples have a very low chance of being MMP+ , hence, an alteration in this locus is not expected. However the samples from paraffin embedded tissue have been previously shown to be MMR+ by Yagci, et al. in 1996. These two samples are definitely not enough to exclude the BAX intragenic repeat alteration on MMR+ cases. Since the aim of this study is to set up the technique rather than the screening of the patiens further samples are not studied.

1 2 3 4 5 6 7 8 9 10 11 12



Alterations

Figure9 *Analysis of the BAT25 locus on the denaturing 10% polyacrylamide gel. Samples are from normal and tumor tissue DNAs of colorectal cancer patients. These are in lanes:*

*1*Smpl80F(tumor), **2.**, Smpl79F(normal), **3.**, Smpl78F(tumor), **4.**Smpl77F(normal), **5.**,Smpl52F(tumor), **6.**Smpl51F(normal), **7.**Smpl50F(tumor), **8.**Smpl49F(normal), **9.** Smpl72P(tumor), **10.**Smpl71P(normal), **11.** Smpl62P(tumor), **12.** Smpl61P(normal)., Marker pBR3332 Digest. The arrows indicate shifted bands, which are taken as evidence of microsatellite instability

3.5 Analysis of the BAT25 locus in colorectal cancer patients

All of the bands are observed within the expected size range (105-112bp). Eventhough the individual bands can not be distinguished due to the high amount of DNA loaded onto the gel, alteration of the BAT25 locus on paraffin-embedded tissue is observed in lanes 9(tumor), 10 (normal), 11(tumor), 12(normal).

4. DISCUSSION:

In this thesis we aimed to implement a non-isotopic diagnostic test for the analysis of microsatellite instability in human tumors. We have carried out PCR amplification of the loci of interest and PAGE analysis which is followed by silver staining. The main point of this study was to develop a non-radioactive visualisation method of DNA fragments on polyacrylamide gels. The best candidate among different visulisation methods was silver staining. Silver staining is a non-expensive, easy, and a reproducible method. The results of silver staining can be obtained in 3 hours as opposed to autoradiography which requires an over night incubation for the exposure of the film to radioactivity. In addition the time necessary to develop the film should also be calculated.

The non-isotopic detection system we have developed can be used for other purposes also. This system is especially suitable for LOH studies, in which there are very minute differences between two alleles in terms of fragment length. With the high resolution we obtained it is easy to differentiate between these two alleles.

12 pairs (normal and tumor) of an allele can be studied in 9 hours by employing this system with a 21 X 50 Bio-Rad sequencing apparatus. Therefore this technique is suitable for routine diagnostic applications.

There were two important problems that we faced in the laboratory. These are: i) the proper amount of the PCR product to be loaded onto the PAGE gel, and ii) developing the gel after silver staining.

i)*The amount of the PCR products to be loaded to the PAGE gel*: This is mainly determined based on the appearance of the fragments under UV light in agarose gels. However this criteria alone is sometimes not sufficient. Therefore it is recommended that in the case of sufficient number of lanes on the PAGE gel, one has to load different volumes of PCR product to optimize the resolution.

ii) *Developing the gel after silver staining*: The most important stage of staining is the development of the gel since this stage will determine the final quality of the gel. There were two cases in which we obtained an excessive background. The gel was either kept too long in the developing solution or all of the developing solution is used in one go to stain the gel. One should avoid carrying out these mistakes. The development reaction should be stopped as soon as the bands are seen on a light background. And the developing solution should be used in two portions. The first portion of the solution should be poured off as soon as it darkens and the second portion of the solution should be used until the bands are seen.

When coupled with good quality of chemicals these two precautions will ensure strong bands on a very faint background.

The world of molecular biology is a world of gels. Today manual techniques based on gels are widely used. However, recent advances show that in the very near future automated systems will replace manual techniques. Even today automated systems are widely used for sequencing and fragment length analysis. A group of automated systems are based on capillary electrophoresis.

An important technique for studying microsatellite instability is automated capillary electrophoresis. In capillary electrophoresis the DNA samples are run trough a polymer filled capillary and the fragments are detected as they pass trough a window on the

capillary. Different methods are used for the detection of the fragments as they pass by the window on the capillary. DNA fragments can be visualized by either by fluorescence or by simple UV absorption. These systems are promising for the near future.

For setting up an automated system one requires a well developed, reliable method which could be used as a control. As a simple, reproducible method, PAGE analysis followed by silver staining is a good candidate for a control method.

There are two capillary electrophoresis apparatus in our department with different detection systems. One requires fluorescent labels and the other detects naked DNA just by reading UV absorption. Based on the experience I gained working on my master project, I want to develop an automated system which is not demanding radioactivity. Manual non-radioactive method will be a good control of the newly developing system.

Since the detection of frameshift mutations in the coding regions of BAX gene (John C. Reed & Manual Perucho et al., 1997) and the TGF-Beta gene (Markowitz et al., 1995), a new era in cancer genetics is opened. Most probably these two genes are not the only genes on the way of carcinogenesis which are altered due to mismatch repair deficiency. A fast, reliable, non-radioactive detection system for fragment length alterations is a good technique to study the coding sequence alterations of candidate genes that contribute to cancer. Both manual and automated systems are suitable for this purpose.

This system is applicable to both for routine diagnosis and for intensive research.

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