ASSOCIATION OF MITOCHONDRIAL DNA 10398 POLYMORPHISM WITH BREAST CANCER AND APOPTOSIS IN MALAY POPULATION

by

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Dedication

In the name of Allah, The Most Gracious, The Most Merciful..

I would like to dedicate this thesis to my parents (Tengku Baharudin Shah and Rukiah Hassan), my siblings (Tengku Mahmud Iskandar, Tengku Mahmud Imran, Tengku Intan Naqiah and Tengku Mahmud Izuddin), my beloved husband (Edinur Hisham Atan) and my little princess (Puteri Elena Aisya), who provided me continuous enriching love, support, encouragement and inspiration to pursue my study.

To myself, hold on the courage, motivation and spirit alive…

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LIST OF ABBREVIATION

ADP	Adenine diphosphate
AIF	Apoptosis inducing factor
ATP	Adenosine triphosphate
Bcl-2	B- Cell lymphoma/ Leukemia-2
Bp	Base pair
CRS	Cambridge reference sequence
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic
H_2O	Water
Kb	Kilo base
M	Molar
$MgCl_2$	Magnesium chloride
mM	Millimolar
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide hydrogenase
NaOH	Sodium hydroxide
nDNA	Nuclear DNA
O_2^{-}•	Superoxide anion
PCR	Polymerase chain reaction
rCRS	Revised Cambridge reference sequence
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
Rpm	Revolution per minutes

RFLP	Restriction fragment length polymorphism
STR	Short tandem repeats
SMAC	Second mitochondria-derived activator caspases
SNP	Single nucleotide polymorphism
Taq	*Thermus aquaticus*
TBE	Tris-borate-ethylene-diamine tetra acetic acid
tRNA	Transfer ribonucleic acid
U	Unit
µg	Microgram
Ml	Microliter
Mm	Micrometer
UV	Ultra violet
VNTRs	Variable number of tandem repeats

Perkaitan polimorfisma 10398 mitokondria DNA dengan kanser payudara dalam populasi Melayu di Semenanjung Malaysia

Abstrak

Polimorfisma 10398 mitokondria DNA telah didapati berkait dengan kanser payu dara dalam beberapa populasi. Dalam kajian ini, polimorfisma 10398 mitokondria DNA telah disaring pada 101 pesakit kanser payu dara wanita Melayu dan 90 wanita yang sihat sebagai kawalan dengan menggunakan kaedah penjujukan mini. Hasil menunjukkan perbezaan statistik yang signifikan dengan nilai P, p = 0.007 (OR, 2.29; 95% CI, 2.252-4.200) dengan jumlah varian G lebih tinggi (73%) daripada varian A (27%) dalam pesakit dan juga dalam kawalan (G = 54%, A = 46%). Tisu kanser payudara kemudian dianalisis menggunakan kaedah immunohistokimia bagi mengkaji perkaitan polimorfisma ini dalam mempengaruhi aras apoptosis sel. Tiada perbezaan signifikan didapati antara aras pengekspresan protein pro (Bax) dan anti (Bcl-2) apoptosis antara pesakit yang membawa varian A (p = 0.48). Bagaimanapun, terdapat perkaitan yang signifikan antara protein-protein ini dalam pesakit yang mempunyai varian G (p = 0.016). Hasil ini menunjukkan polimorfisma 10398 mtDNA mungkin berguna sebagai penanda risiko kanser payu dara dalam populasi ini.

Association of mitochondrial DNA 10398 polymorphism in breast cancer and apoptosis in Malay population

Abstract

The mitochondrial DNA 10398 polymorphism has been observed to associate with breast cancer in several populations. In this study, mitochondrial DNA 10398 polymorphism was screened in 101 Malay female patients with invasive breast cancer and 90 age-matched healthy female controls using minisequencing method. The results showed a statistically significant difference with P value of $p = 0.007$ (OR, 2.29; 95% CI, 1.252-4.200) with the proportion of G variant was higher (73%) than A variant in patients (27%) as well as in controls (G = 54%, A = 46%). The breast cancer tissues were then analyzed using immunohistochemistry method to investigate the relation of this polymorphism in affecting apoptotic level in breast cancer. No significant difference was observed between the expression of pro (Bax) and anti-apoptotic (Bcl-2) proteins among patients carrying A variant ($p = 0.48$). However, significant difference was observed in patients with G variant ($p = 0.016$). These results indicate that mtDNA 10398 polymorphism may be useful as a breast cancer risk marker in this population.

CHAPTER 1

INTRODUCTION

1.1 Human Genome

The human cell consists of two interdependent genomes; the nuclear genome which specializes in cellular and tissue structure, and mitochondrial genome which specializes in energy production (Figure 1.1). Nuclear DNA (nDNA) differs from mitochondrial DNA (mtDNA) in its location, sequence, quantity in the cell and mode of inheritance. Nuclear DNA which is located within the nuclei of the cell constitutes of 23 pairs of chromosomes. Half of the genome is inherited from the biological mother and another half from the biological father. On the other hand, mitochondrial DNA is found within the mitochondria organelle with hundreds to thousands of copies per cell and inherited maternally (Giles *et al*., 1980).

1.1.1 Mitochondrial genome

Mitochondria (singular mitochondrion) are located in the cytoplasm outside the nucleus of the cell. They are known to evolve from prokaryotic organisms based on the endosymbiotic theory (Sagan, 1967; Raven, 1970; Kuntzel and Kochel, 1981; Yang *et al*., 1985). Mitochondria are bacterium size organelle (0.5 x 1.0 μm), mobile and capable of profound changes in size, shapes and location (Avers *et al*., 1969; Sohal and Bridges, 1978; Knight *et al*., 1981). The numbers of mitochondria varies by organism and tissue type. Several cells have only single mitochondrion and some contain thousands of mitochondria. Eukaryotic cells typically contain about 2,000 mitochondria which is one fifth of the total cell volume.

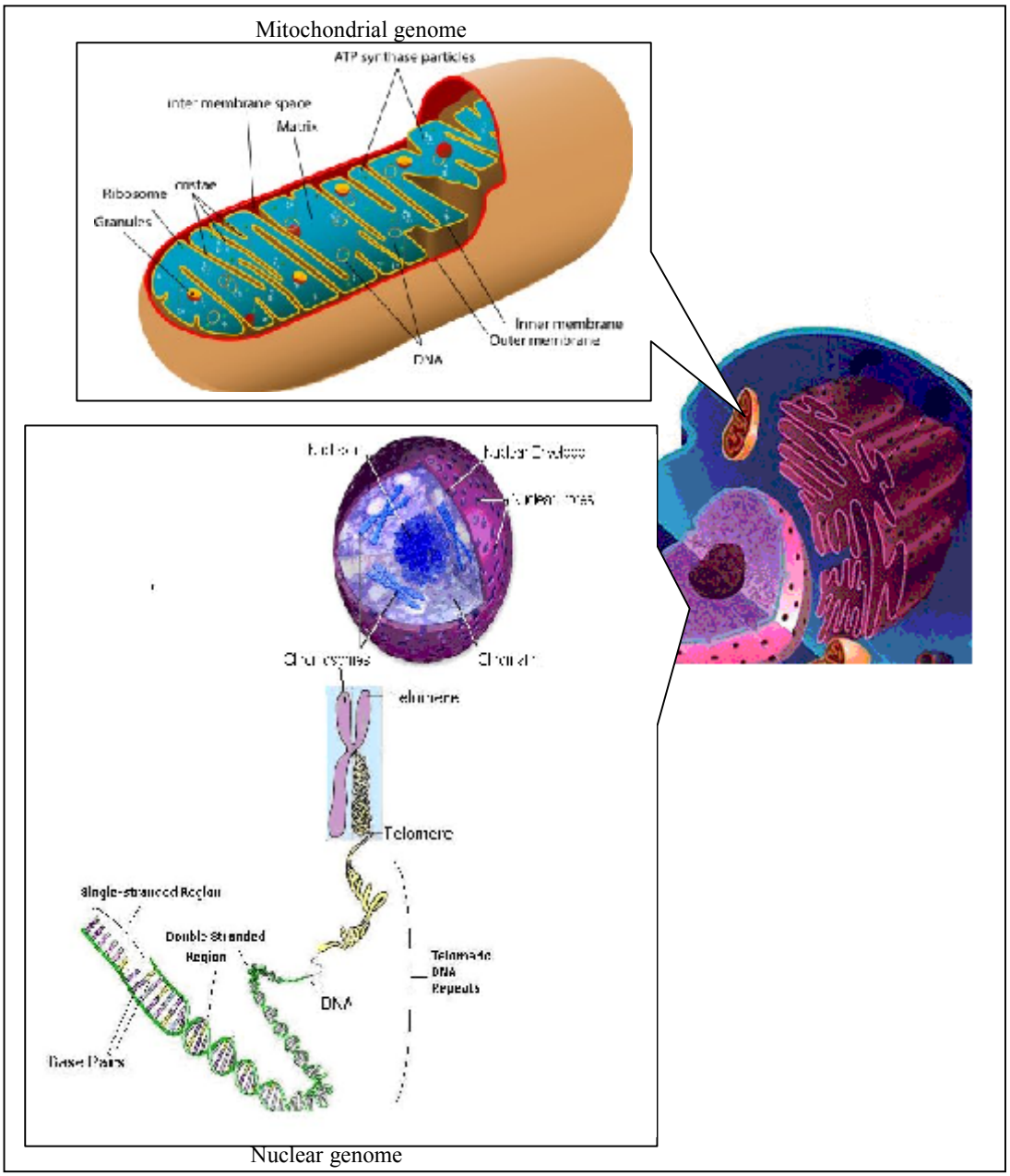

Figure 1.1: Nuclear and mitochondrial genome in human cell.

(Modified from http://www.thescienceexperts.com [Accessed on 5th October 2008]; http://www.pauldos.com/musings/main.php [Accessed on 9th May 2008] and http://www.micro.magnet.fsu.edu/cells/animals/nucleus.html) [Accessed on 24th January 2009]

The mitochondria are ellipsoidal bodies bounded by a smooth outer membrane and an extensively invaginated inner membrane that divides the mitochondria into two components; intermembrane space and matrix. Cristae are formed by the infoldings of the intermembrane that expand the surface area. On this site, food (sugar) is combined with oxygen to produce energy (ATP) for the cell. The matrix is a gel-like solution containing high concentrations of soluble enzymes of oxidative metabolism as well as substrates, nucleotides cofactors and several copies of DNA which are known as mitochondrial DNA (mtDNA) (Margulis, 1981; Voet *et al*., 1998; Lodish *et al*., 1999).

mtDNA is a double stranded circular molecule of 16,569 base pair (bp) (Brown *et al*., 1978; Wiesner *et al*., 1992). The entire mtDNA were first sequenced in 1981 in Frederick Sanger's lab in Cambridge, first authored by Stan Anderson. Resequencing of mtDNA was performed later to eliminate minor errors and the revised version is known as the Cambridge Reference sequence (CRS). mtDNA apparently are lack of protective histones and due to the paucity of DNA repair mechanism, the rate of mutation in mtDNA is calculated to be about ten times greater compared to the nDNA (Monnat and Loeb, 1985;Yakes and Van Houten, 1997). This high mutation rate leads to a high variation between mitochondria, not only among different species but even within the same species (Winstead, 2002).

This bioenergetics molecule consists of two regions which are the coding and control region (Figure 1.2). The coding region codes for 37 genes. From these 37 genes, 2 genes code for 12S and 16S ribosomal RNAs and 22 for transfer RNA. The RNAs help in assembling protein building blocks (amino acid) into functioning proteins (Ingman *et al*., 2000). The remaining 13 genes codes for the electron transport chain subunits which are complex I (NADH dehydrogenase), complex III (ubiquinol: cytochrome C oxyreductase), complex IV (cytochrome C oxidase) and complex V (ATP synthase).

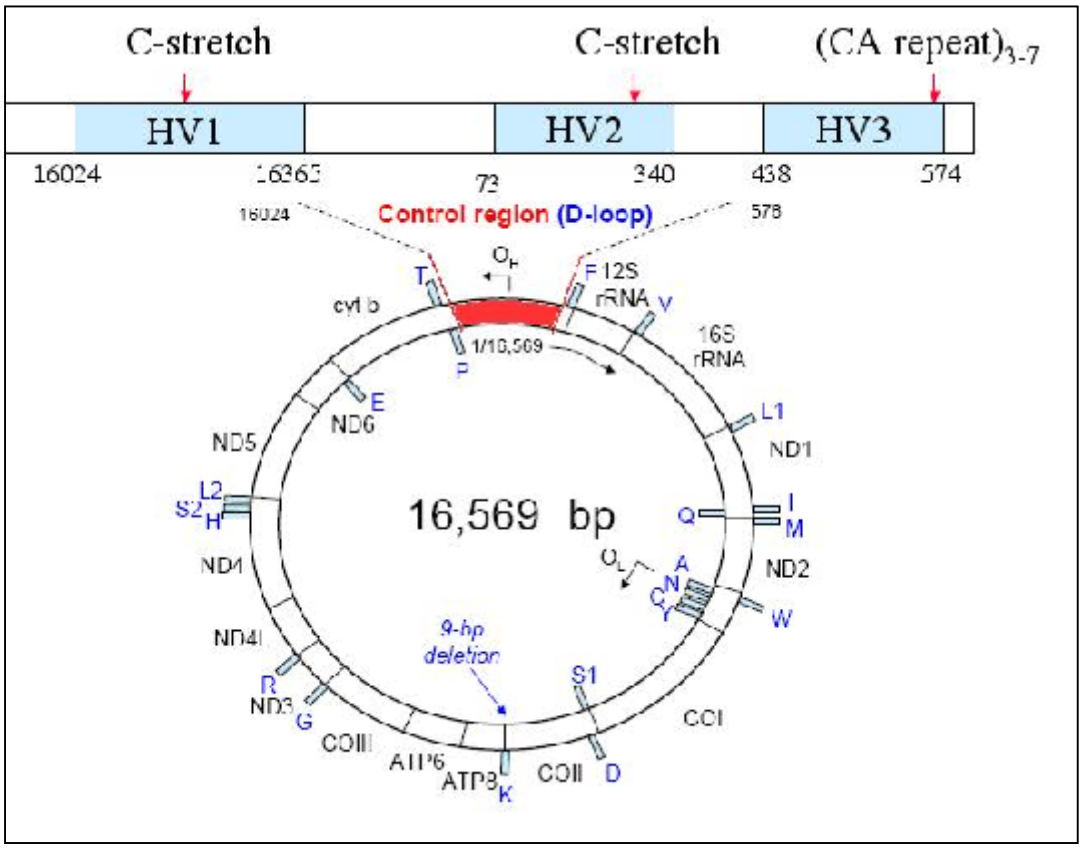

Figure 1.2: Mitochondrial DNA map.

(Modified from http://www.alphabiolab.com/page4/files/picture-12.png) [Accessed on 28th January 2009]

The control region which is also known as D-loop or non-coding region constitutes 7% of the mitochondrial genome with 1,124 base pair (bp) in size (16,024-576). This region is made up of three important regions; i) Hypervariable region I (HV1) expands from nucleotide position (np) 16,024-16,383 ii) Hypervariable region II (HV2) from np 57-372 and iii) Hypervariable region III (HV3) from np 438-574.

mtDNA comprised of 2 strands, Cytosine-rich light (L)-strand and Guanine-rich heavy (H)-strand. Each strand are transcribed from one predominant promoter, PL (for L-strand) and PH (for H-strand), located at the control region (Chang and Clayton, 1984; Chang and Clayton, 1986). This region also contained the origin of replication for the H-strand (O_H) which begins with the synthesis of a daughter H-strand in a continuous and unidirectional fashion. This results in displacement of the parental H-strand. After 2/3 of the parental H-strand has been displaced, the L-strand becomes exposed as a single-stranded and the synthesis of L-strand begins in an opposite direction.

1.1.2 Mitochondria as cellular power plants

Mitochondria are the indispensable energy producers of human cells. There are at least 87 proteins involved in oxidative energy production. A total of 13 proteins are encoded by the mtDNA. The remainders are coded by the nuclear DNA which are synthesized in the cytosol and imported into the mitochondria (Leaver *et al*., 1983).

Oxidative phosphorylation is a process where a cells' main energy, adenosine triphosphate (ATP), is created to allow cells to proliferate, be motile and generate signals for tissues and organs to function appropriately. The oxidative process is accomplished by series of protein complexes including coenzymes and cytochromes known as respiratory chain (Figure 1.3).

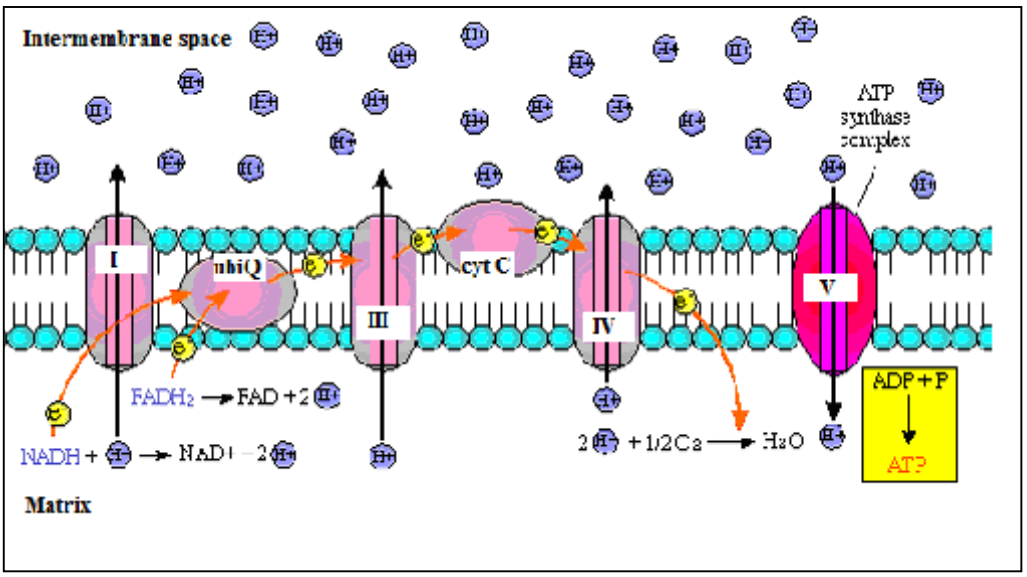

Figure 1.3: Oxidative phosphorylation and electron transport represent a series of multisubunit complexes within the mitochondrial inner membrane.

(Available from http://www2.nau.edu/~fpm/bio205/chapter5.2.html.) [Accessed on 15th October 2008]

Electrons generated from NADH which is produced by oxidation of nutrients such as glucose through citric cycle, enter this array of electron carriers and being shuttled to the first carrier, NADH dehydrogenase (Complex I). At this site, NADH is oxidized to NAD^+, which returns to the site of the citric acid cycle to be reduced again. Electrons are then shuttled to complex III (ubiquinol-cytochrome C reductase) via ubiquinol that moves freely along the membrane, then to complex IV, where oxygen is reduced to form H_2O by the electrons mobilized by a protein electron carrier, cytochrome C. As electron flows along the protein complexes, a high concentration of protons are pumped from the mitochondrial matrix to the intermembrane space at three complexes (I, III and IV), creating a hydrogen ion (H^+) gradient. At the final step in the multiprotein complex V (ATP synthase), such a gradient is utilized as energy source to synthesize ATP energy, condensation of ADP by attaching phosphorus atoms to ADP molecules to form ATP. The ATP is subsequently transported out of the mitochondrial matrix through a special channel. This final-energy producing process is called the oxidation phosphorylation process (Saraste *et al.,* 1999; Cohen and Gold, 2001; Denniston *et al.,* 2001; Ballinger, 2005).

1.1.3 Mitochondria integrate apoptosis

Apoptosis is a term introduced by Kerr, Wylie and Currie (1972) describing a programmed cell death process. Apoptosis is a Greek word meaning a natural cell death (Kerr *et al*., 1972). Apoptotic cells show a very characteristic morphology. The membrane of cells undergoing apoptosis at the early stage will shrink causing lose of interaction with the normal neighbouring cells. The cytoplasm appears dense, and the organelles are tightly packed. The cell's chromatin undergoes condensation followed by compaction of chromatin to the nucleus membrane before fragmentation. Due to the degradation of DNA, the nucleus breaks into several discrete chromatin bodies or nucleosomal units. The cell membrane shows irregular buds known as blebs and makes it breaks apart into several vesicles called

apoptotic bodies, which are then phagocytosed (Newmeyer *et al*., 1994). The organelle's morphology was maintained, even though cell's cytoplasm was condensed (Figure 1.4).

Apoptosis is controlled by a diverse range of cell signals, either extracellularly or intracellularly. Extracellular signals may include hormones, growth factors or cytokines while intracellular initiation of apoptosis may be in response of stress, specifically mitochondrial stress.

Mitochondria is considered as a key organelle regulating apoptosis since pro-apoptosis protein such as cytochrome C, AIF, endogenous G or SMAC (second mitochondria-derived activator caspases) resides in the mitochondria (Liu *et al.*, 1996; Kluck *et al*., 1997; Shimizu *et al*., 1999). Upon receiving signals, these pro-apoptotic proteins trigger a series of biochemical events leading to activation of apoptosis signaling cascades (Figure 1.5).

The release of cytochrome C from the mitochondria, due to increased permeability of the outer membrane, activates the apoptotic cascade in the presence of ATP and dATP. Cytochrome C binds to Apaf-1 (pro-apoptotic protease activating factor) that is free floating in the cell's cytoplasm using the energy provided by ATP. The complex then binds to pro-caspase-9, which is also a floating protein. These complexes aggregate to form a protein complex known as apoptosome. Apoptosome cleaves the pro-caspase to its active form of caspase-9 and activates other caspases such as the effectors of caspase-3 and caspase-7. The activation of these caspases cleaves the proteins of mitochondria membrane causing the membrane to break down and start a reaction of protein denaturation that leads to phagocytosis of the cell.

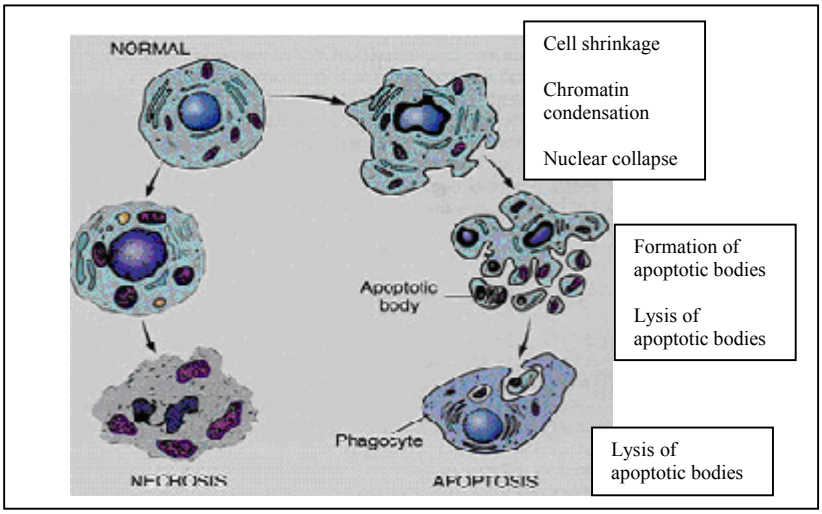

Figure 1.4: Morphology of cells undergoing apoptosis and necrosis.

(Available from http://home.inje.ac.kr/~lecture/immunology/immch2immtissue/immch2.htm) [Accessed on 28th October 2008]

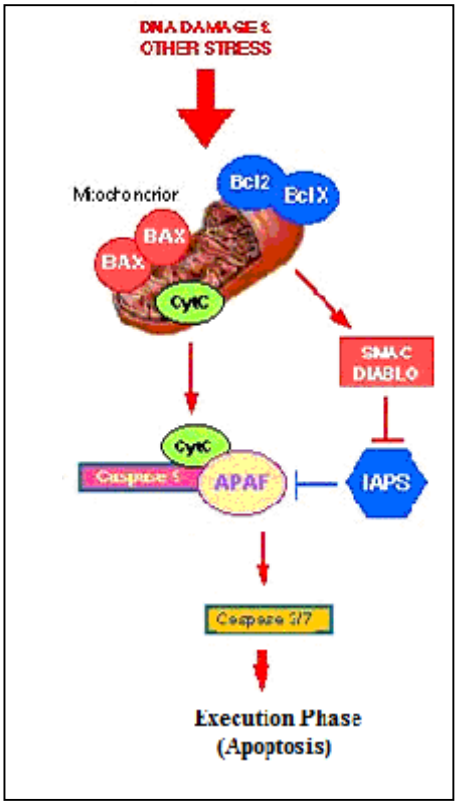

Figure 1.5: Apoptosis activation by intrinsic or extrinsic pathways.

(Available from http://www.apoptosisworld.com) [Accessed on 14th October 2008]

1.1.3.1 Mediation of ROS induces apoptosis

Reactive oxygen species (ROS) is a natural byproduct of the normal metabolism of oxygen in the electron transport chain (Boveris, 1973; Turrens, 2003). During the electron mobilization, although the oxygen reduction by cytochrome oxidase at complex I and III is almost orchestrated with great rapidity and precision, 1-4% electron may occasionally escape or leak. Normally, electron within each orbital of oxygen atom is paired with the same spin of electron.

The leakage of the unpaired electrons will straightly react with oxygen by joining the electrons of the different spin causing the oxygen to be partially reduced and formed reactive oxygen species, primarily superoxide anion (O_2^{-}•) (Figure 1.6).

At moderate, the elevation of reactive oxygen species (ROS) was known to participate in mediating cell signaling (Green and Martin, 1995; Higuchi *et al.,* 1998; Suzuki *et al.,* 1999; Moon *et al.*, 2002). The formation of ROS may reduce cytochrome C in the intermembrane space, or converted to oxygen and H_2O_2 in both matrix and intermembrane. Bcl-2 protein at the surface of the mitochondria membrane will detect these damages and activates protein Bax, a pro-apoptotic protein, and punch holes at the mitochondrial membrane to release the cytochrome C (Yang *et al*., 1997; Shimizu *et al*., 1999). As described in Section 1.1.3, the released cytochrome C will induce the cell signaling.

1.1.3.2 Key elements of Apoptosis

The Bcl-2 family is among the critical proteins besides *ced*-9 gene, *c-myc* gene and tumor suppressors such as p53 that regulate the release of pro-apoptotic factor, cytochrome C (Oltvai *et al*., 1993; Korsmeyer *et al*., 1993; Craig, 1995; Desagher and Martinou, 2000) and governed the machinery of the cell death pathway (Boise *et al*., 1993; Eissa, 1995; Parton *et*

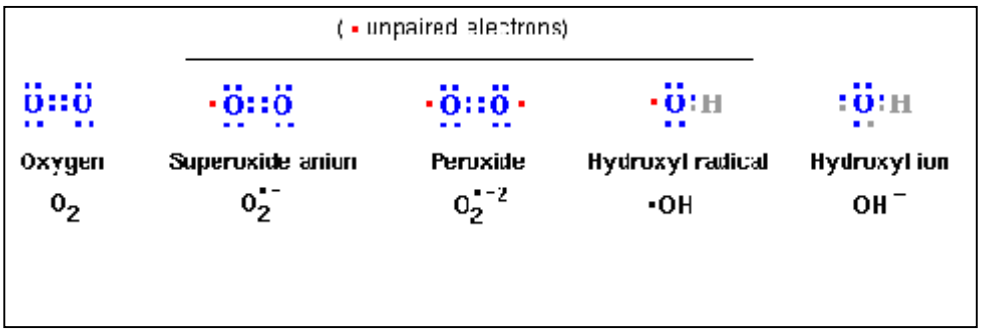

Figure 1.6: Reactive oxygen species formed by unpaired electrons.

(Available from http://www.arbl.cvmbs.colostate.edu/hbooks/pathphys/misc_topics/radicals.html) [Accessed on 27th December 2008]

al., 2001). The Bcl-2 (B-Cell Lymphoma/ Leukemia-2) gene is the pioneering prototype of a large family of pro-apoptotic such as Bax, Bak, Bad, Bcl-Xs, Bim and Bid, and antiapoptotic factors such as Bcl-2, Bcl-X, Bcl-W that directly or indirectly regulate apoptosis (Oltvai *et al*., 1993; Harrington *et al*., 1994; Reed, 1996).

Bcl-2 protein is the first anti-apoptotic members of Bcl-2 family identified (Vaux *et al*., 1988). Bcl-2 resides in the cytoplasmic surface of outer mitochondrial membrane as well as in endoplasmic reticulum and nuclear membrane (Monaghan *et al*., 1992; Krajewski *et al*., 1993; Boise *et al*., 1993; Liu *et al.*, 1996).

Bcl-2 consists of BH1-4 domains which can homodimerize with itself or forms heterodimerizers with a homologous protein (Yin *et al*., 1994; Hanada *et al*., 1995). The pro-apoptotic members are separate into two structurally distinct subfamilies; multidomain proteins for example Bax and Bak that consist of BH1-3 domains, and BH3-only proteins such as Bim and Bid. Bax, which is structurally similar to the anti-apoptotic proteins, is translocated to the mitochondria prior to apoptosis induction, where it disrupts the mitochondrial membrane causes the efflux of cytochrome C (Liu *et al*., 1996; Wolter *et al.*, 1997; Adams and Cory, 1998; Gross *et al*., 1999).

The regulation of apoptosis by the Bcl-2 family in response to apoptotic stimuli is defined in 3 different models as propose in Figure 1.7. First, BH-3 only protein will bind to and neutralizes the anti-apoptotic Bcl-2 which will then releases Bax and Bak. Second, BH3-only protein displays a selective binding to specific Bcl-2 family and since more than one BH3-only protein are needed to initiate the releasing of pro-apoptotic proteins, excessive Bcl-2 may inhibits apoptosis by antagonizing the activated conformation of Bax, preserves Bax in an inactive conformation and prevents the translocation of the pro-apoptotic protein to the mitochondria. Third, BH3-only protein may directly bind to and activates Bax protein.

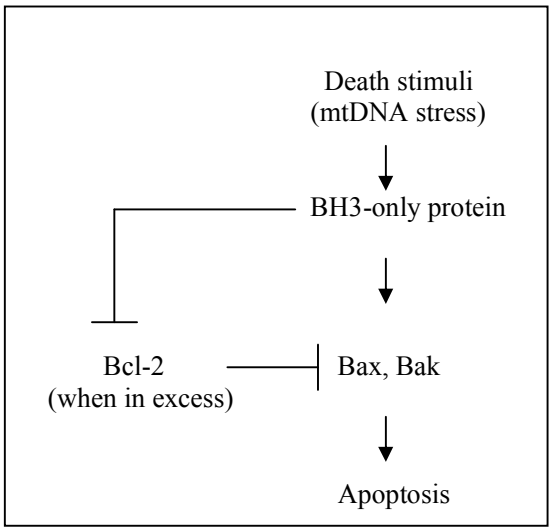

Figure 1.7: Regulation of apoptosis by Bcl-2 family proteins. Death stimuli responses results in activation of BH3-only protein, which will antagonize with Bcl-2 protein or directly activate Bax and Bak. Dimerization of activated conformation of Bax and Bak with Bcl-2 disrupts mitochondrial membrane potential causes the efflux of cytochrome c and activate the death pathway. Opposed function took place when excessive Bcl-2 occurred where Bcl-2 protein will associates with the activated conformation of Bax and block the flux of cytochrome C.

The conformational changes of Bax and Bak, enables them to integrate into mitochondrial membrane, allowing various degrees of dimerization with Bcl-2 proteins and results in release of pro-apoptotic factor, cytochrome C which leads to activation of caspases pathway followed with cell death (Oltvai *et al*., 1993; Wolter *et al*., 1997; Shore and Viallet, 2005).

The ratio between anti- and pro-apoptotic proteins is crucial to dictate cell's sensitivity towards apoptosis (Green and Martin, 1995; Komaki *et al.,* 1998; Parton *et al.*, 2001). Over expressed of Bax counters the death repressor action of Bcl-2 and accelerates apoptosis (Oltvai *et al*., 1993; Hunter and Parslow, 1996) while over expressed of Bcl-2 will induce cell survival that leads to carcinogenesis (Beger *et al.*, 2002; Tombor *et al.*, 2003; Zunfu *et al.*, 2005). In humans, Bcl-2 has been a good prognostic feature in breast cancer since it was expressed in about 80% of breast cancers. This is supported by a study by Bargou *et al*., (1995), showing that levels of pro-apoptotic protein, Bax, were reduced in breast tumor compared to the normal tissue whereas an appropriate balance of pro-apoptotic and anti-apoptotic proteins is present in normal tissue.

1.1.4 Mitochondria DNA dysfunction

Alterations in mtDNA genes or in nuclear genes, that are most likely controlling mtDNA replication and encode numbers of mitochondrial enzyme, will interrupt and inhibit their normal functions. Most mtDNA mutations which lead to mitochondrial dysfunction is increasingly recognized as an important cause of human pathology such as in cancers, since 80% of mtDNA codes functional proteins (Zeviani and Di Donato, 2004; Taylor and Turnbull, 2005; Nomura *et al*., 2007).

Mitochondrial DNA is more prone to somatic mutations. The accumulation of somatic mutations may induce mitochondrial exposure to ROS and leads to errors in the mtDNA-encoded polypeptides which later affect the complexes of the mitochondrial electron

transport chain (Yakes and Van Houten, 1997; Droge, 2002). Under normal conditions where the rates of ROS production and scavenging capacity are essentially constant and in balance, cell and tissues are in stable state. However, oxidative stress generated by environmental factors, will increased the leakage of ROS. Practically, amino acids may serve as target of oxidative attack of ROS and caused modifications of proteins. mtDNA is vulnerable to the effect of these molecules and has limited ability to repair itself due to the lack of histone proteins and mechanism of repair. Therefore, the excessive formation and continuous accumulation of ROS contribute to a 'vicious cycle' and buildup of additional mtDNA mutations. The chronic, persistent and extensive ROS exposure may directly or indirectly damage cellular macromolecule. This could lead to cellular stress response and inhibit the cell apoptosis, which is essential for tissue development and homeostasis (Yakes and Van Houten, 1997; Higuchi *et al*., 1998; Bulkley, 2002). Several findings showed that defects and reduction in the apoptosis threshold can extend cell life span, contributing to continuous proliferation that may leads to cancer development (Thompson, 1995; Reed, 1996; Reed, 1999; Lebedeva *et al.,* 2002; Shore and Viallet, 2005). However, the exact role of mtDNA mutations in inhibiting apoptosis either by suppression of pro-apoptotic genes or by activation of anti-apoptotic genes has not been defined (Carew and Huang, 2002).

1.1.4.1 Mitochondria DNA dysregulation in cancers

The role of mitochondria in cancer development has been in question recently. The subsequent accumulation of mtDNA mutations have been suspected in the initiation of carcinogenesis (Penta *et al.*, 2001; Verma and Kumar, 2007). Different mtDNA mutations have been identified in different tumor tissues and cell lines, including cancers of bladder, head and neck (Fliss *et al*., 2000), cervical (Sharma *et al*., 2005), pancreatic, colorectal (Aral *et al*., 2006), breast (Tseng *et al*., 2006), lung (Jin *et al.,* 2007), prostate (Petros *et al*., 2005) and gastric carcinomas (Carew and Huang, 2002). About 48% of mitochondrial instabilities have been found in 40 pairs of breast cancer tissues (Richard *et al*., 2000). Substantial data

suggested most of the mitochondrial aberrations resided in the mtDNA-encoded complexes I, III, IV, V as well as in the hypervariable regions (Penta *et al*., 2001).

1.2 Cancer

Cancer was known to exist since the early beginning of human history development of human evolution based on the written record and inspection done on mummies (Pardee and Reedy, 1986). The term *cancer* was coined by Hippocrates in the fifth century BC, describing a family of diseases in which a cell population divides beyond normal process without restriction until the normal cell space became confluent and metastasize to distant areas of the body (Coupal, 1927; Kardinal and Yabro, 1979; Gallucci *et al*., 1985). Cancerous cells can arise in a variety of tissues and organs, and each of these sites contains different cell types that may be affected. Cancers are usually classified according to the tissue from which the cancerous cells originated (Pardee and Reedy., 1986).

1.2.1 Breast Cancer

Breast cancer is a cancer of breast tissue. The breast is a collection of glands (also known as lobule) and fatty tissue that lies between the skin and the chest wall. The milk glands of each breast are divided into 15 to 20 lobes, each possessing a duct that runs separately into the nipple. Typically, most common types of breast cancer originate from the epithelial cells of the milk glands (ductal carcinoma) or lobe of the milk glands (lobular carcinoma). Breast cancer is the most common malignancy and the second most fatal cancer in women.

In Malaysia, cancer is a major morbidity and mortality concern. Based on National Cancer Registry data, Malaysian population is estimated to bear a cancer burden of about 40,000 new cases per year (Looi *et al*., 2004). Breast cancer is one of the most prevalence cause of

cancer death in Malaysia (Hisham and Yip, 2004; Aina, 2008). The incidence rate for breast cancer was estimated approximately 1 in 1327 in 2006 and the incidence continues to rise (Yip *et al*., 2006). Breast cancer composed 30.4% of all female cancers with 1 in 9 of Malaysian female having risk for the cancer (Lim *et al*., 2003). This cancer seems to be predominant among Chinese followed by Indians and Malays (Omar *et al*., 2006). A research showed that family history is among factors that significantly associated with breast cancer in Malaysian females but do not associated with age (Norsa'adah *et al*., 2005).

1.2.2 Mutation studies in Breast Cancer

Breast cancer tends to cluster in families. This clustering can be explained by inherited mutations on specific genes that confer high risk of disease (The Breast Cancer Association, 2006). Several alterations and mutations on certain gene and protein have been conferred in nuclear and mitochondrial genome with breast cancer susceptibility (Kristensen and Borresen-Dale, 2000; Richard *et al.*, 2000; Mimori *et al.*, 2002; Beckmann *et al.*, 2007; Oldenburg *et al.*, 2007). A total of 87.5% of nuclear and mitochondrial DNA instabilities were found in breast cancer patients (Richard *et al.,* 2000). Today, there is a broad agreement that there are many genes involved in the process of breast carcinogenesis. The risk for breast cancer increases with each relevant genetic alteration present. Few definitive common susceptibility alleles have been associated with breast cancer including the *BRCA 1* and *BRCA 2*, which are known as the tumor suppressor genes, reside in the nuclear DNA and widely expressed in many tissues (Macdonald *et al.,* 2004; Oldenburg *et al.*, 2007). Mutations at protein p53 and the 'guardian of the genome' TP53 were also frequently studied in association with breast cancer (Buyru *et al.*, 2007; Singh *et al*., 2007).

Alterations or mutations of mtDNA are now emerging as new molecular markers for detecting a variety of cancers (Tseng *et al*., 2006; Levenson, 2007). Various mutations cited both in the non-coding and coding region of the mitochondrial genome showed risk towards

breast cancer (Tseng *et al.*, 2006; Wong *et al.*, 2007). A study by Zhu *et al.,* (2005), showed that most mutations and polymorphisms in breast cancer patients are located in the coding region in contrast with a study by Tan *et al*., (2002) which showed that most mutations and polymorphisms occurred in the D-loop region. Deletion at 4977 bp and D310 polymorphism (a polynucleotide extending from 303-315 nucleotide positions within the non-coding region) have been identified as the most frequent mutation hotspots found in malignant breast tissue (Bianchi e*t al.,* 1995; Tan *et al.*, 2002; Zhu *et* al., 2005; Aral *et al.*, 2006). Several studies reported the association of mtDNA 10398 polymorphism (substitution of A allele to G allele at np 10398) with breast cancer in several populations (Canter *et al*., 2005; Darvishi *et al*., 2006).

Despite the established role of the identified genes in both nuclear and mitochondrial DNA in breast cancer susceptibility, the list of genetic factors involved in this disease is still incomplete and more effort is needed to identify more bio-markers and genetic alterations that would allow the selection of individuals with increase risk for intensive screening, prevention and treatment programs (Kato *et al*., 2001; Esserman *et al*., 2007; Levenson, 2007).

1.2.3 Mitochondria DNA 10398 polymorphism

There are 2 major respiratory regions where ROS are produced, which are complex I and III of the mitochondrial electron transport chain subunits. An A to G polymorphism at np 10398 has resulted in a nonconservative amino acid substitution of threonine (encoded by the A allele) to alanine (encoded by the G allele) within the NADH dehydrogenase (ND3) subunit of Complex I (Anderson *et al.,* 1981: Kazuno *et al*., 2006). This polymorphism has also been reported in altering both mitochondrial pH and intracellular calcium levels (Kazuno *et al*., 2006; Kazuno *et al*., 2008), which have been associated with the modulation of ATP

production and apoptosis (Bernardi, 1992). The structural alteration and impairment of Complex I are associated with the increased production of free radicals and increased risk of several mitochondrial disorders such as Parkinson's disease (Shoffner *et al*., 1993; Kosel *et al*., 1998; Van der Walt *et al*., 2003) and bipolar disorders (Kato *et al*., 2001). This polymorphism in combination with other polymorphisms has showed significantly increased risk in patients with diabetes (Bhat *et al*., 2007; Rai *et al*., 2007) and also been reported in thyroid carcinomas (Yeh *et al*., 2000).

The association of mitochondrial DNA 10398 polymorphism with breast cancer, was first studied by Canter *et al*., (2005) and had shown that the risk of invasive breast cancer was significantly higher for African-American women carrying the 10398A allele than non-carriers. However, no association was observed among Caucasians. This result is also in concordance with a study by Mims *et al*., (2006) in African-American men of prostate cancer. The association of this polymorphism with risk towards breast cancer was also presented in a study done on Indian population. This study had shown that the 10398A allele carrier having risk towards breast cancer and esophageal cancer (Darvishi *et al.,* 2006). In a recent study carried on a larger sample group of the African-American women, no association was detected between the 10398 polymorphism and breast cancer (Setiawan *et al*., 2008). Opposite results were obtained in Polish population where the 10398G variant showed significant high percentage in breast cancer patients compared to controls (Czarnecka *et al.,* 2009). Similar results were also obtained in non-Jewish European American in combination with other variants (Covarrubias *et al*., 2008).

1.3 Single Nucleotide Polymorphisms (SNPs)

SNP is referred as sequence variation between individuals at a particular locus in the genome. A single locus may be changed by substitution, deletion or insertion of a single nucleotide. These variations are considered as SNP when at least 2 alleles have frequency of

more than 1% in a large or random population. Most SNPs are biallelic because the mutation rate at a particular locus in the genome is extremely low, which is estimated 1 SNP per every 100-300 bp throughout the human genome (Bugalho *et al*., 2002; Borsting *et al*., 2007). Currently, there are abundant of common SNPs available in each individual and 90% is in the form of point mutation (a single base change) (Carracedo and Sanchez, 2005).

In mtDNA, SNP is termed by position according to the revised Cambridge reference sequence (rCRS) (Andrews *et al*., 1999). Polymorphisms sites may confined within the coding sequence or non-coding regions of genes (Ingman and Gyllensten, 2001). SNP within coding region may not necessarily changes amino acid sequence of the protein that is produced. Changes of amino acid sequence that forms the same protein is known as synonymous or silent mutation while changes that leads to different polypeptide sequence is called nonsynonymous. These changes may either be missense (produce different amino acid) or nonsense (result in a premature stop codon). SNPs found in the mtDNA coding region however are more likely to alter the biological of a protein. They were highly abundance and have low mutation rate. Therefore, polymorphisms in coding region were seen as best candidate to be expanded as mtDNA markers (Edrogan *et al*., 2001).

1.3.1 Minisequencing analysis of SNPs

Since the discovery of polymorphisms, a diverse range of DNA typing techniques have continuously being developed (Jiang *et al.,* 2004; Endicott *et al*., 2009). The techniques include hybridization based (Jiang *et al*., 2004), enzyme based, primer extension (Li *et al*., 1999) and sequencing.

Minisequencing is a much simplified version of sequencing applied in screening and analyzing the different SNP loci within the mitochondrial genome (Quintans *et al*., 2004; Carvalho and Pena, 2005). This method is based on polymerase-mediated single base

extension of oligonucleotide primer. Minisequencing does not need high quantity of DNA templates and can withstand degraded samples (Bugalho *et al.*, 2002; Quintans *et al.,* 2004; Borsting *et al*., 2007). Minisequencing analysis such as solid-phase (Syvanen *et al*., 1990), microarray based (Liljedahl *et al*., 2004) and SNaPshot™ (Krone *et al*., 2002) are among the emerging techniques for mtDNA SNPs analyses.

SNaPshot™ is an elevated sensitivity technique for SNP screening that provides a rapid, robust and high accuracy result. The processing and data analysis is fully automated and can be easily interpreted (Krone *et al*., 2002). SNaPshot™ analysis can interrogate up to 10 SNPs at known locations on 1 to 10 DNA templates in a single tube. The primer for this analysis was designed to be just one nucleotide shorter from the polymorphic site. In multiplex reaction, the primer length differed by 2-4 nucleotides to avoid overlapping between the SNaPshot™ products on the electropherogram. The primer will be extended by a single fluorescent labeled ddNTP complimentary to the variant base in the template. Application of this technique enables rapid detection of polymorphisms and suitable as a mutation screening method for several diseases.

1.3.2 Immunohistochemistry analysis (IHC)

Detection of cell death in cells and tissues has becoming important in cancer studies as defects in the thresholds has shown to results in several diseases and cancers. Apoptosis occurs through a complex regulated signaling cascades leading to changes of cell's morphology, biochemical and molecular, thus provides many opportunities to exploit the key elements regulating apoptosis. Many assays have been developed to measures the apoptosis elements such as DNA fragmentation, membrane alterations, caspases and mitochondrial changes. The methods were successfully used to detect apoptosis in population of cells and individual cells. However, most of the methods required a large quantity of cells and not applicable to histological sections of archival paraffin-embedded