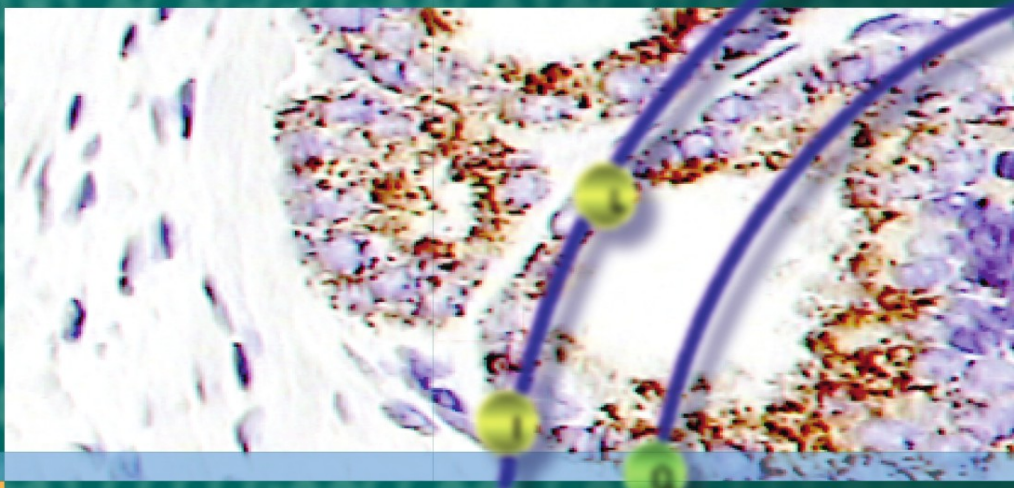

Mitochondrial Genetics and Cancer

Gabriel D. Dakubo

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Preface

With very few exceptions, eukaryotic cells possess two interdependent genomes, chromosomal and extra-chromosomal. Over the past several decades, cancer research has focused primarily on deciphering the intricate alterations in the chromosomal genome, with until recently, very little attention to its cytoplasmic counterpart. In spite of the enormous complexity of the nuclear genome, which we now fully appreciate after completion of the human genome project, the efforts of cancer researchers are commendable in terms of the tremendous gains made in unraveling the numerous genetic changes in cancer. These changes include discoveries of tumor suppressor genes, oncogenes, and caretaker genes that are often mutated in cancer. Recent studies of genomic profiles are uncovering even more altered and mutated genes in cancer. Besides these findings, several therapeutic targets for chemotherapy are currently made from studies of altered nuclear genetic pathways. Inspite of all these positive efforts, the war on cancer, declared in 1971 by Richard Nixon, is far from being worn. Indeed, the failure of chemotherapy is obvious to clinicians, oncologists, and their patients alike. Moreover, the global incidence and prevalence of cancer continue to rise. What are we missing? Which direction should we be taking? Of course, modern integrated nuclear genomics, proteomics, and metabolomics should provide important clues to carcinogenesis, but the contribution of cytoplasmic genetic alterations to carcinogenesis cannot be neglected.

There is extensive bidirectional trafficking and cross talk between the two eukaryotic cell genomes, and these pathways are exploited by the altered cancer cell genetics to its advantage. Thus, targeting just one component of the intricate changes seems insufficient in defeating the cancer cell, because it will quickly adapt using the other genome to sustain its existence. Ignorance of the role of mitochondria in cancer had been a huge oversight and I am pleased that this era is quickly coming to an end. Indeed, Otto Warburg recognized cancer several decades ago as a mitochondrial genetic disease. Since his initial hypothesis says that the genesis of cancer is the damage to the respiration inside the cell, recent evidence conclusively suggests that mitochondrial genetic damage indeed underlies the carcinogenic process.

As a medical student in the late 1980s and early 1990s, all the knowledge we acquired about the mitochondrion was nothing beyond its essential roles in energy production, metabolism of food substrates and inborn errors of metabolism and the diseases they caused. The mitochondrial genome and human diseases were never mentioned in medicine and oncology lectures. This was not accidental and therefore not surprising because the mitochondrial genome had just been published at about that era (1981), and mitochondrial mutagenesis in aging was provided in 1984, with the involvement of mitochondrial genetic alteration in human neurodegenerative disease being published in 1988. Following these discoveries, our knowledge on mitochondrial cytopathies has expanded considerably. Mitochondrial genetics in diseases such as progressive external ophthalmoplegia (PEO), Pearson syndrome (PS) and Kearns-Sayre syndrome (KSS), mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), Leber's hereditary optic neuropathy (LHON), and neuropathy, ataxia, and retinitis pigmentosa (NARP) syndromes are well established and form part of clinical diagnostic algorithms in pediatrics and neurology (*see Mitochondrial Medicine* by DiMauro, Hirano and Schon). However, mitochondrial genome changes in cancer have lagged behind this progress in mitochondria and human diseases simply because mitochondrial mutations associated with cancer were only available in the late 1990s and the plethora of data on mitochondrial genetic alterations and possible causation of cancer is now just appearing at an exponential rate.

A rekindled scientific effort in the fight against cancer is required that integrates the genetics of both genomes. Not only will this renewed direction open up avenues to attack the cancer cell, but it also holds tremendous opportunity for us to appreciate the complexity of this disease. "*Mitochondrial Genetics and Cancer*" aims at making available in a composite form the genetic alterations of mitochondria to carcinogenesis, especially the contributions of this genome to cancer development and progression. The clinical importance and utility of these genetic alterations are not overlooked. It is my hope that cancer researchers, oncologists, clinicians, medical students, researchers in the pharmaceutical industry, and all interested in the fight against cancer will find this book useful and interesting. It is also my hope that this book will awaken our thinking about cancer research and therapy and stimulate young scientists into considering research in this exciting area. *Mitochondria and Cancer* (by Singh and Costello) is a complementary book to *Mitochondrial Genetics and Cancer*, and the reader is encouraged to consult the previous book as well, for some in-depth synthesis of mitochondrial metabolic alterations in cancer.

The chapters in this book are divided into three parts for convenient reference. Part 1 addresses general concepts including mitochondrial genetics, bioenergetics, metabolism, and control of apoptosis. It also examines three levels of communication between the mitochondria and nuclear genomes including tricarboxylic acid cycle enzyme mutations and cancer, nuclear-mitochondrial stress signaling in cancer, and the carcinogenic potential of nuclear integration of mitochondrial DNA fragments. Part 2 deals with mitochondrial genome alterations in cancer, including the contribution of these genetic changes to carcinogenesis. Part 3 focuses

on the clinical utility of mtDNA changes, with emphasis on mitochondrial genome changes in clinical specimens, early detection of cancer using mitochondrial genome changes, methodologies and methodological issues of measuring mtDNA changes, and finally the strategies and agents explored for targeting mitochondria to treat cancer.

This work could not have been completed without the tremendous sacrifice of my family – Zaneta, Bernard, Ethan, Collins, and my lovely wife Crescentia, to whom I am very grateful.

November 2009 Gabriel D. Dakubo

Contents

Part I
General Overview of Mitochondrial Genetics and Functions

Chapter 1
Basic Mitochondrial Genetics, Bioenergetics, and Biogenesis

Abstract Mitochondrial genetics is very unique and important in understanding mitochondrial diseases. This chapter addresses the basic science of mitochondria and mitochondrial genetics and functions. Without being too exhaustive, it serves to provide a general overview of mitochondrial biology necessary for the reader to appreciate mitochondrial genome changes in diseases, especially cancer. Thus, the organelle and its genomic structure, functions, and importantly our emerging knowledge of nuclear mitochondrial genomic linkages are summarized.

1.1 Introduction

Mitochondria are unique, indispensable eukaryotic cellular organelles that orchestrate several vital homeostatic functions of the cell. The eukaryotic cell in a symbiotic relationship with an α-proteobacteria acquired mitochondria putatively over 10 billion years ago, probably for the primary purpose of energy production. However, it is now evident that this organelle has evolved to assume several important cellular functions beyond that of a mere production of adenosine triphosphate (ATP).

Mitochondria possess four compartments: an inner space called the matrix, a unique protein-rich inner membrane, an inter-membrane space, and an outer membrane. The inner membrane houses the respiratory chain (RC) components that generate almost all the energy required for normal cellular functions. The operation of the RC is inherently coupled with the production of reactive oxygen species that are detrimental to biomolecules or can induce signaling pathways involved in the initiation and progression of cancer. Whereas the mitochondrion depends heavily on nuclear-encoded proteins for its functions, it possesses a semi-autonomous genome that contributes modest, albeit, critically important proteins that complement the nuclear imported components for energy production. Inherited entirely from the mother, the mitochondrial genome is only 16,568 bp, which is very small in comparison to the nuclear genome. A well-coordinated network of

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nuclear and mitochondrial proteins regulates the biogenesis of mitochondria. This chapter summarizes the basic genetics of mitochondria, and highlights how mitochondrial biogenesis is regulated. Of note, the current knowledge on mitochondrial genetics is still at its adolescence. The future should be filled with fascinating and surprising revelations with a tremendous impact on human health.

1.2 The Mitochondrion

Although often observed as elongated tubular structures, mitochondria are actually dynamic cellular organelles that undergo regular division (fission) and adjoining (fusion). Similar to other organelles, mitochondria are bound by an outer membrane. However, because of their unique functions in energy production, they also possess a protein-rich inner membrane, thus creating two separate membrane-bound spaces. The compartment in between the membranes is the inter-membrane space, and that inside the inner membrane is the mitochondrial matrix. The mitochondrial compartments house the mitochondrial genome and over thousand proteins for mitochondrial functions. The outer membrane contains several integral membrane proteins, including proteins that participate in cell death, those that form the translocase of the outer membrane (TOM) involved in mitochondrial protein import, and the voltage-dependent anion channel (VDAC). The inner membrane has a very large surface area, which is organized into convoluted structures called cristae (Fig. 1.1). Apart from the protein complexes of the RC, the inner membrane houses the adenine

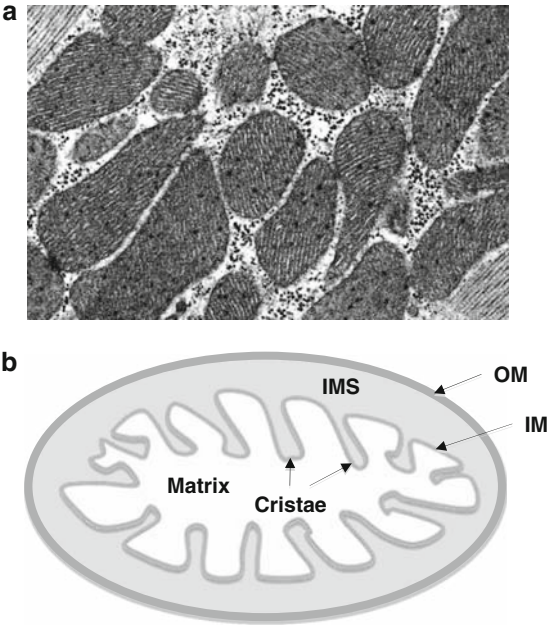

Fig. 1.1 *Mitochondria*. (**a**) An electron micrograph of mitochondria in cardiac myocyte. Note the tightly packed cristae. (**b**) A schematic drawing of mitochondrion illustrating the various compartments. *OM* outer membrane, *IM* inner membrane, *IMS* intermembrane space

nucleotide translocase (ANT), and proteins that form the translocase of the inner membrane (TIM), another component of the mitochondrial protein import machinery. The mitochondrial genome and its transcription and translational machinery (i.e., the tRNAs, rRNAs, and enzymes), as well as several metabolic enzymes involved in processes, such as β-oxidation and the tricarboxylic acid (TCA) or Krebs cycle, are resident in the matrix.

Well known as the power plants or powerhouses of the cell because of their central role in energy production, mitochondria are recognized to play vital roles in cell physiology and homeostasis that have important functional implications for numerous diseases, including cancer. These functions include intermediary metabolism, a synthesis of biomolecules, including steroids, lipids, cholesterol, heme, and amino acids. Mitochondria also regulate energy metabolism such as the TCA cycle, the RC, β-oxidation, and the urea cycle. Mitochondria help to maintain the redox balance of the cell, and together with the endoplasmic reticulum, regulate intracellular Ca^{2+} homeostasis. Mitochondria are also critical regulators of a number of signaling pathways that modulate cell proliferation, differentiation, and death, all of which are cardinal features central to the processes of malignant transformation (Kroemer 1997; Augenlicht and Heerdt 2001). Indeed, the mitochondrion should be regarded as the "master of the orchestra" controlling myriads of metabolic and signaling pathways important in the initiation and perpetuation of several disease states, including the malignant phenotype.

1.3 Contrasting Features of Mitochondrial and Nuclear Genetics

Unlike other organelles, the mitochondrion possesses a semi-autonomous genome. However, mitochondrial genetics deviates in several aspects from the classical Mendelian biparental mode of inheritance (Table 1.1), because this cytoplasmic genome is surprisingly primarily transmitted via the ovum. The unique features of mitochondrial genetics are readily observed in the structural organization of the genome, its mode of transmission, expression, and replication and importantly in the manner in which mutations in this molecule reflect in disease phenotypes. Moreover, mitochondrial diseases tend to have variable phenotypic expressions and therefore, are often more difficult to diagnose than diseases from nuclear genetic alterations. Hence, patients with mitochondrial cytopathies are usually correctly diagnosed by clinicians with either a high index of suspicion of mitochondrial cytopathy or those who have special interest in this field, and they are primarily neurologists and pediatricians. The contrasting features of mitochondrial and nuclear genetics are outlined below and summarized in Table 1.1.

Small Circular Genome: The human mitochondrial genome is a closed-circular 16,568 bp molecule, which is a minuscule entity compared to the staggering nuclear genome with ~three billion base pairs (Andrews et al. 1999; Venter et al. 2001). It is, therefore, relatively economical to sequence the mitochondrial genome for

Table 1.1 Features of michondrial genetics

Unique features	Brief description	Important implications
Small genome	Compared to the three billion base pairs of the haploid human nuclear genome, mtDNA is very small, being just a 16.6 kbp, making mtDNA look like a single nuclear gene. But the mtDNA molecule houses 13 different important structural genes, two rRNAs and 22 tRNAs	The small size of this genome means that it is reasonably cheap to analyze the entire molecule at a population level, or even for purposes of clinical diagnosis
Compact genome	Unlike the nuclear genome, the mitochondrial genes are compactly organized such that there are no intervening introns and other untranslated sequences	The compact genomic organization without intervening introns explains why coding genes are frequently mutated. However, disease is often not overt until a certain threshold of mutant copies is reached. Thus, accumulated mutations without functional decline or cell death make this genome a perfect "biosensor" of impending overt disease, including cancer
Maternal transmission	Transmission of mtDNA is nonMendelian, being primarily from the mother to her children	The clonal nature and hence, lack of recombination make this genome useful in phylogenetic and genealogical studies
Polyploidy	Compared to the diploid nuclear genome, each cell possesses 100–1,000s of mitochondrial genomes depending on the energy requirement	This high copy number of mtDNA facilitates easy assaying of biomarkers in clinical specimens with low cellularity
Heteroplasmy	The polyploid nature of the mitochondrial genome implies that mutations in some genomes will lead to the admixture of both maternally inherited wildtype and mutant copies, in a state referred to as heteroplasmy	Heteroplasmy is a hallmark of mitochondrial diseases, and in cancer, could be responsible for the adaptive bioenergetic pathways employed for survival
Homoplasmy	Usually, all copies of the inherited maternal genomes are the same, a state referred to as homoplasmy. In fast-dividing cells with heteroplasmic mutants, random segregation can lead to homogenous clonal mutants, also leading to homoplasmy, though this is a rare situation in disease states	In cancer, the near homoplasmic nature of some mutations could have important effects on the biology of the specific cancers. For example, an important mutation in complex I gene that is near homoplasmy can exert severe redox imbalances in such cell with accompanying altered signaling pathways that favor cancer survival
Threshold effect	This refers to the levels or percentage of mutant genomes in a heteroplasmic state that enables manifestation of disease	Because of the high mtDNA copy number per cell, cells can tolerate mutations up to ~80% before respiratory chain defects – hence, this genome serves as a repository of disease markers amenable to early detection and monitoring

High mutation rate	The mtDNA molecule has an accelerated mutation rate compared to the nuclear genome, probably as a result of poor protection, inefficient repair capability, and residence in vicinity to elevated reactive oxygen species	D-loop and coding region mutation rate can be as high as 10–17 fold that of the nucleus. The high mutation rate is due to the vulnerability of the molecule to ROS and other genotoxic substances
Unique genetic code	Mitochondrial genetics uses a code similar but not identical to the universal code; there are four codon differences between the mitochondrial genetic code and that of the nucleus	The unique mitochondrial genetic code implies, even if nuclear embedded mitochondrial genes were transcribed, that they will not be properly translated into mitochondrial proteins. Also, this unique genetic code requires that nuclear transfection of mitochondrial genes be preceded by sequence conversion for faithful mitochondrial protein translation
Stochastic segregation	During cell division, mitochondrial genomes are distributed randomly to daughter cells, suggesting in states of heteroplasmy; mutant and wildtype copies will randomly populate daughter cells. This is referred to as stochastic or mitotic segregation	Stochastic segregation can result in clonal homoplasmic mutant genomes in rapidly dividing cells, with possible adverse effects
Relaxed replication	Unlike the nuclear genome, mitochondrial genomes can replicate in postmitotic cells under nuclear genome control. This is referred to as relaxed replication	Relaxed replication in postmitotic cells could be important in maintaining normal respiratory functions in these cells
Genetic bottleneck	This phenomenon describes the process by which mutant mitochondrial genomes are transmitted from the mother to the offspring, such that phenotypic expressions in different generations are different	Knowledge of the mitochondrial genetic bottleneck is important in mitochondrial genetics because, if well established, it will enable proper genetic counseling in probands with severe mitochondrial genetic diseases. However, at present, this is very sporadic
Fusion/fission	These are dynamic mitochondrial activities that determine their shapes, and contents. Conjoining of different mitochondria constitutes fusion, while fission describes the fragmentation of a mitochondrion	Mitochondrial fusion will enable complementation

clinical diagnostics and other genetic studies at the population level. The small genome size also suggests that mining this genome for the discovery of diagnostic, prognostic, and companion diagnostic biomarkers is relatively easier than targeting the nuclear genome. Hence, the overlooked economic potential of this genome is worth considering.

Compact Genome Structure: Compared to the nuclear genome, the minuscule mtDNA is very compact with regard to how genes are organized. The manner of organization of the mitochondrial genome and transcriptional products differ in several respects from the nuclear genome. For example, nuclear genes are interrupted by large noncoding sequences referred to as introns, and the transcribed mRNAs contain short noncoding sequences at their 5′ and 3′ ends referred to as untranslated regions (UTRs). Mitochondrial genes lack these intervening sequences. Transfer RNA genes primarily punctuate the mitochondrial DNA genes encoding the various polypeptides. These tRNA genes facilitate processing of the polycystronic transcript to release the individual mRNAs for translation. Also, mitochondrial rRNAs are smaller and unmethylated and have relatively short polyA tails (∼1–10 adenine residues) compared to their cytoplasmic counterparts (Dubin et al. 1981). Similarly, mitochondrial tRNAs are smaller (averaging ∼70 bp) than those in the cytoplasm.

Uniparental Inheritance: The rules of biparental Mendelian inheritance are not obeyed by mitochondrial transmission. Mitochondrial genomes only follow maternal descents. Sperm contributes about 100 mtDNA during fertilization, but these molecules are actively depleted by ubiquitin-mediated degradation (Sutovsky et al. 1999, 2000). Therefore, only maternal mitochondrial genomes are inherited by the offspring (Giles et al. 1980). Thus, in contrast to nuclear genetics, there is no recombination associated with the transmission of mtDNA. This mode of mitochondrial inheritance has proven useful in evolutionary and genealogical studies, as well as forensic applications, including identification of missing people and victims of mass disasters. However, paternal transmission of mitochondrial genomes has been observed (Schwartz and Vissing 2002), and such inheritance patterns, though rare, require further studies because they could have important implications for some mitochondrial diseases.

Polyploidy, Homoplasmy, Heteroplasmy, and the Threshold Effect: Mitochondrial genetic material constitutes only ∼1% of the total cellular DNA, however, each cell possesses several copies of mtDNA, which is in contrast to the diploid nuclear counterpart. A eukaryotic cell contains hundreds of mitochondria, and each mitochondrion houses 2–10 genomes, implying that hundreds to thousands of mtDNA are present in each cell (Shuster et al. 1988; Wiesner et al. 1992). The high copy number makes this genome much easier than its nuclear counterpart to assay for biomarkers, especially in samples with low cellularity (Jones et al. 2001).

In healthy states or in the absence of somatic mutations, all copies of mtDNA in a cell or cells are the same as those inherited from the mother. This state of identical polyploid mitochondrial genomes in a particular cell or tissue is referred to as *homoplasmy* (clonal population of mtDNA). In normal aging, or disease states, however, some mitochondrial genomes sustain mutations, which can randomly or

selectively increase to various proportions in affected tissues. The coexistence of mutant and wildtype mtDNA molecules in cells or tissues is referred to as *heteroplasmy*, which is a known hallmark of mitochondrial diseases. It should be noted that homoplasmy could also be achieved when mutant genomes selectively or randomly segregate to homogeneity in a particular cell or tissue. This aspect of mitochondrial genome segregation raises the important issue of comparing disease sequences to that representative of the maternal inherited genome to correctly score somatic mitochondrial mutations.

Because of the high copy number per cell, different levels of mutant mtDNA copies will indicate different levels of mitochondrial dysfunction in various tissues and organs. Thus, depending on the type and genomic location of a mutation, as well as on tissue energy requirements, a critical threshold of mutant copies will lead to clinically overt disease. This threshold effect can vary substantially from about 60 to 80% (Lightowlers et al. 1997; Zeviani and Antozzi 1997). Emerging sensitive sequencing technologies that permit low levels of mutant mtDNA copies to be detected are commendable because they will enable early disease detection and monitoring as well as the implementation of available chemoprevention strategies (Tzen et al. 2007).

High Mutation Rate: The mitochondrial genome evolves at a much faster rate than the nuclear genome (Brown et al. 1979). The mtDNA mutates at a rate that is 10–17-fold higher than that of the nucleus. This high mutation rate is partly attributable to the inadequate protection of mtDNA molecules by histone and non-histone proteins (Richter et al. 1988), coupled with the probably low efficiency of DNA proofreading and repair mechanisms compared to the nuclear genome (Pinz and Bogenhagen 1998). Notably, mitochondria have an efficient base excision repair, and recently, a mismatch repair factor, YB-1, was uncovered in mitochondria (de Souza-Pinto et al. 2009). However, mitochondrial functions lead to the production of most of the cellular nucleic acid damaging reactive oxygen species, and therefore, the presence of poorly protected genomes should lead to increased mutagenesis that might outstrip the operations of the available repair systems, and hence, the accumulation of mutations with aging as well as early in disease evolution. The high mutation rate, coupled with the uniparental inheritance, has made the mtDNA molecule attractive for human population genetic and evolutionary studies (Stoneking 1994). It is becoming evident that this early and accelerated mutation rate is a feature of carcinogenesis as well.

Modified Genetic Code: Mitochondrial genes are transcribed and translated in the mitochondrion using mitochondrial translation machinery, which employs a slightly different code from the universal genetic code (Table 1.2). The universal code chain termination codon, UGA, codes for tryptophan in mitochondria, while two arginine codes, AGA and AGG, are chain terminators in mitochondria. Codon AUA in mitochondria codes for methionine instead of isoleucine as dictated by the universal code, and finally, AUU codes for isoleucine during chain elongation, but can code for methionine to be used for chain initiation, as is the case of mitochondrial ND2 gene. These unique features of mitochondrial translation imply that studies employing genetic engineering of mitochondrial structural genes to be

Table 1.2 The vertebrate mitochondrial genetic code

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

[a]Nuclear code; °ND2 initiation code

expressed in the nucleus and translated in the cytosol require sequence conversion to that of the universal code for an accurate mitochondrial protein synthesis.

Relaxed Replication: The replication of nuclear genome is linked to the cell cycle. On the contrary, mtDNA molecules undergo continuous replication even in postmitotic cells (Bogenhagen and Clayton 1977). Mitochondrial DNA replication is, therefore, uncoupled from the cell cycle, a condition referred to as relaxed replication. This mode of replication has important implications for the propagation of mutant mtDNA molecules. In a heteroplasmic state, mutant and wildtype molecules could potentially replicate at different rates, which can change the threshold of mutant copies. For instance, expansion of functionally important mtDNA mutations in postmitotic cells appears to result from positive selection. In this case, it is believed that the defective mitochondria preferentially proliferate as a result of nuclear-regulated compensatory mechanisms to overcome the energy deficiency of the cell. Similarly, large-scale genome deletions result in the formation of smaller circular molecules. Experimental evidence indicates that these deleted molecules repopulate cells faster than wildtype molecules under relaxed replication (Diaz et al. 2002).

Stochastic Mitotic Segregation: Mitochondrial genomes undergo stochastic segregation to daughter cells at cell division. During mitosis, a cell first duplicates its nuclear genome such that following cytokinesis, each daughter cell acquires identical nuclear genes as the parental cell. Roughly, equal numbers of organelles are distributed randomly to each daughter cell during this process, which implies that mitochondria and their genomes will segregate to daughter cells in an equal but unpredictable manner. Thus, mutant and wildtype mtDNA molecules will randomly populate daughter cells (Fig. 1.2). In contrast to postmitotic cells where relaxed replication appears to control mitochondrial genotypes, in proliferating epithelial

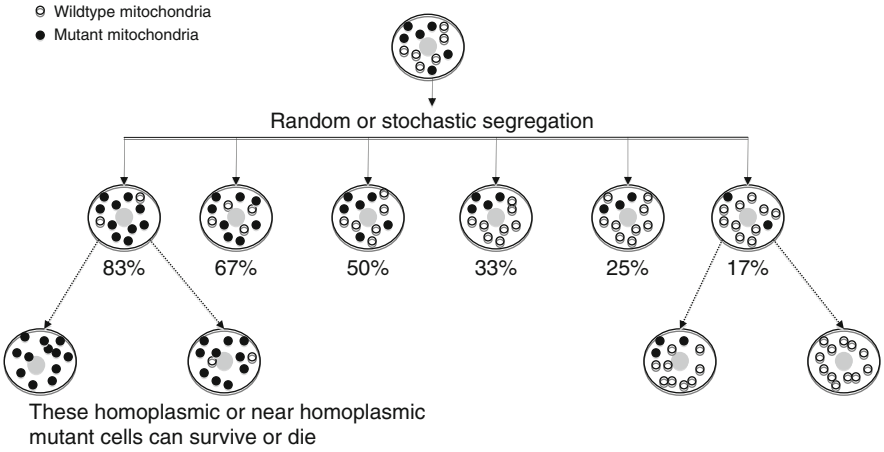

Fig. 1.2 *Mitotic segregation of heteroplasmic mtDNA molecules*. As cells divide, mitochondria and their genomes are distributed randomly to daughter cells, leading to various levels of heteroplasmy. Subsequent cell divisions can result in homoplasmic clones containing either wildtype or mutant genomes. Homoplasmic mutant clones may be selected for or against

and tumor cells, stochastic segregation linked to the cell cycle can fix mutations resulting in new homoplasmic genetic signatures in some cells (Fig. 1.2). These cells can then proliferate, leading to an expansion of mutant clones. On the contrary, functional selection can eliminate mutant clones, resulting in low levels of mutations as occurs in some mitochondrial diseases (Fig. 1.2).

Mitochondrial Genetic Bottleneck: Inherited pathogenic mitochondrial mutations show differences in phenotypic expressions in people of the same maternal descent. These generational differences in mitochondrial allele distribution and especially the variability in siblings with mitochondrial diseases, are partly explained by the mitochondrial "genetic bottleneck" phenomenon (Poulton et al. 1998). Studies in mice and humans indicate that this process occurs early in oogenesis (Jenuth et al. 1996). Although the mechanisms by which mitochondrial genetic bottleneck occurs are not fully understood, two possible events were first proposed to account for this; first, during embryonic oogenesis, mitotic segregation randomly separates heteroplasmic mitochondrial alleles into specific cells, eventually achieving homoplasmic wildtype and mutant genomes in specific oocytes; this is followed by intraovarian elimination of oocytes that harbor very severe defective mitochondrial genomes. However, recent work by Wai et al. (2008), whereby the evolution of variant mtDNA genotypes during mice oogenesis were directly followed, led to the conclusion that this phenomenon may occur during postnatal folliculogenesis and not during embryonic oogenesis.

While this phenomenon operates to partly reduce the frequency of pathogenic mutations in the population, it will appear that the process is not perfect in eliminating defective mitochondrial genomes because heteroplasmic mtDNA mutations are common in oocytes, with heteroplasmic levels estimated to be up to 50% in some cases (Jacobs et al. 2007). Moreover, there is a high prevalence

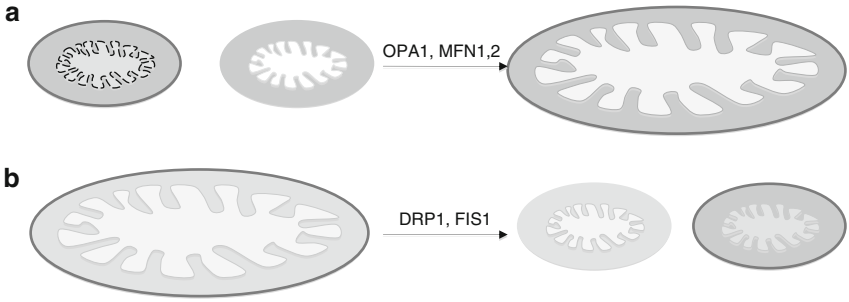

Fig. 1.3 *Mitochondrial fusion* (**a**) *or fission*. (**b**) Mitochondrial fusion can lead to functional complementation

(16.5 per 100,000) of pathogenic mtDNA variants in asymptomatic first-degree relatives of patients with mitochondrial diseases (Schaefer et al. 2008). Finally, at least one in every 200 healthy individuals carries potentially inherited pathogenic mtDNA mutations (Elliott et al. 2008).

Mitochondrial Fusion and Fission: Although mitochondria are commonly observed as elongated or tubular structures in the cytosol, they are actually highly motile and dynamic organelles that undergo constant fusion and fission (Fig. 1.3). These processes are coordinated in response to cellular needs and are observed in conditions of cell death. The shapes of mitochondria in cells depend on the equilibrium state of fission and fusion. Expectedly, increased fission or fusion will lead to mitochondrial fragmentation or elongation, respectively. Mitochondrial fusion can cause inter-mitochondrial complementation, which is an important requirement for a possible restoration of function in damaged mitochondria. These processes appear to control normal mitochondrial functions as well.

GTPase proteins of the dynamin family control mitochondrial dynamics (Fig. 1.3). Mammalian optic atrophy protein 1 (OPA1), and mitofusions 1 (MFN1) and 2 (MFN2) regulate fusion (Chen et al. 2003; Cipolat et al. 2004) and dynamin-related protein 1 (DRP1), fission 1 (FIS 1), and potentially several other proteins control fission (Smirnova et al. 2001; Yoon et al. 2003). Deficiency of MFN1 causes cells to lose their mitochondrial membrane potential, and ablation of MFN2 similarly reduced the mitochondrial membrane potential as well as decreased mitochondrial oxidation and downregulation of nuclear components involved in RC activity and energy production (Chen et al. 2003; Pich et al. 2005). It has been shown that PGC-1β that regulates mitochondrial biogenesis also controls fusion through its induction of MFN2 (Liesa et al. 2008). It is still unclear what role these processes play in cancer biology; however, because mitochondrial dynamics mediate some aspects of apoptosis, these processes are implicated in carcinogenesis. Lack of mitochondrial fission was shown to enhance the levels of mutant mitochondrial genomes in cells (Malena et al. 2009). This finding suggests that stable nondividing mitochondria in a cell with mtDNA mutations will favor the accumulation of mutant genomes to pathogenic proportions.

1.4 Mitochondrial Genomic Structure and Organization

Mitochondria are the only mammalian organelles with their own genomes. However, mitochondrial functions require complementary proteins from the nucleus. Evolutionarily, mitochondria originated from an α-proteobacterium (the proto-mitochondrion) that invaded and established a symbiotic relationship with the primitive eukaryotic cell several billion years ago (Gray 1992; Lang et al. 1999). Prior to this union, the free-living α-proteobacterium encoded all the genes required for its independent existence. The establishment of the union subsequently led to reorganization of the two genomes with defined functions. Several genes of the α-proteobacteria were transferred to nuclear chromosomes, such that currently, ~1,500 of the functional mitochondrial proteins are nuclear encoded, translated in the cytosol, and subsequently imported into mitochondria. The remaining mitochondrial genome contains 37 genes (Fig. 1.2); 24 ribonucleic acids (2 ribosomal and 22 transfer RNAs) that participate in mitochondrial protein synthesis, and 13 polypeptide genes that complement nuclear encoded products necessary for RC activity. The 13 structural genes include NADH dehydrogenase subunits 1, 2, 3, 4L, 4, 5, and 6 of the 46 complex I polypeptides, Cytochrome b of the 11 complex III polypeptides, cytochrome c oxidase subunits I, II, and III of the 13 complex IV polypeptides, and ATPase subunits 6 and 8 of the 16 complex V proteins. The four subunits of succinate dehydrogenase that form complex II are entirely encoded by the nuclear genome.

Nass and Nass (1963) were the first to demonstrate the presence of DNA in mitochondria in the early 1960s. However, it was not until the early 1980s that the Sanger group deciphered the sequence of the human mtDNA molecule (Anderson et al. 1981), making it the first human genome to be fully sequenced. This molecule is a compact closed-circular double-stranded DNA composed of 16,569 bp (but 16,568 base counts because base 3,107 is a deleted nucleotide that is still maintained in the revised Cambridge Reference Sequence (rCRS) as a gap denoted with an "n" such that the historical nucleotide numbering is undisturbed (MITOMAP, Brandon et al. 2005). The two strands have different G-C contents that enable them to be separated by a denaturing gradient into two densities; a G-rich heavy or H-strand and a C-rich light or L-strand. With the exception of ND6, the H-strand codes for all the polypeptides, the two rRNAs, and 14 tRNAs. The L-strand contains genes for ND6 and the remaining eight tRNAs (Clayton 1982; Attardi 1985). Compared to nuclear DNA, the super-coiled mtDNA is poorly associated with proteins.

The mtDNA molecule has two noncoding regions. Extending from nucleotide position 16,024 to 576 and flanked by $tRNA^{Phe}$ and $tRAN^{Pro}$ is a 1,121 bp noncoding control region or displacement loop (D-loop), which is quite variable in some regions. This region of the genome houses important regulatory elements for the replication, transcription, and maintenance of the mtDNA molecule (Fig. 1.4). These *cis*-acting elements include the H and L-strand promoters (P_H and P_L), H-strand origin of replication (O_H) and transcription factor binding sites, and highly conserved sequences, that is, three-conserved sequence blocks (CSB I, II, III) important in generating primers for H-strand replication, and termination-associated sequences (TASs) that prematurely stop replication to produce a 7S DNA in the D-loop. Given

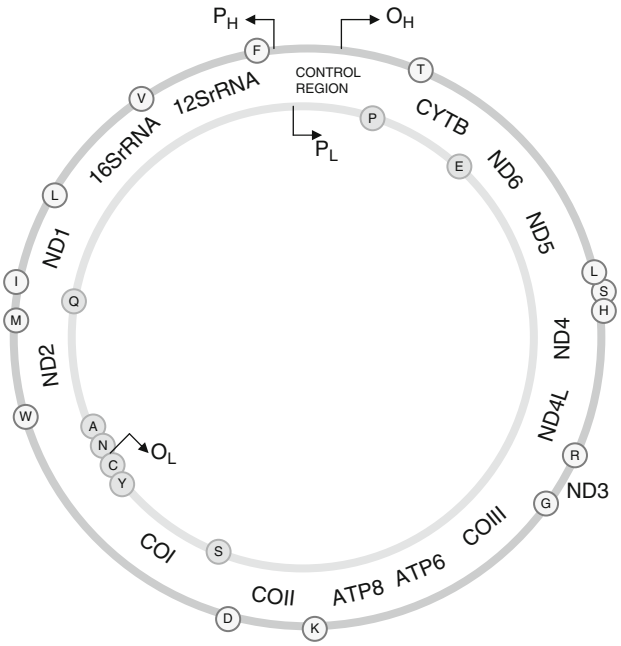

Fig. 1.4 *The human mitochondrial genome*. The circular double-stranded mtDNA molecule has 13 genes that are transcribed and translated within the mitochondrion. Transcription factors interact with the heavy strand (P_H) and light strand (P_L) promoters and induce the production of polycistronic transcripts, which are processed into 22 tRNAs, (*small circles*), two rRNAs, and 13 protein complements of OXPHOS. Replication of the molecule starts at the heavy strand origin of replication (O_H) and proceeds in a clockwise direction producing a daughter H-strand molecule. After copying about 2/3rds of the H-strand, the light-strand origin of replication (O_L) is encountered and the parental H-strand is displaced exposing the O_L, which then commences replication in the opposite direction

the functional importance of these *cis*-acting elements, mutations affecting these sites cannot be regarded as unimportant. Within the D-loop are highly polymorphic regions referred to as hypervariable segments (HVS) I (np 16,024–16,386), and II (np 57–372). Also present within HVS II is a homopolymeric cytosine (C-stretch) region known as D310 (Marchington et al. 1997). The second noncoding segment of the mitochondrial genome is about 30 nucleotides long, located two-thirds from O_H. This region contains the L-strand origin of replication (O_L).

1.5 Mitochondrial Microsatellites and D310

Highly repetitive DNA structures, referred to as satellite DNA, are scattered throughout the human genome. They are called satellite DNAs because when total DNA is fractionated by buoyant density ultracentrifugation, they form satellite

bands compared to the main DNA band. There are two main classes of satellite DNA, minisatellites and microsatellites. Minisatellites are tandem repeats of between 10 and 100 bp. They can be found elsewhere in the genome, but tend to be more common near chromosomal telomeres. Microsatellites, also known as simple sequence repeats (SSRs), are repeats of one to six nucleotides in length. The number of repeats varies considerably between alleles, thus making these units highly polymorphic. They are scattered in the genome and can be found in coding genes as well. Microsatellites have proven useful as molecular markers for genetic studies, and somatically acquired alleles, constituting microsatellite instability, have been demonstrated in a variety of cancers.

Similar to the nuclear genome, the mitochondrial genome contains numerous mononucleotide and dinucleotide microsatellite repeats. Instability at a particular mononucleotide repeat in the D-loop, referred to as D310, has been extensively studied in several cancers. Marchington et al. (1997) first used the term D310 to describe this segment. D310 is a mononucleotide tract of poly(C) that varies from 12 to 18 Cs [$(C)_{12–18}$], interrupted at nucleotide position 310 by a T (CCCCCCC TCCCCC). It is located between np 303 and 318 in CSB II in HVS II. The number of Cs before the T can vary from 7 to 9 in normal people (normal polymorphic variants). Also, extending from bp 16,184–16,193 in the D-loop, is another poly(C) tract interrupted by a T at bp 16,189 (CCCCCTCCCC). Preferably, this locus should be referred to as D16189, to be consistent with Marchington's nomenclature. In some normal individuals, a T→C transition at 16,189 results in an uninterrupted poly(C) tract. Another D-loop mitochondrial microsatellite locus that is commonly studied in cancer is a dinucleotide repeat $(CA)_n$ beginning at bp 514 (CACACA CACA). Several microsatellite loci exist in and outside the D-loop in coding genes, and instability in some of these loci have also been studied in various cancers (see Chaps. 7 and 8).

1.6 Mitochondrial Pseudogenes

Pseudogenes are homologues of genes that are not expressed. Whereas there is no report on the integration of nuclear genetic material into the mitochondrial genome, the reverse is an ongoing and a highly pervasive phenomenon. Nuclear colonization by mtDNA gives rise to nuclear mitochondrial pseudogenes or numts (pronounced "new might"), a term that was first introduced by Lopez et al. (1994). Nuclear mitochondrial pseudogenes are demonstrated in almost all eukaryotic organisms. The vast majority of human numts possesses high sequence homology (70–100%) to the rCRS, and they are distributed on all chromosomes (Woischnik and Moraes 2002). Phylogenetic analyses of authentic mtDNA and numts sequences indicate that nuclear colonization by mitochondrial genetic material is an ancient process that is still ongoing (Ricchetti et al. 2004). Nuclear mitochondrial pseudogenes are useful for the study of ancient mtDNA as well as nuclear genome evolution.

Nuclear mitochondrial pseudogenes are, in general, not transcribed. Even if they were transcribed, they will not be correctly translated because of the inherent inability to use the universal genetic code for the translation of mitochondrial transcripts. An important issue concerning numts is the fact that they can interfere with the genetic analysis of the mitochondrial genome. However, studies based on mitochondrial transcripts and proteins are unlikely to be confounded by numts interference. Caution is, therefore, warranted in mtDNA analysis. Some earlier studies of mitochondrial diseases resulted in erroneous data being reported as disease-specific genetic alterations (Davis et al. 1997; Hirano et al. 1997).

1.7 The Respiratory Chain

Normal cell physiology depends on various bioenergetic processes, and it is well established that over 90% of the energy needed by the cell to accomplish these activities is produced in the mitochondria, making the respiratory function of this organelle probably its most important role in the cell. Together with the other numerous vital functions of the cell, it is not surprising that each cell possesses several mitochondria and mitochondrial genomes. The respiratory machinery resides in the unique inner mitochondrial membrane, and is organized into five protein complexes; complexes I–IV of the electron transport chain (ETC) and the ATP synthase or complex V of oxidative phosphorylation (OXPHOS). In addition to the four complexes, ubiquinone (conenzyme Q10, CoQ) and cytochrome c participate in an electron shuttle through the RC (Fig. 1.5).

Cellular energy in the form of ATP is generated from calories derived from food. Acetyl CoA from the metabolism of carbohydrates (glycolysis), lipids (β-oxidation), and proteins (oxidative deamination) (Fig. 1.6), is oxidized in the Krebs cycle (Fig. 1.7) to generate electrons in the form of NADH, and succinate ($FADH_2$), which are the principal ingredients necessary for establishing a proton gradient that can be coupled for energy production, or if required, uncoupled to generate heat.

1.7.1 The Electron Transport Chain

Five unique protein complexes participate in energy production (Table 1.3). Complexes I–IV, CoQ, and cytochrome c perform electron transport activity resulting in the establishment of an electrochemical gradient mediated primarily by complexes I, III, and IV (Fig. 1.5). Complex V uses the electrochemical gradient or proton gradient produced for energy production or if needed by the cell, uncoupled from OXPHOS and dissipated as heat.

- Complex I (NADH dehydrogenase or NADH:ubiquinone oxidoreductase) is the largest of the RC complexes. It is a 1,000 kDa molecule assembled from about

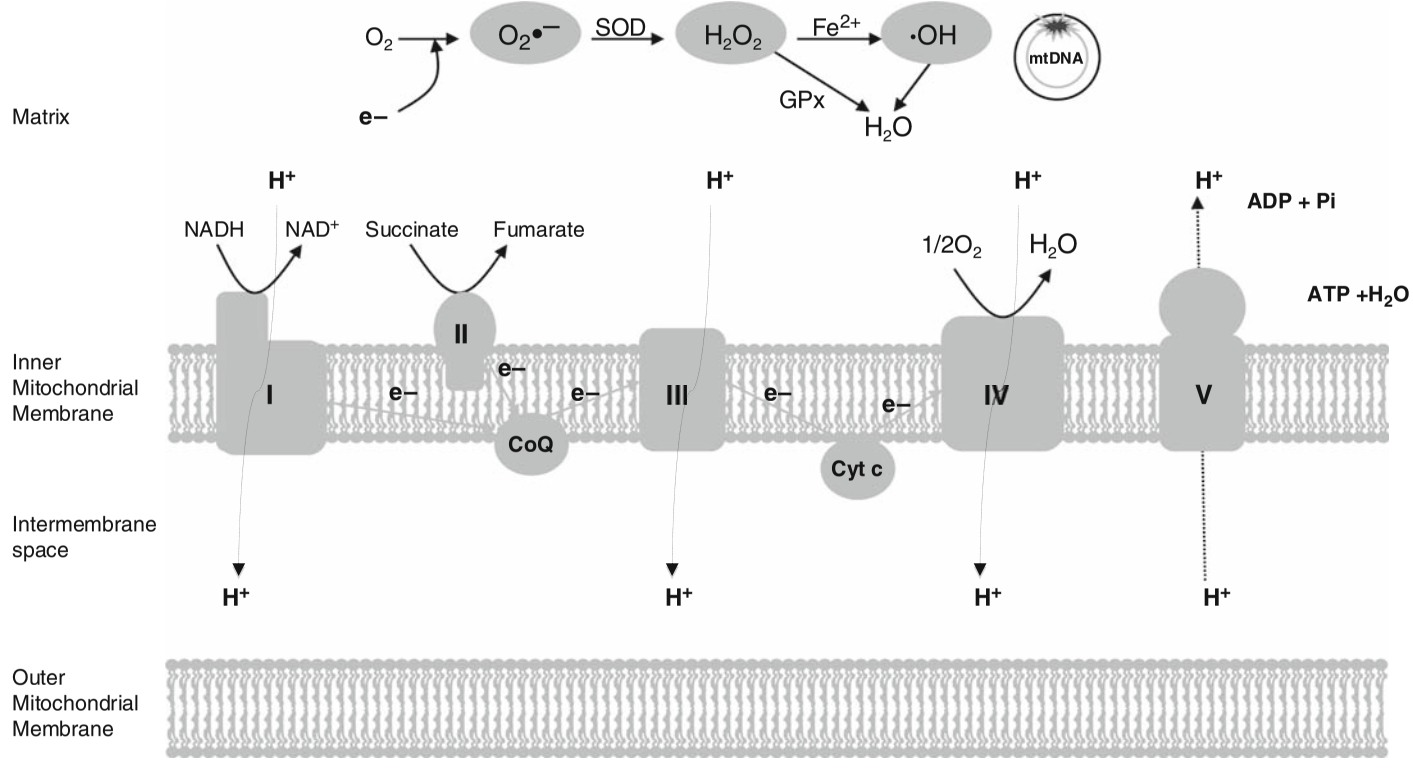

Fig. 1.5 *Mitochondrial OXPHOS and ROS production.* ATP production by the respiratory chain complex is associated with the generation of ROS as electrons leak and interact with oxygen. In normal cells, the levels of the various free radicals are kept in balance by the activities of various antioxidant mechanisms (maintenance of redox homeostasis). However, excess production of free radicals can overwhelm the detoxifying mechanisms, leading to damage to the nearby mtDNA molecules, mitochondrial membranes, and proteins

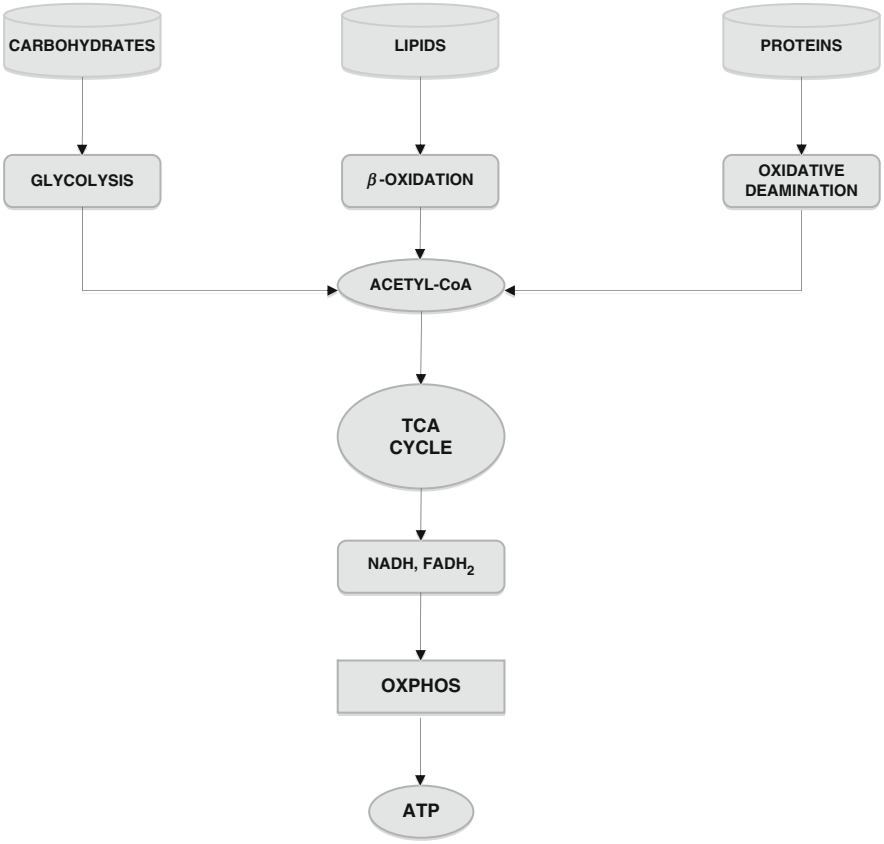

Fig. 1.6 *The basic food substrates, carbohydrates, lipids, and proteins sustain mitochondrial functions*. Metabolism of food substrates yields acetyl CoA to sustain TCA cycle activity, and hence, energy production. More caloric input translates into more supply of reducing equivalents for energy production. Thus, increased caloric intake that overwhelms the activities of the respiratory chain will lead to electron leakage and hence, elevated ROS production and mitochondrial genome damage and its subsequent sequellea

46 protein subunits, seven of which are mitochondrial encoded. Complex I contains one molecule of flavin mononucleotide (FMN) and six to seven Fe-sulfur clusters that participate in electron flow in the molecule. It oxidizes NADH to NAD+, and in the process reduces FMN to $FMNH_2$. The electrons received from NADH are transferred to CoQ, a nonprotein electron carrier, to form ubisemiquinone (CoQH•), and then ubiquinol ($CoQH_2$). The process is associated with the transfer of protons from the matrix to the intermembrane space, thus contributing to the establishment of the proton gradient.

- Complex II (succinate dehydrogenase, SDH) is assembled from four nuclear-encoded subunits; SDHA, SDHB, SDHC, and SDHD. Similar to NADH dehyrogenase, complex II accepts electrons from succinate in the TCA cycle

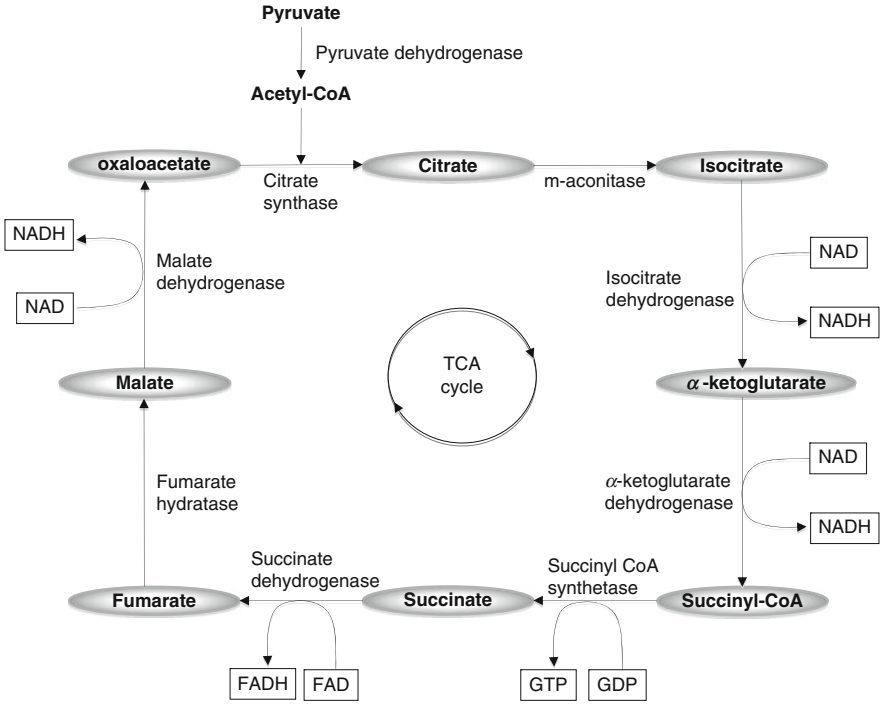

Fig. 1.7 *The Krebs or TCA cycle (certainly not a rectangle!)*. Pyruvate in the mitochondria is converted to acetyl CoA by pyruvate dehydrogenase complex. The resulting acetyl CoA is completely metabolized in the TCA cycle to generate reducing equivalents for respiratory chain activities

and similarly transfers them to ubiquinone (CoQ). However, unlike complex I, succinate dehydrogenase does not pump protons. In addition to its functions in electron transport, complex II catalyzes the conversion of succinate to fumarate in the TCA cycle.

- Complex III (ubiquinol:cytochrome c oxidoreductase) is assembled from 11 subunits with only one, cytochrome b, contributed by mitochondria. Complex III has several heme prosthetic groups, the domains of which possess different absorbance spectra. Two different spectral species of this complex are sometimes called cytochrome b, or cytochrome c_1. Complex III accepts electrons from ubiquinol and transfers them to cytochrome c in the intermembrane space. Protons are pumped across the membrane at this level, thereby contributing to the establishment of the electrochemical gradient.
- Complex IV (cytochrome c oxidase) comprises 13 protein subunits with three (CO I, II, and III) being of mitochondrial origin. This is the final RC complex involved in electron transfer. It accepts electrons from cytochrome c and passes them on to molecular oxygen, the final electron acceptor, which uses them to produce water. Similar to complexes I, and III, complex IV pumps protons across the membrane.

Table 1.3 Respiratory chain protein complexes

Complexes	I	II	III	IV	V
Enzyme	NADH dehydrogenase	Succinate dehydrogenase	Ubiquinol:cytochrome c oxidoreductase	Cytochrome c oxidase	ATP synthase
Number of polypeptides	46	4	11	13	~16
Mitochondrial encoded	7	0	1	3	2
Nuclear encoded	39	4	10	10	14
Flavoproteins	FMN	FAD (SDHA)			
Iron protein	FeS	FeS (SDHB)	Rieske FeS; Heme	Heme	
Functions	Electron transfer to CoQ	Electron transfer to CoQ	Electron transfer to Cyt c	Electron transfer to oxygen	ATP synthesis
Electrochemical gradient	Yes	No	Yes	Yes	No

1.7.2 Coupling of the Proton Gradient with Oxidative Phosphorylation

The Nobel Prize winner in Chemistry, Peter D. Mitchell, proposed the chemiosmosis-coupling hypothesis in which the proton gradient created by the ETC is tightly coupled with OXPHOS (Mitchell 1966). The ETC creates both pH and proton gradients, which are necessary for complex V (F_0F_1ATP synthase) to produce ATP. Complex V is composed of up to 18 subunits. The integral membrane component, F_0, has at least ten subunits, including ATP6 and ATP8. The other component of this complex is the peripheral, or F_1 moiety, and is encoded entirely by the nuclear genome.

Oxidative phosphorylation is accomplished by F_0F_1 subunits of the synthase. The F_0 subunit serves as an ion channel for protons to be pumped back into the matrix. This process is associated with the release of free energy from oxidation of the electron carriers (e.g., NAD+). The free energy is harnessed for ATP synthesis (condensation of adenosine diphosphate – ADP and inorganic phosphate – Pi), catalyzed by the F_1 subunit of complex V. The ATP is exchanged for cytosolic ADP by the inner membrane ANT and the outer membrane VDAC. The coupling of ETC with OXPHOS is the norm for energy production in many cells. However, in certain tissues (e.g., brown adipose tissue) or environments (e.g., habitation in cold climates), the ETC can be uncoupled from OXPHOS. These processes require an alternative pathway for the return of protons into the matrix, accompanied by thermogenesis or heat production. In these circumstances, the cells express mitochondrial uncoupling proteins that mediate the process.

1.7.3 The Respiratory Chain and Reactive Oxygen Species Production

The activities of the RC are inevitably linked to the generation of most of the cellular ROS, namely, superoxide anion ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH). Excess electrons in complexes I, II, and III can leak from the RC and interact with unpaired electrons in oxygen to produce the first family member, $O_2\bullet^-$, which can be dismutated to H_2O_2 by mitochondrial manganese superoxide dismutase (MnSOD). The H_2O_2 generated is usually detoxified by the antioxidant activities of cytosolic glutathione peroxidase and peroxisomal catalase. However, in the presence of reduced transition metals (e.g., Fe^{2+}), H_2O_2 is converted to one of the most reactive forms of the free radicals, the •OH via the Fenton reaction. Reactive oxygen species production increases in conditions of excess electron build-up at the initial steps of the RC. This can occur in conditions such as increased caloric intake and/or defective RC activity. Thus, functionally important mtDNA mutations in several diseases, including cancer, are expected to cause

elevated mitochondrial ROS production that can alter cellular signaling and/or damage to nucleic acids, proteins, and lipids (see Chap. 10).

1.8 Mitochondrial Biogenesis

1.8.1 Mitochondrial Genome Replication

The mitochondrial genome divides during cell division, but replication also occurs in postmitotic cells (relaxed replication). Mitochondria may replicate their genomes in response to the bioenergetic demands of the cell, or in response to changes in nutrients, hormones, temperature, exercise, and hypoxia. There are currently two proposed models of mitochondrial replication, the traditional and well-characterized strand-displacement, and the recently proposed strand-synchronous or strand-coupled modes.

The conventional strand displacement model requires an initial L-strand transcription, and hence, depends on the *cis*- and *trans*-acting factors that regulate mitochondrial transcription. Replication begins at O_H in the D-loop and proceeds in a clockwise direction generating a new daughter H-strand or leading strand. Upon replication of about 60% of the H-strand, O_L is encountered, and the displacement of the parental H-strand at this locus exposes the initiation sites for L-strand synthesis. Replication of L-strand then commences and proceeds in the opposite direction, generating the lagging strand.

Mitochondrial DNA replication requires three conserved sequences; a termination-associated sequence (Doda et al. 1981), and CSBs I, II, and III (Walberg and Clayton 1981). The termination-associated sequence is a short (~15 nucleotides) sequence that functions to prematurely terminate mtDNA replication, leading to the formation of the triple D-loop structure. The CSBs contribute to the formation of RNA primers necessary for replication.

The strand-displacement mode of replication occurs in the following fashion: after recognition of bound transcription factors (TFAM, TFBM1, TFBM2) to P_L in the D-loop, human mitochondrial RNA polymerase (h-mtRPOL) begins transcription of the L-strand (Fig. 1.8a). The newly synthesized RNA hybridizes to the DNA at the CSBs and forms a stable R-loop structure (R-loop structure is the pairing of nascent RNA with a single-stranded DNA to form an RNA-DNA hybrid). This R-loop structure is recognized and cleaved by a mammalian RNase, mitochondrial RNA processing (MRP) enzyme, to generate an RNA primer that is used by the DNA polymerase to begin a synthesis of the leading H-strand (Fig. 1.8a). In many circumstances, this process terminates at TAS, generating the 7S DNA that forms the triple D-loop structure, which is the region where the mtDNA molecule is anchored to the inner mitochondrial membrane (Clayton 1982). When required by the cell, replication proceeds past this block, leading to copying of the entire H-strand. At O_L, the parental H-strand is displaced, resulting in an unpaired single

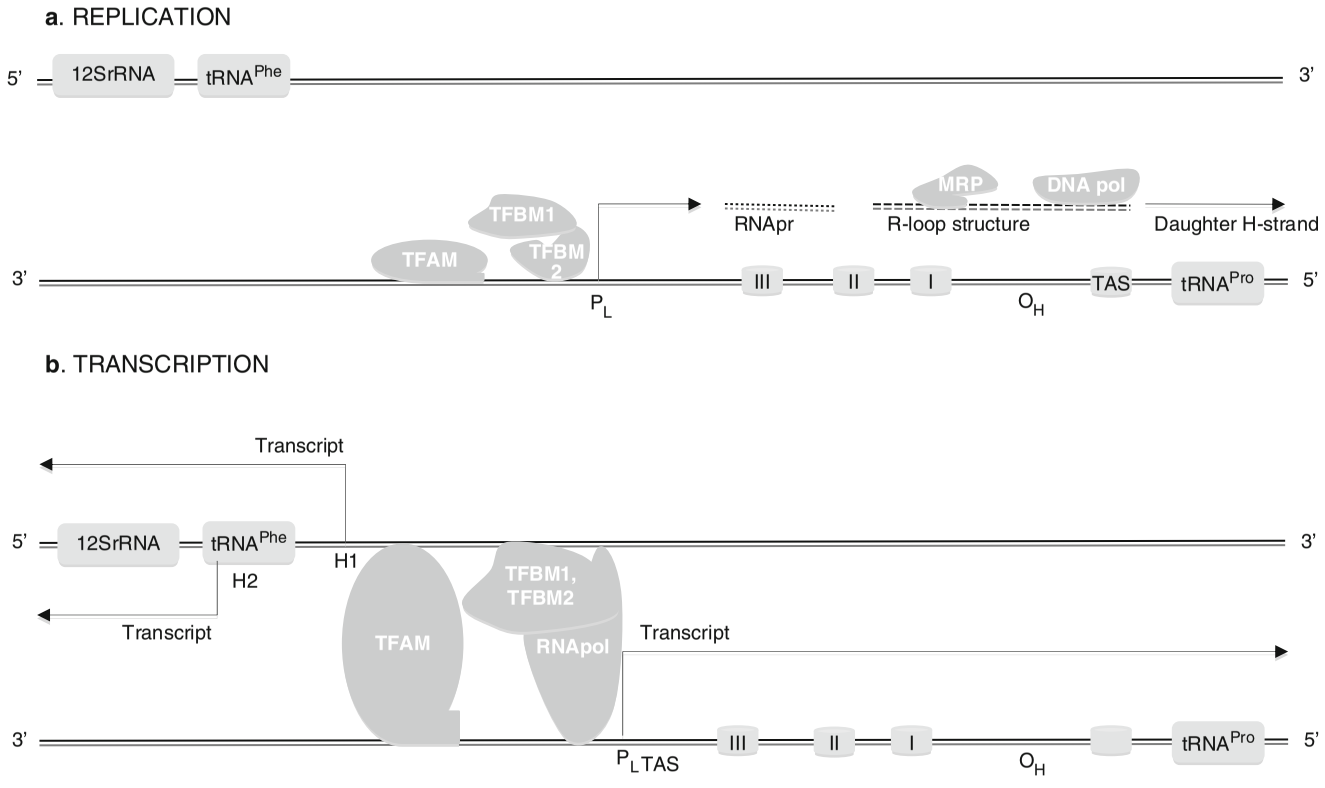

Fig. 1.8 *Mitochondrial biogenesis*. (**a**) Mitochondrial replication. The mitochondrial genome replicates using *trans*- and *cis*-acting elements needed for transcription. Once replication of the heavy strand passes by O_L, primers are generated and used for light-strand replication, which proceeds in the opposite direction. Replication is complete with a synthesis of both strands. Replication can stop at TAS, resulting in the production of the 7S DNA that forms the triplet D-loop structure. (**b**) Mitochondrial transcription. Interaction of transacting elements can lead to polycistronic transcription of both H- and L-strands. More frequently, the activity of PH1 terminates in $tRNA^{Leu1}$ leading to partial genome transcription. *RNApr* RNA primer, *DNApol* DNA polymerase, *RNApol* RNA polymerase, *TAS* termination-associated sequence, I, II, III conserved sequence blocks

L-strand that assumes a stem-loop configuration. An RNA primer is generated in the stem loop and used for L-strand synthesis. The entire process ends with the production of two nascent and complementary mtDNA molecules. As a consequence of the asymmetric mode of DNA synthesis, some daughter molecules are generated with incomplete L-strands in regard to the daughter leading strands, creating molecules known as "gapped circles." These molecules are so called because of the presence of gaps (regions of single-stranded H-strand) in these molecules as a result of an incomplete L-strand synthesis.

Holt and colleagues proposed the strand-synchronous mode of replication (Holt et al. 2000). Their model suggests that replication occurs symmetrically with synchronous synthesis of both leading and lagging strands progressing bi-directionally from multiple replication forks. Their hypothesis was based on two-dimensional (2D) gel electrophoresis analysis of restriction fragments of mtDNA that included the origin of replication and replication forks. On 2D gel analysis, the first dimension separates DNA based on size, while the second dimension separates DNA based on strand configuration. Typical synchronous H- and L-strand syntheses appear on the 2D gel as specific structures referred to as "y-arcs," which were observed by Holt and colleagues. A reanalysis of mitochondrial DNA replication by Brown et al. (2005) identified alternative L-strand initiation sites that he suggested to have accounted for the 2D gel observation by Holt and coworkers. Thus, Brown et al. (2005) suggest that the 2D gel evidence is not inconsistent with the classical strand-displacement mode of mtDNA replication.

1.8.2 Mitochondrial Genome Transcription

Similar to replication, mitochondrial transcription is controlled by several factors, including energy demands. Both strands of the mitochondrial genome are transcribed and processed, resulting in 37 mitochondrial gene products. Transcription is initiated at P_H and P_L in the D-loop and proceeds bi-directionally, generating two polycistronic transcripts that are processed to liberate the mRNAs.

1.8.2.1 Mitochondrial Transcription Initiation Machinery

The promoters of mitochondrial transcription have identical upstream enhancer recognition sites for TFAM, an HMG-box protein that binds and unwinds DNA. The mitochondrial transcription machinery is an assembly of distinct proteins with defined functions. These include those that recognize the *cis*-acting elements (TFAM), those that initiate the transcription (TFBM1, or TFBM2) (Falkenberg et al. 2002; McCulloch et al. 2002), the human mitochondrial RNA polymerase (h-mtRPOL) that mediates the transcription process (Tiranti et al. 1997), and finally, a termination factor that halts transcription (mTERF) (Fernandez-Silva

et al. 1997). Mitochondrial transcription requires interaction of the transcription factors at three *cis*-acting elements in the D-loop; two (PH1 and PH2) for H-strand transcription represent P_H and one, P_L for L-strand transcription (Fig. 1.8b). PH1 is located a few nucleotides upstream of $tRNA^{Phe}$ (np 545–567). This promoter is frequently functional, but only results in the production of four RNAs; $tRNA^{Phe}$, $tRNA^{Val}$, and the two rRNAs. Its termination point (np 3,229–3,256) is well characterized and found to be located in a specific tridecamer sequence in $tRNA^{Leu(1)}$, and also requires the activity of mTERF. PH2 (np 645) is located close to the 3′ end of 12SrRNA gene. This promoter operates at a much slower rate than PH1, but produces the H-strand polycistronic transcript that is processed to yield the 12 polypeptides and the rest of the 12 H-strand tRNAs. The light strand promoter, P_L (np 392–445), is located at about 150 nucleotides downstream of PH1 initiation point. Transcription initiation at this locus generates L-strand polycistronic transcript that is also processed to give one polypeptide (ND6) and eight tRNAs. The different rates at which PH1 and PH2 operate partly explain the variant levels of mitochondrial ribosomal RNAs and mRNAs (Montoya et al. 1982, 1983).

1.8.2.2 Mitochondrial Transcripts

The mitochondria genome contributes mRNA, tRNA, and rRNA during transcription of the molecules. Mitochondrial mRNAs are complete transcripts of mitochondrial structural genes. The matured RNAs are processed with the addition of poly (A) tails of about 50–80 nucleotides to the 3′ ends (Perlman et al. 1973). Unlike nuclear mRNAs, mitochondrial mRNAs lack 5′ untranslated regions, 3′ 7-methylguanylate end cap structures, and polyadenylation signals (Montoya et al. 1981). Mitochondrial tRNAs are generally smaller than those from the nucleus, averaging about 70 nucleotides in length. Apart from their translational function, tRNAs are involved in the initiation of L-strand replication, transcription termination, and the processing of polycistronic transcripts. Mitochondrial rRNAs are also involved in intramitochondrial translation. They are much smaller than cytoplasmic rRNAs, are methylated, and possess much shorter (1–10 nt) 3′ poly (A) tails than cytoplasmic rRNAs.

1.8.2.3 Mitochondrial RNA Processing

Mitochondrial RNAs require enzymatic processing to produce a mature RNA. The RNA punctuation model explains the release of all mRNAs, except for ATP6/8 and ND4L/ND4 genes. The model suggests that folding of tRNAs into the cloverleaf structure provides signals for RNA processing enzymes to perform an endonucleolytic cleavage on both sides of the tRNA. The enzyme RNase P is responsible for the 5′ end cleavage reaction; however, the enzyme for the 3′ end cleavage is unknown. It is conceived that similar tRNA secondary structures exist between ATP6/8 and ND4L/ND4 that provide signals for their cleavage.

Mitochondrial rRNA and mRNAs are polyadenylated by mitochondrial poly (A) polymerase (Tomecki et al. 2004; Nagaike et al. 2005). This enzyme also stabilizes some RNAs and generates stop codons. Mitochondrial tRNAs are processed with the addition of CCA to their 3′ ends by an ATP(CTP)-tRNA-specific nucleotidyl-transferase.

1.8.2.4 Mitochondrial RNA Turnover

With the exception of rRNAs, the polycistronic transcription of mtDNA suggests that the levels of all processed transcripts from each strand should be stoichiometrically identical. This appears to be true for mitochondrial tRNAs, because despite the differences in the rates of transcription, the steady-state levels of tRNAs in the fast-growing HeLa cells were uniform (King and Attardi 1993). However, the steady-state concentrations of all mitochondrial transcripts are different. Indeed, all mitochondrial RNAs are metabolically unstable, though the half-life of rRNAs is 2–6 times longer than those of mitochondrial mRNAs (Gelfand and Attardi 1981). The differences in the steady-state levels of rRNAs and mRNA can be explained by the higher rate of synthesis, coupled with increased stability of rRNAs. Because PH1 promoter is frequently operational and terminates downstream of 16SrRNA, more $tRNA^{Phe}$, $tRNA^{Val}$, and the two rRNAs will be synthesized compared to the other mitochondrial transcripts regulated by PH2 and P_L. It has been shown that in contrast to mRNAs, rRNAs in polysomes have a much higher half-life compared to those from total mitochondria, suggesting that polysomes provide some protection to rRNA (Gelfand and Attardi 1981). Moreover, the L-strand is transcribed at a rate 2–3 times higher than that of the H-strand; however, L-strand transcripts have a much shorter half-life than those from the H-strand.

Several mechanisms appear to account for the metabolic stability of mitochondrial transcripts. For instance, in the analysis of ten mitochondrial transcripts, Piechota et al. (2006) identified three groups of mitochondrial transcripts based on their steady-state levels and half-life. COII and ATP6/8 transcripts had the longest half-life and were, therefore, most abundant. ND1, ND2, COI, and CYTB had an intermediate half-life of 2–3 h, with ND3 having the shortest half-life of 1 h, and hence, the lowest steady-state amounts. Further analysis revealed that ND2, ND3, and CYTB were significantly stabilized by the inhibition of mitochondrial translation. Possibly, mitochondrial translation mediates deadenylation and hence, the destruction of such transcripts. Moreover, COII and ATP6/8 were significantly more stable than the rest of the mitochondrial mRNAs, putatively because of some secondary structures or interaction with proteins (Piechota et al. 2006).

The role for polyadenylation and stability has been addressed. Poly(A) tails protect eukaryotic RNAs from degradation, but in plant mitochondria, bacteria, and chloroplasts, poly(A) directs RNAs for destruction. With the discovery of human mitochondrial poly (A) polymerase (hmtPAP), findings are reported with regard to the length of poly (A) and mitochondrial RNA stability (Tomecki et al. 2004; Nagaike et al. 2005). Silencing of hmtPAP indicated that oligoadenylation was

associated with increased stability (Tomecki et al. 2004). While work by Nagaike et al. (2005) appeared contradictory to these findings, the same conclusion as that of Tomecki et al. (2004) is arrived at when loading controls are taken into consideration.

1.8.2.5 Translation of Mitochondrial Messenger RNAs

Translation of mitochondrial proteins occurs in the mitochondria employing components of both mitochondrial and nuclear-encoded subunits. Its most important function is to produce the structural proteins that complement nuclear genes for energy production via the RC. Whereas it is well known that mutations in genes that contribute to this process can cause diseases, it will appear that mitochondrial translation is dispensable for cell survival when enough glucose is provided for the cell to thrive on glycolysis.

The translational machinery is composed of two ribosomal and 22 tRNAs encoded by the mitochondrial genome, as well as several imported proteins, including tRNA synthases, ~50 ribosomal proteins, and translation initiation, elongation, and termination factors. Mitochondria use a unique codon that is different from the universal genetic code (Table 1.2). Mitochondrial translation is similar to that of bacteria in some aspects. For instance, some antibiotics such as tetracycline that act on ribosomes can inhibit mitochondrial translation. Moreover, mitochondrial translation uses *N*-fomylmethonyl-tRNA for initiation (Galper and Darnell 1969; Epler et al. 1970). Despite this similarity, mitoribosomes are different from bacterial ribosomes, being much larger and more massive with many more subunits than bacterial ribosomes.

The mechanisms of human mitochondrial translation are not well understood. For example, it employs only 22 tRNAs instead of the minimum of 30 required by the wobble hypothesis. Structures such as 5′ UTRs, and 3′ end caps that enable ribosomal binding and scanning to identify the initiation codon are absent in mitochondrial mRNAs. Ribosomal RNA binding is not sequence-specific and does not require initiator tRNA.

The formation of the initiation complex begins with the binding of the initiation factor (e.g., mtIF2) to the small ribosomal subunit. This interaction facilitates the binding of *N*-fomylmethonyl-tRNA. Guanosine triphosphate (GTP) hydrolysis releases mtIF2, and the large ribosomal subunit then binds to complete the initiation complex. The mechanism of chain elongation is quite well characterized. Three mitochondrial elongation factors, EFG, EFTu, and EFTs mediate elongation. These factors are GTPases. During chain elongation, EFG catalyzes the movement of peptidyl-tRNA-mRNA complex on the ribosome. This is accomplished by the transfer of a nascent peptidyl-tRNA from the ribosomal amino acyl or A site to the ribosomal peptidyl or P site, associated with the removal of the tRNA from the P site to the ribosomal exit site. Activated EFTu (GTP:EFTu) moves an aminoacyl-tRNA onto the ribosomal A site and is released from the ribosome as EFTu:GDP, which forms a substrate for EFTs to catalyze the exchange of GDP with GTP.

Termination, though poorly understood, is mediated by the human mitochondrial termination factor, HMTF1L.

1.9 Control of Mitochondrial Biogenesis

Mitochondrial biogenesis is regulated at several levels. Upstream are environmental cues that dictate whether food substrates should be used for energy or heat production. These environmental factors include heat, cold, starvation, increased caloric intake, growth factors, hormones, and metabolic signals. Mitochondrial DNA replication, transcription, and bioenergetics must be regulated in a complex and tissue-specific fashion because there are differences in energy requirements by cells and tissues. For example, brown adipose tissue is designed to conserve heat and hence, will have to uncouple respiration to generate heat. On the contrary, cardiac cells that are in high-energy demand will need a tightly coupled respiration for ATP production, and yet, the skeletal muscle has the adaptive capability to couple or uncouple respiration. Advances in recent years are shedding some light on how this complex phenomenon is controlled. The myriads of environmental signals induce nuclear and mitochondrial gene transcription that coordinates mitochondrial metabolism, proliferation, energy production, transcription, and replication. It is noteworthy that some factors such as hormones can induce mitochondrial biogenesis independent of protein biosynthesis.

1.9.1 Hormonal Control

The two principal hormones with well-defined roles in regulating energy metabolism are thyroid hormone and glucocorticoids. High levels of thyroid hormones induce increased metabolism, oxygen consumption, and heat production, while low levels have the opposite effect. Cortisone and cortisol control the response of the body to increasing energy requirements such as stress. Both glucocorticoids and thyroid hormones have short-term and long-term effects on mitochondrial biogenesis. The long-term effects of glucocorticoids and thyroid hormones involve both nuclear and mitochondrial gene expression and uncoupling proteins. The short-term effects are rapid and occur without gene expression changes. Thyroid hormone, T3, for instance, binds to its receptors to modulate both mitochondrial and nuclear gene expression. As well, T3 target genes include nuclear respiratory factor 1 (*NRF1*) and *PPARγ Coactivator 1α* (*PGC-1α*) that, on activation, enter the nucleus to amplify biogenic gene expression by regulating a series of T3 target genes.

In recent times, estrogens have emerged as important regulators of mitochondrial biogenesis in several different tissue types. The activities of 17β-estradiol (E2) coordinate both nuclear and mitochondrial genome transcription for mitochondrial RC functions. The 17β-estradiol receptors (ERα and ERβ) are found in mitochondria,

and appear to directly modulate mtDNA transcription (Chen et al. 2004; Chen and Yager 2004). In a classical mode of signaling, E2 interacts with their receptors to regulate gene expression. However, it is also becoming evident that E2 can interact with plasma membrane-associated ERs to trigger intracellular signaling cascades via protein kinase-mediated phosphorylation. Among other targets, estrogen signaling regulates *NRF1* (Mattingly et al. 2008). Another set of nuclear receptor super family, the estrogen-related receptors (ERR) without identified ligands (the so-called orphan nuclear receptors), are uncovered to modulate mitochondrial biogenesis. These orphan nuclear receptors, including ERRα, ERRβ, and ERRδ, are suggested to mediate the activities of PGC-1 coactivators (Giguere 2008).

1.9.2 Nuclear Respiratory Factors

Nuclear respiratory factors 1 and 2 (NRF-1, NRF-2) are two important transcription factors that control the expression of several nuclear-encoded proteins that regulate mitochondrial biogenesis at several levels (reviewed in Kelly and Scarpulla 2004; Scarpulla 2008b, a). Nuclear respiratory factor 1 was identified and named by Evans and Scarpulla (1989) as a molecule with *cis*-acting sites in the promoter of cytochrome c gene. It is a 68 kDa polypeptide with a *C*-terminal transcription activation domain that binds DNA as a homodimer, and an *N*-terminal serine phosphorylation site that, upon phosphorylation, enhances DNA binding and transactivation functions.

Nuclear respiratory factor 1 regulates the transcription of genes that encode the subunits of the respiratory complexes, including NADH dehydrogenase subunit 8 of complex I, SDHB, SDHC, and SDHD of complex II, and cytochrome c oxidase subunit IV of complex IV. The genes involved with the assembly of respiratory apparatus (TFAM, and TFB) contain NRF-1 binding sites, and the MRP endonuclase involved in mtDNA replication is controlled by NRF-1. Nuclear respiratory factor 1 regulates the transcription of mitochondrial and cytosolic enzymes regulating heme biosynthesis, as well as the components of the mitochondrial protein import system, including TOM20, TOM70, and TOMM34. The levels of NRF-1 and TFAM increase in mtDNA-depleted cells, and in situations of oxidative damage, possibly as a compensatory mechanism to overcome the respiratory defects in these cells.

Nuclear respiratory factor-2 was also identified as a transcription factor with sites in the promoter of cytochrome c oxidase gene (Virbasius and Scarpulla 1991). It has a DNA binding subunit α and four other subunits (β_1, β_2, γ_1, γ_2) that do not bind DNA but complex with subunit α when it binds DNA. Nuclear respiratory factor-2 controls several genes that are also controlled by NRF-1. However, some genes such as the COX promoter do not have NRF-1 binding sites, suggesting some distinct regulatory functions for NRF-2.

A number of genes that regulate mitochondrial biogenesis do not have NRF-binding sites, suggesting that other transcription factors control mitochondrial biogenesis as well. The general transcription factor, Sp1, is known to interact with

cytochrome c promoter and augments its activity (Evans and Scarpulla 1989). Similarly, *cis*-acting elements in cytochrome c promoter recognize ATF/CREB family of transcription factors, and these promoters are shown to bind ATF/CREB both in vitro and in vivo (Evans and Scarpulla 1989; Gopalakrishnan and Scarpulla 1994; Herzig et al. 2000). While COX genes in many tissues are ubiquitously expressed, in muscle, COX subunit genes depend on MEF-2 and E-box consensus elements for expression (Wan and Moreadith 1995). Finally, the transcription factor YY1 is demonstrated to control cytochrome oxidase expression (Basu et al. 1997; Seelan and Grossman 1997).

1.9.3 Peroxisomal Proliferator Activator Receptor Gamma Co-Activator-1α

Peroxisomal proliferator activator receptor gamma coactivator 1 α (PGC-1 α) is a regulator of mitochondrial biogenesis upstream of NRF-1, NRF-2, and TFAM (Fig. 1.9). PGC-1α was cloned by the Spiegelmans's group as a factor in brown adipose tissue that interacts with the adipogenic nuclear receptor, PPARγ (Puigserver et al. 1998). PGC-1α is a direct transcriptional coactivator of PPARγ. As a transcriptional coactivator, it serves multiple functions in regulating gene expression, including chromatin structural modification and mRNA processing. PGC-1α can recruit additional coactivators that have histone acetylase activity to coactivate its targets.

High levels of PGC-1α are observed in tissues with high-energy demands such as heart and kidneys. The expression of PGC-1α is highly inducible by factors that require increased ETC activity such as cold, exercise, and fasting. PGC-1α has multiple effects on mitochondrial biogenesis: First, it can induce the expression of mitochondrial uncoupling protein 1 (UCP-1), leading to heat production, especially in brown adipose tissue. Second, it can induce the marked expression of TFAM, NRF-1, and NRF-2. Third, it can upregulate genes involved in mitochondrial fatty acid metabolism, including the targets of PPARγ_1 and NRF-1. Finally, it can stimulate increased mitochondrial proliferation in cardiac and skeletal muscles. PGC-1α interacts with several factors to mediate its functions; these include thyroid hormone receptors, PPARα, retinoid receptors, glucocorticoid receptors, and estrogen receptors. Other partners include HNF-4, FOX-01, MEF2C as well as orphan nuclear receptors such as the ERR (ERRα and ERRγ). A homologue of PGC-1α, PGC-1β has similar tissue distribution but different expression patterns (Lin et al. 2002).

1.9.4 Regulation of PGC-1

PGC-1 is regulated by a complex series of events and signaling pathways (Fig. 1.7). ATF/CREB induces PGC-1 expression by binding to CRE sites in its promoter. Similarly, the transcription factor MEF2, upregulates PGC-1 expression. Calcineurin

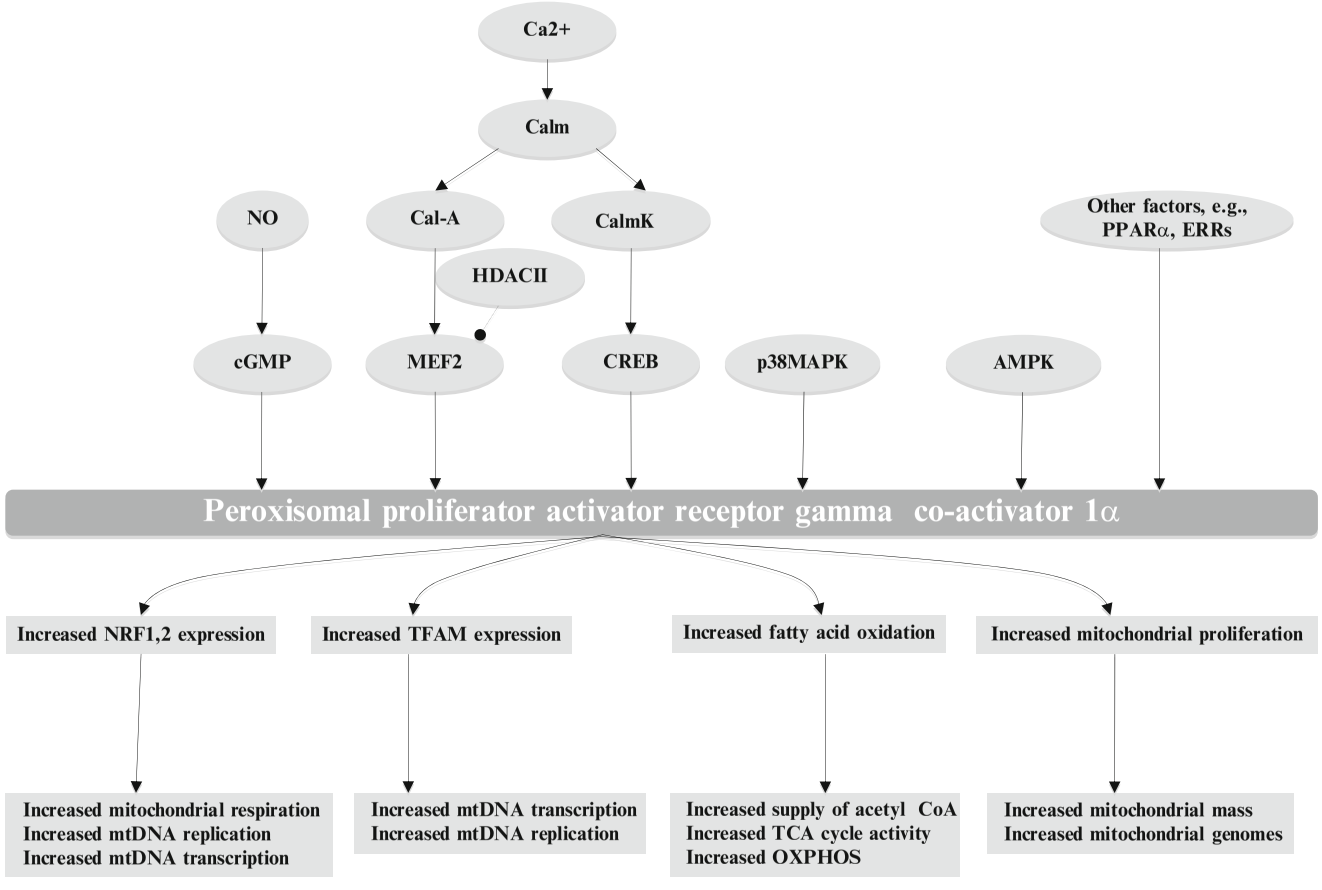

Fig. 1.9 *Regulation of mitochondrial biogenesis by PGC-1α.* The transcriptional coactivator, PGC-1α controls mitochondrial functions at several levels partly through the induction of NRFs and TFAM expression. Glucose, insulin, and cytokine signaling control PGC-1α expression using a number of coordinated intracellular pathways, including p38MAPK, *AMPK* adenosine monophosphate-activated protein kinase, *CalmK* calcium/calmodulin-dependent protein kinase, *Cal-A* calcineurin-A

A through MEF2, and calcium/calmodulin-dependent protein kinase via CREB binding sites induce PGC-1 expression. P38MAPK signaling pathway regulates the expression of PGC-1α and PPARα and thus, fatty acid oxidation. Nitric oxide activates mitochondrial biogenesis via cGMP and PGC-1α. The mTOR pathways is shown to regulate mitochondrial biogenesis via YY1-PGC-1α complex (Cunningham et al. 2007). Finally, class II histone deacytalases (HDAC) repress the activities of PGC-1α, probably through the inhibition of MEF2.

1.9.5 PGC-1α-Related Coactivator

An emerging member of the coactivator family involved in mitochondrial biogenesis is PRC (PGC-1α-related coactivator). PRC, identified by Scarpulla's group, has some functional domains as PGC-1, including an acidic *N*-terminal LXXLL motif that interacts with nuclear receptors (Andersson and Scarpulla 2001). This domain conservation strongly implicates PRC in coactivator functions as PGC-1. PRC is not as inducible as PGC-1α, is ubiquitously expressed, and controls the cell cycle as well. Some functional studies, however, show that PRC interacts with NRF-1, suggesting a possible regulation of mitochondrial biogenesis.

1.9.6 Myc Oncogene and Mitochondrial Biogenesis

The Myc oncogene is a transcription factor that controls the growth, proliferation, and death of cells via the regulation of several genes. Myc forms a dimmer with Max to be activated, and the complex then binds to E-box DNA motifs (canonical CACGTG; CATGTG) to induce transcription. Overexpression of Myc is demonstrated in several cancers. Myc directly targets and activates several genes involved in mitochondrial functions such as metabolism, apoptosis, and OXPHOS. In vitro and in vivo ectopic expression of Myc resulted in an increased expression of genes controlling the mitochondrial structure, function, and biogenesis (Li et al. 2005). Indeed, Kim et al. (2008) demonstrated that ~107 of the promoters of genes that control mitochondrial functions were occupied by Myc. Specifically, Myc directly regulates the expression of *TFAM* and *PGC-1β*, and possibly *POLG*, *NRF1*, and *TFBM1* (Kim et al. 2008). These findings demonstrate the importance of Myc deregulation in modulating mitochondrial functional alteration to favor cancer cell behavior.

1.10 Import of Nuclear-Encoded Proteins into Mitochondria

Mitochondrial protein import is critical to normal mitochondrial functions, because ~1,500 nuclear-encoded mitochondrial proteins complement the few mitochondrial proteins. These nuclear-encoded mitochondrial proteins are translated in the cytosol

and imported through established import channels (reviewed by Stojanovski et al. 2003; MacKenzie and Payne 2007). Cytosolic mitochondrial preproteins have mitochondrial target signals that enable the recognition of mitochondrial surface receptors. Upon interaction, the preproteins enter through the TOM and those destined for the inner membrane or matrix channel via one of the two translocases of the inner membrane (TIM22 or TIM23). Normal functioning of the TIMs requires the presence of a membrane potential. The majority (~70%) of the mitochondrial targeting sequences consists of 15–40 amino acids located at the N-terminus that are rich in basic and hydroxylated amino acids. The remaining preproteins have their targeting signals embedded in the mature protein sequence. Once in the mitochondria, the unfolded preproteins are processed by proteolytic removal of the targeting signals, followed by delivery into the matrix or insertion into membranes.

1.11 Mitochondrial Import of Cytosolic Transfer RNA

The import of tRNA into mitochondria has been known for decades in organisms such as yeast, masupials, trypanosomatids, tetrahymena thermophilia, and plants (Mirande 2007; Tarassov et al. 2007). Whereas the mechanisms of tRNA import are not well established, ATP hydrolysis is implicated. In yeast, some membrane, as well as cytosolic proteins such as enolase and lysyl-tRNA synthetase, participate in mitochondrial tRNA import. Also well known are the different mechanisms by which $tRNA^{Lys}$ and $tRNA^{Glu}$ are imported into yeast mitochondria. Transfer RNA^{Glu}, for instance, is imported into yeast mitochondria without any dependency on protein factors.

The question whether tRNA import into human mitochondria is operational, is just being addressed. It has been a common belief that under normal physiologic conditions, human mitochondria do not import cytosolic tRNAs (Mirande 2007). However, under special conditions, it was demonstrated that yeast and human $tRNA^{Lys}$ could be imported into human mitochondria, raising an important possible mechanism to treat some mitochondriopathies due to dysfunctional mitochondrial tRNA mutations (Kolesnikova et al. 2004; Mahata et al. 2006). Rubio et al. (2008) demonstrated that $tRNA^{Glu}$ could be imported into human mitochondria without the need for any soluble cytosolic factors. This import of tRNA into human mitochondria required ATP and ATPase activity and was independent of protein import pathway.

1.12 Conclusion

Knowledge on mitochondrial genetics is quite well advanced with regard to gene organization, inheritance, maintenance, and pathogenic changes that underlie several mitochondrial cytopathies. However, there are a number of outstanding issues concerning the genetics of mitochondria. Still poorly understood is how the

polycistronic transcript is processed. For example, how are the 3′ ends of tRNAs released? Similarly, genes without tRNAs are processed by mechanisms that are still not fully explained, although tRNA-like structures are suggested to mediate such processing.

The mechanisms by which mutations in this genome accumulate with disease are still poorly understood. For example, do mutant genomes in a cell, at a specified heteroplasmic level, cause cellular distress, which in turn results in compensatory overproduction of these molecules? Are some mutations advantageous to the cell, such that such a cell and its genomes are preferentially selected for by clonal expansion? This is important to know because in some cancer or disease states, mitochondrial mutations accumulate to nearly homoplasmic levels, and yet, these cells appear to be very fit.

The pivotal role of mitochondria in energy production and control of several cellular functions indicates that mitochondrial dysfunction contribute to various diseases and cancer. The dawn of mitochondrial medicine and oncology is now. The small size of the genome should make it practically easy and economical to assay. The polyploid nature and high copy numbers per cell imply that disease mutations can accumulate in a cell without compromising cellular function. Such mutations function as biosensors of impending disease and can easily be assayed in clinical samples. Understanding the genetics of mitochondria is important in medicine and oncology.

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Chapter 2
The Warburg Phenomenon and Other Metabolic Alterations of Cancer Cells

Abstract The altered metabolism of cancer cells was recognized and pioneered by the elegant works of Otto Warburg in the 1920s and popularized in the 1950s. Presently, it is well recognized that over sixty percent of all cancers are glycolytic. The Warburg effect or phenomenon is discussed, and the molecular understanding of some of Warburg's statements is provided with regard to modern knowledge of carcinogenesis. In addition, molecular explanation of aerobic glycolysis of the cancer cell is detailed. Other metabolic alterations of the cancer cell including glutaminolysis and lipogenesis are discussed. The altered citrate metabolism specifically associated with malignant transformation of peripheral zone prostate epithelial cells, and the potential clinical applications of such metabolic alterations conclude this chapter.

2.1 Introduction

> Cancer cells originate from normal body cells in two phases. The first phase is the irreversible injury of respiration.
>
> - Otto Warburg (1956)

One of the main distinguishing features between the normal and cancer cell is in their intermediary metabolism. Cancer cells have an altered metabolism, a characteristic that was recognized decades ago by Otto Warburg, for which initial molecular explanation was offered by Peter L. Pedersen and others. These bioenergetics and metabolic features do not only permit cancer cells to survive under adverse conditions such as hypoxia, but enable their proliferation, progression, invasiveness, and subsequent distant metastasis. Compared to normal cells, malignant transformation is associated with an increased rate of intracellular glucose import, and a higher rate of glycolysis associated with reduced pyruvate oxidation and increased lactic acid production. In addition, the cancer cell has increased gluconeogenesis, increased glutaminolytic activity, reduced fatty acid oxidation, increased de

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novo fatty acid synthesis, increased glycerol turnover, modified amino acid metabolism, and increased pentose phosphate pathway activity. These metabolic alterations are useful and have been explored for diagnostic, prognostic, and therapeutic targeting in cancer management. This chapter addresses some tenets of the Warburg phenomenon or effect in regard to current scientific understandings of cancer biology. Molecular explanations of the Warburg effect are examined, as well as other different cancer cell metabolic changes. Finally, the unique metabolism of prostate cancer (PCa) cells and the clinical utility implications are addressed. Uncovering the intricate and myriad mechanisms employed by the cancer cell to achieve these metabolic switches also holds tremendous companion diagnostic and therapeutic opportunity.

2.2 Bioenergetics of Normal Cells

The metabolism of the basic energy substrates (carbohydrates, proteins, and lipids) in normal cells generates several metabolic intermediates that are used to synthesize nucleic acids, nonessential amino acids, glycogen, and other biomolecules required for normal body functioning. Importantly, because energy is critical to all biologic processes, metabolism partly results in the production of acetyl CoA that is oxidized in the tricarboxylic acid (TCA) or Krebs cycle to produce reducing equivalents for energy production by the respiratory chain. Carbohydrates are broken down to glucose, which is taken up by cells and metabolized via the fundamental biochemical process known as glycolysis (Fig. 2.1). As well, glucose in the blood can arise from noncarbohydrate sources such as gluconeogenesis. Glucose enters the cell through specific glucose transporters. In the cytosol, several enzymes catalyze glycolysis that ends with the production of pyruvate and two molecules of ATP per glucose. Pyruvate enters the mitochondria and is converted to acetyl CoA by pyruvate dehydrogenase (PDH). Acetyl CoA condenses with oxaloacetate (OAA) to form citrate, which is completely oxidized in the TCA cycle to generate reducing equivalents for the respiratory chain. The respiratory chain produces ~34 more ATP molecules per glucose, bringing the total number of ATPs produced per complete oxidation of glucose to ~36, which accounts for over 90% of the energy requirements of the normal cell. Thus, in normal cellular intermediary metabolism, glycolysis, TCA cycle, and respiratory chain activities are tightly linked and regulated.

2.3 The Crabtree Effect

In 1926, Herbert G. Crabtree made an observation on the utility of carbohydrates by tumors (Crabtree 1926). He observed that, for normal cells, the presence of glucose slightly increased respiration or had no effect on oxygen consumption. On the contrary, glucose decreased oxygen uptake by tumor cells. This respiratory

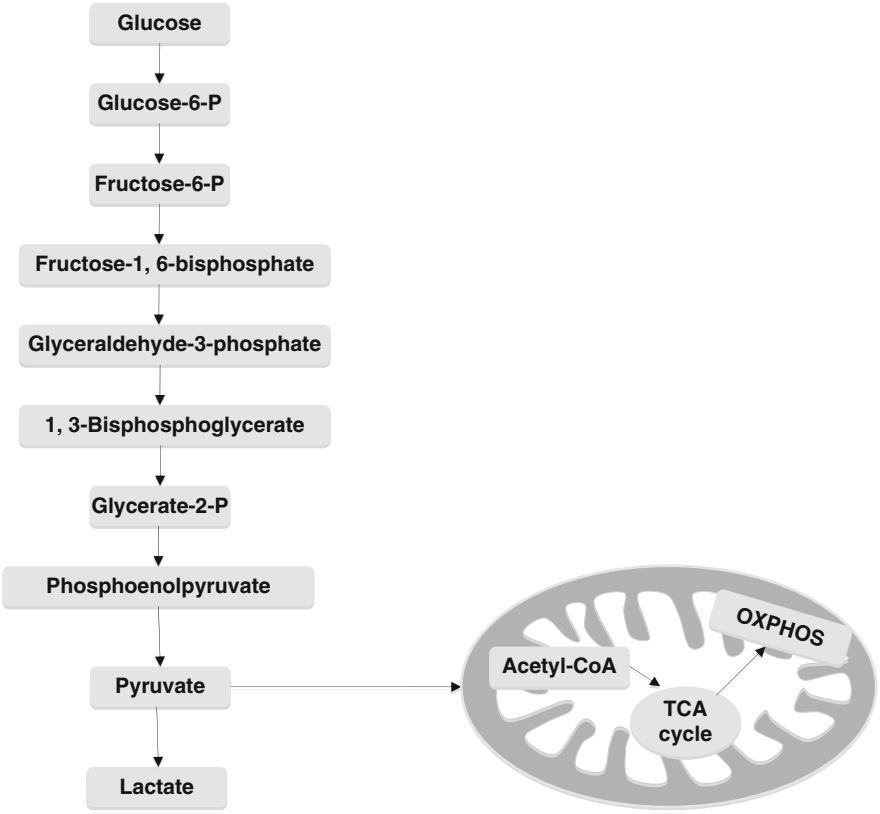

Fig. 2.1 *Glycolysis*. The sequence of reactions that produce pyruvate is known as glycolysis. Under anaerobic conditions, glycolysis produces minimal energy in the form of ATP. This mode of glycolysis or fermentation is usually not coupled to the TCA cycle and OXPHOS. However, under aerobic conditions, glycolysis, TCA cycle activity, and OXPHOS generate considerable energy for cellular functions

inhibition of cancer cells by glucose is called the Crabtree effect, named after Herbert Crabtree. It is now known that this metabolic transformation of cancer cells is not a specific feature of carcinogenesis, but appears to be a requirement of rapidly dividing cells such as proliferating thymocytes, spermatozoa, intestinal mucosal cells, renal cells, and embryonic stem (ES) cells (Wojtczak 1996). This phenomenon is also reported in bacteria and yeast. Apart from rapid proliferation, an important characteristic shared by all these cells, as will be expected from respiratory impairment, is a high rate of glycolysis. Another observation of the Crabtree effect is the initial increase in respiration following the provision of glucose. Indeed, it appears that other hexoses can induce this effect in cancer cells as well.

The mechanism of the Crabtree effect is not completely understood; however, several explanations have been provided. These include the following: (1) A competition between glycolysis and OXPHOS for available ADP + Pi can cause

respiratory impairment by increased glucose availability (Sussman et al. 1980). (2) Increased lactic acid production with decreasing cytosolic pH and inhibition of oxidative enzymes (Heinz et al. 1981). (3) Disruption of coupled respiration by calcium; increased mitochondrial calcium levels by glucose caused an increased association of the inhibitory subunits of F_1F_0 to the ATP synthase to inhibit coupled respiration (Wojtczak 1996). (4) Glucose metabolism increases ROS production that damages mitochondrial membranes and depresses respiration (Yang et al. 1997). (5) It is possible that the Crabtree effect might be regulated by multifaceted mechanisms in cancer cells, possibly involving changes in ATP/ADP ratio, Pi, glucose-6-phosphate, cytosolic pH among others (Rodriguez-Enriquez et al. 2001). Irrespective of the mechanism, cancer cells are glycolytic and this is associated with partial mitochondrial impairment (i.e., suppressed respiration, less oxygen consumption, and low ATP production).

2.4 The Warburg Phenomenon

While it can be conceived that fast-growing cancer cells will require more energy than normal cells, it is ironic to realize that, in contrast to normal cells, cancer cells use a primitive inefficient reaction, aerobic glycolysis, to generate considerable amounts of their energy. A possible reason for this bioenergetic alteration is the requirement to produce other metabolic end products to support their rapid growth and proliferation under low oxygen tension, and the possible adaptation to evade death in toxic environments or due to the effects of cytotoxic agents.

Probably the most important treatise ever provided for mitochondrial dysfunction and its possible causative role of cancer is that provided several decades ago by the Nobel Laureate and Biochemist, Otto Warburg. In his series of experiments on respiration and metabolism of cancer cells, coupled with his in-depth analysis of reported works from other investigators at the time, by an approach reminiscent of what Watson and Crick employed in deciphering the DNA double helical structure, Otto Warburg was able to unwaveringly hypothesize that neoplastic transformation originated as a consequence of irreversible damage to mitochondrial respiration. Cancer cells are, therefore, compelled to rely on the inefficient glycolytic mode of ATP synthesis (2 ATPs/glucose), rather than respiration that produces substantially more ATP/glucose (~36 ATPs/glucose). Warburg observed that, in conditions of normal oxygen tension, normal cells produced most of their energy via mitochondrial respiration. In contrast, over 50% of cancer cell energy was generated in the cytosol via glycolysis, with the remainder from the mitochondrial respiratory chain. This bioenergetically inefficient glycolytic reliance of cancer cells for most of their energy production is not primarily due to lack of oxygen, because it operates even in the presence of adequate oxygenation. The bioenergetically inferior nature of glycolysis implies that cancer cells must adopt a mode of increased glucose import to meet their energy demands.

In his seminal presentation in German on May 25th 1955 to the German Central Committee for Cancer Control, which was later translated into English and published in Science, Otto Warburg made several important landmark observations with regard to the origin of cancer, as implied in the title of his publication *On the origin of cancer cells* (Warburg 1956). Extracts from this publication are presented below and discussed in the context of modern scientific understanding of the development and progression of cancer.

> The irreversible injury of respiration is followed, as the second phase of cancer formation, by a long struggle for existence by the injured cells to maintain their structure, in which a part of the cells perish for lack of energy, while another part succeed in replacing the irreversibly lost respiration energy by fermentation energy. Because of the morphological inferiority of fermentation energy, the highly differentiated body cells are converted by this into undifferentiated cells that grow wildly – the cancer cells.

The body of evidence in support of respiratory damage to cancer cells is currently overwhelming and compelling. Almost every cancer investigated to date demonstrates some components of mtDNA mutations, ranging from somatic single nucleotide mutations, polymorphisms, large-scale deletions, to content alterations, in association with varying levels of respiratory dysfunction and ROS production. Given that mitochondria contributes 13 subunits for four of the five respiratory chain complexes, significant heteroplasmic levels of functionally important mutations will inhibit or slow down respiratory chain activity of cancer cells. Severe damage to some cells should trigger apoptosis (which will be consistent with Warburg's statement *in which a part of the cells perish for lack of energy*).

Glycolysis, TCA cycle, and the respiratory chain are functionally tightly coupled. The TCA cycle is mainly regulated by substrate availability, and inhibited by product accumulation and other cycle intermediates. Loss of respiration will ultimately lead to the accumulation of reduced nicotinamide adenine dinucleotide (NADH) and other critical regulators of the TCA cycle such as OAA, succinyl CoA, and citrate. NADH and succinyl CoA inhibit citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate, which are TCA cycle rate-limiting enzymes. As well NADH inhibits PDH while citrate inhibits citrate synthase. Clearly then, respiratory damage will reduce pyruvate conversion to acetyl CoA and a generalized reduction in TCA cycle activity. Even in the presence of oxygen, glycolysis will become the obvious mode for the cancer cell to obtain sufficient energy for cellular functions (*while another part succeeds in replacing the irreversibly lost respiration energy by fermentation energy*).

> . . .fermentation – the energy-supplying reaction of the lower organisms – is morphologically inferior to respiration. Pasture said in 1876 in the description of these experiments, if there should arise in the mind of an attentive hearer a presentiment about the causes of those great mysteries of life which conceal under the words youth and age of cells. Today, after 80 years, the explanation is as follows: the firmer connection of respiration with structure and the looser connection of fermentation with structure.

Aging is undeniably associated with structural and functional decline in all tissues, but in humans, it is very evident in the skin as a loss of rigidity and elasticity,

which shows as wrinkles. Part of the reason that as we age we lose structure is because of apoptotic cell loss, presumably as a consequence of severe damage to the mitochondrial genome. Indeed, Harman and colleagues proposed several decades ago that the aging process is caused by ROS-mediated damage to macromolecules, giving birth to the free radical theory of aging (Harman 1956). A refinement of this theory by Miquel et al. (1983) led to the mitochondrial theory of aging. While there are several theories on the aging process, the free radical/mitochondrial theory is well studied and has achieved popularity. The basic tenet of this theory is the mitochondria serving as the source as well as target of ROS. The mitochondrial genome and structure can be damaged by ROS. Mitochondrial dysfunction, partly as a consequence of mtDNA mutations, is a hallmark of the aging process. Indeed, experiments have shown that cutaneous aging is associated with significant age-dependent differences in the mitochondrial functions of keratinocytes, and that the aged skin is functionally anaerobic (Prahl et al. 2008). Consistent with this recent observation in the skin is the statement by Warburg about *the looser connection of fermentation with structure*.

> The mysterious latency period of the production of cancer is, therefore, nothing more than the time in which the fermentation increases after a damaging of the respiration. This time differs in various animals; it is especially long in man and here often amounts to several decades, as can be determined in the cases in which the time of the respiratory damage is known – for example, in arsenic cancer and irradiation cancer.

Cancer is an aging disease and therefore, a majority of cancers are diagnosed in people after middle age. Indeed, if we live long enough, almost all of us will develop some form of cancer. Chemical carcinogens have characteristically lengthy periods, averaging decades in some cases, between exposure and appearance of first primary tumors. Carcinogens act via several mechanisms. However, they can covalently bind to DNA to form adducts and cause mutations in the genome. In many circumstances, these DNA adducts and mutations are repaired to maintain genomic integrity. Given the poor repair capability of the mitochondrial system, mutations will likely accumulate in this genome overtime after exposure. Nonetheless, chromosomal instability and mutations in oncogenes, tumor suppressor, and caretaker genes can occur and initiate cancer. But, the noted effects of chemical carcinogens in causing increased ROS production and apoptosis strongly implicate the mitochondrial respiratory injury in chemical carcinogenesis.

> The first notable experimental induction of cancer by oxygen deficiency was described by Goldblatt and Cameron, who exposed heart fibroblasts in tissue culture to intermittent oxygen deficiency for long periods and finally obtained transplantable cancer cells, whereas in the control cultures that were maintained without oxygen deficiency, no cancer cells resulted.

Intermittent oxygen exposure simulates ischemic-reperfusion, and in chronic states, can injure cells. Ischemia is associated with hypoxia, increased glycolysis, lactic acidosis, decreased intracellular pH, increased ROS production, and calcium signaling. Reperfusion following ischemia causes most of the cellular damage by exposing ischemic cells to acute oxygen burst, which can induce excessive

mitochondrial ROS production in association with the depletion of antioxidants. The massive ROS in mitochondria of such cells can inevitably cause mtDNA damage and impairment of respiration with subsequent mitochondrial depletion and initiation of tumorigenesis.

The events of ischemic reperfusion are well studied in organ transplants. Myocardial infarction is a major problem with heart transplants. Ischemic pre or postconditioning is usually performed to prevent or reduce the occurrence of myocardial ischemic injury. Ischemic preconditioning involves inducing short sublethal ischemic episodes interspersed with reperfusion prior to prolonged ischemic insult (Murry et al. 1986). On the other hand, ischemic postconditioning involves short ischemic episodes at the beginning of reperfusion (Zhao et al. 2003). Both manipulations are found to offer protection to ischemic injury via activation of the pro-survival PI3K/AKT signaling pathway (Tong et al. 2000; Mocanu et al. 2002; Hausenloy et al. 2005), probably through the reduction in the levels of the tumor suppressor protein, phosphatase and tensin homolog (PTEN) (Cai and Semenza 2005). It is worth noting that chronic activation of the PI3K/AKT signaling pathway can cause unwanted cellular growth and malignant transformation. Thus, could this be the mechanism by which Goldblatt and Cameron obtained their transplantable cancer cells via chronic intermittent oxygen deficiency?

> Rajewsky and Pauly have recently shown that the respiration linked with the grana can be destroyed with strong doses of X-rays, while the small part of the respiration that takes place in the fluid protoplasm can be inhibited very little by irradiation. Carcinogenesis by X-rays is obviously nothing else than a destruction of respiration by elimination of the respiring grana.

Ionizing radiation generates considerable amounts of ROS. Ogawa et al. (2003) found that ROS formation occurred immediately after cellular irradiation and continued for several hours, resulting in oxidative DNA damage. Perhaps, this high ROS production was due to mtDNA damage following irradiation. Excess ROS can trigger mitochondrial apoptosis; however, depending on the specific types and levels of ROS, some cells can evoke alternative survival pathways. Among the intracellular targets of ROS damage, the mitochondrial genome is more vulnerable. Importantly, the mitochondrial genome is packed with coding genes without introns; hence, damage easily involves coding genes, leading to respiratory impairment (*respiration linked with the grana*[1] *can be destroyed with strong doses of X-rays*). As cancer is a genetic disease, and the nuclear genome is less likely to sustain the major insults from ionizing radiation, the conclusion reached by Warburg that *Carcinogenesis by X-rays is obviously nothing else than a destruction of respiration by elimination of the respiring grana*, is quite interesting and compelling, and should not be viewed with skeptism.

> On the other hand, we have found that the fermentation of the body cells is greatest in the very earliest stages of embryonal development and that it then increases gradually in the course of embryonal development. Under these conditions, it is obvious – since

[1]Grana refers to mitochondria.

> ontogeny is the repetition of phylogeny – that the fermentation of body cells is the inheritance of undifferentiated ancestors that have lived in the past at the expense of fermentation energy

Recent works provide ample evidence in support of the fact that cancer cells are identical to their undifferentiated ancestors or ES cells, not only in their metabolism, but also in the molecular pathways they invoke. Cancer cells and normal human ES cells share similar properties such as self-renewal, pluripotency, and teratoma formation. In support of these characteristics, several cancer cells express molecular signatures that define the pluripotent state as well. These molecular markers include *POU5F1* (*OCT4*), *NANOG, SALL4, TDGF1 (CRIPTO), LECT1, BUB1, SOX2,* and *LIN28*. Indeed, Ben-Porath et al. (2008) demonstrated the preferential overexpression of genes characteristic of ES cells in histologically poorly differentiated tumors. In breast cancer, the ES-like gene signature expression was associated with poor outcome. Apart from these pluripotent markers, it is clear that several signaling pathways employed for embryonic tissue patterning are equally deregulated in cancer cells. These include myriads of signaling networks, including the HH, WNT, NOTCH, BMP, and FGF pathways. Therefore, Otto Warburg's statement that "it is obvious – since ontogeny is the repetition of phylogeny – that the fermentation of body cells is the inheritance of undifferentiated ancestors that have lived in the past at the expense of fermentation energy", is very interesting in the light of our understanding of ontogeny and oncology, and we are only beginning to understand the molecular underpinnings of these observations.

2.5 Molecular Basis of the Warburg Phenomenon

Advances in molecular biology continue to shed tremendous amount of light on the physiologic relevance of the Warburg effect to cancer biology. The well-established increased intake of glucose by cancer cells has found widespread clinical utility in the imaging of cancer using ^{18}(F)-Fluorodeoxyglucose (2-fluoro-2-deoxy-D-glucose – FDG) in positron emission tomography (PET) scans. A glucose analog, 2-fluoro-2-deoxy-D-glucose, is, therefore, taken up at higher concentrations in cells with increased glucose transport such as malignant cells. Intracellular FDG is phosphorylated by hexokinases (HK) to FDG-6-phosphate, which cannot be metabolized further in glycolysis. Thus, FDG-6-phosphate accumulates in the cell and the radioactive isotope fluorine-18 enables imaging of the cell before radioactive decay. The technology is used in the diagnosis, staging, and monitoring of several cancers, including lymphoma, melanoma, and cancers of the colon, breast, and lung. Increased glucose intake by cancer cells is associated with poor prognosis (Lopez-Rios et al. 2007). FDG-PET is also used for diagnosis of Alzheimer's disease. Apart from imaging, the emerging understanding of the molecular

networks controlling the Warburg effect is beginning to reveal exploitable molecular targets for cancer prevention and therapy. It is becoming more evident that aerobic glycolysis is an adaptive mechanism involving several coordinated pathways that maintain the phenotypic features of cancer cells, including survival in hypoxic conditions, metastatic propensity, and evasion from apoptosis. A few interconnected signaling pathways and dysfunctional mutations provide enough evidence for the glycolytic phenotype of cancer cells, and an overview of these events is provided in Table 2.1.

Table 2.1 Control of glycolysis in cancer cells

Regulatory factors	Function	Role in cancer cell	Glycolytic regulation
Hexokinase	Glycolytic enzyme; Converts glucose to glucose-6-phosphate	Expression is increased in several cancers	Rate-limiting enzyme and first to phosphorylate glucose once inside the cell. Enables more glucose entry into the cell via its concentration gradient
PI3K/AKT	Oncogene signaling pathway	Activated in cancer cells by several mechanisms, including *PTEN* mutations	Increases expression and plasma membrane clustering of glucose transporters Increases expression and activity of hexokinase II Promotes association of hexokinase with mitochondria Increases expression of phosphofructokinase
MYC and MondoA	Transcription factors		Induce expression of glycolytic genes such as hexokinase II, enolase, lactate dehydrogenase, and phosphofructokinase
HIF	bHLH transcription factors	Stabilized in cancer even under normoxic conditions	Increases expression of glucose transporters Induces expression of all glycolytic enzymes Induces expression of both lactate dehydrogenase and monocarboxylate transporter 4 Inhibits pyruvate dehydrogenase through activation of PDK1
P53	Tumor suppressor gene; guardian of the genome	Mutated in several cancers	Through loss of TIGAR and SCO2

2.5.1 HK and Glycolysis

The classical role of HK in providing an initial molecular explanation to aerobic glycolysis deserves special attention. The HK gene family comprises four isoforms named HK I–IV (reviewed in Mathupala et al. 2006; Pedersen 2007). All the HKs have structural and functional similarity but different kinetics with respect to substrate utility. HKs I–III are 100 kDa proteins with low Michaelis constants (Km) (~0.02–0.03 mM; i.e., they have high affinities for glucose even at very low glucose concentrations). On the contrary, HK IV, also known as glucokinase, is a 50-kDa protein with only one catalytic site, instead of two as in HK II. Therefore, it has a high Km (~5–8 mM) for glucose that is several hundred times that of HKs I–III. The HKs also differ in their regulatory properties, expression patterns, and subcellular localizations. HK IV is almost exclusively expressed by adult hepatocytes. On the other hand, HK II is silenced in many normal mammalian tissues, except in muscle, fat, and lung tissues where low amounts are expressed (Wilson 1997, 2003).

Work by several groups, including the Pedersen's laboratory that sought explanation for the Warburg's observations, provided the first critical molecular explanation of the Warburg effect. These groups demonstrated that an early metabolic event in carcinogenesis of liver and pancreatic cells was the increased expression of high affinity HK II, and to a lesser extent, HK I in association with the downregulation of HK IV (Rempel et al. 1994; Mathupala et al. 1997; Mayer et al. 1997; Pedersen et al. 2002). In the presence of ATP, HK II catalyzes the rate-limiting and committed step of glucose metabolism in the cell, that is, the ATP-dependent phosphorylation of glucose to glucose-6-phosphate. This reaction does not only create a concentration gradient for the influx of glucose into the cell but also dictates the fate of intracellular glucose. HK I and II directly interact with mitochondria at specific voltage-dependent anion channels (VDACs) (Bustamante and Pedersen 1977). VDACs and ANTs move adenine nucleotides between the mitochondrial matrix and the cytosolic compartments. The interaction of HK II with VDAC can serve dual functions; first, it can inhibit the functional role of VDAC in shuttling ATP from the inter-membrane space into the cytosol, and second, it can interfere with BAX/BAK interaction with VDAC and therefore, inhibit apoptosis. Thus, the elevated levels of HK II in hepatomas and other cancers could contribute to an increase in apoptotic resistance as well as a build-up of ATP at VDAC. The accumulated ATP can be harnessed by HK II to phosphorylate and therefore, commit glucose for metabolism inside the cell. A normal mitochondrial function should couple the glycolytic end product, pyruvate, under aerobic conditions, to the TCA cycle and respiratory chain for energy production. However, defects in the mitochondrial genome, or other signaling pathways in cancer cells that adversely affect mitochondrial respiration imply that the pyruvate produced by glycolysis will be converted to lactate, even in the presence of oxygen (the Warburg phenomenon: i.e., the irreversible damage to cancer cell respiration and therefore, the dependence of cancer cells on glycolysis for energy production even in the presence of adequate oxygen).

2.5.2 The PI3K/AKT Signaling Pathway and Glycolysis

Several oncogene signaling cascades are deregulated in cancer cells, and all appear to control altered metabolism (Table 2.1), apoptosis, and several other phenotypic features of cancer cells.

Phosphoinositide 3-kinase (PI3K) is a heterodimeric protein with two functional subunits, an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The PI3K signaling pathway is activated by prosurvival signals such as cytokines, growth factors, hormones, and oncogenic Ras. Upon activation by G protein-coupled receptors or tyrosine kinase receptors, the 85 kDa subunit interacts with phosphorylated tyrosine residues on the receptor through a Src-homology 2 (SH2) domain. The 110 kDa catalytic subunit then transfers a phosphate group to membrane phospholipids. Sequentially, this process involves the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2)/PIP2 into a second messenger, phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P3)/PIP3. The intracellular second messenger recruits and activates 3-phosphoinositide-dependent kinase 1 (PDK1) that activates protein kinase B (AKT/PKB), which then triggers downstream signaling events.

Protein kinase B/AKT is a serine/threonine kinase that was discovered as a cellular homologue of a viral oncogene. Protein kinase B is downstream of the PI3K signaling cascade, and is activated by phosphorylation. Possibly, the most frequent mode of PKB/AKT activation is through PTEN loss of function. The *PTEN* tumor suppressor is a negative regulator of PI3K. PTEN, which are ubiquitously expressed in cells, dephosphorylate PtdIns(3,4,5)P3 back to PtdIns(4,5)P2 and therefore, block PI3K/AKT signal activation. Heterozygous *PTEN* knockout mice develop numerous tumors. PTEN are mutated in a vast majority of human cancers, and thus, cause PI3K/AKT activation in the absence of growth factor receptor stimulation. Protein kinase B/AKT is amplified and activated in several cancers. Constitutive activation of PKB/AKT in cancer cells can occur indirectly via growth factor stimulation or amplification of the PI3K pathway.

Once phosphorylated and activated, PKB/AKT phosphorylate target intracellular substrates, such as CREB, E2F, NF-κB in the nucleus, and caspase 9, BAD, and GSK-3β in the cytosol. Protein kinase B/AKT has pleiotropic functions. This signaling pathway primarily promotes cell proliferation and survival. It also regulates glucose uptake and hexokinase activity as well as the maintenance of mitochondrial membrane potential. The functions of PKB/AKT are mediated by several downstream components of the signaling cascade. Importantly, PKB/AKT activates mTOR (mammalian target of rapamycin) that is deregulated in several cancers as well.

Protein kinase B/AKT pathway stimulates metabolic conversion of cancer cells toward aerobic glycolysis in several different ways. First, PKB/AKT signaling induces increased expression and plasma localization of glucose transporters, and this increases glucose import and consumption by cancer cells. Second, activated PKB/AKT stimulates increased expression and activity of HK II and

thus, phosphorylation of glucose, which in turn will facilitate more glucose entry into cells along its concentration gradient. Several growth factors promote association of HK with mitochondria by using PI3K/AKT/mTOR pathway. Finally, PKB/AKT signaling induces the expression of another glycolytic enzyme, phosphofructokinase.

Interestingly, PKB/AKT signaling can be activated by mitochondrial respiratory dysfunction (e.g., mtDNA mutations). Decreased respiration from mitochondrial dysfunction inactivates PTEN, leading to PKB/AKT activation. Mitochondrial DNA depletion also activated PKB/AKT signaling accompanied by glycolysis, increased invasiveness, and evasion from apoptosis/anoikis (Moro et al. 2008). Thus, apart from growth factor stimulation or amplification of PI3K and *PTEN* mutations or downregulation, loss of mitochondrial respiratory functions that are pervasive in cancers augments PKB/AKT activation.

2.5.3 The MYC Oncogene and Glycolysis

The MYC oncogene is a transcription factor that controls growth, proliferation, and death of cells via the regulation of several genes. MYC forms a complex with its partner, MAX, and the activated complex binds to E-box DNA motifs (canonical CACGTG, CATGTG) to induce transcription of target genes. Overexpression of MYC is demonstrated in several cancers.

MYC induces glycolysis in a number of ways. Overexpression of MYC increases the metabolism of glucose via the activation and expression of several glycolytic enzymes (Osthus et al. 2000). Hexokinase II, glyceraldehyde-3-phosphate dehydrogenase, enolase 1, pyruvate kinase, and lactate dehydeogenase are highly overexpressed in several cancers (Altenberg and Greulich 2004; Mathupala et al. 2006). MYC has been shown to bind genes-encoding glycolytic enzymes such as HK II, enolase, and lactate dehydrogenase A (Kim and Dang 2005). Lactate dehydrogenase converts pyruvate to lactate, a metabolic pathway that is very active in glycolytic cancer cells. Lactate dehydrogenase expression is directly induced by oncogenes such, as MYC, and indirectly by activation of HIF1α. The activity of MYC leads to increased ROS production that could damage mtDNA, impair respiratory chain activity, and thus, facilitate aerobic glycolysis via several established mechanisms.

Another transcription factor that functions similar to MYC in the regulation of glycolysis is MondoA. MondoA is a basic helix-loop-helix leucine zipper transcription factor that heterodimerizes with its partner, M1x (Billin et al. 1999). This complex shuttles between the mitochondrial outer membrane and the nucleus. It appears that the complex senses the mitochondrial energy requirements of the cells, and relays this to the nucleus to influence the required gene expression. In the nucleus, MondoA:M1x complex interacts with CACGTG E-box motifs to activate the transcription of target genes. Three key glycolytic enzymes, lactate dehydrogenase A, HK II, and 6-phosphofruto-2-kinase/fructose-2,6-bisphosphatase 3, are direct targets of MondoA:M1x complex (Billin et al. 2000; Sans et al. 2006).

2.5.4 *Hypoxia-Inducible Factor Pathway and Glycolysis*

The growth of cancer cells is associated with different levels of oxygen deficiencies. Because solid tumors grow rapidly, cells at the periphery will have access to vasculature and therefore, more likely to have adequate oxygenation. However, progressively deep into the tumor will be cells exposed to decreasing levels of oxygen that could range from hypoxia to even anoxia. The reason for the reduced oxygen is primarily due to poor blood supply from rapid growth that outstrips vascular supply. Indeed, as little as 300 cancer cells can induce hypoxia as a consequence of inadequate vascularization. One mechanism to survive these adverse conditions is the induction and stabilization of hypoxia-inducible factors (HIFs), which partly restore new blood vessels to the tumor.

The HIFs are transcription factors with basic helix-loop-helix and Per/ARNT/Sim (PAS) domains. They were first identified as factors that regulate increased expression of erythropoietin in response to hypoxia and hence, so named (Semenza and Wang 1992). The two family members, HIFα and HIFβ become active upon the formation of heterodimers. Hypoxia-inducible factor α has three subunits, namely, HIF1α, HIF2α, and HIF3α. Hypoxia-inducible factor 2α is ∼48% homologous to HIF1α, and is expressed and stabilized under hypoxic conditions as well (Tian et al. 1997). On the contrary, HIF3α is a dominant negative regulator of HIF because it dimerizes with HIF1β to form a transcriptionally inactive heterodimer, thus, reducing the activity of HIFs. Hypoxia-inducible factor β is an aryl hydrocarbon receptor nuclear translocator (HIF1β/ARNT) with two homologues, ARNT2 and ARNT3. All three HIF1β homologues are constitutively expressed and can heterodimerize with HIFα subunits (Maynard and Ohh 2004). Whereas both HIF1α and 2α are functionally active and can interact with hypoxia-responsive elements (HREs: canonical CCATG sequences) in gene promoters, it appears HIF1α preferentially induces HREs in glycolytic gene promoters (Hu et al. 2007).

2.5.4.1 Regulation of Hypoxia-Inducible Factor

Regulation by Hypoxia: Hypoxia-inducible factor 1α is stabilized under hypoxic conditions. Thus, in normoxia, the levels of HIF1α are low in cells because of ubiquitin-mediated proteasome degradation. In this process, the protein is first hydroxylated on proline 402 and 564 in the oxygen-dependent degradation (ODD) domains by HIF1α prolyl hydroxylases. The von Hippel–Lindau (VHL) protein products (Schofield and Ratcliffe 2005) in a complex with Cul-2, Elongin B, and Elongin C (Linehan et al. 2003) recognize the hydroxylated proteins. The VHL protein is an E3 ubiquitin ligase (together with NEDD8) that mediates the proteasome degradation of hydroxylated HIF1α. Under hypoxic conditions, HIF1α cannot effectively be hydroxylated by prolyl hydroxylases as their Km for oxygen is very high. Therefore, under such circumstances, HIF1α is stabilized and enters the nucleus to form an active transcription factor by heterodimerizing with HIF1β subunits. The heterodimeric complex then binds to HREs and induces the expression

of several genes, including those involved with glucose metabolism, angiogenesis, tumor invasion, and survival. In normal physiology, hydroxylation is the primary mode of HIF1 regulation. However, hypoxia and pseudohypoxia are hallmarks of fast-growing solid tumors. Cancer cell microenvironment can stabilize HIF1α via hydroxylation. It has recently become evident, that apart from hypoxia, the levels and activity of HIF1α can be increased under normoxic conditions by cancer cells (pseudohypoxia).

Regulation by Mutations in VHL Protein: VHL is a tumor suppressor gene and mutations in this gene are associated with the VHL syndrome where patients develop tumors in multiple organs, including the kidneys. VHL gene mutations in renal cell carcinomas cause constitutive stabilization of HIF1α and HIF2α even under normoxic conditions (Kaelin 2002) because of loss or degradation of these molecules. This leads to loss of tumor suppressor activity of VHL, and constitutive transcription of several tumor promoting target genes.

Regulation by Oncogene Signaling: Oncogene signaling is common in almost all cancers. Ras-MAPK pathway activation caused increased levels of HIF1α in several model systems (Sheta et al. 2001). Similarly, the expression of Src resulted in the accumulation of HIF1α in nomoxic conditions (Jiang et al. 1997), and the activation of PKB/AKT increases HIF1α protein translation by AKT/FRAP/mTOR pathway (Laughner et al. 2001; Plas and Thompson 2005). Finally, receptor tyrosine kinase signaling through MAPK/ERK1/2 can phosphorylate and activate HIF1α. Thus, an important function of activated oncogenic pathways in cancer is to promote tumor progression via HIF pathway activation.

Regulation by TCA Cycle Enzymes: Enzymes of the TCA cycle have been recently shown to provide a feedback mechanism to stabilize HIF1α. Mutations in succinate dehydrogenase and fumarate hydratase genes cause paragangliomas and leiomyomas, respectively (see Chap. 4). The functions of the two enzymes in the TCA cycle result in the generation of reducing equivalents for the respiratory chain. Therefore, loss of enzyme activity leads to reduced mitochondrial respiration and stalled TCA cycle activities that lead to the accumulation of succinate and/or fumarate, which can cause a condition known as pseudohypoxia (see Sect. 4.2.3). During hydroxylation of the proline residues in HIF1α, the prolyl hydroxylase converts α-ketoglutarate or 2-oxoglutarate and oxygen to succinate and carbon dioxide (Kaelin 2005). Accumulated succinate or fumarate can leak into the cytosol and inhibit the activity of HIF1α prolyl hydroxylases. Tumors with loss of fumarate hydratase activity are highly vascularized with increased HIF1α expression (Pollard et al. 2005a, b), and increased expression of glycolytic enzymes (Vanharanta et al. 2006). Succinate dehydrogenase mutations in tumors cause HIF pathway activation and gene expression profiling of pheochromocytomas with *SDHB, SDHD,* or *VHL* mutations reveals a pseudo-hypoxic HIF gene signature pattern (Dahia et al. 2005). Pyruvate and lactate have also been shown to stabilize HIF1α (Lu et al. 2005).

Regulation by Mitochondrial Reactive Oxygen Species: It has been suggested that mitochondrial ROS is an important signal that mediates HIF1α stabilization. Mitochondria may function as oxygen sensors and signal under low oxygen tension to stabilize HIF1α by releasing ROS into the cytosol (Simon 2006). Consistent with

this suggestion, ROS from complexes II and III have both been implicated in HIF1α stabilization (Guzy et al. 2005, 2008; Klimova and Chandel 2008). Hydrogen peroxide, in particular, can oxidize Fe^{2+} in the Fenton reaction to Fe^{3+} associated with the production of hydroxyl radical. Because Fe^{2+}, but not Fe^{3+} is a cofactor for HIF1α prolyl hydroxylases, decreased Fe^{2+} will decrease the activity of this enzyme and therefore, reduced hydroxylation of HIF1α for ubiquitination and degradation, leading to its stabilization.

2.5.4.2 Regulation of Glycolysis and Mitochondrial Functions by Hypoxia-Inducible Factor

HIFs contribute to the glycolytic phenotype of cancer cells in several ways (Fig. 2.2). There is more glucose in the blood than in cells; however, the intake of

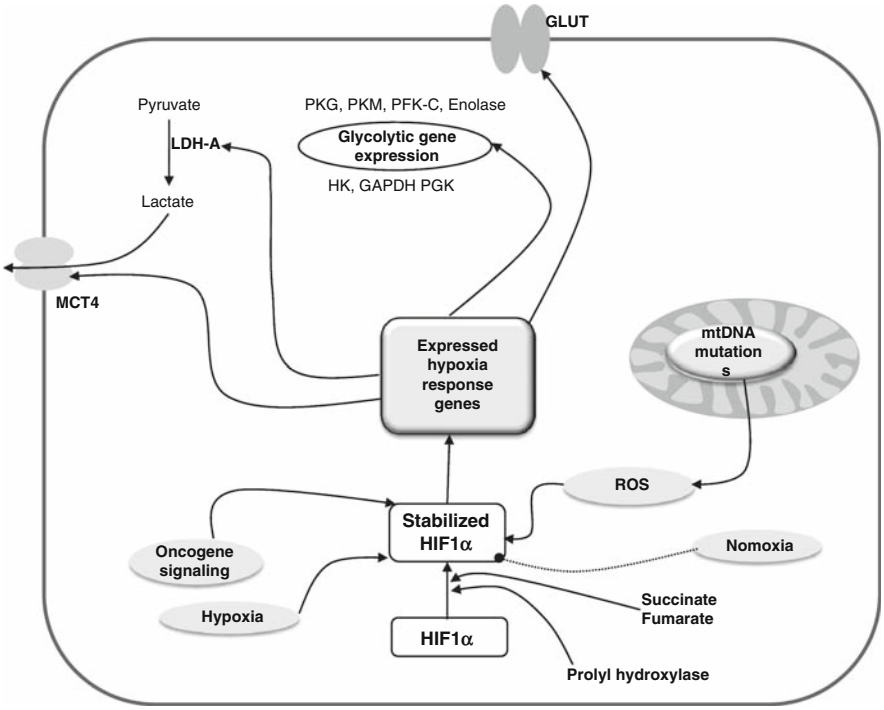

Fig. 2.2 *Regulation of glycolysis by HIF activity*. In nomoxia, HIF1α is hydroxylated, ubiquitinated, and destroyed. However, cancer-cell phenotype, such as hypoxia, oncogene signaling, and accumulation of tricarboxylic cycle intermediates (e.g., succinate and fumarate), stabilize HIF1α. Stabilized HIF1α has several glycolytic effects, including the activation of several glycolytic enzyme gene expressions. Other important genes induced by stabilized HIF1α are glucose transporters (GLUT) to import glucose into the cell, lactate dehydrogenase to convert pyruvate to lactase, and monocarboxylate transporter 4 (MCT4) to remove toxic lactate from the cell into the extracellular space

glucose into cells requires specific transporters. There are 13 members of the glucose transporter proteins; however, GLUT1 and GLUT3 appear to mediate most intracellular import of glucose because of their high affinity for glucose and the number of copies of these per cell (Brown 2000). Hypoxia-inducible factor 1 targets and induces GLUT1 and GLUT3 expression. Once in the cell, glucose is phosphorylated by HK to glucose-6-phosphate, the initial step of glycolysis. However, glucose-6-phosphate can adopt other fates in the cell. It can enter the pentose phosphate shunt to produce ribose for nucleic acid biosynthesis, or used to synthesize glycogen for storage or for a biosynthesis of structural glycoproteins. However, stabilized and activated HIF facilitates glycolysis by its induction of HK I and importantly, HK II, as well as all glycolytic enzymes, thereby biasing the metabolism of glucose-6-phosphate towards glycolysis.

Under normal cellular oxygenation, pyruvate from glycolysis enters the mitochondria and is converted to acetyl CoA by PDH. Acetyl CoA is metabolized in the TCA cycle to produce reducing equivalents for energy production by the respiratory chain. Because of impaired respiration of cancer cells, pyruvate and NADH accumulate in the cytosol. Stabilized HIF1 in cancer or hypoxic cells induces lactate dehydrogenase and plasma membrane monocarboxylate transporter 4 (MCT4). Lactate dehydrogenase converts pyruvate and NADH to lactate and NAD+. The lactate is then shuttled into the extracellular space by MCT4 while the NAD+ is fed back into glycolysis to be used by glyceraldehyde-3-phosphate dehydrogenase.

Hypoxia inducible factor 1 depresses mitochondrial respiration in addition to stimulating glycolysis. Indirectly, HIF1 inhibits PDH, the enzyme that catalyzes the irreversible conversion of pyruvate to acetyl CoA that is subsequently metabolized by the TCA cycle to produce NADH and $FADH_2$ for the electron transport chain. Pyruvate dehydrogenase kinase 1 (PDK1) is one of the four family members of protein kinases that phosphorylate and inactivate PDH (Patel and Korotchkina 2001), thereby attenuating the entry of carbon from pyruvate into the TCA cycle. PDK1 expression results in the inhibition of pyruvate dehydrogenase α (PDHα) subunit through phosphorylation. It is now well established that PDK1 is a direct target of HIF1 in cancer cells (Papandreou et al. 2006). Consistent with this finding, PDK1 is overexpressed in tumors in which HIF1 is stabilized (Koukourakis et al. 2005). Knockdown of PDK 1 with short hairpin RNA reduced PDHα phosphorylation and restored PDH activity. This treatment also reversed the Warburg effect, decreased normoxic HIF1α stabilization, reduced hypoxic cell survival, decreased cancer cell invasiveness, and inhibited tumor growth (McFate et al. 2008).

Oxygen insufficiency and/or depressed respiration will favor glycolysis as a means to obtain energy. Two examples illustrate this in cancer cells.

- In renal carcinomas with VHL mutations in which HIF1 is chronically stabilized, a different mechanism of modulating mitochondrial respiration is postulated. This mechanism involves the MYC oncogene. In normal cells, MYC dimerizes with MAX, and the active complex regulates increased expression of genes, including TFAM, which controls mitochondrial transcription and

replication (Li et al. 2005). In chronic hypoxia, HIF1 activates a different MYC family member, MAX interactor 1 (MXI1), a negative regulator of MYC. MAX interactor 1 displaces MAX and therefore, inactivates MYC, thereby reducing TFAM expression, mitochondrial biogenesis, and oxygen consumption (Zhang et al. 2007).

- Hypoxia-inducible factor 1 also regulates mitochondrial utilization of oxygen through its modulation of cytochrome c oxidase (COX) expression and activity. COX is the final respiratory chain complex that donates electrons to oxygen. Fukuda et al. (2007) demonstrated in mammalian cells that oxygen controls COX4-1 and COX4-2 expression. These two isoforms modulate COX activity differently. Indeed, in hypoxia, HIF1 induces the expression of COX4-2 isoform and mediates the destruction of COX4-1 by inducing the expression of LON, a mitochondrial protease that degrades COX4-1. It is suggested that COX4-2 enables more efficient utilization of limited oxygen in hypoxic conditions.

2.5.5 P53 and Glycolysis

In addition to its well-known functions, such as maintenance of genomic integrity, cell cycle arrest, and apoptosis, recent evidence suggests that p53 regulates mitochondrial respiration and glycolysis. Bensaad et al. (2006) identified TP53-induced glycolysis and apoptosis regulator (TIGAR) as a p53 target gene. In wildtype cells, TIGAR expression is normal and functions to decrease the levels of fructose-2, 6-bisphosphate, which suppresses glycolysis by diverting glucose-6-phosphate to the pentose phosphate pathway. Pentose phosphate pathway activation increases the levels of NADPH, which increases the levels of reduced glutathione to scavenge ROS. Therefore, in normal TIGAR expressing cells, there is an overall decrease in ROS. Loss of p53 and therefore, TIGAR expression, causes increased glycolysis at the expense of pentose phosphate pathway, as well as increased ROS production.

Matoba et al. (2006) also identified a synthesis of cytochrome oxidase 2 (SCO2) as a p53 downstream target. Expression of SCO2 was observed to mirror the levels of p53, decreasing in levels from p53+/+ to p53+/− to p53−/− cells. SCO2 is required for the assembly of COX complex. Interestingly, expression of SCO2 in p53−/− cells restored respiration and disruption of SCO2 in p53+/+ cells impaired respiration. Loss of p53 leads to loss of SCO2 expression and improper COX assembly and a consequent loss of respiration. Loss of respiration will force cells to depend on glycolysis for energy production, and also activate PKB/AKT, setting up an amplification loop for glycolysis.

Phosphoglycerate mutase (PGM) is a glycolytic enzyme that catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate. The activity of PGM is high in cancers of the lung, colon, and liver. Kondoh et al. (2007) demonstrated that p53 suppresses the expression of PGM. Therefore, loss of p53 in cancer cells will lead to increased PGM activity and enhanced glycolysis.

2.6 Glutamine Metabolism in Cancer Cells

Glutamine is the most abundant naturally occurring amino acid in the body. It can be synthesized from metabolic intermediates such as α-ketoglutarate and OAA. However, in catabolic conditions, such as injury or chronic illness, glutamine becomes conditionally essential and has to be ingested to meet the demands of the stressed body.

Glycolysis may function to meet the energy demands of the cancer cell, especially in hypoxic conditions. However, adaptive accelerated glutamine metabolism by cancer cells appears to provide substrates for increased lipogenesis and nucleic acid biosynthesis that are critical to the proliferative phenotype of the cancer cell (Fig. 2.3). Cancer cells exhibit increased glutamine intake and metabolism that far exceeds its role as the amino group donor for many biosynthetic pathways, including being the precursor for the synthesis of proline, ornithine, and arginine.

The majority (∼60%) of glutamine in the cancer cell is used to generate lactate and alanine in a reaction that involves the malic enzymes and hence, NADH

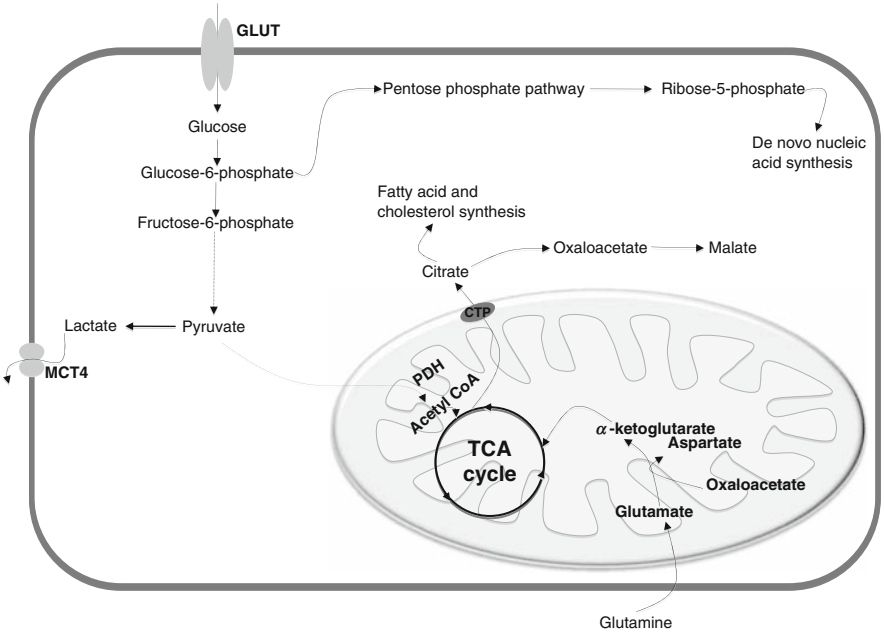

Fig. 2.3 *Increased glycolysis, glutaminolysis, and lipogenesis of the cancer cell.* Increased glucose uptake powers glycolysis, but because of the inefficient utilization of glycolytic end products by the cancer cell, pyruvate is converted to lactate that is removed from the cancer cell by the increased activity of MCT4. Cancer cells take in more glutamine that feeds the tricarboxylic acid (TCA) cycle leading to more citrate production. Citrate is transported into the cytosol mediated by citrate transport proteins (CTP). Cytosolic citrate is converted to acetyl CoA that supports lipid and cholesterol biosynthesis

production. NADH is a critical requirement for lipogenesis. Glutamine metabolism also generates OAA via an anaplerotic reaction (DeBerardinis et al. 2007). The OAA feeds into the TCA cycle and is used to produce citrate. Because, the citrate cycle is truncated in cancer cells, citrate is exported into the cytosol via the activity of citrate transport proteins (CTPs). To augment the transport process, the activity of CTP is increased in cancer cells. Cytosolic citrate is used for lipogenesis. Thus, glutamine metabolism provides both NADH and citrate for increased lipogenesis of the cancer cell. Glutamine carbon in the TCA cycle has been detectable in aspartate, which can be used to synthesize nucleotides, aspartate, and arginine (DeBerardinis et al. 2007).

MYC regulates the transcription of genes involved in mitochondrial glutamine metabolism, independent of PI3K/AKT signaling pathway (Wise et al. 2008). Glutamine metabolism was adequate to supply energy for cancer cells, in association with the reduced utilization of glucose in the TCA cycle as well as glucose for mitochondrial-dependent synthesis of phospholipids (Wise et al. 2008).

2.7 Lipid Metabolism in Cancer Cells

In addition to aerobic glycolysis and increased glutaminolysis, cancer cells have altered lipid metabolism and elevated de novo fatty acid biosynthesis. Studies have shown that irrespective of the concentration of extracellular lipid, fatty acids in cancer cells are mainly synthesized de novo. The reason for this is unclear, but could include perpetuation of the malignant phenotype such as increased proliferation and evasion from apoptosis.

Cytosolic acetyl CoA for lipogenesis appears to come from at least two sources. First, in the TCA cycle, mitochondrial acetyl CoA condenses with OAA to form citrate that can enter the cytosol where it is converted back to acetyl CoA for lipogenesis. Perhaps, the cancer cell adapts increased glutaminolysis in order to accelerate citrate production via the supply of OAA generated in an anaplerotic reaction. Second, lipogenesis can depend on uptake by cancer cells of acetate from blood. In the cell, acetate is converted to acetyl CoA by cytosolic acetyl coenzyme A synthase. In support of this source of substrate for lipogenesis, plasma acetate levels were found to be lower in cancer patients compared to healthy control subjects (Pomare et al. 1985).

Lipogenesis is established in cancer cells as a result of increased expression and activity of a number of lipogenic enzymes, including fatty acid synthase (FASN), ATP citrate lyase, acetyl CoA carboxylase α (ACCα), and Spot14. FASN was first identified as breast cancer-associated protein OA-519. It is overexpressed in a variety of other cancer cells. FASN is a multifunctional enzyme that catalyzes the synthesis of palmitate from acetyl CoA and malonyl coenzyme A. This is a major enzyme that mediates the anabolic synthesis of fatty acids from carbohydrates in cancer cells. FASN is mainly expressed in normal hepatocytes where it mediates the de novo synthesis of lipids for export to adipose tissues for storage or to actively

metabolizing tissues for use in energy production. Many normal tissues rely on circulating lipids and hence, do not express FASN. Many cancers, especially aggressive tumors constitutively express very high levels of FASN. Meanwhile, expression of FASN is an early event in carcinogenesis, and the levels mirror disease progression from early to late stage cancers, and correlates with cancer prognosis.

A complex regulatory network controls the expression of FASN. It is well established that steroid hormones induce its expression in hormone-dependent prostate, breast, and endometrial cancer cells, and this appears to involve MAPK, MEK1/MEK2, and PI3K/AKT signaling pathways. The PI3K/AKT pathway plays an important role in lipogenesis. Activated PKB/AKT signaling stimulates lipogenic gene expression via activation and nuclear localization of the lipogenic transcription factor, sterol response element binding protein-1. Furthermore, activated PI3K/AKT pathway stimulates fatty acid synthesis by a direct activation of ATP citrate lyase and inhibition of β-oxidation by downregulating the expression of CPT1A. FASN expression activates PKB/AKT in a subset of thyroid cancers and inhibition of FASN suppresses growth and induces cancer cell death (Uddin et al. 2008).

ACCα is a rate-limiting enzyme that catalyzes the formation of malonyl CoA from acetyl CoA and carbon dioxide. ACCα is highly expressed in breast and PCa cells, and when inhibited, results in a marked reduction in lipogenesis and induction of apoptosis. Spot14 regulates several lipogenic enzymes, including ACCα and FASN. Spot14 levels in breast cancer correlate with invasive phenotype and poor outcome.

2.8 Citrate Metabolism by Prostate Glandular Epithelial Cells

The intermediary metabolism of the prostate gland is uniquely different from other body cells. PCa development is associated with an early metabolic switch from citrate secreting normal epithelial cells to citrate oxidizing cancer cells. Lower zinc levels in the transforming cells as a consequence of decreased expression of zinc transporters mediate this metabolic switch. In view of these observations, Leslie C. Costello and Renty B. Franklin have advocated for the exploitation of this metabolic transformation for the early detection, diagnosis, chemoprevention, and treatment of PCa. The altered metabolism of PCa cells is summarized here. For an in-depth discussion on this topic, see the numerous reviews by L. C. Costello and R. B. Franklin (Costello and Franklin 2000, 2006; Costello et al. 1999, 2004, 2005).

Benign Prostate Epithelial Cells: The intermediary metabolism of the human prostate gland is not identical in all glandular epithelial cells because of their different embryonic sources (Costello and Franklin 2000). According to McNeal, the prostate gland can be divided into three zones. The part that surrounds the proximal urethra is called the transition zone, and accounts for 10% of all PCas. The central zone surrounds the transition zone and extends to the angle of the urethra

and base of the bladder. Only ~5% of all PCas originate from this region. The bulk of the prostate gland consists of the peripheral zone, which contains ~75% of prostatic glandular tissue. Consistent with this histology, the peripheral zone is the site of most prostate malignancies and the part of the gland that is metabolically important. The epithelial cells in the peripheral zone are highly specialized citrate secreting cells. In contrast to cells in the central zone, the peripheral zone cells accumulate very high levels of zinc (3–10-fold higher) (Costello and Franklin 2000). This is due mainly to the normal activity of the zinc uptake transporter in these cells (Franklin and Costello 2007). The presence of high zinc levels in the mitochondria of the peripheral zone cells inhibits the activity of mitochondrial aconitase (m-aconitase), thereby blocking citrate oxidation in the Krebs cycle. The accumulated citrate is secreted in the prostatic fluid. Thus, normal prostate glandular epithelial cells have truncated Krebs cycle, low citrate oxidation, low respiration, and are therefore, bioenergetically inefficient (~60% less ATP production compared to PCa cells).

PCa Cells: Apart from the prostate gland, normal epithelial cells completely oxidize citrate in the TCA cycle to produce reducing equivalents for energy production in the respiratory chain. On the other hand, most tumor cells demonstrate the Warburg effect (i.e., bioenergetically inefficient aerobic glycolysis). Therefore, tumorigenesis, in general, is associated with a metabolic switch from energy-efficient benign cells to energy-inefficient tumor cells. On the contrary, and indeed very intriguingly, an early metabolic switch from rather energy-inefficient benign cells to energy-efficient tumor cells marks the malignant transformation of peripheral zone prostate glandular epithelial cells. An early indication of this metabolic alteration is the downregulation of the gene that encodes the zinc uptake transporters in the transforming epithelial cells (Desouki et al. 2007). This event leads to a dramatic reduction in zinc levels (70–80% lower than that in normal peripheral zone cells) in these cells. The low zinc levels in the mitochondria of cancer cells remove the inhibition of zinc on m-aconitase and therefore, enable the complete oxidation of citrate through the TCA cycle. This metabolic pathway generates 36 ATP/glucose metabolized, which is over two times higher than that of the normal peripheral zone cells that only produce ~14 ATPs per glucose metabolized. Thus, malignant prostate cells completely oxidize citrate and hence, generate more energy than their benign counterparts. Indeed, citrate levels in PCa cells are significantly lower than in benign epithelial cells. It should be noted that part of the citrate in these transforming cells is used for lipogenesis, which is a necessary requirement of rapidly dividing cells.

2.9 Clinical Implications of Altered PCa Metabolism

PCa is the most commonly diagnosed malignancy in men. However, mortality is low compared to many other cancers. One reason for the low mortality is that PCa is a slow-growing tumor, and importantly, it is completely curable when diagnosed

early. Because of the indolent nature of PCa, it is more frequently diagnosed in men over the age of 50 years, although autopsy series have revealed the presence of this tumor in men as young as 20 years of age. The long latency of PCa development provides an adequate window for early detection and chemoprevention. The metabolic transformation of PCa cells is an early event in PCa evolution. hZIP gene downregulation and therefore, derepression of the activity of m-aconotase to oxidize citrate, are all observed in the very early stages of PCa development. The metabolic transformation can be targeted in imaging and/or biochemical assay development for the detection and management of PCa. They also have a tremendous implication in the treatment and prevention of PCa.

2.9.1 Diagnostic Imaging of PCa

Accurate local cancer staging, intraprostatic localization, and assessment of tumor volume, are all important factors considered in making PCa treatment decisions. Currently, a combination of clinical parameters, such as the extent of disease on biopsy, number of positive cores, Gleason score, and serum PSA, are used in the initial assessment of patients. Conventional pelvic imaging using MRI/CT scans are also useful, but they are insensitive and costly procedures.

The dramatic decrease in citrate levels observed in PCa is associated with increased choline and creatine levels. Importantly, these changes are not observed in normal prostate or benign prostatic hyperplasia. Magnetic resonance spectroscopy is an imaging modality that uses strong magnetic field to assess metabolites in tissue. This technique is developed to obtain the metabolic information of citrate and choline, and the citrate–choline ratio has served as a sensitive detection method of PCa. The metabolic spectra can be coupled with MRI to give precise in situ anatomic and metabolic map of the prostate for accurate intraprostatic localization and assessment of tumor volume. Currently, endorectal MRI/MRSI are standardized and used to improve PCa diagnosis and staging (Jung et al. 2004).

Compared to TRUS-guided prostate biopsy for cancer detection, endoMRI/MRSI was more sensitive in tumor detection. In a study involving 42 men with at least two previous negative biopsies, 31 of them had metabolic abnormalities reminiscent of PCa that was revealed by endoMRI/MRSI (Prando et al. 2005). Umbehr et al. (2008) performed a systemic review and meta-analysis of MRI/MRSI in PCa diagnosis. They found that for men suspected of PCa, the sensitivity and specificity of MRI/MRSI were 82 and 88%, respectively. Importantly, in low-risk patients, MRS/MRSI was not sensitive, but was highly specific (91%) at detecting PCa. This finding is probably because MRI/MRSI can detect tumors that are 0.5 cm or larger, and is better at detecting Gleason score 8–10 tumors than lower Gleason score tumors (Zakian et al. 2005).

2.9.2 Screening for PCa Using Biofluids

The screening for early detection of PCa still relies on PSA and digital rectal examination of men 50–70 years. However, the PSA test is not specific, because serum PSA is also elevated in other nonmalignant prostatic conditions such as prostatitis and benign prostatic hyperplasia. Therefore, there has been a need to identify and validate accurate biomarkers for PCa screening. Yet, no such biomarker exists at present. The specific PCa-associated metabolic transformations appear very attractive for an accurate detection of PCa using biofluids such as PMF or first-catch postDRE urine (Costello and Franklin 2009). The normal PMF citrate (~40–150 mM) and zinc (~8–10 mM) levels are several folds higher than in PCa (citrate; ~0.2–2 mM, and zinc; ~0.4–0.8 mM). These figures strongly suggest that biochemical assays developed and validated for PCa screening using these metabolites should increase sensitivity and specificity over PSA and possibly other emerging biomarkers.

2.9.3 PCa Prevention and Treatment

An early event in PCa development is hZIP downregulation leading to low zinc levels and hence, increased activity of m-aconitase and citrate oxidation. Another important role of zinc is the induction of apoptosis in PCa cells. Zinc treatment of PCa cells increases the BAX/BCL-2 ratio leading to BAX-mediated cytochrome c release and induction of apoptosis via caspase activation (Feng et al. 2008; Franklin and Costello 2009). Thus, can restoration of zinc in premalignant cancer cells reverse them to normal states or will repopulation of PCa cells with normal zinc levels eliminate them via apoptosis? There are models to increase zinc levels in PCa cells.

- The simplest approach will be to increase zinc levels in the extracellular milieu of prostate cells through zinc supplementation. Unfortunately, the epidemiologic literature does not support the basic science findings. In some studies, high doses and prolonged zinc supplementation have been associated with an increased risk of PCa. An Italian study suggested that increased zinc intake adversely influenced the risk for developing advanced PCa (Gallus et al. 2007). However, in other reports, moderate to low levels of zinc, especially in the elderly, is effective against PCa. Gonzalez et al. (2009) showed that the risk of developing significant PCa was reduced with increased zinc intake. Larger and well-designed studies on the effect of zinc in PCa are therefore, necessary to clarify its role in PCa prevention.
- Reactivate the expression of the zinc transporters. This approach is currently not possible because more work needs to be done to uncover the mechanisms by which these transporters are shut down in PCa.

- Because one of the major aims is to inhibit citrate oxidation by m-aconitase, the activity of this enzyme can be targeted in PCa cells. Inhibiting its expression is probably inappropriate to reduce the activity of the enzyme, because protein levels do not account for the decreased enzymatic activity in normal prostate cells (Singh et al. 2006). Hence, an effective agent should directly target the enzymatic activity of m-aconitase. Fluoroacetate can inhibit m-aconitase, but it will need PCa-specific targeting as it is toxic to normal cells.

2.10 Conclusion

The cancer cell metabolism is unquestionably altered. The initial theories of Warburg and his contemporaries are being unequivocally explained by modern science. Not only is the cancer cell glycolytic, but also this metabolic phenotype is intricately linked to other metabolic conversions such as increased de novo lipid synthesis and glutaminolysis. Some of these metabolic changes continue to be invaluable in clinical diagnostics, and are opening up targets for chemotherapy. Part of the reason we are still incapable of fighting cancer successfully is because of our incomplete knowledge on the metabolic changes of the cancer cell. The future should establish a complete metabolic and metabolomic map of the cancer cell. Undoubtedly, such developments will prove very useful in the diagnostic and selective elimination of cancer cells from the human body.

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Chapter 3
Mitochondrial Control of Apoptosis and Cancer

Abstract Cell death processes are important to cancer initiation, progression and chemotherapy resistance. While the importance of the extrinsic apoptotic pathway is not diminished in this chapter, the primary focus is on the intrinsic or mitochondrial apoptotic pathway. The molecular orchestration and how oncogenic signaling and viruses regulate mitochondrial apoptosis in particular are addressed. Because cancer cells have the innate propensity to commit suicide, and yet are able to survive even in adverse micro-environmental niches, the mechanisms of apoptotic evasion by neoplastic transformation are important in cancer biology. Finally, how the myriads of mitochondrial genetic defects in cancer modulate intrinsic apoptosis is illustrated to give an insight into the importance of mitochondrial genetic changes in carcinogenesis.

3.1 Introduction

Cell death processes are morphologically and biochemically characterized mechanisms by which various cells are killed. Apoptosis, autophagy, and necrosis have distinct structural features that define them. However, biochemically, there could be crosstalk between these cell death mechanisms. Irrespective of how it is executed, cell death is a normal physiologic as well as a pathologic feature of living organisms. For instance, apoptosis is a normal physiologic cell death process that operates during the development of an organism to facilitate normal tissue patterning, and in adult tissues to maintain normal tissue homeostasis. In pathologic conditions, apoptosis serves an important function of removing severely damaged cells safely without causing adverse effects to neighboring cells. In order for cancer cells to survive in toxic environmental niches, they modulate apoptotic pathways using mechanisms, including adaptive signaling pathways. De novo inhibition of apoptosis can initiate neoplastic transformation, and established tumors have altered molecular signatures that enable evasion of apoptosis, either spontaneously or when challenged with chemotherapy or radiotherapy.

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Mitochondrial DNA mutations and deletions leading to mtDNA depletion are very common in tumors. It is becoming apparent that part of the mechanism of cancer cell resistance to apoptosis is through mitochondrial genome depletion. It is noteworthy that some chemotherapeutic agents can kill cancer cells only following mtDNA depletion. Thus, how mtDNA depletion modulates apoptosis in cancer cells requires further clarification. Nonetheless, current understanding of the myriads of the ways in which apoptotic induction occurs, and the signaling networks employed by cancer cells to evade apoptosis, have provided insights into acquired chemoresistance and avenues for targeted therapies.

3.2 Cell Death Processes: The Conundrum of Semantics

Several cell death processes have been identified and described. Traditionally, a point of no return was used as the *sin qua non* of cell death. This pivotal point of cell demise was defined as caspase activation, dissipation of mitochondrial electrochemical gradient, mitochondrial outer membrane permeabilization, and exposure of phosphotidylserine moieties by the dying cells that enable neighboring cells to destroy them. Whereas these processes can lead to cell death, they are not necessarily sufficient to kill a cell. In view of this obvious difficulty, the Nomenclature Committee on Cell Death (NCCD) recommends that a cell should only be considered dead when at least one of the following biochemical or morphologic features is met: (1) the cell loses it membrane integrity, (2) the cell is completely fragmented into apoptotic bodies, and/or (3) engulfment of the remains of the cell by nearby cells has taken place (Kroemer et al. 2009).

Apoptosis, necrosis, autophagy, and cornification are distinct modes of cell death. Specific morphologic features are used to delineate these cell death pathways. For example, the term "apoptosis" was coined by Kerr et al. (1972) to describe a form of morphologic features associated with cell death. Apoptotic cell death involves withdrawal of pseudopods, leading to rounding up of cells, plasma membrane blebbing, cellular pyknosis, nuclear fragmentation (karyorrhexis), and phagocytic removal of the cell. In necrosis, cytoplasm and its organelles swell up, leading to plasma membrane disintegration. This process could be associated with some nuclear condensation. The hallmark of autophagic cell death is massive cytoplasmic vacuolation with accumulation of autophagic bodies without nuclear condensation and phagocytosis (Kroemer et al. 2009).

Other cell death processes are described as well. These include anoikis, Wallerian degeneration, mitotic catastrophe, excitotoxicity, paraptosis, pyroptosis, pyronecrosis, and entosis. These atypical cell death processes either involve apoptosis or are poorly defined and therefore, require clarity in terms of terminology. For instance, "mitotic catastrophe" can culminate in necrotic or apoptotic morphology; hence, the NCCD prefers the use of phrases such as "cell death preceded by multinucleation" or "cell death occurring during metaphase" instead of mitotic catastrophe (Kroemer et al.

2009). Similarly, Wallerian degeneration is not a true cell death process because only the axons, but not the cell body, die.

Biochemically and molecularly, however, it appears that cell death processes might be on a continuous spectrum. For instance, the same molecules could regulate more than one morphologic cell death process. An example is the regulation of apoptosis and autophagy by ATG6/BECLIN 1. Autophagy is regulated by the ATG group of evolutionary conserved genes (Meijer and Codogno 2004; Yorimitsu and Klionsky 2005). It has been demonstrated that ATG6, also known as BECLIN 1, interacts with BCL-2 (Liang et al. 1999) and BCL-xL via its BH3 domain. Thus, in addition to antagonizing BAX-mediated apoptosis, prosurvival BCL-2 family members prevent autophagy by interfering with BECLIN 1.

3.3 Physiologic Importance of Apoptosis

Apoptosis is an evolutionarily conserved process of cell self-sacrifice. Apoptosis occurs in normal development and in pathologic states to remove unwanted cells. In normal development, apoptosis controls the size and shapes of tissues and organs. In pathologic conditions, whereby cells are severely damaged (e.g., damage to cell structure or nuclear or mitochondrial genomes), apoptosis operates to eliminate such dysfunctional cells without harming the neighboring cells. Thus, several signals, including developmental cues, cellular stress, and injury from cytotoxic drugs, irradiation, heat shock, and infectious agents, can trigger apoptotic cell death. Apoptosis culminates in morphologic and biochemical changes, including cell shrinkage, nDNA fragmentation, and membrane blebs (Hengartner 2000). It is well established that apoptosis plays important roles in cancer development, therapy, and drug resistance. It is noteworthy that almost all current cancer treatments, including γ-irradiation, chemotherapy, gene therapy, and immunotherapy, target apoptotic pathways to kill cancer cells.

The term apoptosis is often loosely used interchangeably with programmed cell death. However, other mechanisms of cell death exist, as discussed above. Whereas the signals mediating the nonapoptotic cell death pathways are poorly characterized, it is realized that among other factors, calpains or cathepsins, lysosomal enzymes, and PARP-1, may be involved. In view of this, it is more appropriate to reserve the term apoptosis to caspase-mediated cell death with the defined morphologic characteristics alluded to above.

3.4 Caspase Cascade

There are two interrelated pathways that control apoptotic cell death, the intrinsic and extrinsic pathways. Both pathways require activation of caspases (cyteinyl aspartate-specific proteases) (Degterev et al. 2003). Caspases are synthesized as

inactive zymogens, referred to as procaspases, and become active following proteolytic cleavage at aspartic acid residues. The proapoptotic caspases fall into two main categories; the initiator caspases comprised of caspases 2, 8, 9, and 10, and the effector caspases made up of caspases 3, 6, and 7. Apoptotic signals first activate the initiator caspases, which in turn activate the effector caspases to execute the death signal. Other targets of caspase cleavages exist in the cytoplasm and nucleus. Overall cleavage of these targets culminates in the morphologic features of apoptosis. The activities of the caspases are modulated by other factors such as the inhibitors of apoptosis (IAPs) that block caspases 3, 7, and 9. The structural homologue of caspase 8, cFLIP has no caspase activity, but can compete with caspase 8 for binding to FADD at DISC (Tschopp et al. 1998).

3.5 The Intrinsic or Mitochondrial Apoptotic Pathway

The intrinsic pathway of apoptosis is controlled by mitochondrial and cytosolic factors. The pathway is activated intracellularly by developmental signals, and extreme cell stress such as hypoxia, irradiation, cytotoxic drugs, and heat shock. At the level of the mitochondria, a balance between the levels of the B-cell leukemia/lymphoma 2 (BCL-2) antiapoptotic (prosurvival) and proapoptotic members decide whether a cell will die or survive via this pathway. The proapoptotic members can cause mitochondrial membrane permeabilization (MMP), a well-established point of no return in the intrinsic pathway (Kroemer et al. 1995). Similarly, MMP can be induced by mitochondrial permeability transition at the mitochondrial permeability transition pore complex without the participation of BCL-2 proteins.

3.5.1 Mitochondrial Membrane Permeabilization

In 1994, work conducted by Guido Kroemer's team uncovered that loss of mitochondrial membrane potential and mitochondrial outer membrane permeabilization were critical components of intrinsic apoptosis (Kroemer et al. 1995; Zamzami et al. 1995). Mitochondrial membrane permeabilization (MMP) leads to exudation of inner mitochondrial membrane space proteins into the cytosol to initiate apoptosis. There are at least two models by which MMP is activated, and they are not mutually exclusive. Mitochondrial membrane permeabilization can occur exclusively by the activity of BCL-2 family members without the participation of the mitochondrial inner membrane or matrix. In addition MMP can be controlled by permeability transition pore complex (PTPC) (discussed below), with or without the participation of BCL-2 proteins.

Mitochondrial permeability transition (mtPT) is a state of the mitochondria when the proton-motive force is disrupted. This process occurs at the PTPC. The PTPC is fairly well characterized as a 600 kDa multi-protein complex (Faustin et al. 2004) located at the contact sites of both mitochondrial membranes (Fig. 3.1). Soluble

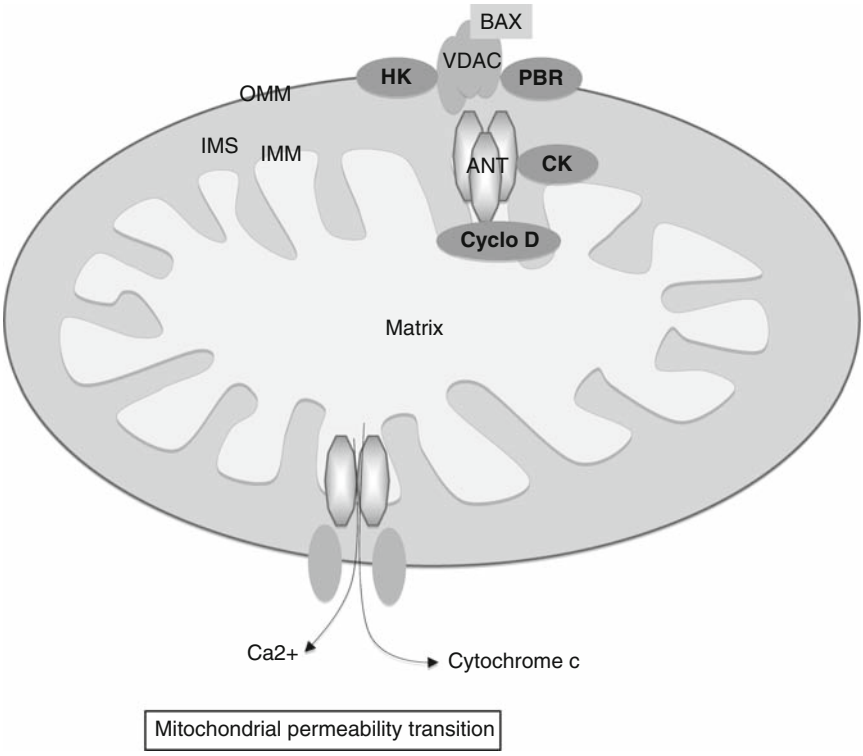

Fig. 3.1 *The mitochondrial permeability transition pore complex (PTPC)*. This conductance pore spans the inner (IMM) and outer (OMM) mitochondrial membranes at the contact sides of both membranes. It consists of the voltage-dependent anion channel (VDAC) on the OMM, the adenine nucleotide translocase (ANT) on the IMM, and cyclophilin D (Cyclo D) in the matrix. As well, proteins such as the peripheral benzodiazepine receptor (PBR), hexokinase (HK), and creatine kinase (CK) are associated with the complex. This physiologic channel opens under pathologic conditions enabling the release of mitochondrial factors, including cytochrome c into the cytosol to induce apoptosis

proteins in the cytosol, intermembrane space, and the matrix as well as integral membrane proteins, constitute the PTPC. The outer membrane voltage-dependent anion channel (VDAC), peripheral benzodiazepine receptor (PBR), also known as translocase protein 18 kDa (TSPO), the inner membrane adenine nucleotide translocase (ANT), creatine kinase (CK), hexokinase (HK), and cyclophylin D, are among the molecular composition of the PTPC (Beutner et al. 1996; Crompton et al. 1998).

The PTPC is a normal physiologic pore that regulates ADP/ATP fluxes and cellular homeostasis. The complex also controls the balance of small solutes (<1.5 kDa), protons, and water between the cytosol and matrix. These functions are regulated by the redox state of the cell, matrix pH, adenine nucleotide pools, and inner membrane electrochemical potential ($\Delta\Psi_m$). In an electrochemical process, ANT moves ATP from the matrix into the intermembrane space from where it is channeled into the cytosol by VDAC, in exchange for ADP.

Mitochondrial permeability transition occurs with increased calcium signaling or oxidative stress, and can be inhibited in vitro by the immunosuppressive drug, cyclosporine A. Apoptotic stimuli primarily induce mitochondrial membrane permeabilization that leads to depolarization and loss of mitochondrial inner membrane integrity and uncoupling of respiration. This process is accompanied by a massive influx of water into the matrix, mitochondrial swelling, cristae remodeling, and subsequent rupture of the outer membrane with the release of several molecules into the cytosol to coordinate apoptotic cell death. It is suggested that the release of cytochrome c is probably not dependent on the rupture of the outer membranes because mitochondria have been observed to release cytochrome c into the cytosol but were still structurally intact (Martinou et al. 1999).

B-cell leukemia/lymphoma 2 family members can modulate PTPC activity by interacting with VDAC and ANT to cause mtPT and hence, MMP. Cyclophylin D-mediated mtPT in apoptosis is disputed. Two groups have suggested that cyclophylin D-mediated mtPT controls necrosis and is not involved in apoptosis, which is reserved for the activities of BCL-2 family members (Baines et al. 2005; Nakagawa et al. 2005). These groups showed that cells from mice lacking cyclophylin D could undergo apoptosis, but were resistant to necrosis induced by ROS and calcium overload, suggesting that mtPT, in this case, regulates necrosis and not apoptosis. This finding provides further evidence for the conundrum with semantics and that molecular control may go far beyond regulating a single cell death process.

3.5.2 BCL-2 Family Members and Regulation of Mitochondrial Membrane Permeabilization

The BCL-2 family members are proteins that control apoptotic cell death processes. These molecules possess the BCL-2 homology (BH) domains. The BH domains are α-helical regions of BCL-2 proteins that specify the structure and therefore, functions of the various family members. There are four BH domains designated as BH1, BH2, BH3, and BH4. The BCL-2 family members fall into two main categories with opposing functions. The anti-apoptotic members (BCL-2, BCL-xL, MCL-1, BCL-W) promote cell survival and usually have all four BH domains. On the contrary, there are two groups of the proapoptotic members. Proapoptotic BAX, BAK, and BOK have BH1, BH2, and BH3 domains, while BID, BIM, BAD, PUMA, NOXA BIK, and HRK, are characterized by the possession of BH3-only domains.

The BCL-2 family members are regulated by protein−protein interaction. This unique mode of control makes these proteins amenable to therapeutic targeting using small molecules (see Chap. 14). Similarly, this mode of control makes the pathways vulnerable to microbial modulation. The BH1, BH2, and BH3 domains of antiapoptotic members form a hydrophobic groove, which interacts with the hydrophobic face of the α-helical BH3 domain of proapoptotic BCL-2 members (Muchmore et al. 1996; Day et al. 2005). The BH4 domain of prosurvival members

enhances the stability of the groove formed by the BH1−3 domains. Interestingly, BIM was shown to bind BAX at an interaction site that is different from the groove occupied by antiapoptotic BCL-2 family members (Gavathiotis et al. 2008). Apart from protein–protein interactions, the BCL-2 proteins are regulated by posttranslational modifications. For instance, antiapoptotic members can be phosphorylated and inactivated, and MCL-1, BIM, and BAD can be ubiquitinated for proteasomal degradation (Haldar et al. 1998; Zhong et al. 2005). These proteins can also be cleaved by caspase-mediated mechanisms (Cheng et al. 1997).

The intrinsic pathway is primarily mediated by BAX and BAK because loss of both proteins in cells blocks cytochrome c release and therefore, resilience to stimuli that induce intrinsic apoptosis (Fig. 3.2). The two proteins trigger cell death by inducing conformational changes followed by homo-oligomerization on the mitochondrial outer membrane, leading to cytochrome c release. BAX is a soluble cytosolic protein that exists as monomers. Following reception of apoptotic signals, BAX translocates and docks on the outer mitochondrial membrane and concentrates in sub-mitochondrial foci, co-localizing with BAK. These two proteins control membrane permeabilization through VDAC and ANT. At the level of the mitochondria, BAX monomers insert multiple α-helical structures into the outer membrane. These multiple-inserted BAX monomers then oligomerize, leading to the permeabilization of the outer mitochondrial membrane. In contrast to BAX, BAK is a resident mitochondrial membrane protein. However, VDAC and possibly, antiapoptotic BCL-2 family members (MCL-1 and BCL-xL), prevent BAK monomers from oligomerization. For instance, VDAC stabilizes the monomeric structure of BAK preventing oligomerization. Apoptotic induction by BAK, therefore, requires the relief of this inhibition to enable oligomerization and mitochondrial outer membrane permeabilization.

Another mechanism by which the BCL-2 family members could trigger apoptosis is through the regulation of calcium dynamics at the endoplasmic reticulum (ER) that is in close proximity to mitochondria. Calcium released from the ER is usually mobbed by mitochondria, and excess mitochondrial calcium can induce membrane permeabilization.

The BH3-only proteins act upstream of BAX and BAK, but are unable to execute apoptosis independent of BAX and BAK (Fig. 3.3). Unlike BAX and BAK that activate apoptosis by direct oligomerization on mitochondrial outer membrane causing the release of intermembrane space molecules, including cytochrome c, the BH3-only proteins regulate apoptosis by diverse mechanisms. They are activated by transcriptional and posttranslational modifications in specific tissues. For example, NOXA and PUMA can be activated transcriptionally by p53, while BAD requires phosphorylation to be active. On the other hand, BID is activated upon cleavage by caspase 8.

Two emerging models that are reconcilable explain how the BH3-only proteins activate BAX and BAK. These are the indirect and direct models. The indirect model relies on the findings that prosurvival BCL-2 family members interact and inhibit BAX and BAK (Fig. 3.3). Hence, the binding of BH3-only proteins to these prosurvival inhibitory proteins relieves their neutralizing effects on BAX and BAK.

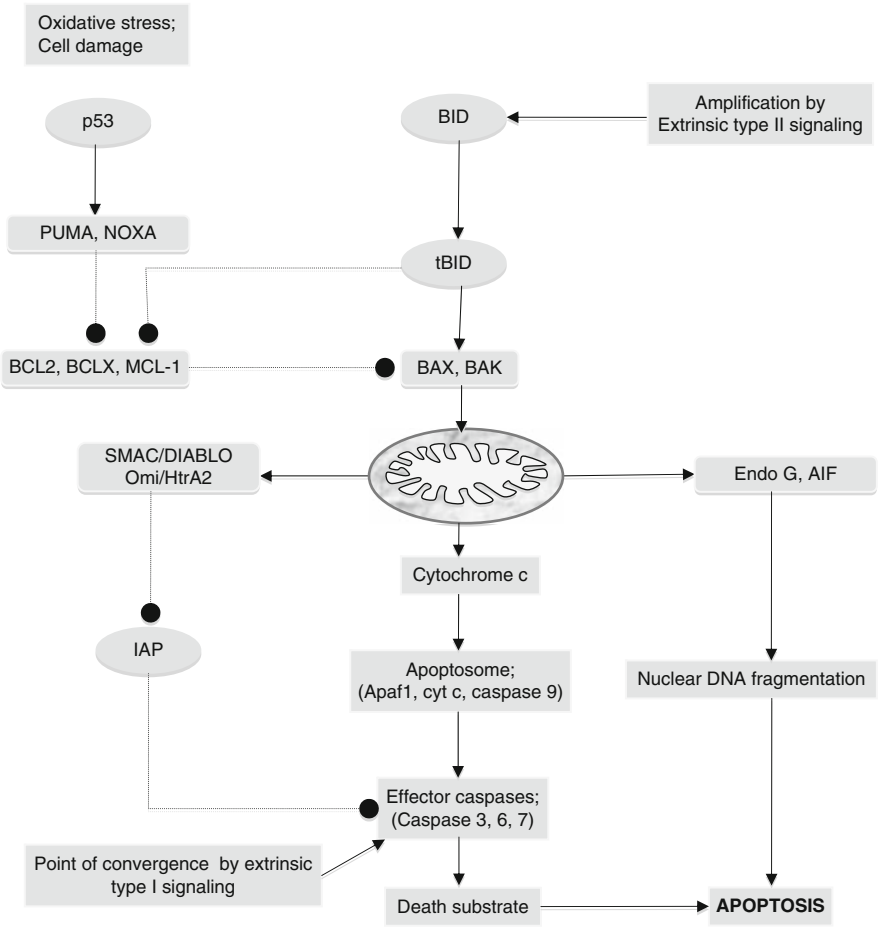

Fig. 3.2 *The intrinsic apoptotic pathway*. Cellular stress, including genomic damage, can trigger this pathway through p53-mediated transcription of BAX, PUMA, and NOXA, which inhibit prosurvival BCL-2 proteins to enable apoptotic induction by BAX and BAK. This pathway is amplified by the extrinsic pathway through caspase 8-mediated processing of BID to tBID. The pathway culminates with the mitochondrial release of apoptotic factors to induce the caspase cascade, leading to cell death. Endonuclease G and AIF enter the nucleus to cause DNA fragmentation, while SMAC/DIABLO inhibit IAPs

BID, BIM, and PUMA are very potent in this regard, because their BH3 domains bind all anti-apoptotic BCL-2 proteins. NOXA and BAD interact with only a subset of the prosurvival proteins. The direct model puts the BH3-only proteins into two classes, activators and sensitizers (also known as inactivators or derepresors). The activators bind both antiapoptotic and proapoptotic proteins, while the sensitizers interact with only antiapoptotic BCL-2 proteins with high affinity. This model posits that BCL-2 prosurvival family members put apoptosis in check by sequestering the BH3-only proteins. Hence, the activators can induce apoptosis when their

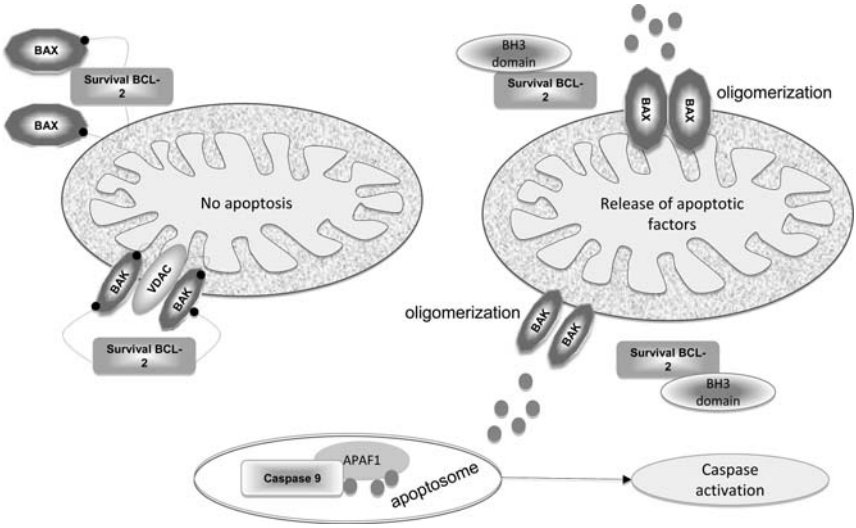

Fig. 3.3 *Induction of apoptosis by the BCL-2 family members*. BAX and BAK are prevented from triggering apoptosis by being antagonized by survival BCL-2 proteins and VDAC. Upon the reception of an apoptotic stimulus, the BH-3 only members of the proapoptotic members antagonize antiapoptotic BCL-2 proteins, facilitating BAX translocation onto mitochondria with subsequent oligomerization of BAX and BAK α-helical domains to cause the release of mitochondrial factors to orchestrate apoptosis

levels exceed the antiapoptotic neutralizing capacity of the BCL-2 prosurvival members. On the other hand, the sensitizers only contribute to apoptosis by binding to the antiapoptotic proteins, thereby enabling the activators, such as BID, BIM, and PUMA, to bind and induce oligomerization of BAX and BAK.

3.5.3 The Execution of Mitochondrial Apoptosis

In addition to cytochrome c, several molecules released by the mitochondria participate in apoptosis (Fig. 3.2). The other mitochondrial-derived molecules include second mitochondrial-derived activator of caspases (Smac), which is also known as "direct inhibitor of apoptosis binding protein with low pI (diablo)." The remaining factors are high-temperature requirement protein A2 (Omi/HtrA2), apoptosis-inducing factor (AIF), Endonuclease G, Opa1, and deafness dystonia peptide (DDP)/TIMM8a.

Cytochrome c is a small heme-containing protein located on the outer surface of the inner mitochondrial membrane that participates in electron transport. When released, cytochrome c mediates an energy-dependent formation of an oligomeric complex with cytosolic apoptotic protease-activating factor 1 (Apaf-1), which contains a caspase recruitment domain (CARD) that binds and processes

procaspase 9 to form a holoenzyme called the apoptosome. Activated caspase 9 in the apoptosome cleaves and activates the effector caspases.

Inhibitors of apoptosis or IAPs negatively control the enzymatic activity of mature caspases. The IAP family includes X-linked IAP (XIAP), cellular IAP1 (c-IAP1), cellular IAP2 (c-IAP2), and melanoma IAP (ML-IAP). X-linked IAP has been the most widely studied. This molecule possesses a RING domain located at its COOH-terminus. This domain possesses an E3 ubiquitin ligase activity for caspases, and hence, mediates caspase destruction. In addition, XIAP possesses three baculoviral IAP repeat (BIR) domains at its NH_2-terminus. Baculoviral IAP repeats 2 and 3 block caspases 7 and 9, respectively, while the linker region between BIR1 and BIR2 inhibits caspases 3 and 7.

Smac/Diablo and Omi/HtrA2 are two molecules that, when released into the cytosol, bind to and reverse the inhibitory effects of IAPs on caspases. Smac/Diablo homodimerizes to interact with IAPs, leading to the release of caspases. It is also believed that Omi/HtrA2 can act in a caspase-independent fashion as a protease. Apoptosis-inducing factor and Endonuclease G translocate into the nucleus to mediate nuclear chromatin condensation and large-scale DNA fragmentation. Apoptosis-inducing factor lacks intrinsic DNase activity, but can cooperate with peptidyl-prolyl cis-isomerase (cyclophylin A) to induce DNA fragmentation (Cande et al. 2004). Finally, mitochondrial membrane proteins Opa1 and DDP/TIMM8a participate in mitochondrial cristae remodeling and fragmentation during apoptosis.

3.5.4 Viral Control of Mitochondrial Apoptosis

Cellular infection by microbes often triggers several host responses targeted at the elimination of the infectious agents. Obviously, the first line of defense by the body is the innate immune system, mediated by phagocytes and natural killer cells. Subsequently, the adaptive immune system is activated, culminating in cellular distraction by the T- and B-lymphocytes and acquired immunologic memory to the agent. These mechanisms effectively defend the host against the possible deleterious effects of the infectious agents. To overcome these host defense mechanisms, microorganisms, especially viruses, have evolved numerous strategies to modulate mitochondrial apoptosis of host cells.

The mode of interaction of viruses with the apoptotic machinery could be direct or indirect and apoptotic modulation can be positive or negative (reviewed by Galluzzi et al. 2008). Antiapoptotic signals will facilitate viral replication, while proapoptotic modulation will kill the cells and thus, enable viral dissemination and infection of neighboring or distant cells. Viruses use various protein products to regulate the apoptotic pathway. Based on the mechanism of modulation, viral apoptotic proteins can be categorized into distinct subgroups.

- Some viral proteins with direct proapoptotic effects directly interact with mitochondrial membranes to induce apoptosis. An example of this mode of

cell death induction by viruses is typified by the mode of CD4+ T cell destruction by HIV-1 virus. The HIV-1 virus encodes numerous proapoptotic proteins. The *C*-terminal amino acids 52–96 of HIV-1 viral protein R (Vpr) can directly interact with mitochondrial ANT and VDAC to induce mtPT and MMP and hence, apoptosis. The synthetic derivative, Vpr52-96, was shown to form large conductance channels with ANT (Jacotot et al. 2000). Numerous other viral proteins can directly induce apoptosis via this mechanism.

- Proteins with indirect proapoptotic effects can induce the host genome to encode or release factors that modulate MMP. Another mode of apoptotic induction by HIV is via this indirect mode. The HIV protein Nef can induce apoptosis via several mechanisms, including permeabilization of lysosomal membranes, leading to the release of cathepsin D into the cytosol. Cathepsin D can activate BAX-mediated MMP and apoptosis.
- There are viral proteins that mimic BH domains and are therefore, capable of apoptotic inhibition. These viral proteins possess sequence and/or structural similarity to BH1-4 multidomains of BCL-2 family members (viral BCL-2 proteins). An example of vBCL-2 homologue is the 19 kDa protein encoded by the adenovirus E1B gene. Similar to antiapoptotic BCL-2/BCL-xL, E1B19kDa associates with proapoptotic members, including BAX and BAK, to inhibit apoptosis (Cuconati and White 2002).
- Finally, other myriads of mechanisms are employed by viruses to modulate apoptosis. Some viruses have unique mechanisms distinct from those that mimic BCL-2 proteins. Viral mitochondrial anti-apoptotic protein (vMAP) of murine gamma-herpesvirus 68 offers one example of a novel mechanism of apoptosis evasion (Feng et al. 2007). The *N*-terminal of vMAP interacts with BCL-2, leading to increased mitochondrial localization and increased binding of BCL-2 to BH-3 only proapoptotic proteins as well as BAX. In addition, the center and *C*-terminal domains of vMAP inhibit apoptosis through interaction with VDAC.

The above mechanisms only serve to illustrate some of the mechanisms by which viruses modulate the intrinsic apoptotic pathway. It should be noted that several viral proteins are employed in diverse ways, and the modulation includes the extrinsic pathway as well, with some viruses using oncogenic signaling pathways.

3.5.5 Regulation of Intrinsic Apoptosis by Signaling Pathways

A number of signaling pathways control the apoptotic pathway (Fig. 3.4). For instance, the PI3K/AKT and ERK1/2 pathways modulate apoptosis in cancer cells using several different mechanisms.

The tumor suppressor gene, p53, regulates both the extrinsic and intrinsic apoptotic pathways. In severe pathologic states when cell injury is virtually irreparable, p53 induces cell death. This is achieved by controlling the expression of

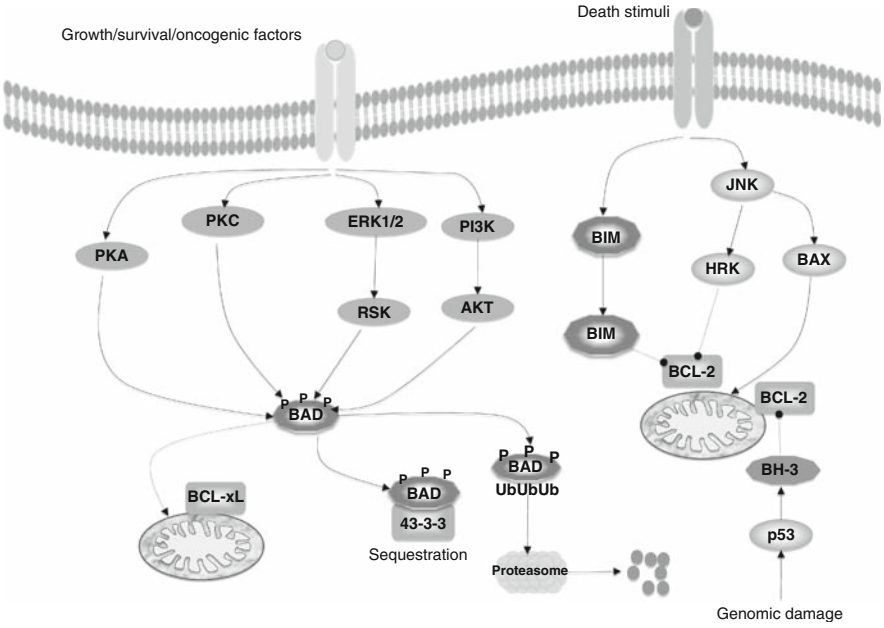

Fig. 3.4 *Regulation of apoptosis by oncogene signaling*. Upon the reception of apoptotic stimuli, cytosolic BAD, BAX, and BIM translocate to mitochondria to antagonize prosurvival BCL-2 proteins. BAD usually forms a complex with BCL-xL. To prevent the induction of apoptosis by BAD, several signaling pathways operate to phosphorylate BAD enabling sequestration by 14-3-3 proteins, and ubiquitination and proteasomal degradation. Death stimuli, including genomic damage, permit mitochondrial localization of proapoptotic factors. These actions are mediated partly by activated p53 and JNK pathways

proapoptotic genes, such as PUMA, NOXA, BAX, APAF-1, FAS, and DR5 and/or inhibition of antiapoptotic proteins such as BCL-2 and BCL-xL.

The prosurvival PI3K/AKT signaling pathway controls apoptosis in several ways. First, PKB/AKT can inhibit cytochrome c release independent of antiapoptotic factors. Second, PKB/AKT signaling can phosphorylate and inhibit the activity of BIM. In addition, PKB/AKT signaling inactivates FOXO3A, thereby preventing BIM transcription. Third, this signaling pathway phosphorylates and prevents cleavage of procaspase 9. Fourth, PKB/AKT pathway phosphorylates and inactivates proapoptotic BAD, so BAD will not be able to interact with BCL-2 proteins. Finally, AKT pathway activation inactivates FKHR and causes it to localize in the cytosol, thus, preventing it from inducing the expression of proapoptotic molecules such as Fas ligand, and BIM. Thus, PKB/AKT signaling that is pervasive in cancers not only regulates aerobic glycolysis but also modulates apoptosis, enabling cancer cell survival.

The nuclear transcription factor, NF-κB controls inflammatory responses, apoptosis, cell cycle progression, and epithelial mesenchymal transition. There are five

Rel/NF-κB family members, namely, p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). These proteins are characterized by the possession of the Rel homology domain (RHD) located at the *N*-terminal regions of the proteins. The RHDs are critical to their functions. The five family members exist as inactive homodimer or heterodimer complexes via their RHD to inhibitor proteins, IKBα and IKBβ. The complex NF-κB:IKB cannot translocate into the nucleus and hence, is prevented from transcriptional regulation of gene expression. NF-κB is activated in two different ways, the classical or canonical and alternative pathways. The inhibitors of IKB kinases (IKK) mediate NF-κB activation. The inhibitors of IKB kinases have two catalytic subunits, IKKα and IKKβ, and a structural subunit, IKKγ/NEMO. In the classical pathway, IKB is phosphorylated by activated IKKβ. The phosphorylated IKBs are then targeted for ubiquitination and proteasomal degradation. Thus, activated IKK frees NF-κB, which then translocates into the nucleus to activate target genes. The alternative pathway involves activated IKKα-dependent phosphorylation and the processing of p100 with nuclear translocation of p52/RelB dimmer.

The inhibitors of IKKs are activated in several cancers where they regulate apoptosis. NF-κB regulates the expression of several BCL-2 family members, including BCL-2, BCL-xL, MCL-1, and BF1-1 at the transcriptional level (Cory and Adams 2002). In pancreatic cancer cell lines, for example, IKK complexes are activated, and it is shown that up to 70% of primary pancreatic cancers demonstrate nuclear localization of Rel/p65 consistent with NF-κB pathway activation (Wang et al. 1999; Liptay et al. 2003; Li et al. 2004a). Nuclear translocation of Rel/p65 is associated with increased expression of NF-κB target genes, including prosurvival BCL-2 family members (Wang et al. 1999; Liptay et al. 2003; Weichert et al. 2007). NF-κB activation is as well related to poor outcomes of pancreatic cancer patients (Weichert et al. 2007). This pathway is targeted for the treatment of pancreatic cancer, in particular. The inhibition of NF-κB is shown to sensitize pancreatic cancer cells to chemotherapeutic agents, and this is mediated partly by target genes such as BCL-xL and cFLIP (Arlt et al. 2001; Fujioka et al. 2003). The regulation of NF-κB is complex, and appears to involve PI3K/AKT, K-ras, and EGF signaling pathways (Wang et al. 1999; Liptay et al. 2003).

Mitogen-activated protein kinase (MAPK) signaling communicates extracellular signals to transcription factors. There are three subgroups of this pathway, extracellular signal-regulated kinases (ERK), c-jun *N*-terminal kinase (JNK), and p38MAPK. The MAPKs are activated sequentially by phosphorylation. Mitogen-activated protein kinase kinase kinase (MAPKKK) phosphorylates MAPK kinase that activates, and in turn, phosphorylates MAPK.

The JNK and p38MAPK are stress-activated protein kinases (SAPKs) and, as such, they mediate stress responses and apoptosis. However, they can mediate proliferation and cancer development in nonstress states. The activation of SAPKs in stressful conditions can lead to apoptosis. For instance, JNK regulates the intrinsic pathway of apoptosis, and both JNK and p38MAPK signaling can phosphorylate p53 to promote apoptosis.

The ERK signaling pathway is activated by growth factors, and the pathway controls cell growth and differentiation. It is now evident that ERK1/2 can prevent

apoptosis or promote cell survival in several ways. ERK and AKT/PKB can inactivate FOXO3A by phosphorylation to suppress BIM transcription. In addition, ERK signaling phosphorylates multiple sites of BIM permitting ubiquitination and proteasome-dependent destruction (Ley et al. 2003). The phosphorylation of BIM by ERK causes it to dissociate from BCL-2 family proteins, BCL-xL and MCL-1 (Ewings et al. 2007a, b). BCL-2, BCL-xL, and MCL-1 expressions are enhanced by ERK signaling. BAD is inhibited by phosphorylation at three sites (Ser^{112}, catalyzed by RSK, Ser^{136}, regulated by PKB/AKT signaling, and Ser^{155}, controlled by PKA). ERK activation causes RSK-mediated phosphorylation of BAD, in addition to PKA and PKB/AKT phosphorylation, causes the sequestration of BAD by the 43-3-3 proteins. Similarly, phosphorylated BAD can be ubiquitinated and subsequently destroyed (Fueller et al. 2008). Finally, MEK inhibition caused apoptosis in pancreatic cancer cells via decreased expression of BCL-2 family members (Boucher et al. 2000).

3.5.6 Mitochondrial Fission and Apoptosis

Mitochondrial shape changes are strongly implicated in the apoptotic cell death process. Apoptosis is coupled with mitochondrial fragmentation or fission and cristae remodeling (Frank et al. 2001). As well, other dynamic changes associated with apoptotic cell death are ultrastructural evidence of tubular cristae junction openings, and cristae fusion (Scorrano et al. 2002). These mitochondrial dynamics can cause cytochrome c release, although the actual molecular mechanisms are not fully elucidated.

Several circumstantial evidences suggest that there is a cross talk between the regulators of apoptosis and mitochondrial dynamics. Pro-apoptotic proteins, BAX and BAK colocalize with DRP1 in scission foci. Also, in cells undergoing stress-induced apoptosis, MFN2, and again BAX, are found to colocalize in fission foci (Karbowski et al. 2002). Loss of FIS1 or DRP1 prevents mitochondrial fission and cytochrome c release following apoptotic induction. Similarly, loss of FIS1 inhibits BAX translocation to mitochondria (Lee et al. 2004b). Loss of DRP1 also prevents cytochrome c release even in cells where BAX has translocated to the mitochondria. Inactivation of MFN1, MFN2, and OPA1, causes increased mitochondrial fragmentation and sensitivity to apoptosis (Olichon et al. 2003; Lee et al. 2004b; Sugioka et al. 2004; Chen et al. 2005). Thus, the mediators of mitochondrial dynamics also control apoptosis at some level.

3.6 The Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway is induced following ligand-receptor interaction. The TNF receptor gene superfamily mediates this pathway. There are over 20 of these receptors, which share identical cysteine-rich extracellular domains, and a

cytoplasmic death domain consisting of ~80 amino acids. The death domains transmit the apoptotic signals following ligand/receptor interaction. The receptors of the extrinsic pathway are Fas (also known as CD95), DR4, and DR5. Fas receptors interact with FasL or CD95L ligands, while DR4 and DR5 bind the Apo2L/TNF-related apoptosis-inducing ligand (TRAIL) (Debatin and Krammer 2004; Fulda and Debatin 2006). Receptor trimerization and death domain clustering occur following ligand interaction. These processes lead to the recruitment of adaptor molecules such as Fas-associated death domain (FADD), and TNF receptor associated-protein with death domain (TRADD), which can recruit initiator procaspases 8 and 10 to form a protein assembly known as CD95 death-inducing signaling complex or DISC. Activated caspase 8 and 10 cleave and activate caspase 9, which then cleaves and activates the effector caspases 3, 6, and 7 to mediate cell death.

Caspase 8 activity and the levels of DISC differ in several cell types. Because of these features, two types of intracellular modes of caspase signaling have been identified, namely, types I and II (Ozoren and El-Deiry 2002). Type I signaling results in the accumulation of sufficient amounts of DISC to activate the effector caspases and execute a complete apoptosis cascade. Type II cells do not generate enough DISC and therefore, rely on an amplification loop involving the intrinsic pathway for full activation. This amplification occurs through the cleavage of the BH3-only protein BID by caspase 8. The requirement of intrinsic pathway activation in some cell types emphasizes the important role of the mitochondria in regulating apoptotic cell death.

3.7 Evasion of Apoptosis by Cancer Cells

Cancer cells have the innate propensity to commit suicide. In a study to identify apoptotic targets for cancer chemotherapy, Yang et al. (2003) examined the expression of apoptotic factors in several cancer and normal cell lines and tissues. High levels of caspase 3 and 8 activities, and caspase 3 fragments, were observed in cancer cell lines and tissues in the absence of apoptotic stimuli. However, high protein levels of survivin and XIAP, two members of the IAP family members were demonstrated in these cancer tissues and cells, suggesting that the lack of apoptosis in the cancer cells was a consequence of the high levels of these counteracting survival factors. Consistent with this conclusion, when the activities of the anti-apoptotic proteins were blocked, cancer cells, but not normal cells, committed suicide. Thus, increased IAP counteracts apoptosis in cancer cells that already have the intrinsic ability to die. In agreement with this conclusion, several groups have shown the lethal effects of interfering with IAP activity on cancer cells (LaCasse et al. 2008). Moreover, several Smac mimetics targeting the activities of IAPs are in development for use in chemotherapy (Sect. 14.2.1.3)

Another important concept of cancer biology is the fact that cancers develop partly because of evasion of apoptosis by mutated and therefore, damaged cells. Also, several tumors develop resistance to therapy partly by adopting antiapoptotic

mechanisms. Understanding the myriads of ways by which tumors escape apoptotic cell death is central to cancer prevention and effective chemotherapy. Whereas it is evident that several molecules of the apoptotic pathway are targets used by cancer cells to evade apoptosis, the final pathways involve the augmentation of antiapoptotic survival signals, and/or the suppression of proapoptotic cues.

For both the extrinsic and intrinsic pathways, apoptosis is modulated by a myriad of mechanisms as summarized above. The processes used to modulate cell death include epigenetic modifications and gene expression changes, downregulation of apoptotic signals, upregulation of survival signals, mutations, instability, and allelic imbalances in specific pathway genes, transport defects or intracellular sequestration of apoptotic factors, blockade of caspase activation, interference with FADD, and Caspase 8 interaction, among others.

3.7.1 Cancer as a Defect in Apoptosis

Pioneering works led by Korsmeyer and colleagues led to the realization in the 1980s that apart from oncogenic stimulation and inactivation of tumor suppressor genes, primary apoptotic suppression can lead to malignant transformation (Raffeld et al. 1987; McDonnell et al. 1989; Nunez et al. 1989; Korsmeyer et al. 1990). BCL-2 was identified through studies of chromosomal translocation in follicular lymphomas. Molecular studies revealed the presence of t(14;18)(q32;q21) translocation in ~85% of lymphomas. Subsequent cloning of the chromosome-14 breakpoint revealed a transcription factor named then as BCL-2 (Bakhshi et al. 1985; Cleary and Sklar 1985; Tsujimoto et al. 1985). Transgenic technology enabled the study of BCL-2 lymphomas in mice. Overexpression of a BCL-2 transgenic construct in mice caused an increase in the number of B-lymphocytes in splenic white pulps (McDonnell et al. 1989). These hyperplastic splenic white pulps of BCL-2 overexpressing transgenic mice progressed to monoclonal B-cell lymphomas in ~15 months period (McDonnell and Korsmeyer 1991). Surprisingly, this finding was not due to differential proliferation rates between transgenic mice and wildtype controls. Instead, it was noted that the BCL-2 overexpressing cells were arrested at G_0/G_1 of the cell cycle (McDonnell et al. 1989). An explanation of the survival advantage conferred by BCL-2 overexpression was later provided when cytokine-dependent cell lines were transfected with BCL-2 overexpressing vectors and observed to survive at G0 without the needed cytokine, IL-3 (Vaux et al. 1988). These findings clearly indicated that the ability to avoid apoptosis could, indeed, lead to malignant transformation.

3.7.2 Intrinsic Apoptotic Pathway Alterations in Cancer

The requirement that cancer progresses from in situ neoplastic to metastatic stages in an environment that is usually toxic and lethal to normal cells, such as hypoxia,

implies that cancer cells must adopt ways to evade cell death processes. Some of the numerous mechanisms employed by cancer cells to evade stimuli that trigger the intrinsic apoptotic pathway are examined below just to illustrate the numerous mechanisms cancer cells adopt to stay alive.

- In general, cancer cells generate excess ROS and the ensuing oxidative stress can cause mtPT and apoptosis. An adaptive mechanism by cancer cells to overcome this stress is the increased production of ROS scavengers, such as glutathione, thioredoxin, and MnSOD (Dvorakova et al. 2002; Pani et al. 2004; Biaglow and Miller 2005). Another mechanism by which apoptosis is prevented by cancer cells is via inhibition of cytochrome c release through redox modulation mediated by altered glucose metabolism. The redox state of cytochrome c dictates its functions. Under conditions of oxidative stress, cytochrome c is oxidized and therefore, activated. In cancer cells, however, cytochrome c is reduced and inactivated by glutathione that is generated as a result of glucose metabolism via the pentose phosphate pathway (Vaughn and Deshmukh 2008).
- Anoikis is associated with the activation of PTPC via BCL-2 proapoptotic family members BID and BAX (Valentijn et al. 2003; Valentijn and Gilmore 2004). Yet, cancer cells must evade this death signal in order to metastasize. Modulation of PTPC in cancer cells has been shown by several groups to mediate apoptotic resistance. Mitochondrial HK interacts with VDAC, such that for BAX to induce apoptosis, it must displace HK from VDAC. To prevent access of VDAC by BAX, cancer cells overexpress HK II (Shinohara et al. 1991; Azoulay-Zohar et al. 2004), as a result of promoter induction and gene amplification (Rempel et al. 1996). Moreover, the outer mitochondrial membranes of cancer cells are more resilient to breakdown than those of normal cells (Green and Evan 2002).
- Hypoxia induces the expression of BCL-2 proapoptotic family members, BNIP and NIX, through the stabilization of HIF (Vande Velde et al. 2000; Sowter et al. 2001; Kubasiak et al. 2002). However, cancer cells are able to prevent cell death mediated by these proapoptotic proteins via several mechanisms. In pancreatic cancer, for example, BNIP3 promoter is hypermethylated and therefore, these cells become resilient to HIF-mediated apoptotic induction (Okami et al. 2004).
- Because the intrinsic pathway is tightly controlled by the PTPC, cancer cells activate factors that prevent permeabilization of this pore, and increase or decrease expression of subunits that form the PTPC. Cancer cells increase the expression of PBR/TSPO, and this is associated with survival, metastasis, and poor patient outcomes in some cancers (Katz et al. 1990; Miettinen et al. 1995; Hardwick et al. 1999). Peripheral-type benzodiazepine receptor/TSPO protein expression directly controls cell survival and proliferation in human breast cancer cells by influencing signaling mechanisms involved in cell cycle control and apoptosis.

- The levels of ANT2 are much higher in many cancers than in normal cells (Bonod-Bidaud et al. 2001). In contrast to ANT1, ANT2 does not activate PTPC, and its overexpression is not associated with apoptosis (Bauer et al. 1999). Hence, this is an excellent mechanism of maintaining normal ANT functions without apoptotic sensitization.
- It is suggested that only VDAC and ANT1 mediate mtPT. Cyclophylin D interacts with ANT1 to suppress mtPT at the pore. To maintain this apoptotic suppression, cyclophylin D is overexpressed by a number of cancers, including breast, ovary, and uterine carcinomas (Lin and Lechleiter 2002; Li et al. 2004b; Schubert and Grimm 2004). Similarly, creatine kinase 1 inhibits PTPC (Schubert and Grimm 2004) and is upregulated in tumors (DeLuca et al. 1981).
- Mutations in BCL-2 family members are found in many tumors. For example, BAX is mutated in cancers, such as T-cell leukemia, and gastric cancers, and BAX coding region mutations are detected in ~50% of colorectal and gastric cancers (Miquel et al. 2005). Somatic mutations in BAD are detected in colon cancers (Lee et al. 2004a). Somatic missense mutations in BIK are common in peripheral B cell lymphomas (Arena et al. 2003). Loss of heterozygosity in BIM is found in mantle cell lymphoma (Tagawa et al. 2005). Similarly, loss of heterozygosity in HRK and associated apoptotic inhibition is demonstrated in glioblastoma (Nakamura et al. 2005). The inactivation of BID in mice leads to myeloproliferative disorders that can progress to chronic myelomonocytic leukemia (CMML) (Zinkel et al. 2003). Finally, Apaf1 expression is lost in ovarian cancers, multiple myelomas, and leukemias (Wolf et al. 2001). Thus, several genetic alterations of the mediators of apoptosis are common in various cancers, and they serve the primary purpose of enabling apoptotic evasion.
- One of the mechanisms of apoptotic evasion by cancer cells is calcium sequestration following the induction of apoptosis. It has been shown that upon apoptotic induction, cancer cells are able to reduce the release of calcium from intracellular sources (Chandra et al. 2002). This calcium sequestration causes the cells to become less sensitive to calcium-mediated mtPT.
- Several signaling pathways, including SHH and WNT, control apoptotic resistance in cancer cells. For example, SHH signaling is activated in several cancers, including basal cell carcinoma, prostate, ovarian, and brain cancers. It is now recognized that one of the consequences of SHH signal activation in cancer is the induction of prosurvival BCL-2 expression (Bigelow et al. 2004).

3.7.3 Extrinsic Apoptotic Pathway Alterations in Cancer

In addition to the intrinsic pathway, cancer cells equally modulate the extrinsic pathway for survival. Some examples of how the extrinsic pathway is altered in cancer cells are discussed.

CD95 and TRAIL receptor genes are silenced by chromatin condensation and CpG-island hypermethylation in colon cancer and neuroblastoma (Petak et al. 2003).

CD95 is mutated in several cancers (Debatin 2004). Also loss of heterozygosity via deletions on chromosome 8p, the locus of TRAIL-R1 and TRAIL-R2 in association with loss of their expression, is observed in a number of cancers (Pai et al. 1998; Dechant et al. 2004). Cancer cells also modulate the surface expression of death receptors. CD95 expression is downregulated in leukemia and neuroblastoma cells resistant to chemotherapy (Friesen et al. 1997; Fulda et al. 1998). Decoy receptor 3 is a soluble ligand that binds and inhibits FasL-mediated apoptotic induction. In primary lung and colon cancers, decoy receptor 3 is amplified and overexpressed (Pitti et al. 1998). Decoy receptor for TRAIL-R3 is also overexpressed in gastric cancers and TRAIL-resistant colon cancer cells are defective in the transport of TRAIL receptors from intracellular sources onto the cell surface.

Phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes-15 kDa (FLIP and PED/PEA-15) can interact with the cytoplasmic domains of death receptors and negatively regulate apoptosis. The sequence of FLIP is homologous to caspase 8 and 10, but FLIP lacks any catalytic activity. It has been observed that several cancers express high levels of FLIP, and the recruitment of these molecules to DISC inhibits apoptosis by interfering with the interaction of DISC with caspases 8 and 10. Increased FLIP expression is also associated with the resistance of cancer cells to CD95- and TRAIL-induced apoptosis. PEA-15 also blocks CD95 and TRAIL-mediated apoptosis by disrupting the interaction of FADD and caspase 8. Protein kinase B/AKT signaling interacts with PEA-15 to mediate chemoresistance in cancer cells, probably through increased PED/PEA-15 expression and/or phosphorylation by PKB/AKT (Trencia et al. 2003; Stassi et al. 2005). These findings illustrate some of the myriads of mechanisms employed by cancer cells to evade extrinsic apoptosis.

3.8 Mitochondrial Genetic Defects and Apoptosis

The intrinsic apoptotic machinery is activated by several extracellular stresses. However, there is enough evidence that mitochondrial genome damage and depletion is a critical regulator of this apoptotic pathway as well. Several cancers have mtDNA mutations and some are associated with marked mtDNA copy number reduction. For instance, estrogen-mediated generation of ROS in breast cancer is associated with multiple mtDNA mutations. Also, it appears that polymerase gamma sustains multiple mutations that impair mitochondrial genome replication, leading to mtDNA depletion in breast cancer cells (Singh et al. 2009).

Mitochondrial DNA-less (ρ0) cell lines are employed by several groups to address the role of mitochondrial mutations in apoptosis. Despite the depletion of mtDNA and several metabolic defects of ρ0 cells, they retain their organelles. However, the structures of mitochondria in ρ0 cells are imperfect, but are capable of establishing high or normal transmembrane potential ($\Delta\Psi_m$), and can generate superoxide anion when treated with menadione. The establishment of $\Delta\Psi_m$ in normal cells depends on proton pumping by the electron transport chain components

as well as ATP hydrolysis by mitochondrial ATP synthase. Because mitochondrial genome contributes 13 polypeptides for respiratory chain complexes I, III, IV, and V, it will be expected that ρ0 cells should lack $\Delta\Psi_m$. How ρ0 cells maintain normal or high $\Delta\Psi_m$ is unclear but postulated to depend on a number of possible factors. Putatively, ATP synthase can function normally in the absence of mitochondrial complements, F_0 subunits (ATPase6 and ATPase8). Thus, F_1-ATPase subunits α and β function normally in ATP hydrolysis in ρ0 cells to help maintain $\Delta\Psi_m$ (Buchet and Godinot 1998; Appleby et al. 1999). Other electrogenic pumps are dependent on nuclear-encoded subunits such as mitochondrial chloride intracellular channel (mtCLIC). The upregulation of mtCLIC in ρ0 cells can function to sustain the $\Delta\Psi_m$ independent of mitochondrial proton transmembrane transport (Arnould et al. 2003). Also, normal functions of ANT in the exchange of adenine nucleotides across the mitochondrial membrane can contribute to $\Delta\Psi_m$ (Buchet and Godinot 1998).

Data on how mtDNA damage modulates apoptosis is conflicting; however, the vast majority of well-designed and executed studies support the notion that loss of mitochondrial genome is associated with various adaptive measures to mediate apoptotic resistance, including increased $\Delta\Psi_m$ (Jacobson et al. 1993; Marchetti et al. 1996; Jiang et al. 1999; Dey and Moraes 2000; Wang et al. 2001; Park et al. 2004; Biswas et al. 2005; Ferraresi et al. 2008; Kulawiec et al. 2009; Yu et al. 2009). Indeed, even when treated with agents such as valinomycin and oligomycin that dissipate $\Delta\Psi_m$, ρ0 cells maintain a normal $\Delta\Psi_m$. Several explanations are provided for the apoptotic resilience of ρ0 cells.

Rho-zero cells overexpress antioxidant enzymes, including MnSOD, catalase, and reduced glutathione that protect them against oxidative stress-mediated apoptosis. For example, MnSOD overproduction by ρ0 cells derived from SK-Hep1 hepatoma cells conferred protection against menadione, paraquat, and doxorubicin-induced superoxide ion production (Park et al. 2004). Similarly, during treatment of ρ0 cells with staurosporine, reduced glutathione levels were elevated in ρ0 cells (Ferraresi et al. 2008). It is suggested that increased antioxidant enzyme expression is an adaptive process that occurs during the production of ρ0 cells such that cells with more efficient antioxidant mechanisms are selected (Ferraresi et al. 2008).

Dey and Moraes (2000) demonstrated that a decrease in caspase 3 activation in ρ0 cells was associated with apoptotic resistance. The ρ0 cells had no changes in PTPC or cytochrome c release. Partially depleted mtDNA in C2C12 cells caused decreased sensitivity to apoptosis, probably through BAX, BID, and BAD sequestration in inner mitochondrial membranes, as well as increased expression of prosurvival BCL-2 family members. Moreover, ρ0 cells could not effectively process p21 BID (Biswas et al. 2005). In several different ρ0 cell lines, including the classical osteosarcoma 143B, hepatoma HepG2, and uterine carcinoma HeLaS3, apoptotic resistance to staurosporine was associated with the loss of proapoptotic death associated protein 3 (DAP3) (Jacques et al. 2006). DAP3 is overexpressed in thyroid oncocytic tumors (Jacques et al. 2009). Furthermore, the resistance of ρ0 cells to chemotherapeutic and other agents that trigger apoptosis

could be due to drug extrusion from the cells. Ferraresi et al. (2008) found increased expression of membrane pumps, such as multidrug resistance 1 (MRP1) gene product, P-glycoprotein in these cells.

Apoptotic resistance is demonstrated not only in cells with complete loss of mtDNA. Indeed, cybrids established from breast cancer cell line with $tRNA^{Leu(CUN)}$ mutation acquired high $\Delta\Psi_m$ and were resistant to etoposide-induced apoptosis. In addition, these cells had metastatic phenotypes associated with PI3K/AKT pathway activation (Kulawiec et al. 2009). Thus, in vivo, mtDNA mutations can confer apoptotic resistance to cells that can contribute to malignant transformation.

3.9 Conclusion

Cell death is a natural process of development and tissue maintenance. Irrespective of the biochemical and morphologic process adapted by a specific tissue for cellular elimination, a mechanism for preventing cancer development is to kill cells with damaged genomes or cellular structures. Indeed, the guardian of the genome, p53, functions to achieve this so as to preserve normal tissues and organs, and to prevent the development of tumors. Since the discovery of the role of mitochondria in apoptosis decades ago, the body of evidence in support of the central role of this organelle in modulating cell survival and death decisions has been overwhelming. An important feat in this regard has been the understanding of the molecular mechanisms of how apoptosis is regulated and importantly, the alterations in cancer cells that have facilitated the development of several anticancer agents. However, several molecular details, including structural organization and regulation of BCL-2 members, subcellular localization of apoptotic factors, and the precise role of PTPC in apoptosis, remain to be elucidated. Conceivably, as is part of scientific enquiry, there are novel players of this pathway yet to be discovered. The future should shed more light on cell death processes because this is central to the development of effective chemotherapies.

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Chapter 4
Mitochondrial-to-Nuclear Communications in Cancer

Abstract Mitochondria are semiautonomous organelles, implying the reliance of these organelles on self and nuclear imported materials for their normal functions. This dependence of mitochondria on the nucleus opens up enormous communication channels and networks between the two genomic compartments. This chapter discusses three levels of inter-genomic communications; 1. Oncogenic defects in nuclear-encoded mitochondrial proteins, specifically succinate dehydrogenase and fumarase, 2. Mitochondrial-to-nuclear signaling mechanisms that operate in stressed cancer cells, and 3. The potential oncogenic effects of the ongoing nuclear integration of mitochondrial genomic fragments are provided.

4.1 Introduction

The two genomes, nuclear and cytoplasmic, of the eukaryotic cell, communicate at several levels. First, the mitochondria depend on imported nuclear-encoded polypeptides to complement their own $\sim$ 1% contribution for respiratory complex protein assembly and activity, as well as other biochemical processes. Second, both genomes play concerted roles in controlling the synthesis, mitochondrial import, and assembly of these proteins. Finally, there is a continuous transfer of mitochondrial genomic fragments into the nucleus with a demonstrated integration of some fragments into the nuclear genome.

Depending on the cell or tissue type, mitochondria contain 1,000–2,000 proteins, a vast majority of which are nuclear-encoded, translated in the cytosol, and imported into the mitochondria. Therefore, the interdependence of the two genomes in normal cell physiology is apparent, and changes in cancer cell genome, metabolism, and signaling pathways reflect in altered mitochondrial-to-nuclear signaling. There is a constant bi-directional flow of information between the two genomic compartments. The nucleus exports proteins into the mitochondria and this function serves as its primary mode of communication. However, the mitochondrion uses several metabolic signals, molecules such as ROS, as well as signal transduction

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pathways to communicate with the nucleus. This chapter examines three levels of intergenomic communication that are of relevance to cancer, including defects in proteins involved with TCA cycle activity and complex II assembly in cancer, signaling between the mitochondria and nucleus in stressed cancer cells, and nuclear integration of mitochondrial genetic material and their potential role in oncogenic induction.

4.2 *Succinate Dehydrogenase* and *Fumarate Hydratase* Mutations and Cancer

Succinate dehydrogenase (*SDH*) and *fumarate hydratase* (*FH*) encode enzymes that participate in the tricarboxylic acid (TCA) cycle (Table 4.1). These genes are recognized as tumor suppressors, because mutations associated with most subunits can cause cancer. In addition to their TCA cycle functions, subunits of SDH form complex II of the electron transport chain (ETC). In the TCA cycle, SDH catalyzes the conversion of succinate to fumarate, which is in turn converted to L-malate by FH. Complex II of the ETC carries electrons from FADH to CoQ. Given that much cellular energy is derived from the activities of the OXPHOS, the critical function of the TCA cycle in providing reducing equivalents for energy production unravels the importance of this biochemical pathway in the maintenance of cellular homeostasis and survival. Several TCA cycle enzyme defects are associated with a number of diseases. However, only *FH* and three subunits of *SDH* mutations cause cancer (Table 4.2). The list of cancers with TCA cycle enzyme mutations includes paragangliomas (PGL), pheochromocytomas, cutaneous and uterine leiomyomas, renal cell carcinomas, and possibly others such as Leydig cell tumors, ovarian mucinous cystadenomas, cerebral carvenomas,

Table 4.1 Genetics and biology of SDH and FH

Gene	Chromosomal location	Protein	Function
SDHA	5p15.33	664aa/~70 kD	Catalytic subunit Flavoprotein subunit of complex II
SDHB	1p35–36.1 PGL4 loci	280aa/~30 kDa	Catalytic subunit Iron-sulfur subunit of complex II
SDHC	1q21 PGL3 loci	169aa/~15kDa	Multi-pass membrane Protein; anchors SDHA, SDHB to the membrane
SDHD	11q23.1 PGL1 loci	159aa/~12kDa	Multi-pass membrane protein; anchors SDHA, SDHB to the membrane
FH	1q43	510aa/~55kDa	Catalyzes conversion of Fumarate to malate

Table 4.2 Disorders form *SDH* and *FH* mutations

Gene	Mutation	Condition	Features
SDHA	Germline homozygous	Leigh syndrome	Severe encephalomyelopathy, cardiomyopathy, myopahty, hepatopathy. Pediatric lethal
SDHB	Germline heterozygous	Hereditary and sporadic pheochromocytoma/ paraganglioma	Features of PGL are described in Table 4.3
		Renal cell and papillary thyroid carcinomas	
		PTEN mutation negative Cowden syndrome	Frequent presentation with breast, thyroid, and renal tumors
SDHC	Germline heterozygous	Hereditary paraganglioma	Common tumors are head and neck, especially glomus vagale tumors
SDHD	Germline heterozygous	Hereditary and sporadic Pheochromocytoma/ paraganglioma	Features of PGL are described in Table 4.3
		Renal cell and papillary thyroid carcinomas	
		PTEN mutation negative Cowden syndrome	Frequent presentation with breast, thyroid, and renal tumors
FH	Germline homozygous	Fumarate hydratase deficiency	Severe neurodegeneration. Lethal condition
	Germline heterozygous	Multiple Cutaneous Leiomyomatosis (MCUL) Hereditary Leiomyomatosis, and Renal Cell Carcinoma (HLRCC) syndrome	Cutaneous and uterine leiomyomas; some also with renal cell carcinoma

and the Cowden syndrome (CS). This list could potentially grow to include several other subtypes of cancers, when the mechanisms of cancer causation by these mutations are fully appreciated.

4.2.1 SDH *Mutations and Cancer*

4.2.1.1 Paragangliomas

The paraganglia are a group of histologically identical neuroendocrine tissues. They are diffusely dispersed throughout the body, ranging from the head and neck region, to the pelvis. They are organized in close association with tissues of the sympathetic and parasympathetic nervous systems. Tumors of the paraganglia, known as PGL, fall into two major anatomic and functional categories; the head and neck paraganglia are often associated with the parasympathetic nervous system

along the cranial nerves and arteries. The remaining paraganglia, including those in the adrenal medulla, are associated with the sympathetic nervous system. These anatomic associations reflect on the biology and biochemistry of diseases that arise from the paraganglia. Thus, PGL fall into two major functional categories: endocrinologically active and inactive tumors. Anatomically, PGL are commonly found in the abdomen (85%), thorax (12%), and head and neck (3%) regions. Frequently, the head and neck parasympathetic PGL tend to be biochemically silent, while abdominal and thoracic tumors are usually functional.

PGL can arise from chromaffin cells in the adrenal medulla, or in the extra-adrenal paraganglia elsewhere in the body. Tumors from the abdominal and thoracic paraganglia are usually hormone secreting. These are principally those associated with the sympathetic nervous system. Clinically, these PGL are referred to as pheochromocytomas, and they secrete large amounts of catecholamines (i.e., epinephrine and norepinephrine) that are useful diagnostic biochemical markers. Most are benign tumors, but $\sim$ 10% are malignant.

Approximately, 800 new cases of pheochromocytomas are diagnosed each year in the US, with $\sim$ 0.1% being among the hypertensive population. It will appear that all hypertensive patients should have at least a biochemical test to rule out this condition, because it is amenable to surgical therapy. The peak incidence occurs in the third to fifth decades, but $\sim$ 10% of all cases are found in childhood. Postmortem series indicate that most pheochromocytomas are not diagnosed clinically, even when they are the primary cause of death. A majority of pheochromocytomas are sporadic, but $\sim$ 25% of all cases are heritable and these usually present with early age of onset, and are often multifocal bilateral tumors.

The head and neck PGL (glomus tumors, chemodectomas) are usually not functionally active. They are often encapsulated benign neuroendocrine tumors, but in rare cases ($\sim$ 3%), they can be malignant and metastasize. Most head and neck PGL occur in the carotid body, the largest paraganglia in this region. Other locations of PGL include jugulotympanic, vagal, and laryngeal paraganglia.

4.2.1.2 Genetics and Clinical Features of PGL

Hereditary PGL are autosomal dominant solitary tumors, but they can occur in association with other familial cancer syndromes. For example, pheochromocytomas are well-known to occur as components of the von Hippel–Lindau (VHL) disease, type II multiple endocrine neoplasia (MEN type II), von Recklinghausen's neurofibromatosis type 1, and retinal cerebellar hemangioblastosis. In rare occasions, head and neck PGL have been associated with VHL disease and MEN type II. It should be noted that there are several sporadic PGLs.

Mutations in the proto-oncogene *RET,* and tumor suppressor genes *VHL*, and *neurofibromin* (*NF1*) cause familial syndromes that are associated with pheochromocytomas. MEN type II is an autosomal dominant disease caused by activating mutations in the RET proto-oncogene. In addition to pheochromocytomas, which occur at a high frequency of 50% in mutation carriers, patients also develop

medullary thyroid cancers, and either parathyroid adenomas (in MEN type II A), or mucosal neuromas (in MEN type II B).

VHL disease is an autosomal dominant disorder caused by mutations in VHL tumor suppressor gene located on chromosome 3p25. One out of every 36,000 births will develop this disorder. Mutation carriers have a predisposition to develop benign and malignant tumors in multiple organs, including kidneys, pancreas, testis, and the central nervous system. Ten to 34% of patients with this disorder also develop pheochromocytomas.

Nuerofibromatosis type 1 is a rare disease caused by inactivating autosomal dominant mutations in the tumor suppressor gene *NF1,* located on chromosome 17q11.2. The disease occurs at a frequency of 1 in 4,000 people, and is characterized by neurofibromas, iris hamartomas, and lightly pigmented birthmarks (café au lait spots). Pheochromocytomas are present in 0.1–5.7% of affected patients, but are substantially enriched in patients with hypertension.

The hereditary patterns of PGL had been known for some time (van der Mey et al. 1989), and the putative genetic locus for type 1 PGL (see below) was subsequently mapped in the early 1990s (Heutink et al. 1992, 1994). However, studies at the turn of the millennium by Baysal et al. (2000) of hereditary paraganglioma (HPGL) patients identified mutations in *SDHD* on chromosome 11q23 as the underlying genetic disorder. Additional mutations on chromosome 1q21 in *SDHC*, and chromosome 1p36 in *SDHB* were immediately identified in HPGL patients (Niemann and Muller 2000; Astuti et al. 2001a, b; Baysal et al. 2002). Currently, it is established that mutations in these genes cause hereditary as well as sporadic PGL and pheochromocytomas. All types of mutations, including missense, nonsense, frameshift, splice site, as well as small and large deletions and insertions, are detected in PGL patients.

The *SDH* mutations are used to classify the paraganglioma syndromes into four subcategories (Table 4.3); mutations in *SDHD* predispose to PGL-1, *SDHC* to PGL-3, and *SDHB* to PGL-4, while mutations in an unidentified gene on chromosome 11 is associated with PGL-2. On the other hand, heterozygous *SDHA* mutation carriers are normal, but homozygous mutations cause Leigh syndrome, a severe neurodegenerative disease (Bourgeron et al. 1995). PGL are not features of *SDHA* mutations (Table 4.3). Briere et al. (2005) explored the possible reason for the lack of PGL in *SDHA* mutation carriers. There are two very similar SDHA isoforms, namely, type I and type II genes. Tomitsuka et al. (2003) discovered the type II gene, and Briere et al. (2005) noted that fibroblasts (and possibly other tissues) express only one of the two isoforms, but the paraganglia express both. Therefore, to develop PGLs, *SDHA* mutation carriers will have to inactivate three alleles, which is a very unlikely occurrence in the population.

The TCA cycle gene mutation database is an important resource for clinicians, geneticists, and researchers interested in hereditary PGLs, MUCL/HLRCC, and patients with SDHA syndromes (Bayley et al. 2005, 2008). The database contains excellent information such as specific mutations, their frequencies, possible pathogenic nature, and even variants based on the patient's country of origin. Because mutation submission is an ongoing process, this is the most complete and up-to-date database to consult for TCA cycle enzyme mutations and diseases.

Table 4.3 Clinical features of paragangiiomas

Clinical features	SDHB – PGL4	SDHC – PGL3	SDHD – PGL1
Prevalence (%)	5	4	5
Penetrance	Late age of onset, but reaches ~80% by age 50	_	Early age of onset, but reaches ~80% by age 50
Median age at diagnosis (yrs)	~30	46	~30
Symptomatic at presentation	84%	_	85%
Presenting symptoms	Headache, pain, palpitations	Cranial nerve palsy (common with jugular PGL)	Neck mass, mass effects such as dysphagia, and hoarseness of voice
Functional tumor	Common	Rare	Less common
Frequent tumor location	Extra-adrenal abdominal	Head and neck, more glomus vagale	Head and neck, more carotid body
Multifocality of tumors	12–28%	Not reported	30–74%
Malignancy	Frequent	Very rare	Very rare, linked to Dutch founder D92Y mutation
Maternal imprinting (or parent of origin effect)	No	No	Yes
Fonder mutations	Possible	Not reported	Yes
Biochemistry			
Epinephrine excess	Yes	Rare	Rare
Norepinephrine excess	Yes	Rare	Rare
Dopamine excess	Yes	–	–

SDHB mutations: SDHB gene located on 1p35–36.1 encodes a 30 kDa protein (Table 4.1). This subunit of complex II contains the iron−sulfur moiety involved in electron transfer. Inactivating autosomal mutations in *SDHB* causes PGL4 and nonsyndromic renal cell carcinomas. The gene has eight exons, and mutations have been reported in exons 1–7, but rarely in exon 8 (Benn et al. 2006). Most notable is the concentration of mutations in exon 2. Compared to *SDHD* mutations, missense mutations are enriched in *SDHB* (Benn et al. 2006). A putative founder mutation (IVS1 1G→T splice site) is reported in four families of Scottish descent (Benn et al. 2006).

Mutations in *SDHB* are very common and the clinical features of PGL4 are well characterized (Table 4.3). The prevalence of *SDHB* mutations is estimated at 4–6%. For mutation carriers, the mean age at diagnosis is ~ 30 years. However, Benn et al. (2006) observed that by the age of 30 years, 29% of mutation carriers will have been diagnosed, and this increases to 45% by the age of 40 years. Many mutation carriers are symptomatic at presentation, with complaints of headaches and tumor-associated pain. The common clinical findings include hypertension and arrhythmias. Biochemically, catecholamine and/or dopamine excess indicative of functional tumors is common among these patients. However, ~ 10% will have nonfunctional tumors

(Timmers et al. 2007). Tumors are more likely found in intraabdominal extraadrenal paraganglia, and are usually solitary but can be multiple with an increased propensity to be invasive and metastatic. Metastatic potential is more prevalent in *SDHB* mutation carriers compared to mutations in the other SDH genes. Overall, 34–70% of patients in this group will develop malignant tumors (Neumann et al. 2004; Amar et al. 2005; Benn et al. 2006; Timmers et al. 2007). Of all pheochromocytoma patients, *SDHB* mutation appears to be an independent predictor of death; thus, 5-year survival was 67% for those without *SDHB* mutations compared to only 36% for those with *SDHB* mutations (Amar et al. 2007).

Germline *SDHB* mutations are also reported in nonsyndromic RCCs (Neumann et al. 2004; Vanharanta et al. 2004; Ricketts et al. 2008). In the series by Ricketts et al. (2008), the prevalence of *SDHB* mutations in RCC was 4.4%. Usually, these tumors are of clear cell histology. Thus, *SDHB* mutation carriers have a chance of developing isolated renal cell carcinomas, which is one important reason why genetic counseling and clinical screening are warranted for these mutation carriers.

SDHC mutations: The *SDHC* gene located on 1q21 encodes a 15 kDa protein, which, together with *SDHD*, anchors the catalytic subunits (SDHA and SDHB) to the inner mitochondrial membrane (Table 4.1). Mutations are reported in all six exons except 3 (Benn et al. 2006). There are much fewer mutations in this gene than in B and D, despite continuous scrutiny in HPGL syndromes by several groups.

The prevalence of *SDHC* mutation is 4% (Schiavi et al. 2005), and clinical presentation occurs at a much younger age than in sporadic PGLs (Table 4.3). Carotid body tumors are common, and multiple tumors occur but are less than that observed in *SDHD* mutation carriers. In contrast to *SDHB* mutations, but similar to *SDHD*, pheochromocytomas or malignant tumors are rare in *SDHC* mutation carriers. Functional and malignant tumor of the carotid body has been reported in a patient with IVS5+1G→T *SDHC* slice site mutation (Niemann et al. 2003).

SDHD mutations: The *SDHD* gene encodes a 12 kDa protein (Table 4.1). Autosomal dominant mutations in *SDHD* cause PGL1 syndrome, which is characterized by familial PGL. Missense, nonsense, and frameshift mutations are demonstrated in all four exons of the gene. Compared to *SDHB*, there are more truncating mutations in *SDHD* (Bayley et al. 2005).

The clinical features of *SDHD* mutations are shown in Table 4.3. The prevalence of *SDHD* mutations is ∼ 5%, and the mean age at diagnosis of PGL1 syndrome is ∼ 30 years (Neumann et al. 2004). By the age of 40 years, a majority of *SDHD* mutation carriers will have been diagnosed. Indeed, some even present clinically and are diagnosed before age 10. For index cases, penetrance at the age of 40 years is 65% for *SDHB* mutations but as high as 84% for *SDHD* mutations. Similarly, when all mutation carriers are considered, penetrance at age 40 for *SDHB* mutations is 45% compared with 73% for *SDHD* mutation carriers (Benn et al. 2006). However, an earlier study suggests that penetrance at age 50 is identical between the two mutation carriers, being ∼ 80% (Neumann et al. 2004).

Extensive studies have demonstrated a possible genotype−phenotype correlation for PGL1. Mutations in this gene are primarily associated with benign head and neck PGL that tend to be multifocal. Less frequently, pheochromocytomas as well

as malignancy can develop. The incidence of pheochromocytoma tends to increase in carriers with nonsense mutations (Astrom et al. 2003).

In contrast to *SDHB* and *SDHC* mutations (except for the Spanish study), founder mutations and genomic imprinting are associated with HPGL from *SDHD* mutations. Three founder mutations, D92Y, L139P, and L95P, are identified in almost all Dutch HPGLs (Baysal 2004). Similarly, founder mutations M1I and P81L, are demonstrated in Chinese and American families, respectively (Taschner et al. 2001; Lee et al. 2003). Founder mutations are demonstrated in a Spanish study as well. These are in *SDHD* (c.129G→A) and *SDHB* (c.166_170delCCTCA) genes (Cascon et al. 2009). *SDHD* mutations also show maternal imprinting whereby only paternal transmission causes disease (Baysal et al. 2000). However, somatic loss of maternal chromosome 11 is suggested as an alternative explanation for the apparent maternal imprinting (Hensen et al. 2004).

4.2.1.3 *SDH* Mutations in Other Cancers

CS or disease is a rare genetic disorder with an incidence of 1 in 200,000. Lloyd and Dennis first reported the condition in 1963, and they named it after their patient, Rachel Cowden (Lloyd and Dennis 1963). The clinical presentation of CS includes multiple harmatomatous neoplasms involving several organs such as the skin, mucous membranes, thyroid, breast, colon, brain, and endocrine glands. Breast cancer, follicular thyroid cancer, and uterine fibroids, are common tumors in CS. The skin is involved in 90–100% of the cases. A majority (> 80%) of patients have *PTEN* mutations. Because 1–5% of patients with *SDHB* and *SDHD* mutations also develop renal cell carcinoma and thyroid cancers, which are also features of patients with CS, mutations in *SDH* genes as a cause for *PTEN* mutation-negative CS patients was sought (Ni et al. 2008). Indeed, germline mutations in *SDHB* and *SDHD* are reported in a subset of *PTEN* mutation-negative CS patients, although functionally, the tumors demonstrated *PTEN* dysfunction as evidenced by increased AKT and/or MAPK phosphorylation. It was also observed that CS patients with *SDH* mutations frequently developed cancers of the breast, thyroid, and kidneys (Ni et al. 2008).

Clinical studies have revealed several non-paraganglioma tumors associated with *SDHB* and *SDHD* mutation carriers. These include renal cell carcinoma, pituitary adenoma, papillary thyroid cancer, parotid adenoma, angiolipoma, ovarian cystic teratoma, gastrointestinal stromal tumors, and adenocarcinoma of the colon (Neumann et al. 2004).

4.2.2 *Genetics and Clinical Features of* Fumarate Hydratase *Mutations*

Fumarate hydratase is a TCA cycle enzyme that catalyzes the conversion of fumarate to L-malate (Table 4.1). Located on chromosome 1q42.3–43, *fumarate hydratase* (*FH*) encodes two isoforms; one with and the other without a mitochondrial

targeting sequence. The mitochondrial isoform is a 510 aa protein that converts fumarate to malate in the mitochondria as part of the TCA cycle operation, while the cytosolic isoform metabolizes fumarate produced in the cytosol. The protein exists as a homotetramer with two substrate-binding sites known as A and B. Site A is formed from three of the four chains and is catalytically active. Site B, which is made of amino acid residues from only one chain, binds substrate but is not catalytically active. Thus, mutations affecting functions of site A are expected to be more deleterious that those of B.

Homozygous mutations of *FH* result in the condition known as fumarate deficiency, which is a severe and lethal neurodegenerative disease. Heterozygous *FH* mutations, however, cause autosomal dominant disorders (Table 4.2). The genetic locus was mapped to chromosome 1q42.3–42, and the first molecular description of *FH* mutation was in 1994 (Bourgeron et al. 1994). However, Tomlinson et al. (2002) first provided evidence that germline heterozygous mutation in *FH* caused the condition known as hereditary leiomyomatosis and renal cell carcinoma (HLRCC). Somatic mutations in *FH* are rare and mainly found in uterine leiomyomas.

Cutaneous leiomyomas are rare, benign tumors of the skin. They arise from the erector pili muscle in hair follicles. Multiple cutaneous and uterine leiomyomas is a genetic disorder in which female patients usually present with multiple skin nodules and uterine fibroids (MCUL). The skin condition is usually clinically evident in teenage years and uterine lesions develop in their active reproductive years, that is, in the thirties. Typically, mean ages at a diagnosis of cutaneous and uterine leiomyomas, are 25 years and 30 years, respectively. The familial uterine leiomyomas are often more severe requiring surgical intervention. Some MCUL families will develop renal cell carcinomas, giving rise to the hereditary leiomyomatosis and renal cell carcinoma (HLRCC) syndrome (Launonen et al. 2001), and others could develop leiomyosarcomas as well.

Prototypical phenotype of *FH* mutations is, however, skin tumors. These cutaneous leiomyomas are sensitive to cold and abrasion. They appear as intradermal papules or nodules measuring $\sim$ 20 mm in diameter. They have disseminated or segmental distribution. Female mutation carriers have increased risk of developing severe uterine fibroids. Rarely, nonsyndromic fibroids have somatic *FH* mutations (Lehtonen et al. 2004). But, *FH* mutations are mainly important in syndromic fibroids.

Renal cell carcinoma in HLRCC tends to be aggressive type II papillary and collecting duct morphology. These tumors usually metastasize before the age of 50 years and are fatal. Lehtonen et al. (2007) reported on finding both clear cell and papillary carcinomas in HLRCC renal cancers. Merino et al. (2007) described the pathology of RCC in HLRCC syndrome. In their analyzes of 40 RCC from 38 HLRCC families, they found that RCC in this syndrome ranged in sizes from 2.3 to 20 cm, and were frequently unilateral. Papillary-type architectural patterns were more common; however, tubulopapillary, tubular, solid, and mixed architectural patterns were also observed. Importantly, their analysis revealed a histopathologic diagnostic nuclear signature of these tumors. They typically possess a large nucleus that has prominent inclusion-like orangiophilic as well as eosinophylic nucleus surrounded by a clear halo (Merino et al. 2007).

4.2.2.1 *Fumarate Hydratase* Mutations in Other Cancers

A patient, who reported sudden loss of consciousness without neurological sequellea was subsequently diagnosed with HLRCC syndrome (Campione et al. 2007). As a diagnostic workout of the nervous system presentation, cerebral carvenomas were diagnosed. The mother of this patient who is also diagnosed with MCUL was found to have similar brain lesions. Familial cerebral carvenoma malformation syndrome was excluded in this family. However, an analysis of *FH* revealed an in-frame duplication of exon 7 in the proband, his mother, and son. The duplicated exon produces a protein that interferes with the 3D structure and therefore, with the function of *FH*. Consistent with this finding, enzymatic activity in lymphocytes of the patients were half that of unaffected family members. Because there is lack of ample evidence linking specific TCA cycle enzyme mutations with specific cancers, it is uncertain whether this exon 7 duplication of *FH* is uniquely related to cerebral carvenomas.

Leydig cell testicular tumors (LCTs) are rare, but the commonest nongerm-cell tumors. A majority of cases occur in adults, yet the molecular genetic alteration in this tumor is probably well-defined in childhood tumors where a hotspot mutation in LH/choriogonadotrophin receptor (LHCGR) gene (D578H somatic substitution) is reported in 70–100% of the cases (Liu et al. 1999; Richter-Unruh et al. 2002). In an attempt to further genetically characterize these tumors, Carvajal-Carmona et al. (2006) analyzed LCTs from one individual with HLRCC as well as 29 sporadic tumors. Loss of heterozygosity in *FH* was demonstrated in the testicular tumor from the familial case, while a specific *FH* mutation, M411I, was identified in one sporadic tumor. The familial tumor had the highest expression of VEGF, while the sporadic tumor with *FH* mutation expressed the highest levels of HIF1α.

In a Polish series, uterine leiomyomas and ovarian cystadenomas were enriched in hereditary ovarian cancer syndrome (Menkiszak et al. 2004), suggesting a possible similar genetic etiology. An analysis of *FH* for mutations in probands with RCC, skin leiomyomas uncovered two mutations in two patients, who also presented with ovarian mucinous cystadenomas (Ylisaukko-oja et al. 2006). Because tumor tissue was unavailable for analysis, the loss of heterozygosity of *FH* could not be ascertained.

Breast, prostate, and bladder cancer as well as skin leiomyosarcomas are possibly associated with MCUL/HLRCC syndromes.

4.2.3 Genetic Testing and Counseling for SDH *and* FH *Mutations*

4.2.3.1 *SDH* Mutations

Genetic testing of sporadic PGL: People presenting with sporadic PGLs often do not have family histories of the disease. There are a couple of reasons for this: First, in spite of the dominant inheritance pattern of these mutations, penetrance is

incomplete. There is an age-related penetrance whereby 50% of *SDHB* and *SDHD* mutation carriers develop tumors by their thirties, but this rises to ~ 80% by the age of 50 years (Neumann et al. 2004). Second, *SDHD* mutations demonstrate maternal imprinting such that children of *SDHD* mutation carrier mothers will not develop disease, but can pass on the mutation to their offspring. Thus, the family history is often missing in many PGLs. Indeed, 63–90% of patients with PGLs involving *SDHB* mutations present as sporadic cases with no evidence of familial linkage (Benn et al. 2006; Timmers et al. 2007). Similarly, about 21% of *SDHD* PGLs are apparently sporadic. In view of these issues, it is recommended that genetic testing be offered to patients presenting with sporadic PGLs. The genes to begin testing will depend on the clinical presentation. For instance, patients presenting with head and neck PGLs, multifocal tumors, or both, should begin with *SDHD* screening. Similarly, extra-adrenal abdominal, malignancy, RCC presentation indicates possible *SDHB* mutations. It is also suggested that presentation with head and neck glomus vagale tumors should begin with *SDHC* mutation testing before *SDHD*.

Pheochromocytoma presentation needs a systematic workflow, because it involves not only SDH genes, but the *RET, NF1*, and *VHL* as well. Jimenez et al. (2006) have provided some guidelines on genetic testing in patients presenting with sporadic pheochromocytoma/PGL. They suggest the following:

- Genetic testing should be provided to all patients presenting with pheochromocytomas, with history or clinical features suggestive of a hereditary syndrome.
- Patients presenting with bilateral or multifocal pheochromocytomas suggest mutations in *VHL* and *RET* genes, and hence, these should be tested before *SDH* genes. It is also indicated that the presence of elevated epinephrine and/or metanephrine should lead to testing the *RET* gene first, while elevated norepinephrine and/or normetanephrine require *VHL* testing first.
- For presentation with sympathetic PGL, prioritized testing is dependent on age. For patients with early onset, (age < 20 years), *VHL* should be tested, followed by *SDHB* and then *SDHD*, and for patients > 20 years, *SDHB* should be tested before *VHL* and *SDHD*.
- For older patients (age > 50 years), the probability of mutations in these susceptibility genes is very low, making genetic testing optional. In this group, testing is not cost-effective.

Tumor Screening in mutation carriers: The question as to whether to screen all asymptomatic mutation carriers of an HPGL family still requires evidence-based studies. Admittedly, early detection of tumors leads to less complications and better treatment outcomes. Hence, it is recommended by many experts that screening for tumors are offered for *SDHB* mutation carriers to prevent possible development of aggressive tumors. Recommendations for screening include the following:

- Provision of genetic counseling to families with identified index case. This should be performed carefully providing useful information on the genetics and natural history of the disease. The strengths and weakness of current testing should be made clear to the family so that they can make informed decisions.

- Genetic testing of all first-degree relatives of the index case should then be offered. The only exception to this testing will be children from a female *SDHD* mutation carrier because of maternal imprinting.
- Offer an annual or biannual screening for mutation carriers. This will include detailed clinical history and physical examination, as well as biochemical and radiologic investigation. These screening regimes should include blood pressure measurements, urinary catecholamines and metanephrines, and computer tomography scans or magnetic resonance imaging of the neck, thorax, abdomen, and pelvis.
- For *SDHB* mutation carriers, screening should commence before 10 years of age, because it is shown that diseases will be detected in 96% of *SDHB* and 100% of *SDHD* mutation carriers by implementing this early intervention strategy (Benn et al. 2006).

4.2.3.2 *Fumarate Hydratase* Mutations

Genetic testing: Genetic counseling and testing are available for fumarate hydratase gene mutations because of the occurrence of fumarase deficiency (FD), which is a rare but severe and lethal neurological disorder. Fumarase deficiency is suggested in babies born with severe neurological disorders without evident metabolic crises. Such findings prompt diagnostic and genetic testing to diagnose or rule out the condition. Biochemical testing of urine organic acid profile will show increased concentration of fumaric acid, and the enzyme activity in fibroblasts or lymphoblasts can be lower than 10% of the normal mean, but typically varies between 10 and 30% of the mean. Molecular genetic testing is used to confirm the diagnosis, as well as detect carriers.

Genetic counseling: Providing genetic information on the nature, inheritance pattern, and effects of the disorder enables families to make informed decisions and choices. Parents of a child born with fumarase deficiency are usually normal heterozygous carriers of *FH* mutations. They certainly require genetic testing for confirmation of these mutations because of the risk of developing MUCL/HLRCC, and the implications for their offspring. Such mutation carriers confer 25% risk of having homozygous mutant (fumarase deficiency), or wildtype (no mutations), and 50% risk of heterozygous children. Female offspring can develop severe fibroids that could require surgery in young age, which have public health implications in terms of fertility and psychosocial effects on such women. These mutation carriers need investigations for the early detection of renal cell carcinoma, because such tumors can be aggressive. Therefore, there is a need for family screening of at-risk individuals for early diagnosis when cure is optimal.

Prenatal testing: As indicated above, if both parents are heterozygous mutation carriers, there is a 25% probability of having a child with fumarase deficiency. Because of the severity of the disease, and the natural course, which currently is almost incompatible with life past the first year, prenatal testing and counseling are

available for such parents. Molecular genetic testing can be performed on fetal cells recovered at gestational age 10–12 weeks via chorionic villus sampling, or at week 15–18 using amniocenthesis. Biochemical testing is possible and available, but some fetuses could demonstrate some fumarase activity, which could confound the interpretation of results.

4.2.4 *Mechanism of Tumor Induction by SDH and* Fumarate Hydratase *Mutations*

The mechanism of tumor formation from mutations in the two TCA cycle enzymes is suggested to include elevated ROS production from defective respiratory chain function, resistance to apoptosis, and establishment of pseudohypoxia (defined as induction of hypoxic gene signature in the presence of adequate oxygen). Mutations in *SDHB* or *SDHD* lead to complete loss of respiratory complex II enzyme activity because of abnormal enzyme assembly. During electron flow through complex II, SDHB, SDHC, and SDHD perform an internal electron flow within the complex. Electrons flow through the iron−sulfur components in SDHB to the CoQ sites associated with SDHC and SDHD. On the contrary, SDHA mediates entry of electrons into complex II by accepting electrons from the TCA cycle. Based on these functional specializations of the subunits, it is suggested that mutations in the various subunits of complex II should lead to different pathophysiologies. Mutations in *SDHB, SDHC*, and *SDHD* will prevent electron flow within complex II and potentially lead to increased ROS production, while mutations in *SDHA* will prevent electrons from entering the ETC at complex II, and therefore, culminate in reduced ATP production and hence, present with more severe phenotypes. However, evidence for oxidative stress is demonstrated to be missing in these tumors (Pollard et al. 2005b; Selak et al. 2005), suggesting other possible mechanisms.

The mechanism with strong evidence in support of tumor causation by mutations in *SDH*, and *FH* is pseudohypoxia. Pseudohypoxia is a general feature of tumors and is associated with HIF activity or pathway expression, angiogenesis, and glycolysis. This hypoxic gene expression signature is achieved by tumors through various mechanisms. The concept of pseudohypoxia in cancer is well established and better understood in the VHL syndrome. The *VHL* is a tumor suppressor gene that is ubiquitously expressed in tissues. It primarily functions to prevent the stabilization of HIF1α and 2α under normoxic conditions. In such scenarios, VHL forms a complex with Elongin B, Elongin C, and Cul-2 and targets HIF1α for degradation. HIFs are transcription factors that can induce the expression of genes involved with angiogenesis, metabolism, as well as cell proliferation and differentiation. For the VHL complex to recognize HIFs, specific prolyl residues must be hydroxylated. This reaction is performed by HIF prolyl hydroxylases that use oxygen and 2-oxoglutarate (α-ketoglutarate) as cosubstrates and Fe^{2+} and ascorbic acid as cofactors (Kaelin 2005). Interference with any of the regulatory factors mediating this process can potentially lead to HIF stabilization. For

example, in hypoxia, the limiting oxygen tension prevents normal functioning of proly hydroxylases, leading to stabilization of HIFs. Importantly, mutations in VHL cause stabilization of HIF in normoxia (psedohypoxia), leading to the formation of tumors, including renal cell carcinomas and pheochromocytomas.

An indication that pseudohypoxic mechanism could underlie tumor formation came from studies of PGL. The incidence of carotid body PGL is high in people who reside on high altitudes where the oxygen tension is low, suggesting a possible link between chronic hypoxia and head and neck PGL (Gimenez-Roqueplo et al. 2001). Baysal et al. (2000) observed phenotypic similarity of PGL with *SDHD* mutations and normal carotid body exposed to chronic hypoxia (Baysal et al. 2000). These findings led Baysal et al. to hypothesize that a component of *SDHD* functions as oxygen sensors, such that inactivating mutations with loss of function should lead to chronic hypoxic stimulation. A link between VHL tumors (pseudohypoxic tumors) and *SDHD* PGLs was quickly entertained. Consistent with this speculation, increased expression of HIF and its downstream target VEGF in HPGL with *SDH* mutations were demonstrated (Gimenez-Roqueplo et al. 2001; Pollard et al. 2005a, b). In addition, gene expression profiling of pheochromocytomas with *SDHB, SDHD,* or *VHL* mutations revealed a pseudohypoxic HIF gene signature pattern (Dahia et al. 2005).

Apart from loss of function of respiratory complex II, it is expected that mutations in SDH should impede TCA cycle activity, leading to possible accumulation of cycle intermediates. Indeed, succinate levels are higher in HPGLs (Selak et al. 2005; Pollard et al. 2005a, b), and *SDHA* mutant fibroblasts accumulate succinate, which facilitates nuclear translocation of HIF (Briere et al. 2005). Thus, HPGLs are succinate-accumulating pseudohypoxic tumors. Selak et al. (2005) provided a mechanistic connection between succinate and pseudohypoxia. They used siRNA to knockdown *SDHD* in cells and demonstrated the accumulation of HIF1α in cells in association with the induction of HIF1 target genes (BNIP3, VEGF). These pseudohypoxic cells accumulated succinate in mitochondria that acted as an intracellular messenger. Succinate moved into the cytosol to inhibit the activity of prolyl hydroxylases, leading to HIF1 stabilization (Fig. 4.1). This product inhibition by succinate occurs because during the hydroxylation of HIF1 by proly hydroxylases, α-ketoglutarate is converted to succinate. Therefore, elevated cytosolic succinate retards this reaction and hence, the activity of prolyl hydroxylases.

A similar mechanism appears to account for how mutations in *FH* cause tumors. The presence of pseudohypoxia in renal cell carcinoma and uterine leiomyomas from HLRCC patients is well established. Pollard et al. (2005b) demonstrated increased expression of HIF1α in RCC from HLRCC subjects. The observed nuclear staining was confirmed by immunoblot showing the presence of HIF1α signal in tumor lysates but not in normal kidney tissue. Isaacs et al. (2005) also used immunohistochemistry to demonstrate elevated expression of HIF 1α and 2α in RCCs (Isaacs et al. 2005). Consistent with the elevated expression and nuclear localization of HIF1, downstream targets were increased. Evidence of psedohypoxia was also demonstrated in uterine leiomyomas from patients with HLRCC syndrome. Using vascular endothelial marker, Pollard et al. (2005a) demonstrated

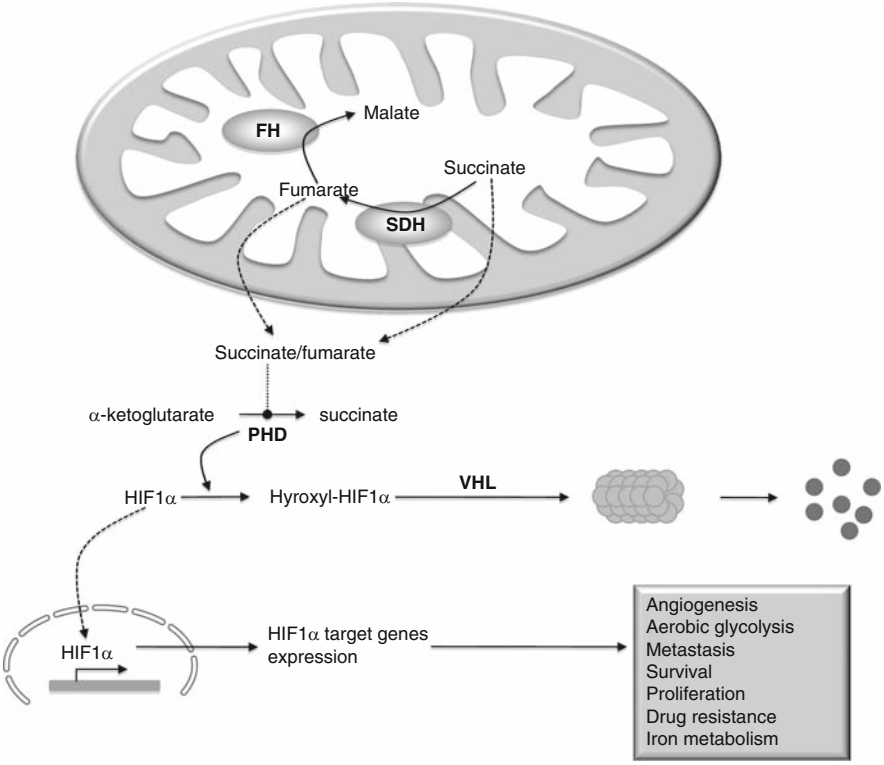

Fig. 4.1 *The mechanism of pseudohypoxia in HPGLs and HLRCC syndromes*. Mutations in *SDH* and *FH* lead to cytosolic accumulation of succinate and fumarate. During hydroxylation of HIF1α for degradation by prolyl hydroxylases (PHD), α-ketoglutarate is converted to succinate. Excess succinate and fumarate inhibit this reaction and hence, the activity of PHD, leading to accumulation and nuclear translocation of HIF1α to induce target gene transcription

a significantly higher microvascular staining in uterine leiomyomas from HLRCC subjects compared to non-leiomyomatous myometrium from the same women (Pollard et al. 2005a). Similarly, microvascular density was much higher in HLRCC uterine leiomyomas than in sporadic uterine leiomyomas. In situ hybridization revealed increased expression of VERF and decreased expression of the anti-angiogenesis factor, TSP1, in HLRCC uterine leiomyomas compared to sporadic cases and normal myometrium from women with and without HLRCC syndrome. Thus, in keeping with the limited evidence of *FH* mutations in sporadic leiomyomas, the mechanism of tumor formation also appears to be different.

Fumarate, and to a lesser extent, succinate levels, are elevated in cancers from *FH* mutations. Analysis of Krebs cycle enzyme levels in leiomyomas from women with HLRCC revealed a dramatically increased enzyme levels in tumors compared to normal myometrium (Pollard et al. 2005b). Isaacs et al. (2005) used genetic manipulation to demonstrate elevated fumarate levels in situations of loss of FH activity. Short interfering RNA was employed to knockdown *FH* in lung carcinoma

cell line A549. The loss of FH activity was rapidly associated with an acute doubling of intracellular fumarate levels. This offers collaborative evidence that the loss of FH activity in renal cell carcinoma and uterine leiomyomas from patients with HLRCC syndrome will be associated with fumarate accumulation in these cancers.

Thus, tumors from *FH* mutations have elevated levels of fumarate in association with pseudohypoxia. What then is the relationship between fumarate and pseudohypoxia? This question was answered by elegant experiments performed by Isaacs et al. (2005). They used the mitochondrial poison, 3-nitropropionic acid, to inhibit the activity of fumarate in A549 cells and observed increased HIF levels in nuclear extracts. A similar experiment performed in the presence of cycloheximide, an inhibitor of protein synthesis, provided identical results. This finding indicates that the elevated levels of HIF in 3-nitropropionic acid treated cells could not be accounted for by gene expression changes. Further analysis revealed that fumarate caused a dramatic reduction in the ratio of hydroxylated HIF to total HIF. Experiments in cell-free systems uncovered an inhibitory interaction of fumarate on 2-oxaloacetate, a required cofactor by proly hydroxylases (Fig. 4.1). Thus, the clear evidence here is that in FH-deficient cells, fumarate accumulates and inhibits 2-oxaloacetate, which then decreases the activity of prolyl hydroxylases, leading to the stabilization of HIF even in normal oxygen conditions.

4.3 Mitochondria-to-Nuclear Stress Signaling in Cancer

Genetic alterations in the mitochondrial genome or metabolic inhibition of mitochondrial functions can induce mitochondrial stress signaling. Any condition that results in a loss of mtDNA integrity will cause mitochondrial genetic stress, while metabolic stress occurs when the effects of compounds inhibit or interfere with mitochondrial functions. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) are common compounds used to induce mitochondrial stress signaling in cells.

Mitochondrial stress induces gene expression changes that mediate various signal activations. The target genes induced by mitochondrial stress appear to depend on how long the stressful event lasts. Li et al. (1995) observed that impairment of respiration had different effects on nuclear regulation depending on the duration of the respiratory defect. Chronic deficiency of OXPHOS by depletion of cellular respiratory genomes gave a distinctively different gene expression profile from acute respiratory impaired cells. Chronically impaired respiration caused expression of genes for inner membrane proteins, intermediate filaments, and ribosomes, and these cells had normal mitochondrial patterns and electrochemical potential. On the contrary, acute mitochondrial respiratory injury was associated with the expression of immediate early genes and heat shock proteins.

Mitochondrial stress signaling results in altered gene expression and cellular phenotypic changes that are mediated by a well-characterized calcium-signaling

pathway. Studies have shown that mitochondrial stress alters calcium homeostasis, leading to the activation of genes that respond to calcium signaling. These genes include *calcineurin, ATF, NFAT, CREB*, and *CEBP/delta*. Signaling pathways, including PKC, MAPK, and NF-κB, and genes controlling glucose metabolism, apoptosis, and oncogenesis, are activated by mitochondrial stress. Luo et al. (1997) induced mitochondrial-to-nuclear stress signaling by treating PC12 pheochromocytoma cells with the mitochondrial-specific ionophore, FCCP. This treatment caused an increase in cytosolic calcium and the activity of extracellular signal-regulated kinase 1 and 2 (ERK1/ERK2). Mitochondrial to nuclear stress signaling in C2C12 myoblasts was established using either mtDNA depletion or treatment of cells with mitochondria-specific inhibitors (Biswas et al. 1999). Both treatments were associated with a disruption of mitochondrial membrane potential, increased cytosolic calcium activation of calcium-responsive factors, and calcineurin.

The effects of the above stress signaling on tumor behavior were provided by two studies by Amuthan and colleagues (Amuthan et al. 2001, 2002). They first induced mitochondrial stress in noninvasive C2C12 myoblasts and human lung carcinoma A459 cells. Their manipulations caused specific nuclear gene expression changes and activation of signaling pathways. There were elevated levels of calcium-dependent protein kinase C and calcineurin-mediated IKBβ in association with increased expression of tumor markers, including cathepsin L and TGFβ. In another study, mitochondrial genome depletion or membrane damage of C2C12 cells conferred much higher invasive propensities to these cells compared to control cells. The genetic, phenotypic, and behavioral characteristics of the stressed cells could be reverse-closed to control cells by reconstitution of the cells with ∼ 70% of the mtDNA from control cells, suggesting that the origin of these effects is loss of mitochondrial genes.

Genetic stress in A459 cells induced increased cytosolic calcium and calcineurin, as well as increased expression of TGFβ and cathepsin L; activation of MAPK and calcineurin-dependent factors, and overexpression of antiapoptotic BCL-2 and BCL-xL, as well as genes involved in de novo glucose synthesis. Moreover, stressed cells were shown to have altered morphology and invasive behavior. Partial mtDNA loss in C2C12 myoblasts induced resistance of the cells to apoptosis because of sequestration of BAX, BID, and BAD in mitochondrial inner membrane, increased BCL-2 and BCL-xL expression, reduced processing of p21 BID to tBID, and decreased the activation of caspases 3, 9, and 8 (Biswas et al. 2005). Thus, mitochondrial genome loss appears sufficient to confer resistance on cells to apoptotic stimuli.

The environmental toxin and carcinogen dioxin may promote tumor development partly by damaging mitochondrial functions. Exposure of C2C12 myoblasts to dioxin-inhibited mitochondrial transcription and translation, altered calcium homeostasis, altered membrane potential, elevated calcineurin levels, and calcineurin-dependent activation of NF-κB signaling. These cells were also resistant to apoptosis and acquired a highly invasive phenotype (Biswas et al. 2008a). As the NF-κB induction was different from the canonical and noncanonical NF-κB signal

induction, a follow-up study was sought to uncover the mechanism (Biswas et al. 2008b). This group had previously shown that mitochondrial stress signaling activates NF-κB via IKKβ inactivation mediated by calcineurin-dependent phosphorylation as a result of direct interaction of calcineurin with IKKβ, which is independent of IKK kinase activation (Biswas et al. 2003). A knockdown of the expression of IKKβ (but not α) decreased mitochondrial stress-induced activation of NF-κB, indicating IKKβ, is the primary mediator of this specific signaling.

Nuclear expression profiles to help explain mitochondrial stress signaling have also been examined in mtDNA depleted cell lines. Rho zero breast cancer cells were shown to exhibit increased oxidative stress and altered expression of several nuclear genes involved in signaling, metabolism, cell morphology, growth, differentiation, and apoptosis (Delsite et al. 2002). Using the established cybrid technology, Singh et al. (2005) further showed that the status of mtDNA greatly influenced nuclear genetic stability. The depletion of cells of their mitochondrial genomes led to the acquisition of transformed phenotypes in association with chromosomal instability, and altered expression of APE1 involved in transcriptional regulation and DNA repair. These features were all reversed by repopulation of ρ0 cells with wildtype mtDNA.

In a well-designed study, Kulawiec et al. (2006) identified proteins responsible for retrograde signaling. Proteomic analysis of ρ0 cells, cybrids with normal repopulated mtDNA, and parental cell line uncovered differential expression of a number of proteins. Rho-zero cells demonstrated decreased expression of respiratory complexes I and III subunits, proteins controlling the cell cycle, molecular chaperons, and increased expression of inosine 5'-monophosphate dehydrogenase type 2 (IMPDH2) that is part of the nucleoside biosynthetic enzymes. These changes were reversible in cybrids with wildtype mtDNA suggesting that they were caused by loss of mtDNA, emphasizing the importance of mtDNA integrity in normal cell physiology.

4.4 Nuclear Integration of Mitochondrial Genome Fragments and Possible Oncogene Induction

Nuclear chromosomal integration of mitochondrial genetic material has occurred throughout human evolution and is still an ongoing process (Ricchetti et al. 2004). Indeed, part of the symbiotic relationship was the downloading of some α-proteobacterial genes on to chromosomes. While these ancient mitochondrial genes are stable and expressed in a normal fashion, the illegitimate ongoing mitochondrial genome integration into the nucleus can be pathogenic. Most of the recent mitochondrial genomic fragments are frequently inserted into nuclear genetic loci that do not encode proteins or regulate gene expression, and hence, remain as nonpathogenic pseudogenes. Occasionally, however, mtDNA fragments integrate into exons or gene control regions and therefore, interfere with gene functions. For example, the Pallister–Hall syndrome is usually inherited as an autosomal dominant mutation

in *GLI3*. Turner et al. (2003) characterized a sporadic case of Pallister–Hall syndrome and showed that a de novo mitochondrial to nuclear transfer of a 72 bp mtDNA insert into exon 14 of *GLI3* caused the disorder. This created a premature stop codon, resulting in disease phenotype. In another study, a 93 bp mitochondrial *ND5* fragment was inserted into the splice site of *MCOLN1* that resulted in improper splicing of the gene, resulting in the disorder, mucolipidosis-IV (Goldin et al. 2004).

In cancer, it can be conceived that a simple model of tumor induction by mitochondrial genomic fragments in the nucleus will be activation of oncogenes, and/or inactivation or suppression of tumor suppressor genes. There are only few circumstantial evidences for the possible tumor induction by mitochondrial-to-nuclear transfer of genetic material. Shay and coworkers demonstrated the contiguous insertion of mitochondrial *COIII* fragments in *MYC* locus in HeLa cells with subsequent transcription of the chimeric mRNA (Shay et al. 1991; Shay and Werbin 1992). The possible induction of malignant transformation by nuclear integration of mitochondrial genome has also been addressed in mouse embryonic fibroblasts (Hu et al. 2000). In this study, nuclear insertion of mitochondrial fragments was achieved using transgenic techniques. HIH3T3 cells with nuclear integrated mtDNA were transformed and could develop tumors in nude mice. Chen et al. (2008) used in situ hybridization to detect mtDNA in cervical cancers, and the frequencies of integration appeared to mirror disease progression from carcinoma in situ to invasive cervical cancer. It should be noted that these studies only provide circumstantial evidence of oncogenic induction by nuclear integration of mtDNA. However, the strong evidence of developmental anomalies caused by such genetic trafficking suggests a possible event in carcinogenesis, given that this process is well known to be ongoing.

4.5 Conclusion

The genetics of TCA cycle enzyme mutations and cancer have been extensively examined. The specific mutations are useful in guiding accurate diagnosis and management of probands, and help in screening and genetic counseling of family members. While the mechanisms of cancer initiation by *SDH* and *FH* mutations are being explored, the underlying reasons for the various cancer types will continue to fascinate scientists. First, not all TCA cycle enzyme mutations cause cancer. While some of these enzyme mutations cause neurodegenerative diseases, only *FH* and *SDH* mutations are linked to specific types of cancers. Second, while it is becoming convincing that cancer evolution from these mutations is due to the accumulation of succinate and fumarate, the contributing role of respiratory chain defects in the etiology of cancer requires clarification. For example, are tumors caused by *SDH* mutations a defect of TCA cycle functions or complex II activity or both? While it is conceptually understandable that *SDHA* isoform variation between tissues of the paraganglia and others accounts for the lack of tumor formation in *SDHA* heterozygous mutations, the reasons for the differing cancer types, locations,

and severity caused by mutations in other *SDH* are unclear. Does the catalytic functions of SDHB explain why mutations in this subunit are associated more with pheochromocytomas and malignant tumors? Finally, are the lethal pediatric encephalopathies from TCA cycle enzyme mutations caused by energetic failure of developing neurons and/or neuronal apoptosis from elevated ROS as a result of defective complex activity? Answers to these questions will enhance our knowledge on PGL.

Mitochondrial stress in cancer cells is either caused by mutations in the mitochondrial genome or the effects of chemicals and toxins on cancer cells. To avoid death, cancer cells respond to such stresses by signaling to the nucleus to evoke survival-signaling pathways. Understanding the myriads of mechanisms such as how cancer cells signal to the nucleus via ROS will open up targets for chemotherapy. Finally, the evidence for oncogenic induction by nuclear integration of mtDNA fragments is not well established. However, the demonstration that mtDNA fragments in the nucleus can cause diseases such as the Pallister–Hall syndrome suggests that abnormal docking and insertion of mtDNA into tumor-causing genes can initiate cancer formation.

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Part II
Mitochondrial Genetic Alterations in Cancer

Chapter 5
Types of Mitochondrial Genetic Alterations in Cancer

Abstract The polyploid nature of mitochondrial genomes associated with the uniparental inheritance makes mitochondrial genetic alterations unique. There are two major types of mitochondrial genome changes, germline polymorphic variants and somatic mutations. Germline variants and haplotypes can confer risk for diseases. Somatic mutations that encompass point mutations, microdeletions and insertions, and large-scale deletions, as well as content changes can be pathogenic at certain heteroplasmic proportions. This chapter describes the various genetic changes in the mitochondrial genome encountered in cancer, and provides an overview of mitochondrial microsatellite instability. Also discussed here are issues with regard to mitochondrial genome analysis and the authenticity of mitochondrial genetic changes in cancer.

5.1 Introduction

Changes in the extra nuclear genome are unequivocally demonstrated in several cancers. Putatively, these mitochondrial genome variations are associated with cancer predisposition, initiation, growth, progression, metastasis, and prognosis. There are two types of mitochondrial genome single nucleotide variants, germline polymorphisms, and somatic mutations. Germline polymorphisms are common in various geographic clusters and evolved as adaptive mutations. Somatic mutations are acquired changes that are not transmitted to the offspring. Other pathogenic mutations in the mtDNA molecule include large-scale deletions and rearrangements. The focus of this chapter is on issues of mitochondrial genetics in carcinogenesis. Specifically, it addresses the concept of mitochondrial haplotypes and how these seemingly neutral polymorphisms influence human health and possibly cancer risk; the concept of mitochondrial microsatellite instability (mtMSI) in cancer; aberant mitochondrial transcripts in cancer; mitochondrial genome rearrangement mutations and associated content changes; issues of research design and analysis that influence the final data quality; and the debate on the authenticity of mtDNA mutations in cancer.

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5.2 Mitochondrial Haplotypes and Haplogroups

Mitochondrial haplotypes are adaptive mutations that evolved as human beings spread across the universe to inhabit diverse climates. For example, residence in a colder climate necessitated the uncoupling of respiration to enable heat production. Hence, the emergence of cold-adapted mitochondrial genomes that enable relatively less ATP production. Many of these adaptive mutations are associated with human health, including longevity, neurodegenerative diseases, and a possible predisposition to cancer.

Unlike the nuclear genome that shows minimal regional variations, mtDNA displays a distinct distribution of alleles in specific geographic locations. The oldest mtDNA lineage is found in Africa. This mtDNA molecule is designed for tight coupling of the respiratory chain, and hence, efficient energy production. This is reasonable to conceptualize because in a hot climate, uncoupled respiration will lead to undesirable heat production. Putatively, as human beings migrated from Africa and occupied colder climates, several modern mitochondrial genomes evolved. It is well conceived that the modern mitochondrial genomes originated from the older ancestors by acquiring de novo mutations. Experimental data analysis estimates that between 10 and 25% of the novel polymorphisms or nucleotide variants in mitochondrial structural genes, tRNAs and rRNAs confer reduced coupling efficiencies of the respiratory chain, thereby enabling heat release to permit habitation in colder climates (Mishmar et al. 2003; Wallace et al. 2003; Ruiz-Pesini et al. 2004). Such "heat-generating" mutations appear to have been positively selected for, and propagated, along with some molecularly linked nucleotide variants that are simply "passengers" or "hitchhikers." These uniquely selected mutations, along with their linked alleles, constitute a mitochondrial haplotype. During the spread of human populations, distinct groups of individuals descended from a closely related mtDNA haplotype or genome. People with identical mitochondrial haplotype constitute a mitochondrial haplogroup. There are several haplogroups and subhaplogroups with variable allele frequencies in various geographic locations.

The oldest haplogroups designated L0, L1, and L2, are characterized by a T at nucleotide position (np) 3,594, and many of these haplotypes also have an A at np 7,521. Distinct polymorphisms subdivide the three into various subcategories; L0 has an A at np 3,516, C at np 13,789 characterizes L1, and a C at np 10,115 defines L2. Collectively, these three haplogroups constitute macrohaplogroup L. The presence of a C (instead of a T) at np 3,594 defines the diverged L3 African lineage from L2. Europe and Asia were colonized by diverged L3 lineages, and macrohaplogroups M and N. Lineage N, defined by T10873C, gave rise to European haplogroups H, I, J, Uk, T, U, V, W, and X, while both M, defined by C10400T, and N account for Asian haplogroups. Asian haplogroups C, D, G, E, and Q arose from M, while N diverged into lineages A, B, F, P, and Y. Finally, the Americas were populated by Eurasian haplogroups A, B, C, D, and X.

As population polymorphisms, it is conceivable that these mutations will be neutral. However, it is now evident that some haplotypes predispose or modify various diseases, including cancer, and that some polymorphisms confer longevity. For example, Leber's hereditary optic neuropathy mutations 11778A and 14484C are associated with European haplogroup J, and 10663C mutation is pathogenic when present in haplogroup J background. The risk of developing some neurodegenerative diseases such as Parkinson's is thought to be elevated in haplogroup H individuals, and decreased in those with J and Uk haplogroups (van der Walt et al. 2003; Ghezzi et al. 2005; Khusnutdinova et al. 2008). Similarly, haplogroup H confers reduced risk of age-related macular degeneration, while J and U increase drusen levels and abnormalities of the retinal pigment epithelium, respectively (Jones et al. 2007). The risk of developing diabetes mellitus is increased in Europeans with haplogroup J (Mohlke et al. 2005; Crispim et al. 2006; Saxena et al. 2006), while in Asian populations, haplogroup N9a confers resistance against type 2 diabetes mellitus (Fuku et al. 2007), N9b protects against myocardial infarction (Nishigaki et al. 2007), and in Japanese women, N9a confers protection against the metabolic syndrome (Tanaka et al. 2007). Haplogroup J increases the life span of Europeans (De Benedictis et al. 1999; Rose et al. 2001; Niemi et al. 2003), Haplogroup D increases the longevity of Asians (Tanaka et al. 1998; Tanaka et al. 2000) and specifically, D4a is associated with extreme longevity in Japanese (Alexe et al. 2007; Bilal et al. 2008). It is apparent that haplogroup polymorphisms are not neutral, but can protect individuals from diseases or carry the risk for development of diseases.

5.3 Mitochondrial DNA Polymorphisms and Somatic Mutations

Single nucleotide changes in the mitochondrial genome can occur as a normal variant within the population (polymorphism) or as cell- or tissue-specific mutation that is rare and different from normal alleles in the population (somatic mutation). In general, mtDNA polymorphisms are detected by comparing the germline sequences of the individual to the reference sequence (rCRS), while somatic mutations are scored by intra-individual sequence comparison (i.e., comparison of tissue specific sequences to the individual inherited germline sequence). Frequently, polymorphisms are homoplasmic, while somatic mutations, which are indicative of the disease, tend to be heteroplasmic. However, it appears that some polymorphic variants can modulate the expression of some diseases or pose as risk factors for the development of diseases, including cancer.

The distributions of several types of cancers reveal some geographic, regional and/or ethnic clustering. For example, the risk of developing cancer of the prostate partly depends on the ethnicity of the individual. The incidence of prostate cancer is much higher among men of African descent compared to Europeans, with Asian men having the lowest risk. Undoubtedly, some of the factors contributing to regional differences in incidence and prevalence of cancers are related to environmental risk

factors and lifestyles. However, this geographic variation in cancer risk could partly be explained by the inherent genetic susceptibility of the defined population to the disease, such as is observed in hereditary cancers. Thus, several founder mutations and nuclear cancer susceptibility genes have been identified to explain some clusters.

In contrast to the nuclear genome, the mitochondrial genome demonstrates considerable geographic and ethnic diversity, and is therefore, an attractive molecule to examine for risk conferring genomic biomarkers of cancer. Several studies have addressed the possible linkage between normal mitochondrial polymorphisms and cancer initiation and progression (see Chaps. 6 and 7). However, some investigators have failed to find any association between mitochondrial polymorphisms and haplotypes with cancer risk or prognosis. Wang et al. (2007) investigated 955 pancreatic cancer patients and 1,102 control individuals for any possible risk-conferring mitochondrial SNPs, by genotyping 24 mitochondrial SNPs, including ten haplotype variants. No SNP was a risk factor for pancreatic cancer. Similarly, Halfdanarson et al. (2008) could not uncover any associations of these mitochondrial SNPs with pancreatic cancer outcomes. In another study, 24 mitochondrial SNPs and 379 tagSNPs on 78 nuclear genes that encode mitochondrial proteins were genotyped in 1,000 prostate cancer patients and 500 controls (Wang et al. 2008). After adjusting for multiple testing, none of the SNPs appeared to be a risk factor for prostate cancer. Analysis of 22 Asian haplogroups of 139 prostate cancer patients and 122 control men without cancer did not reveal any haplogroup-bias for prostate cancer in a Korean study (Kim et al. 2008). Recent examination of middle-aged European men could not identify any haplogroup or control region polymorphisms with prostate cancer (Mueller et al. 2009). Similarly, the studies implicating G10398A polymorphism with breast cancer invasiveness in African-American women could not be reproduced in one independent study (Setiawan et al. 2008).

There are obviously several reasons to account for the incomparable study outcomes or lack of reproducibility of the above studies. Study design, including participant recruitment and sample sources, can bias a study outcome. Regional haplotype variation, especially with regard to age differences in cases and controls, caused by the changing demographics from immigration, was suggested as a possible reason for the disparity between the studies of Booker et al. (2006) and Mueller et al. (2009). Another reason that could have accounted for discrepant results in the two prostate cancer studies is the fact that the age-matched controls used by Mueller et al. (2009) had biopsy proven benign prostate disease. Given that prostate needle biopsy has such a high sampling error, some of the control group subjects could still be patients with prostate cancer. Regardless of the confounding variables, the power to uncover any disease-associated polymorphisms depends considerably on geographic variation in haplotype distribution and study sample size. For example, with complex human diseases, Samuels et al. (2006) used simulation-based models to generate power curves for an analysis of European haplogroups. Their conclusion was that for such studies, very large cohorts are necessary to reveal any possible disease associations.

5.4 Mitochondrial Microsatellites

Mitochondrial instability at D310 and other mononucleotide and dinucleotide repeats in the D-loop and coding genes have been studied in several cancers. D310 is a highly polymorphic region in CSB II that functions to form the RNA–DNA hybrid or R-loop to generate primers for mtDNA replication or formation of the 7S D-loop triplet structure. Thus, this region of mtDNA plays important roles in mitochondrial biogenesis. In many cancers, somatic insertions or deletions of one or two base pairs are observed at this region. Such small nucleotide changes that retain the somatic sequence within the normal polymorphic sequence length ($C_{12–18}$) are thought to have negligible effects on function. However, in some cancers, major deletions or insertions of up to ten bases are observed, and these could alter the D310 structure and therefore, interfere with mtDNA biogenesis.

5.4.1 Discovery of D310 Instability as a Mutational Hot-Spot in Cancer

Sanchez-Cespedes and coworkers were first to identify D310 as a mutational hot spot in cancer (Sanchez-Cespedes et al. 2001). They screened a large panel of tumors, including gastric, head and neck, breast, colorectal, lung, bladder, ovarian, and prostate cancers and their matched lymphocytes for D310 changes. With the exception of prostate and ovarian cancers that had no alterations, somatic D310 variants were detected in 22% of the rest of the tumors, with the head and neck having the most (37%), followed by breast (29%) and colorectal (28%) cancers. To prove that the mutations were tumor-specific, normal adjacent tissues to tumors were also examined and were found to have similar D310 patterns as matched lymphocytes. Since two dysplastic lesions with D310 mutations eventually progressed to cancer, suggested they could be early indicators of tumor progression. Since this initial report, several groups have examined D310 instability in a variety of cancers. For example, analysis of mtMSI at D310 in the colon, stomach, endometrium, breast, lung, and prostate cancers uncovered mutation frequencies of 23, 17, and 11% in colon, gastric, and endometrial cancers, respectively (Schwartz et al. 2006). These frequencies mirror those of tumors with defective mismatch repair. Conceivably, it will be expected that mtMSI will parallel nMSI, a finding that is not consistently reported. Similarly, in the series by Schwartz et al. (2006), more advanced colon cancers were more likely to harbor more than one nucleotide changes, and two tumors with a T > C mutation that restored the homopolymeric repeat, had mutations of up to four and six nucleotides. The findings from this group suggested that the high mutation rate at poly C stretches is dependent on the repetitive structure of the DNA molecule and thus, mutations in the (C)(n) repeat are not a consequence of

selective pressure during tumorigenesis. Mitochondrial MSIs in various cancers are addressed in Chaps. 6 and 7.

5.4.2 Mechanism of mtMSI

The initial works by Sanchez-Cespedes et al. (2001) indicated that the levels of D310 heteroplasmy in lymphocytes correlated with somatic D310 mutations in tumors, leading the authors to suggest that mechanisms responsible for the selection of alleles in oocytes (mitochondrial genetic "bottleneck") similarly operate in cancer, leading to homoplasmic somatic D310 mutations. Earlier, Richard et al. (2000) had provided possible mechanism of mtMSI. Mitochondrial MSIs in their breast cancer series were due to polymerase gamma errors and less so to damage by ROS. Mambo et al. (2003) tested the possible mechanism of D310 instability. They treated cells with DNA-damaging agents and examined the entire mitochondrial genome for DNA mutations. D310 sustained more mutations than the rest of the mitochondrial genome, with the best-protected region being $tRNA^{Gly}$. There was also a delay in D310 repair and homoplasmy was reached only after 7 days of exposure to DNA-damaging agents. These studies suggested that D310 is a fragile site to DNA-damaging agents, including ROS, and that inefficient repair contributes to the accumulated damage.

5.4.3 Clinical Utility of mtMSI

D310 changes can be detected in samples with as few as six cells, implying that it can be a useful marker to assay in clinical samples such as biofluids. To demonstrate the clinical value of D310 measurements for cancer diagnosis, Parrella et al. (2003) first analyzed 56 cancers composed of breast, bladder, cervical, and endometrial cancers. A total of 13 D310 alterations were detected. Identical D310 changes were also demonstrable in all matched urine and fine needle aspirate (FNA) from four bladder and three breast cancer patients, respectively. In another study, free DNA isolated from ductal lavage and NAF from noncancer individuals with known BRCA1 status was analyzed for D310 changes (Isaacs et al. 2004). Three of the nine ductal lavage samples and one of four NAF from BRCA1 carriers had D310 mutations, while none of the noncarriers had D310 changes. In a larger breast cancer cohort (64 samples), D310 was detected at a frequency of 19% (Parrella et al. 2001). Importantly, identical D310 changes in cancer were demonstrable in all five FNA, and the four metastatic tissues also demonstrated identical D310 changes. Ha et al. (2002) demonstrated D310 changes in early stage head and neck cancers, and the incidence mirrored tumor progression. These and many other studies of mtMSIs discussed in Chaps. 6 and 7 strongly suggest that mtMSI analysis has clinical value and should be implemented for patient care.

5.5 Mitochondrial Genome Rearrangements

The mitochondrial genome is characterized by several repeats, and these serve as break-points that characterize large-scale deletions. A majority (∼ 90%) of the deletions are flanked by the repeat sequences and these repeats are unequally distributed in the genome. The region of the mtDNA molecule spanning clockwise from *CYTB* to *COI*, and flanked by O_L and O_H, contains disproportionately several repeats, and therefore, is most frequently deleted. For that reason, this region is referred to as the *major deletion arc*. On the contrary, extending counterclockwise from np 547 in the D-loop to about np 5,443 in the *ND2* harbors less repeats and hence, deletions, and this region is appropriately designated as the *minor deletion arc* (Fig. 5.1).

Mitochondrial DNA deletions are either flanked by repeats or lack repeats, and this forms the basis of deletion classification. About 60% of all deletions fall into the class I category whereby deletions are flanked by two short homologous direct repeats. One of the repeats is removed by the deletion. About a third of all deletions

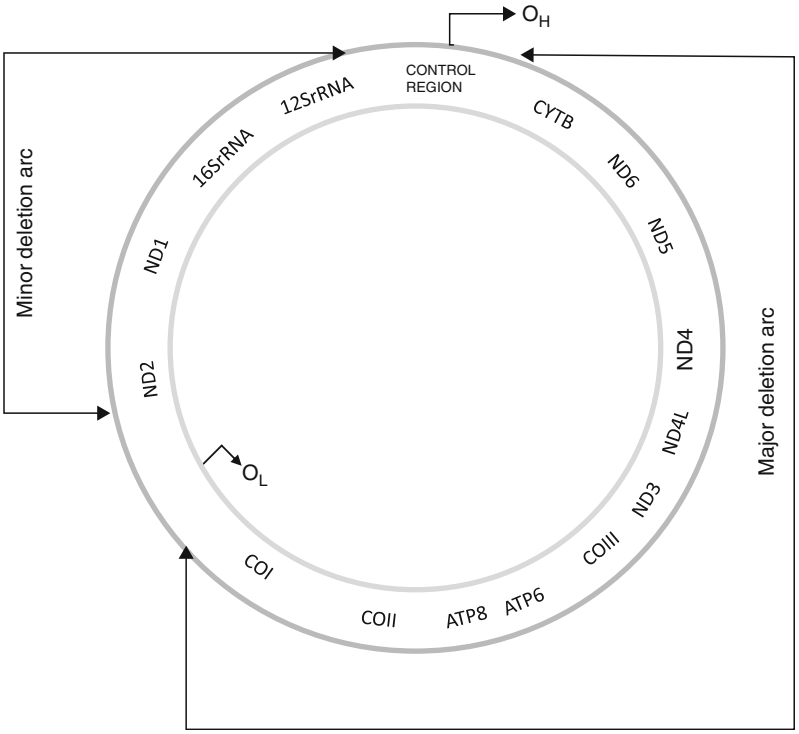

Fig. 5.1 *The major and minor mitochondrial deletion arcs*. These two regions of the mitochondrial genome sustain most deletions. Though not immune to deletions, the regions around O_H and O_L are less affected by deletions

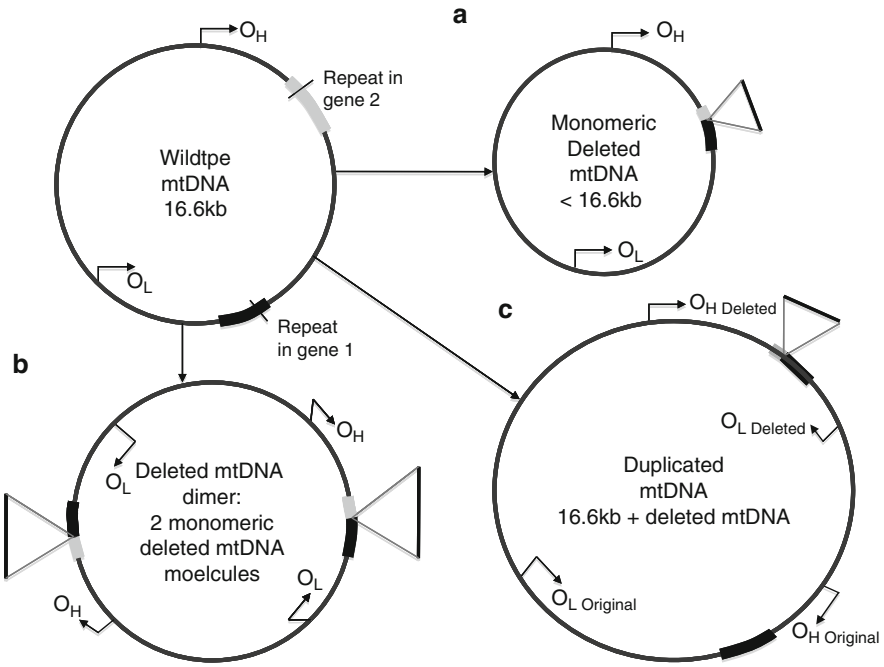

Fig. 5.2 *Types of mtDNA rearrangements*. Shown here are the wildtype mtDNA and three rearranged molecules. (**a**) This is a simple monomeric deleted mtDNA whereby a portion of the genome is lost and the remaining recircularized. (**b**) This is a deleted mtDNA dimer where two deleted mtDNA molecules recombine. (**c**) A duplicated mtDNA molecule is formed when deleted mtDNA molecule integrates into a wildtype genome

have imperfect repeats and these are called class II deletions. Class III deletions, which are in the minority, are without repeats.

The part of the mitochondrial genome removed from the wildtype mtDNA molecule (usually without replication origins) is referred to as the *deleted region*, and the remaining mtDNA molecule that is capable of replication, is called *deleted mtDNA*. The deleted molecule can undergo a number of rearrangements (Fig. 5.2).

- It can simply join at its break points to form a *deleted mtDNA monomer*.
- Two or more such deleted mtDNA molecules could recombine to form *deletion dimmers or multimers*.
- One or more deleted mtDNA may recombine with a wildtype mtDNA molecule to form *partial duplication, triplication*, and possible higher orders of multiple repeat regions.

Other rare rearrangements are reported, including inversions, simple insertions of small bases of up to 300, and complex rearrangements with kb inserts generating multiple molecules of different sizes, some up to 20 kb. However, the most frequent deletion encountered is the simple monomeric deleted mtDNA.

5.6 Mitochondrial DNA Copy Number (Content) Changes

Mitochondrial genome is polyploid, with hundreds of copies per cell. This contrasts with the diploid nuclear genome. While this copy number enables ease of assaying mitochondrial genome for diagnostic purposes, it is also established that changes in mtDNA copy number underlie certain pathologic conditions, and are demonstrated in several cancers as well (see Chap. 8).

Mitochondrial DNA copy number simply refers to the number of functional genomes per cell or tissue. Empirically, mtDNA copy number or content is measured by quantitative PCR assay targeting total mtDNA and normalized to input template determined by amplifying a nuclear target, though this approach has recently been found to be inaccurate in copy number determination (see Chap. 13, Sect. 13.4.2).

Any factors or agents that cause increased mtDNA destruction, and/or that adversely affect mtDNA propagation, may lead to mtDNA depletion. Mitochondrial DNA deletions, for example, can ultimately lead to mtDNA depletion. Similarly, mutations in nuclear genes involved in mtDNA maintenance can cause mtDNA depletion. Thus, mutations in *DGUOK, C10ORF2, POLG, TFAM*, and *MPV17*, are associated with mtDNA depletion syndrome (MDDS), which is a condition that usually presents in infancy and can be lethal. The most severe form of MDDS is the hepatocerebral form characterized by severe hepatic failure, neurologic abnormalities, hypoglycemia, and elevated bodily fluid lactate levels. This condition, which is incompatible with life, is caused by the depletion of over 88% mtDNA molecules. Apart from mutations, there are other inherited genetic factors that control mtDNA copy number in an individual.

Copy number changes are important in cancer because they are demonstrated to modulate cancer initiation, cause nuclear genome instability, and therapy response. Apart from these biologic effects, assaying copy number changes can have diagnostic utility. For example, mtDNA copy number changes are easily measured in saliva rinses from patients with head and neck cancer, and these changes mirror disease progression and therapy response (Kim et al. 2004; Jiang et al. 2006).

5.7 Novel Mitochondrial Transcripts in Cancer

The complexity of mitochondrial genome structure and changes in cancer is further illustrated by the differential expression of novel mitochondrial transcripts in cancer cells. Specifically, two types of novel mitochondrial transcripts are demonstrated in cancer. The first is a group of non-coding mitochondrial RNAs (identical to microRNAs), and the second is a large group of mitochondrial fusion transcripts (Villegas et al. 2000, 2002a, 2002b, 2007; Burzio et al. 2009; Parr et al. 2009).

Non-Coding Mitochondrial RNA: Elaborate experiments by Luis Burzio's group have identified a novel non-coding mitochondrial RNA (ncmtRNA) in the

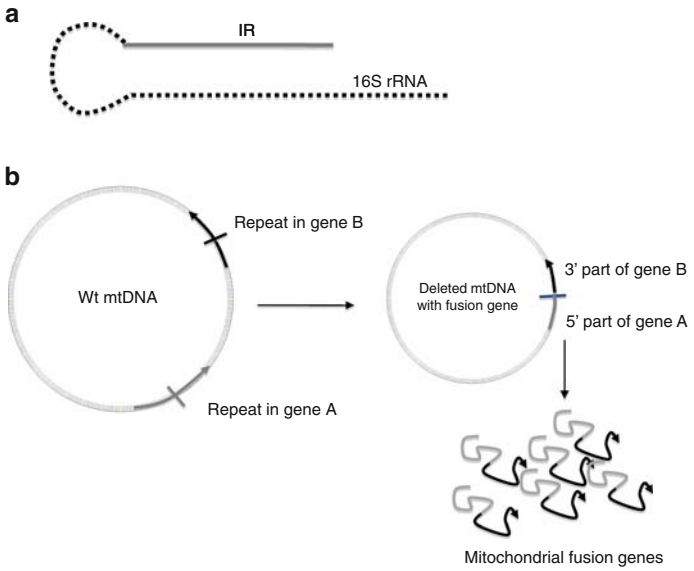

Fig. 5.3 *Aberrant novel mitochondrial transcripts in cancer*. (**a**). Construct of the human ncmtRNA of 2,374 nt long. The inverted repeat (IR) is linked to the 5' end of 16SrRNA gene. (**b**). Formation and expression of mitochondrial fusion genes. In this illustration, repeats in gene A and gene B recombine to form a deleted mtDNA molecule that contains a new gene comprised of 5' end of gene A and 3' end of gene B. The novel gene is expressed and the transcripts can be detected using gene-specific probes

mammalian genome. The ncmtRNA is comprised of an inverted repeat sequence that is covalently linked to the 5′ end of 16SrRNA. The human ncmtRNA is 2374 nt long that is organized into a stem-loop structure composed of ~ 820 bp double stranded stem, which is resistant to RNase A digestion, and a 40 nt loop (Fig. 5.3a). Confocal microscopy analysis found the co-localization of this transcript with cytochrome c and endonucleases G in the mitochondria. Initial molecular analysis by these investigators revealed the almost exclusive association of this transcript with proliferating cells. In a follow-up study, Burzio et al. (2009) uncovered the presence of both sense and antisense variants of this ncmtRNA. The antisense transcripts are two different inverted repeats that are linked to the 5′ ends of the antisense 16SrRNA transcribed from the L-strand.

Expression analysis of the sense and antisense transcripts in normal and cancerous tissues revealed an interest-ing profile (Burzio et al. 2009). As expected form previous experiments, non-dividing cells did not express any of the transcripts, however, normal proliferating cells expressed both transcripts. Intriguingly, analysis of 15 different cancer cell lines, and 273 cancer biopsies comprised of 17 different cancers revealed the complete absence of the antisense tran-script, but as in previous experiments, the sense transcript was expressed. Thus, the absence of the antisense transcript appears to be a tumor-specific marker.

Mitochondrial Fusion Transcripts: Following large-scale deletions in the mitochondrial genome, the deleted mtDNA recircularizes, such that at the break point, parts of two genes become continuous (Fig. 5.3b). The upstream 5′ and downstream 3′ genes retain their initiating and termination codons respectively. Many of these genes connect in frame giving rise to novel mitochondrial fusion genes. We and other workers have demonstrated that these fusion genes are transcribed (Nakase et al. 1990; Parr et al. 2009). In addition to being transcribed, they are also post-transcriptionally polyadenylated. Analysis of several primary tumors and cancer cell lines reveal the differential expression of the fusion genes between cancer and normal cells (Parr et al. 2009). While the functional importance of these genes is unknown at the moment, the differential fold changes in the amounts between cancer and normal cells could have diagnostic value.

5.8 Pitfalls Associated with Scoring Mitochondrial DNA Mutations

The scoring of disease-specific mutations for clinical uses must be accurate. Apart from the established requirements of good laboratory practices to prevent sample contamination or mix-up in mitochondrial genetic studies, there are other major issues concerning how somatic mtDNA mutations are scored. Two major types of mtDNA point mutation variants are associated with cancer. Germline polymorphisms are observed to confer various risks in a number of cancers, including prostate, breast, endometrial, and renal cancers, while somatic mutations are demonstrated in almost all cancers. It is very important that the correct sequence comparisons are made to enable accurate delineation of mitochondrial genome changes. Germline mtDNA population polymorphisms and haplotypes are uncovered by comparing the normal inherited maternal sequence to the reference sequence (rCRS). In many instances, sequences from circulating lymphocytes suffice as accurate surrogates for this comparison. On the other hand, to uncover cancer-specific or disease-specific somatic mutations, intrapersonal pairwise comparison of tumor/disease and germline/wildtype sequences is required. Given that different mtDNA haplotypes exist in different people, there is no universal wildtype sequence, hence, the need to make an intraindividual pairwise sequence comparison. It is unfortunate to note that in some studies, tumor sequences have been compared to the rCRS for scoring somatic mutations. Such comparisons will introduce, albeit probably minor, some erroneous mutation data into the literature.

Another important concern that may adversely influence the scoring of authentic somatic mutations is the choice of control tissue to represent the wildtype sequence. It is evident in the literature that some studies have used the normal adjacent tissue to the tumor as the control sequence, while others have used a different postmitotic tissue

altogether. Understandably, it is difficult, and even impossible in some cases to obtain normal tissues for comparison. However, molecular field cancerization is demonstrated in almost all cancers (Dakubo et al. 2007). Thus, the adjacent histologically benign sample could possess somatically acquired mutations identical to or different from the tumor, making sequences from such tissues unsuitable for comparison. Ideally, it is recommended that lymphocyte sequences from the same individual be obtained, as they have served as an excellent representation of maternal sequence. Finally, because of nuclear integration of mitochondrial sequences with various degrees of homology (up to 100%) to the mitochondrial genome, a BLAST check must be conducted on all primers, and they must also be tested on DNA from cells devoid of mtDNA (rho-zero cells) to preclude co-amplification of nuclear loci.

5.9 Criticisms of Somatic Mitochondrial DNA Mutations in Cancer

There are issues with the literature on mtDNA mutations in cancer, as discussed in Sect. 5.8. Beyond what is indicated in the above session is the outright criticisms for the authenticity of a number of mtDNA mutations reported in various cancers (Salas et al. 2005; Bandelt and Salas 2009). Therefore, the obvious question is, are the numerous mtDNA mutations demonstrated in cancer authentic or they are just a catalog of inaccurate mutations? While the concerns of Salas and Bandelt are somehow legitimate and have introduced some level of vigilance in data acquisition, it cannot be denied that mtDNA mutations are authentic somatic changes in cancer.

The criticism of Salas and Bandelt is based on the phylogenetic approach whereby the presence of cancer somatic mutations at polymorphic loci of known haplotypes are putatively considered an error from sample mix-up or contamination. Using this approach, over ten published mtDNA somatic mutation data are considered erroneous. Indeed, Bandelt and Salas (2009) criticized, probably unfairly, the work of Prior et al. (2006) on oral squamous cell carcinoma samples. As replied by Lewis et al. (in Bandelt and Salas 2009), they exercised extreme caution in the analysis of their oral squamous cell samples. These investigators apparently sequenced each sample multiple times in both directions, and upon the request of reviewers, further confirmed these mutations by performing more independent sequencing reactions. Yet, this data was criticized as being a consequence of sample mix-up. It is possible that the constraints that generated polymorphic site haplotype variants with human evolution and migration are similarly operational in the carcinogenesis process, and hence, somatic mutations at polymorphic sites cannot be discarded as unimportant to cancer. The analyzes by Brandon et al. (2006) and Zhidkov et al. (2009) (see Sect. 5.10) provide an explanation for this conclusion.

5.10 Natural Selection Explains Mitochondrial DNA Mutations in Cancer

An extensive comparative analysis of mtDNA mutations in various cancers to known population mtDNA variants led Brandon et al. (2006) to propose two distinct types of mtDNA mutations in cancer; pathogenic (possibly carcinogenic) and adaptive mutations. Mutations that are more likely to severely compromise mitochondrial bioenergetics and therefore, lead to increased ROS production, are considered pathogenic. These mutations include missense mutations, chain termination mutations, and insertions and deletions of extremely conserved amino acids. Adaptive mutations are less deleterious mutations that are also observed in the population, the aging process, and some diseases. These adaptive mutations are not neutral mutations, but they serve the important function of enabling cancer cells to adapt to new microenvironments, especially when they metastasize to different locations. This is analogous to the adaptive mutations that permitted human survival in colder climates that are different from the hot Africa climate.

Zhidkov et al. (2009) demonstrated that tumor mtDNA mutations were under similar selective constraints as those fixed in ancient human evolution, and therefore, concluded that these mutations potentially have functional importance. They arrived at this conclusion by comparing the patterns of mutations in mtDNA of cancer and natural populations. Cancer mutations clustered with ancient human population variants. Contrary to general belief, the combination of a number of de novo cancer mtDNA mutations were synonymous or involved D-loop region, which could be interpreted as passenger mutations. These combinations of apparently innocuous mutations are selected for "fitness" of the cancer cell without any effect on the protein structure. Intriguingly, one specific group of combined de novo cancer mtDNA nucleotide changes involved seven changes that were a mirror image of the macrohaplogroup R. Several mutation combinations from cancer defined other haplogroups as well. These findings suggest that the criticisms of Salas and Bandelt cannot be accepted in all cases.

5.11 Conclusion

The unique genetics of the mitochondria are reflected in how mutations occur in this genome, and importantly, what these mutations mean with regard to disease phenotype. The simple phenotypic features associated with nuclear genome mutations are not demonstrated by mitochondrial mutations. For example, large-scale deletions in mtDNA result in conditions such as progressive external ophthalmoplegia (PEO), Pearson syndrome (PS), and Kearns-Sayre syndrome (KSS), while point mutations in some genes cause mitochondrial encephalomyopathy, lactic

acidosis, and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF) Leber's hereditary optic neuropathy (LHON) and neuropathy, ataxia and retinitis pigmentosa (NARP) syndromes. Noteworthy are the considerable overlaps between these conditions with regard to clinical presentations, simply because of the concept of heteroplasmy and possibly, nuclear genetic modifiers.

While the nuclear genome is diploid and thus, amenable to simple mutational analysis, the polyploid mtDNA is not so easily comprehended. Single nucleotide mutations and large-scale deletions, though pathogenic, will only manifest disease at certain mutational loads (levels of mutant genome heteroplasmy). Also important in mitochondrial genetics and disease is the uniquely packed intron-less genes, and the semiautonomous maintenance of the molecule (i.e., its dependence on self and nuclear contributions). Thus, mutations in tRNA and rRNA genes tend to affect the global functions of mitochondria, while structural gene mutations are mostly pathogenic with regard to the respiratory chain activity.

Two unique features of mtDNA nucleotide variations and disease are the varying geographic polymorphisms of this genome, and the differing copy numbers or content levels in various individuals, dictated possibly by personal genetic composition and somatic mutations as well as nuclear genomic regulation. While individuals with varying levels and types of mitochondrial genomes are normal, they appear to have different disease predispositions, and life spans.

The illustrated complexity of mtDNA genome changes in human disease calls for an in-depth understanding of mitochondrial genetics and human health, including cancer.

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Chapter 6
Mitochondrial Genetic Alterations in Cancer I

Skin, Head and Neck, Salivary Glands, Thyroid, Breast, Lung, Esophageal, Gastric, Colorectal, Pancreatic, Liver and Gallbladder Cancers

Abstract Mitochondrial genome changes are pervasive in all cancers examined so far. Mutation frequencies and heteroplasmic levels differ considerably because of numerous factors not the least being the proportion and part of the genome examined, methodological issues including sensitivity of sequencing techniques used, and the type of sequence used as control or germline for scoring somatic mutations (normal adjacent to tumor or rCRS). While being critical of the individual studies, this chapter describes mitochondrial risk haplotypes and polymorphisms, mitochondrial microsatellite instability, somatic point mutations, and expressional mitochondrial genome changes in skin, head and neck, salivary glands, thyroid, breast, lung, esophageal, gastric, colorectal, pancreatic, liver and gallbladder cancers.

6.1 Introduction

Mitochondrial mutations are frequent in cancer. Several somatic point mutations, small insertions and deletions, as well as large-scale rearrangements have been reported in all cancers examined. In addition, germline single nucleotide polymorphisms (SNPs) and population polymorphic variants are postulated to modulate the risks of developing certain cancers and may offer prognostic predictions of some malignancies. While several current efforts are directed at unraveling novel mtDNA mutations and the mechanisms responsible for their causation, the functional importance of these mutations to tumor development are becoming apparent as well, and are discussed in Chap. 9. Due to the rich population diversity of mtDNA, the search for somatic mutations associated with cancer requires careful sequence comparison between patient's blood and matched tumor tissue or noninvasively collected body fluids.

Mitochondrial DNA mutation frequencies vary considerably for any given type of cancer and this can be accounted for by a number of reasons. In general, the smaller the percentage of the mitochondrial genome examined, the lower the frequencies of

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Table 6.1 Somatic mtDNA mutations in cancer

Cancer	Somatic mutation frequency (%)
Skin	16–75
Head and neck	49–78
Thyroid gland	23–100
Breast	30–93
Lung	43–79
Esophageal	5–55
Gastric	18–81
Colorectal	16–70
Pancreatic	16–92
Liver	40–68

mutations. Similarly, interrogation of only the hyper-mutable D-loop region will potentially yield more mutations than similar base-sized sequence of the coding region. Moreover, different sequencing methods have differing sensitivities at heteroplasmy detection. Also pervasive in the literature is the use of mtDNA sequences from matched normal tissues in the proximity to tumors as germline for pairwise comparison, and some studies even compare tumor sequences to the rCRS (these issues are examined in Chap. 5). In spite of all these known limitations, the sheer volume of reported evidence on mitochondrial genome changes in cancer cannot be ignored. The outcry by several groups for the use of mitochondrial genome changes, as cancer biomarkers require careful reexamination, validation, and clinical translation. This and the next chapter summarize mitochondrial genome changes in various cancers. For each tumor type, evidence for mtDNA polymorphisms as risk factors for cancer, microsatellite instabilities, somatic point mutations, as well as mitochondrial gene expression changes are addressed. Table 6.1 summarizes mtDNA somatic mutation frequencies in the various cancers covered here. Large-scale deletions and copy number changes are examined separately in Chap. 8.

6.2 Skin Cancer

The global burden of both nonmelanoma and melanoma skin cancers has consistently been on the rise over the past decades. Each year, between two and three million nonmelanoma skin cancers and 132,000 melanoma skin cancers are diagnosed globally. Skin cancer is so frequent that one in every three cancers diagnosed is cancer of the skin. This explains the fact that most cancers are a result of preventable external carcinogenic insults, in this case the over-exposure to ultraviolet radiation (UVR).

The skin is the largest organ of the body that is often exposed to a potent mutagen in the form of UVR in sunlight. Such mutagen is postulated to be responsible for cancers of the skin. As the ozone layer is being depleted, more solar radiation is emitted onto the earth, indicating that the incidence of skin cancer

is likely to continue to increase. Mitochondrial DNA mutations accumulate in the skin with photo-mediated aging and cancer. Mitochondrial genome damage is potentially a sensitive biomarker of UV exposure and a potential biosensor of cutaneous aging and tumors. Ultraviolet radiation is known to cause ROS production, which can lead to various types of cellular damage known to contribute to malignant transformation and aging phenotypes. Cutaneous cancers fall into two main categories; melanoma and nonmelanoma skin cancer.

6.2.1 Melanoma

In the US alone, 62,000 people are diagnosed with melanoma each year, with about 8,000 deaths. While melanoma accounts for the minority of all skin cancers, it is responsible for the majority ($\sim$ 80%) of all deaths from cutaneous cancers. Early detection of melanoma that is confined to the skin can be totally cured, although some can still progress and be fatal. Therefore, molecular markers for early detection and prediction of melanoma behavior are needed.

Melanomas arise from neural crest-derived melanocytes in the epidermis or dermis. The major risk factors for melanoma are fair complexion, red or blond hair, blue eyes, freckles, and inability to tan properly as well as ease of sunburn. Hence, it is prudent clinical practice to have people at high risk such as those with the typical high-risk phenotype, and those with other genetic markers of increased risk such as melanocotin 1 receptor gene variants (Arg151Cys, Arg160Trp, and Asp294His) to undergo regular check- ups and possible molecular tests for evidence of early progressive pigmented lesions.

Somatic mitochondrial microsatellite instability in melanoma: Somatic mtMSI are addressed in just a single study of melanoma. Deichmann et al. (2004) examined microsatellite changes in cutaneous cancers. In this series, 69 melanoma resection specimens and corresponding normal tissues were analyzed for somatic mtMSI at two microsatellite loci in a 157 bp segment of the D-loop (np 270–425). Overall, sequence alterations were present in 16% of the cancers, with the majority found at two microsatellite loci.

Somatic mitochondrial DNA point mutations in melanoma: A number of investigators have examined melanoma specimens for mtDNA somatic mutations. The work by Deichmann et al. (2004) was probably the first to address mtDNA mutations in melanoma samples. Of the 16% mutation rate uncovered by this study, only four samples demonstrated a UV-specific alteration outside the mtMSI loci indicated above. Poetsch et al. (2004a) addressed a similar question of whether UV-radiation caused mtDNA mutations in melanoma. Their limited examination of the mitochondrial genome did not reveal frequent mtDNA mutations in melanoma specimens, and there were no UV-specific DNA changes as well. These observations led the authors to conclude that mtDNA mutations in melanoma were UV-radiation independent events. Takeuchi et al. (2004) studied

the D-loop region in a panel of melanoma samples and surprisingly uncovered more mutations in melanoma samples (41%) than those of Deichmann et al. (2004) (16%) and Poetsch et al. (2004a) (12%). These mutations were detectable in paired plasma samples, especially those from patients with advanced stage disease, but the mutations did not correlate with clinicopathologic parameters (Takeuchi et al. 2004). A much more comprehensive analysis of mtDNA mutations in melanoma was undertaken by the Califano's group at Johns Hopkins University. They used the more sensitive MitoChip v2.0 to sequence the entire mtDNA in melanoma specimens. In comparison to germline mtDNA sequences, they demonstrated a relatively high frequency (75%) of mtDNA mutations in melanoma specimens (Mithani et al. 2008). This finding contrasts with previous limited studies and emphasizes the need to sequence the entire mitochondrial genome in cancer studies. Whereas most mutations occurred in the D-loop region, 56% of melanomas harbored nonsynonymous mutations in coding genes. Of interest was the disproportionately high clustering of mutations in complex I genes that implicates complex I dysfunction and oxidative stress in melanoma carcinogenesis (Mithani et al. 2008).

The work by Govindarajan et al. (2007) on mtDNA mutations and melanoma is quite enlightening in regards to the role of ROS in melanoma progression. This group transfected a noninvasive melanoma cell line, with a plasmid that overexpressed PKB/AKT and therefore invoked AKT signaling pathway in this cell line. Compared to parental cells, the AKT-overexpressing melanoma cells acquired invasive phenotypes in association with increased oxidative stress, angiogenesis, and glycolytic metabolism consistent with oncogenic AKT signaling. Mitochondrial complex I functions were markedly reduced in AKT-overexpressing cells. Mitochondrial DNA sequence analysis of the cell lines revealed 85 mutations, 50 of which were nonsynonymous changes with disproportionately high clustering in complex I genes, which is consistent with the work of Mithani et al. (2008) on primary melanoma samples. Analysis of the possible effects of the nonsynonymous mutations on protein structure and function indicated that 11 of the complex I amino acid changes were conserved among all species, indicating the functional importance of these mutations, which could account for the defective complex I activity and oxidative stress in these cells. The cells with extensive mtDNA mutations were cultured for a long period of time, and did not overexpress extra-mitochondrial ROS-generating enzymes, the NOX family of enzymes. On the contrary, when cultured for a short time (1–2 months), the AKT-transformed cells increased expression of *NOX4*. Subsequent analysis of primary melanoma samples using temperature gradient capillary electrophoresis did not reveal significant levels of mtDNA mutations. These findings led the authors to propose two possible models for AKT-mediated conversion of radial growth melanoma cells into vertical or invasive melanoma cells. First, AKT signaling is suggested to stabilize cells with extensive mtDNA mutations (increased ROS production) and thus prevent them from apoptotic cell death; second, AKT signaling could induce *NOX4* expression and therefore ROS production. Irrespective of the mechanism, in both cases, ROS signaling is the common denominator mediating the acquisition of invasive phenotype.

6.2.2 Nonmelanoma Skin Cancer

Nonmelanoma skin cancer (NMSC) comprises of basal cell carcinomas (BCC) and squamous cell carcinomas (SCC). They are the most common skin cancers diagnosed worldwide. Basal cell carcinoma accounts for the majority (70–90%) of NMSC, and they are less invasive compared to SCC. Squamous cell carcinoma accounts for only ~ 20% of all NMSC but have the potential to metastasize, and hence are responsible for the majority of mortalities associated with NMSC. The incidence of NMSC continues to rise. The most significant risk factors for the development of NMSC are UVB exposure from sunlight. Other important risk factors for NMSC are fair completion, red or blond hair, blue eyes and inability to tan properly.

Basal cell carcinoma originates from epidermal basal cells, while SCC develops from epidermal keratinocytes. Actinic keratosis and cheilitis are hyperkeratotic papules on sun-exposed skin, and both are premalignant lesions of SCC. Similar to melanoma, genetic skin markers of UV damage will help people adopt safer sun lifestyles to minimize the incidence of skin cancers. There is a need for such UV sensitive biomarkers, and that developed by Genesis Genomics Inc. (skinphysical™) is an initial step towards achieving such goals.

Somatic mitochondrial microsatellite instability in nonmelanoma skin cancer: Durham et al. (2003) first investigated both mitochondrial genome mutations and deletions in NMSC. Mutation analysis indicated no differences in the sequence profiles between tumor and perilesional skin in the D-loop, *ND1*, *ND5* and *16S rRNA*, with the exception of a heteroplasmic mutation in one SCC. A study by this same group discovered frequent genetic linkage between a specific mtDNA deletion (a 3895 bp deletion, see Chap. 8, Sect. 8.3.14) and the age-associated T414G mutation (Birket and Birch-Machin 2007). Prior and coworkers examined the mtDNA mutational status in the D-loop of a series of 36 NMSC (Prior et al. 2009). Overall, 13 nucleotide substitutions were uncovered in 25% of the samples, and these mutations had no correlation with positive p53 staining.

6.3 Head and Neck Cancer

Head and neck squamous cell carcinoma (HNSCC) encompasses cancers that originate in the mucosal epithelial lining of the head and neck region. These include a vast number of cancers such as those of the nasal cavities, sinuses, lips, oral cavity, salivary glands, pharynx, and larynx. Clinically and possibly molecularly, salivary gland tumors are excluded from these cancers because of their different behaviors.

The global burden of HNSCC remains high being the fifth most common tumor worldwide. Of all cancers diagnosed in Western Europe and the United States, up to 10% are HNSCC. Over half a million new cases are diagnosed globally, with oral and laryngeal cancers being the common ones in Europe and North America while

nasopharyngeal cancers are the common types in the Mediterranean. The etiological risk factors for developing HNSCC clearly are tobacco and alcohol use. These etiologic agents can set up a precancerous field in the upper aerodigestive tract in the form of erythroplakia and leukoplakia that can progress to invasive cancers. Well-known in head and neck cancers is the occurrence of synchronous and metachronous tumors. Conceivably, alcohol and/or tobacco carcinogens will exert oxidative DNA damage to cells, and hence the mitochondrial genome is an attractive biosensor of HNSCC. The ability to detect and track early mtDNA lesions indicative of disease progression in head and neck mucosa should enable closer surveillance for early cancer detection.

Mitochondrial genome polymorphisms and risk of head and neck cancer: Polymorphisms at np 12308, 11467, 10400, and 10398 were examined as possible risk factors for oral cancers and leukoplakia (Datta et al. 2007). These sites were analyzed using PCR and RFLP methods in 310 cancer, 224 leukoplakia, and 389 control samples. Allele A at np 12308 in $tRNA^{Leu}$ and allele A at np 11467 in *ND4* appeared to increase the risk for oral cancer in male smokers. As well, alleles G at np 10398 and T at np 10400 (haplogroup M allele) in *ND3* significantly increased the risk for oral cancer in all smokers. The effect of the three alleles in smoking-related oral cancer deserves more studies.

Somatic mitochondrial microsatellite instability in head and neck cancer: A number of investigators have demonstrated D310 sequence abnormalities in HNSCC. Ha et al. (2002) studied D310 alterations in premalignant head and neck lesions. Overall, 37% of the patients demonstrated somatic D310 mutations. Interestingly, there was a trend towards increased frequency of D310 changes with disease progression. Whereas only 22% of normal hyperplastic lesions harbored these changes, as many as 62% of in situ squamous cell carcinomas were unstable at D310. Thus, as tumors progress, they appear to acquire D310 changes and this finding could have utility in early detection of malignant progression. Mitochondrial MSI at six loci were also tested in this study for patients with synchronous and metachronous tumors. Most synchronous and metachronous cancers showed clonal relationships with the primary tumor, indicative of field cancerization.

Using the sensitive temporal temperature gradient electrophoresis (TTGE) and direct sequencing methods, D310 alterations were demonstrated in 44% of oral cancers (Tan et al. 2003). Poetsch et al. (2004b) investigated the frequency of mutations in *ND1, ND5* and a portion of the D-loop. Sixty-seven, primary HNSCC from 56 patients and 40 lymph node metastatic tissues from 23 of these 56 patients, as well as matched normal tissues were investigated. The sequences of ten metastatic tumors were identical in both the primary and the metastatic tumors. A total of 12 primary tumors had somatic mutations that were absent in paired normal tissues. The mtMSI were compared to nMSI in *IGFIIR, hMSH3, hMSH6* and five other dinucleotide repeats in the same tissues. The frequency of mtMSI observed in this series (42%) was identical to those demonstrated by Ha et al. (2002) and Tan et al. (2003) and this frequency was much higher than nMSI (36% low nMSI, and 13% high nMSI). There was no correlation between nuclear and mitochondrial MSI, suggesting that independent mechanisms might underlie their formation (Poetsch et al. 2004b).

Mutations in the D-loop, mostly at D310, were found in 21% of HNSCC patients (Lievre et al. 2006). In this series, the frequency of D310 changes positively correlated with the number of D310 cytosines in normal tissues. Prior et al. (2006) characterized somatic mtDNA changes in *ND2* and the D-loop in smoking related oral squamous cell carcinomas. D310 changes were present in 37% of the patients, further confirming the high frequency of D310 changes in head and neck cancers (Prior et al. 2006). In support of smoking-mediated D310 instability, Tan et al. (2008) observed mtDNA length variations in normal bucal cells from 17% of smokers, with 9% being at D310. These limited but consistent findings suggest D310 profiling has potential for identification of people at high risk for developing HNSCC.

Somatic mitochondrial dna point mutations in head and neck cancer: A series of pioneering works from Johns Hopkins University sparked interest in several groups to question the clinical importance of mitochondria genome alterations in cancer. The work by Fliss et al. (2000) included 13 head and neck cancer samples, and mutations were present in six tumors. These mutations were detected in nine paired saliva samples. Using TTGE and sequencing of heteroduplex sites, Tan et al. (2003) uncovered somatic mutations in 78% of oral cancers. Of these mutations, six were in coding genes, including three missense mutations in *ND2*, one frameshift mutation and a deletion in *COIII*. In the study by Prior et al. (2006) of *ND2* and the D-loop in smoking-related oral squamous cell carcinomas and paired normal tissues, 67% of tumors had mutations of which six point mutations were in *ND2*. The majority of changes were at nps 146, 152, 186, and D310. The D-loop mutations demonstrated some gender-associations, being predominantly high in male smokers, but rare in female nonsmokers (Prior et al. 2006).

The Califano's group has reported on mitochondrial genome changes in a number of cancers including HNSCC. A microarray-based re-sequencing of the entire mitochondrial genome was conducted on a panel of 83 HNSCC and mutations were present in 49% of the tumors (Zhou et al. 2007). The mutations were distributed in a nonrandom fashion, because they predominantly clustered in complex I genes. There was a significant positive correlation of the mutations with *p53* mutation status. Sequencing of dysplastic adjacent tissues of one tumor revealed an identical mutation in both tumor and margin samples, suggesting mtDNA mutations occurred early in this tumor, and could be involved in disease progression. The study by Mithani et al. (2007) focused on the timing of mtDNA mutations in HNSCC using array-based re-sequencing of mitochondrial genome. Overall, 23 mutations in 12 tumors were uncovered. Dysplastic lesions and normal tissues adjacent to the tumors were also examined. In two tumors, the same mutations were found in tumors and the adjacent normal and dysplastic lesions, consistent with the field theory of carcinogenesis widely demonstrated in tumors of the aerodigestive tract. Importantly, eleven of the 12 tumors had nonsynonymous mutations. It was noted that tumor mutations significantly differed from those of the adjacent dysplastic and normal tissues. Allegra et al. (2006) reported on the presence of five silent polymorphisms and 8 somatic mutations in ten head and neck cancer cell lines derived from nine patients. Of the eight somatic mutations, three were nonsynonymous changes (Allegra et al. 2006).

A limited but interesting study suggests mutations in HNSCC appear to originate from clonal as well as random expansion of preexisting mtDNA alleles (Gekeler et al. 2009). Analysis of mtDNA changes in HNSCC revealed not only heteroplasmy in the patients' blood, but also homoplasmic changes in the tumors of two patients. Intriguingly, the homoplasmic mutation of one of the tumors was demonstrated to be a reversal to the wild type allele of this individual. Whole genome sequencing indicated the changes observed were at np 152 and 16187.

Analysis of 23 nasopharyngeal cancers uncovered polymorphisms at 72 nps in the D-loop and six in parts of the coding region (Pang et al. 2008). A substitution at np 15970 in $tRNA^{Pro}$ could be functionally important because it changes the position of the proline residue during translation.

In summary, somatic mtDNA mutations are obviously prevalent in HNSCC, and these mutations are demonstrable in precancerous fields, suggestive of their use in early cancer detection studies. The mtDNA alterations in HNSCC are worthy of validation and establishment of clinical utility for translation.

6.4 Salivary Gland Cancer

Salivary gland tumors can originate from the major salivary glands (parotid, submandibular and sublingual) or the minor glands scattered in the submucosa of the upper aerodigestive tract. Malignant transformation is common in submandibular, sublingual, and minor salivary glands, with most parotid tumors being benign in nature. There is no specific risk-associated agent established for salivary gland tumors.

Somatic microsatellite instability in salivary gland cancer: Very few studies have addressed mtDNA somatic alterations in salivary gland tumors. Many studies have focused on salivary gland oncocytomas. D310 was studied in oncocytic neoplasm of the parotid gland (Capone et al. 2002). Of 21 tumors composed of oncocytomas, oncocytosis, and oncocytic carcinomas, only a single oncocytoma showed D310 alterations. It appears D310 changes are very rare in parotid oncocytomas, but more studies are required.

6.5 Thyroid Cancer and Parathyroid Adenoma

Thyroid cancers are the most common endocrine cancers, with an annual incidence of $\sim$ 1 per 10,000. The incidence increases with age, and they are more common in women than men. The well-differentiated thyroid cancers (papillary and follicular thyroid cancers) have a more favorable outcome than the anaplastic cancers. Papillary thyroid cancer (PTC) accounts for the vast majority of well-differentiated thyroid cancers, and has a better prognosis than follicular thyroid cancer (FTC). Childhood neck irradiation and exposure to other ionizing radiations are established risk factors for developing thyroid cancers.

Parathyroid adenomas are benign tumors of the parathyroid glands. The parathyroid glands maintain calcium, phosphorus, and vitamin D homeostasis. Parathyroid adenomas can result from secondary hyperparathyroidism, and parathyroid adenomas can give rise to primary hyperparathyroidism. These tumors are common in women over 60 years of age, and in people with head and neck irradiation.

Somatic microsatellite instability in thyroid cancer: Mitochondrial microsatellite instability in thyroid cancer has been examined by a number of groups. Lohrer et al. (2002) examined HVS II sequence alterations in radiation-associated and sporadic thyroid cancers. In this series, D310 mutations were scored at a dismal low frequency of 2 out of 126 radiation-associated and 6 of 40 sporadic cancers, and the frequencies supposedly correlated with age, suggesting mtMSI is infrequent in thyroid cancer. This low frequency and suggested age association of mtMSI in thyroid cancer contrasts with those of other investigators. Lima et al. (2003) critically examined this issue and uncovered the possible error in this study. Apparently, Lohrer et al. (2002) did not compare their tumor sequences to sequences of normal germline, but the adjacent noncancerous tissues, which obviously could have similar alterations as the tumors, thus underscoring the mutation frequency in this study (of note, many investigators have used normal adjacent to tumor samples as germline controls, however, the extent of mutation masking by this type of study design depends on the level of field cancerization in the specific cancer in question).

Tong et al. (2003) examined D310 status in 72 thyroid cancers that comprises papillary, medullary, anaplastic, and follicular thyroid cancers and insular carcinoma. D310 mutations were rare, being found in only 7% of all the tumors. Maximo et al. (2005) similarly found D310 instability in 7% of thyroid cancers. In another study of 66 thyroid cancers, the frequency of D310 instability was very high (30%), compared to instability at two other loci, D568 (4.6%), and D514 (22.7%). These mutations were not specific to cancers as they were also found in benign adenomas. The great disparity in the frequency of D310 changes in thyroid cancer calls for further studies to clarify the actual mutation frequency.

Somatic mitochondrial DNA point mutations in thyroid cancer: Several investigators have examined mtDNA somatic changes in thyroid cancer given its possible redox-mediated etiology. Yeh et al. (2000) used two-dimensional gene scanning technique to study mtDNA mutations in thyroid cancers and control tissues of thyroid and nonthyroid origins. Three somatic missense mutations were identified in thyroid cancers, with significant differences in polymorphic sites between tumor and controls. Most of the variants were in complex I genes. Polymorphisms at np 10398 and 7521 identified as risk factors in other cancers were among the changes. This study suggests that both somatic mutations and polymorphisms appear to influence thyroid carcinogenesis.

In 2002, Maximo et al. (2002) performed a comprehensive analysis of mtDNA mutations in a panel of tumors from 59 patients by sequencing 70% of the mitochondrial genome comprising mRNA genes. Tumors from all patients demonstrated sequence variants when compared to adjacent thyroid parenchyma. Fifty-seven somatic mutations were scored in 34 cancers. Follicular and papillary type

thyroid cancers harbored more nonsilent mutations in complex I genes than adenomas. There was also increased prevalence of complexes I, and IV mutations in the normal adjacent tissues. ATP synthase 6 sequence variants were more prevalent in Hurthle than non-Hurthle cell cancers. Abu-Amero et al. (2005) sequenced the coding regions of 24 thyroid cancers, mostly PTC, as well as four thyroid cancer cell lines. Somatic mutations were found in seven PTCs and one multinodular hyperplastic thyroid lesion. All cell lines had sequence variations, mainly transitions in complex I genes, in association with defective complex I activity. The presence of possible pathogenic mutations in precursor lesions (hyperplasia), suggests the occurrence of these mutations early in cancer evolution.

Medullary thyroid cancer can occur sporadically or as part of a familial syndrome in multiple endocrine neoplasia type II. Abu-Amero et al. (2006) examined mtDNA changes in sporadic and familial MTCs in comparison to their matched normal tissues. The entire mtDNA coding gene region was sequenced. Overall, twenty (77%) of the tumors harbored nonsynonymous mutations (69% of sporadic and 85% of the familial cancers). Intriguingly, transversion mutations were enriched in familial cases (76%) in contrast to transition mutations in sporadic cancers (77%). Majority of the mutations were in complex I genes, and appeared to affect moderately or highly conserved amino acids. Functional analysis revealed compromised mitochondrial functions in cell lines with mutations at nps 4917 and 11720. In addition, MTC patients with mutations showed compromised mitochondrial functions in their peripheral blood lymphocytes. Witte et al. (2007) analyzed the entire mitochondrial genome of paired papillary and follicular cancer thyroid tissues using high performance liquid chromatography and sequencing. Mutations were found in 49% of PTCs and all three FTCs. Thus, Somatic mtDNA mutations are frequent in thyroid cancers, and they appear to cluster in complex I genes causing mitochondrial functional impairment.

Mitochondrial gene expression changes in thyroid cancer: Haugen et al. (2003) examined mitochondrial gene expression in PTCs using differential display PCR. Overexpression of mitochondrial *ND5, ATP6, CYTB, COI, COIII* and nuclear *TFAM* were observed in PTCs. Analysis by immunohistochemistry further confirmed the increased expression of nuclear-encoded mitochondrial proteins. Thus, in PTC, mitochondrial- and nuclear-encoded mitochondrial proteins are increased compared to normal thyroid tissues. This could reflect a compensatory mechanism from mtDNA mutations with associated respiratory impairment in these cancers.

Somatic mitochondrial DNA mutations in parathyroid adenoma: Whole genome sequencing of the mtDNA was conducted on 30 parathyroid adenomas comprised of 18 chief cell and 12 oxyphil cell adenomas (Costa-Guda et al. 2007). Eight primary chief cell hyperplastic tissues, five normal parathyroid glands, and one thyroid gland and matched blood samples of all patients were sequenced as well. This well-designed study uncovered 27 somatic mutations in 50% of the adenomas, but not in any of the hyperplastic cells, suggesting that mtDNA mutations play a role in the pathogenesis of parathyroid adenoma. The distribution of the mutations in the adenomas was skewed, with significantly more mutations in oxyphil (9/12 – 75%) than chief cell adenomas (6/18 – 33%).

6.6 Breast Cancer

One out of every eight women will develop breast cancer in their lifetime, making it the commonest female cancer. The global burden of breast cancer continues to rise with over one million new cases diagnosed and 400,000 deaths occurring each year in women. Compared to several cancers, however, breast cancer has a relatively low case fatality of 36%. Similar to many nonpediatric cancers, the incidence of breast cancer rises with age, increasing rapidly during the fourth decade but slows in the fifth and sixth decades.

Breast cancer is an endocrine-dependent cancer, being influenced by hormonal exposure. Common risk factors are age at menarche, first full-term pregnancy, and menopause. Elevated risk is associated with early menarche, late first full-term pregnancy and late menopause. Clearly, the period of estrogen exposure is important in breast carcinogenesis. Also increased caloric intake is a risk factor for breast cancer. High calorie diet could alter mitochondrial metabolism, bioenergetics, redox balance and induction of mtDNA mutations. Similarly, estrogen signaling at the mitochondria can alter mitochondrial genome and functions (Chen et al. 2009), that could lead to tumor induction.

Mitochondrial genome polymorphisms and risk of breast cancer: A specific mitochondrial polymorphism that is well known to be associated with degenerative diseases, G10398A, appears to modulate cancer risk in some ethnic backgrounds. The A allele at np 10398 is a nonsynonymous mutation in *ND3* that changes Ala→Thr at codon 114, and appears to alter complex I structure. The G10398A genotype is probably one of the evolved nucleotides required for uncoupled respiration, because it is more frequent in Caucasians ($\sim$ 80%) than people of African heritage ($\sim$ 5%), who predominantly have the A10398G allele (Torroni and Wallace, 1994).

The evidence in support of a modulating role of G10398A allele in African–American women with advanced breast cancer is quite compelling, but will need more validation studies. In a pilot study by Canter et al. (2005), there was a strong association of the A allele with breast cancer invasiveness in African–American women. Data from this study was used for power calculation, and the design of a larger study. After adjusting for other breast cancer risk factors, the A allele remained an independent risk factor in these women. Analysis of a large cohort of white women failed to reveal any association of this allele with breast cancer invasiveness (Canter et al. 2005). In an independent study of organ confined prostate cancer in African–American men, the frequency of the A allele was much higher in cases (13.6%) than in controls (0%) (Mims et al. 2006), providing collaborative evidence for the A allele in modulating endocrine-related tumors in people of African descent. However, a recent multicentre analysis of G10398A polymorphism failed to reveal any association with breast cancer risk in African– American women (Setiawan et al. 2008). Further studies should help resolve the discrepancy, and validated data can serve as risk assessment screening to identify and monitor people at high risks for these cancers.

While the A allele at np 10398 appears to be detrimental for people of African descent, a recent study indicates that the G allele is probably equally pathogenic for

Caucasians. Evidence for the possible predisposition of the G allele to cancer etiology was provided by a Polish study. In Polish breast cancer patients, A10398G polymorphism was present in 23% compared to only 3% of control individuals without cancer, and this difference in frequencies was highly significant (Czarnecka et al. 2009). The possible interaction of A10398G polymorphism and alcohol consumption as risk factors for breast cancer was also recently uncovered (Pezzotti et al. 2009). This association was based on a case control study of 1,501 cases and 2,209 controls. The findings could not be reproduced in a low-powered follow up study, thus more studies with larger samples are required to confirm these associations.

Darvishi et al. (2007) provided further supporting data for the G10398A genotype in conferring cancer risk in an Indian population. Their analysis of complete mitochondrial genomes belonging to people in haplogroup N revealed a breast cancer risk associated with this haplotype. This result was then confirmed in a case control study of 124 sporadic breast cancer patients and 273 control Indian women. A study of type 2 diabetes individuals from Northern India by Bhat et al. (2007) further revealed the possible pathogenic nature of the G10398A polymorphism in this population. Their findings suggest that susceptibility to type-2 diabetes is ROS dependent because both the pro-ROS generating G10398A polymorphism and the reduced ROS response polymorphism, T16189C, were shown to be independent risk factors for type 2 diabetes.

In a Chinese series, the germline polymorphism, T16189C, is suggested to convey increased risk for breast cancer in light of the high frequency observed in breast cancer patients (Wang et al. 2006). Ye et al. (2008) also performed a large study examining (CA)n dinucleotide repeat polymorphisms in 1,058 Chinese women with breast cancer and 1,129 age-matched controls for possible association with the risk of developing breast cancer. While no association was uncovered in this study, some prognostic association was noted. Patients with homoplasmic (CA)n dinucleotide repeat sequences had better outcomes than those with heteroplasmic changes. Covarrubias et al. (2008) sought for possible interactions of mtDNA polymorphisms as possible risk factors for breast cancer. Seventeen different mitochondrial variants were examined in European–Americans and ethnic matched controls using logistic regression. They uncovered that variants A12308G and A10398G interaction significantly elevated the risk of breast cancer. In this study, other loci interactions were noted but did not reach significance.

The above findings provide ample evidence that mitochondrial genome polymorphisms could modulate mitochondrial functions, and possible interactions of such polymorphic loci, as well as their modulation of hormonal effects and diet might underlie the etiology of some breast cancers. The role of np 10398 in breast cancer of different ethnic groups requires clarification, validation and clinical translation.

Somatic mitochondrial microsatellite instability in breast cancer: D310 alterations as diagnostic markers for breast cancer have been studied in primary breast cancers, fine needle aspirates, and metastatic lymph node specimens. Parrella et al. (2001) examined the frequency and distribution of mutations in the mitochondrial

genome of primary breast cancer samples. Twelve somatic mutations were demonstrated in a majority of the cancers, with five mutations at D310. Rapid screening of D310 alterations in additional 46 primary breast cancer samples uncovered seven more mutations. All available five fine needle aspirates and four lymph node metastatic tissues demonstrated clonal D310 changes to the primary tumors. In a follow up study, Parrella et al. (2003) examined D310 mutations in the breast, bladder, cervical and endometrial cancers. In this series, D310 alterations were more frequent in cervical cancer followed by bladder cancer, breast cancer and endometrial cancer. In addition, all paired urine samples of four bladder cancer patients and all fine needle aspirates from three breast cancers possessed identical D310 mutations (Parrella et al. 2003). Tseng et al. (2006) sequenced and scored somatic mutations in breast cancers from 60 Taiwanese women. Mutations were found in 30% of tumors, with the majority (72%) at D310. The D-loop mutations were associated with advanced age (> 50 years), negative estrogen and progesterone receptor status, as well as poorer disease-free survival. A study from China of D310 mutations in familial breast cancer both recorded extremely high frequencies. In one study of 23 familial breast cancer compared to 18 normal breast controls, D310 mutations were detected in all cases of familial breast cancer cases compared to 16 of 30 sporadic breast cancers (Yu et al. 2008).

Alazzouzi et al. (2003) examined nMSI and mtMSI in breast ductal adenocarcinomas. None of the 40 dinucleotide and two mononucleotide repeats of the nuclear genome demonstrated instability in these tumors. On the contrary, mtMSI was detected in 11% of the tumors, none of which was present in the normal matched tissue suggesting somatic acquisition of mtMSI by tumor cells. Moreover, microsatellite heteroplasmy in normal tissues did not correlate with mtMSI in tumors.

The D514 (CA)n microsatellite site and four *Mnl*I loci between nps 16108 and 16420 were examined in 40 breast cancer and matched normal tissues (Richard et al. 2000). *Mnl*I site alterations were demonstrated in 48% of the tumors and this frequency was 216-fold higher than the spontaneous mutation rate in the germline. Similarly, mtMSI occurred in breast cancers and this was also 16-fold above the expected spontaneous mutation rate at this site in the germline. Overall, mitochondrial genetic instability was found in 65% of the breast cancer tissues. These changes however, did not correlate with nuclear genomic instability. Wang et al. (2006) examined mtMSI at 12 loci in breast tumors. Instability was observed at four of the 12 loci. Three of the four markers were located in the D-loop, with the fourth located in *12SrRNA*. Mitochondrial MSI was observed at a rate of 29% in breast cancer. Ye et al. (2009) also examined *Mnl*I sites between nps 16106 and 16437 in breast tissues of 501 women with cancer and 203 with benign breast disease. Matched normal appearing breast tissues from 120 cancer and 59 benign cases were included. Somatic *Mnl*I site changes were significantly higher in breast cancer (28%) than in benign breast disease (15%). Proliferative benign breast disease samples also harbored more alterations (13%) than nonproliferative benign breast tissues (7%). It was concluded that this finding had utility in early detection of breast cancer in high-risk women.

The relationship between xenobiotic metabolizing enzymes, (Glutathione S-transferase isoforms μ (GSTM-1), and τ (GSTT-1), and *N*-acetyl transferase 2) and mtMSI was examined in 94 breast cancer tissues (Pavicic et al. 2009). The rationale was to link a possible lack of carcinogenic ROS scavengers to mtDNA instability in breast cancer. In general, alterations at the microsatellite site beginning at bp 514 and/or *Mnl*I site occurred in 40% of cancers. Histopathologic analysis revealed a significant association of mtMSI with invasive lobular carcinoma (74%) compared to invasive ductal carcinoma (19%). Correlation analysis also indicated a significant association between mtMSI and *GSTM1* null genotype, suggesting a possible mechanism between loss GSTM1 activity and mtMSI in breast cancer.

Somatic mitochondrial DNA point mutations in breast cancer: A large body of evidence suggests somatic mtDNA mutations play a role in breast carcinogenesis. Tan et al. (2002) performed a comprehensive study involving sequencing the entire mitochondrial genome for identification of somatic mutations and reported a relatively high frequency of mutations (74%) in breast tumors. Mutations were scored against matched histopathologic normal tissue in this study. Of the 27 mutations, 22 (82%) were located in the D-loop. Likewise, in the study by Parrella et al. (2001) of 18 primary tumors, they detected somatic mutations in 61% of breast cancer when appropriate sequence comparison to the maternal sequence was made suggesting authentic somatic mutations are frequent in breast cancer. Rosson and Keshgegian (2004) investigated the differences between breast tumor and normal tissues in 15 patients using laser-capture microdissection and reported that all samples had mutations. Despite the comparison of tumor sequences to the revised Cambridge reference sequence (rCRS, Brandon et al. 2005), the consistent demonstration of high frequency of mtDNA mutations by all three investigators suggest some authenticity of their findings.

Zhu et al. (2005) examined the mutation rates in normal and malignant breast tissues. Nipple aspirate fluid (NAF) obtained from the malignant breast was sequenced for np 15,331–2,463 that harbored mutations in tissue. Of 15 cancer samples, 14 (93%) had sequence alterations, consistent with previous investigators. The majority of these mutations were in coding regions and 11 were nonsynonymous changes. Four NAF samples recorded a single mutation each that were also found in the matched tumor. Wang et al. (2007) sequenced mtDNA from ten early stage breast cancer samples as well as the normal adjacent and distant normal tissues. Heteroplasmic transition mutations were found in two patients. An A8601G mutation was present in both cancer and normal adjacent tissues, while a T2275C was only uncovered in the cancer of a different patient. Whole genome sequencing of 36 breast cancer tissues and matched normal with known *p53* mutations revealed somatic mtDNA mutations in 42% of cancer samples (Gochhait et al. 2008). These mutations correlated significantly with mutations in the DNA-binding domains of *p53*. Both mtDNA and *p53* mutations were predominantly found in late stage, high grade and estrogen and progesterone receptor negative breast cancer cases.

In summary, somatic mtDNA mutations are frequent in breast cancer, and their presence in adjacent normal tissues close to tumors, as well as the possible detection in nipple aspirate fluids opens up opportunities for their utility in early detection studies.

6.7 Lung Cancer

Lung cancer is probably the worse cancer in the world, leading in incidence of 1.35 million cases, mortality of 1.18 million, and an extremely high case fatality of 87%. The dismal outcome in lung cancer management is primarily due to late diagnosis, because only 15% of lung cancers are localized tumors with the rest having spread at diagnosis. The 5 year survival rate for early detected localized tumors is $\sim$ 50% compared to only $\sim$ 2% for advanced tumors. Several investigators are exploring accurate screening methods for early detection of lung cancer, using molecular signatures. An important concept of lung carcinogenesis is the presence of precancerous fields, such that validated biomarkers for lung cancer should enable early detection of a carcinogenic process in clinical samples such as sputum. Identification of high-risk individuals for monitoring will lead to early diagnosis. To this end, the high copy number advantage of the mitochondrial genome appears attractive for analysis of such samples with low cellularity (see Chap. 11).

Somatic mitochondrial microsatellite instability in lung cancer: A 336 bp fragment of the D-loop including, D310 was examined in 55 nonsmall cell lung cancers (NSCLC) with their matched histologically normal tissues, as well as 28 lung cancer cell lines (Suzuki et al. 2003). Homoplasmic mutations were found in 20% of the primary lung cancer samples, and 50% of the cell lines.

Somatic mitochondrial DNA point mutations in lung cancer: As lung cancer is associated with smoking, the genotoxins of tobacco smoke can damage the vulnerable mtDNA. Hence, several groups have studied mtDNA mutations in lung cancer. Fliss et al. (2000) were the first to conduct a study of mtDNA mutations in lung cancer, and assessed the possible presence of these mutations in body fluids that could serve as diagnostic biomarkers. Using manual sequencing, 6 of 14 lung cancer samples possessed mutations, which could be detected in bronchoalveolar lavage specimens. The same group later analyzed 27 primary lung cancer samples and detected mutations in only nine specimens (Sanchez-Cespedes et al. 2001). The prevalence of mtDNA mutations in lung cancer has further been addressed (Jin et al. 2002) using whole genome sequencing. These investigators observed mutations at a higher frequency of 62% than those from previous studies. Identical mutations were also demonstrable in eight corresponding normal lung tissues in the proximity of a tumor, consistent with precancerous fields in the lung. The same group later investigated the mutational spectra in 55 lung cancer patients (Jin et al. (2007). They identified 56 mutations including 19 nonsynonymous nucleotide changes in 60% of the patients, consistent with their earlier results. Nine of the 13 coding genes harbored the 19 nonsynonymous nucleotide changes. Tissues from 202 NSCLC and associated normal lung tissues were examined for mutations (Matsuyama et al. 2003). This group sequenced only the D-loop and neighboring rRNA regions and scored mutations in 35% of the samples. The levels of D-loop mutations were correlated with tumor grade, and lymph node metastasis. The authors suggested that preoperative analysis of the D-loop region could have prognostic value.

Atypical adenomatous hyperplasia (AAH) is often found in close proximity to lung adenocarcinoma and is a precursor lesion of invasive adenocarcinoma of the lung. The possible correlation of AAH and lung cancer was determined by D-loop sequence comparison between tumor and matched precursor lesions (Morandi et al. 2007). Different mutation patterns were observed, suggesting that the tumors and corresponding AAH were genetically different. While many mtDNA mutations in cancer, cluster in the hypermutable D-loop region, it was observed that the majority of lung cancer mutations were in the coding region (Jakupciak et al. 2005). Recent sequencing of the entire mitochondrial genome in eight lung cancers revealed mutations in six samples (79%). The mutations were clustered in complex I genes and the D-loop (Jakupciak et al. 2008). An ATP6 mutation, G8836C, that affects a moderately conserved amino acid was present in all three lung cancer samples as well as 80% of normal mucosal biopsies in these patients. This same mutation is detected in LHON and thyroid cancer, affirming to its functional role (Dasgupta et al. 2009). Thus, mtDNA mutations are frequent in lung cancer as demonstrated by whole genome sequencing. Larger studies comprising of tumor and corresponding biofluids should shed more light on the possible clinical value of mitochondrial genome changes as adjunct to early detection of lung cancer, especially in high-risk individuals.

6.8 Esophageal Cancer

Excess alcohol consumption, cigarette smoke, and Barrett's esophagus (chronic gastritis) are well-known risk factors for development of esophageal squamous cell carcinoma (ESCC) and adenocarcinoma. While the incidence of ESCC is on the decline in the US, adenocarcinoma following Barrett's esophagus appear to be on the rise.

Mitochondrial genome polymorphisms and risk of esophageal cancer: The breast cancer risk genotype, G10398A, appear to carry some risk in the develpemnt of squamous cell carcinoma of the esophagus (Darvishi et al. 2007). This finding was demonstrated in a pilot study of Indian patients with squamous cell carcinoma of the esophagus and matched controls.

Somatic mitochondrial microsatellite instability in esophageal cancer: Kumimoto et al. (2004) examined the somatic mutation rate of esophageal cancer in a panel of 38 tumors. Mutations were uncovered in 34% of tumors, with the majority (11/14) being D310 alterations. Another study found the frequency of D310 mutations to be 14% when 82 esophageal cancers were studied (Kose et al. 2005). Using microdissection, samples from ESCC, noncancerous mucosa, metastatic lymph node deposits, and esophageal muscle tissue were obtained from 72 patients for D310 analysis (Lin et al. 2009). Somatic D310 changes were found in 56 patients and these changes were significantly associated with decreased survival. Notably, there was evidence of field cancerization in this study, because of the 56 patients

with somatic D310 mutations, 51 also harbored such changes in the corresponding noncancerous mucosa.

Somatic mitochondrial DNA point mutations in esophageal cancer: Hibi et al. (2001b) were the first to investigate mitochondrial D-loop alterations in esophageal cancers. Of 37 tumors analyzed in comparison to the adjacent normal tissues, only two (5%) tumors had mutations. In a subsequent study to that of Hibi et al. (2001b), Kumimoto et al. (2004) examined the somatic mutation rate of esophageal cancers. This group found mutations in 34% of the tumors, which is nearly sevenfold greater than that seen by Hibi et al. (2001b). Abnet et al. (2004) analyzed 21 esophageal cancers for the presence of mutations in the D-loop. Sequences from cancers were compared to those from matched adjacent normal tissues as well as blood. All samples were genotyped using the ABIAmpFISTR Profiler plus PCR multiplex assay as an added quality control measure. Mutations were found in 33% of the cancers consistent with the frequency unvealed by Kumimoto et al. (2004). Tan et al. (2006) examined mitochondrial genomic alterations in 20 esophageal cancers using TTGE and direct sequencing. Fourteen somatic mutations, mostly in the D-loop were uncovered in as many as 55% of the cancers. Two missense mutations in *ND4L* and *ATP6*, and a frameshift mutation in *ND4* were uncovered in the tumors. Gochhait et al. (2008) found an overall somatic mtDNA mutation rate of 39% in esophageal cancer, and these mutations were primarily in coding genes and correlated with *p53* mutation status. Miyazono et al. (2002) studied the presence of D-loop mutations in Barrett's esophagus and esophageal cancer. Mutations were found in 40% of these samples. Interestingly, mutations were observed in both tumor and Barrett's esophagus, suggesting the presence of precancerous esophageal fields. Sui et al. (2006) also examined seven Barrett's esophagus in their quest for early genetic changes in tumor progression. All seven precancerous lesions harbored at least one somatic mutation.

6.9 Gastric Cancer

Globally, ~ 870,000 new cases of gastric cancers are diagnosed each year, but gastric cancer accounts for a vast majority of cancer deaths, being second only to lung cancer for all cancer mortalities. The global incidence of gastric cancer has, however, declined considerably with the exception of countries such as Ireland, Japan, Chile, and China. Dietary carcinogen exposure early in life seems to be a risk factor for the development of gastric cancer. Prolonged consumption of foods rich in nitrates is implicated as etiologic agents. The nitrates are converted into carcinogenic nitrites in the stomach by bacteria. *Helicobacter* pyloric gastritis, and reduced gastric pH from several causes favor the growth of nitrite-converting enzymes.

The majority of gastric cancers are adenocarcinomas (a few are lymphomas and GISTs). The cellular behaviors and organizations of the cancer cells in adenocarcinoma have given rise to two distinct classes of gastric adenocarcinoma. In the diffuse type adenocarcinoma, the cells disperse throughout the stomach wall that

leads to thickening of the stomach wall with loss of distensibility (linitis plastica). This type of gastric cancer is common in young people and carries an unfavorable outcome. The intestinal type of gastric adenocarcinoma comprises of cohesive neoplastic cells organized into tubular glandular structures.

Somatic mitochondrial microsatellite instability in gastric cancer: Sanchez-Cespedes et al. (2001) found 62% of gastric cancers harbored mtMSI. Hiyama et al. (2003a) examined the possible relationship between D310 mutations in *Helicobacter pylori* gastritis and gastric cancer. This study found D310 changes in 16% of gastric cancers, 7% of *Helicobacter pylori* gastritis and none in controlled *Helicobacter pylori*-negative subjects. In another study, 105 gastric cancers were examined for nMSI-H and D310 mtMSI (Hiyama et al. 2003b). Nuclear MSI-H and mtMSI were detected in 14 and 16 tumors, respectively. Only two tumors had both mutations. D310 and dinucleotide repeat instabilities were present in 59% of gastric cancers and 41% of the adjacent normal mucosa, indicative of field cancerization in gastric epithelium (Han et al. 2003). Mononucleotide and dinucleotide repeat mtMSIs were also found in 63% of 31 gastric cancers (Lee et al. 2005).

Ling et al. (2004) demonstrated the accumulation of mtMSI with gastric cancer progression from chronic gastritis to invasive cancer. They examined mtMSI in 40 chronic gastritis, 30 intestinal metaplasia, 20 dysplastic lesions, and 68 cases of advanced cancer. The frequency of mtMSI increased from chronic gastritis, intestinal metaplasia, dysplasia to advanced cancer. Noted in this study was the significant association of mtMSI with intestinal type histology and distal gastric cancer. The authors had previously shown that the levels of IL-8 were significantly higher in mtMSI-positive than mtMSI-negative cancers (Ling et al. 2003).

RUNX3 is a transcription factor with tumor suppressor functions. The promoter of this gene is hypermethylated in several cancers including about 50% of gastric cancers. Gargano et al. (2007) investigated a possible relationship between *RUNX3* methylation, mtMSI and nMSI in gastric cancers. Nuclear MSI was examined according to National Cancer Institute Bathesda panel, and mtMSI was genotyped using D310. Overall, mtMSI occurred in 11% of gastric cancers while nMSI-H was present in 13% of cancers. Mitochondrial MSI significantly correlated with TNM staging of cancers. Interestingly, an important association between *RUNX3* promoter methylation, nMSI-H and mtMSI was revealed, suggesting a possible mechanistic link between these events in gastric carcinogenesis. In another study, nMSI at BAT26 and mtMSI at D310 were examined in 74 gastrointestinal stromal tumors (Kose et al. 2006). As expected, MSI was much higher at D310 that BAT26. In an earlier study, this group found D310 mutations in 15% of 96 gastric cancers examined (Kose et al. 2005).

Somatic mitochondrial DNA point mutations in gastric cancer: Habano et al. (2000) determined that mitochondrial gene mutations might play a role in the pathogenesis of intestinal-type gastric carcinomas. Mitochondrial genome sequences were recovered from 62 primary gastric cancers and the matched normal tissues. They sequenced *ND1*, *tRNA*^{Leu}, *ND4*, *ND5*, *COXII*, *CYTB*, *ATP6* and *ATP8*. Single-stranded conformational polymorphism (SSCP) was used to identify sites of

mismatch, which were then directly sequenced. Eleven samples (18%) had mutations. One of the coding region changes resulted in a truncation of ATP6 protein, while another had a similar effect on ND5. In a similar study, 32 gastric carcinomas and matched normal tissues were examined for mutations (Maximo et al. 2001). Using SSCP, the authors characterized the mutation patterns in five mitochondrial genes namely, *ND1, ND5, COI,* $tRNA^{Leu}$ and $tRNA^{Ser}$, and parts of the D-loop. Mitochondrial genome mutations were seen in 81% of the samples. The majority of the changes were in the D-loop and complex I genes. Both tRNA genes sequenced in these tumors were free of mutations.

Zhao et al. (2005) investigated D-loop alterations in 20 gastric cancers and matched normal mucosa in relationship to ROS, cell cycle and apoptosis. Mutations were present in 35% of the tumors. Proliferation, apoptosis and ROS production were significantly increased in mutation positive samples compared to controls. The mutation frequency in the D-loop of 31 gastric cancers and the corresponding benign mucosa was examined (Wu et al. 2005). Somatic D-loop mutations were found in 48% of these cancers. In another study, D-loop mutations were uncovered in 52% of gastric cancers, 63% of which were D310 changes (Lee et al. 2005). It was also found that gastric intraepithelial neoplasia without *Helicobacter pylori* infections harbored more D-loop mutations than *Helicobacter pylori*-positive gastritis (Rigoli et al. 2008), suggesting mutation accumulation in gastric mucosa with gastric cancer progression. A recent analysis of 31 gastric cancers revealed mutations in 23% of the cancers (Hung et al. 2009). Five of the changes (G3697A, G4996A, G9986A, C12405T, T13015C) were homoplasmic and three (5895delC, 7472insC, and 12418insA) were heteroplasmic. Half of these mutations involved conserved sequences and were nonsynonymous changes. In vitro studies using gastric cancer SCM1 cell lines indicated that ROS generated as a consequence of mtDNA mutations may contribute to gastric cancer progression (Hung et al. 2009).

6.10 Colorectal Cancer

Colorectal cancer (CRC) is the third most frequent cancer diagnosed worldwide. Globally, 940,000 new cases are diagnosed each year, and in the US, colon cancer is the second leading cause of death. Most colon cancers develop in people after the age of 50 years. Animal fat consumption, inflammatory bowel disease, familial adenomatous polyposis, Turcot's syndrome, Gardner's syndrome and hereditary nonpolyposis syndrome (Lynch syndrome) are all established risk factors of CRC.

There are three types of colonic polyps; (1) juvenile polyps are nonneoplastic hamartomas, (2) hyperplastic polyps are due to hyperplastic mucosal proliferation, and (3) adenomatous polyps are premalignant growth of colonic mucosa. Irrespective of the etiologic agent, most colon cancers develop from adenomatous polyps.

The progression of normal colonic mucosa toward cancer is well delineated in colon cancer. Molecular analysis of normal mucosal tissues, adenomas, dysplastic lesions and carcinoma in situ, has given rise to the possible mutations that lead to colon

cancer progression. Dr. Bert Vogelstein has proposed sequential genomic aberrations in *APC*, *K-ras, p53*, and BAT-26 in colon cancer progression. Where colon cancers show mutations in *K-ras*, allelic loss at *APC* (chromosome 5q21), *DCC* (chromosome 18q), and *p53* (chromosome 17p) loci, the sequence of events leading to colon carcinogenesis is unclear.

Somatic mitochondrial microsatellite instability in colorectal cancer: Mitochondrial MSI at D310 is probably frequent in colorectal cancers. Heteroplasmic mutations in HVSI and HVSII of the mitochondrial D-loop were found at a rate of 23% in colorectal cancer (Alonso et al. 1997). Mitochondrial MSI at D310 was the site of 60% of these CRC mutations. Sanchez-Cespedes et al. (2001) detected D310 mutations at a frequency of 28% in CRCs. Habano et al. (1998) analyzed nine mtMSI sites in 45 sporadic CRCs. Twenty or 44% of the cancers had mtMSI, some of which were frameshift mutations at poly (A)8 or poly(C)6 tracts in complex I genes that resulted in truncated complex I proteins. In a follow-up study, the 45 CRCs were examined by whole genome scanning. Single-stranded conformational polymorphism analysis revealed complex I mutations in 16% of tumors. These mutations included three mononucleotide repeat alterations, two missense mutations, and one 15 bp deletion. Six of the seven tumors with mutations also had mtMSI. Guleng et al. (2005) analyzed D310 and repeats in *ND1* and *ND5* in a cohort of 95 colorectal cancer patients and 95 control patients without gastrointestinal cancer and made appropriate comparisons to sequences from the patients corresponding blood. Of the 95 cancer patients 34% had D310 alterations compared to only 2% in the noncancerous controls. None of the mutations were in complex I genes. D310 changes were not associated with nMSI in these cancers.

The D-loop of 365 colorectal cancer samples was sequenced and compared to matched-healthy colon tissues (Lievre et al. 2005). Sequencing results were compared to the rCRS to determine polymorphisms, and differences between tumor and normal tissues were scored as somatic mutations. D-loop alterations were observed in 38% of these samples the majority (132 out of 142) of which were mtMSI at D310. Interestingly, the 3-year survival rate was significantly lower for those patients with D-loop mutations and may play a role in stage III colon cancer resistance to fluorouracil-based adjuvant chemotherapy, because adjuvant therapy only benefited patients without D-loop mutations, and elevated the risk of death in patients with mutations (relative risk of death was 4.30).

Mitochondrial MSI was present in 90% of 25 CRCs examined by Lee et al. (2005). Mutations at D310 and in coding genes (*ND1, ND5*) were examined in 38 rectal and 25 sigmoid colon cancers (Pinheiro et al. 2009). Consistent with reports in several cancers, mtMSI at D310 was much frequent (34–38%) than mtMSI in coding genes (3–8%) in these cancers. In a large panel of CRC samples (138 specimens) examined, only 8% harbored D310 changes (Kose et al. 2005). Legras et al. (2008) examined the prevalence of D310 in 64 colorectal adenomas and 36 metastatic tissues. They further performed a case control study of the possibility of D310 polymorphisms being a risk factor for colonic adenomas in 613 cases and 572 controls. D310 was mutated in 27% of the adenomas and 33% of the metastatic

cancers. However, germline D310 polymorphisms were not found to be risk factors for developing colorectal adenomas. Kim et al. (2006) analyzed nine mitochondrial microsatellite sites in the D-loop and five in the coding regions in 48 sporadic colorectal cancer epithelia and stroma. In addition, nine nuclear microsatellite loci were examined in the stromal samples, all of which were obtained by laser-capture microdissection. Mitochondrial MSI occurred at a frequency of 31 and 10% in cancer epithelia and stroma respectively. Stromal mtMSI neither correlated with stromal nMSI nor cancer epithelial mtMSI, suggesting independent events.

Somatic mitochondrial DNA point mutations in colorectal cancer: Polyak et al. (1998) were among the first to perform a comprehensive whole genome analysis of mtDNA mutations in CRC. Homoplasmic mitochondrial genome mutations were demonstrated in 70% of colorectal cancer cell lines. The mutations were mainly transitions at purine suggestive of ROS-mediated mechanism. The somatic mutations were present in the primary tumors from which the cell lines were derived. Hibi et al. (2001a) sequenced the D-loop of primary CRCs and then used a more sensitive mismatch ligation assay to screen for the same mutations in the patient's serum. Seven of the 77 primary tumors had somatic mutations, one of which could be detected in the patient's serum. Aikhionbare et al. (2004) used high-resolution restriction endonuclease and sequencing to examine mtDNA mutations in 25 adenomas and 27 CRCs and their matched normal adjacent tissues. In comparison to matched surrounding tissues, adenomas and colorectal cancers had more mutations. Of the 22 mutations uncovered in this study, nine were nonsilent. The mutations were mainly homoplasmic and found in ribosomal genes.

The known effects of inflammation-associated ROS in cancer were examined in colitic colorectal cancer (Nishikawa et al. 2005). Samples from ulcerative colitis-related cancer, colitis without cancer and normal controls were examined for mtDNA mutation rate. Mutations were much higher in ulcerative colitis than normal colonic mucosa, and colitic cancer also harbored significantly more mutations than the other controls. Sui et al. (2006) examined a possible role of mtDNA mutations in mediating progressive tumor formation in the colon. Four adenomas and three colitic-associated dysplasias were studied. Using MitoChip for whole genome sequencing, all the cancers had at least one somatic mutation. More common genes involved were *COI, ND4* and *ND5*. In this study, preneoplastic lesions without dysplasia even demonstrated mutations, emphasizing the early role of mitochondrial genome changes in cancer evolution. Chang et al. (2009) examined the D-loop and *p53* for mutations in a panel of 194 sporadic CRCs. D-loop mutations occurred significantly in CRC with *p53* mutations (39%) compared to tumors without *p53* mutations (22%). The frequency of D-loop mutations was also examined in 40 CRC and 150 control samples (Akouchekian et al. 2009). Mutations, scored by comparison to the rCRS, were much higher in CRCs than in controls.

Mitochondrial gene expression changes in colorectal cancer: Heerdt et al. (1990) uncovered a progressive decrease in *COIII* expression in colon adenomas and carcinomas in comparison to normal colonic mucosa. These changes in mitochondrial genome expression were not due to mtDNA content changes. Upon

differentiation, it was found that HT29 human colonic adenocarcinoma cells had a restored *COIII* expression to normal levels. In a follow-up study, *COIII* expression was examined as a possible risk factor for progression of familial polyposis and hereditary nonpolyposis coli to adenocarcinoma (Heerdt and Augenlicht 1991). Indeed, *COIII* expression decreased with disease progression, thus increasing the risk of cancer development. Nuclear complements of complex IV were however unchanged. An interesting finding in this study was the induced expression of *COIII* in HT29 cells by un-branched short chain fatty acids (SCFAs), a main energy source of colonic cells. In the gut, fermentation of fiber mediated by colonic microflora produces SCFAs. Interventional study of high-risk patients (HNPCC) who were put on 1,500 mg of CaCO3 per day, *COIII* expression increased in seven of 20 participants within a 7 months period.

6.11 Pancreatic Cancer

Pancreatic cancer has a high mortality rate because of late diagnosis, due to the absence of well-established risk factors. Cigarette smoking is the most well associated risk factor. High caloric diet is implicated as risk factor, as well as long-standing diabetes and chronic pancreatitis.

Somatic mitochondrial DNA point mutations in pancreatic cancer: Mutations in the mitochondrial genome have been demonstrated in a number of pancreatic cancers, and are suggested to be potential targets for early cancer diagnosis as well as having prognostic value. The initial work of Jones et al. (2001) involved sequencing the entire mitochondrial genomes of ten pancreatic cancer cell lines and five xenographs. They found 80% of their samples contained homoplasmic mtDNA mutations, and cancer cells had increased mitochondrial mass at about six- to eightfold, compared to normal pancreatic cells. The investigators explained that the high copy number of mtDNA accounted for the ease with which they could uncover these mutations. It was suggested based on mathematical modeling that the homoplastic nature of the mutations occurred as a result of random genetic drift rather than a selective advantage. In conformity with the conclusion that mitochondrial mass in pancreatic cancer is larger than normal cells, Maitra et al. (2004) easily detected pancreatic cancer mutations in corresponding pancreatic juices of two patients. Navaglia et al. (2006) observed D-loop mutations to be a rare event in pancreatic cancer, being detected in only 16% of cases. But the following polymorphisms, T152C, T16189C, T16519C, and A73G were more frequent in cancer than controls. Survival analysis of only locally advanced cancer revealed the association of the T allele of 16519 to be associated with shorter life expectancy. This allele also correlated with diabetes mellitus. Contrary to Navaglia et al. (2006), Mu et al. (2006) uncovered a high frequency of D-loop mutations (11/12 cancers) in pancreatic cancer. Also, the mutation rate appeared to correlate with the degree of hyperplasia, being 33% in PanIN1 and 75% in PanIN3. Thus, D-loop mutations might mirror histopathologic progression to pancreatic adenocarcinoma.

It is more likely that the frequency of mutations in pancreatic cancer is indeed high. A comprehensive analysis of mtDNA using the sensitive mitochondrial-resequencing array that covers the entire genome revealed somatic mutations in all 15 pancreatic cancer samples (Kassauei et al., 2006). Of the 71 mutations detected by this method, 31 involved the D-loop, rRNAs, and tRNAs and 18 were nonsynonymous mutations in coding genes. Commonly involved genes were *ND4, COI* and *CYTB*. Incidentally, an A→G transition at np 841 in *12SrRNA* was found in 3 independent tumors. Depending on their levels and specific locations, the mutations have important biologic consequence on mitochondrial functions.

Mitochondrial DNA mutations are frequent in pancreatic cancer and may have value in early detection, disease monitoring and prognostication. The high mtDNA copy number observed by Jones et al. (2001) in pancreatic cancer coupled with the ease of mitochondrial genome measurement in pancreatic juices suggests a realistic clinical value, of assaying quantitative mitochondrial genome content and sequence variances for early detection. More research and validated work are required for clinical translation.

6.12 Hepatocellular Carcinoma

Cancer of the liver is one of the most frequently diagnosed malignancies in the world, with annual incidence of ~ 560,000. Hepatocellular carcinoma (HCC) usually develops in a cirrhotic liver. The incidence is particularly high in sub-Saharan Africa and Asia, probably because chronic hepatitis B, and hepatitis C viral infections are prevalent in these regions. Also endemic in these geographic regions is aflatoxin B1 infections that are known to be associated with a G→T nucleotide change at codon 249 of the *p53* tumor suppressor gene. Since chronic viral hepatitis is a causative agent in over 80% of all liver cancers, mtDNA mutations is expected to underlie this type of cancer, hence one has to search for causative mtDNA mutations in HCC.

Somatic mitochondrial microsatellite instability in hepatocellular carcinoma: Somatic mtMSI had not been a major focus in mtDNA mutation studies in HCC. Fang et al. (2004) examined nMSI at BAT26 and mtMSI at D310 in 52 HCCs. Twenty-one percent and 6% of HCCs harbored mtMSI and nMSI respectively, and the two MSIs were not correlated. Eight of 13 D-loop mutations in HCCs were at D310 (Nomoto et al. 2002), and Lee et al. (2005) observed that 11/27 D-loop mutations in HCC were at D310.

Somatic mitochondrial DNA point mutations in hepatocellular carcinoma: Liver cancer commonly follows chronic inflammation induced by hepatitis B or C viral infection and cirrhosis. As inflammation is inevitably associated with ROS production, it can be conceived that DNA damage should underlie the development of hepatic tumors. Thus, several groups have addressed various research questions in

relation to mtDNA damage in liver cancer, probably because of its increased susceptibility to damage by oxidative stress. The D-loop region was sequenced in 20 HCCs and the normal adjacent tissues, and mutations correlated with the levels of oxidative stress (Huang et al. 2005). Mutation rate was 40%, with most mutations being transitions, suggestive of ROS-mediated damage. Consistently, oxidative stress was much higher in tumors that controls, and was associated with D-loop mutations. Nishikawa et al. (2001) characterized the mitochondrial D-loop mutation profile in HCC samples, in comparison to matched normal samples from 71 individuals. Fifty-nine patients had chronic hepatitis C while the remaining 12 had chronic hepatitis B viral infections. In addition, control liver tissue was obtained from seven individuals without hepatitis B viral infection and HCC, but had other liver pathologic changes. In an initial study phase, the entire genome was sequenced from two poorly differentiated HCCs, matched normal and one normal control. The D-loop region between nps 100 and 600 harbored the greatest number of mutations and this region was sequenced in the remaining samples. Twenty-eight of the 71 (39%) cancer patients had a greater number of mtDNA mutations in cancerous than in noncancerous tissues. Interestingly, a precancerous liver field is suggested by the higher incidence of mutations in normal liver tissues from liver with malignancy, when compared to liver samples free of HCC.

An important clinical aspect of HCC is the ability to differentiate between relapse and a second primary tumor. In an attempt to resolve this dilemma, liver samples from 19 patients with multiple HCCs were collected, including adjacent histologically normal controls (Nomoto et al. 2002). These samples were characterized by mitochondrial D-loop sequencing. Matched plasma from each patient was also investigated for corresponding D-loop alterations. Thirteen of the 19 patients (68%) had D-loop mutations. Three patients demonstrated identical mutations in multiple tumors, suggesting tumors were monoclonal in origin. Plasma was available from ten of 13 mutation-positive individuals. Interestingly, the corresponding HCC mutations were found in mtDNA from the matched plasma in eight of ten (80%) cases. Okochi et al. (2002) examined liver samples from 50 patients with HCC. D-loop somatic mutations occurred at a rate of 34%. Similar to Nomoto et al. (2002) the same mutations were demonstrated in five of 15-paired sera. Lee et al. (2004) also analyzed the mutation rate in the D-loop of 61 HCCs and the adjacent normal tissues, and found mutations in 24 (39%) of the samples. Yin et al. (2004) reported a D-loop mutation frequency of 22% in HCC. The work by Wheelhouse et al. (2005) uncovered the percentage of D-loop mutations in control subjects to be 11%, which is significantly lower than in adjacent normal (49%) and tumor (59%) samples. A study involving 54 HCCs with 47 surrounding normal tissue samples and five samples with metastatic foci, but lacking inflammation were sequenced for only D-loop alterations. It was observed that the number of mutations increased with loss of differentiation, demonstrating a progressive pattern of accumulating D-loop mutations with cancer (Tamori et al. 2004). While this is not a generally reported phenomenon, studies are warranted to establish the role of mtDNA mutations with tumor progression.

6.13 Gallbladder Cancer

Somatic mitochondrial microsatellite instability in gallbladder cancer: An extensive study addressed the importance of D310 changes in gallbladder cancers (Tang et al. 2004). DNA extracts from 123 tumors, 53 dysplastic tissues, and 90 histologically normal tissues adjacent to cancer, chronic cholecystitis, as well as 15 normal gallbladder tissues were analyzed for D310 changes by PCR and sequencing. D310 mutations were quite frequent in tumors (38%), dysplastic lesions (57%) and normal adjacent tissues (46%). The matched dysplastic lesions and normal mucosa adjacent to tumors showed clonal relationships to tumors based on D310 changes. Furthermore, when compared to normal mucosa, D310 mutations were more frequent in dysplastic and normal mucosa adjacent to chronic cholecystitis, suggesting D310 changes are potential early indications of gallbladder carcinogenesis.

6.14 Conclusion

It is evident that mtDNA mutations are present in almost every cancer examined. While the literature is still at its infancy, knowledge about mtDNA damage in cancer needs rigorous studies, because the potential of risk assessment, early detection, and definitive diagnosis of cancer can easily be achieved assaying alterations of mitochondrial genetic changes. Inherited polymorphisms or haplotype variants and copy numbers per specific tissues, as well as acquired somatic mutations in the mitochondrial genome appear to influence various aspects of carcinogenesis. Also relevant are the effects of some mutations on cancer prognosis and therapy response. The future deserves indebt analysis and validation of these changes for oncologic translation.

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Chapter 7
Mitochondrial Genetic Alterations in Cancer II

Renal, Urinary Bladder, Prostate, Ovarian, Endometrial, Cervical, Nervous System, Hematologic, and Connective Tissue Cancers

Abstract This chapter is a continuation of the previous chapter. It addresses similar mitochondrial genetic changes in renal, urinary bladder, prostate, ovarian, endometrial, cervical, nervous system, hematologic, and connective tissue cancers. Identical differences in mutation frequencies between various cancers are encountered in these cancers as well.

7.1 Introduction

This chapter examines mtDNA alterations in genitourinary, gynecologic, nervous system, hematologic system and connective tissue cancers. The organization is consistent with that of Chap. 6. For each cancer, the evidence for mtDNA polymorphisms as risk factors for cancer, mitochondrial microsatellite instabilities, somatic mutations, and mitochondrial gene expression changes are examined. Table 7.1 summarizes the mutation rate in various cancers examined.

7.2 Renal Cancer

Renal cell carcinoma (RCC) afflicts 190,000 people worldwide each year, with over 49,000 new cases and 11,000 deaths estimated for the US alone for 2009. RCCs are the commonest kidney cancers in adults with renal pelvic tumors being of urothelial origin and hence behave similarly to bladder cancers. Wilms tumor is a pediatric renal cell cancer, diagnosed in children under the age of 5. RCC is diagnosed usually in people over the age of 40. Males are more affected than females. Other risk factors for RCC include prolonged dialysis, hypertension, obesity, occupational chemical exposure, and importantly smoking. Smokers have a twofold increased risk for developing RCC compared to nonsmokers. Germline mutations

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Table 7.1 Somatic mtDNA mutations in cancer

Cancer	Somatic mutation frequency (%)
Renal	27–79
Urinary bladder	64–100
Prostate	19–88
Ovarian	20–80
Endometrial	9–63
Cervical	38–90
Nervous system	Upto 35
Hematologic malignancy	40–50
Connective tissue cancer	Upto 70

in the von Hippel-Lindau gene, SDH, and FH can predispose to the development of RCC as well.

Somatic Mitochondrial Microsatellite Instability in Renal Cancer: Wada et al. (2006) addressed the issue of mtMSI in RCC. This group determined the somatic mutation rate in Japanese patients with RCC by sequencing the D-loop of the mitochondrial genome, as well as assessing the levels of 8-OHdG, a marker of oxidative DNA damage. In this series, only 14% of tumor sequences had mutations that were primarily D310 changes. Interestingly, 8-OHdG levels were significantly elevated in malignant tissues in comparison to matched normal renal tissues. However, in many of the tumors with oxidative stress, D-loop mutations were absent. It will appear reactive oxygen species (ROS) does little damage to mitochondrial D-loop in renal cancer, but this conclusion is premature because technical issues of this study cannot be ignored.

Somatic Mitochondrial DNA Point Mutations in Renal Cancer: Somatic mutations have been the focus of many studies of mtDNA mutations in RCC. Probably the first mtDNA alteration in solid human tumors was demonstrated in renal oncocytomas. Welter et al. (1989) used five restriction endonucleases to examine mutation patterns in six renal oncocytomas. A *Hinf*1 restriction pattern revealed an additional 40 bp band in *COI* that appeared specific to renal oncocytomas because this band was absent in normal renal tissues and RCC samples. Tallini and coworkers (Tallini et al. 1994) were unable to demonstrate changes in restriction patterns in *COI* as well as in the D-loop in 19 renal oncocytomas. However, employing whole mitochondrial genome sequencing, Gasparre et al. (2008) uncovered eight mutations (seven somatic and one polymorphism) in all nine renal oncocytomas, with disproportionate number of mutations in complex I genes. Similarly, Mayr et al. (2008) found frameshift mutations in *ND1, ND4,* or *ND5* in 60% of oncocytomas they examined. Thus, somatic mtDNA mutations are present in renal oncocytomas and appear to cluster in complex I genes with possible contribution to the malignant phenotype (see Sect. 9.5.7).

Restriction fragment length polymorphism analysis of mtDNA in chromophobe RCCs revealed altered patterns in just 27% of the tumors (Kovacs et al. 1992). The tumors also had loss of heterozygosity at chromosomes 3p, 5q, 17p, and 17q. Nagy et al. (2002) sequenced the entire mitochondrial genome of chromophobe RCCs and matched normal renal tissues. Six somatic mutations were uncovered in 63% of

tumors. A "C" deletion in *ND5* and a "T" insertion in *ND3* caused frameshift mutations in two tumors. With the exception of a homoplasmic 9 bp deletion, all mutations were heteroplasmic. RCC specimens and nontumoral renal tissues from patients with end-stage renal disease were examined by whole genome sequencing (Nagy et al. 2003). Nine somatic alterations were found in 79% of the tumors. The mutations were distributed in both coding genes and the noncoding regions. Interestingly, the majority of transitions involved purines in contrast to the more frequently observed pyrimidine exchanges in other studies. Meierhofer et al. (2006) investigated the mtDNA mutation spectrum using the sensitive DHPLC method in RCCs in comparison to normal renal tissue obtained from nephrectomies. Overall 46% of the tumors had at least one heteroplasmic somatic mutation. Indeed, half of the tumors harbored heteroplasmies below 25%, which could easily be missed by conventional DNA sequencing. The mutations were in the D-loop and ribosomal genes. Importantly, nonsynonymous mutations in *ND2, ND5, ND4L* and *COI,* genes were also present in these tumors. In particular, one tumor had somatic MELAS A3243G mutation. Interestingly a study by Sangkhathat et al. (2005) had uncovered the MELAS A3243G mutation in rare pediatric RCC that also harbored a *TFE3* translocation. Jakupciak et al. (2008) detected somatic mutations in 69% of early stage renal cancers, and in 33% of matched urine samples. Thus, somatic mutations are frequent in RCCs, and could have clinical utility. The role of these mutations in the pathology of RCCs deserves further investigation.

Mitochondrial Gene Expression Changes in Renal Cancer: Selvanayagam and Rajaraman (1996) first reported on the loss of *ND3* transcripts which were seen in 62% of RCCs and 83% renal cancer cell lines. Using Northern blotting, Faure Vigny et al. (1996) performed a comprehensive expression analysis of both mitochondrial and nuclear genes involved in energy production. Mitochondrial transcripts were downregulated in renal tumors and tumor cell lines compared to normal cell lines. However, nuclear genes encoding OXPHOS proteins were upregulated in oncocytomas and in one advanced tumor. Importantly, ANT2 was highly expressed in these tumors. It should be noted that in contrast to ANT1, ANT2 cannot induce mitochondrial permeability transition and apoptosis (Bauer et al. 1999). Hence, overexpression will maintain normal ANT functions without apoptotic sensitization.

Mitochondrial enzymes, DNA and proteins involved in OXPHOS were studied in renal cell cancers of different aggressiveness from 25 patients (Simonnet et al. 2002). Mitochondrial enzymes and DNA content were significantly different in the various tumors compared to normal renal tissues. Respiratory chain protein levels were low, and this was associated with respiratory impairment and tumor aggression. Defective complex V assembly and stability resulted in decreased complex V content in clear cell and chromophilic RCC. But ATPase activity was not consistently depressed in these tumors, and tumors with decreased ATPase activity tended to be more aggressive. A subsequent study by Simonnet et al. (2003) found complex I activity and protein levels to be decreased specifically in renal oncocytomas. In general, there is a downregulation of mitochondrial proteins in RCCs and the biologic relevance of these findings requires clarification.

7.3 Bladder Cancer

Annually, over 300,000 people are diagnosed with bladder cancer worldwide. In the US, bladder cancer is the fourth most common cancer in men and the seventh in women. It is more common in men and especially whites than in women and blacks, but death rates tend to be higher in women and blacks than in white men. It is an age-associated cancer with the majority are diagnosed after the age of 80. In general, the incidence of bladder cancer is on the rise, but mortality is on the decline. The most common type of cancer is transitional cell carcinoma, but ~10% are squamous cell carcinoma and adenocarcinoma.

There are numerous risk factors for the development of bladder cancer. Environmental and occupational exposures to chemicals such as aromatic amines, bacterial, fungi, and viral infections of the bladder, bladder calculi, and adverse effects of chemotherapy can all initiate bladder cancer formation. However, the single most important risk factor is tobacco smoke. Smokers have an elevated risk of between five and sevenfold than that of individuals who have never smoked. Given that the risk factors of bladder cancer include chemicals, inflammations, and toxins that can all damage nucleic acids, early mtDNA involvement in bladder carcinogenesis calls for diverse investigation.

Somatic Mitochondrial Microsatellite Instability in Bladder Cancer: Changes in mitochondrial genome in bladder cancer have been addressed in just a handful of studies, and the primary focus had been the possible detection of these changes in matched urine samples, suggestive of clinical translation. Parrella et al. (2003) found a high frequency of D310 alterations in bladder cancer. However, the work by Wada et al. (2006) revealed D-loop mutations, mainly at D310, in just 23% of bladder cancers. In this study, 8-OHdG levels were significantly higher in bladder cancer than adjacent non-cancerous tissues, but many cancers with oxidative stress had no mtDNA mutations (Wada et al. 2006).

Somatic Mitochondrial DNA Mutations in Bladder Cancer: Fliss et al. (2000) were the first to demonstrate somatic mtDNA mutations in bladder cancer. In this study, conventional sequencing was employed. Even with this less sensitive method, somatic mutations were demonstrated in 64% of the tumors. Recent chip-based resequencing of whole genome revealed somatic mutations in all bladder cancers examined (Maitra et al. 2004; Jakupciak et al. 2008). While the number of tumors investigated in both studies are small, it appears whole genome sequencing of bladder cancers using sensitive technologies will reveal a high frequency of somatic mtDNA mutations.

7.4 Prostate Cancer

It is estimated that for 2009, ~190,000 new prostate cancers and ~27,000 deaths from the disease will occur in the US. Prostate cancer is thus one of the leading causes of cancer morbidity and mortality among men in the industrialized world.

Many men over the age of 50 years are diagnosed with prostate cancer, and the incidence increases with age. As an age-associated disease, the incidence of prostate cancer will remain on the rise, mirroring the aging Western population. Similar to other cancers, prostate cancer is debilitating when diagnosed late. Surgical resection of organ-confined tumors is the best available curative method. Therefore, to obtain optimal cure, it is imperative that methods are developed that enable early detection and monitoring of this disease. Epigenetic nuclear gene silencing and alterations in both nuclear and mitochondrial genomes precede histopathologic changes of prostate cancer.

Oxidative stress is strongly implicated in the etiology and pathogenesis of prostate cancer. Inflammatory cells produce and secrete excess ROS, and prostate inflammation is a prostate cancer risk factor. Proliferative inflammatory atrophy is a precursor lesion of prostate cancer. Promoter hypermethylation and loss of the antioxidant enzyme, *GSTP1,* is linked to the etiology of prostate cancer and high plasma concentration of antioxidants, such as carotenoid lycopene reduces the risk of this disease. As a major source of ROS in the cell, it is reasonable to assume an etiologic role for the mitochondrion in prostate carcinogenesis. Indeed, the mitochondrial genome is highly susceptible to oxidative damage and it serves as a useful repository of early disease-associated mutations. Hence, evaluation of mitochondrial alterations in prostate cancer should be considered as an important complement in patient diagnosis and management.

Mitochondrial Genome Polymorphisms and Risk of Prostate Cancer: Considerable amount of work by Petros et al. (2005) suggests that men with germline polymorphisms in the mitochondrial *COI* may have elevated risk of developing prostate cancer. This conclusion was reached after extensive epidemiological study of SNPs in *COI*. Analysis of 260 patients and 54 benign control men without cancer revealed a high frequency of *COI* polymorphisms in the cancer cohort compared to the control group. Subsequent analysis of mtDNA sequences from a much larger control population found the frequency of *COI* polymorphisms to be much lower than that observed in the cancer cohort. As *COI* polymorphisms are more frequent in African mitochondrial macrohaplogroup L than the rest of the world, data from only men of European descent were reanalyzed. Consistent with a possible risk-association, *COI* polymorphisms in the patients were still much higher than the control subjects without cancer and the general European population. The over-representation of *COI* changes in prostate cancer patients compared to individuals without cancer implicates polymorphisms in *COI* as possible risk factors for prostate cancer. Further evidence that *COI* alterations could confer prostate cancer risk among African–American men was recently provided (Ray et al. 2009). Sequencing of the entire *COI* in 132 patients and 135 control African–American men revealed 102 SNPs including 15 missense mutations. Two polymorphisms, a synonymous substitution (T6221C) and a haplogroup L substitution (T7389C) were significantly associated with prostate cancer in this cohort. In another investigation by the same group (Booker et al. 2006), PCR and restriction digest analysis was used to genotype white men with prostate and renal cancers and control white men without cancer. An over-representation of mitochondrial haplogroup U was

observed in cancer patients compared to control individuals. This haplogroup was shown to convey a significant risk for prostate cancer (odds ratio of 1.95) and even much higher risk for renal cancer (odds ratio of 2.52). When validated, these mtSNPs could serve as risk markers for individual's susceptibility for developing prostate cancer. Such high-risk people can then receive close surveillance for early detection of curable cancer. Alternatively, they could be offered chemoprevention therapy such as 5-α reductase inhibitors.

Somatic Mitochondrial DNA Point Mutations in Prostate Cancer: Mitochondrial genomics and proteomics play an integral role in the pathogenesis of prostate cancer. Oxidative stress might be responsible for somatic mtDNA mutations in prostate cancer as indicated by the frequent observation of T–C and G–A base transitions. As reviewed by Dakubo et al. (2006), several heteroplasmic and homoplasmic somatic mtDNA mutations have been reported in prostate cancer. Most of these studies focused on the D-loop region that is well known to be a mutational hotspot. Since mutations in coding and tRNA genes are equally pathogenic, we embarked on whole mitochondrial genome sequencing to uncover the complete mitochondrial genome mutation burden in prostate cancer. Using laser-capture microdissection (LCM), Parr et al. (2006) procured pure population of prostate cancer cells, normal looking adjacent and distant epithelial cells for sequencing using capillary electrophoresis method. As a control, normal prostate samples from individuals with benign biopsies were sequenced. DNA template from each patients' blood and those from their maternal relatives when available, were sequenced and used for pairwise comparison to score somatic mtDNA mutations. Intriguingly, somatic mtDNA mutations were frequent in all prostate samples from a malignant prostate, but very rare in prostate without cancer. Indeed, most of the mutations in the control group were found in the D-loop, with virtually no mutations in coding genes.

Interestingly two independent studies suggest that mtDNA mutations in prostate cancer might frequently be linked on the same molecule. Using LCM and mutant-specific PCR methodology, Chen et al. (2003) demonstrated that mtDNA mutations in prostate lesions were linked. We cloned and sequenced specific amplicons from patients, and observed six linked mutations in one patient sample (Parr et al. 2006). It is possible that mtDNA mutations occur simultaneously in prostate cancer cells, probably following extreme oxidative stress.

Mutations in the mitochondrial genome might occur early in prostate cancer development raising the possibility of early disease detection. Jeronimo et al. (2001) demonstrated identical homoplasmic mtDNA mutations in cancer and corresponding precursor lesion, prostatic intraepithelial neoplasia. Moreover, mtDNA mutations do not correlate with the patient's age, Gleason score or tumor stage, suggesting they are early mutation events that do not appear to modulate with age or disease progression. In addition, mtDNA mutations are present in tumors and the normal-appearing adjacent glands (Parr et al. 2006). The presence of mutations in normal cells close to tumor foci is consistent with prostate precancerous fields.

Mitochondrial Gene Expression Changes in Prostate Cancer: Mitochondrial genome alterations in prostate cancer reflect at the protein level as well. Evidence

for this was provided by the study of complex IV subunits by Herrmann et al. (2003). Quantitative proteomic analysis of normal, premalignant, and invasive prostate tumor tissues uncovered an increase in the ratio of nuclear encoded mitochondrial subunits IV, Vb, and VIc to mitochondrial encoded subunit I and II in several tumors. More importantly, these protein alterations were observed at the premalignant stage and increased with disease progression to invasive cancer, indicating the potential usefulness of such proteins as biomarkers for early prostate cancer detection, as well as targets for therapeutic intervention. Analysis of eight mitochondrial genes and nuclear genes involved in mitochondrial metabolism by RT-qPCR revealed significant decreases in *12SrRNA, COII*, and *ATP6* expression in prostate cancer compared to matched, normal adjacent tissues (Abril et al. 2008). Mitochondrial proteomics in prostate cancer requires validation for clinical translation.

7.5 Ovarian Cancer

It is estimated that in 2009, ~21,000 new cases of ovarian cancer will be diagnosed in the United States alone, with ~14,000 deaths expected. An important reason for the high mortality associated with ovarian cancer is that many patients present themselves with the advanced stage of the disease, making curative treatment almost impossible. Survival among late stage diseases ranges from 25 to 37% (Berek 2005). Early diagnosed cases have a better outcome with cure rate being ~90% (Hoskins 1995; Berek 2005). There are currently no effective screening tools for ovarian cancer. Therefore, there is a need to identify and develop molecular markers that can be detected in the blood of early stage diseases, or that which offer molecular imaging of ovarian tumors.

Somatic Mitochondrial Microsatellite Instability in Ovarian Cancer: Wang et al. (2005) performed extensive analysis of mtMSI in gynecological samples. First, mtMSI alterations in 262 pairs of female cancers and matched normal tissues were studied. The cancers included cervical, endometrial, ovarian and breast cancers. Of 12 mtMSI sites examined, loci beginning at np 303, 514, 956 and 16,184 were informative, with the majority (96%) of mtMSI located in D-loop microsatellite loci. Subsequently, Wang et al. (2006) evaluated mtMSI at these sites in cervical, ovarian, endometrial and breast tumors. Consistent with their previous findings, the four sites were unstable in 25% of cervical, 48% of endometrial, 22% of ovarian and 29% of breast cancers. These preliminary findings call for more studies of mtMSI in ovarian cancer, and what role they may play in disease etiology and prognosis.

Somatic Mitochondrial DNA Point Mutations in Ovarian Cancer: The presence of mtDNA D-loop mutations in primary ovarian cancers has been examined (Liu et al. 2001). Compared to matched-normal tissues as controls, somatic mutations were found in only 20% of tumors. A complete genome analysis of additional ovarian cancers, in parallel with matched normal ovarian tissues, increased the

frequency of mutations to 60%. The mutations were mainly homoplasmic T → C, and G → A transitions, and preferentially clustered in a 3 kb region encompassing *16SrRNA, 12SrRNA, CYTB* and the D-loop. This finding is in contrast with other cancers where complex I genes are disproportionately mutated. Shi and coworkers (Shi et al. 2002) sequenced the D-loop of a number of ovarian cancers and matched normal tissues. Twenty-six mutations were identified in 32% of the cancers. In a follow up study, the effect of chemotherapy on mtDNA mutations in ovarian cancer was examined (Shi et al. 2005). Whole genome sequencing of treated and untreated ovarian cancers identified 17 mutations with a rate of 71% of untreated and 56% of treated tumors. Nonsynonymous mutations were however much higher in those with chemotherapy than those without. The rate of polymorphisms was significantly higher in the treated than untreated groups as well. Van Trappen et al. (2007) examined mutations in the D-loop of primary ovarian samples from 35 patients, with 17 of them presenting with bilateral tumors and metastasis. Matched control tissue was used from each patient. Nine different homoplasmic mutations were found in 26% of the tumors. Four of the 17 bilateral tumors had different mutation patterns indicating different clonal origins of these tumors. A metastatic tumor harbored identical mutation to at least one bilateral primary tumor.

Li et al. (2003) examined mtDNA mutations in gynecological cancers including ovarian cancers and their matched normal tissues as well as normal ovarian tissues from donors. Whereas the rate of polymorphisms was identical between normal (50%) and gynecological cancers (56%), somatic mutation rates were quite different, with 69% and 25% of tumors and normal tissues harboring mutations. Aikhionbare et al. (2004) demonstrated an accelerated mutation rate within a 3.3 kb region of the mitochondrial genome (D-loop, *12SrRNA*, $tRNA^{Phe}$, $tRNA^{Val}$, $tRNA^{Ser}$, $tRNA^{Asp}$, $tRNA^{Lys}$, *COI, COII, ATP6* and *ATP8*). Three hundred and fifty-two sequence variants were uncovered, with three sites in *12SrRNA* (772, 773, and 780) being found at a relatively high frequency in stage IIIC endometriod cancers. A high frequency (81%) of D310 changes was demonstrated in early stage serous type tumors. In a follow up study, two regions of the mitochondrial genome (nps 5,317–7,608, and 8,282–10,110) were examined in relationship to the patient's age and ethnicity (Aikhionbare et al. 2008). One hundred and eighteen frozen tissues of three subtypes of epithelial ovarian cancer made up of serous, endometriod, and mucinous as well as matched normal control ovarian tissues were included in the study. Thirty-nine sequence variants were uncovered with only one somatic mutation (C9500T). Some interesting mutation frequencies in tumor subtypes and ethnic backgrounds were noted. A C → T change at np 7,028 was present in only 8% of borderline tumors, but increased to 75% in stage III/IV cancers. Variant T8548G occurred in 72% of serous type stage III/IV while C7256T and G7520A were found in 54% of endometriod type cancers but not in serous epithelial ovarian cancers. Variants C7028T and C7520T were disproportionately found at a higher frequency in African–American women compared to Caucasian women. C7028T was found in 86% African–American compared to only 43% of Caucasian women; C7520T was enriched in 74% of African–American but found in only 26% of Caucasian women. For women under the age of 40, variant T8548G was present in 95% of

African–American and 22% Caucasian women. Somatic mtDNA mutations are frequent in ovarian cancer, and specific variants might be associated with people from different ethnic backgrounds or tumor stage.

7.6 Endometrial Cancer

The global annual incidence of endometrial cancer is 188,000, with an estimated 42,160 new cases and 7,780 deaths expected for the US in 2009. They are more frequently diagnosed in women over the age of 50, but the incidence in younger women is on the rise. These are primarily adenocarcinomas.

White women are at a higher risk than African–American women, and women with hereditary colorectal cancer are disproportionately at risk as well. Unopposed estrogenic effects on the endometrium are probably the single most important risk factor. Obese women have more estrogen than non-obese women because estrogen is made in adipocytes as well. Hence obesity is a risk factor. Similarly, women on hormone replacement therapy composed primarily of estrogen have elevated risk of endometrial cancer. HRT composition including progesterone lowers the risk and is now the recommended therapy for postmenopausal women. The estrogenic effects of tamoxifen pose a risk, but the benefits of tamoxifen for breast cancer treatment outweighs the risk of developing endometrial cancer.

Mitochondrial Genome Polymorphisms and Risk of Endometrial Cancer: A few studies have examined the role of polymorphic variants as risk factors for developing endometrial cancer. Xu et al. (2006) implicates haplogroup D alleles with increased risk of endometrial cancer in Chinese women; however this conclusion is based on only 49 cases and 31 controls. Similarly, in a different study of Asian women, the T → C polymorphism at np 16,189 is suggested as a risk factor for endometrial cancer (Liu et al. 2003a). Recently in a Polish series, the defining polymorphism, C7028T, of haplogroup H was significantly rare in endometrial cancer patients leading the authors to suggest a protective role for this polymorphism (Czarnecka et al. 2009). In this Polish study, three polymorphisms, 16223C (80% of patients), 16126C (23% of patients) and 207A (19% of patients) were significantly overrepresented in cancer patients. Studies of this nature are important for identifying high-risk women for close monitoring.

Somatic Mitochondrial Microsatellite Instability in Endometrial Cancer: Wang and colleagues have addressed mitochondrial genome alterations in several gynecologic cancers including endometrial cancer (Liu et al. 2003a, b; Wang et al. 2006, 2007). Liu et al. (2003b) investigated the mutation spectrum of a portion of the mitochondrial genome in endometrial carcinomas. Primary endometrial carcinomas were amplified and sequenced through the D-loop as well as the two rRNA genes. Over half of the cancers had at least a single mutation, with the majority (78%) of the changes in the D-loop. Of the D-loop mutations, 44% were mtMSI at D310. Wang et al. (2006) subsequently evaluated mtMSI in a number of endometrial cancers, in addition to other female cancers. Of the four gynecological cancers

investigated by this group, endometrial cancer had the highest frequency of instability. Overall, 48% of the cancer samples had somatic mtMSI changes.

Somatic Mitochondrial DNA Point Mutations in Endometrial Cancer: The mutation patterns in the D-loop, *12SrRNA* and *16SrRNA* of the mitochondrial genome in endometrial carcinomas, were investigated (Liu et al. 2003a). Primary endometrial carcinomas and paired normal control tissues (cervical or lymphocytes) were amplified and sequenced. Somatic mutations were demonstrated in 56% of the cancers. Semczuk et al. (2006) analyzed a set of endometrial tumors collected in parallel with normal tissues (cervix, omentum or blood) for mtDNA sequence variants. All or parts of HVSI, *16SrRNA*, $tRNA^{Leu1}$, *ND2*, *ND3*, *ND4*, *ND4L* and *ND5* were sequenced. Mutations were found at low frequencies in both early (9%) and advanced stage endometrial cancers (12%). In contrast to Semczuk et al. (2006), Pejovic et al. (2004) uncovered mutations in 63% of uterine serous carcinomas, using single-strand conformation polymorphism (SSCP) analysis. Malignant tissues were compared with adjacent matched, histopathologically normal endometrium. Wang et al. (2007) demonstrated the randomness of occurrence and independent expansion of mtDNA mutations in endometrial cancer. Using LCM they obtained cells from different areas of the same endometrial cancer, and demonstrated a heterogeneous heteroplasmic mutation pattern among the samples.

7.7 Cervical Cancer

Cancer of the uterine cervix is one of the most common gynecologic cancers, though it is associated with lower mortality than others such as ovarian cancer. Globally 470,000 cases are diagnosed annually, with just ~11,000 cases and ~4,000 deaths each year in the US. A single most important etiologic agent is human papilloma virus (HPV) infections of the cervix. Thus, male or female promiscuity increases the probability of infection and hence of cervical cancer. Not all women with HPV infections develop cervical cancer, therefore modulating factors such as genetics, lifestyle such as smoking, and other exposures may play a role. Unlike many cancers that are diagnosed commonly in people over 50, cervical cancer mostly affect younger-aged women, between the ages of 30–55 years. Most (~90%) are squamous cell carcinomas, but adenocarcinomas are occasionally found.

Somatic Mitochondrial Microsatellite Instability in Cervical Cancer: Wang et al. (2006), in addition to their work on breast cancer, evaluated mtMSI in a number of cervical cancers. Mitochondrial MSI at two loci was demonstrated in 25% of these tumors. Chen and Zhan (2009) found that 27% of D-loop mutations in cervical cancer were mtMSIs.

Somatic Mitochondrial DNA Point Mutations in Cervical Cancer: Displacement-loop sequence alterations were examined in 19 patients with cervical cancers (Sharma et al. 2005). Mutations were detected in 95% of the patient samples, and these mutations appeared to correlate with HPV infections. The authors indicated that mutation frequency was however lower in lymphocytes and normal cells from

the patients, indicating mutations were scored with sequence comparison to the rCRS. Analysis of D-loop sequence in 24 cervical cancers and matched normal tissues uncovered 30 mutations in 38% of the cancers (Chen and Zhan 2009).

Mitochondrial genome mutations as prognostic markers in women with invasive cervical cancer were sought by Allalunis-Turner et al. (2006). They sequenced mitochondrial complex I genes in a 10-year retrospective invasive cervical cancer samples and found that a third of the cancers harbored multiple mutations. Univariate and multivariate analysis revealed significant poor outcomes in patients with eight or more mutations, and the log-rank analysis indicated a significant difference in overall survival, suggesting the importance of multiple mtDNA mutations in coding genes as prognostic markers.

7.8 Nervous System Tumors

Somatic Mitochondrial Microsatellite Instability in Nervous System Tumors: Kirches et al. (2001) used LCM to recover glioblastoma cells from tumors in parallel with adjacent normal brain tissues for mtDNA analysis. Alterations at D310 were found at a low frequency of 9%. The entire D-loop was sequenced in glioblastomas and compared to the patient's blood for scoring somatic mutations. Instability at other repeats was detected in as high as 35% of the cancers, suggesting that the frequency of 9% was due to mutations being overshadowed from inappropriate use of control tissues. In two cancers, identical mutations were present in low-grade tumors, suggesting the early onset of these changes. D310 as a clonal marker for glioblastoma cerebri was later addressed in two patients (Kirches et al. 2003). Mutated mtDNA was able to determine the clonal relationships of the tumors. In one patient, the mutations were identical in tumors with *p53* mutations.

Somatic Mitochondrial DNA Point Mutations in Nervous System Tumors: Few studies have examined somatic mtDNA point mutations in nervous system tumors, including medulloblastoma, gliomas, astrocytomas and neurofibromas. Analyzes of the entire genome in medulloblastomas, revealed at least one somatic mutation, in 40% of the tumors (Wong et al. 2003). Seven matched cerebrospinal fluid samples harbored mutations, some of which were shared with the tumors. These findings suggest the potential use of mtDNA changes in the management of brain tumor patients. Montanini et al. (2005) found somatic mutations in the D-loop in 36% of gliomas. In this study, mtDNA mutations were easily detected in surgical cavities suggesting the possible utility of mtDNA changes in follow up management of patients. The entire mitochondrial genome sequencing of pilocytic astrocytomas and matched blood revealed somatic mutations in as many as 84% of the tumors. Of the 34 mutations uncovered, half involved OXPHOS genes (Lueth et al. 2009). Kurtz et al. (2004) scanned the entire mitochondrial genome of neurofibromas from neurofibromatosis type 1 patients. Somatic mutations were present in 37% of patients with cutaneous neurofibromas and 50% of patients with plexiform neurofibromas. These frequencies were much higher than mutations in the non-tumor control tissues.

All the mutations were in the D-loop region. Cutaneous neurofibromas from different locations harbored the same mutations indicating clonal origin of these tumors.

Mitochondrial Gene Expression Changes in Nervous System Tumors: Dmitrenko et al. (2005) screened cDNA libraries of glioblastoma and normal brain samples and uncovered 80 differentially expressed genes, 30 of which corresponded to mitochondrial *ATP6, COII, COIII, ND1, ND4,* and *12SrRNA*. Compared to normal adjacent to tumor tissues, these transcripts were all downregulated in glioblastomas. Serial analysis of gene expression revealed lower content of tags for all mitochondrial genes in glioblastoma libraries.

7.9 Hematologic Malignancy

The bone marrow is a known target of severe pathogenic mtDNA heteroplasmies, demonstrated by the Pearson syndrome. Although rare, this syndrome is characterized by exocrine pancreas insufficiency, lactic acidosis, and severe refractory anemia that can only be curtailed by repeated transfusions. The genetic disorder is large-scale mtDNA deletions. Pearson syndrome is so lethal that most affected children never live past their third birthday. However, some survive and progress to develop Kearns–Sayre syndrome, while others almost recover and become even transfusion-independent.

Myelodysplastic syndrome (MDS) is a clonal hematopoietic stem cell disorder affecting hematopoietic cell proliferation and differentiation. They are heterogeneous disorders defined by cytopenias from ineffective hematopoiesis. Primary MDS is usually due to environmental insults such as benzene exposure or radiation, while secondary MDS arise from chemotherapy, especially involving combined modalities. Most therapy-related MDS will eventually lead to refractory acute myeloid leukemia. However, leukemic transformation is more common in refractory anemia with excess blasts (RAEB) subclass of MDS. Acquired sideroblastic anemia is MDS labeled as refractory anemia with ring sideroblasts. The WHO however, has several categories of MDS.

The leukemias and lymphomas are hematologic neoplastic transformation of bone marrow and lymphoid cells respectively. There are several subcategories of these cancers with different behaviors.

Somatic Mitochondrial DNA Point Mutations in Hematologic Malignancy: Lombes et al. (1998) demonstrated the first pathogenic mutation in lymphoma. Using electron microscopy, they initially observed abnormal forms of mitochondria (seen as large cytoplasmic inclusions) in lymphocytes from patients with splenic lymphoma. Subsequent mutation scanning was then pursued using denaturing gradient gel electrophoresis as a screening tool. A G → A transition at np 4,450 in $tRNA^{Met}$ was demonstrated. The pathogenic nature of this mutation was revealed by the transfer of the mutation into ρ0 cells that resulted in severe respiratory chain dysfunction.

He et al. (2003) studied the incidence of somatic mutations in patients with adult-onset leukemia, by sequencing the entire mitochondrial genome from bucal

epithelial cells (germline) and leukemic cells. Pairwise sequence comparison revealed mutations in ~40% of the patients. Carew et al. (2003) examined the relationship between mtDNA mutations, oxidative stress and clinical parameters in chronic lymphocytic leukemias (CLL). Sequence analysis of mtDNA from primary CLL cells uncovered significantly higher heteroplasmic mutations in treated than untreated cases. Similarly, patients refractory to chemotherapy harbored higher mutations that those responsive to treatment. These mutations were associated with increased oxidative stress and persisted several months after treatment. In another study, this group observed significantly increased levels of both mitochondria and nitric oxide (NO) in CLL compared to normal lymphocytes (Carew et al. 2004). Factors mediating mitochondrial biogenesis such as NRF1 and TFAM were upregulated in CLL. NO appear to induce mitochondrial mass because treatment of B cells with exogenous NO caused an increase in mitochondrial mass. Cells with greater mitochondrial content were observed to be less sensitive to fludarabine chemotherapy. Yao et al. (2007) examined the D-loop profile in single cells derived from healthy people and patients with leukemia. In total, 3,534 cells from normal cells and blasts from 18 patients and ten controls were studied. Complex somatic mutation pattern was observed in leukemic blast cells. However, in four patients there was a high frequency of cells with hits at np 189, 260, 16,150 and 16,488.

To study the role of mtDNA mutations in the evolution of MDS to acute myeloid leukemia, Linnartz and coworkers used SSCP and sequencing to study the entire mtDNA molecule of blood from ten patients with MDS (Linnartz et al. 2004). Mutations were found in 50% of these patients, and mutation load appeared to mediate transformation of MDS to AML. Wulfert et al. (2008) addressed the issue of the spectrum and frequency of somatic mutations in bone marrow of people with premalignant hematologic diseases. In this study, 104 patients with MDS, three with acute myeloid leukemia that developed from MDS, and 36 people with myeloproliferative diseases were included in this study. Using the sensitive DHPLC method with 67 fragments covering the entire mitochondrial genome, coupled with sequencing of heteroduplex fragments, heteroplasmic somatic mutations, mainly transitions, were uncovered in 56 and 44% of people with MDS and myeloproliferative disease respectively. The mutation frequency in people with MDS appeared to increase with age and advanced stage disease. Although less frequently hit, ~50% of mRNA mutations involved conserved amino acids.

Mitochondrial iron overload is a feature of acquired idiopathic sideroblastic anemia (AISA), a MDS characterized by refractory anemia with ring sideroblasts. Gattermann et al. (1997) thought that this disorder could relate to mtDNA mutations and defective iron metabolism. Analysis of samples from two patients with AISA uncovered two heteroplasmic nonsynonymous transition mutations in *COI*. Both mutations involved conserved amino acids and affected the same transmembrane helix. The mutations were present in bone marrow, blood, platelets, and granulocytes but not in T- and B-lymphocytes, bucal cells or fibroblasts of patients. In a patient with RAEB, 40–50% of bone marrow mtDNA contained a $tRNA^{(Leu(UUR))}$ mutation (G3242A) (Gattermann et al. 2004). The levels of the mutation were much higher in CD34+ cells than in whole peripheral blood sample.

The mutation was even undetectable in blood using heteroduplex analysis, but was detected in CD34+ cells at about 50% heteroplasmy.

Cytochrome oxidase subunits I and II genes were examined for mutations in bone marrow, peripheral blood and bucal samples from patients with MDS and age-matched controls (Reddy et al. 2002). Mutations in *COI* occurred in 65% of the patients, while *COII* mutations were at a similar frequency of 60%. Two healthy individuals had *COII* mutations as well. Most common sites identified in this study were np 7,264 (25%) and 7,289 (15%) in *COI*, and 7,595 (40%) and 7,594 (30%) in *COII*. Whole genome sequencing of samples from patients with MDS and controls uncovered similar frequencies of polymorphisms in both groups (Shin et al. 2003). However, nucleotide variants in patients with MDS resulted in more amino acid changes than in controls. Overall, 30 nucleotide substitutions were found in patients, five of which were nonsynonymous changes. Apparently, none of the mutations in the control group involved changes in amino acids. Hou et al. (2008) also sequenced *COI* and *COII* between np 7,181 and 7,709 of patients with MDS. DNA from both bucal and bone marrow cells were used for sequencing. Only three mutations were observed, all of which were nonsynonymous changes (T7674C, A7353G, and a frameshift G insert at np 7,702).

Complex Mitochondrial DNA Structures in Hematologic Malignancy: Several groups have studied mtDNA structure in hematologic malignancies using electron microscopy and other assays. Clayton and Vinograd (1967) were first to report abnormal mitochondrial genomes in leukocytes from leukemic patients. In this initial report, circular dimers, catenated dimers, and catenated trimes were demonstrated in blood from leukemic patients. The circular dimers were duplicated mtDNA while the catenated forms were interlocking pairs of mtDNA. Subsequent study of these structural forms in leukocytes from patients with acute and chronic granulocytic leukemias revealed the presence of these complex mtDNA molecules in all samples, while normal healthy leukocytes from three individuals were free of them (Clayton and Vinograd 1969). Intriguingly, the authors demonstrated a decrease in the proportion of the abnormal mtDNA molecules in some patients following chemotherapy. In one individual patient with chronic granulocytic leukemia, a 3-week treatment reduced the levels of abnormal mitochondria from 21 to just 7%. In a larger study by the same group, leukocytes from leukemic patients and patients with other solid tumors were examined (Clayton and Smith 1975). In general, leukemic patients harbored greater percentages of circular dimers than patients with other forms of solid tumors. The levels of circular dimers were much higher in leukocytes from patients with acute and chronic granulocytic leukemias than patients with acute and CLL. Catenated forms and higher oligomers were identical between granulocytic and lymphocytic patients. Again, chemotherapy reduced the levels of circular dimers in this cohort.

Other groups have demonstrated abnormal mitochondrial genomes in hematologic malignancies as well. Firkin and Clark-Walker (1979) used electron microscopy to study mtDNA isolated from leukocytes of patients with acute leukemia and poorly differentiated lymphocytic lymphoma. While normal leucocytes contained

monomeric circular mtDNA molecules, leukocytes from patients had circles that were twice the size of normal, as well as multiple interlocking circles in some cases of untreated acute leukemia and lymphoma. Strikingly, up to 47% of the mitochondria in patients' leukocytes were in this complex form. Robberson et al. (1981) studied patients with acute myelogenous leukemia. In addition to circular dimers, which were the predominant forms, they also observed pentameric mtDNA structures composed of five circular mtDNA molecules. Importantly, this group was first to demonstrate the origin of these abnormal mtDNA as being in the bone marrow.

Miguel et al. (1982) studied the structure of mtDNA isolated from normal lymphocytes and lymphocytes stimulated with phytohemagglutinin as well as Burkitt's lymphoma cell lines. Compared to normal lymphocytes that contained monomeric forms, the other cell types contained catenated mtDNA as well. Christiansen et al. (1983) uncovered a high frequency (9–15%) of catenated dimmer, circular dimers or oligomers in Epstein–Barr-virus transformed lymphoblastoid cell lines compared to lymphocytes (1.3–4.6%), and primary lymphocytes (1.1%). Burkitt's lymphoma cell line possessed intermediate levels (5–7%).

Oncogenic transformation in lymphoid cells appears to be associated with the formation of mtDNA concatemers. An attempt to study extrachromosomal oncogenic viral DNA in primary lymphomas led to the identification of mitochondrial genome concatemers (Bedoya et al. 2009). Detailed analysis of an AIDS-associated lymphoma revealed tandem head-to-tail duplication with insertions of "C" residues near the origin of replication. Importantly, these abnormal forms of mtDNA were present in several other AIDS-associated lymphomas. The lymphomas had reduced mitochondrial mass and activity with increased ROS production, and were resistant to Fas-mediated apoptosis (Bedoya et al. 2009).

Gene Expression Changes in Hematologic Malignancy: Transcription of mtDNA was studied in two murine large cell lymphoma cell lines. Specifically, *ND5* was highly overexpressed in the highly metastatic cell line compared to the one with low metastatic potential (LaBiche et al. 1992). Clark et al. (2002) examined the role of complex mtDNA forms in leukemogesis by studying differential gene expression. Gene expression in isogenic cell lines containing monomer and dimmer mtDNA was examined using Affymetrix gene chip. Differential expression profiles were observed, especially among genes involved with cell surface and extracellular remodeling.

7.10 Connective Tissue Cancer

Only a single study addresses mtDNA mutations in sarcomas. The work of Guo and Guo (2006) involved sequencing of the D-loop of DNA extracted from osteosarcomas from patients. Appropriate sequence comparison to blood was made and lack of primer amplification of targets from ρ0 was ensured. Mutations, mainly in HVSI and II were found in 70% of patients' samples.

7.11 Conclusion

Mitochondrial DNA alterations in cancers covered in this chapter are consistent with those in Chap. 6. The polymorphic variants, instabilities at microsatellites, somatic point mutations and gene expression changes all have biological implications for the various cancers, and offer opportunities for early detection, and disease monitoring. The functional importance of mitochondrial genome changes in cancer needs to be established, because this could offer powerful novel pharmacological approaches to treatment.

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Chapter 8
Mitochondrial Genome Rearrangements and Copy Number Changes in Cancer

Abstract Mitochondrial genome alterations (besides point mutations) are presented in this chapter. Large-sale mtDNA deletions are demonstrated in several cancers. Importantly, several groups have assayed the age-associated common deletion in various cancers, and the emerging trend is the reduced levels or virtual absence of the deletion in cancer, in contrast to its accumulation in postmitotic tissues with aging. An overview of mitochondrial genome deletion disorders, large-scale deletions and copy number or content alterations in cancer are discussed. This chapter ends with a description of the putative mechanisms that mediate deletion formation, and how mtDNA deletions and copy number changes are regulated.

8.1 Introduction

Large-scale mitochondrial DNA (mtDNA) deletions are ubiquitous in both mitotic and post-mitotic tissues. Many mitochondrial cytopathies including progressive external ophthalmoplegia (PEO), Pearson syndrome (PS), Kearns–Sayre syndrome (KSS), and mitochondrial neurogastrointestinal encephalopathy (MNGIE), and the aging process are associated with pathogenic large-scale mtDNA deletions. In the past few decades, several novel, as well as reported mtDNA deletions have been identified in different types of cancers. In particular, the role of the common mtDNA deletion in cancer is well characterized. Whereas the pathogenic nature of these mutations are being deciphered at the moment, the preferential increase or decrease in frequency of these deletions in tumors compared to normal and perilesional tissues seems to implicate them in the carcinogenic process. That apart, quantitative measurement of mtDNA deletions in tumors and available body fluids could serve as biomarkers for disease detection and monitoring. mtDNA copy number or content differences between cancer and normal tissues are also demonstrated in a number of cancers, and are potentially useful for cancer diagnosis and monitoring. This chapter examines large-scale mtDNA deletions and associated content changes in cancer.

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8.2 Overview of mtDNA Deletion Disorders

Since the initial report by Holt et al. (1988) of mtDNA deletions in patients with mitochondrial myopathies, large-scale deletions in the mitochondrial genome in several diseases have appeared in the literature. mtDNA rearrangements are predominant in rare mitochondrial diseases such as PEO, PS, KSS, and MNGIE. Moreover it is well proven that the aging process is linked to multiple mtDNA deletions in association with cell death and functional decline of affected tissues and organs.

A hallmark of diseases characterized by large-scale mtDNA deletions is mitochondrial respiratory dysfunction, especially in tissues with high-energy demands such as nervous tissues and muscle. Many mtDNA deletion disorders present with PEO and mitochondrial proliferation in skeletal muscles often observed as ragged-red fibers. PEO can occur as an autosomal dominant disorder caused by inactivating mutations in *polymerase gamma, ANT1, or PEO1/C10orf2* (formally called twinkle), or as an autosomal recessive disorder with two phenotypes, autosomal recessive cardiomyopathy and ophthalmoplagia (ARCO), and MNGIE.

The most severe phenotype of multiple mtDNA deletion disorders is the PS where patients usually develop sideroblastic anemia and dysfunctional exocrine pancreas. This disease is mostly fatal during the first year of life. A milder phenotype called the KSS is a disease that involves many systems and has an onset at an early age. PEO, pigmentary retinal degeneration and at least an association with cerebellar ataxia, cardiac conduction defects or elevated CSF proteins above 100 mg/dl characterize the clinical presentation of KSS.

Another mitochondrial pathology characterized by site-specific point mutations, multiple deletions and depletion of mtDNA is MNGIE. This is an autosomal recessive disorder caused by a mutation in nuclear-encoded thymidine phosphatase (TP) gene. Although not a mitochondrially targeted protein, mutations in TP are associated with generalized elevated serum levels of deoxythymidine and deoxyuridine leading to mitochondrial nucleotide imbalances and hence mtDNA damage. MNGIE is characterized by PEO, ptosis, gastrointestinal dysmotility, cachexia, peripheral neuropathy, myopathy and leukoencephalopathy.

Whereas these well-established disorders appear to result primarily from nuclear gene mutations leading to mitochondrial genome deletions and depletion, they emphasize the pathogenic nature of mtDNA deletion mutations. Thus, tissue-specific somatic deletion and depletion of mtDNA could have specific pathologies depending on the heteroplasmic levels of these mutations.

8.3 Mitochondrial Genome Deletions in Cancer

Several different deleted mtDNA molecules are reported in various cancerous tissues. The common deletion (CD), however, is the most extensively studied deletion by many groups. The CD is a class I deletion (see Chap. 5) in the major

deletion arc flanked by 13 bp direct repeats. The deletion occurs between np 8,469 and 13,447, and removes *ATP, COIII, ND3, ND4, ND4L* and parts of *ND5*. Variants of this classic deletion are also reported in other tissues and disease states. The CD was initially reported in tissues of older individuals (Cortopassi and Arnheim 1990), and several groups have demonstrated increased amounts in various post-mitotic tissues with advancing age, hence it is well-deserved designation as the age-associated mtDNA deletion. Interestingly, the CD is reported to occur at various frequencies and quantities in different cancers compared to matched normal tissues (Tables 8.1–8.3). In contrast to the aging process, several tumors rather harbor less amounts of the deletion than normal tissues. Apart from the CD, several other deletions in the major and minor deletion arcs, as well as tandem duplications, triplications, and other rearrangements are reported in a number of cancers.

Table 8.1 Mitochondrial DNA deletions in skin, head and neck, thyroid gland, and parotid gland cancer

Cancer	Findings	References
Skin cancer	Amount of deletion significantly lower in BCC/SCC than normal epidermal cells	Kamenisch et al. (2007)
	De novo CD found in 10% of melanomas. Duplications and CD found in 20% of melanomas	Poetsch et al. (2004)
	Deletion much higher in BCC/SCC and sun exposed skin than non-exposed skin. Rapidly growing SCC had lower levels than normal skin	Yang et al. (2004)
	Deletion present at various levels in NMSC, but absent in perilesional epidermis of BCC patients	Durham et al. (2003)
Head and neck cancer	Deletion levels more frequent in normal adjacent tissues than tumor	Dani et al. (2004)
	A de novo common deletion detected in 25% of cancers	Poetsch et al. (2004)
	Deletion levels much lower in oral cancer than pre-cancer tissues	Shieh et al. (2004)
	Novel common deletion more frequent in tumors than matched blood	Shao et al. (2004a, b)
	Deletion levels lower in oral tumors than benign oral tissues	Lee et al. (2001)
Thyroid gland cancer	Deletions high in Hurtle cell tumors. Deleted molecules higher in tumors with D-loop mutations	Maximo et al. (2002)
	Deletion increased in radiation-associated papillary thyroid tumors but not sporadic PTCs	Rogounovitch et al. (2002)
	Deletion is the same in oncocytic thyroid and renal tumors	Tallini et al. (1994)
Parotid gland cancer	Deletion present in 10% of Warthins tumors and all parotid tumors	Lewis et al. (2000)
	Deletion levels much higher in oncocytic tumors	

Table 8.2 Mitochondrial DNA deletions in breast, lung, esophageal, gastric and colorectal cancer

Cancer	Findings	References
Breast cancer	Deletion lower in cancer and benign breast disease tissues than normal adjacent	Ye et al. (2008)
	Deletion higher in non-tumor tissue	Tseng et al. (2006)
	Deletion more frequent in normal adjacent tissue than tumor	Dani et al. (2004)
	Deletion not specific to breast cancer	Zhu et al. (2004)
Lung cancer	No significant difference between tumor and non-tumor tissue; deletion correlated with age and smoking	Dai et al. (2006)
Esophageal cancer	Deletion frequency mirrored degree of dysplasia, but low in cancer	Tan et al. (2009)
	Deletion detected in 92% of tumor and normal adjacent tissue but not in blood	Abnet et al. (2004)
Gastric cancer	CD correlated with ROS and MnSOD production in cancer	Wang and Lu (2009)
	Deletion more frequent in normal adjacent tissues than tumor tissues	Kamalidehghan et al. (2006a)
	An ~8.9 kb deletion more frequent in tumors than normal adjacent tissues	Kamalidehghan et al. (2006b)
	Deletions more frequent in benign tissues than tumor tissues	Wu et al. (2005)
	Deletion more frequent in normal adjacent tissues than tumor tissues	Dani et al. (2004)
	Deletions inversely related to point mutations. Deletions more frequent in nMSI-negative tumors	Maximo et al. (2001)
Colorectal cancer	Deletion more frequent in normal adjacent tissues than tumor tissues	Dani et al. (2004)

Possibly, yet to be discovered are cancer-specific deletions outside these conventional major and minor deletion arcs that have been the focus of numerous investigations.

Ultraviolet Radiation and mtDNA Deletions in Skin: By analysis of multiple skin samples taken from different areas of an 86-year-old man, it was observed that the frequency of the CD depended on sun exposure and cellular growth rates (Pang et al. 1994). Their analysis of samples taken from various sites of this male farmer with skin cancer revealed various levels of the CD in tissues from different skin sites. The levels of the CD differed between sun exposed body parts and cancer specimens. Sun exposed skin harbored higher levels of the CD than non-exposed areas of the skin. This implicates induction of the CD by ultraviolet radiation from sunlight. Faster growing precancerous and cancerous tissues harbored less CD. Thus, the deletion is probably intolerable by normal cells, hence its elimination as cells divide. In addition to the CD, four novel deletions (7,031, 7,150, 7,288, and 7,485 bp) were present in skin samples.

Yang et al. (2004) uncovered a 7,436 bp deletion as well as D-loop tandem duplications (260, 200, and 150 bp duplications) in sun-exposed skin. The deletions

Table 8.3 Mitochondrial DNA deletions in liver, renal, prostate, and endometrial cancer

Cancer	Findings	References
Liver cancer	Deletion more frequent in normal liver and normal adjacent to tumor liver tissues than HCC	Wheelhouse et al. (2005)
	Novel CD (4,981 bp) more frequent in HCC that normal adjacent tissue	Shao et al. (2004a, b)
	Deletion in benign liver of alcoholics higher than non-alcoholics	Yin et al. (2004)
	Deletion much lower in HCC compared to normal liver	Kotake et al. (1999)
	Deletion significantly decreased in tumors compared to normal liver	Fukushima et al. (1995)
	Multiple deletions in cirrhotic liver, but not in HCC	Yamamoto et al. (1992)
Renal cancer	A 264 bp deletion appeared specific to the etiology of renal cancer	Horton et al. (1996)
Prostate cancer	The number of unidentified deletions correlated with age	Jessie et al. (2001)
	A specific deletion (3.4 kb) is quantitatively higher in cancer than normal prostatic tissue	Maki et al. (2008)
	mtDNA deletions conferred androgen-independence in prostate cancer	Higuchi et al. (2006)
Endometrial cancer	Deletion to wildtype mtDNA ratio much higher in normal endometrial tissues than cancer in women 50–60 years. Amount of deletion significantly different between stage II and III cancers	Futyma et al. (2008)

appeared by the age of 30 and the 200 bp duplication was more frequent in the skin of the elderly. The question as to whether mtDNA deletions in skin were associated with chronological or photo aging was addressed using the CD (Birch-Machin et al. 1998). A significant increase in the levels of the CD was found in sun-exposed skin compared to sun-protected, and the frequency was not related to age. Split skin sample analysis revealed the almost dermal accumulation of the CD with several epidermal tumors being free of the deletion. This further confirms the avoidance of the CD by fast growing cells. Ray et al. (2000) demonstrated the occurrence of deletions in the epidermis with increasing sun exposure. They used long-extension PCR method to scan the spectrum of deletions in purified mitochondria from split skin samples. They demonstrated significant increases in deletions with sun-exposure in the epidermis but not the dermis.

Melanoma: mtDNA deletions in perilesional skin samples from melanoma patients have been studied. Hubbard et al. (2008) analyzed the CD as well as a putatively UVB-induced 5,128 bp deletion in normal skin close to melanoma. The deletions correlated with the patient's age. However, when patients were put into three categories based on skin pigmentation and response to sunlight exposure (low, intermediate and high phenotypic indices), the deletions, were found to be much more abundant in the intermediate group. Interestingly, people in this group were also more likely to develop melanoma later in life. Could the deletion be protective against melanoma? mtDNA copy number was much higher in the high-risk (low phenotypic index) group than the other groups.

DNA fragmentation and mtDNA deletions were examined in skin samples from melanoma patients, and the results correlated to skin specimen classification, as to *glutathione-S-transferase M1* (*GSTM1*) wildtype or null genotype, with or without sun exposure (Steinberg et al. 2009). *Glutathione-S-transferase M1* null individuals with sunburn history had increased DNA fragmentation and mtDNA deletions compared to *GSTM1* wildtype people with little or no sun exposure. Solar radiation causes persistent DNA damage increasing the risk of skin cancer.

Non-Melanoma Skin Cancer: Durham et al. (2003) were the first to investigate mtDNA deletions in NMSC. Using long range PCR, the major and minor deletion arcs were amplified from a series of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) samples as well as corresponding normal skin samples. Multiple deletions were observed in the major arc, and expectedly with a smaller number uncovered in the minor arc. Intriguingly, a deletion of ~4 kb in size was found in all samples that harbored deletions. The CD was identified in the dermis of four of five BCC matched normal skin samples, but in only two of the five corresponding tumors, suggestive of the idea that tumors do not tolerate the CD. Moreover, the CD was absent in the epidermis of all BCC samples. This finding is possibly because the epidermis turns over more quickly. In a subsequent study the deletion was identified as a specific 3,895 bp deleted sequence in the minor arc (Harbottle et al. 2004; Harbottle and Birch-Machin 2006). This deletion was identified in 80% of NMSC samples used by Durham et al. (2003) compared to the presence of the CD in only 40% of the samples (Harbottle and Birch-Machin 2006). Not surprisingly, the amount of the 3,895 bp deletion was found to increase at body sites with greater sun-exposure (Krishnan et al. 2004). Thus, the 3,895 bp deletion is a UV-inducible deletion. This deletion accumulates in the dermis, and the amounts in this skin layer indicate cumulative chronic UV damage to mtDNA. Interestingly, acute sun exposure induces the deletion in the epidermis as well, and this serves as a sensitive measure of acute mtDNA damage from sun exposure. Thus, dermal and epidermal measurements of this deletion accurately identify areas of the skin chronically and acutely damaged by sun exposure.

A 7,436 bp deletion as well as D-loop tandem duplications observed in sun-exposed skin was shown not to be specific to NMSC because the levels were similar to sun-exposed skin, but significantly higher than non-exposed skin (Yang et al. 2004). Rapidly dividing SCC cells had less of the CD than normal skin as expected. Eshaghian et al. (2006) conducted a comprehensive study of mtDNA deletion spectrum in NMSC and photo aged skin using PCR and sequencing. The number of deletions from perilesional skin correlated with the patient's age. Deletions were rare in tumors, with significant levels in margin tissues. Two specific deletions (3,715 and 6,278 bp) were present in skin samples from multiple people with frequencies similar to the CD. To really clarify the levels of mtDNA CD in NMSC, Kamenisch et al. (2007) used LCM to obtain pure cells from BCC, SCC and normal cells for analysis. As the CD is supposedly induced by the aging process and sunlight exposure, an intra-patient normalization of the levels in cancer and

non-tumor epidermis was performed. The CD was detectable in 86% of BCC and 95% of SCC. But for both tumors, the amount of the CD was significantly lower than in normal non-tumor tissues.

Head and Neck Cancer: Several investigators have analyzed the levels of the CD in head and neck squamous cell carcinoma (HNSCC) samples and the general trend observed is the decreased frequency and levels of the deletion in tumors compared to non-tumoral samples. Another general observation is the increased levels in precursor lesions compared to cancerous tissues.

Lee et al. (2001) examined the content of the CD in oral cancer and matched non-cancer samples from patients with or without betel quid chewing habits. In general, the deletion was lower in the tumors than the normal samples. When compared to their matched normal samples, patients with betel quid chewing habits harbored much lower levels in their tumors (13-fold) than patients without betel quid chewing history (fivefold). The levels of the CD were also significantly much higher in normal oral samples from people with betel quid than those without betel quid chewing history. Using laser capture microdissection, Shieh et al. (2004) procured cells from oral cancer, pre-cancer and matched normal submucosal tissues for CD analysis. All the samples demonstrated higher proportion of the deletion in tumors compared to lymphocytes. Pair wise comparison revealed much less levels in cancerous than precancerous tissues, and the levels were much higher in stroma compared to cancerous epithelia. It was however, uncovered that the amounts of the CD decreased when the disease progressed from pre-cancer to cancer. Using a multiple of techniques including microdissection, limiting dilution PCR, qPCR, and in situ hybridization, Dani et al. (2004) noted a generalized pattern of marked decreases in the amounts of the CD in a variety of cancers including HNSCC. Their observation led them to conclude that cancer cells are virtually devoid of the CD, and that the observations made in cancer specimen are a result of contamination with normal cells.

Shao et al. (2004b) uncovered a high frequency of a 4,981 bp deletion in nasopharyngeal cancer (93%) compared to nasopharyngitis samples (60%), and very rarely in matched blood. Poetsch et al. (2004) reported a de novo CD in 25% of HNSCC. Han et al. (2004) demonstrated the CD frequency of 57% in laryngeal SCCs. All the mutations were in, well and moderately differentiated cancers with none in poorly differentiated tumors. In nasopharyngeal cell lines, the CD, but not point mutations, was induced by radiation treatment, and the levels of the deletion positively correlated with the degree of apoptosis, suggesting a possible role for the CD in cell death during head and neck cancer radiotherapy (Pang et al. 2008). Work by Tan and coworkers revealed multiple large-scale deletions in oral cancers (Tan et al. 2003).

Salivary Gland Tumors: A number of salivary gland tissues have been examined for the population frequency and quantitative levels of the CD. Lewis et al. (2000) quantified the levels of the deletion in salivary oncocytomas (Warthin's tumors) and age-matched normal parotid glands. All the tissues examined harbored the deletion, but the levels were significantly higher in salivary oncocytomas, and

a trend towards increased levels in smokers was detected. Because of their initial observations, and the fact that smoking is a known risk factor for salivary gland tumors, Lewis et al. (2002) subsequently examined the amounts of the CD in parotid glands of smokers and non-smokers. Whereas point mutations were found in five of the 23 glands from smokers, the levels of the CD were not different in smokers and non-smokers. Thus, smoking related induction of salivary gland tumors might not involve the CD. Using fluorescence in situ hybridization analysis, the CD was found in 10% of Warthin's tumors. By quantitative PCR analysis, the CD was present in all parotid tissue but levels were much higher in oncocytic tumor cells, as expected.

Thyroid Cancer: Tallini and coworkers were first to address the question of mtDNA deletions in thyroid oncocytomas (Tallini et al. 1994). These investigators failed to demonstrate a difference in frequency of the CD between tumor and normal control thyroid samples. Maximo and Sobrinho-Simoes (2000) analyzed various thyroid tumors including adenomas and carcinomas with Hurthle cell, papillary, and follicular patterns. The CD was observed in all Hurthle cell tumors, 33% of the adenomas and only 18% of papillary tumors without Hurthle cells. The normal adjacent thyroid tissues to cancer contained low levels of the deletion. These investigators later performed a comprehensive analysis of the levels of the CD in 79 benign, 43 Hurthle and 36 non-Hurthle cell cancers (Maximo et al. 2002). The CD was present in all Hurthle cell tumors. The amounts, which were as high as 16%, were significantly higher in tumors with D-loop mutations. Rogounovitch and coworkers examined a series of samples comprised of radiation-associated and sporadic papillary thyroid tumors (Rogounovitch et al. 2002). The CD was present in all tissue types irrespective of oxyphil histology. The quantitative levels of the CD however, were elevated predominantly in tumors from radiation-exposure and not sporadic PTC. In addition, other large-scale deletions, but not the CD, in radiation-associated tumors correlated with mtDNA content.

Breast Cancer: The first report of mtDNA mutations and deletions in breast cancer was by Bianchi et al. (1995). They examined breast samples from seven women. Both cancer and normal adjacent samples harbored heteroplasmic mtDNA changes. The CD was present in breast tissues of two older women with DCIS, and a younger patient with medullary carcinoma.

As sensitive technologies were developed, it became evident that the levels of the CD are probably much lower in breast cancer compared to normal breast epithelial cells. Dani et al. (2004) demonstrated up to a 100-fold reduction in the CD in tumor compared to normal adjacent breast cancer tissues. Similarly, Tseng et al. (2006) observed higher rates of the CD in non-tumor tissues (47%) compared to breast cancer (5%) samples. On the contrary, the CD was detected in all breast samples, with levels ranging from as low as 0.000149 to 7% (Ye et al. 2008). However, the amounts of the deletion were lower in cancer and benign breast diseases than the normal adjacent tissues, though this finding was not significant. Microdissected breast cancer, matched normal adjacent cells, and true normal breast tissues were

examined for mtDNA deletions (Zhu et al. 2004). In addition to the CD, three other deletions (3,938, 4,388, and 4,576 bp) were uncovered in breast tissues. The 4,576 bp deletion appeared to be associated with breast cancer, because it was absent in true normal breast tissue, but present at a frequency of 13 and 77% in normal adjacent breast tissue and breast cancer respectively.

The NAD(P)H:quinone oxidoreductase 1 (NQO1) enzyme protects cells against oxidative stress. Hence, the possible association of the CD with NQO1 enzyme polymorphism (C609T) in breast cancer was questioned (Tseng et al. 2009). The rate of detection of the CD in tumor and normal adjacent tissues was similar to those reported earlier by the same group. In spite of the low frequency of the CD in breast cancer samples, it was associated with NQO1 "C/T" (61%) and "T/T" (65%) genotypes, and less frequently with the "C/C" (16%) genotype.

Lung Cancer: As a known risk factor for lung cancer, smokers were found to have 5.6 times, mtDNA, 2.6 times, nuclear gene damage, and seven times the amount of the CD than non-smokers (Ballinger et al. 1996). In lung tissues, the CD was present in 54% of cancer, 60% of normal looking lung tissue adjacent to cancer, and only 30% of normal lung tissues, but the differences were not significant (Dai et al. 2006). The deletion appeared to correlate with age and smoking. These findings suggest that smoking causes accumulation of the CD in lung tissue, such that following malignant transformation both cancer and normal, adjacent to cancer tissues continue to harbor similar frequencies of the deletion.

Esophageal Cancer: Abnet et al. (2004) were first to address the frequency of the CD in esophageal cancer. The deletion was present in as many as 92% of the tumors and the normal adjacent to tumor samples, but not in the corresponding peripheral blood samples. In another study, the frequency of the CD increased with the degree of dysplasia from normal esophagus to Barrett's esophagus, to low-grade dysplasia and to high-grade dysplasia and the peritumoral tissue (Tan et al. 2009). Interestingly, the frequency in cancer was much lower, being comparable to those of Barrett's esophagus. These findings, though preliminary, support the thesis that the levels of the CD first increase with progressive cellular transformation, but drastically decline as cells become cancerous and achieve maximal proliferation rates.

Gastric Cancer: Burgart et al. (1995) uncovered a 50 bp somatic deletion mutation in four gastric cancers mostly from the esophagogastric regions. This deletion included CSBII and was flanked by 9 bp repeats. Maximo et al. (2001) examined the relationship between the CD, nMSI and mtDNA mutations in 32 gastric cancers. The CD appeared to be exclusive to nMSI-negative tumors, and inversely correlated with mtDNA mutations. Quantitative levels of the CD were lower in gastric cancers compared to the normal adjacent to tumor tissues (Dani et al. 2004). Similarly, the CD was more frequent in noncancerous gastric tissues (55%) compared to tumors (9%) (Wu et al. (2005). Kamalidehghan et al. (2006a) found the CD to be less frequent in gastric cancer compared to the normal adjacent tissues. However, in a separate study, a deletion of about 8.9 kb in size was opposite in frequency, being more in cancer than the adjacent normal to cancer tissue

samples (Kamalidehghan et al. 2006b). Wang and Lu (2009) observed a correlation between the CD and ROS as well as MnSOD production in gastric cancers. Their findings suggest that loss of anti-oxidant activity leads to increased ROS production in association with increased frequency of the deletion.

Colorectal Cancer: Two initial reports suggested the absence of mtDNA deletions in colorectal cancer (Heerdt and Augenlicht 1990; Cui et al. 1991). Cui et al. (1991) used 13 different restriction endonucleases and Southern blotting to screen 15 CRCs and eight adenomas. Since they failed to find differences in electrophoretic pattern between tumors and the adjacent normal tissues, the authors concluded there were no large-scale deletions, insertions, rearrangements, and even single base mutations in the detection region (problem could be field cancerization of colonic mucosa). However, Habano et al. (1999) also used whole genome amplification to screen for mtDNA deletions in CRC samples and failed to identify any. Similarly, Nishikawa et al. (2005) screened CRC cell lines for large-scale deletions using long-extension PCR and were unsuccessful in identifying any deletion. Dani et al. (2004) however, reported on the presence, although at lower levels, of the CD in colorectal cancer cells, and Savre-Train et al. (1992) had even demonstrated the presence of transcribed deleted mitochondrial molecules in human colorectal cancer cells. Interestingly in human colonic tissue, large-scale deletions in the major arc of mitochondrial genome could not be demonstrated even in cytochrome c-deficient cells that harbored pathogenic mtDNA mutations (Taylor et al. 2003). It will be interesting to know what protects colonic cells from this deletion.

Liver Cancer: In addition to somatic point mutations, large-scale deletion mutations have been extensively studied in hepatocellular carcinoma (HCC) as well. HCC and non-tumor cirrhotic liver samples were screened for mtDNA deletions (Yamamoto et al. 1992). Multiple deletions were uncovered in non-tumor cirrhotic liver tissues but not in tumors. These deletions included a 7,079 bp deletion with no repeats, and a 7,436 bp deletion flanked by 12-bp repeats. The CD was detected in 66% of non-tumor adult liver tissues but absent in cirrhotic liver tissues from Pediatric cases (Fukushima et al. 1995). When compared to normal liver, the CD frequency was significantly lower in HCC. The levels of a 7,436 bp deletion and the CD were the same in normal and chronic hepatitis liver samples, but significantly lower in livers with HCC (Kotake et al. 1999). Shao et al. (2004a) examined the frequency of a 4,981 bp deletion of the mitochondrial genome in HCC, corresponding non-cancerous control tissue and hepatocellular nodular hyperplasias (HNH). The deletion was found in 85% of HCCs, 83% of HNHs, and 57% of adjacent normal samples. Non-cancer hepatic tissues from liver cancer patients with prolonged alcohol use contained higher levels of the CD compared to those from non-alcohol users (Yin et al. 2004). Wheelhouse et al. (2005) found that the frequency, of the CD was again significantly higher in control liver and non-cancer liver from patients with hepatitis B infection (100 and 95%), than in liver with cancer (28%). Lymphocyte mtDNA deletion was associated with hepatitis B infection and risk of developing HCC (Wu et al. 2009).

Renal Cancer: Horton et al. (1996) uncovered a 264 bp *ND1* intragenic deletion in one primary renal cell cancer. This deletion involved 50% of the mitochondrial genomes of this tumor and could not be found in the normal adjacent renal tissues, indicating its tumor specificity, and possible pathogenic nature. The truncated *ND1* was also preferentially transcribed.

Prostate Cancer: Jessie et al. (2001) first reported on mtDNA rearrangement mutations in prostate cancer. They examined clinical specimens from patients ranging in age from 41 to 81 years. They demonstrated several mtDNA deletions in cancerous tissues that appeared to correlate positively with the age of patients. The absence of normal tissue controls in this study confounded the delineation of deletions due to the aging process from those related to prostate cancer pathology. Maki et al. (2008) embarked on a comprehensive study of mtDNA deletions in prostate cancer and have identified and quantified the levels of a 3,379 bp deletion that is associated with prostate cancer. This mtDNA deletion also demonstrates field cancerization in prostatic tissues, and therefore appears to have value in helping resolve false-negative prostate needle biopsy specimens.

Higuchi et al. (2006) had showed that large-scale mtDNA deletions and mtDNA depletion might underlie the development of androgen independence in prostate cancer. Androgen dependent LNCaP cells were observed to contain fewer deletions than the androgen-independent C4-2 cell line. As a consequence of increased deletions, C4-2 cells consumed less oxygen than LNCaP cells. The androgen dependence of LNCaP cells was blocked by mtDNA depletion, and this was reversible by reconstitution of the cells with normal mtDNA. mtDNA may play a role in the development of androgen independence by prostate cancer cell. In addition, cells depleted of mtDNA could form tumors in athymic mice.

Endometrial Cancer: The heteroplasmic frequency of the CD in endometrial cancer has been addressed (Futyma et al. 2008). This group compared deletions in 38 endometrial cancers and matched normal adjacent tissues. The CD was found in 80% of endometrial cancers and 84% of matched noncancerous tissues. But interestingly, the intensities of the bands from the PCR products were quantitatively significantly higher in normal adjacent samples than cancer. The authors concluded that the CD is not specific to endometrial cancer. Again, this conclusion is not surprising, given the pervasive lack of knowledge on "field cancerization." But the finding of higher amounts of the deletion in normal adjacent tissues compared to cancer is consistent with findings in other cancer.

Hematologic Malignancy: The development of acute myeloid leukemia following radiation insults is preceded by the development of preleukemia or secondary myelodysplastic syndrome (sMDS). Hatfill et al. (1993) showed that clonal deleted mtDNA by chloramphenicol in bone marrow created identical hematologic abnormality as sMDS. Clayton and Vinograd (1967) had earlier reported on the presence of circular dimer forms of mtDNA in leukemic leukocytes, and the levels of these dimers decreased with treatment (Clayton and Vinograd 1969). These abnormal mtDNA molecules, confirmed by several other investigators, were smaller than the wildtype genomes, suggesting they were deleted mtDNA.

8.4 Is the CD a Tumor Suppressor?

A general trend observed in many cells is that the levels per cell of the CD are much lower in cancer than normal cells (Tables 8.1–8.3). Dani et al. (2004) even suggest the complete absence of this deletion in cancer cells, and that the low levels observed in cancer tissues are due to contamination from normal cells. In support of this thesis, numerous studies have reported decreased frequencies and levels of the CD in cancer compared to non-cancer tissues. Using LCM to capture pure tumor and normal cells, Kamenisch et al. (2007) observed a highly significant reduction ($p = 0.0005$) in the levels of the CD in non-melanoma skin cancer samples compared to normal epidermal cells. Similar findings were observed in oral cancer using LCM (Shieh et al. 2004). Thus, Dani et al. (2003) have hypothesized that the CD has a tumor suppressor function. Traditional tumor suppressor genes encode proteins that function to prevent DNA damage and hence carcinogenesis. How a mitochondrial genome deletion will function as a tumor suppressor requires clarification.

8.5 mtDNA Content Alterations in Cancer

mtDNA content changes are reported in several cancers (Tables 8.4 and 8.5). In head and neck, papillary thyroid, esophageal and prostate cancers and expectedly oncocytomas, mtDNA content is demonstrably higher than the respective normal tissues. On the contrary, breast, lung, gastric, colorectal, liver and renal cancers possess decreased amounts of mtDNA.

Table 8.4 Cancers with increased mtDNA copy number or content changes

Cancer	Copy number (%)	References
Brian tumors	Increased (87)	Liang (1996) Liang and Hays (1996)
Head and neck cancer	Increased	Kim et al. (2004) Jiang et al. (2005)
Papillary thyroid carcinoma	Increased (65)	Mambo et al. (2005) Rogounovitch et al. (2002)
Lung cancer	Increased in ~50%, but decreased with tumor progression	Lin et al. (2008) Lee et al. (2005)
Esophageal cancer	Increased (55)	Tan et al. (2006) Lin et al. (2009)
Oncocytomas	Increased	Tallini et al. (1994) Selvanayagam and Rajaraman (1996) Heddi et al. (1996)
Prostate cancer	Increased (78)	Mizumachi et al. (2008)
Endometrial cancer	Increased	Wang et al. (2005)
Ovarian cancer	Increased	Wang et al. (2006)

Table 8.5 Cancers with decreased mtDNA copy number or content changes

Cancer	Copy number (%)	References
Breast cancer	Decreased (63–80)	Mambo et al. (2005) Tseng et al. (2006) Yu et al. (2007) Fan et al. (2009)
Gastric cancer	Decreased (~55)	Wu et al. (2005) Lee et al. (2005)
Hepatocellular carcinoma	Decreased (60–77)	Lee et al. (2004) Lee et al. (2005) Yin et al. (2004) Yamada et al. (2006)
Colorectal cancer	Decreased (30–40)	Lin et al. (2008a, b) Lee et al. (2005)
Renal cell carcinoma	Decreased (60–90)	Meierhofer et al. (2004) Heddi et al. (1996) Selvanayagam and Rajaraman (1996)

8.5.1 Cancers with Increased mtDNA Content

Brain Cancer: Liang (1996) first questioned mtDNA content changes in brain tumors. The content of mtDNA in low-grade gliomas was analyzed using cDNA homologous to mtDNA nps 1,679–1,946 and 2,017–2,057. Compared to normal brain tissue controls, gliomas had increased mtDNA copy number. Fluorescent in situ hybridization analysis using mtDNA-specific probes revealed enhanced nuclear signal in gliomas with increased mtDNA copy number, but not in glioblastoma and normal brain with low mtDNA content. In a follow up study, 87% of gliomas comprised of both low-grade and high-grade tumors demonstrated up to 25-fold increase in mtDNA content (Liang and Hays 1996). This frequency was much higher than amplification of erb-B that was present in only 18% of these tumors.

Head and Neck Cancer: Smoking and alcohol consumption are among the risk factors for head and neck cancers. mtDNA changes are demonstrated in smokers and in primary head and neck cancers. The content of mtDNA was found to be elevated in exfoliated cells in saliva of smokers compared to non-smokers (Jiang et al. 2006). Previously, Kim et al. (2004) had shown a progressive model of mtDNA content changes in various histopathologic grades of head and neck cancers. mtDNA content increased from mild, to moderate then to severe dysplasia. Cancer tissues, however, contained significantly higher levels of mtDNA compared to normal mucosal cells. Jiang et al. (2005) had demonstrated an increase in content of mtDNA in head and neck cancers as well as salivary samples from the patients, and these findings were independent of age and smoking. Treatment has effects on mtDNA content changes as well. Salivary mtDNA content was significantly lower in post treatment compared to pretreatment saliva, and was equally significantly

lower in saliva of patients who received radiation treatment compared to those without radiotherapy (Jiang et al. 2006).

Thyroid Cancer: The content of mtDNA is increased in papillary thyroid cancer (PTC) specimens compared to matched normal adjacent tissues and benign thyroid neoplasia (Mambo et al. 2005). Rogounovitch et al. (2002) examined four different parts of the mitochondrial genome for content changes in PTC and follicular adenomas. mtDNA content was increased in these tumors irrespective of their association with radiation exposure.

Lung Cancer: Lee et al. (2005) observed decreased mtDNA copy number in 23% of lung cancer samples. A collection of neoadjuvant treated stage III NSCLC resection specimens were analyzed for mtDNA content changes (Lin et al. 2008a). Decreased mtDNA content and oxidative stress as measured by 8-OHdG correlated with disease progression after treatment. Decreased copy number and oxidative stress was much pronounced in the advanced stage NSCLC after treatment. It was suggested that decreasing mtDNA content and mass, prevented an excess ROS production and hence cytotoxicity of advanced tumors. Bonner et al. (2009) hypothesized that the increased affinity of polycyclic aromatic hydrocarbons for mtDNA could cause mtDNA damage leading to a compensatory increase in mtDNA content. To address this issue, they measured mtDNA content in saliva of 122 cases and 121 control subjects from Xuan Wei, China. Using mtDNA copy number cutoff of 157 per cell, the higher content conferred greater risk for developing lung cancer.

Esophageal Cancer: Tan et al. (2006) demonstrated a mixed pattern of content changes in esophageal cancer, when they compared tumor to matched non-tumor tissues. Overall, mtDNA content was increased in 11 cases and decreased in nine. A larger number of samples comparing non-matched normal esophageal mucosal cells to esophageal cancer are needed to uncover the content alterations in this type of cancer. Using microdissection, mtDNA copy number was found to increase from noncancerous mucosa (0.59), to ESCC (0.192) to lymph node metastasis (0.206) (Lin et al. 2009). This trend was significantly more pronounced in cigarette smokers and heavy wine drinkers.

Oncocytoma: Oncocytomas are characterized by increased mitochondrial mass due to increased mitochondrial proliferation. Works by several groups show a relative increase in mtDNA content in these tumors consistent with their enormous mitochondrial mass. Tallini et al. (1994) observed mtDNA to apparently be intact in oncocytomas, despite an earlier demonstration of *COI* alteration in six renal oncocytomas (Welter et al. 1989). However, mitochondrial content was fivefold higher than normal controls. Selvanayagam and Rajaraman (1996) uncovered a 200-fold increase in mtDNA content in benign renal oncocytomas compared to normal adjacent kidney tissues. Similar increases in mitochondrial content in renal and salivary oncocytomas were observed (Heddi et al. 1996). While the levels of mitochondrial transcripts increased in salivary oncocytomas in parallel with the increased content, mitochondrial transcripts paradoxically were reduced in renal oncocytomas. Nuclear genes encoding respiratory chain components were induced

in oncocytomas. The functional importance of these content changes and other mtDNA abnormalities in oncocytomas is discussed in Sect. 9.5.7.

Genitourinary Cancer: An increase in mtDNA copy number is also associated with prostate cancer pathogenesis (Mizumachi et al. 2008). Using single cell qPCR assay, a widely varied mtDNA content per LNCaP clones of cells were uncovered. Laser-capture microdissection was then used to capture pure single primary cancer cells and normal adjacent cells for analysis. Cancer cells demonstrated a higher skewed distribution of mtDNA copies per cell. Consistent with these findings, mtDNA content was increased in seven of the nine prostate cancer compared to the normal adjacent tissues.

Gynecological Cancer: Wang et al. (2005) examined the possible relationship between mtMSI at D310 and content changes in endometrial cancer. Using LCM, pure endometrial cancer cells from 65 patients and normal endometrial glandular epithelial cells from 41 control subjects without cancer were obtained and used for the analysis. Extensive variations in mtDNA copy number were uncovered in the cancer group. However, the overall mtDNA content was significantly higher in endometrial cancer compared to normal endometrium. Cases with mtMSI also harbored higher content than mtMSI-negative cancers. In another study by the same group, mtDNA content was shown to be much higher in ovarian cancer than normal ovarian tissues (Wang et al. 2006). Importantly, the copy number in low-grade tumors was significantly much higher than in high-grade carcinomas. Similarly, mtDNA copy number was significantly higher in type I (mucinous carcinomas, low-grade serous and endometriod carcinomas and clear cell carcinomas) than type II (high-grade serous and endometriod carcinomas and undifferentiated carcinomas) ovarian cancers (Shih Ie and Kurman 2004; Bell 2005; Wang et al. 2006).

Hematologic Malignancy: The mitochondria in etoposide-resistant human leukemic cells were found to be swollen but with intact cristae (Tokue et al. 1991). Further analysis uncovered a fourfold increase in mtDNA content in leukemic cells compared to the parental cell line. Boultwood et al. (1996) studied quantitative mtDNA levels relative to nuclear targets in hematologic cancers. mtDNA to nuclear target ratio was $\sim$1 in peripheral blood obtained from 20 healthy control individuals. In 25 acute myeloid leukemic cases, the ratio was very high, ranging from 2 to 50, with 13 cases having a ratio of 8 or higher. Analysis of four cases with mtDNA/nuclear ratio of between 3 and 10 at presentation indicated a normal ratio at clinical remission. Interestingly, 18 cases of chronic granulocytic leukemias had no increased mtDNA, but showed increased mtDNA content during the transformation from chronic phase.

A hypothesis tested by Lan et al. (2008) was that, high mtDNA content in peripheral blood of healthy people might convey increased risk for non-Hodgkin's lymphoma. To test this, mtDNA content in blood was measured in 104 cases and 104 healthy controls in a prospective study. They observed a dose–response between mtDNA copy number and risk of developing non-Hodgkin's lymphoma especially in CLL/small lymphocytic lymphoma subtype.

8.5.2 Cancers with Reduced mtDNA Content

Breast Cancer: mtDNA copy number is significantly decreased in up to 80% of breast cancers (Mambo et al. 2005; Tseng et al. 2006; Yu et al. 2007; Fan et al. 2009). This mtDNA content reduction in breast cancer is likely associated with older patients and hormone receptor status. One study observed that decreased mtDNA content was significantly associated with poor overall survival (Yu et al. 2007). Low copy number was also associated with tumors that contained D-loop mutations, especially at D310 or close to H-strand origin of replication (O_H). Recently, decreased mtDNA copy number in breast cancer was associated with *polymerase gamma* mutations (Singh et al. 2009).

Gastric Cancer: In two studies, decreased mtDNA content was detected in 55% of gastric cancers (Lee et al. 2005; Wu et al. 2005). The depletion in mtDNA was significantly associated with Bormann's type III and IV types of gastric cancers.

Colorectal Cancer: Lee et al. (2005) observed that mtDNA content decreased in only 28% of CRCs. Lin et al. (2008b) examined mtDNA content, TFAM and beta-F1-ATPAse expression in 153 CRCs, and the effects of these on 5-fluorouracil therapy response was also examined in HCT116 cells. In general, CRCs with low TFAM and beta-F1 ATPase expression had low mtDNA content. At the same time, mitochondrial content, TFAM and ATPase expression were cancer-stage dependent. Response to 5-FU treatment positively correlated with high ATPase expression. Decreased mitochondrial mass and content also decreased 5-FLU response by cancer cells. Mitochondrial content in 194 sporadic CRCs were examined (Chang et al. 2009). Decreased mtDNA content was significantly associated with tumor stage and *p53* mutations. Compared to patients with normal mitochondrial content (61%), the 5 years disease-free rate was only 39% in patients with reduced mtDNA content.

Liver Cancer: mtDNA content alteration was reduced in rat hepatoma cells (Luciakova and Kuzela 1992; de Heredia et al. 2000). A number of studies of primary human liver tumors also indicate significant reduction in mtDNA copy number in HCC compared non-tumoral liver tissues (Lee et al. 2004, 2005; Yin et al. 2004; Yamada et al. 2006). mtDNA copy number was much reduced in cancers with D-loop mutations (Lee et al. 2004), and appeared to be sex-dependent, being significantly low in female than male HCC patients (Yin et al. 2004). Yamada et al. (2006) observed that decreased copy number correlated with tumor size and cirrhosis, and a trend towards low copy number and poor outcome was noted. The normal adjacent liver tissue to HCC in alcoholics had much reduced mtDNA content than similar tissues from non-alcoholics (Yin et al. 2004). This finding suggests that alcohol may create a preconditioned field in the liver from which cancers develop consistent with field cancerization.

Genitourinary Cancer: Using a subtractive hybridization procedure, Selvanayagam and Rajaraman (1996) observed a loss of mtDNA and mRNA for *ND3* in about 62% of renal cancers and five of six renal carcinoma cell lines. Heddi et al. (1996) demonstrated a marked decrease in mtDNA content and mtRNAs in renal cell carcinoma. mtDNA content and enzyme activity was much reduced in about 92% of renal cancers compared to controls, but this feature did not correlate with tumor

type or progression (Meierhofer et al. 2004). It is suggested that mtDNA content is highly heritable, and that people who inherit low levels have increased risk for renal cancers (Xing et al. 2008). Consistent with this observation, low mtDNA content was demonstrated in peripheral blood lymphocytes of renal cancer patients compared to normal controls.

8.6 Possible Reasons for mtDNA Repletion or Depletion in Cancer

8.6.1 Increased mtDNA Copy Number

Simple deleted mtDNA molecules often have at least one origin of replication to enable replication and propagation of these molecules. In some instances, both origins of replication are preserved in the deleted mtDNA. Deleted mtDNA are smaller than wildtype genomes, and evidence indicates that a smaller mtDNA molecule has a replicative advantage over wildtype and does repopulate organelles faster under relaxed replication conditions (Diaz et al. 2002). Thus, in postmitotic cells or cells with low proliferation rates deleted mitochondrial genomes will accumulate to higher levels than wildtype, and this can lead to mitochondrial repletion.

How mtDNA content is modulated is complex, but involves both nuclear and mitochondrial genetic loci. Nuclear genetic loci and gene expression profiles control mtDNA content (Curran et al. 2007). These investigators identified chromosome 10q to be strongly linked to mtDNA content in a population study. Importantly, this loci houses TFAM and TIMM23 involved in mtDNA replication and protein import respectively. Also this study uncovered several genes controlling mtDNA content. This study suggests that, for example in cancer, mtDNA content will be under the control of the myriads of signaling pathways activated.

Cancer cell proliferation also appears to control mtDNA content (Trinei et al. 2006). Mitochondria replicate their genomes prior to S-phase of the cell cycle before cell division. Trinei et al. (2006) demonstrate that this process is controlled by growth factors and oncogenic signaling pathways. Oncogenic Ras activity is unchecked in many cancers leading to uncontrolled proliferation in the absence of growth signals. It is demonstrated by this group that Ras signaling also controls mtDNA replication. Thus, Ras-mediated hyper-proliferation of cancer cells will be associated with hyper-replication of mtDNA. P66Shc regulates receptor signaling, mitochondrial activity and is a genetic determinant of lifespan. In addition to Ras, these investigators demonstrate that p66Shc also induces mtDNA replication.

Factors controlling mitochondrial biogenesis are elevated in cancer. In type I endometrial cancer, citrate synthase activity, mtDNA content and TFAM levels were twice that of normal endometrial tissue (Cormio et al. 2009). This finding was associated with increased expression of mitochondrial biogenic genes, PGC-α and NRF-1. The role of these genes in mitochondrial content regulation deserve in-depth study.

8.6.2 Decreased mtDNA Copy Number

Several reasons appear to account for mtDNA depletion, and the mechanisms could be different in different diseases. For example, mutations in genes (*DGUOK*, *TK2*, *SUCLA2*) involved in the salvaging pathway of dNTPs, required for mtDNA synthesis play important roles in the mitochondrial depletion syndrome. In cancer, redox stress, mutations in mtDNA regions important in replication (D-loop, OL, or OH) can affect mtDNA replication. In cancer cells, it is more likely that mutations in genes such as p53 and polymerase gamma mediate mtDNA depletion.

Evidence of *polymerase gamma* mutations and mtDNA depletion in breast cancer has been demonstrated (Singh et al. 2009). The coding exons (2–23) and flanking intron/splice junctions of *POLG* was sequenced in breast tumors. Overall POLG gene was mutated in 63% of breast tumors with mutations affecting all three domains of the POLG protein. It is expected that these dysfunctional mutations will affect mtDNA biogenesis. Consistent with this conclusion, mtDNA content was decreased in primary breast tumors examined by this group.

Mutations in the p53 tumor suppressor gene are common in many types of cancers. A number of studies provide a role for p53 in mtDNA maintenance. Heyne et al. (2004) identified a p53 binding sequence in human mtDNA. Consistent with this finding, Achanta et al. (2005) demonstrated that p53 translocates into mitochondria, where it directly interacts with mtDNA and polymerase gamma. This interaction enhanced mtDNA replication. Loss of p53 made mtDNA more vulnerable to mutations that could be abrogated by transfection of wildtype p53. A role for p53 in mtDNA content maintenance was provided in a separate study of mtDNA depletion syndrome. Bourdon et al. (2007) studied seven unrelated families with the MDDS, and uncovered nonsense, missense, and splice-site mutations as well as in-frame deletion in *Ribonucleotide Reductase M2 B* (*RRM2B*). *Ribonucleotide Reductase M2 B* encodes a cytosolic p53-inducible ribonucleotie reductase subunit, which supplies deoxyribonucleotides for DNA repair in cells arrested at G_1 or G_2. Consistent with these family studies, mtDNA depletion was demonstrated in Rrm2b−/− mouse tissues. These findings strongly suggest that p53R2 plays important roles in supplying dNTP for mtDNA synthesis. In another investigation, *p53*−/− mouse and *p53* knockdown human primary fibroblasts harbored less mtDNA and decreased mitochondrial mass (Lebedeva et al. 2009). The mtDNA depletion in these fibroblasts was associated with decreased mtTFA protein and decreased mRNA and protein of p53R2. Thus, the p53 pathway critically control mtDNA content, and this is a likely mechanism of mtDNA depletion in many cancers.

8.7 Postulated Mechanisms of Deletion Formation

The mechanism by which deletions are produced in the mitochondrial genome is poorly understood and debatable making any proposed mechanism subject to more validation before approval. However, based on experimental data on deletion

formation and sequence structures observed at deletion junctions, several suggested models are proposed. These include slipped-strand mispairing, illegitimate elongation, homologous recombination, and DNA degradation and mispairing as an error of repair of double strand breaks. The first two models can only occur during asynchronous mtDNA replication, the latter during repair of double strand breaks, while recombination can occur at any time provided the appropriate spatial arrangement of mtDNA is provided.

The slipped mispairing model requires asynchronous mtDNA replication beginning at the O_H. This must move past an upstream direct repeat. When replication encounters the downstream repeat, the upstream repeat of the parental H-strand displaces the nascent daughter H-strand and mispairs with the complementary L-strand downstream repeat. Immediately downstream of the H-strand annealed upstream repeat, the single-stranded parental H-strand loop breaks and is degraded. The double stranded misannealed repeat is ligated. Elongation of the truncated H-strand followed by L-strand replication generates a deleted mtDNA molecule.

Similar to the slipped mispairing model, illegitimate elongation model requires asynchronous replication that must move past an upstream repeat. However, in this instance, when replication proceeds past the second downstream repeat, the upstream repeat of the daughter H-strand mispairs with the downstream L-strand repeat. This rearrangement creates a shortened daughter H-strand, which can be elongated coupled with L-strand replication to produce deleted mtDNA molecules. In both the slipped mispairing and illegitimate elongation models, some secondary structural arrangements must occur to bring the H-strand upstream and L-strand downstream repeats together.

Nishigaki et al. (2004) observed the presence of micro-deletions at deletion breakpoint junctions that could not be accounted for by the two previous models. They also observed deletions lacking O_L and O_H, which are not adequately explained by the replication models because at least they require the sparing of O_H. To help account for their observations, a third model was proposed, intra-molecular homologous genetic recombination similar to what occurs in the nucleus during meiosis. This mode of deletion formation requires that homologous segments of mtDNA align, which can be enhanced by DNA super-coiling or the electrostatic properties of DNA. In this conformation, strand breaks and exchange between upstream and downstream repeats creates a Holliday-type structure. Branch migration extends the crossover point followed by cleavage and repair of the Holliday intermediate structure to produce recombinant deleted mtDNA molecules.

Krishnan et al. (2008) disputed the conventional slipped-strand misparing model and hence proposed a new model that suggests mtDNA deletions occur as a result of the repair of double-strand breaks (DSB). Their model calls for DSB between two direct repeats, such that the activity of a $3' \rightarrow 5'$ exonuclease removes the $3'$:$5'$ intervening sequences to generate single strand DNA between the repeats. Mispairing of the upstream and downstream repeats can then occur with floating unpaired single strands degraded and the misannealed double strand ligated to produce a deleted mtDNA.

These models only account for class I deletions. Class II deletions with imperfect repeats and class III deletions that do not have any repeats must occur by a very different mechanism. However, Krishnan et al. (2008) dispute this arguing that mtDNA deletions occur irrespective of the presence or absence of repeats and/or length of repeat sequences.

8.8 Control of mtDNA Deletions

Apart from the mechanisms elaborated above, it appears mtDNA integrity and therefore the factors controlling deletion formation are under nuclear gene regulation. Nucleoside imbalances in the mitochondria can cause deletions by several possible mechanisms. For instance impaired mtDNA maintenance and replication, can occur leading to replication stalling, DSB, deletions and point mutations.

The nucleotide pools in the cytosol and mitochondria are physically separate. As the inner mitochondrial membrane prevents passive movement of charged molecules, cytosolic nucleotides enter the mitochondria via transport pathways. Cytosolic nucleotide pools are very low in non-dividing cells, and therefore import into mitochondria is expected to be low as well. Mitochondria appear not to be able to perform de novo nucleotide biosynthesis. Yet mitochondria in quiescent cells continue to replicate. This scenario suggests how important mitochondrial nucleotide salvage is to mtDNA maintenance. Two of the four-deoxyribonucleoside kinases that initiate the salvage reaction are mitochondrial proteins. Deoxyguanoside kinase (DGUOK) and thymidine kinase 2 (TK2) efficiently participate in mitochondria nucleotide salvage. DGUOK can phosphorylate deoxyguanosine and deoxyadenosine, while TK2 phosphorylates deoxythymidine, deoxycytidine and deoxyuridine. The importance of these enzymes in mitochondrial nucleotide turnover is provided by the fact that the cytosolic TK isoform (TK1) is only highly active in diving cells while TK2 is constitutively expressed.

Our knowledge on nuclear causes of mtDNA mutations primarily come from mitochondrial diseases characterized by large-scale mtDNA deletions resulting from specific nuclear gene mutations. Specifically, in mtDNA depletion syndromes, deoxyribonucleoside kinase (DGUOK and TK2) mutations are demonstrated (Mandel et al. 2001; Saada et al. 2001). Similarly, mutations in thymidine phosphorylase (TP) underlie the autosomal recessive mitochondrial disorder, MINGIE (Nishino et al. 1999). In PEO that is characterized by large-scale mtDNA deletions, *polymerase gamma* mutations appear to be the underlying cause of many severe cases (Van Goethem et al. 2001), although mutations in *ANT1*, and *PEO1* or *C1Orf2* (formerly known as twinkle) are reported.

Cancer cells are often seen dividing, so nucleotide salvage might not be important for mitochondrial maintenance in these cells.

8.9 Conclusion

mtDNA deletions and copy number changes are demonstrated in several cancers. In addition to the CD, there are numerous other unique deletions that occur in the mitochondrial genome of cancer cells. However, these deletion mutations are currently understudied. It will be important in future to establish the myriads of deletion heteroplasmies for each cancer, and how these relate to content alterations and nuclear gene mutations. For example, how will *p53* or *polymerase gamma* mutations relate to mtDNA content changes? Do they have synergistic effects in some cancers, or are they mutually exclusive in cancers? Do these mutations determine tumor behavior such as the propensity to become invasive?

Mitochondrial genome deletions ultimately lead to mitochondrial depletion. Expectedly, loss of mtDNA will adversely affect mitochondrial functions, including defective OXPHOS with increased ROS production. Mitochondrial deletions, and depletions also affect other cancer cell biology such as epigenetic changes, chromosomal instability and altered signaling pathways (see Chap. 9). Other effects of mtDNA deletions are their nuclear integration and interference with gene expression, and possible oncogene induction.

Finally, the changes in mitochondrial genome can be targeted for diagnosis and monitoring of cancer. For example, certain heteroplasmic level of deletions can be strongly associated with cancer. Also possible is the specificity of some deletions to cancer that are virtually absent in normal tissues. mtDNA content alterations appear useful in the detection and therapy monitoring of head and neck cancer. There are several clinical utilities of mtDNA deletions and content alterations and this should be explored for patient care.

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Chapter 9
Functional Importance of Mitochondrial Genetic Alterations in Cancer

Abstract Do the plethora of reported mitochondrial genetic alterations in cancer have any biologic relevance to carcinogenesis? Answerers to this question form the bedrock of this chapter. An attempt is made to provide elaborate evidences of various mitochondrial genome alterations in contributing to cancer risk predisposition, initiation, proliferation and growth, as well as progression to advanced stage and subsequent metastasis. Mitochondrial genome changes may underlie hormonal resistance in endocrine tumors and such changes can confer resistance of cancer cells to chemotherapy. The emerging importance of mitochondrial genome changes in the pathogenesis of oncocytomas is of interest. Of similar interest to cancer research is the idea that intrinsic mitochondrial membrane potential changes may modulate cancer initiation and progression.

9.1 Introduction

The catalog of mutations illustrated in the previous three chapters conclusively affirms the fact that cancer cell mitochondrial genomes are severely damaged. It is conceivable that some cancer cells with unacceptably high levels of mutated mtDNA will undergo apoptosis. However, depending on the levels of heteroplasmy, these mutations could confer other biologic characteristics that may positively contribute to the cancer cell existence even in adverse conditions. It is quite evident that mutations in the genome will alter the redox balance and even changes in membrane potential, bioenergetics, and metabolism of the cancer cell. The Warburg effect and other metabolic adaptations are partly caused by the damaged cancer cell mitochondrial genome. Apart from these obvious effects from damaged cancer cell mitochondrial genome, a body of evidence strongly implicates the cancer cell mitochondrial genetic changes in the risk of developing cancer, as well as cancer initiation, proliferation, growth, metastasis, and acquisition of chemoresistance following treatment. Indeed, six hallmark features are required for a normal cell to undergo neoplastic transformation: self-sufficiency

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in growth signals, insensitivity to antigrowth signals, limitless ability to replicate, sustained angiogenesis, evasion of apoptosis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). These features are undeniably imparted by mitochondrial genome changes. The pivotal role of cancer cell mtDNA mutations in cancer biology is the basis of this chapter.

9.2 Mitochondrial DNA Mutations in Cancer: Cause or Consequence?

The role of mitochondrial genome mutations in carcinogenesis, including the induction of global genomic instability, disease progression, and prognosis has been demonstrated in all cancers. The frequency and increased prevalence of mitochondrial genome mutations in early stage cancer, and the findings that in most tumors the mutations do not correlate with disease stage or other clinical and pathologic factors, suggest possible causative effects. However, the inherent oxidative stress of cancer cells also implies that the mutations in the mitochondrial genome could just be a consequence of the carcinogenic process. Evidences for both possibilities are discussed.

Small albeit important amounts of ROS are produced under normal physiologic mitochondrial function of energy production. The virtually unprotected mtDNA molecules are more liable than nuclear genomes to sustain the initial nucleic acid damage from such ROS, especially when detoxifying mechanisms are less efficient. Such initial mtDNA damage can lead to cellular distress and apoptosis or evasion from apoptosis by transformation. Indeed, homoplasmic ATP6 np 8,993 or np 9,176 mutations conferred enhanced initial growth advantage to cybrids, probably due to evasion of apoptosis (Shidara et al. 2005). Apart from SNP, ROS can cause large-scale deletions in the mitochondrial genomes leading to mtDNA depletion. Such depletions can cause epigenetic nuclear gene changes, chromosomal instabilities, and hence, initiation of carcinogenesis. The decrease in respiration from elevated mtDNA mutations can cause metabolic switch to aerobic glycolysis, which is an eminent cancer phenotype.

Evidence for cancer causation by mitochondrial genome damage includes molecular field effect demonstrated by mtDNA changes. Mitochondrial genome alterations, including somatic mutations, large-scale deletions, and content changes, are demonstrated in histologically normal cells in the proximity to tumors. These findings of mtDNA field effect indicate early genetic damage that precedes histopathologic changes indicative of cancer. However, nuclear genome changes in histopathologically normal cells close to tumors are similarly reported, suggesting that the two genomes may play synergistic roles in malignant transformation. Because genotoxins first damage mtDNA before having any effect on the protected nuclear genome, it is possible that mitochondrial genome mutations precede and may contribute to nuclear genome instability and the initiation of cancer.

Because of the polyploid nature of mtDNA, it is conceivable that if malignant progression caused mitochondrial genome changes, then mutation load should accumulate with disease evolution. However, most cancer mtDNA mutations do not increase or decrease with advanced stage disease and do not correlate with other pathologic features of cancer.

Cancer initiation and progression is akin to somatic evolution whereby a number of somatic mutations are acquired sequentially to enable uncontrolled cellular proliferation. Historically, it is estimated that 4–6 stochastic nuclear genome mutations are needed for tumor formation (Renan 1993). These mutations usually involve tumor suppressor genes, oncogenes, and caretaker genes, with associated genomic instability and defective DNA repair mechanisms. If these events are sufficient to initiate tumor formation, then the redox stress that ensues following malignant transformation could potentially cause mtDNA mutations. In this case, mtDNA mutations will augment other features of tumor development or simply remain as a genetic signature of the cancer with no biologic significance. In colorectal cancer, for instance, D310 changes were early events in adenomas but not causative of colorectal cancer (Legras et al. 2008).

9.3 Functional Consequences of Mitochondrial DNA Mutations on Mitochondrial Biogenesis in Cancer

Do the numerous mtDNA mutations reported have any physiological impact on mitochondrial function? Notably, every component of the compact mitochondrial genome appears to be critical to mitochondrial functions, an element that further explains the redundancy of this genome (need for multiple copies) in the cell. The *cis*-acting elements in the noncoding region are critical for mitochondrial transcription and replication; the structural proteins regulate mitochondrial respirations and the ribosomal genes mediate mitochondrial protein synthesis. Whereas the expression of mitochondrial genes is dispensable in cell culture models in which sufficient nutrients are provided for anaerobic existence, it is possibly not the case in vivo. At specific levels of mutant copies, cellular functions are impaired requiring adoption of alternate pathways for cell maintenance.

Mitochondrial DNA mutations in cancer are, in general, heteroplasmic. Homoplasmic mutations have been reported, but the homoplasmic nature of these mutations are likely due to the possibility that mutant copies are disproportionately higher than wild type, coupled with insensitive sequencing methods at detecting heteroplasmy. Many cancer cells are alive partly because the normal functional wildtype mtDNA prevents cellular demise driven by lethal mutant copies, as well as adaptive pathways of avoiding dependency on mitochondrial function.

Mitochondrial polypeptide genes contribute important products that function in OXPHOS, including electron transport chain activity and ATPase functions. Given that this feature of mtDNA is critical to cell functions not only in the production of the energy required for biochemical activities but also in its important functions of

maintaining redox balance, mutations in these genes should be regarded as pathogenic. However, because some nonsynonymous mutations in structural genes appear nonpathogenic and seem as normal polymorphisms, what is important in deciding the pathogenic nature of mutations is the conservation index (CI), that is the level of conservation of the altered amino acid between species (Ruiz-Pesini et al. 2004). High and low CI indicate pathogenic and neutral mutations, respectively. For example, Petros et al. (2005) sequenced the entire mtDNA of one prostate cancer sample and uncovered several nucleotide changes including three missense mutations. One of the mutations, an A14769G in *CYTB*, altered amino acid N8S with low CI of 20.5%, and hence was suggested to have minimal effect on tumorigenesis. However, an ATP6 C8932T mutation that altered amino acid P136S with a CI of 64% could have contributed to carcinogenesis of this particular tumor.

Mutations in mitochondrial rRNA genes are reported in several cancers, and deletions in the minor arc tend to encompass these genes. While the heteroplasmic levels and hence pathogenic nature of these mutations are yet to be established in cancer, the shear abundance of rRNA molecules makes it appear that mutations in these genes in a subset of mitochondrial genomes can be dispensable and disregarded as pathogenic. Molecular evidence of mitochondrial ribosomal gene mutations in disease etiology are mainly provided using the pathogenic A1555G and C1494T mutations associated with nonsyndromic and aminoglycoside-induced sensorineural deafness (Guan et al. 2000; Zhao et al. 2005; Hobbie et al. 2008a, b). Guan et al. (2000) used cybrid technology to demonstrate that aminoglycosides target the A1555G mutation, to alter mitochondrial protein synthesis. This leads to decreased mitochondrial proteins below a normal functional threshold. They also observed that the nuclear background determined the phenotypic expression of nonsyndromic deafness from the A1555G mutation (Guan et al. 2001). The molecular mechanisms of the pathogenic nature of rRNA gene mutations were recently provided (Hobbie et al. 2008a, b). These invstigators used bacterial hybrid ribosomes that contained different versions of the mitochondrial-encoded regions of rRNA to show that the A1555G and C1494T mutations decreased the fidelity of translation. This defect in mitochondrial translation increased the susceptibility of the ribosomal site to aminoglycosides (Hobbie et al. 2008b). Indeed, they also demonstrated that the mutations increased the binding of the drugs to this site, which interfered with the translational functions of the ribosomes, thus inhibiting protein synthesis (Hobbie et al. 2008a). Thus, rRNA mutations can be pathogenic as well in cancer cells, but this is yet to be proven.

Several cancers have tRNA mutations, but the heteroplasmic levels of the mutant genomes are not well established. Mitochondrial tRNAs perform several functions including processing and translation of the polycistronic transcript. Some of the effects exerted by tRNA mutations on cellular functions have been revealed using established pathogenic tRNA mutations in cybrids (see Sect. 9.4.2). The A3243G transition mutation in $tRNA^{Leu(1)}$ of MELAS patients and A8344G transition mutation in $tRNA^{Lys}$ of MERRF patients have been used as cytoplasmic donors to generate cybrids, and the effects were examined in comparison to cybrids with wildtype mtDNA. At a heteroplasmic proportion of >85%, these mutations impair

intramitochondrial protein synthesis. The global reduction in mitochondrial proteins induced by the $tRNA^{Leu(1)}$ mutation could be a result of premature transcription termination. Recall that the PH1 promoter that generates $tRNA^{Phe}$, $tRNA^{Val}$ and the two rRNAs has a termination sequence in $tRNA^{Leu(1)}$ (np 3,229–3,256) which includes the A3243G MELAS mutation. Potentially, this mutation could interfere with the functions of mTERF. However, Chomyn et al. (2000) failed to observe differences among mTERF interaction with both mutant and wildtype sequences. One major defect created by this mutation is decreased aminoacylation of $tRNA^{Leu(1)}$ (Chomyn et al. 2000; Park et al. 2003). At 70–75% heteroplasmy, mutant cybrids were shown to have reduced sizes of mitochondrial polysomes, reduced aminoacylation of $tRNA^{Leu(1)}$ with decreased association of mitochondrial mRNAs with ribosomes accompanied by pronounced mRNA degradation (Chomyn et al. 2000).

The mechanism of impaired protein synthesis is probably defective processing of primary mitochondrial transcripts (King et al. 1992; Kaufmann et al. 1996). Cybrids carrying the MELAS mutation demonstrated consistent increase in steady-state concentration of partially processed contiguous RNAs including 16SrRNA, $tRNA^{Leu(1)}$, and ND1. Although the steady-state levels of the normally processed matured RNAs of these genes were unaffected by the mutation, the cells carrying the mutation had respiratory impairment. This finding raised the possibility that the partially processed 16SrRNA molecules were incorporated into mitoribosomes leading to defects in translation (King et al. 1992; Schon et al. 1992; Koga et al. 1995). In other studies, cell lines carrying various mutations including A8344G mutation in $tRNA^{Lys}$, C3256T transition mutation in $tRNA^{Leu(1)}$, and G5703A mutation in $tRNA^{Asn}$ all revealed marked decreases in steady-state levels of affected tRNAs possibly due to nucleolytic digestion (Enriquez et al. 1995; Masucci et al. 1995; Hao and Moraes 1996, 1997). An obvious effect of tRNA mutation is conformational changes and defective wobbling during translation. Because the effects tRNA mutations are demonstrated in other cells and disease conditions, definite biological effects of tRNA mutations in cancer are needed.

9.4 Techniques Employed to Study the Contribution of Mitochondrial DNA Mutations in Cancer

Several techniques are available for studying the contribution of mitochondrial genetic alterations to disease pathogenesis. In contrast to the nuclear genome that is relatively easily manipulated using various transgenic technologies, the unique genetic features of the mitochondrial genome require different approaches to study the effects of specific mitochondrial mutations to disease processes. Three common strategies are employed:

- For the study of the contribution of mtDNA to the carcinogenic process, especially retrograde signaling, some workers have relied on destroying mtDNA in cells and comparing the biochemical functional alterations in these cells to normal

cells. A major concern with this approach is that the agents used to destroy mtDNA can also damage nuclear DNA making the comparisons inappropriate.
- A much better approach is the generation of two types of cells with the same nuclear genetic background, but that differ in their mitochondrial genomes (i.e., cells with specific mtDNA mutations and cells without the mutations).
- A novel approach is nuclear integration, expression, and import into mitochondria of desired mitochondrial proteins.

9.4.1 Generating ρ0 Cells

Rho-zero (ρ0) cells are in vitro constructed eukaryotic cells with mitochondria that are devoid of their genomes. Historically, ρ0 stands for "ρ-depleted" cells, because the first observed cytoplasmic nucleic acids were called "ρ-nucleic acids" (Ephrussi et al. 1949). These cells can be stably established in culture without demise because mtDNA is important in coding for the minority of respiratory chain complement of proteins, which are dispensable in cells with alternative means of energy production. Thus, by providing the appropriate substrates to cells for ATP synthesis (i.e., to enable glycolytic mode of ATP synthesis) without mtDNA should enable sustained survival. However, the viability of ρ0 cells depends on their maintenance in culture enriched with pyruvate and uridine, because these cells are auxotrophic for both molecules. Given the other critical mtDNA-independent functions of mitochondria to cell physiology such as carbohydrate, protein, lipid, and nucleotide metabolism among others, these mtDNA-less cells, however, retain their mitochondria, although they are not perfect ρ structures.

King and Attardi first generated human ρ0 cells from osteosarcoma cell lines, and they named these 143B101 and 143B206 (King and Attardi 1989). This was achieved by chronic exposure of parental cell line to ethidium bromide (King and Attardi 1989; Hayashi et al. 1991). Ethidium bromide is positively charged and hence concentrates in the negatively charged mitochondrial matrix, thus minimizing any adverse effects on the nuclear genome. Ethidium bromide is a strong inhibitor of mitochondrial RNA polymerase. Mitochondrial replication requires mitochondrial RNA polymerase for the generation of primers necessary for mitochondrial DNA polymerase activity. Therefore, the loss mitochondrial RNA polymerase shuts off mtDNA replication and the subsequent loss of mtDNA in these cells. Ethidium bromide is also a cationic mutagen that intercalates DNA during replication, and hence interferes with mtDNA replication.

Apart from ethidium bromide, treatment of cells with methotrexate, or rhodamine 6-G can deplete their mtDNA. Similarly, exposure of cells to dideoxynucleoside analogs used to treat HIV infections can cause mtDNA depletion, but this effect is reversible when the drug is removed. Expression of a dominant-negative polymerase γ can also create ρ0 cells in culture.

Rhoz-zero cells have several uses in mtDNA research. DNA extracted from ρ0 cells are used extensively to screen primers to preclude coamplification of

pseudogenes or numts. Though imperfect, they are used to study the nuclear response to loss of mtDNA. Importantly, they are used to generate cybrids that enable unequivocal study of the contribution of mitochondrial genome changes to carcinogenesis and other mitochondrial diseases.

9.4.2 Producing Transmitochondrial Hybrids (Cybrids)

Cybrid constructs have been one of the real breakthrough technologies in mitochondrial molecular medicine. Cybrids are cells frequently used in the studies of the pathogenic nature of mitochondrial mutations. For direct comparative study of mitochondrial mutations, without nuclear DNA background effects, cybrids are constructed to carry mitochondrial genomes of interest with the same nuclear background as cells carrying wild-type mtDNA. These cells therefore permit unbiased study of the effects of mtDNA-specific mutations in various diseases, because the two comparative cell lines carry the same nuclear but different mitochondrial genomes.

The term "*cybrid,*" which stands for "cytoplasmic hybrid" was introduced by Bunn et al. (1974) to describe their work on fusing cytoplasts of chloramphenicol-resistant mouse A9 cells with nucleated chloramphenicol-sensitive mouse LMTK cell line. This pioneering works of Bunn and Wallace are currently broadly used by many investigators in the study of the role of specific mtDNA mutations in diseases. Currently, simply fusing an enucleated cell carrying mutant mtDNA of interest or wild-type mitochondrial genome (*mtDNA donor*) with ρ0 cells (*nuclear donor*) enables the generation of cybrids (Fig. 9.1). These two cells only differ in their mitochondrial genomes, because their nuclear genome is the same as the parental ρ0 cells.

Other mtDNA or cytoplasmic donors include platelets and synaptosomes that can be obtained from patients with specific mtDNA defects (Inoue et al. 1997). Platelets are nuclear-free fragments of megakaryocytes, and hence when derived from patients with the disease of interest can serve as a source of mtDNA for the generation of cybrids. Chemical enucleation by exposure to actinomycin D has also been described as a convenient way of making cybrids (Bayona-Bafaluy et al. 2003). Actinomycin D treatment causes a permanent irrecoverable defect in nuclear genome transcription and replication. Treatment of cells with the cell-permeable mycotoxin, cytochalasin B, can lead to nuclear extrusion. Following centrifugation, these cytoplasts can be harvested and used to generate cybrids.

The cybrid technology has played revolutionary roles in elucidating the necessity and sufficiency of defective mtDNA mutations in several diseases. Currently, numerous groups use cybrids to study the relevance and effects of specific mtDNA mutations in carcinogenesis. Several such evidences of the contribution of mitochondrial genome to cancer are discussed in this chapter.

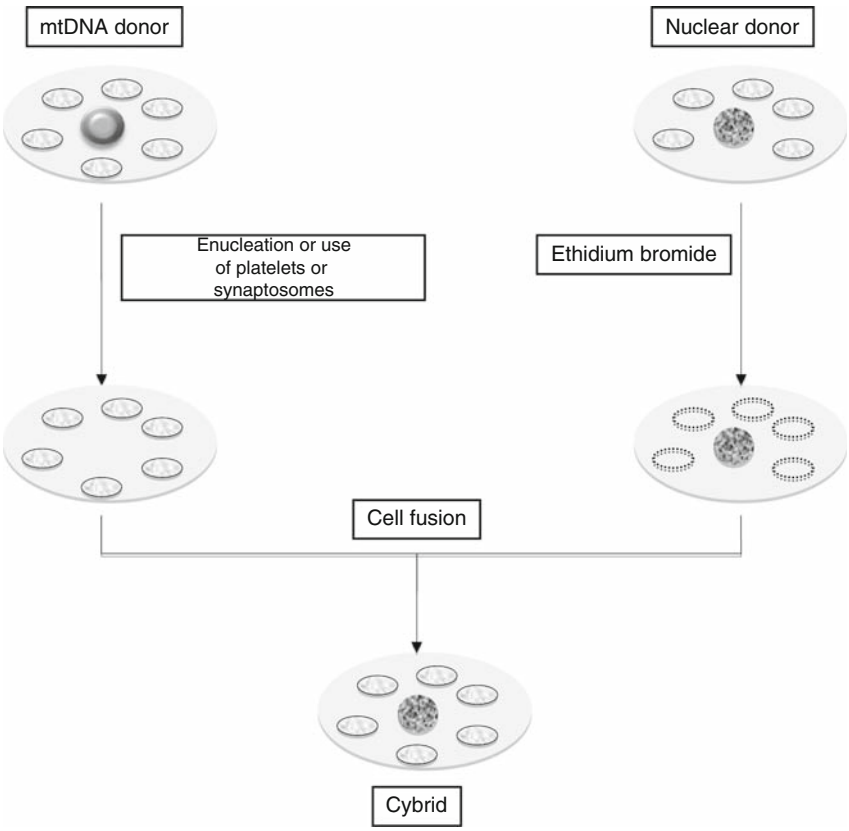

Fig. 9.1 *Production of cybrids*. Cybrids are constructed by fusing two cells, namely, mtDNA donor that contains the mtDNA genomes of interest and a nuclear donor

9.4.3 Nuclear Transfection of Mutant Mitochondrial DNA

Another technique of studying mtDNA mutations associated with defective proteins relies on the well-established model of nuclear genetic transfer (Manfredi et al. 2002). However, to integrate mitochondrial genes into the nucleus for it to be expressed and imported into mitochondria requires overcoming two challenges. First, the proteins must be properly engineered and so they can be imported into mitochondria. Second, the targeting sequence must be constructed to enable proper translation using the universal genetic code. The vast majority of mitochondrial proteins are encoded in the nucleus and translated in the cytoplasm before being imported into the mitochondria via protein translocation machineries located in the outer and inner mitochondrial membranes. These mitochondrial pre-proteins usually have a mitochondrial target signal that once inside the mitochondria gets cleaved to release the functional protein, which is then folded into its functional

structure. Therefore, by providing these mitochondrial targeting signals should enable the import of the engineered pre-proteins of interest into mitochondria. The second hurdle is resolved by converting the sequence of interest into a sequence recognizable by the nuclear code. Thus, long-range gene synthesis can be employed to synthesize mitochondrial genes of interest containing sequences recognizable by universal genetic code. This construct can then be cloned into a plasmid vector such as pCMV/myc/mito (Invitrogen) that contains mitochondrial targeting sequence (Zhou et al. 2007).

9.5 Contribution of Mitochondrial DNA Changes to Cancer Biology

Mitochondrial genome polymorphisms, somatic mutations, and content changes are demonstrated to contribute to the carcinogenic process (Table 9.1).

9.5.1 Cancer Risk Predisposition

Aging is a well-known risk factor for developing various cancers, and the aging process itself is associated with mtDNA mutations and functional decline. Thus, age-related mtDNA damage could be a carcinogenic risk factor. Apart from somatic mtDNA mutations, various groups have examined the possibility that there are potential cancer risk-conferring mtDNA polymorphisms in the general population. It has been entertained that some mitochondrial haplotypes may carry elevated risk

Table 9.1 Effects of mtDNA changes on carcinogenesis

Cancer biology	mtDNA changes	Possible mechanisms
Risk or cancer predisposition	Polymorphisms and haplotypes	Redox stress
	Copy number changes	Altered signaling pathways
Cancer initiation	mtDNA depletion	Epigenetic gene alterations
		Chromosomal instability
Cancer cell proliferation and growth	Somatic mtDNA mutations	Redox stress
		Altered nuclear gene expression
		Activated oncogenic signaling
		Apoptotic resistance
Cancer progression and metastasis	Somatic mtDNA mutations	Redox stress
	mtDNA depletion	Activated oncogenic signaling
Hormonal independence and chemoresistance	mtDNA deletions	Apoptotic resistance

for some specific cancers, and some of the evidences from various groups are compelling. Studies of mtDNA polymorphisms and haplotypes in relationship to cancer risks can be found in Chaps 6 and 7.

Mitochondrial DNA content might also play an enabling role in malignant transformation. Xing et al. (2008) examined the possible genetic heritability of mtDNA genomes. By studying twins, they demonstrated a high level of heritability of mtDNA content. In a case control study, low mtDNA content was associated with a statistically significant risk of developing renal cell carcinoma (Xing et al. 2008).

The biologic effects of some polymorphisms are demonstrated. The effects of the G10398A genotype, which is associated with breast cancer invasiveness in African-American women, were revealed recently using cybrid technology (Kulawiec et al. 2009). Cybrids were established using cells derived from African–American women with breast cancer and the G10398A genotype. In comparison to the G genotype, cybrids established with G10398A had slower growth rates, increased complex I activity and ROS production, with depolarized membrane potential. However, these cybrids were more resistant to etoposide-induced apoptosis, and this effect was mediated by AKT/PKB pathway. In addition, the G10398A cybrids demonstrated metastatic propensities as indicated by increased formation of anchorage-independent colonies in vitro as well as metastasis in mice.

9.5.2 Cancer Initiation

Epigenetic changes in the nuclear genome are well-established to mediate malignant transformation of various tissues. Data from recent studies implicate mitochondrial genome in modulating epigenetic changes in nuclear DNA. Mitochondrial DNA depletion in LNCaP cells (LNρ0-8) was associated with promoter hypermethylation at CpG islands in endothelin B receptor, O6-methylquanine-DNA methyltransferase, and E-cadherin genes, compared to parental LNCaP cells (Xie et al. 2007). Inhibition of the enzyme mediating the methylation process, DNA methyltrasnferase 1 in LNρ0-8 cells alleviated the methylation and therefore restored normal expression of these genes. Smiraglia et al. (2008) also investigated the role of mtDNA depletion in epigenetic nuclear gene modifications. They demonstrated significant changes in methylation patterns of several nuclear genes in mtDNA depleted cells, and these changes were reversible by restoration of wild-type mtDNA in these cells. Therefore, a role for the normal mitochondrial genome in preventing epigenetic changes in the nucleus is suggested by these studies.

Evidence for the possible involvement of mitochondrial genome damage in causing nuclear instability in cancer was provided by a separate group of investigators (Veatch et al. 2009). This group showed that in yeast loss of mtDNA induced nuclear genome instability through a process involving cell-cycle arrest and selection, which the authors referred to as a “cellular crisis.” This “cellular crisis” correlated with a reduction in the mitochondrial membrane potential, and not loss of respiration, and is a consequence of defective biogenesis that needs normal

mitochondrial function. Thus, mitochondrial dysfunction stimulates nuclear genome instability by inhibiting the generation of iron–sulfur cluster-containing proteins required for maintenance of nuclear genome integrity

It is also becoming evident that loss of mtDNA can confer oncogenic phenotype to cells. Initially, ρ0 cells were thought to be unable to form tumors. In this study, however, ρ0 cells with reintroduced wild-type mtDNA acquired tumor-forming capabilities when injected into nude mice (Hayashi et al. 1992). Subsequent works indicated that ρ0 cells had the innate ability to form tumors. More recently, the study by Shidara et al. (2005) provided an explanation for this conflicting data. They observed tumor formation by ρ0 cells at a much lower frequency, and demonstrated that tumor formation by ρ0 cells was a function of their viability following transplantation. Several other investigators have shown the tumor formation propensity of ρ0 cells. For example, transient depletion of mtDNA by Amuthan et al (2001) caused expression of nuclear genes involved in tumorigenesis and invasiveness. Loss of mtDNA caused transformed phenotypes with chromosomal instability (Singh et al. 2005). Rho-zero cells demonstrated increased lipid peroxidation, oxidative damage to nuclear genome associated with impaired DNA repair (Delsite et al. 2003). Although ρ0 cell nuclear might be damaged by ethidium bromide treatment and therefore potentially contribute to the transformed phenotypes, in many of these studies cybrids were used and therefore nuclear contribution to acquisition of transformed phenotypes is nullified. The bona fide genome contributing to the transformed phenotypes in these experiments is that of mtDNA.

Chromosomal instability is a hallmark of several cancers, and a balance of dNTP pools appears to maintain chromosomal stability. In a study of cellular repair in mtDNA-depleted cells, Desler et al. (2007) found marked decrease in dNTP pools in association with chromosomal instability. It is therefore plausible that mitochondrial loss can initiate or promote tumor formation through reduction in dNTP pools. An independent evidence that mtDNA regulates chromosomal stability is provided by another study (Kulawiec et al. 2008). In this study, the effects of mitochondrial depletion on tumorigenesis and nuclear gene induction were addressed. Loss of mtDNA in breast epithelial cell lines conferred malignant phenotypes to these cells, in association with differential regulation of *fibronectin 1* (*FN1*) and *p53* gene networks. Fibronectin causes cell adhesion and migration processes that occur during embryogenesis, wound healing, blood coagulation, and cancer metastasis. Aberrant expression of *structural maintenance of chromosomes 4* (*SMC4*) and *Werner syndrome, RecQ helicase-like* (*WRN*), two members of the p53 network involved in chromosomal and DNA maintenance, indicated possible chromosomal instability of these mtDNA-less breast epithelial cells. This conclusion was confirmed by the demonstration of DNA double-strand breaks and abnormal chromosomal rearrangements. Furthermore, downregulation of two other p53 network members, claudin 1 and 7 appeared to mediate malignant transformation in these cells. Additionally, claudin 1 and 7 were downregulated in primary breast tumors (Kulawiec et al. 2008).

Another evidence for a role of dysfunctional mitochondria in carcinogenesis is provided by the tumor forming propensities caused by mutations in nuclear-encoded

mitochondrial complex II subunits as well as TCA cycle enzymes. Nuclear gene mutations and defects in carcinogenesis are documented for paraganliomas and pheochromocytomas (Chap. 4). Mutations in *SDHB, SDHC,* and *SDHD* appear to cause a situation known as pseudohypoxia that facilitates cancer formation. To clarify the mechanism by which complex II subunits cause cancer, Cervera et al. (2008) used gene-silencing technology to specifically reduce the expression and activity of SDHB. This caused impaired respiration, and proliferation with a switch of the cells toward glycolytic pathway for ATP production. Reactive oxygen species production was however normal in these *SDHB*-silenced cells consistent with complex I/III being the major sources of ROS production. As well, these cells showed evidence of pseudohypoxia by the increased expression of HIF1α and HIF2α. The cells also adopted a malignant phenotype since they demonstrated greater capacity to adhere to fibronectin and laminin. Transient silencing of HIF1α partly reversed the adherent phenotype.

9.5.3 Cancer Cell Proliferation and Growth

A well-established hallmark of cancer is uninhibited proliferation and growth with or without growth factor stimulation. Several lines of evidence suggest these processes are partly controlled by the mitochondrial genome alterations in cancer.

Multiple linked and unlinked somatic mtDNA mutations occur in several cancers, making the contribution of any specific mutation to the carcinogenic process difficult to ascertain. However, several groups have used well-established specific pathogenic mtDNA mutations to convincingly demonstrate the functional contributions of these mutations to cancer cell growth. The ATP6 gene T8993G transversion and T9176C transition mutations that cause Leigh syndrome and neuropathy, ataxia, and retinitis pigmentosa have been used as models for a number of studies. Enucleated cells from these patients are fused to ρ0 HeLa cells to generate cybrids with homoplasmic mutant or homoplasmic wild-type clones.

The work by Shidara et al (2005) indicated that cybrids with mutations at np 8,993 and 9,176 grew faster in culture and promoted tumor growth when transplanted into nude mice. When wild-type ATP6 gene was transfected into the cybrids with mutant genomes, the tumor phenotypic features were curtailed. Confirmatory evidence that mutant ATP6 gene caused the observed changes in cybrids was provided with a collaborative experiment. Transfection of mutant ATP6 into wildtype cells conferred accelerated tumor growth characteristics to these cells in association with compromised respiration.

A role for mitochondrial ROS in controlling prostate cancer growth was provided by experiments conducted by Petros et al. (2005). In this study, a missense mutation (ATP6 C8932T) in prostate cancer was modeled to study the effects of such mutations in cancer development. To achieve this, the well-characterized ATP6 mutation (T8993G) that is a few amino acids upstream of the prostate cancer somatic mutation (ATP6 C8932T) was used. Cybrid cell lines were established and

when injected into nude mice it was observed that the mutant cells consistently grew larger than wildtype cells. Immunohistochemical analysis demonstrated elevated superoxide anion production by tumor cells, consistent with known ROS-producing T8993G mutation. Another study by this group showed the effect of the T8993G mutation in promoting prostate cancer growth in bone marrow environment (Arnold et al. 2009). Subcutaneous injection of cybrids with mutant and wild-type mtDNA into nude mice in the presence or absence of stromal cells revealed that tumors grew more aggressively in the presence of mtDNA mutations and stromal cells. Similar findings were demonstrated when injections were performed in bone morrow cavity. Tumor growth was associated with differential expression of 37 genes including overexpression of fibroblast growth factor 1 (FGF1) and focal adhesion kinase (FAK). Focal adhesion kinases mediate cell growth, proliferation, survival, and migration, and this gene is overexpressed in a number of primary cancers including invasive cancers (Golubovskaya et al. 2009).

Mutations in the mtDNA associated with respiratory defects can provide survival advantage to cancer cells via activation of oncogenic signaling pathway. Treatment of HL-60 leukemic, and Raji lymphoma cells with ethidium bromide led to multiple mtDNA deletions in *ND1* and in the D-loop with associated reduced levels of COII, ND4, and ATP6 (Pelicano et al. 2006). This respiratory dysfunction caused increased levels of NADH, which through redox-mediated inactivation of PTEN, activated AKT/PKB. This mitochondrial genetic defect made the cells to adopt the glycolytic phenotype of cancer cells, drug resistance, and enhanced survival in hypoxia (Pelicano et al. 2006).

Li et al. (2008) introduced mtDNA from colorectal cancer cell line, SW480 into NIH3T3 cells. The NIH3T3 cells with SW480 mtDNA acquired enhanced growth, increased colony formation rate and survival. Thus, colorectal cancer mtDNA promotes the malignant phenotype. Namslauer and Brzezinski (2009) also investigated the functional importance of colorectal cancer mtDNA mutations. Two mutations (Ser458Pro and Gly125Asp in COI) were engineered in Rhodobacter sphaeroides. The Se458Pro mutation was not expressed, probably because of severe enzyme structural alteration. However, the Gly125Asp mutation had a structurally normal enzyme, but with markedly reduced activity. Functional analysis revealed Gly125Asp mutation caused a proton leak that obviously could alter mitochondrial membrane potential ($\Delta\Psi m$) and mitochondrial bioenergetics. This finding is interesting in view of the findings of Heerdt et al. (2005, 2006, see Sect. 9.5.6) that changes in intrinsic $\Delta\Psi m$ in colorectal epithelial cell lines confer malignant potentials to the cells.

Independent studies investigating *CYTB* and *ND2* mutations provide supporting evidence for altered mitochondrial genome in promoting tumor growth (Zhou et al. 2007; Dasgupta et al. 2008, 2009). First, the functional importance of a 21 bp microdeletion in *CYTB* that is associated with primary bladder cancer was studied in murine xenograph and human bladder cancer models (Dasgupta et al. 2008). The mutation was overexpressed in the nucleus of uroepithelial cell lines, and this caused increased oxygen consumption, increased ROS production, and the acquisition of the glycolytic phenotype of cancer cells. These cells with mutant mtDNA had enhanced tumor growth in vitro and in vivo, partly via increased nuclear factor-kappa B2

signaling. In a follow-up study following transfection of the mutant *CYTB*, the uroepithelial cells had increased mitochondrial mass, in association with apoptotic resistance, demonstrated by cytoplasmic localization of BAX, lack of cytochrome c release, and failed cleavage of apoptotic poly(ADP-ribose)polymerase (PARP) (Dasgupta et al. 2009). In another study, mutation screening of the entire mitochondrial genome in head and neck squamous cell carcinoma revealed more nonsynonymous mutations in *ND2, ND5, COIII, CYTB*, and *ATP6* (Zhou et al. 2007). To investigate the possible roles of these mutations in tumor biology, mutations in *ND2* of complex I were cloned. This construct and wildtype *ND2* were transfected into HeLa cells. The mutant cells that expressed defective *ND2* demonstrated significant increase in growth rates in association with elevated ROS production. The impaired complex I activity was associated with accumulation of pyruvate and lactate as well as stabilization of HIF1α via induction of pseudohypoxia.

A role for mitochondrial genome on cancer cell growth was investigated in breast cancer using cybrid technology (Ma et al. 2009). Compared to cybrids containing normal mitochondria, breast cancer cell mitochondria conferred significant reduction in cell growth when metabolically stressed. In addition, the cells had reduced respiratory chain activities and energy production. Though not conclusive, tRNA mutations identified in two breast cancer cell lines were speculated to have accounted for the respiratory impairment (Ma et al. 2009).

9.5.4 Cancer Progression and Metastasis

An elegant experiment by Ishikawa et al. (2008) in mouse tumor models reveals an important contribution of mitochondrial mutations to tumor metastasis. They observed that complex I activity was normal in cell lines with low metastatic potential such as Lewis lung carcinoma cell line, P29, and fibrosarcoma cell line, B82, but much reduced in similar cell lines with invasive propensities including Lewis lung carcinoma (A11) and fibrosarcoma (B82M). In colon cell lines, however, invasive potential was not associated with defective complex I activity, suggesting that alternative signaling and metabolic pathways mediate colon cancer invasiveness.

To investigate the possible contribution of defective complex I activity in tumor invasiveness, the cybrid technology was employed. By reciprocal transfer of mtDNA between P29 and A11 cell lines, it was uncovered that the complex I defects were caused by possible mutations in mtDNA and not the nDNA subunits of this complex. By injection of these cybrids into tail veins and under the skin, and counting metastatic nodules in the lung, it was shown that the A11 mtDNA, irrespective of the nuclear DNA background (P29 or A11), conveyed the metastatic phenotype. Interestingly, the ability of mtDNA to confer metastatic potential was equally transferable to other tumor types. This was demonstrated by transferring mtDNA from A11 cells into B82 fibrosarcoma cells to generate B82mtA11 cybrids, and mtDNA from B82M cells into P29 cells to generate P29mtB82M cybrids. Irrespective of the cancer background, the two cell lines (low metastatic potential)

with mtDNA from highly invasive cells demonstrated defective complex I activity and high metastatic potential. Similarly, transfer of mtDNA from highly metastatic human breast cancer cell line, MDA-MB-231, into HeLa cells with low metastatic ability caused complex I defects in association with increased ROS production and acquisition of high metastatic phenotype.

Features of the malignant phenotype including increased ROS production and expression of antiapoptotic (myeloid cell leukemia 1 (MCL-1) and angiogenic (HIF1α and VEGF) genes were observed in A11 and B82M cells. Two mutations in *ND6* were uncovered when mtDNA sequence comparison between low (P29 and B82) and high (A11 and B82M) metastatic cells were made. A missense mutation, G13997A was seen in A11 cells, and a frame-shift mutation, 13885insC in B82M cells. Mechanistically, it was later showed that the ND6 mutation in A11 cells induced HIF1α overexpression through redox-mediated activation of PI3K/AKT and PKC signaling cascades, involving histone deacetylase (HDAC) (Koshikawa et al. 2009).

A role for mtDNA damage in tumor metastasis has also been investigated in human cell lines (Naito et al. 2008b). Loss of mtDNA in breast (MCFρ0) and prostate (LNρ0-8) cell lines acquired invasive phenotypes in association with the expression of mesenchymal cell markers. Thus, loss of or reduced mtDNA appears to induce epithelial–mesenchymal transition and invasive propensity. This phenotype was mediated by TGFβ signaling through the Raf/MAPK signaling cascade.

9.5.5 Acquisition of Hormonal Independence and Chemoresistance by Cancer Cells

Chemoresistance is an adaptive mechanism of cancer cells for survival under adverse conditions. Several investigators have studied the role of mitochondrial genome changes in cancer cell lines with regards to refractory response to hormonal therapy, as well as chemoresistance. Higuchi's group examined changes in the mitochondrial genome in relationship to androgen-independent phenotype. Almost all prostate cancers will respond to androgen deprivation initially, but subsequently develop resistance in order to sustain their survival. The androgen-independent C4-2 cell line was noted to contain more large-scale mtDNA deletions in association with reduced normal mitochondrial content compared to the androgen-dependent cell line, LNCaP. The androgen-independent phenotype could be generated in LNCaP cells by depleting their mitochondria, and was reversible by the reintroduction of normal mtDNA. Furthermore, mtDNA-depleted cells injected into nude mice developed tumors whereas LNCaP cells failed to form tumors. The study by Moro et al. (2008) addressed many more questions in regard to mtDNA depletion in prostate cancer cell lines. The androgen-independent prostate cancer cell lines, PC-3, DU145, and C4-2 possessed less mtDNA than the minimally invasive androgen-dependent LNCaP cells. The depletion of mtDNA in these androgen-independent

prostate cancer cells caused a reduction in the mitochondrial membrane potential, acquisition of metastatic potential demonstrated by enhanced migration into laminin-1, decreased chemosensitivity to paclitaxel, and decreased expression of poly(ADP-ribose)polymerase 1 (PARP-1). These phenotypic characteristics were specifically induced by mitochondrial depletion because they could be reproduced in LNCaP cells following depletion of their mtDNA. Importantly, decreased mtDNA content caused a significant decrease in PARP-1 protein levels.

Cancer mitochondrial genome contributes to acquired chemoresistance. Mizutani et al. (2009) generated cybrids with cytoplasts derived from human pancreatic cancer cell lines as well as normal healthy human cells. In vitro treatment of mutant and wild-type cybrids with the apoptosis-inducing agent (staurosporine) and anti-cancer agents (5-fluorouracil and cisplatin) demonstrated increased resistance of cybrids with cancer mitochondrial genomes to both apoptosis and chemotherapy. These in vitro findings were confirmed using in vivo model systems. Thus, cancer cell mtDNA may be the target to modulate for increase chemosensitivity.

In head and neck cancer cell lines, increase in mtDNA content was shown to induce resistance to docetaxel treatment. Exposure of docetaxel to human laryngeal cancer cell lines, HEp2 enabled the establishment of a doxetaxel-resistant cell line, DRHEp2, for study. DRHEp2 cells had very high mtDNA, but paradoxically low ROS production. Reduction of mtDNA in DRHEp2 cells by ethidium bromide treatment reduced drug resistance in these cells. Similarly, drug resistance was decreased and ROS production restored via interference with respiratory chain activity through blockade of F_0-ATPase, but not other respiratory chain subunits (Mizumachi et al. 2008).

Hormonal therapy of breast cancer is associated with depletion of mtDNA and subsequent development of hormone resistance. Long-term culture of human breast adenocarcinoma cells, MCF-7 in the presence of hydroxytamoxifen (4-OHT) led to mtDNA depletion and acquisition of hormone resistance (Naito et al. 2008a). To investigate whether mtDNA depletion caused the drug resistance, MCF-7 cells were depleted of their mtDNA by treatment with ethidium bromide. The mtDNA-depleted MCF-7 cells became resistant to hormonal therapy, which could be restored by reconstitution of these cells with wild-type mtDNA. It was also uncovered that withdrawal of 4-OHT in the long-term cultures of MCF-7 cells restored mtDNA content and susceptibility to hormonal therapy in these initially mtDNA-depleted cells.

9.5.6 Contribution of Mitochondrial Genome Changes to the Development of Oncocytic Tumors

Oncocytes are unique cells found in some cancers with mitochondrial aberrations. Oncocytic tumors are characterized by increased presence of oncocytic cells in the tumor. The levels of oncocytes in these tumors could be as high as 80%. A fundamental biologic observation in oncocytes is the disproportionate increase

in mitochondria as a consequence of increased mitochondrial proliferation. The basic nature of oncocytes therefore renders oncocytic tumors their characteristic granular eosinophilic appearance in conventional hematoxilin and eosin staining. Oncocytes are found in several cancers including cancers of the thyroid, salivary, and pituitary glands, and in renal, adrenal, ovarian, pancreatic, and breast tissues. They are very frequent in follicular thyroid cancers, where they are associated with increased aggression and poor prognosis.

Several investigators have demonstrated mitochondrial defects in oncocytic tumors, including increased mtDNA content, loss of electron transport components, accompanied by deficient ATP synthesis, and impaired mitochondrial protein synthesis. The molecular mechanisms mediating these observations are recently been uncovered. Abnormal restriction bands, that might be specific to renal oncocytomas, were detected when mtDNA was digested (Kovacs et al. 1989; Welter et al. 1989). While the pathogenic nature of the abnormal mtDNA bands was unknown at the time, it is now becoming evident that defects in respiratory chain complex activity underlie this pathology. Simonnet et al. (2003) detected low complex I protein and activity in renal oncocytomas compared to non-oncocytic renal tumors. Bonora et al. (2006) were the first to examine the bioenergetic efficiency of thyroid oncocytomas in relationship to mtDNA mutations. They studied follicular thyroid oncocytomas (XTC.UC1) in comparison to non-oncocytic thyroid follicular tumors (TPC-1). They observed that the oncocytic XTC.UC1 cells survived poorly when forced to rely on OXPHOS for energy production. This poor survival was associated with reduced oxygen consumption, ATP synthesis, and elevated ROS production. The ability to transfer these bioenergetic failures into cybrids containing XTC.UC1 mtDNA suggested that the defect was resident in the mitochondrial genome. Mitochondrial sequence analysis of XTC.UC1 cells detected two heteroplasmic pathogenic mutations in complex I and III subunits. A frameshift mutation, C insertion at np 3571 of *ND1* caused the production of truncated ND1 protein, and this was associated with a complete loss of ND1 protein levels as assayed by Western blotting. The other mutation is a missense mutation, G15557A in *CYTB*, the only mitochondrial complement of complex III. Importantly, the mutation affected a catalytic site of *CYTB* involved in electron shuttle.

In another study by the same group, the sequences of full mitochondrial genomes from 45 thyroid and five breast oncocytomas were compared to those from 52 non-oncocytic thyroid, breast and glial tumors. Nonsense and frameshift mutations were common in complex I genes – *ND1, ND2, ND4*, and *ND5*. Interestingly, the two oncocytic tumors harbored the same 3571insC that was uncovered in their earlier study of oncocytic cell line XTC.UC1. In an attempt to study the pathogenecity of these mutations, primary cultures of oncocytic tumors and corresponding normal tissues were established. Surprisingly, the oncocytic tumors lost the pathogenic mutations and oncocytic phenotype in culture, providing another confirmatory evidence that oncocytic phenotype depends on mtDNA complex I mutations and function.

Two independent groups studied renal oncocytomas with reference to mitochondrial mutations and defects in respiratory complex activities (Gasparre et al. 2008;

Mayr et al. 2008). Nuclear and mtDNA analysis unraveled defective complex I mtDNA mutations as the only genetic abnormalities in all 9 renal oncocytic tumors that were studied (Gasparre et al. 2008). These mutations were clonally amplified in many of these tumors, suggesting a possible selective advantage for tumor growth. Mayr et al. (2008) examined the activities of respiratory chain enzymes and also sequenced mtDNA from 15 renal oncocytomas. Renal oncocytic tumors demonstrated unassembled multi-subunit complex I in association with loss of its enzymatic activity. Frameshift mutations were revealed in *ND1, ND4*, or *ND5*, suggesting their pathogenic role in defective complex assembly and activity. Immunohistochemical analysis of all OXPHOS enzymes in 19 thyroid oncocytomas revealed a specific loss of complex I expression in tumors compared to the normal adjacent tissues (Zimmermann et al. 2009).

Thus, respiratory complex I, and possibly III defects, appear to mediate oncocytogenesis. The importance of these mutations in oncocytomas requires in-depth study.

9.6 Mitochondrial Membrane Potential and Cancer

The mitochondrial membrane potential ($\Delta\Psi m$) is established by the inner mitochondrial membrane as a consequence of proton or hydrogen ion pumping across the membrane during operation of the respiratory chain. The $\Delta\Psi m$ is critical to the viability of cells because dissipation is associated with apoptotic cell death. For example, overexpression of gelsolin prevented loss of $\Delta\Psi m$ and cytochrome c release (Koya et al. 2000). Energy and ROS production, as well as protein import into mitochondria, depend on normal $\Delta\Psi m$. Also, the translation of mitochondrial transcripts and stability of mitochondrial proteins require intact $\Delta\Psi m$. Thus, it is obvious that many normal cellular processes require intact and normal $\Delta\Psi m$.

The $\Delta\Psi m$ is very dynamic, changing in response to micro-environmental cues, cellular localization with respect to other cells, intracellular localization of specific mitochondria, and in response to induction of intrinsic apoptotic partway. Heerdt and coworkers have examined the effects of changes in intrinsic cellular $\Delta\Psi m$ among stably established colonic cells on tumor initiation and progression (Heerdt et al. 1998; Heerdt et al. 2003; Heerdt et al. 2006). In the normal colon, cell turnover mediated by proliferation and apoptosis is an ongoing process, and an imbalance in this process can lead to malignant transformation. Also known is the increased $\Delta\Psi m$ in colonic cancer cells compared to normal colonic cells. Indeed, Heerdt et al. (2003) observed that in human colonic cancer cell line SW620, the initiation of apoptosis and G_0/G_1 cell cycle arrest by butyrate (a short-chain amino acid) were both inhibited by a collapse in $\Delta\Psi m$, suggesting that $\Delta\Psi m$ plays a critical role in the homeostatic balance of colonic epithelial cell turnover and that disturbance in $\Delta\Psi m$ could potentially trigger malignant transformation. By establishing single-cell clones from SW620 cells, the authors further demonstrated that various colonic cells in culture possessed

stable, but significant differences in their intrinsic ΔΨm (Heerdt et al. 2005). While these differences in ΔΨm did not interfere with mitochondrial functions and cell viability, cells with increased ΔΨm also had increased VEGF expression compared to parental cells from which they were derived. Similarly, cells with low ΔΨm had lower VEGF protein levels than the original cell population. Noteworthy, VEGF expression is linked to colorectal cancer liver metastasis and poor outcomes. Additionally, similar findings of intrinsic ΔΨm and MMP-7 expression as well as invasive propensities were demonstrated (Heerdt et al. 2005). Further studies indicated that changes of ΔΨm in colonic epithelial cells were associated with adaptive response to hypoxia, cell survival, anchorage-independent growth, and propensity to invade and metastasize (Heerdt et al. 2006). Thus, ΔΨm changes in colonic cells and possibly other cell types can initiate tumor formation and promote progression.

9.7 Collaborative Evidences from Clinical Studies

Changes in mitochondrial genome and clinicopathologic parameters have been addressed by a number of investigators. In some of these studies, mtDNA changes appear to correlate with disease process and outcomes. A few of these studies are exemplified here to illustrate the importance and potential of mitochondrial genome changes in clinical outcomes of cancer.

Mitochondrial genome changes may confer prognosis in some cancers. In colon cancer, D-loop mutations were noted to be associated with poor prognosis, as well as response to 5-Fluorouracil treatment (Lievre et al. 2005). Patients without D-loop mutations had a significantly better 3-year-survival rates, and in stage III colon cancers, D-loop mutations were associated with chemoresistance, and therefore only benefited patients without these mutations. Yamada et al (2006) observed in liver cancer that decreased mtDNA copy number significantly correlated with tumor size and presence of cirrhosis. A survival analysis indicated that patients with decreased tumor mtDNA content had poor 5-year survival compared to patients with high tumor mtDNA copies. In breast cancer, D-loop mutations, especially at D310 were associated with older age, negative estrogen, and progesterone receptor status, as well as poorer disease-free survival (Tseng et al. 2006). Mitochondrial genome mutations as prognostic markers in women with invasive cervical cancer have been demonstrated (Allalunis-Turner et al. 2006). This group sequenced mitochondrial complex I genes in a 10-year retrospective invasive cervical cancer samples. Univariate and multivariate analysis revealed significant poor outcomes in patients with eight or more mtDNA mutations, and the log-rank analysis indicated a significant difference in overall survival, suggesting the importance of multiple mtDNA mutations in coding genes as prognostic markers for cervical cancer.

Other studies suggest that mtDNA mutations are associated with various pathological parameters of cancer. In a study of breast cancer mtDNA mutations,

histopathologic analysis revealed a significant association of microsatellite instabilities with invasive lobular carcinoma (74%) compared to invasive ductal carcinoma (19%) (Pavicic et al. 2009). This is interesting because mtMSI may influence the pathogenesis of the various breast cancer subtypes. In ovarian cancer, specific mtDNA variants are associated with cancer subtypes as well as ethnic background of patients (Aikhionbare et al. 2008). A C7028T change was present in only 8% of borderline ovarian tumors, but increased to 75% in late stage III/IV cancers. Similarly, variant T8548G occurred in 72% of serous type stage III/IV, while C7256T and G7520A were found in 54% of endometriod type cancers but not in serous epithelial ovarian cancers. C7028T was found in 86% African-American compared to only 43% of Caucasian women; C7520T was uncovered in 74% of African-American but found in only 26% of Caucasian women.

Mitochondrial genome changes in biofluids also appear to correlate with clinical outcomes. Mehra et al. (2007) reported that the levels of circulating mitochondrial nucleic acids in advanced stage prostate cancer patients could be used as a predictor of disease behavior. In this series, patients with high levels of circulating mitochondrial DNA and RNA survived poorly compared to those with low levels. The levels of mtRNA appeared to be an independent predictor of 2-year survival. Ellinger et al. (2008) also examined the levels of mtDNA fragments in serum of prostate cancer patients in relation to disease progression. They observed high levels of short mtDNA fragments in the serum of patients with early PSA recurrence after radical prostatectomy, and this biomarker was the strongest predictor of PSA recurrence in a multivariate analysis.

In summary, there is evidence that mitochondrial genetic alterations modulate clinical outcomes of cancer patients. These findings have important implications in cancer detection, staging, and prognostication. Clinical validation of such mtDNA alterations is needed.

9.8 Conclusion

Recent genetic manipulations are providing important evidences for the contribution of mtDNA alterations in cancer biology. Especially, the cybrid technology makes it convincing to draw unequivocal conclusions on the effect of specific mutations to cancer biology. It is hoped that specific mtDNA mutations, including tRNA, rRNA, and D-loop mutations, will be modeled to illustrate their specific roles in cancer. Not only will these findings provide avenues for developing effective chemotherapies, but they will also enable risk assessment and chemoprevention. With increasing technological advancement, it will become possible in future to model these mutations in mouse models so as to directly test the in vivo functional importance of mtDNA mutations.

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Chapter 10
The Role of Mitochondrial Reactive Oxygen Species in Cancer

Abstract Reactive oxygen species (ROS) are oxygen-derived free radicals generated in the cell as byproducts of normal mitochondrial metabolism, peroxisomal reactions or cytochrome P-450 activity. The interactions of ionizing radiation with biomolecules can produce ROS, and enzymatic processes such as the NADPH oxidase system produces significant levels of ROS in some cells. Evidence, however, suggest that most of the cellular ROS is generated in the mitochondria as byproducts of normal energy production via the respiratory chain. The unpaired electrons in free radicals are highly unstable and therefore react with biomolecules such as nucleic acids, proteins and lipids. Such interactions can cause cellular damage leading to cell death or malignant transformation. Apart from their deleterious effects, free radicals mediate several intracellular signaling in normal physiology. Because redox signaling can be beneficial to the cell, it is imperative for the cell to establish a balance of the amounts of free radicals needed for normal physiology without cellular injury. Such redox homeostasis, or redox balance is achieved in biologic systems by the activity of detoxifying enzymes and/or antioxidant systems. Discussed in this chapter are ROS generated by mitochondrial functions, and their role in carcinogenesis.

10.1 Introduction

Free radicals are molecules, ions, or atoms with unpaired electrons in their outermost shell of electrons. The reactive oxygen species (ROS) are oxygen-derived free radicals generated in cells as byproducts of normal mitochondrial metabolism, peroxisomal reactions, or cytochrome P-450 activity. ROS can originate from interactions of ionizing radiation with biomolecules, or by enzymatic processes. ROS are very reactive as a consequence of the presence of unpaired electrons in their outermost electrons shells. As such, ROS can readily react with and damage biomolecules including nucleic acids, lipids, and proteins that could culminate in cellular destruction or transformation. However, it is now very clear that the effects

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of ROS are probably far more reaching in other cellular activities than mere cytotoxicity or cancer initiation. ROS can modulate cellular proliferation, differentiation, apoptosis, and senescence as well as other aspects of cell physiology. It is possible that the effects of ROS in a particular cell are dependent on the levels, types, and kinetics of the particular ROS. Genomic damage in normal cells by low-level ROS can effectively be repaired to maintain genome integrity. Cellular transformation is associated with elevated levels of ROS. More troubling is the fact that moderate levels of ROS can induce cellular proliferation and apoptotic suppression, thereby favoring malignant transformation. Exceptionally high levels of ROS in malignant cells could cause cell death. Endogenous enzyme systems and mitochondrial bioenergetics, as well as exogenous processes such as chemical and physical carcinogens, growth factor stimulation, and cytokine signaling, can generate ROS production in a cell. Whereas there are several diverse endogenous sources of ROS in the cell, including the NADPH oxidase (Nox) system, the primary functions of mitochondria in energy production is inevitably linked with ROS production that can play important roles in cancer cell initiation and progression.

There is now convincing evidence that almost all types of cancers are characterized by mitochondrial genome mutations in association with elevated ROS. The functional importance of these mutations and associated oxidative stress in the pathogenesis of cancer are becoming apparent. Recent studies using cell lines and other transgenic technologies are shedding some light on the important consequences of mitochondrial genome mutations and ROS production in cancer initiation and progression. Mitochondrial genome changes could cause increased oxidative stress leading to epigenetic modification of nuclear genes and resistance of cells to apoptosis, as well as other adaptive mechanisms of cancer cells that enable survival in various adverse environments. Elevated ROS in cancer cells could alter signaling pathways to perpetuate the malignant phenotype. These demonstrable cellular changes associated with mitochondrial genome mutations implicate this organelle in the pathophysiology of cancer. This chapter focuses on ROS generated by mitochondrial functions and the role of these molecules in the carcinogenic process.

10.2 ROS Production by Mitochondria

Isolated mitochondria have served as models for several studies of where and how mitochondria produce ROS. As the first family member to be produced, the $O_2^{\bullet -}$ plays an important role in determining the production of other family members (Fig. 10.1). Superoxide anion is generated by a single electron reduction of O_2 by electron donors. This reaction occurs at several sites in the mitochondria; however, a number of experiments in isolated mitochondria suggest that the major source of $O_2^{\bullet -}$ production occurs at mitochondrial complex I of the respiratory chain (Murphy 2009). The electron donors are redox-active groups within proteins such as reduced coenzyme Q10 (CoQ).

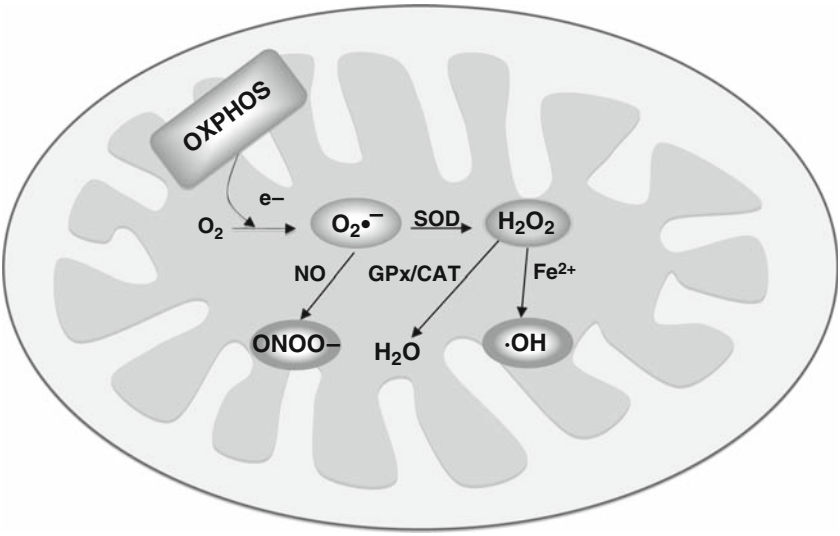

Fig. 10.1 *ROS production by mitochondria*. Electrons leak from the respiratory chain (OXPHOS) to reduce molecular oxygen to produce superoxide anions, which can be dismutated to hydrogen peroxide and removed by catalase (CAT) and glutathione peroxidase (GPx) systems. However, hydrogen peroxide can react with transition metals such as iron and copper to produce the most reactive ROS, the hydroxyl ion. Superoxide anions can react with nitric oxide to produce peroxynitrite

ROS production in mitochondria is regulated by several factors operating in concert. These include: (1) the concentration of O_2 because it is known that excess O_2 favors $O_2^{\bullet -}$ production; (2) the concentration of enzymes or proteins with redox-active sites, because exposed redox-active sites similarly will enhance $O_2^{\bullet -}$ production; (3) an increased ratio of NADH/NAD+ favors the production of $O_2^{\bullet -}$; (4) an increased CoQH/CoQ ratio equally enhances $O_2^{\bullet -}$ production; (5) the need for ATP production verses heat production. Low ATP requirements will decrease respiratory chain activity favoring the build-up of NADH and hence $O_2^{\bullet -}$ production, especially when coupled with increased caloric intake; and finally (6) an increase in the CoQH/CoQ ratio in the presence of high proton-motive force (Δp) favors $O_2^{\bullet -}$ production by reverse electron transport (RET). However, two primary mechanisms basically determine the ROS production in mitochondria; (1) a high NADH/NAD+ ratio, and (2) a high CoQH/CoQ ratio in association with high Δp.

Mitochondrial complex I is a 1,000-kDa protein complex composed of 45 polypeptides, a flavin mononucleotide (FMN) and seven iron–sulfur (FeS) clusters. The FMN component accepts electrons from NADH and passes them via the FeS clusters to CoQ. The electron centers in FMN and CoQ are usually shielded from O_2 because of their propensity to donate electrons to O_2 that could initiate the production of ROS. Superoxide anion production occurs at two sites of complex I:

- The reaction of O_2 with reduced FMN, whose levels are controlled by NADH/NAD+ ratio (Kussmaul and Hirst 2006; Hirst et al. 2008). This ratio will be

elevated in conditions such as mutations in genes that affect respiratory chain functions, or in situations of low energy demands. In support of this mode of $O_2^{\bullet-}$ production, expression of yeast *NDI1* that encodes the internal NADH dehydrogenase in cells increased NADH oxidation and decreased $O_2^{\bullet-}$ production (Seo et al. 2006). Indeed, in cells without *NDI1* expression, inhibition of complex I with rotenone caused elevated ROS production in association with lipid peroxidation and mtDNA mutations.

- The second site of $O_2^{\bullet-}$ production in complex I is at the quinone-binding site during RET. In conditions when electron flow to CoQ by complex I is decreased, coupled with high Δp, electrons can flow back from CoQH into complex I and putatively reduce NAD+ to NADH at FMN leading to $O_2^{\bullet-}$ production. This mechanism is referred to as RET (Adam-Vizi and Chinopoulos 2006). Whereas the site of $O_2^{\bullet-}$ production during RET is unclear, it is demonstrated that this mode of $O_2^{\bullet-}$ production critically depends on Δp, because an elevated $O_2^{\bullet-}$ production occurred in mitochondria isolated from liver, heart and brain under high Δp coupled with electron supply to CoQ via complex II (Adam-Vizi and Chinopoulos 2006). Mitochondrial complex II is composed of four polypeptides encoded entirely in the nucleus. It accepts electrons from succinate and passes them onto CoQ. Damage or mutated complex II can produce $O_2^{\bullet-}$; however, in normal succinate oxidation, $O_2^{\bullet-}$ is produced primarily via RET (Liu et al. 2002).

Another site of $O_2^{\bullet-}$ production in the electron transport chain is at complex III. Complex III is a 240 kDa monomer composed of 11 polypeptides. It has three hems and one FeS center. It receives electrons from CoQ and passes them on to cytochrome *c*, during which it transiently interacts with CoQ at its Q_i and Q_o sites. When the Q_i site of CoQ was inhibited by antimycin, $O_2^{\bullet-}$ production increased as O_2 reacted with ubisemiquinone at the Q_o site. However, damage to distal respiratory chain, e.g., cytochrome *c*, does not increase $O_2^{\bullet-}$ production by complex III (Turrens et al. 1985). It is likely that complex III contributes little $O_2^{\bullet-}$ under normal physiologic conditions, but could be an important source under pathologic conditions.

Finally, NADH and CoQ can interact with other redox-active sites to produce $O_2^{\bullet-}$ in the mitochondrial matrix. Reduced NADH in association with α-ketoglutarate increases $O_2^{\bullet-}$ production from α-ketoglutarate dehydrogenase. This occurs because the dihydrolipoamide dehydrogenase component of α-ketoglutarate dehydrogenase possesses a flavin that donates electrons to NAD+ during enzymatic reaction (Starkov et al. 2004; Tretter and Adam-Vizi 2004). Thus, in conditions of limiting NAD+, electrons can leak from flavin to O_2, leading to increased $O_2^{\bullet-}$ production. Consistent with this conclusion, decreased dihydrolipoamide dehydrogenase in mouse fibroblasts was associated with decreased $O_2^{\bullet-}$ production (Starkov et al. 2004; Tretter and Adam-Vizi 2004). Finally, fatty acid oxidation in mitochondria can also be associated with hydrogen peroxide production.

Once produced, $O_2^{\bullet-}$ is dismutated to H_2O_2, which in the presence of sufficient antioxidant enzymes such as calalase and peroxidase, is detoxified. However, in the presence of transition metals H_2O_2 is converted to the most reactive ROS, the hydroxyl radical (Fig. 10.1).

In addition to ROS, mitochondria generate peroxynitrite ($ONOO^-$), a potent free radical from nitric oxide (NO) and $O_2^{\bullet -}$ anion (Fig. 10.1). Nitric oxide is synthesized from L-arginine and O_2 in a reaction catalyzed by nitric oxide synthase (NOS). There are at least three NOS isoforms; neuronal NOS (nNOS/NOSI), inducible NOS (iNOS/NOSII) and endothelial NOS (eNOS/NOSIII). Mitochondrial NOS (mtNOS) is nNOS-α that is posttranslationally modified and imported into the mitochondria. There are other posttranslational variants of NOS in addition to the above mentioned. NOS I and III are constitutively expressed by many cells and can be induced by Ca^{2+} pulses. On the contrary, iNOS/NOSII is not expressed constitutively and must be activated by factors such as cytokines (e.g., TNF-α), and transcription factors (e.g., NF-κB). The steady-state concentration of mitochondrial NO is controlled by the activity of mtNOS and influx of NO from the cytosol. In general, cytosolic NO is bound to molecules such as myoglobulin, and therefore only small amounts enter the mitochondria. However, mitochondrial NO levels can increase sunstantially following induction of cytosolic NOS or increased activity of mtNOS. Elevated mitochondrial NO in the presence of $O_2^{\bullet -}$ anion can lead to the formation of $ONOO^-$. Peroxynitrite is more reactive than NO and $O_2^{\bullet -}$ and oxidizes biomolecules via direct oxidative modification through a one- or two-electron oxidation processes. It can react with thiol groups in proteins, iron–sulfur centers, and zinc fingers, thereby interfering with the normal functions of these molecules.

An established important source of ROS in the cell is the Nox system. While the various enzymatic sources of ROS may operate independently, it is possible that mitochondrial ROS and Nox systems interact at some levels. Lee et al. (2006) revealed an intriguing relationship between mitochondrial ROS and Nox1-mediated ROS production. In human 293T cells, serum withdrawal induced increased ROS production from both mitochondrial and Nox1 systems. It was uncovered that the immediate response to serum withdrawal was mitochondrial production of ROS, which indirectly triggered the Nox1 system. This indirect activation of Nox1 was via activation of both phosphoinositide 3-kinases (PI3K) signaling and Rac1. Indeed, Rac1 can induce Nox1-mediated ROS production by forming a multimeric complex with Nox1 and its regulatory subunits NOXO1 and NOXA1 (Cheng et al. 2006).

10.3 Biomolecular Targets of ROS

Apart from their physiologic functions, ROS can oxidize biomolecules such as nucleic acids, lipids, and proteins. Oxidative modification of nucleobases and sugar–phosphate backbone of nucleic acids could lead to epigenetic modifications and mutations in the genome. Guanine, which has a low reduction potential, is the most reactive base that is often oxidized to 8-oxoguanine. ROS can induce lipid peroxidation of membranes, liposomes, and lipoproteins by removing a hydrogen atom from polyunsaturated fatty acids. Lipid peroxidation leads to loss of membrane integrity and hence permeabilization. Among other protein components, ROS can

oxidize thiol groups (Cys-SH) in proteins to sulfenic acid (Cys-OH), which can react with another thiol to form disulfide residues. These oxidized molecules can be reversed by various reducing agents back to Cys-SH. Thiol modifications of proteins is an important mechanism of ROS-mediated altered signal transduction pathways in cancer. For example, inactivation of protein tyrosine phosphatases by thiol oxidation can lead to enhanced phosphotyrosine-dependent signaling. In addition, several serine/tryonine kinases including protein kinase A, protein kinase G, protein kinase C, PI3K/AKT/PKB, and S6 kinase contain Cys-SH in their active domains. Cys-SH motifs in transcription factors such as c-fos, c-jun, NF-κB and HIF1 are the likely targets of ROS. Oxidation of critical cysteine groups may inactivate many enzymes involved in bioenergetics including creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, mitochondrial complexes I–III and V. Importantly, low-molecular-weight thiol groups such as glutathione and thioredoxin can be oxidized by ROS, and these interactions help lower the levels of toxic ROS, thus maintaining redox balance in states of excess ROS production.

10.4 Modulation of Intrinsic Apoptosis by ROS

An overview of the intrinsic apoptotic pathway is given here for convenience. For detailed discussion, see Chap. 3. The mitochondrial pathway of apoptosis is activated intracellularly by extreme cell stress such as hypoxia, irradiation, and cytotoxic drugs. Central to this apoptotic pathway is mitochondrial membrane permeabilization (MMP) and the release of inter-membrane space (IMS) proteins into the cytosol. These mitochondrial proapoptotic factors include cytohcrome c, procaspase 9, Smac/Diablo, apoptosis-inducing factor (AIF), and endonuclease G. Cytochrome *c* interacts with Apaf1 and procaspase 9 to form an apoptosome, which then activates the executioner caspase (caspase 3). Smac/Diablo activates caspase 3 via inhibition of inhibitors of apoptosis (IAPs). AIF and endonuclease G translocate into the nucleus to induce DNA fragmentation.

A balance between the BCL-2 antiapoptotic/pro-survival (BCL-2, BCL-xL, MCL-L, BCL-W) and proapoptotic (BAX, BAK BID, BIM, BAD) members decides whether a cell will survive or die. The proapoptotic members cause MMP leading to a point of no return in the intrinsic pathway. Mitochondrial permeability transition (mtPT) occurs with increased calcium signaling or oxidative stress. Primarily, these apoptotic stimuli induce opening of the permeability transition pore complex (PTPC) leading to the influx of water into the matrix causing mitochondrial swelling and dissipation of $\Delta\Psi m$ and physical rapture of the outer membrane with the release of IMS proteins. Amongst other molecules, PTPC is formed by VDAC on the outer membrane and ANT located in the inner membrane.

ROS can cause cell death directly via interaction with the mtPT pore complex to cause mtPT. Adenine nucleotide translocase (ANT) has three redox-sensitive reduced cysteine residues (Cys^{57}, Cys^{160}, and Cys^{257}) that can undergo thiol oxidation by ROS (Costantini et al. 2000). Oxidative induced cross-linking of

Cys^{160}, and Cys^{257} alters the conformation and binding ability of ANT to nucleotides resulting in the influx of calcium into the matrix. Pore opening occurs because ANT and cyclophylin D form a complex in the presence of calcium (Halestrap et al. 2004). ROS, especially $O_2^{\bullet -}$, interacts with voltage-dependent anion channel (VDAC) to induce outer membrane permeabilization. Both $O_2^{\bullet -}$ and H_2O_2 can also mediate cytochrome *c* release by calcium-mediated permeability transition (Madesh and Hajnoczky 2001). It is also suggested that, in addition to interaction with VDAC, ROS may enhance dissociation of cytochrome *c* from cardiolipin, which usually binds cytochrome *c* in the inner mitochondrial membrane. Indirectly, ROS can modulate apoptosis by activating signaling pathways such as mitogen-activated protein kinase (MAPK) and PI3K (see Chap. 3 on how apoptosis is modulated by these oncogenic pathways).

10.5 ROS and Hypoxia-Inducible Factor Stabilization in Cancer

The reduced vascularizaion with tumor growth causes varying degrees of hypoxia in solid tumors. A means by which cancer cells survive these adverse conditions is the induction and stabilization of HIF, leading to the formation of new blood vessels.

The hypoxia-inducible factors (HIFs) are transcription factors that possess basic helix–loop–helix and Per/ARNT/Sim (PAS) domains. Semenza and Wang identified them as factors that regulate increased expression of erythropoietin in response to hypoxia (Semenza and Wang 1992). There are two family members, HIFα and HIFβ that become active upon heterodimerization. In normal oxygen conditions, the protein products of HIF1α are degraded following hydroxylation of specific proline residues catalyzed by prolyl hydroxylases. In hypoxia, however, prolyl hydroxylases are unable to effectively hydroxylate HIF1α leading to accumulation and stabilization of the protein. Hypoxia-inducible factor pathway activation promotes carcinogenesis through transcriptional regulation of several genes involved in angiogenesis, metabolism, proliferation, differentiation and metastasis.

The paradox of increased ROS production in conditions of low oxygen is explained by the work of Guzy et al. (2005). ROS in such conditions is primarily generated at mitochondrial complex III. Guzy et al. (2005) used RNAi to knockdown the expression of Rieske iron–sulfur protein of complex III, and this manipulation was associated with decreased ROS production and HIF1α stabilization. Conceivably, decreased oxygen delays the transfer of electrons from ubiquinol to cytochrome *c*1 by Rieske iron–sulfur protein leading to electron buildup and leakage to form ROS. Mitochondria thus functions as oxygen sensors in situations of decreased partial pressure of oxygen to release ROS into the cytosol to stabilize hypoxic transcription factors. Specifically, H_2O_2 in the cytosol inhibits the activity of prolyl hydroxylases thereby permitting the stabilization and accumulation of HIF1α.

10.6 ROS and p53 Functions

The tumor suppressor protein, p53, is well known as the guardian of the genome, primarily because the wild-type protein functions to prevent accumulation of genomic damage and loss of cellular integrity. Cellular stress that causes DNA damage activates p53 to control genes involved in cell cycle arrest, senescence, apoptosis, and DNA repair. This serves to prevent accumulation of mutations in the genome. The functions of p53 are controlled in part by oxidative stress, but the manner in which ROS controls specific set of p53-inducible genes is not well understood. P53 has redox-sensitive cysteine groups that can undergo thiol oxidation. Oxidized p53 abrogates its DNA-binding capacity. Velu et al. (2007) demonstrated that oxidant treatment of cells caused a disulfide bond formation between GSH and p53 by the cross-linking of p53 Cys^{124} or Cys^{141} to Cys^{182}. The balance between GSH and GSSH controls this *S*-glutathionylation of p53. Importantly, p53 with conjugated thiol residues were present in the nucleus. ROS can also indirectly mediate p53 phosphorylation and therefore its stabilization, accumulation, and activation. The phosphorylation of p53 is through ROS activation of protein kinases such as MAPK and ataxia-telangiectasia mutated (ATM) protein (Kurz and Lees-Miller 2004; Bragado et al. 2007). Also, well known is the control of ROS levels by p53. Normal physiologic amounts of p53 are required to maintain redox balance. Low or high levels of p53 induces oxidative stress via the control of expression of antioxidant and pro-oxidant genes (Liu et al. 2008). In addition to its nuclear localization and functions, p53 translocates into the mitochondria (Marchenko et al. 2000). Under oxidative stress, mitochondrial p53 induces ROS production and apoptosis by binding and inhibiting MnSOD (Zhao et al. 2005). P53 in mitochondria also protects mtDNA in minimally stressed cells. It can interact with both mtDNA and polymerse gamma to facilitate mtDNA replication (Achanta et al. 2005).

10.7 ROS and Oncogenic Signaling Pathways

ROS production is stimulated by cytokines, peptide growth factors, agonist receptors, and phobol esters. Transforming growth factor β, epidermal growth factor, and platelet growth factor stimulate mostly H_2O_2 production (Sundaresan et al. 1995). Downstream signal mediators of stimulated ROS production include NF-κB, AP-1, MAPK, PI3K, phospholipase A2, phospholipase D, protein kinase C, and increased cytosolic calcium.

Extracellular signals such as growth factors orchestrate numerous cellular functions through interactions with G proteins or receptor tyrosine kinase (RTK). Receptor tyrosine kinases are glycoprotein membrane receptors with several family members. Activated RTK activates downstream signal pathways such as the Ras-MAPK, PI3K/PKB, and PKC pathways. Receptor tyrosine kinase signaling therefore

controls virtually every cellular function including cell proliferation, differentiation, metabolism, and survival. Deregulated RTK signaling is a hallmark of cancer cells. Cancer cells overexpress oncogene RTK and their growth factors to maintain increased proliferation rates and apoptosis suppression. ROS mediate several signaling by RTKs. For example, through oxidation of Cys-SH groups in tyrosine phosphatases such as PTEN, ROS can activate AP-1 and AKT/PKB, which otherwise will be inactivated by PTEN (Leslie et al. 2003).

10.7.1 MAPK Pathway

MAPK signaling pathway conveys extracellular signals to specific transcription factors. There are three subgroups of this pathway: extracellular signal-regulated kinases (ERK), c-jun *N*-terminal kinase (JNK), and p38MAPK. Jun *N*-terminal kinase and p38MAPK are stress-activated protein kinases (SAPKs) and hence mediate stress responses and apoptosis. Activation of SAPKs in stressful conditions can lead to apoptosis. For instance, JNK regulates the intrinsic pathway of apoptosis, and both JNK and p38MAPK signaling can phosphorylate p53 to promote apoptosis.

ROS activate SAPKs by several mechanisms. Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAPKKK family that activates SAPKs in response to stress. In non-stressed cells, ASK1 is associated with thioredoxin and therefore inactive. Oxidative stress dissociates ASK1 from thioredoxin, so it can activate downstream SAPKs (Liu et al. 2000). Glutathione S-transferase pi (GSTPi) is a JNK inhibitor in non-stressed cells (Adler et al. 1999a). ROS was shown to trigger detachment of JNK from GSTPi permitting JNK activation (Adler et al. 1999b). Finally, H_2O_2 may activate JNK via downregulation of JNK phosphatases (Chen et al. 2001).

Receptor tyrosine kinase activation by growth factor activates ERK via small G proteins such as Ras, Raf1 kinase, and MERK. As well, extracellular stress including oxidative stress activates ERK. ERK signaling induces cell proliferation via activation of cyclin D1 expression and increased AP-1 activity. Signaling via ERK pathway can be pro-apoptotic or antiapoptotic depending on the type of stimulus and the kinetics of activation. ERK signaling probably through the activation of pro-apoptotic p53 can cause cell death (Kaji et al. 2002). In this study, $ONOO^-$-induced p53-dependent apoptosis was independent of p53 phosphorylation, but rather p53 accumulation required p21(ras)-MAPK-p19(ARF) signaling pathway activation (Kaji et al. 2002). Several anticancer agents induce cell death via redox-mediated activation of MAPK signaling. Indeed, sustained activation of ASK1-MEK-JNK signaling plays critical regulatory role in ROS-mediated apoptosis. Furthermore, Kuo et al. (2007) have shown that isoobtusilactone A induces ROS production that activates ASK1 to mediate JNK and p38MAPK signaling, and apoptosis. The glioma pathogenesis-related protein 1 can cause ROS-mediated JNK signaling to induce apoptosis (Li et al. 2008).

ROS-mediated activation of ERK is important in cancer because ERK signaling modulates the BCL-2 proteins to promote cell survival. BAD, a proapoptotic BCL-2 family member is inhibited by phosphorylation at three sites; Ser^{112} (catalyzed by RSK), Ser^{136} (by PKB), and Ser^{155} (by PKA). ERK activation mediates RSK-catalyzed phosphorylation of BAD leading to ubiquitination and proteasome degradation (Fueller et al. 2008), thereby promoting cell survival. ERK activation can directly cause an increase in the expression of BCL-2 antiapoptotic proteins (BCL-2, BCL-xL and MCL-1). Consistent with this observation, MAP kinase kinase or MEK inhibition caused apoptosis in pancreatic cancer cells via decreased expression of BCL-2 family members (Boucher et al. 2000).

10.7.2 PI3K/AKT Pathway

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a protein tyrosine phosphatase with essential roles in regulating the PI3K signaling pathway. PI3K activation controls a number of downstream effector proteins including AKT/PKB. Activated PI3K signaling regulates growth, survival, proliferation, and metastasis of cancer cells. PTEN, which is ubiquitously expressed in all cells, negatively regulates PI3K by dephosphorylation of PtdIns(3,4,5)P3, thereby preventing activation of downstream effector proteins. PTEN is mutated in several cancers. It appears that, apart from mutations, oxidative stress that is inherent in cancer cells can activate PTEN. The protein tyrosine phosphatase enzyme family members possess a highly reactive active site cysteine residue that is important for their catalytic activity. This site is also highly susceptible to oxidation. Hydrogen peroxide oxidizes PTEN by causing the formation of disulphide bond between Cys^{124} and Cys^{71}. Oxidized PTEN can be reduced by thioredoxin. PTEN phosphatase activity is abrogated by H_2O_2 in vitro and in vivo. Oxidative inhibition of PTEN leads to increased PtdIns(3,4,5)P3 levels and activation of downstream AKT/PKB (Leslie et al. 2003; Silva et al. 2008).

10.7.3 Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells Pathway

The redox-sensitive transcription factor, NF-κB controls inflammatory responses, apoptosis, cell cycle progression, and epithelial–mesenchymal transition. The NF-κB family comprises five members, namely, p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). The five family members are inactivated when they form homodimers or heterodimers with inhibitor proteins, IκBα, and IκBβ. The complex NF-κB:IκB cannot enter the nucleus to regulate gene expression. The inhibitors of IκB kinases (IKK) can antagonize IκB to activate this signaling

pathway. The IKKs are activated in several primary cancers and they modulate apoptosis in such cancers. NF-κB regulates expression of several BCL-2 family members at the transcriptional level (Cory and Adams 2002).

ROS-mediated activation of NF-κB and AP-1 was associated with tumor progression (Gupta et al. 1999), and it is well established that several agents activate NF-κB by inducing oxidative stress in cells. Takada et al. (2003) provided a possible mechanism for this activation. Tyrosine phosphorylation of IκBβ needed for dissociation and phosphorylation of NF-κB was induced by H_2O_2, acting via Syk protein tyrosine. Specifically, this mechanism involved serine phosphorylation of p65/RelA leading to its nuclear translocation and induction of gene expression.

10.7.4 Activator Protein 1

Activator protein 1 (AP-1) is a redox-sensitive transcription factor that comprises of a mixture of dimers formed among members of the Jun (Jun, JunB, and JunD) and Fos (Fos, FosB, Fra-1, and Fra-2) DNA-binding proteins. While members of the Fos family only form heterodimers, those of the Jun family form both homo- and heterodimers. Nuclear modulation of gene expression induced by signaling pathways such as MAPK, and TGFβ are controlled by AP-1. Not surprisingly, therefore AP-1 plays essential roles in carcinogenesis (Matthews et al. 2007). Several tumors have increased AP-1 activity, and inhibition of AP-1 decreased tumor progression to invasive cancers (Leaner et al. 2009). AP-1 is a target of antioxidants. The redox regulation of AP-1 is via APE1/Ref-1, a well-known redox regulator of transcription factors including p53, and NF-κB. Ref1 enhances DNA-binding activity of transcription factors via reduction of critical cysteine residues. It was recently shown that this process occurs by direct reduction and also enhancement of thioredoxin and glutathione-mediated reductions of cysteine moieties (Ando et al. 2008).

10.7.5 Protein Kinase C

Protein kinase C is a family of over ten serine/threonine kinase isoforms with unique functions in carcinogenesis. They were discovered as intracellular receptors for phorbol ester-mediated tumor development. Several cellular functions including survival, proliferation, differentiation, motility, and migration are regulated by PKCs. The PKC family is divided into three groups based on structure and cofactor requirements for activation. The classical or conventional PKC isoforms (α, βI, βII, and γ) require both phospholipids and calcium for activation, the novel group (ε, δ, θ, and η) is activated independent of calcium, and the atypical isoforms (ζ and ι/λ) can be activated without the need for both calcium and phospholids. It is becoming apparent that the functions of PKCs are mediated by specific isoforms. For

example, PKC-α, PKC-ε, as well as atypical PKCs, appear to control cellular proliferation and survival, while novel isoform PKC-δ mediates cell death (Reyland 2009).

Protein kinase C and ROS have reciprocal activation pathways. It appears that ROS-mediated epithelial–mesenchymal transition requires PKC-α. Wu et al. (2006) demonstrated that tetradecanoylphorbol-13-acetate (TPA), a potent activator of PKC, requires ROS for PKC activation, because scavengers of ROS abrogated PKC activation by TPA. The sustained oxidative stress induced by TPA caused activation of PKC and ERK to mediate epithelial–mesenchymal transition in HepG2 cells, partly through the induction of Snail and suppression of E-cadherin expression.

10.7.6 Myc Oncogene

ROS can be detrimental to cancer cells. Laurent et al. (2005) demonstrated that in CT26 colon cancer cells, and Hepa 1–6 liver cancer cells, high levels of mitochondrial ROS are produced. Noteworthy, low levels of H_2O_2 induce cellular proliferation. In normal cells, superoxide dismutase (SOD) increases H_2O_2 production to signaling levels that modulates controlled cellular proliferation. However, in cancer cells SOD appears to have lethal effects as it elevates H_2O_2 to toxic levels. Cancer cells have amplification and altered expression of c-Myc, and this may be one mechanism of maintaining redox homeostasis. The c-Myc oncogene is a transcription factor that controls cellular growth, proliferation, and apoptosis via the regulation of several target genes. Cancer cells stabilize oxidative stress in part using c-Myc (Benassi et al. 2006). These investigators showed that H_2O_2 enhanced recruitment of phosphorylated c-Myc to gamma-glutamyl-cysteine synthetase (gamma-GCC), the rate-limiting enzyme in glutathione biosynthesis. Gamma-GCC, and hence glutathione expression and biosynthesis are enhanced by the interaction of c-Myc with Gamma-GCC. Thus, c-Myc contributes to oxidative stress resistance in cancer cells.

10.8 ROS and Carcinogenesis

The delirious effects of free radicals in carcinogenesis are an established fact. Free radicals cause cancer through their damaging effects on nucleic acids, nuclear chromosomal modification, and gene expression changes. Oxidized bases can be repaired to maintain genome stability; however, persistent mutations can potentially overwhelm the repair mechanisms leading to activation of oncogenes and/or loss of tumor suppressor gene activity.

ROS cause epigenetic changes in the genome that could alter gene expression or lead to mutations. CpG island methylation of E-cadherin promoter occurs in several

cancers and this is associated with disease progression and invasiveness. ROS are shown to cause hypermethylation of E-cadherin promoter by inducing the expression of the transcriptional repressor, Snail to recruit histone deacetylase, and DNA methyltransferase 1 (Lim et al. 2008).

Damage of DNA by ROS can generate 8-hydroxyguanine, which alters methylation of adjacent cytosines to guanines. The reaction of hydroxyl radicals with CH_3 groups of 5-methylcytosine in the genome causes demethylation of m5C to C, and/or deamination of m5C to thymine. Deamination converts m5CG to TA transition mutations. Oxidation of m5C can generate 5-formylcytosine which is also mutagenic because it changes C to either G, A, or a T (Kamiya et al. 2002).

There is ample evidence for the importance of ROS in general and mitochondrial ROS in particular in carcinogenesis (several of these biologic effects are examined in Chap. 9). The potential mechanistic processes for ROS to transform cells or enhance tumor progression to invasiveness are addressed by a number of studies. Suh et al. (1999) overexpressed Mox1 that encodes a homologue of the catalytic subunit of NADH oxidase in NIH3T3 cells and this resulted in an increase in $O_2^{\bullet -}$ production in association with increased growth and acquisition of malignant potential including anchorage-independent growth. Transplantation of the transformed cells into nude mice produced tumors. Platelet growth factor induces *Mox*1 expression, thus providing a link between PDGF and carcinogenesis. Arnold et al. (2001) investigated the effects of H_2O_2 on cellular transformation. Nox1-expressing cells had significantly elevated H_2O_2 production, increased proliferation, and formed tumors in athymic mice. The oncogenic properties of these cells were reversible with catalase expression. A mutation in *SDHC* caused elevated $O_2^{\bullet -}$ production in association with apoptosis (Ishii et al. 2005). These investigators established a transgenic cell line with *SDHC* mutation, which caused increased oxidative stress and apoptosis associated with cellular transformation. Injection of the transformed cells into nude mice established flourished tumors.

A number of experiments demonstrate the relevance of mitochondrial ROS and carcinogenesis (see Chap. 9). For example, cloning and nuclear transfection and expression of mutant *ND*2 resulted in ROS production coupled with increased tumor growth, HIF1α stabilization, and aerobic glycolysis (Zhou et al. 2007). Mitochondrial DNA damage and increased ROS production can confer metastatic potential to cancer cells. Ishikawa et al. (2008) used cybrid technology to exchange the mitochondrial genomes between a poorly metastatic and a highly metastatic mouse tumor cell lines. This manipulation uncovered an intriguing observation. The recipient tumors acquired the metastatic capabilities of the transferred mtDNA. The highly metastatic cancers had mutations in the mitochondrial *ND*6 that compromised respiratory complex I functions leading to elevated ROS production. Treatment of the cells with ROS scavenger *N*-acetyl cysteine, suppressed the metastatic propensities of these cells. Pelicano et al. (2009) induced mitochondrial dysfunction by interfering with respiratory chain activity, and this caused increased ROS production in some clones of breast cancer cells. These clones had increased proliferation rates, had high cellular motility and acquired invasive phenotypes. ROS via AP-1 induced expression of CXCL14, and binding of CXCL14 to inositol

1,4,5-trisphosphate receptor on endoplasmic reticulum caused increased cytosolic calcium that promoted cell motility.

Oxidative stress initiates and/or aggravates carcinogenesis. The few illustrations here are to give an insight into the importance of ROS in cancer etiology and progression.

10.9 Ameliorating the Harmful Effects of ROS

Antioxidants are agents or enzyme systems with the capability of slowing down the oxidation processes in the body that will otherwise generate intolerable free radicals. The agents include molecules such as tocophenols (vitamin E), ascobic acid (vitamin C), albumin, uric acid, and other dietary phytochemicals such as flavonoids, carotinoids, organosulfur compounds; the enzyme systems include glutathione peroxidase, thioredoxin, catalase, and SOD.

There are several available antioxidant supplements being used by many people to ameliorate the harmful effects of ROS. While it may seem simple that reducing ROS levels in physiologic systems will obviously translate into beneficial outcomes like cancer prevention, the verdict from the literature is complex. Notably, in addition to their harmful effects when in excess, ROS have beneficial effects to some cells including mediating the pharmacologic actions of some chemotherapeutic agents and inducing apoptosis in precancerous cells. However, in normal tissues, elevated ROS levels are detrimental because they are linked to the causation of several diseases including cancer, emphysema, cataracts, cardiomyopathies, and neurodegenerative diseases, among others. For example, an OXYS Wistar rat strain with overproduction of hydroxyl radicals was shown to have increased lipid peroxidation, DNA rearrangements, in association with development of degenerative diseases, preneoplastic lesions, and aging (Salganik et al. 1994). This finding suggests that sustained elevated ROS underlies aging and age-related diseases including cancer.

Should cancer patients be given antioxidant supplements? There are no straightforward answers to this question. First, it will appear that reducing ROS levels in cancer patients is not a good idea. Experiments in mouse as a model of brain tumor lend support to this assumption (Salganik et al. 2000). The TgT_{121} transgenic mice develop tumors of the choroid plexus due to tissue-specific inactivation of retinoblastoma tumor suppressor pathway. In contrast to control transgenic mice fed on regular diet, TgT_{121} mice fed on diet depleted of antioxidants had increased ROS levels specifically in the tumor cells, and this was associated with increased tumor cell apoptosis and reduced tumor size. This tumor cell death from increased ROS in the antioxidant-depleted mice was not observed in normal tissues. Similarly, depletion of vitamin A and E in diets fed to mice that develop de novo mammary alveolar adenocarcinomas caused reduced tumor growth and lung metastasis (Albright et al. 2004). Thus, increased ROS can specifically kill cancer cells. But antioxidants may benefit cancer patients. Antioxidants, such as *N*-acetylcysteine

and vitamin C can diminish tumorigenesis by decreasing HIF1 levels in cancer patients, which is very different from the general concept of antitumoral effects of antioxidants in preventing DNA damage (Gao et al. 2007).

Should people at high risk for developing cancer be given antioxidant supplement? There are several clinical trials that demonstrate the effects of antioxidants in cancer prevention. Two large chemoprevention trials provide ample evidence for the benefits and harmful effects of antioxidants in high-risk population. The alpha-tocopherol, beta-carotone (ATBC) cancer prevention study is a randomized double-blind placebo-controlled study conducted among male smokers from Southwestern Finland (The ATBC Cancer Prevention Study Group 1994). This study included 29,133 men on daily 50 mg alpha-tocopherol alone, 20 mg beta-carotene alone or a combination of both. The participants were then followed for 5–8 years and monitored for the development of various cancers. The initial study focused on lung cancer prevention, and it was uncovered that there was no reduction in lung cancer. Indeed, people on beta-carotene had increased incidence and mortality from lung cancer. However, there were fewer cases of prostate cancer in those taking alpha tocopherol. A follow-up study confirmed the decline in both incidence and mortality of prostate cancer in men on alpha-tocopherol (Heinonen et al. 1998). Beta-carotene again had adverse effects in both the incidence and mortality of prostate cancer. These supplements had no beneficial effects on the development of cancers such as pancreatic, gastric, bladder, and other urinary tract cancers, but rather vitamin E had modest preventive effect in colorectal cancer (Rautalahti et al. 1999; Albanes et al. 2000; Malila et al. 2002; Michaud et al. 2002).

A similar study to ATBC is the CARET (Beta-Carotene and Retinol Efficacy Trial) based in the US. This trial included 18,314 high-risk men and women receiving daily supplements of 30 mg beta-carotene and 25,000 IU of vitamin A (Omenn et al. 1996). The initial trial that focused on lung cancer had to be stopped prematurely because of lack of any beneficial effects, but rather a trend towards excess lung cancer incidence and mortality in the case group. Neuhouser et al. (2003) noticed that fruits and vegetables were effective in lung cancer prevention in only people not receiving antioxidant supplements (the placebo arm of the CARET study).

Should healthy people be taking antioxidant supplements? There are a number of studies that provide evidence for the protective role of antioxidants in healthy individuals. For example, in nonsmokers, antioxidants such as vitamin C, E, and carotene protect against lung cancer, in spite of their harmful effects on smokers (Byers and Perry 1992). Similarly, people on vitamin E had lower colon cancers (Bostick et al. 1993), and vitamin E and beta-carotene lowered the incidence of gastric cancer (Hwang et al. 1994). Beta-carotene, vitamin E, and selenium supplementation for over 5 years was associated with decreased incidence of gastric and esophageal cancer in a Chinese Cancer Prevention Study (CCPS) (Blot et al. 1993, Blot et al. 1994). Ten years after cessation of supplementation in the CCPS study, the beneficial effects of selenium, vitamin E, and beta-carotene on mortality were still evident and were much pronounced in younger individuals (Qiao et al. 2009).

The reasons for the mixed outcomes in these clinical trials are many and include: (1) the possible irreversible genomic damage caused by genotoxins in tobacco – it has been demonstrated that previous smokers retained mtDNA mutations at the same levels as current smokers, suggesting the genomic damage were never repaired; (2) the loss of protective (proapoptotic) functions of ROS in high-risk and cancer patients on antioxidants; (3) the lack of knowledge on baseline levels of antioxidants in an individual prior to supplementation (e.g., beta-carotene appears to reduce the incidence of prostate cancer in people with low baseline antioxidant levels (Cook et al. 1999); (4) the lack of knowledge on the right combination of antioxidants – could some antioxidants be synergistic and others detrimental when combined? (answer to such questions will help explain some of the disparities); (5) normal human genetic variation influences how different people respond to therapy (e.g., polymorphisms or mutations in ROS-scavenging enzymes can influence the effects of supplements).

10.10 Conclusion

ROS production by mitochondria is an inevitable process of mitochondrial function. Because of the proximity of the naked mitochondrial genome to the genotoxic compounds, coupled with the inefficient repair mechanisms, it can be conceived that mitochondrial damage occurs as a natural process of aging. Pathogenic mutations that accumulate to high heteroplasmic levels will lead to defective complex assembly and function, thus setting in motion a vicious cycle of oxidative stress in the cell. Indeed, several cancers demonstrate mitochondrial coding gene mutations in association with ROS production.

ROS have pleiotropic effects in the cell. Redox imbalance can cause epigenetic modification and altered gene expression, mutations to nucleic acids, modulation of apoptosis, HIF1α stabilization, and thiol oxidation leading to deregulated signal transduction cascades. All of these processes can modulate cancer initiation, progression, acquired resistance to therapy, and invasiveness. Thus, the decision for a cell to transform and survive appears to depend in part on the levels and types of ROS and the signaling pathways they invoke. Normal antioxidant mechanisms, however, operate to prevent the development of diseases including cancer.

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Part III
Clinical Applications of Mitochondrial Genome Changes in Cancer

Chapter 11
Mitochondrial DNA Measurement in Exfoliated Cells for Cancer Detection and Monitoring: The Copy Number Advantage

Abstract Mitochondrial genetic alterations are demonstrated in several primary tumors, and efforts by many investigators indicate tumor specific mtDNA mutations can be detected in bodily fluids. Prostate and liver cancer, as well as melanoma mtDNA SNP are detectable in whole blood and serum. Additionally, circulating mitochondrial nucleic acids (DNA and RNA) are measured in blood of prostate and testicular cancer patients. Importantly, such circulating mitochondrial genetic materials in blood have prognostic utility. The high copy number of mitochondrial genome in tissues enables easy measurement of mutations in biofluids such as saliva, sputum or BAL, nipple aspirate fluids and urine. When demonstrated that mitochondrial mutation load is indicative of cellular progression towards malignancy, such mutation load measurements in biofluids will enable early cancer detection. This chapter examines the clinical applications of mtDNA mutations in biofluids of cancer patients.

11.1 Introduction

In general, the global burden of cancer remains high with associated devastating mortalities and unacceptable morbidities despite tremendous achievement made in cancer care in recent years. Therefore, efforts at curbing this devastating disease should include early detection, chemoprevention, and adequate management strategies. This feat is achievable by screening high-risk populations and monitoring cancer patients using sensitive molecular markers of cancer, preferably in noninvasively collected bodily or biological fluids (biofluids). Early detection enables application of curative treatment measures, such as organ-confined surgical resection and chemoprevention to ameliorate incurable late disease with dismal outcomes. Biofluids offer an acceptable noninvasive means of obtaining samples for screening and monitoring purposes, and hence studies of its utility are ongoing efforts by many industries and academia. Given the low cellularity and nucleic acid

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content in biofluids, the high copy number of the mitochondrial genome and its noted early imprints in cancer make this molecule more sensitive than nuclear targets to measure. In spite of the limited data available, mitochondrial genome changes in biofluids of cancer patients have been demonstrated in a wide variety of cancers.

11.2 Tumor Signatures in Biofluids

Cancer detection and monitoring using altered nucleic acids in biofluids was explored decades ago, and has obtained much attention in recent years with the surge in the discovery of cancer-specific or related biomarkers. Mandel and Metais (1947) first recognized the presence of free nucleic acids in the circulation; however, the diagnostic potential of plasma nucleic acids in oncology had to await three decades before resurgence. In 1977, Leon and coworkers reported on the use of radioimmunoassay to measure the levels of free circulating DNA in the serum of cancer patients compared to healthy control individuals (Leon et al. 1977). In this initial study, all their patients had received some form of treatment; however, free DNA levels were much higher in serum of cancer patients than in those of control subjects. Analysis of cancer progression and free DNA levels indicated that patients with metastatic disease had a much higher levels than those with localized cancer. That apart, the levels of free serum DNA in patients correlated with various clinicopathologic parameters. Total serum DNA was up to micrograms per milliliter in cancer patients compared to just less than 200 ng/ml in healthy individuals with the majority of noncancer individuals demonstrating levels below 50 ng/ml to undetectable. Whereas the sources of circulating nucleic acids in cancer patients were unknown, Stroun and coworkers first demonstrated that they originated from cancer cells in cancer patients (Stroun et al. 1987, 1989).

Sidransky et al. (1991) pioneered the successful use of a molecular genetic approach to assay tumor-associated *p*53 mutations in the urine of bladder cancer patients. They employed multiple techniques including the polymerase chain reaction, mutant-specific oligonucleotide hybridization and sequencing techniques to screen for mutations in clinical samples as a diagnostic possibility. These pioneering works served as catalysts that fueled further studies in the field. Currently, the available body of evidence suggests that early cancer detection and management using genetic information in biofluids is an invaluable clinical tool that is in translation.

Several groups have analyzed various tumor-genetic signatures in biofluids such as blood (including serum and plasma), saliva, sputum, bronchoalveolar lavage (BAL), ductal lavage, nipple aspirate fluids (NAF), urine, and cerebrospinal fluid (CSF) for the diagnosis and monitoring of different types of cancers (Belinsky et al. 2005; Muller and Brenner 2006; Gormally et al. 2007). It is unclear how tumor nucleic acids enter the circulation. Umetani et al. (2006) suggested that circulating

DNA in healthy individuals was from apoptotic cells, while in cancer patients they were derived from cancer cells that died through different cell death processes. Consistently, this argument provided explanation for the shorter nucleic acids in the circulation of healthy subjects compared to the longer sequences in the blood of people with cancer. It is also possible that tumor nucleic acids enter biofluids as a result of exfoliated tumor cells or free DNA released from necrotic or apoptotic tumor cells (Jahr et al. 2001).

Irrespective of the mode of entry into biofluids, tumor-derived molecular targets are usually present at very low levels, especially in the early stage cancers, and therefore require target enrichment and/or sensitive methods for detection. In some instances, tumor signatures in biofluids are dependent on the stage, size, and location of the tumor, making their detection in late metastatic stages easier than early stage cancers. Not surprisingly therefore, an inherent issue with assaying nuclear targets in many biofluids is the lack of sensitivity, though specificity has generally been high (Hu et al. 2002), partly because of the functional importance of signature nuclear genome mutations in cancer. Several studies of nuclear targets that produced potentially promising results faced difficulties in reproducibility, and therefore have not motivated their extension into validation studies. For instance, *K-RAS* mutations in sputum/BAL for the detection of lung cancer failed to produce encouraging results to merit clinical translation (Mills et al. 1995; Ahrendt et al. 1999). However, *K-RAS* mutations are detected with high sensitivity and specificity in stool samples using mutant-enriched PCR techniques. Also, attempts at assaying promoter methylation of *GSTPi*, and *TMPRSS2-ETS* fusion transcripts for prostate cancer detection in urine are met with low sensitivity (Cairns et al. 2001; Laxman et al. 2006). Future refinement of these assays should enable translation of some of these targets for patient management using clinical samples with low cellularity. For example, with the recent development of more sensitive nucleic acid amplification techniques, some tests based on measurement of nuclear genetic changes in biofluids are becoming clinically useful. The refined uPM3 urine test by Gen-Probe, the APTIMA-PCA3, is a useful adjunct to prostate cancer management (Groskopf et al. 2006). With all these technical difficulties, it is obvious that a biomarker that occurs in abundance should be more sensitive to measure in biofluids. RNA is such an attractive target because of its multiple copies per cell compared to DNA, but RNA tends to be unstable and is also technically more difficult to handle. However, endogenous RNA in the circulation can be associated and protected by small particles in plasma (Ng et al. 2002). Owing to their stability, protein targets have been extensively explored for the detection of cancer (Hundt et al. 2007), but not many cancers secrete specific tumor-associated protein markers. The mitochondrial genome that exists as multiple copies per cell is one such target of interest. Indeed, it has been estimated that mutated mtDNA is 19–220 times as abundant in biofluids as a mutated nuclear target (Fliss et al. 2000). In addition, Jones et al. (2001) found the "mass" advantage of mtDNA useful in detecting mtDNA mutations in pancreatic cancer. Accurate mtDNA markers of cancer should enable facile detection in clinical samples with low cellularity.

11.3 Mitochondrial Genome Changes in Biofluids of Cancer Patients

Analyses of mtDNA changes in biofluids can be used for early cancer detection, monitoring of disease progression and response to therapy, or prognostic stratification of patients for treatment. Field cancerization, simply defined as the process whereby genetically altered but histologically normal-appearing cells precede the development of neoplasia or coexist with malignant cells, is an important concept of tumor biology that can be explored for the discovery of early detection markers for cancer. Whereas considerable work on field cancerization focused on nuclear genomic alterations such as allelic imbalances, a careful review of a number of mitochondrial genome studies including mutations, large-scale deletions and content changes indicate similar field changes in cancer (Dakubo et al. 2007) (also see Chap. 12), providing evidence for tracking these changes in biofluids for early cancer detection. It is becoming obvious that mitochondrial genome alterations may modulate the behavior of or be associated with some tumors. For example, mtDNA content changes are clinicopathologically correlated with head and neck, ovarian and endometrial cancers (Jiang et al. 2005; Wang et al. 2005, 2006); D-loop mutations are associated with poor outcomes in breast and colorectal cancers (Lievre et al. 2005; Tseng et al. 2006); levels of circulating mitochondrial genomes appear to have prognostic significance in prostate cancer (Mehra et al. 2007), and modulate therapeutic responses (Naito et al. 2007).

The elegant demonstration of mtDNA mutations in cancer by the Vogelstein's group, followed by the seminal work by the Sidransky's group of mitochondrial genome changes in biofluids convincingly proofed that analysis of mtDNA mutations in biofluids is an easy, feasible and practical means of tracking early genomic alterations in cancer (Polyak et al. 1998; Fliss et al. 2000). Currently, several other investigators have shown that tumor-specific mutations in mtDNA are detectable in biofluids representative of the specific organ associated with the cancer. Mitochondrial genome changes detected in biofluids of various cancers are summarized in Table 11.1, and discussed.

11.3.1 Blood (Serum and Plasma)

The possibility of detecting tumor-derived mtDNA mutations in circulating body fluids has been explored in melanoma, colorectal and liver cancers. Takeuchi et al. (2004) examined mtDNA mutations in the D-loop region of melanoma samples and matched plasma. Forty-two percent of the tumors had D-loop mutations, of which two mutations were detected in matched plasma samples.

Following a successful demonstration of *K-RAS* and *p*53 mutations in plasma of colorectal cancer patients, Hibi and coworkers extended their studies to include mtDNA D-loop alterations (Hibi et al. 1998, 2001). Analysis of seven

Table 11.1 Mitochondrial genome changes assayed in various biofluids of cancer patients

Biofluids	Primary tumor	Detection method	Mutation frequency in biofluids (%)	MtDNA changes in biofluids	References
Plasma/serum	Liver cancer	MLA	5/15 (33)	Somatic mutations	Okochi et al. (2002)
		MLA	8/10 (80)	Somatic mutations	Nomoto et al. (2002)
	Melanoma	CE	2/5 (40)	Somatic mutations	Takeuchi et al. (2004)
	Colorectal cancer	MLA	1/7 (14)	Somatic mutations	Hibi et al. (2001)
	Prostate cancer	MLA	3/3 (100)	Somatic mutations	Jeronimo et al. (2001)
		qPCR	–	Increased mtDNA and mtRNA associated with poor outcomes	Mehra et al. (2007)
	Prostate cancer	qPCR	–	Increased short mtDNA fragments associated with poor outcomes	Ellinger et al. (2008)
	Ovarian	qPCR	–	High mtDNA in cancer patients	Zachariah et al. (2008)
	Testicular	qPCR	–	High mtDNA in cancer patients	Ellinger et al. (2009)
Saliva	Head and neck cancer	Manual sequencing	6/9 (67)	Somatic mutations	Fliss et al. (2000)
		MitoChip	10/13 (78)	Somatic mutations	Mithani et al. (2007)
		qPCR		Increased mtDNA content in cancer patients	Jiang et al. (2005)
		qPCR		Decrease mtDNA content with therapy	Jiang et al. (2006)
Bronchoalveolar lavage/Sputum	Lung cancer	MLA	5/6 (83)	Somatic mutations	Fliss et al. (2000)
		MitoChip	3/7 (43)	Somtic mutations	Jakupciak et al. (2008b)
Nipple aspirate fluid	Breast cancer	CE	4/10 (40)	Somatic mutations	Zhu et al. (2005)
Urine	Bladder cancer	MitoChip	1/3 (33)	Somatic mutations	Jakupciak et al. (2008a, b)
		MitoChip	4/5 (80)	Somatic mutations	Maitra et al. (2004)
		Manual sequencing	3/3 (100)	Somatic mutations	Fliss et al. (2000)
	Renal Cancer	MitoChip	4/12 (33)	Somatic mutations	Jakupciak et al. (2008a, b)
	Prostate cancer	MLA	3/3 (100)	Somatic mutations	Jeronimo et al. (2001)
CSF	Medulloblastoma	TTGE/CE	7/8 (88)	Somatic mutations	Wong et al. (2003)
Pancreatic juice	Pancreatic cancer	MitoChip	2/4	Somatic mutations	Maitra et al. (2004)

MLA – mutant-specific mismatch ligation assay; CE – capillary electrophoresis sequencing; qPCR – quantitative polymerase chain reaction; TTGE – temporal temperature gradient gel electrophoresis; MitoChip – microarray resequencing of mitochondrial genome

colorectal cancer patients with mtDNA mutations identified only one in matched serum sample. Several groups have demonstrated mitochondrial genome mutations in colorectal cancer. Therefore, analysis of exfoliated colonocytes in stool samples may increase the sensitivity of detecting colorectal tumor-derived mtDNA mutations.

Nomoto et al. (2002) examined mtDNA alterations in hepatocellular carcinoma (HCC) and matched plasma samples. Tumor-specific mtDNA mutations were successfully detected in 80% of plasma samples. In a follow-up study, Okochi et al. (2002) uncovered tumor-specific mutations in only 33% of plasma samples from HCC patients. These studies imply that freely circulating mtDNA contain tumor-specific genomic alterations.

Employing the sensitive mutant-specific mismatch ligation assay, Jeronimo et al. (2001) were able to detect all three primary prostate cancer mutations in matched plasma. Because the majority of men diagnosed with prostate cancer have a favorable outcome, an important requirement for prostate cancer diagnosis and management is the ability to predict tumor behavior. It has been shown that the levels of mitochondrial nucleic acids in circulating blood might have a prognostic utility in managing prostate cancer patients (Mehra et al. 2007; Ellinger et al. 2008). A study of men with advanced prostate cancer found that those with high levels of plasma mitochondrial DNA and RNA had a poor 2-year survival compared to men with low levels. Importantly, the levels of mitochondrial RNA appeared to be an independent predictor of survival. Ellinger et al. (2008) amplified both large and short cell-free circulating mtDNA in 100 patients with prostate cancer and 18 with benign prostatic hyperplasia. Similar levels were observed in both groups. However, patients with short mtDNA fragments had an increased risk of biochemical recurrence after prostatectomy.

Ellinger et al. (2009) evaluated the potential utility of cell-free circulating nucleic acids in the management of patients with testicular cancer. In an earlier study, actin-beta DNA fragments were analyzed and DNA integrity, defined as the ratio of the large to the smaller fragments assessed. The study subjects comprised 39 patients with seminomas, 35 with nonseminoma testicular cancer and 35 healthy controls. Cell-free DNA fragments were significantly increased in circulation of patients compared to controls. In addition, DNA integrity was significantly decreased in patients compared to controls. This assay could differentiate patients from healthy controls with a sensitivity and specificity of 87 and 97% respectively. The levels of circulating mtDNA also tended to be more elevated in high-grade tumors. In a follow-up study of these same subjects, mtDNA fragments comprised of 79 and 220 bp fragments in 12*SrRNA* were amplified, and DNA integrity defined as previously (levels of 220 bp fragments/levels of 79 bp fragments). Again, both fragments were significantly elevated in cancer patients compared to controls; however, mtDNA integrity was similar in both groups. The assay was accurate at distinguishing between patients and controls with a sensitivity of 59.5% and a specificity of 94.3% with the area under a receiver operating characteristic curve of 0.787. Importantly, using both nuclear and mitochondrial DNA analysis, 31 patients with normal levels of testicular cancer serum markers (alpha-fetoprotein,

human chorionic gonadotrophin, placental alkaline phosphatase and lactate dehydrogenase) could be distinguished from healthy subjects with a very high degree of accuracy. Thus, cell-free circulating nucleic acids appear to be valuable in testicular cancer detection, especially in people with normal levels of established testicular cancer markers.

Free circulating nuclear and mitochondrial DNA content in serum and plasma were analyzed in 104 female patients who had epithelial ovarian cancer, benign epithelial ovarian tumors and endometriosis (Zachariah et al. 2008). Patients with epithelial ovarian cancer had significantly high nuclear and mtDNA fragments in their plasma compared to healthy control subjects and women with benign ovarian tumors. For patients with endometriosis, only cell-free mtDNA could significantly separate them from epithelial ovarian cancer subjects. These findings indicate a diagnostic potential for the measurement of circulating nucleic acids in ovarian cancer diagnostics.

11.3.2 Salivary Rinses

As an established risk factor for head and neck, esophageal, lung and bladder cancers, the toxins in tobacco smoke can damage mtDNA and possibly contribute to the development of these cancers. Mitochondrial DNA content changes were measured in the saliva of smokers and nonsmokers. Saliva from smokers, including those who had stopped smoking contained significantly high levels of mtDNA compared to those from people who never smoked (Masayesva et al. 2006).

Mitochondrial DNA copy number or content alterations have been measured in head and neck cancers and matched saliva using quantitative polymerase chain reaction (qPCR). In an initial study of head and neck tumor tissues of various clinical grades reminiscence of disease progression, it was found that increased mtDNA content correlated positively with histopathologic grade (Kim et al. 2004). In a follow-up study, oral rinse samples were collected from 94 patients with head and neck squamous cell carcinoma (HNSCC) as well as 656 individuals without cancer for mtDNA content study (Jiang et al. 2005). Mitochondrial DNA levels were significantly higher in the saliva of cancer patients than controls. Importantly, primary tumors also had higher levels of mtDNA than pretreatment saliva, suggesting the elevated levels of mtDNA in the saliva of cancer patients were tumor-derived. In another study, the effect of treatment on mtDNA levels was examined in 79 patients with HNSCC (Jiang et al. 2006). Analysis of pre- and posttreatment saliva revealed a significant decrease in mtDNA content in posttreatment samples. In addition, saliva from patients who had radiation treatment and those who never smoked contained lower levels of mitochondrial genomes than in saliva from nonirradiated and smoking counterparts. Thus, the harmful effects of smoking on mtDNA appear to be chronic and irreversible.

Mutations in the mitochondrial genome are present in early stage head and neck cancers. Quite recently, microarray-based resequencing of the entire mitochondrial

genome was conducted on a panel of 83 HNSCC and mutations were found in nearly half of the tumors (Zhou et al. 2007). Sequencing of dysplastic adjacent tissues of one tumor identified the same mutation in both tumor and margin samples, suggesting that mtDNA mutations occurred early in this particular tumor, and could be involved in disease progression. To demonstrate the possible utility of mtDNA mutations as a tool for early cancer detection in clinical samples, Fliss et al. (2000) used manual sequencing to study mtDNA mutations in primary head and neck cancers, and matched available saliva. Six of the thirteen solid tumors had mutations that were also detected in six of the nine saliva samples. MitoChip resequencing of salivary rinses from patients with known tumor mtDNA mutations were studied by Mithani et al. (2007) for the possible presence of the same mutations in noninvasively collected biofluids. Using a proprietary algorithm, heteroplasmic mutations could be detected at even a dilution of 1:200. This sensitive approach enabled these investigators to uncover tumor mutations in the saliva of 77% of the patients. Thus, mtDNA content changes and mutations can be measured in saliva for diagnosis and management of HNSCC.

11.3.3 BAL and Sputum

Lung cancer has a high morbidity and case fatality rate compared to other cancers. In addition to avoiding tobacco smoke, early detection of small tumors amenable to curative resection will help reduce the fatalities. Because smoking is a strong risk factor for lung cancer, and the genotoxins in tobacco smoke can cause damage to the mitochondrial genome, mtDNA damage and the levels of the 4,977 bp common deletion (CD) were measured in BAL from smokers and nonsmokers (Ballinger et al. 1996). Not surprisingly, smokers had a highly significant damage in their mitochondrial genomes compared to nonsmokers. Although the levels of the CD were much higher in smokers than nonsmokers, this difference was insignificant. In another study, Ahrendt et al. (1999) were able to detect, though with limited sensitivity, nuclear gene mutations and methylation in early stage lung cancer and the corresponding BAL. The study by Fliss et al. (2000) also included 14 lung cancer samples and matched BAL. Six of the fourteen tumors had mutations. Using a sensitive detection method, eight of the ten lung cancer-mutations were easily detectable in matched BAL samples. Jakupciak et al. (2008b) uncovered three somatic mutations in BAL from seven patients with lung cancer. Sputum and/or BAL appear to be a useful source of exfoliated bronchial epithelial cells for mtDNA analysis for early cancer detection in high-risk population.

11.3.4 Nipple Aspirate Fluid and Ductal Lavage

Although death rates have declined partly because of the early detection strategies, breast cancer is still the most commonly diagnosed female cancer, and the second

cause of all cancer deaths in women. Early detection is critical to achieving high cure rates. Ductal lavage and NAF offer a practical possibility of early detection of breast cancer. As a proof of principle, free DNA from 26 ductal lavage and six NAF samples from nine BRCA1 carriers and five noncarriers were studied for genomic alterations (Isaacs et al. 2004). Somatic mutations at mtDNA D310 locus were found in three ductal lavage samples form three BRCA1 carriers, and in none of the noncarriers. Four NAF samples were successfully analyzed with one having a mutation that was also present in the ductal lavage sample. Zhu and coworkers examined approximately 25% of the mitochondrial genome in NAF samples from women with breast cancer, and found four identical mutations in both NAF and cancer samples (Zhu et al. 2005). Importantly, NAF sequences from five tumors that did not have a mutation were also free of mutations. Thus, tumor-specific mtDNA mutations are detectable in ductal lavage and NAF from corresponding breast. We explored the possibility of using NAF for early breast cancer detection with chip-based resequencing of mtDNA (Jakupciak et al. 2008a). We were successful in sequencing the entire mitochondrial genome from 19 NAF samples with both microarray and capillary electrophoresis sequencing. Although after clinical investigation all our study participants had benign breast lesions, somatic mtDNA mutations were uncovered in four of the NAF samples. However, it is unknown whether these findings indicate disease progression towards malignancy. As a concept, mitochondrial genome sequencing using NAF is a relatively easy process that can have real clinical translation.

11.3.5 Urine

Urine is a suitable sample for detection and monitoring of urogenital cancers. Fliss et al. (2000) were the first to study mtDNA mutations in urine samples and matched bladder cancers. Somatic mutations were observed in 64% of the primary bladder cancers. Interestingly, the available three matched urine samples contained tumor-derived mutations as well. Using a sensitive and reliable microarray approach, the entire mitochondrial genome was interrogated in five bladder cancer samples and matched biofluids (Maitra et al. 2004). Tumor-derived heteroplasmic mutations were easily assayed in four of five urine samples, confirming the ease with which bladder cancer mtDNA mutations can be detected in urine. Recently, Jakupciak et al. (2008b) analyzed urine from three patients with bladder cancer, and 12 with renal cancer using the Mitochip. One and four somatic mutations were found in bladder and renal cancer urine samples respectively. Thus, chip-based detection of cancer in biofluids has a real possibility of clinical translation.

Prostate cancer remains the most commonly diagnosed, and the second cause of cancer deaths in American men, despite increased survival rates. The improved outcomes are partly due to early detection of organ-confined tumors from PSA and digital rectal examination screening programs. It is noteworthy that the PSA test lacks accuracy; hence, the relentless search for sensitive and specific molecular

targets. There is paucity of studies on mitochondrial genome analysis in biofluids of prostate cancer patients; however, the available evidence from studies of primary tumors points to early mtDNA damage in prostate cancer (Jeronimo et al. 2001; Parr et al. 2006), suggesting targeting mitochondrial genome changes in biofluids can serve as a screening tool. Whereas manual sequencing of mtDNA in urine and saliva was feasible for mutation detection in bladder and head and neck cancer patients, respectively (Fliss et al. 2000), this method of sequencing was not possible for the detection of prostate cancer mutations in plasma and urine. However, employing the sensitive mismatch-ligation assay, all three primary prostate cancer mutations were successfully detected in matched plasma and urine (Jeronimo et al. 2001). It is possible that enriching urine with prostate-derived nucleic acids by collecting post-DRE urine, prostate massage fluids, or semen will enable the application of even less-sensitive, but reliable and clinically suitable methods such as qPCR for prostate cancer early detection.

11.3.6 Cerebrospinal Fluid

Because CSF is obtained by invasive methods, it is unsuitable as a sample for the diagnosis of brain tumors. However, for the purposes of disease monitoring, CSF can be a valuable sample to assay for mtDNA changes. Wong et al. (2003) investigated the possible utility of mtDNA changes in CSF for the prognostic evaluation of patients with medulloblastoma, a pediatric brain tumor. They examined 15 primary tumors, six of which had somatic mutations. Seven matched CSF samples (including five with somatic mutations in tumors) were analyzed. A primary tumor mutation in one patient could be demonstrated in CSF at the end of therapy when conventional laboratory methods failed to identify any sign of persistent disease. This patient subsequently relapsed, suggesting the real possibility of using mtDNA changes to monitor disease progression. This finding is important because mtDNA mutations are present in other types of brain tumors (Kirches et al. 2001, 2002). Several other mutations uncovered by Wong et al. (2003), especially at the D310 region, were different between tumor and CSF, meaning these tumors could be of polyclonal origin, consistent with the concept of field cancerization (Dakubo et al. 2007).

11.3.7 Other Biofluids

There are other biofluids that can be obtained for mtDNA analysis, though these have not yet been examined. For example, gastric juice, pancreatic juice, biliary juice, peritoneal fluid, cervical secretions or swab and menstrual fluid are all potential for the development of mtDNA markers for early detection and management of

organ-associated cancers. Mitochondrial genome analysis of pancreatic cancer mutations detected two of four mutations in the biofluids (Maitra et al. 2004).

11.3.8 Exfoliated Skin Cells for Skin Cancer Detection

Exfoliated cells from the surface of the skin can be obtained using various techniques. The patented Epidermal Genetic Information Retrieval (EGIR™) technology from DERMTECH International enables noninvasive acquisition of cells for gene expression analysis. The process of the technology is by rolling a 6-cm piece of adhesive on lesions such as moles to recover cells for diagnostic analysis. A panel of gene expression markers developed by this company enables diagnosis of melanoma. Genesis Genomics Inc. has developed a similar technique using cotton swab to retrieve enough cells for the measurement of acute mtDNA damage from UV radiation. Facile application of this technique to detect other mtDNA mutations in skin lesions is obviously implied.

11.4 Clinical Utility of Mitochondrial DNA Changes in Biofluids

An important aspect of mtDNA measurements in cancer is the ability to demonstrate the practical utility of the changes. To show that specific cancers have signature-mtDNA mutations will be an important breakthrough in mitochondrial genetic alterations in cancer. With the exception of content changes, and large-scale deletions, mtDNA point mutations in cancer appear, at the moment, to occur at random. However, there is emerging evidence that indicate the nonrandom distribution of mtDNA point mutations in cancer. Given the pivotal functional role of this molecule, minor alterations often carry significant consequences. It is, however, comforting to realize that the small size of this genome makes it economically feasible to sequence the entire molecule for diagnostic purposes.

Tumor genetic material is found in biofluids as either cell-free nucleic acids, or in exfoliated tumor cells. Free circulating DNA is reported to be much higher in cancer patients than healthy individuals. Similar elevated levels of nucleic acids could be in other organ-derived biofluids such as urine, BAL and NAF. The mechanism of elevated free DNA in plasma/serum of cancer patients remains unknown. It is estimated that metastatic tumors can shed up to a million cells per gram of tumor per day into the circulation (Folkman 2001). Thus, lysis of circulating tumor cells could account for the elevated free DNA. However, work done by Sorenson et al. (1994) and Chen et al. (1999) demonstrate that the levels of free DNA in plasma of cancer patients cannot easily be accounted for by the lysis of circulating tumor cells. Several mechanisms including apoptosis, necrosis and active DNA release have been proposed. Since high levels of free DNA are

associated with other nonmalignant diseases, measurement of free DNA for the early cancer detection will lack specificity, but can be valuable for disease management. With the exception of free DNA, methods that enable enrichment of biofluids for tumor cells should be explored. For instance, technologies such as CellSearch™ (Veridex) enable the capture of circulating tumor cells for molecular analysis.

For screening purposes, biofluids such as blood, saliva, sputum, urine and NAF that are obtained by noninvasive or minimally invasive procedures are acceptable. Invasively acquired biofluids including CSF, BAL, ductal lavage, pancreatic and gastric juices should be reserved for extremely high-risk individuals or used as an adjunct to disease management. An important aspect of using biofluids for cancer detection is how representative these fluids are of the specific cancer in question. Saliva (for head and neck cancer), sputum/BAL (for lung cancer), NAF/ductal lavage (for breast cancer), urine (for urogenital cancers), pancreatic juice (for pancreatic cancer) and gastric juice (for stomach cancer) are reasonable representations of their respective cancers.

Mutations in the mitochondrial genome and mtDNA content changes are not specific for any cancer. Thus, apart from managing disease progression and therapeutic responses, blood is probably not likely to be the fluid of choice for screening using mitochondrial genome changes. However, if ever demonstrated that early mtDNA changes can be measured in blood prior to disease evolution or at the genesis of cancer, their use in screening using blood will be of tremendous importance. Though there is no definite scientific proof, post-DRE urine is more likely a better fluid than ordinary urine for prostate cancer screening.

Cancer is a devastating disease, and therefore early detection strategies such as screening of high-risk populations using acceptable noninvasive clinical samples are invaluable. With the recent surge in genomic research and technology development, sensitive measurement of cancer signatures in biofluids should be available. Cancer DNA changes in plasma of cancer patients were demonstrated three decades ago. It is therefore questionable why testing genomic changes in biofluids for cancer detection and monitoring is not currently a widespread clinical practice. There are surely several explanations for this. For example, many of the studies lack sensitivity and reproducibility, which may partly be accounted for by different research protocols, including analytical procedures, as well as the heterogeneous nature of cancer. In addition, many of the data reported appear to be from academic research institutions, which might not have intellectual property protection for these findings. Such disclosures often discourage investments in further validation and translation of the results. However, with the establishment of technology transfer offices in virtually all institutions of higher learning, with constant intellectual property protection, this trend will quickly be reversed. Irrespective of the explanation, the current literature on genomic changes detectable in biofluids of cancer patients is convincing enough to warrant more studies with prospective validation and translation.

Analysis of mitochondrial genome in cancer-derived biofluids is still a research endeavor, but has the potential to significantly contribute to cancer detection and management. Several of the studies examined here involve very small sample sizes.

As a proof of principle, these studies have affirmed the practical utility of mitochondrial genome analysis in clinical practice. Large scale studies with prospective validation are needed. If promising, these should be clinically translated to help curb the endless disastrous endings of cancer.

11.5 Conclusion

For noninvasive detection and management of cancer, targeting the altered cancer genome in biofluids is a real possibility. Assaying nuclear mutations/changes is a laudable endeavor that ought to be actively pursued. However, because of the "mass" advantage of the mitochondrial genome, the clonal expansion of some mtDNA mutations, and the possible homoplasmy of these mutations, attention must be paid to the analysis of mitochondrial genome changes in biofluids. Whereas nuclear mutations tend to be specific for cancers, mitochondrial genome point mutations appear random, at the moment, probably as a consequence of the nonspecific effects of ROS and other genotoxins on this genome. However, the small size of mtDNA molecule makes it economical and practical to interrogate the whole molecule for mutations. Importantly, modern technologies such as microarray resequencing should make it clinically feasibly to rapidly and reliably sequence mtDNA for clinical diagnosis. Noteworthy is the strong association of mtDNA copy number or content alterations, as well as large-scale deletions associated with numerous cancers. Therefore, relatively inexpensive quantitative assays such as qPCR should equally be considered in the measurement of mitochondrial genome changes in biofluids for cancer management. With this optimistic view, measurement of genomic changes in biofluids for cancer detection requires further studies and prospective validation of potential biomarkers. Mitochondrial genome analysis in biofluids is feasible and relatively easy. Tumor-specific changes are observed in biofluids of cancer patients. Given the numerous advantages with the analysis of this genome, future cancer detection efforts should consider complementary measurements of mtDNA changes in biofluids.

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Chapter 12
Early Cancer Detection and Monitoring Using Changes in the Mitochondrial Genome as Biosensors

Abstract The concept of field cancerization described in 1953 by Slaughter offers considerable opportunity for cancer management. While nuclear genetic alterations are extensively studied with regard to field cancerization, there is ample evidence of mitochondrial genome changes in premalignant and normal cells adjacent to tumors. The concept of field cancerization, as well as mitochondrial and nuclear genome changes in various cancer fields, and the clinical applications of such molecular alterations are addressed.

12.1 Introduction

The numerous cumulative genetic changes that drive a cell toward malignancy often occur over several years. Of note, it takes a cell several mutational events to undergo neoplastic transformation, which suggests that tissues with numerous mutations are more likely to be transformed. Additional mutations in such tissues can transform them into preneoplastic lesions, and then to frank malignancies. In view of this, the American Association for Cancer Research Task Force on the Treatment and Prevention of Intraepithelial Neoplasia (IEN) has recognized the importance of targeting the treatment of early cancerous lesions to prevent or regress carcinogenesis (O'Shaughnessy et al. 2002). Considerable research has identified molecular markers of IEN that serve as useful targets or endpoints for chemoprevention (Kelloff et al. 2000; O'Shaughnessy et al. 2002; Tsao et al. 2004). This laudable effort by the task force can be complemented by the identification of biomarkers in normal tissues adjacent to tumors (what is referred to as pretumoral or peritumoral cancer fields, consistent with Slaughter's field cancerization, and such tissues usually have high background levels of mutations). Validated biomarkers from pretumoral or peritumoral cancer fields are useful for clinical management of patients including primary chemoprevention.

The conceptual idea that cancer begins with multiple cumulative epigenetic and genetic alterations that eventually transform a cell or a group of cells in a particular

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organ is important in understanding and controlling carcinogenesis. The early genetic events could lead to clonal expansion of preneoplastic daughter cells in a particular cancer field. Subsequent genetic changes in some cells could drive them toward the malignant phenotype. These transformed cells are currently diagnosed histopathologically as cancers owing to changes in cell morphology (e.g., nuclear and cytoskeletal changes). Conceivably, a population of daughter cells with early genetic changes without histopathologic alterations precede tumor formation or remain in the organ concurrent with the tumor, demonstrating the concept of field cancerization or precancerous fields.

The idea of field cancerization was first conceived by Slaughter almost a decade prior to introducing the term in 1953. In an earlier publication, he clearly stated, "cancer does not arise as an isolated cellular phenomenon, but rather as an anaplastic tendency involving many cells at once" (Slaughter 1944). The term "lateral cancerization" was subsequently used by Slaughter to explain the lateral spread of tumors. In his opinion, lateral tumor spread was a function of progressive transformation of cells adjacent to the tumor, rather than the invasion and destruction of the adjacent epithelium by preexisting cancer cells (Slaughter 1946). In a more extensive histopathologic review of 783 oral cancer patients, Slaughter and coworkers then used the term "field cancerization" to describe the existence of generalized carcinogen-induced early genetic changes in an epithelium from which multiple independent lesions develop, leading to the development of multifocal tumors (Slaughter et al. 1953; Fig. 12.1). In some cases,

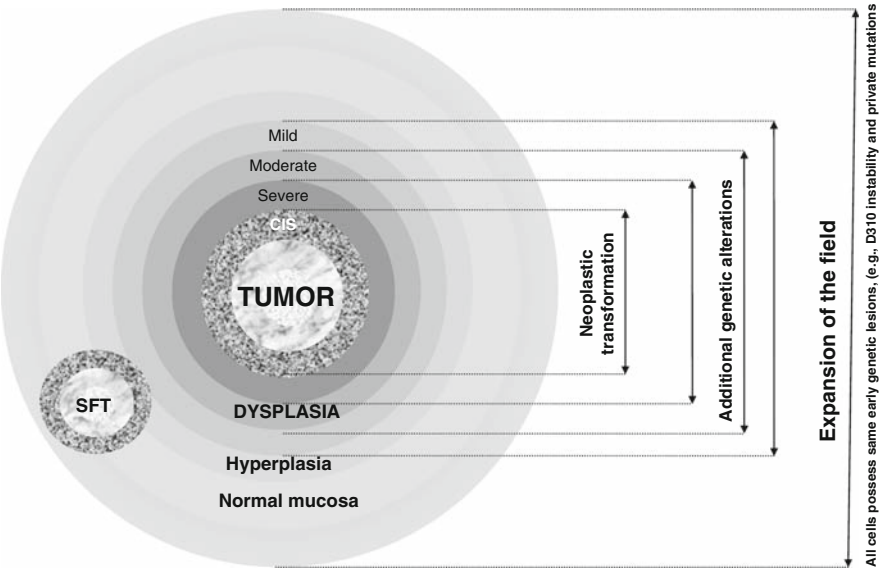

Fig. 12.1 *Model of field cancerization in a contiguous epithelium, such as the urinary bladder*. Surrounding the primary tumor are cells with various degrees of dysplasia and histologically normal cells but with genetic alterations. Second field tumors (SFT) can develop within such epithelium

multiple contiguous tumor foci coalesced that partly explained the lateral spread of squamous cell cancers. It was also observed that normal-looking cells in close proximity to malignant cells were histologically abnormal and therefore were part of the transformed cells in a particular tumor field, and consequently were responsible for the occurrence of local tumor recurrences. These observations were made at about the same era when the DNA double helix was discovered by Watson and Crick, and hence, in the absence of modern molecular genetic technologies. More recent studies using various genetic analyses have provided unequivocal evidence in support of the work of Slaughter and colleagues (Braakhuis et al. 2003).

An important unanswered question of field cancerization is with regard to how exactly do these cancer fields develop.

- Could a particular carcinogen induce multiple distinct genetic alterations in different cells resulting in polyclonal preneoplastic lesions in a particular tissue from which multiple tumors develop?
- Could a single genetic event occur in a cell that subsequently clonally expand and laterally spread to replace the normal epithelium, and therefore create a vast expanse of preneoplastic clonal field from which multiple tumors develop?
- Alternatively, does a particular early genetic event (e.g., *p53* mutation) occur simultaneously in a group of cells such that subsequent different genetic lesions drive some cells toward malignancy?
- Are peritumoral cancer fields created by communication between cancer cells and the histologically normal adjacent cells, probably through epithelial–stromal signaling?
- Is it also plausible that some field changes are created during organogenesis, when some altered cells proliferate to generate a vast area of preconditioned epithelial surface, such that subsequent genetic lesions in some cells result in multiple cancers?

Whereas all these modes of field cancerization are possible, molecular data accrued from studies of head and neck, esophagus, and bladder cancers support the clonal expansion model (Prevo et al. 1999; Simon et al. 2001; Tabor et al. 2002). This model could explain how multiple tumors develop in organs such as skin, colon, esophagus, stomach, bladder, cervix, and vulva that have contiguous epithelium. Though possible, it is less likely that the spread of monoclonal cells is responsible for the development of multiple tumors in glandular tissues like the breast, ovary, pancreas, and prostate that have convoluted glandular epithelial organization, even when exposed to the same carcinogen. Multiple tumors in these organs are likely to originate from polyclonal tumor stem cells, although these clones may have identical genetic alterations. For example, patterns of loss of heterozygosity (LOH) in primary ductal carcinoma in situ (DCIS) were distinct from those in the corresponding cancers, suggesting that some DCIS and subsequent adenocarcinoma in the same breast developed from genetically divergent clones (Smeds et al. 2005). Furthermore, a study of microdissected normal glands and their coexisting malignant epithelia from breast cancer specimens

demonstrated that only one had identical LOH to the cancer, with the majority harboring LOH at different genetic loci from the tumor (Larson et al. 2002).

Notwithstanding the mechanism of precancerous field evolution, the multistep model of tumorigenesis is probably well understood by field cancerization studies. Importantly, genetic information gained at various stages of cancer development continues to advance our knowledge and understanding of cancer biology, and how such molecular markers can be used clinically. Recent advanced molecular techniques have enabled detection of alterations at all genomic levels. Epigenetic gene silencing, chromosomal anomalies, LOH, DNA sequence analysis for SNP and mutation detection, altered gene expression (transcripts and proteins), and mitochondrial genome changes have all been demonstrated in both precancerous lesions and normal-appearing cells close to tumors (Deng et al. 1996; Ha et al. 2002; Tabor et al. 2002, 2004; Chandran et al. 2005; Shen et al. 2005; Heaphy et al. 2006; Sui et al. 2006). DNA structural modification, indicative of possible neoplastic transformation, has even been demonstrated (Malins et al. 2003, 2004a, b, 2005). One can also infer a metabolomic field cancerization profile in various tissues. It could be surmised that the early genetic changes will remain with the tumor cells as well as preneoplastic cells from the same organ. If well characterized, such early molecular alterations should have great value in risk assessment, early cancer detection, monitoring of disease progression, and chemoprevention.

The focus of this chapter is on identified biomolecules in normal adjacent cells or precancerous cells that differ from real normal tissue samples. Hence, the term "field cancerization" is used to loosely and broadly mean "the process whereby cells in a particular tissue or organ acquire genetic changes, such that genetically altered but histologically normal-appearing cells predate the development of neoplasia or coexist with malignant cells, irrespective of their clonal or multiclonal origin." Specifically, this definition seeks to identify altered genetics and molecular pathways in histologically normal looking cells, which are indicative of tumorigenesis.

12.2 Field Cancerization Demonstrated by Mitochondrial Genome Changes

Genetic changes preceding cancer development and/or remaining in normal histologically looking and possibly untransformed cells close to cancer cells is unquestionably pervasive in many cancers. Despite the apathy to recognize and translate these findings into clinical benefits of patients, a deaf ear cannot be turned on these findings. With regard to mitochondrial genetic changes, several evidences are illustrated in Chaps. 6, 7 and 8. A few of these studies will be discussed here to illustrate the almost ubiquitous nature of mitochondrial genetic changes preceding or occurring in histological normal cells adjacent to the gold standard histopathologic indications of cancer. The importance of mitochondrial genome changes in cancer detection and monitoring is resident in its obvious copy number advantage that

enables facile detection of mutations in clinical samples with low cellularity such as tissue-associated biofluids (illustrated in Chap. 11).

Skin Cancer: Field cancerization is well documented in skin using *p53* mutations. Corroborative evidence for field cancerization in the skin has been provided by mtDNA analysis. In nonmelanoma skin cancer (NMSC), both tumors and the normal tissue adjacent to tumor (i.e., perilesional skin) contained homoplasmic UV-induced mtDNA mutations (Durham et al. 2003). In another study, mtDNA deletions were present in both tumors and margin samples, with margin tissues harboring more deletions than tumor (Eshaghian et al. 2006). Thus, perilesional skin tissue might contain expanded mutant mtDNA keratinocytes, as has been demonstrated using nuclear DNA markers (Jonason et al. 1996). This suggests that the conventional use of histologically normal perilesional skin in NMSC studies may have several limitations. This is important when one considers that the majority of studies involving nuclear DNA damage and skin cancer/skin disease often use perilesional skin as a control tissue.

Head and Neck Cancer: Field cancerization in head and neck squamous cell carcinoma (HNSCC) has been addressed using mtDNA markers. Ha et al. (2002) analyzed 137 premalignant lesions from 93 patients and demonstrated the presence of mtDNA C-tract alterations in 34 patients compared to their germline mtDNA. Notably, these mutations increased with increasing severity of dysplasia, suggesting acquired mitochondrial genome alterations might drive or indicate disease progression. Normal adjacent mucosal samples to dysplastic lesions were also analyzed. Identical mtDNA mutations were found in perilesional tissue of 3/8 lesions that had mtDNA alterations. Mutations persisted in 3/7 metachronous lesions, while 8/18 synchronous lesions had an identical pattern of mitochondrial mutations (Ha et al. 2002). These findings lend support to the monoclonal nature of some head and neck cancer fields. In another study by this group, mitochondrial content alteration was observed in premalignant lesions and appeared to increase with disease progression to malignancy (Kim et al. 2004). These changes in mtDNA content could be detected in saliva (Jiang et al. 2005), and the levels decreased with treatment (Jiang et al. 2006), indicating the possibility of a noninvasive means of early detection and monitoring of head and neck cancers using mtDNA markers. The levels of the 4,977-bp common mtDNA deletion has been investigated by quantitative PCR in laser-capture microdissected tissues from paired oral cancers, their precancerous lesions, and adjacent submucosal stroma in comparison to lymphocytes microdissected from lymph node biopsies (Shieh et al. 2004). The deletion was higher in lesions compared to lymphocytes. Interestingly, precancerous lesions had higher levels of the deletion than cancerous tissue, and in both cases the adjacent submucosal stroma harbored more deletions than the lesions (Shieh et al. 2004). Thus, the levels of the common deletion are increased in precancerous oral lesions, and appear to decrease with disease progression to invasive cancer. These observations are consistent with what other investigators have demonstrated in other cancers.

Lung Cancer: Field cancerization is well documented in the lung using various nuclear genetic markers. Indeed, the entire airway epithelium is preconditioned by

the most important risk factor of lung cancer, tobacco smoke. Thus, smoking creates several clonal patches in the lung from which multifocal and second primary tumors (SPTs) develop. The role of mitochondrial genome changes as clonal markers of airway patches and cancer has been addressed in an elaborate study (Dasgupta et al. 2009). In this study, 25 different suspicious (by autofluorescence bronchoscopy) but histologically normal biopsies, and corresponding tumors and normal lymph node (used as germline control) tissues from three patients were sequenced using the version 2 MitoChip. As well, mtDNA copy number was examined in this study. As expected from smoking-induced field cancerization, the histologically normal airway mucosal biopsies from all the lung cancer patients harbored multiple clonal mtDNA mutations as the corresponding primary tumor. One patient developed two tumors in two different lung locations (right upper and left lower lobes) within a period of 2 years. Intriguingly, the clonal relationships of these two tumors were evident, because both shared 20 identical mtDNA mutations. In addition, the mtDNA copy number was higher in cancers and histologically normal lung tissues than the matched normal lymph nodes (Dasgupta et al. 2009).

Esophageal Cancer: Sui et al. (2006) performed an array-based sequencing of mtDNA from 14 preneoplastic lesions of the gastrointestinal tract (seven Barrett esophagus, four colonic adenomas, and three inflammatory colitis-associated dysplasia). Mitochondrial DNA mutations were observed in all 14 preneoplastic samples. Two colonic adenocarcinomas and the synchronous dysplastic lesions harbored identical genetic changes, suggesting a possible field cancerization (Sui et al. 2006). This finding suggests that whole genome mtDNA profiling might help early detection of gastrointestinal tumors.

Gallbladder Cancer: In a well-designed study, a panel of normal, preneoplastic and neoplastic gallbladder samples was analyzed for mitochondrial D310 alterations. Whereas D310 abnormalities were infrequent in normal samples, they increased in frequency in dysplastic lesions and normal-appearing tissue adjacent to a tumor (Tang et al. 2004). This finding calls for comprehensive analysis of mitochondrial genome alterations in premalignant gallbladder lesions.

Prostate Cancer: Although not directly questioned, the concept of field cancerization is indicated in a number of mtDNA mutation studies of prostate cancers. Mutations in the mitochondrial genome were present in a coexisting precursor lesion, prostatic IEN and matched tumor (Jeronimo et al. 2001). In a comprehensive study of laser capture microdissected tissues from prostatectomy specimens, we demonstrated some aspects of field cancerization using mitochondrial genome markers (Parr et al. 2006). Pure populations of cells from a malignant focus and the normal-appearing cells at two distinct locations, immediately proximal to the tumor and further away from the tumor were obtained. MtDNA mutation load in tumors and matched normal-appearing glands were identical and these were significantly different from those obtained from age-matched control individuals without cancer (Parr et al. 2006). In a follow-up study, a single large-scale mtDNA deletion associated with prostate cancer was observed to increase in frequency in normal-appearing glands adjacent to a tumor (Maki et al. 2008).

Hematologic Malignancy: Mitochondrial DNA heteroplasmy is an early sign of disease, and in oncology, this probably is an early indicator of field cancerization. The dynamics of mitochondrial genome changes was shown in association with the progression of myelodysplastic syndrome (MDS) to acute myeloid leukemia (AML) (Linnartz et al. 2004). MDS are clonal myeloid disorders that can transform to AML. A heteroplasmic mtDNA mutation in MDS was observed to increase with disease evolution until the final stage of AML when the mutant copy became homoplasmic. This sequencing data was confirmed using restriction digest analysis by showing that the mtDNA mutation load positively correlated with progression from MDS to AML (Linnartz et al. 2004). These findings are indeed very interesting, and importantly emphasize the need for sensitive sequencing methods for heteroplasmy detection.

12.3 Field Cancerization Is a General Carcinogenic Phenomenon: Evidence from Nuclear Genetic Markers

An important physiologic function of epithelia is their protective role that inevitably exposes them to environmental substances, including carcinogens that can create a vast area of genetically altered cancer fields. Epithelial cells frequently self-renew and can undergo abnormal proliferation. Hyperplastic epithelia could form the basis of neoplastic transformation leading to the formation of the most common types of cancers of the human body. Molecular signatures of field cancerization have been documented for several epithelial tumors including those of the head and neck (Roesch-Ely et al. 2006), esophagus (Wong et al. 2001), stomach (Kim et al. 2006b), lungs (Grepmeier et al. 2005), skin (Jonason et al. 1996), cervix (Chu et al. 1999), vulva (Rosenthal et al. 2002), bladder (Kakizoe 2006), colon (Shen et al. 2005), breast (Heaphy et al. 2006), ovary (Furlan et al. 2006), pancreas (Kitago et al. 2004), and the prostate (Hanson et al. 2006). It has even been conceptualized in brain and hematologic malignancies (Kros et al. 2002; Brodsky and Jones 2004). A brief synopsis of nuclear genetic markers used in field cancerization studies of several cancers is provided followed.

Skin Cancer: The organ, most exposed to environmental carcinogens including ultraviolet radiation (UVR), is skin. Gene mutations caused by UVR play a critical role in the development of skin cancer. Different skin neoplasms are associated with signature gene mutations and alterations in gene expression. Precursor lesions such as actinic keratosis is associated with *p53* mutations (and moderately increased *p16* expression); squamous cell carcinoma (SCC) is associated with *p53* mutations, increased *p16* expression, activation of the mitogenic ras pathway, reduced expression of FasR (CD95-R), and increased expression of FasL; and basal cell carcinoma (BCC) is associated with mutations in *PTCH* (from the sonic hedgehog pathway) and *p53*. The contiguous nature and ease of accessibility of skin has made this organ suitable for studies of the mechanisms of how field

cancerization develops. In fact, field cancerization in skin has been described as a process "whereby the whole neighborhood is affected." As described above, mutations in *p53* are common in skin cancer and as such have been used as biomarkers of clonality in the skin. In one study, *p53* mutations were present in nonmelanoma skin cancer (NMSC) as well as the normal-appearing perilesional skin of eight patients (Kanjilal et al. 1995). Using whole-mount preparation, Jonason et al. (1996) studied the clonal evolution and spread of p53 mutant keratinocytes arising from the dermal–epidermal junctions and hair follicles. Clones comprised of 60–3,000 cells, and were larger and more frequent in sun-exposed than in sun-shielded skin (Jonason et al. 1996). These genetically altered clones might await other genetic alterations to fully demonstrate the malignant phenotype. The carcinogenic effect of high-dose therapy of psoriasis with UVA and psoralens is known. In a study of 69 tumors, *p53* mutations were present in 54% of cases (Christensen et al. 1991). These mutant cells in multiple tumors from the same patient were heterogeneous, suggesting they arose from different somatic stem cell clones in cancer fields created by the UVA and psoralen treatment.

Head and Neck Cancer: Because field cancerization was first described in oral cancers, it is not surprising that considerable molecular genetic studies have been conducted in head and neck tumors in an attempt to explain the mechanisms and importance of this phenomenon (van Oijen and Slootweg 2000; Braakhuis et al. 2003; Ha and Califano 2003). Whereas the debate on the clonal nature of these fields is ongoing, carefully designed experiments with strict definition of clonality (i.e., using cytogenetic markers, microsatellite instability, and mutation analysis) seem to support the notion that the majority of HNSCC originates from contiguous monoclonal preneoplastic fields (Tabor et al. 2002). Indeed, a field size of over 7 cm has been mapped in these cancers (Tabor et al. 2002), and about 62.5% of HNSCC second primary tumors (SPT) recurrences are from similar clonal fields left behind after resection (Tabor et al. 2004). Recently, abnormal protein profiles were demonstrated in various head and neck cancer samples in contrast to samples from healthy individuals. Profiling of mucosa from 73 healthy individuals, 113 HNSCC, 99 tumor-distant, and 18 tumor-adjacent, it was discovered that 72% of tumor-adjacent and 27.3% of tumor-distance samples harbored aberrant protein profiles indicative of field cancerization. Interestingly, these altered protein profiles in tumor-distant samples were significantly associated with tumor relapse, indicating that proteomic analysis of peritumoral samples might have prognostic utility (Roesch-Ely et al. 2006). It appears that carcinogen exposure creates a large molecularly altered preneoplastic field in the epithelium of the aerodigestive tract from which multiple tumors develop, and the remaining cancer fields left after resection give rise to local recurrences.

A role for genomic alterations in stromal tissue in modulating the development of HNSCC has been provided. Using laser-capture microdissection, tumor epithelial and stromal cells were obtained form 122 patients for whole genome LOH and allelic imbalance analysis using 366 microsatellite markers, and the results were compared to clinicopathologic parameters at presentation. Tumor size and nodal metastases were linked to three stroma-specific loci, whilst two epithelial loci were

associated with nodal invasion (Weber et al. 2007). Stromal genetic alterations, therefore, appear to control the growth and spread of HNSCC.

Lung Cancer: Genotoxins in tobacco smoke are established carcinogens of lung cancer. In an attempt to map out smoking-related cancer fields, Franklin et al. (1997) sampled tissues from the entire tracheobronchial tree of one individual, who had 50-pack-years of smoking history but without lung cancer, for *p53* mutation analysis. They found that a transversion in codon 245 was present in 7/10 sites in both lungs, indicating a carcinogen-targeted mutation in widespread lung epithelium of this individual (Franklin et al. 1997). Clone sizes in lung epithelium have been studied using 12 microsatellite markers on microdissected tumors and associated normal-appearing bronchial epithelium. Clone sizes of up to 90,000 cells were identified (Park et al. 1999). Several sites of normal-looking epithelium contained genetic abnormalities as well. Tumors from an individual tended to be homogeneous with respect to molecular markers; however, preneoplastic clonal patches were often heterogeneous (Park et al. 1999). LOH, especially at chromosome 12p12 was demonstrated in normal bronchial epithelium of long-term smokers, and deletion hotspots at two chromosomal regions (2q35-q36, 12p12p13) were observed in non-small cell lung cancer (NSCLC) and matched normal bronchial epithelial cells (Grepmeier et al. 2005). These findings suggest LOH could indicate susceptibility to or potential presence of cancer and may be a hallmark of progression of apparently phenotypically normal preneoplastic cells to cancer (Pan et al. 2005). Thus, similar to the aerodigestive tract, it appears that carcinogens cause an early genetic change in widespread tracheobronchial epithelium from which multiple tumors develop. Given that about 72% of lung cancers diagnosed have already spread (Muller et al. 1996), noninvasive molecular profiling of sputum for biomarkers from tracheobronchial epithelial cancer fields in high-risk population should aid early detection.

Breast Cancer: Glandular epithelial cells of the breast undergo cyclical proliferation, which favors neoplastic transformation. DCIS is a precancerous lesion of invasive breast cancer. Several groups have demonstrated genomic instability in normal breast lobules adjacent to cancer focus, and in DCIS. LOH in normal breast tissue adjacent to breast cancer was reported in 8/30 cases, all of which possessed the same missing allele as the corresponding carcinoma (Deng et al. 1996). Analysis of LOH was performed on fine needle aspiration (FNA) biopsy samples obtained from 30 asymptomatic (11 with normal cytology and 19 with proliferative cytology) women with known risk of breast cancer. LOH was observed in 2 and 14 patients with normal cytology and abnormal cytology respectively (Euhus et al. 2002). These findings suggest that random FNA biopsy sampling of breast tissue for molecular screening could potentially be useful in individualized medicine. In another study, normal-appearing tissue samples were obtained from breast quadrants of 21 patients with known breast cancer for studies of LOH and allelic imbalances (Ellsworth et al. 2004). Genomic instabilities were higher in outer breast quadrants than inner quadrants. Thus, the increased frequency of breast cancer in outer quadrants is related to the presence of elevated genomic instabilities (Ellsworth et al. 2004). LOH was also studied on samples from 30 women with grade 1 and grade 3 DCIS, and six patients who subsequently developed invasive cancer

(Smeds et al. 2005). At four chromosomal loci (6q, 11p, 17p and 17q), there were higher losses in grade 3 compared to grade 1 DCIS; however, the fractional allelic loss at 19 loci was significantly higher in grade 1 than in grade 3 DCIS. As well, LOH in DCIS and corresponding invasive breast cancer were heterogeneous (Smeds et al. 2005). On the contrary, a previous study had indicated the possible progressive nature of DCIS to invasive ductal carcinoma (IDC) using similar LOH analysis. In this study, samples from seven women with DCIS who subsequently developed breast cancer in the same breast were examined. At 50 loci, LOH in DCIS and IDC were concordant, and LOH appeared to accumulate with disease progression from DCIS to IDC (Amari et al. 2003). Alterations in telomere DNA content and allelic imbalances were demonstrated in histologically normal breast tissue located at 1 cm distance from visible tumor margins. These changes decreased as a function of distance from the tumor margin (Heaphy et al. 2006).

Epigenetic gene silencing in breast cancer has been studied. Methylation of the *cyclin D2* promoter appeared to be specific to breast cancer; however, promoter methylation of *APC, RARbeta2* and *RASSF1A* in normal breast from breast cancer patients was associated with increased breasts cancer risk (Lewis et al. 2005). In a comprehensive study of methylation of *RASSF1A* promoter in breast tissue samples, it was uncovered that primary tumors had significantly higher promoter methylation than control reduction mammoplasty tissue, with adjacent normal samples having intermediate levels. Interestingly, global profiling of DNA methylation revealed more methylated genes in normal adjacent samples than in normal donor control samples (Yan et al. 2006).

Esophageal Cancer: Barrett's esophagus is a preneoplastic condition of esophageal cancer, and thus has served as a useful model for studies of clonality and tumor progression in field cancerization. Using *p53* mutation as a clonal marker, Prevo et al. (1999) mapped 213 endoscopic biopsies from 58 patients, and demonstrated that 50% were clonal and occupied cancer fields ranging from 1 to 9 cm (Prevo et al. 1999). LOH at 9p21 (p16 locus) and 17p13 (p53 locus) has also been used to map fields in the esophagus (Galipeau et al. 1999). In 61 patients with 404 samples, LOH at one or both loci was observed in 73% of cases. Clone sizes were heterogeneous, with many clones showing incomplete expansion; however, some were as large as 2 cm, and others occupied the entire Barrett's segment (Galipeau et al. 1999). In another study, it was observed that clone expansion correlated with p16 status, being 1.5 cm in p16+/+, 6 cm in p16+/−, and 8 cm in p16−/− clones. Indeed, mutant p16 clones could expand to involve about 17 cm of the esophagus (Wong et al. 2001). Epigenetic gene silencing of *APC*, *CDH1, ESR1,* and *p16* has been studied in Barrett's esophagus. Hypermethylation in large contiguous fields were observed, further confirming molecular field cancerization in Barrett's metaplasia (Eads et al. 2000). In a prospective study where 267 patients were followed, it was demonstrated that clone sizes (which is an index of clones multiplied by the length of Barrett's segment they occupied) with *p53* LOH and ploidy, were a better predictor of progression towards adenocarcinoma (Maley et al. 2004). Clones with p16 lesions did not carry much risk for tumor development in the absence of *p53* LOH. Thus, clone expansion with added genetic instability seems to indicate

disease progression in Barrett's esophagus. Recently, a clonal cellular diversity theory appears to explain progression of Barrett's esophagus towards adenocarcinoma. By applying the principles of ecology and evolution (mutation rate, population size of evolving clones, rate of natural selection or clonal expansion), clonal diversity was a strong predictor of disease progression, even after controlling for known risk factors such as *p53* LOH and ploidy abnormalities (Maley et al. 2006; Merlo et al. 2006). This finding is clinically relevant with regard to disease surveillance.

Gastric Cancer: Genetic evidence has been provided for the multicentricity of synchronous multiple gastric cancers. Analysis of mutation pattern in *APC, MCC,* and *p53* genes in multiple tumors from 13 patients (ten with double tumors, two with triple tumors and one with quadruple tumors), concluded that there was discordance in mutation patterns in these tumors (Kang et al. 1997). Thus, independent genetic events in a preconditioned epithelium might have given rise to these multiple lesions. Epigenetic silencing via CpG island hypermethylation of *LIMS2*, a gene involved in cell dispersion, has been demonstrated in 53% of gastric cancers (Kim et al. 2006b). Interestingly, *LIMS2* methylation was observed in normal-appearing gastric tissue suggesting that this could be an early genetic event in the development of gastric neoplasia (Kim et al. 2006b). C-erb amplification was observed in a subset of tumors and the normal mucosa close to tumor margin (Kim et al. 1997), and aneuploidy was frequent in normal mucosa at about 3 cm distance from the tumor margin (Kim and Cho 2000).

Colorectal Cancer: Colorectal cancers lend themselves to the study of field cancerization owing to the contiguous nature of the epithelium. Carcinoembryonic antigen (CEA) staining intensities in normal colonic mucosa adjacent to tumor was similar to that of the tumor, but this decreased at a distance of 1 cm, and staining at 5 cm from the tumor margin was identical to mucosa without tumor (Jothy et al. 1996). Thus, a gradient of CEA expression in peritumoral colon epithelium was observed. Evidence for field cancerization in colorectal cancer has also been provided by analysis of promoter methylation in the DNA repair gene, *O^6-methylguanine-DNA methyltransferase* (*MGMT*) (Shen et al. 2005). In this well-designed study, methylation in tumors was observed to be associated with methylation in normal adjacent mucosa, and normal-appearing colorectal mucosa located 10 cm away from tumor margins were methylated in 10/13 tumors. Normal mucosa located 1 cm from tumor margin was more likely to be hypermethylated than those 10 cm away (Shen et al. 2005). Epigenetic silencing of *MGMT* thus creates a preconditioned genetic field from which colorectal tumors develop. Indeed, epigenetic events are proving to be useful biomarkers of the molecular process leading to colorectal cancer (Grady 2005). In another study, *K-RAS* oncogene was mutated in 30% and activated in 26% of adenomas. Importantly, several downstream targets of K-RAS were overexpressed in adenomas (Wang et al. 2006), indicating that these can be evaluated for early diagnosis and risk assessment.

Pancreatic Cancer: Multiple intraductal papillary tumors of the pancreas are common, and a precancerous lesion of pancreatic adenocarcinoma is ductal

hyperplasia. Therefore, could pancreatic cancers arise from genetically transformed but normal ductal epithelium? Mutations in *K-RAS* are early important events in pancreatic ductal carcinoma and non-neoplastic pancreatic ductal lesions. Therefore, these markers have been used for several studies of pancreatic cancer evolution. Microdissected tumors and associated ductal hyperplastic tissue from 37 patients for *K-RAS* mutation and X-chromosome inactivation analyses demonstrated distinct genomic abnormalities in hyperplasia as well as pancreatic cancer. This study concluded that polyclonal multicentric pancreatic cancers originate form epithelium with early genetic changes (Izawa et al. 2001). In a study of microdissected samples from 20 intraductal papillary-mucinous tumors (IPMT) and seven ductal adenocarcinoma, *K-RAS* mutations were noted in 66.7% of peritumoral tissue and 62.5% of separate IPMT lesions, and at least one identical mutation was demonstrated in the tumor and peritumoral tissue in all IPMT patients with those lesions (Kitago et al. 2004). *K-RAS* mutation as a marker of disease progression has been demonstrated. Analysis of 46 different histologic grades of IPMT from 16 patients and nine with ductal adenocarcinoma revealed an increased frequency of *K-RAS* mutation from 16.7% in normal epithelium and papillary hyperplasia to 57.1% in high-grade dysplasia, carcinoma in situ and invasive cancer (Z'Graggen et al. 1997).

Bladder Cancer: As the final recipient and reservoir of urine, the bladder urothelium is inevitably exposed to carcinogens, and therefore is at a high risk of developing large cancer field from which multifocal tumors could arise. Whole organ mapping of bladder cancer fields has been studied using a combination of LOH, *p53* mutation, and fluorescence in situ hybridization (FISH) analysis (Denzinger et al. 2006). Cells were microdissected from various parts of a cystectomised bladder for analysis. Several abnormalities, not observed in normal bladder, were present in bladder with cancer. Molecular and histopathologic data comparison suggested monoclonality of multifocal lesions in bladder cancer (Denzinger et al. 2006). In another investigation, 32 tumors from six bladders were analyzed using chromosomal markers (Simon et al. 2001). Interestingly, multiple tumors from the same bladder harbored identical chromosomal alterations in addition to private chromosomal changes, as is expected of the multistep model of tumorigenesis. In fact, it is likely that in the bladder, lesions occur in genetically transformed but histologically normal urothelium (Hoglund 2006).

Prostate Cancer: Compared to breast, relatively little work has been conducted on field cancerization in prostate cancer. Prostate cancer is often multifocal, and it is likely that multiple tumors arise from an organ genetically altered by a particular carcinogen. Genomic instability, gene expression studies and analysis of mitochondrial genome alterations have recently been reported to show field cancerization in prostate cancer. Methylation in *GSTP1* and *RARbeta2* was present in prostate cancer, adjacent stroma and adjacent normal glands close to tumor, and were absent in normal epithelia from benign prostatic hyperplasia (Hanson et al. 2006). Telomere content alterations were observed in normal-appearing tissue close to tumors, and were shown to be a good predictor of prostate cancer recurrence (Fordyce et al. 2005). Injection of prostate cancer cell lines into athymic nude

mice caused cytogenetic abnormalities in stromal cells (Pathak et al. 1997), suggesting that at least in prostate cancer, tumor cells might have the potential of transforming adjacent normal glands. Gene expression signatures in normal tissue adjacent to a tumor focus closely resembled those of tumors and were different from normal donor prostate samples (Yu et al. 2004; Chandran et al. 2005). Interestingly, early prostate cancer antigen (EPCA), which is expressed in normal tissues close to a tumor, was shown to have elevated expression in normal prostate glands of individuals who subsequently developed cancer years later (Dhir et al. 2004; Uetsuki et al. 2005). The possible clonal nature of multifocal prostate cancer is suggested from gene expression analysis. Laser-capture microdissection was used to procure pure glandular epithelial cells from multifocal tumors for gene expression studies. In any particular individual, *ERG, ETV1*, and *ETV4* were either overexpressed or not expressed in all samples, suggesting alteration in these genes could be early events in prostate cancer evolution (Tomlins et al. 2007). Expression of genes in prostate cancer and adjacent normal-looking epithelial cells were different from prostatic tissues without cancer (Haaland et al. 2009).

Prostate cancer is an age-associated disease. Interestingly, a prostate cancer DNA phenotype, likely induced by an age-associated oxidative DNA damage, was found in some older men and in normal prostate glands adjacent to tumors (Malins et al. 2003; Malins et al. 2005). This cancer DNA conformation is likely an early event in prostate cancer development because it occurs long before tumors develop (Malins et al. 2004a). Indeed, a metastatic prostate cancer DNA phenotype was also demonstrable in metastasizing tumors and the normal glands surrounding these tumors, and this phenotype was different from that of primary cancer phenotype (Malins et al. 2004b). Some of the molecular changes in prostate cancer appear to have excellent potential utility as early diagnostic biomarkers (Perry et al. 2006).

Ovarian Cancer: The most common ovarian cancer, serous ovarian carcinoma, can develop following serous borderline ovarian tumors (BOTs), suggesting a carcinogen can genetically precondition the ovarian epithelium from which tumors develop. Moreover, it has been the conception that multifocal and recurrent ovarian tumors are monoclonal. A study of clonality using *p53* and *K-RAS* mutation analysis in 8 patients with BOTs who later developed serous carcinomas concluded that the tumors were unrelated (Ortiz et al. 2001). Likely, distinct genetic alterations in a preconditioned epithelium may lead to BOTs and serous carcinomas independently. Analysis of 13 primary and corresponding recurrent ovarian tumors using four markers of genomic instability indicated 10/13 were different, with the rest being identical (Buller et al. 1998). Promoter methylation status of *hMLH1, CDKN2A,* and *MGMT* in synchronous independent ovarian and endometrial cancers was studied for the possible origin of these tumors from a cancerized field. High frequency of promoter methylation of *CDKC2A* and *MGMT* were found in both endometrial and ovarian carcinomas, suggesting the epigenetic silencing of these genes could be an early event in the evolution of these synchronous cancers (Furlan et al. 2006).

Vulval and Cervical Cancers: Vulval intraepithelial neoplasia (VIN) is often clonal and contiguous with vulval squamous cell carcinoma (VSCC). This suggests

VIN may be a precursor lesion of VSCC. X-chromosome inactivation analysis of 9 cases of VIN, 10 cases of VSCC with contiguous VIN and 11 cases of VSCC with noncontiguous VIN indicate that the majority of VIN and VSCC were monoclonal in origin. Two cases of VIN with noncontiguous VSCC, however, had molecular patterns consistent with separate clonal origins (Rosenthal et al. 2002). Allelotyping of three markers on chromosome 3p in microdissected samples from low- and high-grade cervical intraepithelial lesions found that microsatellite instabilities were common in low-grade lesions associated with invasive cancers, suggesting premalignant and malignant lesions were of monoclonal origin (Chu et al. 1999). Thus, biomarkers in early stage vulval and cervical lesions seem useful for early detection and monitoring of these cancers.

Nervous System Tumors: Gliomatosis cerebri (GC) is a rare neoplasm of the brain with extensive distribution, usually involving both lobes and even occasionally infratentorial regions. Could this lesion therefore arise from a vast field of preconditioned neural tissue? In one study of GC, 24 tissue samples were randomly obtained from several areas of the brain for study. Genome-wide scan for chromosomal aberrations, *p53* mutation analysis and LOH were performed on all samples. Mutations in *p53* were present in 20/24 samples, with chromosomal loses and allelic imbalances in several other tumor samples (Kros et al. 2002). In a separate series, mtDNA was used as a clonal marker for GC, and consistent band losses were observed in all tumor samples from two individuals, one of whom also had *p53* mutations (Kirches et al. 2003).

Hematologic Cancer: It has been proposed that generalized insults to bone marrow can lead to simultaneous generation of many abnormal bone marrow clones. Therefore, admixture of cells representing severe aplastic anemia, acute promyelocytic leukemia, chronic myeloid leukemia, and MDS can be present in the same bone marrow, and that proliferative advantage of one clone results in its dominant appearance and thus dictate a specific diagnosis. The term "field leukemogenic effect" was used to describe this phenomenon (Brodsky and Jones 2004). It will be interesting to know whether the ecological and evolutionary concepts used to predict progression in Barrett's esophagus to adenocarcinoma (Maley et al. 2006) applies to the evolution of these hematologic malignancies.

12.4 Clinical Importance of Cancer Field Molecular Signatures

The myriads of demonstrated mitochondrial and nuclear genetic changes preceding carcinogenesis offer great opportunity for early cancer detection, chemoprevention, and disease monitoring. High-risk population identified by lifestyle for example, can be profiled for field changes using possibly samples obtained by noninvasive means such as urine or sputum. Early detection of genetic footprints indicative of cancer field development will indicate closer monitoring for diagnosis of curable cancers.

12.4.1 Appropriateness of Control Tissue in Cancer Studies

An important resounding message from the above bird's-eye view of early cancer genetic signatures is that using histologically normal-appearing samples, as the sole control tissue in cancer research is probably inappropriate (Braakhuis et al. 2004). The use of donor tissues (ideally obtained under similar conditions as tumor) will serve as better controls for tumor-specific biomarker discovery. Donor control, in addition to normal adjacent to tumors, precancerous lesions, and tumor samples will provide the best sample set for resolution of genetic alterations predictive of disease progression. If normal adjacent to tumor tissue must be used as the only normal control, it should first be examined for the absence of genetic abnormalities (Braakhuis et al. 2004). In skin cancer studies, for example, additional analysis of UV-induced damage in sun-protected skin from the same individual would provide a complementary isogenic control.

With regards to mtDNA analysis, a number of investigators have used sequences form the normal-appearing cells in the presence of tumors as normal maternal germline control sequence for pairwise comparison to tumor sequences to score somatic mtDNA mutations (Chen et al. 2002; Maximo et al. 2002; Tan et al. 2002, 2006; Suzuki et al. 2003; Lee et al. 2005; Zhu et al. 2005; Gomez-Zaera et al. 2006; Meierhofer et al. 2006). Obviously, this can lead to erroneous mutations being associated with the cancer, because the adjacent normal tissue in a cancerized field may sustain somatic mtDNA mutations as well. Comparison to the mtDNA profile of the individual's blood, which is determined to be the authentic germline mitochondrial genome, is necessary.

12.4.2 Risk Assessment, Early Cancer Detection, Chemoprevention, and Disease Monitoring

Genetic changes present in tumors and not in normal-appearing cells close to a tumor are thought to be useful for early detection of cancer. Usually, such tumor-specific biomarkers are validated and used for screening to diagnose organ-confined tumors that have better treatment outlook. In some instances, early detection strategies such as molecular profiling of circulating tumor cells rather indicate the presence of metastasis (Stemke-Hale et al. 2006). Future biomarker discovery and validation efforts should focus on the identification of molecular genetic biosensors that signal the genesis of disease, rather than biomarkers of the disease. Such biosensors will be useful in risk assessment, early detection, disease monitoring, and chemoprevention. For example, LOH in normal breast epithelial cells obtained by random fine-needle aspiration biopsy from women at known risk of breast cancer was used to predict breast cancer risk (Euhus et al. 2002). In this study, the Gail risk model predicted a mean lifetime breast cancer risk of 16.7% for women with no LOH compared to 22.9% for women with LOH (Euhus et al. 2002). These markers thus correlate with individual risk of developing breast

cancer, and thus are useful for early detection and risk assessment of breast cancer. Genetic changes present in normal-appearing cells can be used for the identification and recruitment of individuals at the risk of developing cancer for primary chemoprevention (i.e., to prevent *de novo* development of cancers). Indeed, epigenetic gene silencing through promoter hypermethylation and transcriptional repression of several tumor-associated genes is an early event in several cancers including breast, prostate, colorectal, gastric, and ovarian cancers. Importantly, reversal of epigenetic events with agents such as hydralazine, 5-Aza-2′-deoxycytidine, zebularine, and magnesium valporate is possible. Knowledge of methylation patterns and their role in malignant transformation should enable controlled use of methylation reversal agents in primary chemoprevention. Similarly, relevant markers present in precancerous lesions will be useful end point measures or targets of secondary chemoprevention (i.e., to prevent the progression of premalignant lesions to invasive cancers). Importantly, biofluids, representative of cells from a particular organ may serve as useful noninvasive or minimally invasive samples for disease surveillance. For instance, genetic changes preceding breast cancer development may be detectable in nipple aspirate fluids.

Multistep field cancerization indicates there are at least two levels of cancer progression; *molecular progression* whereby histologically normal-looking cells undergo sequential cumulative acquisition of genomic damage (without histopathologic changes), and *phenotypic progression* whereby a neoplastic cell accumulates genetic alterations and undergoes further phenotypic changes (e.g., from IEN to various stages of invasive cancer). Functionally relevant pathways altered at the molecular progression phase should uncover useful biosensors for early detection and monitoring of cancer. It is also well known that some precancerous lesions do not progress to invasive cancers (O'Shaughnessy et al. 2002). Thus, molecular profiling of early lesions using appropriate control tissue will enable important pathways or biomarkers that predict disease progression to be deciphered. In a recent elegant study, laser-capture microdissection was used to procure pure population of cells at various stages of prostate cancer development for gene expression profiling. Using a novel analytical approach referred to as "molecular concept model," several genes and pathways were identified that represented molecular progression of prostate cancer from benign through prostatic IEN to prostate cancer. Increased expression of genes involved in cell cycle regulation, and on chromosome 8q was associated with disease progression (Tomlins et al. 2007). The estimated progression of an atypical hyperplasia to an adenocarcinoma may occur over a period of 5–20 years. This prolonged time course provides opportunities for early detection within an activated pre-cancerous epithelial field (Kelloff et al. 2000; O'Shaughnessy et al. 2002).

An important clinical utility of field cancerization is in complementary evaluation of pathologic biopsy specimen. Currently, biopsies for cancer diagnosis are reviewed by the histology, gold standard, and absence of abnormal cells often precludes the diagnosis of cancer. However, histologically normal biopsy specimens that possess molecular signatures of cancer fields suggest either there is a concurrent tumor missed by the biopsy procedure, or that some cells in the tissue or

organ are progressing towards malignancy. Such high-risk patients will require chemoprevention or close surveillance for early detection of disease.

12.4.3 Tumor Margins and Recurrences

Tumor recurrences are common in surgical oncology. Depending on the type of tumors, recurrent rates could be as high as 50% (Hockel and Dornhofer 2005). There are two types of local tumor recurrences; those that occur at the primary site of surgical resection (local or scar recurrence), and those that occur at a distance from the surgical scar in the residual organ left after resection of the primary tumor (in situ recurrence, SPT, or second field tumors (SFT). In situ recurrences that are genetically similar to the primary tumor are referred to as SFT, and those that are genetically dissimilar to the primary tumor are identified as true SPT. Field cancerization may have an etiologic role in a substantial number of recurrences. For example, a surgical resection margin that includes a genetically altered field can explain the occurrence of scar recurrence. This explanation suggests that molecular profiling of surgical margins will help reduce scar recurrences. Because multiple independent patches of cancer fields may be present in the same organ exposed to the same insult, clean molecular margins may not necessarily prevent recurrences in the residual organ. Nonetheless, complementary molecular assessment of tumor margins should at least help reduce tumor recurrences, because such information can impart management. For instance, in pancreatic cancer, analysis of *K-RAS* codon 12 mutation in histologically normal surgical margin tissues from 70 patients could have prognostic significance. In this study, 53% of patients with positive molecular margins had unfavorable overall survival outcome (Kim et al. 2006a).

12.5 Conclusion

Field cancerization is a well-studied and well-documented process of malignant transformation. Several studies confirm the importance of this phenomenon in tumor development. With technological advancement, the future should benefit from well-designed studies aimed at identifying genetic markers and pathways useful in disease management. An obvious shortcoming in almost all the studies of field cancerization is the lack of extensive genome-wide scans that will enable early and important genetic changes in tumor evolution to be uncovered. Many studies have relied heavily on known markers associated with a particular tumor. Such preselected tumor markers might be later acquisitions in the disease process, and will be missed in peritumoral samples or precancerous lesions. Comprehensive high-throughput analyses for the discovery of early and relevant genetic changes that extend across global networks and represent modular alterations of multiple targets (or surrogates) of terminal histologically differentiated stages of cancer subtypes will be essential for early detection, risk assessment and primary chemoprevention.

Laser-capture microdisection and high-throughput genomic technologies should enable carefully designed studies using appropriate control tissue for identification of important molecular signatures in these genetically transformed but histologically normal cells. Such tumor-specific biomarkers should have excellent clinical utility in areas such as risk assessment, early cancer detection, monitoring of tumor progression, and definition of tumor margins. The multiplicity of mtDNA copies per cell should enable ease of detection in biofluids and hence ease of clinical translation using tissues with less cells. Also, because of the high copy number, mitochondrial genome sustains tremendous amounts of damage without cell demise which is an invaluable feature for using this genome as a biosensor of carcinogenesis.

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Chapter 13
Analysis of Mitochondrial Genome Alterations in Cancer

Abstract Clinical measurements of mitochondrial genome changes in cancer is as important as assessing this genome in mitochondrial cytopathies. Polymorphisms, somatic point mutations, as well as large-scale deletions and content changes are all applicable in cancer management. Proper sample preparation and attention to technical details are important issues in assaying authentic mitochondrial genome changes in clinical specimens. This chapter examines several available methods of measuring mtDNA mutations as well as quantitative measurements of content changes and large-scale deletions. Important precautions needed for good assay performance are provided as well.

13.1 Introduction

The mitochondrial genome sustains several diverse mutations in cancer as detailed in previous chapters. These mutations are usually single nucleotide insertions or deletions, micro-deletions or insertions (few bases), and large-scale deletions (>500 bp) and rearrangements. The D-loop region often sustains most of the mutations, partly because of its function of anchoring the genome to the inner mitochondrial membrane, and possibly because of the inherent DNA conformational structure of this region. Also evident in some cancers are clustering of mutations in complex I genes. Many of the large-scale deletions occur in the major arc, partly because it contains numerous repeats, but other important deletions are demonstrated in the minor arc. Aside from these somatic mutations, there are several geographic polymorphisms that appear to carry risk for developing cancer. Circulating mitochondrial nucleic acids could have prognostic utility in cancer management. In addition, mitochondrial genome copy number differences between cancer and non-cancer tissues, as well as the role of copy number changes in cancer risk, are demonstrated.

There is enough evidence now that implicates the role of the mtDNA in carcinogenesis. While clinical translation of mitochondrial changes in practice

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has been slow, it is the hope of this chapter to demonstrate some of the methodologies available for clinical measurement and hence, facilitate use of mitochondrial genome changes in cancer management. Sensitive and accurate methods for mitochondrial genome measurements should have clinical value and deserve translation. The focus of this chapter is on methodologies and technologies with clinical potential for measuring mtDNA changes, and also issues that could confound accurate measurement of mtDNA changes in cancer.

13.2 Preparation of Clinical Samples for Mitochondrial Genome Analysis in Cancer

Clinical specimens are useful for measurement of mtDNA mutations, content changes, and circulating nucleic acids. The types of specimens required for mtDNA mutation analysis depend on whether the specific sequence change being assayed is a polymorphism or somatic mutation. Any normal tissue is suitable for polymorphism and haplotype analysis because the sequence should be representative of the maternal inherited sequence. In many instances, circulating lymphocytes have been used, but other normal tissues are equally suitable for such analysis. Both frozen and formalin-fixed and paraffin-embedded tissues are suitable for analysis.

Clinical samples such as bodily fluids, including prostate massage fluid, post-digital rectal examination void urine, nipple aspirate fluid, ductal lavage, bronchoalveolar lavage, or saliva that are enriched for organ-specific exfoliated epithelial cells, are suitable for analysis of mtDNA somatic mutations, deletions, content changes, and gene expression alterations. Sequences from these samples must be compared with normal maternal germline sequences to score somatic mutations. Circulating nucleic acids in plasma or serum are used for quantitative measurements of altered mtDNA sequences or levels.

The recovery of mtDNA from any of these samples can be accomplished by means of commercially available DNA extraction kits. For qualitative analysis, such as sequencing or genotyping, small starting total DNA template can be enriched for mtDNA using amplification techniques such as the REPLI-g mitochondrial DNA amplification kit from Qiagen. This kit uses high fidelity DNA polymerase in an isothermal reaction to uniformly amplify the entire mitochondrial genome from very small starting material. Typically, 10 μg total DNA, which contains just nanogram amounts of mtDNA, will yield ~4 μg mtDNA. A major advantage of this procedure is the enrichment of the sample for mitochondrial genomes that will reduce the likelihood of pseudogene coamplification. Because of the high copy number of mtDNA per cell, it is recommended that an appropriate dilution of starting template be made for quantitative analysis in order to enable resolution between normal and diseased samples. In addition, for each analytical procedure, the recommended template amounts should be adhered to, so as to avoid erroneous data acquisition.

13.3 Analysis of Mitochondrial Genome Point Mutations in Cancer

One important aspect of mtDNA analysis in clinical samples is sensitive detection of heteroplasmy, which is a hallmark of mitochondrial diseases. Low levels of mutant mitochondrial genomes signal early events in disease evolution. Indeed, mtDNA mutation load as low as 0.1% can be clinically relevant for disease detection and monitoring. Given that several mtDNA mutations occur early in cancer, identifying and tracking the accumulation of somatic mtDNA mutant copies could potentially be very useful in early cancer detection and monitoring. High-throughput and sensitive methods of heteroplasmy detection, which are amenable to automation, should be clinically useful. Currently, there is no specific somatic mutation characteristic of any cancer. However, the mutation load is higher in several tumors than in the normal tissues. Thus, an analysis of the entire mtDNA for mutation load might seem to be a reasonable diagnostic approach.

Single nucleotide polymorphisms or somatic point mutations can be targeted for detection or screened for using several methods, each of which has its strengths and weaknesses, but have all proven useful in mtDNA mutation analysis. Restriction fragment length polymorphism (RFLP), allele-specific oligonucleotide, single-strand conformation polymorphism, hetreoduplex assays, and direct sequencing are all amenable to mtDNA mutation detection. However, many of these methods are currently inadequate for sensitive heteroplasmy detection, hence, the search for newer technologies. Microarray-based resequencing, denaturing high-performance liquid chromatography (DHPLC), and pyrosequencing, are some newer methods that hold tremendous promise to increase the sensitivity of heteroplasmy detection. Methodological considerations and some technologies for analysis of mtDNA point mutations in cancer, are examined.

13.3.1 Diagnostic Approaches

Analysis of haplotype markers, polymorphisms, and known disease mutations is a simple laboratory practice. These mutations often change or create new restriction sites such that simple PCR amplification of the known region, followed by radioactive or fluorescent RFLP analysis of the amplicon using nondenaturing gel, enable a straightforward diagnosis or genotyping. PCR-RFLP analysis has a sensitivity of ~5% for heteroplasmy detection, probably because of heteroduplex species that form after PCR, and therefore, interfere with complete restriction analysis. The traditional capillary electrophoresis sequencing method is a very robust, but an insensitive and labor-intensive process that is not easily automated. With a sensitivity of 25% for heteroplasmic detection, low-level heteroplasmies are missed using this method. Other suitable methods for mtDNA mutation analysis include enzymatic and chemical cleavage, heteroduplex assays (denaturing gradient gel

electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), temporal temperature gradient gel electrophoresis (TTGE), and DHPLC), single-strand conformation polymorphism, use of allele-specific oligonucleotide probes, and ligase-mediated detection. However, with the exception of the DHPLC, these methods are generally time-consuming, labor-intensive, and not easily adaptable to automation and require small-sized nucleic acid fragments for detection. GeneChip® resequencing of the entire mitochondrial genome has enormous potential for translation into clinical practice. Pyrosequencing and other emerging sequencing methods are potentially useful for mtDNA mutation analysis. Quantitative PCR technology is useful for quantification of mtDNA deletions and copy number. The methodologies and the utility of some of these technologies for mtDNA mutation detection and content changes in cancer diagnostics, are described.

13.3.1.1 Restriction Fragment Length Polymorphism

RFLP enables the detection of specific known mutations using PCR and restriction digest analysis. The method requires modest steps, including PCR amplification of the genomic fragments encompassing the mutation, and restriction digest with an enzyme that discriminates between wildtype and mutant fragments. This process is followed by electrophoresis of the sample on nondenaturing polyacrylamide gels, staining, and visualization. Mutant fragment length differs from wildtype, allowing straightforward interpretation (Fig. 13.1).

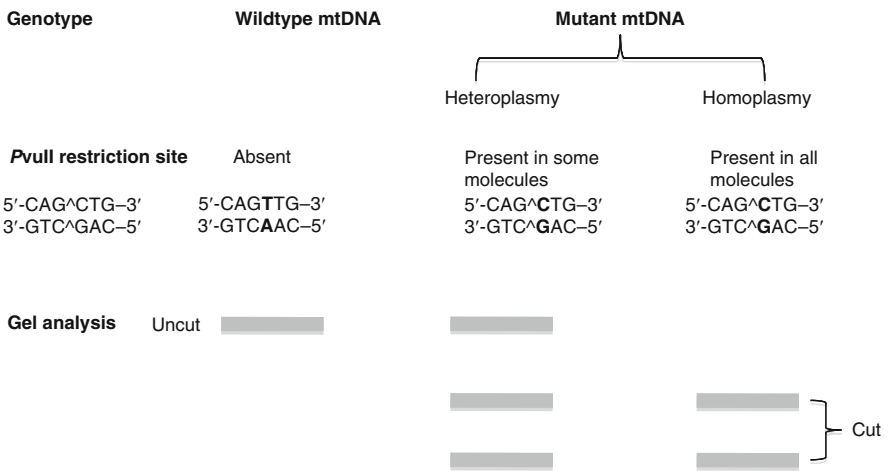

Fig. 13.1 *Restriction fragment length polymorphism analysis of mtDNA*. In this example, a mutation (T→C) creates a *Pvu*II restriction site. Polymerase chain reaction amplification of this fragment followed by *Pvu*II digest and gel analysis reveals an uncut band in the wildtype, two cut bands in the homoplasmic state and both uncut and cut fragments in the sample with heteroplasmic mutation

13.3.1.2 Allele-Specific Oligonucleotide

Allele-specific oligonucleotide (ASO) is one of the sensitive methods employed for the detection of known mtDNA point mutations in clinical specimens such as urine, saliva, or blood. This method is well established for mutation analysis in several mitochondrial mutation disorders such as mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), Leber's hereditary optic neuropathy (LHON) and neuropathy, ataxia and retinitis pigmentosa (NARP). The method requires the synthesis of allele-specific oligonucloetides. These ASO probes are fluorescent, radioactive, or enzymatic labeled 19–21 mers, used in dot blot analysis of amplified DNA samples. The PCR products are immobilized as dots on a positively charged nylon membrane. The number of dots prepared depends on the number of mutations being targeted. Hybridization with the labeled ASO probes is performed overnight, followed by stringent washes, before the detection of signal intensities for each dot position. Wildtype mtDNA hybridizes to wildtype ASO only, and similarly, homoplasmic mutations hybridize only to mutant probes. However, heteroplasmic mutations will reveal hybridization to both mutant and wildtype ASOs (Fig. 13.2).

ASO-PCR, also known as amplification refractory mutation system (ARMS), enables simultaneous detection and quantitation of mutations in clinical samples. This is a quantitative PCR performed using nonradioactive-labeled ASOs as primers. The basic principle is that only perfectly matched strands can be amplified (Bai and Wong 2004).

13.3.1.3 Single-Strand Conformation Polymorphism Analysis

This method has been used extensively to score mtDNA mutations. It involves the amplification of target sequences with incorporated radiolabeled dATP. The

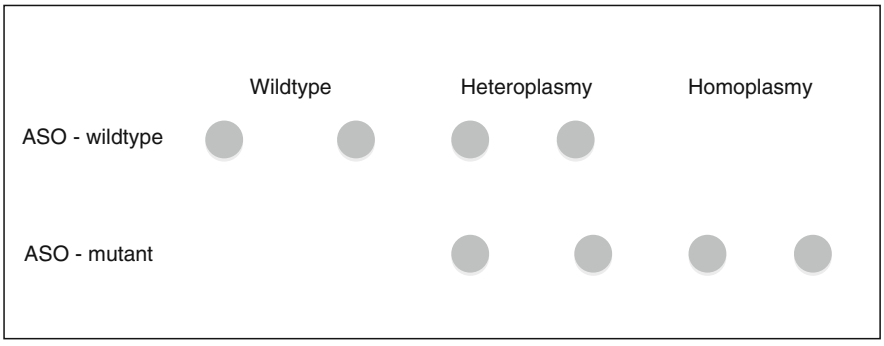

Fig. 13.2 *Allele specific oligonucleotide (ASO) analysis of mtDNA*. The use of wildtype and mutant ASO enables detection of both heteroplasmic and homoplasmic mutations

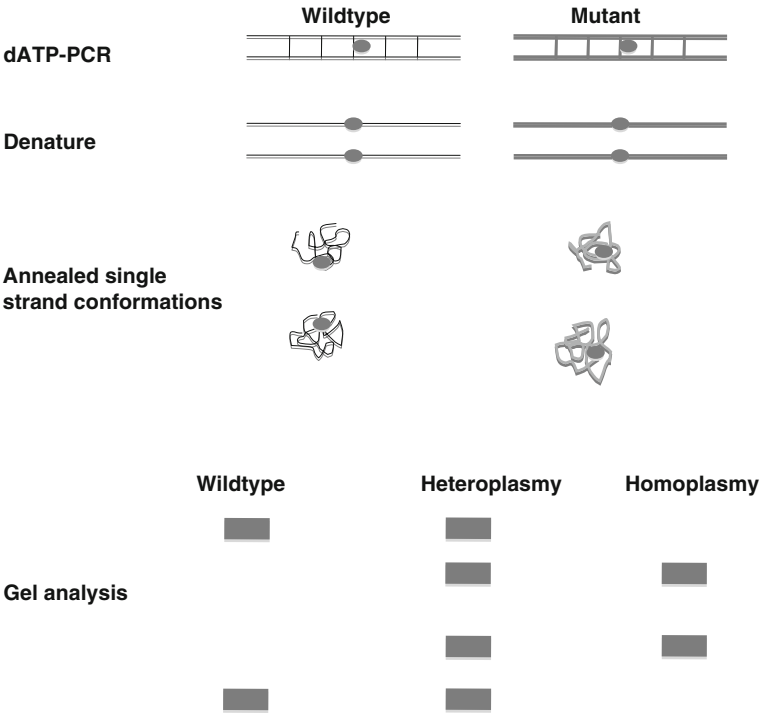

Fig. 13.3 *Single-strand conformation polymorphism analysis*. Single point mutations in the mitochondrial genome can create major differences in structural conformations of single stranded DNA. Following electrophoresis, these strands can be detected because of the differences in their electrophoretic mobility

amplified products are first denatured into single DNA strands, and electrophoresed on nondenaturing polyacrylamide gels. In the process of electrophoresis, the single strands assume conformations dependent on their sequences. These sequence-specific conformational fragments migrate at different patterns, enabling their detection (Fig. 13.3). Fragments with mutations can be sequenced to uncover the specific mutation.

13.3.1.4 Heteroduplex Assays

The basic principle of heteroduplex assays is the detection of hybridizations between wildtype and mutant strands following reannealing of denatured PCR products. Because mtDNA mutations are mostly heteroplasmic, PCR amplification of targeted sequence followed by reannealing enables heteroduplex formation between mutant and wildtype single strands (Fig. 13.4). These reannealed products denature and separate during analysis because of their melting characteristics that are dependent on their specific sequences. Several methods are available for

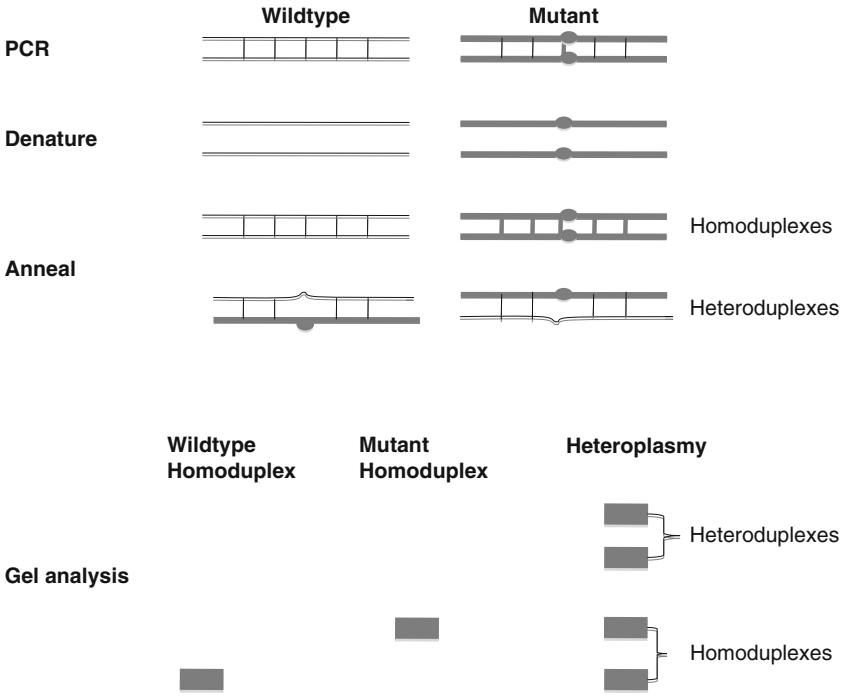

Fig. 13.4 *Principle of mtDNA mutation detection using heteroduplex methods*. Amplification of target sequence, followed by denaturation and annealing leads to the formation of homo- and heteroduplexes. These are separated and visualized using different techniques

performing heteroduplex analysis. These methods are different based on sensitivity, the size of fragments that can be analyzed, ease of automation, and avoidance of radioactive substances. Some of the methods are denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), temporal temperature gradient gel electrophoresis (TTGE) and denaturing high-performance liquid chromatography (DHPLC). It should be noted that these methods can be used to uncover novel mutations as well, but such heteroduplexes need to be sequenced to reveal the specific sequence change.

TTGE: First introduced in 1991 by Yoshino et al (1991), TTGE separates DNA on the basis of differences in the melting behavior of mutant verses wildtype in a temporal temperature gradient that increases in a linear fashion throughout the duration of the electrophoresis. The denaturant is, however, a constant urea concentration in the gel, which contrasts with the gradient urea concentration used in the original DGGE. Also, the temperature gradient increases with time during the electrophoresis, which contrasts with the constant temperature gradient employed in TGGE. The gradual increases in temperature (usually 0.5°–3°C/h) cause partial denaturation of DNA that results in retarded mobility through the gel. Because of sequence differences, mutant and wildtype molecules denature at different temperatures and hence, run at different speeds and separate on the gel. The presence of

two homoduplex and two heteroduplex bands is indicative of heteroplasmy; however, (Fig. 13.4), partial separation can occur leading to fewer than four bands. Homoplasmy may or may not cause a change in the single band. TTGE can analyze DNA fragments of up to 1.2 kb in size.

DGGE: Fischer and Lerman (1979) invented DGGE. Using a uniform temperature of 60°C in a concentration gradient of a urea-formamide mixture, they were able to separate Eco RI fragments of lambda or *Escherichia coli* DNA. Importantly, their experiments demonstrated that this separation was dependent upon the specific nucleotide sequences of DNA, but not on the sizes of the fragments. This method requires the use of GC-clamped primers for PCR amplification, and its major limitation is the size of DNA that can be examined (limited to 200–300 bp). It is, however, as sensitive as TTGE.

TGGE: TGGE is similar to DGGE; however, the DNA molecules are separated on the basis of temperature-mediated melting behavior of the fragments, which depends on their primary sequences. The basic principle is similar to that of TTGE, except that TGGE uses a constant temperature gradient throughout the process of electrophoresis to separate molecules. Hence, this method has a much lower sensitivity than that of TTGE.

13.3.1.5 Denaturing High-Performance Liquid Chromatography

DHPLC is one of the most sensitive heteroduplex methods available for heteroplasmy detection (O'Connell et al. 2003). This technique of mtDNA sequence analysis does not allow identification of specific sequence variants; however, it is an excellent method for haplotype analysis and detection of known polymorphisms and mutations associated with specific diseases. A sample with unknown mutations that demonstrate heteroduplex profiles can however, be sequenced to identify the specific sequence variants. Additionally, DHPLC is valuable in screening clinical samples for the total suite of sequence variants or mutation load. Unlike the other heteroduplex methods of mutation detection that require small-sized DNA fragments, DHPLC allows mutation detection in larger fragments of up to 1.5 kb. The WAVE® DNA Genetic Analysis System is an automated and cost-effective DHPLC system developed by Transgenomic Inc. This instrument can rapidly screen the entire mitochondrial genome in <8 h.

The template preparation for heteroduplex formation depends on the type of mtDNA mutation or polymorphism being targeted. To analyze genetic variants in the nuclear genome, two separate amplifications of wildtype and mutant sequences are performed and used for heteroduplex formation. For somatic mtDNA mutations, the molecules with pathogenic mutations coexist with normal wildtype genomes in cells and tissues (heteroplasmy). Therefore, single-tube PCR is adequate for obtaining mutant and wildtype products for heteroduplex formation. However, nonpathogenic polymorphisms and haplotype markers are often homoplasmic. For the analysis of such markers in cancer, a known rCRS sequence needs to be amplified separately and used as the wildtype reference sequence for

heteroduplex formation. Also, some somatic mtDNA mutations in cancer are homoplasmic and these differ from the germline variant of the individual. Thus, amplification of both tumor tissue and normal germline mtDNA (e.g., from normal white blood cells) will be required for heteroduplex formation. In instances where two separate amplifications have been performed, a ratio of 1:1 wildtype to mutant templates is used for subsequent analysis.

Whole mitochondrial genome analysis is possible. To achieve this, the entire mtDNA is amplified in small fragments with overlapping primers for a direct DHPLC analysis. Alternatively, larger fragments encompassing the entire mitochondrial genome can be amplified using multiple overlapping primers. These big fragments are then cut into smaller-sized fragments using appropriate restriction sites for a multiplex DHPLC analysis. Because mtDNA demonstrates polymorphisms in individuals, restriction sites could be modified, resulting in different end products that could have different melting temperatures. Thus, a gel analysis should be performed on digested PCR products prior to downstream DHPLC analysis. The MitoScreen™ assay kit from Transgenomic Inc. employs 19 primer pairs, the products of 15 of which are restricted prior to analysis. A detailed protocol for using this assay can be found at http://www.transgenomic.com.

The mixed templates are denatured and allowed to cool slowly so that the mtDNA strands reanneal. This process, although simple, is a critical step for accurate mutation detection. Two main types of reannealed products, homoduplex (complementary wildtype strands, and mutant strands in perfect matches) and heteroduplex species (complementary wildtype and mutant strands that contain mismatch base pairs) will form. Approximately, 5–8 μl of the samples are then injected into a flow column of the WAVE® system that is filled with alkylated nonporous poly(styrene-divinylbenzene) beads. The flow path has an oven that contains a patented DNASep® Cartridge. The cartridge temperature in the oven is initially set to partially denature the reannealed products. In the presence of triethylamine acetate (TEAA) buffer, the partially denatured templates are anchored to the beads via the interaction of the negatively charged phosphate backbone of the partially denatured DNA with the positively charged ammonium groups in the TEAA, whose hydrophobic parts are anchored to the hydrophobic parts of the beads in the cartridge. The bound mtDNA is then eluted from the column with increasing concentrations of acetonitrile (ACN) at optimized mobile phase temperatures for each mtDNA fragment. The optimal temperatures and ACN gradients can be determined empirically or by means of the Navigator™ software. Heteroduplexes with internal mismatch base pairing display reduced column retention compared to homoduplexes, and therefore, elute first, followed by homoduplexes. Absorbances of mtDNA fragments are recorded over time as they pass through a UV (or fluorescence) detector. The Navigator™ software analyzes and records the information as chromatograms. The presence of a single peak means the absence of mutation; however, high percentage heteroplasmic mutations give two peaks, the first peak representing the heteroduplexes and the second one the homoduplexes. Very low percentage heteroplasmy often appears as a small "bump" in the single major peak (Fig. 13.5).

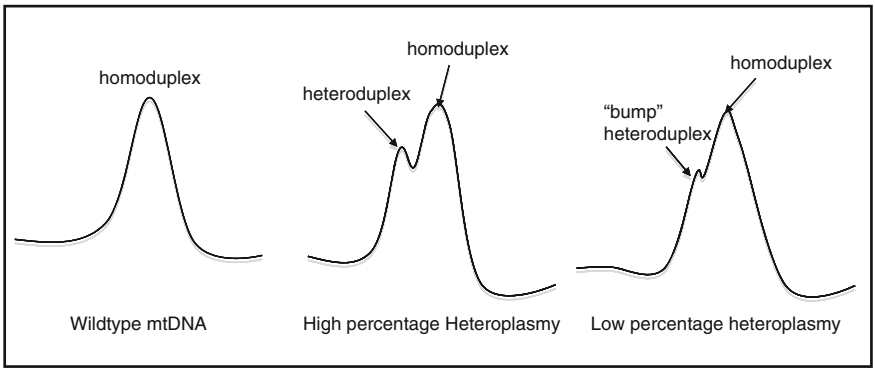

Fig. 13.5 *Detection of mtDNA mutations using DHPLC*. The appearances of high and low frequency of heteroplasmic mutations are shown in these chromatograms

The high sensitivity of DHPLC makes it an attractive method to scan the entire mtDNA for heteroplasmy. The reliability, reproducibility, and sensitivity of DHPLC for mtDNA mutation detection have been demonstrated. Using various mixtures of known percentages of mutant and wildtype mtDNA molecules, DHPCL has been able to resolve heteroplasmies as low as 1% (Meierhofer et al. 2005), making it one of the best methods currently available for mtDNA sequence analysis.

13.3.1.6 Microarray Resequencing of Mitochondrial DNA

The GeneChip® Human mitochondrial resequencing array version 2.0 (MitoChip v2.0) is a sensitive, reliable, and high-throughput oligonucleotide microarray that is commercially available from Affymetrix. Unlike the first generation MitoChip v1.0 (Maitra et al. 2004), which lacked the D-loop, the MitoChip v.2.0 enables bi-directional sequencing of the entire mitochondrial genome (Zhou et al. 2006). This chip is fabricated using standard Affymetrix photolithography and solid phase DNA synthesis and has a feature size of 8 μm, each of which contains 10^6 copies of 25 mer probes selected from the human revised Cambridge Reference Sequence (rCRS). Eight probes (four for each strand) interrogate each base position. The four features that query a particular base on a strand contain identical 25-mer probes that only differ at the central or 13th base, each of which contains one of the four nucleotides, A, T, C, or G. In addition to the 16,568 bases of the human mitochondrial genome, the MitoChip v2.0 contains additional tiling of sequences for 500 of the most common variants in the hypervariable regions I and II of the genome, including single-nucleotide substitutions, insertions, and deletions.

Detailed assay procedures can be found in the Affymetrix GeneChip® Custom-Seq® Resequencing Array Protocol. Good assay performance requires the use of high quality template. Depending on the amount and quality of starting material, the

entire mitochondrial genome can be amplified using one primer pair in a long extension PCR. Because of polymerase read errors when amplifying long templates, it is recommended that a very high fidelity DNA polymerase be used for whole genome amplification. In many applications, three overlapping primers suffice coverage of the entire mitochondrial genome. However, formalin-fixed and paraffin-embedded clinical samples that usually contain degraded nucleic acids require multiple overlapping primers to cover the entire genome. When more than one primer pair is used for amplification, equimolar amounts of amplified templates should be pooled for subsequent procedures to ensure that equal mtDNA targets are available for each probe set. We have been able to obtain equivalent hybridization and good sequence data using REPLI-g (Qiagen) DNA template. The fragmentation step should be carefully monitored to avoid over or under fragmentation, which otherwise affects assay performance. Over-fragmentation will cause excessive background and under-fragmentation could interfere with hybridization efficiency.

The Affymetrix GeneChip® CustomSeq® Resequencing Array Protocol suggests that 100 ng of DNA be used for sequencing; however, dilution experiments performed at the National Institute of Standards and Technology (NIST) indicate that <1 ng, representing <100 cells is sufficient for obtaining equivalent hybridization to the array (Vallone et al. 2007). Once the template is labeled and hybridized, the chips are washed on an Affymetrix fluidics station using the preprogrammed CustomSeq® Resequencing array wash protocols. After washing, the arrays are scanned and the Affymetrix GSEQ software generates a raw pixel data (.DAT) file, which is automatically converted to a .CEL file for batch analysis.

There are open source softwares as well as proprietary softwares for subsequent analysis. However, batch analysis can be performed using free software available at http://www.dpgp.org (RA tools). This software is a modified version of ABACUS developed by Cutler et al. (2001) for making genotype calls using microarray technology. The software uses a statistical algorithm to assign a quality score for each base. Initially, a series of statistical models are developed based on the assumption that various genotypes are present or absent in the sample. For a given genotype, the likelihood of each statistical model is computed independently for each strand and then combined for the overall likelihood of the model. Using these models, a quality score, which is the difference between the log(10) likelihood of the first and second best fitting statistical models for assigning a genotype at any position on the chip, is provided. To make a call, a given base must exceed the total quality score threshold (totThresh), which is empirically determined. Increasing totThresh will result in fewer calls and decreasing it will lead to ambiguous calls. When a base fails to reach this threshold, an "n" is assigned. An important built-in concept of this software is that the quality of base calls improves with multiple uses due to the adaptive nature of the software. Thus, the software uses arrays from multiple runs to factor-in the differences in background that occurs as a result of unequal levels of cross-hybridizations at each site.

Once batch analysis is complete, the Affymetrix GSEQ software generates a report and .CHP files. In addition to other assay information, including quality

metrics, these files contain the nucleotide sequences of the samples analyzed as well as the corresponding rCRS nucleotides, making it easy to read and score sequence variants. Pairwise comparison of patient sequences to the rCRS enables polymorphic loci to be scored and haplotype analysis. To identify somatic mutations, the sequences must be compared to known normal maternal mtDNA sequences. Traditionally, sequences from normal peripheral white blood cells of the same individual or a maternal relative suffice this comparison.

Microarray-based resequencing technology has displayed higher sensitivity and automation capabilities compared with conventional sequencing technologies. In an experiment conducted at the NIST, the MitoChip v2.0 detected heteroplasmy at a level of 1–2%, which is an order of magnitude more sensitive than capillary electrophoretic fluorescent DNA sequencing. MitoChip v2.0 has >99.99% reproducibility and 99.999% accuracy. Through its development as a rapid and reliable approach to sequencing mtDNA, this array is currently an important research tool. Our preliminary results on the use of the MitoChip to measure the mutation load (the aggregate of genomic mutations in a sample) in noninvasively collected body fluids, such as urine, sputum, and nipple aspirate fluids, have demonstrated the feasibility of translating the technology into the clinical laboratory (Jakupciak et al. 2008a, b).

13.3.1.7 DNA Sequencing by Synthesis-(Pyrosequencing)

Pyrosequencing is another reliable and sensitive DNA sequencing method that has been used to resolve mtDNA heteroplasmy in clinical samples. This technology allows simultaneous detection and quantification of incorporated nucleotides, and is therefore, a very useful method for analyzing gene copy number and mtDNA heteroplasmy. Pyrosequencing is simply described as DNA sequencing by synthesis (Ronaghi et al. 1998). There are two main pyrosequencing techniques: liquid and solid phases.

The liquid phase pyrosequencing method uses four enzymes, a DNA polymerase, apyrase, ATP sylfurylase, and luciferase, in a reaction cascade during which DNA synthesis is coupled with light emission that is detected and quantified. The template to be sequenced is first amplified by PCR and then, an enzymatic template preparation process is used to generate primed single-stranded DNA for sequencing. Exonuclease I and nucleotide-degrading enzymes are added to the PCR product and incubated at room temperature. This process removes the nucleotides (nucleotide-degrading enzyme activity) and PCR primers (exonuclease I activity). The sequencing primer is then added to the mixture and the mixture then heated to inactivate the enzymes. Primed templates are formed by rapidly cooling the mixture.

In solid-phase pyrosequencing, biotinylated PCR products are captured onto streptavidin-coated magnetic beads, followed by washing and alkali denaturation to generate single-stranded DNAs, which are then primed and used for sequencing. The process employs the same enzymes as liquid phase sequencing, except the

nucleotide-degrading enzyme, apyrase. Therefore, a washing step is performed between cycles to remove unincorporated nucleotides.

During the sequencing process, DNA polymerase synthesizes a complementary strand by sequentially adding the correct base. The four nucleotides are added in a defined order,for example, A, C, T, and G. If the first base, A, added is not the correct complement of the first targeted base in the sequence, it is quickly removed by the DNA-degrading enzyme, apyrase (in liquid phase), or washed off (in solid phase). The other bases are sequentially added until the correct base is incorporated. This base pairing is associated with the release of pyrophosphate (PPi), which is used by ATP sylfurylase to generate energy in the form of ATP. This energy is harnessed by luciferase to oxidize luciferin, and in the process, produces light, which is captured by a camera and displayed in a Pyrogram as peaks, in conjunction with a record of the incorporated nucleotides. Apyrase then degrades ATP and the other nucleotides and the light switches off. The cycle is repeated several times. The peaks in the Pyrogram are proportional to the number of nucleotides incorporated at a particular position, thereby allowing some aspects of quantification. There are a number of automated instruments and software from Qiagen for a simultaneous analysis of multiple samples.

Pyrosequencing is a rapid and robust method of detecting mtDNA point mutations in clinical specimens. The technology has been successfully applied in forensics and clinical diagnosis of known disease-associated mtDNA mutations. The ability for this technique to detect and quantify heteroplasmies in formalin-fixed and paraffin-embedded clinical specimens is an obvious advantage for this method to be considered in the clinics for mitochondrial molecular genetics in cancer. With a sensitivity and specificity of 100% as well as the capability to detect mtDNA mutations at a 1% heteroplasmic level, this technology holds considerable promise for clinical translation in analyzing known mtDNA disease mutations.

One major limitation of pyrosequencing is the short read lengths, due to misincorporation errors and inhibitory substances in the reaction. For whole genome sequencing, a long read length is advantageous because it will permit fewer reactions to cover the entire 16,568 bases of the mitochondrial genome. Thus, complete mitochondrial genome sequencing might require multiple runs. This technology, however, appears useful for haplotype and SNP analysis, and because it is fast, cost-effective, and sensitive, it can prove very useful in clinical diagnostic applications.

13.4 Analysis of Mitochondrial Genome Deletions and Content Changes

Large-scale mtDNA deletions are associated with several cancers and might be one of the mediators of androgen independence in prostate cancer. Furthermore, circulating mitochondrial nucleic acids could potentially hold prognostic value in advanced stage cancers. Therefore, quantitative measurement of mtDNA deletions

and circulating nucleic acids in clinical specimens can be useful in the diagnosis and prognostic evaluation of cancer patients. Southern blotting and quantitative PCR allow the detection and quantification of mtDNA deletions.

13.4.1 Southern Blotting

Traditionally, Southern blotting is used to detect mtDNA rearrangements. In this assay, total DNA is isolated and linearized using preferably enzymes that cut mtDNA once. The linearized genomic DNA is separated on an agarose gel and then transferred onto a membrane, usually nitrocellulose or nylon membrane. The membrane is hybridized with a labeled mtDNA probe and the mtDNA bands detected using autoradiography. The sizes of bands reveal whether they are partial duplications (larger bands than the 16.6 kb wildtype genome) or simple deleted molecules (smaller than the wildtype mtDNA band). Commonly used restriction enzymes are *Bam*H1, *Pvu*II, *SnaB*I, or *Eag*I that cut mtDNA once, or *Hind*III, which cuts the genome thrice but is useful when smaller deletions are to be visualized.

13.4.2 Quantitative Polymerase Chase Reaction

There are multiple ways to screen for mtDNA deletions. Conventionally, Southern blotting is the method of choice for the detection and confirmation of mtDNA deletions. However, Southern blotting is insensitive, requiring large amounts of template, which will not be easily attainable when clinical samples such as saliva specimens are used for diagnostic testing. Moreover, low heteroplasmic levels of mtDNA deletions that are clinically useful for early detection and monitoring of cancer, will not be resolved by Southern blotting. Quantitative polymerase chain reaction (qPCR) is a sensitive, reliable, and high-throughput method for easy quantification of mtDNA deletions, copy number, or content in clinical specimens.

An important aspect of qPCR analysis of mtDNA deletions is primer and probe design as well as the choice of appropriate control targets to be used for normalization and accurate quantification. Primer design depends on whether the deletion is known or unknown. For known deletions, three types of primer sets are required for the quantification of mtDNA deletions and content alterations:

- Mitochondrial DNA deletion-specific primers that will only measure the amount of deleted molecules. This target amplification is accomplished by designing one primer or probe to span across the deletion junction (bridging primer). This primer is then paired with a downstream or upstream primer to target a small amplicon. Alternatively, flanking primers on either side of the deletion junction that target a very small product can be used, but the qPCR extension time must be short to preclude coamplification of wildtype molecules.

- Primers targeting total mtDNA (both wildtype and deleted mtDNA) molecules. These primers should be located outside the deleted region and preferably in a region of the mitochondrial genome known to sustain negligible deletions in the tumor. The D-loop can be targeted; however, because this region is highly polymorphic, primer locations should be in the least polymorphic sites.
- Nuclear primers to control for input template and mtDNA content analysis. In choosing nuclear targets, avoid genes with several homologues (e.g., β-actin) or multiple copies (18SrRNA). Single copy genes that are not amplified in the specific cancer must be used. The use of nuclear targets for normalization has been questioned because of the differences in extraction efficiencies between nuclear and mitochondrial DNA (Myers et al. 2009). It is suggested that exogenous ϕX174 DNA added to samples, extract with similar efficiencies as mtDNA, and hence, is a better target for normalization.

To screen for unknown deletions, there are a number of primer pairs that can be used. Primers can be designed to specifically screen for deletions in the major or minor deletion arcs. Alternatively, whole mitochondrial genome amplification with a pair of primers will enable the detection of deleted molecules (bands smaller than 16.6 kb; Fig. 13.6). The deletion location will depend on where the primer pair is located. Primers in the minor deletion arc will primarily amplify deletions in the major deletion arc and vice versa.

The basic principle of real-time PCR is the monitoring of product accumulation during each PCR cycle through the emission of fluorescence. The detection of PCR products progresses from initial template detection through an exponential phase and finally, a nonexponential or plateau phase. Because the choice of chemistry is quite important in qPCR assay design with regard to cost, sensitivity, specificity, and ease of assay performance, the basic chemistries are examined. Several chemistries for the detection of amplicon accumulation are suitable for mtDNA quantification. The four chemistries that are currently available are TaqMan hydrolysis probes, hybridization probes such as molecular beacons and scorpion primer-probe combo, and DNA intercalating dyes such as SYBR green assay. TaqMan probes, Molecular Beacons, and Scorpions assays depend on Förster resonance energy transfer (FRET) to generate the fluorescent signal through the coupling of a fluorogenic dye molecule and a quencher moeity to the same or different oligonucleotide substrates. However, SYBR Green is a fluorogenic dye that exhibits very little fluorescence in solution, but emits a strong fluorescence when bound to double-stranded DNA, which occurs during PCR process.

TaqMan Assay: TaqMan probes have been widely used for mtDNA quantification. These probes are short single-stranded oligonucleotides that are complementary to a sequence in the target amplicon (Fig. 13.7). They contain a fluorescence reporter dye at the 5′ end and a quencher dye at the 3′ end. The close proximity of these two dyes prevents the emission of fluorescence. With the accumulation of amplicons during the PCR cycling process, TaqMan probes anneal to denatured target amplicons, and as the polymerase replicates a template with bound TaqMan probe, the reporter dye is cleaved using its 5′ exonuclease activity, and this releases

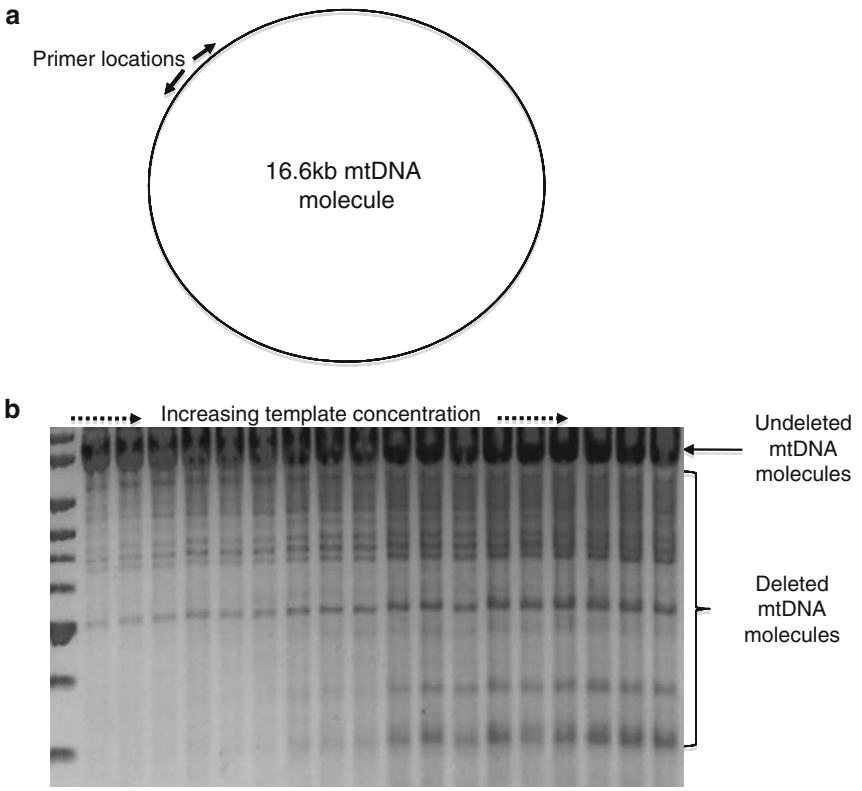

Fig. 13.6 *PCR amplification of whole mitochondrial genome to detect deleted molecules.* (**a**) Wildtype mtDNA molecule with indicated primer locations used for PCR. (**b**) Gel image of PCR products. The wildtype (16.6 kb) mtDNA molecules are indicated – the band intensity shows the abundance of these molecules in this sample. The submolar bands reveal deleted molecules detected by this assay. Their intensities indicate they are at low heteroplasmic levels

it from the quencher, thereby resulting in irreversible fluorescent signal emission (Fig. 13.7). Because the cleaved reporter dye is irreversible, this introduces a background noise in the assay. However, well-designed TaqMan probes require minimal optimization and are useful for PCR analysis. TaqMan probes can also be used for multiplex assays by designing each probe with unique fluorescence and quencher pair. A limitation of this assay is the cost to synthesize TaqMan probes because they can be expensive.

Molecular Beacons: Molecular beacon probes are designed such that about six bases at the 5′ and 3′ ends of the single-stranded sequence that contain reporter dye and quencher, are complementary to one another and the middle portion is complementary to the target amplicon. Thus, in an unbound state, the complementary ends anneal to form a double-stranded stem, while the middle portion remains as a single-stranded loop (Fig. 13.8). In this hairpin configuration, the reporter and quencher are in close proximity precluding fluorescent emission (FRET occurs).

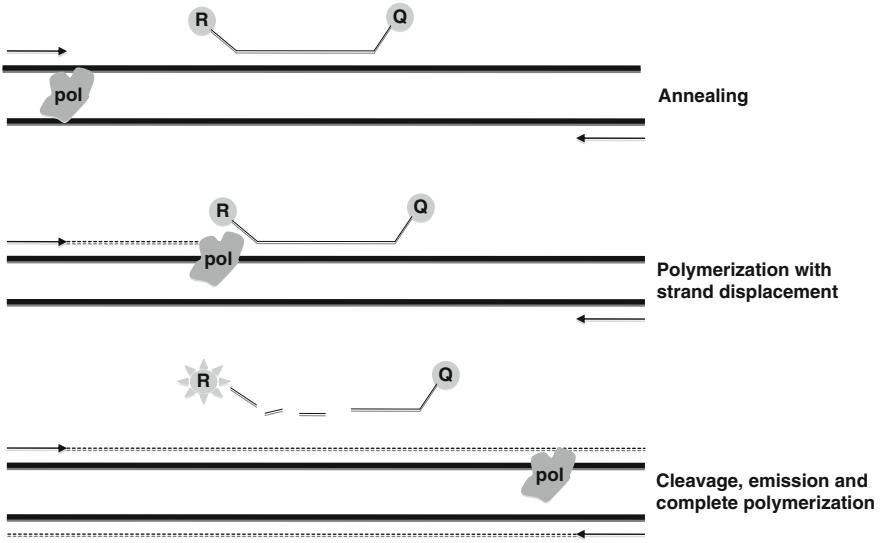

Fig. 13.7 *The basic principle of qPCR analysis of mtDNA using TaqMan probes*. These probes are designed such that reporter ® and quencher (Q) are close enough for FRET to occur. Amplicon extension, strand displacement, and probe cleavage release the reporter to emit fluorescence

However, the probes are designed to bind to the amplicon at a specified temperature. This interaction, which is thermodynamically more stable than the hairpin structure, leads to destabilization of the stem and thus, the emission of fluorescence (Fig. 13.8). Unlike TaqMan probes, molecular beacons produce less background due to the reversibility of the fluorescence at each cycle. This low background makes molecular beacons probably more suitable for multiplex PCR assay designs. These probes are also expensive to synthesize.

Scorpions: With regard to TaqMan and Molecular beacon probes, targets with repetitive elements, as is inherent in the mitochondrial genome, may form secondary structures preventing probe interactions. To overcome such problems, scorpions are designed to combine probe and primer in the same oligonucleotide. Scorpion primers are attached to scorpion probes at their 5′ ends. The probes are similar to beacon probes with a complementary stem that forms a hairpin structure bringing the 5′ end reporter and 3′ end quencher together, as well as an intervening loop sequence that is complementary to sequences in the newly synthesized amplicon (Fig. 13.9). Following the first PCR reaction, the newly synthesized amplicon, which is attached to the probe via the primer as a single molecule, will contain complementary sequences to the loop sequence of the probe. An intra-molecular hybridization between the probe and target amplicon occurs during the second PCR cycle of denaturation and annealing (Fig. 13.9). This internal hybridization occurs because denaturation of the hairpin loop requires less energy than required by the new DNA duplex formed. This opens up the hairpin loop and prevents FRET from occurring.

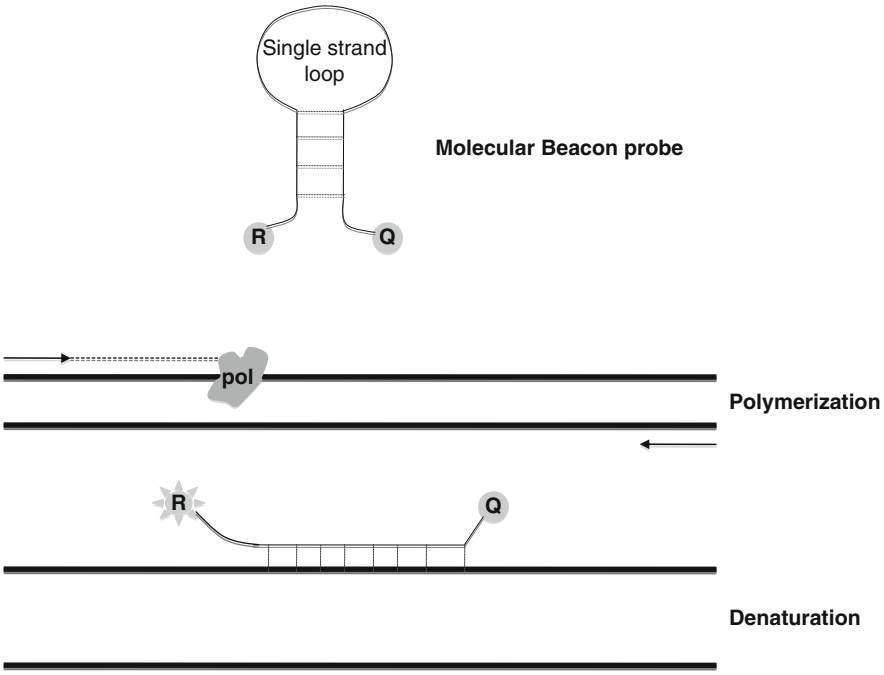

Fig. 13.8 *The basic principle of qPCR analysis of mtDNA using molecular beacon probes*. In solution, complementary base pairing at the 5′ and 3′ ends of the probe leads to the formation of a hairpin structure. The close proximity of reporter to quencher leads to FRET. The single strand loop sequences are complementary to one strand of the PCR product. As amplicons accumulate, denaturation permits the hybridization of the single strand loop to its complementary segment in the PCR product. In this extended state, the reporter is dissociated from the quencher leading to fluorescent emission

SYBR Green: Double-stranded DNA intercalating dyes such as SYBR green are less expensive, easy to use, and sensitive. These dyes emit fluorescence when bound to double-stranded DNA. As PCR products accumulate, more dye intercalates and more fluorescence emission occurs when excited. SYBR green assays are less specific than the probe-based assays, because the dyes will interact with any double-stranded DNA, including primer-dimers and nonspecific PCR products. Melting curve analysis, however, allows this to be easily realized. When fully optimized, the inexpensive dye-binding assays perform equally well as hydrolysis and hybridization probe detection methods.

Several PCR quantifications of mtDNA levels have relied on TaqMan and SYBR green assays. Many of these assays employ multiple tubes for different targets. However, judicious use of precious clinical material and accurate normalization is achievable with multiplex PCR.

Quantification: Real-time PCR assays often include a passive dye used as an internal reference to normalize or correct for background fluorescence. Rn

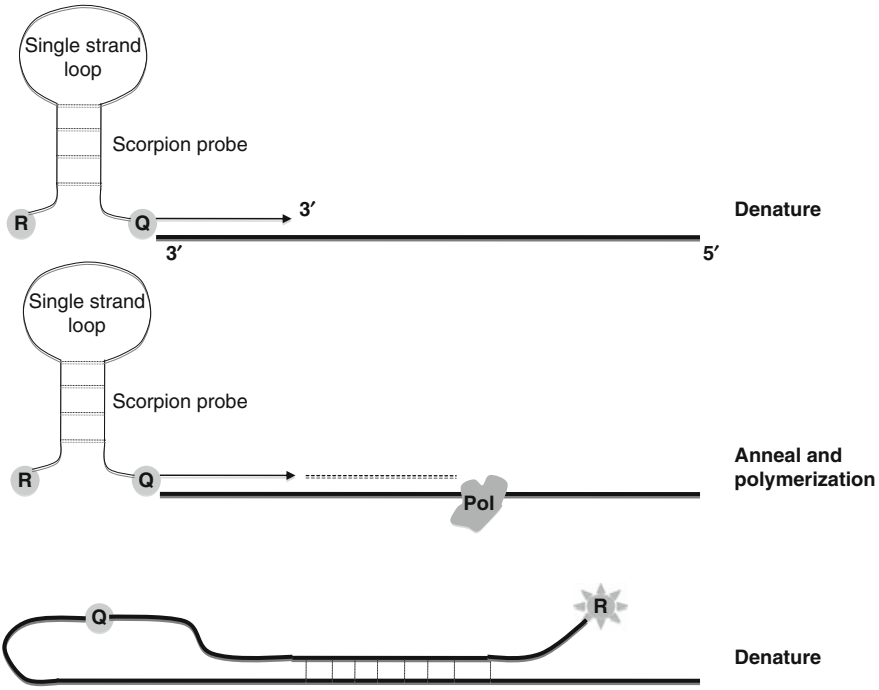

Fig. 13.9 *The basic principle of qPCR analysis of mtDNA using scorpion probes*. Scorpion probes are identical to molecular beacons except that they are attached to one PCR primer at their 3′ ends (i.e., 5′ end of the primer). Target amplification results in production of molecules that contain scorpion probes. During denaturation, the loop sequences in the probe hybridize to their complementary sequences in the PCR amplicon, thus separating reporter from quencher and hence fluorescent emission

designates the ratio of emission intensities of reporter dye to passive reference dye. The normalized emission intensity of a reaction containing all reagents and target template is referred to as Rn+ and that of a no template control as Rn−. The difference between Rn+ and Rn− is ΔRn. The PCR cycle at which the device software first detects significant reporter fluorescence (Rn+) above the threshold, is referred to as the cycle threshold (Ct), and this is the critical parameter used for quantification.

Computation of the levels of a target molecule is accomplished using either a standard curve of Ct values obtained from serial dilutions of standard target molecules (absolute quantification) or by comparison of the differences in the Ct between two samples or targets (relative quantification). Relative quantification usually makes several assumptions, which when unfulfilled, lead to erroneous results. Moreover, results from relative quantification tend to differ depending on the method and reference target gene chosen. In clinical diagnostics of mtDNA alterations, it is recommended that the "absolute" quantification method is used,

and this will be described. "Absolute" quantification aims at accurately measuring the number of target molecules in a sample. Whereas this is a difficult claim to confirm in many assays, it offers a means of closely measuring target molecules. An important requirement for absolute quantification is that an accurate standard template is prepared. This is achieved by cloning the PCR product of each target sequence into a plasmid vector, followed by sequencing to confirm identity of the cloned fragment. A single clone is then selected, DNA extracted, and quantified by absorbance. The copy number of the target is computed on the basis of the molecular weight of the cloned plasmid DNA sample.

$$\textit{Target copy number} = (\textit{cloned plasmid DNA concentration})/(\textit{weight of cloned plasmid molecule})$$

$$\textit{The weight of the cloned plasmid} = (S_{cp} \times 6.6 \times 10^5 g/\textit{mole})/(6.02 \times 10^{23}\ \textit{molecules/mole})$$

$$S_{cp} = \textit{size of cloned plasmid } (\textit{plasmid} + \textit{cloned fragment})\ \textit{in base pairs}$$

$$6.6 \times 10^5 g/\textit{mole is the molecular weight of double stranded DNA}$$

$$6.02 \times 10^{23}\ \textit{molecules/mole is the Avogadro's number}$$

For each PCR reaction, serial dilutions of the stock solution are included and the Cts from these are plotted on a standard curve, which shows the target copy numbers and corresponding Cts. This curve is used to compute copy numbers or levels of target molecules in experimental samples using their various Cts. Assay reliability is influenced by the PCR efficiency. It is possible to certify an optical density (OD) value of a quantity of DNA to serve as a standard that can measure the PCR efficiency.

The heteroplasmic levels of deleted mtDNA molecules are determined by computing the ratio of deleted to total mtDNA copies. It is very important to note that this computation is only possible when the primers used to quantify the total mtDNA molecules are not within a region of the mtDNA that is deleted in the specific cancer. The appropriate region can be determined empirically by the analysis of a subset of tumor and benign samples with these primers, and ensuring that there is no statistically significant resolution in the total mtDNA copies between the two groups. It is equally important to use a diluted template for this experiment because a concentrated template will shadow differences, if any between samples. It is also recommended that two separate primer pairs be used for this quantification. Similarly, mtDNA content (normal, depletion, or over-replication) is computed from the ratio of the copies of the total mtDNA to nuclear target. Again, it is imperative that this nuclear target is single copy and not amplified in the specific cancer.

13.5 Quality Assurance Issues in Mitochondrial DNA Analysis

In spite of the importance of mtDNA mutations in cancer, the mutation literature is criticized for a possible presence of erroneous data (Salas et al. 2005; Bandelt and Salas 2009). Accurate molecular diagnostics depends on several factors, including instrument performance, assay optimization, training, and skills of technicians as well as good sample handling to avoid contamination or mix-up. Sample contamination is a major quality assurance issue in clinical molecular diagnostics. This issue is even of a major concern with regard to mtDNA analysis, given its high copy number per cell. Because many of the methods described above involve genomic amplification, good laboratory practices are required to prevent sample contamination, especially when the same mtDNA fragment is amplified repeatedly over a long period of time. Sample contamination can occur at several levels, beginning with specimen procurement through all the analytical steps. Aerosol means of template spread in the laboratory is one major cryptic mode of contamination. Another important concern when analyzing mtDNA is taking the necessary precautions to avoid amplifying nuclear-embedded mtDNA copies, some of which are 100% homologous to the authentic cytoplasmic DNA. The following minimum recommendations should be observed in clinical molecular diagnostics of mtDNA mutations:

- The instruments used in molecular diagnostics should be maintained and calibrated periodically by trained personnel following the manufacturer's recommendations and an established instrument maintenance standard operating procedure (SOP). This will require periodic instrument maintenance by an expert from the manufacturer.
- There is the need to have at least three distinct designated laboratory areas for (1) reagent storage and preparation, (2) DNA extraction and PCR set up, and (3) PCR amplification and downstream analysis. Each of these areas should have its own dedicated equipment. The PCR amplification and analysis room is a highly contaminated area, and to minimize aerosol spread of template from this to other rooms, (1) the pressure in this room must be sub-atmospheric, (2) upstream flow of people, equipment, and other materials, including gowns should be avoided, and (3) surfaces in this room should be decontaminated frequently after work with sodium hypochlorite or UV radiation.
- The various assays for each molecular target must be optimized and an SOP developed and followed at all times. For instance, the diagnostics DHPLC quality assurance collaborative group has developed two separate SOPs for instrument operation/maintenance and the detection of a specific mutation using DHPLC (Schollen et al. 2005). Similarly, each assay should include a standard reference material with known performance to be used to minimize assay-to-assay variability and to measure assay sensitivity.
- Dedicated gowns and gloves in each room should be worn at all times when handling samples, reagents, equipment, and instruments. These should be disposed of, or left in each room before departing.

- Clean reagents, equipment, and instruments must be used for sample processing and DNA extraction.
- To prevent co-amplification of pseudogenes, a BLAST search of mtDNA primers must be performed initially to ensure lack of homologous nuclear sequences, and these primers should then be tested on DNA template from cells lacking in mtDNA to confirm the lack of pseudogene co-amplification.
- It is also recommended that a high-fidelity DNA polymerase be used to prepare template for sequencing, so as to prevent mutation artifacts.
- All analytical steps must include appropriate controls.
- An important issue with mtDNA analysis is the occasional sample mix-up or cross-contamination. In such situations, Short Tandem Repeat (STR) and Phylogenetic analyzes should be performed.
- If there is any doubt about the authenticity of the sequence, it should be compared to the sequences of laboratory personnel, because they are potential sources of contamination.

13.6 Conclusion

Mitochondrial DNA mutations are useful for early detection and monitoring, risk assessment, and prognostic stratification of patients for therapy. The methods discussed here can be used to detect and monitor mtDNA changes in cancer. Mitochondrial DNA studies should focus on the utility of minimally invasive or noninvasive samples. It should be noted that despite the extensive body of literature on the biology of mitochondria and mtDNA mutations in a wide variety of human diseases, an exact role for mtDNA mutations in the etiology of cancer is currently being explored. Mutations that alter mitochondrial gene structure and function, replication, and/or transcription should be considered as significant when evaluating their physiological impact, while silent mutations that are hitchhikers or passengers might be important for assessing the mutation load. Automation is a standard requirement and practice of many clinical diagnostic laboratories. Hence, simple methods amenable to automation are more likely to be translated. However, sensitive quantitative methods cannot be ignored because heteroplasmic mutations can signal early disease.

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Chapter 14
"Mitocans": Agents Targeting Mitochondria to Kill Cancer Cells

Abstract Mitochondrial alterations perpetuate the malignant phenotype, such that any perturbations or reversal of such alterations to normalcy result in cancer cell demise. Cancer cells have altered apoptotic pathway expressions, metabolism of basic food substrates, redox imbalances, membrane potential changes, and diverse receptor expressions. While considerable efforts are geared towards developing agents that target apoptosis, the potential of abrogating glycolysis, and exploitation of redox status and membrane potential changes to kill the cancer cell are equally valuable. Differential receptor expression also provides targets used to specifically deploy mitochondrotoxic substances to the cancer cell. The mode of action and clinical applications and stage of development of several agents are provided in this chapter

14.1 Introduction

The seemingly hypothetical but indeed practical reality of targeting mitochondria to specifically kill cancer cells is based on the simple premise of the numerous differences between mitochondria of cancer and normal cells. "Mitocans" is a term coined by Stephen J. Ralph and coworkers to describe the anticancer drugs that are specifically geared toward mitochondria of cancer cells (Ralph et al. 2006). The mitochondria of cancer cells have altered structures, genomes, proteomes, bioenergetics, metabolism, and membrane potentials, all of which are exploitable targets for chemotherapy and even chemoprevention. However, currently, considerable efforts are focused on agents that interfere with apoptosis, especially the BCL-2 family members. The glycolytic switch of cancer cells noted decades ago by Otto Warburg is a feature of a majority of cancer cells, and agents targeting these changes are in development, and so are "mitocans" targeting redox and membrane potential changes, and other alterations of cancer cell mitochondria. Novel approaches to kill cancer cells rely on the overexpression of surface receptors by cancer cells. These receptors are exploited to target delivery of molecules that are

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toxic to mitochondria. This chapter examines a number of agents developed or under development for specific targeting of cancer cell mitochondria.

14.2 Chemotherapy Targets

There are numerous approaches to selectively kill cancer cells targeting molecular alterations in their mitochondria. However, considerable efforts are placed on the apoptotic and metabolic changes of cancer cells. The mitochondrial permeability transition pore complex (PTPC), BCL-2 proteins, and substances released to mediate the apoptotic pathway provide clues for drug targeting. Similarly, glycolysis, lipogenesis, and glutaminolytic addiction of the cancer cell offer opportunity to target and kill cancer cells. Redox imbalances, changes in bioenergetics, thiol oxidation, and membrane potential differences are also explored. While there are marked differences between the mitochondrial genomes of cancer and normal cells, this has yet to be specifically targeted for therapy. However, it is still a possibility to target and deplete mtDNA of cancer cells to enhance chemotherapy. Finally, mitochondriotoxic compounds can be delivered to cancer cells using the altered expression of tumor-specific surface receptors.

14.2.1 The Intrinsic Apoptotic Pathway

This section provides a brief overview of mitochondrial apoptotic pathway; for detailed discussion refer to Chap. 3. Several chemotherapeutic agents target the apoptotic pathway to kill cancer cells. This strategy is possible because of the deregulated expression and activity of mediators of apoptosis in cancer cells. Whereas conventional chemotherapeutic agents such as cisplatin, paclitaxel, and doxorubicin induce mitochondrial membrane permeabilization (MMP) by activating proapoptotic factors such as Fas/FasL and p53 pathways, several “mitocans” directly attach to mitochondrial components to trigger apoptosis. The intrinsic apoptotic pathway requires the release of active agents from the inter membrane space (IMS) into the cytosol to begin the cascade of events leading to cell death. The release of mitochondrial factors requires MMP, a process that occurs in two possible ways, though both are not mutually exclusive.

Cellular stress such as radiation, chemotherapy, and heat shock, as well as calcium overload can trigger a process at the inner mitochondrial membrane (IMM) known as mitochondrial permeability transition (mtPT). mtPT causes loss of membrane potential, influx of solutes and ion into the matrix, and physical rupture of the membrane causing MMP. Permeability transition occurs at a specialized pore referred to as permeability transition pore complex (PTPC). The PTPC is a cyclosporine A (CsA) sensitive channel formed at the contact sites of the

IMM and the outer mitochondrial membrane. This pore is primarily formed by voltage-dependent anion channel (VDAC) on the outer membrane and adenine nucleotide translocase (ANT) on the inner membrane. Interacting with VDAC at the surface of the mitochondria are translocator protein 18 KDa (TSPO, also known as peripheral-type benzodiazepan receptor-PBR) and hexokinase (HK). Similarly IMS creatine kinase and matrix cyclophylin D interact with ANT to cause mtPT.

Cancer cells have deregulated expression of PTPC proteins, and these can be exploited for therapeutic purposes. Also, the inherent redox imbalance of cancer cells indicates a possible ease of targeting vicinal thiol-containing proteins such as ANT, which together with VDAC have served as attractive targets of many anticancer drugs. HK, cyclophylin D, and TSPO are also possible targets, but these agents tend to have other unwanted effects because they have other functions besides being part of the PTPC.

MMP occurs as the balance in the levels and activities of the pro-apoptotic BCL-2 family members tip against the antiapoptotic family members. The BCL-2 proteins are characterized by the presence of one or more of the four BCL-2 homology (BH) domains. Conventionally, the prosurvival members, BCL-2, BCL-xL, MCL-1, contain all the four domains; however, the proapoptotic members fall into two categories on the basis of their BH domains. The multidomain members, BAX, BAK, and BOK, possess BH1-3 domains, and the BH3 only members, possess only BH3 domain. BAX and BAK mediate MMP, and both the anti-apoptotic BCL-2 and the BH3 only members modulate their activity. Activating BH3 only members, including BIM and BID, can heterodimerize with BAX/BAK to mediate MMP. On the other hand, derepressor BH3 only members including BAD/BIK/PUMA bind and sequester antiapoptotic BCL-2 family members, thereby freeing BAX/BAD to interact with the activators to induce apoptosis.

Cancer cells have increased expression of antiapoptotic family members, and this feature contributes to their survival as well as chemoresistance. Therefore, chemotherapeutic strategies have included downregulation of the expression or inhibition of the activities of the BCL-2 prosurvival proteins.

14.2.1.1 Agents Targeting Mitochondrial PTPC

The agents targeting the mitochondrial PTPC to induce apoptosis are summarized in Table 14.1. This list is just a snapshot of currently available agents at various levels of development. Novel compounds are not included.

CD437 (*6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carbo-xylic acid*): CD437, a naphthalene carboxylic acid is a synthetic retinoic acid receptor gamma (RARγ) agonist. It induces cell death in all-trans retinoic acid resistant leukemic cells (Hsu et al. 1997), and was demonstrated to cause apoptosis of cells independent of its RAR interaction (Costantini et al. 2000). It has a direct effect on ANT to induce mtPT and release of cytochrome *c*, a feature that can be blocked by CsA (Marchetti et al. 1999; Belzacq et al. 2001). The effects of three agents that target PTPC including CD437 in temozolomide-resistant glioblastoma cell line (ADF)

Table 14.1 Chemotherapeutic agents targeting permeability transition pore proteins

Agent	Specific actions	Phase of development
CD437	Inhibits ANT	Preclinical
Betulinic acid	Interacts with ANT Increase ROS	Clinical trials
Lonidamine	Inhibits ANT	Launched for treating lung, breast, cervical, and prostate cancer; In clinical trials for other cancers
Arsenic trioxide (TRISENOX)	Inhibits ANT and VDAC via vicinal thiol oxidation	Launched for treating promyelocytic leukemia; Clinical trials for multiple myloma and leukemia
PK11195	TSPO antagonist, increases caspase activity	Preclinical
FGIN-1-27	TSPO antagonist	Preclinical
RO5-4864	TSPO ligand	Preclinical
BBL22	TSPO ligand	Preclinical
GSAO	Inhibits ANT	Preclinical
3BrPA	Redox stress Membrane depolarization	Preclinical
Mastoparan	Penetrates inner membrane Redox stress	Preclinical

were investigated by Lena et al. (2009). All the drugs produced cytostatic and cytotoxic effects on ADF cell lines in a concentration-dependent manner. Lonidamine and betulinic acids induced apoptosis as well in these cell lines. In ovarian adenocarcinoma SKOV3 cells, CD437 induced apoptosis by modulating intracellular calcium levels to upregulate thioredoxin-binding protein 2 (TBP2), which antagonized thioredoxin thereby enabling activation of ASK1 and downstream cJun N-terminal kinase (Matsuoka et al. 2008). In another study by the same group, CD437 cytotoxicity on SKOV3 cells was partly via induction of endoplasmic reticulum stress signaling (Watanabe et al. 2008). Other pathways including the AP-1 signaling pathways are implicated in CD437 induction of apoptosis. In human melanoma cell line A375, CD437 caused marked apoptosis that involved retinoic acid inducible gene 1-mediated NF-κB activation (Pan et al. 2009). These agents are in preclinical development.

Betulinic acid: Betulinic acid is a naturally occurring pentacyclic triterpenoid. It has a selective cytotoxic effect on neuroectodermal and epithelial cancers, and has been shown to inhibit the growth of melanoma and ovarian cancer xenographs in mice (Eiznhamer and Xu 2004). Betulinic acid directly induces mtPT, probably via interaction with ANT. It also causes loss of mitochondrial membrane potential that is not inhibited by caspase inhibitors, suggestive of its actions on PTPC (Costantini et al. 2000). Betulinic acid induces ROS production that is linked to proapoptotic p38MAPK signaling. Reports have indicated that betulinic acid may modulate expression of BCL-2 family members in a context-dependent fashion. However, a recent study indicates that betulinic acid purely induces cytochrome *c* release via mtPT and not modulation of BCL-2 proteins (Mullauer et al. 2009). In this study, BAX/BAK deficient cells were as sensitive to betulinic acid as their

wildtype controls, indicating that cytochrome *c* release was independent of BCL-2 proteins. As a chemotherapeutic agent, betulinic acid is presently in clinical trials for the treatment of dysplastic nevi with malignant potential.

Lonidamine: Lonidamine is a dichlorinated derivative of indazole-3-carboxylic acid. It inhibits ANT and oxygen consumption and therefore reduces ATP production (Stryker and Gerweck 1988; Ravagnan et al. 1999; Belzacq et al. 2001). Lonidamine causes loss of membrane potential and release of cytochrome *c* from isolated mitochondria. This effect is blocked by CsA, which suggests that it targets the PTPC. Lonidamine also inhibits HK and therefore interferes with glucose metabolism and increased sensitivity of cancer cells to apoptosis. The agent is well tolerated with little toxicity, has been launched for the treatment of lung, breast, cervical, and prostate cancers, and is in clinical trials for the treatment of other cancers. For example, it is in phase II trials in combination with diazepam for the treatment of glioblastoma multiforme (Oudard et al. 2003).

Arsenic trioxide (TRISENOX): Arsenic is a toxic metalloid. Oxidation of arsenic produces arsenic trioxide (As_2O_3). TRISENOX directly targets PTPC to cause mtPT and apoptosis (Belzacq et al. 2001). The primary targets of PTPC are oxidation of vicinal thiol groups in VDAC and ANT. In addition arsenic trioxide interferes with the respiratory chain leading to increased ROS production and cytochrome *c* release in cancer cells. Arsenite is in clinical trials for treatment of resistant multiple myeloma, and is in use for the treatment of promyelocytic leukemia. TRISENOX is used in conjunction with interferon-α and zidovudine in a phase II trial for the treatment of newly diagnosed chronic adult T-cell leukemia/lymphoma patients. It demonstrated an impressive quick response rate with minimal hematologic toxicities (Kchour et al. 2009).

Translocator protein 18 kDa antagonists and ligands: PK11195, FGIN-1-27, RO5-4864, and BBL22 are antagonist and ligands of TSPO (PBR), in development for the teatment of various cancers.

PK11195 is an isoquinoline carboxamide that antagonizes TSPO. This agent was initially shown to preferentially bind to TSPO of gliomas with no binding to TSPO of normal adjacent cells to cancer cells (Starosta-Rubinstein et al. 1987). PK11195 enhanced apoptosis induced by radiotherapy and chemotherapy in BCL-2/BCL-xL overexpressing human cholangiocarcinoma cells (Okaro et al. 2002). The agent blocked P-glycoprotein-mediated drug efflux in a TSPO-independent, ATP-binding cassette (ABC) transporter-dependent mechanism (Walter et al. 2005). Thus, in combination therapy, PK11195 can induce apoptosis via TSPO-mediated mechanism while helping retain, and therefore, increase the efficacy of the other chemotherapeutic agents in cancer cells.

FGIN-1-27 is an indoleacetamide derivative and a TSPO antagonist. The three TSPO ligands, PK11195, RO5-4864, and FGIN-1-27 were demonstrated to inhibit colorectal and esophageal cancer cell proliferation in a dose-dependent fashion (Maaser et al. 2002; Sutter et al. 2002). In addition to increased caspase three activities, FGIN-1-27 decreased membrane potential to induce apoptosis. The three ligands also decreased cell cycle arrest at G_1/G_0 phase. It appears that the induction of apoptosis and cell cycle arrest was by means of activation of p38MAPK pathway

(Sutter et al. 2003). Inhibition of prosurvival MAPK/ERK kinase enhanced the activities of TSPO ligands (Sutter et al. 2004).

RO5-4864 is a synthetic TSPO ligand. Wang et al (1984) observed that the binding of RO5-4864 to peripheral-type benzodiazipine receptors (TSPO) of mouse thymomas inhibited proliferation. The degree of receptor binding correlated with the level to which proliferation was inhibited. Pawlikowski et al. (1988) extended these findings in human gliomas. All benzodiazepines tested including RO5-4864 inhibited glioma cell proliferation and this effect was much pronounced for peripheral-type ligands. Compared to cells grown in serum free media that had normal looking mitochondria, gliomas treated with PBR ligands including RO5-4864 had increased mitochondria that were distributed in a perinuclear fashion. These mitochondria were vacuolated and swollen (Shiraishi et al. 1991). The expression of TSPO was demonstrated in breast cancer cell lines MCF-7 (Carmel et al. 1999). Treatment of these cell lines with PBR ligands (RO5-4864 and PK11195) inhibited proliferation of the cells, while central-type benzodiazepine receptor ligand, clonazopan did not have such an effect. MCF-1 cells were arrested at G_1/G_0 and G_2/M phases of the cell cycle in PBR ligand-treated cells (Carmel et al. 1999). Decaudin et al. (2002) investigated the role of PBR ligands in Fas/APO-1/CD95-induced apoptosis in cell lines. RO5-4864, PK11195, and diazepam were shown to induce in vitro apoptosis of T-cell Jurkat cells. However, only RO5-4864 was able to induce apoptosis in the other cell lines including neuroblastoma SHEP, osteoblastoma 143N2, and glioblastoma SNB79 cells. In cells of mesenchymal origin, PBR ligands appear to operate in a PBR-independent fashion. Both fibroblasts and fibrosarcoma cells express high levels of PBRs. Treatment of these cells with PK11195 and RO5-4864 inhibited the proliferation and cell cycle arrest at G_1/G_0 without inducing apoptosis. Peripheral-type benzodiazepine knockdown by RNA interference failed to interfere with the inhibitory effects of both drugs suggesting a possible novel mechanism. In fibroblasts, inhibitory effects were correlated with decreased activation of ERK and c-Jun (Kletsas et al. 2004).

2-amino 9-chloro-7-(2-fluorophenyl)-5H-pyrimidol[5,4-d][2]benzazepine (BBL22) is a benzazepine that selectively inhibits the growth of several epithelial and hematologic cancers. Growth arrest at G_2/M phase of the cell cycle was associated with apoptosis, especially in prostate and breast cancer cell lines. BBL22 significantly reduced growth of PC3 prostate cancer xenographs without much toxicity (Xia et al. 2000).

4-(N-(S-glutathionylacetyl)amino) phenylarsonous acid (GSAO): GSAO is a derivative of the thiol crosslinking agent, phenyl arsenoxide (PAO). This small synthetic molecule is poisonous to mitochondria and targets angiogenic endothelial cells (Don et al. 2003). Specifically, the trivalent arsenical of GSAO reacts with and perturbs the functions of ANT of the IMM of endothelial cells, leading to growth arrest. GSAO inhibits tumor angiogenesis in vivo. At high doses, GSAO is toxic to the kidney. Mechanistically, it appears that the gamma-glutamyl residue of GSAO is cleaved at the endothelial cell surface by gamma-glutamyl transpeptidase (gamma-GT), because GSAO is an efficient substrate for gammaGT. Cleavage leads to

endothelial cell accumulation and subsequent antiproliferative activity of GSAO. The resulting metabolite of the cleavage of GSAO, 4-(N-(S-cysteinylglycylacetyl) amino) phenylarsonous acid (GCAO) is probably processed by dipeptidases in the cytosol to 4-(N-(S-cysteinylacetyl)amino) phenylarsonous acid (CAO), which is the active metabolite that reacts with mitochondrial ANT (Dilda et al. 2008). GSAO is currently in preclinical development.

3-Bromopyruvate (*3BrPA*): 3BrPA inhibits cancer cell energy production via glycolysis and thus depletes ATP levels in cancer cells with respiratory defects. In addition to inhibition of HK, 3-BrPA induces mitochondrial depolarization. The energy depletion in cancer cell was followed by dephosphorylation of BAD at Ser^{112}, translocation of BAX to mitochondria, and induction of apoptosis (Xu et al. 2005b). The glycolytic inhibition was much pronounced in colon cancer and lymphoma cells in hypoxic conditions. Glycolytic inhibition also induced apoptosis in multidrug-resistant cancer cells (Xu et al. 2005b).

Mechanistically, it was shown that 3BrPA causes a covalent modification of HKII protein that then mediates its dissociation from mitochondria, where it is physically associated with apoptosis-inducing factor (AIF), resulting in the release of AIF into the cytosol and hence induction of apoptosis (Chen et al. 2009). It is also possible that cell death induced by 3BrPA involves ROS-mediated mechanisms as it was blocked by *N*-acetylcysteine. In combination with cisplatin or oxaliplatin, 3BrPA synergized to induce massive cell death (Ihrlund et al. 2008). Zhang et al. (2009) demonstrated that 3BrPA significantly prolonged the survival of murine model of malignant pleural mesothelioma. This survival effect was enhanced when 3BrPA was combined with cisplatin, which alone had no or little effect. Geschwind et al. (2002) initially demonstrated the in vivo inhibition of liver cancer cells. Local intraarterial delivery to liver-implanted rabbit tumors led to almost complete lethality of the cancer cells. Systemic delivery suppressed growth of lung metastasis with no toxic effects on other organs (Geschwind et al. 2002). The therapeutic dose and method of infusion of 3BrPA were determined in rabbits, where it demonstrated a favorable biodistribution with negligible effects on normal tissues (Vali et al. 2008).

Mastoparan: Mastoparan is a 14-amino-acid α-helical amphipathic peptide obtained from wasp venom. The helical structure penetrates membranes via mechanisms dependent on membrane potential. Unlike proapoptotic BH3 peptides, mastoparan targets IMMs to cause mtPT. Mastoparan is also a calmodulin antagonist, and an inhibitor of phosphoinositide hydrolysis. The agent appears to have a bimodal mode of action depending on concentration. At submicromolar or low concentration, the agent causes mtPT that can be blocked by CsA, and this action requires calcium. At higher concentrations, the actions of mastoparan are independent of calcium and insensitive to CsA (Pfeiffer et al. 1995). Mastoparan was noted to enhance the cytotoxicity of bleomycin A2 in leukemic L1210 cells, probably by increasing DNA damage or loss of DNA repair. This effect was not evident with other DNA-damaging agents such as etoposide and X-irradiation (Lazo et al. 1985). Mastoparan is toxic to normal cells. It caused sarcolemal disruption and collapse of myofibril arrangement in normal skeletal muscle cells (Rocha et al. 2008). To

overcome these toxic effects on normal cells, Yamada et al. (2005) delivered mastopran in a capsule of transferrin-modified liposomes with a pH-sensitive fusogenic peptide to specifically target leukemic cells.

14.2.1.2 Agents Targeting Prosurvival BCL-2 Family Members

As indicated above, a balance between prosurvival and proapoptotic BCL-2 family members is what keeps apoptosis in check. In normal cells, the prosurvival members effectively antagonize the proapoptotic members to prevent apoptosis. Because cancer cells have the innate propensity to commit suicide due to genomic damage and/or altered signaling pathways, they tend to overexpress prosurvival BCL-2 proteins to offset these death signals, and to even overcome the effects of chemotherapy and radiotherapy. Thus, an effective strategy to kill cancer cells has therefore been to inhibit the BCL-2 members (Table 14.2). Several small molecules targeting the BCL-2 proteins are in various phases of clinical trials, as single agents or combination therapies.

Oblimersen (*Genasense*): Oblimersen is an 18 mer phosphorothioate antisense oligonucleotide that blocks the expression of BCL-2 mRNA and therefore reduces its protein levels. It is shown in preclinical studies to be cytostatic on growth of melanoma and breast cancer xenograph transplants (Jansen et al. 1998). Oblimersen chemosensitized melanoma cells by downregulation of BCL-2, and synergized with dacarbazine in patients with advanced melanoma (Jansen et al. 2000; Bedikian et al. 2006). Oblimersen improved the survival of patients on both therapies, and this was especially pronounced in patients with normal baseline serum lactate dehydrogenase (Bedikian et al. 2006). In a phase III trial in patients with relapse or refractory chronic lymphocytic leukemia, oblimersen synergized with fludarabine and cyclophosphamide (O'Brien et al. 2007). This combination therapy improved complete and nodular partial responses, particularly in fludarabine-sensitive patients. Oblimersen is currently in phase II trials for the treatment of nonHodgkin's lymphoma and other cancers.

Table 14.2 Chemotherapeutic agents targeting BCL-2 prosurvival proteins and IAPs

Agent	Specific actions	Phase of development
Oblimersen (Genasense)	BCL-2 antisense oligonucleotide	Clinical trials
(-)Gossypol (AT-101)	BCL-2, BCL-xL, MCL-1, BCL-w antagonist	Clinical trials
ABT-737 and ABT-263	BCL-2, BCL-xL, BCL-w antagonist	Clinical trials
Antimycin A	BCL-xL antagonist	Preclinical
SAHB	BH3 peptide, BCL-2 antagonist	Preclinical
GX15-070 (Obatoclax)	Pan BCL-2 antagonist	Clinical trials
BH3I-2, Chelerythrine, HA14-1	BH3 mimetic, antagonizes BCL-2	Preclinical
EM20-25	BCL-2 antagonist	Preclinical
TW-37	BCL-2 antagonist	Preclinical
Smac mimetics, e.g., LBW242	Antagonizes IAPs	Preclinical

(-)-*Gossypol* (*AT-101; R*-(-)-*gossypol is a derivative of gossypol*): Gossypol is a natural polyphenolic aldehyde extracted from cottonseed with direct effects on mitochondria. It binds and inhibits BCL-2, BCL-xL, MCL-1, and BCL-w, and was shown to enhance the efficacy of radiation treatment of human hormone-independent prostate xenograph transplant (Kitada et al. 2003; Xu et al. 2005a). Gossypol induced apoptosis in chemosensitive and chemoresistant bladder cancer cell lines, and synergized with gemcitabine and carboplatin to kill chemoresistant bladder cancer cells. These effects were associated with downregulation of prosurvival BCL-xL and MCL-1 as well as increased expression of BH3 only proteins, BIM and PUMA (Macoska et al. 2008). In a phase I/II clinical trials of castrate-resistant prostate cancer, AT-101 was well tolerated with some patients showing prostate specific antigen decline (Liu et al. 2009). A derivative of gossypol, apogossypolone (ApoG2) was demonstrated to have three- to sixfold more potency than the parental gossypol in blocking growth of prostate and breast cancer cell lines, probably via induction of cell cycle arrest (Zhan et al. 2009). Clinical trials in combination with docetaxel and androgen-deprivation are ongoing.

ABT-737and ABT-263: These are two BCL-2 inhibitors that have shown promise in cancer treatment, because unlike many of the BCL-2 inhibitory molecules that tend to be nonspecific and are intolerable, these molecules act differently. They are small molecule BH3 mimetics that antagonize prosurvival BCL-2, BCL-xL, and BCL-w.

Oltersdorf et al. (2005) used NMR-based screening, parallel sequencing, and structure based design to discover ABT-737. Initial mechanistic study indicated it did not initiate apoptosis, but synergized with radiation and other chemotherapeutic agents. ABT-737 binds the hydrophobic groove of BCL-2 with high affinity, and is shown to cause apoptosis in lymphoma and small cell lung cancer cell lines. ABT-737 treatment decreased glutathione levels and increased ROS production, activated caspase-dependent apoptosis in Jurkart human acute lymphocyte leukemia and HeLa cells (Howard et al. 2009).

In a study of six BCL-2 inhibitors (obatoclax, gossypol/apogosypolone, EM20-25, ABT-737 and chelerythrine), only ABT-737-induced apoptosis could be completely blocked by lack of BAX/BAK and caspase-9. The other members were not specific to BCL-2 inhibition. However, all the compounds induced some aspects of apoptosis including cytochrome *c* release and caspase activation. Ultrastructural analysis revealed that only ABT-737 caused classic apoptotic changes such as rupture of the outer mitochondrial membrane. The other BCL-2 inhibitors caused mitochondrial swelling with formation of phospholipid inclusions (Vogler et al. 2009).

ABT-263 is a modified version of ABT-737 with 20-fold improvement in its pharmacologic properties when given orally (Park et al. 2008). Similar to ABT-737, this molecule is a BAD-like BH3 mimetic that can disrupt BCL-2/BCL-xL interaction with proapoptotic BIM protein. ABT263 induces BAX translocation and cytochrome *c* release. It is orally efficacious against human small cell lung cancer xenographs, where it caused a complete tumor regression. In an in vitro study of a panel of cell lines in a pediatric preclinical testing program, ABT-263 demonstrated

activity in several cancer cell lines with acute lymphocytic leukemia xenographs showing the greatest sensitivity (Lock et al. 2008). This agent is currently in phase I trials for the treatment of SCLC and hematologic malignancies.

Antimycin A: Antimycin A is a small molecule peptidomimetic that binds to BH3 domain of BCL-xL. It also inhibits electron transport chain activity to induce oxidative stress in cells. Antimycin treatment caused apoptosis in BCL-2 and BCL-xL overexpressing cells (Tzung et al. 2001), and inhibited growth of lung cancer cells probably via G1 cell cycle arrest and apoptosis (Han et al. 2008). The agent was effective in enhancing colon cancer cell killing through an inhibitor of glucose metabolism, 2-deoxyglucose, and this effect was mediated by oxidative stress in cancer cells (Fath et al. 2009).

Stabilized alpha helix of BCL-2 domains (*SAHB*): Walensky et al. (2004) used a chemical strategy known as hydrocarbon stapling to uncover SAHB. These are BH3 peptides that have maintained their specificity of native BH3 death ligands. They are protease resistant, cell-permeable compounds with increased affinity for BCL-2 proteins. The agent can directly initiate BAX-mediated apoptosis, and induces apoptosis of Jurkart cells and growth inhibition of leukemic xenographs (Walensky et al. 2004).

GX15-070 (*Obatoclax*): This small molecule antagonist is a pan BCL-2 inhibitor. It targets the BH3 binding groove of BCL-2, causing the release of BIM and BAK from antiapoptotic BCL-2 family members. This agent has shown promising efficacy against several hematologic malignancies. In a phase I trial of advanced chronic lymphocytic leukemia (CLL), it showed biological activity and modest effects as a single agent against heavily treated CLL patients. One patient in this study showed improvement (O'Brien et al. 2009). In another phase I study of refractory leukemia and myelodysplasia, the drug was well tolerated and again one patient with acute myeloid leukemia showed complete 8 months remission. Moreover, three patients with myelodysplasia became independent on RBC and platelet transfusion (Schimmer et al. 2008). Currently obatoclax is undergoing various clinical trials in hematologic cancers.

BH3I-2, chelerythrine, and HA14-1 are small organic molecules that can bind BH3 domains of BCL-2. In addition to inducing apoptosis, these agents have toxic effects on respiration, depending on their concentration. At low concentration, they uncouple respiration, but inhibit respiration at high concentration. At much lower concentrations below respiratory uncoupling, these agents sensitize PTPC opening in intact cells and isolated mitochondria (Milanesi et al. 2006).

BH3I-2 is a 3-iodo-5-chloro-N-[2-chloro-5-((4-chlorophenyl)sulphonyl)phenyl]-2-hydroxybenzamide. It was shown to cause IMM damage and release of cytochrome *c* (Feng et al. 2003). It was also demonstrated to synergize with Apo2L/TRAIL to induce apoptosis in prostate cancer cell lines (Ray et al. 2005), and reactivate apoptosis in TRAIL-resistant cell lines (Hetschko et al. 2008a).

Chelerythrine is a benzophenanthridine alkaloid with several effects on cancer cells. It was uncovered as a selective antagonist of calcium/phospholipid-dependent protein kinase C (Herbert et al. 1990), and later found to induce apoptosis in HL-60 human promyelocytic leukemic cells (Jarvis et al. 1994). Recently, Chelerythrine

was shown to affect the cell cycle, as well as induce mitochondrial apoptosis and necrosis in HL-60 cells (Vrba et al. 2008). In human CEM T-leukemic cells, chelerythrine induced apoptosis that was associated with cytochrome *c* release, caspase processing, ROS production, and loss of membrane potential, but no changes in BCL-2 family members in the mitochondrial fraction (Kaminskyy et al. 2008).

Wang et al. (2000), identified HA14-1 using computational chemical modeling of the 3D structure of BCL-2. As a BH3 mimetic, it inhibits BCL-2 to induce apoptosis in cancer cells, and caspase inhibitors can block its actions. It was shown to reactivate autophagic cell death in anoxia-resistant malignant gliomas (Hetschko et al. 2008b), and sensitized cervical cancer cells to heavy-ion radiation (Hamada et al. 2008). The resistance of renal cancer cells to mitochondrial apoptosis was reconstituted by HA14-1 treatment (Heikaus et al. 2008). HA14-1 enhanced the apoptotic effects of cisplatin (Arisan et al. 2009), synergized with genistein to dowregulate survival factors, and induced apoptosis in malignant neuroblastoma cell lines (Mohan et al. 2009).

EM20-25 (5-(6-chloro-2,4-dioxo-1,3,4,10-tetrahydro-2H-9-oxa-1,3-diaza-anthracen-10-yl)-pyrimidine-2,4,6-trione) is an HA14-1 analog from the Hoffmann-La Roche chemical library that was identified by Milanesi et al. (2006). This agent is devoid of respiratory impairment that is common in other small molecule BCL-2 inhibitors, including BH3I-2, HA14-1, and chelerythrine. Mechanistically, it can induce opening of PTPC and disrupt BCL-2/BAX interaction. EM20-25 activated caspase in BCL-2 expressing cells and sensitized such cells from leukemic patients to apoptosis induced by staurosporine, chlorambucil, and fludarabine (Milanesi et al. 2006). Similar to many hematologic cancers, CLL overexpresses BCL-2 and primary CLL tend to be resistant to oncolysis by vesicular stomatitis virus (VSV). In view of this it was hypothesized that treatment with inhibitors of BCL-2 might sensitize CLL cells towards VSV ocolysis. Consistently, EM20-25 treatment synergized with VSV to kill CLL and B-lymphoma cells (Tumilasci et al. 2008).

TW-37: Mohammad et al. (2007) used structure-based strategy to develop a new nonpeptide small molecule inhibitor of BCL-2, named TW-37. This agent is a benzenesulfonyl derivative that binds the BH3-binding groove in BCL-2 where proapoptotic members normally interact. TW-37 has shown antiproliferative effects in chemoresistant lymphoma cell lines as well as primary lymphoma cells from patients, with no effect on normal lymphocytes. These agents also synergize with other chemotherapeutic drugs used to treat lymphoma.

14.2.1.3 Targeting the Mediators of Apoptosis

MMP leads to the release of several molecules from the mitochondria into the cytosol. These molecules include cytochrome *c*, Smac/diabolo, Omi/HtrA2, AIF, and endonuclease G. Cytochrome *c* forms a complex with apoptotic protease-activating

factor 1 that binds and process procaspase 9 to form a complex known as the apoptosome. The apoptosome mediates downstream caspase cascade to induce apoptosis. IAPs block the enzymatic activity of caspases keeping apoptosis in check. Hence, many cancers overexpress IAP that helps confer chemoresistance. Smac and Omi/HtrA2, when released from mitochondria induce apoptosis by interfering with IAPs. Specifically, the N-terminal AVP1 binding motif of Smac interacts with IAPs. Endo G and AIF translocate into the nucleus to cause DNA fragmentation.

Smac mimetics: One therapeutic strategy in advanced development is the use of potent mimetics of Smac that target IAP-overexpressing cancer cells. There are two types of Smac mimetics, monovalent and divalent compounds. The monovalent Smac mimetics simulate the binding of a single AVP1 motif to IAPs, and the divalent molecules possess two such motifs linked together. Whereas the divalent Smac mimetics are more potent (100–1,000 fold) than the monovalent counterparts, both are efficacious at inducing apoptosis in cancer cells.

There are several petidic and non-peptidic Smac mimetics being developed, and preliminary in vitro cell line functional assays indicate that they inhibit growth of cancer cell lines (Peng et al. 2008; Zhang et al. 2008; Sun et al. 2009). In breast cancer, multiple IAPs were concomitantly expressed (Foster et al. 2009). IAP antagonists promoted apoptosis and reduced the cell turnover index of breast cancers when used in combination therapy with ErbB antogonist. Similarly, when used in combination with some pro-apoptotic agents such as TRAIL, there was synergistic enhancement of apoptosis of breast cancer cells (Foster et al. 2009). Zobel et al. (2006) developed Smac mimetics that bind Smac binding sites on IAP BIR3 of XIAP, cIAP1, cIAP2 as well as melanoma-IAP (ML-IAP). Those targeting cIAP1 and ML-IAP were the most potent agents. These agents induced caspase 3 and 7 activation in breast and melanoma cell lines and synergized with doxorubicin to induce apoptosis. Chauhan et al. (2007) developed another agent that mimics Smac. This agent, labeled LBW242 induced apoptosis in multiple myeloma cells resistant to conventional chemotherapies. Its mechanism of action includes activation of caspases 8, 9, and 3 followed by PARP cleavage. LBW242 is well tolerated and synergizes with other agents to kill myeloma cells. There are likely multiple mechanisms by which Smac mimetics induce cell death, one of which is targeting IAPs for degradation leading to NF-κB activation, TNFα expression, and caspase activation. Whereas several Smac mimetics are available, results from clinical trials are awaited for only a few.

14.2.2 Targeting Glycolysis to Kill Cancer Cells

The Warburg phenomenon of cancer cell reliance on glycolysis, as well as other metabolic alterations of the cancer cell, has been targeted for chemotherapy. Aerobic glycolysis is observed in $\sim$ 70% of cancers. Because this is not a feature of normal cells, pharmacologic inhibition of glycolysis should have pronounced

Table 14.3 Chemotherapeutic agents targeting glycolysis, redox, and mitochondrial membrane potential differences

Target	Agent	Specific actions	Phase of development
Glycolysis	Lonidamine	Inhibits HK	Launched/clinical trials
	3BrPA	Inhibits HK	Preclinical
	Glufosfamide	Toxic conjugate glucose analog	Clinical trials
	2-Deoxyglucose	Inhibits HK	Clinical trials
	Mannoheptulose	Inhibits HK	Preclinical
	5-thioglucose	Inhibits HK	Preclinical
Redox	2-Methoxyestradiol	Inhibits MnSOD	Preclinical
	Arsenic trioxide	Interferes with electron transport chain activity	Launched/clinical trials
	Bistetrahydrofuranic acetogenins	Blocks complex I	Preclinical
	Imexon	Thiol depletion	Clinical trials
$\Delta\Psi m$	F16	Interferes with mitochondrial functions	Preclinical
	Dequalinium	Membrane depolarization ROS production ATP depletion	Preclinical
	MKT-077	Accumulates in mitochondria, but also targets Ras, Mortalin, and telomerase	Clinical trials
	Rh123	Accumulates in mitochondria; Could interfere with mitochondrial functions	Preclinical

effects on malignant cells. Thus, several small molecules that target various components of glycolysis are in various stages of trials. Favorable targets include HKs, lactate dehydrogenase, phosphofructokinase, and glyceraldehyde 3-phosphate dehydrogenase. However, inhibitors of glycolysis with therapeutic potential that have progressed through various phases of development include lonidamine (see above), 3-BrPA, (see above), glufosfamide, and 2-deoxyglucose. These and other promising targets are discussed (Table 14.3).

Glufosfamide (*Beta-D:glucosylisophoramide mustard*; D-*19575*): Glufosfamide is an alkylating agent with a covalently linked ifosfamide moiety to beta-D:glucose. Metabolism of ifosfamide results in the release of a cytotoxic compound, isophospharamide to kill cancer cells. Cancer cells because of their high glucose intake preferentially take up more glufosfamide than normal cells. Specifically, the agent is possibly transported into cancer cells using Na-D-glucose cotranspoter SAAT 1 that are overexpressed by many cancer cells (Veyhl et al. 1998). Hence, glufosfamide will preferentially accumulate in cancer cells. Glufosfamide has undergone phase II studies in pancreatic cancers and non-small cell lung cancer (NSCLC) with modest activities (Briasoulis et al. 2003; Giaccone et al. 2004; Chiorean et al. 2009). In recurrent glioblastoma multiforme, glufosfamide did not show significant antitumor activity (van den Bent et al. 2003). The primary concerns with this drug are hematologic and renal toxicities, which were somehow more pronounced when

combined with gemcitabine, but were mild when administered alone (Briasoulis et al. 2003; Chiorean et al. 2009). Further studies on dose and combination therapies are needed.

2-Deoxyglucose: 2-Deoxyglucose is a glucose analog that is taken up in excess by cancer cells. Once inside the cell, it is metabolized by HKs to 2-deoxyglucose-phosphate that accumulates in the cell because it cannot be metabolized further. 2-deoxyglucose inhibits glucose metabolism by product inhibition of HKs. Cancer cells in hypoxic conditions and those with defective OXPHOS are easily killed by 2-deoxyglucose-mediated ATP depletion (Liu et al. 2001; Maher et al. 2004). Whereas alone this agent has minimal effects on tumor growth, when combined with adriamycin or paclitaxel in mouse models of human osteosarcoma and NSCLC, there was a significant synergistic effect on tumor growth (Maschek et al. 2004). 2-Deoxyglucose has entered various clinical trials.

Hexokinase II inhibitors: HK II, which is usually expressed by normal skeletal muscle and adipose tissues is overexpressed in a number of cancers, and performs functions that are primarily beneficial to the cancer cell. First, HK II enhances the glycolytic phenotype by phosphorylating glucose for metabolism. Second, HK II interacts with VDAC, preventing apoptosis sensitization by preventing the deployment and docking of BAX on VDAC. Inhibition of HK II thus will both affect glucose metabolism and increase the sensitivity of cancer cells to apoptosis. HK II is inhibited by a number of agents including lonidamine, 3-BrPA, mannoheptulose, 2-deoxyglucose, and 5-thioglucose.

Phosphofructokinase inhibitors: Phosphofructokinase (PFK) is a rate-limiting glycolytic enzyme that converts fructose 6-phosphate to fructose 1,6-bisphosphate. Inducible PFK catalyzes the phosphorylation of fructose 6-phosphate to fructose 2,6-bisphosphate, which is an allosteric activator of glycolysis. Hence iPFK is constitutively expressed by tumors, and aggressive colon, breast, ovarian, and thyroid cancers are shown to overexpress this gene (Atsumi et al. 2002). To facilitate glycolysis, iPFK is also induced by hypoxia. In animal models, inhibition of iPFK decreases tumor growth (Chesney 2006).

Lactate dehydrogenase inhibitors: Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate with the oxidation of NADH that can be used by GAPDH. Knockdown of LDH-A using shRNA decreased cancer cell proliferation under hypoxia (Fantin et al. 2006). In hereditary leiomyoma and renal cell carcinoma syndrome where tumors overexpress LDH-A, it was shown that inhibition of LDH-A resulted in increased ROS-mediated apoptosis in fumarate hydratase deficient cells (Xie et al. 2009).

ATP citrate lyase: Aerobic glycolysis is linked to lipid biosythesis in cancer cells. Citrate in the cytosol is a potent inhibitor of glycolysis. Cancer cells are able to overcome glycolytic inhibition of cytosolic citrate by activation of the PI3K/AKT/mTOR pathway (Bauer et al. 2005). Signaling via this pathway is shown to increase phosphorylation and activation of ATP citrate lyase, which shunts citrate into lipid biosynthetic pathway, thus permitting glycolysis to continue uninhibited. Targeting ATP citrate lyase as a novel mode of killing cancer cells has been demonstrated. Inhibition of ATP citrate lyase with RNAi or using a chemical

agent, SB-204990 decreased in vitro and in vivo proliferation and survival of glycolytic cancer cells (Hatzivassiliou et al. 2005).

14.2.3 Targets of Redox Homeostasis

Oxidative stress is inherent in cancer cells as a consequence of damage to their mitochondrial genomes, existence in hypoxic conditions, and activated signaling pathways. To overcome such stresses, cancer cells increase antioxidant mechanisms such as superoxide dismutase, catalase, and reduced glutathione. The redox imbalance and adaptive mechanisms of the cancer cell are not identical to those of the normal cell. Hence, this offers opportunities for therapeutic targeting. There are at least two ways to target redox status to kill the cancer cell.

- The antioxidant systems can be targeted to prevent detoxification and hence mtPT and apoptosis. 2-methoxyestradiol (2ME2; Panzem) is an endogenous estrodiol-17β metabolite devoid of any estrogenic effects. Work by Huang et al. (2000) using cDNA and microarray analysis revealed that this molecule targets and inhibits the functions of mitochondrial MnSOD, thereby leading to the build-up of superoxide anions to cause mtPT and cell death. It however, appears that the natural product, 2-ME does not inhibit MnSOD (Kachadourian et al. 2001). A chemically modified version at the second carbon (2-OH, 2-OCH3) inhibits MnSOD and has shown utility in preclinical studies by being a selective cytotoxin to leukemic cells (Huang et al. 2000). Panzem is also antimitotic and antiangiogenic. This agent is in phase II clinical trials for the treatment of solid and hematologic cancers.
- Because cancer cells are already stressed, the threshold to reach lethal levels of oxidative stress is low. Hence, agents that will augment oxidative stress should be more lethal to the cancer cell than normal cells. An example of such an agent is arsenic trioxide. Apart from its interaction with PTPC, arsenic trioxide interferes with the electron transport chain causing enhanced ROS production, mtPT, and apoptosis. Acetylated bis-tetrahydrofuranic acetogenin is a modified acetogenin that inhibits electron transport at complex I. It is shown to inhibit growth of breast, lung liver, and colon cancer cell lines (Chahboune et al. 2006).
- A third and attractive model will be to combine the above two modes, to inhibit the antioxidant enzymes and increase oxidative stress simultaneously.

Imexon: Imexon is an aziridine-containing iminopyrrolidone compound that is well known for its immune enhancing effects on lymphocytes. Researchers at the Arizona Cancer Center discovered that the agent demonstrated antitumor activity against multiple myeloma, lymphoma, and pancreatic cancer cell line models both in vitro and in vivo (Hersh et al. 1992, 1993; Salmon and Hersh 1994). Mechanistically, imexon reacts with sulfhydryl groups leading to cellular thiol depletion and hence depletion of antioxidant molecules such as glutathione and thioredoxin. In addition, the agent increases oxidative stress, causes mitochondrial swelling,

mtDNA mutations, and apoptosis (Dvorakova et al. 2001). Imexon is undergoing clinical trials.

14.2.4 Targeting Membrane Potential Changes

In general, cancer cells exhibit increased membrane potentials compared to normal cells. The difference in membrane potential between the normal and cancer cell is at least 60mV. The high membrane potential of cancer cells enables selective uptake of some agents into the cancer cell.

F16: This agent was identified by Fantin et al. (2002) as a small molecule with selective antiproliferative effects on mammary epithelial, neu-overexpressing cells, as well as, mouse and human mammary tumors. F16 accumulates in mitochondria of cells driven by membrane potential, to induce apoptosis and thus reduce tumor cell growth. In cells overexpressing pro-survival BCL-2, F16 can induce necrosis instead of apoptosis (Fantin and Leder 2004).

Dequalinium: Dequalinium is a selective antitumor agent because it preferentially accumulates in mitochondria of cancer cells. In one study, a high dose induced mitochondrial changes within 30 min of treatment. The changes included membrane depolarization, ROS production, ATP depletion, and release of cytochrome *c*. After 16 h, apoptosis via the intrinsic pathway ensued (Sancho et al. 2007).

MKT-077: MKT-077 (formerly known as FJ-776) is a highly water soluble rhodacyanine dye that accumulates in mitochondria and inhibits growth of many cancer cells (Koya et al. 1996). Some of the changes induced in mitochondria of cancer cells include ultrastructural changes, depressed respiratory activity, and selective loss of mtDNA (Modica-Napolitano et al. 1996).

Rh123: The uptake and retention of this dye depends on plasma and mitochondrial membrane potentials. It is shown in human breast cancer cell lines (MCF-7) that when plasma membrane is depolarized, uptake and retention are mediated by mitochondrial membrane potential (Davis et al. 1985). Rh123 is an F_0F_1 ATPase inhibitor.

14.2.5 Cancer Mitochondrial DNA Depletion as a Strategy

The mitochondrial genome is another target of some chemotherapeutic agents. While this field is novel, future developments should unravel its potential in cancer treatments. It is well known that chemotherapeutic agents cause mtDNA damage, and this has been viewed primarily as a toxic side effect on normal mitochondria. While this is true, especially in mitochondria in tissues with high energy demands such as cardiac cells, it is probably the primary mode of action of some drugs. Lo et al. (2005) examined the contribution of mtDNA to the cytotoxic effects of DNA-interactive agents. By comparing the response of A549 NSCLC cell line and its

ρ0 derivative to chemotherapeutic agents, it was observed that in comparison to parental A549 cell line, the ρ0 derivative was resistant to both daunorubicin and the rhodacyamine derivative, MKT-077. The sensitivity to both drugs was restored when ρ0 cells were repopulated with wildtype mtDNA using cybrid technology. These findings suggest that, these and other chemotherapeutic agents kill cancer cells targeting mitochondrial genome depletion. Several agents including antiviral nucleoside analogs, ciprofloxacin, adriamycin, and lipophilic cations such as dietercalinium induce mtDNA depletion.

The agents targeting mtDNA are not specific to cancer cells. Because of the inherent toxicity of these agents on normal cell mtDNA, specific delivery to cancer cells will reduce their toxic effects. There are several agents in development for specific delivery of chemotherapeutic and mitochondrotoxic agents to cancer cells. For example, delocalized lipophilic cations (DLC) target mitochondrial inner membrane and accumulate in mitochondrial matrix. The mitochondrial targeting of DLC is dependent on the membrane potential. The high membrane potential of cancer cells enables the preferential accumulation of DCL in cancer cell mitochondria (Summerhayes et al. 1982; Modica-Napolitano and Aprille 2001).

14.2.6 Delivery of Toxic Compounds to Mitochondria Using Differential Receptor Expression by Cancer Cells

A novel approach to deliver mitochondrotoxic compounds specifically to cancer cells is the exploitation of the differential overexpressed genes by cancer cells. Various nanotechnology approaches are explored not only for the delivery of mitochondrotoxic compounds but also lethal compounds in general, specifically targeting cancer cells. For example, several tumors overexpress HER-2 and Luteinizing hormone-releasing hormone (LHRH) receptors, and these have been studied by a number of groups. Dharap et al. (2003) constructed a chimeric peptide with a moiety specific to LHRH receptor and a synthetic BH3 domain as an apoptotic inducer. The LHRH-BH3 peptide was efficacious in killing several cancer cell lines. In a subsequent study, Dharap et al. (2005) developed both targeted and non-targeted drug delivery systems for study using human ovarian cancer cell lines because they overexpress LHRH. The three compounds used for constructing the conjugates were poly(ethylglycol) (PEG) polymer used as a carrier, camptothecin (CPT) as an anticancer agent, and modified LHRH peptide targeting its receptor. Three conjugates tested were untargeted (CPT-PEG), and two were targeted (PEG-LHRH, CPT-PEG-LHRH). As expected, the LHRH targeting peptides enhanced chemotherapeutic efficacy with minimal effects on normal cells.

Ellerby et al. (1999) designed a fusion peptide consisting of 47 amino acid residues with two functional domains, one specifically targeting tumor vasculature, and the other targeting apoptosis. The tumor vessel targeting peptide served to guide the peptide to tumor vessels and also facilitated internalization of the agent into

tumor cells. The pro-apoptotic peptide induced apoptosis once inside the cell, but was nontoxic in the extracellular milieu. This compound showed effective antitumor activity in mice by destroying mitochondrial membranes and triggering apoptosis.

14.3 Conclusion

The plethora of "mitocans" under development demonstrates the level of activity by various researchers and pharmaceutical companies. Several molecules are in various phases of clinical trials or preclinical development, while others are in the discovery phase. It is expected that the list will continue to increase because, for example the number of small molecule BCL-2 agents is very large, and there is an increase in patent filings on BCL-2 inhibitors and BH3 mimetics. Moreover, the use of computational, mathematical, and structure-based modeling should enable discovery of more specific and potent molecules. Similarly, with refinement of delivery methods, several mitochondrotoxic agents that are specifically targeted at the mitochondria will be developed. These agents are currently being trialed singly and in combination with conventional chemotherapy. In many instances, the "mitocans" synergize with the established agents. It will appear that an approach targeting mitochondria as well as other altered signaling pathways will offer maximum efficacy in chemotherapy of cancer. Clearly skewed are the types of cancers currently in clinical trials. The vast majority are hematologic malignancies, but many of the agents are also effective against solid tumors. Thus, it is hoped that, as these molecules get translated into clinical practice, a day will come when the ability to kill the cancer cell, irrespective of its location in the human body will be a facile clinical accomplishment.

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