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IMPACT OF MITOCHONDRIAL DNA MUTATION ON SPORADIC BREAST CANCER IN SULAIMANIYAH PROVINCE

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Dedication

Every challenging work needs self-efforts as well as guidance and support of those who are close to our hearts:

To my beloved Havyar and Helin for their Understanding

To my Mother and father for making me who I am

My Husband Hoshyar for giving me a life time support

To my brothers and sisters

Friends and teachers

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ABSTRACT

Background and objectives:
Through history nuclear genome and its mutations were the subject of interest for cancer researchers to determine the molecular bases of carcinogenesis, however recently mitochondrial genome attracted more attention as they are more susceptible to mutations and these mutations may contribute in a mitochondrial dysfunction affecting the OXPHS resulting in excess toxic reactive oxygen species production causing further mitochondrial DNA mutations and more ROS production that may eventually affect the nuclear DNA as well. In this study non-familial (sporadic) breast cancer samples were used as a model to screen mtDNA mutation profile, identify relation of mitochondrial haplogroup and SNP to breast cancer incidence

Methods:
Whole mtDNA was extracted from 30 breast cancer tissue samples and 20 benign breast lesions as a control were amplified in four overlapping fragments using 4 forward and 4 reverse primers; sequencing was done for 20 out of the 30 cancerous tissues and all the control samples using another 19 reverse primers. Haplogrep 2.0 was used for Haplogroup identification; odds ratio was calculated as well as Chi-square and Fishers Exact test were used for calculating p values

Results:
Most common type of mutation was base pair substitution, concentrated mostly in the protein coding region, mainly of complex I (57%) and least was observed in tRNA. Sporadic mutations were significantly higher in cancer samples than the control samples with a p value of 0.000. A statistically significant association was identified between haplogroup HV and breast cancer using Chi-square (p value=0.002) and Fishers exact (p value=0.006) and Odd ratio for (HV/H) was greater than one (OR=28.00). Twenty one novel mutations were observed among the breast cancer samples 15 were in the protein coding region and almost all were asynchronous. A significant relation between incidence of SNP (A8860G) and breast cancer was identified with odd ratio greater than 1 and p value less than 0.05

Conclusions:
There is a significant relation between cancer and sporadic mtDNA mutations which in general affect the structure of mitochondrially coded proteins of the respiratory chain mainly complex I, as well as structure of the tRNA and rRNA impairing their proper interaction resulting in a mitochondrial dysfunction. Other important findings in this study are the significant incidence of breast cancer among the mitochondrial haplogroups (HV) and relation between SNP A8860G and breast cancer in the current study's population

Key words: breast cancer, mtDNA, haplogroup, SNP, complex I

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LIST OF ABBREVIATION

8-OHdG	8-hydroxy-2′-deoxyguanosine
ACO2	Aconitase
ADP	Adenosine diphosphate
AMH	anatomically modern human
ATP	Adenosine triphosphate
BER	Base excision repair
BH3	BCL-2 homologues 3
BMI	Body mass index
BRCA 1 and BRCA2	Breast cancer susceptibility 1 and 2
C I, II, III, IV and V	Complex 1-5
CK	Cytokeratin
CL	Cardiolipin
CMA	Chaperon mediated autophagy
Co1-3	Cytochrome C oxidase
Cox	Cytochrome oxidase
CS	Citrate synthase
DBD	DNA-binding domain
DCIS	Ductal carcinoma in situ
DDR	DNA damage response
Dnm2	Dynamin2
Drp1	Dynamin-related protein 1
DSBs	Double strand breaks
EA	European Ancestry

EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ER	Estrogen receptor
ETC	Electron transport chain
ETS	Environmental tobacco smoke
FAO	Fatty acid β-oxidation
FH	Fumarate hydratase
HD	helical domain
Her 2	Human epidermal growth factor receptor 2
HR	Homologous recombination
HRT	Hormone replacement therapy
HSP	heavy-strand promoter
HV1, HV2, HV3	Hypervariable region 1, 2, 3
IARC	International agency of research on cancer
IDH	Isocitrate dehydrogense
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
IMM	Inner mitochondrial membrane
LCIS	Lobular carcinoma in situ
LOH	Loss of heterozygosity
LSP	Light strand promoters
MCU	Mitochondrial calcium uniporter
MDH	Malate dehydrogenase

Mfn1	Mitofusine1
Mfn2	Mitofusine2
MMEJ	Microhomology-mediated end joining
MOMP	Mitochondrial outer membrane permeabilization
mtDNA	Mitochondrial DNA
mtDNA-CN	Mitochondrial DNA copy number
Mt-LSU	Mitochondrial large subunit
mtSNP	Mitochondrial single nucleotide polymorpism
mtSSB	Mitochondrial single-stranded DNA-binding protein
Mt-SSU	Mitochondrial small subunit
NAD	Nicotinamide adenine dinucleotide
NATs	N-acetyltransferase
nDNA	Nuclear DNA
NER	Nucleotide excision repair
NGS	Nottingham Grading System
NGS	Next-generation sequencing
NHEJ	Non-homologous end joining
NLSs	Nuclear localization signals
NOS	Not otherwise specified
OB	oligonucleotide/oligosaccharide-binding
O_H	Heavy chain origin of replicaation
O_L	Light chain origin of replication
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1

OR	Oral contraception
OXPHOS	Oxidative phosphorylation
PA	Phosphatidic acid
PAH	Polycyclic aromatic hydrocarbons
POL γ	Polymerase γ
POLRMT	Mitochondrial RNA polymerase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RING	Really Interesting New Gene
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SNP	Single nucleotide polymorphism
SOCE	Store operated Calcium Entery
SOD	Superoxide dismutase
SSBR	Single strand break repair
ssDNA	Single strand DNA
TCA	Tricarboxylic acid
TEFM	Mitochondrial transcription elongation factor
TNBC	Triple-negative breast cancer
TOM	Translocases of the outer membrane
TSGp	Tumor suppressor gene
UCP	Uncoupling proteins
α -KGDH	α -ketoglutarate dehydrogenase

LIST OF AMINOACID ABBREVIATIONS

Ala	Alanine
Arg	Arginine
Asn	Aspargine
Asp	Aspartic acide
Cys	Cysteine
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proleine
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

INTRODUCTION

INTRODUCTION:

According to the GLOBOCAN 2020 data, breast cancer is one of the most diagnosed cancers and the 5th cause of cancer-related deaths with an estimated number of 2.3 million new cases worldwide (Sung et al., 2020). Breast cancer incidence is rising in our locality as well and being the top recorded type of cancer in Iraq according to Iraq Cancer Registry (Al Alwan, 2022). By definition breast cancer is a complex, heterogeneous disease of various clinical, histological and molecular features (Leong and Zhuang, 2011), and over the past decade, major progresses have been made regarding molecular mechanisms responsible for breast carcinogenesis, since nuclear genome was in the center of the proposed molecular models (Aaron et al., 2011; Russnes et al., 2017), as a result several susceptibility genes accusable of breast carcinogenesis were identified with various penetrance potentials; however only 5-10% of breast cancers can be explained by these susceptibility genes, yet the bulk is explained by non-genetic factors causing somatic mutations occurring during life time as a result of intrinsic cellular errors or extrinsic environmental insults. Among the well-known susceptibility genes with high penetrance potential were *BRCA1* and *BRCA2*, still these are implicated in only 20-25% of hereditary breast cancers (van der Groep, van der Wall and van Diest, 2011).

During the past 30 years, interest of the molecular studies was deviated towards the mitochondrial genome, as a contributor in the carcinogenic process. Otto Warburg, a German physiologist, medical doctor, and Nobel laureate (Krebs, 1972) was the first one who accused malfunctioning mitochondria of malignant cell behaviour as he observed that cancer cells show increased glycolysis and lactic acid production with reduced oxygen consumption a state, he called aerobic glycolysis (Koppenol, Bounds and Dang, 2011).

Mitochondrial DNA (mtDNA), the second and smaller cellular genome, is a double stranded circular molecule present within the matrix of mitochondrion in form of 10^3–10^4copies, composed of 16569 bp, encodes 37 genes, for 13 essential proteins for the respiratory chain subunits, 2 rRNAs (12S and 16S) and 22 tRNAs (Jime'nez-Morales et al., 2018). Furthermore, a non-coding region is also present which is composed of 1100 bp, contains the promoters for mtDNA transcription and the origin of replication (Dalla Rosa et al., 2017).

An intact Mitochondrial DNA is essential for a proper cellular respiration, ATP production through the OXPHS process, modulation of oxidation–reduction (redox) status, maintaining balanced level of ROS, controlling cytosolic calcium and apoptosis. Evidently distortion in any

of these activities may convert a stable, terminally differentiated cell to an actively dividing malignant cell (Wallace, 2012).

Nevertheless, mutations are common in mtDNA and these mutations can be the result of intrinsic cellular errors during DNA replication or repair or it could be the result of exposure to environmental mutagens and its unquestionable that the rate of mutation in mtDNA is several times higher than that of the nuclear DNA (Li et al., 2016); this is because mtDNA lack the protective histone proteins found in nuclear DNA, have limited DNA repair system, and lack introns, furthermore these genomes are in a close proximity to the electron transport chain which continuously emits reactive oxygen species(ROS) (Jime'nez-Morales et al., 2018).
Accordingly it's proposed that mutagenic environmental exposures will affect the mtDNA more than the nuclear DNA, resulting in a faulty OXPHOS with excess ROS production that affects replication and transcription of mtDNA, resulting in a decline in mitochondrial function which in turn leads to enhanced ROS production, predisposing to further mtDNA mutation and mitochondrial dysfunction (Cui, Kong and Zhang, 2012); and this may activate cytosolic signalling (retrograde signalling) pathways that will eventually change nuclear gene expression (Hsu, Tseng and Lee, 2016).
Furthermore, neoplastic transformation and cancer progression are highly affected by mitochondrial retrograde signalling that is greatly affected by levels of reactive oxygen species, Ca^{+2} and oncometabolites (Hsu, Tseng and Lee, 2016).
Point mutations and copy number changes are the two most common mitochondrial DNA alterations observed in cancers (Lee, Chang and Chi, 2010). However, one important point to clarify is that mtDNA mutations show phenotypic effects only when the mutant variants of mtDNA are the dominant copy, as every cell contains many (thousand) copies of mtDNA (Patananan et al., 2016).
In addition, human mitochondrial DNA is regarded as a rich source of genetic data in human evolution and classification of population genetics, as it is maternally inherited, rapidly evolving, non-recombining and present in a high copy number per a cell (Kivisild, 2015). Worldwide studies revealed the presence of significant differences (variation) in mtDNA among populations of different geographical regions as a result of natural selection. (Kloss-Brandstatter et al., 2010) Accordingly the mitochondrial haplogroups models were reconstructed (Ingman et al., 2000).

In the current study, sporadic breast cancer samples were used to minimize the effects of hereditary susceptible nuclear genes and to screen for possible breast cancer related sporadic mitochondrial DNA mutation, as well as to identify mitochondrial haplogroup and Single nucleotide pleomorphism that are risky for breast cancer development

CHAPTER -1

LITERATURE REVIEW

1.1. BREAST CANCER

1.1.1. Epidemiology and background of breast cancer:

Breast cancer is the commonest malignancy and leading cause of cancer death among women in both economically developed and developing countries (Larsen et al., 2014). According to the American cancer society 2022, there will be an estimated 287,850 (31%) new cases of invasive breast cancer and an additional 51,400 cases of in situ ductal carcinoma (DCIS) diagnosed in women. Female breast cancer incidence rates have been increased slowly by about 0.5% per year since the mid-2000s, with an estimated 43,250 breast cancer deaths (15%) in 2022 (American Cancer Society, 2022). This increase in the incidence rate was observed among Iraqi women as well with an Annual Percent change (APC) of breast cancer of 3.192 (Al-Hashimi, 2021).

Breast cancer develops because of malignant proliferation of the milk-secreting glands (acini) or from the ducts transferring them to the nipple. Less commonly breast cancer may originate from supporting stromal tissue, fibrous and adipose tissue (Feng et al., 2018).

1.1.2. Anatomy and Histology of breast:

Breasts (mammary glands) are bilateral unique organs in mammalian species; they are epidermal appendages, derived from the apocrine glands with a specific function in mammalians which is the production of milk for the nourishment of newly bourn offspring's (Lteif and Javed, 2013).

Histologically human breast consists of parenchyma and stroma, originating from ectodermal and mesodermal elements respectively. Breast parenchyma forms a branching system of ducts (ending as terminal ducts surrounded by clusters alveoli) and a stroma consists mainly of a supporting adipose tissue. Terminal ducts and alveoli form lobules, and are lined by two layers of epithelial cells, luminal and basal intermingled with myoepithelial layer resting on a basement membrane. These building blocks of breast are identified during the embryonic stage of human development (Lteif and Javed, 2013). Mammary gland undergoes profound architectural, structural, and functional modulations throughout different physiological stages of life because of change in gene expression and hormonal influence, and while most body organs get into a nearly mature state at birth, mammary gland reaches its mature functional state only during the pregnancy and lactation cycle (Hassiotoul and Geddes, 2013).

Lobules are formed within 1–2 years after onset of the first menstrual period (lobule type 1), while differentiation completion of lobule types 2, 3, and 4 is a progressive process of new

alveolar sprouting, occurs over many years. They increase in number from approximately 11 in lobule type 1 to 47 and 80 in lobules type 2 and type 3, respectively (Russo and Russo, 2014), (Fig.1-1). Mammary gland reaches its maximum developmental stage during pregnancy, in which the distal elements of the ductal system proliferates resulting in the formation of ductules that are called acini at this stage, and hence progression of a lobule type 3 into a lobule type 4 with an increase in number of the epithelial cells and their cytoplasmic size (Russo and Russo, 2014).

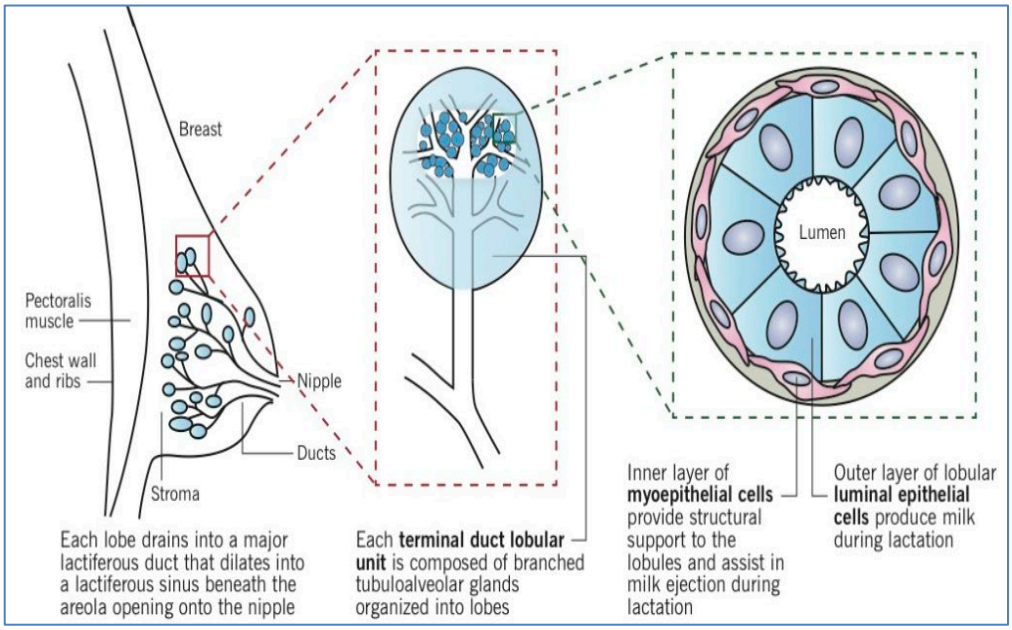

Figure 1-1: Structure of the mammary tissue (PANDYA and MOORE, 2011)

1.1.3. Classification of breast cancer:

Breast cancer involves a complex heterogeneous group of diseases with different clinical, histological, and molecular properties and this heterogeneity could be the result of genetic, epigenetic and transcriptom changes (Eliyatkin et al., 2015). To sort the heterogeneity of breast cancer many classification systems are developed to categorize breast cancer and these classification systems are in a continuous evolution to give an optimal aid in breast cancer therapy and prognosis (Malhotra et al., 2010)

During the beginning of the last century all breast cancer patients were treated as one group with a uniform treatment but different patient's response to these treatments with different prognosis, made pathologists start identifying different morphological types and led to development of the traditional classification system (Eliyatkin et al., 2015).

Traditional breast cancer classification: The old traditional breast cancer classification depends on morphological architecture and histological detail, according to which breast cancer can be divided broadly into lobular carcinoma when the tumor arises from the lobular epithelial cells and ductal carcinoma when tumor arises from the ductal epithelial cells. Each one of these tumours (lobular and ductal carcinoma) is further subdivided in to non-invasive (in situ) and invasive tumours (Makki, 2015).

In the recently published WHO classification (2019) breast tumors in general are divided in to epithelial, mesynchymal, mixed epithelial and mesynchymal, neuroendocrine and hematolymphoid tumors; the epithelial tumors in turn are divided in to non-invasive (in situ lobular and ductal), microinvasive and invasive breast carcinoma (Agarwal and Blanco, 2022)

Epithelial breast cancers:

A-In Situ (Non-Invasive) Mammary cancer:

1-Ductal carcinoma in situ (DCIS): DCIS is malignant ductal epithelial cell proliferation that is limited to the epithelial component without stromal invasion. The incidence of DCIS is much higher than that of lobular carcinoma in situ (LCIS). It is considered a precursor of invasive ductal carcinoma. In these ducts myoepithelial cells are preserved however they may show some attenuation or a decrease in number (Malhotra et al., 2010). On the bases of their architectural pattern DCIS are further subdivided in to comedo, cribriform, solid, micropillary and papillary (Malhotra et al., 2010).

2-Lobular carcinoma in situ (LCIS): Is an intra-lobular epithelial proliferation originating in the terminal ductal-lobular unites; in general, they are composed of uniform, small, loosely cohesive cells (Makki, 2015). Lobular carcinoma in situ (LCIS) is regarded a risk factor and a non-obligate precursor of breast cancer with a relative risk of invasive carcinoma being approximately 9-10 times that of general population (Reed et al., 2015; Wen and Brogi, 2018).

B-Invasive Mammary Carcinoma:

Invasive carcinoma includes a large heterogeneous group of breast cancers with different cyto-architectural features classifying them into different subgroups, among which infiltrating duct carcinoma or not otherwise specified (NOS), this is the commonest and constitutes about 40-75% of all breast cancers (Makki, 2015). NOS breast cancers include all tumours that do not

have a distinctive morphological feature or behaviour, to categorize them into specific subclasses (Sinn and Kreipe, 2013; Russnes et al., 2017). Other types of invasive breast carcinoma account for about 25% of all breast cancers and according to the last version of the World Health Organization classification, at least 17 distinct histological special types have been identified (Viale, 2012; Yilmaz et al., 2018). The specialized types of invasive ductal carcinoma are named after their cytoarchitectural appearance, hence a tumour with a predominant tubular differentiation is named tubular ductal carcinoma and is the same regarding lobular, cribriform, mucinous, oncocytic, and so on (Russnes et al., 2017). Special types of breast cancer include the following: Lobular, Tubular, Mucinous, lipid rich, Micropapillary, Cribriform, Papillary, Apocrine, Metaplastic, Secretory, Oncocytic, Adenoid cystic carcinoma, Acinic cell carcinoma) (Sinn and Kreipe, 2013; Makki, 2015; (Agarwal and Blanco, 2022). In any of the mentioned special types, the distinct architectural pattern should comprise not less than 90% of the tumour to be called after it (Makki, 2015)

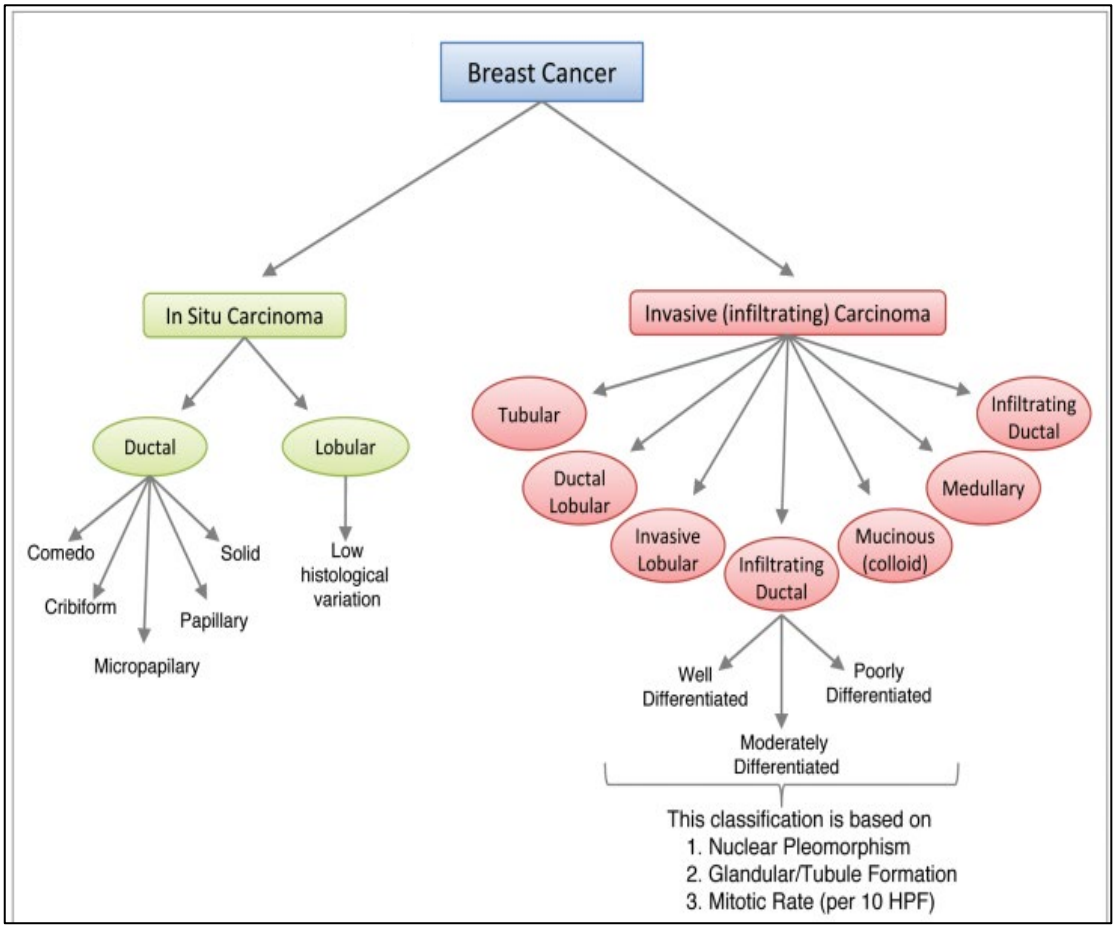

Figure 1-2: Histological classification of breast cancers (Malhotra et al., 2010)

Molecular classification of breast cancer: As it's noticed histopathological examination of breast tumor plays a crucial role in the diagnosis of breast cancer, determination of the histological type (whether it's invasive ductal carcinoma or lobular carcinoma) and to assess the histological grade. These are important parameters to guide a clinical management but with the aid of molecular analyses further diagnostic, prognostic and predictive information are obtained for a better management and prognostic outcomes (Heng et al., 2016). With the introduction of the molecular techniques as gene expression profiling a new era of breast cancer typing is developed which is the molecular classification (Eliyatkin et al., 2015)

The first molecular profiling of human breast tumor was published by Perou and colleagues in 2000 using RNA derived from 65 breast tumors with complementary DNA microarrays representing 8102 human genes and the data analysis revealed a great variation in gene expression profile, about 550 genes were identified and named the intrinsic genes (Aaron et al., 2011; Russnes et al., 2017). They had a pervasive order in the expression showing a relationship between specific gene expression and specific tumour types (Aaron et al., 2011).

According to their work 4 classes or groups of breast cancer were identified with distinct molecular features; oestrogen receptor positive/luminal-like, basal-like, HER2/*neu* positive and normal breast-like (Aaron et al., 2011; Shawarby, Al-Tamimi and Ahmed, 2013).
Recent studies have divided breast cancer in to six molecular subclasses Luminal A, Luminal B, Triple negative (basal-like), HER-2 type, Claudin-low, and normal-like (Dias et al., 2017). (Fig. 1-3) shows the summary of molecular classification of breast cancer.

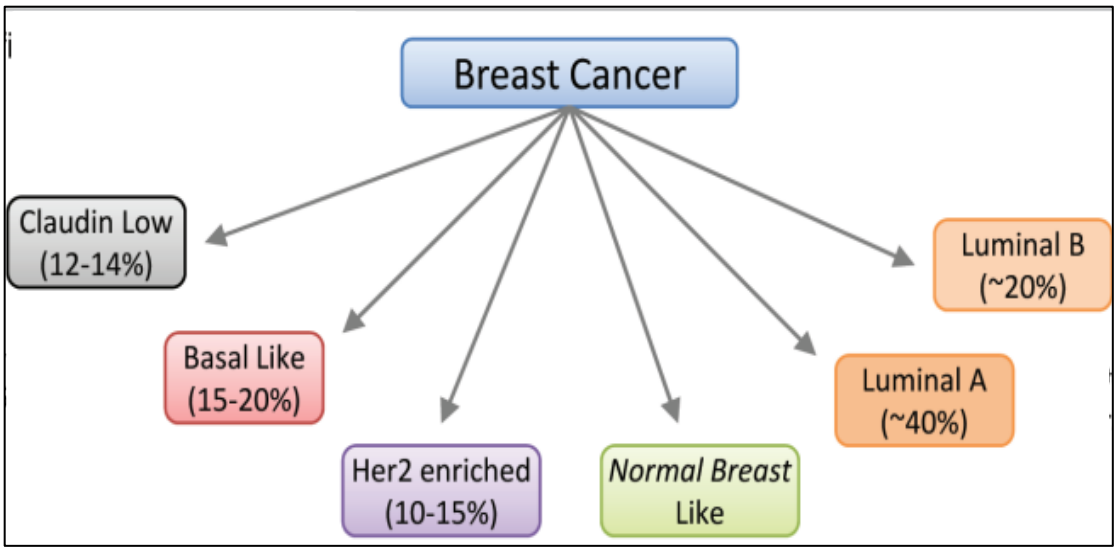

Figure 1-3: Molecular classification of breast cancers (Malhotra et al., 2010)

1) Luminal A: Luminal A is the commonest molecular subtype of breast cancer, accounts for about 40% of breast cancers. Tumors within this subtype are of low histological grade, with low mitotic activity and low degree of nuclear pleomorphism. Luminal-A subtype is defined as oestrogen receptor (ER) positive and/or progesterone receptor (PR) positive tumours with negative HER2 and low Ki67 (proliferating cell nuclear antigen) index (Yersal, 2014; Trop et al., 2014). Luminal A tumours include breast cancer of special histological type as tubular, invasive cribriform, mucinous, and lobular (Yersal, 2014; Makki, 2015)

2) Luminal-B: Luminal-B tumours account for about 20% of breast cancers, when it is compared to the Luminal A subtype, luminal B tumours are of higher histological grade with a high proliferative index, having a more aggressive phenotype and a worse prognosis (Makki, 2015; Yersal, 2014; Trop et al., 2014). Bulk of Luminal B tumors are ER+/HER2-/ with high Ki-67 expression profiles, another class of luminal B tumors are ER+/HER2+ (Bediaga et al., 2016; Fragomeni, Sciallis and Jeruss, 2018). About 30% of HER2-positive tumors are assigned to the luminal-B subtype tumours by immunohistochemistry (Yersal, 2014)
Luminal B breast tumours have a prognosis similar to that of non-luminal cancers (including the HER2−enriched and base-like subtypes) (Li et al., 2016)

3) HER2 enriched (over-expressed): HER2 enriched breast cancers account for 10-15% of all invasive breast cancers. Tumours within this group are showing overexpression of HER2/neu, high ki-67 expression and commonly TP53 mutation (Makki, 2015; Yersal, 2014).
In addition to HER2/neu gene, HER-enriched tumors show amplification of other genes in the RAS pathway which are involved in cell signalling as well and enhance cell proliferation and tumorigenesis (Trop et al., 2014).

4) Basal-like breast cancer subtype: The basal-like intrinsic breast cancer subtype represents about 15% of invasive ductal breast cancers (Badowska-Kozakiewicz and Budzik, 2016; Milioli et al., 2017). Basal-like breast cancers expression profile is similar to that of the basal-myoepithelial layer of the normal breast and hence derived their name basal-like (Kittaneh, Montero and Glück 2013; Hubalek, Czech and Müller, 2017). They express basal myoepithelial markers as CK5, CK 14, CK 17, epidermal growth factor receptor (*EGFR*) and laminin (Yersal, 2014).

On the other hand, they do not express ER, PR and HER2 (luminal cytokeratins CK8, 18, 19), hence referred to as triple-negative breast cancers (TNBC) (Milioli et al., 2017; Hubalek, Czech and Müller, 2017). American College of Pathology have defined breast cancers with less than 1% tumor cells expressing ER and PR via IHC as TNBC (Hubalek, Czech and Müller, 2017). TNBC accounts for approximately 15% to 30% of all breast cancers, and it is more associated with early recurrence and poorer prognosis than non-TNBC (Kim et al., 2017).

Approximately 75% of TNBCs are basal-like, with the other 25% comprising all other subtypes (Hubalek, Czech and Müller, 2017). Despite the interchangeably used names TNBCs and Basal-like breast cancer are not exact synonyms and at molecular levels Basal-like breast cancers are more homogenous than the immunohistochemically defined more heterogeneous TNBCs (Milioli et al., 2017).

Furthermore basal-like breast cancers are frequently showing TP53 mutation, high mitotic index Ki-67 as well as evidence of genomic instability, inactivation of the *retinoblastoma (Rb)* pathway and integrin expression defects (Badowska-Kozakiewicz and Budzik, 2016; Yersal, 2014).

5) Claudin-low breast cancer subtype: Claudin-low tumours account for 14% of all invasive breast cancers, they show low expression of genes involved in tight junctions and epithelial-epithelial adhesion as claudin 3, 4 and 7 and E-cadherin and occludin (Sabatier et al., 2014; Dias et al., 2017). In addition claudin-low breast cancers show low expression of luminal markers. Pathologic examinations have shown a high percentage of medullary-like and metaplastic tumours within this subtype (Kittaneh et al., 2013; Dias et al, 2017).

6) Normal breast-like: These tumors account for about 5%-10% of all breast carcinomas. They have the same signature as fibroadenomas and normal breast samples and express genes specific for adipose tissue. Normal breast like carcinomas lack the expression of ER, PR and HER2, they can be classified as triple-negative (Tang and Tse, 2015).

Apart from these subtypes, two more subtypes are mentioned in some references:

-Luminal C exhibiting two different clusters of gene (clusters G and cluster D) (Kittaneh et al., 2013; Dvorkin-Gheva and Hassell, 2014)

-Apocrine breast carcinoma, is a rare primary breast cancer, constitute less than 1% of all breast cancers with a distinctive genetic profile that is ER and PR negative and androgen receptor (AR) positive (Vranic, Feldman and Gatalica, 2016).

1.1.4. Histological tumour grade:

Histologic tumour grade is a simple and low-cost classification method using the degree of tumour differentiation as parameters of classification (Rakha, and Ellis, 2011)

Histologic grading as measured by the Nottingham Grading System (NGS) is based on evaluation of 3 important biology-dependent morphologic features: (i) degree of tubule or gland formation, (ii) nuclear pleomorphism, and (iii) mitotic count (Fig. 1-4); accordingly, grading of the invasive breast carcinoma is classified into a three-point scale: Grade 1 (low grade, well-differentiated carcinoma), Grade 2 (intermediate grade, moderately differentiated carcinoma) and Grade 3 (high grade, poorly-differentiated carcinoma) (Dimitropoulos et al., 2017)

Histologic grade represents the morphologic assessment of tumour biological characteristics and has been shown to be able to generate important information related to the clinical behaviour of breast cancer (Rakha, and Ellis, 2011).

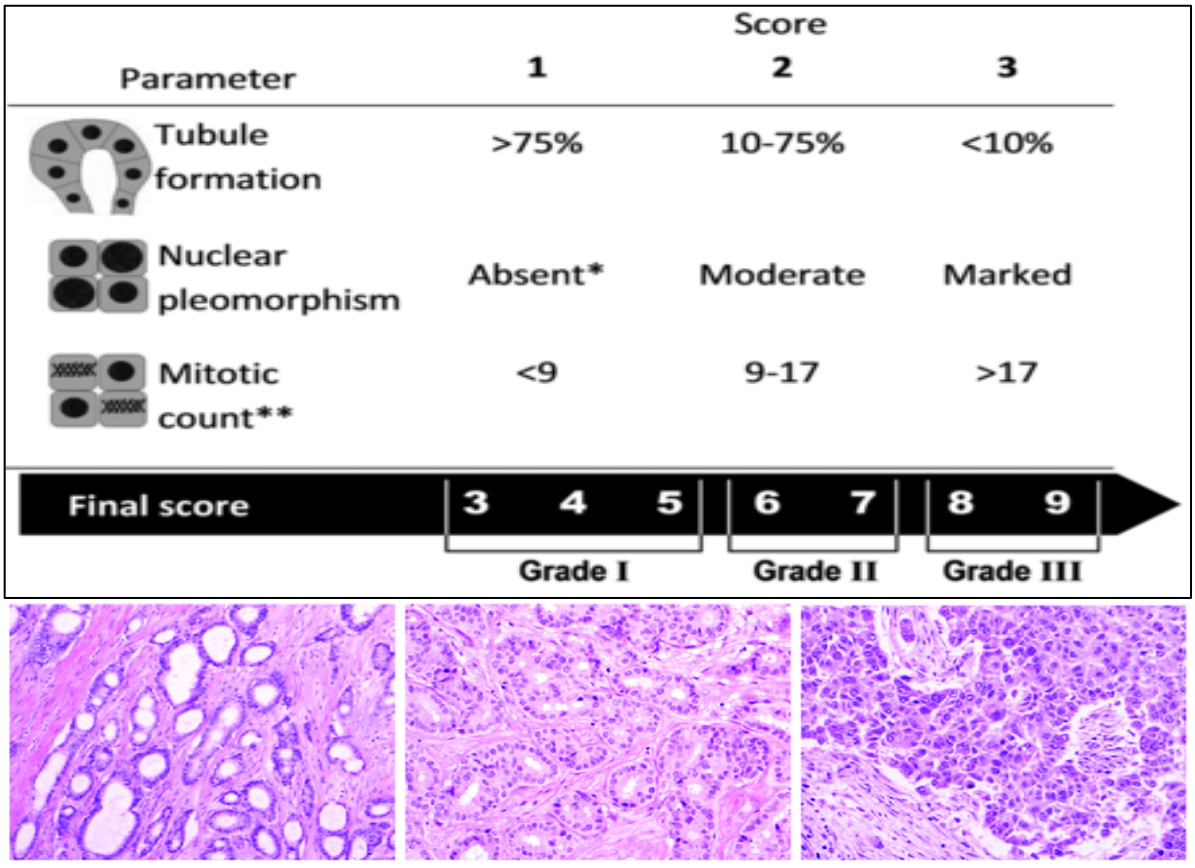

Figure 1-4: Nottingham histological grade: criteria for scoring each grading parameter (Santos et al., 2015)

1.1.5. Risk factors of breast cancer:

Breast cancer is a multifactorial disease, occurs as a result of interaction between different genetic and non-genetic factors (Mavaddat et al., 2010; Apostolou and Fostira, 2013). Approximately 5–10% of all breast cancers have a hereditary background (Larsen et al., 2014). Many factors are implicated in the development of breast cancer; broadly the predisposing causes or risk factors can be divided in to:

a. Genetic factors

b. Non-genetic (environmental) factors

A. Genetic Factors :

Several genes are implicated in causing breast cancer and are responsible for the well-known hereditary or familial breast cancer. One of the most important risk factors for developing breast cancer is hereditary or genetic factor, it was in the mid of 19th century were familial aggregates of breast cancer first reported (Shiovitz and Korde, 2015).

There is not a precise definition for 'familial' breast cancer, but some generally accepted features as: having at least three breast and/or ovarian cancer cases in a family, having two breast cancer cases in close relatives, with at least one diagnosed before age of 50, having two breast cancer cases in a family diagnosed before 40 years of age, any male breast cancer with a family history of ovarian cancer or early onset female breast cancer. Ashkenazi Jewish ancestry with breast cancer, particularly triple-negative breast cancer diagnosed before the age of 60 are categorized as familial, as well as having breast and ovarian cancer in the same patient (Shiovitz and Korde, 2015).

Approximately 10–30% of breast cancers cases are attributed to familial factors of this group only 5%–10% are identified with a strong inherited component (Apostolou and Fostira, 2013).

In general genes that are responsible for familial breast cancer are divided into high penetrance, moderate penetrance and low penetrance variants (Apostolou and Fostira, 2013; Mavaddat et al., 2010)

1. High penetrance gene; In this group familial clustering of breast cancer occurs as a result of alleles with high risk, examples in this group are breast cancer susceptibility gene BRCA1 and BRCA2 gens, PTEN, TP53, CDH1 and STK11 (Mavaddat et al., 2010).

-*BRCA1* and *BRCA2* genes:

Totally, about 20-25% of hereditary breast cancers and 5-10% of all breast cancers are caused by *BRCA1/2* mutations (Nakagomi et al., 2017; Sun et al., 2017). Mutations in BRCA1 and BRCA2 genes are associated with increased risk of breast and other cancers as ovarian cancer, leading to the development of hereditary syndromes called HBC-SS (Hereditary Breast Cancer Site Specific) or HBOC (Hereditary Breast Ovarian Cancer) syndrome, which manifests themselves in the form of breast and/or ovarian cancer (Kamińska et al., 2015). BRCA1 and BRCA2 proteins are collectively called tumour suppressor gene protein (TSGp) (Mavaddat et al., 2010; Shiovitz and Korde, 2015). Estimates in the range of 40% to 87% for *BRCA1* and 18% to 88% for *BRCA2* mutation carriers have been reported for breast cancer (Mavaddat et al., 2013).

BRCA1: BRCA1 breast cancers are usually diagnosed in young age group; pathologically they tend to be invasive ductal carcinoma with a high tumour grade, lymphocytic infiltration and 'pushing' margins (Beirne et al., 2015). It is located on chromosome 17, composed of 22 exons and codes a multi-domain protein of 1,863 amino acids (Godet and M. Gilkes, 2017). It's usually mutated in three domains or regions: the N-terminal RING domain (Really Interesting New Gene), exons 11-13 comprises 65% of BRCA 1 peptide sequence, and the BRCT (tandem BRCA1 carboxy-terminal repeats) domain (Clark et al., 2012). Many proteins that are functioning in different cellular processes bind to exon 11-13 including Rad50 and Rad 51(important DNA repair proteins), transcription factor c-Myc and cell cycle regulator Retinoblastoma Rb (Orr and Savage, 2015; Prakash et al., 2015; Mahdavi et al., 2018). Figure (1-5) shows the structure of BRCA1 gene, domains and binding proteins

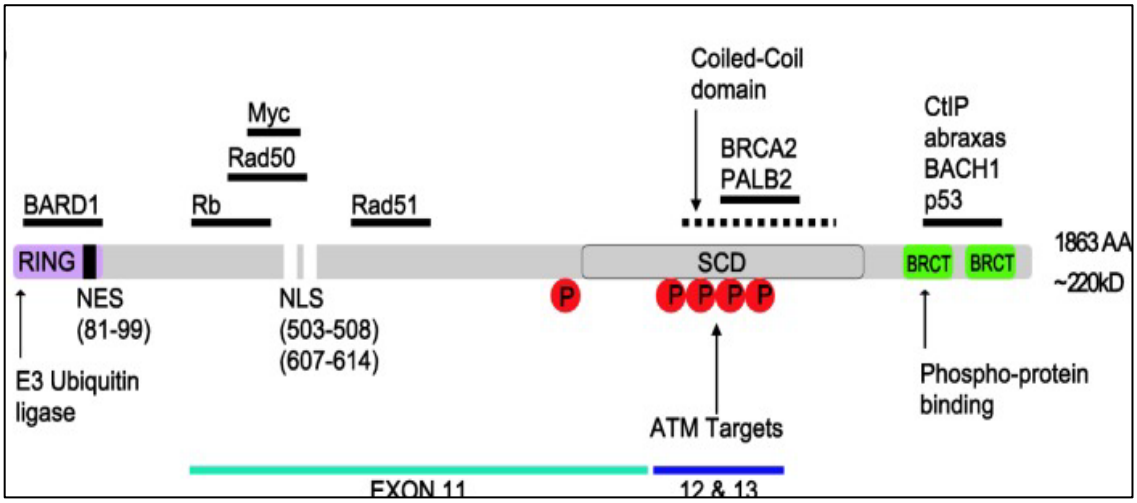

Figure 1-5: Structure of BRCA1 gene (Clark et al., 2012)

BRCA2: BRCA2 breast cancers do not have a clear histopathological feature that distinguishes them from sporadic breast cancers (Beirne et al., 2015). *BRCA2* is located on chromosome 13, consists of 27 exons and codes for a large protein of 3,418 amino acids; functional domains are BRC repeats, which consist of eight conserved motifs of about 35 amino acids, the DNA-binding domain (DBD) composed of a long helical domain (HD) and three oligonucleotide/oligosaccharide-binding (OB) folds and finally the C-terminal TR2 domain. BRCA2 is predominantly nuclear protein and its subcellular localization is controlled by two distinct nuclear localization signals (NLSs) (Fradet-Turcotte et al., 2016). Figure (1-6) shows the structure of BRCA2 gene

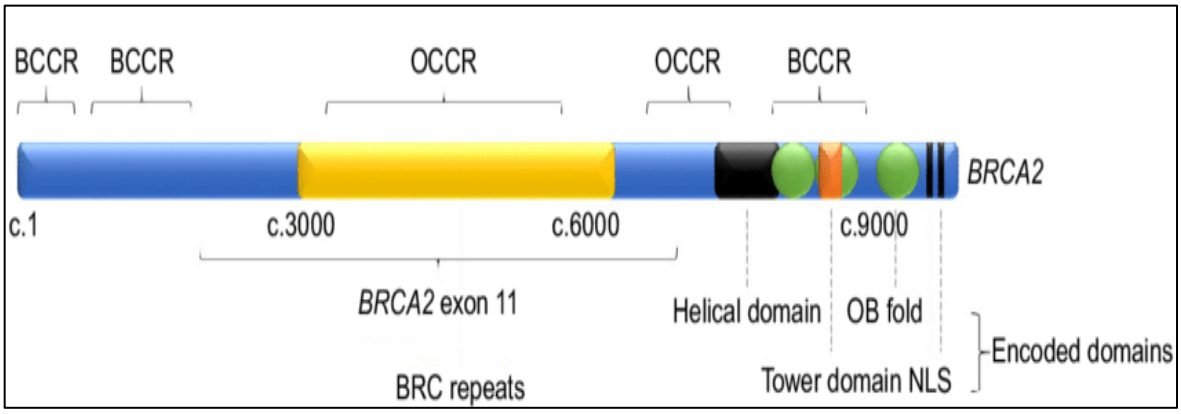

Figure 1-6: Structure of BRCA2 (Hollis, Churchman and Gourley, 2017)

BRCA1 and *BRCA2* dysfunction:

BRCA1 and BRCA2 dysfunction mostly arises from germ-line mutations, promoter methylation, and somatic mutations (Mahdavi et al., 2018). Mutations are inherited in an autosomal dominant fashion, but act recessively on the cellular level as tumour suppressor genes (Shiovitz and Korde, 2015). They have important role in ensuring genomic stability by signalling DNA damage and enhancing DNA repair (Larsen et al., 2014; Mavaddat et al., 2010). Both have a role in repairing double strand breaks (DSBs) (which can occur as by-products of DNA replication or during exposure to ionizing radiation and other genotoxic compounds) through homologous recombination (HR) and by interactions with RAD51 (Roy, Chun, and Powell, 2016; Prakash et al., 2015). The protection of the genome by HR involves damage recognition, signal mediation by CHK2 and BRCA1 and initiation of repair by the

effectors BRCA2 and RAD51 (Apostolou and Fostira, 2013; Walsh, 2015; Roy et al., 2016). Figure (1-7) Simply illustrates the steps of homologous recombination.

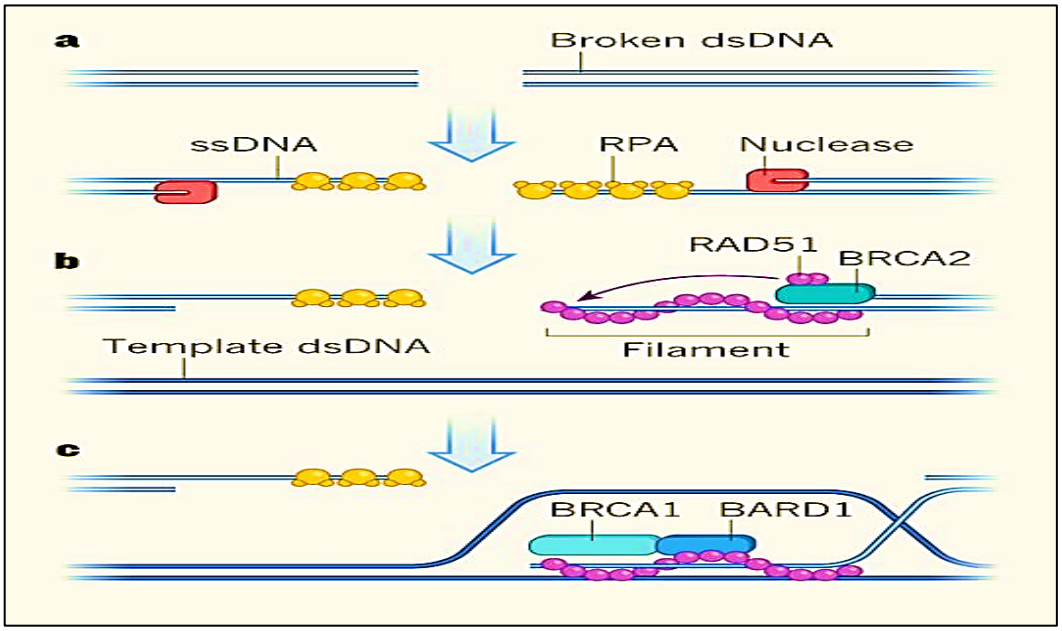

Figure 1-7: Mechanism of homologous recombination (Cejka, 2017)

So cells which are BRCA1 or BRCA2 deficient are unable to repair double strand breaks by the error-free HR, and repair will be by the error-prone non-homologous end-joining (NHEJ) pathway introducing chromosomal instability (deletions and translocations) (Venkitaraman, 2002; Prakash et al., 2015).

Mutations are detected across the entire coding sequences of BRCA1 and BRCA2. The well-known pathogenic mutations include small deletions, insertions or nonsense mutations which consequently lead to the production of truncated (non-functioning) proteins (Larsen et al., 2014; Apostolou and Fostira, 2013). Currently, more than 1700 mutations have been identified in the *BRCA1* gene of which, 858 have been confirmed as being pathologic (Godet and M. Gilkes, 2017; Mahdavi et al., 2018).

Regarding BRCA2, about 2000 mutations have been identified, that include frame shift deletions, insertions, or nonsense mutations that are leading to premature truncation of proteins (Godet and Gilkes, 2017)

One of the important factors influencing mutations with high penetrance like BRCA1 and BRCA2 and worth's mentioning is founder mutation. A founder mutation is a genetic variation identified in a high frequency (recurrently) in a group that share the same geographical and cultural background, and this genetic variation was carried by one or more of the ancestors

(Heramb et al., 2018). As a consequence, hundreds of different alterations are identified in the genomic sequence that causes the disease and the risk of cancer can vary according to the mutation, country of residence, and family history (Ossa and Torres, 2016)

Additional rare, but highly penetrant genes include PTEN (Zhang et al., 2013; Apostolou and Fostira, 2013), TP53 (Schon and Tischkowitz, 2017) and STK11 (Lipsa et al., 2019).

2. Moderate penetrance gene variants:

In this group are genes moderately increasing breast cancer risk (about two folds) and are relatively of low frequency (Shiovitz and Korde, 2015). Genes of this group are generally involved in cell cycle regulation and DNA repair. It's now accepted that these moderately penetrant genes all together with negative BRCA mutations account for <3% of familial breast cancer (Mavaddat et al., 2010; Shiovitz and Korde, 2015).

Members of this group are CHEK2, BRIP1 (BACH1), ATM, and PALB2 (Apostolou and Fostira, 2013).

- *CHEK2*:

CHEK2 is a tumor suppressor gene, encodes a serine/threonine kinase protein. This gene plays role in DNA repair, cell cycle regulation and apoptosis in response to DNA damage. Mutations in CHEK2 have been implicated in various types of cancer including breast cancer (Apostolou and Papasotiriou, 2017). Activated CHEK2 stabilizes p53 and interacts with BRCA1 (Shiovitz and Korde, 2015).

-BRIP1, ATM and PALB2

Other moderately penetrance genes as; BRIP1 gene encodes a protein that interacts with BRCA1 C-terminus (BRCT) domain (Shiovitz and Korde, 2015), ATM gene encodes for a large serine-threonine kinase that has a crucial role in detecting DNA double-strand breaks (Marouf et al., 2017). Pathogenic ATM variants are found in 1%–2% of the population and a lifetime risk of breast cancer in these individuals will be likely greater than 25% (Jerzak, Mancuso and Eisen, 2018).

Partner and localizer of breast cancer 2 (PALB2) gene, encodes for proteins participating in double strand DNA repair by interacting with BRCA2 during homologous recombination (Apostolou and Fostira, 2013). Recently, it was reported that *PALB2* carriers have a high risk of

developing breast cancer, and that by age of 70 the cumulative risk of a mutation carrier is about 34% (Nakagomi et al. 2017)

3. Low Penetrant gene:

A group of breast cancer susceptibility genes contribute to breast cancer risk in a polygenic fashion and act synergistically with environmental factors. Some of these SNPs are known to serve as modifiers for BRCA1 and BRCA2. About 90 officially known SNPs are recognized. Mutations in RAD51C and RAD51D genes belonging to RAD51 group are detected in breast or ovarian cancer. The mechanism of increased cancer risk may be through activation of growth-promoting genes rather than inactivation of DNA repair, which is the most common mechanism seen for moderate or high-penetrance genes. On average, each allele only mildly increases risk and is additive per allele rather than multiplicative (Mahdavi et al., 2018).

There are other known genes related to breast cancer as *HER2* gene encodes for HER2 protein a transmembrane tyrosine kinase receptor. 15-20% of breast cancer patients are HER2 positive with overexpression and or gene amplification (Furrer et al., 2018), EGFR also known as HER1 encodes a cell surface glycoprotein of tyrosine kinase family, downstream signalling pathways of EGFR promote cell proliferation (Sun et al., 2017).

MYC is an oncoprotein acting as a master regulator of many cellular signalling and metabolic pathways; it is overexpressed in 30–50% of high-grade tumors (Sun et al., 2017).

B. Non-Genetic (Environmental) Factors:

Environmental factors are important breast cancer-predisposing factors as the genetic factor can explain only a quarter of breast cancer cases (Figure 1-8) and the only explanation for almost fivefold difference in breast cancer incidence across countries; is the difference of their environmental elements (Hiatt and Brody, 2018).

Furthermore low penetrance susceptibility genes require environmental triggers and even in case of high penetrance genes as BRCA1 and BRCA2 somatic mutations are required for the loss of heterozygosity. So, breast cancer is more likely to be caused by complex interactions between genetic and environmental factors as well as possible endocrine factors; environmental factors involve everything that is not genetic including exogenous and endogenous hormonal exposure, chemical substances, lifestyle factors etc. (Strumylaitė, Mechonošina and Tamašauskas, 2010).

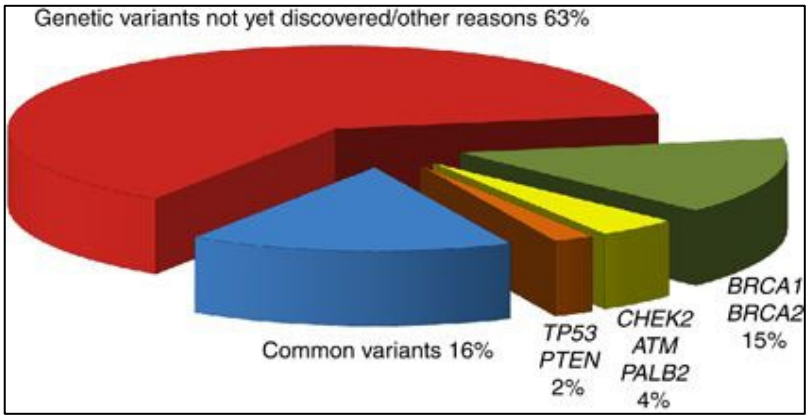

Figure 1-8: Genetic and non-genetic factor distribution (Rudolph, Chang-Claude and Schmidt, 2016)

Non-genetic or environmental risk factors are summarized in the following topics:

1. Endogenous and exogenous hormonal exposure: Evidences exist that estrogen is an important determinant of breast cancer risk. Ovaries are the principle source of estradiol (a main type of oestrogen) the circulating hormone acting of distal targets in premenopausal women, but in postmenopausal women it is produced in a number of extragonadal sites, acting locally at these sites as; mesenchymal cells of adipose tissue including that of the breast, osteoblasts and chondrocytes of bone, the vascular endothelium and aortic smooth muscle cells, and numerous sites in the brain (Simpson, 2003).

Early menarche and late menopause are two well known risk factors in breast cancer in both conditions exposure to endogenous oestrogen are prolonged (Strumylaitė, Mechonošina and Tamašauskas, 2010). There is a strong association between increasing concentrations of sex hormones in postmenopausal women as in case of hormone replacement therapy (HRT) and a higher breast cancer risk (Okoh, Deoraj and Roy, 2011; Kamińska et al., 2015). Estrogen-plus-progestin therapy for more than 5 years significantly increases risks of lobular and ER positive-PR positive breast cancer compared to non-users of hormone therapy (Engin, 2017).

Still the relationship between oral contraception (OC) and risk of breast cancer remains controversial, and in a study, there was a small increase in the relative risk of developing breast cancer in women taking oral contraceptive pills for 10 years (Hulka and Moorman, 2008).

Experimental studies support the hypothesis that oxidative metabolites of estrogens have genotoxic, mutagenic, transforming, and carcinogenic potential and thus could cause the initiation or progression of carcinogenesis in humans (Strumylaitė, Mechonošina and Tamašauskas, 2010).

2. Lifestyle:

-**Dietary habits and obesity:** High dietary fat intake and obesity are two lifestyle related factors that are associated with an increase in breast cancer incidence (Hulka and Moorman, 2008).

Obese women have elevated risks of ductal and ER-positive-PR-positive breast cancer compared to lean subjects (Engin, 2017). Obesity as measured by body mass index (BMI) has long been known to be associated with an elevated risk of postmenopausal breast cancer, and decreased risk of premenopausal breast cancer; this paradoxical relationship of obesity in pre- versus postmenopausal women may be due to the differential frequency of estrogen receptor positive/progestin receptor positive (ER+/PR+) tumors in these two age groups. It is important to remember the BMI may not be the critical measure of body fatness, rather abdominal fat, which is thought to be biologically more important and critical in terms of insulin resistance and cancer risk (Hiatt and Brody, 2018). Furthermore, Obesity is associated with an increased risk of breast cancer recurrence and cancer death (Ecker et al., 2019). Eating processed products containing chemical additives for food preservation and flavour enhancement as well may promote the neoplastic transformation process in mammary gland cells (Kamińska et al., 2015).

-**Physical activity:** Most epidemiologic studies have shown that regular participation in physical activity can reduce the risk of breast cancer (Loprinzi et al., 2012) and (Hiatt and Brody, 2018). On average, a 20–25% reduction in breast cancer risk has been observed among physically active women in comparison to the least active ones (Ferrini, 2015; Godinho-Mota et al., 2019)

The mechanism probably responsible for protective effect is through reducing the level of body fatness; but may also work through changing sex hormone levels, immune function and insulin-related factors (Loprinzi et al., 2012)

-**Parity and nursing:** There is strong evidence that not having children or having them after 35 years and avoiding breast feeding all increase the incidence of mammary tumours (Mc Pherson et al., 2000; Hulka and Moorman, 2008; Ferrini, 2015). Possible underlying mechanisms are differentiation of the mammary epithelial cells, decreased number of mammary stem cells, altered mammary response to oestrogen, and reduced levels of circulating hormones (Opdahl et al., 2011).

-Light at Night: Exposure to light at night which is usually associated with shift works, has been repeatedly associated with increased breast cancer rates, in 1978 it was suggested that disruption to circadian rhythm could result in higher circulating oestrogen levels and thereby increase the risk of breast cancer, hence in 1987 it was proposed that increase in breast cancer incidence might be explained by increasing exposure to artificial light. (Johns et al., 2018)

The International Agency for Research on Cancer (IARC) has classified shift work as probably carcinogenic for breast cancer (Hiatt and Brody, 2018). The mechanism is unclear but could work through the suppression of melatonin, which normally rises during the darkness of night (Hiatt and Brody, 2018). Melatonin is known to have antiproliferative effect in breast cancer cells *in vitro* and inhibit the growth of mammary tumors in rats; furthermore different other anticancer effects were identified such as inducing apoptosis, antiestrogenic effect, inhibition on invasion and angiogenesis (Li et al., 2017). Experimental studies provide results which support the value of melatonin as an oncostatic drug for reducing the risk of ER+ breast cancer (Gonzalez-Gonzalez, Mediavilla and Sanchez-Barcelo, 2018).

3. Chemical exposure:

Many chemicals are implicated in predisposing breast cancer, here we mention chemicals that are in a closer contact with modern day humans:

-Alcohol consumption: It has been well documented as a causal factor for breast cancer using many studies from different countries around the world (Schmidt, 2012; McDonald, Goyal and Terry, 2013)

A meta-analysis based on 53 epidemiological studies indicated that intake of 35-44 grams of alcohol per day can increase the risk of breast cancer by 32% (Sun et al., 2017). It appears that alcohol consumption has more effect on ER+/PR+ tumors and not on ER−/PR− ones. However the exact mechanism of action of alcohol in tumorigenesis is unclear but possible formation of genotoxins such as acetaldehyde or alterations of hormones, hormone receptors, or other mechanisms are suggested (Hiatt and Brody, 2018).

-Tobacco smoke: It contains more than 20 components that are known carcinogens and these substances can be found in the breast fluid and tissue of smoker ladies (Hiatt and Brody, 2018). The updated (IARC) Monographs stated that smoking has a positive association with breast cancer (Goldvaser et al., 2017). The risk of smoking related breast cancer is particularly increased among women who started smoking at adolescent or peri-menarcheal ages and the

relative risk of breast cancer was greater for women with a family history of the disease (Jones et al., 2017)

-**Air Pollution:** Air pollution includes a mixture of many compounds as polycyclic aromatic hydrocarbons (PAHs), metals, and benzene (White, Bradshaw and Hamra, 2018)
These compounds have genotoxic, estrogenic and antiestrogenic effects (Hiatt and Brody, 2018). The most widely studied of these are the polycyclic aromatic hydrocarbons (PAHs), representing a large class of chemicals formed by the incomplete combustion of coal, oil and gas, as well as grilled meats, tobacco smoke, and other substances to which humans are exposed. (PAHs) have been linked to breast cancer in animal models through their genotoxic effect causing DNA damage by oxidative stress, as well as their weakly estrogenic effects (White, Bradshaw and Hamra, 2018). Motor vehicle density which is an important contributor of air pollution, being a major source of Nitrogen oxides (NO2 and NO); is another chemical that is positively related to breast cancer incidence rates in different geographic areas (Chen and Bina, 2011)

4. Radiation: Ionizing radiation (both diagnostic and therapeutic) is a well-known risk factor for the development of primary breast cancer through direct damaging effect on DNA, with a clear positive dose-risk relation (Drooger et al., 2015). In addition to DNA damage, ionizing radiation may induce changes in breast cancer related serum hormones and proteins that may be implicated in carcinogenesis (Grant et al., 2011)
The risk of developing breast cancer increases if the exposure to radiation was during the rapid breast formation period in the young age groups (Drooger et al., 2015; Hiatt and Brody, 2018). Nowadays the most important sources of ionizing radiation exposure are from diagnostic medical imaging, including radiographs, fluoroscopy, and computed tomography (Hiatt and Brody, 2018). Exposure to ionizing radiation at an early age in individuals with BRCA1/2 mutation increases the risk of developing breast cancer, so accordingly its recommended to use non-ionising radiation imaging techniques (such as MRI) as the main tool for surveillance in young BRCA1/2 mutation carriers. (Pijpe et al., 2012; Drooger et al., 2015)

5. Aging: Aging is a well-known risk factor of breast cancer, as the incidence of breast cancer is highly related to increasing age (Tesarova, 2016). In 2016, approximately 99.3% and 71.2% of all breast cancer-associated deaths in America were reported in women over the age of 40

and 60, respectively (Sun et al., 2017). The risk of breast cancer increases with age doubling with every 10 years until menopause. (Mc Pherson et al., 2000).

Seemingly most of the above mentioned non-genetic (environment related) risk factors (that are making the bulk of breast cancer risk factors) collectively contributing in free radicals and toxic chemical formation that will affect fundamental cellular components as (DNA molecules).
The current study focuses on the mitochondrial DNA (mtDNA), hypothesizing that breast cancer could be the result of cumulative mtDNA mutations resulted from the effect of environmental factors, as mtDNA are more prone to mutations than nuclear DNA (nDNA), thus disrupting the proper mitochondrial function and electron transport chain, creating more toxic free radical products and hence resulting in further mutations in mitochondrial as well as nuclear DNA causing carcinogenic changes in the breast tissue. Next part of the literature review will cover important facts about mitochondria, mitochondrial genome and their possible roles in carcinogenesis and breast cancer

1.2 MITOCHONDRION:

Mitochondria are fundamental organelles within cell, almost all eukaryotic cells contain mitochondria, present as numerous, mobile, and polymorphic organelles providing critical cellular function and are the only cytoplasmic organelles having their own genome; semiautonomously living and reproducing inside human body (Picard et al., 2011)

1.2.1 Origin of Mitochondria: According to the generally accepted concept of endosymbiosis, mitochondria originated in the early stages of eukaryotic evolution from an endosym-biotic α-proteobacterium an Alphaproteobacteria around 1.5–2 billion years ago (Ryzhkova et al., 2018). During the process of evolution, genetic information's not any more necessary for the endosymbiont survival were deleted (lost) and others were transferred (uni-directionally) from the mitochondrial genome to the nuclear chromosome through an incomplete transfer process that has left mitochondria with a vestigial genome (Choi, Liu and Adams, 2006; Wang, 2012).

1.2.2 Structure of Mitochondria: Mitochondria are double membrane bound organelles, having an inner with an outer membrane and two aqueous compartments, intermembrane space and matrix in the center (Bohnert et al., 2012; McCarron et al., 2013), (Fig 1-9). The mitochondrial membranes with their phospholipid and protein components being in a homeostatic state are essential for mitochondrial shape, interaction with other organelle and function (Schenkel and Bakovic, 2014). The outer mitochondrial membrane (OMM) separates mitochondria from cell cytoplasm and has specialized region for interacting with other cellular organelles (Cogliati, Enriquez and Scorrano, 2016). The OMM is freely traversed by ions and uncharged molecules (smaller than 10000 Dalton). Regarding large molecules especially proteins need special translocases to be imported, these translocases of the outer membrane complex are responsible for importing nearly all nuclear encoded proteins, from the cytosol (Jimenez-Morales et al., 2018).

Inner mitochondrial membrane (IMM) protrudes into the mitochondrial matrix and is of two parts, peripheral part adjacent to the outer membrane (inner boundary membrane) and tube-like invaginations (cristae) having extensions to the matrix. In contrast to the outer one, the inner membrane is a tight barrier to ion and molecule diffusion, transfer of molecules is only through specific and selective membrane bound transport proteins. Because of this ion selectivity, an electrochemical membrane potential of about 180 mV is built up across the inner mitochondrial membrane (Kühlbrandt, 2015).

Mitochondrial matrix is the inner most part in mitochondria, surrounded by the inner mitochondrial membrane, it is composed of a densely packed mass of about 50% protein, (Wiederkehr et al., 2009; Goodsell, 2010).

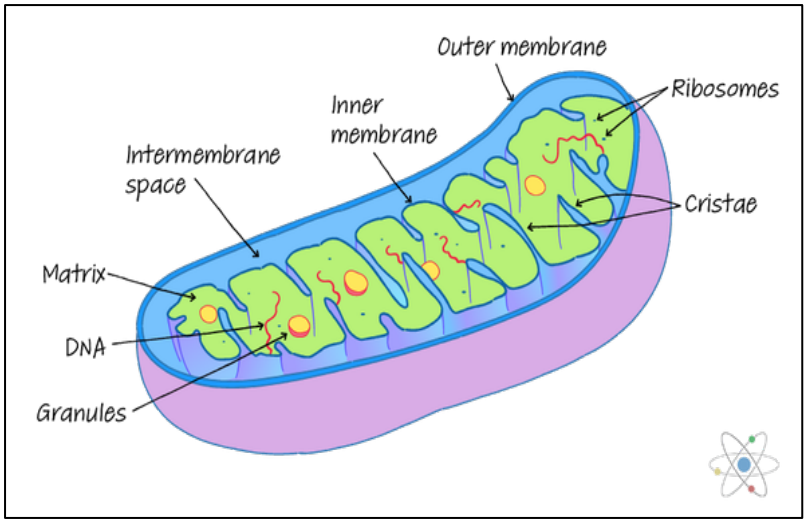

Figure 1-9: Structure of Mitochondria

1.2.3 Mitochondrial morphology and dynamics (fission and fusion): Mitochondrial morphology is widely variable according to cell type and functional state; it could be long filaments as in fibroblasts, or spherical-ovoid as in hepatocytes (Osellame et al., 2012). This on-going dynamic morphology is achieved through the combined actions of two opposing processes fission and fusion, the balance between them ensures proper mitochondrial number, size, positioning and hence will impact mitochondrial bioenergetics state and play a critical role in maintaining functional mitochondria state (Detmer and Chan, 2007; Scott and Youle, 2010). Fission is the division of one mitochondrion into two daughter mitochondria, it is essential for biogenesis and inheritance, for growing and dividing cells, as well as for mitophagy (specialized autophagy) (Parone et al., 2008; van der Bliek, Shen and Kawajiri, 2013). On the other hand, fusion is the union of two mitochondria resulting in one mitochondrion and is important for ensuring a uniform organelle population and proper mDNA expression (Picard et al., 2011). Disturbance in the equilibrium of fission and fusion may lead to diseases (Ranieri et al., 2013).

1.2.4. Functions of Mitochondria: mitochondria are involved in performing many fundamental body functions as ATP production, apoptosis, Ca^{+2} regulation and heat production.

i) ATP synthesis and bioenergetics: ATP production is the main function of mitochondria achieved by the cellular respiration which is the process of the final oxidation of carbohydrates, fats and proteins transforming them to consumable energy (ATP) (Osellame et al., 2012). Cellular respiration starts by catabolizing sugar, fats and proteins into acetyl-CoA which enters the Citric acid cycle (Bonora et al., 2012). Mitochondria contain the fundamental enzymes required for the three main pathways participating in ATP generation: the tricarboxylic acid (TCA) or Kreb's cycle, oxidative phosphorylation (OXPHOS) and fatty acid β-oxidation (FAO) (Nsiah-Sefaa and McKenzie, 2016).

Oxidative phosphorylation (OXPHOS); Is the process of ATP production, involves the transfer of electron from NADH (produced in the citric acid cycle), to oxygen through a series of large protein complexes in the inner mitochondrial membrane, creating a transmembrane electrochemical gradient by pumping protons across the membrane and the flow back of protons into the matrix via a proton channel in the ATP synthase leads to conformational changes in the nucleotide binding pockets and the formation of ATP (Sousa et al., 2018).

-*Complex I (CI) NADH: ubiquinone reductase* (H+ translocation): an L-shaped enzyme the largest among OXPHOS complexes, composed of the 45 subunits, seven of which are mtDNA-encoded, (Alexeyev et al., 2013; Nsiah-Sefaa and McKenzie, 2016). In general, dysfunctions of CI cause generation of reactive oxygen and nitrogen species. (Nsiah-Sefaa and McKenzie, 2016)

-Complex II (CII) succinate dehydrogenase (quinone): is composed of four highly conserved nuclear encoded subunits and participates in both OXPHOS and the TCA cycle. It accepts electrons from FADH2. CI and CII then reduce ubiquinone, the substrate of CIII (Zhao et al., 2019).

-Complex III (CIII) quinol-cytochrome c reductase: composed of 11subunits, largely encoded by the nDNA with only one being mtDNA encoded which is cytochrome b; it

transfers electrons from reduced ubiquinone to cytochrome c. (Nsiah-Sefaa and McKenzie, 2016).

-*Complex IV (CIV) cytochrome oxidase (COX):* possesses 13 subunits, three of which are encoded by mtDNA. These three mtDNA-encoded subunits are CO1which is the largest catalytic subunit, CO2 and CO3 (Nsiah-Sefaa and McKenzie, 2016; Signes and Fernandez-Vizarra, 2018). Interestingly, mutations to these three subunits are rare. To date about ten conditions with mtCO2 variants have been reported (Roos et al., 2018).

-*Complex V (CV):* Complex V phosphorylates ADP to produce ATP by utilizing the electrochemical gradient produced during CI to CIV electron transfer (Nsiah-Sefaa and McKenzie, 2016). Subunits a (MT-ATP6) and A6L (MT-ATP8) are encoded in the mtDNA, whereas all the rest of CV components are nDNA encoded (Signes and Fernandez-Vizarra, 2018).

Respirosome; Is the supercomplex that performs the respiratory action, consuming electron donors and oxygen and generates water and energy, its composed of a high-order structure, interacting proteins namely formed by complex I, II, III and IV (Guo et al., 2016)

ii) Apoptosis: is a regulated (programmed) form of cell death in living tissue; it was during the mid-nineties when the central role of mitochondria in apoptotic cell death was proposed. Initiation of this process is through two different pathways, the extrinsic (death receptors on the surface of cell) and the intrinsic pathway (mitochondrial) (Otera and Mihara, 2012). Most trigger induced apoptotic processes are through the intrinsic pathway and the fundamental step in which is the mitochondrial outer membrane permeabilization (MOMP) (Lopez and Tait, 2015). Following MOMP, cytochrome *c*, is released into the cytosol which in turn activates caspases. Cytochrome C is a highly conserved hemeprotein in plants, animals, and organisms. Normally cytochrome C resides in the inner mitochondrial membrane as one of the main components of the electron transport chain, shuttling electrons between complexes III and IV. The release of cytochrome C from the mitochondria to the cytosol is the trigger for establishing the apoptotic cascade (Hüttemann et al., 2012). When cytochrome C in the cytosol reaches cytotoxic levels, it activates pro-caspase-9 that in turn cleaves and activates the executioner caspases-3 and -7. Executioner caspase activity effectively kills the cell within minutes through the parallel cleavage of hundreds of different substrates (Wang and Youle, 2009). In addition to cytochrome c,

apoptosis inducing factor (AIF) is another death pathway when is released into the cytoplasm initiates a caspase-independent, apoptosis and works through chromatin condensation and DNA fragmentation (Nguyen and Pandey, 2019). MOMP the fundamental step of no return in apoptosis is highly regulated by members of the BCL-2 protein family (Otera and Mihara, 2012).

iii) Calcium signalling: Ca^{2+} is a second messenger that regulates different cellular processes as gene transcription, muscle contraction and exocytosis, endoplasmic reticulum is the biggest store of Ca^{2+} in cell (Raffaello et al 2016). When there are stimuli intracellular Ca^{2+} increases through two different mechanisms, either mobilization of intracellular Ca^{2+} stores, mainly the endoplasmic reticulum (ER), or influx of Ca^{2+} from extracellular space through the plasma membrane Ca^{2+} channels (Patergnani et al., 2011). However, the regulation of Ca^{2+} homeostasis is mediated by fine network of interaction between ER, plasma membrane and other intracellular organelles as mitochondria and lysosomes (Raffaello et al 2016). Mitochondrial Ca^{2+} regulation is essential for performing aerobic metabolism as three dehydrogenases reactions of the Krebs cycle have been shown to be modulated by Ca^{2+}. Furthermore, mitochondrial Ca^{2+} uptake shapes cytosolic $Ca^{2+}$and are important for cell survival. An overload in mitochondrial Ca^{2+} level has been implicated in apoptosis and necrosis in several pathological conditions (Marchi and Pinton, 2014; Raffaello et al 2016). Moreover, malignant cells may exert their anti and pro-apoptotic activists by manipulating Ca^{2+}levels. (Marchi and Pinton, 2014).

iv) Heat production: Mitochondria generate most of the heat in endotherms. Given some impedance of heat transfer across protein-rich bioenergetic membranes, mitochondria must operate at a higher temperature than body temperature in mammals and birds (Lane, 2018). The process of oxidative phosphorylation is not perfectly coupled with ATP synthesis, because some of the energy produced by the oxidation of nutritional substrates is lost as heat instead of changing to ATP and hence the name (mitochondrial uncoupling) This heat is produced by the re-entry of H^+ into the matrix, through pathways independent of ATP synthase (proton-leak). (Busiello, Savarese and Lombardi, 2015).

v) Immune modulation; A recently observed effect of mitochondria in immune modulation is when in an infected cell the apoptotic signalling pathway is engaged at a low level, with failure of inducing apoptotic cell death; this partial activation is found to stimulate the

infected cell with the gain of an independent (autonomous) immune response through the secretion of cytokines (Brokatzky et al., 2019).

1.3 MITOCHONDRIAL GENOME:

1.3.1. Structure of Mitochondrial Genome:

Mitochondrial DNA (mtDNA) was first discovered in 1963, completely sequenced in 1981 and sequence was revised in 1999 (Moraes et al., 2002; Nicholls and Minczuk, 2014). DNA of mitochondria is a compact, double stranded, supercoiled, circular molecule of 16569bp containing 37 genes, located in the mitochondrial matrix, in multiple copy numbers with values of 1000-10,000 have been reported (Chinnery, and Hudson, 2013; STEFANO and KREAM, 2016). It has been established that mtDNA does not have histones and are packaged in protein–DNA complexes with more than 20 proteins, known as nucleoids (100 nm in diameter) of varies size which are layered structures consisting of a core of condensed one or more copy of mtDNA, where replication and transcription of mtDNA occur, and peripheral regions, where translation of mitochondrial transcripts and assembly of newly synthesized polypeptides into respiratory complexes occur (Gilkerson et al., 2013). The strands are designated as heavy purin rich (H-strand) and light strand pyremidin rich (L-strand) according to their nucleotide composition, guanine rich and cytosine-rich respectively (Schon et al., 2012). In general, mitochondrial genome is divided in to two main parts coding and non-coding parts. Within the coding parts are 37 genes coding for two rRNAs, 22 tRNAs and 13 polypeptides, most of which are encoded on the heavy (H) strand, including genes for two rRNAs, 14 tRNAs, and 12 polypeptides, while the light (L) strand codes for 8 tRNAs and a single polypeptide (Alexeyev et al., 2013; Sharma and Sampath, 2019). All 13 protein products are constituents of the enzyme complexes of the oxidative phosphorylation system; of which seven are subunits of complex I (NADH dehydrogenase) and these are ND1-6 and ND4L, three are subunits of complex IV (cytochrome *c* oxidase) subunit I-III (COX1-3), two are subunits of complex V adenosine triphosphate 6 (ATP6) and ATP8, and cytochrome B of respiratory complex III (Alexeyev et al., 2013; Stefano and Kream, 2016). (Fig.1-10)

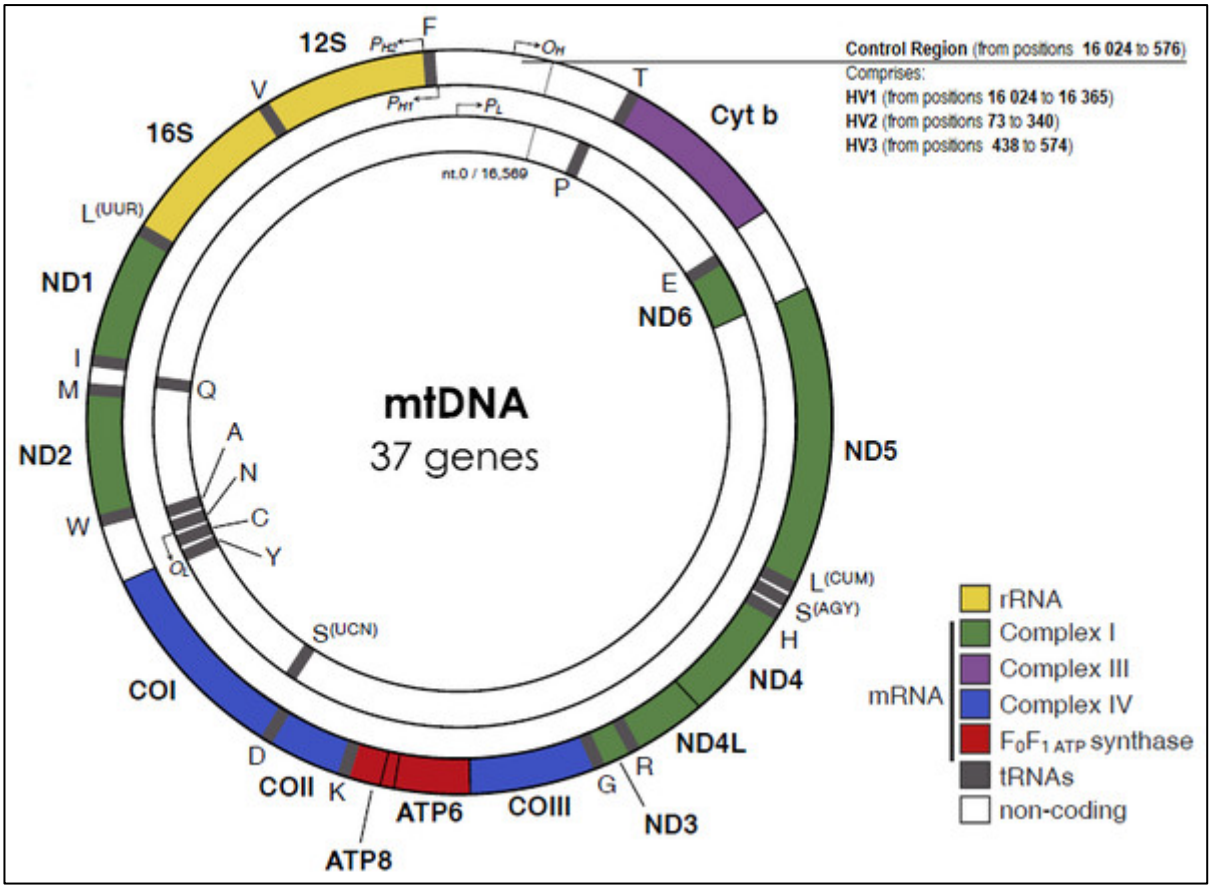

Figure 1-10: Mitochondrial DNA genome with genes and control regions (Amorim et al., 2019)

1.3.2. Mitochondrial genetic economy: The density of genetic information within the mtDNA is high, this is because mitochondrial DNA is organized in an economic pattern as genes lack introns, intergenetic sequences are absent or not exceeding few bases, rRNA and tRNA molecules are unusually small (Alexeyev et al., 2013). Genes coding for proteins show overlapping and occasionally parts of termination code are absent and are post-transcriptionally produced by polyadenylation of mRNA (Taanman, 1999).

1.3.3 D-Loop:

The main non-coding regions (control region) in mitochondrial DNA is of 1.1kb located between tRNA genes of phenylalanine and proline, contains the origin of replication of the heavy strand and promoters of transcription of genes on both H and L strands (heavy strand promoters (HSP) and light strand promoters (LSP). A large part of this non-coding region is incorporated in to a linear third DNA strand which is formed during the process of new Heavy strand formation; as mitochondrial DNA starts replication the newly synthesized heavy, or H,

strand which is of about 700nt known as (7S DNA) is formed and it remains attached to the parental L-strand and displaces the parental H strand as a loop, known as displacement loop or D-loop (Nicholls and Minczuk 2014; Falkenberg, 2018)

The control region or D-loop is characterized by a rapid evolutionary change (5-10) times, with high polymorphic regions known as hyper-variable regions, namely hypervariable I (HVR I) 342 bp located within length of 16,024-16,365, hypervariable II (HVR II) 268 bp within the length of 73-340 and hypervariable region III (HVR III) 137 bp within the length of 438-574. The sequences of HVR regions are powerful discrimination tools for human identification especially the HVR I region (Sangthong et al., 2014; Verma et al., 2018). As for its important regulatory function regarding replication and transcription it's logical to imagine that major alterations in the sequence of control region can affect the overall mitochondrial function, impair ATP production, and promote excess free radical production which may in turn contribute to carcinogenesis (Guo et al., 2016).

1.3.4. Mitochondrial DNA replication: Unlike nuclear DNA replication and repair which needs a wide range of polymerases only one polymerase (polymerase γ) is found in mitochondria and is believed to be responsible for all the replication and repair processes that are taking place within mitochondria. Pol γ is a heterotrimeric complex consisting of two nuclear-encoded components (Bailey and Doherty, 2017).

To start with Polymerase γ (POL γ) cannot use double-stranded DNA as a template so a DNA helicase is required at the mitochondrial replication fork. The DNA helicase TWINKLE travels in front of POLγ, unwinding the double-stranded DNA template. TWINKLE forms a hexamer and requires a fork structure (a single-stranded 5′-DNA loading site and a short 3′-tail) to load and initiate unwinding (Falkenberg, 2018)

One of the presented models of replication called the displacement model presented in 1972; is the most widely accepted longest standing model for mammalian mtDNA replication, according to this model replication is in an asymmetric fashion, thus there is two origins of replication for each strand (O_H and O_L). Replication starts in the D-loop O_H, L strand is used at first as a template to synthesize a new H strand by DNA polymerase γ, after copying two thirds of the genome the replication fork exposes the O_L, initiating L-strand DNA synthesis. From this point, H- and L-strand synthesis proceeds continuously until the two strands have reached full circle (Stumpf and Copeland, 2010). A second model called the strand-coupled bidirectional replication model (coupled leading H strand and lagging L-strand synthesis in a discontinuous,

bidirectional mode) L strand is the lagging strand and initiates at multiple sites, requiring multiple primers (Stumpf and Copeland, 2010)

1.3.5 Protein Biosynthesis in Mitochondria: Gene expression is a complicated process that extends from genetic organization to transcription; mRNA processing, to fully synthesized and assembled protein. Mitochondrial genome is transcribed in to a polycistrons from either the light-strand promoter (LSP) or the heavy-strand promoter (HSP) by mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription elongation factor (TEFM) complex (Gammage and Frezza, 2019). Through their own mRNA translation machinery, mitochondrially coded proteins are synthesized. Components of the translation machinery as rRNA and tRNA, are mitochondrially encoded while all the other proteins necessary for the translational machinery are encoded in the nDNA and are imported from the cytosol to the mitochondria (Ott et al., 2016).

Human mitoribosome contains three RNA molecules and 80 molecule proteins. The rRNA molecules include a large subunit (mt-LSU), small subunit (mt-SSU) and mt-tRNA Valine (Amunts et al., 2015). Mammalian mt-SSU is composed of 12S mt-rRNA and 30 proteins, of which 14 are specific to mitochondria and the mammalian mt-LSU composed of 16S mt-rRNA and 53 proteins, of which 22 are specific to mitochondria (Greber and Ban, 2016; Mai et al., 2016). Mammalian mitochondrial ribosome components are mainly protein, bulk of this extra protein is in the periphery acting as a protective shield for mt-RNA and preventing potential damage caused by the high ROS levels found within the organelle and ensure the preserved functional core of the mitoribosom, which are mt-mRNA recognition site on the mt-SSU and peptidyl transferase centre in the mt-LSU (Mai et al., 2016; Ott et al., 2016). Translation cycles in mitochondria are of three steps: initiation, elongation and termination, AUG is the most common start codon in mammalian mitochondria; AUA and AUU are used as start codon as well (Mai et al., 2016). Terminates occurs when a translating ribosome encounters one of four stop codons: AGA, AGG, UAA or UAG (Ott et al., 2016).

1.3.6. Mitochondrial genetic codes: The mitochondrial genetic code is slightly different from the eukaryotic code; UGA codes for tryptophan rather than being a STOP codon; AGA and AGG, normally code for arginine, are STOP codons; AUA is methionine and not isoleucine; and the ubiquitous AUG start codon is sometimes replaced by AUA or AUU in mitochondrial genes (Anderson et al., 1981). (Fig. 1-11)

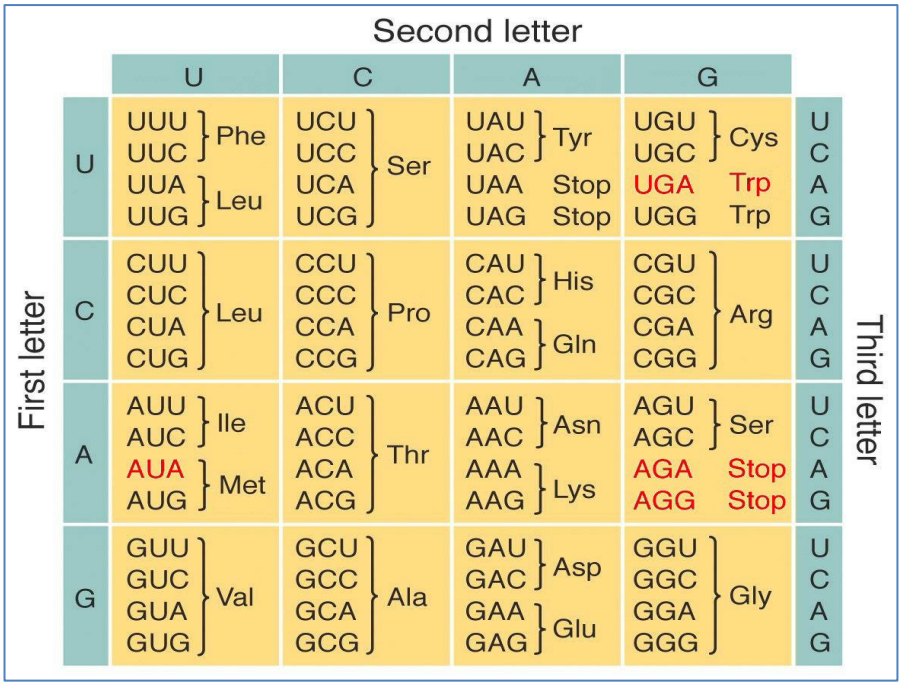

Figure 1-11: Vertebrate mitochondrial DNA (mtDNA) genetic code (Wikimedia)

1.3.7. Mitochondrial inheritance: It is widely accepted that the inheritance of mtDNA in all animals is uniparental or maternally inherited which means they received DNA only from the mitochondria of the oocyte alone. In mammals, sperm-derived paternal mitochondria generally enter the oocyte cytoplasm after fertilization and temporarily co-exist in the zygote alongside an excess of maternal mitochondria (Sato and Sato, 2013). In almost all eukaryotes, paternal mtDNA and mitochondria themselves are selectively eliminated or degraded from the embryonic cytoplasm; thus, mitochondrial genes are inherited mainly from the maternal parent (Ding and Yin, 2012). Evolved maternal transmission is important to prevent heteroplasmy (existence different mitochondrial population) which is involved in mitochondrial diseases (Ladoukakis and Zouros, 2017)

1.3.8. Mitophagy:

Mitophagy is the mitochondria-specific autophagy; in general, it is a lysosome-mediated degradation of intracellular defective or excess organelles (Saito and Sadoshima, 2015; Paz et al., 2016). Mitophagy plays a fundamental role in mediating mitochondrial quality control and maintaining mitochondrial health by selectively targeting the damaged mitochondria for lysosomal degradation (Tan et al., 2019).The continuous production of ROS and their damaging effect on mitochondria necessitates turnover and replacement of mitochondria every 10-25 days and during energy stress, amino acids and fatty acids that are recovered through

cellular degradation by autophagy can be recycled to generate ATP (Saito and Sadoshima, 2015). Mitophagy is important in modulating the percentage of heteroplasmy of the mitochondrial DNA mutations (different mitochondrial population within a cell, wild and mutant), and is directly related with the pathophysiology of the resulted disease (Paz et al., 2016). Since mitochondria are not isolated organelles but are within a dynamic network, the dysfunctional mitochondrion needs to be separated from the healthy network, requiring a tight coordination between fusion, fission and mitophagy machinery (Vara-Perez, Felipe-Abrio and Agostinis, 2019). Pink1-Parkin–dependent is one of the well-known pathways of (macroautophagy) autophagy. PINK1 is stabilized on the outer membrane of damaged mitochondria, where it facilitates recruitment of cytosolic Parkin, PINK1and Parkin accumulate on damaged mitochondria (Fig. 1-17), promote their detachment from the mitochondrial network, and target these organelles for autophagic degradation in a process that requires Parkin-dependent ubiquitination of mitochondrial proteins (Saito et al., 2019) and (Ashrafi and Schwarz, 2012). Most of the proteins involved in mitophagy have been shown to be dysregulated in cancer cases, but their precise effect being tumor promoter or tumor suppressor is largely determined by the cancer subtype.

Growing evidence indicates that autophagy supports the metabolic plasticity of cancer cells, by providing virtually all essential components of carbon metabolism through the degradation of carbohydrates, proteins, lipids and nucleotides (Vara-Perez, Felipe-Abrio and Agostinis, 2019).
Other important role of mitophagy is reported in aging process; during aging autophagy declines suggesting a contribution in aging process. The expression of many of autophagy genes and proteins decline in brain and liver of aging humans; conditions activating autophagy as caloric restriction, have shown beneficial effects on delaying the aging-related degeneration process (Ding and Yin, 2012)
In addition to the mentioned roles, mitophagy plays an essential role in the destruction of paternal mitochondria in the fertilized oocytes. (Ding and Yin, 2012)

Apart from lysosomal degradation, mitochondria have their own proteolytic system, two ATP dependant AAA protease complexes in the inner membrane, whose function is to degrade unfolded membrane proteins (Gerdes, Tatsuta and Langer, 2012).

1.4. MITOCHONDRIAL DNA AND MUTATION:

1.4.1. Mitochondrial DNA Mutations: Mutation rate in mtDNA is about 10-17 folds higher than that of nDNA. Mitochondrial genomes are more susceptible to damage than nuclear DNA,

because repair systems in mtDNA are not as competent as that of the nDNA, they lack protective histone proteins, and finally because they are close to the respiratory complexes where reactive oxygen species (ROS) are produced by the oxidative phosphorylation (Tuppen et al., 2010).

When a mitochondrial DNA damage is not repaired, disruption in the electron transport chain ensures causing an increase in the generation of ROS, possibly resulting in vicious cycle of ROS production and mitochondrial DNA damage, leading to energy depletion and ultimately cell death (A., 2011). There are many deletions and point mutations in mtDNA, some of them are associated with serious human disorders, such as ophthalmoplegia, migraine, dysphagia, sensorineural hearing loss, cognitive decline, and others (Kaarniranta et al., 2019). Unfortunately, the discussion of every single disease caused by mitochondrial DNA mutation is beyond the scoop of this review. Mitochondrial mutations are either germline or somatic, germline mutations are heritable from mother to offspring and are constitutively found throughout the body of the offspring while the somatic mutations cannot be inherited by offspring but can be found in subsequently proliferating populations of cells (Hertweck and Dasgupta, 2017). Fortunately, most of the mtDNA mutations are neutral polymorphisms and are useful in tracking human origin and migrations (Wallace, 2008). The first pathogenic mtDNA mutation was identified in 1988 and since then, over 300 pathogenic mtDNA mutations (point mutations and rearrangements) have been identified and linked to various diseases (Park and Larsson, 2011; Li et al., 2019).

In addition to ROS there are other sources for mtDNA damage; alkylating damage, hydrolytic damage, adducts formation as estrogens and tobacco smoke, mismatches due to replication errors or incorporation of nucleotides containing modified, e.g., oxidized, bases during replication and DNA strand breaks, these come in the form of both single-strand breaks (SSBs) and double-strand breaks (DSBs) (Alexeyev et al., 2013; Omar García-Lepe and Ma Bermúdez-Cruz, 2019).

1.4.2. Mitochondria and Free Radical: Free radicals are molecules or fragments of molecules with one or more unpaired electrons in their outer orbit and are capable of independent existence; they are uncharged, highly reactive, and short-lived molecules. Our body contain about 10000-20000 free radicals that attack body component's (Phaniendra, Jestadi and Periyasamy, 2014; Ahmad, 2018). Free radicals are either of reactive oxygen species (ROS) as hydroxyle radical ($OH^{\bullet}$), superoxide ion radical ($O^{\bullet}_2$), peroxyl ($ROO^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$);

or reactive nitrogen species (RNS), as nitric oxide radical ($NO^{\bullet}$) (Engwa, 2018). Another group of non-radical but highly reactive molecule that can easily be converted to reactive species are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), singlet oxygen ($^1O_2$), nitrous acid (HNO_2), nitrosyl cation (NO^+), nitroxyl anion (NO^-), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), nitronium (nitryl) cation (NO_2^+) (Phaniendra et al., 2014)

Sources of these free radicals could be external (exogenous) as environmental pollution, cigarette smoking, alcohol, radiation UV light, pesticides, some drugs, etc. (Phaniendra, Jestadi and Periyasamy, 2014), or could be of endogenous source, generated because of enzymatic body reactions. Respiratory chain reaction in mitochondria is an important endogenous source of free radical, P450 system and during inflammation in phagocytes free radicals are generated as well. There are certain physiological conditions in which free radicals are formed as stress, mental status, emotion, and aging (Engwa, 2018). Hydroxyl radical (OH) is the most potent and aggressive oxidant substance which is primarily responsible for oxidative damage of DNA bases. It is well known that $^{\cdot}OH$ is generated from H_2O_2 and $O_2^{\cdot-}$ catalyzed by iron through the Haber-Weiss reaction. Ionizing radiation decomposing H_2O is another source of $^{\cdot}OH$ formation (Zorov, Juhaszova and Sollott, 2014)

Fortunately, there are defence systems of antioxidant that counteract the free radicals by removing them either enzymatically through: superoxide dismutase, catalase, and glutathione peroxidase or non-enzymatically through molecules with scavenging and reducing capacity as ascorbic acid, tocopherols, uric acid and glutathione (GSH) (Gurer-Orhan et al., 2018).

Free radicals are double edged swards as they have both beneficial and toxic effects. At moderate and low levels ROS have beneficial effects and are involved in normal biochemical pathways as cellular response against infections, intercellular recognition, and induction of mitogenic response (Phaniendra, Jestadi and Periyasamy, 2014; Georgieva et al., 2017). ROS are important second messengers in several signal transduction pathways critical for cell growth and proliferation and they influence the activity of key cellular enzymes (tyrosine kinases, serine-threonine kinases, and protein phosphatases) (Pajares et al., 2018)

Some reports indicate that ROS plays a signalling role in mitochondrial p53 migration and regulates p53-mediated cell apoptosis (Gu et al., 2014). However, in high concentrations free radicals will result in oxidative stress and potential damage to the biomolecules. Oxidative stress occurs when the balance between pro-oxidants and antioxidants is impaired in favour of the prooxidant (Zorov, Juhaszova and Sollott, 2014; Gurer-Orhan et al., 2018).

Biomolecules prone to oxidative damage are lipids, proteins, and DNA. mtDNA is highly subjected to effects of oxidative damage because of its location (close to the ETC); they may be in the form of small modifications to the nitrogen bases and the deoxyribose ring, apurinic/apyrimidinic (AP) sites, strand breaks, chemical adducts of bases, and others. Apurinic/apyrimidinic sites can be converted to single-strand breaks (SSBs); these modifications together form the principal form of mtDNA damage (Kaarniranta et al., 2019).

Among all the nucleobases, guanine is highly prone to oxidation by ROS (Fouquerel et al., 2019). When guanine is exposed to ROS ($OH\cdot$ or $^1O2$) either in DNA or freely as 2-deoxyguanosine5-triphosphate (dGPT), an oxygen is added to the C-8 carbon to generate 8-oxo-7,8-dihydroguanine (GO) or 8-oxo-dGPT. This results in an increase in the occurrence of A-T to C-G or C-G to A-T transversion mutation after two rounds of replication (Nakabeppu, Ohta and Abolhassani, 2017). 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoG), is widely used as an indicator of DNA damage (Dizdaroglu and Jaruga, 2012; Ríos-Arrabal et al., 2013)

1.4.3. Mitochondrial Repair System:

The mitochondrial antioxidant pathways represent the first line of defence that protects mitochondrial genome integrity which includes the already mentioned superoxide dysmutases (SODs), and the glutathione (GSH) peroxidase (Saki and Prakash, 2017).

In general the repair system in mtDNA was previously thought to be an insufficient system and to be limited to short-patch base excision repair (BER), but now it's well-established that other repair mechanisms as long-patch (BER), mismatch repair, single strand break repair (SSBR), microhomology-mediated end joining (MMEJ), and homologous recombination dependent repair (HRR) are available; plus sanitation of the mitochondrial deoxynucleotide triphosphate (dNTP) pool and selective degradation of heavily damaged mitochondrial DNA or even degradation of the whole organelle (Omar García-Lepe and Ma Bermúdez-Cruz, 2019) .

However one of the effective repair mechanisms which is believed to be absent in mtDNA repair system is nucleotide excision repair (NER) (A., 2011; Omar García-Lepe and Ma Bermúdez-Cruz, 2019). NER is a multistep process involving identification and removal of wide spectrum (bulky damages), bulky covalent adducts, these are created by nitrogenous bases affected by UV light, ionizing irradiation, electrophilic chemical mutagens, some drugs, and chemically active endogenous metabolites, including reactive oxygen (Petruseva, Evdokimov and Lavrik, 2014).

DNA damage response (DDR) which is an evolutionary reaction to DNA damage, in this mechanism the process of sending genetic information from one generation to the next will be

disrupted. DDR as well has been recognized as less efficient in mitochondria than in the nucleus, again predisposing the mtDNA to a higher mutation rate (Kaarniranta et al., 2019).
It worth's mentioning that many of the proteins participating in the mtDNA repair system have to be transcribed and translated from nDNA where they are encoded and imported into the mitochondrion (A., 2011). Tumor suppressor gene p53is one of the main nuclear codded proteins participating in mtDNA repair mechanisms as in BER it senses damage and enhances its removal, through exonuclease activity (Park et al., 2016).

1.4.4. Maintaining Healthy Population of Mitochondria: As previously mentioned, cells contain thousands of mtDNA molecules (copies) and in the majority of them the sequence is identical, a state known as Homoplasmy. When a new mutation occurs in mtDNA copy sequence it creates a state of intracellular Heteroplasmy where there is coexistence of the mutated sequence and wild type sequence (Chinnery and Hudson, 2013; Wallace and Chalkia, 2013). Surprisingly low-level of mitochondrial heteroplasmies are common findings in healthy individuals and the advent of next-generation sequencing (NGS) technologies showed that about 25-65% of the general population are carriers of at least one heteroplasmic variant across the entire mitochondrial genome (Errichiello and Venesio, 2018).
Heteroplasmy can vary between cells in the same tissue or organ, from organ to organ within the same person, and between individuals in the same family (Stewart and Chinnery, 2015)
In some conditions, the initial mutant mtDNA becomes enriched within some cells; ultimately the mutant variant or sequence becomes the predominant one and may influence phenotypic alteration at cellular or clinical level. The mechanism by which this enrichment occurs in either germline or somatic cells remains a mystery (Wallace and Chalkia, 2013). As a result of continuous mtDNA replication during cell cycles the mutated and wild copies of mtDNA will be transmitted unevenly and causing variation in the mtDNA population between the daughter cells and as a consequence of genetic drift or selection heteroplasmy can shift either up or down (McMahon and LaFramboise, 2014; Germain et al., 2015; Filograna et al., 2019). For any given disease caused by a mutation in mtDNA, the ratio of normal to mutant mtDNAs within the cells of the body can substantially impact the clinical presentation, penetrance, and severity of the disease phenotype (Sobenin et al., 2014; Bussard and Siracusa, 2017). Usually a pathogenic mutation must occur at a level high enough to contribute to a pathological phenotype—in several cases that level is determined as 85% (Errichiello and Venesio, 2018; Kaarniranta et al., 2019).

mtDNA Heteroplassmy or mosaicism eventually will result in a bioenergetic mosaicism that is differently appreciated by different organs, according to their sensitivity; brain is the most sensitive to partial bioenergetic defects followed by heart, muscle, kidney, and endocrine systems. Thus minor systemic mitochondrial defects can cause organ-specific symptoms, but it is important to remember that the mtDNA heteroplasmy level in blood cells can be quite different from that of brain, heart, muscle, or kidney (Wallace and Chalkia, 2013).
Heteroplasmic mtDNA segregation is a random process so with aging and further cellular division great alteration occurs in the proportion of mutant and wild mtDNA sequence.(Li et al., 2019)

1.4.5. Mitochondrial haplogroups: Human mitochondrial DNA is regarded as a rich source of genetic data in human evolution and classification of population genetics, because it is present with high copy number per cell, maternally inherited, rapidly evolving and non-recombining (Richards 1998; Pakendorf and Stoneking, 2005; Kivisild, 2015).

Worldwide studies revealed the presence of significant differences in mtDNA among populations of different geographical regions as a result of natural selection (Kloss-Brandstatter et al., 2010). To determine balance between heat production and ATP production mitochondria adapted with environmental changes and climatic fluctuations through history resulting in a great effect on demographic human population history (Cheng et al., 2013). Variations of mtDNA among populations (single nucleotide polymorphism)was of great help in reconstructing models of human origin and their line of distribution, these distribution lines gave up different mitochondrial DNA lineages and were revealed to be continent specific, (Ingman et al., 2000). According to a work done by Cann et al 1987 on mitochondrial DNA, it was concluded that anatomically modern human (AMH) first evolved in Africa about 150000 years ago, migrated out of Africa around 100000 years ago, but the Anatomically modern humans' (AMH) long lasting settlement in Eurasia started 60000-70000 years before present in the south western Asian corridor (Quintana-Murci et al., 2004; Roostalu et al., 2006). Haplogroups were named using capital letters A to Z in order of their discovery, for subgroups or subclades numbers are used following the original capital letter designation and indicate that they derive from the ancestral capital letter haplogroup (Bussard and Siracusa, 2017). Accordingly the oldest super-haplogroups L1, L2 and L3 characteristically found in African population and out of these only L3 migrated out of Africa in the form of haplogroups M and N 60 000 YBP (Richards et al., 2000; Kivisild, 2015). (Fig 1-11)

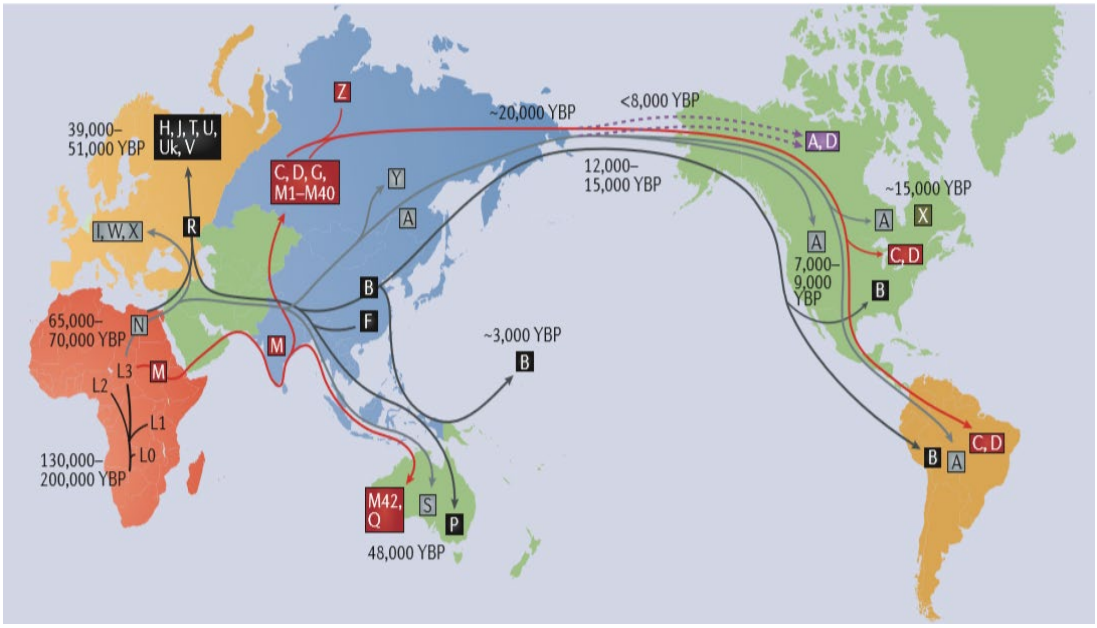

Figure 1-12: Major mitochondrial haplogroups and their migration (Stewart and Chinnery, 2015)

Recently more attention was attracted towards the relation between (SNP) s and different pathological conditions as neurodegenerative disease, cardiovascular disease and cancers; as these mtDNA polymorphisms may predispose to changes in mitochondrial respiratory chain activity and free radical production, predisposing individuals or a population of the same mtDNA genotype, to an earlier onset of apoptotic processes, accumulation of somatic mtDNA mutations and mitochondrial impairment or predispose them oppositely to a less ROS production and better mitochondrial function (Ienco et al., 2011; Chinnery and Gomez-Duran, 2018; Veronese et al., 2019).

1.4.6. Mitochondrial DNA Mutation and Aging: Aging is a progressive loss of physiological integrity over time, during which there will be functional impairment and increased vulnerability to death (Zhang et al., 2017). Through history aging was a big mystery, human wanted to unreveal and avoid; targeting the mitochondria as one of the important answers for this mysterious process resulted in a great advance in this field. According to mitochondrial free radical theory, it has been postulated that ageing is caused by the toxic effect of ROS, creating a vicious cycle, as damage to mtDNA and other mitochondrial components leads to respiratory chain dysfunction, in turn increasing generation of ROS and facilitating more mtDNA damage (Kazachkova, 2013; Lagouge and Larsson, 2013; Stuart et al., 2014).

Accumulation of mtDNA mutation is associated with reduced life expectancy and early onset aging related features as weight and hair loss, kyphosis and osteoporosis (Ortiz et al., 2018). Apart from mutation accumulation in mtDNA two more aging related phenomenon were observed; an increase in the incidence of mtDNA heteroplasmy and a decrease in mitochondrial copy number (Mengel-From et al., 2014;). Experimentally mtDNA depleted
mice by replication blockage showed aging phenotype as hair lose kyphosis and skin changes, surprisingly they showed complete recovery after removal of the blockage effect (Bonora and Pinton, 2018). Mitochondria are closely related to processes associated with aging, as senescence, immune decline, and inflammation (Zhang et al., 2017).

Cellular senescence is a state were cells stop dividing after a set number of divisions, caused by telomere shortening and hence losing the ability to protect the ends of their chromosomes. Recently a relationship between elevated levels of ROS and telomere has been confirmed that directly connects the two processes through chemical damage to guanine bases (Fouquerel et al., 2019). Mitochondrial metabolism defects predisposes to senescence as well through altered TCA level and increased pyruvate oxidation, that will predispose to generation of increased mitochondrial ROS and entry into the senescent (Sun et al., 2016).

Studies on muscle of aged mice proved the impairment of mitochondrial quality control, according to which Drp1and PINK1 proteins are deceased, Drp1 mediates fission which is an important step in the process of mitophagy and thus decreased Drp1 may lead to decreased mitochondrial fission and subsequent removal through autophagy (Zhou et al., 2017).

1.4.7. Mitochondrial DNA Mutation and Cancer: Cancer cells undergo metabolic reprograming in order to fulfill their basic needs, which are rapid ATP production, metabolic precursor supply and maintenance of an appropriate cellular redox status (Vara-Perez, Felipe-Abrio and Agostinis, 2019).

Otto Warburg was the 1st one in (1956) who observed that cancers ferment glucose in the presence of oxygen, using aerobic glycolysis, proposing that abnormalities in mitochondrial respiration may be responsible for cancer production (Mohamed Yousoff et al., 2019; Zong et al., 2016). Two decades ago the 1st somatic mtDNA mutation associated with human cancer was reported and the last twenty years showed many trials and studies to explore the effects of mitochondrial mutation in different types of cancer and most of these studies initially were focusing on the non-coding part of the genome however analysis of the coding part is now the issue of most of such studies.

When normal, non-proliferating cells are submitted to mitochondrial respiration inhibition with unlimited glucose supply they upregulate the hypoxia inducible factor 1α (HIF α) which in turn switch on the glycolytic pathway for ATP production; while highly proliferating tumor cells with low oxygen and glucose concentrations are likely to enhance autophagic pathways to produce adenosine triphosphate (ATP) by rapidly degrading endogenous substrate (Kim, 2014; Santana-Codina, Mancias and Kimmelman, 2017). In addition to being highly glycolytic, mitochondria in cancer cells show altered cristae, membrane composition and membrane potential, resulting in an aberrant mitochondrial function influencing ROS production and apoptosis (van Gisbergen et al., 2015). ROS can induce various DNA changes (mutations) which can be regarded as the initiation step in the process of carcinogenesis, it can interact with surface and intracellular receptors as well and modulate signalling pathways; disrupt physiological mechanisms related to proliferation, apoptosis and angiogenesis, which can be characteristic for the promotion step in carcinogenesis (Sainz, Lombo and Mayo, 2012; Gisbergen et al., 2015; Hecht et al., 2016).

However other theories indicate that changes in the biochemical processes accompanying carcinogenesis as the aerobic glycolesis do not impair mitochondrial function but are rather essential for cancer cell viability (Germain et al., 2015; Li et al., 2019). It is true that this switching off the oxidative phosphorylation will result in less efficient energy production per molecule of glucose but tumour cells will have the privilege of having more available substrates needed for their rapid growth, the hypoxic environment they create activates factors essential for their growth, invasion, metastasis and finally anaerobic glycolysis will reduce the chance of cellular senescence by decreasing the reactive oxygen species (Larman et al., 2012).

Another important role of mtDNA mutation in cancer is the single nucleotide polymorphism mutations which determine the haplogroups and their relation with susceptibility to develop cancer. Accordingly specific polymorphisms within mtDNA from different populations may be associated more often with cancer patients than healthy controls (Xu et al., 2013; Bussard and Siracusa, 2017).

Apart from the relation of mtDNA quality with cancer, mtDNA quantity (copy number) (mtDNA-CN) as well became a subject of interest in many cancer related studies (Reznik et al., 2016). Interestingly, alteration of mtDNA-CN has been observed in many types of cancers, and accumulating evidence has implied that mtDNA-CN alterations (depletion or increase) play a crucial role in the development of cancer (Errichiello and Venesio, 2018) and (Sun et al., 2018). The molecular basis upon which mtDNA-CN changes in cancer is not yet fully understood; however in a study on colorectal cancer it has been suggested that hypomethylation

of specific sites on CpG islands of the D-loop promoter may be involved in the regulation of mtDNA copy numbers (Errichiello and Venesio, 2018). Another study on colorectal cancer explained that mutations in the poly cytosine tract of D-loop region of mtDNA directly affect replication and transcription of the genome (Kumar et al., 2017).

A recent study showed significant depletion of mtDNA content in solid tumors and other diseases in comparison to healthy individuals, suggesting that mtDNA-CN could differentiate healthy controls from cancer and other diseases but not cancer from other diseases (Memon et al., 2017)

1.4. 8.Mitochondrial DNA Mutation and Breast Cancer: Despite the great advances made in exploring the genetic background and molecular basis of breast cancer yet the available data's are not enough to understand the whole process of tumorogenesis in breast cancer; however there are many facts supporting mitochondrial contribution in breast cancer through ROS production. It is well-known that oxidative damage biomarker 8-OH-dG, is detected in a higher concentration in breast cancer tissue than normal tissues of same patients (Rohan et al., 2010) and (Gurer-Orhan et al., 2018). Furthermore, Superoxide dismutase (SOD) is a mitochondrial antioxidant enzyme and acts as a tumor suppressor protein, changing superoxide to H_20_2; is shown to decrease cellular proliferation in breast cancer when it's overexpressed (Weydert et al., 2006).

One of the well-known and important risk factors in breast cancer is oestrogen exposure and carcinogenicity of estrogen is suggested to be mediated by two pathways: Estrogen receptor (ER) mediated (ER signaling pathway) and ER-independent (Gurer-Orhan et al., 2018).

ER independent effect is through estrogen induced mitochondrial ROS formation resulting in DNA damage and carcinogenesis (Okoh, Deoraj and Roy, 2011; Gurer-Orhan et al., 2018). ROS particularly H_2O_2, seems to play a dual function, causing oxidative damage and inducing cellular apoptosis at high doses but at low doses produce genomic instability as well as transduce signals for cell growth, cell transformation and cell invasion (Okoh, Deoraj and Roy, 2011). Another example supports mitochondrial and ROS contribution in breast cancer is that BRCA1, one of the main genes implicated in hereditary breast cancer is known to upregulate the expression of genes contributing in antioxidant response, including glutathione-S transferase (GST), glutathione peroxidase, oxidoreductases, and other antioxidant genes (Ríos-Arrabal et al., 2013; Hecht et al., 2016; Gurer-Orhan et al., 2018).

Finally, considering the free radical theory of aging, the sum of the free radical damage associated with suboptimal living conditions (HARMAN, 2006). The contribution of a group of

genes involved in aging process in breast cancer occurrence and aggressiveness is well-established (K Mishra, 2013).

CHAPTER -2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1. SAMPLE SELECTION:

-Mitochondrial study in breast cancer:

During the period between March 2017 to March 2018, thirty breast cancer specimens (mastectomy and wide local excision samples) were selected, all of them were known cases of invasive ductal carcinoma (Grade II and III) diagnosed histologically by core biopsy, none of them had a family history of breast cancer. Concurrently twenty samples were taken from benign breast lesions in comparable, healthy individuals as well as their blood samples as control. This study was approved by the Ethical Committee of the Faculty of Medical Sciences/University of Sulaimani (Number 44), and verbal consents were obtained from participants.

-Haplogroup study of Sulaymaniyah city resident:

Using the blood samples of the 20 control cases with another 16 mitochondrial DNA samples (unpublished data from healthy individuals from Sulaymaniyah city center), a total of 36 samples were used to study their haplogroups and determine their historical demography as a representative sample from the city.

2.2 LOCATION OF THE PROJECT:

This study was carried out in molecular biology laboratory at Kurdistan Institute for Strategic Studies and Scientific Research in Sulaimani (KISSR).

2.3 MATERIALS: materials were divided in to two groups, materials used for the histopathological study and those used for the molecular study.

a. **Materials used for the histopathological study:**

-Equipment's and instruments used for histopathology slide preparation, table (2-1)

-Chemicals and stain material, table (2-2)

Table 2-1: Histopathology equipment's

No.	Equipment	Company name
1.	Tissue autoprocessor	Leica
2.	Rotary microtome	Sakura
3.	Tissue embedding system	Sakura
4.	Oven	Memmert
5.	Binocular light microscope	Leica
7.	Water bath	Sakura
8.	Tissue processing capsule	Sakura
9.	Embedding moulds	Sakura
10.	Slide	Sakura
11.	Cover slide	-
12.	Slide holders	-
13.	Staining jars	-

Table 2-2: Chemicals and stain used in histopathology procedures

No.	Chemicals
1.	Alcohol (ethanol) 70-100% concentration
2.	Xylene
3.	Paraffine
4.	Distilled water
5.	DPX
6.	Haematoxylin and Eosin stain

b. Materials used for the molecular procedure: these are sorted in three categories.

- Equipment's: including instruments and glassware's, table (2-3)

-Chemicals and buffers: chemicals and buffers used during the procedures table (2-4).

-Enzymes and kits; table (2-5).

Table 2-3: Molecular Equipment's

	Equipment name	Company
1	Biophotometer	Eppendorf
2	Centrifuge (5417R)	Eppendorf
3	Concentrator (AG22331)	Eppendorf
4	Deep freezer (-40)	GFL
5	Deep freezer (-70)	GFL
6	Autoclave	Systec
7	Distilled water system	
8	Electerophoresis Unit	Biometra
9	Refrigerator +4C^0	Arcelik
10	Microwave oven	Sharp
11	Micropipette variable size (0.5□l -1ml)	Eppendorf
12	Micropipette tips variable size	Eppendorf
13	Microtubes	Eppendorf
14	Sensitive balance	Sartorious
15	Thermomixer	Eppendorf
16	Vortex	IKA
17	Veriti Thermocycler (PCR)	Applied biosystem
18	UV cabinet	Clean view
19	Water bath	GFL
20	Cuvettes	

Table 2-4: Chemicals and buffers

	Chemicals and materials	Company
1	DNA loading dye	DSBIO
2	Agarose	GeNet Bio
3	TBX buffer	GeNet Bio
4	DNA ladder 1Kb and 15	DSBIO
5	Ethanol 96%	J.T.Baker
6	Ethidium bromide	SIGMA
7	Safe dye	GeNet Bio
8	DEPC treated water	GeNet Bio

Table 2-5: Enzymes and kits

Enzymes and Kits	Company
GeNet Bio Genomic DNA extraction from tissue	GeNet Bio
GenNet Bio Genomic DNA extraction from blood	GenNet Bio
Long Taq PCR kit	Dongsheng Biotech
FS^{TM} Mix PCR kit	Dongsheng Biotech
PCR purification kit	NORGEN BIOTEK CORP

- Primers: This study was performed using two groups of already designed primers used in a previous thesis (Rashid, 2014), 1st group composed of eight (4 forward and 4 reverse) primers, table (2-6) and 2nd group of primers were used for the purpose of chromosomal walking and sequencing composed of 19 reverse primers, table (2-7). The stock and working concentration of the primers were 100 picomole and 5 picomole respectively

Table 2-6: 1st group of primers

Name	Polarity	Sequence	Position	$T_{annealing}$
Mt.A.F	Forward	5`-AGG TCT ATC ACC CTA TTA ACC ACT CA-3'	7-32	54C^0
Mt.B.F	Forward	5`-CAA GAG CCT TCA AAG CCC TCA GTA-3'	5535-5558	52 C^0
Mt.C.F	Forward	5`-ACG CCA CTT ATC CAG TGA ACC ACT-3'	11002-11025	52 C^0
Mt. D.F	Forward	5`-CCT AGC AAT AAT CCC CAT CCT CCA-3'	15646-15669	52 C^0
Mt.2.R	Reverse	5`-TGA GCA AGA GGT GGT GAG GTT GAT-3'	1251-1228	52 C^0
Mt. 9.R	Reverse	5`-GGG CAC CGA TTA TTA GGG GAA CTA-3'	6171-6148	52 C^0
Mt.16.R	Reverse	5`-TAT GAG AAT GAC TGC GCC GGT GAA-3'	11707-11684	52 C^0
Mt.23.R	Reveres	5`-CGT GAT GTC TTA TTT AAG GGG AAC GT-3'	16566-16541	54 C^0

Table 2-7: 2nd group of primers

Name	Polarity	Sequence	Position	T_m
Mt.1.R	Reverse	5'-TGA ACT CAC TGG AAC GGG GAT GCT-3'	723-700	54 C^0
Mt.3.R	Reverse	5'- GCA GAA GGT ATA GGG GTT AGT CCT-3'	1852-1829	52 C^0
Mt.4.R	Reverse	5'- ATG CCT GTG TTG TGA GAG TGA-3'	2439-2416	52 C^0
Mt.5.R	Reverse	5'-TCT TGT CCT TTC GTA CAG GGA GGA-3'	3138-3115	52 C^0
Mt.6.R	Reverse	5'-CTG AGA CTA GTT CGG ACT CCC CTT-3'	3934-3911	54 C^0
Mt.7.R	Reverse	5'- CGG TTG CTT GCG TGA GGA AAT ACT-3'	4665-4642	52 C^0
Mt.8.R	Reverse	5'- GGA GTA GTG TGA TTG AGG TGG AGT-3'	5385-5362	52 C^0
Mt.10.R	Reverse	5'-GGA GTG TGG CGA GTC AGC TAA ATA-3'	6885-6862	52C^0
Mt.11.R	Reverse	5'- AAG GGC ATA GAG GAC TAG GAA GCA-3'	7711-7688	52 C^0
Mt.12.R	Reverse	5'- AGG GAG GTA GGT GGT AGT TTG TGT-3'	8477-8454	52 C^0
Mt.13.R	Reverse	5'- GGG GTC ATG GGC TGG GTT TTA CTA-3'	9258-9235	54 C^0
Mt.14.R	Reverse	5'- TAT AGG GTCGAA GCC GCA CTC GTA-3'	10190-10167	54 C^0
Mt.15.R	Reverse	5'- GTG AGG GGT AGG AGT CAG GTA GTT-3'	10986-10963	54 C^0

Mt.17. R	Reverse	5'- TAG GGA AGT CAG GGT TAG GGT GGT-3'	12381-12358	54 C^0
Mt.18. R	Reverse	5'- AGT GCT TGA GTG GAG TAG GGC TGA-3'	13089-13066	54 C^0
Mt.19. R	Reverse	5'- AAT CCT GCG AAT AGG CTT CCG GCT-3'	13733-13710	54 C^0
Mt.20. R	Reverse	5'- GCT ATT GAG GAG TAT CCT GAG GCA-3'	14454-14431	52 C^0
Mt.21. R	Reverse	5'- TGC AAG CAG GAG GAT AAT GCC GAT-3'	15112-15089	52 C^0
Mt.22. R	Reverse	5'- GGT AGC TTA CTG GTT GTC CTC CGA-3'	15782-15759	54 C^0

2.4. METHODS:

2.4.1. Sample processing:

For the molecular study a small slice was taken from the deepest focus of grossly malignant looking mass (tumor) from nearly fresh* (mastectomy and wide local excision) samples (Fig.2-1), about 0.3 cm in maximum dimension, placed in normal saline and stored in -20 C^0freezer until time of DNA extraction.

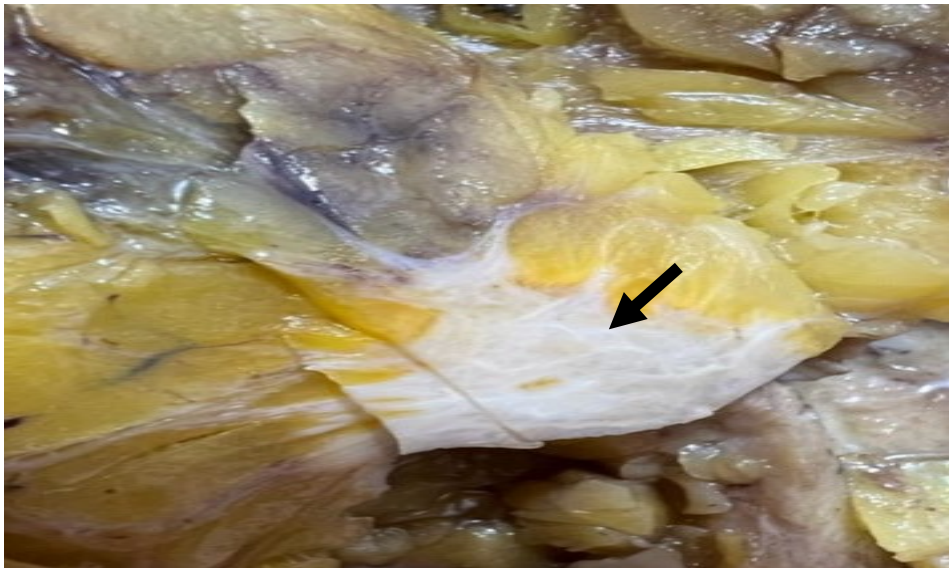

Figure 2-1: Gross appearance of malignant-looking focus in mastectomy specimen, radiating, ill-defined

Two other sections lateral to the selected molecular section were taken for histopatological examination to confirm presence of malignant cells within the selected sample for the molecular study. Paraffine blocks were prepared from the histopathology sections, stained and were examined by two pathologists under light microscope, Nottingham scoring system was used for grading.

***Nearly fresh, specimens were sampled within 2-3houres from the surgery and samples were taken from the deepest portion of the tumor with minimal formalin penetration**

2.4.2. DNA extraction from tissue:

Whole DNA (nuclear and mitochondrial DNA) was extracted from the tissue samples using (*GeNet* bio/South Korea) for Genomic DNA extraction Kit (from tissue) which includes the following component:

(Proteinase K solution, Buffer TL (tissue lysis buffer), Buffer GB, Wash buffer GW1, Wash buffer GW2, Elusion buffer GE) (steps of work according to the manufactures manual)

2.4.3. DNA extraction from blood:

Whole DNA (nuclear and mitochondrial DNA) was extracted from the blood of control samples using (*GeNet* bio/South Korea**)** for Genomic DNA extraction Kit (from blood) which includes the following component: **(**Proteinase K solution, Buffer GB, Wash buffer GW1, Wash buffer GW2, Elusion buffer GE), (Steps of the work are mentioned in Appendix)

2.4.4. DNA concentration measurement:

The photometer was turned on for a while for warming up, 50µl of nuclease free water was placed in disposable cuvettes as control and read by the photometer, then after 5µl of the nuclease free water was removed from the cuvette and 5µl of the extracted DNA placed and mixed well by pippeting with the remaining Nuclease free water. Finally the mixture read by the photometer, giving the concentration of DNA in ng/µl.

2.4.5. PCR amplification of human mitochondrial genome:

The entire mitochondrial genome was amplified in the form of four overlapping PCR-fragments A, B, C and D

PCR protocol

Two types of PCR kits were used for the amplification process:

A. Long Taq kit (Dongsheng Biotech/China), components of this kit were: Long reaction mix (dNTPs, $MgCl_2$, 0.02% bromophenol blue), PCR enhancer, Long Taq DNA polymerase

This kit was used for the amplification of A, B, C fragments as these fragments were > than 2kb, table (2-8).

Table 2-8: Components of PCR reaction for fragments A, B and C with there concentration percentage

	Reaction component	**Volume**	**Concentration**
1	Long reaction mix	12µl	1X
2	PCR enhancer (10X)	2.5	1X
3	Long Taq DNA polymerase	0.5	1.25U
4	Forward primer	0.5	0.5µM
5	Reverse primer	0.5	0.5µM
6	Nuclease free water	6.5µl	-
7	Template	2.5µl	25pg/µL
	Total volume	25µl	

B. FS^{TM} Mix (Dongsheng Biotech/China), components of which were:

- 2xFS^{TM} Mix (containing FS^{TM} Taq DNA Polymerase, dNTPs, and all other PCR components). This kit was used for D fragment amplification, table (2-9).

Table 2-9: Component of PCR reaction for D fragment with their concentration:

	Reaction component	**Volume**	**Concentration**
1	PCR mix	12.5µL	1X
2	Nuclease free water	9µL	-
3	Forward primer	0.5µL	0.5µM
4	Reverse primer	0.5µL	0.5µM
5	Template	2.5	25pg/µL
	Total volume	25µL	

PCR program:

The PCR program was the same except for the alterations in the annealing temperature and the extension time which were manipulated according to the type of primers used and fragment sizes respectively (2-10)

Table 2-10: PCR program

Cycle steps	Temperature	Duration	No. of cycles
Initial Denaturation	94C^0	*	1cycle
Denaturation	94 C^0	40Seconds	35cycle
Annealing	**	40 seconds	
Extension	72 C^0	***	
Final extension	72 C^0	5minute	1cycle
Hold	4 C^0	∞	1cycle

***Changed according to the size of the fragment, **Changed according to the type (component) of the primers, ***Changed according to the size of the fragment, ∞ infinity**

2.4.6 DNA visualization on agarose gel electrophoresis:

1% agarose gel was prepared for visualizing amplified DNA and measure the fragment length

Protocol of Agarose gel preparation:

Gel was prepared using 1.5gm of agarose powder was added to 150ml of (1X TBE) in a flask, then the mixture was heated in microwave for 2minutes, until the mixture changes into a homogenous clear solution, after cooling in room temperature, 6µl of 1Mg/ml of Ethidium bromide was added to the solution and stirred. The solution is then poured into a gel tray with its combs placed and left to solidify at room temperature for 30-45minute.

Solidified gel was placed in to the electerophoresis chamber and filled with 1X TBE buffer and lastly the comb was removed. DNA sample or PCR product was mixed with 1µl of loading buffer and placed in separate wells; appropriate DNA ladders were used according to the used product.

Electerophoresis was turned on voltage of 90 after a period ranged between minutes to hours (according to the sample) the gel was visualized under ultraviolet light.

2.4.7. PCR purification:

The PCR products were cleaned up from residual element using norgen biotek corp PCR purification kit**,** composed of the following components: (Binding buffer C, Wash solution A, Elution buffer B) steps of work are mentioned in Appendix

2.5. SEQUENCING:

Sequencing was done for 20 out of the 30 cancer samples and all the 20 control samples. Four PCR product fragments (A, B, C and D) were used as templates for sequencing with primers listed in tables (2-4 and 2-5), by using BigDye^{TM} Terminator v3.1 cycle sequencing kit and depending on the cycle sequencing technology (dideoxy chain termination; Sanger sequencing)on 6 Applied Biosystems 3730x1 and 9 ABI 3700 in Macrogene: http://dna.macrogen.com/eng/

2.6. ALIGNMENT OF SEQUENCES AND DATA ANALYSIS:

Homology searches were conducted using NCBI BLAST between the sequences of standard Revised Cambridge Reference Sequence (rCRS) of the Human Mitochondrial DNA from the Entrez database; accession number NC_012920. https://www.mitomap.org/MITOMAP website which is a comprehensive database for the human mitochondrial DNA was used for allocation of mutations, identifying their types and determines their effect on amino acids.

2.7. STATISTICAL PROCEDURES:

Different charts and columns were used to show proportions and percentages between categories of different mutations in different mtDNA regions. The algorithm implemented in the HaploGrep 2.0 was used for identification of haplogroups (Kloss-Brandstätter et al,

2011), neutrality test and Tajima's D (Tajima 1989) were used to historical demography determination in the control sample

Chi-square and Fishers Exact test were used to calculate p values. Odd ratio was used for determining the relation between specific haplogroups and breast cancer, occurrence of specific SNPs among breast cancer cases, and difference in the percentage of SNPs and sporadic mutations in breast cancer cases.

CHAPTER -3

RESULTS

3.1. HISTOPATHOLOGICAL ASSESSMENT OF SAMPLES:

Collected samples (patients) age ranged between 28-68 years oldm, and all were in the 2nd and 3rd stage of the disease.

All the 30 cancer samples were invasive ductal carcinomma not otherwise specified , 25 of them were poorly diffrentiated (grade III) and the remaining 5 were moderately differentiated (grade II), (Fig 3-1and 3-2). Regarding the pathological examination of the twenty control samples, 11 of them were non-proliferative fibrocystic disease and 9 were fibroadenomas.

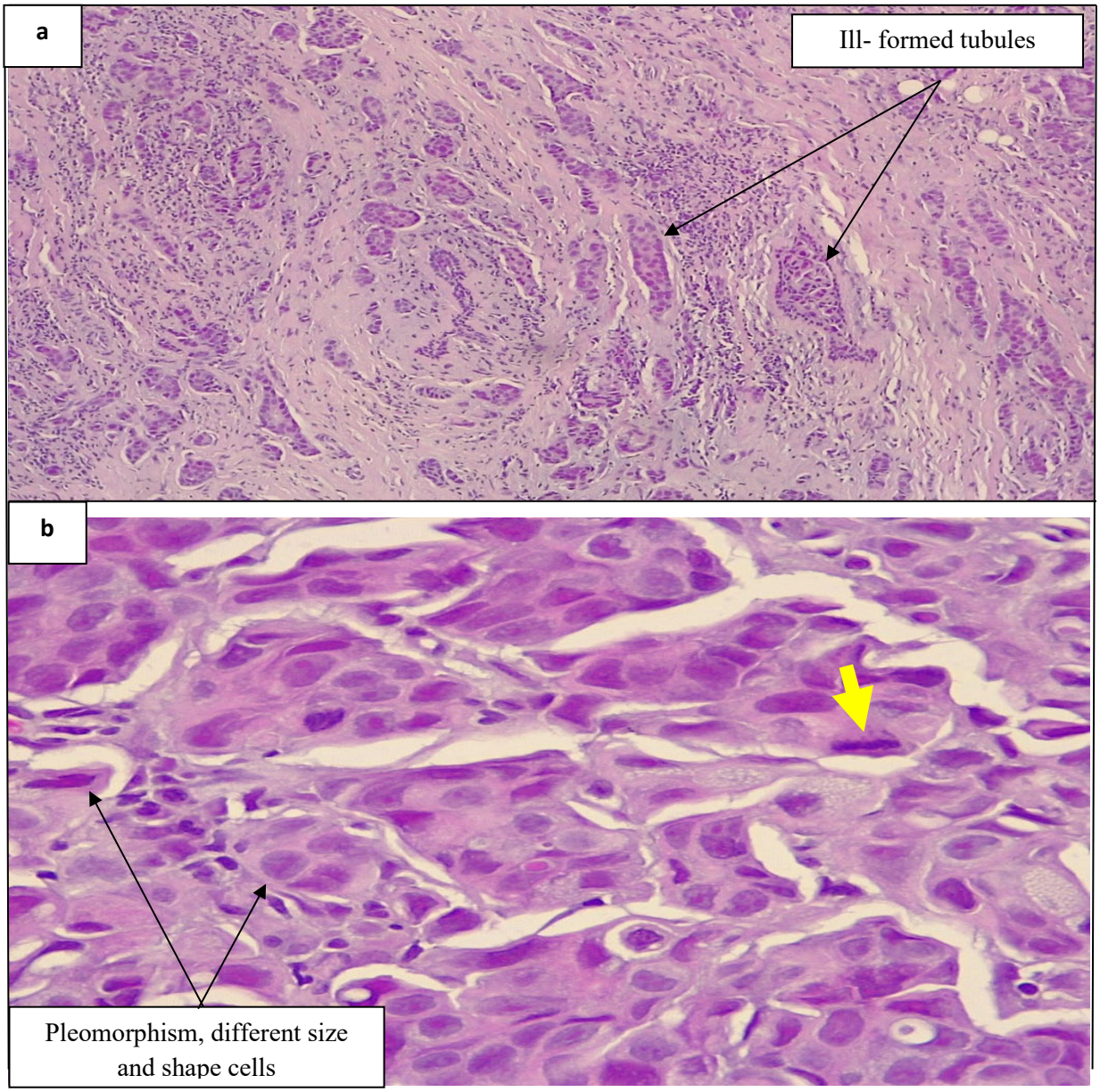

Figure 3-1: Microscopical apperance of breast cancer (grade II), a 10x, b 40x, yellow pointer is pointong to an atypical mitotic figure

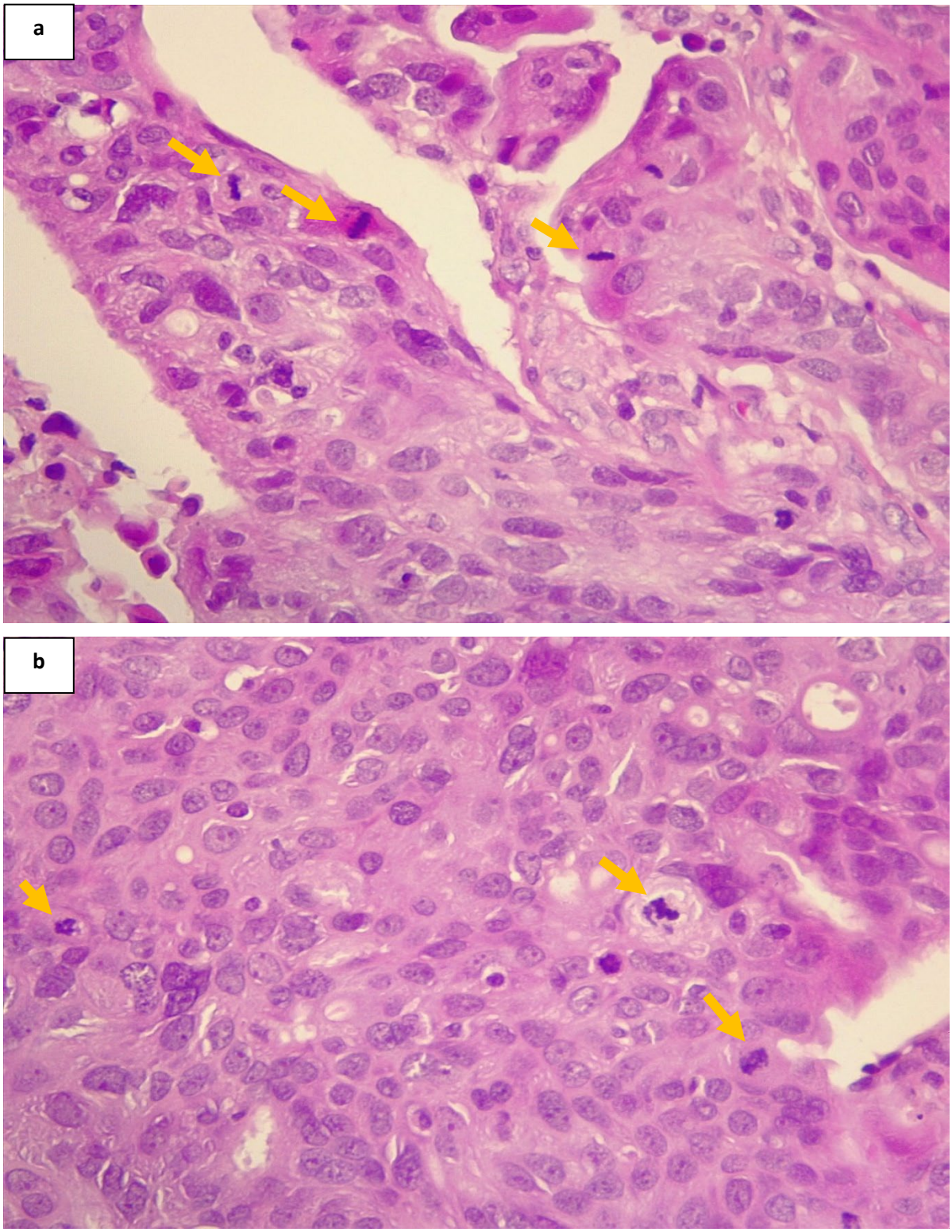

Figure 3-2: Microscopical apperance of breast cancer (grade III), a, b 40x, yellow pointers are pointong to an atypical mitotic figures

3.2. MITOCHONDRIAL DNA AMPLIFICATION:

Mitochondrial DNA of each case was extracted and amplified in the form of four overlaping fragmets (A, B, C and D) (Fig.3-3) and were subsequently sequenced. The cases were named numerically (1-30).

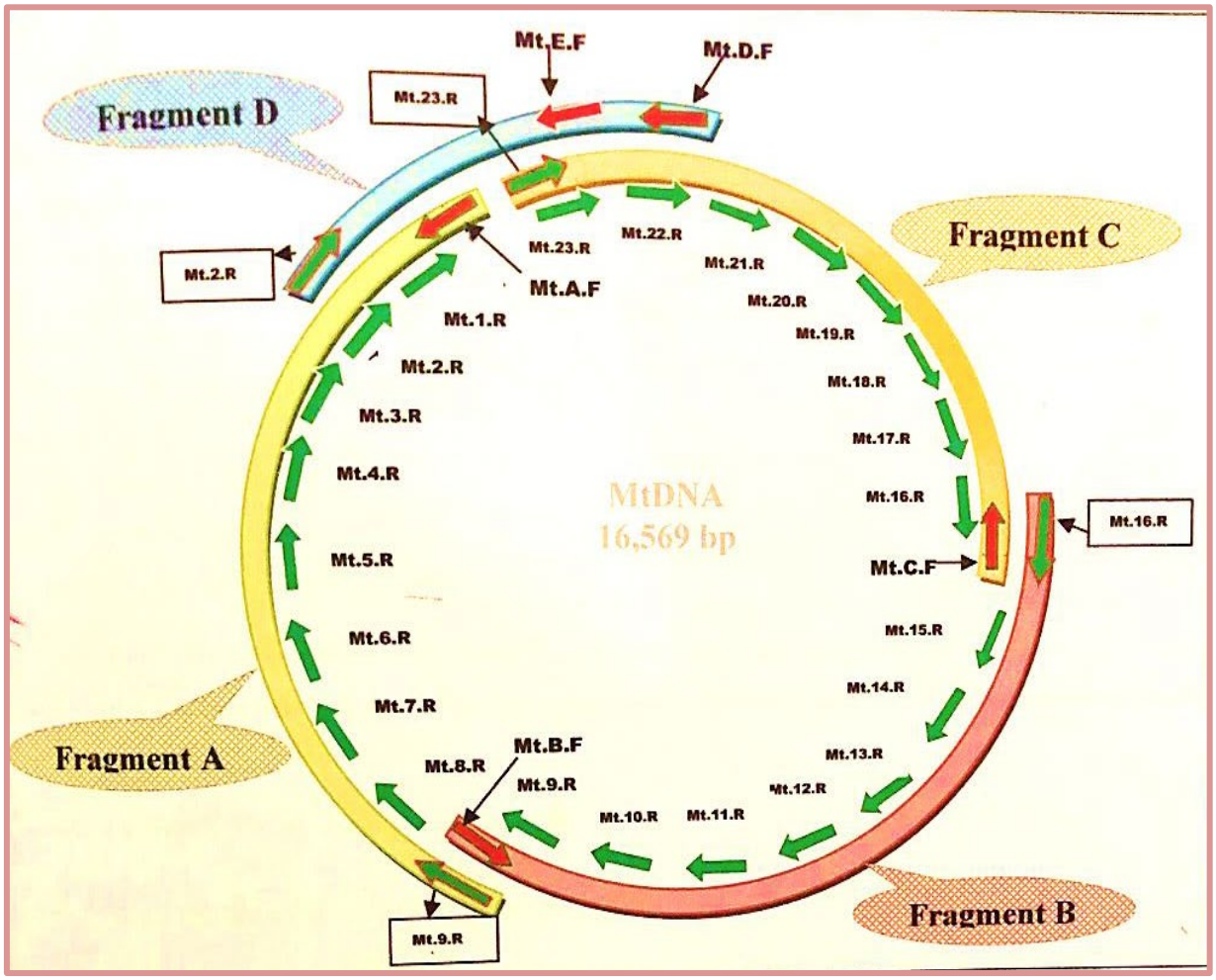

Figure 3-3: Diagram representing mitochondrial genome with locations of both amplifications and internal reverse primers (Rashid, 2014)

Upon the PCR amplification, three pattern of PCR fragment amplification (three groups) were identified in breast cancer cases, compared to control samples;

1. First group showed partial amplification of mitochondrial genome (failure of amplification of fragments B, C or both out of the 4 overlapping fragments)
2. Second group showed no amplification (Mt-DNA Copy number defect) of the entire mitochondrial genome (failure of amplification of the 4 overlapping fragments)
3. Third group showed intact amplification of the 4 overlapping fragments

3.2.1. Partial mtDNA amplification:

One fourth of the breast cancer cases were in this group (1st mutant group), and charactristically showed an intact amplification of the two overlapping PCR fragments (A & D) with 2 distinct bands (6000 bp & 2000 bp respectively) were visualized on agarose gel (Fig. 3-4). However the 2 other overlapping fragments (B & C) were either poorly amplified (faint band) or not amplified compared to the control samples as well as fragments (A & D) of the same extracted DNA of the cancer samples (Fig.3-5 and 3-6).

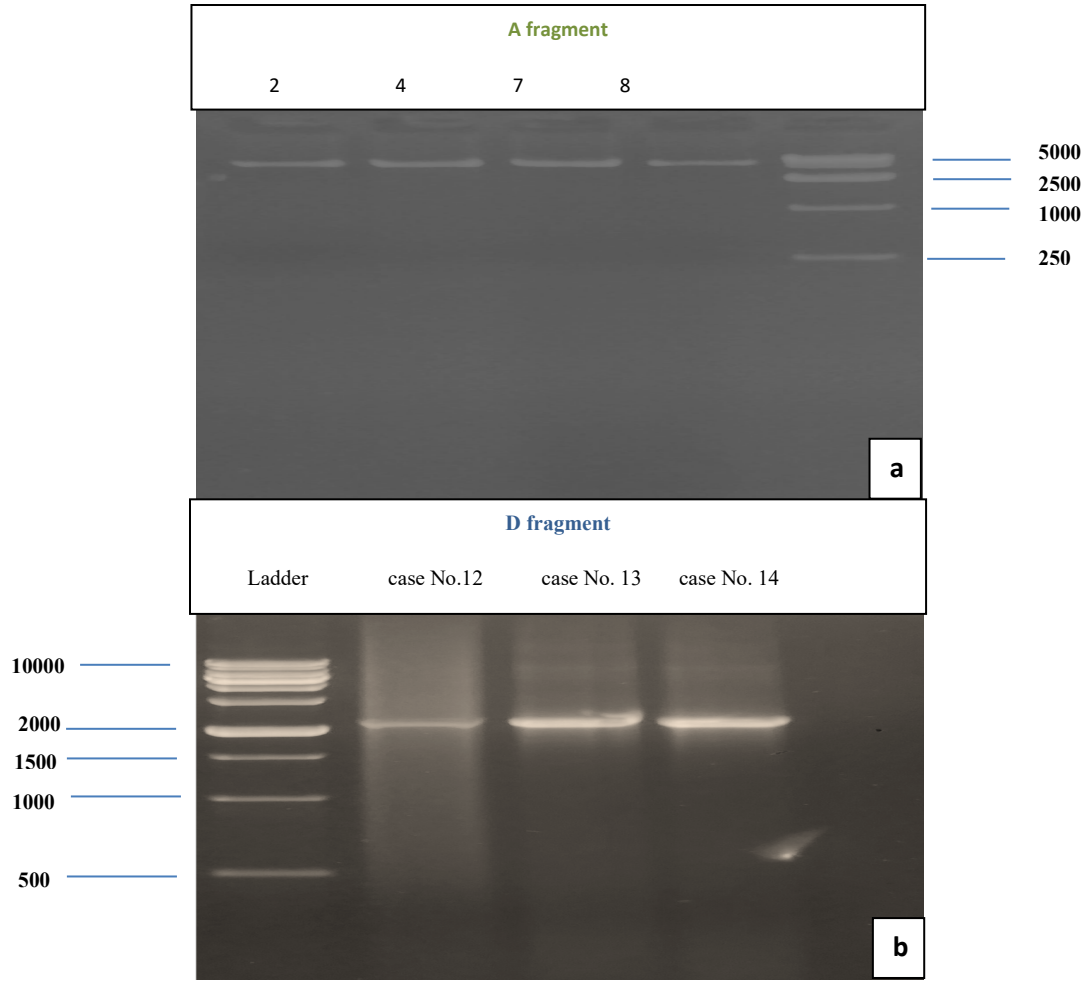

Figure 3-4: Agarose gel electrophoresis, fragment A (a) and fragment D (b) amplification with DNA ladder 15kb and 1kb respectively

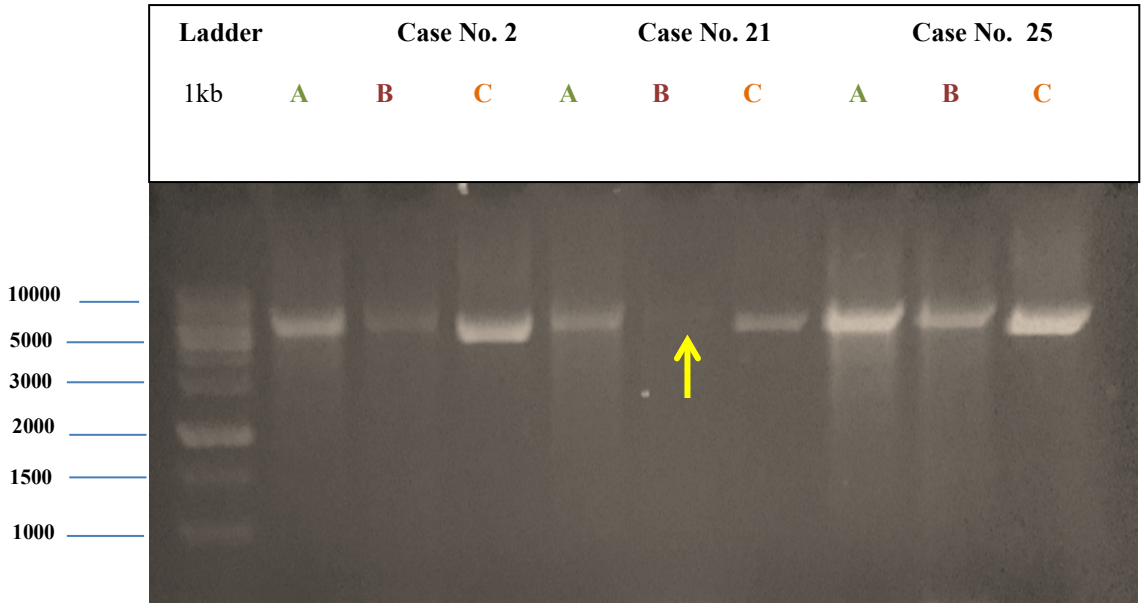

Figure 3-5: Agarose gel electrophoresis, faint to no B fragment amplification in case No.21

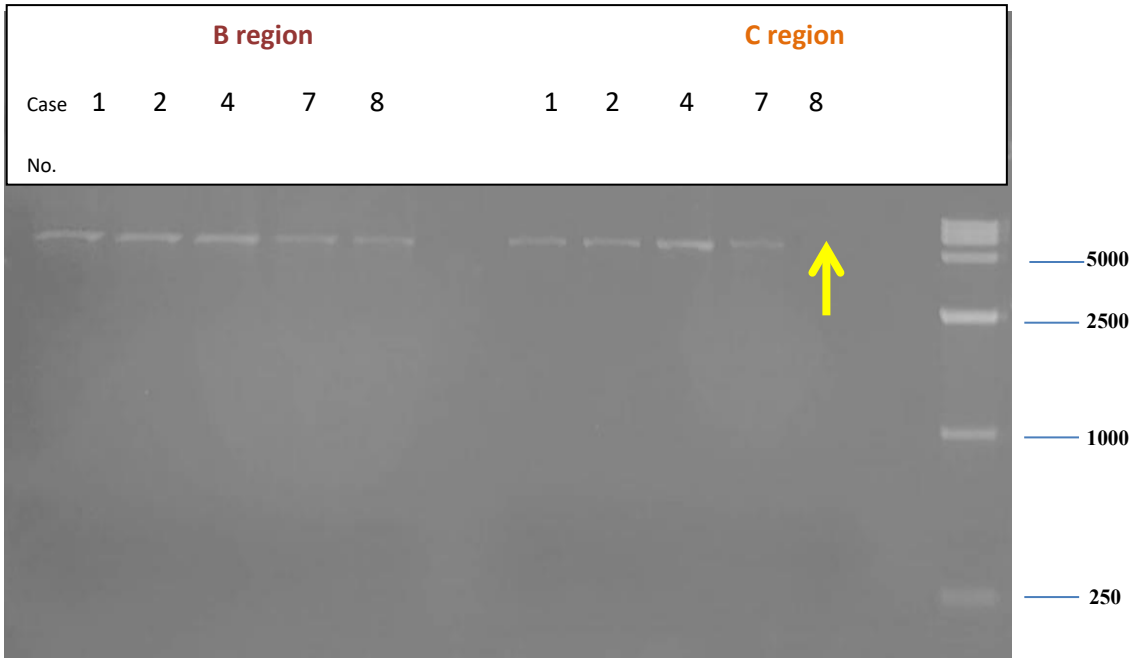

Figure 3-6: Agarose gel electrophoresis, faint to no C fragment amplification in case No.8

The partial PCR amplification (defective B and C fragment amplification) in this group of cancer cases indicates the presence of a mutation. In this aspect, there are 3 possible types of mutation : either a point mutation at the 3'site of the primers or an insertional mutation

leading to elongated fragment, in which the impaired amplification might be due to short extension time of the RCR programs, and the last possible mutation is a deletion mutation at the primer site in a way that the primers have no complimentary sequences for annealing and subsequent amplification. Therdefore, in order to clarify the type of the mutation, PCR based chromosomal walking was performed with different reverse primers for both fragments (Fig 3-3).

PCR based Chromosomal Walking

PCR based chromosome walking was performed for cases with impaired fragment B (Mt.B_F-16_R) amplification using three different reverse primers one internal within the B fragment (15_R) and two externals (17_R and 18_R). (Fig. 3-7).

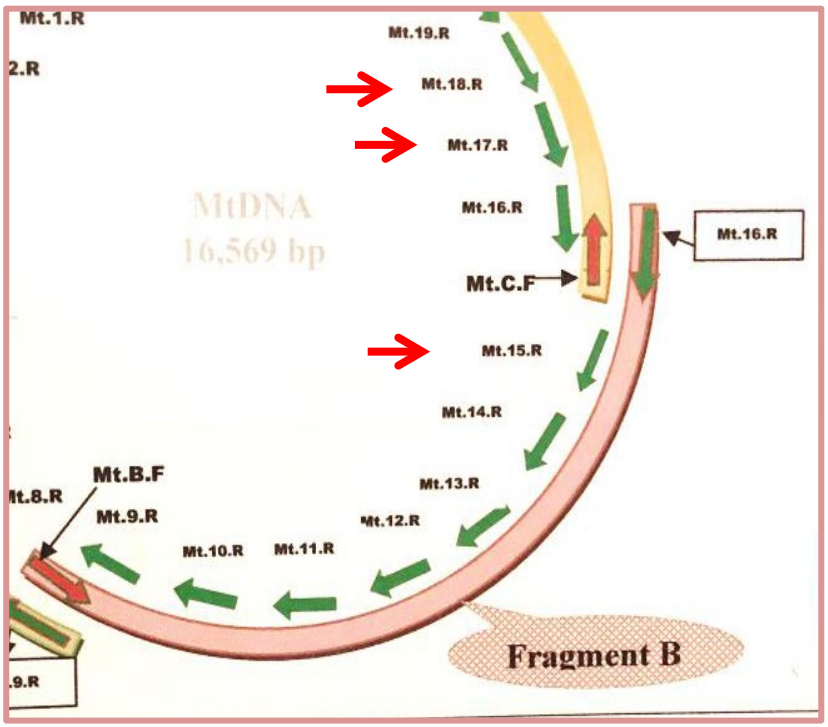

Figure 3-7: Fragment B with PCR based chromosomal walking (reverse primers marked by a red arrow)

The patterns of amplification as shown in figure (3-8), reveales distinct bands of the B fragment with the reverse primer 15_R and 18_R, while with reverse primer 17_R the amplification

band was faint. These results indicate the presence of mutation within the 1000bp (1kb) region were primers 16_R and 17_R are located.

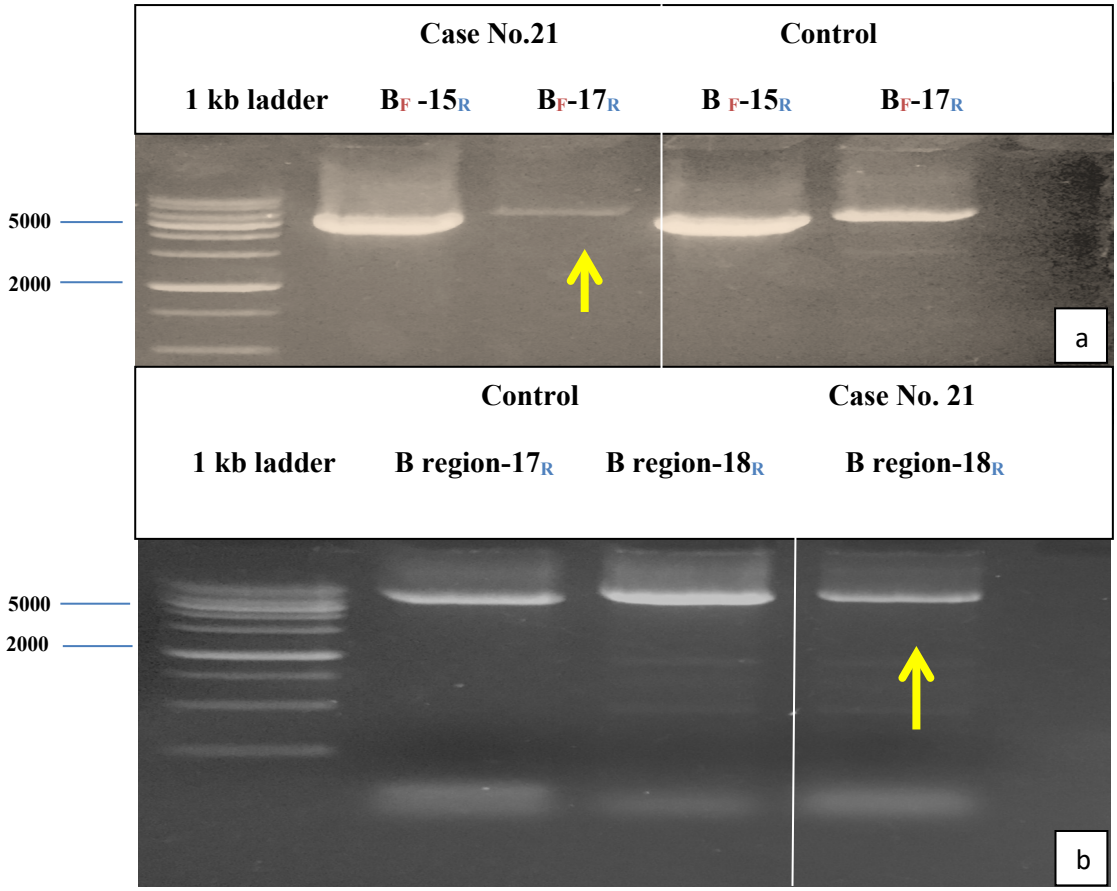

Figure 3-8: Agarose gel electrophoresis, PCR based chromosomal walking product

a. B region amplification result with reverse primers 15_R and 17_R
b. B region amplification result with reveres primer 18_R

Regarding cases with impaired C fragment amplification, expected mutations were more likely to be located in the reverse primer region, rather than forward primer region, as the forward primer is overlapping with the reverse primer of fragment B and most of these cases had readily amplified B fragments (Fig. 3-6). Therefore PCR based chromosome walking was performed for the fragment C (Mt.C_F-23_R) with 2 reverse (1_R) an external and (22_R) an internal primer as indicated in (Fig.3-9 a). The amplification pattern was as shown in figure (3-9 b), distinct bands of the fragment C were observed with the reverse primer (22_R) while with the reverse primer (1_R) there was no amplification.

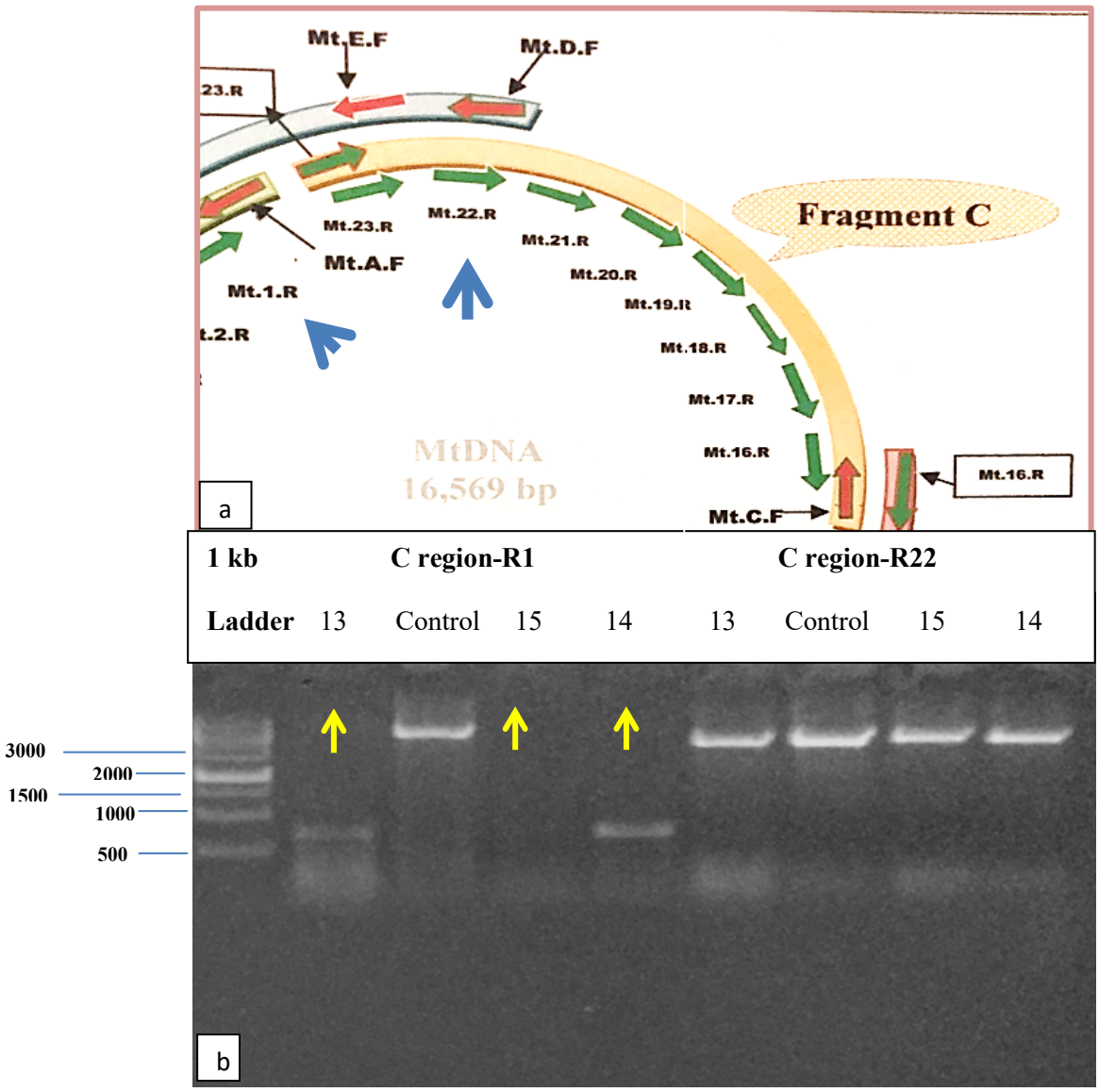

Figure 3-9: PCR based chromosomal walking:
a. Schematic illustration of overlapping area between A and C fragment (blue arrow refers to the site of the used reversed primers)
b. Agarose gel electrophoresis, amplification result of fragment C with the reverse primers 1_R and 22_R

To clarify the defect, PCR based walking with two other reverse primers (2_R and 3_R) was performed to screen the area between C and A fragments. (Fig. 3-10 a). Surprisingly amplification of the area between fragment A and C was readily amplified (Fig.3-10 b), indicating presence of a mutation in the site where reverse primers 23_R and 1_R are located

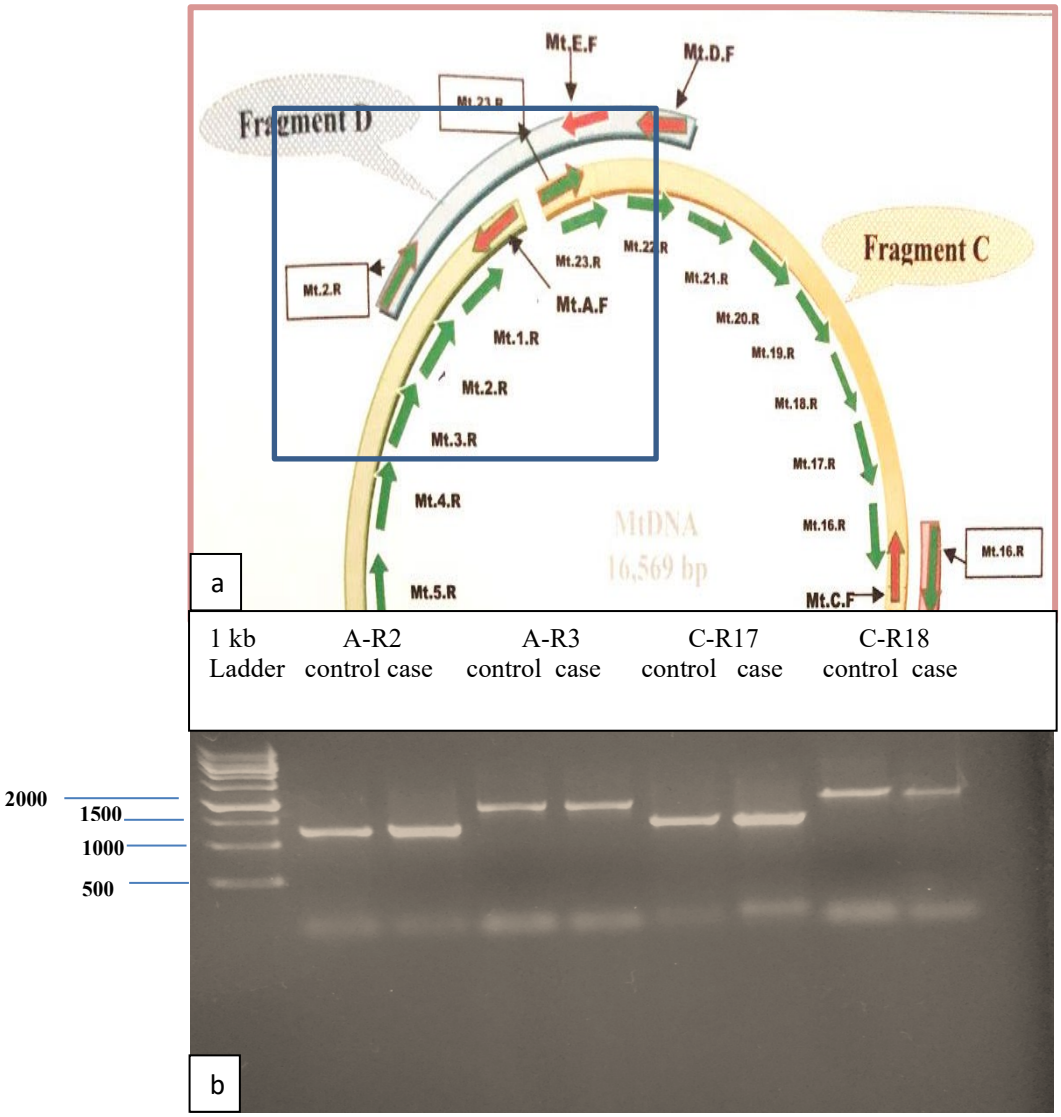

Figure 3-10: PCR based chromosomal walking:

a. Schematic illusturate of a mtDNA segment, area the blue frame thoroughly studied by chromosomal walking

b. Agarose gel electrophoresis, A region with 2_R, 3_R, and C region with 17_R and 18_R

3.2.2. mt- DNA copy number (No amplification of mitochondrial genome):

The second group of mitochondrial mutational defect (dysfunction) in cancer cases of the current study was the failure of amplification of all the 4 overlapping fragments A, B, C and D (Fig 3-11).

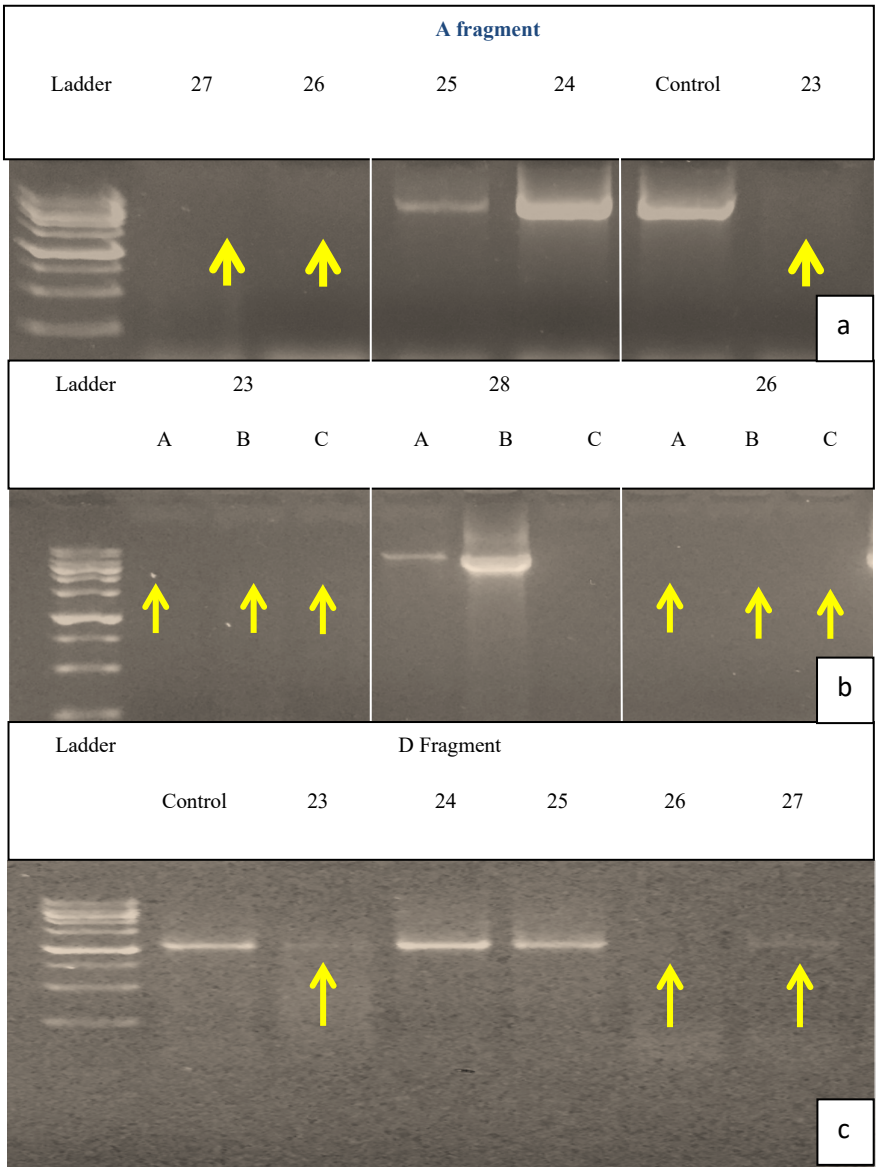

Figure 3-11: Agarose gel electrophoresis of impaired PCR reaction products**, a.** Fragment A, **b.** Fragments A, B and C and **c.** Fragment D

Failure in amplification of mitochondrial genome (all the four overlapping fragments) in this group of cases was supported by error minimizing techniques as repeating PCR fragment amplification in two different experiments and using control samples with every amplification steps, furthermore the possibility of inhibitors was eliminated as well by performing multiplex

PCR for chromosomes (13, 18 and XY) for the extracted DNA and the fragments were readily amplified. (Fig. 3-12).

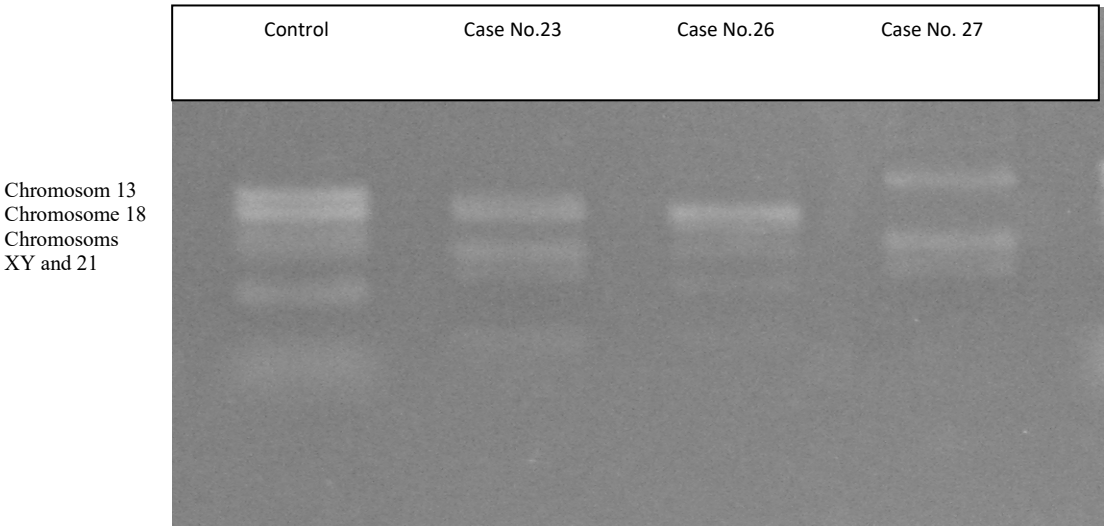

Figure 3-12: Multiplex PCR reaction for chromosomes 13, 18 and XY 21

3.2.3. Intact amplification of 4 fragments of mitochondrial genome:

Twenty cancer cases in the current study were within this group, showing an intact amplification of all the 4 overlapping fragments (A, B, C and D). Therefore, all the fragments of each case were sequenced with the control samples to identify population related mutations (Single nucleotide polymorphism "SNP"), haplogroups and their possible relation to breast cancer as well as pathogenic mutations that cause mitochondrial dysfunction and possibly predisposing to carcinogenesis.

3.3 PATTERN OF MITOCHONDRIAL MUTATION:

According to the results, 203 mutations were detected from the 20 control samples and 344 mutations were detected from 20 breast cancer samples*.

Nucleotide substitution (point mutation) was the commonest type of mutation, constituting 100% of mt-tRNA mutations, 99% of protein coding region and 94% of mt-rRNA mutations. While mutation in the non-coding region were mixed and composed of 90% nucleotide substitution, 7.5% insertion and 2.5% deletion (Fig 3-13).

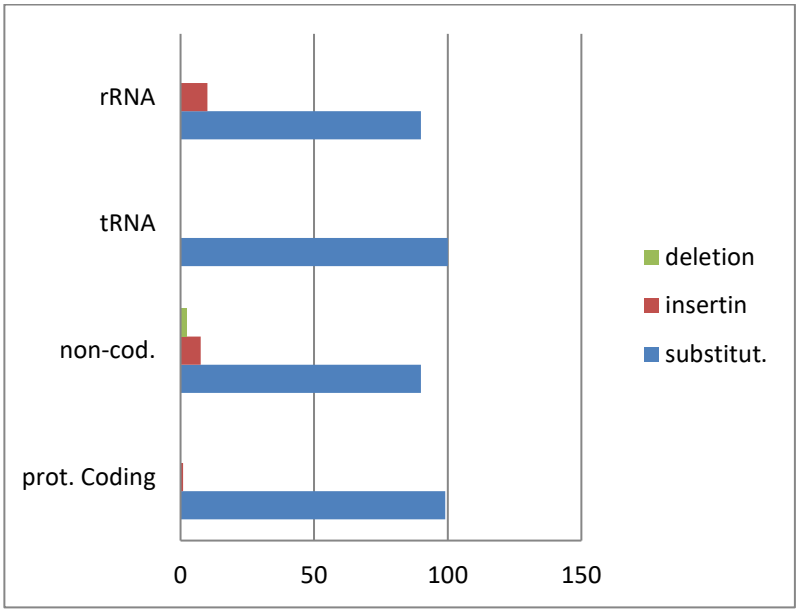

Figure 3-13: Distribution of different kinds of mutation throughout the mtDNA in breast cancer cases

*This number included the population related mutations and pathogenic mutations

In general mutations were concentrated mostly in the protein coding region then followed by HV1, HV2, rRNA, tRNA and least in the HV3, as shown in Figure (3-14)

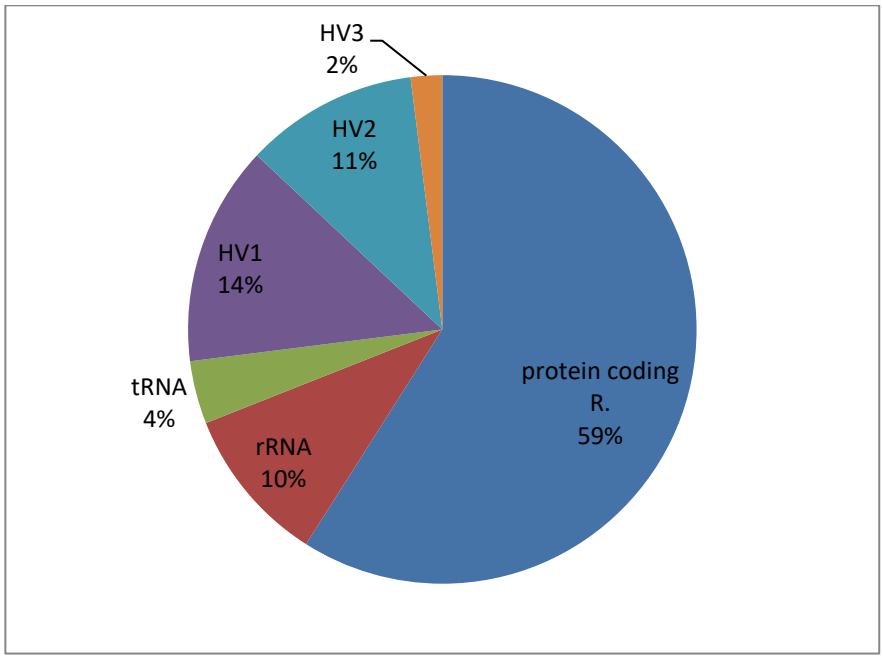

Figure 3-14: Percentage presentation of mutation across the whole coding and non-coding mtDNA regions

In general, the mutations were categorised into three groups:

1. Single nucleotide polymorphism* (SNP), most of the identified mutations belonged to this category (74% among the breast cancer cases and 90% among control samples)
2. Variant mutations**, in the current study these types of mutations have been identified in 20% of breast cancer cases while in the control samples they were identified in only 7.8%.
3. Unique mutations***: these types of mutation in breast cancer samples and control samples were 6% and 2.2% respectively. (Fig 3-15)

*Also called germ-line mutations, are population related
**these types of mutations are recorded in the genebank, either in studies related to population or disease-related, yet of unknown potentials.
***these are mutations that have not been yet recorded in the genebank and are of unknown pathological potentials.

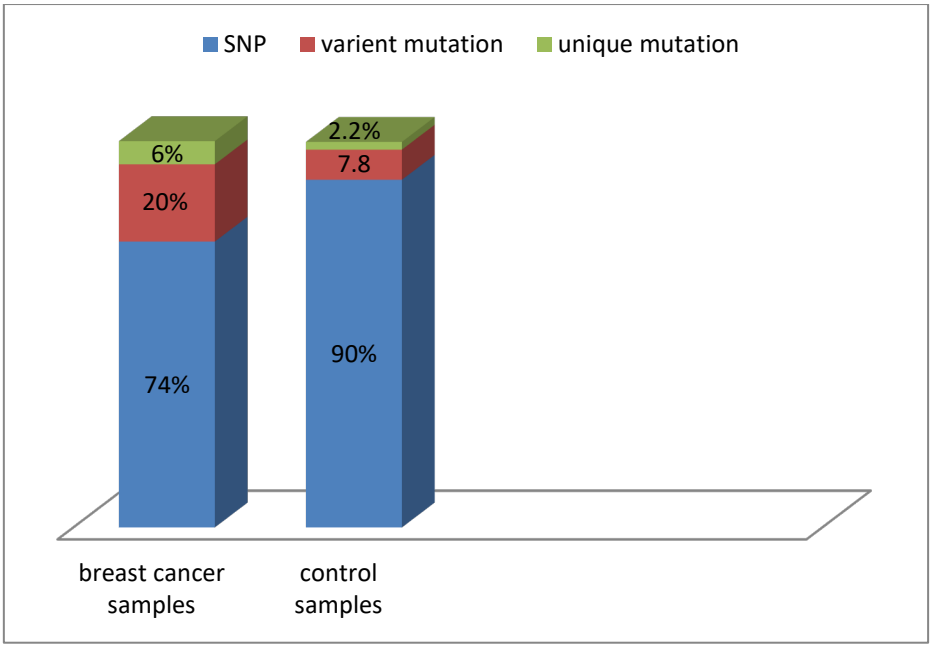

Figure 3-15: Categories of mutation distribution in breast cancer cases and controls

Nevertheless, mutations in the unique category are prone to jump to the variant category at any time as there is an on-going and continuous interest in mitochondrial DNA mutation studies.

3.3.1. Haplogroups and Breast Cancer:

In order to find correlations between the haplogroups and the breast cancer in the current study, based on the total (SNP) mutations and by using the Haplogrep 2.0 program, nine haplogroups and their subclades were identified in the samples of the current study (both cancerous and control samples) and all the nine haplogroups were among the Western Eurasian haplogroups, indicating that they are the dominant haplogroups in the Kurdish population in Sulaymaniyah city. Interestingly while H haplogroup was the dominant haplogroup among the control samples (60%), HV and N haplogroups were the dominant ones among the cancer samples constituting 35% and 25% respectively (table 3-1).

Table 3-1: The identified Haplogroups in breast cancer (A) and control (B) subjects

(A)

Breast cancer samples		
Haplogroups	Frequency of occurrence	%
HV	7	35%
N	5	25%
U7	2	10%
R0	2	10%
J	1	5%
U1	1	5%
T	1	5%
H	1	5%
Total	20	100%

(B)

Control samples		
Haplogroups	Frequency of occurrence	%
H	12	60%
HV	2	10%
N	1	5%
R0	1	5%
J1	1	5%
T1a	1	5%
U1a	1	5%
U7	1	5%
Total	20	100%

Table 3-2: Statistical analysis of haplogroups

Statistical test for haplogroup analysis	P value	OR for HV/H
Chi square	0.002	28.00
Fishers Exact	0.006	

A statistically significant association was identified between haplogroup HV and breast cancer, with an odd ratio for (HV/H) greater than one (table 3-2), indicating that (HV) is a high-risk factor for the occurrence of breast cancer among the studied population.

3.3.2 Haplogroup study of Sulaymaniyah city residents:

Using the 20 control samples (blood) with another 16 mitochondrial DNA samples (unpublished data from healthy individuals from Sulaymaniyah city center), a total of 36 samples were used to study their haplogroups and determine their historical demography as a representative sample from the city. Western Eurasian haplogroups were the only determined groups in the studied samples, in which haplogroup H was the most common (41.6%), and the

second most common haplogroup was HV (19.4%), while macro-haplogroup N which represents the dominant Western Eurasian haplogroups and its descendants as R0, U, T and J were less common in the current study. Not to mention three haplogroups (T1a, J1 and U1a) were singletons (Table 3-3)

Table 3-3: Haplogroups and sub-haplogroups and their percentage among the 36 samples as an illustrative group of the city

Haplogroups	Frequency of occurrence	Percentage
H	15	41.6%(14%H+28%H2a
HV	7	19.4%(17%HV+3%HV1)
N	6	16.6%
RO	3	8.3%
J1	1	2.7%
T1a	1	2.7%
U1a	1	2.7%
U7	2	5.5%
Total	36	100%

Tajima's D value was calculated for demographic expansion determination, and it was strongly negative (−2. 155811, p <0.01) table (3-4)

Table 3-4: Results from Tajima's neutrality test

m	s	Ps(s/n)	Ɵ	Л	D
36	186	0.011228	0.0027108	0.001157	-2.155811

(**m** number of samples, **n** total number of sites, **s** number of segregating sites, Ps stands for proportion of polymorphic sites and its = s/n, Ɵ Ps/a1, Л nucleotide diversity) D Tajima's test statistic (Thomas 2001)

3.3.3. Single nucleotide polymorphism (SNP):

Most of the population related mutations (SNP of Kurds/Sulaymaniyah) were within the coding region of mitochondrial genome 61%, out of which 78% were in protein coding region, 18% were in mt-rRNA and 4% in mt-tRNA region (table 3-5). The reminder 39% of population related mutations were in the non-coding region (HV1, 2 and 3), (table 3-6), the patterns of distribution of SNP across the whole mitochondrial genome are illustrated in (Fig. 3-16).

Table 3-5: SNP and mutation positions in coding region of cancer samples, red coloured sites are repeated more than once

Coding region	**List of mutations**
Protein coding region	T3394C, G3834A, C3741T, C4011T, T4216C, A4769G, A4917G, C7028T, C8137T, C8684T, G8697A, A8860G, G9755A, T9899C, C10142T, G10586A, A11251G, A11467G, G11719A, G12372A, A12612G, G12618A, C12705T, T12879C, A13104G, C13188T, G13368A, T13500C, G13708A, A14139G, G14368A, G14569A, G14905A, C14766T, G15148A, A15326G, C15452A and T15607C
tRNA	A12308G, A10463C
rRNA	G709A, A750G, T980C, A1438G, A1811G, G1888A, C2259T, G3010A, A2706G,

Table 3-6: SNP and mutation positions in non-coding region (hypervariable region) of cancer samples, red coloured sites are repeated more than once

Non-coding region	List of mutations
HV1	T16086C, T16172C, C16186T, C16187T, T16189C, C16192T, C16193T, T16217C, C16223T, G16274A, T16209C, C16234T, T16249C, C16291T, C16294T, A16309G, A16318T, T16362C and T16519C
HV2	T146C, C151T, T152C, T195C, A263G, G417A, G499A
HV3	C462T and T489C

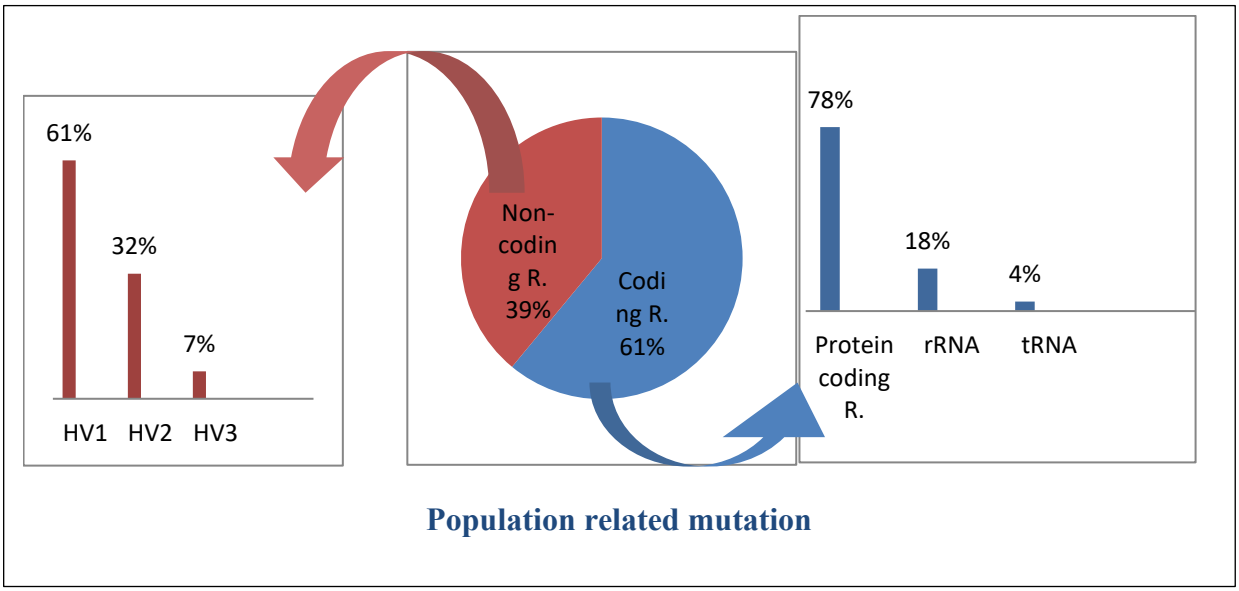

Figure 3-16: Distribution of SNP through the regions of mtDNA

A specific transition mutation (A8860G) was identified in all breast cancer samples (100%) (Figure 3-17) compared to the control samples (20%). By comparing the transition mutation with three other randomly selected SNPs (A750G, A1438G and C7028T), a statistically significant association was identified (using chi-square and Fishers exact test), indicating that SNP (A8860G) represents a risk factor for breast cancer development (Table 3-7).

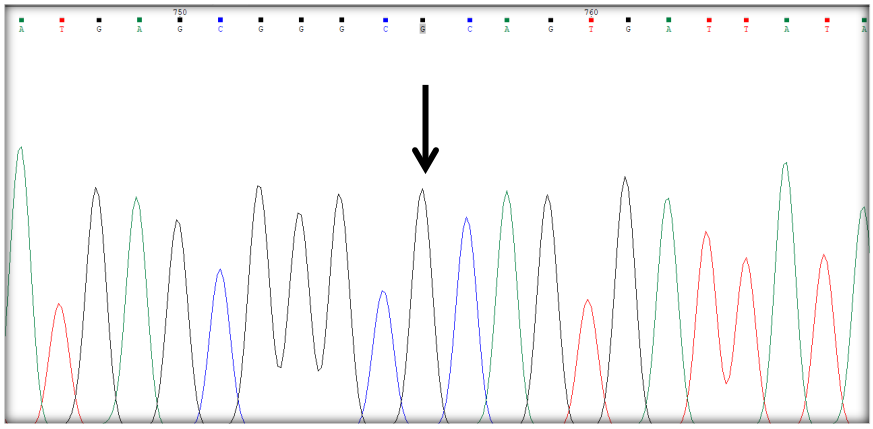

Figure 3-17: Sequencing electropherogram of (A8860G) mutation

Table 3-7: Calculated odd ratios and p values for SNP A8860G and three randomly selected SNPs

SNPs	Odd ratio	P value (chi-square	P value (Fishers exact)
8860/750	4.722	0.000	0.000
8860/1438	5	0.009	0.013
8860/7028	5	0.011	0.021

3.3.4. Sporadic mutations:

Sporadic mutations, non-population related mutations including both variants and unique mutation; among the breast cancer cases, 26% of the mutations were sporadic while only 10% of the mutations in control samples were of sporadic type. Calculated P value using both chi-square and Fishers exact was equal to 0.000 indicating a significant relation between occurrence of sporadic mutation and breast cancer. The odds ratio for (sporadic/population) was equal to (3.62) indicating that sporadic mutation occurrence is a high-risk factor in breast cancer among the studied samples.

a. Variant mutation:

Among breast cancer samples, 20% of mutations were in this category, aggregated mostly in genes coding for mitochondrial proteins 69%, (Fig. 3-18) demonstrates variant mutations across mtDNA regions.

Table 3-8: Percentage of variant mutations across the whole mitochondrial DNA

mtDNA region	Percentage of variant mutation
Protein coding region	69%
HV2	13%
rRNA	7.5%
HV1	6%
tRNA	3%
HV3	1.5%
Total	100%

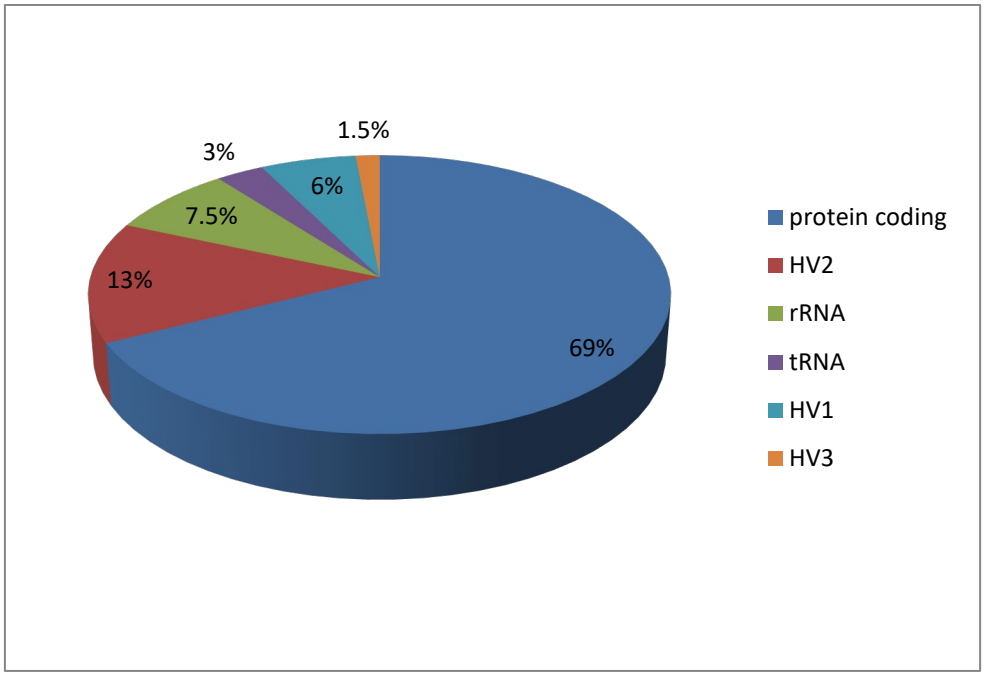

Figure 3-18: Percentage of variant mutation distribution across mtDNA regions

Most of the variant mutations were in protein coding region (69%) out of which (63%) were synchronous that means the mutation (nucleotide substitution) is not leading to amino acid change; while the reminder (33. %) of variant mutation were non-synchronous (leading to amino acid change)

Surprisingly, in the current study none of the variant mutations were observed among breast cancer cases or the control samples. Also, common sporadic mutation was neither identified in cancer samples nor in control samples except for (C9011T) mutation which was observed in two of the breast cancer samples.

b. Unique (not recorded) mutations:

Mutations of this category have not been reported previously, and therefore they are regarded as private mutations according to the mtSNP database. Thus, its pathophysiological significance is difficult to be predicted, however in the present study (6%) of mutations in breast cancer sample belonged to this category, of which (96%) were nucleotide substitution with only a single insertion mutation. Furthermore, most of the mutations were in protein coding region and almost all were nucleotide substitutions, (93%) of the nucleotide substitutions were asynchronous, resulting in change in the coding amino acid, distributed

mostly in the Cyto-B followed by ND5 (Table 3-9); figures (3-19 to 3-32) show the unique mutations in protein coding regions

Table 3-9: Unique (unrecorded) mtDNA mutations in the protein coding region

UNIQUE MUTATION	LOCATION	EFFECT	No. of Fig.
C4068G	ND1	synch	3-19
C4126G	ND1	Arg-Gly	3-20
A4590G	ND2	Ile-Val	3-21
C7418G	Cox 1	Phe-Leu	3-22
C7687G	Cox 2	Ile-Met	3-23
9956-57 T insertion	Cox 3	Frame shift	3-24
T9965A	Cox 3	Tyr-stop	3-25
A10784C	ND4	Ile-Leu	3-26
CA13166 and 67GG	ND5	Thr-Stop	3-27
A13862C	ND5	Asn-Thr	3-28
A14500T	ND6	Tyr-Asn	3-29
T14868C	Cyt-B	Leu-Pro	3-30
A15414G	Cyt-B	Tyr-Cyt	3-31
C15587G	Cyt-B	Leu-Val	3-32
C15590G	Cyt-B	Arg-Gly	3-32

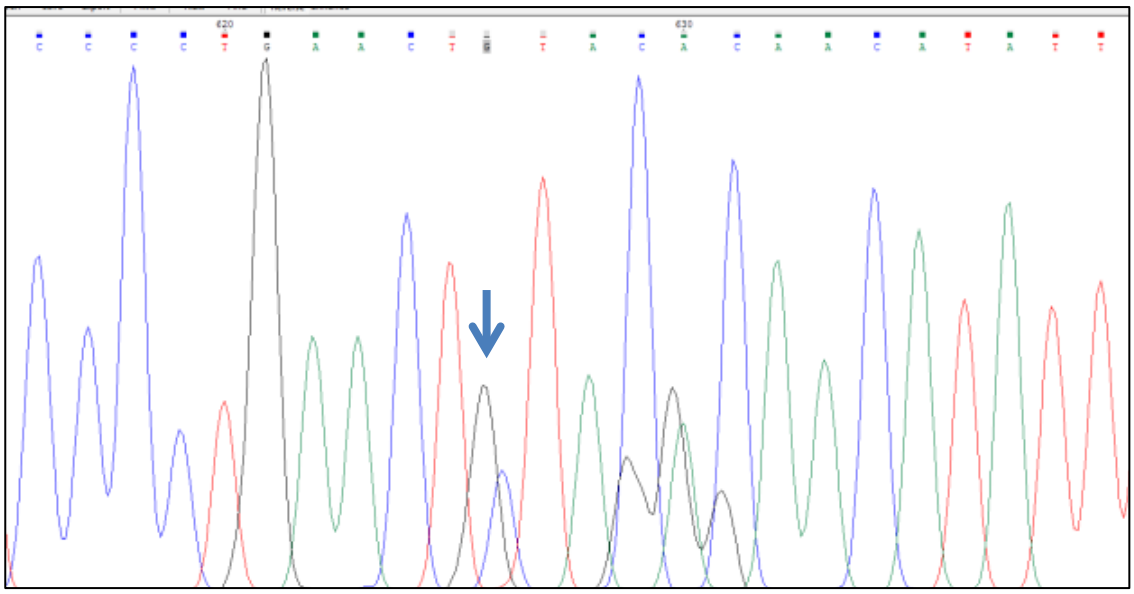

Figure 3-19: Sequencing electropherograms of novel mutation: substitution of C by G at position 4068

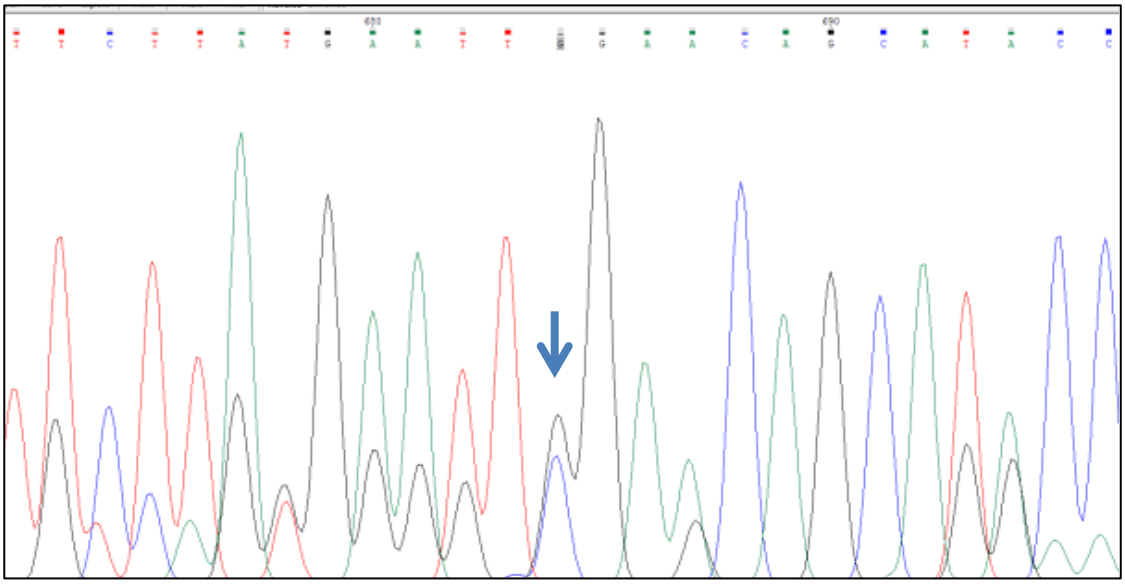

Figure 3-20: Sequencing electropherograms of novel mutation: substitution of C by G (Arg to Gly), at position 4126

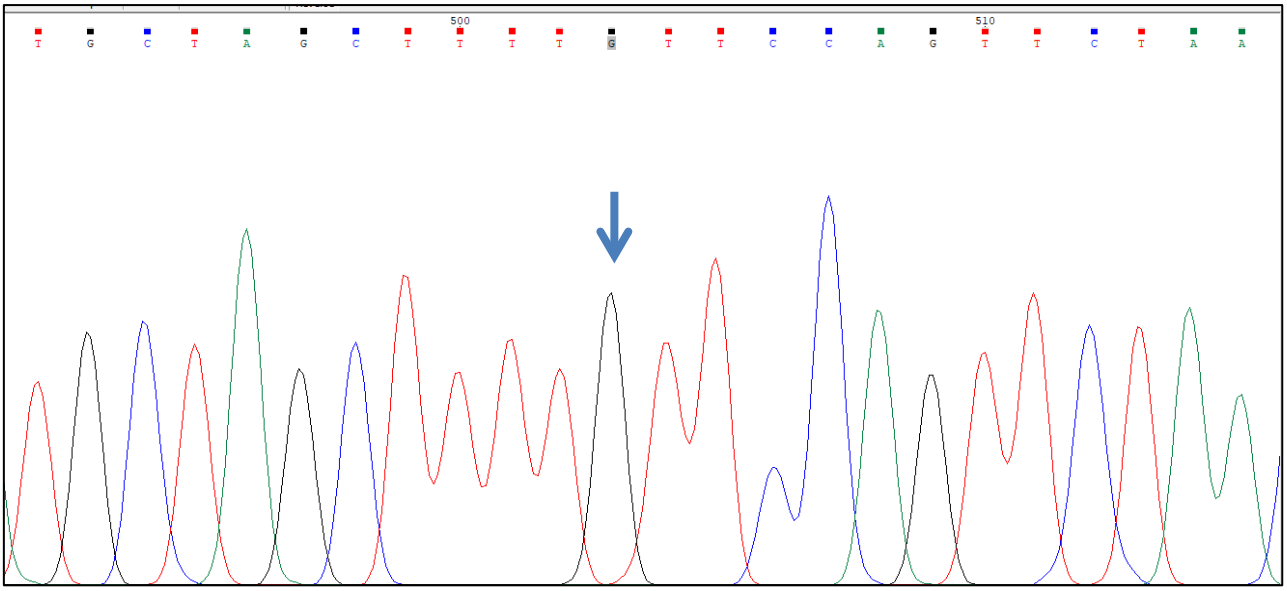

Figure 3-21: Sequencing electropherograms of novel mutation: substitution of A by G(Ile change to val), at position 4590

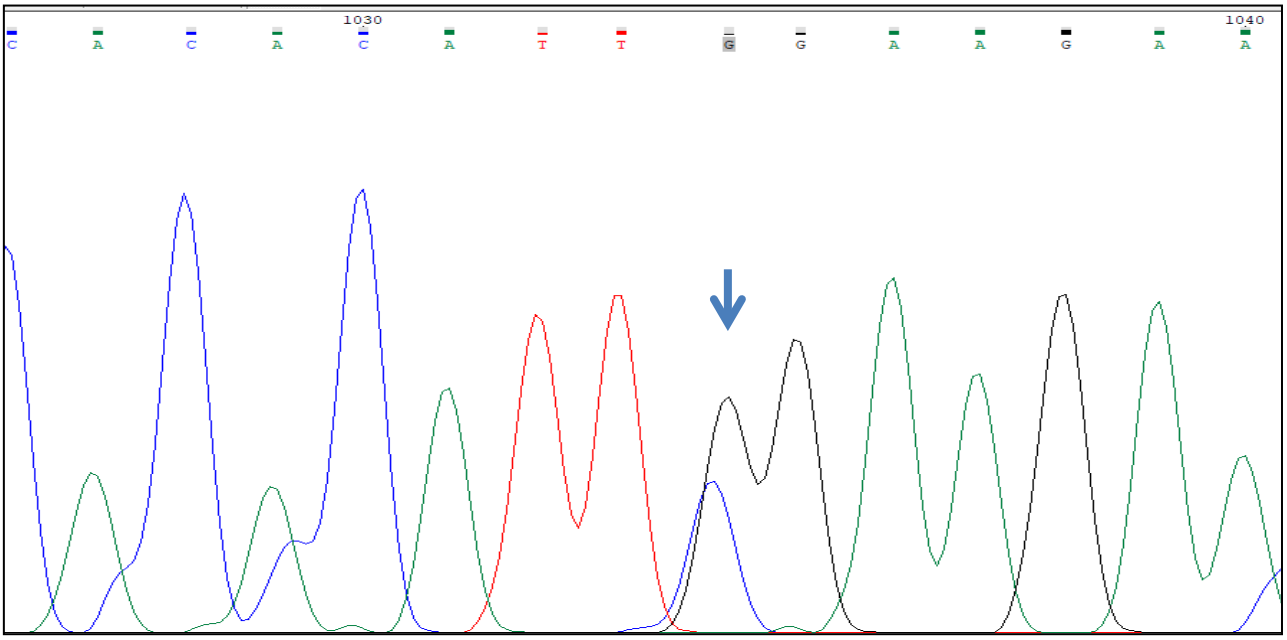

Figure 3-22: Sequencing electropherograms of novel mutations: substitution of C by G (Phe change to Leu), at position 7418

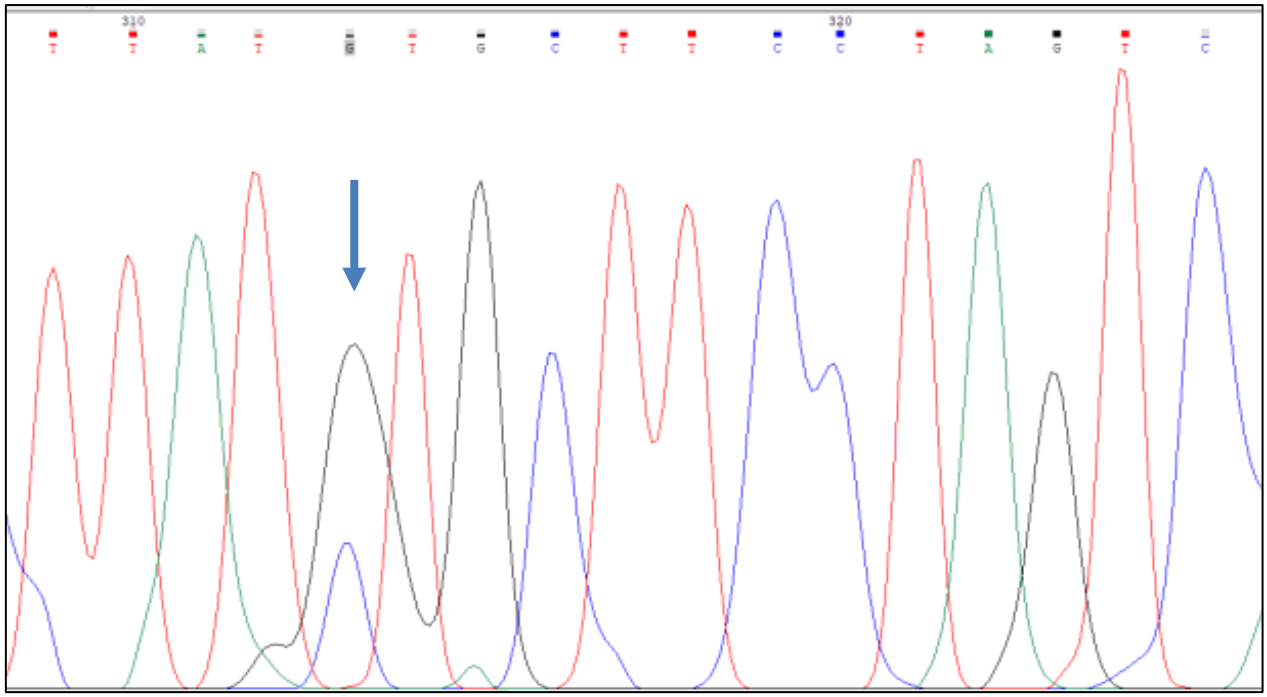

Figure 3-23: Sequencing electropherograms of novel mutations: substitution of C by G (Ile-Met), at position 7687

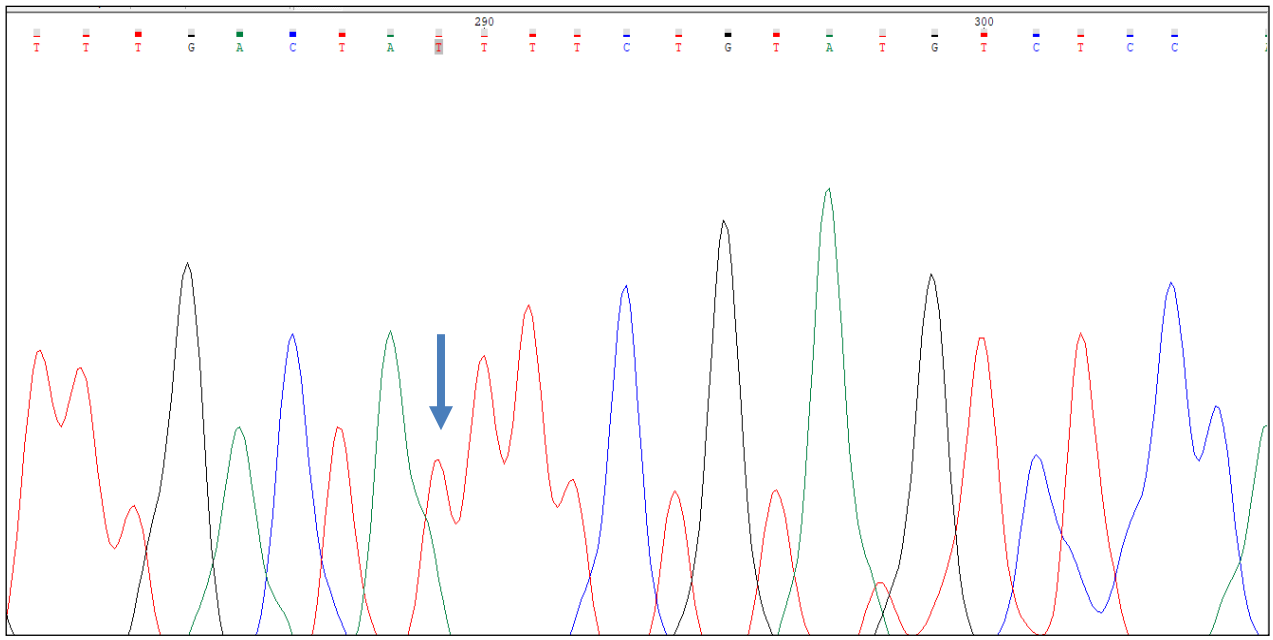

Figure 3-24: Sequencing electropherograms of novel mutations: T insertion at position 9956-9957

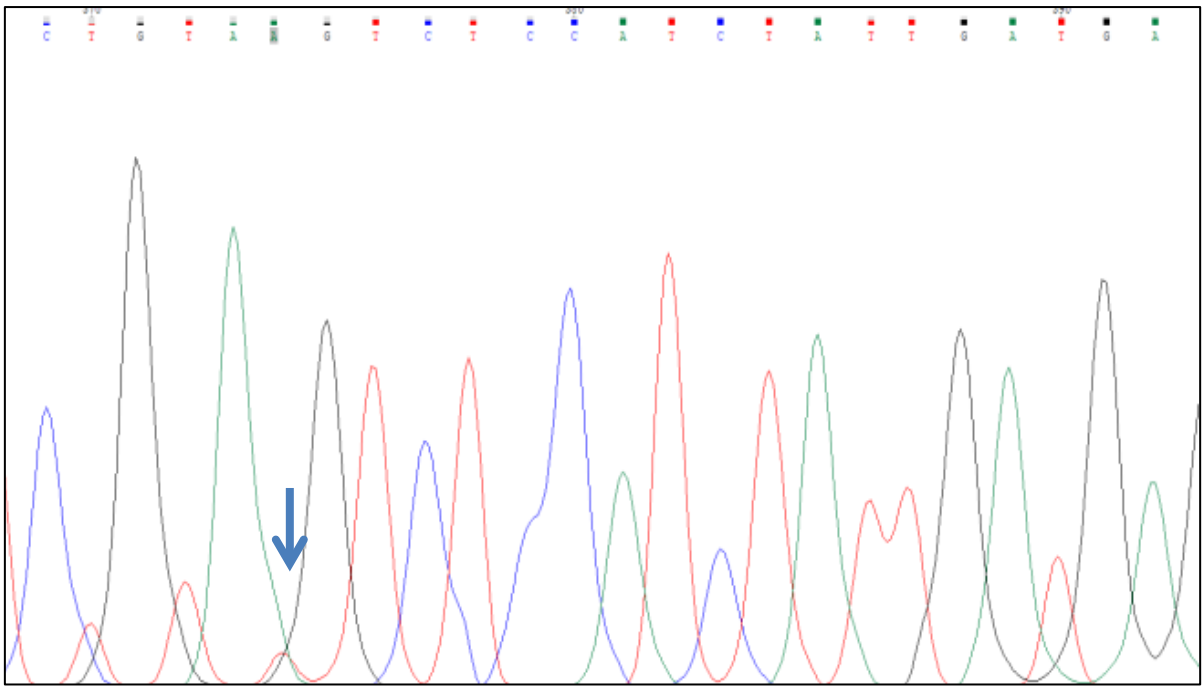

Figure 3-25: Sequencing electropherograms of a novel mutation: substitution of T by G (Tyr to stop codon), at position 9965

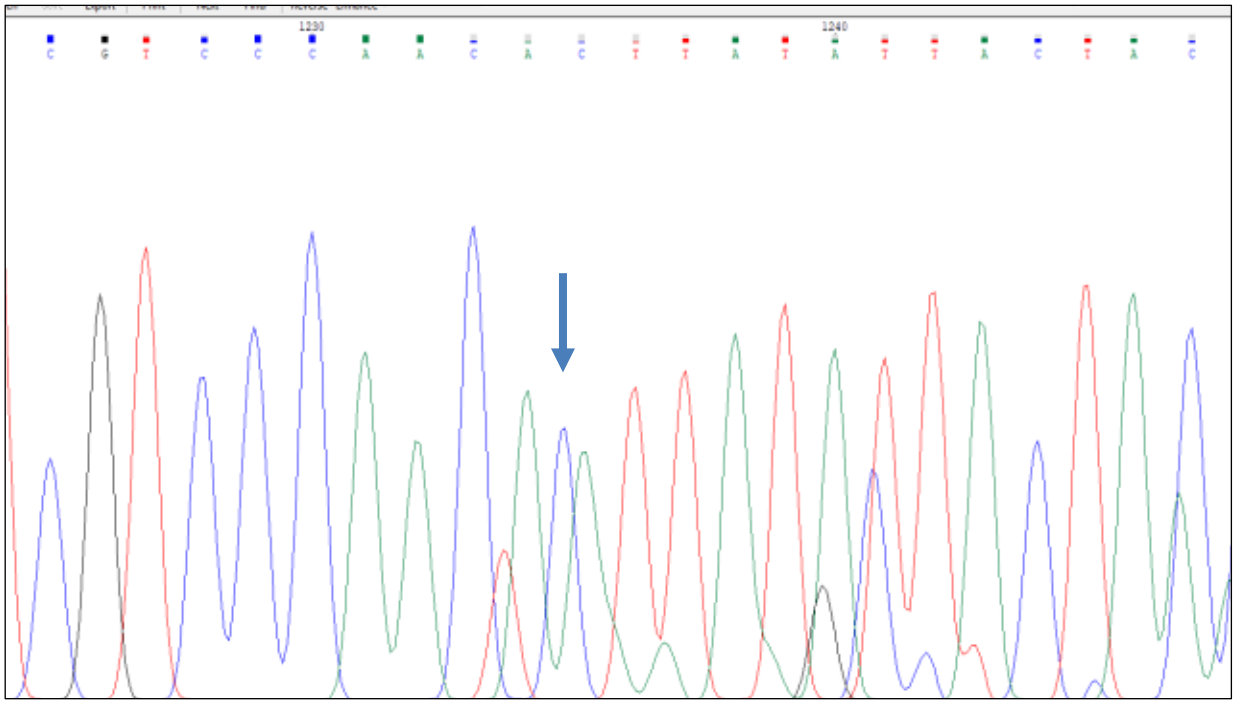

Figure 3-26: Sequencing electropherograms of a novel mutation: substitution of A by C (Ile to Leu), at position 10784

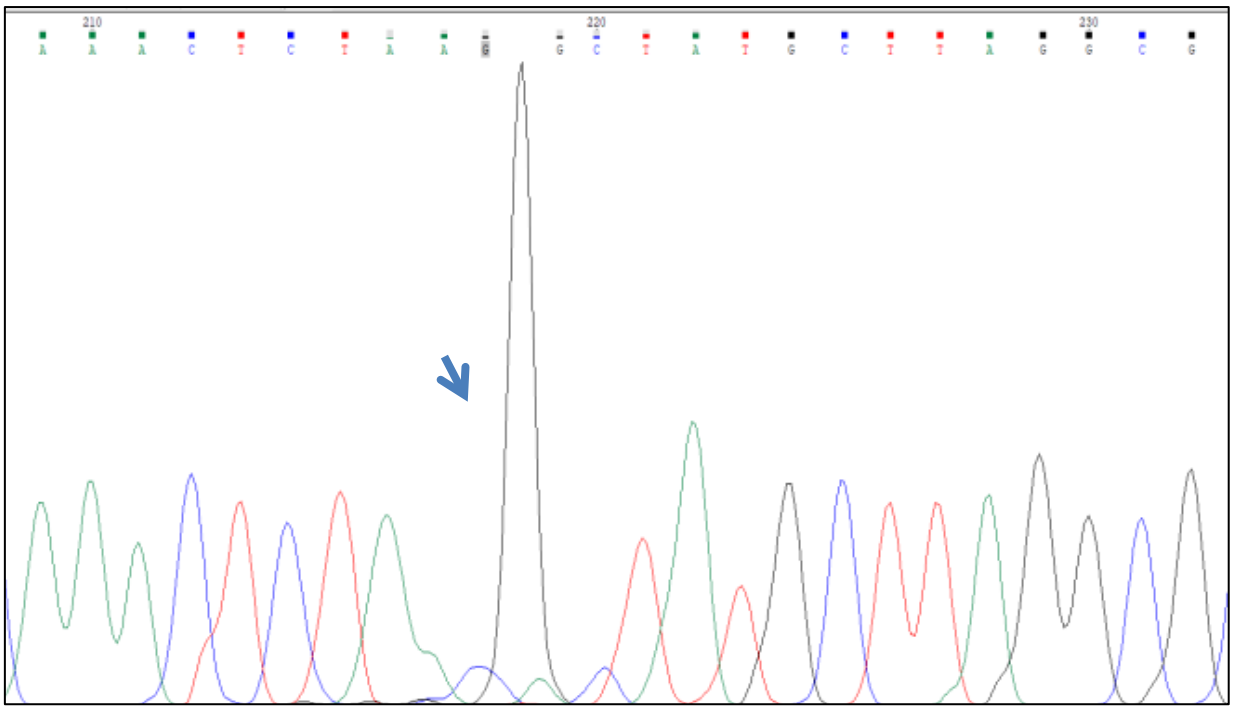

Figure 3-27: Sequencing electropherograms of a novel mutation: substitution of CA by GG (Thr to stop codon) at position 13166 and 13167

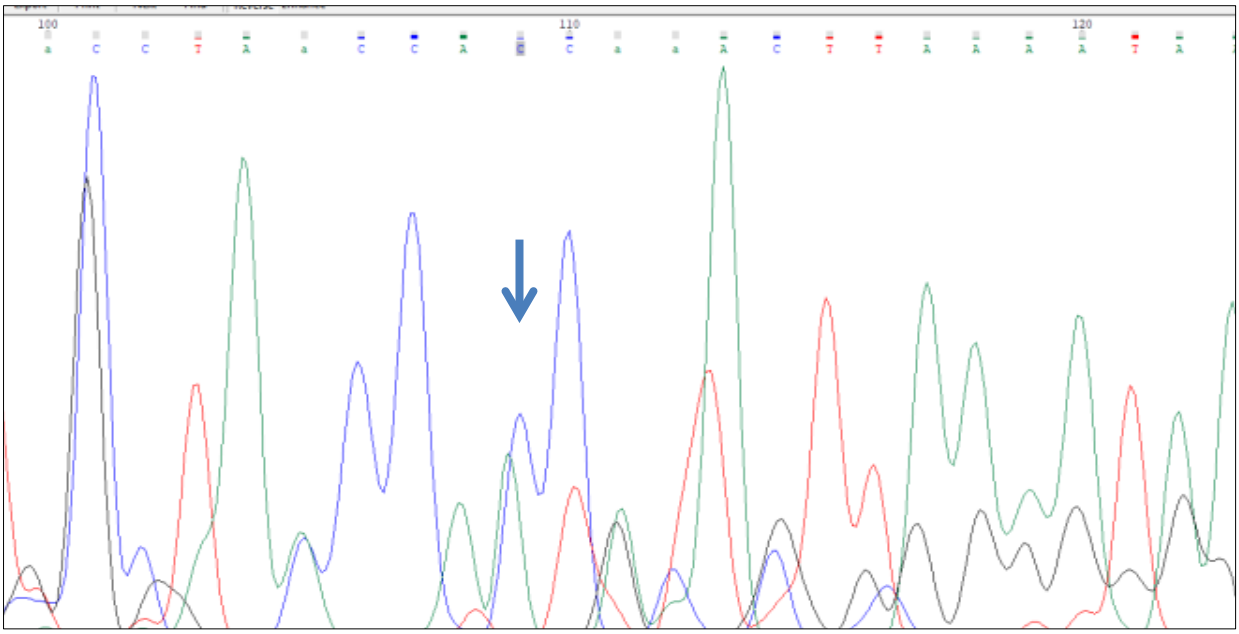

Figure 3-28: Sequencing electropherograms of a novel mutation: substitution of A by C (Asn to Thr) at position13862

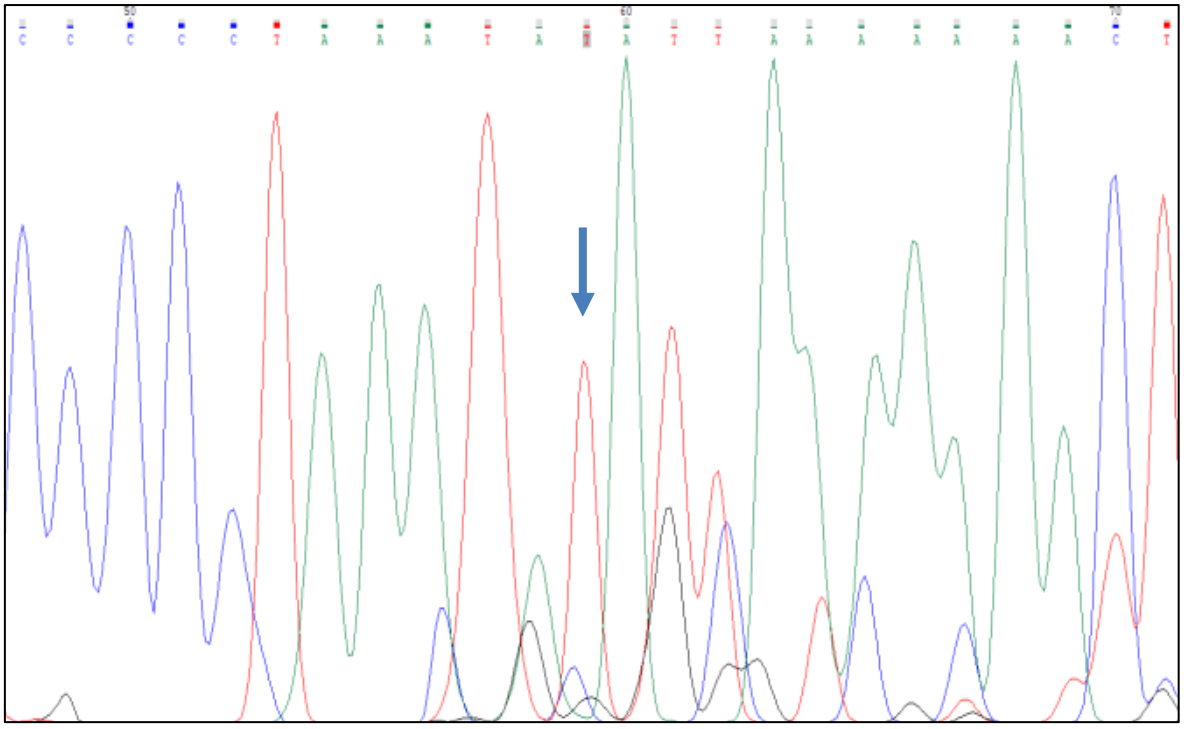

Figure 3-29: Sequencing electropherograms of a novel mutation: substitution of A by T (Tyr to Asn) in 14500

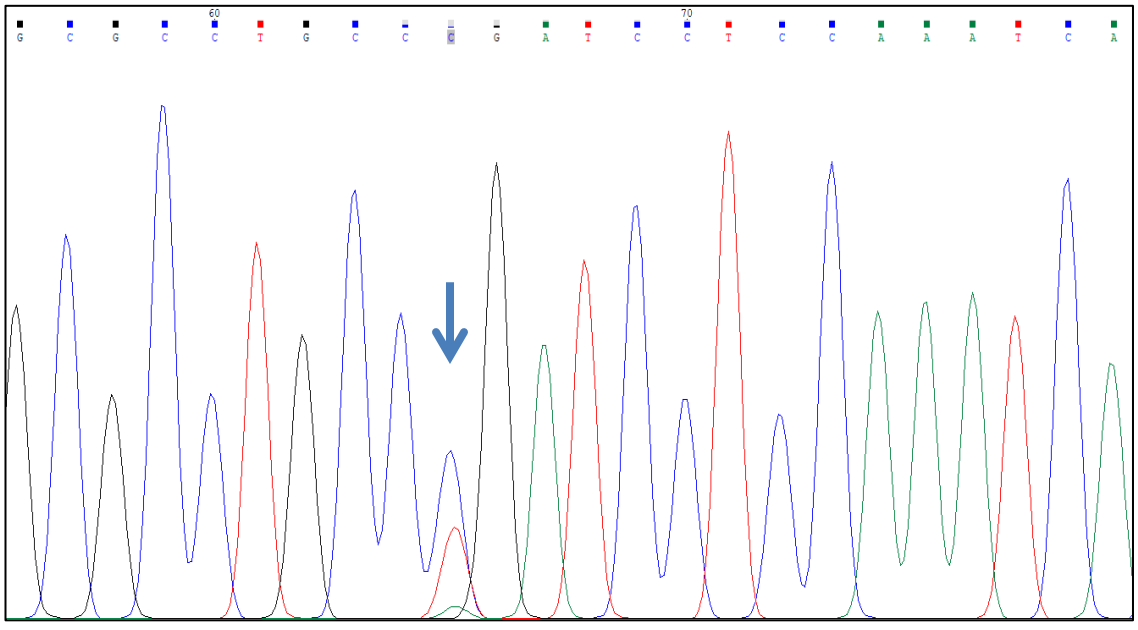

Figure 3-30: Sequencing electropherograms of a novel mutation: substitution of T by C (Leu to pro) at position 14868

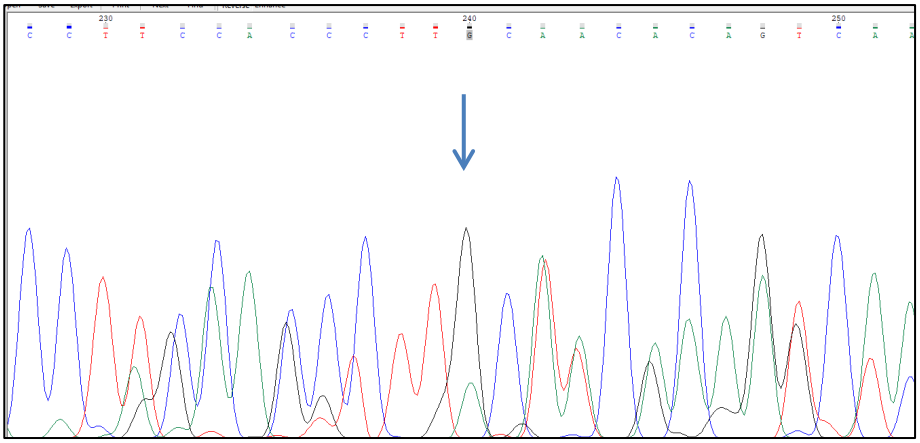

Figure 3-31: Sequencing electropherograms of novel mutation: substitution of A by G (Tyr to Cys) at position 15414

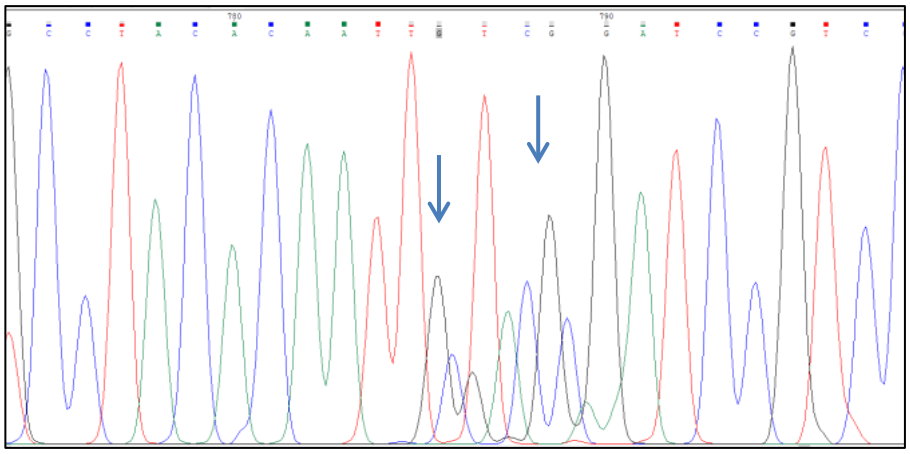

Figure 3-32: Sequencing electropherograms of novel mutation:

Substitution of C by G (Leu toVal) at 15587, and C by G (Arg to Gly) at position 15590

In general mutations (SNP and sporadic mutations, including unique type) predominated in protein coding region, and these were commonly populated in the ND5 coding region, followed by Cyt-B and least (zero) in ATP 8 (table 3-10)

Table 3-10: Percentage of mutations (SNP and sporadic) across the protein coding mtDNA regions

	Protein coding	Mutation percentage	Synchronous percentage	Non-synchronous percentage
1	ND5	28%	75%	25%
2	Cyt-B	18%	33%	67%
3	ND1	8%	50%	50%
4	ND6	7%	28%	72%
5	Cox3	7%	57%	28.5%+**14.2% insertion**
6	ATP 6	7%	57%	43%
7	ND4	6%	67%	33%
8	ND2	6%	50%	50%
9	Cox1	5%	80%	20%
10	Cox2	5%	80%	20%
5	ND4L	1%	100%	0%
6	ND3	1%	100%	0%
13	ATP 8	0%	0%	0%

Table 3-11: Asynchronous protein coding sporadic (variant and unique) mutation and their effects

No.	**MUTATION**	**PROTEIN CODING REGION**	**AMINO ACID CHANGE**	**EFFECT**
1	C9011T	ATP 6	Ala-Val	Same group
2	C4126G	ND1	Arg-Gly	Positively charged to non-polar aliphatic
3	A4136G	ND1	Tyr-Cys	Non-polar aromatic to polar uncharged
4	A4590G	ND2	Ile-Val	Same group
5	G5262A	ND2	Ala-Thr	Non-polar aliphatic to polar uncharged

6	C7418G	Cox1	Phe-leu	Non-polar aromatic to non-polar aliphatic
7	C7687G	Cox2	Ile-Met	Same group
8	A9336G	Cox3	Met-Val	Same group
9	9956-57 C insertion	Cox3		
10	T9965A	Cox3	Tyr-stop	Stop coding
11	A10784C	ND4	Ile-Leu	Same group
12	A12950G	ND5		
13	CA13166 and 67GG	ND5	Thr-stop	Stop coding
14	C13658T	ND5	Thr-Ile	Polar uncharged to non-polar aliphatic
15	A13862C	ND5	Asn-Thr	Same group
16	C13912T	ND5	Leu-phe	Non-polar aliphatic to non-polar aromatic
17	C13999A	ND5	Leu-Met	Same group
18	A14500T	ND6	Tyr-Asn	Non-polar aromatic to polar uncharged
19	G14544T	ND6	Gln-His	Polar uncharged to positively charged
20	A14566T	ND6	Thr-Ser	Same group
21	T14634C	ND6	Met-Val	Same group
22	T14868C	Cyt-B	Leu-Pro	Non-polar aliphatic to polar uncharged
23	C14891G	Cyt-B	Leu-Val	Same group
24	G14960A	Cyt-B	Asp-Asn	Negatively charged to polar uncharged
25	T15394G	Ccyt-B	Asp-Glu	Negatively charged to polar uncharged
26	A15414G	Cyt-B	Tyr-Cys	Non-polar aromatic to polar uncharged
27	A15422G	Cyt-B	Ile-Val	

28	C15587G	Cyt-B	Leu-Val	Same group
29	C15590G	Cyt-B	Arg-Gly	Positively charged to non-polar aliphatic
30	T15674C	Cyt-B	Ser-Pro	Same group

3.3.5. Mutation in mt-tRNA in breast cancer cases:

In the present study, seven nucleotide substitutions were identified in mt- tRNA that comprises 4% of the total mutations in mt-DNA (genome). Two of the mutations, tRNA arg (T10463C) and tRNA leu (A12308G) were population related (SNP Kurds), and two mutations, tRNA Asp (T7581C) and tRNA Gly (T10045C) were in the variant category, while the reminder three mt – tRNA mutations tRNA phe (A623C), tRNA Leu (A3269T) and tRNA lys (C8305A) were in the unique categories that are not yet recorded in the genebank. Table (3-11) shows the mutation and position of each of the mt-tRNA mutants; the electropherogram and the structure of the unique mt-tRNA mutations are shown in (Figure 3-33, 3-34 and 35).

Table 3-12: Types of mutations in tRNA gene

tRNA mutations	Mutation category	tRNA type
A623C	unique	Phenyl alanine
A3269T	unique	Lucien
T7581C	Variant	Aspartate
C8305A	unique	Lysine
T10045C	Variant	Glycine
T10463C	Population related	Arginine
A12308G	Population related	Lucien

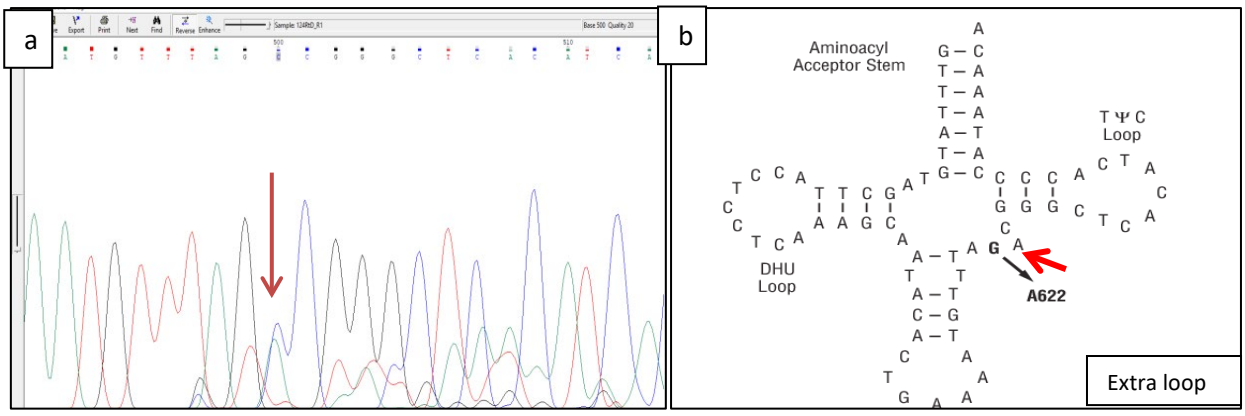

Figure 3-33: Novel mutation in tRNA (phenyle alanine)

a. Electropherogram, substitution of A by C at position 623
b. Structure of Phenyl alanine with position of mutation

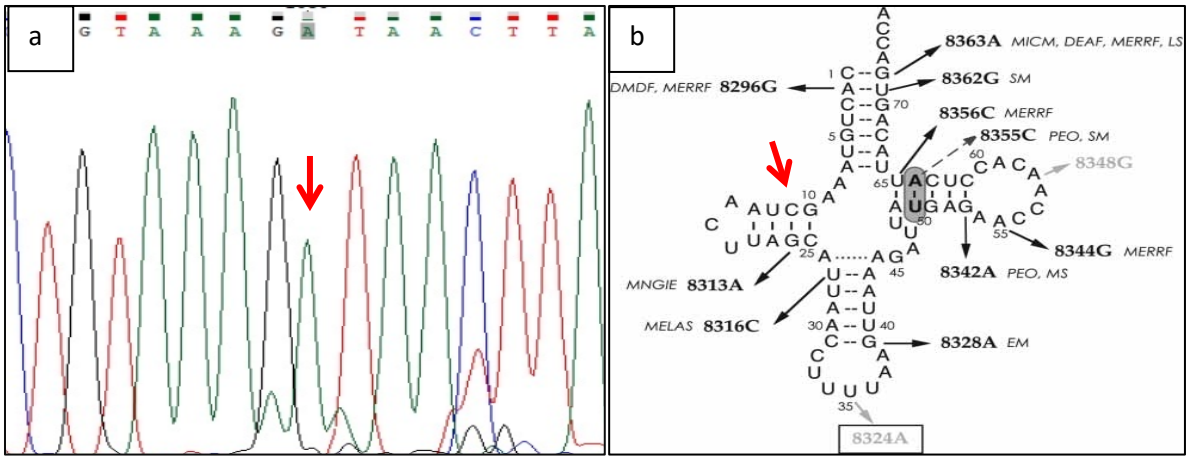

Figure 3-34: Novel mutation in tRNA (lysine)

a. Electropherogram, substitution of C by A at position 8305
b. Structure of tRNA Lysine with position of mutation, D-stem

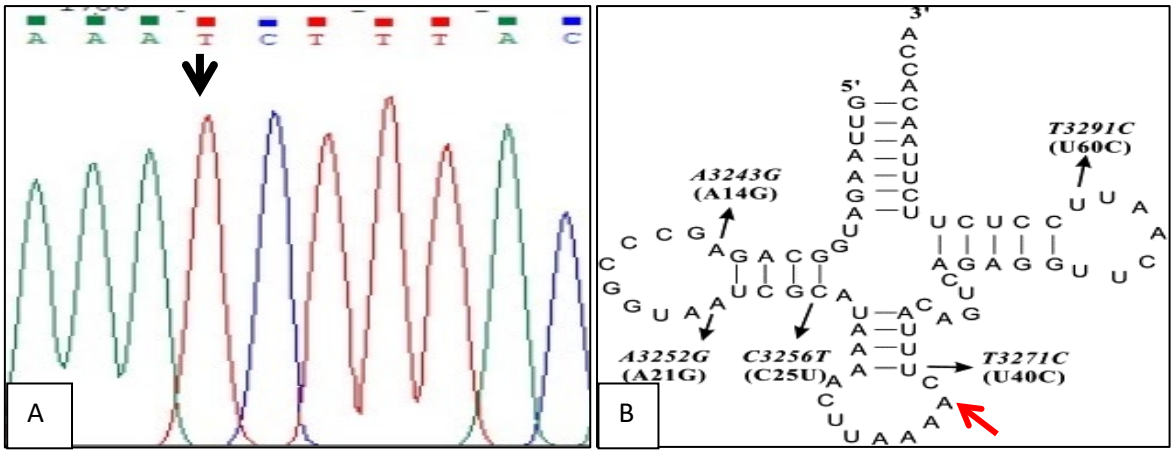

Figure 3-35: Novel mutation in tRNA (Lucine)

a. Electeropherogram, ubstitution of A by T at position 3269
b. Sttructure of tRNA Lucine with position of mutation

3.3.6. Mutations in mt-rRNA in breast cancer cases:

Ten percent (10%) of all the mutations in breast cancer samples were in mt-rRNA coding region, of which 94% were nucleotide substitution and only 6% were in the form of insertion mutation. Nine of the identified nucleotide substitution mutations (G709A, A750G, A1438G, T2706C, T980C, A1811G, G1888A, G3010A and C2259T) were population related (SNP Kurds)

Moreover, 2 unique mutations were also identified, nucleotide substitution A1152G of the RNA1 gene coding for mt – 12s rRNA (Figure 3-36), and insertion mutation C1784ins of RNA2 gene coding for mt-16s rRNA (Figure 3-37).

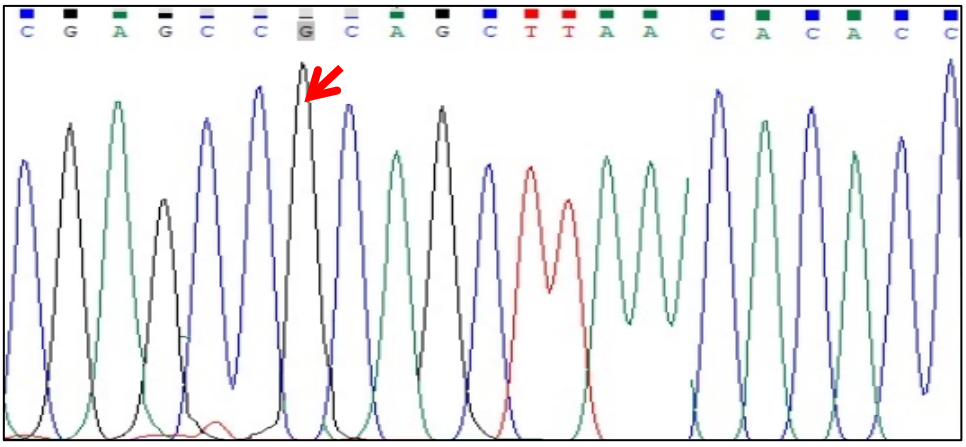

Figure 3-36: Novel point mutation in rRNA, substitution of A by G at position 1152

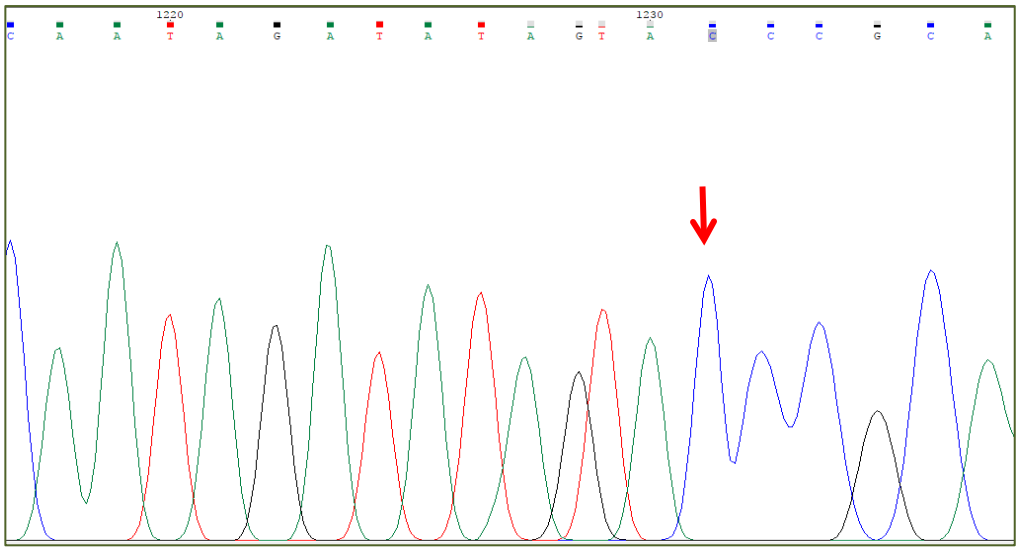

Figure 3-37: Novel insertion mutation in rRNA, insertion of a C at position 1784

3.3.7. Mutations in the non-coding (hypervariable regions):

Twenty seven percent (27%) of all the mutations in mtDNA sequence in breast cancer samples were in the non-coding region, 14% were in the HV1, 11% were in HV2 and 2% in HV3 region (Fig. 3-14).

Most of the mutations in the non-coding region were population related (64%). One of the well-known mutations a polycytocine stretch (C-tract) was repeatedly observed in both control (10 samples) and in cancer samples (17 samples) where there was insertion of C in two close positions (309-310) and (310-311). Figure (3-38) shows the sequence and the electropherogram of the poly C insertion in one of the breast cancer samples in form of 8CT6C, other forms as 7CT6C and 7CT7C were also observed.

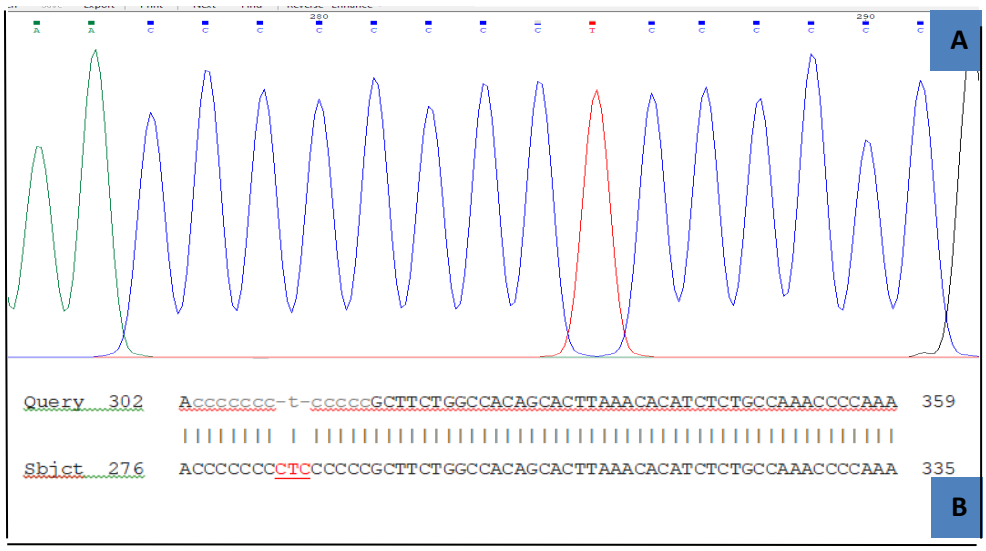

Figure 3-38: Sequence electeropherogram of poly C insertion mutation

Only a single unique mutation was identified in HV2 region, a (GC) insertion in position 512 (Fig.3-39).

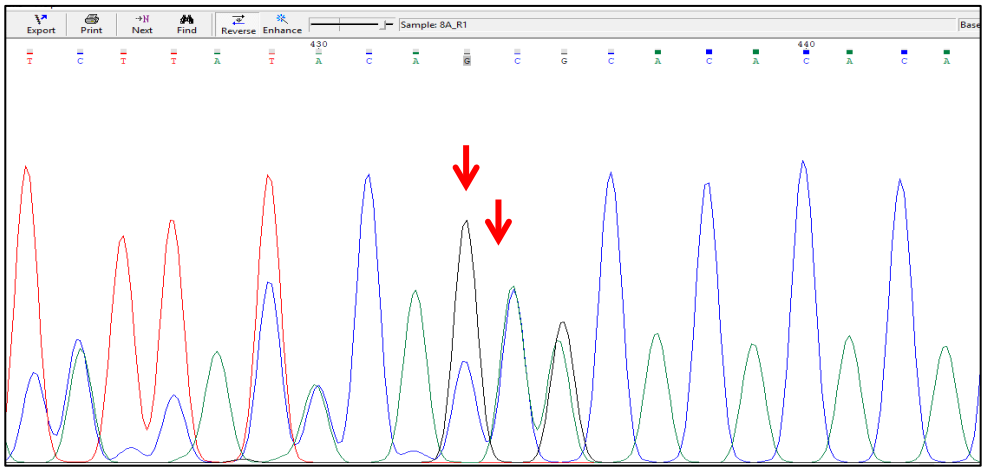

Figure 3-39: Novel mutation in hypervariable region, GC insertion at position 512

CHAPTER -4

DISCUSSION

DISCUSSION:

The aim of this project was to identify common accusable mutations in mtDNA of breast cancer tissues, relying on an assumption built up upon the effects of environmental impacts and the consequence of free radical production on mtDNA. Recalling back the list of the non-genetic risk factors of breast cancer as obesity, ionizing radiation, tobacco smoke and alcohol all are related to ROS production and are implicated in breast carcinogenesis (Gurer-Orhan et al., 2018), accordingly environmental impacts can result in free radical formation, affecting mtDNA and causing mutations that in turn will disrupt the OXPHS and enhance more free radical production further damaging the mtDNA and eventually damaging nuclear DNA as well. Nevertheless, the results were much more complicated than what was expected and they will be demonstrated in the following topics

4.1. AMPLIFICATION DEFECTS:

Starting with difficulties in amplifying regions of mtDNA lying between base pairs 5535 to 16541 (fragments B and C), (Fig 3-2), these fragments were either weekly amplified, indicating a heteroplasmy trait (i.e some of the mtDNA copies are wild type and others are mutated) or there was complete absence of amplification (homoplasmy in favour of the mutated copy); these results raised the suspicion of mtDNA 4977 deletion, a well-known deletion mutation eliminating 8470 to 13447 of the human mitochondrial genome (Mohamed Yusoff et al., 2019). mtDNA 4977-bp deletion is a highly non-specific mutation and has been observed in degenerative disorders, various mitochondrial disorders and related to their severity (Zhang et al., 2015), identified also in different cancers (Chen et al., 2011) including breast (Zhu, Qin and Sauter, 2004), aging, aging related disease (Zabihi Diba et al., 2015) as well as in healthy tissues (Nie et al., 2013).

But the results of PCR based chromosomal walking for the defective fragments with the use of multiple reverse primers, excluded large scale deletions as mutations were limited to a narrow range. According to the results, mtDNA 4977-deletion was neither detected in cancer samples nor in controls and these results were compatible with those of (Tan, Bai and Wong, 2002) from USA, (Aral et al., 2009) from Turkey and (A.R. Dhahi, Abdul Jaleel and Adnan Mahdi, 2016) from Iraq; but are not compatible with those of (Dimberg et al., 2015) from Vietnam and (Zhu, Qin and Sauter, 2004) from USA. Surprisingly in other studies regarding breast cancer, the mtDNA 4977 deletion was identified but was of no significant relation to breast cancer (Ye et al., 2007).

Absence of 4977-bp deletion in our samples (cancerous and control) may be explained by population variation as this mutation in breast samples were absent to low in European ancestry (EA) population and present in Asian and other population (Nie et al., 2013).

4.2. LACK OF AMPLIFICATION:

Failure of amplification of mtDNA in some of the cases raised the suspicion of possible reduction in mitochondrial DNA copy number (mtDNA-CN), in this regard, a possible explanation for the impaired amplification in this group of cancer cases was a copy number defect of mitochondrial genome.

Therefore, two different PCR experiments were performed; in the 1st one DNA template in the PCR reaction was increased, but still results were negative, and no amplification was observed.

In the 2nd experiment the number of PCR reaction cycles was increased from 35 to 45 cycles; this second experiment was obtained for only one of the fragments (fragment D). As it shown in (Fig. 4-1) a faint band of fragment D visualized on agarose gel, this result obviously supports the low copy number of mitochondrial DNA in this group of cancer cases.

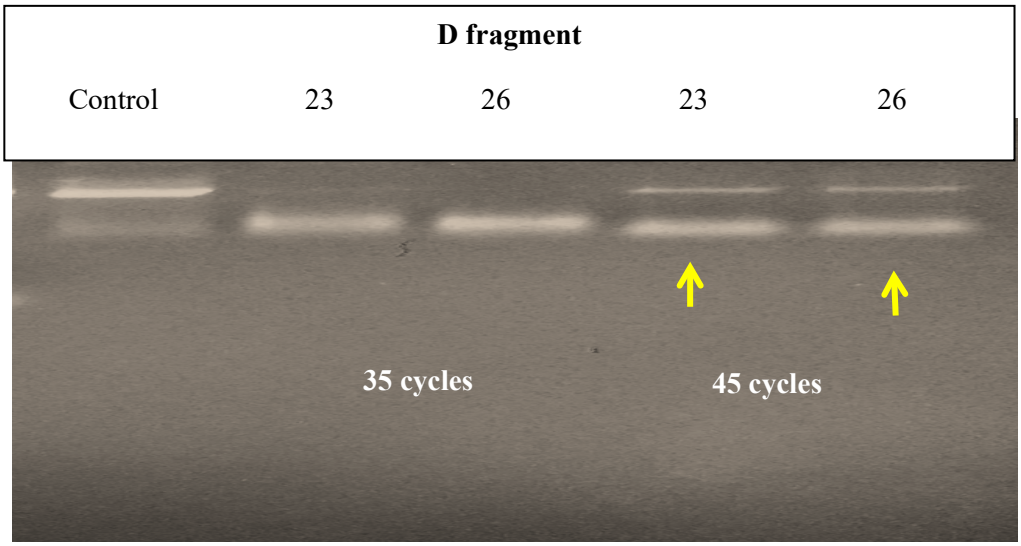

Figure 4-1: Agarose gel electrophoresis, PCR product of D fragment with increasing the PCR reaction cycles

A low mtDNA-CN results in defective oxidative phosphorylation, enhancing generation of ATP by glycolysis; this is the natural history in cancer development. In addition, reduced mtDNA in cancer cells makes them more resistant to apoptosis, and facilitates their epithelial-mesenchymal transition (EMT), in which epithelial cells lose their cell polarity, cell-cell adhesion and acquire mesenchymal (fibroblast-like) characteristics, gaining the migratory and invasive properties (invasive and metastatic properties) (Hu, Yao and Shen, 2016). mtDNA-CN in cancer was and still the subject of interest for many researchers willing to be used one day as a tumor biomarker. Results of the present study showed low mtDNA-CN and were incompatible with most of the available data's that showed inversely, a high mtDNA-CN in breast cancer cases (Shen et al., 2010; Thyagarajan et al., 2013; Lemnrau et al., 2015 and Shen et al., 2015). A single study on peripheral blood (Xia et al., 2009) and another one on malignant breast tissue (Yu et al., 2007) showed results compatible with the current results. These differences can be explained by the fact that the baseline peripheral blood mtDNA content in breast cancer significantly changes with tumor progression and stage (Xia et al., 2009; Iqbal et al., 2017). The high mtDNA-CN in peripheral blood leukocytes could be a compensatory process in the late stages, as in late stages of cancer there will be mtDNA damage and dysfunction caused by the high oxidative stress, impaired aerobic metabolism and excess ROS production (Mi et al., 2015).

According to a meta-analytic study including 36 studies from different tissue cancers there were no obvious relation between mtDNA content and cancer risk in the overall analysis, because of the heterogeneity among different cancer types however in subgroup analysis the outcome and the relation with copy number changed according to cancer type (Hu, Yao and Shen, 2016).

4.3 SNP FINDINGS AND HAPLOGROUPS:

Regarding the mutations identified through the whole mitochondrial sequencing, population related mutations (SNP) were the commonest category of mutations among control samples (90%) as well as in the breast cancer samples (74%), (Fig. 3-14).

Interestingly, the (SNPs) were aggregated in the coding region of mtDNA, both in cancer and control samples (61% and 58%, respectively), interpreted with other compatible results (Lan et al., 2019)

This shows the importance of whole genomic sequencing for precise haplogroup determination and reducing the value of the traditionally used SNPs in the hypervariable regions for forensic purposes (Fridman et al., 2011; Weng et al., 2013).

According to the identified (SNP) s through the whole mitochondrial sequence all the samples (breast cancer and control) belonged to the Western Eurasian haplogroups with lack of Eastern Eurasian and sub-Saharan African lineage, supporting the results of (Zarai and Rajabi-Maham, 2016).

As mentioned in the results Haplogroup (H) was the most encountered haplogroup among the control samples, 60% among the 20 control samples, and descends to 41.6% among the 36 control samples (20 +16 unpublished data). This finding was comparable to the European range (43.7%) yet inconsistent with Near East results (25%) (Richards et al. 2000; Achilli et al. 2004).

Regarding the neighbour populations, the current results regarding haplogroup H were incompatible with Iraqis in general (19.9%) and Iraqi Arabs in particular (16.9%) (Al-Zahery et al. 2013; Azzawi and Oleiwi 2013), as well as with the Turks (20.98%) and Persians (28.6%) (Derenko et al. 2013; Serin et al. 2016). is believed to originate in Southwestern Asia some 20000 to 25000 years ago (Achilli et al. 2004).

While haplogroup HV was the 2nd most common among control samples, it was the commonest among the cancer samples (35%); HV is the ancestral clade of H and V, originated between West and Central Asia; it reaches its highest incidence in the Iranian Plateau, Mesopotamia and South Caucasus and it is recognized as a crucial component of early human spread in Eurasia (Shamoon-Pour et al. 2019)

The presence of a relation between haplogroups and different types of cancer was and still a matter of controversy; however, many specific mtDNA haplogroups have been identified to be associated with the risk of developing prostate, breast colonic and gastric cancer, as well as myelodysplastic syndromes, etc. (Xu et al., 2013; Bussard and Siracusa, 2017; Jimenez-Morales et al, 2018). This can be explained by the fact that through history haplogroup determining (SNP) s are contributed in human survival by manipulating the OXPHOS equilibrium, and consequently individuals of different haplogroups would have differences in their metabolism and susceptibility to cancer (Bayona-Bafaluy et al., 2011). In breast tissue, Chinese women of haplogroups M and subhaplogroup D5 had shown a higher incidence for cancer (Fang et al, 2010; Ma et al, 2017), while no such a remarkable relation was identified between cancer and specific haplogroups in European and Caucasian women, but a significant relationship between the occurrence of haplogroup U and control was identified suggesting them as protective factors against breast cancer (Gutiérrez Povedano et al., 2013). Still

haplogroup K showed a significant association with breast cancer in European-American women (Bai et al, 2007). Nevertheless, in the current study a significant relation between haplogroup HV and breast cancer was identified with p value = 0.002 and 0.006 for Chi square and Fisher's exact test respectively and OR of 28.

In addition to the haplogroups, several distinct SNPs have been previously discovered to be associated with breast cancer (Covarrubias, Bai, Wong and Leal, 2008). T to C substitution at position 16189 (located in D-loop) was one of the earliest polymorphisms found to be significantly high in breast cancer (WANG et al., 2006; Jimenez-Morales et al., 2018), however in the current study only one breast cancer sample showed this substitution making it incompatible with the results of Wang et al.

A10398G is another well-known SNP in breast cancer detected in European-American, Malaysian and African-American women (Mims et al, 2006; Bai et al, 2007; Darvishi et al, 2007; Covarrubias, Bai, Wong and Leal, 2008; Tengku Baharudin 2012; and Jahaniet al, 2019); in addition, SNPs G9055A and T16519C were also identified as risk factors for breast cancer in European-American females (Bai et al, 2007), but with the exception of T16519C which was identified in only one case (5%) none of the other two were detected in this study.

A12308G which is a polymorphism in anticodon loop of leucine tRNA gene, defines the mtDNA superhaplogroup U/K (Gutiérrez Povedano et al., 2013), in the current study the percentage of A12308G polymorphism was higher (35%) in breast cancer samples when compared to the control group (0%), the relation of A12308G polymorphism with breast cancer was suggested in other previous studies as well (Covarrubias, Bai, Wong and Leal, 2008; Grzybowska-Szatkowska and Slaska, 2012; MA Mohammed et al., 2015; Meng et al., 2015), however in a study with induced A12308G mutation, no significant differences in ROS production between the cells containing the wild type or A12308G mtDNA variant were detected (Kulawiec, Owens and Singh, 2009).

Furthermore, several other germ line mutations as 2463 A-deletion, C6296A, 6298 T-deletion, A8860G, and 8460-13327deletion, were detected in chines women with breast cancer (Li et al, 2016).

Although many SNPs were identified in breast cancer samples in the current study, but the only mutation showed a significantly high incidence among breast cancer samples compared to the control samples was homoplasmic SNP (A8860G). This mutation is a non-synchronous mutation in the Mt-ATP 6 gene that was detected in all 20 breast cancer samples while only in 4 of the control samples and this result was compatible with Li et al, 2016. This gene

encodes ATP synthase 6 (681 amino acids), a subunit of complex V, whose mutation results in substitution of a polar uncharged amino acid (threonine) with a non-polar aliphatic amino acid (alanine); this may affect hydrophobic interactions and hence the structure of the protein. However, such a prediction of protein structure is not absolute as these mutations may be followed by other compensatory mutations (suppressor mutations) in order to minimize the initial mutation's effect (Schaefer and Rost, 2012), these compensatory and suppresser mutations may explain the presence of the mutation A8860G in 20% of phenotypically healthy control samples.

Still A8066G mutation in combination with other (SNP) s of haplogroup (HV) and (N) could have a synergistic effect on the mitochondrial function, especially as this mutation was registered as a risk factor in other breast cancer studies (Li et al., 2016), and causes a nonsynchronous amino acid change, in one of the essential protein of OXPHS process, this mutation may be the trigger of mitochondrial dysfunction, excess ROS production and the enhancer of all the other mutations observed in this study.

Neither the previous studies nor the current study have been able to identify a definite relation between mitochondrial haplogroups and breast cancer. This could be due to heterogeneous and wide-ranging result outcomes, variations which can possibly be explained by the effect of other parameters on the mitochondrial genome, such as individual physiology and influence of geographical location.

4.4. VARIANT AND UNIQUE MUTATION EFFECTS:

In this group are mutations that are not yet population related (variant and unique) and are either recorded in gene banks (in studies related to population or a pathology) or never been recorded (unique). These mutations were identified in a higher percentage among the breast cancer samples (26%) than in control samples (10%); most of these mutations were point mutation in the coding region. Correspondingly these mutations could be of pathological effect as they alter the mitochondrial protein-coding genes consequently affects the function of the respiratory chain complex to which the corresponding protein belongs (Tuppen et al., 2010).

Not surprisingly none of the sporadic mutations were common among the breast cancer samples and all were single tone except for (C9011T) mutation which was seen in two of the breast cancer samples. 9011 C>T mutation is located in ATPase 6 causing a non-synchronous mutation with change of Alanine amino acid to Valine, both are non-polar amino acids, this

mutation was pointed out in previous studies in relation with intracellular Ca regulation (Kazuno et al., 2006; Kazuno et al., 2008).

Still as there is no adequate, available data regarding SNPs of Sulaymaniyah population, probably some of the pointed out variant mutations are in fact SNPs specific to this population, hence mutations of the current study are not necessarily pathological but still they are of undetermined pathological potential, recorded either in population studies or in pathology related studies as chronic diseases and cancers. Many further investigations on mtDNA in this locality are required to identify (SNP) that are specific to this population.

4.5. PROTEIN CODING REGION MUTATION EFFECTS:

As indicated most of the mutations were in the protein coding region and asynchronous base pair substitution was the predominant type of mutations, changing the code of amino acids, resulting in a premature (early) stop codon, or a switch from polar to nonpolar amino acid, a positively charged amino acid to a negatively charged one or vice versa. Defects in the mitochondrially encoded proteins of the OXPHOS represent the center of this project as dysfunctional mitochondria will impair p53 gene and hence suppresses apoptosis in cancer cells (Compton et al., 2011). On the other hand, activity of p53 decreases glycolysis and stimulates mitochondrial respiration through the activation of specific proteins required for the assembly of the cytochrome oxidase (*COX*) complex, thus the loss of p53 results in an increasing glycolysis and a decreasing mitochondrial respiration, contributing to the Warburg effect (Weigl, Paradiso and Tommasi, 2013)

In addition, dysfunctional mitochondria will result in excess free radical production, resulting in oxidation of many fundamental cellular components, among which PTEN which is very sensitive to oxidation, suppression of PTEN phosphatase activity leads to activation of the oncogenic Akt pathway in cancer cells (Pelicano et al., 2006).

As pointed out previously sporadic mutations predominated in protein coding region, commonly populating in the ND5 coding region, followed by Cyt-B, these results were compatible with those of (Liu et al., 2017) and least (zero) in ATPase 8. More than half (57%) of the protein coding region mutations were in the genes coding for complex I subunits (ND6, ND5, ND4, ND3, ND2, ND4L), which is consistent with other studies result's (Fendt et al., 2010). This is important because Complex I is a fundamental element of the respiratory chain, essential for ATP production, preserves NAD^+/NADH ratio, affects the level of (ROS) and creates a membrane potential in mitochondria, therefore these mutations for sure will

predispose a mitochondrial dysfunction (Hashizume et al., 2012), that may participate in carcinogenesis.

Although not all the identified sporadic asynchronous point mutations are predictably causing major structural changes, yet most are causing changes in charge, polarity and completion of the proteins (Table 3-11). Substitution of T by A, at position 9965 in Cox3 gene Tyrosine changes to a stop codon (Fig. 3-25) and substitution of CA by GG at position 13166 and 67 in ND5 gene, threonine changes to a stop codon (Fig.3-27), both mutations are novel and have a major effects causing premature termination of the coding proteins.

One of the unique protein coding region mutations was an insertion mutation; T insertion in 9956-9957 in the gene coding for Cox3 (Fig.3-24). This mutation causes a frame shifting with an early (premature) protein termination by a stop codon, as its shown in (Fig. 4-2) the original codons from 9956 are as follow; (phe., leu, Tyr, Val, Ser, Ile, Tyr, trp, Trp, Gly, Ser) while after the insertion of the T, shifting occurs with a great change in the codons following the insertion as follow; (phe, Ser, Val, Cyt, Leu, His, Leu, Leu, Met, stop codon AGG), resulting in a truncated protein.

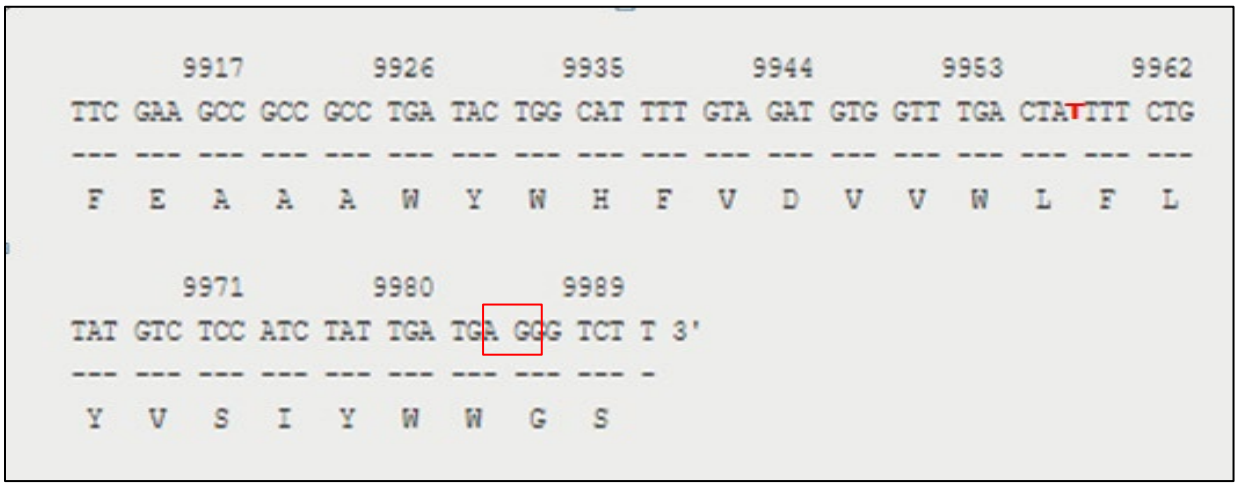

Figure 4-2: Effect of T insertion with frame shift and an early stop codon (AGG)

4.6. tRNA MUTATION EFFECT:

Other fundamentally important regions in mtDNA are the genes coding for mt-tRNA s, pathogenic mutations in tRNA may cause defect in overall mitochondrial translation process (protein synthesis) and impair OXPHS process (Tuppen et al., 2010). In general, these mutations will affect the tertiary structure of mt-tRNA affecting its stability and ability to

interact with other important enzymes and proteins required for the folding modifications (Giordano et al., 2015). Mitochondrial tRNA mutation are uncommon and mt-tRNA coding genes are not hotspots for mutation, because they are under strong selection pressure, still some polymorphisms and other mutations are identified in relation to breast cancer (Grzybowska-Szatkowska and Slaska, 2012); in the current study as well, mt-tRNA mutations were not common, only 4% and five of the mutations were non-population related (tRNA phe, tRNA Leu, tRNA Asp, tRNA Lys and tRNA Gly) and three out of the five were newly discovered (unique) mutations (fig. 3-33, 34, 35).

Two gremlin mutations (polymorphisms) were identified; the previously mentioned (A12308G) mutation and (T10463C) mutation which is located in the acceptor stem, related to haplogroup H and U (Vilmi et al., 2005), recorded among the current breast cancer samples but not the controls, A12308G mutation was related to breast cancer in other previous studies (Covarrubias, Bai, Wong and Leal, 2008; Grzybowska-Szatkowska and Slaska, 2012; Meng et al., 2015).

T7581C a variant mutation recorded once in breast cancer samples of the current study, is another mutation in aspartate tRNA gene and is suggested to have a connection with breast cancer (Grzybowska-Szatkowska and Slaska, 2012; Meng et al., 2015). In general data's regarding mt-tRNA mutations in breast cancer are very limited and their effects and phenotypic expression are unpredictable, because the steps of mt-tRNA processing and modifications are affected by other parameters as environment, mitochondrial genetic background, and interaction with nuclear encoded proteins (Giordano et al., 2015)

4.7. EFFECT OF rRNA MUTATIONS:

As its stated before mitochondrial rRNA genes encode the RNA component of mitochondrial ribosomes or mitoribosomes; mutations in these genes are uncommon in comparison to the D-loop and the protein coding genes, still many variants of the mitochondrially encoded rRNA mutations are identified in different previous studies, but established pathogenic effects of these mutations are unclear and evidences supporting mutation consequences are nothing but circumferential, this is because the mitochondrial translation apparatus is ungovernable (Smith et al., 2013; Elson et al., 2015).

To date only two RNA mutations are known with an established relation with a disease, these are 908A>G (m.1555A>G) and 847C>U (m.1494C>T) mutations and they are known to cause hearing loss (Hema Bindu and Reddy, 2008). Identified RNA mutations of the current study were either population related or somatic, among the somatic mutations two variants

(T2158C) and (T1005C) were observed, both are recorded in previous population related studies and cancer concerning studies in colorectal (Webb et al., 2008) and ovarian (Liu et al., 2001) respectively.

Like other regions of mtDNA, novel mutations were found in mitochondrially coding rRNA, as A1152G mutation in RNA1 encoding for S12 RNA (Fig 4-3) and the insertion of cytosine at position 1784 in RNA2 (Fig. 4-4), encoding for 16S RNA, however as indicated earlier the effect of these mutations are only subjects of prediction.

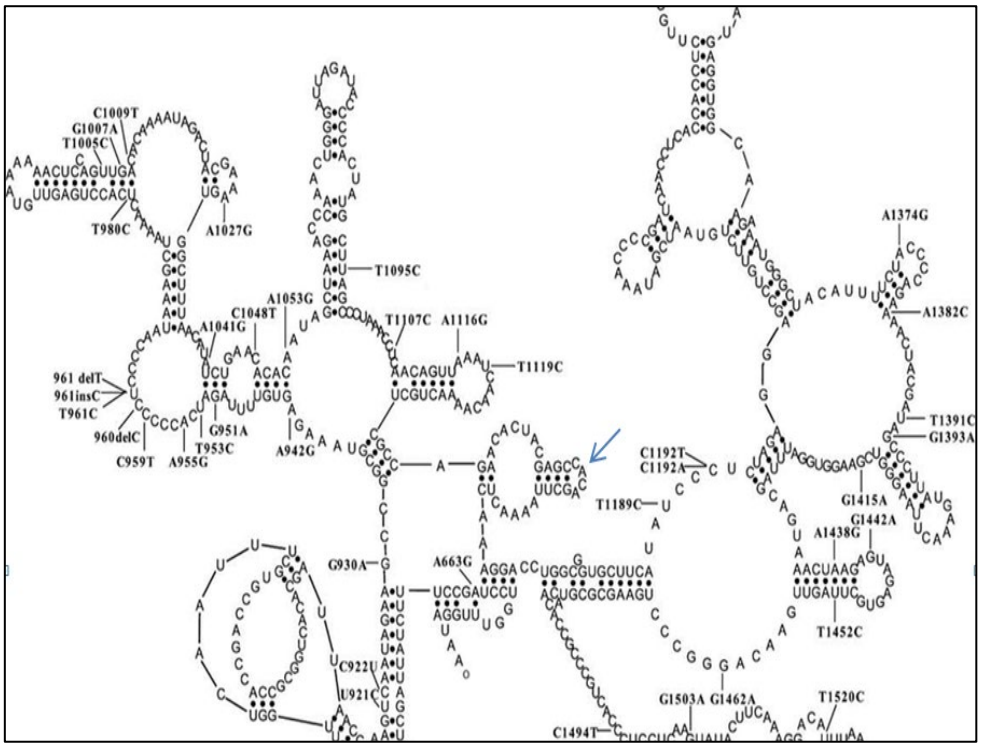

Figure 4-3: Part of 12S rRNA structure (Shen et al, 2011); the A1152G mutation site pointed to with a blue pointer

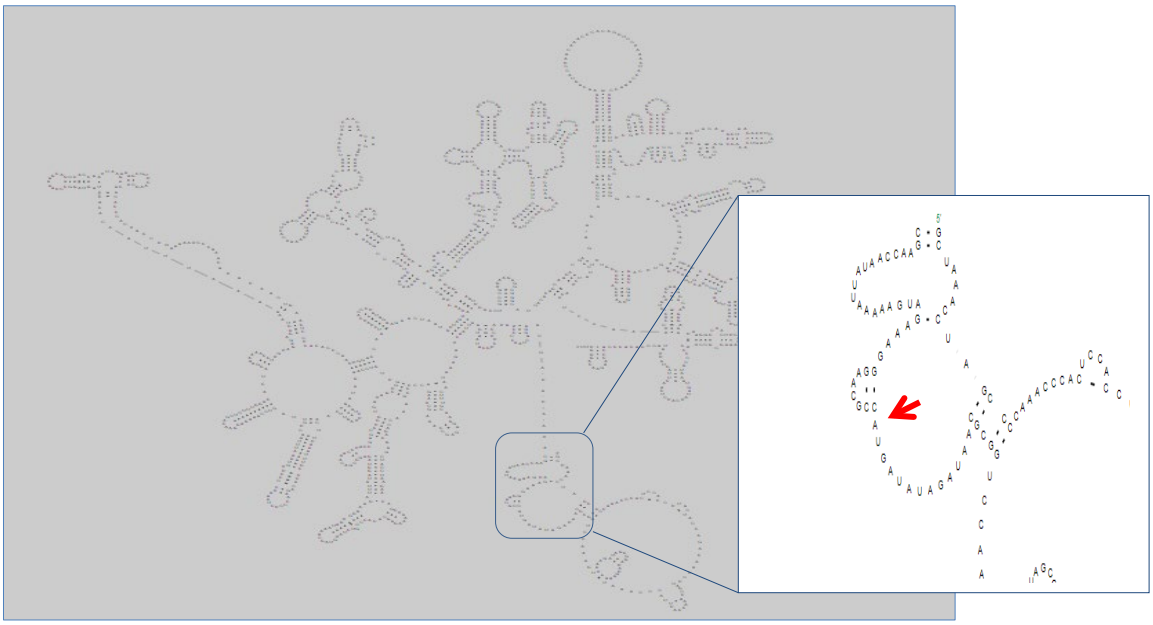

Figure 4-4: Secondary structure of human 16S rRNA (RNA central web site. https://rnacentral.org/rna/URS000049359E/9606); site of mutation pointed out by a red pointer

4.8. EFFECTS OF MUTATION IN HYPERVARIABLE REGION:

D-loop or hypervariable region in mtDNA harbours fundamental regulatory sites for mtDNA replication and transcription; hence mutation in this region in cancer tissues may affect the mtDNA copy number. This region is a well-known hot spot for somatic mutation in breast and other cancer tissue (Yin et al., 2010; Tseng et al. 2011; Lai et al., 2013; Lee, 2014), however in the current study it was the 2nd most common (27%) site of mutation after the protein coding region. Most of the mutations in hypervaariable region were population related. One of the common mutations in this region which was observed in the current samples as well (both in cancer and control samples) is the nucleotide repeats at 303–315 poly-C stretch in the HV2 region (fig. 3-38) which includes 12–18 C bases, usually interrupted by a T base at position 310 that's why also called (D310) mutation (Zhao et al., 2010) a highly susceptible focus for oxidative damage with ineffective repair mechanisms, justifying there frequent occurrence in different cancer types (Mambo et al., 2003). Only a single unique (novel) mutation a GC insertion in position 512 was observed in the hypervaribale region (Fig. 3-39).

According to what have been discussed so far, intact mitochondrial DNA is essential for cellular respiration, maintaining healthy ROS level and cellular apoptosis, presence of wide,

complex, and varied types of mutations in different parts of this genome in the current breast cancer tissue samples, including genes encoding proteins of the respiratory chain, mitochondrially encoded tRNA and rRNA. Finally the non-coding or hypervariable region which is important for the mitochondrial genome replication and transcription, is undoubtedly predisposing to a state of mitochondrial dysfunction either through reducing mtDNA copy number, or changing the tertiary structure of the mitochondrially encoded electron transport chain proteins, tRNA and rRNA impairing the proper interaction between these structures, resulting in faulty protein production, which are components of the respiratory chain, excess ROS production, shifting to aerobic glycolysis (Warburg phenomenon) and reduced apoptosis. High percentage of non-germline mutations in the breast cancer samples in comparison to the control samples in the current study is a possible indicator of an environmental hit, especially as all the cases in this study were sporadic and had no family history of breast cancer. According to the Iraqi cancer registry 2012, the last 10 years showed rising levels of breast cancer incidence in our locality, one of the major environmental hits that have a direct damaging effect on the cellular genome are the free radicals. There are many sources of free radical in this region and they are continuously increasing, as progressive population crowding, air pollution due to previous wars, increase in number of automobiles, oil and gas industry (Amin, 2017), water pollution with industrialisation, and rudimentary garbage management (Hassan, 2010); in one hand and having a sedentary lifestyle with an increase in BMI (Shabu, 2019) on the other hand, are other factors that are possibly responsible for the rising levels of breast cancer cases in our locality.

As was indicated previously of the cellular genomes, mitochondrial one is much more affected and damaged than nuclear because of lack of protective histones, inefficient repair mechanisms as well as their position, being close to the respiratory chain and free radicals, furthermore lack of introns, all are contributing in making mtDNA susceptible to mutation and damage.

Nevertheless, no single somatic cancer causing mutation (carcinogenic mutation) was identified in the examined samples, however many contributable mutations were observed predictably resulting in major defects in mitochondrial functions, as point mutation causing early protein termination, change in structure of proteins especially aggregated in the proteins of the Complex I, tRNA and rRNA with change in mtDNA copy number as a result of mutations in the hypervariable regions.

Furthermore population related mutations were not totally innocent as in many studies specific SNP and haplogroups were found to be related to breast cancer as A8860G and (HV) haplogroup in the current samples, probably making individuals with a specific combination of SNP more susceptible to carcinogenic process.

CHAPTER -5
CONCLUSIONS
AND RECOMMENDATIONS

CONCLUSION AND RECOMMENDATIONS

Mitochondrial DNA is a very rich source of information and a big piece of an unsolved puzzle in the process of carcinogenesis; this study ascertained few of many unproved other facts.

5.1. CONCLUSION

1. In the current study, individuals with SNP (A8066G) are at high risk of developing mitochondrial dysfunction and breast cancer.
2. Other risky groups for breast cancer in the current population are individuals with haplogroups HV and N, combined with SNP A8066G may have a synergistic effect
3. Base pair substitutions were the commonest type of mutation in all mitochondrial regions.
4. There is no single common somatic mitochondrial mutation responsible for the process of carcinogenesis in breast cancer.
5. Mitochondrial dysfunction represented by variant mtDNA mutations are significantly higher in cancer samples in comparison to control samples.
6. Breast cancer may be associated with decreased copy number.
7. Protein coding region shows the highest rate of SNP mutation both among breast cancer samples and control samples.
8. Nearly half of the protein coding region substitutions were asynchronous
9. Fifteen new (novel) mutations were identified in protein coding region and almost all were asynchronous
10. Three new mutations were observed in tRNA, phenyl alanine (A623C), Lucien (A3269T) and Lysine (C8305A)

5.2. RECOMMENDATION:

1. Further studies on mitochondrial DNA mutations are recommended in our community to create a general knowledge about common haplogroups, SNP and their phenotypic effects
2. A larger population of comparable cancer and control samples to be used for more precise statistical results
3. For a concise somatic mutation identification, a comparable tumor sample, and control from adjacent non-tumor tissue of same individual to be taken

4. Conducting further studies regarding the relation of SNP A8066G with breast cancer.
5. Include protein coding region in population studies as many SNP are found in this region.
6. More studies on mitochondrial DNA mutations using different tissue cancers, to find out whether mutations are tissue specific or regardless of the cancer type similar mutational changes ensue.

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الخلّاصه

الخلفية والأهداف: عبر التاريخ ، كان الحمض النووي للنواة وطفراته موضع اهتمام الباحثين في مجال السرطان لتحديد الأسس الجزيئية للتسرطن ، ومع ذلك جذب الحمض النووى للمايتوكوندريا في الآونة الأخيرة مزيدًا من الاهتمام لكثرة تعرضه للطفرات ،وقد تساهم هذه الطفرات في احداث خلل وظيفي في المايتوكوندريا و يؤثر على الفسفره التأكسديه مما يؤدي إلى زيادة فى إنتاج الشوارد الحرة السامة والتي تؤدى الى المزيد من الطفرات فى الحمض النووي للميتوكوندريا وبالتالي انتاج المزيد من الشوادر التأكسديه التي قد تؤثر في نهاية المطاف سلبيا على الحمض النووي للنوات أيضا. وقد تم الاستعانة بالانسجه السرطانية الغير وراثيه للثدي كنموذج في هذه الدراسة لتحديد الطفرات الموجودة بالحامض النووي للمايتوكوندريا و التعرف على طبيعه هذه الطفرات و علاقة هابلوگروب المايتوكوندريا و والتعددات الشكليه للنيوكليوتايد الاحادي(SNP) بحدوث سرطان الثدي

طريقة العمل: تم استخراج الحمض النووى للمايتوكوندريا من ٣٠ نسيج سرطاني للثدي و ٢٠ نسيج حميد للثدي كعينات تحكميه، وتم تكبيره في أربع اجزاء متداخلة باستخدام primer٤ امامية التفاعل و ٤ عكسية ؛وقد تم قراءةالتسلسل الكامل للحمض النووى للمايتوكوندريا ل ٢٠ من أصل ٣٠ عينة سرطاانيه ولكل العينات التحكميه ، وذلك باستخدام ١٩ primers عكسي. استعمل برنامج Haplogrep 2.0 لتعريف الهابلوگروبات و تم استخراج نسبة (odd)و اسخدام كل من chi-square و Fishers exact لحل قيم ال (p value)

النتائج: استبدال الزوج الأساسي هي الطفرة الاكثر شيوعا في حالات السرطان ، والتي تركزت معظمها في منطقة تشفير البروتين ، خاصةً البروتين المركب رقم ١ (٥٧٪) وقلة منها لوحظت في منطقة tRNA. نسبة الطفرات المتفرقه كانت أعلى بكثير في عينات السرطان بالمقارنة مع العينات الضابطة بقيمة p =0.000 وقد تم التعرف على ٢١ طفرة جديدة بين عينات السرطان, ١٥منها في منطقة تشفير البروتين وجميعها تقريبا كانت غير متزامنة. واخيرآ تبين وجود علاقه كبيره (مؤثره) بين هابلوغروب (HV) و حدوث سرطان االثدي فى العينات المدروسه, كما تبين ارتفاع نسبة التعدد الشكلي الاحادي A8860G بشكل كبير في عينات سرطان الثديOdd ratio اكثر من واحد و قيمة p اقل من 0.05

الاستنتاجات: هناك علاقة فعالة بين السرطان والطفرات الجسدية (غير الوراثيه) في الحمض النووى للمايتوكوندريا التي تؤثر بشكل عام على بنية البروتينات المشفرة بالميتوكوندريا في السلسلة التنفسية المعقدة بشكل رئيسي البروتين المركب رقم ١ ، وكذلك بنية الحمض الريبي النووي النقال (tRNA) والحمض النووي الريبوزي الرابوسومي (rRNA) التي تضعف تفاعلهما السليم مما يؤدي إلى خلل وظيفي في الميتوكوندريا. من النتائج المهمة الأخرى في هذه الدراسة ارتفاع معدل الإصابة بسرطان الثدي بين مجموعة هابلوگروب الميتوكوندريا (HV) مع وجود علاقة كبيرة بين SNP A8860G وسرطان الثدي في مجتمع الدراسة الحالي.

الكلمات الرئيسية: سرطان الثدي ،الحمض النووى للمايتوكوندريا ، هابلوگروب ، التعدد الشكلي الاحادي ، المركب رقم ١

حكومة اقليم كوردستان- العراق

وزارة التعليم العالي و البحث العلمي

جامعة السليمانية

كلية الطب

تأثير طفرات الحمض النووي للمايتوكوندريا على سرطان الثدي الغير وراثي في محافظة السليمانيه

رسالة مقدمة الى مجلس كلية الطب - جامعة السليمانية كجزء

من متطلبات نيل درجة الدكتوراه في علم الامراض

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پوخته

پاشخان و مەبەستەکان: بە درێژای مێژوو ماددەی بۆماوەی ناووک وبەزینە کانی جێی بایەخ و خویندنەوەی توێژەران و زانایانی شیرپەنجە بوو بۆ دیاری کردنی بنەما گەردیەکانی دروست بوونی شێرپەنجە ، لەگەڵ ئەوەشدا لە چەندساڵی ڕابردوودا بایەخەکان زیاتر ڕوویان کردە ماددەی بۆماوەی وزە ماڵە (مایتۆکۆندریا) بەهۆی زۆری بەزینە جینیەکان لەم ماددەیەدا ،کە دەبێتە هۆی تێکچوونی کردارەکانی وزە ماڵە و تێکچونی کرداری هەناسەدانی خانە و زۆر بوونی توخمە ئۆکسجینیە ناجێگیرەکان کە ئەمانەش دەبنە یارمەتیدەریک بۆ زیاتر ڕووداتی بەزینی جینی لە مادەی بۆماوەوەی وزەماڵە کە ئەمەش دیسانە وە هۆکاریکە بۆ زیاتر بوونی توخمە ئۆکسجینیە ناجێگیرەکان کە لە ئەنجامدا بە هەمان شێوە کاریگەری بۆ سەر ماددەی بۆماوەی ناوکیش دەبێت. لەم توێژینەوەیەدا شێرپەنجەی مەمک وەک نمونەیەکی شانەی شێرپەنجەی بەکار هێنراوە بۆ ناسینەوەي بەزینەکاني بۆماوە ماددەي وزەماڵە لە شیرپەنجەی مامکی نابۆماوەیی ، وە ئاشنا بوون بە جۆری بەزینەکان و پەیوندی نێوان هاپلۆگروپی وزە ماڵە و تاکە نیوکلیوتایدی هەمەچەشنی (SNP) و دروست بوونی شێرپەنجەی مەمک

ڕێچکەکان: ماددەی بۆماوەی مایتۆکۆندریای ل۳۰ شانەی شێرپەنجەی مەمک و ۲۰ شانەی گرێی پاکي مەمک وەک گروپی کۆنترۆل جیاکرایەوە ، چەندجار گەورەکردنی ئە ماددە بۆماوەییە(PCR) لە شیوەی چوار پارچەی بەیەکداچوو بە بەکارهێنانی ٤ پرایمر (primer) ڕاستەوانەو ٤ ی پێچەوانە کرا ، ڕیزبەندی بۆتەواوی ماددەی بۆماوەی مایتۆکۆندریای بۆ ۲۰ لە شانە شێرپەنجەییەکان و بۆ هەموو گروپی کۆنترۆل کرا بە بەکارهێنانی ۱۹ primerی پێچەوانەی تر. بەرنامەی Haplogrep 2.0 بەکارهێنرا بۆ دیاری کردنی هە پلۆ گروپەکان ،دیاري کردني ڕێژەي (odd) ، هەروەها بەکارهێناني بەرنامەی Chi-square و Fishers-Exact بۆ دۆزینەوەی پەیوەندیەکان و دیاری کردنی نرخی (P value)

ئەنجامەکان: زۆرترین جۆری بەزین بریتی بوولە ئالوگۆری تفتە نایترۆجینیەکان کە بەزۆری لە و بەشەدا کۆبۆتەوە کە کۆدی دروستکرنی پرۆتینەکانی پێوست بە کرداری هەناسەدانی خانەی تیایە، بەتایبەت کۆمپلێکسی ۱ (۵۷٪) وە کەمترین ئاستی بەزینن لە tRNA بەدی کرا. بەزینە دەگمەنە کان (کە پەیوەندی بە هەپلۆگروپەوە نیە) بەشێوەیەکی بەرچاو زیاتر بوو لە شانە شیرپەنجەییەکان بە بەراورد بە شانەی گرێ پاکەکان و (p 0.000=). لەم توێژینەوەیەدا ۲۱ جۆری تازە (تۆمار نەکراو) لە بەزینی بۆماوە ماددەی وزە ماڵە بەدی کرا کە ۱۵ یان دە کەوتە ناوچەی کۆدی پرۆتینی وە بە نزیکەی هەمووی لە جۆری لێکنەچوو (asynchronous) بوو. بوونی پەیوەندییەکی کاریگەر لەنێوان هاپلۆگروپی (HV) و شێرپەنجەی مەمک هەروەها بوونی پەیوەندی کاریگەر لە نێوان تاکە نیوکلیوتایدی هەمەچەشنەی A8860G و ڕووداتی شێرپەنجەی مەمک لە نمونە پشکنینکراوەکانی ئەم توێژینەوەیە بەنرخی(p value) ی بچووکتر لە(0.05) وە (Odd ratio)گەورەتر لە یەک.

دەرئەنجامەکان: بوونی پەیوەندی یەکی کاریگەر لە نێوان شیرپەنجە و بەزینە دەگمەنە کانی بۆماوە ماددەی وزە ماڵە کە بەشێوەیەکی گشتی کاردەکاتە سەر پیکهاتەی ئەو پرۆتینانەی لە ڕێی بۆماوەماددەی وزە ماڵەوە کۆد دەکرین کە بەشدارن لە ئاڵۆزە کرداری هەناسەدان (OXPHS) بەتایبەتی کۆمپلیکسی ۱وە هەروەها کاردەکاتە سەر پێکهاتەي (tRNA) و

(rRNA) وه به تێکچونی ئەم پێکهاتانه کارلێکی نێوانیان کەم دەکات ودروستکردني پرۆتین تیک دەچێت. له دەرئەنجامەگرنگەکانی تری ئەم توێژینەوەیه ، بوونی پەیوەندیەکی راستەوانه له نێوان هاپلۆگروپی (HV) وه تاکه نیوکلیوتایدی هەمەچەشنەی A8860G لەگەڵ شێرپەنجەی مەمک له نمونه کانی ئەم توێژینەوەیه

ووشه سەرەکیەکان: شێرپەنجەی مەمک، ماددەی بۆماووەی وزه ماڵه ، هاپلۆگروپ ، تاکه نیوکلیوتایدی هەمەچەشنه ، کۆمپلێکسی ١

حکومەتی هەرێمی کوردستان - عێراق

وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی

زانکۆی سلێمانی

کۆلێژی پزیشکی

کاریگەری بەزین لە بۆماوە ماددەی وزە ماڵە لەسەر شێرپەنجەی مەمکي نابۆماوەي لە پارێزگای سلیماني

تێزێك پێشكه شه به ئه نجومه نی كۆلێژی پزیشكی زانكۆی سلێمانی وه ك به شێك له جێبه جێكردنی پێویستییه كانی وه رگرتنی بڕوانامه ی دكتۆرای فەلسەفەی له نەخۆشی زانی .

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