Ministry of Higher Education

and Science Research

University of Misan

College of Science

Department of Biology

Genetic Polymorphism Study of the Genes Related to Breast Cancer Prediction

A Thesis

Submitted to the Council of the College of Science / University of Misan as Partial Fulfillment of the Requirements for the Master Degree in Biology

By

Zainab Zamil Gataa

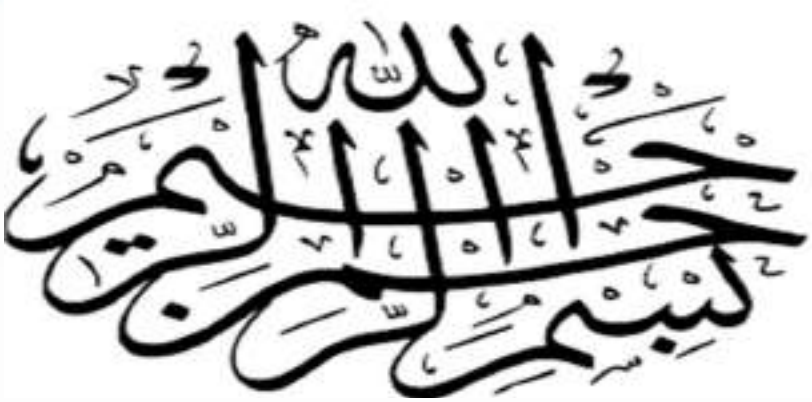
B. Sc. Biology (2012)

Supervised

Assist. Prof. Dr. Maytham Abdulkadhim Dragh

January 2022 A.D

Jumada al-thani 1443 A.H



فَتَعَٰلَى ٱللَّهُ ٱلْمَلِكُ ٱلْحَقُّ ۗ وَلَا تَعْجَلْ بِٱلْقُرْءَانِ مِن قَبْلِ

أَن يُقْضَىٰٓ إِلَيْكَ وَحْيُهُۥ ۖ وَقُل رَّبِّ زِدْنِى عِلْمًا ﴿١١٤﴾

صدق الله العلي العظيم

سورة طه (الآية ١١٤)

Supervisor's Certificate

We certify that this thesis entitled " Genetic Polymorphism Study of the Genes Related to Breast Cancer Prediction"

" has been prepared under my supervision at the college of Science, University of Misan : as a partial fulfillment of the requirements for the degree of Master of Biology"

Signature

Assist. Prof. Dr. Maytham Abdul Kadhim Dragh

Department of Biology

College of Science/Misan University

Date : / / 2021

Recommendation of Head of Biology Department

In view of the available recommendations ; I forward this thesis to debate by the examining committee.

Signature

Assist. Prof. Dr. Maytham Abdul Kadhim Dragh

Head of Department of Biology

College of Science/Misan University

Date : / / 2021

Dedication

To the master of humanity, the messenger of mercy dedicated to our master Muhammad and his good and pure family... and his faithful companions

To my country with everything in it .....

To those who stayed up nights and drowned me with their kindness and tenderness and taught me honesty, patience, perseveranc, giving and loyalty my mother and father

To my support in my life .....my brothers and sisters

For everyone who taught me

To my friends and colleagues

And to everyone who stood with me, even with a glimmer of hope

I dedicate the result of my effort

Zainab Zamil

Acknowledgments

Praise be to Allah Almighty, first and foremost, who gave me the blessing of patience, ability, and perseverance to complete the work. I thank God, the Exalted, the Majestic, a great, good, and blessed one who fills the heavens and the earth for what he has honored me with from completing this study, I hope you are satisfied about her. Thank you to the Messenger of Mercy and Humanity, Muhammad Al-Hadi Al-Amin, and his good and pure family... and his faithful companions, who taught us to perform honesty and seek knowledge.

I am pleased as I put the last fingerprints of my thesis to express my heartfelt thanks and gratitude to my esteemed **Assis. Prof. Dr. Maytham Abdul Kadhim Dragh**, who kindly supervised my thesis and suggested the subject of the thesis, and his continued encouragement to me in all stages of the research work, I wish him all success and continued health, and may God reward him on my behalf.

I also extend my sincere thanks to the officials in the Genetic Engineering Laboratory, **Assis. Prof. Dr. Maytham Abdul Kadhim Dragh, Assis. Prof. Dr. Salah Hassan Faraj and M. Sc. Shaima Rabie Banoon** (College of Science) for their approval of graduate students work in the laboratory and to provide them with information and support, wishing them success.

I also extend my heartfelt thanks to the esteemed Head of the department of Biology, my distinguished teachers, and to the Deanship of the College of Science, especially the esteemed Dean. I also thank my friends and my colleagues.

Much deep thanks to **Assis. Prof. Dr. Salah Hassan Faraj** (College of Science) for his cooperation with me in drawing some 3D protein. Much deep thanks to Maysan Health Directorate/ AL-Sadder Teaching Hospital /AL-Shifa Tumor Treatment Center especially **Prof. Dr. Haider Sadun Qasim** and all the staff of the /AL-Shifa Tumor Treatment center for facilitating the approval of the collection of blood samples from breast cancer patients. My thanks and appreciation to all breast cancer women patients and normal people for their consent to draw blood samples from them, much deep thanks to Saeed Sayed Ahmed, Ikhlas zyarra lazeem, for their cooperation with me in Al-Sader Teaching Hospital. Deep thanks to M. Sc. Hassan Ghaly Abdul Hassan, for his cooperation with me in entry of DAPI stain in to Iraq. Much deep thanks to Baghdad laboratory for cooperation with me in some tissues samples collection.

I extend my deep thanks and gratitude to my family, especially my father, mother, brothers and sisters, may God reward them with the best reward , praying to God to bless them with health, wellness and healing.

Zainab Zamil

Summary

Summary

The current study was conducted to compared the genetic polymorphisms of *ABCG2*, *ABCB1*, *BRCA1*, *ER-α*, and *miRNA-152* genes between breast cancer patients (BC) and control individuals, and determined some cytogenetic changes of breast cancer tissues, and compare it with normal tissues. One-hundred seventeen of blood specimens were collected from participants attended AL-Sadder Teaching Hospital and AL-Shifaa Tumor Treatment Center in Maysan province. The study period was from 3rd of September 2020 to 15th of September 2021. RFLP-PCR, PCR-RAPD-PCR technique (utilize for the first time), nucleotide sequencing and gene expression measuring were used to study genetic polymorphisms. In this study a specific primers (ABCG2, ABCB1, BRCA1, ER-α, and mi-RNA 152 primers), two types of restriction enzymes (MboI, EcoRI) in RFLP technique, four RAPD primers (OPAA11, OPU15, OPAA17, and OPD18) in PCR-RAPD-PCR technique were used. Regarding cytogenetic study, four types of flourescent stains (DAPI, DCFH-DA, Mito-Sox, and Mito-Tracker) were used to determine the reactive oxygen species (ROS), mitochondrial dysfunction and the relationship of genetic mutations to ROS in the tissues of BC patients and their comparison with normal participants. The study was done in the Genetic Engineering laboratory at of the Department of Biology, College of Science, University of Misan, with the aim of extracting DNA from all blood samples and amplification by using polymerase chain reaction (PCR) technique, and use their products as template for study the genetic polymorphisms by PCR techniques and nucleotide sequencing via Macrogen Korea company to analyze the sequences of nitrogenous bases. All results were analyzed by Chi-square test.

In RFLP-PCR technique the results of the statistical analysis of BC genotypes by using X^2 (HWE) show there were differences from the Hardy-Weinberg equilibrium in *ABCG2*, *ABCB1* genotypes of BC patients compared to the healthy peoples, and the genotypes of *BRCA1* shows somewhat similarity in the BC patients and healthy controls. There were statistically significant differences in the distribution of alleles (C, T) for *ABCG2*, *ABCB1* between the BC patients and the control group; without

statistical significant differences in the distribution of alleles(C, T) for the *BRCA1* gene between two groups. The statistical analysis figured significant differences in the genotypes of *ABCG2*, *ABCB1* ($p < 0.05$) between BC females and control at CC, CT genotypes. Besides, the findings documented no significant differences in the genotypes of the *BRCA1* (TT, TC, CC) ($P > 0.05$) between the BC patients and the control. There were statistically significant differences in the distribution of the dominant model for *ABCG2* and *ABCB1* genes between the BC patients and the control group; with no statistical association in the distribution of the recessive model, and no statistically significant differences in the distribution of the dominant model and recessive model of the *BRCA1* gene were observed.

In relation to the PCR-RAPD-PCR technique, our findings revealed the total number of *ABCG2*, *ABCB1*, *BRCA1*, *ER-α*, and *miRNA 152* bands were higher in the patient's group than the other group, with significant differences ($P < 0.05$) between two groups in a total of the polymorphic, unique and monomorphic band of *ER-α*, *mi-RNA-152*, and no significant differences ($P > 0.05$) in *ABCG2*, *ABCB1*, *BRCA1*. The molecular weight (band size) is (20-30) bp of the polymerization of the OPD18 of the *ABCG2* gene, OPAA 17 of *ABCB1* gene, OPAA 17 of the *BRCA1* gene, whereas OPAA11 in the *ER-α* gene showed bands with a molecular weight (40-50) bp, OPAA17 and OPD18 in the *mi-RNA-152* gene showed bands with molecular weight (20-40) bp. These bands appeared in the BC group and did not appear in the healthy group, which is a positive indicator, and thus the primers can be nominate to be a distinctive indicator of BC at the level of the RAPD indicators. Therefore, these primers can be adopted to distinguish BC genetically at the molecular level.

Regarding nucleotide sequencing of the *ABCG2* gene, there are 11 new genetic mutations (SNPs) were obtained, 6/11 (G71T), (T141C), (T148C), (G150C), (T169C), (G172C) were non-synonymous mutations that caused changes in the structure of the three-dimensional protein as a result of changing amino acids in BC patients and three of which (G38A), (G2559C), (G96428A) are synonymous mutations, two of which (163 G^{Del}), (167 A^{Del}) are frameshift mutations. The nucleotide sequencing of the

ABCB1 gene figured eight genetic mutations, six of which (G55C), (T59G), (C89A), (C90G), (A113C), (A139T) are non-synonymous, two (C153T), (C323T) are synonymous mutations. The *BRCA1* gene sequencing documented three genetic mutations, 3 of which (C213T, T248C, T261C) are a non-synonymous mutations.The nucleotide sequencing of the *ER-α* gene revealed that six genetic mutations, 4/6 of which (C114T), (C244G), (C344G), (C424T) are synonymous mutations, 1/ 6 which (T440C) is non-synonymous mutation, 1/6 (454 A^{Del}) is frameshift mutation.

In the cytogenetic section the reaction to DAPI, DCFH-DA, Mito-Sox, and Mito-Tracker increased and expressed in the BC tissues compared with normal tissue due to increased ROS production, defect of mitochondria function and structure, besides, genetic mutations.

List of contents

Subject		Page No
Summary		I-III
List of Contents		IV-VII
List of Tables		VIII-XI
List of Figures		XI-XIV
List of diagrams		XIV
List of Abbreviations		XV-XVII
Chapter One		
1	Introduction	1-3
Chapter Two		
2	Literature Review	4-35
2-1	Cancer	4
2-2	Breast cancer	5
2-2-1	Etiology of breast cancer	5-7
2-3	Genetics basis of breast cancer	7-32
2-3-1	Gene expression	7-9
2-3-2	Genetic mutation	9-11
2-3-3	Genetic polymorphism	12-32
2-3-3-1	The most common polymorphism in human being breast cancer	12-16
2-3-3-1-1	Single nucleotide polymorphisms	12-16
2-3-3-1-1-1	Effects of SNPs location	13-14
2-3-3-1-1-2	Types of SNPs in exons	14-16
2-3-3-1-2	Insertion / deletion polymorphism	16
2-3-4	Techniques for study and determining genetic polymorphism	17-20
2-3-4-1	Restriction fragment length polymorphism(RFLP)	17-18
2-3-4-2	Random amplified polymorphic DNA(RAPD)	18-19
2-3-4-3	DNA sequencing	19-20
2-3-4-3-1	Bioinformatics methods	21-24
2-3-4-3-1-1	Genetic prediction	22-23
2-3-4-3-1-2	Three-dimensional protein (3D)	23
2-3-5	Genes that related to cancer	24-25
2-3-5-1	Oncogenes in breast cancer	24-25
2-3-5-1-1	Oncotype DX	24
2-3-5-2	Tumor suppressor gene in breast cancer	25
2-3-6	Cancer driver genes	25-32
2-3-6-1	*ABCB1* gene	25-27

2-3-6-2		*ABCG2* gene	27-28
2-3-6-3		*BRCA1* gene	28-29
2-3-6-4		*miRNA-152* gene	29-30
2-3-6-5		*ER-α* gene	30-32
2-4		Programmed cell death (Apoptosis)	33
2-5		Reactive Oxygen species (ROS) in cancer	34-35
Chapter Three			
3	Materials and methods		36-59
3-1	Collection of samples		36
3-1-1	Blood samples		36
3-1-2	Breast tissue sample		36
3-2	Chemicals		37
3-3	Equipments and their suppliers		38
3-4	Consumer materials		39
3-5	Laboratory kit		40
3-6	Primers used in present study		41-42
3-6-1	The specific primers for genes that related to breast cancer		41
3-6-2	The RAPD primers		42
3-7	Design of experiment		43
3-7-1	Sterilization Methods		44
3-7-1-1	Sterilization by autoclaving		44
3-7-1-2	Sterilization by dry heat		44
3-7-2	Preparation of stains		44-45
3-7-2-1	Preparation of DAPI stain		44
3-7-2-1-1	Stock solution		44
3-7-2-1-2	Work solution		44
3-7-2-2	Preparation of DCFH-DA stain		45
3-7-2-3	Preparation of Mitotracker stain		45
3-7-2-4	Preparation of Mitosox stain		45
3-7-3	Preparation of Buffers or solutions		45-46
3-7-3-1	Tris Borate EDTA (TBE) buffer		45-46
3-7-3-1-1	Stock Buffer solution (10X)		45
3-7-3-1-2	Work Buffer solutions(1X)		46
3-7-3-2	Permeabilization solution		46
3-7-3-3	Ladder preparation for use in Gel electrophoresis		46
3-7-4	Genetic and molecular study		46-57
3-7-4-1	DNA extraction		46-48
3 -7-4-2	Detection of DNA by Agarose Gel Electrophoresis		48-49
3-7-4-3	Column purification		49
3-7-4-4	Preparation of primers solution		49

3-7-4-5	Master mix	49
3-7-4-6	Polymerase Chain Reaction Protocol	49-50
3-7-4-7	Agarose Gel Electrophoresis	51
3-7-4-8	Extraction of PCR product from the gel	51-53
3-7-4-9	Column purification	53
3-7-4-10	Restriction fragment length polymorphism (RFLP)	53-54
3-7-4-11	PCR-RAPD-PCR	54-55
3-7-4-11-1	Molecular weight index	55-56
3-7-4-12	Determine the sequence of nitrogen bases	56-57
3-7-4-13	3 D protein drawing	57
3-7-5	Very simple cytogenetic study	57
3-7-5-1	Tissue preparation	57-59
3-7-6	Statistical Analysis	59
Chapter Four		
4	Results	60-121
4-1	DNA extraction and amplification	60
4-2	Genetic polymorphism	61-114
4-2-1	PCR-RFLP	61-76
4-2-1-1	Detection of the genetic polymorphism of the BC gene by RFLP technique	61-62
4-2-1-1-1	Detection of the genetic polymorphism of *ABCG2* gene by RFLP technique	62-66
4-2-1-1-2	Detection the genetic polymorphism of *ABCB1* gene by RFLP technique	67-71
4-2-1-1-3	Detection of the genetic polymorphism of *BRCA1* gene by RFLP technique	72-76
4-2-2	PCR-RAPD	77-103
4-2-2-1	PCR-RAPD-PCR (PRP)	77-103
4-2-2-1-1	Detection of the genetic polymorphisms of the BC gene by PCR-RAPD-PCR technique	78-105
4-2-2-1-1-1	Detection of the genetic polymorphisms of the *ABCG2* gene by PRP	79-83
4-2-2-1-1-2	Detection of the genetic polymorphisms of the *ABCB1* gene by PRP	84-88
4-2-2-1-1-3	Detection of the genetic polymorphisms of the *BRCA1* gene by PRP	89-93
4-2-2-1-1-4	Detection of the genetic polymorphisms of the *ER-α* gene by PRP	94-98
4-2-2-1-1-5	Detection of the genetic polymorphisms of the *miRNA-152* gene by PRP	99-103
4-2-3	Detection polymorphism of BC related gene by nucleotide sequencing	104-114

4-2-3-1	*ABCG2* gene	104-106
4-2-3-2	*ABCB1* gene	107-109
4-2-3-3	*BRCA1* gene	110-111
4-2-3-4	*ER-α* gene	112-114
4-2-4	Cytogenetic study of breast cancer tissues	115-119
Chapter Five		
5	Discussion	120-145
5-1	Study genetic polymorphism of genes related to breast cancer by RFLP technique	120-127
5-1-1	Study genetic polymorphism of the *ABCG2* gene by RFLP technique	121-123
5-1-2	Study genetic polymorphism of the *ABCB1* gene by RFLP technique	123-125
5-1-3	Study the genetic polymorphism of the *BRCA1* gene by RFLP technique	126-127
5-2	PCR-RAPD-PCR	128-132
5-2-1	Detection of the genetic polymorphisms of the BC genes by PCR-RAPD-PCR technique	128-132
5-3	Detection genetic polymorphism of BC related genes by nucleotide sequencing	133-141
5-3-1	*ABCG2* gene	134-135
5-3-2	*ABCB1* gene	135-137
5-3-3	*BRCA1* gene	137-139
5-3-4	*ER-α* gene	139-141
5-4	Cytogenetic study of breast cancer tissue	142-145
Chapter six		
6	Conclusions and Recommendations	146-148
6-1	Conclusions	146-147
6-2	Recommendations	148
Chapter seven		
7. References		149-181
Appendixes		

List of Tables

Table (3-1): List of chemicals, and their suppliers	37
Table (3-2): List of equipments and their suppliers	38
Table (3-3): List of consumer materials and their suppliers	39
Table (3-4): Types of kits applied in the current study and their company	40
Table (3-5): Primers sequences used for breast cancer related genes amplification	41
Table (3-6): RAPD primers sequences used for PCR-RAPD-PCR amplification	42
Table (3-7) : The components of PCR mixture	50
Table (3-8) : PCR program of *ABCB1*, *ABCG2*, *BRCA1*, *ER-α* and *miRNA 152* genes	50
Table (3-9) The components of RFLP mixture	53
Table (3-10) The components of PCR-RAPD-PCR mixture	54
Table (3-11) PCR-RAPD-PCR program for *ABCB1*, *ABCG2*, *BRCA1*, *ER-α* and *miRNA-152* genes	55
Table (4-1) Clinical and pathological characteristics of Maysan breast cancer women	61
Table (4-2) Allele frequency and genotype frequency in the *ABCG2* gene of the breast cancer patient and control	62
Table (4-3) Alleles and genotype percentages of *ABCG2* polymorphism among patients and controls	63
Table (4-4) Alleles frequency of *ABCG2* gene among breast cancer patients and control	63
Table (4-5) The genotype frequencies of the *ABCG2* gene among the breast cancer patients and control group	64
Table (4-6) The observed and expected frequencies under Hardy-Weinberg equilibrium in the *ABCG2* gene of the control	65
Table (4-7) Genotype distribution and frequencies of *ABCG2* polymorphism among breast cancer patients and control	65
Table (4-8) Distribution of the *ABCG2* genotypes in the breast cancer patients and control under dominant model	66
Table (4-9) Distribution of the *ABCG2* genotypes in the breast cancer patients and control under recessive model	66
Table (4-10) Allele frequency and genotype frequencies in the *ABCB1* gene of the breast cancer patient and control	67
Table (4-11)Alleles and genotype percentages of *ABCB1* gene polymorphism among patients and control	67

Table (4-12)) Alleles frequency of *ABCB1* gene among breast cancer patients and control	68
Table (4-13) The genotype frequencies of the *ABCB1* gene among the breast cancer patients and control group	68
Table (4-14) The observed and expected frequencies under Hardy Weinberg Equilibrium in the *ABCB1* gene of the control	69
Table (4-15) Genotype distribution and frequencies of *ABCB1* polymorphism among breast cancer patients and control	70
Table (4-16) Distribution of the *ABCB1* genotypes in the breast cancer patients and control under dominant model	70
Table (4-17) Distribution of the *ABCB1* genotypes in the breast cancer patients and control under recessive model	71
Table (4-18)) Allele frequency, and genotype frequency in the *BRCA1* gene of the breast cancer patients and control	72
Table (4-19) Alleles and genotype percentages of *BRCA1* polymorphism among patients and control	72
Table (4-20) Allele frequency of *BRCA1* gene among breast cancer patients and control	73
Table (4-21) The genotype frequencies of the *BRCA1* gene among the breast cancer patients and control group	73
Table (4-22) The observed and expected frequencies under Hardy-Weinberg Equilibrium in the *BRCA1* gene of the control	74
Table (4-23) Genotype distribution and frequencies of *BRCA1* polymorphism among breast cancer patients and control	75
Table (4-24) Distribution of the *BRCA1* genotypes in the breast cancer patients and control under dominant model	75
Table (4-25) Distribution of the *BRCA1* genotypes in the breast cancer patients and control under recessive model	78
Table (4-26) The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ABCG2* gene of breast cancer patient sample	79
Table (4-27) The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ABCG2* gene of control sample	80
Table (4-28) Statistical analysis for polymorphic,unique and monomorphic bands of *ABCG2* gene between patient and control samples	83
Table (4-29)The numbers and percentages of the total band, polymorphic, unique, monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ABCB1* gene of breast cancer patient sample	84

Table (4-30) The numbers and percentages of the total band, polymorphic, unique, monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ABCB1* gene of control sample	85
Table (4-31) Statistical analysis for polymorphic, unique and monomorphic bands of *ABCB1* gene between patient and control samples	88
Table (4-32) The numbers and percentages of total band, polymorphic, unique , monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *BRCA1* gene of breast cancer patient sample	89
Table (4-33) The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *BRCA1* gene of control sample	90
Table (4-34) statistical analysis for polymorphic, unique and monomorphic bands of *BRCA1* gene between patient and control samples	93
Table (4-35) The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ER-α* gene of breast cancer patient sample	94
Table (4-36) The numbers and percentages of the total band, polymorphic, unique, monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ER-α* gene of control sample	95
Table (4-37) Statistical analysis for polymorphic, unique and monomorphic bands of *ER-α* gene between patient and control samples	98
Table (4-38) The numbers and percentages of the total band, polymorphic, unique, monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *mi RNA-152* gene of breast cancer patient sample	99
Table (4-39) The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *miRNA-152* gene of control sample	100
Table (4-40) Statistical analysis for polymorphic, unique and monomorphic bands of *mi-RNA 152* gene between patient and control samples	103
Table (4-41) Nucleotide changes, the type of mutations and the resulting amino acid changes and their impact on the translation process of the *ABCG2* gene	105
Table (4-42) Nucleotide changes, the type of mutations and the resulting amino acid changes and their impact on the translation process of the *ABCB1* gene	108
Table (4-43) Nucleotide changes, the type of mutations and the resulting amino acid changes and their impact on the translation process of the *BRCA1* gene	110

Table (4-44) Nucleotide changes, the type of mutations and the resulting amino acid changes and their impact on the translation process of the *ER-α* gene	113

List of Figures

Figure (2-1) Showing general steps of gene expression	9
Figure (2-2) Single nucleotide polymorphism for two alleles	13
Figure (2-3) Structure of gene and SNP location	14
Figure (2-4) showing an example for mutation was found in human cancer using Sanger sequencing	20
Figure (2-5) Bioinformatics is the application of techniques from computer science to the problems from Biology	21
Figure (2-6) Three-dimensional structures of the *BRCA1*	23
Figure (2-7) Molecular structure of *ABCB1* gene	27
Figure (2-8) Shows microRNA biogenesis and modulation of miRNA activity	30
Figure (2-9) ESR1 mutations in breast cancer cause widespread transcriptional changes due to constitutive of the receptor altered chromatin	32
Figure (2-10) Summary of the pathways of Apoptosis	33
Figure (2-11) The electron transport chain (ETC) in mitochondria	35
Figure (2-12) Showing the Warburg effect in the cancer cell	35
Figure (4-1) Amplification of the *ABCG2*, *ABCB1*, *BRCA1*, *ER-α*, and *miRNA-152* obtained from BC patient and control	60
Figure (4-2) PCR RFLP results for *ABCG2*, *ABCB1*, and *BRCA1* after digested with MboI, MboI, EcoRI restriction enzyme.	62
Figure (4-3) The comparison among the numbers and percentages of the total CC, CT and TT genotype frequencies of the *ABCG2* gene between BC patients and control group	64
Figure (4-4) The comparison among the numbers and percentages the CC, CT and TT genotype frequencies of the *ABCB1* gene between BC patients and control group	69
Figure (4-5) The comparison among the numbers and percentages of the CC, CT and TT genotype frequencies of the *BRCA1* gene between BC patients and control group	74
Figure (4-6) Extraction of PCR product from the gel, its used to estimate the amount of DNA in PCR mixture after amplification and then choose the appropriated PCR mixture as a template for PCR-RAPD-PCR.	77
Figure (4-7) PCR-RAPD-PCR profile generated by four primer (OPAA11, OPU15, OPAA17, and OPD 18). The first lane from the right (M) is DNA ladder marker 100-1500bp in size, the last lane from the left (M) is DNA ladder marker 25-300bp in size and the other lanes are PCR-RAPD-PCR bands on 2% agarose gel electrophoresis. Red circles refer to unique bands.	78

Blue arrow refers to the lane of the polymorphic band, green lane refers to monomorphic band	
Figure (4-8) Total number of bands in *ABCG2* gene	80
Figure (4-9)(A, B) Size range of the fragments in *ABCG2* gene of BC patient and control	81
Figure (4-10) (A, B) Primer efficiency and discriminatory power in *ABCG2* gene of BC patient and control	82
Figure (4-11) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *ABCG2* gene between BC patient and control	83
Figure (4-12) Total number of bands in *ABCB1* gene	85
Figure (4-13)(A,B) Size range of the fragments in *ABCB1* gene of BC patient and control	86
Figure (4-14) (A, B) Primer efficiency and discriminatory power in *ABCB1* gene of BC patient and control	87
Figure (4-15) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *ABCB1* gene between BC patient and control	88
Figure (4-16) Total number of bands in *BRCA1* gene	90
Figure (4-17) (A, B) Size range of the fragments in *BRCA1* gene of BC patient and control	91
Figure (4-18)(A, B) Primer efficiency and discriminatory power in *BRCA1* gene of BC patient and control	92
Figure (4-19) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *BRCA1* gene between BC patient and control	93
Figure (4-20) Total number of bands in *ER-α* gene	95
Figure (4-21) (A, B) Size range of the fragments in *ER-α* gene of BC patient and control	96
Figure (4-22) (A, B) Primer efficiency and discriminatory power in *ER-α* gene of BC patient and control	97
Figure (4-23) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *ER-α* gene between BC patient and control	98
Figure (4-24) Total number of bands in *miRNA-152* gene	100
Figure (4-25)(A, B) Size range of the fragments in *miRNA-152* gene of BC patient and control	101
Figure (4-26) (A,B) RAPD primer efficiency and discriminatory power in the *miRNA 152* gene of BC patient and control	102
Figure (4-27) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *miRNA-152* gene between BC patient and control	103

Figure (4-28) Single nucleotide polymorphisms (SNPs) at the studied sites of the *ABCG2* gene	105
Figure (4-29) Three-dimensional shapes of the ABCG2 protein (3D protein) in breast cancer patients	106
Figure (4-30) The percentages of mutations in the *ABCG2* gene	106
Figure (4-31) Single nucleotide polymorphisms (SNPs) at the studied sites of the *ABCB1* gene	108
Figure (4-32)Three-dimensional shapes of the *ABCB1* protein (3D protein) in breast cancer patients	109
Figure (4-33) The percentages of mutations in the *ABCB1* gene	109
Figure (4-34) Single nucleotide polymorphisms (SNPs) at the studied sites of the *BRCA1* gene	110
Figure(4-35)Three-dimensional shapes of the *BRCA1* protein (3D protein) in breast cancer patients	111
Figure (4-36) The percentages of mutations in the *BRCA1* gene	111
Figure (4-37) Single nucleotide polymorphisms (SNPs) at the studied sites of the *ER-α* gene	113
Figure (4-38) Three-dimensional shapes of the *ER-α* protein (3D protein) in breast cancer patients	114
Figure (4-39) The percentages of mutations in the *ER-α* gene	114
Figure (4-40) Fluorescent microscopy. (A) DCFH-DA stained cell, green fluorescence indicates the amount of reactive oxygen species (ROS), white arrows indicate high ROS. (B) nucleus stained with DAPI, blue fluorescence indicates the nucleus, white arrows indicate the abnormal nucleus. (C) Merged images.	115
Figure (4-41) Fluorescent microscopy. (A, D) DCFH-DA stained cell with green fluorescence indicates the amount of reactive oxygen species ROS, arrows indicate high. (B, E) Fluorescent microscopy of Mito-Tracker stained mitochondria, red fluorescence indicate a defect of mitochondrial morphology, arrows indicate mitochondrial structure irregular. (C, F) Merged images. Error bars indicate Standard Error Mediation (SEM). Analysis of variance (ANOVA/Dunnett: * P <0.05, ** P <0.01, *** P <0.001).	116
Figure (4-42) Fluorescent microscopy. (A) DCFH-DA stained cell, green fluorescence indicates the amount of reactive oxygen species ROS, (White) arrow indicate normal breast tissue; (Black) arrow indicate breast cancer tissue. (B) Mito-tracker stained cell, arrow indicate the mitochondrial morphology, arrows show (yellow) normal and (white) affected tissues. Error bars indicate SEM. Analysis of variance (ANOVA/Dunnett: * P <0.05, ** P <0.01, *** P <0.001).	117
Figure(4-43). Fluorescent microscopy.(A) Mito-Tracker stained mitochondria, red fluorescence indicates defect of mitochondrial morphology**,** arrows indicate mitochondrial structure irregular. **(B)** DCFH-DA stained cell, green fluorescence indicate	118

the amount of reactive oxygen species ROS, arrows indicate high ROS. **(C)** Mito-Sox stained cell, red fluorescence indicate the amount of mitochondrial reactive oxygen species, arrows indicate high mitochondrial ROS. Error bars indicate SEM. Analysis of variance (ANOVA/Dunnett: * *P* <0.05, ** *P* <0.01, *** *P* <0.001).	
Figure(4-44). Fluorescent microscopy. **(A)** DCFH-DA stained cell, green fluorescence indicates the amount of reactive oxygen species (ROS), (White) arrows indicate normal breast tissue. **(B)** Mito-Sox stained cell, (White) arrows indicate the normal mitochondrial ROS. Error bars indicate SEM. Analysis of variance (ANOVA/Dunnett: * P <0.05, ** P <0.01, *** P <0.001).	119

List of diagrams

Diagram (2-1) Schematic illustration of causes of mutations	11
Diagram (2-2) Schematic illustration of SNPs regions and types of SNPs	16
Diagram (2-3) Schematic illustration of some types of restriction enzymes and target sequence in PCR-RFLP	18
Diagram (2-4) Schematic illustration of tools used in Bioinformatics	22

List of Abbreviations

Abbreviations	Meaning
A	adenine
AA	Amino acid
ABC	Adenosine triphosphate Binding
ABC	ATP - binding cassette
ABCB1	Adenosine triphosphate Binding Cassette superfamily B1
ABCG2	Adenosine triphosphate Binding Cassette superfamily G
ADP	Adenosine diphosphate
AP	Arbitrary primed PCR
ATP	Adenosine triphosphate
B.C.	Before christ
BC	Breast cancer
BCRP	Breast cancer resistance protein
Blast	Basic Local Aligment search tool
bp	base pair
BRCA1	Breast cancer 1
BSA	Bovine serum albumin
C	Cytosine
C SNPs	Synonymous SNP
°C	Degree Celsius
CI	Confidence interval
CCP	Contraceptives
d.f	Degree of freedom
DAPI	Di amidino phenyl indole
DCFH-DA	Dyechloro methyl dichlorofluorescein diacetate
ddNTPs	Dideoxynucleotide phosphate (ddNTPs)
DDR	DNA damage response
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Documentation
DFS	Disease free survival
EcoRI	Escerichia coli RY 13
EDTA	Ethylene diamine tetra acetic acid
ER	Estrogen receptor
ERs	Estrogen receptors
ER-α	Estrogen receptor alpha

eSNP	Expression single nucleotide polymorphisms
ESR1	Estrogen receptors 1(α)
Et al.	et alli (and others)
ETC	Electron transport chain
FS	Frameshift
G	Guanine
GE	Gene expression
gm	gram
H W E	Hardy Weinberg Equilibrium
Het Exc	Heterozygote excess
HRT	Hormone replacement therapy
i.e	Id est
Indel	Insertion / Deletion
LnCRNA	Long non coding RNAs
MboI	Moraxella bovis
MDR1	Multi drug resistance
MHT	Menopausal hormone therapy
ml	militer
mRNA	Messanger RNA
mt ROS	Mitochondrial ROS
NBD	Nucleotide binding domain
NCBI	National Center for Biotechnology Information
NFS	Non-frameshift
ng	Nanogram
NHR	Nuclear Hormone Receptor superfamily
No	Number
NS	No significance
OR	Odd ratio
OS	Overall survival
P	Probare(P-value)
PB	Polymorphic band
PBS	Phosphate Buffer saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
P-gp	Permeability glycoprotein
PH	Power of hydrogen force
Phyre 2	Protein Homology analogy Recognition engine V.2.0
PRP	PCR-RAPD-PCR
Psi	Per square inch
RAPD	Random Amplified Polymorphic DNA

RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
Sec	Second
SNP	Single nucleotide polymorphisms
T	Thymine
TBE	Tris Borate EDTA
TME	Tumor microenvironment
TNB	Triple negative breast cancer
MBC	Metastatic breast cancer
TSGs	Tumor suppressor genes
U	Uracil
U	Unit
UTR	Un-translated region
UV	Ultraviolet
X^2	Chi Square
µg	Microgram
µl	Microliter
%	Percent
+	Positive
>	Greater than
<	Less than
~	Approximately
3 D	Three Dimension

Chapter one

Introduction

1. Introduction

Breast cancer is the most common type of cancer in women and the second leading cause of death from cancer in some countries (Testa *et al*, 2020). About 5% to10 % of BC is inherited, whereas 90% to 95% is sporadic, this revealed randomly not predetermined genetically (Antoniou *et al*., 2003; Al Hannan *et al*., 2019; Sabra *et al*., 2020).

At the molecular level, BC is a heterogeneous disease, characterized by high genetic instability evidenced by somatic genetic mutations, copy number alterations, chromosome structural rearrangements, defects in DNA damage repair, transcription, and DNA replication (Testa *et al*., 2020). The process of developing this cancer involve multiple cellular activities and signaling pathways (Kim *et al*., 2018; Karaayvaz *et al*., 2018; Pei *et al*., 2020).

Genetic counseling has become an important tool for the health care system which provide information and support to families at risk of developing a genetic disorder (Silva, 2019).

The genetic polymorphism or variation in DNA can affect susceptibility to diseases, and future response to the anticancer agents (Sukhumsirichart, 2018). The tumor cells or tissues contain the nucleic acids, besides, many proteins, thus the central dogma of molecular biology show that these proteins are associated with nucleic acids. Therefore, proteins are another important can predict the occurrence of cancer. Similarly, protein as an important biomarker make a great contribution to the diagnosis of BC (He *et al*., 2020).

The genetic variations are important predictors for breast cancer risk (Wen *et al*., 2016), contribute to the development of chemotherapy (Daniyal *et al*., 2021), and play role in influencing protein expression (Šarmanová *et al*., 2004). Therefore, the prediction of disease for individuals through the use of genetic

information and genetic profiling tests can play role in cancer management (George *et al*., 2016; Tokunaga *et al*., 2021).

The malignant transformation of cells involves multiple genes that act with lifestyle, and several environmental factors (Sas-Korczyriska *et al*., 2017; Mampunye, 2020). The BC development of breast is subject to several genetic factors such as cumulative mutations of different genes (Xie, 2020), including *ABCG2*, *ABCB1*, *BRCA1*, *ER-α, and miRNA-152*. The overexpression of ABC transporters affect resistance to chemotherapy (Manandhar *et al*., 2017; Nayak *et al*., 2020). The *BRCA1* is cause inherited mutations and a high risk of BC (Huo *et al*., 2021), and associated with many cellular processes including genomic stability, cell cycle checkpoint, DNA damage repair, and apoptosis (Meshram, 2019). *ER-α* receptor superfamily play a major function in cancer development and progression, it is stimulate proliferation and differentiation of mammary epithelial tissue through combining with estrogen (Kalinina *et al*., 2021). Therefore, *ER-α* is involve in breast carcinogenesis by increased gene expression (Shah *et al*., 2014; Kalinina *et al*., 2021). The *Mi-RNA 152* gene has the main function in regulating biological processes such as differentiation, apoptosis, and *miR-152* may be involve in chemoresistance (Safi *et al*., 2021).

According to the previously mentioned information and due to lack of studies in Iraq about the genetic polymorphism of genes that related to BC, the current study aimed to shed light on the axes the following:

1- Identification of a number of genes (*ABCG2*, *ABCB1*, *BRCA1*, *ER-α,* and *miRNA-152*) which are directly or inversely relate to the growth and development of BC.

2- Determination of genetic polymorphisms of the identified genes.

3- Analysis of genetic variation and 3D protein of the selected genes using bioinformatics programs which is depending on gene sequencing .

4- Determination of cytogenetic alterations as chromosome condensed, the shape of mitochondria, and produced ROS in the tissues of BC using fluorescent microscopy.

5- Determination relationship between genetic mutations and cytogenetic alterations in BC patients.

Chapter two

Literature Review

2- Literature Review

2-1: Cancer

The term "cancer" is currently used for a collection of diseases characterized by abnormal and uncontrolled cellular growth caused primarily by genetic mutations (Stratton, 2011; Martínez-Jiménez *et al*., 2020). The genetic changes (mutations) in genes that control cell growth and division can be hereditary (germline changes) or caused by exposure to environmental toxins during an individual's lifetime (Somatic changes) (Hanahan and Weinberg, 2011; Nyqvist *et al*., 2020).

Generally, cancer cells are developed from normal cells due to damage of DNA, most of the time whenever DNA is damaged, the body is able to repair it, unfortunately, in cancer cells damaged DNA is not repaired, these damaged cells ignore the normal signals for a cell to stop dividing and avoid programmed cell death (apoptosis). Humans can also inherit damaged DNA from parents, which accounts for inherited cancers (Sudhakar, 2009; Chandira *et al*., 2019). Detection and repair of DNA damage by repair mechanisms play an important role in preventing carcinogenesis, maintaining genome integrity and protecting against mutations (Shadrina *et al*., 2016).

Cancer progression can be summarized across four different steps as follows: cancer initiation, tumor propagation, metastasis to distant organs, and resistance to chemotherapy (Lytle *et al*., 2018; Colaprico *et al*., 2020).

The four main genetic drivers of cancer include tumor suppressor genes (inhibit cell growth and division), proto-oncogenes (stimulate cell growth and division), DNA repair genes (fix damaged DNA) (Hanahan and Weinberg, 2011; Nyqvist *et al*., 2020) and genes for apoptosis (Kiraz *et al*., 2016). Cancer progression is accelerated by the accumulation of genomic abnormalities to two different categories of cancer driver genes, oncogenes or tumor suppressors (Vogelstein *et al*., 2013; Colaprico *et al*., 2020).

2-2: Breast cancer

Breast cancer (BC) is the most common cancer in women around the world, accounts for 15% of cancer deaths among women, which is the leading cause of mortality in females worldwide (Jin, 2020). It occur in both gender, although male BC is very rare (Rodney, 2003; Chandira *et al*., 2019). It is sporadic in more than 90% of cases and inheritance in a bout 5-10% (Antoniou *et al*., 2003; Al Hannan *et al*., 2019; Sabra *et al*., 2020).

In Iraq, BC is the commonest pattern of female malignancy accounting for one-third of the registered female cancers according to the Iraqi Cancer Registry Board in 2004 (Mohammed, 2011; Ismaeel, 2013).

The mechanisms underlying breast carcinogenesis are not completely understood, but various factors are define for the disease, including induction of DNA damage by endogenous or exogenous agents (Shadrina *et al*., 2016), failure in repairing damage in the chemical structure of DNA play an important role in cancer progression (AlMutairi *et al*., 2015).

2-2-1: Etiology of breast cancer

The initiation of breast BC remain unknown but a woman's probabilities of developing mammary malignancy are associate with a broad range of risk factors (Torre *et al*., 2015). There are many risk factors including:

- Environmental factors play a major role in the development and survivability of certain cancers including BC (Tweed *et al*., 2018; Riba *et al*., 2019).
- Income and education are the major issues in the delineation of mortality of BC (Lundqvist *et al*., 2016; Newman, 2017; Coughlin, 2019).

- Family history of BC in close relatives especially mothers and siblings increase the risk of developing the BC. Approximately 5% are inherited by faulty genes, usually *BRCA1* or *BRCA2* (Adebayo *et al*., 2019).
- The extent and duration of exposure to sex hormones have been consistently identified as risk factor include endogenous sex hormones relate to the menstrual cycle, as well as exogenous hormones derive from contraceptives (CCP), hormone replacement therapy (HRT) and diet (Tan, 2015).
- Alcoholic drinkings associate with the risk of cancers (Dumitrescu and Shields, 2005). At the molecular level, alterations of the cell cycle leading to hyperproliferation, modulation of cellular regeneration or induction of cytochrome P-450 leading to the generation of reactive oxygen species (ROS) that may explain the association between alcohol intake and increased BC risk (Mahmoud, 2016).
- Smoking increases the risk of various types of cancer, chemical carcinogens in tobacco smoke can cause mammary tumors, metabolites of tobacco smoke have been created in the breast fluid or tissues of smokers. Thus, it is biologically plausible that exposure to tobacco smoke (Kori, 2018).
- Breast cancer increases with age, thus half of the women diagnosed annually being aged 61 and older (Adebayo *et al*., 2019).
- Obesity can increase the risk for developing BC due to higher levels of endogenous estrogen in obese women because adipose tissue is an important source of estrogen (Howell *et al*., 2014). BC rates are increase slightly from 2006 to 2015, and this change is attributed to the prevalence of obesity and lower parity among women (Cronin *et al*., 2018).
- Lack of exercise can be associated with an increasing risk of BC, whereas some research show a link between regular exercise at a moderate or intense level for 4 to 7 hours per week and a lower risk of BC (Kori, 2018).

- Certain non-cancer breast problems can be increase the risk of BC, like benign breast conditions, for example fibroadenomas, hyperplasia, adenosis, phyllodes tumors, papillomas, duct ectasia, and granular cell tumors (Kori, 2018).

- Oxidative reaction and reactive oxygen species (ROS) that are naturally present within the cell can develop a hereditary diseases and sporadic cancer, inherently predisposed reaction of DNA damage due to extrinsic and intrinsic factor (Perrone *et al*., 2016).

2-3: Genetics basis of breast cancer

The gene is referred to as the molecular unit of heredity, the organisms inherit their complete set of genes through reproduction. Mutations occurring in gene sequence lead to the different variants of the same gene. Variations of a gene are called alleles. Alleles may produce variations of trait inside a population. Alleles are either dominant or recessive, the dominant allele is common in nature and is called as the wild type. The recessive allele is comparatively rare and is called the mutant type (Panawala, 2017).

Several genes are found related to BC (Sun *et al*., 2017). The susceptible genes are critical risk factors for both hereditary and sporadic BC. The incidence of carcinoma for carriers with mutated related genes might increase in comparison with that of the normal population. Therefore, the development of breast cancer is usually attributed to the accumulative mutations of several genes (Xie, 2020).

2-3-1: Gene expression

Gene expression (GE) is the synthesis of a functional gene product using the information provided by DNA (Perdew *et al*., 2006; Singh *et al*., 2018), Ribonucleic acid (RNA) is synthesized from DNA through the process of transcription, and it is a part of the process of GE (Koleck and Conley, 2016; Singh *et al*., 2018). In addition,

proteins are the final products of gene expression, which determine the phenotypes and biological processes (Narrandes and Xu, 2018). Cancer is a genetic disease where genetic variations cause abnormally functioning genes that appear to alter GE (Narrandes and Xu, 2018).

The protein formation of the GE involve four main steps includings: transcription, RNA splicing, translation and post translational modification (Watson *et al*., 2014), as shown in figure (2-1). Transcription is controlle by transcription factors, epigenetic marks and chromatin topology (Koch, 2015); mRNA processing is control by splicing, poly adenylation and modifications, while transport and degradation are regulate by RNA-binding proteins, and non-coding RNAs (Hentze *et al*., 2018).

The organism responds to changes in its cells, and the differences among populations could be explain by small changes in the regulatory mechanisms associate with alterations in GE (Koleck and Conley, 2016; Singh *et al*., 2018). Therefore, detecting GE levels can be using for cancer diagnosis, prognosis, and management prediction at clinical setting (Narrandes and Xu, 2018).

Many methods have been standardized to identify the variation in GE, including genetic sequencing. GE analyses are beneficial in determining the prognosis and selection of therapy for early-stage BC, and the purpose is to provide maximum benefit with minimum side effects (Güler, 2017).

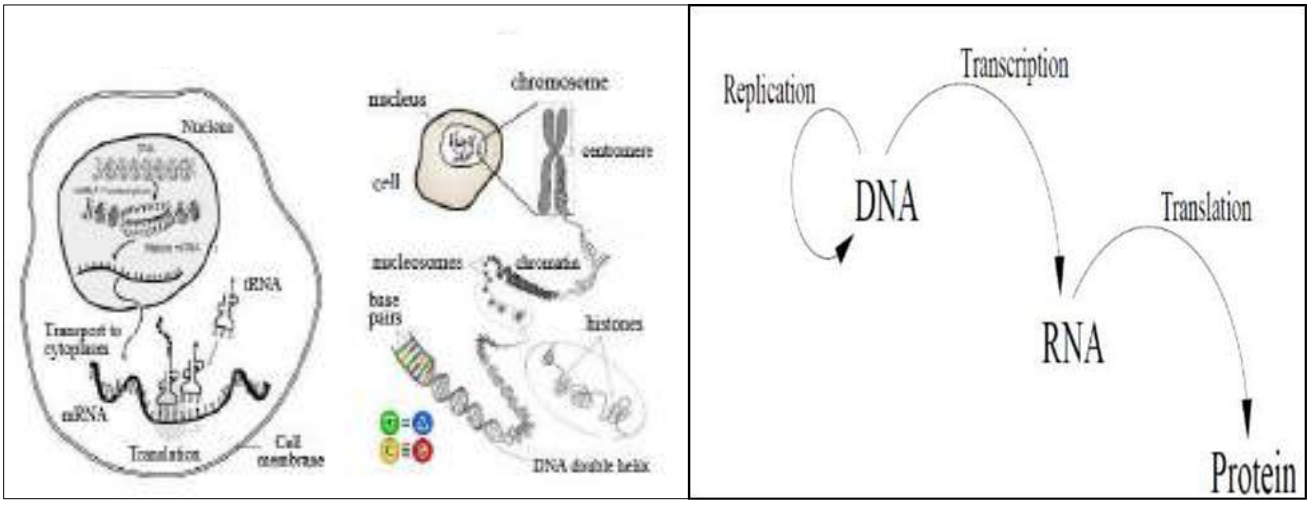


Figure (2-1) Showing general steps of gene expression (Pevsner, 2009; Lahti, 2011)

2-3-2: Genetic mutation

Preserving genomic sequence information in living organisms is important for the perpetuation of life. It is known that DNA, the basic unit of inheritance, is highly susceptible to chemical modifications by endogenous and exogenous agents (Wolters and Schumacher, 2013; Chatterjee and Walker, 2017). DNA mutagenesis occurs spontaneously in nature or as a result of mutagens (agents with a predisposition to alter DNA). However, it can lead to cancers and heritable diseases (Zhang and Vijg, 2018). Mutagenesis occur as a result of endogenous and exogenous causes (Durland and Ahmadian-Moghadam, 2021), as shown in diagram (2-1). There are many molecular genetic techniques such as polymerase chain reaction (PCR) have revolutionized how mutations are obtained and studied (Zhang and Vijg, 2018).

There are various types of mutations, such as silent, missense, nonsense, and frameshift mutations:

1. A Silent mutation is a nucleotide substitution that codes for the same amino acids. Therefore, there is no changes in the amino acid sequence or protein function (Ribeil *et al*., 2017).
2. A missense mutation is when a nucleotide substitution results in an amino acid change. Missense mutations have variable effects but can lead to a decreased or altered protein function (Ribeil *et al*., 2017).
3. A nonsense mutations are when a nucleotide substitution results in a new stop codon, which includes UGA, UAA, and UAG. The protein products are truncated and frequently non-functional (McHugh *et al*., 2018).
4. A frameshift mutation occurs due to the addition or deletion of nucleotides not divisible by 3 bases, resulting in the misreading of the downstream nucleotides. These proteins may be shorter or longer, and protein function may be disrupted or altered (Tabebordbar *et al*., 2016).

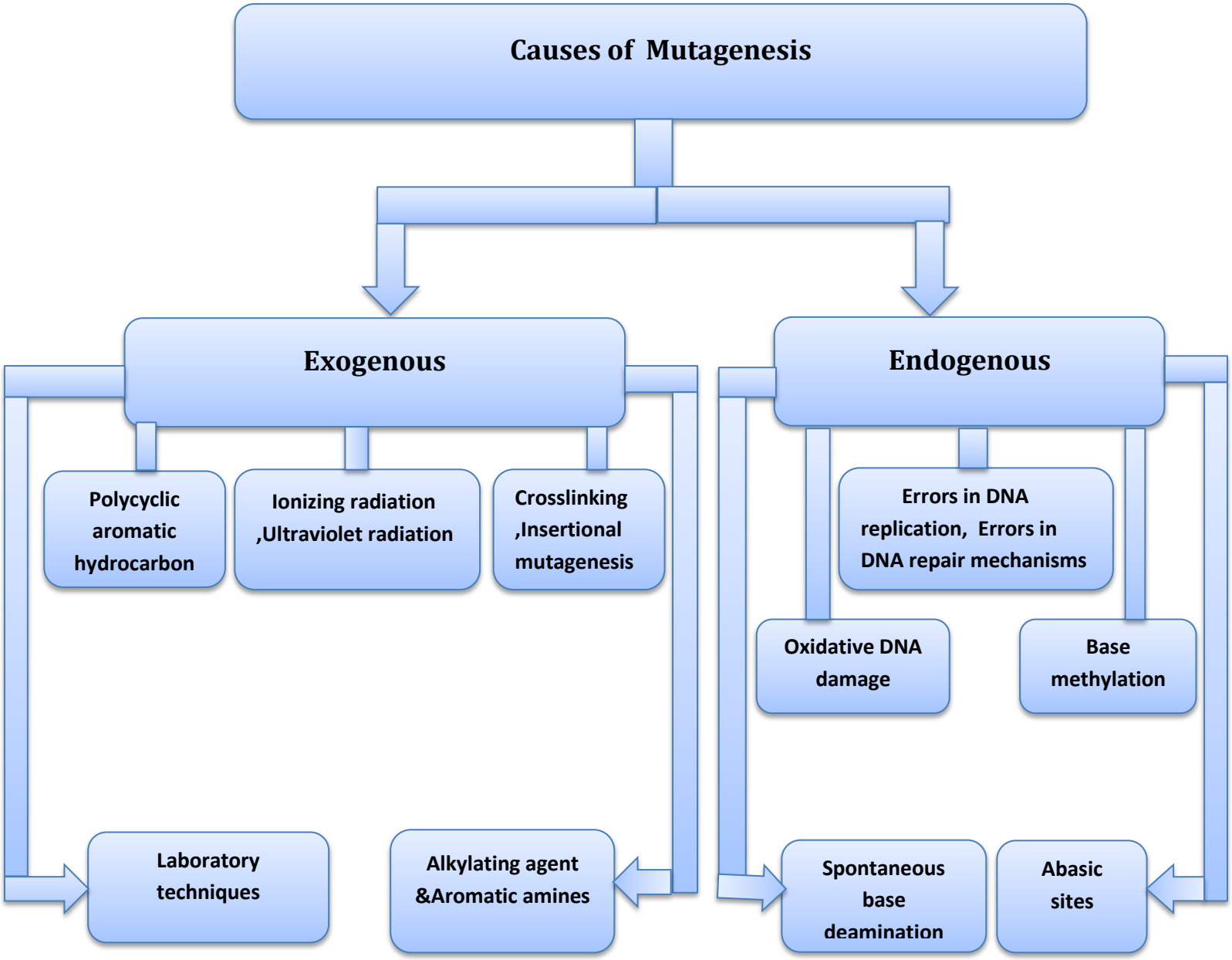


Diagram (2-1) Schematic illustration of causes of mutations (Durland and Ahmadian-Moghadam, 2021)

2-3-3: Genetic polymorphism

DNA polymorphisms are the different DNA sequences among individuals, groups, or populations. Polymorphism at the DNA level includes a wide range of variations from single base pair change, many base pairs, and repeated sequences (Buckingham, 2012; Teama, 2018), insertions, deletions, and recombination (Ismail and Essawi, 2012).

Genetic polymorphism is the existence of at least two variants with respect to gene sequences, chromosome structure, or phenotype (gene sequences and chromosomal variants are seen at the frequency of 1% or higher). The human genome comprises 6 billion nucleotides of DNA packaged into two sets of 23 chromosomes, one set inherited from each parent. The probability of polymorphism DNA in humans is great due to the relatively large size of the human genome (Buckingham, 2012; Teama, 2018). Genetic polymorphisms may be the result of chance processes or may have been induced by external agents such as radiation and other factors (Ismail and Essawi, 2012).

2-3-3-1: The most common polymorphism in humans

There are two types of genetic polymorphisms includes :

2-3-3-1-1: Single nucleotide polymorphisms (SNPs)

Genetic differences are a major source of diseases in human being like cancers, SNPs have a significant impact on the formation and proliferation of cancers such as BC (Ghosh *et al*., 2021).

A single nucleotide polymorphism is a genetic substitution of a base pair at a single position of the genome, as shown in figure (2-2). SNPs are a common phenomenon and influence mRNA expression. They are extensively studied to distinguish genetic

expression and protein synthesis (Ghosh *et al*., 2021). Single nucleotide polymorphisms in DNA repair genes may change the effects of DNA repair and protein function and additionally may affect the development of various cancers including BC (Patrono *et al*., 2014).

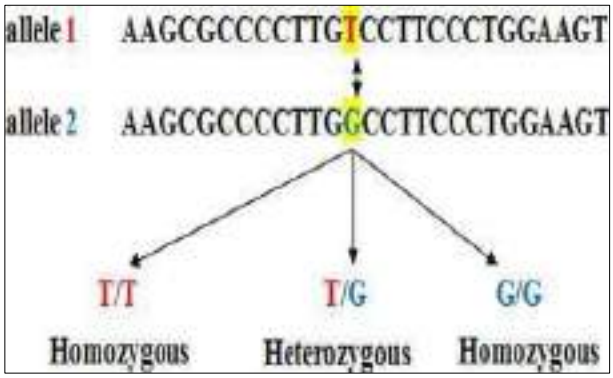


Figure (2-2) Single nucleotide polymorphism for two alleles (AL-Koofee and Mubarak, 2019)

2-3-3-1-1-1 : Effects of SNPs location

SNPs are located in different regions of genes as promoters, exons, introns as well as 5′ and 3′ UTRs. Therefore, alterations in GE and their effect on cancer susceptibility vary depending on the location of the SNPs:

- The SNPs of the promoter region affect GE by altering promoter activity, transcription factor binding, DNA methylation and histone modifications (Schirmer *et al*., 2016; He *et al*., 2016).
- The exonal SNPs are affecting cancer susceptibility by suppressing gene transcription and translation (Fang *et al*., 2014; Deng *et al*., 2017).
- SNPs in intron regions generate splice variants of transcripts and promote or disrupt binding and function of long non-coding RNAs (lncRNAs) (Xiong *et al*., 2015).
- SNPs in the 5′-UTR affect translation, whereas SNPs in the 3′-UTR affect microRNA (miRNA) binding (Xu *et al*., 2014).

- SNPs are in non-coding regions of gene, or in the intergenic regions may affect gene splicing (SNPs at intron region), transcription factor binding (SNPs at 5′ untranslated region), messenger RNA degradation, or the sequence of non-coding RNA (Sukhumsirichart, 2018).
- The type of SNPs located upstream or downstream from the gene that affects gene expression is refer to an expression SNP (eSNP) (Sukhumsirichart, 2018), as shown in figure (2-3), diagram (2-2).

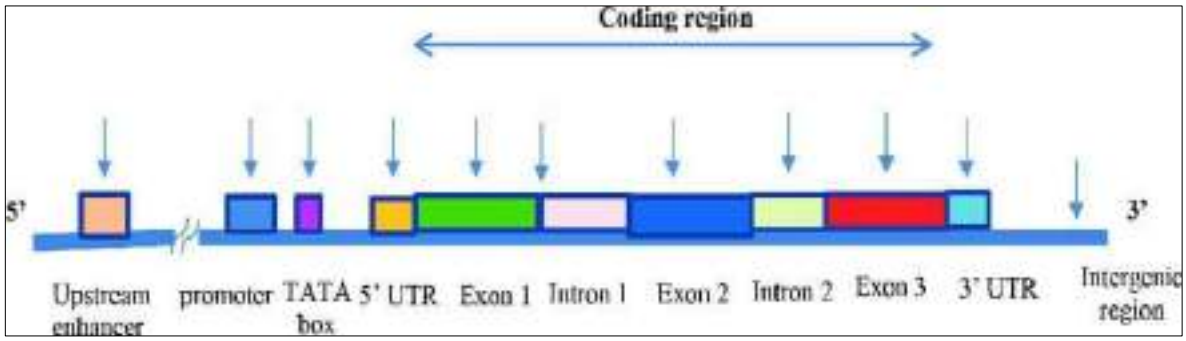


Figure (2-3) Structure of gene and SNP location (Sukhumsirichart, 2018)

2-3-3-1-1-2: Types of SNPs in exons

The SNPs may fall within coding sequence of gene, or non-coding region, or in the intergenic region (regions between genes). The SNPs in the coding region of the gene are divided into two types: synonymous and nonsynonymous SNPs (Sukhumsirichart, 2018), as shown in diagram (2-2).

The effect of SNPs on the translation process is as follows:

1- Synonymous cSNPs do not alter the amino acid sequence of the encoded protein. Recently, several studies showed that synonymous cSNPs affect gene function by changing the expression of neighboring genes, messenger RNA (mRNA) splicing, and protein folding. A synonymous SNP in multidrug transporter (MDR1) gene alter

the expression of the critical drug transport protein (P-glycoprotein), and this affect its expression and function thereby impacting drug resistance (Koehn *et al*., 2008; Deng *et al*., 2017).

2- The nonsynonymous SNPs are divided into two types:

- A missense SNP is single nucleotides change results in a codon that codes for a different amino acid, resulting in protein non-function (Deng *et al*., 2017).
- Nonsense SNP is a point mutation sequence of DNA that changes to a stop codon results in a non functional protein product. Alterations in the amino acid sequences can alter the secondary structure of the proteins by increasing or decreasing hydrogen bonding and phosphorylation, which affects protein interactions and functions. As a consequence, these changes alter cell signaling pathways as well as the level of oncogenic and tumor suppressor proteins (Deng *et al*., 2017).

More than 13,000 known SNPs are in exons of various genes, of which 58% are non-synonymous cSNPs (Tennessen *et al*., 2012; Deng *et al*., 2017). Non-synonymous SNPs influence cancer susceptibility due to changes in the structure and function of the encoded proteins (Deng *et al*., 2017).

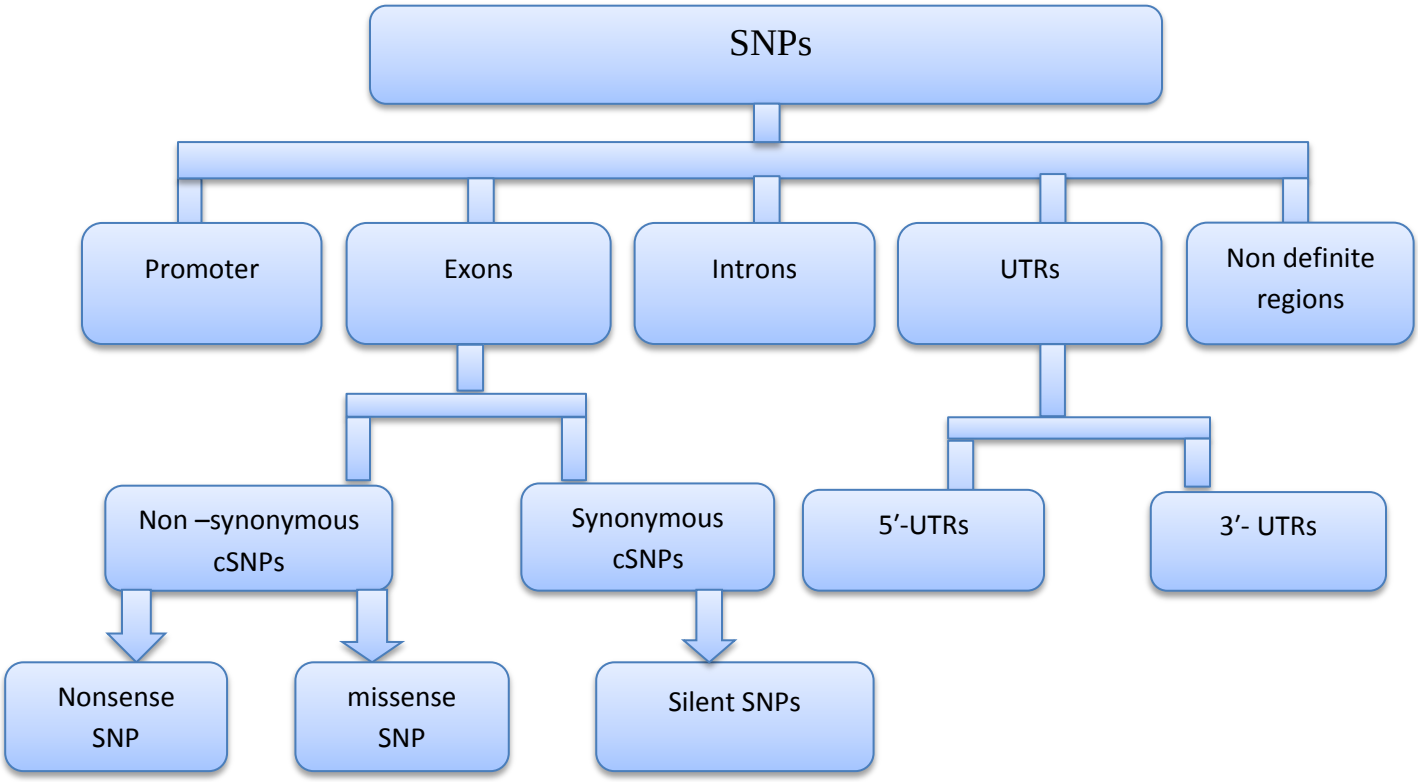


Diagram (2-2) Schematic illustration of SNPs regions and types of SNPs (Sukhumsirichart, 2018), and types of SNPs (Deng *et al*., 2017).

2-3-3-1-2: Insertion /deletion polymorphism

Insertions and deletions (Indels) are addition or deletions of one or more nucleotides in DNA sequence. Indels are highly abundant in human genomes, second only to single nucleotide polymorphisms (SNPs), and make up 15-21% of human polymorphisms (Mullaney *et al*., 2010; Lin *et al*., 2017). Many studies have demonstrated the role of indels in the development of a number of cancers including breast (Ye *et al*., 2016; Lin *et al*., 2017).

2-3-4: Techniques for study and determining genetic polymorphism:

2-3-4-1: Restriction fragment length polymorphism (RFLP)

RFLP is discovered in 1984 by the English scientist "Alec Jeffreys" while working on area of hereditary diseases. RFLP is a molecular biology tool that reveals the difference between samples of homologous DNA molecules from differing locations of restriction enzymes sites (Chaudhary and Maurya, 2019). PCR-RFLP is perhaps the simplest method for detection of known mutations in cancer related genes and for genotyping a wide range of other human diseases. However, its application can be detect mutant alleles that are present in more than 5-10% of wild-type alleles (Liu *et al* ., 2003).

In this technique, the DNA sample is digested by many specific restriction enzymes which cut at specific sites on DNA, as shown in diagram (2-3). In RFLP, the length of restriction fragments of a certain type of restriction enzyme differs between individual organisms and species which helps to identify and study genetic polymorphisms as well as inter and intra specific variations. It help in analysis of genes involved in genetic or hereditary diseases among family members, detection of the mutated gene, it can be using in the analysis of genetic diversity in populations, and the identification of single nucleotide polymorphisms (SNPs) (Chaudhary and Maurya, 2019).

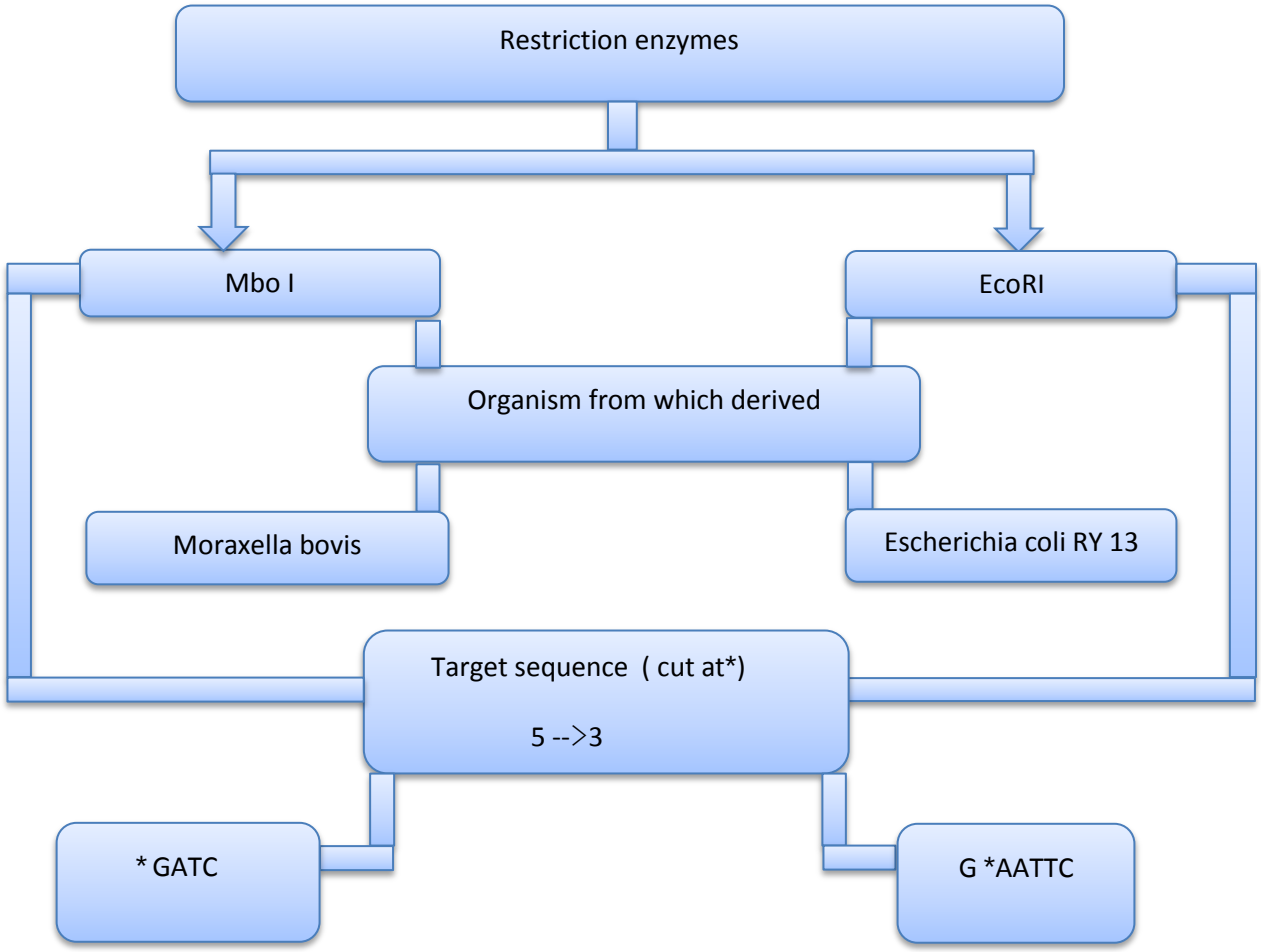


Diagram (2-3) Schematic illustration of some types of restriction enzymes and target sequence in PCR-RFLP: MboI (Pingoud *et al*., 2005); EcoRI (Senthilkumar *et al*., 2016).

2-3-4-2: Random amplified polymorphic DNA (RAPD)

RAPD is a PCR based technique for identifying genetic variation. The randomly amplified polymorphic DNA (RAPD) was developed independently by Williams (Williams *et al*., 1990), and by Welsh and McClelland (Welsh and McClelland, 1990). The technique was developed independently by two different laboratories and called RAPD and AP-PCR (Arbitrary primed PCR), respectively. It is a commonly used molecular marker in genetic diversity studies and determining genetic variation

(Rahiman *et al*., 2015). RAPD-PCR analysis has been use as a means for identifying and investigating genetic instabilities associate with human tumors development and revealed frequent occurrence of genetic polymorphisms in various types of tumors such as BC (Papadopoulos *et al*., 2002; Ismaeel, 2013).

RAPD-PCR involve the use of a single arbitrary primer in a PCR reaction, resulting in the amplification of many discrete DNA products. The standard RAPD technique uses short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by polymerase chain reaction (PCR) (Rahiman *et al*., 2015).

2-3-4-3: DNA sequencing

DNA sequencing is the process of determining the number and order of nucleotides within a DNA molecule (Fernandez-Cadenas *et al*., 2003; Bamanga *et al*., 2018), as shown in figure (2-4).

There are two methods for detection of DNA sequencing:

- Maxam Gilbert sequencing is a chemical method that chemically modifies the DNA nucleotides and subsequently cleave the DNA backbone at the sites neighboring to the modified nucleotides. As a results to technical complexity and the use of hazardous chemicals, and this method is not currently use for standard molecular biology (Haldar, 2019).
- Sanger sequencing is the method of DNA sequencing in which dideoxynucleotide phosphates (ddNTPs) are incorporated by DNA polymerase during in vitro DNA replication. Modified ddNTPs terminate DNA strand elongation since they lack a 3′-OH group require for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to stop the extension of DNA. This technique

is known as dye terminator sequencing. Each of the four ddNTPs (where N = A/T/G/C) is label with fluorescent dyes that emit light at different wavelengths. Therefore, can be capture in the form of colored peaks called a chromatogram (Haldar, 2019).

The importance of DNA sequencing can be see in the determination of the causes of diseases. For example, some human diseases can be caused by a silent mutation at a splice site which implies that the resulting amino acid translated and coded for the same but the spliceosome does not recognise the splice site because the nucleotide has changed on the DNA (Fernandez-Cadenas *et al*., 2003; Bamanga *et al*., 2018). A point mutation can also be detect by using DNA sequencing methods to determine the genetic implications of other diseases (Bao *et al*., 1996; Mahdieh and Rabbani, 2013). The missense and nonsense mutation was identified using the DNA sequencing methods (Sparrow *et al*., 2003). Finally, genomic sequencing data help to predict the chance of genetic risk in human diseases and helps to take preventive strategies, and assess to decide on which therapeutic decisions should take in the management of diseases (Lokuge and Ganegoda, 2018).

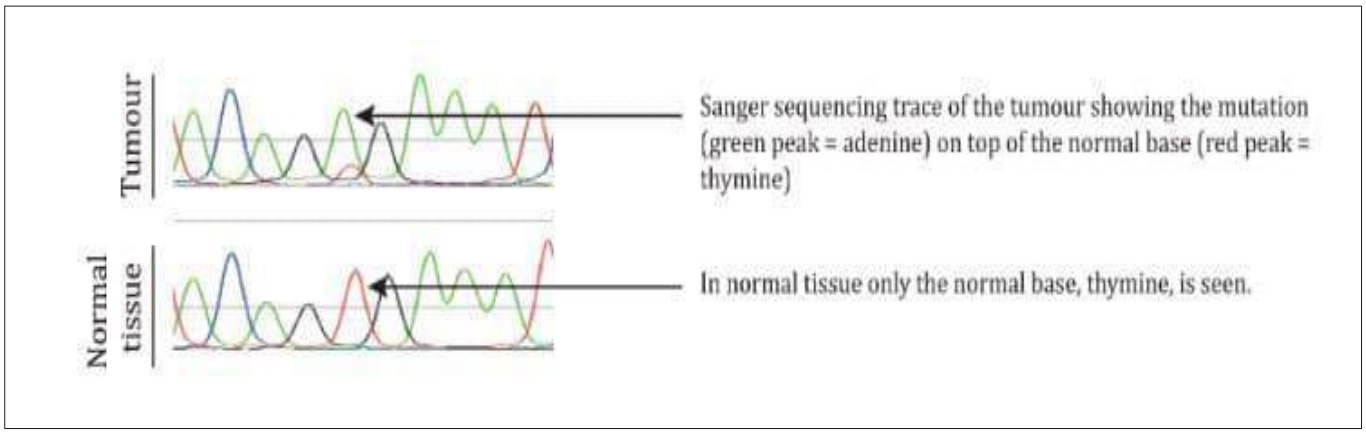


Figure (2-4) Showing an example for mutation was found in human cancer using Sanger sequencing (Behjati and Tarpey, 2013).

2-3-4-3-1: Bioinformatics methods

Bioinformatics is the use of computers, mathematics, and statistics in molecular biology to analyze big quantities of biological data quickly, freely and accurately (Dorn *et al*., 2014). Bioinformatics refer to the field which deal with the collection and storage of biological information. All matter which is concerned with biological databases is termed as bioinformatics (Rana and Vaisla, 2012; Thapar, 2018). Bioinformatics as a science has developed to meet computational challenges related to the analyses of large amounts of data generated by DNA sequencing technique (Harris *et al*., 2016), as shown in figure (2-5). Sequence analysis is referred to understanding various constituents of a biomolecule such as DNA, RNA, and proteins which give them their characteristic function (Mehmood *et al*., 2014).

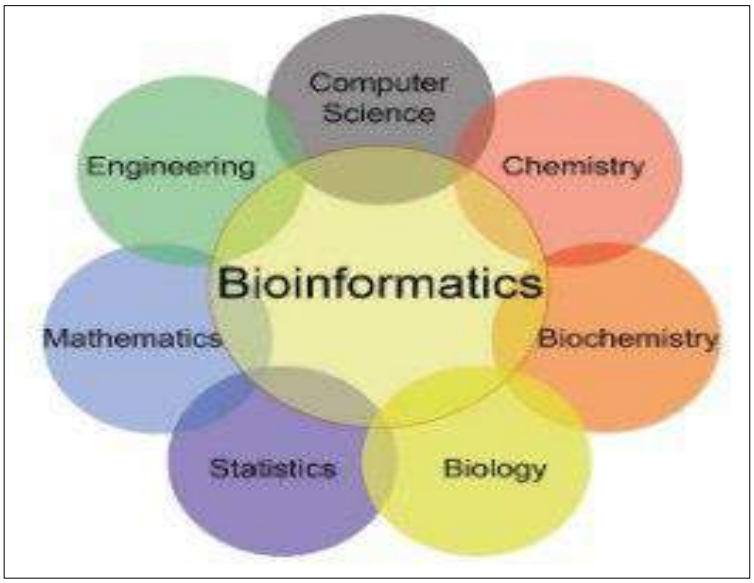


Figure (2-5) Bioinformatics is the application of techniques from computer science to the problems from Biology (Lopresti, 2008).

Depending upon the feature to be used, various tools have been develop. The tools and and databases of bioinformatics that are the backbone of genetic and proteomic research

can be classify (Thapar, 2018), as shown in diagram (2-4).

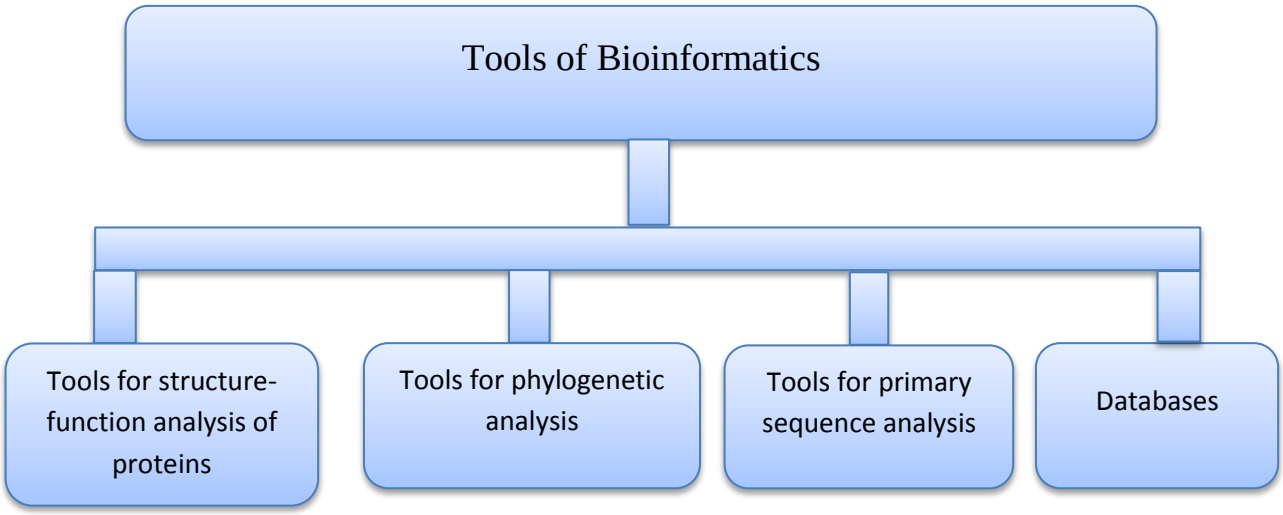


Diagram (2-4) Schematic illustration of tools used in Bioinformatics (Thapar, 2018)

2-3-4-3-1-1: Genetic prediction

Gene prediction defines the process of identifying regions of genomic DNA that contain genes. These genes may be of different variety such as protein coding genes, RNA genes and may also contain genes performing other functions such as regulatory genes (Mathé *et al*., 2002). It s a complex process involve mathematical modeling of sites instrumental in classifying gene in a DNA sequence. Both the contents i.e. site essential to identifying genes and mathematical model to represent those sites accurately are important in gene prediction (Shah *et al*., 2013).

Predicting genes from DNA sequence is a challenging task in genomics, predicting genes provide many insightful hints regarding genes as deducing the protein products code, tracing hereditary, and detecting genetic malfunctioning (Shah *et al*., 2013). DNA in a gene control the sequence of nucleotide in an RNA molecule that controls the structure of protein (Pevsner, 2009).

2-3-4-3-1-2: Three-dimensional protein (3 D)

The protein are present in all living systems, performing a variety of fundamental functions. The nature of a function performed by a certain protein is strictly related to its adopted conformation or folding. The folding of an amino acid sequence is further restricted by several types of non covalent bonds originated by interactions between different parts of the amino acid chain, these forces involve atoms in the polypeptide backbone as well as atoms located in the amino acid side chains (Consortium *et al*., 2015; De Lima Corrêa *et al*., 2018).

The prediction of the three-dimensional (3D) structure of proteins or polypeptides is one of the most important and challenging problems in structural bioinformatics (Dorn *et al*., 2014).

Each protein is identified by a unique sequence of chained amino acids that under some physiological conditions fold into a particular three-dimensional shape (3 D) (Anfinsen, 1973; LaPelusa and Kaushik, 2020).

The bioinformatics analysis of BRCA1 protein variants can provide information essential for cancer diagnosis or therapy (Lafta, 2020), as shown in figure (2-6)

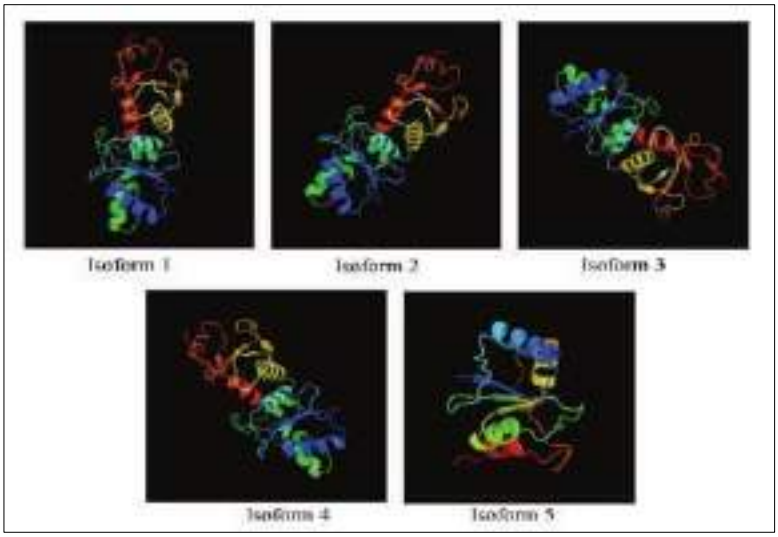


Figure (2-6) Three-dimensional structures of the *BRCA1* (Lafta, 2020).

2-3-5: Genes that related to cancer

Cancer is a medical condition which has a molecular basis (Kontomanolis *et al*., 2020). The susceptible genes are critical risk factors for both hereditary and sporadic cancers (Xie, 2020).

2-3-5-1: Oncogenes in breast cancer

Mammalian genomes contain a range of proto-oncogenes which control normal cell differentiation and proliferation. Proto-oncogenes are the first regulatory factor of this biological process. They act in transmitting signals, resulting as growth factors. Modifications of these genes called oncogenes that lead to the appearance of cancer cells. The activation process leading to proto oncogenes are chromosomal translocation, and point mutations (Kontomanolis *et al*., 2020).

The physical mutations that lead to the activation of the proto-oncogenes is lead to differences in the structure of the encoded proteins and deregulation of protein expression (Abel and DiGiovanni, 2011; Jan, 2019).

2-3-5-1-1: Oncotype DX

The Oncotype DX is a quantitative assay of the expression of 16 tumor-related genes and 5 reference genes that predicts the potential of adjuvant chemotherapy benefit in estrogen receptor (ER)-positive early breast cancer patients (Fayaz *et al*., 2020). The Oncotype DX RS was validated both as a prognostic and a predictive tool, given its capacity to identify patients with node- negative, hormone receptor-positive and HER2 negative breast cancer whose prognosis reduced the likelihood of a benefit gained in potential chemotherapy, 21-gene Oncotype DX assay significantly impacted the clinical decision for treating early breast cancer by changing the reclassification of originally intermediate-risk patients to low-risk. Moreover, the approach contributes to avoiding

unnecessary chemotherapy and identifying patients with high risk; especially those in need for HER2 monoclonal antibodies (Al-Naqqash, 2020).

2-3-5-2: Tumor suppressor gene in breast cancer

In the normal cell, in addition to oncogene there are tumor suppressor genes which play a major role in the normal growth and differentiation of the cell and block the development of cancer. Tumor suppressor genes form a huge group that exhibit one common characteristic: somehow, every single one of these genes protects the organism from neoplasia. Both copies of a tumor suppressor gene must be in an inactive state before a cancer cell can proliferate or survive further. Lack or inactivation due to mutations of tumor suppressor genes lead to developing cancer (Nenclares and Harrington, 2019).

2-3-6: Cancer driver genes

Generally, 5-10 % of BC are inherited (ALHannan *et al*., 2019); while 90-95 % is sporadic (Antoniou *et al*., 2003; Sabra *et al*., 2020). The susceptible genes are critical risk factors for both hereditary and sporadic BC (Xie, 2020). Many genes are found related to BC (Sun *et al*., 2017).

2-3-6-1: Adenosine triphosphate Binding Cassette superfamily B1 (*ABCB1)* gene

ABCB1 was initially cloned by Riordan in 1985 (Riordan *et al*., 1985). It is also known as multidrug resistance gene (*MDR1*). Its located on chromosome 7q21.12 and comprises a core promoter region and 28 exons (Sheng *et al*., 2012; MUTLU İÇDUYGU *et al*., 2020), and 28 introns (Tulsyan *et al*., 2016), as shown in figure (2-7). *ABCB1* encoding a 170 kilo Dalton transmembrane glycoprotein called permeability glycoprotein (P-gp) (Abuhaliema *et al*., 2016; MUTLU İÇDUYGU *et al*., 2020).

On the other hand, the physiologic role of P-gp an ATP-driven transporter mediates to transport many metabolites and harmful substances from inside to outside through plasma membrane, this make the intracellular levels of toxic compounds are keep low (Abuhaliema *et al*., 2016; MUTLU İÇDUYGU *et al*., 2020). The permeability glycoprotein of *ABCB1* is responsible for the energy ATP-dependent efflux of drugs (Sheng *et al*., 2012; MUTLU İÇDUYGU *et al*., 2020).

The *ABCB1* gene is highly polymorphic, till date ˜ 66 coding single nucleotide polymorphisms, out of these 22 are synonymous and 44 non-synonymous (Wolf *et al*., 2011). Single nucleotide polymorphisms of MDR1 gene can be affect expression and function of P-gp (Stańko *et al*., 2016; Yin *et al*., 2016; Mijac *et al*., 2018).

The changes in P-gp transport function may trigger mutagenesis through accumulation of harmful substances and increase the risk of various diseases including BC (Stańko *et al*., 2016; Yin *et al*., 2016; Mijac *et al*., 2018).

The occurrence of multidrug resistance is one of the main obstacles to the successful chemotherapeutic treatment of cancer, the development of MDR in cancer is mediated by an increasing expression of the ATP-binding cassette (ABC) transporters, the transporters utilize the energy of ATP to actively pump a wide variety of structurally and mechanistically unrelated drugs out of cancer cells and consequently lower their intracellular accumulation (Fojo *et al*., 1987; Li *et al*., 2020). Therefore, an increasing in the expression of ABC transporters is often associate with poor prognosis (Tang *et al*., 2011; Li *et al*., 2020).

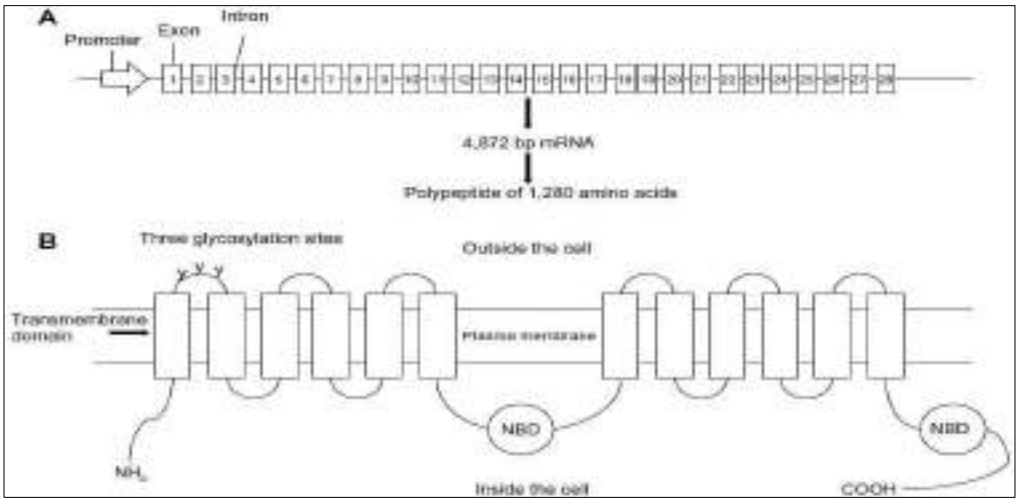


Figure (2-7) Molecular structure of *ABCB1* gene. **A**. Containing 28 exons and 28 introns; encoding P-gp of 1280 amino acids. **B**. Secondary structure of P-gp protein: This has a single polypeptide chain with both the N and C termini located inside the cytoplasmic region while the 12 transmembrane domains are located inside the plasma membrane. It also consists of two nucleotide-binding domains (NBD), which act as ATP binding sites. The first extracellular loop contains three glycosylation sites (Tulsyan *et al*., 2016).

2-3-6-2: Adenosine triphosphate Binding Cassette superfamily G (*ABCG2)* gene

ABCG2 was first discovered in 1998 in BC (Ni *et al*., 2010). The *ABCG2* gene is locate on human chromosome at the locus 4q22.1 and consist of approximately 141 kb of genomic sequence with 16 exons and 15 introns (Ni *et al*., 2010; Haider *et al*., 2011; Chen *et al*., 2019). *ABCG2* is encode a 655 amino acid proteins (Wong *et al*., 2016).

The genetic variants of the ATP-binding cassette, superfamily G, member 2 (*ABCG2*) is known to be involving in the developing cancer risk and inter individual differences in chemotherapeutic response (Wu *et al*., 2015).

Breast cancer resistance protein (BCRP) belonging to the superfamily G of the adenosine triphosphate binding cassette (ABC) transporters, and plays a critical role in protecting cells against xenobiotics and toxic compounds including (pro) carcinogens. BCRP is expressed in many tissues (Wu *et al*., 2015). The mutation of drug targets, overexpression of ABC transporters can enhance drug inactivation mechanism, subsequently contribute to drug resistance (Manandhar *et al*., 2017; Nayak *et al*., 2020). The Single nucleotide polymorphisms (SNPs) of *ABCG2* can change the gene expression and/or reduce their products activity which may affect an individual's susceptibility to xenobiotics and the development of carcinoma (Niebudek *et al*., 2019).

2-3-6-3: Breast cancer 1 (*BRCA1)* gene

In 1994, Miki and others were identified and cloned *BRCA1*. The genetic basis underlying common for some hereditary breast and ovarian cancer was discovered in early 1994 with the identification of the BC early onset genes (Miki *et al*., 1994; Paual and Paual, 2014). It's the major cause of familial BC, 5-10% of BC is inherited (ALHannan *et al*., 2019).

BRCA1 is the most common genes in autosomal dominant and high penetrance form of breast and ovarian cancer (Ayub *et al*., 2014; Mehrgou and Akouchekian, 2016). Its situated on chromosome 17q21 (Rebbeck *et al*., 2016; Ndiaye *et al*., 2020). BRCA1 is structurally sized 100 Kb (Ewald *et al*., 2011). Its consists of 22 exons (60% of the gene corresponding to exon 11), its encode a protein of 1,863 amino acids (Sallum *et al*., 2018). Its work at different stages in the DNA damage response (DDR), checkpoint activation, DNA repair, and apoptosis (Roy *et al*., 2012; Meshram, 2019).

BRCA1 gene is produce tumor suppressor proteins, called as TSGs gene. The changes or mutations in *BRCA1* gene can lead to an increased risk of developing breast, ovarian, and prostate cancer (Ayub *et al*., 2014; Mehrgou and Akouchekian, 2016).

The germline mutations of *BRCA1* is usually point mutation (Ewald *et al*., 2011). Germline mutation of *BRCA1* contribute almost equally in the generated BC (Shah *et al*., 2018). Single nucleotide polymorphism (SNPs) of *BRCA1* is associated with the development and progression of various types of tumor (Prosperi *et al*., 2014; Liu *et al*., 2021). Genetic changes of high penetrance gene *BRCA1* is inherited (Van der Groep *et al*., 2011; Jara *et al*., 2017). Therefore, early detection of the *BRCA1* mutations is important in counseling for targeted family members and in reducing the incidence of BC (Neamatzadeh *et al*., 2015).

2-3-6-4: *mi RNA-152* gene

In 2002, *miRNA-152* was first identified in mouse colon (Lagos-Quintana *et al*., 2002). It is a member of the *miR-148/152* family. In human being, its located at 17q21.32 (Liu *et al*., 2016). It is a class of small, single strand and non-coding RNAs, typically 19-25 nucleotides in length that are known to play a critical role in gene regulation (Di Leva *et al*., 2014; Ge *et al*., 2017).

Specifically, *miRNAs* can regulate gene expression at the post transcriptional level by repressing translation of protein coding gene. The *miRNA-152* is abnormally expressed in a variety of diseases, including various cancers, more evidence suggests that *miR-152* is a tumor suppressor related to cell proliferation, migration, and invasion of cancer (Kindrat *et al*., 2016). *MiRNAs* play an important role in tumor formation; they can act as oncogenes or guardian of the genome when tumor suppressor genes or oncogenes are targeted, respectively (Farazi *et al*., 2013). Single nucleotide polymorphism (SNP) in *microRNA* genes can have potentially wide-ranging influences on BC development (Nguyen-Dien *et al*., 2014). Furthermore, it has been proposed that they can mediate metastasis and chemoresistance (Safi *et al*., 2021). The *microRNA* genesis, shown in figure (2-8).

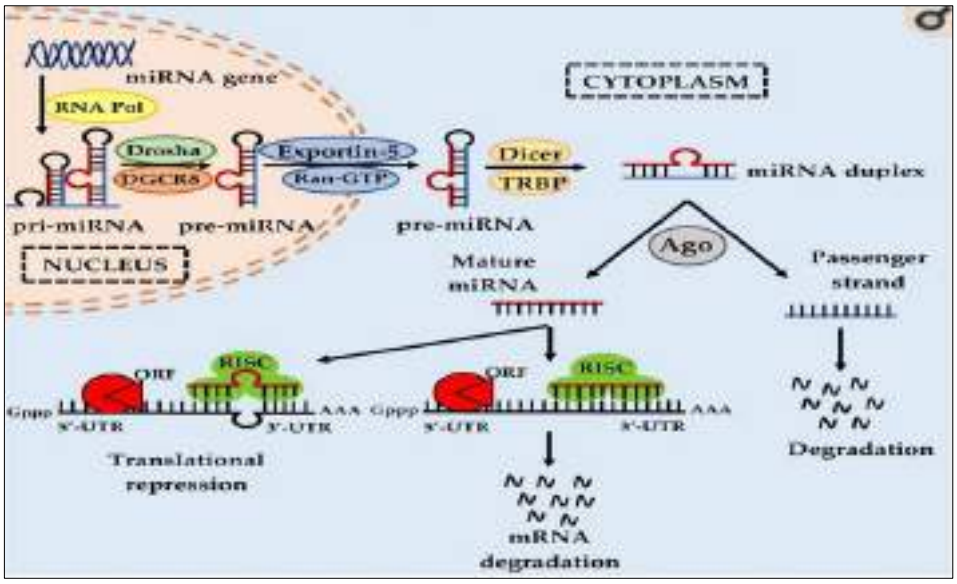


Figure (2-8) Shows microRNA biogenesis and modulation of miRNA activity. *MiRNA* genes are transcribed to produce primary miRNA transcripts (pri-miRNA) by RNA polymerase II (Loh *et al.*, 2019).

2-3-6-5: Estrogen Receptor (*ER-α)* gene

The first report of estrogen receptor was in 1997 when Fuqua and colleagues described non-synonymous mutations in BC (Zhang *et al*.,1997; Alluri *et al*., 2014). Estrogen is a steroid hormone that play critical role in reproductive development, estrogen also promotes breast, ovarian and endometrial tumorigenesis (Hua *et al*., 2018). Abnormal ER signaling lead to development of a variety of diseases cancer (Jia *et al*., 2015).

Estrogen exposure is a widely known risk factor for BC, and the interaction of estrogen with estrogen receptor (ER) plays an important role in BC development (Dai *et al*., 2019).

ER-α gene is located on chromosome 6q25.1, its consists of 8 exons and 7 introns. The human *ER-α* gene (ESR1) is a large genomic segment that spans ~ 300 kb. Its

encode the full-length 66 kDa protein that is composed of 595 amino acids (Koš *et al*., 2001; Sand *et al*., 2002).

ER-α gene encodes to estrogen receptor alpha that is belongs to nuclear receptor super family (NHRs family) (Ayaz *et al*., 2019). Estrogen receptor alpha act as transcription factors that are involved in the regulation of many complex physiological processes in humans (Jia *et al*., 2015). Polymorphism of the *ER1* gene includes a change in the expression of the gene by altering the transcription factor binding sites and affecting alternative splicing of the *ESR1* gene (Deroo and Korach, 2006). Estrogen receptor alpha (ER-α) is play important role in development and progression of cancer, also in addition, it stimulate breast epithelial tissue proliferation and differentiation through combining with estrogen (Abd Ellatif *et al*., 2016; AL-Amri *et al*., 2020).

ER mutations are rarely found in primary ER+ breast cancers but have been observed often in metastatic tumors (Arnesen *et al*., 2021). *ESR1* gene is a low-penetrance BC susceptibility gene, may increase the likelihood of accumulation of genetic mutations occurring throughout cellular division, functions as a ligand and stimulates cell proliferation and controls cell growth and death of by binding to both endogenous and exogenous hormones. SNPs within ESR1 that are associated with predisposition to BC (Deroo and Korach, 2006; Ramalhinho, 2014).

The effect of estrogens on DNA as: initially, estrogen act on target tissues by binding to parts of the cells called estrogen receptors. Estrogen receptor is a protein molecule found inside those cells that are targets for estrogen action (inner lining of the breast). Estrogen receptors contain a specific site to which only estrogens can bind. The target tissues affected by estrogen molecules that contain estrogen receptors (Sikdar *et al*., 2019). Therefore, when estrogen molecules circulate in the bloodstream and move

throughout the body, they exert effects only on cells that contain estrogen receptors (Simpson *et al*., 2005; Contrò *et al*., 2014).

In the absence of estrogen molecules, receptors are inactive and have no influence on DNA. But when an estrogen molecule enters a cell and passes into the nucleus, the estrogen bind to its receptor, causing the shape of the receptor to change. The estrogen receptor complex then bind to specific DNA sites called estrogen response elements which are locate near genes that are control by estrogen. After it has become attach to estrogen response elements in DNA, this estrogen receptor complex bind to coactivator proteins, and nearby genes become active (Fuentes and Silveyra, 2019). The active genes produce messenger RNA (mRNA), which guide the synthesis of specific proteins. These proteins can then influence cell behavior in different ways. Estrogen receptors (ERs) functionally act as transcription factors to initiate target gene expression (Heldring *et al*., 2007), as shown in figure (2-9).

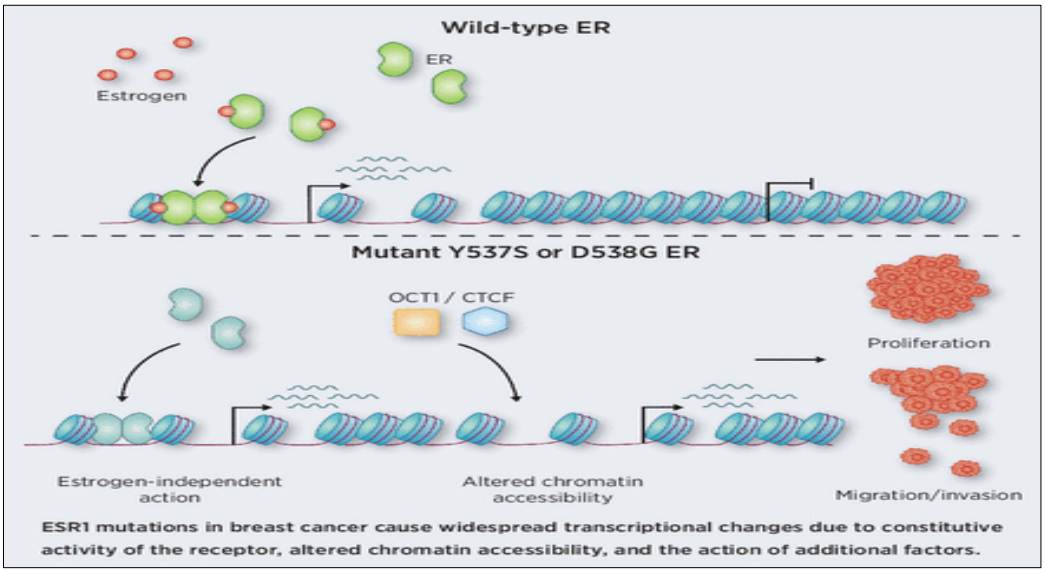


Figure (2-9) ESR1 mutations in breast cancer cause widespread transcriptional changes due to constitutive activity of the receptor altered chromatin accessibility, and the action of additional factors (Arnesen *et al*., 2021).

2-4: Programmed cell death (Apoptosis)

Apoptosis is a form of programmed cell death that is finely regulated at gene level (Fuchs and Steller, 2011; Pistritto *et al*., 2016). Both the intrinsic and extrinsic pathways use caspase to carry out apoptosis through the cleavage of hundreds of proteins. In cancer, the apoptotic pathway is typically inhibited through a wide variety of means including overexpression of anti-apoptotic proteins and under expression of proapoptotic proteins (Pfeffer and Singh, 2018). Apoptosis can be triggered by signals from within the cell, as the binding of ligands to cell surface death receptors. Deregulation in apoptotic cell death machinery is an hallmark of cancer. Apoptosis alteration is responsible not only for tumor development and progression but also for tumor resistance to chemotherapy (Pistritto *et al*., 2016).

Apoptotic cell death inhibits oncogenesis at multiple stages, ranging from transformation to metastasis. Consequently, apoptosis must be inhibit in order for cancer to develop and progress (Lopez and Tait, 2015), as shown in figure (2-10).

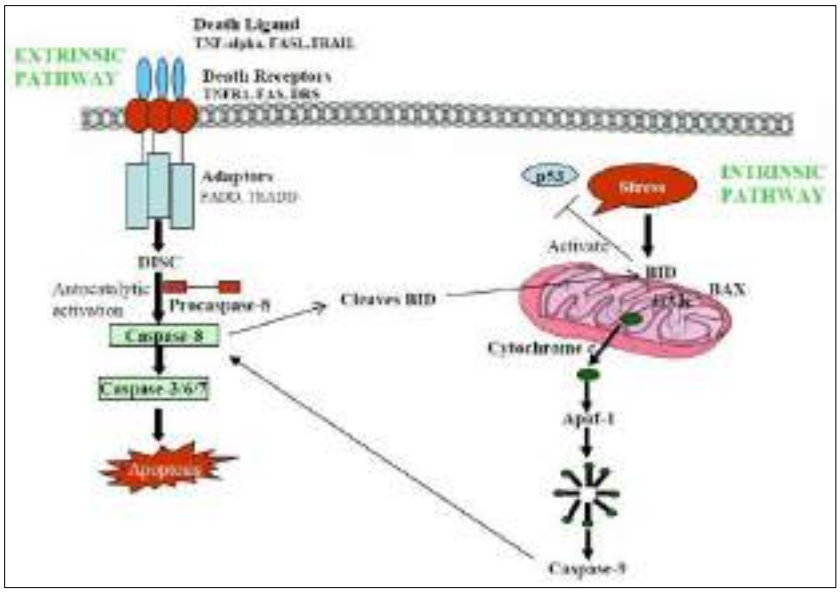


Figure (2-10) Summary of the pathways of Apoptosis (Rampal *et al*., 2012).

2-5: Reactive oxygen species (ROS) in cancer

Reactive oxygen species (ROS) are molecules capable of freelance existence, containing at least one oxygen atom and one or more unpaired electrons, excessive formation of free radicals contributes to causing damage at the molecular and cellular level as well as changes to proteins, carbohydrates, and nucleotides (changes in the DNA structure), these changes contribute to the development of many free radical mediated diseases (Jakubczyk *et al*., 2020).

ROS play an important role in the metastasis, migration, and invasion of cancerous cells (Takeuchi *et al*.,1996; Yang *et al*., 2018).

In eukaryotic cells, ROS produced through aerobic metabolism (Forman *et al*., 2014; Perillo *et al*., 2020). Its considered as byproduct during mitochondrial electron transport in aerobic respiration and have numerous deleterious effects (Chitty *et al*., 2018). Some electrons are directly transferred to generate (ROS) in electron transport chain (ETC) (Zhao *et al*., 2019).

The production of ROS is elevated in tumor cells as a consequence increased metabolic rate, gene mutations and relative hypoxia (Perillo *et al*., 2020). Excessive ROS produced by environmental carcinogens, mitochondrial (ETC) or NADPH oxidases induce DNA damage including de-purination, de-pyrimidination, single-double-stranded DNA breaks, bases modification, DNA and protein changes (Barnes *et al*., 2019), as shown in figure (2-11).

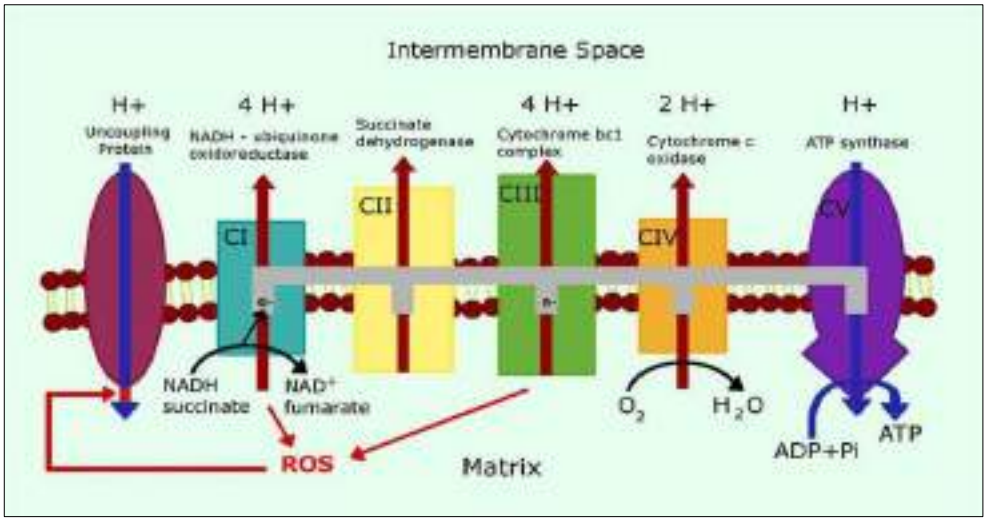


Figure (2-11) The electron transport chain in mitochondria. ROS generated by complex I and III. (ADP, adenosine diphosphate; ATP, adenosine triphosphate; ROS, reactive oxygen species) (Cronshaw *et al.*, 2019).

In pathophysiology of cancer: the high levels of ROS generated by ongoing aerobic glycolysis followed by pyruvate oxidation in mitochondria (Warburg effect) induce genetic instability (Finkel, 2011), as shown in figure (2-12).

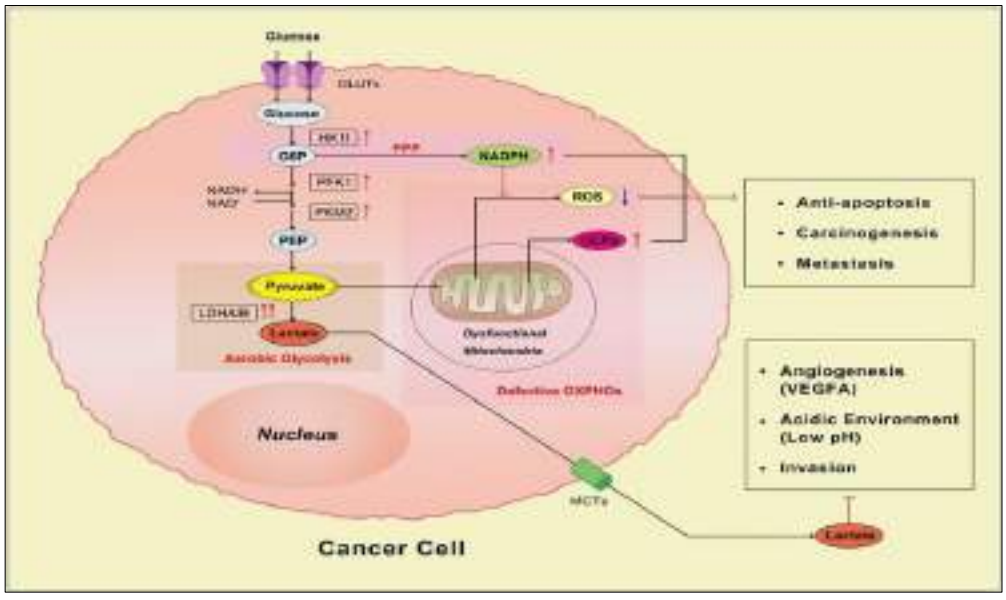


Figure (2-12) Showing the Warburg effect in the cancer cell (Fu *et al.*, 2017)

Chapter Three

Materials and Methods

3- Materials and Methods

3-1: Collection of samples

The present study was carried out in the Department of Biology, College of Science, University of Misan. The study included one hundred-thirty samples were collected from different clinical cases and healthy control. They were distributed between (117) blood and (13) tissue samples, during period from 1st September 2020 to the 30th March 2021 from Maysan Health Directorate/AL-Sadder Teaching Hospital and Al-Shifa Tumor Treatment Center in Al-Amarah city (sub-districts of Maysan province/south of Iraq), and Baghdad laboratory in/Al-Amarah city. The DNA extraction and genes amplification examination were performed on all blood samples.

3-1-1: Blood samples

Eighty seven blood samples, from which 1 to 3 ml were collected from BC females and 30 blood samples, 5ml were collected from healthy women. Blood samples were collected through the median cubital vein or carpal veins in EDTA containing tubes, and the labelled tubes were stored at -20 °C for DNA extraction. The only best 20 PCR products were selected for BC patients and healthy individual were subjected to the complete study by PCR techniques.

3-1-2: Breast tissue sample

Thirteen tissue samples, divided into eight samples of BC as a case group and five sample of healthy women as control group were collected at Baghdad laboratory. Tissue samples were collected into labeled containers (containing 10 % formalin). The tissue samples were transferred to Genetic Engineering laboratory/Department of Biology/College of Science/University of Misan and stored at room temperature away from sunlight until use.

3-2: Chemicals

The chemicals used in the present study are listed in table 3-1

Table (3-1): List of chemicals, and their suppliers.

Chemicals	Company	Country of origin
10X TBE (Tris base -Boric acid -EDTA)	Promega	USA
1X TBE (Tris base -Boric acid- EDTA)	Promega	USA
Absolute Ethanol	Scharlau	Spain
Agarose	Promega	USA
DAPI Stain	SIGMA	USA
DCFH-DAStain	Abcam	CHINE
DEPC Fixation	Invitrogen	USA
Di methyl sulfoxide (DMSO)	Canvax	USA
Distal Water		
DNA Ladder(100-1500 bp), (25-300 bp)	Promega	USA
DNA Ladder(25-2000 bp)	Bioneer	South Korea
Ethidium Bromide	Promega	USA
Glycerol	RPI	USA
Loading dye (6X)	Promega	USA
Loading dye	Promega	USA
Mitotracker stain	Thermo fischer	USA
nuclease free water (De-ionized water)	Promega	USA
Phosphate Buffer Saline (P B S)	Irvine Scientific	USA
Tween 20 X	Canvax	USA

3-3: Equipments and their suppliers

Table (3-2): List of equipments and their suppliers.

Instrument	Company	Country
Autoclave	Hirayama	Japan
Biosafety cabinet	Human Lab	Korea
Centrifuge	Eppendorf	Germany
Cooling centrifuge	Eppendorf	Germany
Distillator	Lab Teach	Korea
Electrophoresis apparatus	Bioneer	Korea
Fluorescent Microscope	Olympus	Japan
Frozen	AUCMA	China
Gel Documentation	Biometra	Germany
Laboratory Shaker	Dragon	China
Magnetic stirrer	Heidolph	Germany
Microcentrifuge spin	SCILOGEX D1008	China
Microwave	Shonic	China
NanoDrop spectrophotometers	Thermofisher	USA
PH meter	HANNA	Romania
Refrigerator	Concord	Lebanon
Sensitive Balance	Sartorius	Germany
Stereo Microscope	KERN	Kern
Thermal cycler apparatus	TECHNE prime	USA
Ultrasonicater	LABLINE	India
UV-Transilluminator	ELETROFOR	Italy
Vortex	CYAN	Belgium
Water bath	Memmert	Germany

3-4: Consumer Materials

Table (3-3): List of consumer materials and their suppliers

Consumer goods	**Company**	**Country**
Anticoagulant tubes	EDTAK2	Chine
Cotton	SHAKEEB	Malaysia
Eppendorf tubes	BDH	UK
Conical flask (500-1000) ml	Lab glas	Japan
Forceps	Hebson	India
Disposible gloves	SHAKEEB	Malaysia
Intravenous cannula	IndiaMART	India
Magnetic bar	IndiaMART	Newdelhi
PCR tubes	BDH	UK
Plain tube	RITTAL	China
Sharp scalpel	LG	USA
Slides	Super star	India
Spatula	Hebson	India
Plastic cups	Shangai Blopak	China
Syringe	CHANGZHOU	Chine
Micropipette tips	BOENMED	Chine
Volumetric flasks (500-1000 ml)	Lab glass	Japan
Plastic cups	Shangai Blopak	China
Micopipettes (0.5-10), (5-50), (100-1000)	Dragon	China

3-5: Laboratory kit

The kits used in the present study are listed in the table (3-4) and some of which their components in appendix (1).

Table (3-4): Types of kits applied in the current study and their company

Name of kits	Purpose of used	Manufactures (origin)
Taq G2Ⓡ Green master mix	For amplified DNA template by PCR and for amplified PCR product in PCR-RAPD- PCR	Promega /USA
g SYNC™ DNA Extraction	Extraction blood genomic DNA	Geneaid /Taiwan
Restriction enzyme protocol (MboI)	Used to digestion of amplified DNA(PCR product)	Promega /USA
Restriction enzyme protocol (EcoRI)	Used to digestion of amplified DNA (PCR product)	Promega /USA
E. Z. N.A ® Gel Extraction kit	Used to nucleic acid purification	OMEGA Bio-TEK/USA
DAPI stain	Used to stain the nucleus	SIGMA /USA
DCFH-DA stain	Used to detection cytoplasmic ROS	Abcam/Chine
Mitotracker stain	Used to detection mitochondrial morphology	Thermofischer/USA
Mitosox stain	Used to detect mitochondrial ROS	Thermofischer/USA

3-6: Primers used in present study

3-6-1: The specific primers for genes that related to breast cancer

The specific primers for *ABCB1*, *ABCG2*, *BRCA1*, *ER- α*, and *miRNA -152* genes which have been chosen in table (3-5) were prepared by the company of Alpha DNA /Montreal, Quebec /Canada.

Table (3-5): Primers sequences used for breast cancer related genes amplification

Name	Primer sequences (5′-3′)		Exons	Size (bp)	References
ABCB1	F	TTGATGGCAAAGAAATAAAGC	26	206	Tazzite *et al*., 2016
	R	CTTACATTAGGCAGTGACTCG			
ABCG2	F	AAATGTTCATAGCCAGTTTCTTGGA	16	302	Wu *et al*., 2015
	R	ACAGTAATGTCGAAGTTTTTATCGCA			
BRCA1	F	CACCTCCAAGGTGTATGAAG	11	465	Gholipoorfeshkecheh and Arjunan, 2014
	R	CTCTAGGATTCTCTGAGCATGG			
ER-α	F	ATGCGCTGCGTCGCCTCTAA	1	500	Sato *et al*., 2008
	R	CTGCAGGAAAGGCGACAGCT			
miRNA -152	F	TCTGTCATGCACTGACTGCTC	-	170	Nguyen-Dien *et al*., 2014
	R	GGGCATGCTTCTGGAGTCTA			

3-6-2: The RAPD primers

The RAPD primer for *ABCB1*, *ABCG2*, *BRCA1*, *ER- α*, and *miRNA 152* genes which have been chosen in table (3-6) were prepared by the company of AUGCT DNA SYN Biotechnology/China.

Table (3-6): RAPD primers sequences used for PCR- RAPD-PCR amplification

Primers	Primers sequences	Length (Base)
OPAA11	ACCCGACCTG	10
OPU 15	ACGGGCCAGT	10
OPAA17	GAGCCCGACT	10
OPD18	GAGAGCCAAC	10

3-7: Design of experiment

The general steps for the current study are shown below in figure (3-1)

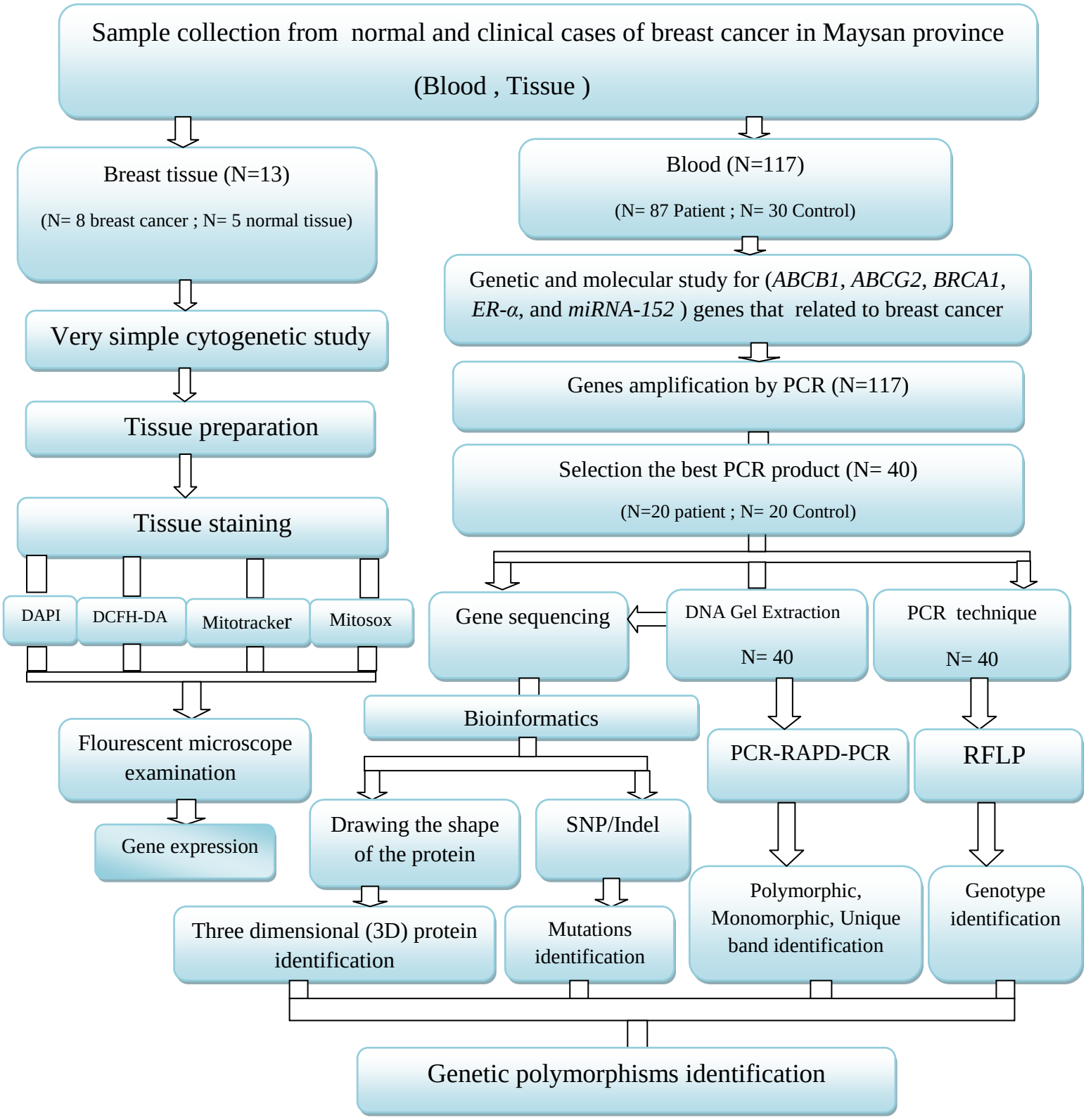


Figure (3-1): The common important steps in current study

3-7-1: Sterilization Methods

3-7-1-1: Sterilization by autoclaving

The micropipette tips and eppendorf tubes were sterilized by autoclave at 121º C for (15-20) minutes under pressure 15 Psi.

3-7-1-2: Sterilization by dry heat

The glassware tools (Volumetric flask, Conical flasks, and others tools) were sterilized by oven at 180 ºC for 1.5-2 hours.

3-7-2:Preparation of stains

The following stains were used in the present study, and prepared as based on the method described in the preparation kit manufactured by the SIGMA-Aldrich/USA; Abcam company/CHINE; Thermofischer/USA with some modification in dissolvent as follows:

3-7-2-1: Preparation of DAPI stain

3-7-2-1-1: Stock solution

The stock solution of the DAPI stain was prepared, freshly in a black bottle via dissolving 1mg of (4, 6-Diamidino-2-phenylindole dihydrochloride), in one ml of nuclease free water.

3-7-2-1-2: Work solution

The work solution of the DAPI stain was prepared from the stock solution, in a black bottle by adding 10 microliter of the stock solution of DAPI stain in 10 ml PBS, and it is used for staining nucleus, especially condensed chromosomes in normal and BC tissue and to determine the differences between them.

3-7-2-2: Preparation of DCFH-DA stain

The DCFH-DA stain was prepared from the stock solution, in a black bottle by adding one ml of the stock solution of DCFH-DA to 50 ml PBS, and it's used for detection of cytoplasmic ROS that indicates alterations in breast tissue.

3-7-2-3: Preparation of Mitotracker stain

The Mitotracker stain was prepared from the stock solution, in a black bottle by adding one ml of the stock solution of Mitotracker stain to 50 ml PBS, and it's used for staining mitochondria and considered as an index for mitochondrial morphology.

3-7-2-4: Preparation of Mitosox stain

The Mito-Sox stain was prepared from the stock solution, in a black bottle by adding 1 ml of the stock solution of Mito-Sox stain to 50 ml PBS, and it's used for detecting mitochondrial ROS and considered as an index for the mitochondrial defect.

3-7-3: Preparation of Buffers or solutions

The following Buffers (solutions) were used in the current study and prepared as described by protocol preparation.

3-7-3-1: Tris Borate EDTA (TBE) buffer

3-7-3-1-1: Stock Buffer solution (10X)

The stock Buffer solution of Tris Borate EDTA **(**TBE) buffer was prepared, freshly in a conical flask via dissolving 121.1 gm of Tris base, 61.8 gm of Boric acid and 7.4 gm of EDTA and then completed the size to 1000 ml of distilled water (D.W), then it was heated by magnetic stirrer and mixing by a clean magnetic bar until all components of stock solution were excellent dissolved, and the stock solution of Buffer was converted to clearless, and the Power Hydrogen force (PH) was measured as 8.

3-7-3-1-2: Work Buffer solutions (1X)

The work Buffer solution of TBE buffer was prepared, freshly in a volumetric flask by adding 100 ml of the stock buffer solution (TBE 10X) to 900 ml of distilled water (D.W) and mixed well.

3-7-3-2: Permeabilization solution

The permeabilization solution was used and prepared by using the method described in the flow cytometry permeabilization protocol/USA. The permeabilization solution of tissue for the simple cytogenetic study was prepared, freshly in a cup container by adding 10 ml of PBS to 10 µl Tween 20 X and immediately mixed by pipetting for 3-4 times, then permeabilization solution was stored at 4 ºC until use.

3-7-3-3: Ladder preparation for use in gel electrophoresis

DNA marker was prepared by Promega company but it was without loading dye. At gel electrophoresis, the loading dye (4µl) was added to (3µl) of known DNA fragment (DNA marker) and immediately mixed by pipetting for 3-4 times, then the first well of gel was loaded with 5 µl of the mixture (DNA marker and loading dye).

3-7-4: Genetic and Molecular study

Polymerase chain Reaction (PCR) assay was performed for identification of BC genes (*ABCB1*, *ABCG2*, *BRCA1*, *ER-α* and *miRNA-152*). This method was carried out as in the following steps:

3-7-4-1: DNA extraction

DNA was extracted from whole blood by using the gSYNC^{TM} DNA Extraction Kit functional test data according to (Geneaid, Taiwan).

Blood Protocol Procedure

Step 1: Sample Preparation

- Transferred 200 µl of whole blood to a 1.5 ml sterile microcentrifuge tube.

- Added 20 µl of Proteinase K, then carefully mix by pipetting, and incubate at 60ºC for 5 minutes.

Step 2: Cell Lysis

- Added 200 µl of GSB Buffer then mix by shaking vigorously.
- The tubes were incubated at 60ºC for 5 minutes, inverting the eppendorf tubes every 2 minutes.

Step 3: DNA Binding

- Added 200 µl of absolute ethanol to the sample lysate and mix immediately by shaking vigorously for 10 seconds.
- GS Column was placed in each a 2 ml sterile collection tube.
- The mixture (including any insoluble precipitate) was transferred to the GS column then centrifuge at 14,000 rpm for 5 sec.
- The 2 ml collection tube containing the flow-through was discarded.
- The GS Column was placed in a new 2 ml collection tube.

Step 4: Washing step

- Added 400 µl of (W1) Buffer to the GS Column and then centrifuge at 14,000 rpm for 30 seconds, then discard the flow-through, and place GS column back in the collection tube (2 ml).
- Added 600 µl of (W2) buffer (make sure absolute ethanol was added) to the mid GS Column, centrifuge at 15,000 rpm for approximately 30 seconds, then discard the flow-through, place the GS column back in the collection tube (2ml).
- The dried GS column was centrifuge at 15,000 rpm for 3 minutes to dry.

Step 5. Elution

- 100-200 µl of pre-heated elution buffer was added into the mid of the column matrix.

- We waited for at least (3 minutes or more) to allow the elution buffer to be perfect completely absorbed.
- The tubes were placed in a centrifuge at 15,000 rpm for 30 second or more to elute the purified DNA.
- Finally, the DNA was stored at -20 ºC to avoid DNA degradation.

3-7-4-2: Detection of DNA by Agarose Gel Electrophoresis

The DNA extraction has been previously carried out, agarose gel was prepared according to (Sambrook and Russel, 2006). Agarose gel electrophoresis was adopted to confirm the ready and integrity of extracted DNA of blood isolates according to the gel electrophoresis protocol which included the following steps:

1- One hundred of 1X TBE buffer were taken in a clean bottle.
2- Agarose powder (1.2 gm) was added to 100 ml of 1X TBE buffer.
3- The solution was heated to boiling by using a microwave until all Agarose particles were dissolved and become clear.
4- Approximately 3.5 -5µl of ethidium bromide were added to the agarose solution, and then the agarose was mixed.
5- The solution was left to cool down at 50-60 ºC.
6- After sealing alike edges of the gel tray with a rubber ruler tape and fixing the comb in 1 cm away from forward edge, the agarose solution was carefully poured into the gel tray.
7- The agarose was allowed to solidify at room temperature for 25-30 minutes or more and then transferred to the refrigerator for minutes.

   The fixed comb was precisely removed and the gel tray was transferred in the gel tank. The tank was previously filled with 1 X TBE buffer, until 1 X TBE buffer reached approximately 1-2 mm up the surface of the gel.
8- The DNA sample was transferred 5 µl from it on sterile sticky tape, 2 µl of loading dye (Blue 6 X Loading dye) added to each DNA sample on sticky tape

and mixing well, then the mixture was loaded into the wells of the agarose gel. Electric current was allowed at 60-70 volt for 30-60 minutes.

9- Finally, the bands of DNA were visualized on a UV transilluminator at wave length 350 nm.

3-7-4-3: Column purification

Genomic DNA was isolated from all blood. The amount of isolated DNA was varied from 52.392 -88.712 ng/µl DNA and an absorbance ratio of A260/280 was obtained in the range of 1.71-1.98.

3-7-4-4: Preparation of primers solution

The oligonucleotide primers were suspended by dissolving the lyophilized product of each primer after spinning down briefly with De-ionized water (nuclease free water) depending on manufacturer's instruction as stock suspension of primers. Working solutions of primer tube was diluted with nuclease free water, and the last picomoles depended on the procedure of each primer used.

3-7-4-5: Master mix

The components of the Taq G2® Green master mix were prepared by the company of Promega/USA.

3-7-4-6: Polymerase Chain Reaction protocol

The protocol used is according to promega manufacturer's instructions. All PCR components were assembled in PCR tube and mixed by microcentrifuge spin for 10 sec.

- The steps were conducted are as follows:

1- Template DNA and specific primers were dissolved before usage.

2- Template DNA, primers, De-ionized water (nuclease free water) and Taq G2® Green master mix were added into the PCR tubes for getting 25 µl reaction volume, as shown in **table (3-7).**

Table (3-7): The components of PCR mixture

PCR Mixture	**Volume (µl)**
Nuclease free water	2.5
Taq G2® Green master mix	12.5
Forward primer	2.5
Reverse primer	2.5
DNA template	5
Final volume	25

3- The components of PCR tubes were completely mixed and spin down either by using pipette up and down several times or by using Mini spin Microcentrifuge 15 sec.

4- The PCR tubes were placed in the TECHEN prime thermocycler and the right PCR cycling program parameter conditions were shown in **table (3-8).**

Table (3-8): PCR program of *ABCB1*, *ABCG2*, *BRCA1*, *ER-α*, and *miRNA -152* genes:

PCR step	*ABCB1*	*ABCG2*	*BRCA1*	*ER-α*	*miRNA-152*	Cycle
Initial Denaturation	95 ºC 5:00	95 ºC 5:00	95 ºC 5:00	95 ºC 5:00	95 ºC 5:00	1
Denaturation	94 ºC 1:00	95 ºC 00:45	95 ºC 00:35	95 ºC 00:40	95 ºC 00:15	35
Annealing	53 ºC 1:00	53.5-58.5 ºC 00:45	55 ºC 00:35	52-58 ºC 00:45	65 ºC 00:15	
Extension	72 ºC 1:00	72 ºC 1:00	71 ºC 1:00	72 ºC 40:00	72 ºC 00:30	
Final Extension	72 ºC 7:00	72 ºC 5:00	72 ºC 5:00	72 ºC 5:00	72 ºC 5:00	1
Final Hold	4 ºC					

3-7-4-7: Agarose Gel Electrophoresis

TBE 1X buffer was added to the tank of electrophoresis tank, tray with agarose which had previously attended was put in electrophoresis tank. Each well is loaded with 5 µl of PCR product. Then standard molecular weight of DNA marker (DNA ladder) (5 µl) is loaded in a first well. Electrophoresis run at 65-70 volt for 1-1.15 hour, the gel was visualized and then photographed by using a gel documentation system (under UV), repeat this method with each gene (Mishra *et al*., 2010).

3-7-4-8: Extraction of PCR product from the gel

Agarose gel extraction was used to isolate and purify the amplified specific gene (PCR product). Desired DNA fragment was extracted from gel by using E. Z. N. A ® Gel Extraction kit according to company instructions (OMEGA Bio-TEK/USA), as follows with some modification in time:

1- Agarose powder (0.6 gm) was added to 50 ml of 1X TBE buffer and heated with microwave until all agarose particles were dissolved and become clearness, 2.5 µl of ethidium bromide were added to the agarose solution. The agarose was stirred in order to get mixed, the agarose solution was put into gel tray, the agarose was allowed to solidify at room temperature for 30 minutes. The fixed comb was carefully removed and the gel tray was carefully placed in the gel tank. The tank was filled with 1X TBE buffer until the buffer reached 2 mm over the surface of the gel.
2- PCR product mixture of the specific gene (5 µl) was loaded into the wells of agarose gel. Electric current allowded at 65-70 volt for 30 min-1 hour.
3- Finally, the bands were visualized on a UV transilluminator at wave length 350 nm.
4- Carefully excised the DNA fragment of interest by using a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose.
5- The volume of the gel slice was determined by weighing a clean 1.5 ml microcentrifuge tube. The volume of gel was derived as follows: The difference between the weight of the eppendorf tube containing gel slice minus the weight of

empty eppendorf tube (weight of the eppendorf tube containing gel slice - weight of empty eppendorf tube). The numbers were rounded to the nearest integer number and the integer number was converted to microliter unit.

6- Added 1 volume Binding Buffer (XP2) to eppendorf tube.

7- The tubes were incubated at 60 ºC for 7 minutes or more until the gel has completely melted. Shake the tube every 2-3 minutes.

8- A HiBind DNA Mini colum was placed in a 2ml collection tube.

9- Added no more than 700 µl DNA/agarose solution from step 6 to the HiBind DNA Mini column.

10-The tubes were placed in centrifuge at 10,000 rpm for 1 minute at room temperature.

11- Discard the filtrate and reuse the collection tube.

12-Repeated steps (8-10) until all of the sample has been transferred to the column.

13-Added 300 µl Binding Buffer(XP2).

14-The tubes were placed in centrifuge at 14,000 rpm for 1minute at room temperature.

15- Discard the filtrate and re use collection tube.

16-Added 700 µl SPW wash Buffer.

17-The tubes were placed in centrifuge at 14,000 rpm for 1 minute at room temperature.

18- Discard the filtrate and reuse collection tube.

19- The empty HiBind DNA Mini column was centrifuge for 2 minutes at maximum speed to dry the column matrix.

20-The HiBind DNA Mini column was transferred to the clean 1.5 ml microcentrifuge tube and was labeled.

21- Added 30 µl of Elution Buffer directly to the mid of the column.

22- We waited for at least 30 minutes or a little more to allow the Elution buffer to be completely absorbed at room temperature.

23-The column tubes were placed in centrifuge at 14,000 rpm for 1 minute.

24- Finally, the DNA (Purified gene) was stored at -20 ºC.

3-7-4-9: Column Purification

PCR product (specific gene) was isolated by extracting DNA from gel, the amount of isolated DNA (PCR product) was varied from 52.392-88.712 ng/µl and absorbance ratio of A260/280 was obtained in the range of 1.71-1.98.

3-7-4-10: Restriction fragment length polymorphism (RFLP)

PCR products for (*ABCB1*, *ABCG2*, and *BRCA1*) genes were digested by using MboI, EcoRI restriction enzyme protocol according to functional test data (Promega/USA) with some modification, as shown in **table (3-9).**

Table (3-9) The components of RFLP mixture

RFLP mixture	**Volume (µl)**
Sterile, deionized water	16.3
RE 10X Buffer	2
Acetylated BSA,10 µg/ µl	0.4
DNA (PCR product),1 µg / µl	3
Restriction Enzyme,10 U/µL	0.6
Final volume	22.3

The total volume of 22.3 µl of the mixture was incubated at 37 ºC in the water bath for 4 hours. After digestion and adding stopping solution, the sample fragments were visualized by electrophoresis at a concentration of 3.5% agarose for (*ABCG2*, *ABCB1*) and 2.5% agarose for (*BRCA1*) (prepared by the same detection of DNA content via agarose gel electrophoresis except 3.5 gm or 2.5gm of agarose powder dissolved in 100 ml of 1X TBE buffer). Finally, the fragments were visualized on a UV transilluminator and by using a gel documentation system at wave length 350 nm, the genotype size was determined by comparing their size with DNA Ladder (25-300) bp in the first well of gel, (100-1500) bp in the last well of gel, and by NUB cutter V 0.2 program.

3-7-4-11: PCR-RAPD-PCR

RAPD primers used in this study mentioned in the previous **table (3-6)**. The PCR-RAPD-PCR was carried out at total volume of 20 µl containing a mixture of 7.5 µl GO Taq G2® Green master mix, 7.5 µl nuclease free water, 2.5 µl PCR product, 2.5 µl (RAPD primers), as the following table **(3-10)**:

Table (3-10) The components of PCR-RAPD-PCR mixture

PCR-RAPD-PCR mixture	**Volume (µl)**
GO Taq G2 Green master mix	7.5
nuclease free water	7.5
PCR product (*ABCG2*, *ABCB1*, *BRCA1*, *ER-α*, and *miRNA-152*)	2.5
RAPD primers **(**OPAA11, OPU15, OPAA17, OPD18)	2.5
Final volume	20

Amplification was carried out for each gene with four RAPD primers in Techne prime thermocycler with program, as follows **(Table 3 -11)**

Table 3-11 PCR-RAPD-PCR program for *ABCB1*, *ABCG2*, *BRCA1*, *ER-α*, and *miRNA-152* genes:

PCR steps	Temperature ºC	Time Sec	Repeat cycle
Denaturation	94	60	3
Annealing	36	45	
Extension	72	45	
Denaturation	94	30	35
Annealing	35-39	45	
Extension	72	90	
Final Hold	10	-	-

The PCR-RAPD-PCR results were electrophoresed on 2.5% agarose gel with ethidium bromide 2.5 µl at 65-70 Volat-Voltage for 60 minute. The DNA ladder (25-300), (100-1500) bp was inserted into the first and last gel well to determine the size of the PCR-RAPD-PCR band. PCR-RAPD-PCR fragments were visualized on a Gel DOC UV Transilluminator.

3-7-4-11-1: Molecular Weight Index

A well-known molecular weight indicator (DNA Ladder 100 bp) was used, consisting of 11 bands as follows (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500) base pair from the company Promega / USA. Also, we used a molecular size indicator (DNA Ladder 25 bp), consisting of 10 bands as follows (25, 50, 75, 100, 125, 150, 175, 200, 250, 300) base pair from the company (Promega / USA).

The following parameters were calculated to take them from the gel images

A - The number of total bands produced from each primer was calculated.

B - The primer efficiency and discriminatory power was calculated from the below law according to (Ismaeel, 2013).

C - The percentage of the primer bands was calculated from the below law.

D - The percentage of polymorphisms (%), Monomorphisms (%), Uniqueness (%) were calculated from the below laws according to (Al-Rawi, 2015):

$$\textbf{Primer efficiency} = \frac{The\ number\ of\ polymorphic\ bands\ produced\ by\ each\ the\ primers}{Total\ number\ of\ bands\ of\ all\ primers}$$

$$\textbf{Primer discriminatory power \%} = \frac{The\ number\ of\ polymorphic\ bands\ produced\ from\ the\ primer}{Total\ number\ of\ polymorphic\ bands\ produced\ from\ all\ primers} \times 100$$

$$\textbf{Percentage of primer bands \%} = \frac{The\ number\ of\ bands\ produced\ by\ the\ primer}{Total\ number\ of\ bands\ of\ all\ primers} \times 100$$

$$\textbf{Polymorphisms \%} = \frac{The\ number\ of\ polymorphic\ bands\ produced\ from\ the\ primer}{Total\ number\ of\ bands\ produced\ from\ the\ primer} \times 100$$

$$\textbf{Monomorphisms \%} = \frac{The\ number\ of\ monomorphic\ bands\ produced\ from\ the\ primer}{Total\ number\ of\ bands\ produced\ from\ the\ primer} \times 100$$

$$\textbf{Uniqueness \%} = \frac{The\ number\ of\ unique\ bands\ produced\ from\ the\ primer}{Total\ number\ of\ bands\ produced\ from\ the\ primer} \times 100$$

40 PCR-RAPD-PCR product (20 patients and 20 controls) are transferred to a (2.5%) agarose gel to separate the resulting bands. The bands that appear in all wells at a certain molecular weight are called monomorphic bands, the bands that appear in some wells and are absent in others at a certain molecular weight are called polymorphic bands, and the single band that appear at a certain molecular weight is called unique band.

3-7-4-12: Determine the sequence of nitrogen bases

After confirming the amplification of the samples, samples of 20 microliters of the PCR product were sent to the Macrogen Company in South Korea to obtaining the real sequences of the nitrogenous bases for the required pieces of genes, as a sequencing process was carried out for single strands of DNA Forward (F), according to our request from the company for the purpose of identifying genetic mutations, and after the results arrived, the identity of the sequence was reviewed in the Gene Bank using Bioinformatics techniques and algorithms as a tool for Blast search. This tool helped to

determine the conformity of records of samples sent and records in the gene bank, sequences lined up and aligned were identified using specialized programs software such as BioEdit, and Genetic mutations were identified using Blast, Geneious V 4.0.20.

3-7-4-13 : 3 D protein drawing

Drawing a three-dimensional protein according to the following programs and steps:

1. Converting DNA sequences in FASTA format to amino acid sequences in the same format using Blast program on the NCBI website (Altschul *et al*., 1990).
2. Using Phyre 2 V.2.0 program to convert amino acid sequences from FASTA format to PDB format and predict the three-dimensional shape of the protein (Kelley *et al*., 2015).
3. Using EzMol V.1.22 program (www.Sbg.bio.ic.ac.uk/ezmol) for the purpose of drawing three-dimensional protein shapes with determination of the location of the genetic mutation on the protein (Reynolds *et al*., 2018).
4. Using Expasy and Swiss-Model program for the drawing 3 D protein of some genes.

3-7-5: Very simple cytogenetic study

All tissue samples were isolated from BC peoples and normal peoples, then processed according to (Dragh *et al*., 2017) with some modifications for fluorescent microscopic study as follow:

3-7-5-1: Tissue preparation

To assess whether or not cancer was present in breast tissue, we used four dyes with fluorescent microscopy (DAPI, DCFH-DA, Mitotracker, and MitoSOX) as follows: we transferred the tissue under a Stereo (dissecting) microscope, then we took a very small portion the size of a pinhead with forceps and place it in a concave glass slide (a slides with deep holes).

1- Fixation done in 10% formalin solution was used to stabilize the samples until use.

2- Washing

The samples were washed with running water to remove the formalin from samples.

3- Transferred the tissue sample under a Stereo or dissecting microscope and took a very small portion the size of a pinhead with forceps and place it in a concave glass slide (a slide with deep holes).

4-Fixation with 4% DEPC fixation for 30 minute.

5-Permeabilization

Samples were treated with 300 µl of permeabilization solution for 15-20 min.

6-The samples were washed with PBS at 3 times for 5 minute.

7-Staining

Using fluorescent dyes such as (DAPI, DCFH-DA, Mitotracker, and Mitosox) as a fluorescent stains have been used to demonstrate chromosome condensed in nucleus, ROS amount, differences in shape of mitochondria, and mtROS as indicators for different features of gene expression between normal and breast cancer tissue.

- Added 300 µl of DAPI stain for 20 minute.
- The samples were washed with PBS at 3 times for 5 minute.
- Added 300 µl of DCFH-DA stain for 20 minute.
- The samples were washed with PBS at 3 time for 5 minute.
- Added 300 µl of Mitotracker stain for 20 minute.
- The samples were washed with PBS at 3 time for 5 minute.
- Added 300 µl of Mitosox stain for 20 minute.
- The samples were washed with PBS at 3 time for 5 minute.

After staining procedures the samples were examined under the fluorescent microscopic under magnification power (400X) and photographs were captured by a computer and Corel Capture and J Image software were used.

3-7-6: Statistical Analysis

The statistical analysis of data was performed by (SPSS version 22). Chi-square used to show the important statistic and significant differences limited on P ≤0.05 of probability (Al-Rawi and Khalaf Allah, 2000).

The expected genotype of HWE was examined manually and then by Michael H.Court (2005-2008) online calculator and the deviation from HWE was accomplished by SPSS version 22 and Michael H. Court (2005-2008). Odds ratios (ORs) and confidence intervals (CIs) of genotypes and alleles were examined by MedCalc statistical software (version 20.0111) (https://www.Medcalc.net/tests/odds ratio php)(Altman,1991). The statistical analysis of the cytogenetic study was performed by GraphPad Prism version 7 program to know the statistical differences in ROS between the tissues of breast cancer patients and control based on the captured image.

Chapter four

Results

4- Results

4-1: DNA extraction and amplification

The results of the DNA extraction are showed that the amount of DNA ranged from 52.392 - 88.712 ng/µl, and the absorbance ratio A 260/280 was obtained in the range 1.71-1.98 (nm) which is within the normal range (1.7-2.0) nm was detected by the nanodrop device. The results of the electrophoresis on 1.2% agarose gel are showed the success of the DNA amplification process, as the primer *ABCG2* was given a band of 302 bp, the primer *ABCB1* was given a band of 206 bp, the primer *BRCA1* was given a band of 465 bp, the primer *ER-α* was given a band of 500 bp, and the primer *miRNA-152* was given a band of 170 bp, as shown in the figure (4-1), respectively.

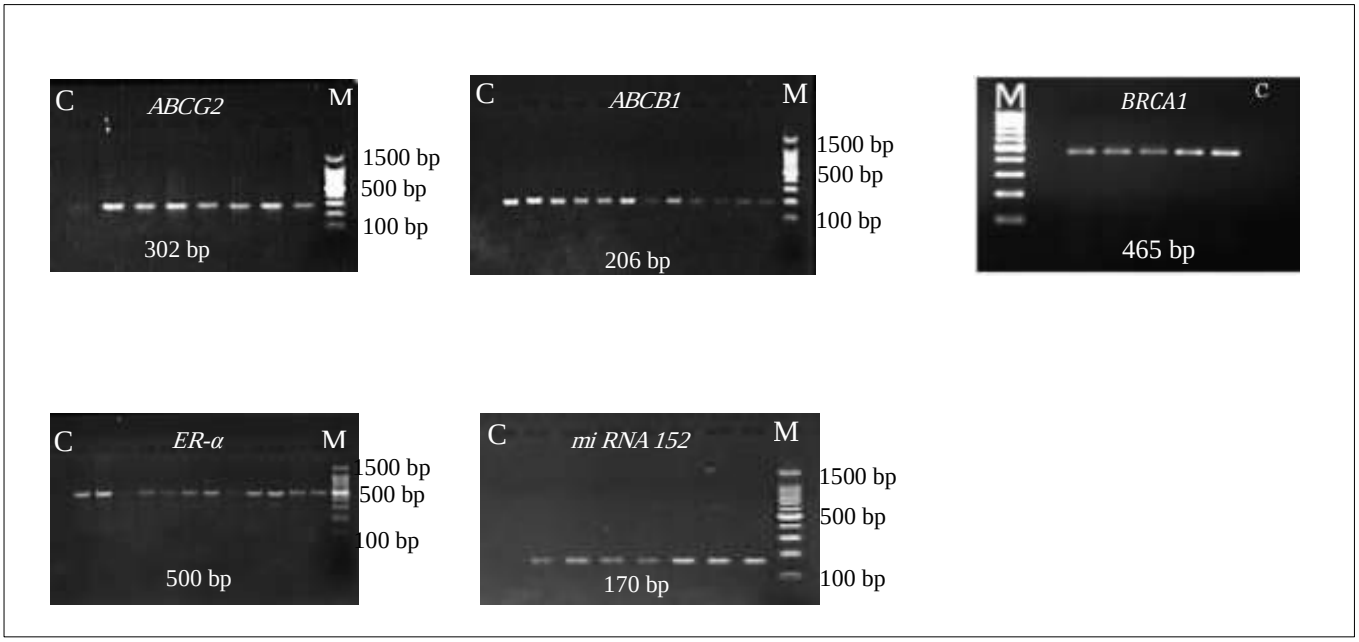


Figure (4-1) Amplification of the *ABCG2* 302 bp, *ABCB1* 206 bp, *BRCA1* 465 bp, *ER-α* 500 bp, and *miRNA-152* 170 bp obtained from BC patient and control. The first lane from the right (M lane) is DNA ladder marker 100-1500 bp in size used as DNA molecular weight marker, the last lane from left (C lane) is negative control (PCR mixture without DNA) and all other lanes are PCR product of BC gene depended on molecular weight.

4-2: Genetic polymorphism

The genetic polymorphism of the (*ABCB1*, *ABCG2*, *BRCA1*, *ER-α,* and *mi-RNA 152)* genes was studied in patients with BC and compared with the control sample by using the PCR-RFLP technique, PCR-RAPD-PCR technique, and Gene sequencing.

4-2-1: PCR-RFLP

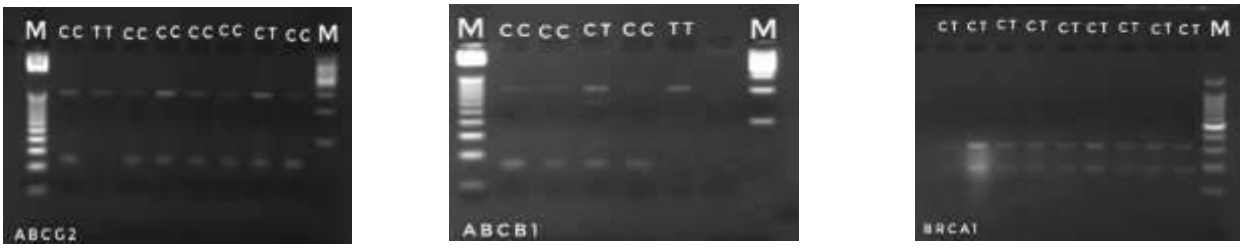
The studied population in the PCR-RFLP technique is composed of 20 verified female breast cancer patients, and 20 control women. The clinicopathological features of patients are listed in the table (4-1).

Table (4-1): Clinical and pathological characteristics of Maysan breast cancer women.

Variables		No (%)	Total No (%)
Age (year)	>45	16(80)	20(100)
	<45	4(20)	
Marital status	Married	19(95)	20(100)
	Unmarried	1(5)	
Family history	Family history	6(30)	20(100)
	Non-Family history	14(70)	
Socioeconomic status	Poor-median	16(80)	20(100)
	Good-excellent	4(20)	

4-2-1-1: Detection of the genetic polymorphism of the BC gene by RFLP technique

The results of the electrophoresis of the digested *ABCG2*, *ABCB1*, and *BRCA1* genes with MboI, MboI, EcoRI, respectively, by RFLP-PCR technique showed the presence of two alleles C, T and three genotypes CC, CT, TT in the BC patients and the control sample. When the band appears in the C field and does not appear in the T field, the genotype is called CC. If the bands appear in the T field and do not appear in the C field, the genotype is called TT, and when the band appears in C and T field, the genotype is called CT, as shown in figure (4-2), appendix (2).



ABCG2 with MboI (A) | *ABCB1* with MboI (B) | *BRCA1* with EcoRI (C)

Figure (4-2) PCR RFLP results for *ABCG2, ABCB1, BRCA1* after digested with MboI, MboI, EcoRI restriction enzyme, respectively. (A) The first lane (M) on left is ladder (25-300) bp in size, fragments were 300 bp and 62 bp for wild-type homozygote CC, while RFLP-PCR product 300 bp, 150 bp and 62 bp for the heterozygote CT, 300 bp for Homozygote mutant variant TT. **(B)** The first lane (M) on left is ladder (25-300) bp in size, fragments were 206 bp and 45 bp for the Wild-type homozygote CC, while 206 bp, 161 bp and 45 bp for the heterozygote CT, and 206 bp for Homozygote mutant variant TT. **(C)** The first lane (M) on right is ladder (100-1500) bp in size, fragments were 302 bp,163 bp for the heterozygote CT.

4-2-1-1-1: Detection of the genetic polymorphism of *ABCG2* gene by RFLP technique

The results of electrophoresis and digestion of the PCR products of the *ABCG2* gene by MboI restriction enzyme was showed that there are two alleles are (C, T), its frequencies in the patients (0.9, 0.1), respectively, and in the control (0.725, 0.275), respectively, and three genotypes CC, CT, TT. Its frequencies in the patients (0.9, 0, 0.1), and in the control (0.45, 0.55, 0), respectively, as shown in table (4-2).

Table (4-2): Allele frequency and genotype frequency in the *ABCG2* gene of the breast cancer patient and control.

Gene	Genotypes	No.Genotypes frequency (Patient)	No.Genotypes frequency (Control)	Alleles	No.Alleles frequency (Patient)	No.Alleles frequency (Control)
ABCG2	CC	18(0.9)	9(0.45)	C	36(0.9)	29(0.725)
	CT	0(0)	11(0.55)	T	4(0.1)	11(0.275)
	TT	2(0.1)	0(0)	-	-	-

The results of the frequency distribution of the C and T alleles of the *ABCG2* gene, using the Hardy-Weinberg equilibrium law, showed different results between the breast cancer patients and the control. The C allele in the BC patients recorded a percentage (90%) compared to the T allele that recorded a percentage (10%), while the C allele in the control sample recorded a percentage (72.5%) compared to the (T) allele that recorded a percentage (27.5%), as shown in the table (4-3).

Table (4-3): Alleles and genotype percentages of *ABCG2* polymorphism among patients and controls.

Gene	Genotypes	Patient%	Control%	Alleles	Patient%	Control%
ABCG2	CC	18(90)	9(45)	C	36(90)	29(72.5)
	CT	0(0)	11(55)	T	4(10)	11(27.5)
	TT	2(10)	0(0)	-	-	-

The table (4-4) shown that the C, T frequency distribution differed significantly (P = 0.05) between BC sample and the control sample, and the results showed the C allele figured a frequency in the BC with a higher percentage (90%) than the control sample (72.5%), while the T allele showed a frequency in the control sample with a greater percent (27.5%) than the BC sample (10%); Odd ratio (OR)= 3.4138; confidence interval (95% CI) = 0.9834-11.8501, as shown in table (4-4).

Table (4-4) Alleles frequency of *ABCG2* gene among breast cancer patients and control.

Gene	Allele	Patient%	Control%	OR	95% CI	P-value
ABCG2	C	36 (90)	29(72.5)	3.4138	0.9834 - 11.8501	0.05*
	T	4(10)	11(27.5)			
% Percentage, OR Odd Ratio, CI Confidence Interval, C (Allele), T (Allele)						
Significance *P <0.05,P <0.01,***P <0.005, NS =No significance, P >0.05**						

There are a high significant differences between the genotypes distribution of the BC patients and control group (P= 0.000), as shown in the table (4-5), figure (4-3).

Table (4-5): The genotype frequencies of the *ABCG2* gene among the breast cancer patients and control group.

Genes	Genotypes	Patient	Control	Total	X^2	P-value
ABCG2	CC	18	9	27	16	0.000***
	CT	0	11	11		
	TT	2	0	2		
	Total	20	20	40		
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS=No significance P >0.05**						

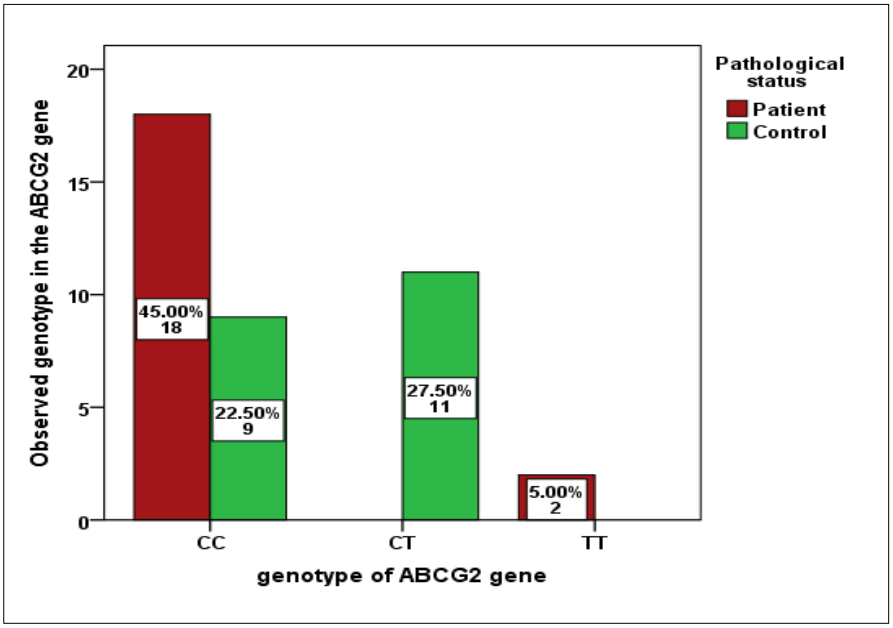


Figure (4-3) The comparison among the numbers and percentages of the total CC, CT and TT genotype frequencies of the *ABCG2* gene between BC patients and control group.

The results of the frequency distribution of the *ABCG2* gene, using the Hardy-Weinberg equilibrium law, showed different results between the genotypes of the BC patients and the control. The distribution in the control group is in the Hardy Weinberg equilibrium (HWE) revealed, no significant deviation (P= 0.0898) was recorded between *ABCG2* observed and expected genotype, as shown in the table (4-6), Appendix (3).

Table (4-6): The observed and expected frequencies under Hardy -Weinberg equilibrium in the *ABCG2* gene of the control.

Gene	Genotypes	Control (Observed)	Control (Expected)	X^2 (H.W.E)	P-value
ABCG2	CC	9	10.5	2.8775	0.0898
	CT	11	8		
	TT	0	1.5		
X^2 Chi-square H.W.E Hardy - Weinberg equilibrium (if P > 0.05 consistent with H.W.E), at 1 d.f.					

The results of the genetic analysis of the RFLP-PCR technique for the *ABCG2* gene, showed three genotypes in the BC sample and the control sample, which are CC, CT, and TT. The genotype (CC) showed a higher percentage in the BC sample compared to the control sample, and the percentages were 90%, and 45%, respectively, and there was a significant difference, (P = 0.0059; OR= 11.0000; and 95% CI= 1.9976 - 60.5734).

The genotype (CT) showed a higher percentage in the control sample compared to the BC sample, and the percentages were 55%, and 0%, respectively, and there was a significant difference, (P= 0.0091; OR= 0.0201; and 95% CI= 0.0011- 0.3789).

The genotype (TT) showed a higher percentage in the breast cancer sample compared to the control group, and the percentages were 10 %, and 0%, respectively, without any significant difference between them (P= 0.2792; OR= 5.5405; and 95% CI= 0.2494-123.0857), as shown in the table (4-7).

Table (4-7): Genotype distribution and frequencies of *ABCG2* polymorphism among breast cancer patients and control.

Genes	Genotypes	Patient%	Control%	OR	95% CI	P-value
ABCG2	CC	18 (90)	9 (45)	11.0000	1.9976 - 60.5734	0.0059***
	CT	0 (0)	11 (55)	0.0201	0.0011 - 0.3789	0.0091**
	TT	2 (10)	0 (0)	5.5405	0.2494 - 123.0857	0.2792
% Percentage, OR Odd Ratio,CI Confidence Interval, CC homozygous, CT heterozygous ,TT homozygous						
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS=No significance P >0.05**						

The findings of *ABCG2* genotype by dominant model showed that the significant differences between breast cancer patients and the control, (P= 0.0059), this significant relation was observed under dominant model, as shown in the table (4-8).

Table (4-8): Distribution of the *ABCG2* genotypes in the breast cancer patients and control under dominant model.

Genes	Genotypes	Patient%	Control%	OR	95% CI	P-value
ABCG2 Dominant model	CC	18(90)	9(45)	11.0000	1.9976 - 60.5734	0.0059**
	TT+CT	2(10)	11(55)			
% Percentage, OR Odd Ratio, CI Confidence Interval, CC homozygous,CT heterozygous ,TT homozygous						
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS=No significance P >0.05**						

The findings of *ABCG2* gene in recessive model showed that there were no significant differences between BC patients and the control, (P= 0.2792), this no significant relation was observed under recessive model, as shown in the table (4-9).

Table (4-9): Distribution of the *ABCG2* genotypes in the breast cancer patients and control under recessive model.

Genes	Genotypes	Patient%	Control%	OR	95% CI	P-value
ABCG2 Recessive model	CC+CT	18 (90)	20 (100)	0.1805	0.0081 - 4.0096	0.2792
	TT	2(10)	0(0)			
% Percentage, OR Odd Ratio, CI Confidence Interval, CC homozygous,CT heterozygous ,TT homozygous						
Significance *P <0.05,P <0.01,***P <0.005, NS=No significance P >0.05**						

4-2-1-1-2: Detection of the genetic polymorphism of *ABCB1* gene by RFLP technique

The results of electrophoresis and digestion of the PCR products of the *ABCB1* gene by MboI restriction enzyme was documented two alleles were (C, T) and its frequencies in the patients (0.575, and 0.425), respectively, and in the control (0.8, and 0.2), respectively, with three genotypes (CC, CT, and TT) and its frequencies in the patients (0.3, 0.55, and 0.15), and in the control (0.7, 0.2, and 0.1), respectively, as shown in table (4-10).

Table (4-10): Allele frequency and genotype frequencies in the *ABCB1* gene of the breast cancer patient and control.

Gene	Genotypes	No.Genotypes frequency (Patient)	No.Genotypes frequency (Control)	Alleles	No.Alleles frequency (Patient)	No.Alleles frequency (Control)
ABCB1	CC	6(0.3)	14(0.7)	C	23(0.575)	32(0.8)
	CT	11(0.55)	4(0.2)	T	17 (0.425)	8 (0.2)
	TT	3(0.15)	2(0.1)	-	-	-

The results of the frequency distribution of the C and T alleles of the *ABCB1* gene showed different results between the BC patients and the control, as shown in table (4-11). The C allele in the BC patients recorded higher percent (57.5%) compared to the T allele that recorded (42.5%), whereas the C allele in the control sample recorded percentage very high percent (80%) compared to the (T) allele that recorded (20%), as shown in the table (4-11).

Table (4-11): Alleles and genotype percentages of *ABCB1* gene polymorphism among patients and control.

Gene	Genotypes	Patient%	Control%	Alleles	Patient%	Control%
ABCB1	CC	6(30)	14(70)	C	23(57.5)	32(80)
	CT	11(55)	4 (20)	T	17(42.5)	8(20)
	TT	3(15)	2 (10)	-	-	-

The table (4-12) shown that the C, T frequency distribution differed significantly (P = 0.033) between the BC sample and the control sample, and the results revealed that the C allele had higher frequency in the control group (80%) than the BC patients (57.5%), while the T allele showed a frequency in the BC group with a higher percent (42.5%) than the control sample (20%), (OR= 0.3382; 95%CI= 0.1249-0.9163).

Table (4-12): Alleles frequency of *ABCB1* gene among breast cancer patients and control.

Gene	Allele	Patient%	Control%	OR	95% CI	P-value
ABCB1	C	23(57.5)	32(80)	0.3382	0.1249 - 0.9163	0.033*
	T	17(42.5)	8(20)			
% Percentage , OR Odd Ratio ,CI Confidence Interval , C(Allele) ,T(Allele)						
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS =No significance , P >0.05**						

There are significant differences between the genotypes distribution of the BC patient and control group, (P= 0.036) in relation to *ABCB1* gene, as shown in the table (4-13), figure (4-4).

Table (4-13): The genotype frequencies of the *ABCB1*gene among the breast cancer patients and control group.

Genes	Genotypes	Patient	Control	Total	X^2	P-value
ABCB1	CC	6	14	20	6.667	0.036*
	CT	11	4	15		
	TT	3	2	5		
	Total	20	20	40		
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS =No significance , P >0.05**						

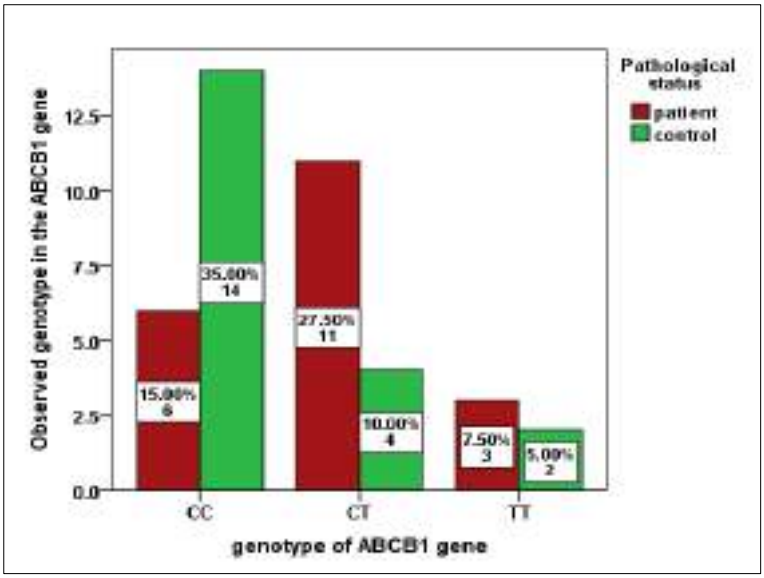


Figure (4-4) The comparison among the CC, CT and TT genotype frequencies of the *ABCB1* gene between BC and control group.

The frequency distribution of the *ABCB1* gene, using the HWE law, showed different findings between the genotypes of the BC patients and healthy control. The distribution in the control group in HWE, is of no significant deviation (P= 0.0935), which is recorded between *ABCB1* observed and expected genotype, as shown in the table (4-14), (Appendix 4).

Table (4-14): The observed and expected frequencies under Hardy-Weinberg equilibrium in the *ABCB1* gene of the control.

Gene	Genotypes	Control (Observed)	Control (Expected)	X^2 (H.W.E)	P-value
ABCB1	CC	14	12.8	2.8125	0.0935
	CT	4	6.4		
	TT	2	0.8		
X^2 Chi-square H.W.E Hardy - Weinberg equilibrium (if P > 0.05 consistent with H.W.E), at 1 d.f.					

The results of the genetic analysis of the RFLP-PCR technique for the *ABCB1* gene, showed three genotypes in the BC sample and the control sample, which are CC, CT, and TT.

The genotype (CC) showed a high percent in the control sample compared to the BC were 70%, and 30%, respectively, (P= 0.0141; OR= 0.1837 and 95%CI= 0.0475-0.7103).

The genotype (CT) showed a strong association in the BC sample compared to the control group, were 55%, and 20%, respectively, (P = 0.0269; OR= 4.8889; 95%CI= 1.1985 - 19.9429).

The genotype (TT) showed a high correlation in the BC patients compared to the control sample, were (15%), and (10%), respectively, (P = 0.6346; OR= 1.5882; 95% CI= 0.2356 - 10.7048), as shown in the table (4-15).

Table (4-15): Genotype distribution and frequencies of *ABCB1* polymorphism among breast cancer patients and control.

Genes	Genotypes	Patient%	Control%	OR	95% CI	P-value
ABCB1	CC	6(30)	14(70)	0.1837	0.0475 - 0.7103	0.0141**
	CT	11(55)	4(20)	4.8889	1.1985 - 19.9429	0.0269*
	TT	3(15)	2(10)	1.5882	0.2356 - 10.7048	0.6346
% Percentage, OR Odd Ratio, CI Confidence Interval, CC homozygous,CT heterozygous ,TT homozygous						
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS=No significance P >0.05**						

The results of distribution of the *ABCB1* genotypes in the breast cancer patients and control under dominant model showed a significant differences (P= 0.0141), as shown in the table (4-16).

Table (4-16): Distribution of the *ABCB1* genotypes in the breast cancer patients and control under dominant model.

Genes	Genotypes	Patient%	Control%	OR	95% CI	P-value
ABCB1 Dominant model	CC	6(30)	14(70)	0.1837	0.0475-0.7103	0.0141**
	TT+CT	14(70)	6(30)			
% Percentage, OR Odd Ratio, CI Confidence Interval, CC homozygous,CT heterozygous ,TT homozygous						
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS=No significance P >0.05**						

The data of distribution of the *ABCB1* genotypes in the breast cancer patients and control under recessive model showed no significant differences between both groups, (P= 0.6346), as shown in the table (4-17).

Table (4-17): Distribution of the *ABCB1* genotypes in the breast cancer patients and control under recessive model.

Genes	Genotypes	Patient%	Control%	OR	95% CI	P-value
ABCB1 Recessive model	CC+CT	17(85)	18(90)	0.6296	0.0934 - 4.2437	0.6346
	TT	3(15)	2(10)			
% Percentage, OR Odd Ratio, CI Confidence Interval, CC homozygous,CT heterozygous ,TT homozygous						
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS = No significance P >0.05**						

4-2-1-1-3: Detection of the genetic polymorphism of *BRCA1* gene by RFLP technique

The findings of electrophoresis and digestion of the PCR products of the *BRCA1* gene by EcoRI restriction enzyme were revealed two alleles(C,T) and the frequencies in the BC patients (0.05, 0.05), respectively, and in the control (0.525, 0.475), respectively, and three genotypes (CC, CT, TT), and the frequencies in the patients (0, 1, 0), and in the control (0.05, 0.95, 0), respectively, as shown in the table (4-18).

Table (4-18): Allele frequency and genotype frequency in the *BRCA1* gene of the BC patients and control.

Gene	Genotypes	No.Genotypes frequency (Patient)	No.Genotypes frequency (Control)	Alleles	No.Alleles frequency (Patient)	No.Alleles frequency (Control)
BRCA1	CC	0(0)	1(0.05)	C	20(0.05)	21 (0.525)
	CT	20(1)	19(0.95)	T	20(0.05)	19 (0.475)
	TT	0(0)	0(0)	-	-	-

The frequency of the C and T alleles of the *BRCA1* gene figured a slight different findings between the BC patients and the control. The C allele in the BC group recorded in 50% compared to the T allele 50%, while the C allele in the control sample recorded a 52.5% compared to the T allele that recorded as 47.5%, as shown in the table (4-19).

Table (4-19): Alleles and genotype percentages of *BRCA1* polymorphism among patients and control.

Gene	Genotypes	Patient%	Control%	Alleles	Patient%	Control%
BRCA1	CC	0 (0)	1 (5)	C	20 (50)	21(52.5)
	CT	20(100)	19(95)	T	20(50)	19(47.5)
	TT	0(0)	0(0)	-	-	-

The table (4-20) shown the C, T frequency distribution not differ significantly (P= 0.8230; OR= 0.9048; 95%CI= 0.3764-2.1749) between the BC sample and the control sample, and the data showed that the C allele frequency in the patient have the same percent 50% in the control sample, whereas the C allele showed a frequency in the control sample with a high percent 52.5% than the control sample 47.5% among alleles frequency of *BRCA1* gene.

Table (4-20): Alleles frequency of *BRCA1* gene among breast cancer patients and control.

Gene	Allele	Patient%	Control%	OR	95% CI	P-value
BRCA1	C	20(50)	21 (52.5)	0.9048	0.3764-2.1749	0.8230
	T	20(50)	19(47.5)			
% Percentage, OR Odd Ratio,CI Confidence Interval,C (Allele),T(Allele)						
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS =No significance , P >0.05**						

There are no significant differences between the genotypes distribution of the patients and control group of the *BRCA1* gene, (P= 0.311), as shown in the table (4-21), figure (4-5).

Table (4-21): The genotype frequencies of the *BRCA1* gene among the breast cancer patients and control group.

Genes	Genotypes	Patient	Control	Total	X^2	P-value
BRCA1	CC	0	1	1	1.026	0.311
	CT	20	19	39		
	TT	0	0	0		
	Total	20	20	40		
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS=No significance P >0.05**						

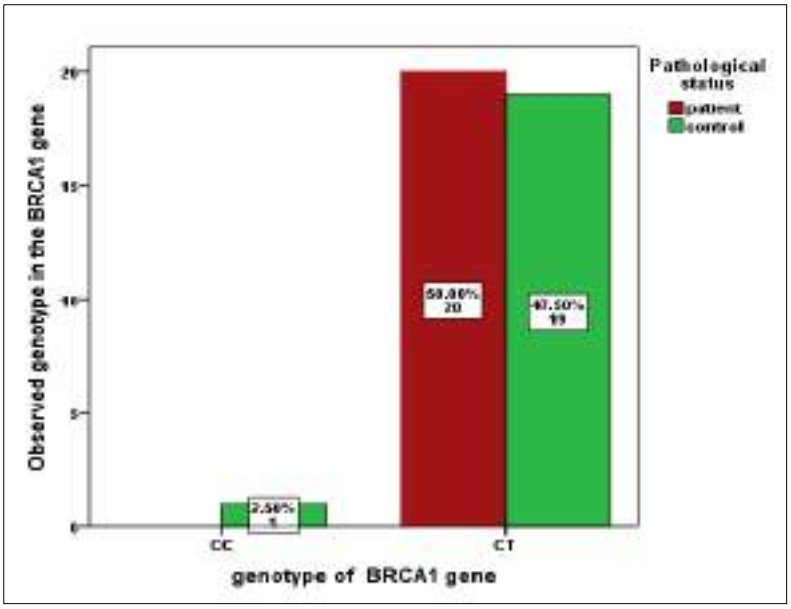


Figure (4-5) The comparison among the numbers and percentages of the CC, CT and TT genotype frequencies of the *BRCA1* gene between BC patients and control group.

Our results showed the genotypes distribution in the BC patient and the control group are no subject to HWE, as significant deviation were recorded between observed and expected genotype in BC patients and control (P= 0.000052), as shown in the table (4-22), (Appendix 5).

Table (4-22): The observed and expected frequencies under Hardy -Weinberg equilibrium in the *BRCA1* gene of the control

Gene	Genotypes	Control (Observed)	Control (Expected)	X^2 (H.W.E)	P-value
BRCA1	CC	1	5.5	16.371	0.000052
	CT	19	10.0		
	TT	0	4.5		
X^2 Chi-square , H.W.E Hardy - Weinberg equilibrium (if P < 0.05 not consistent with H.W.E) at 1 d.f.					

The genetic analysis of the RFLP-PCR technique for the *BRCA1* gene, revealed three genotypes in the BC sample and the control sample, are CC, CT, and TT.

The genotype (CC) have a higher percent in the control sample compared to patients, with 5%, 0%, respectively, and there was no significant difference (P= 0.4898; OR= 0.3171; 95%Cl= 0.0122-8.2610).

The genotype (CT) have a higher percent in the BC patient compared to the control sample, 100%, 95%, respectively, and there was no significant difference between them, (P= 0.4898; OR= 3.1538; 95% CI= 0.1211-82.1697).

The genotype (TT) documented in the same percent in the both sample, 0% without any significant difference (P= 1; OR= 1; 95% CI= 0.0189-52.8531), as shown in the table (4-23).

Table (4-23): Genotype distribution and frequencies of *BRCA1* polymorphism among breast cancer patients and control.

Genes	Genotypes	Patient%	Control%	OR	95% CI	P-value
BRCA1	CC	0 (0)	1(5)	0.3171	0.0122-8.2610	0.4898
	CT	20(100)	19 (95)	3.1538	0.1211-82.1697	0.4898
	TT	0(0)	0 (0)	1	0.0189-52.8531	1.0000
% Percentage , OR Odd Ratio ,CI Confidence Interval,CC homozygous,CT heterozygous ,TT homozygous						
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS=No significance P >0.05**						

Distribution of the *BRCA1*genotypes in the breast cancer patients and control under dominant model showed no any significant differences between both groups, (P= 0.4898), as shown in the table (4-24).

Table (4-24): Distribution of the *BRCA1* genotypes in the breast cancer patients and control under dominant model.

Genes	Genotypes	Patient%	Control%	OR	95% CI	P-value
BRCA1 Dominant model	CC	0(0)	1(5)	0.3171	0.0122-8.2610	0.4898
	TT+CT	20(100)	19(95)			
% Percentage, OR Odd Ratio, CI Confidence Interval, CC homozygous,CT heterozygous ,TT homozygous						
Significance *P <0.05 ,P <0.01 ,***P <0.005 , NS=No significance P >0.05**						

Distribution of the *BRCA1* genotypes in the breast cancer patients and control under recessive model showed no significant differences between two groups, (P= 1). This no significant relation was observed under recessive model, as shown in the table (4-25).

Table (4-25): Distribution of the *BRCA1* genotypes in the breast cancer patients and control under recessive model.

Genes	Genotypes	Patient%	Control%	OR	95% CI	P-value
BRCA1 Recessive model	CC+CT	20(100)	20(100)	1	0.0189-52.8531	1.0000
	TT	0(0)	0(0)			
% Percentage, OR Odd Ratio, CI Confidence Interval, CC homozygous,CT heterozygous ,TT homozygous						
Significance *P <0.05,P <0.01,***P <0.005, NS=No significance P >0.05**						

4-2-2: PCR-RAPD

The DNA gel extraction of PCR product showed the amount of DNA of PCR product (specific gene) ranged from 52.392 to 88.712 ng/µl and absorbance ratio of A260/280 was obtained in the range of 1.71 to1.98 (nm) which was within the normal range (1.7-2.0) nm, using the NanoDrope device for detection. The DNA gel extraction showed a success of the specific gene amplification process. Therefore, PCR products can be use instead of DNA as a template in the RAPD technique. Therefore, this technique is called PCR-RAPD-PCR, as shown in figure (4-6).

Figure (4-6) Extraction of PCR product from the gel, its used to estimate the amount of DNA in PCR mixture after amplification and then choose the appropriated PCR mixture (product) as a template for PCR-RAPD-PCR.

4-2-2-1: PCR-RAPD-PCR (PRP)

The studied population in the PCR-RAPD-PCR technique is composed of 20 verified female BC patients, and 20 control women.

4-2-2-1-1: Detection of the genetic polymorphisms of the BC gene by PCR-RAPD-PCR (PRP) technique

The electrophoresis of the BC gene (PCR product) using RAPD primers showed a clear difference in the number of amplified gene bands and a clear difference in their molecular weights, depending on the primer used. The four primers (OPAA11, OPU 15, OPAA17, and OPD18) were used to study the possibility of determining the genetic polymorphisms of the *ABCG2*, *ABCB1*, *BRCA1*, *ER-α, and mi-RNA 152* gene between both groups. The genetic polymorphisms in the two groups upon the presence or absence of bands resulting from the amplification of certain segments of the genes of samples and the molecular weights of those bands. The results of gene amplification showed that all primers (OPAA11, OPU15, OPAA17, and OPD18) amplified the *ABCG2, ABCB1, BRCA1, ER-α, and mi-RNA 152* genes of the patient and the control group and type of band was determined depending on the size and site of the bands in the field of the gel, as shown in the figure (4-7).

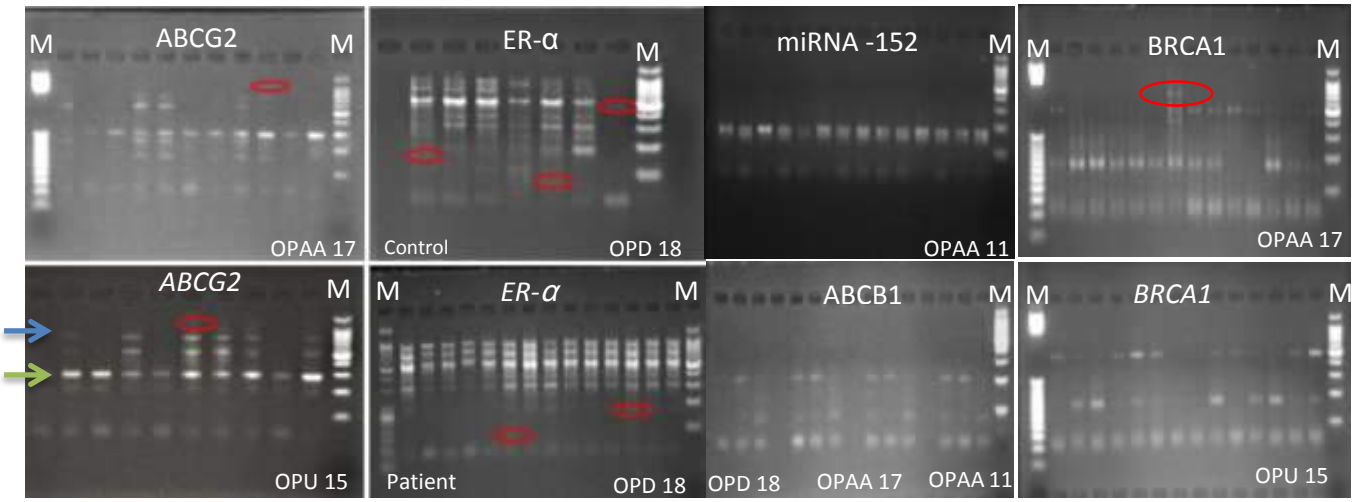


Figure (4-7) PCR-RAPD-PCR profile generated by four primer (OPAA11, OPU15, OPAA17, and OPD 18). The first lane from the right (M) is DNA ladder marker 100-1500bp in size, the last lane from the left (M) is DNA ladder marker 25-300bp in size and the other lanes are PCR-RAPD-PCR bands on 2% agarose gel electrophoresis. Red circles refer to unique bands. Blue arrow refers to the lane of the polymorphic band, green lane refers to monomorphic band.

4-2-2-1-1-1: Detection of the genetic polymorphisms of the *ABCG2* gene by PRP

A marked increase in the number of total bands in the sample of patients compared to the healthy sample, (214 vs.162).

In our findings showed the total number of total bands of *ABCG2* in the patient group was 214 band, the primer OPAA11 was given the highest number of bands (58) bands with highest ratio (27.10%) among other the primers in generating the total bands and the primer OPAA17 was the least one (21.49%), as it gave only 46 bands, compared with 162 bands in the control sample. The OPAA11 primer gave the highest number of bands (45, 27.78%) bands. The primer OPD18 was the least percentage (23.46%), as it gave only 38 bands, as shown in table (4-26), (4-27), figure (4-8).

Table (4 -26): The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ABCG2* gene of breast cancer patient sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of polymorphic bands	Polymorphism%	Primer efficiency	Primer discriminatory power %	Total No. of unique bands	Uniqueness%	Total No. of Monomorphic bands	Monomorphisms%
ABCG2 patient	OPAA11	58	27.10	4	6.8965	0.018	26.66	1	1.724	3	5.17
	OPU 15	56	26.17	6	10.714	0.028	40	0	0	3	5.35
	OPAA17	46	21.49	3	6.5217	0.014	20	5	10.86	3	6.52
	OPD 18	54	25.23	2	3.7037	0.009	13.33	4	7.407	3	5.56
Total		214		15	7.0093			10	4.672	12	5.607

Table (4-27): The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ABCG2* gene of control sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of polymorphic bands	Polymorphism%	Primer efficiency (PB)	Primer discriminatory power %	Total No. of unique bands	Uniqueness%	Total No. of monomorphic bands	Monomorphism%
ABCG2 Control	OPAA11	45	27.78	6	13.333	0.037	42.85	2	4.4444	4	8.89
	OPU15	39	24.07	4	10.256	0.024	28.57	2	5.1282	4	10.25
	OPAA17	40	24.69	2	5	0.012	14.28	0	0	4	10
	OPD18	38	23.46	2	5.2631	0.012	14.28	2	5.2631	4	10.52
Total		162		14	8.6419			6	3.7037	16	9.876

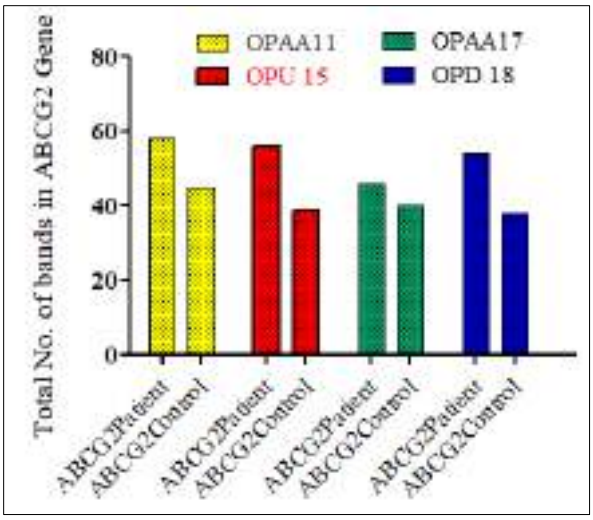


Figure (4-8) Total number of bands in *ABCG2* gene.

In the patient sample, the smallest size fragments were recorded at OPD 18 (20-30) bp, whereas the highest size fragments were recorded at OPAA 11and OPU 15 (990-1000) bp in size, as shown in figure (4-9A). In comparison with control sample the smallest size fragments were recorded at (60 -70) bp at all primers and the highest size fragments were recorded at OPU 15 (1090 - 1100) bp in size, as shown in figure (4-9B). Besides, the primer OPD 18 showed the molecular weight (20-30) bp of the

ABCG2 bands in the patient group and its absence in the other group, as shown in figure (4-9 A, B).

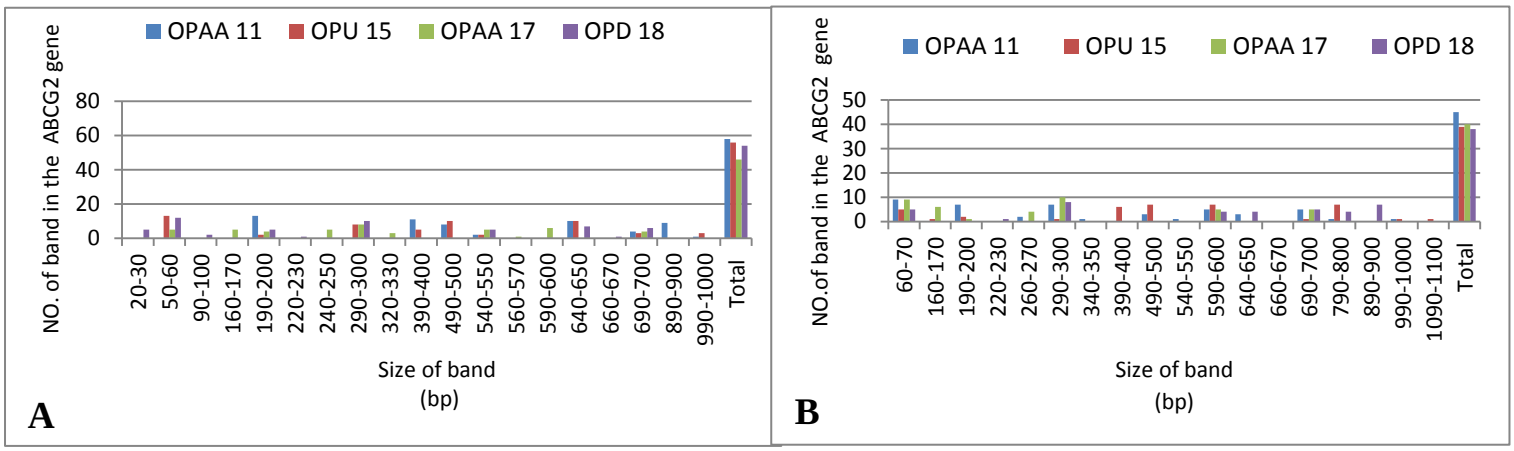


Figure (4-9) (A) Size range of the fragments in *ABCG2* gene of BC patient**. (B)** Size range of the fragments in *ABCG2* gene of control.

The total number of polymorphic bands was 15 bands, polymorphisms 7.00%. The primer OPU15 gave the highest number of polymorphic band (6), compared with the control sample (14, 8.64%) bands. The primer OPAA11 gave the highest number of polymorphic band 6 bands.

The OPU15 have the highest efficiency (0.028). This primer gave the highest percentage of polymorphisms amounted to (10.714%) with a primer discriminatory power of 40%, which is the highest discriminatory power, compared with the control sample. The primer OPAA11 gave the highest number of polymorphic band (6) bands and this is the highest efficiency (0.037). This primer gave the highest percentage of polymorphisms amounted to 13.333% with a primer discriminatory power of 42.85%, which is the highest discriminatory power, as shown in figure (4-10 A, B).

The primer OPD 18 was the least efficient in generating polymorphic bands (0.009), as it gave only two polymorphic bands in BC group. This primer gave the lowest percentage of polymorphisms (3.7037%) with a primer discriminatory power of (13.33%), which is the least a primer discriminatory power, compared with the

control sample. The primer OPAA17 and OPD18 were the least efficient in generating polymorphic band (0.012), which were given only two polymorphic bands for each one. The primer OPAA17 gave the lowest percentage of polymorphisms 5% with a primer discriminatory power of (14.28%) which showed the least a primer discriminatory power, as shown in figure (4-10 A, B).

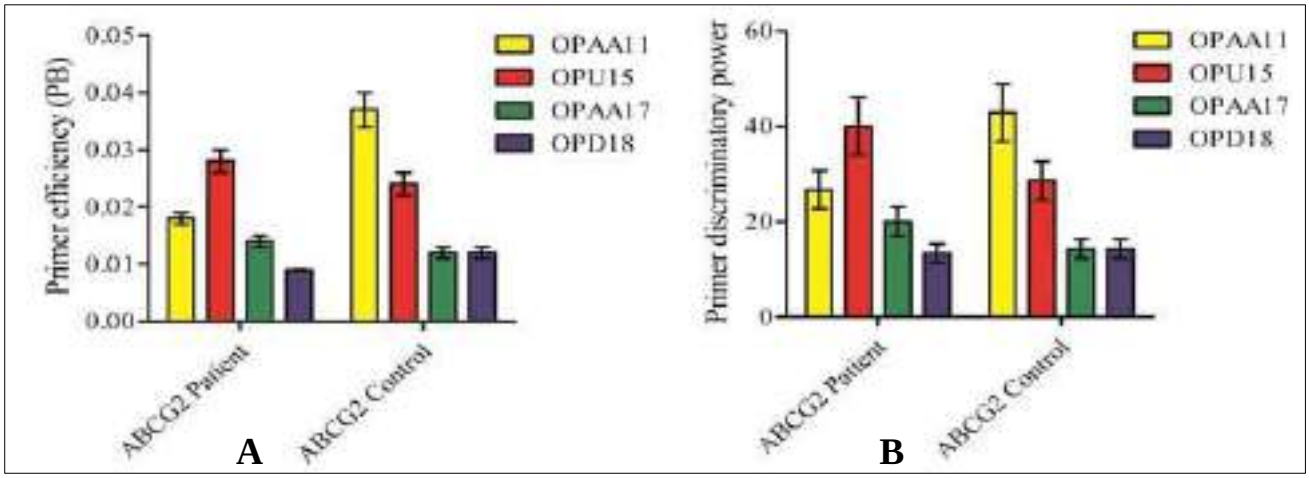


Figure (4-10) (A) Primer efficiency in *ABCG2* gene of BC patient and control. **(B)** Primer discriminatory power in *ABCG2* gene of BC patient and control.

The highest total number of unique band (10) bands in the patient sample compared with (6) bands in the control sample. While the highest total number of monomorphic band (16) band in control sample compared with (12) band in the patient sample.

In the patient sample, the primer OPAA17 was given the highest percentage of uniqueness and monomorphisms reached (10.86% and 6.52%), respectively. The primer OPU15 was given the lowest percentage of uniqueness amounted to 0%, while the primer OPAA11 was gave the lowest monomorphisms that amounted to (5.17%), as shown in table (4-26).

In the control sample, the primer OPD18 gave the highest uniqueness and monomorphisms reached (5.2631% and 10.52%), respectively. The primer OPAA17 was given uniqueness amounted to 0%, while the primer OPAA11 was given the lowest monomorphisms that amounted to (8.89%), as shown in table (4-27).

Although, there was no significant differences in the types of bands between both groups (P=0.451), as shown in table (4-28), figure (4-11).

Table (4-28) Statistical analysis for polymorphic, unique and monomorphic bands of *ABCG2* gene between patient and control samples.

Gene	Type of band	Overall total band	Total No.of band (patient)	Total No.of band (control)	X^2	P-value
ABCG2	Polymorphic band	29	15	14	1.593	0.451
	Unique band	16	10	6		
	Monomorphic band	28	12	16		
	Overall total	73	37	36		
Significance *P <0.05,P <0.01,***P <0.005 , NS=No significant P >0.05**						

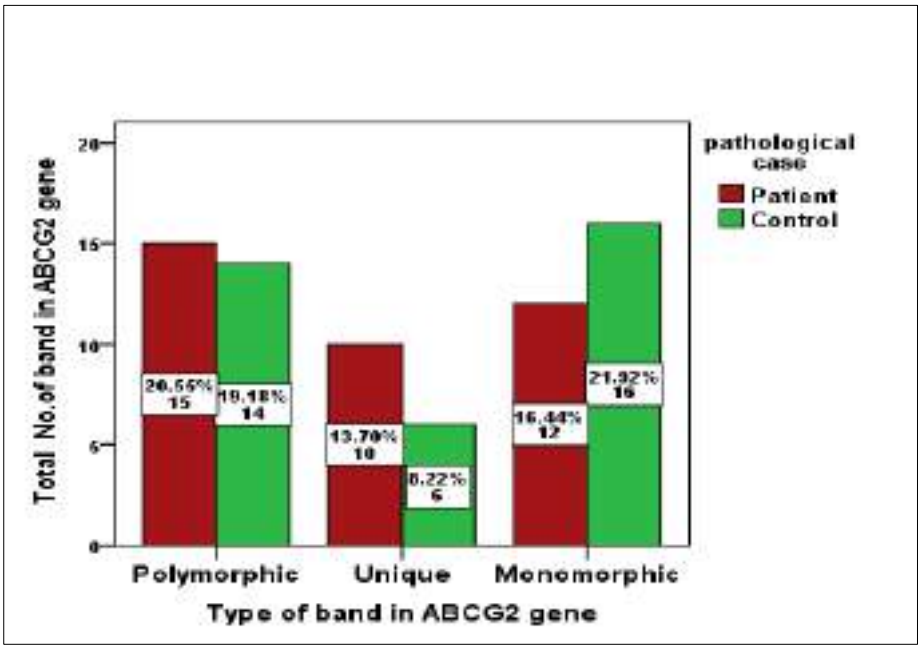


Figure (4-11) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *ABCG2* gene between BC patient and control.

4-2-2-1-1-2 Detection of the genetic polymorphisms of the *ABCB1* gene by PRP

A marked increase in the number of total bands in the sample of patients compared to the control sample (215 vs. 114) bands.

The total number of total bands of *ABCB1* gene in the patient sample was 215 band. The primer OPAA11 was given the highest number of (57) bands and it has the highest primer ratio (26.51%) in generating the total bands, while the primer OPU15 was the least (23.73%), as it gave only (51) bands, compared with control sample (114) bands, the primer OPD 18 was given about (34) bands out of the total bands and it has highest ratio (29.82%) among others, while the primer OPU 15 was the least ratio (19.30%), as it gave only (22) bands, as shown in table (4-29), (4-30), figure (4-12).

Table (4-29): The numbers and percentages of the total band, polymorphic, unique, monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ABCB1* gene of breast cancer patient sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of polymorphic bands	Primer efficiency (PB)	Polymorphism %	Primer discriminatory power %	Total No. of unique bands	Uniqueness %	Total No. of Monomorphic bands	Monomorphisms %
ABCB1 patient	OPAA11	57	26.51	6	0.027	10.5263	24	2	3.5087	1	1.75
	OPU 15	51	23.73	5	0.023	9.80392	20	1	1.9607	1	1.96
	OPAA17	54	25.12	6	0.027	11.111	24	2	3.7037	1	1.85
	OPD 18	53	24.65	8	0.037	15.0943	32	3	5.6603	1	1.89
Total		215		25		11.6279		8	3.7209	4	1.860

Table (4-30): The numbers and percentages of the total band, polymorphic, unique, monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ABCB1* gene of control sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of polymorphic bands	Polymorphism %	Primer efficiency	Primer discriminatory power %	Total No. of unique bands	Uniqueness%	Total No. of monomorphic bands	Monomorphisms %
ABCB1 Control	OPAA11	32	28.07	4	12.5	0.035	30.76	0	0	1	3.125
	OPU15	22	19.30	4	18.1818	0.035	30.76	1	4.5454	1	4.55
	OPAA17	26	22.81	1	3.84615	0.008	7.69	2	7.6923	1	3.85
	OPD18	34	29.82	4	11.7647	0.035	30.76	0	0	1	2.94
Total		114		13	11.4035			3	2.6315	4	3.508

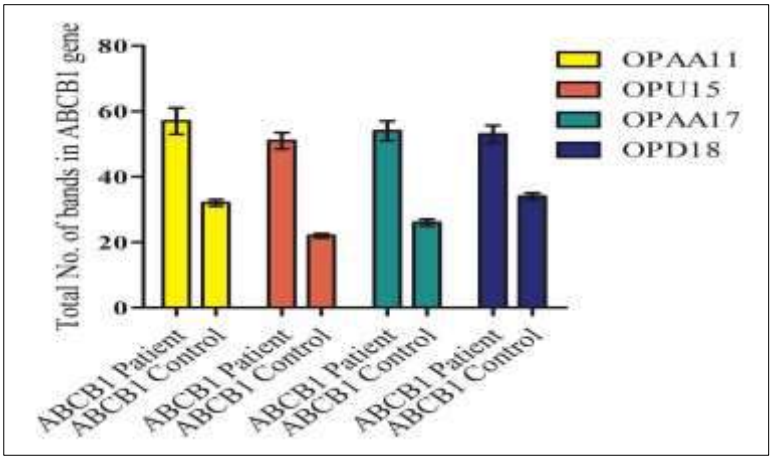


Figure (4-12) Total number of bands in *ABCB1* gene.

In the patient sample, the smallest size fragments were recorded at OPAA 17 (20 - 30) bp while the highest size fragments were recorded at OPAA 11, OPD18 (990-1000) bp in size, as shown in figure (4-13 A), compared with the control sample the smallest size fragments were recorded at OPAA 11, OPU 15, OPD 18 (20-30) bp and the highest size fragments were recorded at OPAA11, OPD18 (290-300) bp, as shown in figure (4-13 B). The primer OPAA 17 showed the molecular weight (20-30) bp of the *ABCB1* bands in the patient group and its absence from the control group, as shown in figure (4-13 A, B).

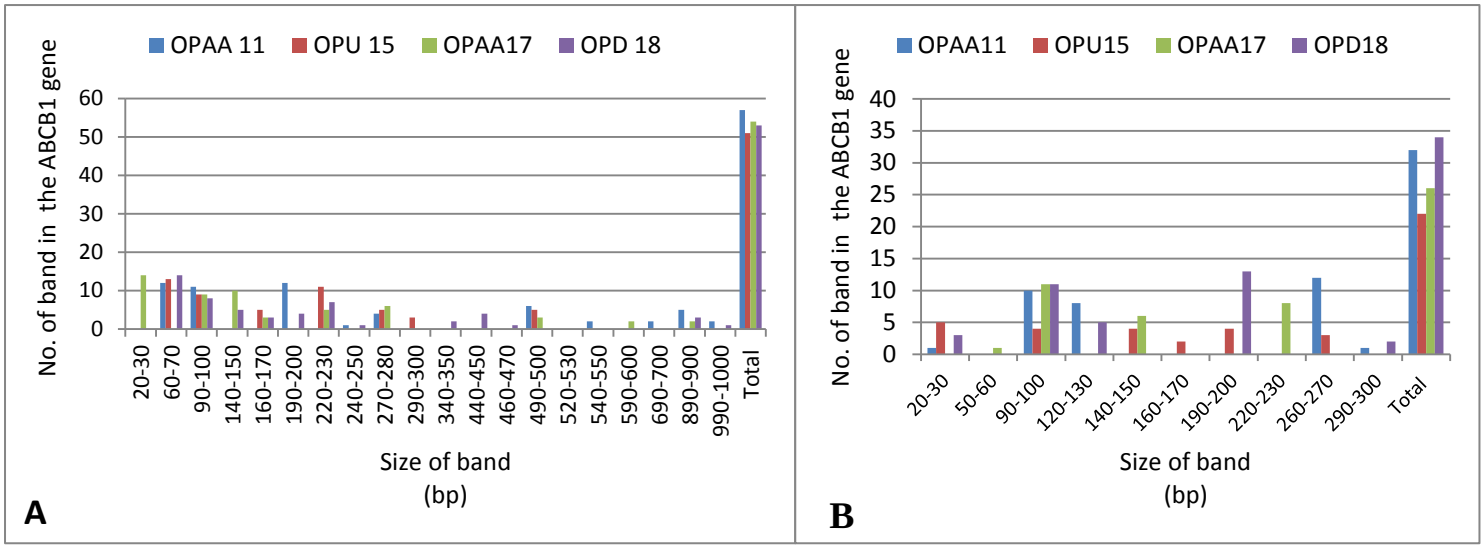


Figure (4-13) (A) Size range of the fragments in *ABCB1* gene of BC patient**. (B)** Size range of the fragments in *ABCB1* gene of control.

In patient sample, the total number of the polymorphic band was 25 band, polymorphisms(11.63%), the primer OPD18 gave the highest number of polymorphic band (8) bands, compared with the control sample the total number of the polymorphic band was (13) band, polymorphisms (11.4 %), the primers OPAA11, OPU15, and OPD18 were given the same number of the polymorphic band (4) bands.

In the patient sample, OPD18 has the highest primer efficiency (0.037). This primer gave the highest polymorphisms amounted to (15.09%) with a primer discriminatory power of (32%), which is the highest discriminatory power, compared with control sample OPAA11, OPU15, and OPD 18 have the highest efficiency (0.035), and the primer OPU15 was given the highest percentage of polymorphisms amounted to (18.18%) with a primer discriminatory power of (30.76 %), which is the highest discriminatory power, as shown in figure (4-14 B).

In the patient sample, the primer OPU15 was the least efficient in generating polymorphic bands (0.023), as it gave only five polymorphic bands, it gave the lowest polymorphisms (9.8%) with a primer discriminatory power of (20%) and it has the least a primer discriminatory power, compared with control sample the primer OPAA17 was the least efficient in generating polymorphic bands (0.008), as it gave

only one polymorphic band, it has the lowest polymorphisms (3.85%) with a primer discriminatory power of (7.69%) that it is the least a primer discriminatory power, as shown in figure (4-14 A, B).

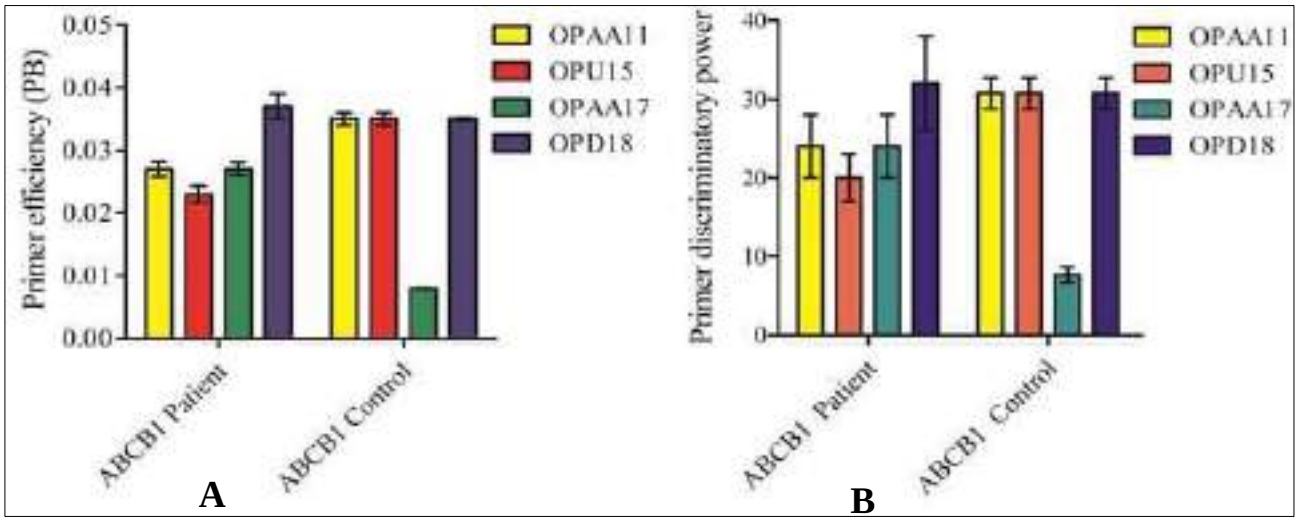


Figure (4-14) (A) Primer efficiency in *ABCB1* gene of BC patient and control**. (B)** Primer discriminatory power in *ABCB1* gene of BC patient and control.

Our results showed that the highest total number of the unique band (8) in the patient sample compared with (3) band in the control sample. While the total number of the monomorphic band was the same (4 bands) in the patient and control sample.

In the patient sample, the primer OPD18 was given the highest uniqueness reached (5.66%), while the primer OPU15 was given the lowest uniqueness amounted to (1.96 %) and the primer OPU15 gave the highest monomorphisms reached (1.96%), while the primer OPAA11 was gave the lowest monomorphisms that amounted to (1.75%), as shown in table (4-29).

In the control group, the primer OPAA17 was given the highest uniqueness reached (7.96%), while the primer OPAA11, OPD18 was not given any uniqueness. The primer OPU 15 gave the highest monomorphisms reached (4.55%), while the primer OPD 18 was given the lowest monomorphisms that amounted to (2.94%), as shown in table (4-30).

Although, there were no significant differences in the types of bands between two samples (P= 0.580), as shown in table (4-31), figure (4-15).

Table (4-31) Statistical analysis for polymorphic, unique and monomorphic bands of *ABCB1* gene between patient and control samples.

Gene	Type of band	Overall total band	Total No.of band (patient)	Total No.of band (control)	X^2	P-value
ABCB1	Polymorphic band	38	25	13	1.089	0.580
	Unique band	11	8	3		
	Monomorphic band	8	4	4		
	Overall total	57	37	20		
Significance *P <0.05,P <0.01,***P <0.005,NS=No significant P >0.05**						

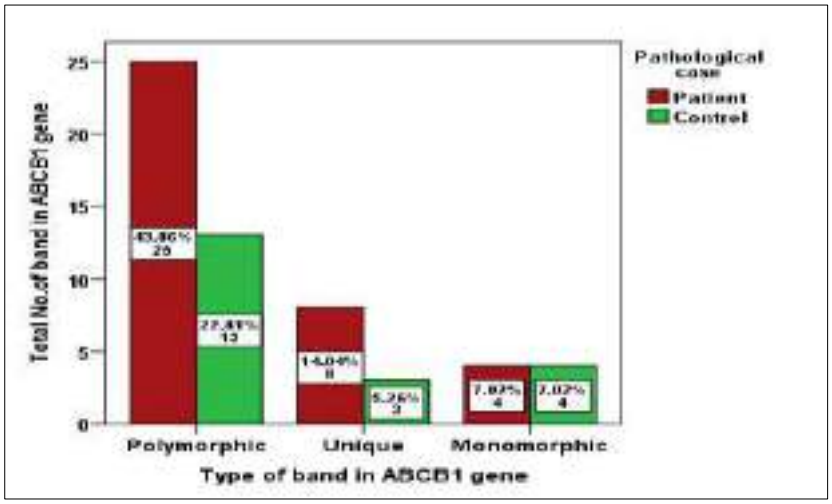


Figure (4-15) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *ABCB1* gene between BC patient and control.

4-2-2-1-1-3: Detection of the genetic polymorphisms of the *BRCA1* gene by PRP

There is a marked increase in the number of total bands in the sample of patients compared to the control sample, (206 vs. 126) band.

The total number of total bands of *BRCA1* gene in patient sample was 206 band, the primer OPU15 was gave the highest number of bands 68 bands, it has highest primer percentage ratio (33.01%) among others, while the primer OPD 18 was the least one (20.39%), as it gave only (42) bands, compared with (126) bands in the control sample. The primer OPAA11 was given the highest number of bands as (42) bands. It has highest primer ratio (33.33%) among the primers, the primer OPD18 was the least (19.84%), as it gave only (25) bands, as shown in table (4-32), (4-33), figure (4-16).

Table (4-32): The numbers and percentages of total band, polymorphic, unique, monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *BRCA1* gene of breast cancer patient sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of polymorphic bands	Polymorphism %	Primer efficiency	Primer discriminatory power %	Total No. of unique bands	Uniqueness %	Total No. of Monomorphic bands	Monomorphism%
BRCA1 Patient	OPAA11	51	24.757	3	5.8823	0.014	15.78	3	5.88235	3	5.88
	OPU 15	68	33.01	5	7.3529	0.024	26.31	3	4.41176	3	4.41
	OPAA17	45	21.84	5	11.11	0.024	26.31	2	4.4444	3	6.67
	OPD 18	42	20.39	6	14.285	0.029	31.57	3	7.14285	3	7.14
Total		206		19	9.2233			11	5.3398	12	5.825

Table (4-33): The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *BRCA1* gene of control sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of Polymorphic bands	Polymorphism %	Primer efficiency	Primer discriminatory power %	Total No. of Unique bands	Uniqueness %	Total No. of Monomorphic bands	Monomorphisms %
BRCA1 control	OPAA11	42	33.33	3	7.14285	0.023	37.5	1	2.38095	3	7.14
	OPU 15	30	23.81	2	6.66666	0.015	25	2	6.66666	3	10
	OPAA17	29	23.06	3	10.3448	0.023	37.5	1	3.44827	3	10.34
	OPD 18	25	19.84	0	0	0	0	4	16	3	12
Total		126		8	6.34920			8	6.34920	12	9.523

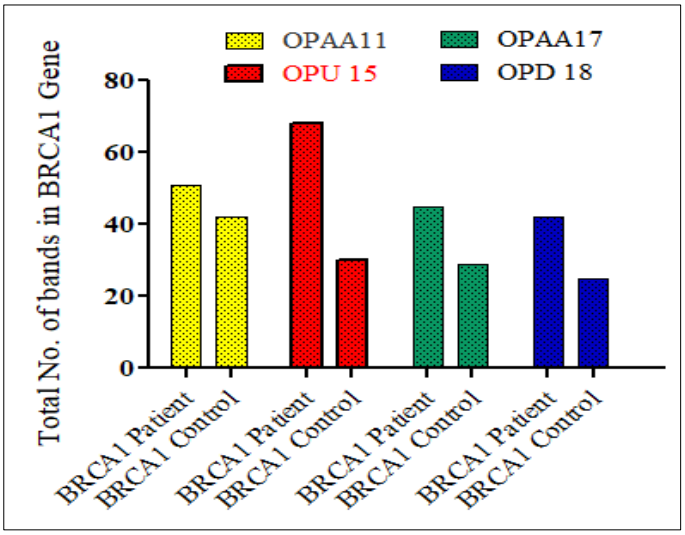


Figure (4-16) Total number of bands in *BRCA1* gene.

In the patient sample, the smallest size fragments were recorded at OPAA17 (20-30) bp, while the highest size fragments were recorded at OPU15 (990-1000) bp in size, as shown in figure (4-17A).When compared with the control sample the smallest size fragments were recorded at all primer (50-60) bp and the highest size fragments were recorded at OPAA17(1090-1100) bp in size, as shown in figure (4-17 B). The primer OPAA17 showed the molecular weight (20-30) bp of the *BRCA1*

bands in the patient group and its missed from the control group, as shown in figure (4-17 A, B).

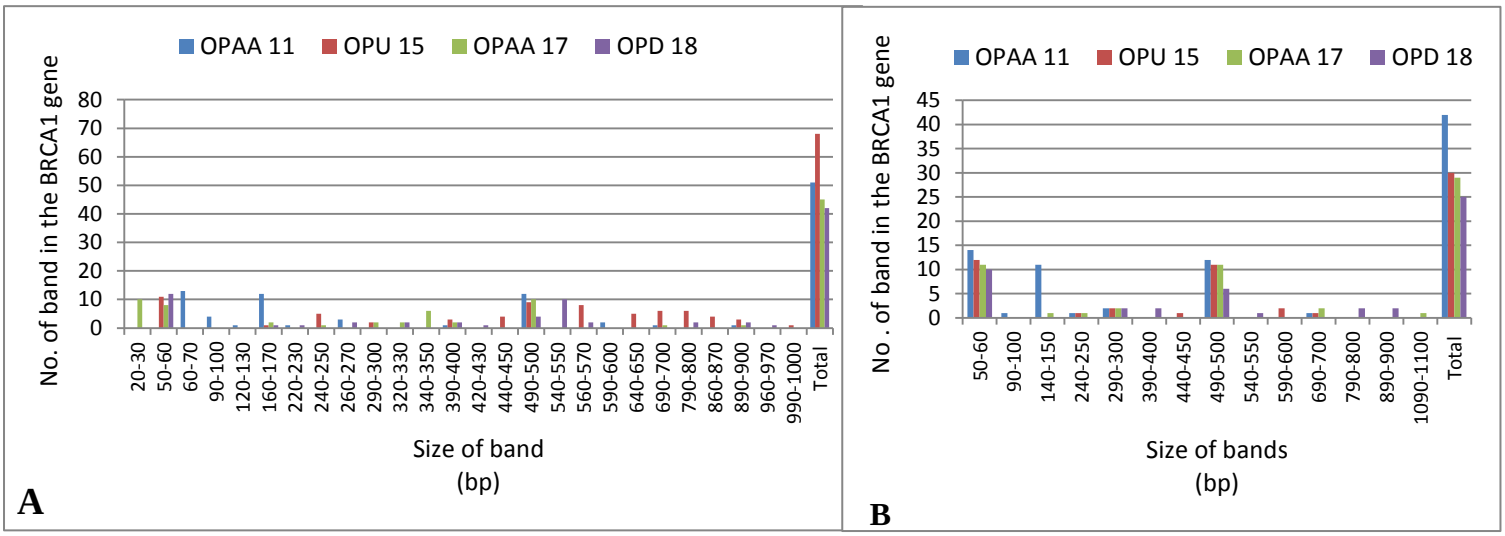


Figure (4-17) (A) Size range of the fragments in *BRCA1* gene of BC patient**. (B)** Size range of the fragments in *BRCA1* gene of control.

In the patient sample, the total number of the polymorphic band was 19 band, polymorphisms (9.22%), the primer OPD 18 gave the highest number of polymorphic band (6 vs.8) bands, polymorphisms was 6.35%, the primer OPAA11, OPAA17 gave the highest number of polymorphic band (3) bands.

In the patient sample, the OPD18 primer has the highest efficiency (0.029). This primer gave the highest polymorphisms amounted to (14.29%) with a primer discriminatory power of (31.57%), which is the highest discriminatory power, compared with the control sample the primer OPAA11, OPAA17 have the highest primer efficiency (0.023). The OPAA17 primer gave the highest polymorphisms amounted to (10.34%) with a primer discriminatory power of (37.5%), which is the highest discriminatory power, as shown in figure (4-18 A, B).

In the patient sample, the primer OPAA11 gave the least efficient in generating polymorphic bands (0.014), as it gave only three polymorphic bands, it gave the lowest polymorphisms (5.88%) with a primer discriminatory power of (15.78%), and it has the least a primer discriminatory power, compared with control sample the

primer OPD18 was the least efficient in generating polymorphic band, it gave no polymorphic bands with no percent and no primer discriminatory power, as shown in figure (4-18 A, B).

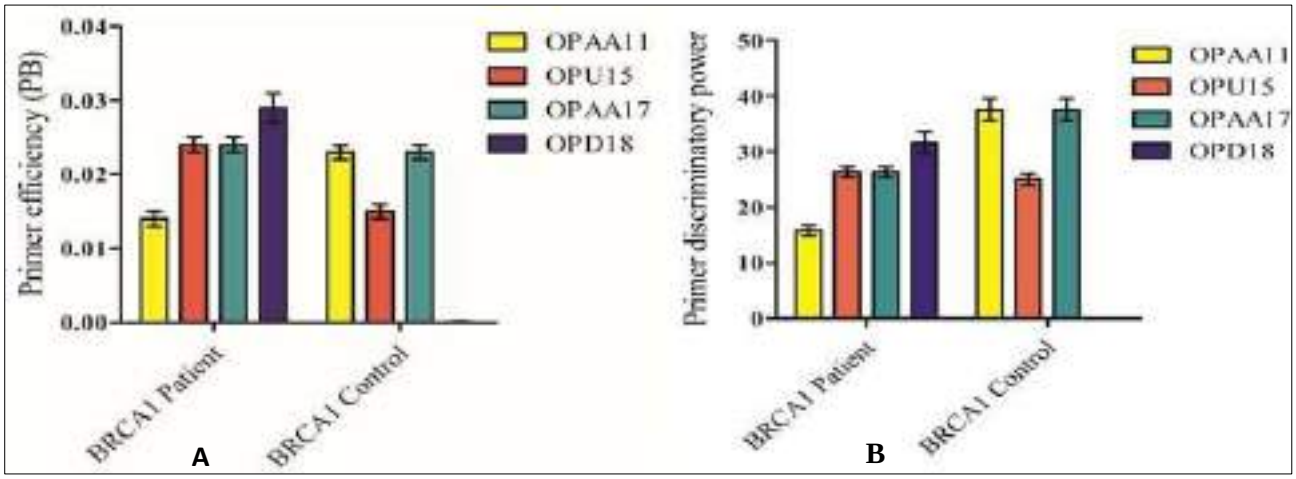


Figure (4-18) (A) Primer efficiency in *BRCA1* gene of BC patient and control. **(B)** Primer discriminatory power in *BRCA1* gene of BC patient and control.

The highest total number of unique band (11) bands in the patient sample, compared with (8) bands in the control sample.Whereas the total number of the monomorphic band was the same 12 bands in both samples.

In the patient sample, the primer OPD18 was given the highest uniqueness and monomorphisms reached (7.14285, 7.14%), respectively, whereas the primer OPU15 was given the lowest uniqueness and monomorphisms amounted to (4.41176, 4.41%), respectively, as shown in table (4-32). In comparison with control sample the primer OPU15 was given the highes uniqueness and monomorphisms reached (16 %,12%), respectively, while the primer OPAA11was given the lowest uniqueness and monomorphisms amounted to (2.38%, 7.14 %), respectively, as in table (4-33).

Although, there were no significant differences in the types of bands between the patient sample and the control sample, (P= 0.325), as shown in table (4-34), figure (4-19).

Table (4-34): Statistical analysis for polymorphic, unique and monomorphic bands of *BRCA1* gene between patient and control samples.

Gene	Type of band	Overall total band	Total No.of band (patient)	Total No.of band (control)	X^2	P-value
BRCA1	Polymorphic band	27	19	8	2.245	0.325
	Unique band	19	11	8		
	Monomorphic band	24	12	12		
	Overall total	70	42	28		
Significance *P <0.05,P <0.01,***P <0.005 , NS=No significance P >0.05**						

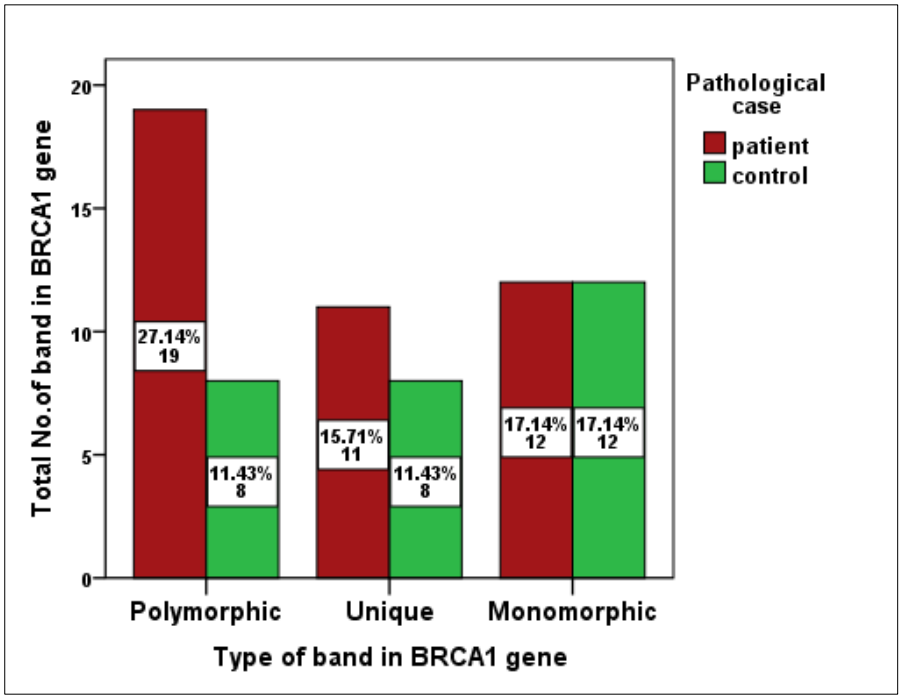


Figure (4-19) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *BRCA1* gene between BC patient and control.

4-2-2-1-1-4: Detection of the genetic polymorphisms of the *ER-α* gene by PRP.

A marked strongest increasing in the number of total bands in the sample of patient compared to the control sample, (298 vs. 128) bands.

The total number of total bands of *ER-α* in the patient group was 298 band , the primer OPD18 was given the highest number of bands (86), it has the highest primer ratio (28.85%) others and the primer OPAA17 was the least (17.44%), as it gave only 52 bands, compared with (128) bands in the control sample, the primer OPD18 was given the highest number of bands (44) and it has highest primer ratio (34.38%) among the primers rates and the primer OPAA11 was the least (14.06%), as it gave only (18) bands, as shown in table (4-35), (4-36), figure (4-20).

Table (4-35): The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ER-α* gene of breast cancer patient sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer band %	Total No. of polymorphic bands	Primer efficiency	Polymorphisms %	Primer discriminatory power %	Total No. of unique bands	Uniqueness %	Total No. of monomorphic bands	Monomorphisms %
ER-α patient	OPAA11	81	27.18	5	0.016	6.1728	27.77	2	2.46913	4	4.93
	OPU15	79	26.51	3	0.010	3.7974	16.66	0	0	4	5.06
	OPAA17	52	17.44	4	0.013	7.6923	22.22	1	3.84615	4	7.69
	OPD18	86	28.85	6	0.020	6.9767	33.33	2	2.32558	4	4.65
Total		298		18		6.0402		5	1.67785	16	5.3691

Table (4-36): The numbers and percentages of the total band, polymorphic, unique, monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *ER-α* gene of control sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of polymorphic bands	Polymorphism %	Primer efficiency (PB)	Primer discriminatory power %	Total No. of unique bands	Uniqueness %	Total No. of monomorphic bands	Monomorphism %
ER-α Control	OPAA11	18	14.06	8	44.4444	0.062	23.52	1	5.5555	0	0
	OPU15	41	32.03	10	24.3902	0.078	29.41	1	2.43902	0	0
	OPAA17	25	19.53	7	28	0.054	20.58	2	8	0	0
	OPD18	44	34.38	9	20.4545	0.070	26.47	3	6.8181	0	0
Total		128		34	26.5625			7	5.46875	0	0

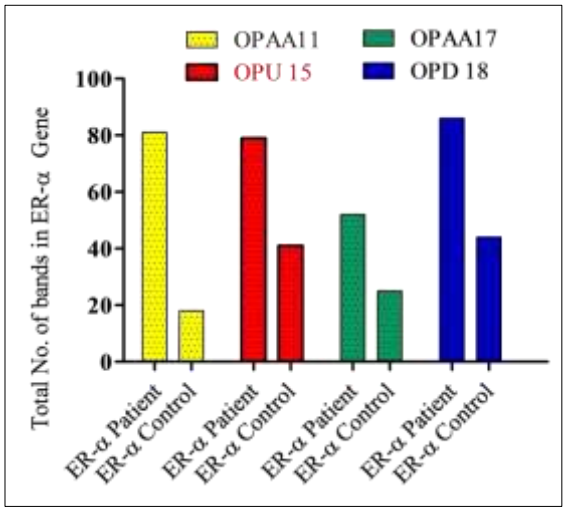


Figure (4-20) Total number of bands in *ER-α* gene.

In the patient sample the smallest size fragments were recorded at OPAA11, OPD 18 (40-50) bp, whereas the highest size fragments were recorded at all primer (890 - 900) bp in size, as shown in figure (4-21A). When compared with control sample the smallest size fragments were recorded at OPU15, OPAA 17, and OPD 18 and the highest size fragments were recorded at OPAA11, OPU15, and OPD 18 (890-900) bp in size, as shown in figure (4-21B). The primer OPAA11 showed the molecular weight (40-50) bp of the ER-α bands in the patient group and its not found in the control group, as shown in figure (4-21 A, B).

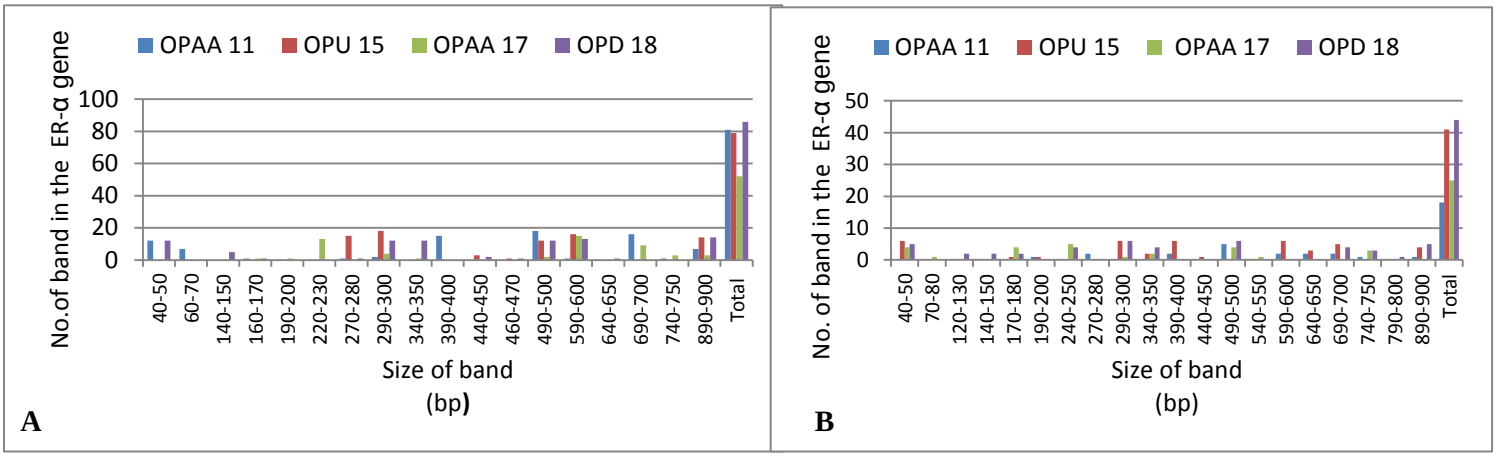


Figure (4-21) (A) Size range of the fragments in *ER-α* gene of BC patient**. (B)** Size range of the fragments in *ER-α* gene of control.

In the patient sample, the total number of the polymorphic band was 18 band, the polymorphisms was (6.04%), the primer OPD18 gave the highest number of polymorphic band (6), compared with the control sample the total number of the polymorphic band was (34) bands, and the polymorphisms was 26.56 %. The primer OPU15 gave the highest number of polymorphic band (10) bands.

In the patient sample, primer OPD18 has the highest efficiency (0.020). Despite this, the primer OPAA17 was given the highest polymorphisms amounted to (7.69 %) with a primer discriminatory power of 22.22%. The highest discriminatory power was 33.33% for OPD18, compared with the control sample the primer OPU15 has the highest efficiency (0.078). Despite this, the primer OPAA11was given the highest polymorphisms amounted to (44.44%) with a primer discriminatory power of 23.52 %.The highest discriminatory power was 29.41% for OPU15, as shown in figure (4-22 A, B).

In the patient sample, the primer OPU15 was the least efficient in generating polymorphic bands (0.010), as it gave only three polymorphic bands, this primer gave the lowest polymorphisms (3.79 %) with a primer discriminatory power of 16.66%, and it has the least a primer discriminatory power, compared with the control sample the primer OPAA 17 was the least efficient in generating polymorphic bands(0.054), as it gave seven polymorphic bands. Despite this, the OPD18 primer gave the lowest

polymorphisms amounted to (20.45%) with a primer discriminatory power of 26.47%. The lowest discriminatory power was 20.58% for OPAA17, as shown in figure (4-22 B).

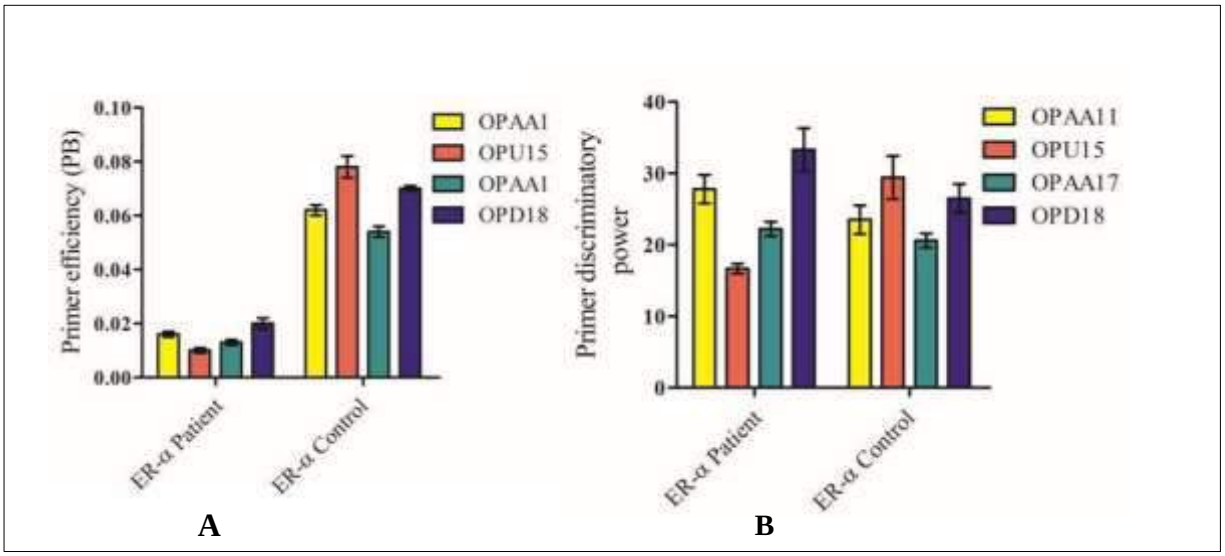


Figure (4-22) (A) Primer efficiency in ER-α gene of BC patient and control**. (B)** Primer discriminatory power in *ER-α* gene of BC patient and control.

The highest total number of the unique band (7) in the control sample compared with five bands in the patient sample, while the highest total number of the monomorphic band was in the patient sample (16) bands compared with no band in the control sample.

In the patient sample, the primer OPAA17 was given the highest uniqueness and monomorphisms reached (3.85%, 7.69%), respectively. The primer OPU15 was given no uniqueness, the primer OPD18 was given the lowest monomorphisms amounted to (4.65%), as shown in table (4-35), compared with the control sample the primer OPD18 gave the highest uniqueness reached (6.82%) and the primer OPAA11 was given the lowest uniqueness amounted to 2.44%, the monomorphisms amounted to zero, as shown in table (4-36).

There were significant differences in the types of bands between both samples, (P= 0.000), as shown in the table (4-37), figure (4-23).

Table (4-37): Statistical analysis for polymorphic, unique and monomorphic bands of *ER-α* gene between patient and control samples.

Gene	Type of band	Overall total band	Total No.of band (patient)	Total No.of band (control)	X^2	P-value
ER-α	Polymorphic band	52	18	34	21.220	0.000***
	Unique band	12	5	7		
	Monomorphic band	16	16	0		
	Overall total	80	39	41		
Significance *P<0.05,P<0.01,***P<0.005 , NS=No significant P>0.05**						

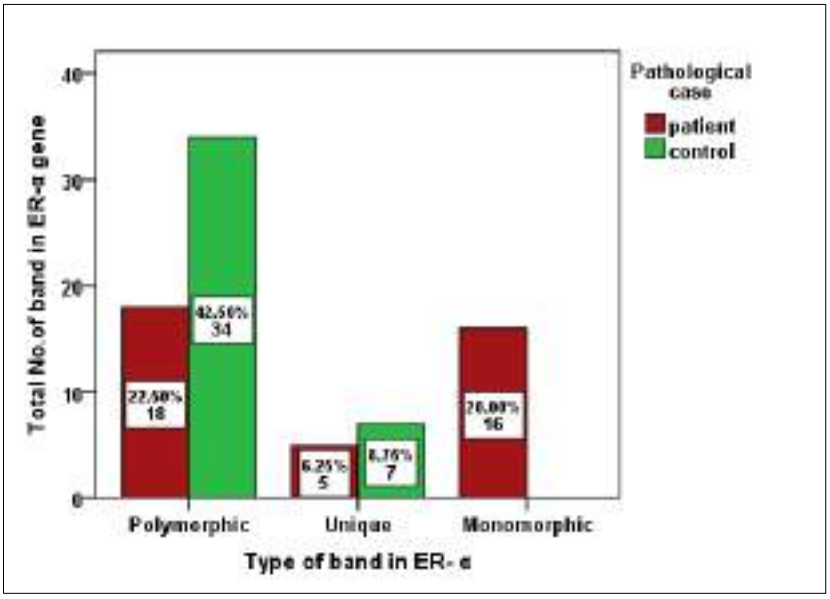


Figure (4-23) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *ER-α* gene between BC patient and control.

4-2-2-1-1-5: Detection of the genetic polymorphisms of the *miRNA-152* gene by PRP

There is a marked increase in the number of total bands in the BC samples compared to the control samples, (149 vs. 93) bands.

The total number of total bands of *miRNA-152* gene was (149) bands, the primer OPAA11 was given the highest number of bands (43), it has the highest primer ratio (28.85 %) among other primers, while the primer OPD18 was the least (21.48%), as it gave only (32) bands, compared with control sample, the total number of total bands were (93) bands, the primer OPAA11 and OPAA17 were given the highest number of bands (24) and they have the highest primer ratio (25.81%) among, while the primer OPD 18 was the least (23.66%), as it gave only (22) bands, as shown in table (4-38), (4-39), figure (4-24).

Table (4-38): The numbers and percentages of the total band, polymorphic, unique, monomorphic band and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *mi RNA-152* gene of breast cancer patient sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer band %	Total No. of polymorphic bands	Polymorphism %	Primer efficiency	Primer discriminatory power %	Total No. of unique bands	Uniqueness %	Total No. of monomorphic bands	Monomorphisms%
miRNA patient	OPAA11	43	28.85	2	4.651162	0.013	25	1	2.32558	2	4.65
	OPU15	38	25.50	2	5.263157	0.013	25	1	2.631578	2	5.26
	OPAA17	36	24.16	2	5.555555	0.013	25	0	0	2	5.56
	OPD18	32	21.48	2	6.25	0.013	25	0	0	2	6.25
Total		149		8	5.369127			2	1.342281	8	5.369

Table (4-39): The numbers and percentages of the total band, polymorphic, unique, monomorphic band, and primer efficiency and primer discriminatory power that produced from amplified four primer RAPD in *miRNA -152* gene of control sample.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of polymorphic bands	Polymorphism%	Primer efficiency (PB)	Primer discriminatory power %	Total No. of unique bands	Uniqueness, %	Total No. of monomorphic bands	Monomorphisms%
miRNA Control	OPAA11	24	25.81	0	0	0	0	0	0	3	12.5
	OPU15	23	24.73	0	0	0	0	0	0	3	13.04
	OPAA17	24	25.81	0	0	0	0	0	0	3	12.5
	OPD18	22	23.66	0	0	0	0	0	0	3	13.63
Total		93		0	0			0	0	12	12.903

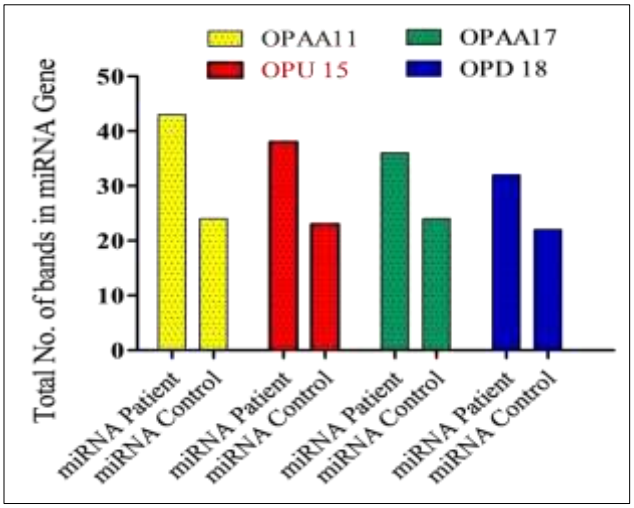


Figure (4-24) Total number of bands in *miRNA-152* gene.

In the patient sample, the smallest size fragments were recorded at OPAA 17, OPD 18 (20-40) bp, whereas the highest size fragments were recorded at OPAA11, OPU 15(290-300) bp in size, as shown in figure (4-25 A). When shifted to the control sample the smallest size fragments were recorded at all primer (50-60) bp and the highest size fragments were recorded at all primer (170-180) bp, as shown in figure (4-25 B). Our results showed that the primer OPAA17, OPD18 showed the molecular weight (20-40) bp of the *mi-RNA 152* bands in the patient group and not found in the control group, as shown in figure (4-25 A, B).

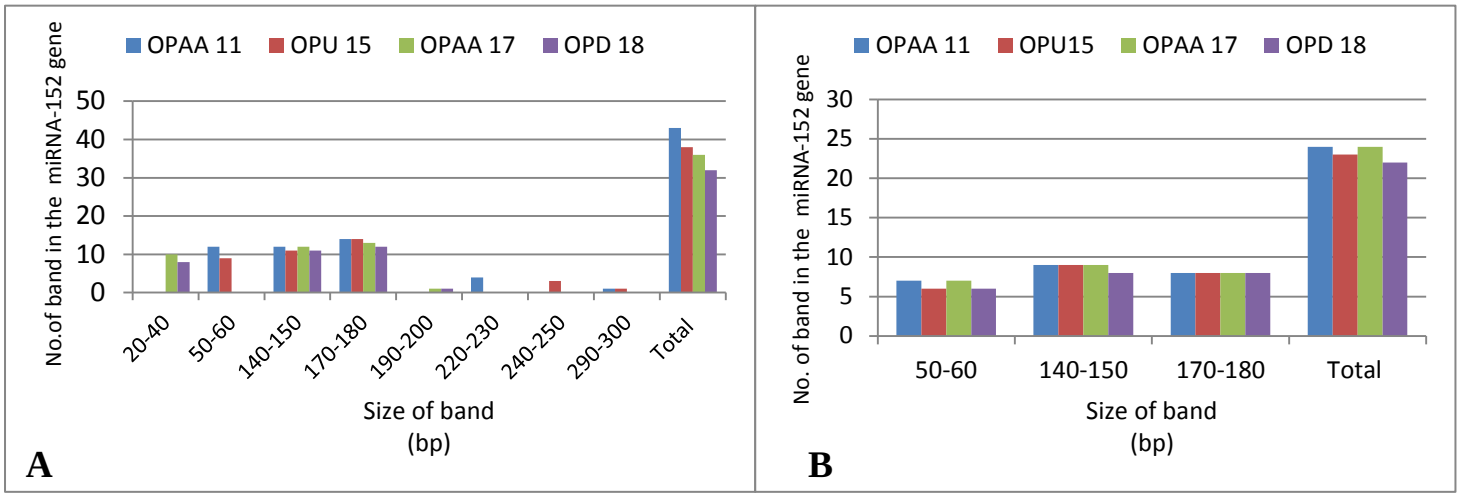


Figure (4-25) (A) Size range of the fragments in *miRNA-152* gene of BC patient. **(B)** Size range of the fragments in *miRNA-152* gene of control.

The total number of the polymorphic band was (8) bands, polymorphisms percentage was 5.37%, the four primer OPAA11, OPU15, OPAA17, and OPD18 were given the same number of polymorphic band (2) band, compared with the control sample, no polymorphic band, the four primer OPAA11, OPU15, OPAA17, and OPD18 were given no polymorphic band.

Approximately, four primers of BC samples have the same primer efficiency (0.013). The primer OPD18 was given the highest polymorphisms amounted to (6.25%) with a primer discriminatory power of (25%), whereas the four primers have the same discriminatory power (25%), compared with the control sample, the four primers have not primer efficiency, and have not primer discriminatory power, as shown in figure (4-26 A, B).

In the patient sample, the four primers have the same primer efficiency (0.013). The primer OPAA11 was given the lowest polymorphisms (4.65%) with a primer discriminatory power of 25%, compared with the control sample the four primers were not given any polymorphisms and primer discriminatory power, as shown in figure (4-26 A, B).

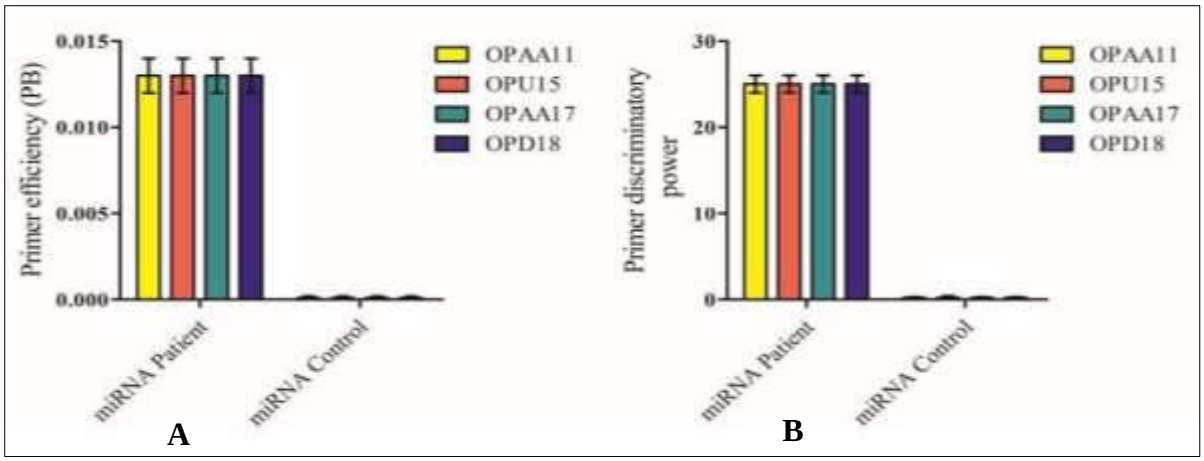


Figure (4-26) (A) Primer efficiency in *miRNA-152* gene of BC patient and control**. (B)** primer discriminatory power in *miRNA-152* gene of BC patient and control.

In the patient sample, the highest total number of unique band and the monomorphic band were (2, 8), respectively, as in the table (4-38), compared with the control sample the highest total number of unique and monomorphic bands were (0, 12), respectively, as in the table (4-39).

In the patient sample, the primer OPU15 gave the highest uniqueness reached (2.63%), the primers OPAA17 and OPD 18 were not given any uniqueness, the primer OPD18 gave the highest monomorphisms reached (6.25%). The primer OPAA11 was gave the lowest monomorphisms amounted to (4.65%), as shown in table (4-38).

In comparison with the control sample, the four primers not gave any uniqueness, the primer OPD18 was given the highest monomorphisms amounted to (13.63%), while the primer OPAA11 and OPAA17 were given the lowest monomorphisms amounted to (12.5%), as shown in the table (4-39).

Furthermore, there were strongest significant differences in the types of bands between both samples, (P= 0.007), as shown in table (4-40), figure (4-27).

Table (4-40) Statistical analysis for polymorphic, unique and monomorphic bands of *mi-RNA 152* gene between patient and control samples.

Gene	Type of band	Overall total band	Total No.of band (patient)	Total No.of band (control)	X^2	P-value
mi-RNA 152	Polymorphic band	8	8	0	10.000	0.007**
	Unique band	2	2	0		
	Monomorphic band	20	8	12		
	Overall total	30	18	12		
Significance*P <0.05,P <0.01,***P <0.005 , NS=No significance P >0.05**						

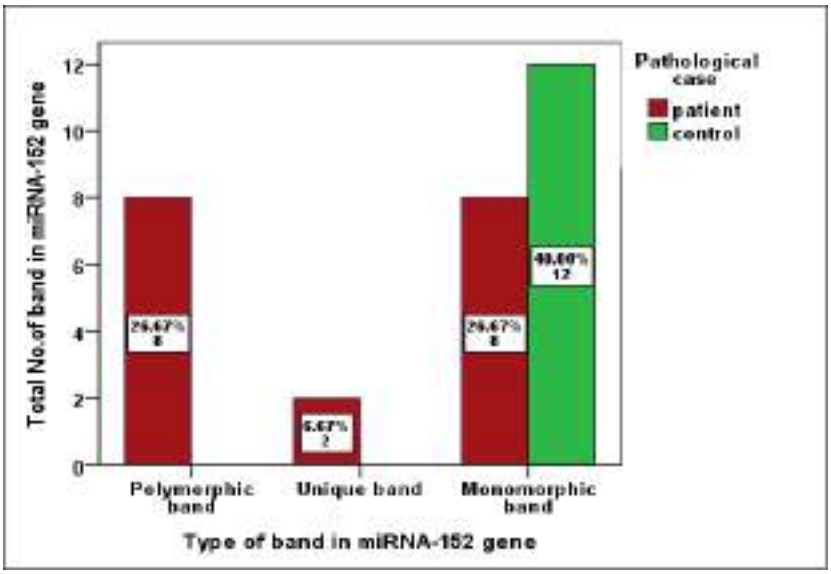


Figure (4-27) Statistical numbers and percentages of the total polymorphic, unique and monomorphic bands of *miRNA-152* gene between BC patient and control.

4-2-3: Detection polymorphism of BC related gene by nucleotide sequencing

4-2-3-1: *ABCG2* gene

The results of the nucleotide sequence analysis of 14 samples revealed the presence of changes in the nitrogenous bases, specifically in the (38, 71, 141, 148, 150, 163, 167, 169, 172, 2559, 96428) sites of the *ABCG2* gene, as the base T changed to C (T141C), (T148C), (T169C), as the base G changed to A, (G38A), (G96428A), as the base G converted to C (G150C), (G172C), (G2559C), as the base G and A deletion at site 163,167, respectively, as shown in the table (4-41), figure (4-28), and appendix(7).

The polymorphisms (G71T), (T141C), (T148C), (G150C), (T169C), (G172C) led to a change in the genetic codes, led to a change of amino acids at the level of the resulting protein from (Serine) to (Tyrosine), (Methionine) to (Serine), (Methionine) to (Valine), (Proline) to (Alanine), (Methionine) to (Valine), (Proline) to (Alanine), respectively, where the mutations were not synonymous (missense mutation), as shown in table (4-41), figure (4-29).

Our results showed that the polymorphisms (G38A), (G2559C), (G96428C), despite the change of genetic codes, but they gave the same amino acid (Glutamic) to (Glutamic), (Isoleucine) to (Isoleucine), (Glutamic) to (Glutamic), respectively, meaning that the mutations are silent, as shown in the table (4-41).

Our results showed that the deletion G and A at sites 163 and 167, respectively, led to a change in the genetic codes, which led to a change of amino acids at the level of the resulting protein, where the mutations were frameshift mutations, as shown in the table (4-41).

Table (4-41) Nucleotide changes, the type of mutations and the resulting amino acid changes and their impact on the translation process of the *ABCG2* gene.

Gene	Site of SNP / InDel polymorphism	Nucleotides (SNPs)	Amino acids	Type of mutation	Effect of mutation on translation	Accession number	Triple code	Missense mutation%	Silent mutation%	Non sense mutation %	Frameshift mutation %
ABCG2	38	G>A	E>E	Transition	Silent	AY333756.1	TCG > TCA	54.54	27.27	-	18.18
	71	G>T	S>Y	Transversion	Missense	HE819179.1	GAA> TAA				
	141	T>C	M>S	Transition	Missense	HE819179.1	ATT> ACT				
	148	T>C	M>V	Transition	Missense	HE963049.1	ACT>ACC				
	150	G>C	P>A	Transversion	Missense	HE963049.1	GCG>CCC				
	163	G	-	Deletion	Frame shift	HE963049.1	-				
	167	A	-	Deletion	Frame shift	HE963049.1	-				
	169	T>C	M>V	Transition	Missense	HE819179.1	ATT> ACT				
	172	G>C	P>A	Transversion	Missense	HE819179.1	GGG> GCG				
	2559	G>C	I>I	Transversion	Silent	AB973570	GGA>CGA				
	96428	G>A	E>E	Transversion	Silent	NG032067.2	TCG >TCA				

SNP:single nucleotide polymorphism ; InDel: insertion /deletion polymorphism ; C: Cytosine ; T:Thymine ; A:adenine ; G :Guanine ; M: Methionine ; A: Alanine ; P: Proline ; V: Valine ; Y: Tyrosine ; S: Serine ; I: Isoleucine; E: Glutamic

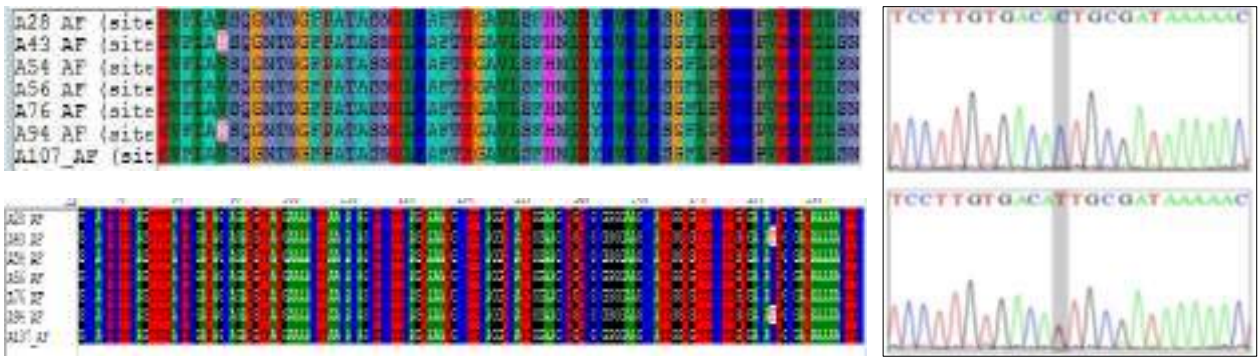


Figure (4-28) Single nucleotide polymorphisms (SNPs) at the studied sites of the *ABCG2* gene.

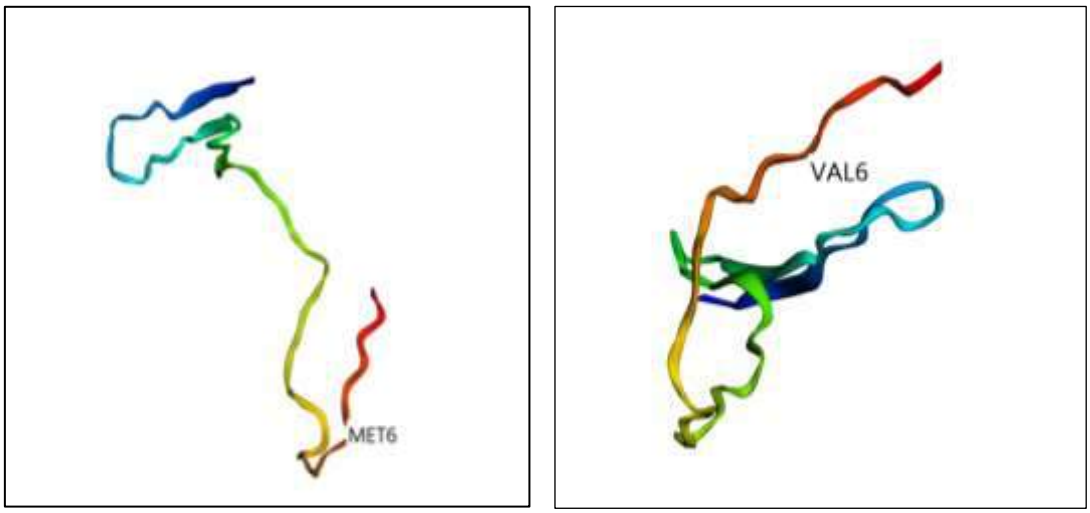


Figure (4-29) Three-dimensional shapes of the ABCG2 protein (3D protein) in breast cancer patients.

Here, the largest proportion of *ABCG2* mutations (54.54%) was in favor of missense mutations, as shown in the table (4-41), figure (4-30).

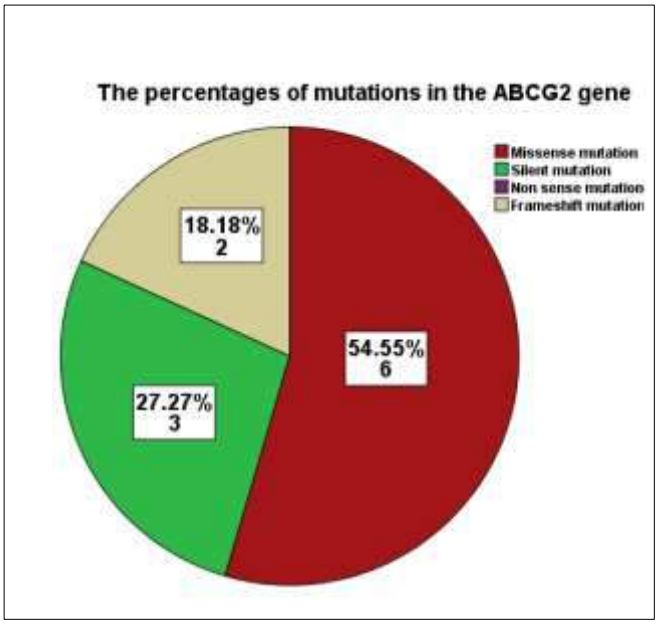


Figure (4-30) The percentages of mutations in the *ABCG2* gene.

4-2-3-2: *ABCB1* gene

The sequencing analysis of 14 samples revealed the presence of changes in the nitrogenous bases, specifically at (55, 59, 89, 90, 113, 139, 153, 323) sites of the *ABCB1* gene, the base G changed to C (G55C), the base T changed to G (T59G), the base C changed to A (C89A), the base C changed to G (C90G), the base A changed to C (A113C), the base A changed to T (A139T), the base C changed to T (C153T), (C323T), respectively, as shown in the table (4-42), figure (4-31) and appendix (7).

The polymorphisms (G55C), (T59G), (C89A), (C90G), (A113C), and (A139T) led to a change in the genetic codes, which led to a change of amino acids at the level of the resulting protein from (Valine) to (Leucine), (Valine) to (Glysine), (Alanine) to (Aspartic acid), (Valine) to (Aspartic acid), (Aspartic acid) to (Alanine), and (Threonine) to (Serine), where the mutations were not synonymous (missense mutation).

In addition, the polymorphisms (C153T) and (C323T), despite the change of genetic codes, but gave the same amino acid (Isoleucine) to (Isoleucine), (Isoleucine) to (Isoleucine), respectively, meaning that the mutations are silent (synonymous), as shown in the table (4-42).

As a result, the 3D protein of *ABCB1* in BC patient, as shown in figure (4-32).

Table (4-42) Nucleotide changes, the type of mutations and the resulting amino acid changes and their impact on the translation process of the *ABCB1* gene.

Gene	Site of SNP / InDel polymorphism	Nucleotides (SNPs)	Amino acids	Type of mutation	Effect of mutation on translation	Accession number	Triple code	Missense mutation %	Silent mutation %	Non-sense mutation %	Frameshift mutation %
ABCB1	55	G>C	V>L	Transversion	Missense	HQ291759.1	GTG>CTG	75%	25%	-	-
	59	T>G	V>G	Transversion	Missense	HQ291759.1	GTC>GGC				
	89	C>A	A>D	Transversion	Missense	HQ29175.1	GCC>GAC				
	90	C>G	V>D	Transversion	Missense	HQ291630.1	GAC>GTG				
	113	A>C	D>A	Transversion	Missense	HQ291612.1	GAC>GCC				
	139	A>T	T>S	Transversion	Missense	HQ291612.1	ACA >TCA				
	153	C>T	I>I	Transition	Silent	HQ291612.1	ATC>ATT				
	323	C>T	I>I	Transition	Silent	FJ158815.1	ATC>ATT				

SNP:single nucleotide polymorphism ; InDel :insertion /deletion polymorphism ; C: Cytosine ; T:Thymine ; A:adenine ; G : Guanine; I: Isoleucine ; S: Serine ; A: Alanine ; T: Threonine ; D: Aspartic acid ; G: Glysine ; L:leucine

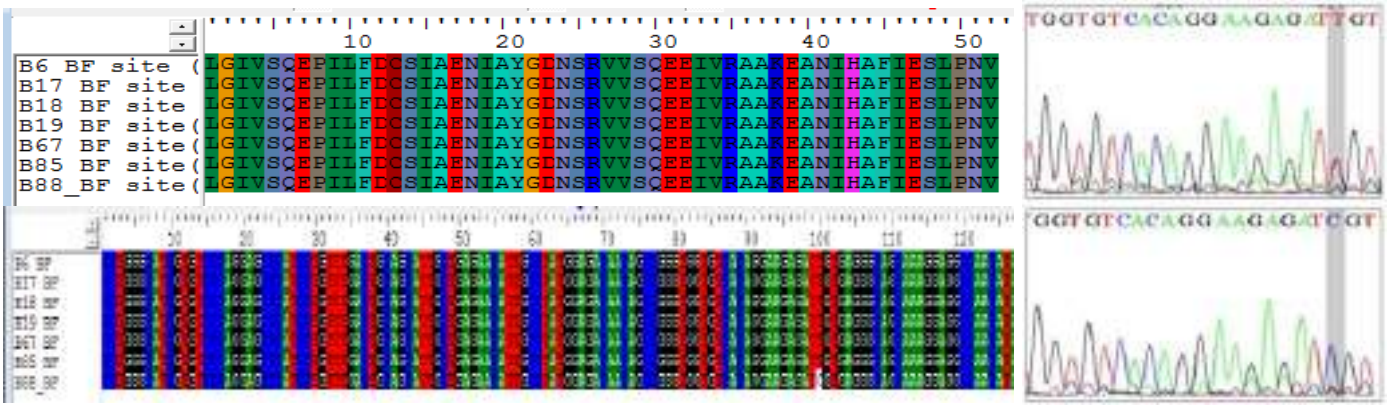


Figure (4-31) Single nucleotide polymorphisms (SNPs) at the studied sites of the *ABCB1* gene.

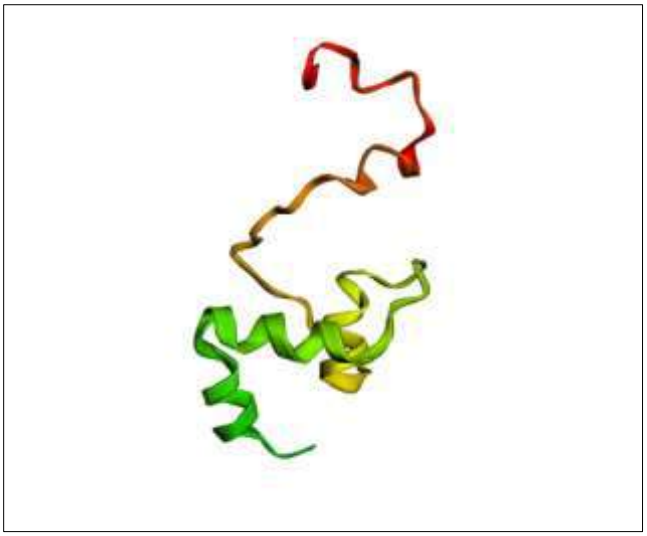


Figure (4-32) Three-dimensional shapes of the ABCB1 protein (3D protein) in breast cancer patients.

Furthermore, the largest proportion of *ABCB1* mutations (75%) was in favor of missense mutations, as shown in the table (4-42) , figure (4-33).

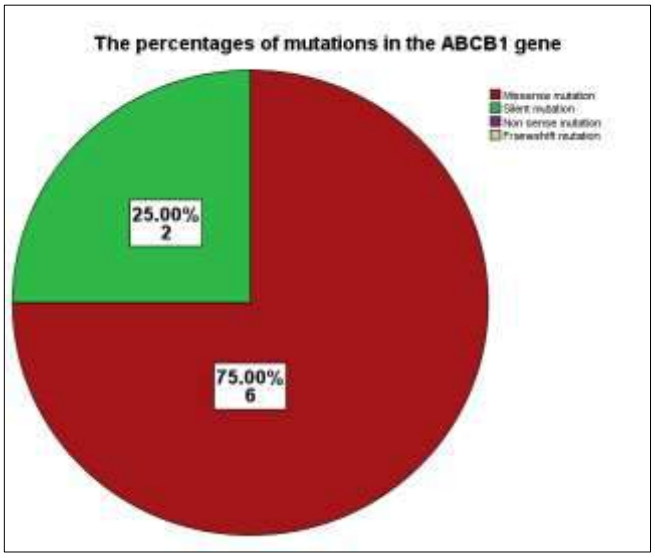


Figure (4-33) The percentages of mutations in the *ABCB1* gene.

4-2-3-3 : *BRCA1* gene

The nucleotide sequence analysis of four samples revealed the presence of changes in the nitrogenous bases, specifically in the sites (213, 248, and 261) of the *BRCA1* gene, as the base C changed to T (C213T), and the base T changed to C (T248C and T261C), as shown in the table (4-43), figure (4-34) and appendix (7).

Moreover, polymorphisms (C213T), (T248C), and (T261C) led to a change in the genetic codes, which led to a change of amino acids at the level of the resulting protein from (Valine) to (Methionine), (Glutamic) to (Glysine), (Threonine) to (Histidine), where the mutations were not synonymous (missense mutations), as shown in the table (4-43). The 3D protein of *BRCA1*, as shown in figure (4-35).

Table (4-43) Nucleotide changes, the type of mutations and the resulting amino acid changes and their impact on the translation process of the *BRCA1* gene.

Gene	Site of SNP / InDel polymorphism	Nucleotides (SNPs)	Amino acids	Type of mutation	Effect of mutation on translation	Accession number	Triple code	Missense mutation%	Silent mutation%	Non-sense mutation%	Frameshift mutation %
BRCA1	213 248 261	C>T T>C T>C	V>M E>G T>H	Transition Transition Transition	Missense Missense Missense	AY304547.1 AY304547.1 -	CAC>CAT TTC>TCC CAT >CAC	100	-	-	-

SNP:single nucleotide polymorphism ; InDel :insertion /deletion polymorphism ; C: Cytosine ; T:Thymine ; A:adenine ; G : Guanine; T: Threonine ; H: Histidine ; M: Methionine ; E: Glutamic; G: Glysine; V: Valine *: The symbol (*) indicates mutation identification by Geneious prime program so it does not have accession number.

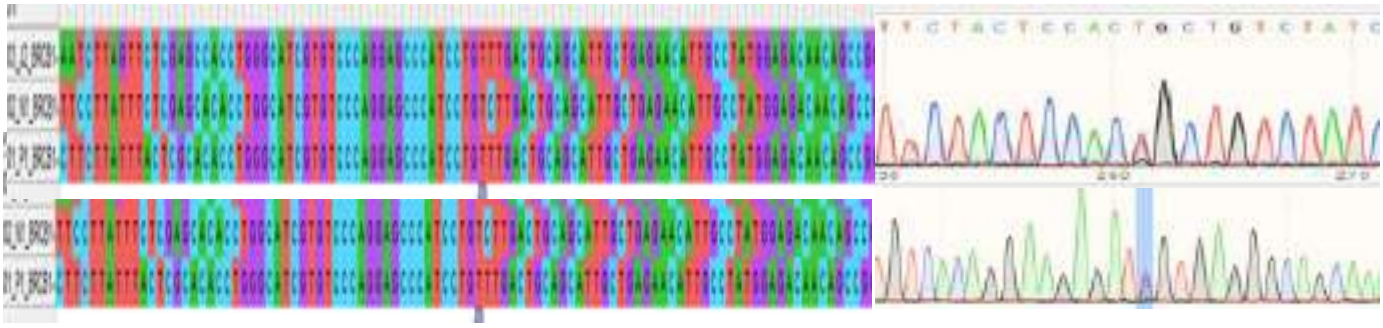


Figure (4-34) Single nucleotide polymorphisms (SNPs) at the studied sites of the *BRCA1* gene.

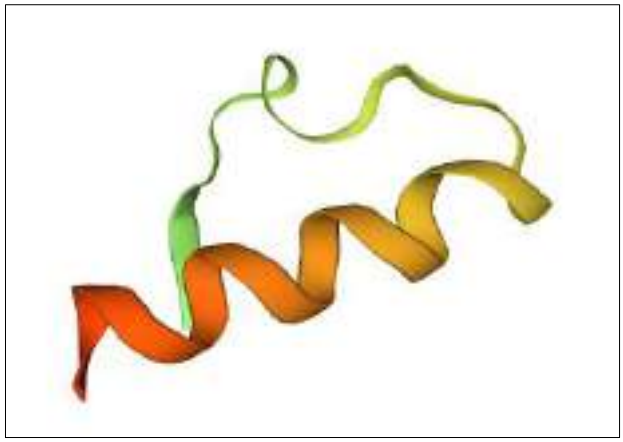


Figure (4-35) Three-dimensional shapes of the BRCA1 protein (3D protein) in breast cancer patients.

Also, the largest proportion of *BRCA1* mutations (100%) were in favor of missense mutations, as shown in table (4-43), figure (4-36).

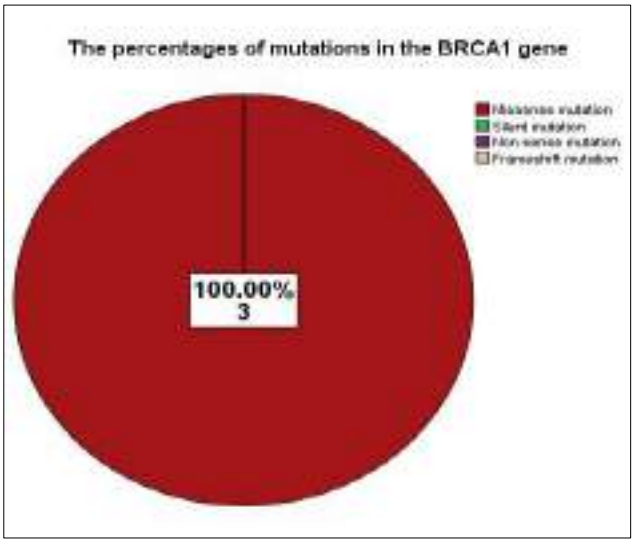


Figure (4-36) The percentages of mutations in the *BRCA1* gene.

4-2-3-4: *ER-α* gene

In the sequencing analysis of seven samples revealed the presence of changes in the nitrogenous bases, specifically in the sites (114, 244, 344, 424, 440, 454) of the *ER-α* gene, the base C changed to T (C114T), the base C changed to G (C244G), (C344G), the base C changed to T (C424T), the base T changed to C (T440C), the base A deletion at 454 site, as shown in the table (4-44), figure (4-37), appendix (7).

The polymorphisms (T440C) led to a change in the genetic codes, from (Leucine) to (Proline), where the mutations were not synonymous (missense mutation), as shown in table (4-44).

The polymorphisms (C114T), (C244G), (C344G), and (C424T), despite the change of genetic codes, but gave the same amino acid (Glycine) to (Glycine), (Glycine) to (Glycine), (Alanine) to (Alanine), (Serine) to (Serine), respectively, meaning that the mutations are silent (synonymous), as shown in table (4-44).

Besides, the deletion A in the site 454 led to a change in the genetic codes, where the mutation is frameshift mutation, as shown in table (4-44).

The 3D protein of *ER-α* in breast cancer patient, as shown in figure (4-38).

Table (4-44) Nucleotide changes, the type of mutations and the resulting amino acid changes and their impact on the translation process of the *ER-α* gene.

Gene	Site of SNP/InDel polymorphism	Nucleotides (SNPs)	Amino acids	Type of mutation	Effect of mutation on translation	Accession number	Triple code	Missense mutation %	Silent mutation %	Non-sense mutation%	Frameshift mutation%
ER-α	114 244 344 424 440 454	C>T C>G C>G C>T T>C A	G>G G>G A>A S>S L>P -	Transition Transversion Transversion Transition Transition Deletion	Silent Silent Silent Silent Missense Frameshift	* * * JX996117 * JX996117	GGC>GGT GGC>GGG GCC> GCG TCC>TCT GTC>GCC -	16.67	66.67	-	16.67

SNP:single nucleotide polymorphism ; InDel: insertion / deletion polymorphism ; C: Cytosine ; T: Thymine ; A: adenine ; G : Guanine ; G: Glycine ; A: Alanine ; L: Leucine ; P: Proline; S: Serine ; *: The symbol (*) indicates mutation identification by Geneious prime program so it does not have accession number.

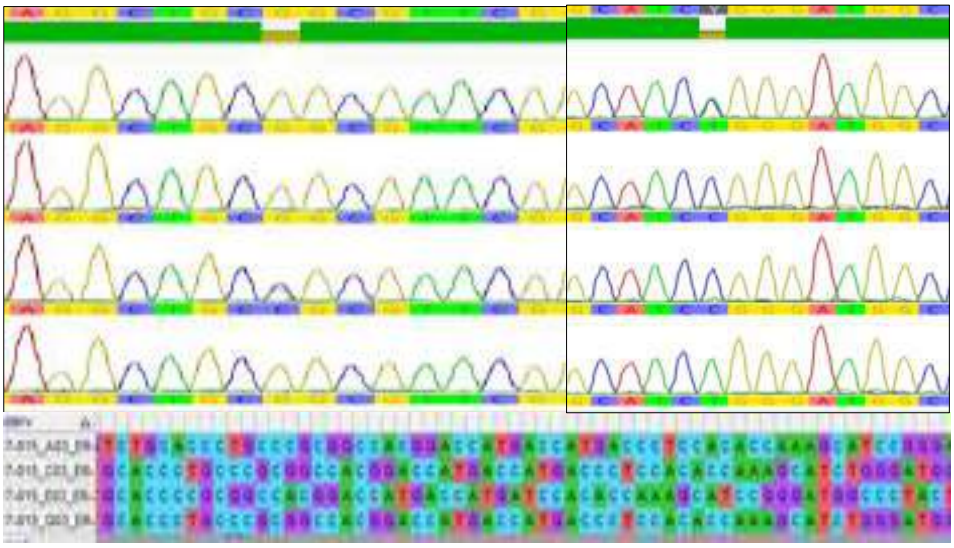


Figure (4-37) Single nucleotide polymorphisms (SNPs) at the studied sites of the *ER-α* gene.

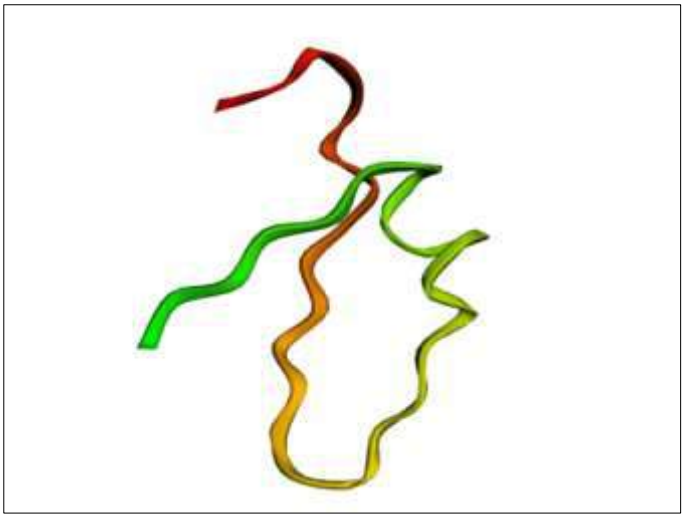


Figure (4-38) Three-dimensional shapes of the ER-α protein (3D protein) in breast cancer patients.

Also, The largest proportion of *ER-α* mutations (66.67%) were infavor of silent mutations, as shown in the table (4-44), figure (4-39).

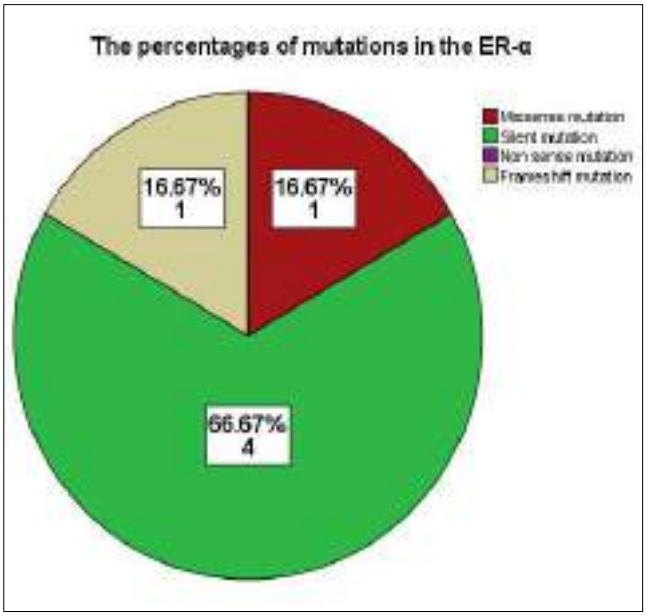


Figure (4-39) The percentages of mutations in the *ER-α* gene.

4 -2-4: Cytogenetic study of breast cancer tissues

Regarding cytogenetic tissues, the investigation were obtained from staining breast tissue with four fluorescent stains as follows: DAPI stain (Blue), was used to detect and stain the nucleus (chromosomes staining); DCFH-DA (Green), was used for cytoplasmic ROS detection; Mito-Tracker (Red) was used to detect mitochondrial morphological defect; Mito-Sox (Red) was used to detect mitochondrial ROS (mtROS), the findings showed after examining stained breast tissues under a fluorescent microscope as follows:

The data under fluorescent microscopy showed the nucleus of the BC cells presenting with a strong reaction with DAPI stain, the nucleus not fragmented; and abnormal shapes of the nucleus (condensed chromatin), as shown in figure (4-40B).

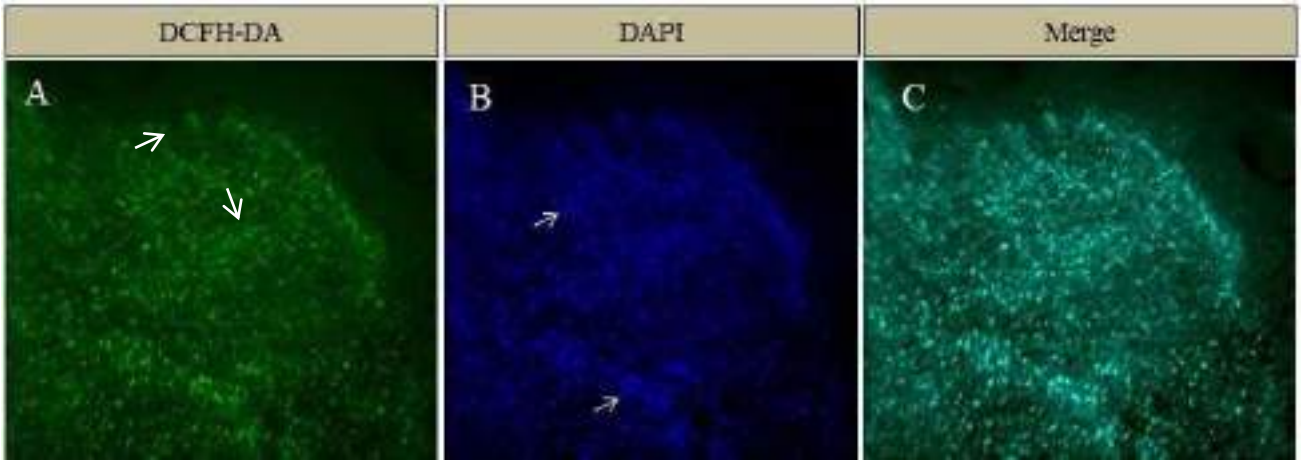
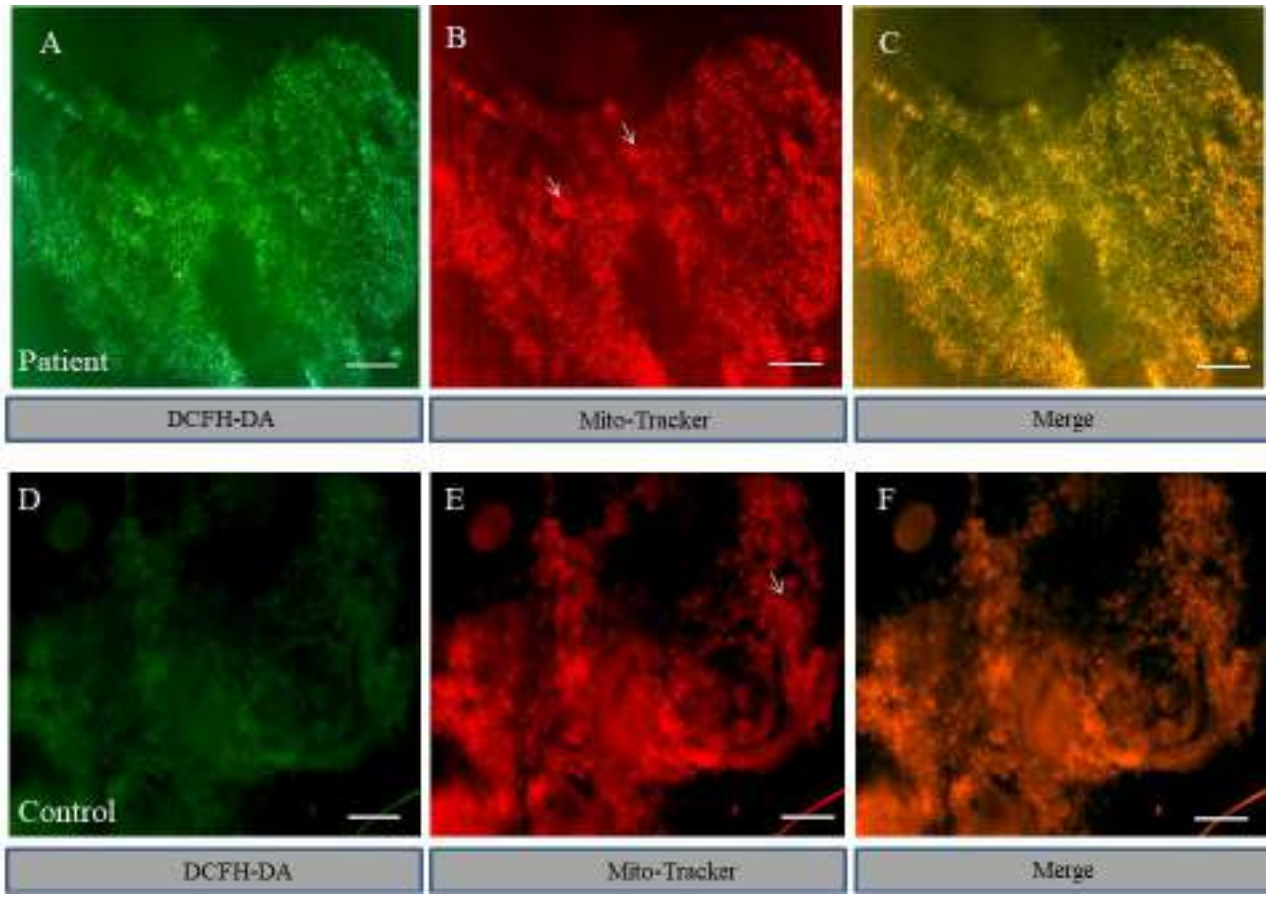


Figure (4-40) Fluorescent microscopy. (A) DCFH-DA stained cell, green fluorescence indicates the amount of reactive oxygen species (ROS), white arrows indicate high ROS. (B) nucleus stained with DAPI, blue fluorescence indicates the nucleus, white arrows indicate the abnormal nucleus. (C) Merged images.

However, the increased ROS in the BC tissues, this tissue gave a strong reaction with DCFH-DA stain in comparison with control breast tissues, as shown in figure (4-41). Mito-Tracker stain revealed the defect of mitochondrial morphology in BC tissue compared with normal tissue, as shown in figures(4-41), (4-42).



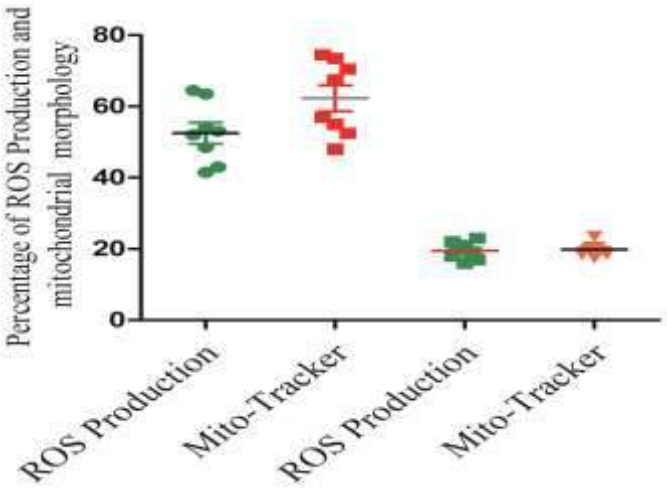


Figure (4-41) Fluorescent microscopy. (A, D) DCFH-DA stained cell with green fluorescence indicates the amount of reactive oxygen species ROS**,** arrows indicate high. **(B, E)** Fluorescent microscopy of Mito-Tracker stained mitochondria, red fluorescence indicate a defect of mitochondrial morphology, arrows indicate mitochondrial structure irregular. **(C, F)** Merged images. Error bars indicate SEM. Analysis of variance (ANOVA/Dunnett: * P <0.05, ** P <0.01, *** P <0.001).

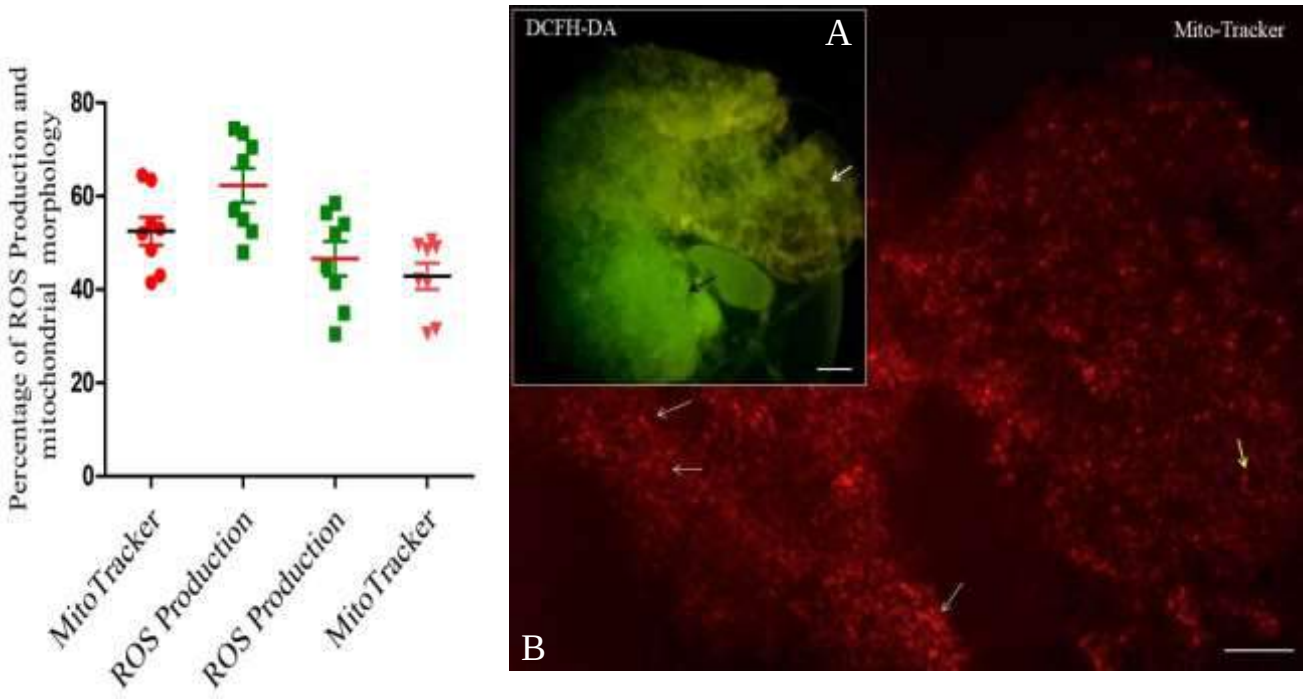
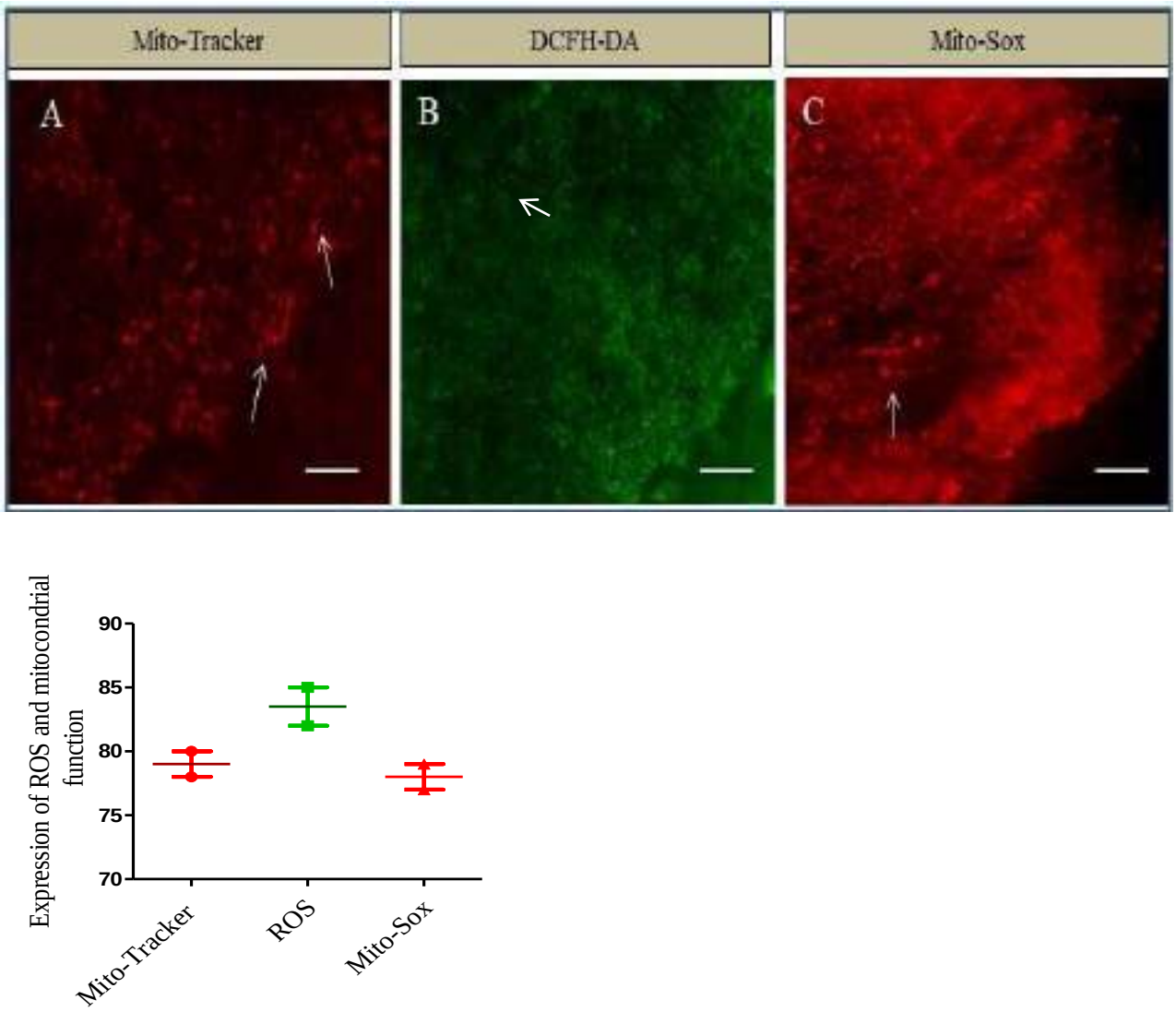


Figure (4-42). Fluorescent microscopy. (A) DCFH-DA stained cell, green fluorescence indicates the amount of reactive oxygen species ROS, (White) arrow indicate normal breast tissue; (Black) arrow indicate breast cancer tissue. **(B)** Mito-tracker stained cell, arrow indicate the mitochondrial morphology, arrows show (yellow) normal and (white) affected tissues. Error bars indicate SEM. Analysis of variance (ANOVA/Dunnett: * P <0.05, ** P <0.01, *** P <0.001).

In addition, the increased mitochondrial ROS (mtROS) in the BC tissues compared with normal tissue, the mitochondria of the BC tissue showing a strong reaction with Mito-Sox stain, as shown in figures (4-43), (4-44).



Figure(4-43). Fluorescent microscopy. (A) Mito-Tracker stained mitochondria, red fluorescence indicates defect of mitochondrial morphology**,** arrows indicate mitochondrial structure irregular. **(B)** DCFH-DA stained cell, green fluorescence indicate the amount of reactive oxygen species ROS, arrows indicate high ROS. **(C)** Mito-Sox stained cell, red fluorescence indicate the amount of mitochondrial reactive oxygen species (mtROS), arrows indicate high mtROS. Error bars indicate SEM. Analysis of variance (ANOVA/Dunnett: * P <0.05, ** P <0.01, *** P <0.001).

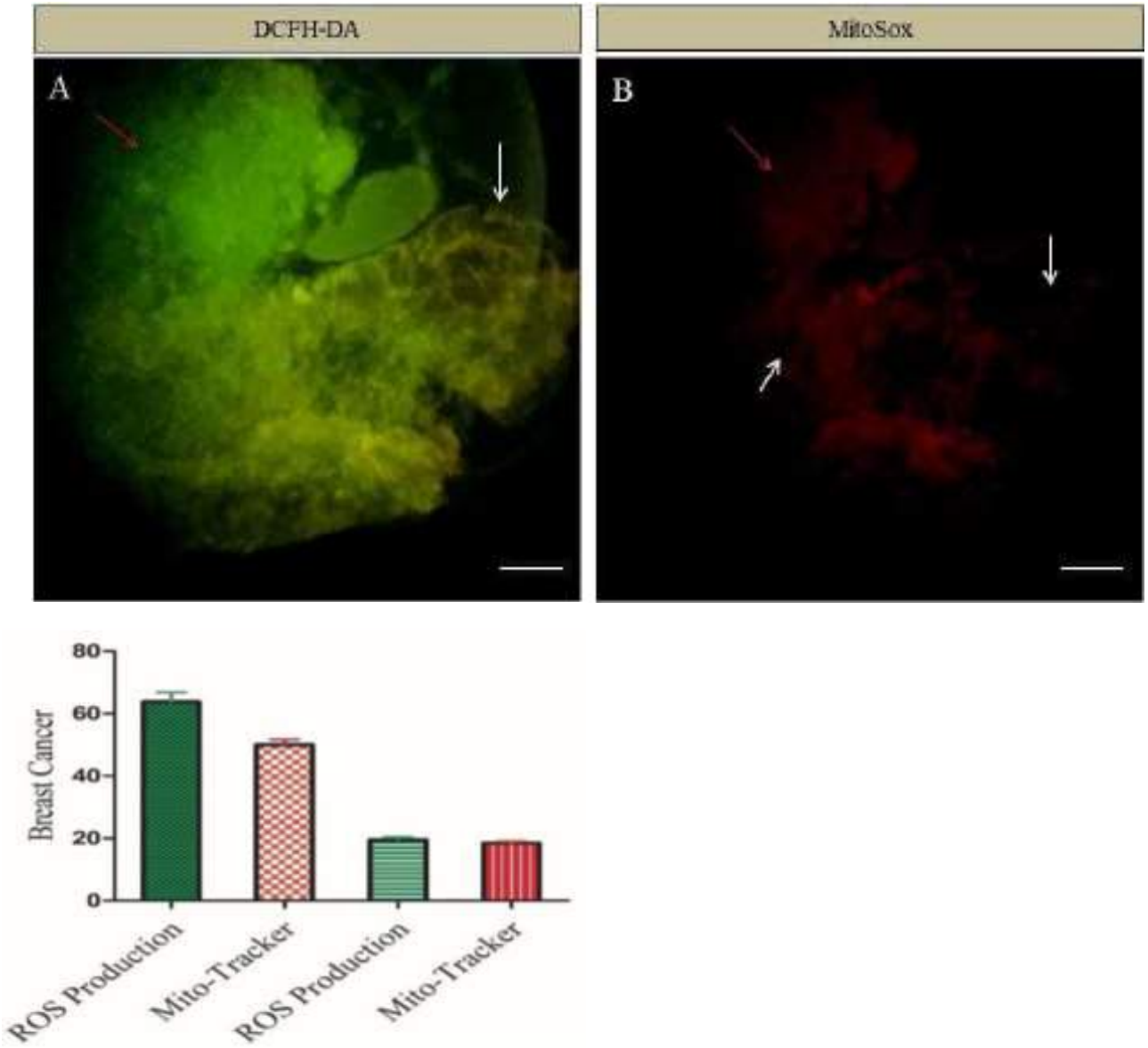


Figure (4-44). **Fluorescent microscopy. (A)** DCFH-DA stained cell, green fluorescence indicates the amount of reactive oxygen species (ROS), (White) arrows indicate normal breast tissue. **(B)** Mito-Sox stained cell, (White) arrows indicate the normal mitochondrial ROS. Error bars indicate SEM. Analysis of variance (ANOVA/Dunnett: * P <0.05, ** P <0.01, *** P <0.001).

The BC patients have tissue with high ROS, also had genetic mutations in their genes of blood samples identified by gene sequencing. Therefore, the results of the fluorescent were identical and closest to gene sequencing analysis.

Chapter five

Discussion

5. Discussion

5.1: Study genetic polymorphism of genes related to breast cancer by RFLP technique

RFLP is a very useful tool for studying the genotypes in the genes related to breast cancer, it has become an essential component of the human disease diagnostic systems.

In our current study, a routine test for HWE was performed in the control sample to detect genotypes frequency and genotype errors, thus allele frequency and genotype differences in both groups could be tested to discover the association between the genotype of the studied genes and the risk of BC. Abramovs *et al*., (2020) reported that the HWE using for estimating the number of homozygous and heterozygous variant carriers based on its allele frequency in populations. Wang and Shete, (2017) stated the HWE in case-control study designs to be better than the traditional methods of testing this test in control groups, whereas that the deviation from the (HWE) in affected individuals could provide evidence for an association between genetic variants and diseases occurrence. In addition, Lee *et al*., (2008) mentioned the large-scale studies have revealed abundant structural differences in the human genome, including copy number, and SNP differences in some sites that may cause deviation from HWE ratio.

Furthermore, genetic association study aims to find statistical associations between genotypes and traits or disease status and thus to identify genetic risk factors (Brunel, 2013).

5-1-1: Study genetic polymorphism of the *ABCG2* gene by RFLP technique

The electrophoresis and digestion data of *ABCG2* PCR products by PCR-RFLP figured two alleles (C, T) and three genotypes (CC, CT, TT) that differ from the alleles and genotypes in the previous studies, this explain by using of a restriction enzyme (MboI) for the first time in the cutting the PCR product of the *ABCG2* gene, which differs from the (BseMI) enzyme used in previous studies in the restriction site because it does not digest PCR products of Iraqi female samples. Therefore, the alleles and genotypes appeared to be quite different from those in the previous studies. Wu *et al*., (2015) mentioned PCR product of *ABCG2* gene were digested with BseMI at 55 ºC overnight.

Our results showed that the members of the control sample were different from patients in HWE, whereas differences in HWE due to significant differences between observed and expected frequencies ($P < 0.05$). Ward and Carroll, (2014) stated that the HWE tests evaluate the degree of difference between observed genotype and expected frequencies, when observed vs. expected frequencies are different to a large extent, the test would turn statistically significant and suggest deviation from HWE assumption. The deviation from HWE in the BC patient group may be due to genetic mutations that may be occurred in the *ABCG2* gene of the patient. Therefore, the HWE between the control sample and the BC sample is important in determining the variation of the genotype frequencies between the patient and the control sample, this may indicate the extent to which the genotype is associated with BC risk.

By comparing the frequency of genotypes and occurrence alleles between the both samples, CC genotype had a higher rate in the BC sample compared to the control sample, which indicate that the homozygous genotype (CC) may be a disease genotype related to BC. Therefore, the CC genotype may be reliable as an

indicator of increased risk and progression of BC. Therefore, it may be responsible for increasing the expression of *ABCG2* gene that has homozygous genotype CC in BC patient that increase the progression and onset of the disease by affecting the function of (ATP-binding cassette) that essentially encodes for p-glycoprotein, which play an important role as a transmembrane efflux pump, and pumping its substrates, and xenobiotic such drug from inside the cell to outside it. Therefore, if the expression of the *ABCG2* gene is blocked by blocking the ATP-binding receptor, the risk of developing and progression of BC may be reduced. The heterozygous genotype CT had a higher rate in the control sample compared to the BC sample, which indicate that the heterozygous genotype may be a protective genotype from BC. Therefore, the heterozygous CT genotype may be associated with prevention of BC risk. Although the homozygous genotype TT had a higher rate in the patients sample compared to the control sample. Therefore, the risk is the same at TT genotype. These results are obtained for the first time to our knowledge using the MboI restricting enzyme for the first time to cutting *ABCG2* gene. Our results showed that the significant differences ($P < 0.05$) in the dominant model between BC patient and control. Therefore, dominant model associated with risk of BC.

Wang *et al*., (2020) reported many studies were shown that the overexpression of *ABCG2* remains a major barrier to successful cancer therapy, because *ABCG2* acts as an efflux pump for chemotherapeutic agents and causes clinical multidrug resistance (MDR).

It is worth mentioning that the significant differences between genotypes of the patient and control group ($P < 0.05$) and their relationship to the risk of developing BC, indicate that there are genetic differences between the two groups that may be attributed to the different environmental factors, social conditions, and the nature of exposure to BC risk factors and geographic distribution. Mbemi *et al*., (2020) concluded that human diseases are not only caused by specific genetic and

environmental factors but also by genetic interactions with the environment, although genetic polymorphisms play an important role in human susceptibility to cancer, and many single nucleotide polymorphisms (SNPs) resulted from somatic mutations from human exposure to environmental stresses.

The results were clear that the genetic polymorphism of *ABCG2* gene is associated with the risk and development of BC. Wu *et al*., (2015) mentioned that the genetic polymorphisms of *ABCG2* gene are involved in the development of cancer risk in the Chines population and interindividual differences in chemotherapy response attributed to polymorphisms of *ABCG2* gene that are associated with BC susceptibility.

5-1-2: Study genetic polymorphism of the *ABCB1* gene by RFLP technique

The *ABCB1* gene polymorphism was studied to evaluate the association between the polymorphism of the *ABCB1* gene and breast cancer risk in the Maysan province. *ABCB1* encodes for P-gp that is a transmembrane transporter protein that extrudes several active compounds outside the cell. It has been expressed in a variety of tissues. Its physiological function is to protect cells from the effects of harmful substances by reducing their accumulation in the cell (Abuhaliema *et al*., 2016). Fang *et al*., (2013); Wang *et al*., (2013) found that the *MDR1* polymorphisms may cause impairment of P-gp expression or function and this lead to a decrease decline in Protective effects of P-gp.

Those populations of the control were underwent to the HWE test, resulting in the differences between observed and expected frequencies were by chance. These significant differences in CC, CT, TT genotype distribution between both groups, besides, their relationship to the risk of BC developing ($P < 0.05$), may indicate genetic differences between the two arms.

The homozygous genotype CC had a higher rate in the control sample compared to the BC sample, which indicate that the homozygous genotype (CC) may be a protective genotype from BC. While CT genotype had a higher rate in the BC sample compared to the control sample, which indicate that the heterozygous genotype (CT) may be a disease genotype related to BC. Although, the homozygous genotype TT had a higher rate in the patients sample compared to the control sample, yet, it is not associated with the risk. Therefore, the risk is same at TT genotype.

The genotype rates in the patient were 30%, 55%, 15% for CC, CT, and TT, respectively, which different from genotype distribution of the other population countries may be due to the race, environment, the type of analysis, and the size of the sample. Salem *et al*., (2014) reported that the distribution of C3435T genotypes varies between populations. Hamidovic *et al*., (2010) reported that the frequency of the C3435T genotype was 22%, 50%, and 28% for CC, CT, and TT genotypes, respectively, in the Caucasian population. Kassogue *et al*., (2013) observed that the genotype frequencies were 39%, 51%, and 10 % for CC, CT, and TT, respectively, in the Morocco population. MUTLU İÇDUYGU *et al*., (2020) suggested that the *ABCB1* C3435T polymorphism may increase the BC risk in Turkish women and significant difference in distribution of C3435T genotypes between the cases and the controls (cases, CC 37.1%, CT 28.6%, and TT 34.3%; controls, CC 25%, CT 65%, and TT 10%, P: 0.023) were observed.

Our results showed there is significant difference in genotype frequencies between the patient and control group ($P < 0.05$). Therefore, the polymorphism of the *ABCB1* was associated with BC risk in the Maysan Iraqi population.

A confirming and agreement with many studies, like Wu *et al*., (2012) which mentioned that the significant association between *ABCB1* polymorphism and BC risk in Chinese breast carcinoma patients ($P < 0.05$, $P = 0.043$). In addition, agree

with Turgut *et al*., (2007) they reported a significant association of the genotype distribution with BC risk in Turkey. Abuhaliema *et al*., (2016) found a higher prevalence of CC genotype in BC patients compared to control ($P < 0.001$) among Jordanian women. Ameyaw *et al*., (2001) that the C3435T polymorphism of *ABCB1* gene varies across different populations due to the modifications in genetic background and lifestyle.

Whereas, our findings disagree with Tatari *et al*., (2009) which reported that there is no association between C3435T genotypes of the *ABCB1* gene and risk factors in Iranian population, and dis agree also with Taheri *et al*., (2010) that mentioned the difference in genotypes between the patient and the control sample was not statistically significant ($P > 0.05$, $P = 0.980$) in the Iranian population.

Tazzite *et al*., (2016) mentioned there is no significant association between the *ABC B1* polymorphism and the development of breast cancer among Moroccan woman, whereas no significant difference in the distribution CC, CT, and TT genotypes between patient and control.

Moreover, our results disagree with Rubiś *et al*., (2012); Macías-Gómez *et al*., (2014); Gutierrez-Rubio *et al*., (2015) in Poland population (Western Europe), and Mexican population (Southern America), respectively, which stated that the differences in the genotype distribution (CC, CT, and TT) between the BC patient and control were not of any evidence.

The differences in our results and the results of other countries may be because life style, environment of the studied sample, sample size and geographic origin.

5.1.3: Study the genetic polymorphism of the *BRCA1* gene by RFLP technique

The *BRCA1* gene polymorphism was studied to evaluate the association between polymorphism of *BRCA1* gene and breast cancer risk in the Maysan city. *BRCA1* is a tumor suppressor gene and has a critical role in maintaining genetic stability. It encode a large protein called tumor suppression protein which plays an important role in tumor suppression and DNA repair activities (Elia *et al*., 2012). Calò *et al*., (2010) mentioned that most changes in the *BRCA1* genes result in protein dysfunction or the diminishing of protein product.

The deviation from HWE law in control may be due to genotyping errors, and small size of the sample. Graffelman *et al*., (2017) observed that several factors, including mutations, can cause deviations from HWE. Garnier-Géré and Chikhi, (2013) found that the geographical location may be a common cause of deviations from HWE. Whereas, Royo *et al*., (2021) reported that the deviation from (HWE) in the human control group can be due to normal factor such as excess selective pressure on the certain genotype, and other factor may be contributed in deviation from (HWE) in control sample such as a technical problems. We excluded these from our experience because our experiment was repeated more than once and getting the same data. In addition, these results practically in the laboratory matched the results of the bioinformatics, where the *BRCA1* gene sequence was cut using the NEB cutter V 2.0 bioinformatics program, as shown in appendix (6).

Gholipoorfeshkecheh and Arjunan, (2014) wrote that the results of the RFLP detected *BRCA1* mutations have a role in BC. Our results disagree with Hasan *et al*., (2013) concluded that the heterozygous genotypes were associated with decreased gene expression of the *BRCA1* gene in the sporadic BC disease. Okada *et al*., (2012) mentioned that the loss of heterozygosity at *BRCA1* locus was

correlated with significantly shorter disease-free survival (DFS), besides overall survival(OS) in a retrospective study of 202 Japanese patients with invasive BC.

It is worth mentioning that the no significant differences between genotypes of the patient and control group (P >0.05) due to the somewhat similar of genotypes distribution between the two groups, and risk the same.

5-2: PCR-RAPD-PCR

5-2-1: Detection of the genetic polymorphisms of the BC genes by PCR-RAPD-PCR technique

Different techniques used to determine the polymorphism of BC genes, although the RAPD technique is considered random with reduced reproducibility, but we chose it due to rapid and low price (Bidet *et al*., 2000). Fu *et al*., (2017) mentioned there are some limitations of RAPD, such as poor reproducibility, and low output. Recently, the technique has been modified with high GC primer content and increased prolonging the RAMP time from the stage of annealing to extension in the thermocycler device to produce more bands for polymorphism for detecting genomic alterations in BC.

In spite of that, we performed a new procedure with some modifications in the traditional RAPD technique, using the amplified gene as a template in the RAPD technique instead of total DNA. This procedure resulted in more accuracy and specific data, we termed as PCR-RAPD-PCR(PRP).

Our PRP technique is a simple PCR product based gene polymorphism assay system using for studying and analyzing the genomic instability or genomic alteration. Novikov *et al*., (2016) mentioned that the RAPD is used in the field of studying genetic alteration of BC. Boysen *et al*., (2019) and Sabra *et al*., (2020) found that the genetic progress towards transformation from normal to malignant cells can be achieved by comparing the RAPD profiles obtained from healthy and malignant cells.

The *ABCG2*, *ABCB1*, *BRCA1*, *ER-α*, and *mi-RNA 152* genes were selected in the study of the genetic polymorphism by PRP technique due to its important role in cancer.

The reasons behind using special genes (PCR products) as template including initially: the extracted total DNA contains exons (a region coding for proteins), and introns (a non-coding region), whereas this the BC genes that used as a template in our experiment contain exons only, and this measurement may be contributed to reducing RAPD randomness. Smith (2005) mentioned that the RAPD primers binding to different DNA sites and consequently detecting different RAPD polymorphisms. The RAPD primers do not discriminate between coding and non-coding regions. Therefore, the sample genome is more randomly selected. The second reason is to elevate the accuracy of the findings and to enhance comparison the relationship between RAPD results of the BC gene with the results of the sequencing in terms of presence or absence of genetic mutations. Smith, (2005) wrote that the RAPD primers detected polymorphisms caused by point mutations. Lastly, to produce more accurate bands for detecting genetic differences in specific BC related genes between two arms.

We found that the number of bands per primer for each gene was high, and this improved PRP technique as successfully increased the number of RAPD bands produced from a given PCR Product. Therefore, we applied the PCR-RAPD-PCR technique with five specific genes related to BC (*ABCG2*, *ABCB1*, *BRCA1*, *ER-α*, and *mi-RNA-152*) to generate more bands for detecting genomic alterations in BC. The total number of bands in (*ABCG2*, *ABCB1*, *BRCA1*, *ER-α* and *miRNA-152*) for the patients group (214, 215, 206, 298, 149), while control group have (162, 114, 126, 128, 93), respectively. Ismaeel, (2013) found that the total number of bands were (69) bands, when extracted total DNA as a template and six arbitrary RAPD primers for the study of genetic polymorphism in DNA of the BC patient by RAPD technique. Al-Askeri, (2016) reported that the total amplified bands were (72) bands in the patient group and (28) band in the control group, when extracted total DNA as a

template and five arbitrary RAPD primers. The number of bands per primer of each gene in the patient sample was higher than control sample may be due to genetic alterations between the two groups. Atienzar *et al*., (2002) observed that the changes in the DNA profile such as (band patterns) reflect DNA changes in the genome from single base change to complex rearrangements.

The total polymorphic bands of the (*ABCG2*, *ABCB1*, *BRCA1*, and *mi-RNA-152*) genes in the patient group were higher than the control group except *ER-α* gene, and the total unique band of the all genes in the patient group was higher than the control group. Also, the total monomorphic bands of the all genes were in the control group either equal or higher than the patient group except *ER-α* gene. The differences in the number of bands between both groups might be due to the nucleotides sequence of the primers and on the genotype of the BC patients. Moreover, the number of compatible sites of primer in the BC gene of the patient is affected by different types of mutations and translocations, this will affect the primer and template interaction sites and may results in the loss or profit of bands. Rocco *et al*., (2014) mentioned that the differences in the number of bands and the variations in their intensity, in the RAPD-PCR profile, are associated with alterations of genetic material. Xian *et al*., (2005) stated that the losses of alleles due to their linkage to genes may decrease of band intensities, and gene amplification or chromosomal aneuploidy lead to increase of band intensities, and mutations at the primer template interaction sites results in the loss or gain of a band, so mutations occurred between the primer-template interaction site, such as deletions or insertions, resulted in a mobility shift of bands. Liu *et al*., (2005) suggested that the modifications of band intensity and lost band are likely to be due to one or a combination of the following events: changes in oligonucleotide priming sites due mainly to genomic rearrangement, point mutations and DNA damage in the primer

binding sites is only 10 base long, and interactions of DNA polymerase with damaged DNA.

There are a marked differences in the primer discriminatory power between both groups of the (*ABCG2*, *ABCB1*, *BRCA1*, *ER-α*, and *mi-RNA152*) genes, this differences may be due to the dependence of the primer discriminatory power on the number of polymorphic bands generated from each primer and the sum of the polymorphic bands generated from all primers. Ismaeel, (2013) reported that the primer capacity to show polymorphisms in comparison to polymorphisms shown by all primers is called primer discriminatory power.

The data showed a marked differences in the primer efficiency between both groups of the (*ABCG2, ABCB1, BRCA1, ER-α, and mi-RNA 152)* genes, these differences may be due to the dependence of the primer efficiency on the number of polymorphic bands generated from each primer and the sum of the bands generated from all primers, furthermore, the primer efficiency was variable between the patient and control group. Newton and Graham, (1997); and Ismaeel, (2013) concluded that the primer efficiency values range from (0 - 1) and are defined as the measure of the primer's ability to produce polymorphisms. Ibrahim *et al*., (2010) reported that the efficiency of the primer can be demonstrated by its ability to give the highest percentage of polymorphic bands compared to the total number of amplified bands. The important characteristic of the efficient primer is the ability to illustrate the polymorphism between normal individuals and patient individuals as in leukemia.

A marked differences in the molecular size of fragments produced from primers between both groups. The primer OPD18 have the molecular weight (20-30) bp of the *ABCG2* bands in the patient group. The primer OPAA17 have the molecular weight (20-30) bp of the (*ABCB1*, *BRCA1*) bands in the patient

group. The primer OPAA11 showed the molecular weight (40-50) bp of the *ER-α* bands in the patient group. The primer OPAA17, OPD18 showed the molecular weight (20-40) bp of the (*mi-RNA-152*) bands in the patient group. Thus, the bands with their primers may rely upon in distinguishing between two groups, genetically, after studying them more in the future. Papadopoulos *et al*., (2002); Xian *et al*., (2005); and Ibrahim *et al*., (2010) found that the polymorphism also include the differences in molecular weights of amplified bands, which resulted from multiple types of mutations and translocations that occurred, thus causing mobility shift of bands and might cause the addition of new band (s).

There were significant differences ($P < 0.05$) between two groups among polymorphic, unique, and monomorphic bands in the *ER-α* and *mi-RNA 152* gene may be due to genetic alteration between the two groups. Singh and Roy, (2001) stated the disappearance (deletion) or appearance (insertion) of an amplified DNA fragment might be associated with genetic rearrangements, or unequal mitotic recombination. The differences between two groups may be attributed to different genetic factors, environmental conditions, hormonal changes, physical and chemical factors, whose interaction with the genetic material may lead to several genetic modifications. Perrone *et al*., (2016) reported that the inherently predisposed reaction of DNA damage due to exogenous and endogenous factors can be the development of hereditary diseases and sporadic cancer.

There are differences between two groups may be related to mutations or modifications in the genes included by our study, this made us directed to do sequencing to find and determine these mutations in our next part of experiments of the research.

5-3: Detection genetic polymorphism of BC related genes by nucleotide sequencing

Existing routine techniques for measuring biomarkers and morphological features in BC patients lack accuracy, leading to mistreatment and overtreatment of women with early BC (Rantalainen *et al*., 2016). Therefore, sequence-based breast cancer diagnosis methods have the potential to provide molecular characterization that aids customized precision medicine in the treatment of breast cancer patients (Rantalainen *et al*., 2016).

Gene sequencing to BC revealed whether the genetic mutations of the BC genes are point mutations or frameshift mutations and their effect on the translation process, in addition, to their effect on the amino acids and 3D protein resulting from the gene expression of the studied genes. Roychowdhury *et al*., (2011) mentioned that the Oncology Sequencing Program could be used to identify potential genomic alterations in various cancers.

In this study, we used blood samples for revealed many single-nucleotide polymorphisms (SNPs) and indel (insertion-deletion) polymorphism and 3 D protein (gene expression) of the BC genes. Aarøe *et al*., (2010) mentioned that some studies have yielded encouraging results in developing a GE profiled by using blood samples for early detection of BC with a prediction accuracy 79.5%, a sensitivity 80.6%, and specificity 78.3%.

We studied the genetic polymorphism of the *ABCG2*, *ABCB1*, *BRCA1*, and *ER-α* genes by sequencing, as the genetic polymorphisms of these genes are believed to underlie the differences between people in their response to chemotherapy and other xenobiotics. Also, contributes to predicting the presence of a previous or later family history of BC disease in the examined people.

5-3-1: *ABCG2* gene

This gene used to identify common *ABCG2* polymorphisms associated with BC risks in a sample of women from Maysan province.

The obtained mutations of the *ABCG2* were substitution (Transition) mutations (G38A, T141C, T148C, T169C) due to the conversion of the nitrogenous base to another base of the same type pyrimidine to pyrimidine (T↔C), (G↔A), while (G71T, G150C, G172C, G2559C, G96428A) were (Transversion) mutations, as a result of the change of the nitrogenous base from the purine group to the pyrimidine group and vice versa.

When comparing with previous studies conducted in other countries on the *ABCG2* gene, showed transition and transversion mutations in the other sites of the *ABCG2* gene. In the Chinese population, Wu *et al*., (2015) found C421A (Q >K), G34A(V >M). Stomka *et al*., (2020) discovered 706 C >T(Arg >Ter), 1714 A >C (ser >Arg); 335 C >A (Pro >Gln), 706 C >A, 1060 G >A (Gly >Arg).

The presence of missense mutation in many sites of *ABCG2*; in the site 71, 141, 148, 150, 169, 172 of *ABCG2*; the missense mutation may be due to converted single nucleotide base, as a result of the substitution transition and transversion; the reading of triple code for Serine amino acid will turn into a triple code for Tyrosine, Methionine into Serine, Methionine into Valine, Proline into Alanine, and this may lead to a change in the protein produced by *ABCG2*, consequently affecting GE. Thus, it may contribute to the development and progression of BC. Heyes *et al*., (2018) mentioned that the polymorphisms affecting *ABCG2* expression or function may have clinically important roles in anti-cancer disposal and efficacy.

Our results showed that the largest proportion of mutations (54.54) % was in favor of missense mutations. Vogelstein *et al*., (2013); Tokheim and Karchin, (2019) reported that missense mutations are the most common protein coding mutations found in cancer genomics.

The presence of a silent mutation in the sites of the *ABCG2*, G38A, G2559C, G96428A; due to converted single nucleotide base Guanine to Adenine and Cytosine; as a result of the substitution transition and transversion, the reading of triple code for Glutamic and Isoleucine amino acid (AA) will change to another triple code but it will code for the same amino acid. Lawrence *et al*., (2013); and Engin *et al*., (2016) mentioned that the silent mutations are often used to figured the mutation rate in carcinogenesis, as most silent mutations are unlikely to alter protein activity. Our results showed that the polymorphisms (163G^{del}, 167 A^{del}) gave frameshift mutation due to the deleting of the nitrogen base G at position 163 and A at position 167, respectively.

A new sites of the SNPs differs from the sites in previous studies on the *ABCG2* gene, Imai *et al*., (2002) found that the presence of the SNPs at the site 376C >T of *ABCG2* leads to the formation of a stop codon (Q126 Stop) resulting in decreased protein expression and may be associated with chemotherapy hypersensitivity. Poonkuzhali *et al*., (2008) concluded that the presence of SNP at the site 15994C >T of the *ABCG2* gene ended with increased Breast Cancer Resistance Protein (BCRP) expression.

The new SNPs in our results may be accompany by a dys-function of *ABCG2* in Maysan women. Noguchi *et al*., (2014) reported that the drug efflux activity of the *ABCG2*/BCRP is influenced by several mutations, which necessarily affect the clinical efficacy of the *ABCG2*/BCRP- transportable anticancer drugs.

5-3-2: *ABCB1* gene

The *ABCB1* gene is highly polymorphic, and these polymorphisms are correlated with P-gp expression, and its contribute substantially to MDR. Daniyal *et al*., (2021) stated that the genetic polymorphisms of the transporter gene can contribute to multidrug resistance because they are responsible for changes that lead to treatment differences of different individuals.

There are four SNPs (C153T, C323T) gave silent mutations despite the change of genetic code, because the AA (Isoleucine) has more than one code. The silent mutation may be related to in defect of function of *ABCB1*gene in Maysan women. Jelen *et al*., (2015) ; and Tulsyan *et al*., (2016) stated that the silent mutation of *ABCB1* may exert an impact on the protein function by changing mRNA splicing, folding and stability or modification of translation efficiency. Antonarakis and Cooper, (2013) wrote that the synonymous SNPs of the *ABCB1* gene have been shown to alter *ABCB1* protein structure and activity, may by changing the timing of protein folding following extended ribosomal pause times at rare codons. On the other hand, the synonymous polymorphisms of the *ABCB1* (*MDR1*) gene can change the structure or function of the protein by coupling with non-synonymous polymorphisms, lead directly to change AA sequences of a protein (Kimchi-Sarfaty *et al*., 2007; Zawadzka *et al*., 2020).

The SNP (G55C, T59G, C89A, C90G, A113C, A139T) gave missense mutation due to a change in the genetic code, that led to a change in AA at the level of the resulting protein from Valine to Leucine, Valine to Glysine, Alanine to Aspartic acid, Valine to Aspartic acid, Aspartic acid to Alanine, and threonine to Serine. Wang *et al*., (2009); Wolf *et al*., (2011) reported the most common form of genetic variation is an SNP, and the SNP in the coding regions of *ABCB1* gene may directly alter a protein structure, producing AA substitutions (non-synonymous SNPs). Kadioglu *et al*., (2020) mentioned that the missense and frameshift mutations of the transporter gene, lead to altered binding of anticancer drugs in the molecular docking approaches. O'connor, (2007); Ankathil, (2017) mentioned that the over-expression of *ABCB1* mRNA expression may cause a fast drug efflux from the cell, decrease in the intracellular drug concentration, and subsequently limits the drug penetrance into intra tumor cells. De Azevedo Delou *et al*., (2017) mentioned that the negative or decreased expression of the *ABCB1* gene in breast tumors rather than being beneficial due to a possible reduced drug efflux appears to indicate more phenotype aggressive, which may occur more common among triple negative BC (TBC).

The sites of the new SNPs differ from the sites in previous studies. Priyadarshini *et al*., (2019) concluded that the SNPs C3435T, C1236T were synonymous due to no alteration in amino acids sequences, Ile >Ile in C3435T and Gly >Gly in C1236T, in the BC patients from southern India.

Also, Kim *et al*., (2001) mentioned that the 1199 G >A polymorphism of the *MDR1* in the Caucasian population was a non-synonymous type with alteration of AA from Serine to Asparagine (Ser >Asn).

A study by Chang *et al*., (2009) reported that the 2677 G >T/A or 3435 C >T polymorphism associated with chemoresistance of the metastatic Breast Cancer (MBC). Whereas Wang *et al*., (2005) documented that the C3435T SNP of *MDR1* is silent mutation, and is associated with decreased mRNA and protein expression levels via unknown mechanisms.

Kim *et al*., (2001); and Fung and Gottesman, (2009) reported that the *MDR1* 3435C >T(I >I), 1236 C >T (G >G) polymorphism is linked to a synonymous type, and 2677G >T/A (Ala >Ser) polymorphism is linked to a non-synonymous type, where they cause effects in cellular systems, alterations in the mRNA levels, and increased drug efflux, subsequently some of the SNPs are associated with altered P- glycoprotein structure and function by altering the protein folding.

Lévy *et al*., (2013) found polymorphisms 129 T >C, 61A >G, 1236C >T, 2677G >T/A, and 3435 C >T in *ABCB1* gene of 101 BC patients receiving doxorubicin and docetaxel, the SNP C3435T is associated with a good pathological response to chemotherapy.

5-3-3: *BRCA1* gene

The *BRCA1* gene is the main cause of familial BC, and it has a very strong relationship with hereditary BC. Thus, this study aimed to determine the presence of polymorphism (SNP or indel) of the *BRCA1* gene by gene sequencing. Then it may contribute in declining the incidence of early familial BC. Surbone, (2011) mentioned that females

who carry a harmful mutation in the *BRCA1* gene may be offered chemoprevention, such as early monitoring and preventive surgery. This is important to strengthen public policies through female population commitment to preventive strategies programs, thus reducing morbidity and mortality associated with hereditary cancers.

Koumpis *et al*., (2011) referred to the importance of identifying females at risk of BC because the inheritance of a harmful *BRCA1* mutation is one of the most important predictors of individual risk.

A study by Mehrgou and Akouchekian, (2016) mentioned the role of *BRCA* genes in early detection of mutation which can play an important role in the prevention of BC. Therefore, female screening of the mutations in predisposing genes, seem to be necessary.

The *BRCA1* polymorphisms (C213T, T248C, and T261C) gave missense mutation due to a change in the genetic code, in return back a change in AA at the level of the resulting protein from Valine to Methionine, Glutamic to Glysine and Threonine to Histidine, possibly depending on the type of AA that has been substituted in the protein associated with BC, the appropriate counseling and treatment can be suggested.

The new SNPs of *BRCA1* gene may contribute to the development and progression of BC in the Maysan women Iraq by affecting the tumor suppressor protein produced from the *BRCA1* gene. The most common types of mutations are frameshift, nonsynonymous, and disruption of splice site leading to entire non-functional *BRCA1* proteins (Karami and Mehdipour, 2013). The mutation of the *BRCA*1/2 lead to gene function silencing or over-activation (Valarmathi *et al*., 2004; Shah *et al*., 2018).

Our findings showed the sites of the new SNPs differs from the sites in previous studies Saleh-Gohari *et al*., (2012) found SNPs of *BRCA1* at site 966 T >C of the breast cancer patients in Kerman province/Iran.

Neamatzadeh *et al*., (2015) found SNPs of *BRCA1* at site 2311 T >C, 4308T >C in *BRCA1* of Iranian female BC patients.

Lara *et al*., (2012) found SNPs of *BRCA1* at site 4427 T >C in *BRCA1* of breast cancer patients in Venezuela.

Mehta *et al*., (2018) found that the novel *BRCA1* polymorphism included 168 T ^{del} of *BRCA1* in North India.

Gajalakshmi *et al*., (2007) found a novel mutation 1307 T ^{del} of the *BRCA1* gene in an Indian family with breast and ovarian cancer.

5-3-4: *ER-α*

Estrogen play a role of progression and development of BC. Polymorphisms in the estrogen receptor alpha gene may be associated with BC risk. Thus, this study objectives to determine genetic polymorphisms in the estrogen alpha gene for the evaluation relationship between the type of polymorphism and its effect on translation in the BC patients. Barzan *et al* ., (2013); and Fjeldheim *et al*., (2016) reported the SNPs in *ESR1* may directly or indirectly lead to variations in its activity, and may have an effect on BC risk. Fuentes and Silveyra, (2019) concluded that the estrogens exert their actions by binding to specific receptors, the estrogen receptors (ERs), which in turn activate transcriptional processes and /or signaling events that finishing in the control of gene expression.

There are four new SNPs (C114T, C244G, C344G, and C424T) gave silent mutations despite the change of genetic code, because the AA (Glycine, Glycine, Alanine, Serine) have more than one code, although these mutations are silent, and they may or may not effect on resulting protein of GE. Thus, may or may not contribute to progression and development of cancer in Maysanian women. Siddig *et al*., (2008) said that the *ER-α* gene polymorphism may indirectly affect the protein function by alteration of the RNA half-life or protein translation, hence indirectly affecting the level of the ERα protein.

They mentioned that silent a SNP affected protein function because it forced the cell to read a different DNA codon than it usually does, while the same protein sequence eventually was made. Thus, this silent change may slow the folding rhythm resulting in an altered protein conformation, which in turn affect function. Herynk and Fuqua, (2004) reported silent mutations of *ER-α* do not directly affect protein sequence, and they may indirectly affect protein function through changes in RNA turnover rates, thus indirectly altering ER-α protein levels.

The new SNPs of *ER-α* (T440C) gave missense mutation due to a change in the genetic code, that led to a change in AA at the level of the resulting protein from (Leucine) to (Proline). Our results showed that the Indel polymorphisms (454 A^{del}) gave frameshift mutation due to the deleting of single nitrogen base A in the position 454 of *ER-α* gene. The missense SNP and indel polymorphisms of *ER-α* gene may contribute to the development and progression of BC by affecting the level of GE. Fuqua *et al*., (2014) reported that the alterations in a protein sequence of the *ER-α* contributed in acquired resistance to hormonal therapy in patients with *ER-α* positive BC. Thomas and Gustafsson, (2015) mentioned that the genetic alterations of the ERs lead to altered protein sequence through altering the conformation of the protein, and increasing the interaction with coactivators, with point mutations in *ESR1*.

The sites of the new SNPs differ from the sites in previous studies. When comparing results with previous studies. Madeira *et al*., (2014) mentioned that the SNP of *ER-α* at site 397, 454 T >C in BC patients.

Siddig *et al*., (2008) mentioned that the SNP of ER-ALPHA at site 325 C >G.

McGuire *et al*., (1992) mentioned that the missense mutation in the ER-ALPHA was determined as a Leucine to proline substitution at AA 296.

We suggest the reason for the differences in the genetic polymorphism for (*ABCG2*, *ABCB1*, *BRCA1*, and *ER-α*) in this study and the previous studies mentioned may be attributed to the differences in environmental conditions, lifestyle and nature of pollutants

to which the population exposed. Therefore, the genetic polymorphisms in this study differed from previous studies in the SNP sites, and the type of polymorphism.

5-4: Cytogenetic study of breast cancer tissue

Reactive oxygen species (ROS) are molecules capable of freelance existence, containing at least one oxygen atom and one or more unpaired electrons may be contributing to the development of many free radical-mediated diseases. It contributes in causing damage at the molecular and cellular level, as well as changes in proteins and nucleotides (Jakubczyk *et al*., 2020).

DAPI is the nuclear stain that can stain deoxyribonucleic acid (DNA). In this study, the nucleus of the BC cell with DAPI stain not fragmented, this may be because of the absence of apoptosis due to the acidic (microenvironment) out of cancer cell that act to prevent or decrease cytochrome C activity of mitochondria and thus fail in activation caspases which in turn fail in the stimulation nucleases. This is leading to the failure of apoptosis in cancer, so the cancer cell will escape from apoptosis and persist in proliferation due to alkaline PH inside the cancer cell and acidic PH outside it (microenvironment). Matsuyama *et al*., (2000); and White *et al*., (2017) reported that apoptosis is caused by the release of cytochrome C from mitochondria, which subsequently activate catabolic enzymes including caspases, that decreased pH (PH_i) and it is an early signal of caspase activation in apoptosis, while increased pH_i blocks apoptosis signals and effected on DNA degradation. Cancer cells have a reversed PH gradient with a slightly elevated intracellular PH (PH_i) despite an acidic microenvironment (PH_e) (Damaghi *et al*., 2013; Swietach *et al*., 2014; Lee, 2021).

The increasing of the green color signal in the BC tissues compared with control may be due to the high level of ROS. DCFH stain is converted to DCF, when it interact with ROS in breast tissue. Malla *et al*., (2021) reported increased ROS in breast tumors and their surrounding tumor microenvironment (TME) cells. Arun, (2015) stated the DCFH-DA reduced to DCFH by the esterases that presenting inside cells, DCFH converted to DCF due to its interaction with the ROS. The cells

with more DCF give more green fluorescence under the fluorescence microscope, the green color signal is a measure of the amount of ROS inside the cell, and more fluorescence indicating increased production of ROS (Arun, 2015).

The findings showed the high ROS in BC cells and its spread in most BC tissues may be due to cancer cell playing a role similar to role atoms or molecules that has an unpaired electron, as the cancer cell that has free radical will collide with another stable cell and convert this cell into an unstable state due to emergence free radical and later on, this transformed cell will attack another stable cell. Therefore, cancer will spread due to free radical and it is an important source of ROS. Free radicals can be considered as a unique property of cancer (Kumari *et al*., 2018).

Our findings showed the defect of mitochondrial morphology in the BC tissue and increase in mtROS may be due to a defect in mitochondrial function, mitochondrial dynamic, hypoxia, acidic microenvironment, and defect in the electron transport chain (ETC). One of the major sources of ROS in epithelial cells of BC is mitochondrial dysfunction coupled with metabolic readaptation for ATP generation (Costa *et al*., 2014). Rohrmann *et al*., (2013) mentioned the elevated metabolic rate in mitochondria, endoplasmic reticulum, and cellular membranes in cancer cells cause a defect in ETC, resulting in elevated electron leakage and increased ROS generation. Chiu *et al*., (2019) assumed the hypoxia in carcinomas due to a lack of oxygen (O_2) as an electron acceptor, causes inefficient electron transfer through the electron transport chain (ETC) in mitochondria, with accumulation of (ROS) which can cause irreversible cellular damage.

In relation to Warburg effect (aerobic glycolysis) of cancer cell give a small amount of ATP, these cell more proliferated to produce more ATP by this rapid process, therefore, the cancer cell resist cell death may be by slowing the consumption of O_2 in hypoxic cell and mild hypoxia can support cell growth. Therefore, they can live a prolonged period without apoptosis. Lewis *et al*., (2005);

and Kumari, (2018) mentioned the cancer cells maintain their high energy levels through glycolysis followed by lactic acid fermentation even in the presence of abundant oxygen (aerobic glycolysis, Warburg effect) followed by oxidation in mitochondria, which is essential for cancer cells to adapt to hypoxic states with defect mitochondria and ROS production.

As a results, when prescribing a treatment or giving a dose of radiation for a cancer patient, one must first measure the microenvironment acidity and then give the treatment or radiation because prescribing treatment and giving radiation to the patient in the presence of acidic environment is considered ineffective and cancer continues to spread. Raghunand *et al*., (2003) discovered in the mouse model the modulation of PH_e can be reduced metastasis and improve survival in BC.

All BC tissues used in this study showed markedly elevated levels of ROS under fluorescent microscopy, and they applied to verify the association between ROS and (SNPs, mutations) in some studied genes associated with BC by comparing the DNA sequencing results of studied genes with the results of fluorescence microscopy of breast tissue.

The results showed that BC tissues showed a high level of ROS, in addition, showed mutations in *ABCB1*, *ABCG2*, *BRCA1*, and *ER-α* genes such as frameshift mutation (deletion and insertion); Point mutation (SNP) (transition, transversion), these mutations may be due to an elevated ROS level in BC tissues. These are agreement with (Lunec *et al*., 2002; Noreen *et al*., 2018) which reported that ROS can cause DNA mutations, which convert Guanine to Thymine or vice versa. Choudhari *et al*., (2014) reported that ROS can cause oxidation of purines and pyrimidines. Brown and Bicknell, (2001); and Noreen *et al*., (2018) concluded the mutations that caused by ROS mostly affect GC bases substitutions, while deletions and insertions mutation are less occurring, whereas AT bases mutations are rare, and all these genetic instabilities resulting in the inactivation of tumor

suppressor genes or enhance the expression of proto-oncogenes that can strengthen cancer.

Findings of gene sequencing showed that an SNPs in the *BRCA1* gene may be the cause of the elevated ROS level within BC tissues. Our results are in agree with (Martinez-outschoorn *et al*., 2012; Gorodetska *et al*., 2019) which reported the mutations in *BRCA1* can increase cellular ROS, and this contribute to the development of BC.

The ROS was significant high in BC tissue compared with healthy/normal tissue may be attributed to many reasons, as the presence of oxidative stress, that produce under the special condition in cells when unbalance of oxidant and anti-oxidant. Legg, (2017) wrote that oxidative stress is an imbalance between oxidants (free radicals) and antioxidants in the body, it can lead to a large number of diseases over time as cancer. Another reason, may be contributed to high ROS levels in BC tissue is food system, patients' socio-economic conditions and the lack of non-enzymatic antioxidants, the antioxidants have electrons in their outer shell that combine with free radicals and thus get rid of free radicals. Adwas *et al*., (2019) stated that antioxidants block production of ROS and scavenge free radicals. Shalaby and Azzam, (2018) reported that antioxidants can donate electrons that neutralize radicals without forming others. Exposure to environmental pollutants such as ionizing and non-ionizing radiation, ultraviolet radiation, high concentrations of ozone, cigarette smoke, and many other compounds in the environment; can result in overproduction of ROS, oxidative damage to cellular components and biomolecules can be involved in many chronic diseases including cancer (Poljšak and Fink, 2014).

Chapter six

Conclusions and Recommendations

6. Conclusions and Recommendations

6-1: Conclusions

1. Both *ABCG2* and *ABCB1* genotypes differs from HWE of BC patients compared to the control.

2. The *ABCG2*, *ABCB1* genotypes differs between BC patients and control, and the CC, CT genotypes of the *ABCG2*, *ABCB1* genes may be associated with the risk of BC development.

3. *BRCA1* genotypes are similar in HWE of the BC patients and the control, the deviations from HWE in the control group may indicate genetic mutations in some healthy individuals.

4. Different dominant model of *ABCG2*, *ABCB1* genotypes between BC patients and control.

5. The primers can be nominate to be a distinctive indicator of BC at the level of the RAPD indicators, and thus these primers can be adopt to distinguish BC genetically.

6. Successful PCR product for the first time to be a template in terms of the number of bands and the accuracy of the result in studying genetic polymorphisms of the BC related genes in PCR-RAPD-PCR technique.

7. Different total number of polymorphic, unique, monomorphic bands of (*miRNA-152*, and *ER-α*) genes between the BC patients and control reverse to the total number of polymorphic, unique, monomorphic bands of (*ABCG2*, *ABCB1*, and *BRCA1*) genes.

8. Nucleotide sequencing analysis revealed 11 mutations in the *ABCG2* gene, seven in the *ABCB1* gene, three in the *BRCA1* gene and six in the *ER-α* gene.

9. Changing the shape of the three-dimensional (3D) protein of the *ABCG2*, *ABCB1*, *BRCA1*, and *ER-α* genes among BC patients.

10. Evidence of a high level ROS and mitochondrial defect in BC tissue than healthy tissue by cytogenetic study.

6-2: Recommendations

1. This study presented genetic informations of the breast cancer related genes and cytogenetic informations on breast cancer tissue, this information can be used as a reference for future studies of these genes and tissues.

2. The results indicated similarities and differences in the genetic polymorphisms of the *ABCG2*, *ABCB1*, *BRCA1*, *ER-α* and *miRNA-152* between breast cancer patients and control and this help in the ongoing struggle to clarify and confirm the differences through several techniques of PCR, nucleotide sequencing and cytogenetic study.

3. Future studies including a larger number of populations to give a clearer picture of the genetic polymorphisms of the breast cancer related genes.

4. Increments the impaction about lifestyle, physiological and environmental factors on the genetic polymorphisms of BC related genes.

5. Elucidation of the role of silent mutations on gene expression of BC related genes.

6. Studying the genetic polymorphisms of the breast cancer related genes in male BC patients.

7. Study the effects of anti-cancer agents on the genetic polymorphisms of BC related genes.

8. The use of bioinformatics and molecular genetics techniques in the field of medical diagnosis and as one of the methods for genetically diagnosing BC, and in the choosing of a suitable treatment.

9. Adopting the use of fluorescent microscopy in diagnosing BC tissues, with more details and information about cancer tissues upon the amount of ROS and defect of mitochondrial morphology.

Chapter seven

References

7. References

Aarøe, J., Lindahl, T., Dumeaux, V., Sæbø, S., Tobin, D., Hagen, N., Skaane, P., Lönneborg, A., Sharma, P., & Børresen-Dale, A. L. (2010). Gene expression profiling of peripheral blood cells for early detection of breast cancer. *Breast Cancer Research*, 12(1), 1-11.

Abd Ellatif, M., Zahran, M. A., Denaiwar, A., Elbaz, A., & Abdel Azeez, H. A. (2016). Study of polymorphism of the estrogen receptor alpha gene as a genetic marker for the risk of breast cancer. *IJCEBS*, *4*, 16-22.

Abel, E. L., & DiGiovanni, J. (2011). Multistage carcinogenesis. In Chemical Carcinogenesis. Humana Press. Philadelphia: USA. PP 440.

Abramovs, N., Brass, A., & Tassabehji, M. (2020). Hardy-Weinberg equilibrium in the large scale genomic sequencing era. *Frontiers in genetics*, 11, 210-220.

Abuhaliema, A. M., Yousef, A. M. F., El-Madany, N. N., Bulatova, N. R., Awwad, N. M., Yousef, M. A., & Al Majdalawi, K. Z. (2016). Influence of genotype and haplotype of MDR1 (C3435T, G2677A/T, C1236T) on the incidence of breast cancer-a case-control study in Jordan. *Asian pacific journal of cancer prevention*, 17(1), 261-266.

Adebayo, R. O., Olaogun, A. A., Loto, O. M., Kolawole, A. A., Alabi, T. O., Ajao, O., & Solarin, A. E. (2019). Assessment of breast cancer risk factors among women of reproductive age group in Oshogbo using Gail model. *International Journal of Nursing and Midwifery*, 11(2), 7-17.

Adwas, A. A., Elsayed, A., Azab, A. E., & Quwaydir, F. A. (2019). Oxidative stress and antioxidant mechanisms in human body. *Journal of Applied Biotechnology & Bioengineering*, 6(1), 43-47.

Al Hannan, F., Keogh, M. B., Taha, S., & Al Buainain, L. (2019). Characterization of BRCA1 and BRCA2 genetic variants in a cohort of Bahraini breast cancer patients using next-generation sequencing. *Molecular genetics & genomic medicine*, 7(7), 771-779.

Al-Amri, R. J., Alotibi, M. K. H., Al-Raddadi, R. I., Shebli, W. T. Y., Fallatah, E. I. Y., Alhujaily, A. S., & Mohamed, H. S. (2020). Estrogen Receptor 1 Gene (ESR1) rs2234693 Polymorphism and Breast Cancer Risk in Saudi Women. *Asian Pacific Journal of Cancer Prevention*, 21(11), 3235-3240.

Al-Askeri, M. A. (2016) . Genetic Polymorphism in Iraqi Females Diagnosed with Breast Cancer Using Random Amplification of Polymorphic DNA Technique. *INTERNATIONAL JOURNAL OF PHARMTECH RESEARCH*, 9(11), 312-316.

Al-Koofee, D. A., & Mubarak, S. M. (2019). Genetic polymorphisms. In *The Recent Topics in Genetic Polymorphisms*. IntechOpen. London: United kingdom. PP135.

Alluri, P. G., Speers, C., & Chinnaiyan, A. M. (2014). Estrogen receptor mutations and their role in breast cancer progression. *Breast Cancer Research*, 16(6), 1-8.

AlMutairi, F., Ali Khan Pathan, A., Alanazi, M., Shalaby, M., Alabdulkarim, H. A., Alamri, A., Al Naeem, A., Elrobh, M., Shaik, J. P., & Khan, W. (2015). Association of DNA repair gene APE1 Asp148Glu polymorphism with breast cancer risk. *Disease Markers*, 2015, 1-10.

Al-Naqqash, M. A. (2020). The 21-gene oncotype DX offers more accurate treatment decisions in early breast cancer. *Gastric Breast Cancer*, 15, 1-7.

Al-Rawi, K. M., and Khalaf Allah, A. M. (2000). Design and Analysis of Agricultural Experiments. University of Mosul. Ministry of Higher Education and Scientific Research. Dar Al Kuttab for printing and publishing. Mosul. Iraq.

AL-Rawi, T. (2015). Study of sex determination of date palm in seeding stage using convential molecular indice (Doctoral dissertation, Baghdad university).

Altman, D. G. (1991). Statistics in medical journals: developments in the 1980s. *Statistics in medicine*, *10*(12), 1897-1913.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, 215(3), 403-410.

Ameyaw, M. M., Regateiro, F., Li, T., Liu, X., Tariq, M., Mobarek, A., Thornton, N., Folayan, G. O., Githang'a, J., & Indalo, A. (2001). MDR1 pharmacogenetics: Frequency of the C3435T mutation in exon 26 is significantly influenced by ethnicity. *Pharmacogenetics and Genomics*, 11(3), 217-221.

Anfinsen, C. B. (1973). Principles that govern the folding of protein chains. *Science*, 181(4096), 223-230.

Ankathil, R. (2017). ABCB1 genetic variants in leukemias: current insights into treatment outcomes. *Pharmacogenomics and personalized medicine*, 10, 169-181.

Antonarakis, S. E., & Cooper, D. N. (2013). Human gene mutation in inherited disease: Molecular mechanisms and clinical consequences. Rimonin: David . Elsevier. PP626.

Antoniou, A., Pharoah, P. D., Narod, S., Risch, H. A., Eyfjord, J. E., Hopper, J. L., Loman, N., Olsson, H., Johannsson, O., & Borg, Å. (2003). Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case series unselected for family history: A combined analysis of 22 studies. *The American Journal of Human Genetics*, 72(5), 1117-1130.

Arnesen, S., Blanchard, Z., Williams, M. M., Berrett, K. C., Li, Z., Oesterreich, S., Richer, J. K., & Gertz, J. (2021). Estrogen receptor alpha mutations in breast cancer cells cause gene expression changes through constant activity and secondary effects. *Cancer Research*, 81(3), 539-551.

Arun, A. (2015). Apoptotic regulation of Cu/Zn SOD in retinal pigment epithelial cells (Master thesis, Kalasalingam University).

Atienzar, F. A., Venier, P., Jha, A. N., & Depledge, M. H. (2002). Evaluation of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 521(1), 151-163.

Ayatollahi, H., & Keramati, M. R. (2018). BCR-ABL fusion genes and laboratory findings in patients with chronic myeloid leukemia in northeast Iran. *Caspian journal of internal medicine*, 9(1), 65-70.

Ayaz, G., Yasar, P., Olgun, C. E., Karakaya, B., Kars, G., Razizadeh, N., Yavuz, K., Turan, G., & Muyan, M. (2019). Dynamic transcriptional events mediated by estrogen receptor alpha. *Front. Bio sci*, 24, 245-276.

Ayub, S. G., Rasool, S., Ayub, T., Khan, S. N., Wani, K. A., & Andrabi, K. I. (2014). Mutational analysis of the *BRCA2* gene in breast carcinoma patients of Kashmiri descent. *Molecular medicine reports*, 9(2), 749-753.

Bamanga, R. A., Ja'afar, J. N., & Gali, A. I. (2018). Progress in DNA sequencing. *Bayero Journal of Pure and Applied Sciences*, 11(1), 110-119.

Bao, Y., Kishnani, P., Wu, J. Y., & Chen, Y. T. (1996). Hepatic and neuromuscular forms of glycogen storage disease type IV caused by mutations in the same glycogen-branching enzyme gene. *Journal of Clinical Investigations*, 97 (4), 921-948.

Barnes, R. P., Fouquerel, E., & Opresko, P. L. (2019). The impact of oxidative DNA damage and stress on telomere homeostasis. *Mechanisms of ageing and development*, 177, 37-45.

Barzan, D., Veldwijk, M. R., Herskind, C., Li, Y., Zhang, B., Sperk, E., Du, W. D., Zhang, X. J., & Wenz, F. (2013). Comparison of genetic variation of breast cancer susceptibility genes in Chinese and German populations. *European Journal of Human Genetics*, 21(11), 1286-1292.

Behjati, S., & Tarpey, P. S. (2013). What is next generation sequencing?. *Archives of Disease in Childhood-Education and Practice*, 98(6), 236-238.

Bidet, P., Lalande, V., Salauze, B., Burghoffer, B., Avesani, V., Delmée, M., Rossier, A., Barbut, F., & Petit, J. C. (2000). Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing Clostridium difficile. *Journal of Clinical Microbiology*, 38(7), 2484-2487.

Boysen, L., Viuff, B. M., Landsy, L. H., Price, S. A., Raymond, J. T., Lykkesfeldt, J., & Lauritzen, B. (2019). Formation and glomerular deposition of immune complexes in mice administered bovine serum albumin: Evaluation of dose, frequency, and biomarkers. *Journal of immunotoxicology*, 16(1), 191-200.

Brunel, H. (2013). Genetic association analysis of complex diseases through information theoretic metrics and linear pleiotropy (Doctoral dissertation, Universitat Politècnica de Catalunya (UPC)).

Brown, N. S., & Bicknell, R. (2001). Hypoxia and oxidative stress in breast cancer Oxidative stress-its effects on the growth, metastatic potential and response to therapy of breast cancer. *Breast cancer research*, 3(5), 1-5.

Buckingham, L. (2012). Chromosomal structure and chromosomal mutation. Molecular Fundamentals Methods and Clinical Applications. 2nd ed. Philadelphia: FA Davis Company, 348-352.

Calò, V., Bruno, L., La Paglia, L., Perez, M., Margarese, N., Di Gaudio, F., & Russo, A. (2010). The clinical significance of unknown sequence variants in *BRCA* genes. *Cancers*, 2(3), 1644-1660.

Chandira, R. M., Prabakaran, M., Jaykar, B., Venkateswarlu, B. S., & Palanisamy, P. (2019). BRCA mutation: A review of breast cancer. *Journal of Drug Delivery and Therapeutics*, 9(4), 750-758.

Chang, H., Rha, S., Jeung, H. C., Im, C. K., Ahn, J., Kwon, W., Yoo, N., Roh, J., & Chung, H. (2009). Association of the *ABCB1* gene polymorphisms 2677G> T/A and 3435C>T with clinical outcomes of paclitaxel monotherapy in metastatic breast cancer patients. *Annals of Oncology*, 20(2), 272-277.

Chatterjee, N., & Walker, G. C. (2017). Mechanisms of DNA damage, repair, and mutagenesis. *Environmental and molecular mutagenesis*, 58(5), 235-263.

Chaudhary, R., & Maurya, G. K. (2019). Restriction fragment length polymorphism. Encyclopedia of Animal Cognition and Behavior. Cham: springer. PP 175.

Chen, L., Manautou, J. E., Rasmussen, T. P., & Zhong, X. B. (2019). Development of precision medicine approaches based on inter-individual variability of BCRP/ABCG2. *Acta Pharmaceutica Sinica B*, 9(4), 659-674.

Chitty, J. L., Filipe, E. C., Lucas, M. C., Herrmann, D., Cox, T. R., & Timpson, P. (2018) . Recent advances in understanding the complexities of metastasis. *F1000 Research*, 7,1-18

Chiu, D. K. C., Tse, A. P. W., Law, C. T., Xu, I. M. J., Lee, D., Chen, M., Lai, R. K. H., Yuen, V. W. H., Cheu, J. W.S., HO, D. W.H., Wong, C. M., Zhang. H., Ng. I. O. L., & Wong, C. C. L. (2019). Hypoxia regulates the mitochondrial activity of hepatocellular carcinoma cells through HIF/HEY1/PINK1 pathway. *Cell death & disease*, 10(12), 1-16.

Choudhari, S, K., Chaudhary, M., Gadbail, A. R., Sharma, A., & Tekade, S. (2014).Oxidative and antioxidative mechanisms in oral cancer and precancer: a review. *Oral Oncology*, 50(1), 10-18.

Colaprico, A., Olsen, C., Bailey, M. H., Odom, G. J., Terkelsen, T., Silva, T. C., Olsen, A. V., Cantini, L., Zinovyev, A., & Barillot, E. (2020). Interpreting

pathways to discover cancer driver genes with Moonlight. *Nature Communications*, 11(1), 1-17.

Consortium, G. P., Auton, A., Brooks, L. D., Durbin, R. M., Garrison, E. P., & Kang, H. M. (2015). A global reference for human genetic variation. *Nature*, 526(7571), 68-74.

Contrò, V., Basile, J. R., & Proia, P. (2015). Sex steroid hormone receptors, their ligands, and nuclear and non-nuclear pathways. *AIMS Mol Sci*, 2(3), 294-310.

Costa, A., Scholer-Dahirel, A., & Mechta-Grigoriou, F. (2014). The role of reactive oxygen species and metabolism on cancer cells and their microenvironment. *Seminars in Cancer Biology*, 25, 23-32.

Coughlin, S. S. (2019). Epidemiology of breast cancer in women. Breast cancer metastasis and drug resistance, Cham: springer, PP 424.

Cronin, K. A., Lake, A. J., Scott, S., Sherman, R. L., Noone, A., Howlader, N., Henley, S. J., Anderson, R. N., Firth, A. U., & Ma, J. (2018). Annual Report to the Nation on the Status of Cancer, part I: National cancer statistics. *Cancer*, 124(13), 2785-2800.

Cronshaw, M., Parker, S., & Arany, P. (2019). Feeling the heat: evolutionary and microbial basis for the analgesic mechanisms of photobiomodulation therapy. *Photobiomodulation, photomedicine, and laser surgery*, 37(9), 517-526.

Dai, Z., Tian, T., Wang, M., Yang, T., Li, H., Lin, S., Hao, Q., Xu, P., Deng, Y., & Zhou, L. (2019). Genetic polymorphisms of estrogen receptor genes are associated with breast cancer susceptibility in Chinese women. *Cancer Cell International*, 19(1), 1-7.

Damaghi, M., Wojtkowiak, J. W., & Gillies, R. J. (2013). pH sensing and regulation in cancer. *Frontiers in Physiology* , 4, 370-380.

Daniyal, A., Santoso, I., Gunawan, N. H. P., Barliana, M. I., & Abdulah, R. (2021). Genetic Influences in Breast Cancer Drug Resistance. *Breast Cancer: Targets and Therapy*, 13, 59-85.

De Azevedo Delou, J. M., Vignal, G. M., Índio-do-Brasil, V., de Souza Accioly, M. T., da Silva, T. S. L., Piranda, D. N., Sobral-Leite, M., de Carvalho, M. A., Capella, M. A. M., & Vianna-Jorge, R. (2017). Loss of constitutive ABCB1

expression in breast cancer associated with worse prognosis. *Breast Cancer: Targets and Therapy*, *9*, 415-428.

De Lima Corrêa, L., Borguesan, B., Krause, M. J., & Dorn, M. (2018). Three-dimensional protein structure prediction based on memetic algorithms. *Computers & Operations Research*, 91, 160-177.

Deng, N., Zhou, H., Fan, H., & Yuan, Y. (2017). Single nucleotide polymorphisms and cancer susceptibility. *Oncotarget*, 8(66), 110635-110649.

Deroo, B. J., & Korach, K. S. (2006). Estrogen receptors and human disease. *The Journal of clinical investigation*, 116(3), 561-570.

Di Leva, G., Garofalo, M., & Croce, C. M. (2014). MicroRNAs in cancer. *Annual Review of Pathology: Mechanisms of Disease*, 9, 287-314.

Dorn, M., e Silva, M. B., Buriol, L. S., & Lamb, L. C. (2014). Three-dimensional protein structure prediction: Methods and computational strategies. *Computational biology and chemistry*, 53, 251-276.

Dragh, M. A., Xu, Z., Al-Allak, Z. S., & Hong, L. (2017). Vitamin K2 prevents lymphoma in drosophila. *Scientific reports*, 7(1), 1-15.

Dumitrescu, R. G., & Shields, P. G. (2005). The etiology of alcohol-induced breast cancer. *Alcohol*, 35(3), 213-225.

Durland, J., & Ahmadian-Moghadam, H. (2021). Genetics, Mutagenesis. In StatPearls. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK560519.

Elia, A. E., & Elledge, S. J. (2012). BRCA1 as tumor suppressor: lord without its RING?. *Breast Cancer Research*, 14(2), 1-3.

Engin, H. B., Kreisberg, J. F., & Carter, H. (2016). Structure-based analysis reveals cancer missense mutations target protein interaction interfaces. *PLOS ONE*, 11(4), 152929-152933.

Ewald, I. P., Izetti, P., Vargas, F. R., Moreira, M. A., Moreira, A. S., Moreira-Filho, C. A., Cunha, D. R., Hamaguchi, S., Camey, S. A., & Schmidt, A. (2011). Prevalence of the BRCA1 founder mutation c. 5266dupin Brazilian individuals at-risk for the hereditary breast and ovarian cancer syndrome. Hereditary Cancer in Clinical Practice, 9(1), 1-8.

Fang, W., Qiu, F., Zhang, L., Deng, J., Zhang, H., Yang, L., Zhou, Y., & Lu, J. (2014). The functional polymorphism of NBS1 p. Glu185Gln is associated with an increased risk of lung cancer in Chinese populations: Case-control and a meta-analysis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 770, 61-68.

Fang, Y., Zhao, Q., Ma, G., Han, Y., & Lou, N. (2013). Investigation on *MDR1* gene polymorphisms and its relationship with breast cancer risk factors in Chinese women. *Medical Oncology*, 30(1), 375-380.

Farazi, T. A., Hoell, J. I., Morozov, P., & Tuschl, T. (2013). MicroRNAs in human cancer. *MicroRNA cancer regulation*, 1-20.

Fayaz, S., Eissa, H. E. S., & Demian, G. A. (2020). Implications of the 21-gene recurrence score assay (Oncotype DX) on adjuvant treatment decisions in ER-positive early-stage breast cancer patients: experience of Kuwait Cancer Control Center. *Journal of the Egyptian National Cancer Institute*, 32(1), 1-7.

Fernandez-Cadenas, I., Andreu, A. L., Gamez, J., Gonzalo, R., Martin, M. A., Rubio, J. C., and Arenas, J. (2003). Splicing mosaic of the myophosphorylase gene due to a silent mutation in McArdle disease. *Neurology* , 61 (10), 1423-1434.

Finkel, T. (2011). Signal transduction by reactive oxygen species. *Journal of Cell Biology*, 194(1), 7-15.

Fjeldheim, F. N., Frydenberg, H., Flote, V. G., McTiernan, A., Furberg, A. S., Ellison, P. T., Barrett, E. S., Wilsgaard, T., Jasienska, G., & Ursin, G. (2016). Polymorphisms in the estrogen receptor alpha gene (ESR1), daily cycling estrogen and mammographic density phenotypes. *BMC Cancer*, 16(1), 1-12.

Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M., and Pastan, I. (1987). Expression of a multidrug-resistance gene in human tumors and tissues. *Proceedings of the National Academy of Sciences*, 84(1), 265-269.

Forman, H. J., Ursini, F., & Maiorino, M. (2014). An overview of mechanisms of redox signaling. *Journal of molecular and cellular cardiology*, 73, 2-9.

Friedlaender, A., Nouspikel, T., Christinat, Y., Ho, L., McKee, T., & Addeo, A. (2020). Tissue-plasma TMB comparison and plasma TMB monitoring in patients

with metastatic non-small cell lung cancer receiving immune checkpoint inhibitors. *Frontiers in oncology*, 10, 142-150.

Fu, S., Cheng, J., Wei, C., Yang, L., Xiao, X., Zhang, D., Stewart, M. D., & Fu, J. (2017). Development of diagnostic SCAR markers for genomic DNA amplifications in breast carcinoma by DNA cloning of high-GC RAMP-PCR fragments. *Oncotarget*, 8(27), 43866-43877.

Fu, Y., Liu, S., Yin, S., Niu, W., Xiong, W., Tan, M., Li, G., & Zhou, M. (2017). The reverse Warburg effect is likely to be an Achilles' heel of cancer that can be exploited for cancer therapy. *Oncotarget*, 8(34), 57813-57825.

Fuchs, Y., & Steller, H. (2011). Programmed cell death in animal development and disease. *Cell*, 147(4), 742-758.

Fuentes, N., & Silveyra, P. (2019). Estrogen receptor signaling mechanisms. *Advances in protein chemistry and structural biology*, 116, 135-170.

Fung, K. L., & Gottesman, M. M. (2009). A synonymous polymorphism in a common MDR1 (*ABCB1*) haplotype shapes protein function. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1794(5), 860-871.

Fuqua , S. A., Gu, G., & Rechoum, Y. (2014). Estrogen receptor (ER) α mutations in breast cancer: hidden in plain sight. *Breast cancer research and treatment*, 144(1), 11-19.

Futreal, P. A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L. M., Haugen-Strano, A., Swensen, J., & Miki, Y. (1994). BRCA1 mutations in primary breast and ovarian carcinomas. *Science*, 266(5182), 120-122.

Gajalakshmi, P., Natarajan, T. G., Rani, D. S., & Thangaraj, K. (2007). A novel BRCA1 mutation in an Indian family with hereditary breast/ovarian cancer. *Breast cancer research and treatment*, 101(1), 3-6.

Garnier-Géré, P., and Chikhi, L. (2013). Population Subdivision, Hardy-Weinberg Equilibrium and the Wahlund Effect. *eLS*, New York, NY: American Cancer Society.

Ge, S., Wang, D., Kong, Q., Gao, W., & Sun, J. (2017). Function of miR-152 as a tumor suppressor in human breast cancer by targeting PIK3CA. *Oncology research*, 25(8), 1363-1371.

George, S. H., Garcia, R., & Slomovitz, B. M. (2016). Ovarian cancer: the fallopian tube as the site of origin and opportunities for prevention. *Frontiers in oncology*, 6, 108-115.

Gholipoorfeshkecheh, R., & Arjunan, S. (2014). Genotyping frequent BRCA1 SNPs in familiar breast cancer in Indian population by restriction fragment length polymorphism and sequencing. *Adv Appl Sci Res*, 5, 262-267.

Ghosh, A., Mukherjee, D., Patel, P., & Mukhopadhyay, D. (2021). The effect of single nucleotide polymorphism (SNP) in glioblastoma multiforme. *OSF*, preprints Article, (1-13).

Gorodetska, I., Kozeretska, I., & Dubrovska, A. (2019). *BRCA* genes: the role in genome stability, cancer stemness and therapy resistance. *Journal of Cancer*, 10(9), 2109-2127.

Graffelman, J., Jain, D., & Weir, B. (2017). A genome-wide study of Hardy-Weinberg equilibrium with next generation sequence data . *Human Genetics*. 136 (6), 727-741.

Güler, E. N. (2017). Gene expression profiling in breast cancer and its effect on therapy selection in early-stage breast cancer. *European journal of breast health*, 13(4), 168-174.

Gutierrez-Rubio, S., Quintero-Ramos, A., Durán-Cárdenas, A., Franco-Topete, R., Castro-Cervantes, J., Oceguera-Villanueva, A., Jiménez-Pérez, L., Balderas-Peña, L., Morgan-Villela, G., & Del-Toro-Arreola, A. (2015). 1236 C/T and 3435 C/T polymorphisms of the *ABCB1* gene in Mexican breast cancer patients. *Genet Mol Res*, 14(1), 1250-1259.

Haider, A. J., Briggs, D., Self, T. J., Chilvers, H. L., Holliday, N. D., & Kerr, I. D. (2011). Dimerization of ABCG2 analysed by bimolecular fluorescence complementation. *PLoS One*, 6(10), 25818-25826.

Haldar, S. (2019). Bioinformatics methods: Application toward analyses and interpretation of experimental data. Advances in biological science research. Academic Press. London. PP 551.

Hamidovic, A., Hahn, K., & Kolesar, J. (2010). Clinical significance of *ABCB1* genotyping in oncology. *Journal of Oncology Pharmacy Practice*, 16(1), 39-44.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, 144(5), 646-674.

Harris, E. Y., Keller, D., Stachura, D., & Wolfe, G. (2016). Bioinformatics sister courses: An interdisciplinary collaborative learning framework to teach bioinformatics. International Conference on Computational Science and Computational Intelligence (CSCI). *IEEE*. USA. PP 385.

Hasan, T. N., Grace, B. L., Shafi, G., & Syed, R. (2013). Association of *BRCA1* promoter methylation with rs11655505 (c. 2265C> T) variants and decreased gene expression in sporadic breast cancer. *Clinical and Translational Oncology*, 15(7), 555-562.

He, C., Xu, Q., Tu, H., Sun, L., Gong, Y., Liu, J., & Yuan, Y. (2016). Polymorphic rs9471643 and rs6458238 upregulate PGC transcription and protein expression in overdominant or dominant models. *Molecular carcinogenesis*, 55(5), 586-599.

He, Z., Chen, Z., Tan, M., Elingarami, S., Liu, Y., Li, T., Deng, Y., He, N., Li, S., Fu, J. & Li, W. (2020). A review on methods for diagnosis of breast cancer cells and tissues. *Cell Proliferation*, 53(7), 12822-12838.

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Strom, A., Treuter, E., & Warner, M. (2007). Estrogen receptors: How do they signal and what are their targets. *Physiological Reviews*, 87(3), 905-931.

Hentze, M. W., Castello, A., Schwarzl, T., & Preiss, T. (2018). A brave new world of RNA-binding proteins. *Nature reviews Molecular cell biology*, 19(5), 327-341.

Herynk, M. H., & Fuqua, S. A. (2004). Estrogen receptor mutations in human disease. *Endocrine reviews*, 25(6), 869-898.

Heyes, N., Kapoor, P., & Kerr, I. D. (2018). Polymorphisms of the multidrug pump ABCG2: a systematic review of their effect on protein expression, function, and drug pharmacokinetics. *Drug Metabolism and Disposition*, 46(12), 1886-1899.

Howell, A., Anderson, A. S., Clarke, R. B., Duffy, S. W., Evans, D. G., Garcia-Closas, M., Gescher, A. J., Key, T. J., Saxton, J. M., & Harvie, M. N. (2014). Risk determination and prevention of breast cancer. *Breast Cancer Research*, 16(5), 1-19.

Hua, H., Zhang, H., Kong, Q., & Jiang, Y. (2018). Mechanisms for estrogen receptor expression in human cancer. *Experimental hematology & oncology*, 7(1), 1-11.

Huo, Y., Selenica, P., Mahdi, A. H., Pareja, F., Kyker-Snowman, K., Chen, Y., Kumar, R., Paula, A. D. C., Basili, T., & Brown, D. N. (2021). Genetic interactions among Brca1, Brca2, Palb2, and Trp53 in mammary tumor development. *NPJ Breast Cancer*, 7(1), 1-12.

Ibrahim M, Saleh N, Archoukieh E, Al-Obaide H, Al-Obaidi M, Said H. (2010). Detection of Novel Genomic Polymorphism Detection of Novel Genomic Polymorphism in Acute Lymphoblastic Leukemia by Random Amplified Polymorphic DNA Analysis. *J. can. Res.*, 6, 19-26.

Imai, Y., Nakane, M., Kage, K., Tsukahara, S., Ishikawa, E., Tsuruo, T., Miki, Y., & Sugimoto, Y. (2002). C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance 1 supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labour and Welfare, Japan, and the Virtual Research Institute of Aging of Nippon Boehringer Ingelheim. 1. *Molecular Cancer Therapeutics*, 1(8), 611-616.

Ismaeel, H. M. (2013). Identification of genomic markers by RAPD-PCR Primers in Iraq Breast Cancer patients. *Iraq J Sci*, 54, 97-104.

Ismail, S., & Essawi, M. (2012). Genetic polymorphism studies in humans. *Middle East Journal of Medical Genetics*, 1(2), 57-63.

Jakubczyk, K., Dec, K., Kałduńska, J., Kawczuga, D., Kochman, J., & Janda, K. (2020). Reactive oxygen species-sources, functions, oxidative damage. *Polski merkuriusz lekarski: organ Polskiego Towarzystwa Lekarskiego*, 48(284), 124-127.

Jan, R. (2019). Understanding apoptosis and apoptotic pathways targeted cancer therapeutics. *Advanced pharmaceutical Bulletin*, 9(2), 205-218.

Jara, L., Morales, S., Mayo, T. D., Gonzalez–Hormazabal, P., Carrasco, V., & Godoy, R. (2017). Mutations in *BRCA1*, *BRCA2* and other breast and ovarian cancer susceptibility genes in Central and South American populations. *Biological research*, 50(35), 1-18.

Jeleń, A. M., Sałagacka, A., Żebrowska, M. K., Mirowski, M., Talarowska, M., Gałecki, P., & Balcerczak, E. I. (2015). The influence of C3435T polymorphism of the *ABCB1* gene on genetic susceptibility to depression and treatment response in polish population-preliminary report. *International journal of medical sciences*, 12(12), 974-979.

Jia, M., Dahlman-Wright, K., & Gustafsson, J. Å. (2015). Estrogen receptor alpha and beta in health and disease. *Best practice & research Clinical endocrinology & metabolism*, 29(4), 557-568.

Jin, Y. (2020). Blood DNA methylation as a surrogate epigenetic biomarker in study of night shift work and breast cancer, (Doctoral dissertation, Yale University).

Kadioglu, O., Saeed, M. E., Munder, M., Spuller, A., Greten, H. J., & Efferth, T. (2020). Effect of ABC transporter expression and mutational status on survival rates of cancer patients. *Biomedicine & Pharmacotherapy*, 131, 110718-110740.

Kalinina, T., Kononchuk, V., Alekseenok, E., Obukhova, D., Sidorov, S., Strunkin, D., & Gulyaeva, L. (2021). Expression of Estrogen Receptor-and Progesterone Receptor-Regulating MicroRNAs in Breast Cancer. *Genes*, 12(4), 582-595.

Karaayvaz, M., Cristea, S., Gillespie, S. M., Patel, A. P., Mylvaganam, R., Luo, C. C., Specht, M. C., Bernstein, B. E., Michor, F., & Ellisen, L. W. (2018). Unravelling subclonal heterogeneity and aggressive disease states in TNBC through single-cell RNA-seq. *Nature Communications*, 9(1), 1-10.

Karami, F., & Mehdipour, P. (2013). A comprehensive focus on global spectrum of *BRCA1* and *BRCA2* mutations in breast cancer. *BioMed research international*, 2013,1-21.

Kassogue, Y., Dehbi, H., Nassereddine, S., Quachouh, M., & Nadifi, S. (2013). Genotype variability and haplotype frequency of *MDR1* (*ABCB1*) gene polymorphism in Morocco. *DNA and cell biology*, 32(10), 582-588.

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N. and Stemberg, M. J. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols*, 10, 845-858.

Kim, C., Gao, R., Sei, E., Brandt, R., Hartman, J., Hatschek, T., Crosetto, N., Foukakis, T., & Navin, N. E. (2018). Chemoresistance evolution in triple-

negative breast cancer delineated by single-cell sequencing. *Cell*, 173(4), 879-893.

Kim, R. B., Leake, B. F., Choo, E. F., Dresser, G. K., Kubba, S. V., Schwarz, U. I., Taylor, A., Xie, H., McKinsey, J., & Zhou, S. (2001). Identification of functionally variant *MDR1* alleles among European Americans and African Americans. *Clinical Pharmacology & Therapeutics*, 70(2), 189-199.

Kimchi-Sarfaty, C., Oh, J. M., Kim, I. W., Sauna, Z. E., Calcagno, A. M., Ambudkar, S. V., & Gottesman, M. M. (2007). A" silent" polymorphism in the *MDR1* gene changes substrate specificity. *Science*, 315(5811), 525-528.

Kindrat, I., Tryndyak, V., de Conti, A., Shpyleva, S., Mudalige, T. K., Kobets, T., Erstenyuk, A. M., Beland, F. A., & Pogribny, I. P. (2016). MicroRNA-152-mediated dysregulation of hepatic transferrin receptor 1 in liver carcinogenesis. *Oncotarget*, 7(2), 1276-1287.

Kiraz, Y., Adan, A., Kartal Yandim, M., & Baran, Y. (2016). Major apoptotic mechanisms and genes involved in apoptosis. *Tumor Biology*, 37(7), 8471-8486.

Koch, L. (2015). Adding another dimension to gene regulation. *Nature Reviews Genetics*, 16(10), 563-563.

Koehn, J., Fountoulakis, M., & Krapfenbauer, K. (2008). Multiple drug resistance associated with function of ABC-transporters in diabetes mellitus: molecular mechanism and clinical relevance. *Infectious Disorders-Drug Targets* (Formerly Current Drug Targets-Infectious Disorders), 8(2), 109-118.

Koleck, T. A., & Conley, Y. P. (2016). Identification and prioritization of candidate genes for symptom variability in breast cancer survivors based on disease characteristics at the cellular level . *Breast Cancer (Dove Med Press)*, 8, 29-37.

Kontomanolis, E. N., Koutras, A., Syllaios, A., Schizas, D., Mastoraki, A., Garmpis, N., Diakosavvas, M., Angelou, K., Tsatsaris, G., & Pagkalos, A. (2020). Role of Oncogenes and Tumor-suppressor Genes in Carcinogenesis: A Review. *Anticancer Research*, 40(11), 6009-6015.

Kori, S. (2018). An overview: several causes of breast cancer. *Epidemiology International Journal*, 2(1), 107-112.

Koš, M., Reid, G., Denger, S., & Gannon, F. (2001). Minireview: genomic organization of the human *ER-α* gene promoter region. *Molecular endocrinology*, 15(12), 2057-2063.

Koumpis, C., Dimitrakakis, C., Antsaklis, A., Royer, R., Zhang, S., Narod, S. A., & Kotsopoulos, J. (2011). Prevalence of *BRCA1* and *BRCA2* mutations in unselected breast cancer patients from Greece. *Hereditary cancer in clinical practice*, 9(1), 1-4.

Kumar, S., Chinnusamy, V., & Mohapatra, T. (2018). Epigenetics of modified DNA bases: 5-methylcytosine and beyond. *Frontiers in genetics*, 9, 640-654.

Kumari, S., Badana, A. K., & Malla, R. (2018). Reactive oxygen species: a key constituent in cancer survival. *Biomarker insights*, 13, 1-19.

Lafta, I. J. (2020). Bioinformatics and Molecular Analysis of the Breast Cancer Susceptibility Gene *BRCA1* in Breast Cancer. *Middle East Journal of Cancer*, 11(1), 59-71.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., & Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. *Current biology*, 12(9), 735-739.

Lahti, L. (2011). Probabilistic analysis of the human transcriptome with side information (Doctoral dissertation, Aalto University).

Lara, K., Consigliere, N., Pérez, J., & Porco, A. (2012). *BRCA1* and *BRCA2* mutations in breast cancer patients from Venezuela. *Biological research*, 45(2), 117-130.

LaPelusa, A., & Kaushik, R. (2020). Physiology, Proteins. StatPearls [Internet].

Lawrence, M. S., Stojanov, P., Polak, P., Kryukov, G. V., Cibulskis, K., Sivachenko, A., Carter, S. L., Stewart, C., Mermel, C. H., & Roberts, S. A. (2013). Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature*, 499(7457), 214-218.

Lee, S., Kasif, S., Weng, Z., & Cantor, C. R. (2008). Quantitative analysis of single nucleotide polymorphisms within copy number variation. *PLoS ONE*, 3(12), 3906-3916.

Lee, S. (2021). Effect of Exogenous pH on Cell Growth of Breast Cancer Cells. *International Journal of Molecular Sciences*, 22(18), 9910-9922.

Legg, T. J. (2017). Everything You Should Know About Oxidative Stress. *Healtline* , https://www.healthline.com/health/oxidative-stress.

Lévy, P., Gligorov, J., Antoine, M., Rezai, K., Lévy, E., Selle, F., Saintigny, P., Lokiec, F., Avenin, D., & Beerblock, K. (2013). Influence of *ABCB1* polymorphisms and docetaxel pharmacokinetics on pathological response to neoadjuvant chemotherapy in breast cancer patients. *Breast Cancer Research and Treatment*, 139(2), 421-428.

Lewis, A., Du, J., Liu, J., Ritchie, J. M., Oberley, L. W., & Cullen, J. J. (2005). Metastatic progression of pancreatic cancer: changes in antioxidant enzymes and cell growth. *Clinical & experimental metastasis*, 22(7), 523-532.

Li, W. J., Chen, X. H., Zeng, J. C., Duan, L. L., Liu, Z. H., & Sheng, X. H. (2020). Theoretical insight into the multiple interactions of quinazoline inhibitors with breast cancer resistance protein (BCRP/ABCG2). *Journal of Biomolecular Structure and Dynamics*, 38(14), 4336-4343.

Lin, M., Whitmire, S., Chen, J., Farrel, A., Shi, X., & Guo, J. T. (2017). Effects of short indels on protein structure and function in human genomes. *Scientific reports*, 7(1), 1-9.

Liu, D., Gao, Y., Li, L., Chen, H., Bai, L., Qu, Y., Zhou, B., Yan, Y., & Zhao, Y. (2021). Single nucleotide polymorphisms in breast cancer susceptibility gene 1 are associated with susceptibility to lung cancer. *Oncology Letters*, 21(5), 1-7.

Liu, W. H., Kaur, M., & Makrigiorgos, G. M. (2003). Detection of hotspot mutations and polymorphisms using an enhanced PCR-RFLP approach. *Human mutation*, 21(5), 535-541.

Liu, W., Li, P. J., Qi, X. M., Zhou, Q. X., Zheng, L., Sun, T. H., & Yang, Y. S. (2005). DNA changes in barley (Hordeum vulgare) seedlings induced by cadmium pollution using RAPD analysis. *Chemosphere*, 61(2), 158-167.

Liu, X., Li, J., Qin, F., & Dai, S. (2016). miR-152 as a tumor suppressor microRNA: Target recognition and regulation in cancer. *Oncology letters*, 11(6), 3911-3916.

Loh, H. Y., Norman, B. P., Lai, K. S., Rahman, N. M. A. N. A., Alitheen, N. B. M., & Osman, M. A. (2019). The regulatory role of microRNAs in breast cancer. *International journal of molecular sciences*, 20(19), 4940-4967.

Lokuge, L. D. C. S., & Ganegoda, G. U. (2018). Review of Prediction of Human Diseases using DNA Sequencing Technologies. *IEEE*, 1-6.

Lopez, J., & Tait, S. W. G. (2015). Mitochondrial apoptosis: killing cancer using the enemy within. *British journal of cancer*, 112(6), 957-962.

Lopresti , D.(2008) . Introduction to Bioinformatics. *Biol*, 95, 1-43.

Lundqvist, A., Andersson, E., Ahlberg, I., Nilbert, M., & Gerdtham, U. (2016). Socioeconomic inequalities in breast cancer incidence and mortality in Europe-a systematic review and meta-analysis. *The European Journal of Public Health*, 26(5), 804-813.

Lunec, J., Holloway, K. A., Cooke, M. S., Faux, S., Griffiths, H. R., & Evans, M. D. (2002). Urinary 8-oxo-2′-deoxyguanosine: redox regulation of DNA repair in vivo?. *Free Radical Biology and Medicine*, 33(7), 875-885.

Lytle, N. K., Barber, A. G., & Reya, T. (2018). Stem cell fate in cancer growth, progression and therapy resistance. *Nature Reviews Cancer*, 18(11), 669-680.

Macías-Gómez, N., Gutiérrez-Angulo, M., Leal-Ugarte, E., Ramírez-Reyes, L., Peregrina-Sandoval, J., Meza-Espinoza, J., Ramos Solano, F., de la Luz, A.-M. M., & Santoyo, T. F. (2014). *MDR1* C3435T polymorphism in Mexican patients with breast cancer. *Genet Mol Res*, 13(3), 5018-5024.

Madeira, K. P., Daltoé, R. D., Sirtoli, G. M., Carvalho, A. A., Rangel, L. B. A., & Silva, I. V. (2014). Estrogen receptor alpha (ERS1) SNPs c454-397T> C (PvuII) and c454-351A> G (XbaI) are risk biomarkers for breast cancer development. *Molecular biology reports*, 41(8), 5459-5466.

Mahdieh, N., & Rabbani, B. (2013). An overview of mutation detection methods in genetic disorders. *Iranian journal of pediatrics*, 23(4), 375.

Mahmoud, E. H. (2016). Role of Adiponectin gene polymorphism and serum level in Breast Cancer (Doctoral dissertation, Cairo University).

Malla, R., Surepalli, N., Farran, B., Malhotra, S. V., & Nagaraju, G. P. (2021). Reactive oxygen species (ROS): critical roles in breast tumor microenvironment. *Critical Reviews in Oncology/Hematology*,160, 103285-103297.

Manandhar, S., Kim, C. G., Lee, S. H., Kang, S. H., Basnet, N., & Lee, Y. M. (2017). Exostosin 1 regulates cancer cell stemness in doxorubicin-resistant breast cancer cells. *Oncotarget*, 8(41), 70521-70537.

Martínez-Jiménez, F., Muiños, F., Sentís, I., Deu-Pons, J., Reyes-Salazar, I., Arnedo-Pac, C., Mularoni, L., Pich, O., Bonet, J., & Kranas, H. (2020). A compendium of mutational cancer driver genes. *Nature Reviews Cancer*, 20(10), 555-572.

Martinez-Outschoorn, U. E., Balliet, R., Lin, Z., Whitaker-Menezes, D., Birbe, R. C., Bombonati, A., Pavlides, S., Lamb, R., Sneddon, S., & Howell, A. (2012). *BRCA1* mutations drive oxidative stress and glycolysis in the tumor microenvironment: Implications for breast cancer prevention with antioxidant therapies. *Cell Cycle*, 11(23), 4402-4413.

Mathé C., M. F. Sagot, T. Schiex and P Rouzé. (2002). Current methods of gene prediction, their strengths and weaknesses. *Journal of Nucleic Acids Research*, 30 (19), 4103-4117.

Matsuyama, S., Llopis, J., Deveraux, Q. L., Tsien, R. Y., & Reed, J. C. (2000). Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nature Cell Biology*, 2(6), 318-325.

Mbemi, A., Khanna, S., Njiki, S., Yedjou, C. G., & Tchounwou, P. B. (2020). Impact of Gene-Environment Interactions on Cancer Development. *International journal of environmental research and public health*, 17(21), 8089-8104.

McGuire, W. L., Chamness, G. C., & Fuqua, S. A. (1992). Abnormal estrogen receptor in clinical breast cancer. *The Journal of steroid biochemistry and molecular biology*, 43(1-3), 243-247.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., & Ding, W. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science*, 266(5182), 66-71.

Mehmood, M. A., Sehar, U., & Ahmad, N. (2014). Use of bioinformatics tools in different spheres of life sciences. *Data Mining in Genomics & Proteomics*, 5(2), 1000158-1000171.

Mehrgou, A., & Akouchekian, M . (2016). The importance of *BRCA1* and *BRCA2* genes mutations in breast cancer development. *Medical journal of the Islamic Republic of Iran*, 30, 369-381.

Mehta, A., Vasudevan, S., Sharma, S. K., Kumar, D., Panigrahi, M., Suryavanshi, M., & Gupta, G. (2018). Germline *BRCA1* and *BRCA2* deleterious mutations and variants of unknown clinical significance associated with breast/ovarian cancer: a report from North India. *Cancer management and research*, 10, 6505-6516.

Meshram, Y. (2019). *BRCA1* and *BRCA2*: Role in breast cancer. *International Journal of Scientific Research and Reviews*, 8(1), 2219-2229.

Mijac, D., Vukovic-Petrovic, I., Mijac, V., Perovic, V., Milic, N., Djuranovic, S., Bojic, D., Popovic, D., Culafic, D., & Krstic, M. (2018). *MDR1* gene polymorphisms are associated with ulcerative colitis in a cohort of Serbian patients with inflammatory bowel disease. *PLoS One*, 13(3), 0194536-194551.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., & Ding, W. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science*, 266(5182), 66-71.

Mishra, A. K., Sharma, K., Misra, R. S. (2010). Isozyme and PCR-based genotyping of epidemic *Phytophthora colocasiae* associated with taro leaf blight. *Archives of Phytophathology and Plant Protection*, 43(14), 1367-1380.

Mohammed, Z. A. M. (2011). Determination of Serum IL-8 Level in Women with Breast Cancer and Their Correlation With Disease Progression. *Iraqi Journal of Cancer and Medical Genetics*, 4(2), 43-46.

Mullaney, J. M., Mills, R. E., Pittard, W. S., & Devine, S. E. (2010). Small insertions and deletions (INDELs) in human genomes. *Human molecular genetics*, 19(2), 131-136.

MUTLU İÇDUYGU, F., ŞAMLI, H., EVRENSEL, T., ÖZGÖZ, A., HEKİMLER ÖZTÜRK, K., CANHOROZ, M., DELİGÖNÜL, A., &

İMİRZALIOĞLU, N. (2020). Association Between *MDR1* (*ABCB1*) Gene C3435T, C1236T, G2677T/A, A2956G Polymorphisms And The Risk Of Breast Cancer Among Turkish Women. *Medical Journal of Suleyman Demirel University*, 27(3), 345-352.

Narrandes, S., & Xu, W. (2018). Gene expression detection assay for cancer clinical use. *Journal of Cancer*, 9(13), 2249-2265.

Nayak, D., Tripathi, N., Kathuria, D., Siddharth, S., Nayak, A., Bharatam, P. V., & Kundu, C. (2020). Quinacrine and curcumin synergistically increased the breast cancer stem cells death by inhibiting *ABCG2* and modulating DNA damage repair pathway. *The international journal of biochemistry & cell biology*, 119, 105682-105725.

Ndiaye, R., Diop, J. P. D., Bourdon-Huguenin, V., Dem, A., Diouf, D., Dieng, M. M., Diop, P. S., Gueye, S. M. K., Ba, S. A., & Dia, Y. (2020). Evidence for an ancient *BRCA1* pathogenic variant in inherited breast cancer patients from Senegal. *NPJ Genomic Medicine*, 5(1), 1-6.

Neamatzadeh, H., Shiryazdi, S. M., & Kalantar, S. M. (2015). *BRCA1* and *BRCA2* mutations in Iranian breast cancer patients: A systematic review. *Journal of research in medical sciences*, 20(3), 284-293.

Nenclares, P., & Harrington, K. J. (2020). The biology of cancer. *Medicine*, 48(2), 67-72.

Newman, L. A. (2017). Breast cancer disparities: socioeconomic factors versus biology. *Annals of Surgical Oncology*, 24(10), 2869-2875.

Newton, C.R. & Graham, A. 1997. Polymerase Chain Reaction. Bios Scientific publishers. Oxford, UK.

Nguyen-Dien, G. T., Smith, R. A., Haupt, L. M., Griffiths, L. R., & Nguyen, H. T. (2014). Genetic polymorphisms in miRNAs targeting the estrogen receptor and their effect on breast cancer risk. *Meta gene*, 2, 226-236.

Ni, Z., Bikadi, Z., F Rosenberg, M., & Mao, Q. (2010). Structure and function of the human breast cancer resistance protein (BCRP/ABCG2). *Current drug metabolism*, 11(7), 603-617.

Niebudek, K., Balcerczak, E., Mirowski, M., Pietrzak, J., Zawadzka, I., & Żebrowska-Nawrocka, M. (2019). The contribution of *ABCG2* G34A and

C421A polymorphisms to multiple myeloma susceptibility. *OncoTargets and therapy*, 12, 1655-1660.

Noguchi, K., Katayama, K., & Sugimoto, Y. (2014). Human ABC transporter ABCG2/BCRP expression in chemoresistance: basic and clinical perspectives for molecular cancer therapeutics. *Pharmacogenomics and personalized medicine*, 7, 53-64.

Noreen, A., Bukhari, D. A., & Rehman, A. (2018). Reactive oxygen species: Synthesis and their relationship with cancer-a review. *Pakistan Journal of Zoology*, 50(5),1951-1963.

Novikov, V. V., Shumilova, S. V., Novikov, D. V., Kalugin, A. V., Fomina, S. G., & Karaulov, A. V. (2016). Genetic Instability in Locus rs5498 E469K (A/G) of ICAM-1 Gene in Patients with Colorectal Cancer and Breast Cancer. *Bulletin of experimental biology and medicine*, 160(6), 811-813.

Nyqvist, J., Parris, T. Z., Helou, K., Sarenmalm, E. K., Einbeigi, Z., Karlsson, P., Nasic, S., & Kovács, A. (2020). Previously diagnosed multiple primary malignancies in patients with breast carcinoma in Western Sweden between 2007 and 2018. *Breast Cancer Research and Treatment*, 184(1), 221-228.

Okada, S., Tokunaga, E., Kitao, H., Akiyoshi, S., Yamashita, N., Saeki, H., Oki, E., Morita, M., Kakeji, Y., & Maehara, Y. (2012). Loss of heterozygosity at *BRCA1* locus is significantly associated with aggressiveness and poor prognosis in breast cancer. *Annals of Surgical Oncology*, 19(5), 1499-1507.

O'connor, R. O. B. E. R. T. (2007). The pharmacology of cancer resistance. *Anticancer research*, 27(3A), 1267-1272.

Panawala, L. (2017). Difference Between chromosome and Gene. *PEDIAA*, 2017 Article, 8.

Papadopoulos, S., Benter, T., Anastassiou, G., Pape, M., Gerhard, S., Bornfeld, N., Ludwig, W., & Dörken, B. (2002). Assessment of genomic instability in breast cancer and uveal melanoma by random amplified polymorphic DNA analysis. *International Journal of Cancer*, 99(2), 193-200.

Patrono, C., Sterpone, S., Testa, A., & Cozzi R. (2014). Polymorphisms in base excision repair genes: Breast cancer risk and individual radiosensitivity. *World J Clin Oncol*, 5(5), 874-882.

Paul, A., & Paul, S. (2014). The breast cancer susceptibility genes (BRCA) in breast and ovarian cancers. *Frontiers in bioscience* (Landmark edition), 19, 605-618.

Pei, J., Wang, Y., & Li, Y. (2020). Identification of key genes controlling breast cancer stem cell characteristics via stemness indices analysis. *Journal of translational medicine*, 18(1), 1-15.

Perdew,G.H., Vanden-Heuvel, J. P., &Peters, J.M. (2006). Regulation of gene expression-Molecular mechanisms.Totowa, NJ:Humana Press. Peters, R. (2006). Ageing and the brain. *Postgraduate MedicalJournal*, 82(964), 84-88.

Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., Castoria, G., & Migliaccio, A. (2020). ROS in cancer therapy: The bright side of the moon. *Experimental & Molecular Medicine*, 52(2), 192-203.

Perrone, S., Lotti, F., Geronzi, U., Guidoni, E., Longini, M., & Buonocore, G. (2016). Oxidative stress in cancer-prone genetic diseases in pediatric age: the role of mitochondrial dysfunction. *Oxidative medicine and cellular longevity*, 2016, 1-7.

Pevsner .J. (2009). Bioinformatics and Functional Genomics, 2nd Edition, Wiley-Blackwell, PP 992.

Pfeffer, C. M., & Singh, A. T. (2018). Apoptosis: a target for anticancer therapy. *International journal of molecular sciences*, 19(2), 448-448.

Pingoud, V., Sudina, A., Geyer, H., Bujnicki, J. M., Lurz, R., Lüder, G., Morgan, R., Kubareva, E., & Pingoud, A. (2005). Specificity changes in the evolution of type II restriction endonucleases: A biochemical and bioinformatic analysis of restriction enzymes that recognize unrelated sequences. *Journal of Biological Chemistry*, 280(6), 4289-4298.

Pistritto, G., Trisciuoglio, D., Ceci, C., Garufi, A., & D'Orazi, G. (2016). Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. *Aging (Albany NY)*, 8(4), 603-619.

Poljšak, B., & Fink, R. (2014). The protective role of antioxidants in the defence against ROS/RNS-mediated environmental pollution. *Oxidative medicine and cellular longevity*, 2014, 671539-671561.

Poonkuzhali, B., Lamba, J., Strom, S., Sparreboom, A., Thummel, K., Watkins, P., & Schuetz, E. (2008). Association of breast cancer resistance protein/*ABCG2*

phenotypes and novel promoter and intron 1 single nucleotide polymorphisms. *Drug metabolism and disposition*, 36(4), 780-795.

Priyadarshini, R., Raj, G. M., Kayal, S., Ramesh, A., & Shewade, D. G. (2019). Influence of *ABCB1* C3435T and C1236T gene polymorphisms on tumour response to docetaxel-based neo-adjuvant chemotherapy in locally advanced breast cancer patients of South India. *Journal of clinical pharmacy and therapeutics*, 44(2), 188-196.

Prosperi, M. C., Ingham, S. L., Howell, A., Lalloo, F., Buchan, I. E., & Evans, D. G. (2014). Can multiple SNP testing in *BRCA2* and *BRCA1* female carriers be used to improve risk prediction models in conjunction with clinical assessment?. *BMC medical informatics and decision making*, 14(1), 1-11.

Raghunand, N., Mahoney, B. P., & Gillies, R. J. (2003). Tumor acidity, ion trapping and chemotherapeutics: II. pH-dependent partition coefficients predict importance of ion trapping on pharmacokinetics of weakly basic chemotherapeutic agents. *Biochemical pharmacology*, 66(7), 1219-1229.

Rahiman, F., Balasubramanian, T., Kumar, P., & Shejina, M. (2015). RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) -A TOOL FOR GENE MAPPING . *National conference on Advances in Laboratory Medicine*, 2, 163-166.

Ramalhinho, A. C. M. (2014). Influence of Low Penetrance Genes in Susceptibility for Breast Cancer in the Portuguese Population of Beira Interior (Doctoral dissertation, Universidade da Beira Interior (Portugal)).

Rampal, G., Khanna, N., Thind, T. S., Arora, S., & Vig, A. P. (2012). Role of isothiocyanates as anticancer agents and their contributing molecular and cellular mechanisms. *Med Chem Drug Discovery*, 3, 79-93.

Rana, J. M. S. & Vaisla, K. S. (2012). Introduction to Bioinformatics-Tools and applications., 2012 Edition; Uttarakhand State Biotechnology Department (Ministry of Science &Technology and Biotechnology). PP 180.

Rantalainen, M., Klevebring, D., Lindberg, J., Ivansson, E., Rosin, G., Kis, L., Celebioglu, F., Fredriksson, I., Czene, K., & Frisell, J. (2016). Sequencing-based breast cancer diagnostics as an alternative to routine biomarkers. *Scientific Reports*, 6(1), 1-10.

Rebbeck, T. R., Friebel, T. M., Mitra, N., Wan, F., Chen, S., Andrulis, I. L., Apostolou, P., Arnold, N., Arun, B. K., & Barrowdale, D. (2016). Inheritance of deleterious mutations at both *BRCA1* and *BRCA2* in an international sample of 32, 295 women. *Breast Cancer Research*, 18(1), 1-19.

Reynolds, C. R., Islam, S. A. and Sternberg, M. J. E. (2018). EzMol: A Web Server Wizard for the Rapid Visualization and Image Production of Protein and Nucleic Acid Structures. *Journal of Molecular Biology*, 430(15), 2244-2248.

Riba, L. A., Gruner, R. A., Alapati, A., & James, T. A. (2019). Association between socioeconomic factors and outcomes in breast cancer. *The breast journal*, 25(3), 488-492.

Ribeil, J. A., Hacein-Bey-Abina, S., Payen, E., Magnani, A., Semeraro, M., Magrin, E., Caccavelli, L., Neven, B., Bourget, P., & El Nemer, W. (2017). Gene therapy in a patient with sickle cell disease. *New England Journal of Medicine*, 376(9), 848-855.

Riordan, J. R., Deuchars, K., Kartner, N., Alon, N., Trent, J., & Ling, V. (1985). Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature*, 316(6031), 817-819.

Rocco, L., Valentino, I. V., Scapigliati, G., & Stingo, V. (2014). RAPD-PCR analysis for molecular characterization and genotoxic studies of a new marine fish cell line derived from Dicentrarchus labrax. *Cytotechnology*, 66(3), 383-393.

Rodney, C. (2003). Breast cancer: A Review of the literature. *Journal of Insurance Medicine*, 35(2),85-101.

Rohrmann, S., Overvad, K., Bueno-de-Mesquita, H. B., Jakobsen, M. U., Egeberg, R., Tjønneland, A., Nailler, L., Boutron-Ruault, M. C., Clavel-Chapelon, F., & Krogh, V. (2013). Meat consumption and mortality-results from the European Prospective Investigation into Cancer and Nutrition. *BMC Medicine*, 11(1), 1-12.

Roy, R., Chun, J., & Powell, S. N. (2012). *BRCA1* and *BRCA2*: different roles in a common pathway of genome protection. *Nature Reviews Cancer*, 12(1), 68-78.

Roychowdhury, S., Iyer, M. K., Robinson, D. R., Lonigro, R. J., Wu, Y. M., Cao, X., Kalyana-Sundaram, S., Sam, L., Balbin, O. A., & Quist, M. J.

(2011). Personalized oncology through integrative high-throughput sequencing: A pilot study. *Science Translational Medicine*, 3(111), 111-121.

Royo, J. L. (2021). Hardy Weinberg Equilibrium Disturbances in Case-Control Studies Lead to Non-Conclusive Results. *Cell journal*, 22(4), 572-574.

Rubiś, B., Hołysz, H., Barczak, W., Gryczka, R., Łaciński, M., Jagielski, P., Czernikiewicz, A., Półrolniczak, A., Wojewoda, A., & Perz, K. (2012). Study of *ABCB1* polymorphism frequency in breast cancer patients from Poland. *Pharmacological Reports*, 64(6), 1560-1566.

Sabra, S. A., Saad, A. A., Abd El Moneim, N. A., Hemida, M. A. E. A., Moussa, N., & Haroun, M. (2020). Evaluation of breast cancer regarding molecular and immunochemical markers. *International Journal of Immunotherapy and Cancer Research*, 6(1), 1-9.

Safi, A., Delgir, S., Ilkhani, K., Samei, A., Mousavi, S. R., Zeynali-Khasraghi, Z., Bastami, M., & Alivand, M. R. (2021). The expression of miRNA-152-3p and miRNA-185 in tumor tissues versus margin tissues of patients with chemo-treated breast cancer. *BMC Research Notes*, 14(1), 1-7.

Saleh-Gohari, N., Mohammadi-Anaie, M., & Kalantari-Khandani, B. (2012). *BRCA1* gene mutations in breast cancer patients from Kerman Province, Iran. *Iranian journal of cancer prevention*, 5(4), 210-215.

Salem, A. H., Ali, M., Ibrahim, A., & Ibrahim, M. (2014). Genotype and allele frequencies of *MDR1* gene polymorphism in Jordanian and Sudanese populations. *American Journal of Medicine Studies*, 2(1), 19-23.

Sallum, L. F., Andrade, L., da Costa, L. B. E., Ramalho, S., Ferracini, A. C., de Andrade Natal, R., Brito, A. B. C., Sarian, L. O., & Derchain, S. (2018). *BRCA1*, *Ki67*, and β-Catenin immunoexpression is not related to differentiation, platinum response, or prognosis in women with low-and high-grade serous ovarian carcinoma. *International Journal of Gynecologic Cancer*, 28(3), 1-11.

Sambrook, J. F. and Russell, D. W. (2006). Detection of DNA in agarose gels. *Cold Spring Harbor Protocols*, 2006(1), pdb-prot4022.

Sand, P., Luckhaus, C., Schlurmann, K., Götz, M., & Deckert, J. (2002). Untangling the human estrogen receptor gene structure. *Journal of neural transmission*, 109(5), 567-583.

Šarmanová, J., Šůsová, S., Gut, I., Mrhalová, M., Kodet, R., Adámek, J., Roth, Z., & Souček, P. (2004). Breast cancer: Role of polymorphisms in biotransformation enzymes. *European Journal of Human Genetics*, 12(10), 848-854.

Sato, H., Nogueira-de-Souza, N. C., D'Amora, P., Silva, I. D., Girão, M. J., & Schor, E. (2008). Intron1 and exon1 alpha estrogen receptor gene polymorphisms in women with endometriosis. *Fertility and sterility*, 90(6), 2086-2090.

Schirmer, M. A., Lüske, C. M., Roppel, S., Schaudinn, A., Zimmer, C., Pflüger, R., Haubrock, M., Rapp, J., Güngör, C., & Bockhorn, M. (2016). Relevance of Sp binding site polymorphism in WWOX for treatment outcome in pancreatic cancer. *JNCI: Journal of the National Cancer Institute*, 108(5), 387-398.

Senthilkumar, C. S., Malla, T. M., Akhter, S., Beg, A., & Ganesh, N. (2016). Restriction digestion of EcoRI in *E.coli* RY13 plasmid DNA. *Biosciences Biotechnology Research Asia*, 5(2), 885-887.

Shadrina, A. S., Ermolenko, N. A., Boyarskikh, U. A., Sinkina, T. V., Lazarev, A. F., Petrova, V. D., & Filipenko, M. L. (2016). Polymorphisms in DNA repair genes and breast cancer risk in Russian population: a case-control study. *Clinical and experimental medicine*, 16(1), 21-28.

Shah, H., Hasan, L., Jan, S., Khattak, A., Ahmad, G., & Hassnain, S. (2013). Understanding Gene Prediction: A Descriptive Analysis, 45(A-1),189-194.

Shah, N. D., Shah, P. S., Panchal, Y. Y., Katudia, K. H., Khatri, N. B., Ray, H. S. P., Bhatiya, U. R., Shah, S. C., Shah, B. S., & Rao, M. V. (2018). Mutation analysis of *BRCA*1/2 mutations with special reference to polymorphic SNPs in Indian breast cancer patients. *The Application of Clinical Genetics*, 11, 59-67.

Shah, R., Rosso, K., & Nathanson, S. D. (2014). Pathogenesis, prevention, diagnosis and treatment of breast cancer. *World journal of clinical oncology*, 5(3), 283-298.

Shalaby, E., & Azzam, G. M. (Eds.). (2018). Antioxidants in Foods and its Applications. BoD. IntechOpen. United kingdom. PP168.

Sheng, X., Zhang, L., Tong, N., Luo, D., Wang, M., Xu, M., & Zhang, Z. (2012). *MDR1* C3435T polymorphism and cancer risk: a meta-analysis based on 39 case-control studies. *Molecular biology reports*, 39(7), 7237-7249.

Siddig, A., Mohamed, A. O., Awad, S., Hassan, A. H., Zilahi, E., Al-Haj, M., Bernsen, R., & Adem, A. (2008). Estrogen receptor α gene polymorphism and breast cancer. *Annals of the New York Academy of Sciences*, 1138(1), 95-107.

Sikdar, S., Nandy, S., Mukherjee, A., Bhattacharyya, R., Pandey, D. K., & Dey, A. (2019). Phytoestrogens as Anticancer Therapeutics: A Retrospective and Future Perspectives. *Journal of Biologically Active Products from Nature*, 9(3), 179-196.

Silva, K. S. F. (2019). Genetic Counseling, Polymorphisms and Breast Cancer. *Family Medicine and Disease Prevention*, 5 (1), 1-7.

Simpson, P. T., Reis-Filho, J. S., Gale, T., & Lakhani, S. R. (2005). Molecular evolution of breast cancer. *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland*, 205(2), 248-254.

Singh, K. P., & Roy, D. (2001). Identification of novel breast tumor-specific mutation (s) in the q11.2 region of chromosome 17 by RAPD/AP-PCR fingerprinting. *Gene*, 269(2), 33-43.

Singh, K. P., Miaskowski, C., Dhruva, A. A., Flowers, E., & Kober, K. M. (2018). Mechanisms and Measurement of Changes in Gene Expression. *Biological research for nursing*, 20(4), 369-382.

Smith, P. J. (2005). Random amplified polymorphic DNA (RAPD). In Stock Identification Methods. Academic Press. PP 387.

Sparrow, J. C., Nowak, K. J., Durling, H. J., Beggs, A. H., Wallgren-Pettersson, C., Romero, N., Nonaka, I., and Laing, N. G. (2003). Muscle disease caused by mutations in the skeletal muscle alpha-actin gene (ACTA1). *Neuromuscular disorder*, 13, 519-531.

Stańko, G., Kamiński, M., Bogacz, A., Seremak-Mrozikiewicz, A., Kosiński, B., Bartkowiak-Wieczorek, J., Kotrych, D., & Czerny, B. (2016). The importance of G2677T/A and C3435T polymorphisms of the *MDR1* gene in the aetiology of colorectal cancer. *Przeglad Gastroenterologiczny*, 11(1), 35-40.

Stomka, M., Sobalska Kwapis, M., Korycka-Machała, M., Dziadek, J., Bartosz, G., & Strapagiel, D. (2020). Comprehensive analysis of *ABCG2* genetic variation in the polish population and Its inter-population comparison. *Genes*, 11(10), 1144-1165.

Stratton, M. R. (2011). Exploring the genomes of cancer cells: progress and promise. *Science*, 331(6024), 1553-1558.

Sudhakar, A. (2009). History of cancer, ancient and modern treatment methods. *Journal of cancer science & therapy*, 1(2), 1-4.

Sukhumsirichart, W. (2018). Polymorphisms. In Genetic Diversity and Disease Susceptibility. IntechOpen. PP154.

Sun, Y. S., Zhao, Z., Yang, Z. N., Xu, F., Lu, H. J., Zhu, Z. Y., Shi, W., Jiang, J., Yao, P. P., & Zhu, H. P. (2017). Risk factors and preventions of breast cancer. *International Journal of Biological Sciences*, 13(11), 1387-1397.

Surbone, A. (2011). Social and ethical implications of *BRCA* testing. *Annals of oncology*, 22, 60-66.

Swietach, P., Vaughan-Jones, R. D., Harris, A. L., & Hulikova, A. (2014). The chemistry, physiology and pathology of pH in cancer. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 369(1638), 369-378.

Tabebordbar, M., Zhu, K., Cheng, J. K., Chew, W. L., Widrick, J. J., Yan, W. X., Maesner, C., Wu, E. Y., Xiao, R., & Ran, F. A. (2016). In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science*, 351(6271), 407-411.

Taheri, M., Mahjoubi, F., & Omranipour, R. (2010). Effect of *MDR1* polymorphism on multidrug resistance expression in breast cancer patients. *Genet Mol Res*, 9(1), 34-40.

Takeuchi, T., Nakajima, M., & Morimoto, K. (1996). Accelerated Paper: Relationship between the intracellular reactive oxygen species and the induction of oxidative DNA damage in human neutrophil-like cells. *Carcinogenesis*, 17(8), 1543-1548.

Tan, A. I. (2015). Researchers Discover New Hormone Receptors to Target When Treating Breast Cancer (Doctoral dissertation, University of MIAMI).

Tang, C., Schafranek, L., Watkins, D.B., Parker, W.T., Moore, S., Prime, J.A., White, D.L., & Hughes, T.P. (2011). Tyrosine kinase inhibitor resistance in chronic myeloid leukemia cell lines: investigating resistance pathways. *Leukemia& Lymphoma*, 52(11), 2139-2147.

Tatari, F., Salek, R., Mosaffa, F., Khedri, A., & Behravan, J. (2009). Association of C3435T single-nucleotide polymorphism of *MDR1* gene with breast cancer in an Iranian population. *DNA and cell biology*, 28(5), 259-263.

Tazzite, A., Kassogue, Y., Diakité, B., Jouhadi, H., Dehbi, H., Benider, A., & Nadifi, S. (2016). Association between *ABCB1* C3435T polymorphism and breast cancer risk: a Moroccan case-control study and meta-analysis. *BMC genetics*, 17(1), 1-11.

Teama, S. (2018). DNA polymorphisms: DNA-based molecular markers and their application in medicine. Genetic Diversity and Disease Susceptibility. IntechOpen. London: United kingdom. PP155.

Tennessen, J. A., Bigham, A. W., O'Connor, T. D., Fu, W., Kenny, E. E., Gravel, S., McGee, S., Do, R., Liu, X., & Jun, G. (2012). Evolution and functional impact of rare coding variation from deep sequencing of human exomes. *Science*, 337(6090), 64-69.

Testa, U., Castelli, G., & Pelosi, E. (2020). Breast cancer: a molecularly heterogenous disease needing subtype-specific treatments. *Medical Sciences*, 8(1), 18-121.

Thapar, P. (2018). Bioinformatics-tools and applications. ACADEMIA, 5044-5047.

Thomas, C., & Gustafsson, J. A. (2015). Estrogen receptor mutations and functional consequences for breast cancer. *Trends in Endocrinology & Metabolism*, 26(9), 467-476.

Tokheim, C., & Karchin, R. (2019). CHASMplus reveals the scope of somatic missense mutations driving human cancers. *Cell systems*, 9(1), 9-23.

Tokunaga, H., Iida, K., Hozawa, A., Ogishima, S., Watanabe, Y., Shigeta, S., Shimada, M., Yamaguchi-Kabata, Y., Tadaka, S., & Katsuoka, F. (2021). Novel candidates of pathogenic variants of the *BRCA1* and *BRCA2* genes from a dataset of 3,552 Japanese whole genomes (3.5 KJPNv2). *PloS One*, 16(1), 0236907-0236925.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J., & Jemal, A. (2015). Global cancer statistics, 2012. *CA: a cancer journal for clinicians*, 65(2), 87-108.

Tulsyan, S., Mittal, R. D., & Mittal, B. (2016). The effect of *ABCB1* polymorphisms on the outcome of breast cancer treatment. *Pharmacogenomics and personalized medicine*, 9, 47-58.

Turgut, S., Yaren, A., Kursunluoglu, R., & Turgut, G. (2007). *MDR1* C3435T polymorphism in patients with breast cancer. *Archives of medical research*, 38(5), 539-544.

Tweed, E., Allardice, G., McLoone, P., & Morrison, D. (2018). Socio-economic inequalities in the incidence of four common cancers: a population-based registry study. *Public Health*, 154, 1-10.

Valarmathi, M. T., Sawhney, M., Deo, S. S., Shukla, N. K., & Das, S. N. (2004). Novel germline mutations in the *BRCA1* and *BRCA2* genes in Indian breast and breast-ovarian cancer families. *Human mutation*, 23(2), 205-205.

Van der Groep, P., Van Der Wall, E., & Van Diest, P. J. (2011). Pathology of hereditary breast cancer. *Cellular oncology*, 34(2), 71-88.

Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S., Diaz, L. A., & Kinzler, K. W. (2013). Cancer genome landscapes. *Science*, 339(6127), 1546-1558.

Wang, D., Johnson, A. D., Papp, A. C., Kroetz, D. L., & Sadee, W. (2005). Multidrug resistance polypeptide 1 (*MDR1*, *ABCB1*) variant 3435C> T affects mRNA stability. *Pharmacogenetics and genomics*, 15(10), 693-704.

Wang, J. Q., Teng, Q. X., Lei, Z. N., Ji, N., Cui, Q., Fu, H., Lin, L., Yang, D. H., Fan, Y. F., & Chen, Z. S. (2020). Reversal of cancer multidrug resistance (MDR) mediated by ATP-binding cassette transporter G2 (*ABCG2*) by AZ-628, a RAF kinase inhibitor. *Frontiers in Cell and Developmental Biology*, 8, 601400-601415.

Wang, J., & Shete, S. (2017). Testing departure from Hardy-Weinberg proportions. In Statistical Human Genetics. Humana Press. New York. NY. PP 115.

Wang, Z., Wang, J., Chong, S. S., & Lee, C. G. L. (2009). Mining potential functionally significant polymorphisms at the ATP-binding-cassette transporter genes. *Current Pharmacogenomics and Personalized Medicine (Formerly Current Pharmacogenomics)*, 7(1), 40-58.

Wang, Z., Wang, T., & Bian, J. (2013). Association between *MDR1* C3435T polymorphism and risk of breast cancer. *Gene*, 532(1), 94-99.

Ward, R., & Carroll, R. J. (2014). Testing Hardy-Weinberg equilibrium with a simple root-mean-square statistic. *Biostatistics*, 15(1), 74-86.

Watson, J. D., Baker ,T. A, Bell, S. P, Gann. A, Levine M., & Losick, R. (2014). Expression of the genome. Molecular Biology of the Gene. 7 th ed. Boston: Pearson. 2014.

Welsh, J., & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic acids research*, 18(24), 7213-7218.

Wen, W., Shu, X., Guo, X., Cai, Q., Long, J., Bolla, M. K., Michailidou, K., Dennis, J., Wang, Q., & Gao, Y. T. (2016). Prediction of breast cancer risk based on common genetic variants in women of East Asian ancestry. *Breast Cancer Research*, 18(1), 1-8.

White, K. A., Grillo-Hill, B. K., & Barber, D. L. (2017). Cancer cell behaviors mediated by dysregulated pH dynamics at a glance. *Journal of Cell Science*, 130(4), 663-669.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic acids research*, 18(22), 6531-6535.

Wolf, S. J., Bachtiar, M., Wang, J., Sim, T. S., Chong, S. S., & Lee, C. G. L. (2011). An update on *ABCB1* pharmacogenetics: insights from a 3D model into the location and evolutionary conservation of residues corresponding to SNPs associated with drug pharmacokinetics. *The pharmacogenomics journal*, 11(5), 315-325.

Wolters, S., & Schumacher, B. (2013). Genome maintenance and transcription integrity in aging and disease. *Frontiers in genetics*, 4, 19-29.

Wong, K., Briddon, S. J., Holliday, N. D., & Kerr, I. D. (2016). Plasma membrane dynamics and tetrameric organisation of *ABCG2* transporters in mammalian cells revealed by single particle imaging techniques. Biochimica et Biophysica Acta (BBA). *Molecular Cell Research*, 1863(1), 19-29.

Wu, H., Kang, H., Liu, Y., Tong, W., Liu, D., Yang, X., Lian, M., Yao, W., Zhao, H., & Huang, D. (2012). Roles of *ABCB1* gene polymorphisms and

haplotype in susceptibility to breast carcinoma risk and clinical outcomes. *Journal of Cancer Research and Clinical Oncology*, 138(9), 1449-1462.

Wu, H., Liu, Y., Kang, H., Xiao, Q., Yao, W., Zhao, H., Wang, E., & Wei, M. (2015). Genetic variations in *ABCG2* gene predict breast carcinoma susceptibility and clinical outcomes after treatment with anthracycline-based chemotherapy. *BioMed Research International*, 2015, 279109-279121.

Xian, Z. H., Cong, W. M., Zhang, S. H., & Wu, M. C. (2005). Genetic alterations of hepatocellular carcinoma by random amplified polymorphic DNA analysis and cloning sequencing of tumor differential DNA fragment. *World Journal of Gastroenterology*, 11(26), 4102-4107.

Xie, Z. (2020). A systematic review: breast cancer susceptibility genes. E3S Web of Conferences. *EDP Sciences*, 218, 1-6.

Xiong, H. Y., Alipanahi, B., Lee, L. J., Bretschneider, H., Merico, D., Yuen, R. K., Hua, Y., Gueroussov, S., Najafabadi, H. S., & Hughes, T. R. (2015). The human splicing code reveals new insights into the genetic determinants of disease. *Science*, 347(6218), 124-125.

Xu, Q., Liu, J., He, C., Sun, L., Gong, Y., Jing, J., Xing, C., & Yuan, Y. (2014). The interaction effects of pri-let-7a-1 rs10739971 with *PGC* and *ERCC6* gene polymorphisms in gastric cancer and atrophic gastritis. *PloS One*, 9(2), 89203-89209.

Yang, H., Villani, R. M., Wang, H., Simpson, M. J., Roberts, M. S., Tang, M., & Liang, X. (2018). The role of cellular reactive oxygen species in cancer chemotherapy. *Journal of Experimental & Clinical Cancer Research*, 37(1), 1-10.

Ye, K., Wang, J., Jayasinghe, R., Lameijer, E. W., McMichael, J. F., Ning, J., McLellan, M. D., Xie, M., Cao, S., & Yellapantula, V. (2016). Systematic discovery of complex insertions and deletions in human cancers. *Nature Medicine*, 22(1), 97-104.

Yin, G., Xiao, Z., Ni, Y., Qu, X., Wu, H., Lu, H., Qian, S., Chen, L., Li, J., & Qiu, H. (2016). Association of *MDR1* single-nucleotide polymorphisms and haplotype variants with multiple myeloma in Chinese Jiangsu Han population. *Tumor Biology*, 37(7), 9549-9554.

Zawadzka, I., Jeleń, A., Pietrzak, J., Żebrowska-Nawrocka, M., Michalska, K., Szmajda-Krygier, D., Mirowski, M., Łochowski, M., Kozak, J., & Balcerczak, E. (2020). The impact of *ABCB1* gene polymorphism and its expression on non-small-cell lung cancer development, progression and therapy-preliminary report. *Scientific Reports*, 10(1), 1-10.

Zhang, L., & Vijg, J. (2018) . Somatic mutagenesis in mammals and its implications for human disease and aging. *Annual review of genetics*, 52, 397-419.

Zhang, Q. X., Borg, Å., Wolf, D. M., Oesterreich, S., & Fuqua, S. A. (1997). An estrogen receptor mutant with strong hormone-independent activity from a metastatic breast cancer. *Cancer research*, 57(7), 1244-1249.

Zhao, R. Z., Jiang, S., Zhang, L., & Yu, Z. B. (2019) . Mitochondrial electron transport chain, ROS generation and uncoupling. *International journal of molecular medicine*, 44(1), 3-15.

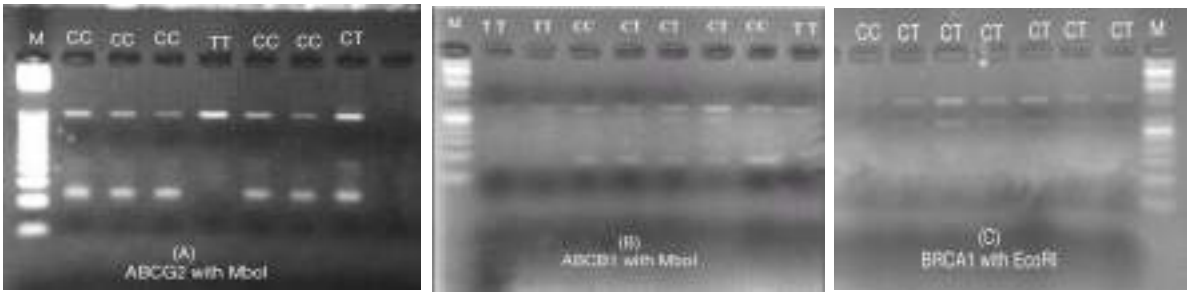
Appendixes

Appendix (1): The component of Go Taq ® Green Master Mix kits that used in the present study

The component of the Go Taq ® Green Master Mix

1	Go Taq ®DNA polymerase	2X
2	Green Go Taq ® Reaction Buffer (PH 8.5)	1X
3	µM dATP, µM dGTP, µM dCTP , µM dTTP	400µM for each
4	Mgcl2	3mM
5	Yellow and blue dyes	Trace

Appendix (2): **PCR RFLP results for *ABCG2, ABCB1, BRCA1* after digested with MboI, MboI, EcoRI restriction enzyme, respectively. (A)** The first lane (M) on left is ladder (25-300) bp in size, fragement were 300 bp and 62 bp for wild- type homozygote CC, while RFLP- PCR product 300 bp, 150 bp and 62 bp for the heterozygote CT, 300 bp for Homozygote mutant variant TT. **(B)** The first lane (M) on left is ladder (25-300) bp in size, fragments were 206 bp and 45 bp for the Wild-type homozygote CC,while 206 bp, 161 bp and 45 bp for the heterozygote CT, and 206 bp for Homozygote mutant variant TT. **(C)** The first lane (M) on right is ladder (25-2000) bp in size, fragments were 302 bp,163 bp for the heterozygote CT, and 302 bp CC for the homozygote CC.



Appendix (3): The observed and expected frequencies under Hardy-Weinberg equilibrium in the *ABCG2* gene of breast cancer patients.

Gene	Genotypes	Patient (Observed)	Patient (Expected)	X^2 (H.W.E)	P-value
ABCG2	CC	18	16.2	20	0.000008
	CT	0	3.6		
	TT	2	0.2		
X^2 Chi-square H.W.E Hardy - Weinberg equilibrium (if P > 0.05 consistent with H.W.E), at 1 d.f.					

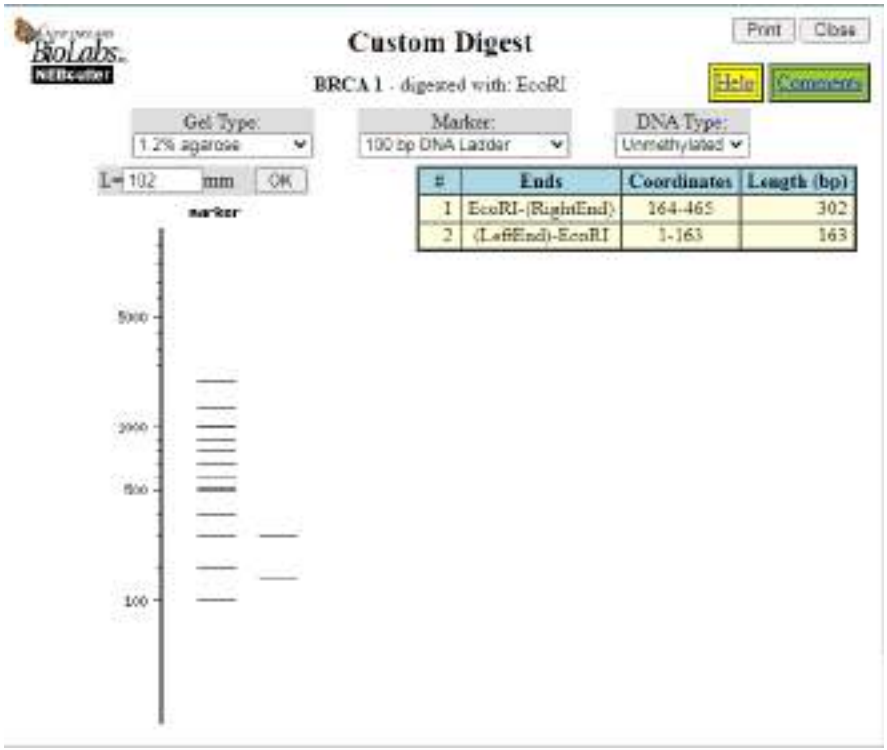
Appendix (4): The observed and expected frequencies under Hardy-Weinberg equilibrium in the *ABCB1* gene of breast cancer patients.

Gene	Genotypes	Patient (Observed)	Patient (Expected)	X^2 (H.W.E)	P-value
ABCB1	CC	6	6.6	0.314	0.575
	CT	11	9.8		
	TT	3	3.6		
X^2 Chi-square H.W.E Hardy - Weinberg equilibrium (if P > 0.05 consistent with H.W.E), at 1 d.f.					

Appendix (5): The observed and expected frequencies under Hardy-Weinberg equilibrium in the *BRCA1* gene of breast cancer patients.

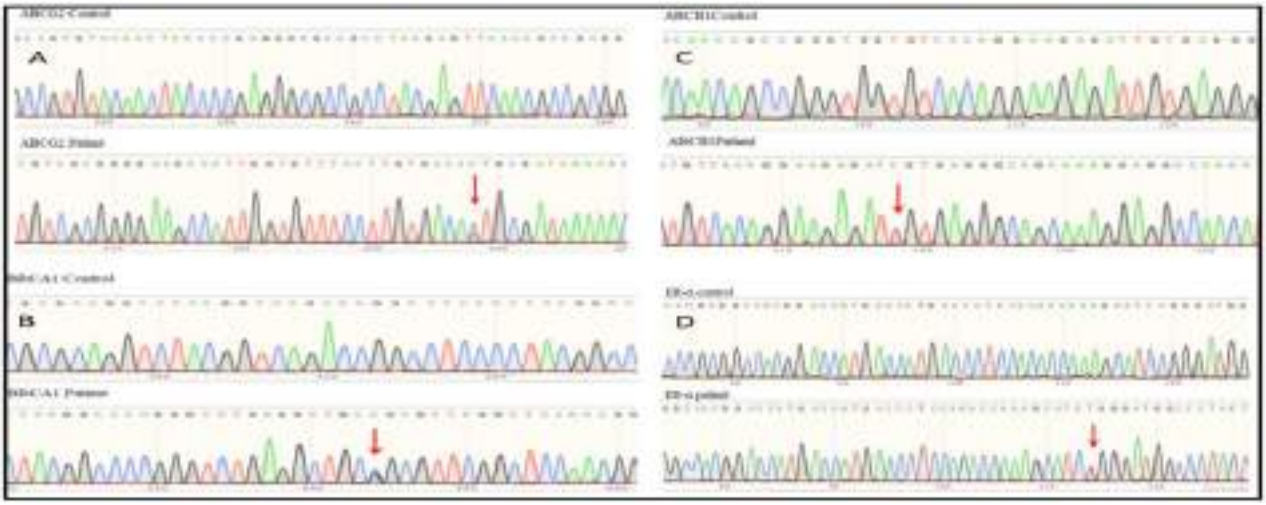
Gene	Genotypes	Patient (Observed)	Patient (Expected)	X^2 (H.W.E)	P-value
BRCA1	CC	0	5.0	20	0.000008
	CT	20	10.0		
	TT	0	5.0		
X^2 Chi-square H.W.E Hardy - Weinberg equilibrium (if P > 0.05 consistent with H.W.E), at 1 d.f.					

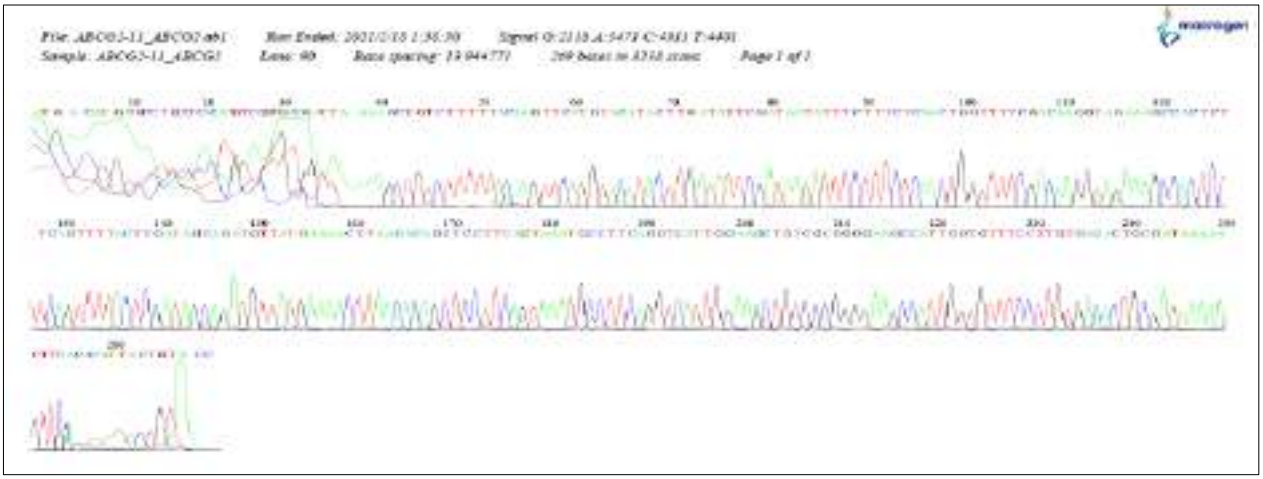
Appendix (6): *BRCA1* digested by NEB cutter bioinformatics program

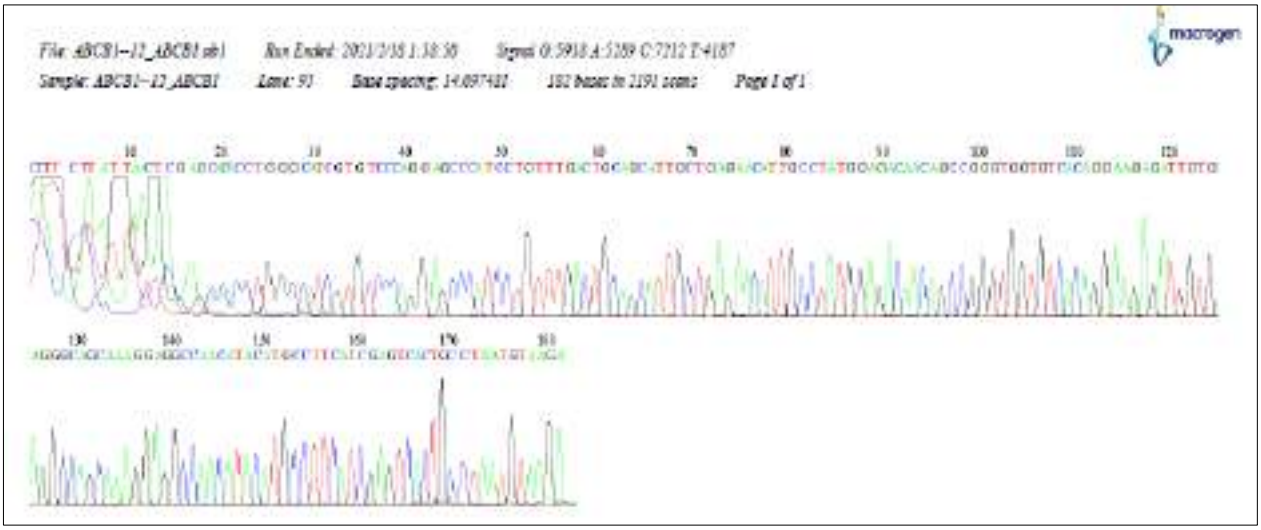


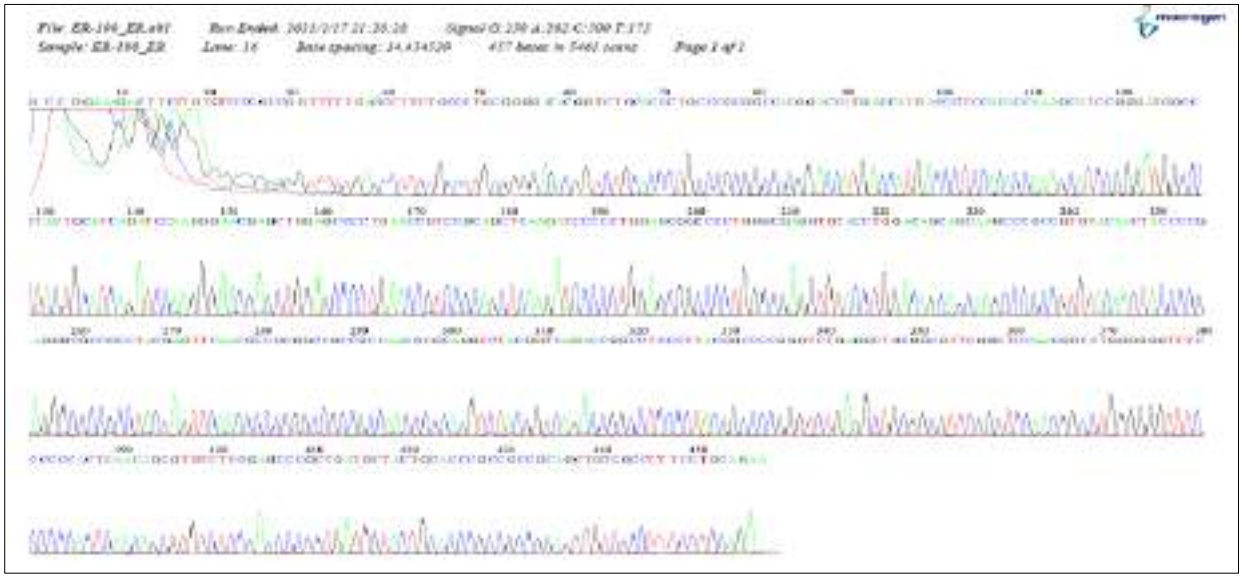
(*BRCA1* gene) 5'CAGTTTCTGCTTATTCCATTCTTTTCTCTCACACAGGGGATCAGCATTCAGATCTACCTTTTTTTCTG TGCTGGGAGTCCGC CTATCATTACATGTTTCCTTACTTCCA GCCCATCTGTTATGTTGGCTCCTTGCTAA GCCAGG CTGTTTGCTTTTATTACAGAATTCAGCCTTTTCTACATTCATTCTGTCTTTAGTGAGT AATAAA CTGCTGTTCTCATGCTGTAATGAGCTGGCATGAGTATTTGTGCCACATGGCTCCACATGCA AGTTTGAAA CAGAAC TACCCTGATACTTTTCTGGATGCCT CTCAGCTGCACG CTTCTCAGTGGTGTTCA AATCATTATT ACTGGGT TGATGATGTTCAGTATTTGTTACATCCGTC TCAGAAAATTCACAAGCAGCTGAAAATATACAA AAATAAC A AGGTACTCAAAAACTGAATTGTCAT TAAAAAAATACATA3'

Appendix (7): DNA sequencing identification for *ABCG2*, *ABCB1*, *BRCA1*, and *ER-α* mutation regions in patient samples (red arrows).









الخلاصة:

أجريت الدراسة الحالية لمقارنة التشكلات الوراثية لجينات (*ABCG2*، *ABCB1*، *BRCA1*، *ER-α*، *miRNA-152*) بين مرضى سرطان الثدي (BC) والاصحاء، وتحديد بعض التغيرات الوراثية الخلوية لأنسجة سرطان الثدي ومقارنتها مع الأنسجة السليمة. تم جمع مائة وسبعة عشر عينة دم من المشاركين في مستشفى الصدر التعليمي ومركز الشفاء لعلاج الأورام في محافظة ميسان. كانت فترة الدراسة من 3 سبتمبر 2020 إلى 15 سبتمبر 2021.

تم استخدام تقنية RFLP-PCR و PCR-RAPD-PCR (لأول مرة) وتسلسل النيوكليوتيدات لدراسة التشكل الوراثي للجينات. في هذه الدراسة تم استخدام بادئات خاصة لجينات (*ABCG2*، *ABCB1*، *BRCA1*، *ER-α* ، *mi-RNA 152*) ونوعان من إنزيمات التقييد (MboI، EcoRI) وأربعة بادئات عشوائية (OPAA11، OPU15، OPAA17، OPD18). فيما يتعلق بالدراسة الوراثية الخلوية، تم استخدام أربعة أنواع من الصبغات المشعة (DAPI ، DCFH-DA ، Mito-Sox ، Mito-Tracker) لتحديد أنواع الأوكسجين التفاعلي (ROS) وخلل المايتوكوندريا وعلاقة الطفرات الوراثية بـ ROS في الأنسجة بين مرضى BC ومقارنتهم مع الاصحاء. أجريت الدراسة في مختبر الهندسة الوراثية في قسم علوم الحياة- كلية العلوم - جامعة ميسان، بهدف استخراج الحمض النووي من جميع عينات الدم وتضخيمها باستخدام تقنية تفاعل البلمرة المتسلسل (PCR)، واستخدام منتجاتها كقالب لدراسة تعدد الأشكال الوراثية للجينات بتقنيات PCR وتحديد تسلسل النيوكليوتيدات عبر شركة Macrogen Korea لتحليل تسلسل القواعد النيتروجينية. تم تحليل جميع النتائج بواسطة اختبارمربع كاي Chi-Square test.

في تقنية RFLP-PCR، أظهرت نتائج التحليل الإحصائي للأنماط الوراثية باستخدام اختبارChi Square لاتزان هاردي واينبرغ ان هناك اختلافات في توازن هاردي-واينبرغ في الأنماط الوراثية لجينات *ABCG2* و *ABCB1* للمصابين بسرطان الثدي مقارنة بالأشخاص الأصحاء، واظهرت الأنماط الوراثية لـجين *BRCA1* تشابهًا إلى حد ما في المرضى المصابين بسرطان الثدي والاشخاص الاصحاء. كانت هناك فروق ذات دلالة إحصائية في توزيع الأليلات (C ، T) لـجينات (*ABCG2* ، *ABCB1*) بين المرضى المصابين بسرطان الثدي والاصحاء، وعدم وجود فروق ذات دلالة إحصائية في توزيع الأليلات (C ، T) لجين *BRCA1* بين المجموعتين. أظهرالتحليل الإحصائي فروق ذات دلالة إحصائية في الأنماط الوراثية (CC ، CT) لجينات (*ABCG2* ، *ABCB1*). إلى جانب ذلك، لم توثق النتائج أي فروق ذات دلالة إحصائية في الأنماط الـوراثية (TT، TC،CC) لجين *BRCA1* بين مرضى سرطان الثدي والاصحاء. كانت هناك فروق ذات دلالة إحصائية في توزيع النموذج السائد (Dominant model) لجينات *ABCG2* و *ABCB1* بين مرضى سرطان الثدي

ومجموعة الاصحاء مع عدم وجود ارتباط إحصائي في توزيع النموذج المتنحي (Recessive model)، وعدم وجود فروق ذات دلالة إحصائية في توزيع النموذج السائد والنموذج المتنحي لجين *BRCA1*.

فيما يتعلق بتقنية PCR-RAPD-PCR ، كشفت نتائجنا أن العدد الإجمالي لحزم (*ABCG2* ، *ABCB1* ، *BRCA1* ، *ER-α* ، *miRNA 152*) كانت أعلى في مجموعة المرضى مقارنة بالمجموعة الأخرى، مع وجود اختلافات كبيرة ($P < 0.05$) بين المجموعتين في مجموع الحزم المتعدد الأشكال والفريد والاحادية الشكل لجينات *ER-α* ،mi-RNA-152، ولا توجد فروق ذات دلالة إحصائية ($P > 0.05$) لجينات *ABCG2* ، *ABCB1* ، *BRCA1*. اظهر البادئان OPD18 ، OPAA 17 حزم ذات وزن جزيئي (20-30) زوج قاعدي في جينات *BRCA1,ABCB1 ,ABCG2* في حين أظهرالبادئ OPAA11 حزم ذات وزن جزيئي (40-50) زوج قاعدي في جين *ER- α* بينما اظهر البادئان OPAA17 و OPD18 حزم ذات وزن جزيئي (20-40) زوج قاعدي في جين*mi-RNA 152* ، ظهرت هذه الحزم في مجموعة المصابين بسرطان الثدي ولم تظهر في مجموعة الاصحاء، وهو مؤشر إيجابي وبالتالي يمكن ترشيح البادئات لتكون مؤشرًا مميزًا لسرطان الثدي على مستوى مؤشرات RAPD. لذلك، يمكن اعتماد هذه البادئات لتمييز سرطان الثدي وراثيًا على المستوى الجزيئي.

فيما يتعلق بتسلسل النيوكليوتيدات لجين *ABCG2*، تم الحصول على 11 طفرة وراثية جديدة 6 منها (G71T) ، (T141C)، (T148C)، (G150C)، (T169C)، (G172C) كانت طفرات غير مترادفة تسببت في تغير بنية البروتين الثلاثي الأبعاد نتيجة لتغير الأحماض الأمينية في مرضى سرطان الثدي، و3 منها (G38A)، (G2559C)، (G96428A) كانت طفرات مترادفة ، و2 منها ($163\ G^{Del}$ ، $167\ A^{Del}$) هي طفرات ازاحة (حذف). أظهر تسلسل النيوكليوتيدات لجين *ABCB1* 8 طفرات وراثية، 6 منها (G55C)، (T59G)، (C89A)، (C90G)، (A113C)، (A139T) كانت طفرات غير مترادفة ، 2 منها (C153T، C323T) هي طفرات مترادفة. وثق التسلسل الجيني لجين *BRCA1* ثلاث طفرات وراثية، (C213T ، T248C ، T261C) جميعها كانت طفرات غير مترادفة. كشف تسلسل النيوكليوتيدات لجين *ER-α* 6 طفرات وراثية، 6/4 (C114T، C244G، C344G، C424T) هي طفرات مترادفة ، 6/1 (T440C) غير مترادفة ، 6/1 ($454\ A^{Del}$) هي طفرة ازاحة (حذف).

الخلاصة :

في الجزء الوراثي الخلوي، زاد التفاعل مع DAPI و DCFH-DA و Mito-Sox و Mito-Tracker في أنسجة سرطان الثدي مقارنة بالأنسجة الطبيعية بسبب زيادة إنتاج ROS، وخلل في تركيب المايتوكوندريا ووظيفتها، بالإضافة إلى الطفرات الوراثية.

وزارة التعليم العالي والبحث العلمي

جامعة ميسان

كلية العلوم

قسم علوم الحياة

دراسة التشكل الوراثي للجينات ذات العلاقة بالتنبؤ بسرطان الثدي

رسالة مقدمة

الى مجلس كلية العلوم/جامعة ميسان

وهي جزء من متطلبات نيل درجة الماجستيرعلوم في علوم الحياة

من قبل

زينب زامل گاطع

بكالوريوس علوم/علوم الحياة (٢٠١٢)

بأشراف

أ.م.د. ميثم عبد الكاظم دراغ

جمادى الثاني ١٤٤٣هـ

كانون الثاني ٢٠٢٢م