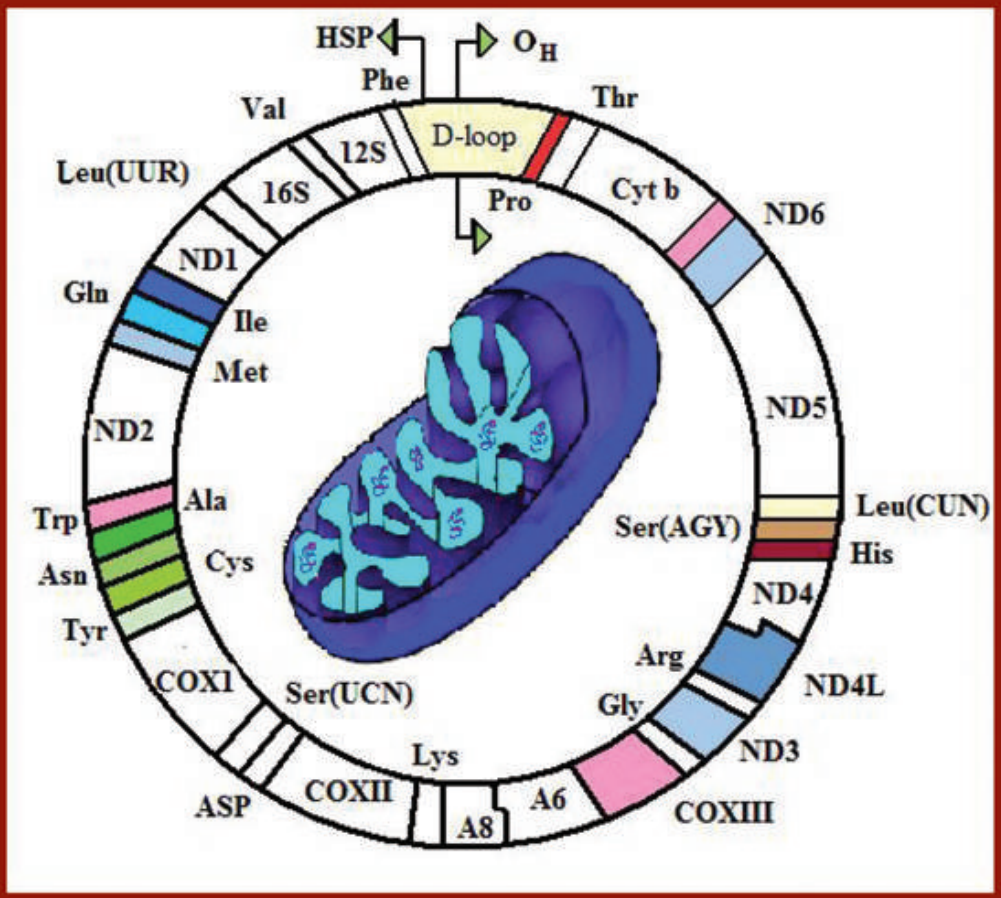
Mansoureh Akouchekian

Investigation of human mitochondrial DNA abnormality in colon cancer



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Hierdurch erkläre ich, dass die Dissertation

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selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.
Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Mansoureh Akouchekian

to my beloved family

Asghar, Mohammad, Amin

and my parents

List of Publications

1. **Mansoureh Akouchekian**, Massoud Houshmand, Simin Hemati, Mehdi Shafa. Appearance of large scale mitochondrial DNA deletion in human colorectal cancer, Accepted for publication in the Archive of Medical Science Journal.

2. **Mansoureh Akouchekian**, Massoud Houshmand, Simin Hemati, Mohammad Ansaripour, Mehdi Shafa. High Rate of Mutation in Mitochondrial DNA Displacement Loop Region in Human Colorectal Cancer, Accepted for publication in the Diseases of Colon & Rectum Journal.

3. **Mansoureh Akouchekian**, Massoud Houshmand. Mitochondrial haplotype analysis in colorectal cancer: identification of high-risk population, Accepted for publication in the Archive of Medical Science Journal.

4. **Mansoureh Akouchekian**, Massoud Houshmand, Behnam Kamalidehghan, Hassan Akbari, Masoumeh Dehghan. Investigation of Mitochondrial ND1 gene mutation in Human Colorectal Cancer, Submitted for publication in the Scandinavian Journal of Gastroenterology.

5. Emilia Daniłowicz, **Mansoureh Akouchekian**, Cord Drögemüller, Bianca Haase, Tosso Leeb, Heidi Kuiper, Ottmar Distl, and FUGATO-consortium IRAS (2008). Molecular characterization and SNP development for the porcine *IL6* and *IL10* genes, Animal Biotechnology 19: 159–165.

6. Houshmand M, Sanati M, **Akouchekian M**, *et al*., (2005). Population screening for association of mitochondrial haplogroups BM, J, K and M with multiple sclerosis: interrelation between haplogroup J and MS in Persian patients, Multiple Sclerosis 11: 728-730.

7. Houshmand M, Sanati M, **Akouchekian M**, *et al*., (2004). Investigation of the mitochondrial haplogroups M, BM, N, J, K and their frequencies in five regions in Iran, Iranian Journal of Biotechnology 2: 1-12.

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Abbreviations:

ATP	adenosine triphosphate
bp	base pair
CPEO	chronic progressive external ophthalmaplegia
CRC	colorectal cancer
Cyt *b*	cytochrome *b*
D-loop	displacement loop
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
ETC	electron transport chain
H-strand	heavy strand
IM	inner membrane
IMS	inter-membrane space
kb	kilobase
KSS	Kearns-Sayre syndrome
LHON	Leber's hereditary optic neuropathy
L-strand	light strand
MELAS	mitochondrial encephalopathy, Lactic acidosis and stroke-like episodes
MERRF	myclonic epilepsy with red ragged fibers
MILS	maternally inherited Leigh's syndrome
MNGIE	mitochondrial neurogastrointestinal encephalomyopathy
mtDNA	mitochondrial DNA
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NARP	neuropathy, ataxia and retinitis pigmentosa
nDNA	nuclear DNA
np	nucleotide pair
nt	nucleotide
OH	H-strand replication origin
OL	L-strand replication origin
OM	outer membrane
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
SSCP	single-strand conformation polymorphism
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TTGE	Temporal Temperature Gradient Gel Electrophoresis

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1 Introduction

Each human cell contains a nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Mitochondria are small cytoplasmic organelles that contain their own DNA, which is a circular genome of 16,569 bp. Mitochondria are unique, because they are responsible for the major energy producing system of the cell, the oxidative phosphorylation enzyme pathway (OXPHOS). This process is accomplished by a series of protein complexes, collectively known as the respiratory chain, encoded by both nDNA and mtDNA. The complete respiratory chain contains at least 87 polypeptides, 13 of which are encoded by mtDNA, which is known for having a high acquired mutation rate at least 10 times higher than that reported for nDNA. It is generally accepted that the high mutation rate of mtDNA is caused by the lack of protective histones, inefficient DNA repair systems and continuous exposure to mutagenic effects of oxygen radicals generated by oxidative phosphorylation (Miyazono, *et al*., 2002). An association between mtDNA mutations and neurologic or metabolic disorders has previously been reported (Wallace, 1992, Armstrong, *et al.,* 2000). However most of the mutations are neutral polymorphisms which have accumulated sequentially in maternal lineages, creating groups of related mtDNA haplotypes.

Tumor development is often associated with mtDNA mutations and alterations in mitochondrial genomic function. Mutations in the mtDNA have been reported to occur in human cancers (Burgart, *et al.*, 1995, Fliss, *et al.,* 2000, Habano, *et al.*, 2000, Brandon, *et al*., 2006).

The mitochondrial electron transport chain has been recognized as one of the major cellular generators of reactive oxygen species (ROS), which include hydrogen peroxide (H_2O_2), the hydroxyl free radical ($\cdot OH$) and superoxide ($O_2\cdot^-$) (Liu, 2002). ROS have deleterious side effects, as they cause damage to nucleic acids, proteins and lipids. Since mitochondrial DNA and membranes are directly exposed to the ROS produced during cellular respiration they are especially vulnerable. ROS are involved in various pathological processes such as carcinogenesis and neurodegeneration.

The aims of this work were to detect new mtDNA mutations, deletions or insertions in patients with CRC to determine the prevalence of known mtDNA mutations, and to estimate the role of mtDNA polymorphisms and haplogroups as a risk factor for CRC.

2 Review of the literature

2.1 Mitochondria

Mitochondria are organelles that are situated in the cytoplasm of eukaryotic cells. They are involved in various physiological processes such as intermediary metabolism and cellular signaling events. The name "mitochondrion" was coined by C. Bender in 1898, and is derived from the Greek *mitos* meaning thread and *chondrion* meaning granule. These small cytoplasmic organelles have been known as "the powerhouses of the cell" for long time. That means the most well-known and best-characterized function of mitochondria is the production of adenosine triphosphate (ATP) through oxidative phosphorylation. This process is accomplished by a series of protein complexes, collectively known as the respiratory chain, encoded by both nDNA and mtDNA. ATP is the unique energy source for performing a wide range of cellular functions. During the last few decades researchers have realized the involvement of mitochondria in other cellular functions as well apoptosis (Desagher & Martinou 2000), cell division (von Wangenheim & Peterson 1998), and possibly aging (Melov 2000, Rustin *et al*., 2000). Our understanding of the role of mitochondria in human diseases is continuously growing.

A typical human cell has several hundred mitochondria, which convert energy to forms that can be used to drive cellular reactions. Mitochondria are thought to derive from α-proteobacteria (Gray *et al*., 1999). It is believed that throughout their evolution mitochondria transferred most of their genes to the nucleus, and, in turn, received various “eukaryotic” genes of the host cell. It is accepted now that mitochondria contain approximately 700 different proteins and provide the cell with important functions such as energy production and apoptosis (Gaucher *et al.,* 2004).

2.1.1 Structure of mitochondria

Mitochondria are 0.5-1 μm in size and are bounded by two membranes, the outer (OM) and inner (IM) membranes, separated by a space called the intermembrane space (IMS). The OM completely encloses the mitochondria, while the IM surrounds a space called the matrix. The outer membrane is permeable to molecules of 10,000 daltons or less in molecular weight. It contains non-selective membrane channels and is composed of equal amounts of protein and lipid.

The IM composition is about 20% lipid and 80% protein, and it has the highest protein fraction among cell membranes. The IM is highly impermeable, and therefore virtually all molecules and ions require special transporters. The mitochondrial inner membrane is organized into convoluted invaginations that project into the matrix and are called cristae (Lodish, 2000). The IM contains the enzyme complexes of oxidative phosphorylation for the synthesis of ATP.

The internal structure of mitochondria is traditionally depicted as a "baffle model" in which the cristae of the IM form folds similar to the bellows of an accordion (Sjöstrand 1956). The mitochondrial matrix contains hundreds of enzymes, including those required for the oxidation of pyruvate and fatty acids and those, which are active in the tricarboxylic acid (TCA) cycle. The matrix also contains several identical copies of the mtDNA, mitochondrial ribosomes, tRNAs and various enzymes required for the transcription and translation of mitochondrial genes. There are contact sites between the outer and inner mitochondrial membranes; these sites are formed during the transport of nuclear-encoded precursor proteins into mitochondria.

2.2 The mitochondrial genome

The human mitochondrial DNA was the first human "chromosome" that has been completely sequenced in 1981. It was reported to be a circular double stranded molecule 16,569 base pairs in length (Anderson *et al*., 1981). mtDNA has two strands, a guanine rich heavy (H strand) and a cytosine rich light (L strand), and encodes 13 proteins, 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA), 12SrRNA and 16SrRNA (Fig 2.1). Due to the presence of many copies per cell, it corresponds to about 1% of the total DNA content in an average cell. The mitochondrial genome of mammals is organized extremely economically, showing a gene organization that is very compact without any introns (Fernandez-silva *et al*., 2003).

The only non-coding segment of mtDNA in all vertebrates is the displacement loop (D-loop) or control region, a region of 1124 bp (position 16024 – 576) on the mtDNA. The control region contains the origin of replication of the H-strand (OH) and the promoters for L and H-strand transcription. Both strands of the mammalian circular mtDNA genome are transcribed from a single major promoter. The D-loop is the region with the most variable sequence among different species, even though it harbors some well conserved elements (Sbisa *et al*., 1997).

Almost the entire H strand is used as a template when producing RNA information for the L strand sequence, and similarly an extensive part of the L strand is used to produce RNA. These polycistronic transcripts are cleaved at precise sites to produce tRNAs, rRNAs and mRNAs. Genes coding for the protein

ND6 and tRNAs for glycine (Gly), alanine (Ala), asparagine (Asn), cysteine (Cys), tyrosine (Tyr), serine (Ser), glutamine (Glu) and proline (Pro) are transcribed from the L chain. The genes encoding the ATPase 6 and 8 proteins are partly overlapping, and in addition, several tRNA genes overlap by 1-3 nucleotides. The frequency of RNA transcription is regulated by nuclear encoded proteins (Ojala *et al*., 1981, Christianson & Clayton 1986, Jeong-Yu & Clayton 1996).

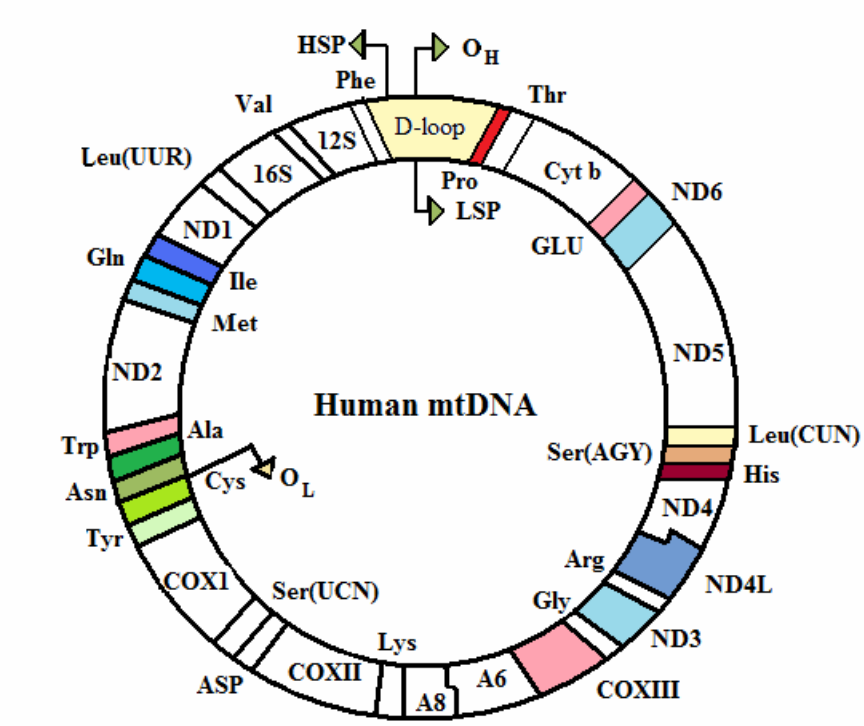

Figure 2.1: Human mitochondrial genome

The mitochondrial genome has several unique features relative to the nuclear genome. The organization of mtDNA is different from that of nDNA as listed in the following: The genetic code of mtDNA is different, replication of mtDNA is independent of the cell cycle, mtDNA is maternally inherited, and the mtDNA pool of a cell is randomly segregated to daughter cells. Also, a certain level of mutant DNA is required before it affects the function of a cell or tissue. This is called threshold effect. The mutation rate of mtDNA is higher than that of nDNA, and each cell harbors a high number of copies of mtDNA molecules, in contrast to

only two to four copies of nDNA. In the following some mitochondrial characteristics are mentioned in details.

2.2.1 Special features of mtDNA

The mitochondrial genome has some distinctive characteristics that are different when compared to the nuclear genome. The first unique feature is the use of a differing genetic code, for this reason the cytosolic translation machinery is not able to translate mitochondrial mRNAs or vice versa (Table 2.I). Second, nuclear genes follow a Mendelian pattern of inheritance, whereas mitochondrial genes are maternally inherited. Third, the nuclear genome is either haploid or diploid while the mitochondrial genome is polyploid since there are thousands of mtDNA molecules per cell. When only one form of mtDNA exists in a cell, the state is called homoplasmy; when two or more co-exist, the state is called heteroplasmy. Another difference of the mtDNA molecule is the higher mutation rate. mtDNA is positioned in the vicinity of the OXPHOS system, which is considered to be the major producer of reactive oxygen species (ROS). This location might explain the increased mutation rate (Wallace, 2005).

Cells contain a high copy number of mitochondrial genomes, the number varying from one cell type to another, between 1,000 and 10,000 copies per cell (Larsson & Clayton 1995, Lightowlers *et al*., 1997). Normally, short-lived human cells such as sperm or leukocytes have a low mtDNA copy number, whereas long-lived cells (e.g. skeletal muscle, brain cells, oocytes) tend to have a high copy number of mtDNA. One hypothesis has suggested that high copy number may protect mtDNA from the accumulation of a critical threshold of mutations (Chinnery & Samuels 1999). One more different between mtDNA and nDNA is the high mutation rate in mtDNA. The rate of mutation in mtDNA is 10 to 17 times faster than that in nuclear genes (Neckelmann *et al.,* 1987, Wallace *et al.,* 1997).

2.2.2 Mitochondrial Haplogroups

Mitochondrial haplogroups are determined by polymorphisms that occurred tens of thousands of years ago and form high-prevalence population-specific substitutions today. These haplogroups are defined by ancient mutations (Wallace, 2003). The changes appeared and survived, therefore, they could not be deleterious mutations. Most of them probably had not any phenotypic effect and were neutral. Some of them had a beneficial effect and were positively selected. However, this positive effect was related to a particular environment and nowadays, in other environmental conditions, may have different effects on the

phenotype (Mishmar, 2003, Wallace, 2003, Ruiz-Pesini, 2004). In the other words human haplogroups are defined by special polymorphisms in human mitochondrial DNA. These haplogroups trace the matrilineal inheritance of modern humans back to the human origins in Africa and reflect the subsequent human migrations across the earth.

Haplotypes are subclusters of haplogroups, and the polymorphisms that determine them are less prevalent and have occurred more recently. Most of the polymorphisms determining haplogroups are continent-specific (Wallace, 1994).
Haplogroups could have important implications for understanding of the relationship between mutability of the mitochondrial genome and disease (Ozawa, 1991, Shoffner, 1993). There is growing evidence that certain mtDNA Haplogroups are associated with distinct disorders (Brown, 1995, Torroni, 1997).

2.3 Mitochondrial DNA replication

The replication and transcription of Mitochondrial DNA take place within the mitochondria. It occurs independently from the cell cycle and from nuclear DNA replication.
Factors involved in mtDNA metabolism are nuclear-encoded, including mtRNA polymerase, mtDNA polymerase γ (Pol γ) and most of the potential factors that regulate mtDNA replication, mtDNA transcription and mtRNA processing (Moraes, 2001).
The generally accepted model for mtDNA replication consists of an asymmetric synthesis. This means that the two mtDNA strands replicate in an asynchronous manner from two independent origins. The synthesis starts at one of the multiple origins of replication of the heavy strand (O_H) at the D-loop region using a short RNA primer. mtDNA synthesis continues until reaching the origin of replication of the light strand (O_L), which is situated approximately two thirds away around the mtDNA molecule. At this time, the synthesis of the light strand starts (Garesse & Vallejo, 2001). Because Pol γ requires short mtRNA primers; replication depends on mitochondrial transcription. Studies with a recombinant human DNA polymerase have estimated that one round of mtDNA replication takes about 1 h (Graves *et al*., 1998).

It was suggested that cells use different synthesis mechanisms under specific physiological conditions (Holt *et al*., 2000). However, these results remained controversial among scientists (Bowmaker *et al*., 2003, Bogenhagen & Clayton, 2003, Holt & Jacobs, 2003, Brown *et al*., 2005).

2.4 Mutations in mtDNA cause several diseases in humans

In the late 1980s the first mtDNA defects associated with diseases were reported (Holt *et al*., 1988, Wallace *et al*., 1988a, Wallace *et al*., 1988b). Since then more than 400 mtDNA mutations and abnormalities (deletions and insertions) have been found (Mitomap, 2008). The severity of disease caused by a mutation in mtDNA depends on the nature of the mutation and on the proportion of mutant and wild-type mtDNAs present in a particular cell type. All cells in an individual have mitochondria, but mutations in mtDNA affect only some tissues. Those most usually affected are tissues that have a high requirement for ATP produced by OXPHOS and tissues that require most of the mtDNA in the cell to synthesize sufficient amounts of functional mitochondrial proteins.

Investigations into pathogenic mtDNA mutations have revealed a complex relationship between the genotype and the phenotype (Schon *et al*., 1997).
Generally, when mutations in mtDNA are found, cells contain mixtures of wild-type and mutant mtDNAs, a condition known as heteroplasmy. Each time a mammalian somatic or germ-line cell divides, the mutant and wild-type mtDNAs will segregate randomly into the daughter cells. Thus, the mtDNA genotype, which fluctuates from one generation and from one cell division to the next, can drift towards predominantly wild-type or predominantly mutant mtDNAs. Since all enzymes for the replication and growth of mitochondria, such as DNA and RNA polymerases, are imported from the cytosol, a mutant mtDNA should not be at a "replication disadvantage"; mutants that involve large deletions of mtDNA might even be at a selective advantage in replication because they can replicate faster.
The phenotypes of diseases vary between mutations, and also between individuals with the same mutation, but it is more difficult to explain a difference in phenotype seen in patients with an identical genetic defect. Patients with mtDNA disease often have heteroplasmic mtDNAs, and the mode of presentation can vary both between and within individuals. The degree of mutation heteroplasmy, tissue-specific differences in the threshold and variable dependence of different organs on oxidative metabolism will partly explain the varied clinical phenotypes seen in patients with mtDNA disease (Chinnery & Turnbull, 2000).

2.4.1 Explanation of threshold effect in mitochondria

In normal tissue, all mtDNA molecules are the same (homoplasmy), however mutations can arise, be amplified to different levels and coexist with wild-type mtDNA (heteroplasmy). As a consequence of this it is common to find a 'threshold effect' in mtDNA related human diseases in which a minimum number

of mutant mtDNA molecules are required to cause mitochondrial dysfunction in a particular tissue (Chinnery & Schon, 2003).

Most pathogenic mtDNA mutations (but not all) are heteroplasmic. It was reported that the individual's phenotype remains normal until a significant proportion of mutant mtDNA is present within the tissue and the threshold for genotype expression is overstepped (Wallace *et al*., 1997a). The threshold is different for various mtDNA mutations and is about 60% for deleted mtDNA (Hayashi *et al*., 1991). It is clear that different tissues have different dependencies on OXPHOS for their normal function. Organs with the highest ATP requirements and the lowest regenerative capacities, such as the brain, heart and skeletal muscle, are the most sensitive to the effects of pathogenic mtDNA mutations (Wallace, 1994, 1995).

2.5. mtDNA abnormalities

The first deletion was found in the skeletal muscle of patients with mitochondrial myopathy (Holt *et al*., 1988), and since then deletions have also been detected in patients with Kearns-Sayre syndrome (KSS), chronic progressive ophthalmoplegia (CPEO) (Lestienne & Ponsot 1988, Moraes *et al*., 1989), Pearson marrow/pancreas syndrome (Cormier *et al*., 1990) and maternally inherited diabetes mellitus and deafness (Ballinger *et al*., 1992b). About a third of all CPEO and KSS patients carry an identical deletion of 4977 bp spanning the interval 8469 - 13,466 (Schon *et al*., 1989). The pathophysiology of KSS and CPEO resulting from deletions appears to be due to a defect in protein synthesis, as all the deletions associated with these diseases remove at least one essential tRNA or protein coding gene (Wallace *et al*., 1997).
Multiple deletions have been found in a number of disorders such as autosomal dominant PEO (adPEO) (Zeviani *et al*., 1989, Moslemi *et al*., 1999), mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (Papadimitriou *et al*., 1998) and the sporadic and inherited forms of inclusion body myositis (Moslemi *et al*., 1997, Jansson *et al*., 2000). The deletions are usually located between the replication origins, with one end lying within the end of the D-loop (Kajander *et al*., 2000). Nuclear mutations have been found to be a cause of multiple deletions.
Large deletions have also been found at a low level in healthy tissues (Ozawa, 1997) and the proportion of mutant DNA appears to increase with age, at least in humans and rodents (Cortopassi & Arnheim, 1990, Yen *et al*., 1991, Simonetti *et al*., 1992).

2.5.1 Point mutations

More than 300 mtDNA point mutations have been identified; most of them are transitions (Mitomap, 2008).
Point mutations may affect any gene of mtDNA. They are usually maternally inherited and affect the nervous system and skeletal muscle.
The most important of pathogenic point mutations in mtDNA encoded OXPHOS subunits are usually linked with five dieases. Leber's hereditary optic neuropathy (LHON), maternally inherited leigh's syndrome (MILS), mitochondrial encephalopathy, Lactic acidosis, and stroke-like episodes (MELAS), myclonic epilepsy with red ragged fibers (MERRF) and neuropathy, ataxia and retinitis pigmentosa (NARP) (Goto *et al*., 1990, Holt *et al*., 1990).

2.6 Mitochondrial function

2.6.1 ATP production by mitochondria

The main mission of mitochondria is to provide energy for cellular processes by forming ATP in the process called oxidative phosphorylation (OXPHOS). The OXPHOS pathway is composed of the electron transport chain (ETC) and ATPase (Fig 2.2). The whole OXPHOS system embedded in the lipid bilayer of the mitochondrial inner membrane is composed of five multiprotein enzyme complexes (I-V) and two electron carriers, coenzyme Q and cytochrome *c*. The enzymes involved in OXPHOS are assembled from multiple polypeptides, which are encoded by the mtDNA or by the nDNA. The compositions as well as the function of these complexes are described as follows.
Complex I (NADH dehydrogenase) is composed of more than 40 protein subunits, of which seven, MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5 and MT-ND6, are encoded by mtDNA. It also includes a flavin mononucleotide (FMN) and six iron-sulphur (Fe-S) centers. It pumps protons across the IM coupled to the oxidation of NADH to NAD^+ and the reduction of ubiquinone to ubiquinol.
Complex II (succinate dehydrogenase) is composed of four subunits, all encoded by nDNA. It contains flavin-adenine dinucleotide (FAD), three Fe-S centers and cytochrome *b*. It is not involved in proton pumping but transfers electrons from succinate to ubiquinone, reducing the latter to ubiquinol.
Complex III (cytochrome bc_1 complex) is composed of eleven subunits, of which one, cytochrome *b* is encoded by mtDNA (MT-CYTB). It also contains a Rieske protein (a Fe-S protein) and cytochrome c_1, which are involved in electron transfer. The rest of the protein subunits are responsible for binding ubiquinone.

Complex III pumps protons across the IM and oxidates ubiquinole back to ubiquinone in a reaction that reduces cytochrome *c*, which transfers electrons from complex III to complex IV.

Table 2.I: The human mitochondrial genetic codes, differences from the nuclear genetic code are highlighted in color (Mitomap database: www.mitomap.org). The mammalian mtDNAs share a unique genetic code where UGA encodes tryptophan instead of termination, AGA and AGG are terminators and not codings for arginine, and AUA encodes methionine instead of isoleucine, AUU codes for isoleucine during elongation but can also code for methinonine for initiation (Peabody 1989).

Leu (1) L (UUA/G)	UUA UUG		**Ala** A	GCU GCC GCA GCG		**Glu** E	GAA GAG
Leu (2) L (CUN)	CUU CUC CUA CUG		**Tyr** Y	UAU UAC		**Cys** C	UGU UGC
Ile I	AUU AUC		**Ter**	UAA UAG		**Trp** W	UGA UGG
Met M	AUA AUG		**His** H	CAU CAC		**Arg** R	CGU CGC CGA CGG
Val V	GUU GUC GUA GUG		**Gln** Q	CAA CAG		**Ser (2)** S (AGU/C)	AGU AGC
Ser (1) S (UCN)	UCU UCC UCA UCG		**Asn** N	AAU AAC		**Ter**	AGA AGG
Pro P	CCU CCC CCA CCG		**Lys** K	AAA AAG		**Gly** G	GGU GGC GGA GGG
Phe F	UUU UUC		**Thr** T	ACU ACC ACA ACG		**Asp** D	GAU GAC

Complex IV of the respiratory chain (cytochrome *c* oxidase) is composed of thirteen subunits, of which three, MT-COX1, MT-COX2, MT-COX3, are encoded by mtDNA, and it also contains cytochrome $a+a_3$, CuA and CuB, which are involved in electron transfer to molecular oxygen, reducing it to water. Complex IV also pumps protons across the IM.
Complex V (ATP synthase) uses the proton gradient formed by complexes I, III and IV to produce ATP, the most usable form of energy in living cells. It has fourteen subunits, of which two, MT-ATP6 and MT-ATP8, are encoded by mtDNA. (Wallace D.C. & Lott M.T., 2002)
The subunits necessary for mitochondrial complexes are summarized in table 2.II.

The mitochondrial mRNAs are translated within the mitochondrion on chloramphenicol-sensitive ribosomes using mtDNA-encoded rRNAs and tRNAs.

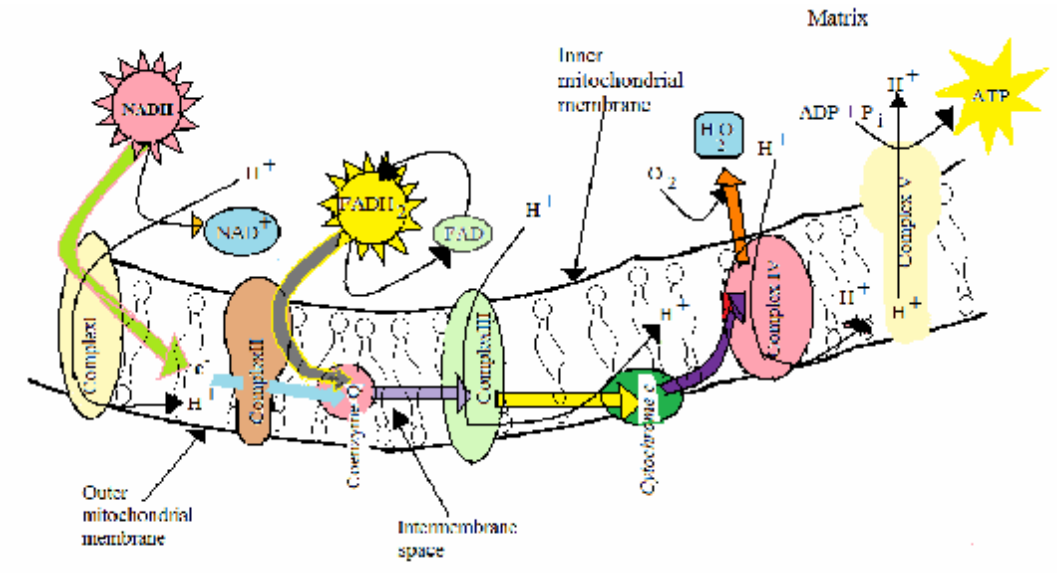

Figure 2.2: Illustration of mitochondrial complexes and place of action and functional organization of oxidative phosphorylation system.

Coenzyme Q, also called ubiquinone, shuttles electrons from complexes I and II to complex III. Complexes I, III, and IV create an electrochemical gradient, used by complex V for ATP generation.

In the mitochondrion the metabolism of one molecule of glucose produces about 30 molecules of ATP, while only two molecules of ATP are produced by glycolysis alone.

Table 2.II: Number of mtDNA and nDNA encoded gene products of electron respiratory chain

Mitochondrial complex	Number of proteins encoded by mtDNA genes	Number of protein encoded by nDNA genes	mtDNA gene products
I	7	36	MT-ND1, ND2, ND3, ND4, ND4L, ND5, ND6
II	0	4	0
III	1	10	Cytochrome *b*
IV	3	10	Cytochrome *c* oxidase I, II and III
V	2	14	ATPase 6 and 8
Total	13	74	

2.6.2 Production of reactive oxygen species

Reactive oxygen species (ROS) are highly reactive derivatives of the oxygen molecule which contain an unpaired electron in the outermost shell of electrons. This is an extremely unstable configuration, and ROS quickly react with other molecules or radicals to achieve a stable configuration. ROS include the superoxide anion ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$). Mitochondria are the major site of ROS production in cells, and the respiratory chain produces ROS at complex I and complex III. ROS can damage other molecules and cell structures, and mitochondrial DNA and mitochondrial membranes are especially vulnerable since they are directly exposed to ROS produced during cellular respiration.

ROS are involved in various pathological processes such as carcinogen and neurodegeneration and in the ageing process (Wei 1998), but the ROS produced by mitochondria have an important physiological role as well, as they affect gene expression and protein phosphorylation by functioning as signalling molecules under subtoxic conditions (Brookes *et al.*, 2002). ROS are also important in cell death transduction pathways, and therefore in apoptosis (Skulachev 1996, Fleury *et al.*, 2002), and they may also induce "mitoptosis", death of individual mitochondria in a cell, and therefore be involved in mitochondrial selection by removing mitochondria that overproduce ROS (Skulachev 1996).

2.7 Cancer

The progression from normal to malignant cells involves multiple steps, during which several distinguishable properties are acquired. Pending normal development and throughout adult life, intricate genetic control systems regulate the balance between cell birth and death in response to growth signals, growth-inhibiting signals, and death signals. Most importantly, cancer cells lose the ability to control growth, and can proliferate almost inevitably. They are able to promote angiogenesis and thus guarantee an unlimited supply of nutrients and oxygen. The telomerase enzyme is reactivated and maintains stable telomeres during repeated cycles of cell proliferation.
The cancer forming process, called oncogenesis or tumorigenesis, is the interplay between genetics and the environment. Most cancers arise after genes are altered by carcinogens or by errors in the copying and repair of genes. Even if the genetic damage occurs only in one somatic cell, division of this cell will transmit the damage to the daughter cells, giving rise to a clone of altered cells.
Cancer cells no longer respond to apoptotic signals (Rudin & Thompson, 1998). In the final stages of progression, cancer cells are able to detach from their original site, travel through blood or lymphatic veins to new destinations, and form new colonies, a process called metastasis.
On the tissue level, cancers can be categorized on the basis of their origin. For example, sarcomas are derived from soft tissue and bone, whereas leukemias originate from blood cells. Carcinoma, derived from epithelial tissue, is the most common type of cancer. It typically starts within a benign, well-differentiated tumor (Muto, 1975), which has a structure resembling that of normal tissue. The next stage is carcinoma *in situ* (local tumor). After reaching the invasive stage, cancer is able to penetrate into the basal membrane, and infiltrate the underlying tissue. Finally, the ability to metastasize is acquired. The clinical stages of carcinoma are illustrated in Figure 2.3.

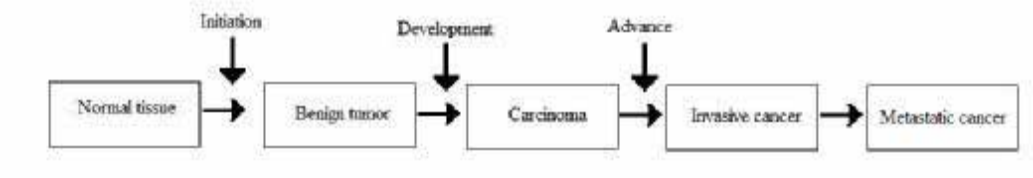

Figure 2.3: Clinical stages of carcinomas

2.7.1 Colorectal cancer

Colorectal cancer is the second most common malignancy as a cause of death in the Western countries (Dunlop, 1992). It is the fourth most common cancer in Iran

(breast, prostate, lung, colorectal). Colon and rectum cancers accounted for 36,804 new cases in 2004 in Iran. Approximately 1 in 1834 or 0.05% of the Iranian population developed CRC in this year.
Most colorectal cancers arise within an adenoma, a pre-neoplastic lesion. The progression from a benign adenoma to a malignant carcinoma passes through a series of well-defined histological stages, and may take 10 to 15 years (Muto, 1975, Jass, 1989). This progress is referred to as the adenoma-carcinoma sequence (Vogelstein *et al*., 1988). Colorectal adenomas are fairly common in the general population, but only 5-10% progress to a malignant tumor (Winawer *et al*., 1990). It was demonstrated that colorectal tumor initiation and progression requires at least seven different somatic changes before a cell can develop into a carcinoma (Kinzler & Vogelstein 1996).
Several environmental factors, such as diets containing high fat, excess of calories, low fiber, alcohol, or inadequate intake of vitamins and minerals, are implicated as risk factors for the development of colorectal cancer (Wargovich & Levin, 1996; Le Marchand *et al*., 1997). Low physical activity has also been associated with an increased risk for CRC (Sternfeld, 1992). A family history of CRC represents an independent risk factor and may account for up to 15% of all cases. The remaining 85% of colorectal cancer cases are considered "sporadic", i.e., without a clear inheritance pattern.

2.7.2 Mitochondrial DNA variation in colorectal cancer

The first study reporting that mtDNA mutations could have a functional significance in cancer came with the report of a middle-aged woman with a renal adenocarcinoma that was heteroplasmic for a deletion of 294 bp in the mtDNA oxidative phosphorylation gene MT-ND1 (Horton *et al*., 1996). Afterwards, a variety of mtDNA coding region and control region mutations have been reported in colon cancer cells, prostate cancer, and a variety of other solid tumors.
For several years, mtDNA mutations have been associated with development and progression of colorectal cancer
This field of research was developed by Vogelstein's group (Fearon & Vogelstein 1990; Polyak *et al*., 1998), who sequenced the mtDNA genome of 10 human colorectal cancer cell lines and found 12 different mutations in 7 of those cell lines. Of the 12 mutations identified, 8 were found in protein-coding genes (MT-ND1, MT-ND4L, MT-ND5, MT-COX subunit II, MT-COX subunit III, cytochrome *b*) and 4 in rRNA genes (12S MT-rRNA and 16S MT-rRNA). These were mostly T>C or G>A transitions. The mutations included 11 nucleotide substitutions and a single base-pair insertion.
Of the 8 mutations in protein encoding genes, 6 were missense mutations, 1 was a nonsense mutation, and 1 was a single base-pair insertion. Three of the cell lines

contained just 1 mutation and 4 lines contained 2–3 mutations (Polyak *et al*., 1998).
Many reports have shown mtDNA mutations in cancer samples. In the work of Alonso *et al.*, 1997, 23% (3/13) of colorectal tumors revealed two AT/GC transitions. Forty-five sporadic colorectal carcinomas were investigated and alterations were detected in a polycytidine tract within the D-loop region in 20 carcinomas (44%), and also frameshift mutations in a polyadenosine (A)8 or polycytidine (C)6 tract within the NADH dehydrogenase (MT-ND) genes were detected (Habano *et al*., 1998).

This research group also analyzed 45 carcinomas with single-strand conformation polymorphism (SSCP) and demonstrated NADH dehydrogenase (MT-ND) gene alterations in 7 carcinomas (16%), including 3 mononucleotide repeat alterations, 2 missense mutations, and 1 small (15-bp) deletion (Habano *et al*., 1999). Six of these 7 carcinomas also exhibited microsatellite instabilities of the (C)n sequence in the D-loop region. This region, which constitutes only 6.8% of the mitochondrial genome, seems to be of pivotal importance, as it is the hotspot for somatic mutations in colorectal tumors.

In their report Zhao *et al*., 2005 emphasized that although the mtDNA D-loop is only 1.12 kb long, numerous mutations are found in cancer patients (18 mutations among 20 patients), as compared with the remaining 15-kb mtDNA. This group indicates that the D-loop of mtDNA is a fragment with a high mutation rate. This hypothesis has also been favored by Lievre *et al*., 2005. Those authors sequenced the D-loop region of 365 patients and found it mutated in 38.3% of the cases. The longitudinal analysis has shown that the 3-year survival rate is 53.5% and 62.1% in patients with and without D-loop mutations, respectively. After adjustment for age, stage, and microsatellite instability status, the relative risk of death in the patients with D-loop mutations was 1.40, as compared with patients who are not harboring D-loop mutations.

2.8 Role of apoptosis in cancer development

2.8.1 Apoptosis

The process in which the cells play an active role in their own death is called apoptosis or programmed cell death, therefore apoptosis is also known as cell suicide. It is a normal process in the development and maintenance of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. Therefore apoptosis and

necrosis are two modes of cell death with distinct morphological and biochemical features.

Upon receiving specific signals instructing the cells to undergo apoptosis, a number of distinctive changes occur in the cell. In the early stages of apoptosis a group of proteins known as caspases are typically activated. These proteins break down essential cellular components, which are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus.

A number of mechanisms are responsible for inducing apoptosis in cells. The sensitivity of cells to any of these stimuli can widely depend on a number of factors such as the expression of pro- and anti-apoptotic proteins such as the Bcl-2 proteins or the inhibitor of apoptosis proteins, the intensity of the stimulus and the stage of the cell cycle.

2.8.2 Role of mitochondria in apoptosis

Mitochondria play an important role in the regulation of cell death. They contain many pro-apoptotic proteins such as apoptosis inducing factor (AIF), Smac/DIABLO and cytochrome *c*. The release of cytochrome *c* is tightly controlled because cytochrome *c* causes the assembly and activation of a big protein complex termed apoptosome. Mitochondria also play an important role in amplifying the apoptotic signalling from the death receptors, with receptor recruited caspase 8 activating the pro-apoptotic Bcl-2 protein, Bid. In the apoptosome, proteases of the caspase-family are activated, and most of the changes that can be observed during apoptosis are the result of caspase-activity. In some but not all situations caspases are necessary to kill the cell but they probably also serve important functions in the disposal of the dead cell. Pro-apoptotic proteins are released from the mitochondria following the formation of a pore in the mitochondrial membrane called the permeability transition pore, or PT pore. These pores are thought to form through the action of the pro-apoptotic members of the Bcl-2 family of proteins, which in turn are activated by apoptotic signals such as cell stress, free radical damage or growth factor deprivation.

2.8.3 Role of Bcl-2 proteins

The most important regulators in apoptosis are the members of the Bcl-2 family. The Bcl-2 proteins are a family of proteins involved in the response to apoptosis. Some of these proteins (such as Bcl-2 and Bcl-XL) are anti-apoptotic, while others (such as Bad, Bax or Bid) are pro-apoptotic. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic proteins. When there is an excess of pro-apoptotic proteins the cells are more sensitive to apoptosis, when there is an excess of anti-apoptotic proteins the cells will tend to be more resistant. An excess of pro-apoptotic Bcl-2 proteins at the surface of the mitochondria is thought to be important in the formation of the PT pore.

The pro-apoptotic Bcl-2 proteins are often found in the cytosol where they act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic Bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome *c* and other pro-apoptotic molecules from the intermembrane space. This in turn leads to the formation of the apoptosome and the activation of the caspase cascade.
The release of cytochrome *c* from the mitochondria is a particularly important event in the induction of apoptosis. Once cytochrome *c* has been released into the cytosol it is able to interact with a protein called apoptotic protease activating factor 1 (Apaf-1). This leads to the recruitment of pro-caspase 9 into a multi-protein complex with cytochrome *c* and Apaf-1 called the apoptosome. Formation of the apoptosome leads to activation of caspase 9 and the induction of apoptosis. The role of mitochondria in the induction of apoptosis is summarized in the figure 2.4.
ARNT, aryl hydrocarbon receptor nuclear translocator; Bad and Bax, pro-apoptotic Bcl-2 family members; Bcl-xL, anti-apoptotic Bcl-2 family member; CBP, cyclic AMP response element binding protein; cyt *c,* cytochrome *c*;; HIF-1, hypoxia-inducible transcription factor; HRE, hypoxia response elements; P, phosphorylation; Pol II, RNA polymerase II; smac/DIABLO second mitochondria-derived activator of caspases /direct IAP binding protein with low pI); XIAP, inhibitor of apoptosis protein X.

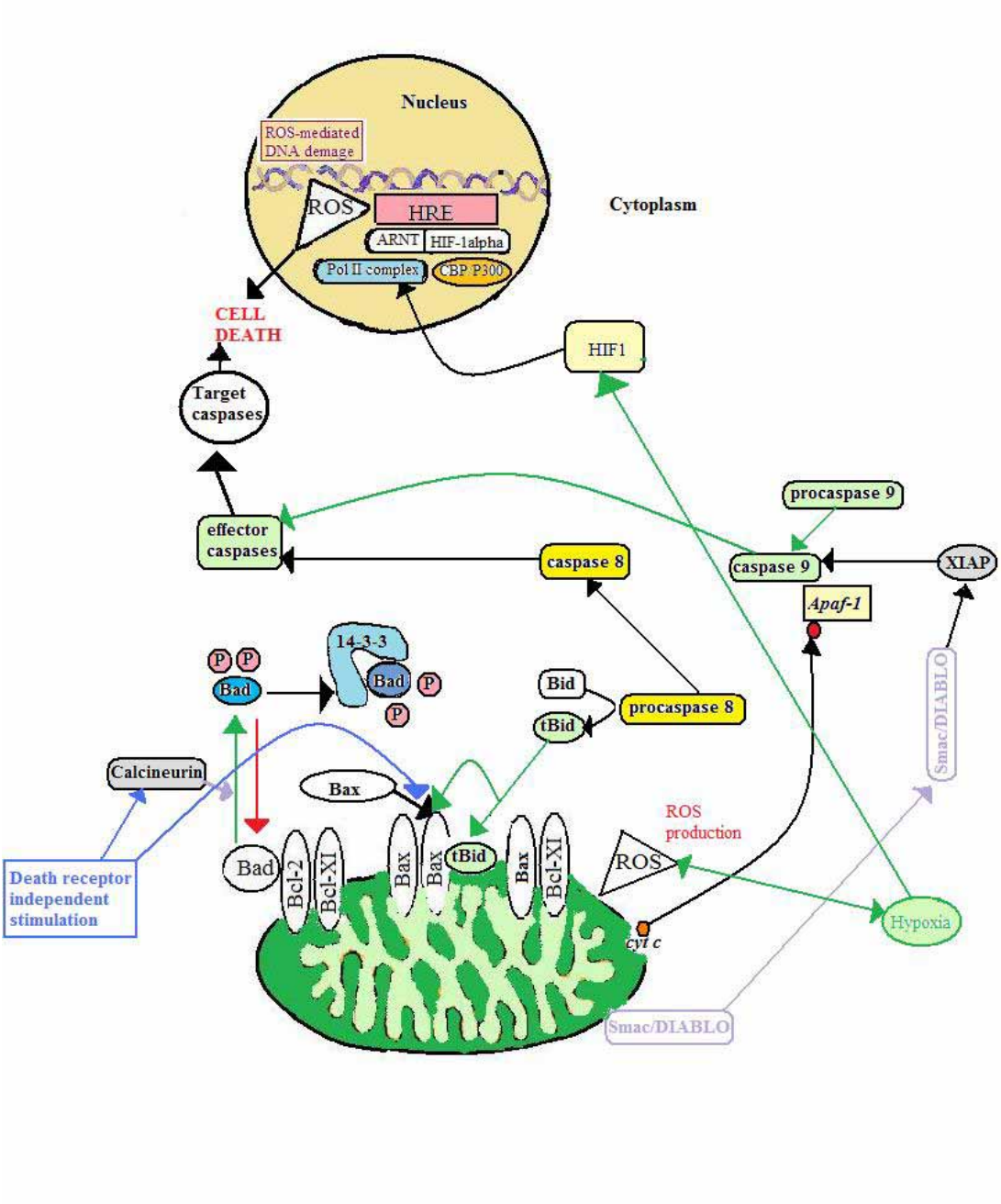

Figure 2.4: Mitochondrial apoptosis pathways and ROS production disruptions in cancer cells

2.9 Importance of the mitochondrial abnormality in tumorgenesis

Mitochondrial dysfunction in cancer has been a subject of great interest and intensive investigation. Although most cancer cells show somatic mutations in

mtDNA, the question of whether such mutations contribute to the promotion of carcinogenesis remains unclear. Some reports seem to help in understanding of correlation between mtDNA mutations and cancer development.

AT8993G point mutation in the mtDNA, resulting in a Leu156Arg substitution in the MT-ATP6 subunit of the mitochondrial F1F0-ATPase, is known to introduce impairment in OXPHOS in 2 mitochondrial disorders: NARP (neuropathy, ataxia and retinitis pigmentosa) and MILS (maternally inherited Leigh's syndrome) (Mattiazzi *et al*., 2004).

A MELAS mutation was reported in a colon tumor, which is known to impair OXPHOS very strongly and confirmed the role of mitochondrial mutations in tumor growth (Lorenc *et al.*, 2003).

It was showed, that the ATP synthase subunit 6 (MT-ATP6) gene mutations contributed to tumor growth, not due to secondary nuclear mutations caused by abundantly produced ROS, but because apoptosis occurred less frequently in the mitochondria with mutant MT-ATP6 versus the wild-type (Shidara *et al*., 2005). This suggests that the pathogenic mtDNA mutations promote tumors not only by causing OXPHOS deregulation and ROS production but also by preventing apoptosis.

Furthermore Modica-Napolitano and Singh 2002 have suggested that because mutant mtDNA is easily detectable in urine, blood and saliva of head & neck and lung cancer patients, mtDNA mutations should be considered as extremely useful biomarkers for the detection of many cancers, and further research can develop standard methods for different types of cancer.

2.10 Aim of the study

The aim of the present study is to clarify the relationship between mitochondrial abnormalities and human colorectal cancer. CRC can be used as an ideal model system to study the development and progression of human tumors, because epithelial cells of the colon mucosa often follow a systematic process of cellular proliferation, differentiation, adenoma formation, and, eventually, cancer transformation (Fig 2.3).

We have focused on understanding the genetic changes that occur in tumorigenesis and the specific aims of presented study are:

- To investigate mitochondrial DNA abnormalities including deletions, duplications and insertions or combinations of them in an Iranian CRC patient collection.
- To estimate the role of mtDNA polymorphisms and haplogroups as a risk factor for CRC.
- To determine mtDNA point mutations in colon cancer cells.

3 Materials

3.1 Patients

3.1.1 Blood samples

A total of 95 blood samples of CRC patients were collected from the Department of Oncology Sayedoalshohadae hospital in Isfahan (Iran). The patients consisted of 40 women and 55 men ranging in age from 26–78 years (mean age 54.8 years). All of them had well differentiated adenocarcinomas.
Demographic, clinical and tumor-related characteristics of patients were recorded based on their hospital documents. These parameters included gender, age at diagnosis, place and date of birth and tumor-related factors such as location, stage, degree of differentiation and mucus production.
All of these patients were interviewed to trace their family history of cancer including occurrence of malignancy in the family, type of cancer and the age at diagnosis of the affected family member.
In the families of fifteen patients we saw occurrence of malignancy but only one case had a history of colon cancer in his family.

Haplogroups are defined by ancient mutations (Wallace, 1994). Therefore we used blood samples to investigate haplogroups and polymorphism in the D-loop region of mtDNA. Most researchers use blood samples to determine the haplogroups (Torroni, 1994, 1995).

3.1.2 Controls

The control group consisted of 150 Iranian people (71 men and 79 women; mean age 43.5 years), who visited the blood donor clinic in Tehran. They answered an extensive questionnaire regarding their current health and medical history, and we chose healthy individuals with neither apparent genetic or metabolic disorders nor any type of cancer.

3.1.3 Tissue samples

Formalin-fixed, paraffin embedded material of 30 surgically resected colorectal cancers and their adjusted normal tissue from the Baghiyatallah hospital in Tehran during the years between 2003- 2005 were collected.

The patients consisted of 13 women and 17 men ranging in age from 28 - 74 years (mean age 50.66 years). Five-µm sections were prepared on slides. Sections were deparaffinized and stained with hematoxylin and eosin (H&E) by standard procedures used for pathological diagnosis. In order to distinguish cancer tissue from normal tissue, specimens were examined under an inverted light microscope used for manual microdissection. None of the patients with colon cancer included in the present series had received chemotherapy or radiation therapy before surgery.
To determine somatic mutations in colorectal cancer we used formalin-fixed, paraffin embedded tumor and normal tissue from the same patients.

3.2 Enzymes

List of enzymes used in this study are summarized in table 3.I.

The enzymes used were either from Fermentas life sciences or from Roche. The *Taq*-DNA-Polymerase was from Sinagene Tehran, Iran.

Table 3.I: The used restriction enzymes

enzyme name	sequence	position	diseases
*Bgl*I	5'-G C C N N N N^N G G C-3'	8344	MERRF
*Apa*I	5'-G G G C C^C-3'	3243	MELAS
*MSP*I	5'-C^C G G-3'	8993	NARP
*Mae*III	5'-N^GTNACN-3'	11778	LHON
*Acy*I	5'-GR^CGYC-3'	3460	LHON
*Nde*II	5'-N^GATCN-3'	14484	LHON
*Mae*III	5'-N^GTNACN-3'	14459	LHON
*BamH*I	5'- G^GATCC-3'	14259	Southern blotting
*Nla*III	5'-CATG^-3'	4216	4216 mutation

3.3 Primers

The sequences of primers used in the study are presented in table 3.II.

Table 3.II: The primer list used in experiments

Name	Sequence 5'→ 3'	Annealing temperature [°C]
mt15340F	ATTCTTGCACGAAACGGGATC	57
mt110R	GCTCCGGCTCCAGCGTCTCG	57
mt5421F (PD1)	GAACATACAAAACCCACCCC	58
mt5740R (PD2)	GGCGGGAGAAGTAGATTGAA	58
mt15000R (PD5)	TTGGCGTGAAGGTAGCGGAT	58
mt8161F (PD3)	CTACGGTCAATGCTCTGAAA	58
mt13640R (PD4)	GGTTGACCTGTTAGGGTGAG	58
mt16150R (PD6)	GTGGTCAAGTATTTATGGTA	58
mt3187F	CTCAACTTAGTATTATACCC	59
mt4650R	GGAAATACTTGATGGCAGCT	59
mt3679F	TCAAACTACGCCCTGATCGG	58
mt8161F	CTACGGTCAATGCTCTGAAA	58
mt8390R	ATACGGTAGTATTTAGTTGGGGCAT TTCACTGTAAAGAGGTGTTGG	58
mt3187F	CTCAACTTAGTATTATACCC	60
mt3550R	GATGGTGAGAGCTAAGGTCG	60
mt8901F	AGCCCACTTCTTACCACAAG	62
mt9239R	TACTATATGATAGGCATGTGA	62
mt11646F	TCGTAGTAACAGCCATTCTC	58
mt11860R	GAGGTTAGCGAGGCTTGCTA	58
mt14184F	CACCAACAAACAATGGTCAACCAG	58
mt14542R	GAATTTTGGGGGAGGTTATATGGG	58
mt14439F	GATACTCCTCAATAGCCATC	57
mt15000R	TTGGCGTGAAGGTAGCGGAT	57
mt3187F	CTCAACTTAGTATTATACCC	60
mt3550R	GATGGTGAGAGCTAAGGTCG	60
mt14441F	TACTCCTCAATAGCCATCGCTGTA GTATATCCAAAGACAACG	59
mt14542R	GAATTTTGGGGGAGGTTATATGGG	59

3.4 Buffers

The solutions are prepared as described in table 3.III

Table 3.III: List of required solutions

Solution	Composition/preparation	Storage	Use
20X SSC	176g NaCl(3M), 88g Na_3Citrate(0.3M):MiliQ to 1L, pH to 7.0 w/1M HCl	15-25°C	transfer
10X Blocking Solution	50ml 10X Blocking Reagent(bottle 10) ,50ml10X maleic acid buffer: MiliQ to 1L	2-8°C	preparation of blocking solution
10X Maleic acid Buffer	116g maleic acid(1X is 0.1 M), 88g NaCl (1X is 0.15M): MiliQ to 1L pH to 7.5 w/solid NaOH	15-25°C	preparation of blocking solution
0.25N HCl	25ml conc. HCl: MiliQ to 1L	15-25°C, stable	depurination
Denaturization Solution	88g NaCl(1.5M), 20g NaOH(0.5N): MiliQ to 1L	15-25°C	denaturization
Neutralization Solution	176g NaCl(3M), 6.7g Tris base(0.5M), 70.2gTris-HCl:MiliQ to 1L	15-25°C	neutralization
10X Detection Buffer	1M Tris-HCl, 1M NaCl pH to 9.5	15-25°C	detection
Washing Buffer	3ml Tween-20 (0.3%), 100ml 10X maleic acid Buffer, 0.15M NaCl: MiliQ to 1L pH 7.5 (20°C)	15-25°C	removed of unbound antibody
Hybidization Buffer	250ml 20X SSC(5X), 100ml 1% lauryl sarcosine (0.1%), 2ml 10%SDS(0.02%), 100ml 10XBlocking reagent (bottle 10): MiliQ to 1L	15-25°C	hybridization
10N NaOH	200g NaOH: MiliQ to 1L	15-25°C	adjustment of pH
2X SSC;0.1% SDS	100ml 20X SSC, 10ml 10% SDS: MiliQ to 1L	15-25°C	washing
0.5X SSC; 0.1% SDS	25ml 20X SSC, 10ml 10% SDS: MiliQ to 1L	15-25°C	washing
2X SSC	50ml 20X SSC: MiliQ to 500ml	15-25°C	washing

Table 3.IV: 50x TAE Buffer

Reagent	Amount	Final Concentration
Tris base	242.0 g	2 M
Acetic acid, glacial	57.1 ml	1 M
0.5 M EDTA, pH 8.0	100.0 ml	50 mM
dH_2O	to 1,000.0 ml	

Table 3.V: 10x TBE Buffer

Reagent	Amount	Final Concentration
Tris base	108 g	0.89 M
Boric acid	55 g	0.89 M
0.5 M EDTA, pH 8.0	40 ml	20 mM
dH_2O	to 1L	

3.5 Gels

In table 3.VI to 3.VIII the used gels including polyacrylamide gel, TTGE gel and SSCP gel are summarized respectively.

Table 3.VI: Polyacrylamide Gel

Total volume	10 ml	20 ml	50 ml
TBE buffer	2 ml	4 ml	8 ml
40% Acrylamide/Bis	2 ml	4 ml	8 ml
TEMED	10 µl	20 µl	50 µl
APS	100 µl	200 µl	500 µl

Table 3.VII: TTGE gel

Reagent	6% Gel	8% Gel	10% Gel	12% Gel
40% Acrylamide/Bis	6.0 ml	8.0 ml	10.0 ml	12.0 ml
50x TAE	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Urea	14.4 g	14.4 g	14.4 g	14.4 g
TEMED	40.0 µl	40.0 µl	40.0 µl	40.0 µl
10% Ammonium persulfate	400.0 µl	400.0 µl	400.0 µl	400.0 µl
Total volume	40.0 ml	40.0 ml	40.0 ml	40.0 ml

Table 3.VIII: SSCP gel

Reagent	**6% Gel**	**8% Gel**
40% Acrylamide/Bis	6.0 ml	8.0 ml
0.75 MTris-Formate buffer pH 9.0	21 ml	21 ml
41% Glycerol	7 ml	7 ml
TEMED	60.0 µl	60.0 µl
10% Ammonium persulfate	600.0 µl	600.0 µl
Total volume	40.0 ml	40.0 ml

4 Methods

4.1 DNA extraction

4.1.1 DNA extraction from blood samples

Total DNA was isolated from blood using the FlexiGene DNA Kit (Qiagen, Hilden, Germany).

4.1.2 DNA extraction from formalin-fixed, paraffin embedded samples

Deparaffinization was carried out by adding 1 mL xylene to the 1.5-mL microtubes containing 10-µm tissue sections for 30 min for two changes, followed by 100 and 75% ethanol for 30 min with two changes. After washing with distilled water for 15 min in two changes, 500 µL of lysis buffer (50 µL proteinase K 20 mg/mL, 10 µL 1 M Tris-HCl solution, 2 µL 0.5 M EDTA, 100 µL 10% SDS, and 838 mL distilled water) was added and incubated at 55°C overnight until all tissue fragments were dissolved completely. Further extraction and purification procedures were performed by the following steps: addition of 500 µL phenol:chloroform:isoamyl-alcohol at 25:24:1 ratio to the dewaxed tissues, followed by mixing by vortex, and centrifugation at RT, 12,000 X g for 10 min. The supernatant fluid was removed to an autoclaved microtube using a 100-µL pipette, and one volume of chloroform was added, mixed by vortexing, and centrifuged at 12,000 X g for 10 min. The upper aqueous supernatant was carefully removed to another fresh microtube, adding 0.1 volume of 3 M sodium acetate and again mixed by vortexing, followed by addition of 1 volume of isopropanol, and incubation at -20°C overnight. The precipitated DNA was centrifuged at 12,000 X g at 4°C. The supernatant fluid was discarded and the precipitate washed once with 75% ethanol. The extracted DNA was collected after further centrifugation. The final yield of DNA was dissolved in 50 µl distilled water after drying completely in a hood (Shi SR *et al*., 2002).

4.2 Multiplex PCR

Multiplex PCR was carried out using five sets of primers (Fig 4.1) PD1/PD2, PD1/PD5, PD3/PD4, PD5/PD3 and PD6/PD3. Primer PD1 located at 5421–5440 bp and primer PD2 located at 5740-5721 bp of the mtDNA were used to amplify a

319 bp fragment in a rarely deleted region as an internal control in each multiplex PCR. Primer PD1and primer PD5 located at 15000–14981 bp were used to amplify an 850 bp region created by the ~8.7 kb deletion. Primer PD3 located at 8161–8180 bp and primer PD4 located at 13640–13621 bp of the mtDNA were used to amplify a 470 bp region created by the ~ 5 kb deletions. Primer PD1 and primer PD4, primer PD3and primer PD6 located at 16150–16131 bp were used to amplify the regions created by 7.5 kb, and 7.4 kb deletions.

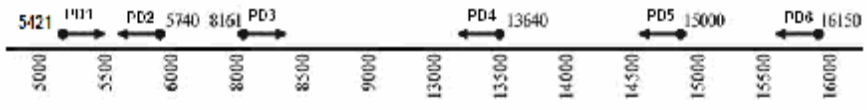

Figure4.1: Diagram shows primers and their position on mtDNA.

All primers summarized in table 3.II.

4.3 Southern Blotting

Total DNA was isolated from blood sample of CRC patients and was digested overnight with *BamH*I at 37°C. The probe was made using PCR product from mtDNA D-loop region and southern blots were carried out as described in instruction manual of DIG DNA labeling and Detection Kit.

4.4 PCR amplification and sequence analysis

PCR amplification was carried out in a final volume of 25 µL containing 200–300 ng total DNA 70 µM of each dNTP, 10 pmol of each primer, 2.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase and 2.5 µl of PCR buffer. The PCR profile was as follows: 94˚C for 5 min; 30 cycles of 94˚C for 50 s, 57˚-62˚C for 50 s and 72˚C for 50 s, followed by 72˚C for 10 min. The sequenced products were analyzed on 1.5% agarose gel.

DNA sequencing was performed using the BigDye terminator kit (Macrogene Seoul, Korea) with primers designed for mtDNA.

The obtained sequences were aligned with a multiple sequence alignment interface CLUSTAL X with comparison to rCRS (http: www.gen.emory.edu/mitomap/mitoseq.html).

4.4.1 Analysis of mitochondrial haplogroup

The primer pair mt15791F primer located at 15791-15810 bp and mt16420R primer located at 16420-16401 bp of the mtDNA were used to amplify a 629 bp sequence encompassing HVS-I in the D-loop of the mtDNA to fetch the 359 bp sequence (16,024–16,383 nt) for HVS-I.

Haplotypes were assigned according to the West Eurasian mtDNA genealogy (Macaulay *et al*., 1999). For assignment the haplogroup proceeded we used the following algorithm (for brevity all numbering is according to Anderson *et al*., 1981, minus 16,000 in the control region of mtDNA): 069T 126C 223C assigned to haglogroup J; 126C 223C 294T assigned to T; 129A 223T 391A assigned to I; 223T 292T assigned to W; 189C 223T 278T assigned to X; 223C 224C 311C assigned to K; 362C assigned to D; 290T and 319A assigned to A; 223T assigned to R; 304C assigned to H1, 189C and 356T assigned to H3, 129A assigned to H4, 221T assigned to H5; 162G assigned to H8; 223C 249C and either 189C or 327T assigned to U1; 129C 223C assigned to U2: 223C 343G assigned to U3; 223C 356C assigned to U4; 223C 270T assigned to U5; 172C 219G 223C assigned to U6; 223C 318T assigned to U7; 223C 298C assigned to V; 067T 223C assigned to HV1; 126C 223C 362C assigned to preHV; 145A 176G 223T assigned to N1b; 223T 278T 390A assigned to L2; and 187T 189C 223T 278T 311C assigned to L1.

4.5 RFLP analysis

The RFLP method was used to investigate known mtDNA mutations responsible for mitochondrial disease in CRC samples. The PCR amplification was carried out for regions which we planned to study. The amplified sequence was digested using the suitable enzyme.

4.5.1 Investigation of the mtDNA "MERRF" mutation in CRC

MERRF disease is a maternally inherited mitochondrial encephalomyopathy characterized by myoclonus epilepsy, generalized seizures, ataxia and myopathy. The syndrome is due to an mtDNA A8344G transition mutation in the gene coding for $tRNA^{lys}$.
This region was amplified using primer pair mt8161F located at 8161-8180 bp and mt8390R located at 8390-8345 (table 3.II). These primers amplified a 229 bp

sequence. The PCR product was digested with *Bgl*I (37°C/overnight). If the mutation is present, the enzyme will cut the PCR product (Shoffner *et al*., 1990).

4.5.2 Investigation of the mtDNA "MELAS" mutation in CRC

MELAS is a clinical entity of mitochondrial diseases. The most important mutation is a transition mutation A3243G in the mitochondrial $tRNA^{leu}$ gene. This mutation inactivates downstream transcription factor terminators of the $tRNA^{leu}$ gene.
This region was amplified using primer pair mt3187F located at 3187-3206 bp and mt3550R located at 3550-3530. These primers amplified a 363 bp sequence. The PCR product was digested with *Apa*I (37°C/overnight). If the mutation is present, the enzyme will cut the PCR product (Goto *et al*., 1990, Jazin *et al*., 1995).

4.5.3 Investigation of the mtDNA "NARP/MILS" mutation in CRC

The mutation T8993G/C causing NARP/MILS disease is in the ATPase6 encoding gene. NARP and MILS are caused by mutations in the same gene and the difference is in the mutation percentage. Individuals harboring more than 90% mutant cells may die of Leigh syndrome.
The region was amplified using primer pair mt8901F located at 8901-8920 bp and mt9239R located at 9239-9219. These primers amplified a 363 bp sequence. The PCR product was digested with *Msp*I (37°C/overnight). If the mutation is present, the enzyme will cut the PCR product.

4.5.4 Investigation of the mtDNA "LHON" mutation in CRC

LHON disease presents in young adults as acute or subacute central vision loss leading to blindness. There are four primary point mutations which are associated to LHON at positions 3460, 11778, 14484, 14459.

To amplify the region containing the G11778A mutation, the primer pair mt11646F located at 11646-11665 bp and mt11860R located at 11860-11841 was used. These primers amplified a 214 bp sequence. The PCR product was digested with *Mae*III (55°C/overnight). If the mutation is present the enzyme will cut the PCR product to 132bp+82bp (Wallace, 1992).

For amplification of the region containing the G3460A mutation, the primer pair mt3187F located at 3187-3206 bp and mt 3550R located at 3550-3530 was used. The amplification product size was 363 bp. It was digested with *Acy*I (37°C/overnight). Mutant mtDNA will not be cut (363 bp) but normal mtDNA will be cut to 273bp + 90bp (Savantaus *et al*., 1992).

The region containing the T14484C mutation was amplified using the primer pair mt14441F located at 14441-14483 bp and mt14542R located at 14542-14519. These primers amplified a 101 bp sequence. The PCR product was digested with *Nad*II (37°C/overnight). If the mutation is present, the enzyme will not cut the PCR product (101bp). The wildtype product will be cut to 58bp + 43bp.

The region containing the G14459A mutation was amplified with the primer pair mt14439F located at 14439-14458 bp and mt15000R located at 15000-14981. These primers amplified a 561 bp sequence. The PCR product was digested with *Mae*III (55°C/overnight). If the mutation is present, the enzyme will cut the PCR product. All digested PCR products were loaded on 8-10% polyacrylamide gel.

All primers are summarized in table 3.II and the restriction enzymes are summarized in table 3.I.

4.6 Statistical Analysis

Initial analysis was carried out using Z-statistic test for each individual SNP. Additionally, for each SNP Fisher's exact test was performed and reanalyzed. The odds ratio (OR) and its 95% confidence interval (95% CI) were calculated. All statistical analysis was implemented using the MedCalc software. The mentioned tests were used to examine the association between patients and control groups. Values of $P \leq 0.05$ were regarded as statistically significant and also if the Z statistics calculated in Z test be more than 1.96 then the difference between two groups was regarded as significant.

4.6.1 Hierarchical clustering

Hierarchical clustering is a way to investigate grouping in data, simultaneously over a variety of scales, by creating a cluster tree. The tree is not a single set of clusters, but rather a multilevel hierarchy, where clusters at one level are joined as clusters at the next higher level. This allowed us to decide what level or scale of clustering is most appropriate in our application.

Hierarchical clustering dendrograms were constructed for cases and controls separately using Euclidean distances to illustrate the relationships of the SNPs that were detected. The Euclidean distance is calculated by:

$$d_{pq} = \sqrt{(p_1 - q_1)^2 + (p_2 - q_2)^2 + ... + (p_n - q_n)^2} = \sqrt{\sum_{i=1}^{n} (p_i - q_i)^2}$$

After calculating the Euclidean distance for each pair wise SNP combination of p and q such that p≠q, this distance is used as an index for clustering the SNPs. At each SNP an individual can be considered either 0 for wild-type or 1 if a variant is present. The Hierarchical clustering dendrograms were made for cases and controls separately using MATLAB software version 7.3.

4.7 Analysis of mtDNA somatic mutation

To identify mutations in genes encoding subunits of the respiratory complex I in CRC patients, the amplified sequences containing each gene of the respiratory complex I of tumor and adjusted normal tissue were investigated by either the TTGE mutation detection method or the SSCP mutation detection system.

4.7 1 Temporal Temperature Gradient Gel Electrophoresis (TTGE)

To identify mutations in genes encoding subunits of the respiratory complex I in CRC patients, the mitochondrial regions containing each gene of CRC samples and adjusted normal tissue were amplified by PCR. The amplified sequences were studied for somatic mutation by TTGE mutation detection method.

According to figure 4.2 during electrophoresis, double-stranded DNA will become partially denatured. When DNA begins to denatureits electrophoretic mobility decreases. Mutant and wild-type molecules are separated on the gel as they begin to denature at different temperatures. The TTGE conditions are presented in table 4.I.

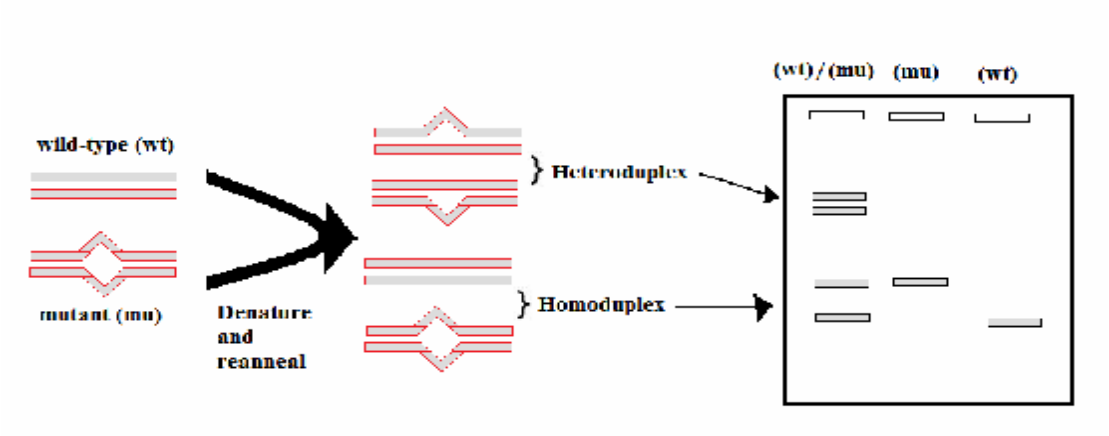

Figure 4.2: Illustrations of TTGE mutation detection system

Table 4.I: TTGE conditions

Region	Forward primer	Reverse primer	Size, bp	Temperature range, °C	Ramp/rate, °C/min	Gel, %
MT-ND1	mt3679F	mt4650R	971	50-66	2-2.5	4.5
MT-ND4	mt11646F	mt11860R	214	48-66	2.5-3	6
MT-ND5	mt13201F	mt13640R	439	56-60	0.5-1	5
MT-ND6	mt14184F	mt14542R	358	54-64	1.5-2	5

4.7.2 Single-Stranded Conformational Polymorphisms (SSCP)

The SSCP mutation detection method was carried out to detect somatic mutation in genes involved in complex I (for methodic detail please see Appendix).

5 Results

5.1 Large scale mtDNA deletion in human colorectal cancer

In this part of the presented study 90 CRC samples were screened for deletions using multiplex PCR, Southern blot, and direct DNA sequencing.

Multiplex PCR was carried out to investigate mtDNA large scale deletion using five sets of primers: PD1/PD2, PD1/PD5, PD3/PD4, PD5/PD3 and PD6/PD3 (Fig 4.1).

Our data indicated the presence of a ~8.7 kb deletion in 11 patients out of 90 studied samples so the percentage of the 8.7 kb deletion in our colon cancer patients was 12.2%.
As control we analyzed 33 unrelated healthy individuals with neither apparent genetic/metabolic disorders nor any type of cancer. We did not find any deletion in this control population.

The 850 bp PCR product generated by primers PD1 and PD5 indicating the presence of the 8.7 kb deletion in our study population is shown in figure 5.1.

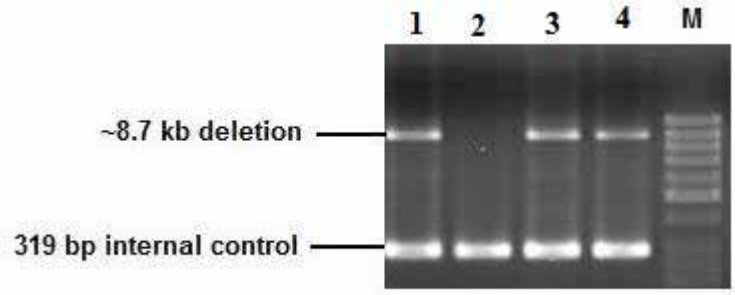

Figure 5.1: Detection of 8.7 kb deletion in CRC patients by multiplex PCR. Lanes 1, 3 and 4 show patients with deletion. Lane 2 shows a sample without deletion and M is 100 bp DNA ladder

We also detected a ~5 kb deletion in 2 patients out of 90 CRC samples in our study population. However, this is not a statistically significant difference between patients and controls.

The overall characteristics of 90 CRC patients with different deletions are summarized in table 5.I.

Table 5.I: Characteristics of 90 CRC patients

	N	Frequency of ~8.7 kb deletion	Frequency of ~5 kb deletion
Patients	90	11	2
controls	33	0	0
p value		0.035	1
Age(years)			
≥50	64	9	2
<50	26	2	0
Sex			
Male	50	6	0
Female	40	5	2

The 8.7 kb deletion is flanked by a repeat sequence and the deletion breakpoint was confirmed in some samples by sequencing. A 9-bp direct repeat (CTACTCCTA) at position 5472/5480–14131/14139 was identified and the deletion was between nucleotide positions 5481 and 14139 (Fig 5.2 A & B).

```
 5361 tactccacct caatcacact actccccata tctaacaacg taaaaataaa atgacagttt gaacatacaa aacccacccc
 5441 attcctcccc acactcatcg cccttaccac gctactccta cctatctccc cttttatact aataatctta tagaaatttа
 5521 ggttaaatac agaccaagag ccttcaaagc cctcagtaag ttgcaatact taatttctgt aacagctaag gactgcaaaa
      ..........................................................................................
14081 gcataattaa actttacttc ctctctttct tcttcccact catcctaacc ctactccta tcacataacc tattcccccg
14161 agcaatctca attacaatat atacaccaac aaacaatgTt caaccagtaa ctactactaa tcaacgccca taatcataca
14241 aagccccccgc accaatagga tcctcccgaa tCaaccctga cccctctcct tcataaatta ttcagcttcc tacactatta
```

Figure 5.2 A: part of human mtDNA sequence. The observed 8.7 kb deletion occurred between positions 5481 and 14139. The 9 bp directed repeat at 5472/5480 and 14131/14139 is highlighted

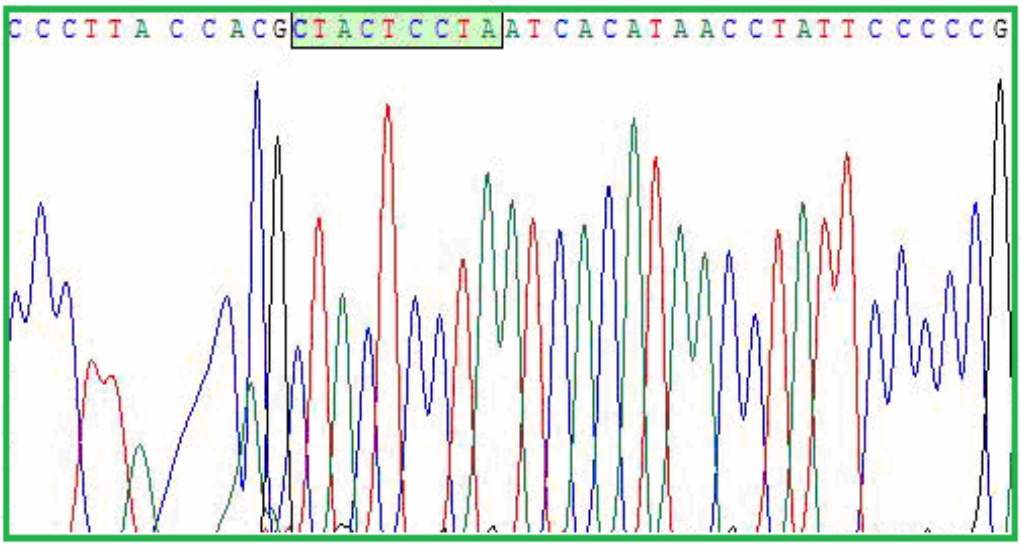

Figure 5.2 B: Electropherogram showing the 9 bp direct repeat flanking the ~8.7 kb deletion. Bases of the repeat sequences are highlighted

Our southern blot analysis confirmed the 8.7 kb deletion in 11 patients (Fig 5.3).

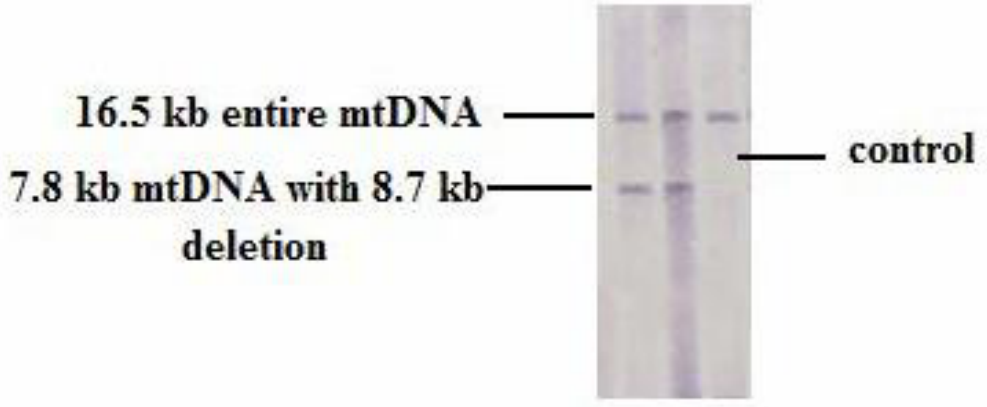

Figure 5.3: Southern blotting shows the 16.5 kb entire mtDNA and mtDNA molecules carrying the 8.7 kb deletion

5.2 Haplotype analysis: identification of a high risk population

Since mitochondria play pivotal roles in carcinogenesis and metabolism of cancer cells (Macaulay, 1999), we analyzed the correlation between cancers and mitochondrial haplogroups.

We examined the relationship between colorectal cancer and each of the 9 major mitochondrial haplogroups in Iranian CRC patients. The mtDNA haplogroups of 95 CRC patients and 100 control subjects were determined by direct sequencing of mtDNA HVS I (table 5.II).

Table 5.II: Characteristics of 95 CRC patients and 100 normal controls

Parameters	No. of Patients	No. of controls
Age		
≤50	28	68
>50	67	32
Sex		
Male	55	71
female	40	29
Tumor differentiation	adenocarcinoma	---

Our results for the haplogroups in 95 CRC patients and 100 normal subjects are demonstrated in table 5.III.

Haplogroup K is significantly more abundant in CRC patients (P=0.001) compared to controls.

5.3 High Rate of Mutation in mtDNA D loop Region of CRC patients

The mitochondrial control region from 40 CRC sample and 150 unrelated age/gender/ethnic matched control subjects was amplified by PCR and the nucleotide sequence of the amplified region was directly determined by automated sequencing.
We found 19 variations in our study population. When each single nucleotide polymorphism (SNP) is tested individually using Fisher's exact test and the Z-statistic test (table 5.IV), the frequencies of eight SNPs were found to be significantly different (P≤0.05) between the CRC patient group and the control

group (A16163G, C16186T, T16189C, C16223T, T16224C, C16295T, T16311C, and T16519C). Our results showed that the D-loop mutation rate in CRC samples was higher than in normal controls ($P \leq 0.05$). Most of the mutations were single base substitutions and most of them were transitions rather than transversions.

Table 5.III: Distribution of mtDNA haplogroups among CRC patients and normal controls

mtDNA haplogroups	CRC patients (%)	Control subjects (%)	P-value
H	4/95 (4)	7/100 (7)	0.538
D	8/95 (8)	13/100 (13)	0.359
L	12/95(13)	15/100 (15)	0.682
J	13/95(14)	19/100 (19)	0.339
T	10/95(11)	15/100 (15)	0.396
U	6/95 (6)	3/100 (3)	0.321
K	9/95 (9.5)	0/100 (0)	0.001
A	2/95 (2)	1/100 (1)	0.613
R	20/95(21)	18/100 (18)	0.717
*Other	16/95(17)	9/100 (9)	0.133

*Others: Haplogroups I, W, X, and V

Table 5.IV: Association analyses for individual SNP loci in CRC samples

SNPs	Cases (n=40)		Controls(n=150)		OR	95% CI		Z statistic	P-value	Fisher's exact test P value
	Positive	%	Positive	%		Lower	Upper			
A16163G	3	7.5	1	0.7	12.081	1.221	119.499	2.131	0.0331	0.0299
C16186T	3	7.5	1	0.7	12.081	1.221	119.499	2.131	0.0331	0.0299
T16189C	11	27.5	19	12.7	2.615	1.124	6.085	2.231	0.0257	0.0291
C16218T	2	5	0	0	19.546	0.919	415.601	1.906	0.0567	0.0434
C16223T	10	25	18	12	2.444	1.025	5.827	2.017	0.0437	0.0472
T16224C	5	12.5	3	2	7.000	1.597	30.692	2.580	0.0099	0.0113
C16261T	4	10	6	4	2.667	0.715	9.951	1.460	0.1443	0.2225
A16269G	2	5	0	0	19.546	0.919	415.601	1.906	0.0567	0.0434
C16292T	2	5	0	0	19.546	0.919	415.601	1.906	0.0567	0.0434
A16293G	2	5	0	0	19.546	0.919	415.601	1.906	0.0567	0.0434
C16294T	3	7.5	4	2.7	2.960	0.635	13.802	1.381	0.1673	0.1630
C16294G	1	2.5	0	0	11.430	0.457	286.021	1.483	0.1381	0.2105
C16295T	4	10	1	0.7	16.556	1.796	152.642	2.476	0.0133	0.0073
A16300G	1	2.5	1	0.7	3.821	0.234	62.463	0.940	0.3471	0.3776
T16311C	10	25	8	5.3	5.917	2.156	16.240	3.451	0.0006	0.0007
A16316G	2	5	2	1.3	3.895	0.531	28.553	1.338	0.1810	0.1956
A16318T	2	5	4	2.7	1.921	0.339	10.885	0.738	0.4607	0.6078
A16335G	1	2.5	1	0.7	3.821	0.234	62.463	0.940	0.3471	0.3776
T16519C	28	70	64	43	3.135	1.482	6.636	2.988	0.0028	0.0024

Sequence comparison also showed one A insertion at position 16266 in a single sample.
The T16519C variation is seen in 28 samples out of 40 patients (70%). The results are summarized in table 5.IV. Thirteen polymorphisms were newly identified in this study population (table 5.V). Our result demonstrates that most of the D-Loop variations in CRC patients were homoplasmic.

Table 5.V: Newly identified polymorphisms in this study population that had not previously been recorded in the human genome database (Mitomap database: http://www.mitomap.org)

	loci	Variation	No. of samples	Reference sequence
1	16026	C→T	1	C
2	16032	T→G	1	T
3	16175	A→T	1	A
4	16266	ins A	1	
5	16289	A→T	1	C
6	16425	C→T	1	C
7	16430	T→A	1	T
8	16446	C→T	1	C
9	16499	A→G	1	A
10	16508	C→T	1	C
11	16510	A→T	1	A
12	16542	C→T	1	C
13	16628	C→T	1	C

As mentioned in section 4.6.1 the hierarchical clustering is a mean to investigate grouping in data. The hierarchical clustering dendrograms were generated in this work to study the correlation between the 19 SNPs for patients and controls. The generated clustering dendrograms are presented in figure 5.4. Among the controls two distinct clusters are apparent in which the distance between clusters is noticeable. One cluster includes only one SNP, which is np16519. The dendrograms respect to patients while the distance between np16519 and all the other clusters approximately remains unchanged some other sub-clusters like the one containing np16311 and np16224 and the other containing np16186 and np16163 are also appeared. In addition the scattering of clusters are increased in hierarchical clustering for patients compared with control samples.

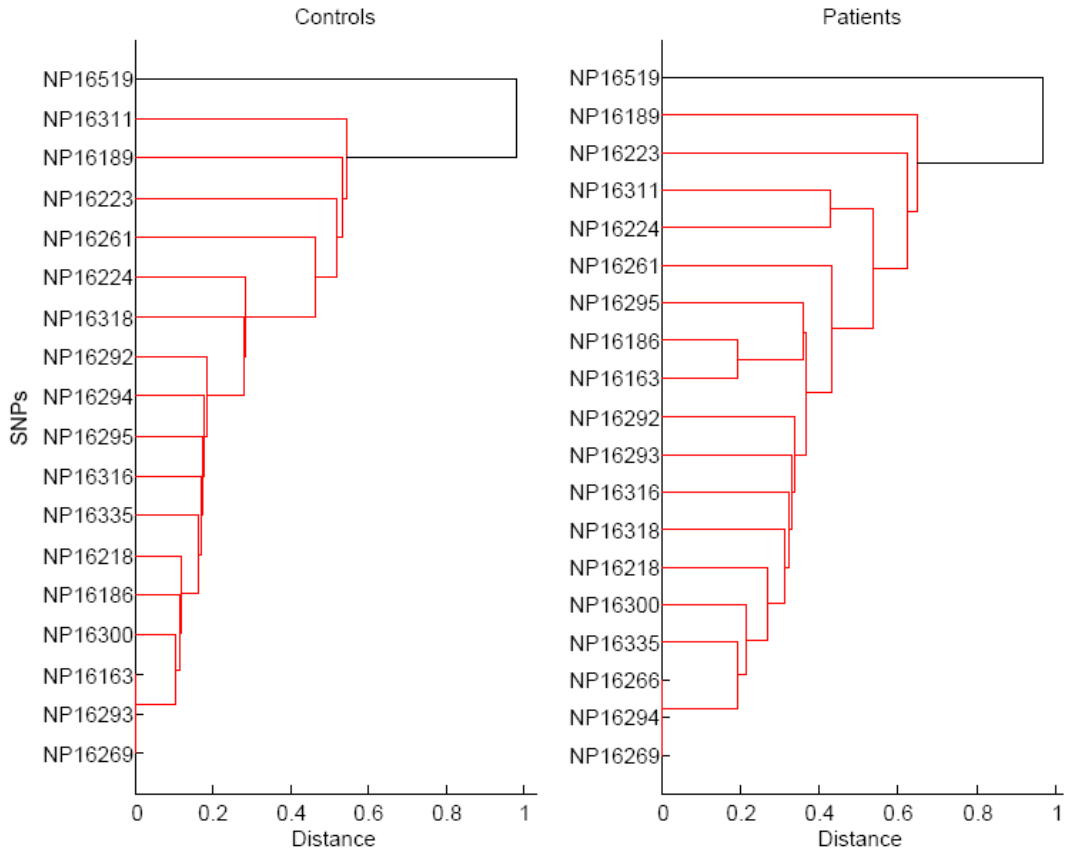

Figure 5.4: Hierarchical clustering dendrograms for CRC patients and controls

5.4. Search for mtDNA mutations responsible for mitochondrial disease in CRC samples

As is explained in previously chapter we searched for mutations responsible for mitochondrial diseases in 30 of the CRC tissues. In the investigated samples we did not find any known mtDNA mutations responsible for mitochondrial diseases. We only found a recently reported mutation T4216C in LHON diseases in the CRC samples. The details are described in following part.

5.5 Analysis of mitochondrial somatic mutation in genes involved in respiratory complex I

The genes involved in respiratory complex I were screened for somatic mutations in CRC tissue samples. Somatic mutations occur in the DNA of certain cells

during a person's lifetime and typically are not passed to future generations. Somatic mutations have been reported in mtDNA in some forms of cancer, including breast, colon, stomach, liver, and kidney tumors.

As mentioned before somatic mutations in mtDNA may increase the production of ROS, which represent potentially harmful molecules. mtDNA is particularly vulnerable to the effects of these molecules and has a limited ability to repair itself. As a result, reactive oxygen species easily damage mtDNA, causing a buildup of additional somatic mutations. Some somatic mutation in MT-ND1 gene was detected in this study (Fig 5.5) & (Fig 5.6).

Figure 5.5: Amplified tumor and adjacent respective non-tumor tissues of MT-ND1 gene. Separation by SSCP run overnight at constant 70 V in 2X TBE buffer on an 6% acrylamide gel with 7% glycerol at 4° C. Lane 1 undenatured non-tumor tissue, lanes 2 and 3 denatured tumor tissue and lane 4 denatured non-tumor tissue. Differences in mobility of the single strands between the tumor DNA with mutation consequence two bands on the gel and the non-tumor DNA arise one band because of no mutation; no difference between single strand mobility was been.

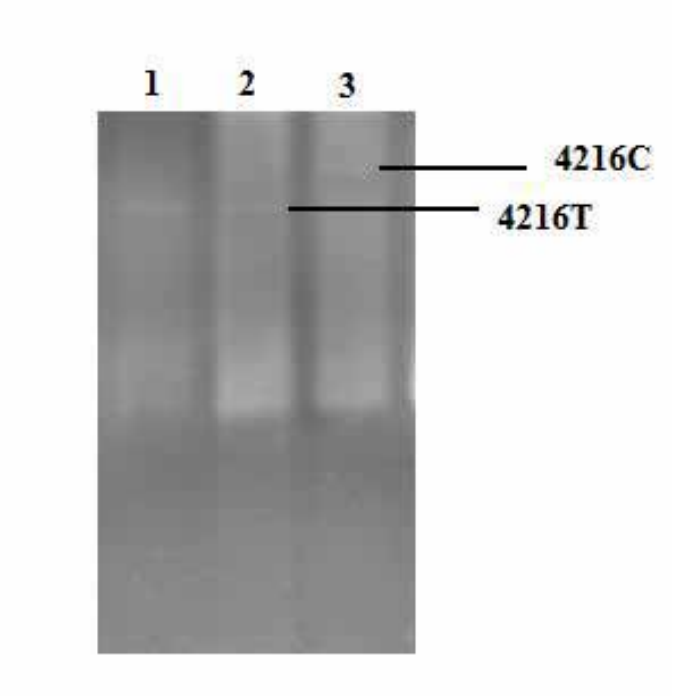

Figure 5.6: Detection of somatic mutation in MT-ND1 gene by the TTGE method. Lane 1 is a control sample, lane 2 is the non-tumor sample and lane 3 is adjacent respective tumor tissues.

5.5.1 Mitochondrial ND1 gene analysis

After detection of mutations in the MT-ND1 gene in CRC patients by SSCP and TTGE methods, the MT-ND1 gene region from each tumor and non-tumor CRC samples was amplified. The amplified sequence was directly determined by automated sequencing, using forward and reverse primer. The obtained mtDNA sequences were analyzed as explained before.

MT-ND1 is encoded by the guanine-rich heavy (H) strand of the mtDNA between nucleotide positions 3307 and 4262 (Anderson *et al*., 1981; Wallace *et al*., 1994). It is maternally inherited along with the mtDNA (Giles *et al*., 1980; Case and Wallace, 1981). The MT-ND1 gene encompasses 955 bp of continuous coding sequence. It contains no introns, has a two-base (AC) 5'-noncoding sequence, an ATA methionine start codon, and ends with the UA of the UAA termination codon (Anderson *et al*., 1981; Montoya *et al*., 1981; Ojala *et al*., 1981). The structure of the MT-ND1 gene is presented in figure 5.8.

Our results demonstrated a mutation T4216C in 8 out of 30 CRC samples. This mutation caused an amino acid change Y→H in the MT-ND1 protein. Furthermore, we found each of the mutations, T3456C, A3480G, C3622T, C3741T, T3777C, and T3847C in one single sample (table 5.VI).
The mutation T4216C was reported recently to be present in LHON (Povalko *et al*., 2005) and also it was reported to be present in an early stage of prostate cancer (Jerónimo *et al*., 2001).

The T→C variant at position 4216 created a restriction endonuclease recognition site for *Nla*III. Therefore the mutation was confirmed with the RFLP method.

The frequency of the T4216C mutation was 26% (8/30) and the mutation was always found to be homoplasmic in the 8 patients (Fig 5.6 A & B).

Table 5.VI: Mutations found in the MT-ND1 gene in CRC patients

Nucleotide Position	Locus	Nucleotide Change	Amino Acid Change	Number of patients
3456	MT-ND1	T→C	syn	1
3480	MT-ND1	A→G	syn	1
3622	MT-ND1	C→T	syn	1
3741	MT-ND1	C→T	syn	1
3777	MT-ND1	T→C	syn	1
3847	MT-ND1	T→C	syn	1
4216	MT-ND1	T→C	Y→H	8

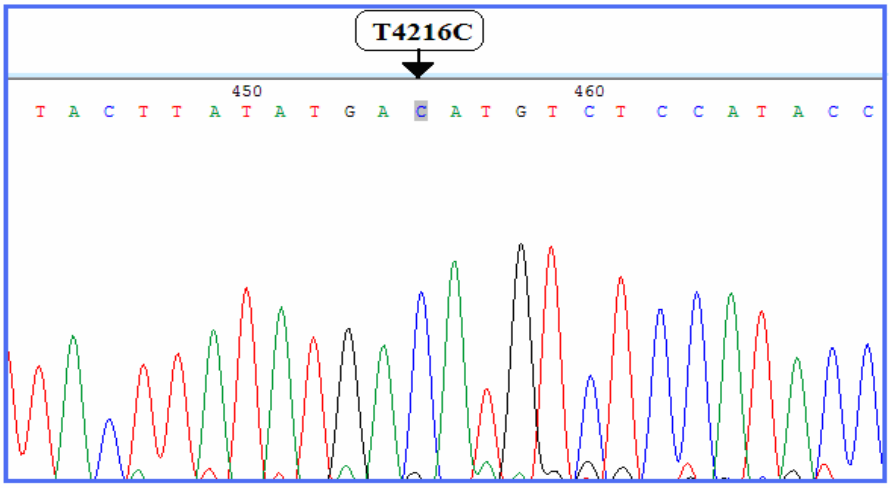

Figure 5.7 A: Electropherogram showing the presence of the T4216C mutation in 8 CRC patients. Altered bases are highlighted in color.

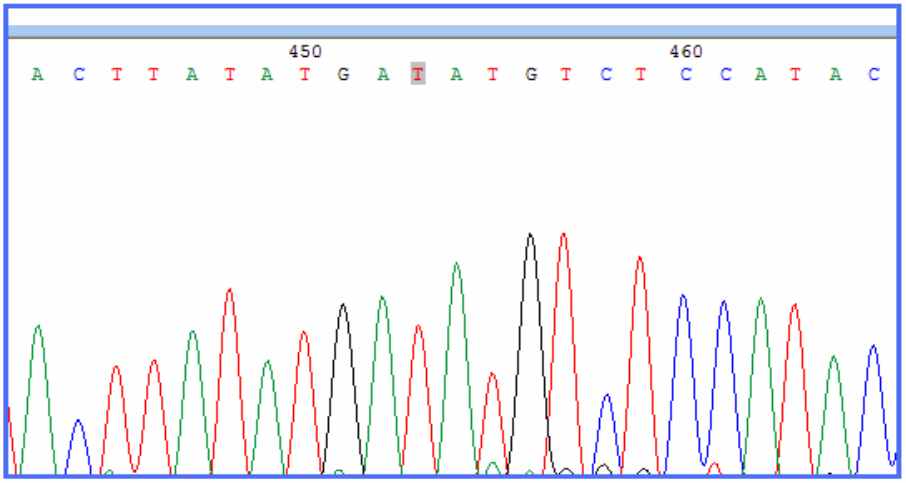

Figure 5.7 B: Electropherogram showing the np 4216 of MT-ND1 gene in non-tumor tissue

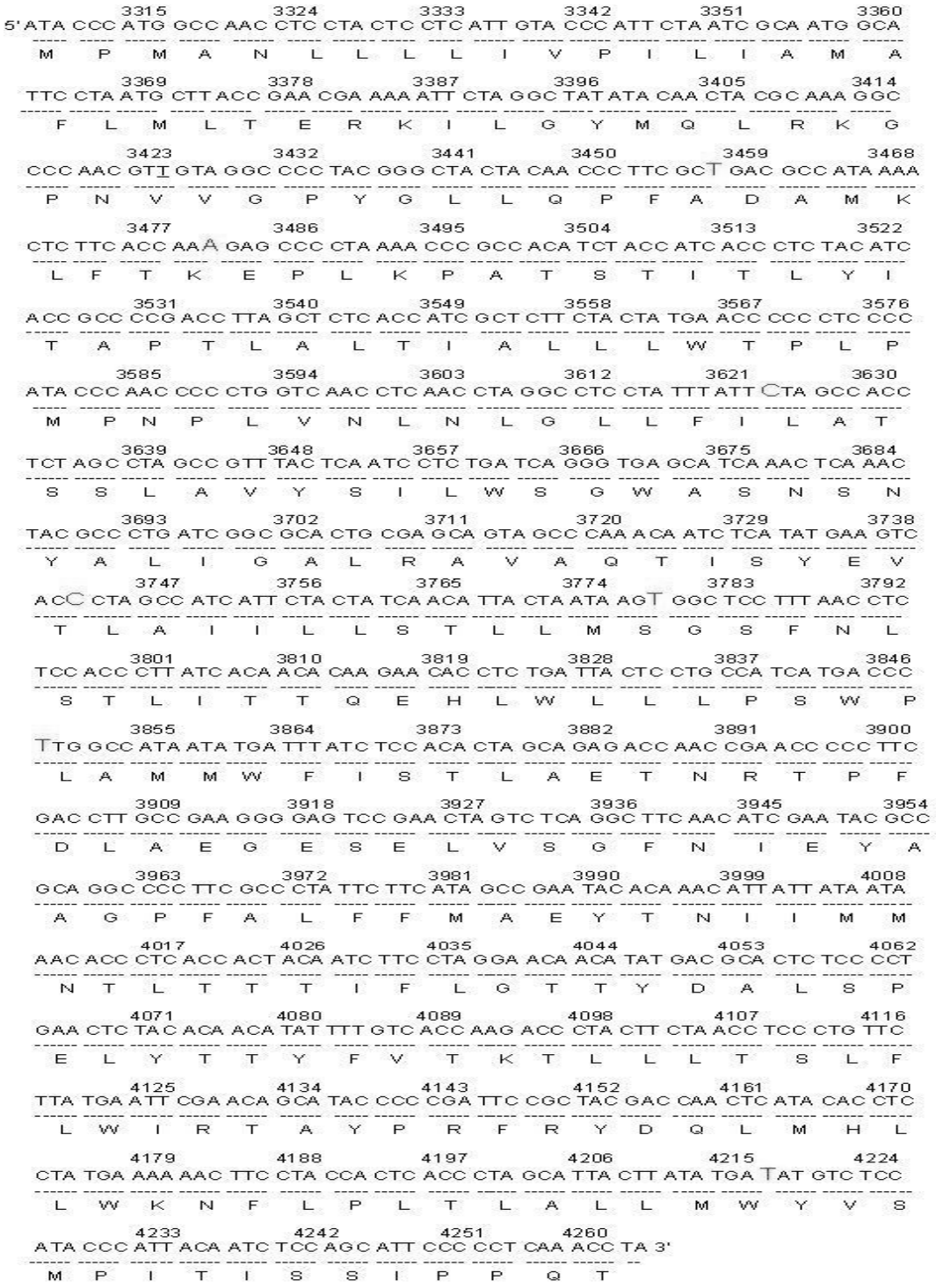

Figure 5.8: The nucleotides and amino acid of the MT-ND1 (mtDNA nt 3307-4262). Altered bases are highlighted in color.

6 Discussion

Our hypothesis is that mitochondrial dysfunction leads to altered mtDNA, which may in turn associated to the carcinogenesis. It could be primary or secondary effects. The specific aims in this work concentrated on (1) determining the extent of mtDNA deletions or duplications in the total mtDNA content, (2) the mitochondrial haplotype analysis in colorectal cancer to identify the high-risk population and (3) detection of new mtDNA mutations in patients with CRC.

6.1 Large scale mitochondrial deletion in colorectal cancer

In the last decade, mtDNA mutations have been reported to be associated with development and progression of cancer and colorectal cancer is probably the most studied cancer type in the mitochondrial field.
Mitochondria are the site of initiation of apoptosis, a fundamental biological process by which cells die in a controlled manner, combination of nuclear and mitochondrial DNA play a causative role in cancer. Different nuclear genes have been investigated in different cancers as Bax, Bak, etc, which play role in apoptosis and their location is on the mitochondrial membrane. The location of mtDNA near to the ROS production site makes it more vulnerable to oxidative injury and can be responsible for a portion of the increased mtDNA mutations often reported in cancer. Previous reports showed that 70% of the examined colon cancers displayed mtDNA mutations (Fliss, 2000).

Our results showed that 11 of 90 CRC patients had a 8.7 kb mtDNA deletion (12.2%) while this deletion was not found in 33 healthy controls (P=0.035).

The 8.7 kb deletion causes a loss or truncation of the structural genes. Defective respiratory enzymes containing protein subunits encoded by the deleted mtDNA may further enhance free radical production, resulting in more profound oxidative damages in patients.
Even mtDNA deletions were found in many diseases such as kern/Sayre syndrome, chronic external ophthalmology, etc, but our patients did not show any neurological as well as muscular disorders. The reason may be depend on threshold effects and heteroplasmy in cancer patient's mtDNA.

In the presence of mtDNA deletions, which may be caused by ROS or free radicals generated during aerobic metabolism, sensitive cells are deprived of ATP (due to the defective respiratory functions of mitochondria) and then they run into a state of energy crisis through a ‘vicious cycle’ as proposed by (Wei 1998). This

'vicious cycle' may have catastrophic consequences and is accelerated by electron leakage from defective mitochondria; as such, it may play an important role in the pathophysiology of CRC patients.

Since deletion in mtDNA is a sporadic event it could confirm probable secondary effect or association between deletions in mtDNA and circumstance of colonic cancer. A specific deletion in mtDNA of 4977 bp, called the "common deletion", has been detected at higher frequency in adult brains and hearts whereas it has not been detected in fetal and young tissues (Cortopassi *et al.*, 1992, Wallace, 1992). The 8.7 kb deletion was not reported in healthy old controls (>50) as we did not found in our controls as well.

So we conclude that the 8.7 kb deletion can be a secondary effect of cancer process which is not inherited. This is the first report on large scale deletion in colon cancer.

6.2 Mitochondrial haplotype analysis in colorectal cancer

We also analyzed the correlation between cancers and mitochondrial haplogroups. The relationship between colorectal cancer and each of 9 major mitochondrial haplogroups were examined in Iranian CRC patients.

This is the first study to trace mtDNA HVSI variants in CRC patients of the Persian population. We concluded from the tested data that haplogroup K is considerably more frequent in CRC patients (P=0.001) (table 5 III).

The mitochondrial polymorphisms in haplogroup K might play a causative role in predisposing to colorectal cancer. Substitutions in the D-loop may be part of a haplotype with mutations elsewhere in the mtDNA. Also mtDNA HVS-I mutations may cause energy deficiency in stressful situations during a vulnerable developmental period (Arnestad, 2002). The hypothesis is that on their own some polymorphisms are selectively neutral, but in specific combinations they act in a synergistic, deleterious manner with established pathogenic mtDNA mutations to increase the risk of disease expression or to produce a more severe clinical outcome. The rich variability within HVS-I compared with the relatively constant constellation within the gene regions provides useful markers for pathogenetic studies.

Our data showed that patients with colorectal cancers clustered in haplogroup K have a significantly higher frequency (9.5%) when compared with controls(0%), implicating a possible association of haplogroup K to colorectal cancer. Of course it can depend on sample size or definition of haplogroup K and its sub-

haplogroups. Our data was done according to European haplogroup agreements. We have not identified any haplogroup K in our control samples and the other studies in our group showed that this haplogroup is also (0%) in Iranian controls (Hassani-Kumleh *et al*., 2006). So all together we can say that this haplogroup is not found in Iranian controls.

Our result for the haplogroup K is consistent with a recent study, in which individuals bearing haplogroup K were reported to have an increased risk for breast cancer (Ren-Kui *et al*., 2007). However, it is interesting to note that mtDNA haplogroups K and J have apparently protective effects on Parkinson's disease (Van der Walt, 2003). Haplogroup J has also been found to increase the risk for disease expression of Leber's hereditary optic neuropathy (Brown, 2002). Also an increased risk for haplogroups K and A to develop multiple sclerosis was previously reported in Iranian patients by our group.

Our findings confirm that the haplogroup K may play some role to develop disease such as cancer, MS and etc.

6.3 Single nucleotide polymorphisms in the mtDNA D-loop region in human CRC

The D-loop of mtDNA is highly polymorphic among individuals. Previous *in vitro* studies showed that mtDNA variations have subtle effects on mitochondrial respiratory chain activity (Polyak, 1998). However, incorporation of many subtle changes may lead to significant consequences.

In this part of the study we focused on mtDNA D-loop mutations in CRC. We directly analyzed the sequences in the D-loop region of mtDNA in 40 CRC in comparison to 150 control samples. Variations in the D-loop region were found in both CRC patients and healthy people but the frequency of SNPs in CRC patients was higher than in control samples. We found 13 new polymorphisms that had not been recorded in mitochondrial database [Mitomap database: http://www.mitomap.org] (table 5.V).

It is possible that mtDNA variations are related to nuclear DNA mutations in CRC. The accumulation of mtDNA mutations seems to be a useful predictor of colorectal carcinogenesis.

The frequencies of eight SNPs (A16163G, C16186T, T16189C, C16223T, T16224C, C16295T, T16311C, T16519C) were found to be significantly different ($P \leq 0.05$) between the samples and controls. It shows that because of high energy

consumption in cancer cell in one side and no repair system in mtDNA in another side, the rate of mutations become higher than in normal cells.
We also detected one T→ C transition at position np16519 in 28 samples out of 40 patients (70%). In a recent study researchers reported that the T16519C variation worsens the outcome of pancreatic cancer patients, possibly because it is involved in altering cellular metabolism (Basso *et al*., 2007). Because we found this mutation frequently in our healthy controls (43%) and could not find any differentiation among our patients with respect to the alleles at this mutation, we conclude that the functional significance of this mutation needs further investigation.

6.4 Search for mtDNA mutations in CRC samples causing mitochondrial disease

We have investigated the previously reported mtDNA point mutations associated to mitochondrial diseases in our CRC tissues samples as well.
In our samples we did not find any known mtDNA point mutations responsible for mitochondrial diseases. We conclude that the known pathogen mutation in mitochondrial disorders can not be causative in colon cancer. Even some report has published for role of those mutations in cancer (Lorence *et al*., 2003).

6.5 Analysis of mitochondrial somatic mutation in genes involved to respiratory complex I

We analyzed the genes encoding subunits of the respiratory complex I., using TTGE mutations detection method and SSCP method.
Seven somatic mutations in the MT-ND1 gene were found. Six of these mutations were synonymous mutations. The T4216C mutation that was detected in 26% of tested samples resulted in the substitution of tyrosine with the amino acid histidine at amino acid position 304.
The T4216C mutation was recently reported in LHON patients (Povalko *et al*., 2005). The patients in our study who carried the T4216C mutation had no known vision problems. It is difficult to speculate about the role of this mutation in CRC. LHON mutations were shown to affect complex I in mitochondria and other investigators found other mutations in different parts of this gene. To show pathogenicity of this mutation or find its role in cancer, more investigations particularly in mtDNA of LHON patients are needed. Because cancer is a multifactorial disease and a causative mutation which leads to blindness and cancer has not been reported, we conclude that this mutation may be a secondary effect or rare polymorphism.

7 Summary

The appearance of mtDNA abnormalities including deletions, duplications, insertions or combinations of them in colorectal cancer (CRC) patients was investigated in this work. Our result showed that 11 of 90 CRC patients had a 8.7 kb mtDNA deletion (12.2%) while this deletion was not found in 33 healthy controls. (P=0.035)
We conclude that the 8.7 kb deletion can be a secondary effect of the cancer process, which is not inherited. This is the first report on large scale deletions in CRC.
We also analyzed the correlation between cancers and mitochondrial haplogroups. The relationship between CRC and each of 9 major mitochondrial haplogroups were examined in Iranian CRC patients. This is the first study to trace mtDNA HVSI variants in CRC patients of the Persian population. Our data showed that patients with CRC have a significantly (P=0.001) higher frequency of haplogroup K (9.5%) when compared with controls (0%). The mitochondrial polymorphisms in haplogroup K might play a causative role in predisposing to CRC.

Variations in the D-loop region were found in both CRC patients and healthy people but the frequency of SNPs in CRC patients was higher than in control samples. We found 13 new polymorphisms that had not been recorded in the mitochondrial database. We also detected one T→C transition at np16519 in 28 out of 40 patients (70%). In a recent study researchers reported that the T16519C variation worsen the outcome of pancreatic cancer patients, possibly because it is involved in altering cellular metabolism. Because we found this mutation frequently in our healthy controls (43%) and could not find any differentiation among our patients with respect to the alleles at this mutation, we conclude that the functional significance of this mutation needs further investigation.
We also analyzed somatic mutations in mitochondrial genes encoding subunits of the respiratory complex I. Seven somatic mutations in the MT-ND1 gene were found. Six of these mutations were synonymous mutations. The T4216C mutation that was detected in 26% of the CRC tested samples resulted in the substitution of tyrosine with the amino acid histidine at amino acid position 304.
The T4216C mutation was recently reported in LHON patients (Povalko *et al.*, 2005). The patients in our study who carried the T4216C mutation had no known vision problems. It is difficult to speculate about the role of this mutation in CRC. LHON mutations were shown to affect complex I in mitochondria and other investigators found other mutations in different parts of this gene. To show pathogenicity of this mutation or find its role in cancer, more investigations particularly in mtDNA of LHON patients are needed. Because cancer is a multifactorial disease and a causative mutation which leads to blindness and cancer has not been reported, we conclude that this mutation may be a secondary effect or rare polymorphism.

7 Zusammenfassung

In dieser Doktorarbeit wurde die Erscheinung von mtDNA-Abnormalitäten einschließlich Deletionen, Duplikationen, Insertionen oder Kombinationen davon untersucht. Unseres Ergebnis zeigte, dass 11 von 90 Patienten mit kolorektalem Krebs (*colorectal cancer*, CRC) eine 8.7 kb-Deletion in der mtDNA aufwiesen (12.2 %), während diese Deletion in der gesunden Kontrollgruppe nicht beobachtet wurde (P=0.035).
Wir schließen daraus, dass die 8.7 kb-Deletion ein Sekundäreffekt des Krebs-Prozesses sein könnte, der nicht vererbt wird. Hiermit werden erstmalig Deletionen dieser Größenordnung in CRC beschrieben.

Es wurde ebenfalls die Korrelation zwischen Krebs und mitochondrialen Haplogruppen analysiert. Die Verbindung zwischen CRC und jeder der 9 wichtigsten mitochondrialen Haplogruppen wurde in iranischen CRC-Patienten untersucht. Dieses ist die erste Untersuchung über die mtDNA HVSI-Varianten in CRC-Patienten der persischen Bevölkerung. Unsere Daten zeigten, dass Patienten mit CRC eine signifikant (P=0.001) höhere Frequenz (9.5 %) der Haplogruppe K im Vergleich zur Kontrolle (0%) haben. Der mitochondriale Polymorphismus in der Haplogruppe K könnte eine ursächliche Rolle in der Prädisposition für CRC spielen. Unsere Ergebnisse bestätigen, dass die Haplogruppe K hinsichtlich der Entwicklung von Krankheiten wie Krebs eine Rolle spielen könnte.

Variationen in der D-loop-Region wurden sowohl in CRC-Patienten als auch in gesunden Kontrollpersonen gefunden, aber die Frequenz von SNPs (*single nucleotide polymorphisms*) in CRC-Patienten war höher als in Kontrollproben. Wir fanden 13 neue Polymorphismen, die zuvor nicht in der mitochondrialen Datenbank registriert worden waren. Wir entdeckten auch einen T→C Übergang an der Position np16519 in 28 von 40 Patienten (70 %). In einer aktuellen Studie berichteten Forscher, dass die T16519C-Variation den Verlauf von Bauchspeicheldrüsenkrebs in Patienten verschlechtert, möglicherweise aufgrund ihrer Beteiligung am Zell-Metabolismus. Da wir diese Mutation auch in unseren Kontrollen fanden (43 %) und hinsichtlich dieser Mutation keinen Unterschied zwischen unseren Patienten feststellen konnten, schließen wir daraus, dass die funktionelle Bedeutung dieser Mutation weiter untersucht werden muss.
Wir analysierten ebenfalls somatische Mutationen in mitochondrialen Genen, die am Atmungskomplex I beteiligt sind. Es wurden sieben somatische Mutationen im MT-ND1 Gen gefunden. Sechs davon sind synonyme Mutationen. Die T4216C Mutation, die in 26 % der untersuchten Proben detektiert wurde, bewirkte den Austausch eines Tyrosins durch die Aminosäure Histidin an der Aminosäure-Position 304.
Die 4216-Mutation wurde kürzlich als ursächliche Mutation im LHON-Patienten beschrieben. Es ist schwierig, über die Rolle dieser Mutation in CRC zu diskutieren. Es wurde gezeigt, dass LHON-Mutationen den Komplex I in

Mitochondrien beeinflussen, und andere Forscher fanden weitere Mutationen in verschiedenen Teilen dieses Genes. Um die Pathogenität dieser Mutation zu zeigen oder ihre Rolle im Krebs zu finden, werden weitere Untersuchungen insbesondere der mtDNA von LHON-Patienten benötigt. LHON wird mit Blindheit in Verbindung gebracht, doch wir haben keine Zeichen von Blindheit bei unseren Patienten festgestellt. Da Krebs eine multifaktorielle Krankheit ist und keine ursächliche Mutation, die zu Blindheit und Krebs führt, beschrieben ist, folgern wir, dass diese Mutation entweder ein Sekundäreffekt oder ein seltener Polymorphismus ist.

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Appendix

A.1 Southern Blot:

Southern transfer and hybridization (Southern 1975) is used to study how genes are organized within genomes by mapping restriction sites in and around segments of genomic DNA for which specific probes are available. Genomic DNA is first digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through a standard agarose gel. The DNA is then denatured *in situ* and transferred from the gel to a solid support (usually a nylon or nitrocellulose membrane). The DNA attached to the membrane is hybridized to a labeled DNA, RNA, or oligonucleotide probe, and bands complementary to the probe are located by an appropriate detection system, for example, by autoradiography.

DNA fragments are carried from the gel in an upward flow of liquid and deposited on the surface of the solid support (Southern 1975). The liquid is drawn through the gel by capillary action that is established and maintained by a stack of dry absorbent paper towels.

The rate of transfer of the DNA depends on the size of the DNA fragments and the concentration of agarose in the gel. Small fragments of DNA ≤ 1 kb are transferred almost quantitatively from a 0.7% agarose gel within 1 hour; larger fragments are transferred more slowly and less efficiently.
For example, capillary transfer of DNAs > 15 kb in length requires at least 18 hours, and even then the transfer is not complete. The efficiency of transfer of large DNA fragments is determined by the fraction of molecules that escape from the gel before it becomes dehydrated. As elution proceeds, fluid is drawn not only from the reservoir, but also from the interstices of the gel itself. This flow reduces the gel to a rubbery substance through which DNA molecules cannot easily pass. The problem of dehydration due to lengthy transfer can be alleviated by partial acid/base hydrolysis of the DNA before capillary transfer (Wahl *et al*., 1979; Meinkoth and Wahl 1984). The DNA in the gel is exposed to weak acid (which results in partial depurination), followed by strong base (which hydrolyzes the phosphodiester backbone at the sites of depurination). The resulting fragments of DNA (-I kb in length) can then be transferred rapidly from the gel with high efficiency.
The depurination reaction must not proceed too far; otherwise, the DNA will be cleaved into small fragments that are too short to bind efficiently to the solid support. Depurination/hydrolysis can also cause the bands of the final autoradiograph to assume a "fuzzy" appearance, presumably because of increased diffusion of DNA during transfer. Therefore, depurination/hydrolysis is

recommended only when it is known ahead of time that the target DNA fragments will exceed 15 kb in length. (Sambrook & Russell, 2001)

A.1.1 Southern blot procedure using DIG DNA labeling and detection kit

1. Labeling DNA probe

10ng-3µg probe DNA (genomic, plasmid or gene clean fragment) was diluted in dsH_2O to a final volume of 15µl. For a control labeling reaction 5µl of control DNA 2 (vial 2) was used and 10µl double distilled water was added.
DNA was denatured by boiling 10 min; it was chilled quickly on ice to prevent reannealing of strands.
2µl of hexanucleotide mix (vial 5) and 2µl dNTPs labeling mix (vial6) and 1µl Klenow enzyme labeling grade (vial 7) were added, mixed, and briefly centrifuged.
The reaction was incubated overnight at 37°C. The reaction was stopped by adding 2 µl 0.2M EDTA (pH 8) and heat inactivation at 65°C for 10 min. The probe was boiled for 10-20 min before using.

2. Determination of labeling efficiency

A series of dilutions of DIG-labeled DNA was applied to a small strip of positively charged nylon membrane.
A part of the nylon membrane was preloaded with defined dilutions of DIG-labeled control DNA (vial 4) which were used as standards.
The nylon membrane was subjected to immunological detection with anti-digoxigenin-AP conjugate (vial 8) and the freshly prepared color-substrate solution.
The color intensities of the dilution series of DIG-labeled DNA and control DNA were compared and amounts calculated.

3. Digestion of genomic DNA

15 µl of genomic DNA was aliquoted into fresh labeled tubes to make up appropriate digestion mixture:
29 µl H_2O, 5 µl enzyme, 5 µl enzyme buffer including BSA
40 µl of digestion mixture were added to each tube and incubated overnight in a 37ºC waterbath.

4. DNA fragment separation by electrophoresis through a standard agarose gel

To prepare 100 ml of a 0.7% agarose solution, 0.7 g agarose was measured into a glass beaker and added 100 ml 1X TBE. The suspension was then microwaved until agarose was dissolved and solution was clear. The solution was allowed to cool to about 55° C before pouring. Gel tray was now prepared. It is positioned vertically such that the teeth are about 1-2 mm above the surface of the tray. The 50° C gel solution was poured into tray to a depth of about 5 mm and is allowed to solidify about 20 minutes at room temperature. To run, the comb was gently removed. Then the tray was placed in electrophoresis chamber and covered with electrophoresis buffer (the same buffer used to prepare the agarose). To prepare samples for electrophoresis, 1 µl of 6x gel loading dye is added for every 5 µl of digestion solution. These samples were mixed and loaded.Now, the samples were electrophoresized at 20-30 volts for approximately 6-7 hours.

5. Transfering DNA from gel to membrane

The gel was transferred to a sealable tupperware container and incubated for 40 min at room temperature in 0.25 HCl to depurinate. Subsequently it was rinsed twice in MilliQ
It was incubated 2X 20 min in denaturation solution to cleave depurination sites and then 30 min in neutralization solution
Capillary transfer was set up as shown in the following figure using 20X SSC as the transfer solution. Transfer was done overnight.

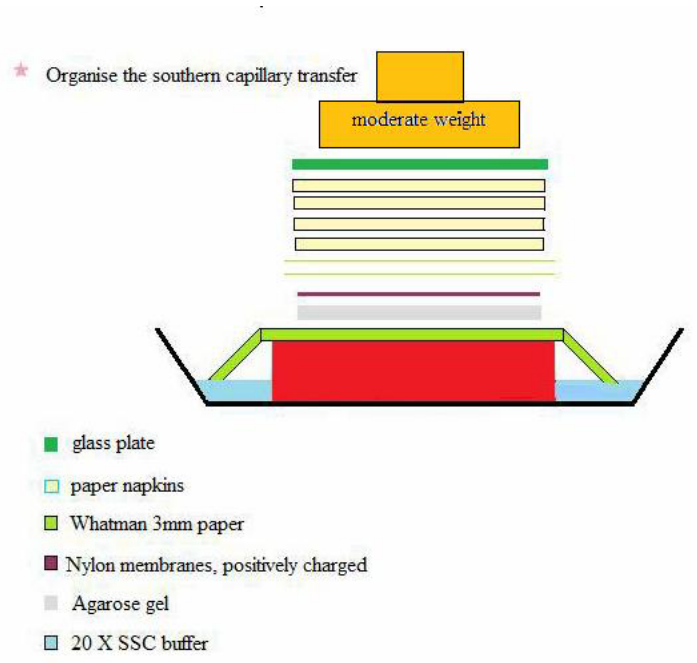

Figure A.1: Capillary transfer of DNA to positively charged membrane

6. Fixation procedure

Top corner of gel and membrane was cut off left for orienting blot. Gel was stained and photographed to assess transfer efficiency.
Membrane was rinsed briefly in 2X SSC and is transfered to sealable tupperware container and was baked at 80°C for 2 h under vacuum

7. Probe Membrane

Membrane was pre-hybridized on rocker for a minimum of 3hr at 68°C in hybridization buffer
Probe was boiled in 40ml hybridization buffer at least 10min to denature. Membrane DNA was hybridized side down overnight at 68°C (42° for lower homology) in boiled hybridization buffer/Probe mix, rocking optional.
Used hybridization buffer/probe mix can be stored at -20°C and used repeatedly

8. Membrane Detection

Membrane was washed 2X 15min at room temperature with 2X SSC, 0.1% SDS on rocker then it was washed 2X 15min at 42°C with 0.5X SSC, 0.1% SDS on rocker and it was rinsed in washing buffer then was incubated 30min at room temperature in blocking solution (rocking is optional).
It was incubated 30min at room temperature with 30ml blocking solution + 2µl anti-DIG-AP (vial 8). Anti-DIG-AP was centrifuged for 5 min at 10000 rpm in original vial prior before each use and the necessary amount was pipette carefully from the surface (1:5000 in blocking solution). It was conjugated (premix before adding to blot).
In the next step the membrane was washed 2X 15min at room temperature with 100ml washing buffer on rocker and then it was equilibrated 5min at room temperature with 20ml detection buffer.
After that the membrane was incubated in 10 ml freshly prepared colorsubstrate solution in an appropriate container in the dark. It was not shaken during color development. The reaction was usually completed after 20 h.
When desired spot or band intensities were achieved the reaction was stopped by washing the membrane for 5 min with 50ml of sterile double dist. water or with TE- buffer. Results were documented by photocopying the wet filter.

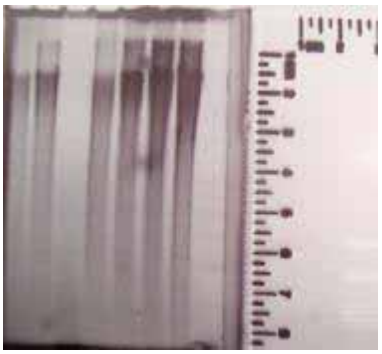

Figure A.2: Agarous gel before transfer DNA to positively charged nylon membrane

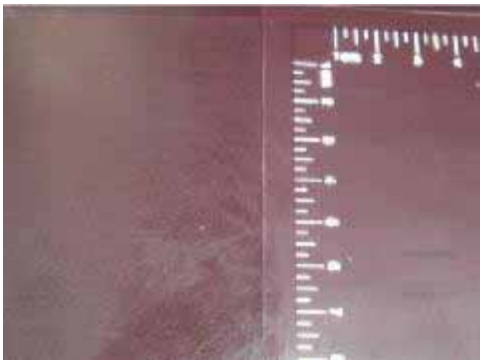

Figure A.3: Agarose gel after transfer DNA to positively charged nylon membrane

A.2 Temporal Temperature Gradient Gel Electrophoresis (TTGE):

Researchers have used different screening methods including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), heteroduplex analysis (HA), single stranded conformation polymorphism (SSCP), chemical mismatch cleavage, enzyme mismatch cleavage (EMC), restriction fragment length polymorphism (RFLP) and Temporal Temperature Gradient Gel Electrophoresis (TTGE).

mtDNA is highly polymorphic, and these single nucleotide polymorphisms are usually homoplasmic. Thus, a unique requirement for the molecular diagnosis of mtDNA disorders is the ability to detect heteroplasmic mtDNA mutations and to distinguish them from homoplasmic sequence variations. TTGE is sensitive and specific for detection of mtDNA heteroplasmy. In addition, each heteroplasmic variation produces a distinctive band pattern.

TTGE was first introduced by Yoshino et al., 1991. It is based on the sequence specific melting behavior of wild-type and mutant DNA in a temporal temperature gradient that increases gradually in a linear fashion over the length of the electrophoresis.

Temporal Temperature Gradient Gel Electrophoresis (TTGE) exploits the principle on which DGGE is based, without requiring a chemical denaturing gradient.

The difference between TTGE and TGGE is that TGGE has a fixed temperature gradient from top to bottom of the gel but in TTGE, the temperature at any location of the entire gel is the same at any given time but changes with respect to time (temporal temperature). Thus, it is easier to modulate the temperature over time and to provide a wider separation range that increases sensitivity. Thus, several fragments with different melting behaviors can be analyzed simultaneously. TTGE has been shown to be a powerful tool for the detection of novel nuclear DNA mutations (Marsh et al, 1998, Bjorheim et al, 1998, Kressner et al, 1998).
Amplified mutant and wild-type DNA from the gene of interest is loaded onto a polyacrylamide gel containing a constant concentration of urea. During electrophoresis, the temperature is increased gradually and uniformly. The result is a linear temperature gradient over the length of the electrophoresis run. Thus, a denaturing environment is formed by the constant concentration of urea in the gel in combination with the temporal temperature gradient. With no chemical gradient required, rapid, high-throughput screening is possible.

According to figure 4.3 during electrophoresis, double-stranded DNA will become partially denatured. When DNA begins to denatureits electrophoretic mobility decreases. Mutant and wild-type molecules are separated on the gel as they begin to denature at different temperatures. TTGE procedure was done using DCode Universal Mutation Detection System. (Bio Rad: Catalog Numbers 170-9080)

A.3 Single-Stranded Conformational Polymorphisms (SSCP):

Single-strand confirmation polymorphism analysis (SSCP) was originally described by (Orita et al. 1989a). SSCP is a powerful and robust method for the detection of DNA sequence changes (single-base substitutions) based on shifts in electrophoretic mobility.
Single-stranded DNA molecules fold into complex three-dimensional structures as a result of intrastrand base pairing. Single strands of equal length but different sequence can therefore vary considerably in electrophoretic mobility as a result of

the looping and compaction caused by intrastrand pairing. Alteration of the nucleotide sequence of the molecule by as little as a single base can reshape the secondary structure, with consequent changes in electrophoretic mobilities through native gels. SSCP exploits the differences in mobility between wild-type and mutant strands of DNA (Orita et al. 1989a, b, 1990; Ainsworth et al. 1991; Dean and Gerrard 1991; Condie et al. 1993; Glavac and Dean 1993). (sambrook & Russell, 2001).

The major advantage of SSCP is that many individual PCR products may be screened for variation simultaneously. Most researchers use SSCP to reduce the amount of sequencing necessary to detect new alleles at loci of interest (Sweetman et al. 1992) or to better estimate allele frequencies of populations.

Double-stranded mutant and wild-type samples are first denatured into single strands and then loaded onto the gel. Differences in mobility of the single strands between the control wild-type DNA and the other samples indicate a mutation. SSCP is a widely used mutation screening method because of its simplicity. However, since experimental conditions cannot be predicted for a particular DNA, it is important to optimize gel electrophoresis conditions. The ability to detect single base changes rests on several factors which optimize band resolution.

1. Fragment size: The estimated efficiency for detecting single base changes is 90–95% for fragments less than 350 bp, but the efficiency will decrease as the length of fragment increases.

2. Gel temperature: Migration differences due to a single mutation are observed at buffer temperatures between 4–25 °C. Optimal temperature must be determined empirically.

3. Gel additives: In some cases, 5–10% glycerol can be added to the gel to improve the mobility differences in fragments. Since glycerol can reduce the mobility of single-stranded DNA fragments at low temperatures, it is typically used with gels run near room temperature.

4. Crosslinking ratio: The acrylamide/bis ratio determines the percent of crosslinking. SSCP gels generally use 1–2 % crosslinking. Acrylamide concentrations will vary from 5% to 10%.

5. Buffer concentration: Gels are run with TBE buffer at concentrations of 0.5x or 2.0x. In some cases, 2x TBE appears to give slightly better results than 0.5x TBE.

Before loading on the gel, double-stranded DNA is denatured by alkali or, more commonly, by a combination of formamide and heat. If the DNA has been

amplified from a heterozygote, a proportion of the denatured complementary strands may reanneal to one another before the DNA is loaded onto the gel. A proportion of these molecules will be heteroduplexes formed by annealing of complementary wild-type and mutant strands. The presence of both single-stranded and heteroduplex DNA in the same sample provides an opportunity to use two scanning methods simultaneously. If the DNA under test has been amplified from a haploid organism or from a homozygous diploid, the formation of heteroduplexes can be promoted by adding an appropriate amount of the corresponding segment of wild-type (Axton and Hanson 1998)

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