Breast Cancer High-Penetrance Genes *BRCA1* and *BRCA2* Mutations Using Next-Generation Sequencing among Kurdish Women in Erbil City

A Dissertation

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DECLARATION

I declare that the Ph.D. dissertation entitled: **Breast Cancer High-Penetrance Genes *BRCA1* and *BRCA2* Mutations Using Next-Generation Sequencing among Kurdish Women in Erbil City**, is my own original work, and hereby I certify that, unless stated, all work contained within this dissertation is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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DEDICATION

My dear mother, for whom I applied for a doctorate.

My dear father, my role model in my studies.

My dear wife, who deserves half of this certificate.

My dear brothers and sisters, my support in life.

My dear daughters, the light of my eyes and my hope in life.

My deceased grandmother, Nazahat Al-Asaadi, who died of cancer.

My mother-in-law, for her continuous prayers.

To all cancer patients and survivors, of which I am one.

You deserve my efforts, and I dedicate this work to all of you.

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ABSTRACT

Breast cancer is the most common type of cancer among women; every year, millions of new cases are detected worldwide, and the cases increase dramatically. Despite the fact that most of the cases are caused by non-genetic factors, hereditary and familial breast cancer also contribute and are considered risk factors that are responsible for about 20% of the cases. The present study aimed to be the first study to investigate the frequency of hereditary breast cancer caused by the high penetrance genes BReast CAncer gene 1 (*BRCA1*) and BReast CAncer gene 2 (*BRCA2*) using net generation sequencing (NGS) among Iraqi Kurdish women in Erbil province. Also, investigate several important parameters that some of them have studied for the first time among Kurdish breast cancer patients in Erbil, Iraq.

The present study included 150 participants who were already diagnosed with breast cancer and registered at Nanakali Hospital for Blood Diseases and Cancer, Erbil, Iraq. For mutation analysis and variant detection, 70 participants were selected for NGS. Samples underwent DNA extraction, estimation of the extracted DNA, polymerase chain reaction (PCR) for amplification of all exomes of the *BRCA1* and *BRCA2* genes, and NGS for sequencing of all coding regions (exomes) through (Illumina Inc., San Diego, CA). Results of NGS obtained in different formats (BAM, BAI, VCF, and FASTA) files. Variant viewing and detection were carried out through the Integrative Genomic Viewer (IGV) and MutationTaster websites. Finally, for interpretation of the clinical significance of the variants, different databases were used, including mainly: NCBI/ClinVar, BRCAExchange, ENIGMA, gnomAD, and COSMIC.

Many variants were detected on these two genes, variants in intronic regions were neglected (except one on *BRCA2* that was not benign). At the end, 42 variants

were included in the present study, 20 (47.6%) on *BRCA1* and 22 (52.4%) on *BRCA2.* Regarding the clinical significance of the variants, 9 (21.4%) of them were clinically significant. On *BRCA1*, 4 (9.5%) pathogenic variants were detected (c.3607C>T, c.3544C>T, c.224_227delAAAG, c.68_69del), while on *BRCA2*, 2 (4.76%) pathogenic variants (c.100G>T, c.1813delA), 2 (4.76%) conflict interpretations of pathogenicity (c.3318C>G, c.1909+12delT), and 1 (2.38%) variant of uncertain significance (c.6966G>T) were detected. Also, 29 (69%) other benign variants were detected on these two genes.

An important finding of the present study was the detection of four new variants, three on the *BRCA1* gene (c.463dupC, c.3190A>C, c.981del) and one on the *BRCA2* gene (c.3787A>G). Those exact variants were not reported in any databases or articles before. Those new variants were submitted to NCBI/ClinVar, and unique accession numbers were obtained for each of them (SCV005196609, SCV005199865, SCV005199845, SCV005196610), respectively. Detecting new variants on these two genes is popular, especially among low- and middle-income countries, where little or no studies have been done among those populations.

Besides the molecular part, several other important parameters were investigated in the present study, including 150 participants. The mean age at the time of diagnosis with breast cancer was 49.5 years of age, with highly significant differences between the age groups (P<0.0001). The level of awareness by assessing previous knowledge about breast cancer was very low; 120 (80%), had no previous information about breast cancer, and the rest had simple knowledge about different aspects of the disease (P<0.0001). Most of the participants, 131 (87.3%) didn't undergo any pre-tests before being diagnosed, and the rest underwent a few attempts or just once during their lifetime (P<0.0001). About half

of the cases 72 (48%) were detected at advanced stages (stages III and IV), followed by stage I, then stage II (P<0.0001).

Many participants 103 (68.7%) indicated that the cases were observed by the patients themselves (P<0.0001), either by feeling a tumor or pain under the armpit. Despite the fact that cancer is known to be a silent disease, especially in its early stages, more than half 89 (59.3%) of the cases stated that they experienced some signs before the disease was detected; the most popular signs were swelling of the breast, while a few cases felt some pain, vomiting, stiffness of the breast, a shortage in breathing, and finally abnormal stuns in the breath and discharges of liquids, seen rarely (P<0.0001). For family history, 49 (32.7%) of the patients had relatives with breast cancer (P<0.0001). Regarding breast removing surgery, 62 (41.3%) already underwent mastectomy (P<0.04); among the rest of them, 73 (82.9%) stated they would take the choice of mastectomy if needed and recommended in the future.

Regarding the results of the psychological impact, 118 (78.7%) stated that the disease had a bad impact on their lives (P<0.0000.); most of them suffered from depression, and the quality of their sleep lowered dramatically after being diagnosed with cancer. For receiving sufficient information about their status, more than one-third, 53 (35.3%) of the participants stated that they were either little informed or not informed by the physician (P<0.0001). Regarding family support, 140 (93.3%) of them stated that they received good family, relatives, and friends' support (P<0.0001). The majority 148 (98.7%) were taking one or two types of medications; chemotherapy was the most popular 129 (86%), followed by mastectomy (P<0.0001).

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LIST OF ABBREVIATIONS

Abbreviations	Full name (description)
ACMG	American College of Medical Genetics and Genomics
ACS	American Cancer Society
AD	Anno Domini
AI	Artificial Intelligence
AMP	Association for Molecular Pathology
ATM	Ataxia Telangiectasia Mutated gene
BAM	Binary Alignment Map
BARD1	BRCA1-associated RING Domain protein 1 gene
BC	Breast Cancer
BCL	Base Call
bp	Base Pair
BRCA	BReast CAncer gene
BRCA1	BReast CAncer gene 1
BRCA2	BReast CAncer gene 2
BRCT	BRCA1 C-Terminal *domain*
BRIP1	BRCA1 Interacting Protein
BSE	Breast Self-Examination
BSGI	Breast Specific Gamma Imaging
BWA	Burrows Wheeler Aligner
CASP8	Caspase 8 *gene*
CAT	Computed Axial Tomography
CDH1	Cadherin 1
cDNA	Complementary DNA
CGH	Comparative Genomic Hybridization
CHEK2	Checkpoint Kinase 2
Chr	Chromosome
CIP	Conflict Interpretations of Pathogenicity
ClinVar	Clinically Relevant Variants
CNV	Copy Number Variation Calling
COSMIC	Catalogue of Somatic Mutations in Cancer
CT	Computer aided Tomography
DCIS	Ductal Carcinoma *in situ*
DNA	Deoxyribonucleic Acid
dsDNA	double-stranded DNA
DWI	Diffusion-Weighted Imaging
EDTA	Ethylenediaminetetraacetic Acid

ENIGMA	Evidence-based Network for the Interpretation of Germline Mutant Alleles
FDG	fluoro-D-Glucose
FGFR2	Fibroblast Growth Factor Receptor 2
GATK	Genomic Analysis ToolKit
GB	Gigabytes
gnomAD	Genome Aggregation Database
HBC	Hereditary Breast Cancer
HGVS	Human Genome variation Society
IDC	Invasive Ductal Carcinoma
IGV	Integrative Genomics Viewer
ILC	Invasive Lobular Carcinoma
LCIS	Lobular Carcinoma *in situ*
lncRNA	long noncoding RNA
LSP1	Lymphocyte Specific Protein 1
MAP3K1	Mitogen-Activated Protein Kinase 1.
MLH1	MutL protein Homolog 1
MLH2	MutL protein Homolog 2
MLPA	Multiplex Ligation-dependent Probe
MPSS	Lynx therapeutics' Massively Parallel Signature Sequencing
MRE	Magnetic Resonance Elastography
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MRS	Magnetic Resonance Spectroscopy
NBN	Nibrin *gene*
NCBI	National Center for Biotechnology Information.
NF1	Neurofibromin1 gene
NG-CHM	Next-Generation Clustered Heat Map
NGS	Next Generation Sequencing
NLS	Nuclear Localization Signals
NPM	Nextera PCR Mix
OB	Oligonucleotide Binding
PALB2	Partner and Localizer of BRCA2 gene
PARP4	Poly [ADP-Ribose] Polymerase 4
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PTEN	Phosphatase and Tensin Homologue Gene
PV	Pathogenic Variants
RNA	Ribonucleic Acid

RNAP	Ribonucleic Acid Polymerase
rRNA	Ribosomal Ribonucleic Acid
RTA	Real-Time Analyzer
SAM	Sequence Alignment Map
SBS	Sequencing by Synthesis
SIFT/PolyPhen	Sorting Intolerant from Tolerant and Polymorphism Phenotyping
SOLID	Sequencing by Oligonucleotide Ligation and Detection
STK11	Serine/Threonine Kinase 11
TAD	Transcriptional Activation Domain
TGFβ1	Transforming Growth Factor-Beta
TNRC9	Trinucleotide-Repeat-Containing 9 gene
TOX3	TOX high mobility group box family member 3 *gene*
TP53	Tumor Protein p53 gene
TR2	Testicular Receptor 2
UTR	Untranslated Region
VCF	Variant Call Format
VUS	Variants of Uncertain Significance
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
ZMW	Zero-Mode Waveguide

1. INTRODUCTION

Breast cancer is among the most three common cancers worldwide, it is the main cause of cancer death among women. Breast cancer is a major global problem because it causes serious health problems that cause death in about 30% of the cases. Besides mortality and health problems, breast cancer causes many other consequences like psychological, social, and economical for the affected individual and their families (Sung *et al.*, 2021, Arnold *et al.*, 2022).

Breast cancer is caused mainly by non-hereditary factors, they are caused by mutations in the somatic cells of the breast that acquired during the lifetime, and they do not cluster in families. Genetic factors are responsible for fewer cases that are estimated at 5%-10% for hereditary and up to 20% for familial breast cancer. The inherited one is caused by mutations in a gene that is related to the breast, while familial one resulted from an interaction of genetic factors with environmental factors, their genetic bases and specific genes not specified yet (De Silva *et al.*, 2019, Petrucelli *et al.*, 2022, Barili *et al.*, 2024).

Hereditary breast cancers are caused through mutation in one or more of the susceptible genes that are related to breast cancer. Until now up to 34 genes are expected to be linked with this disease. Some genes are proved to have a direct link to the disease, while other genes have no clear evidence and only suggested by studies to be linked with increasing the risk for developing breast cancer (Barili *et al.*, 2024). In genetics, penetrance refers to the proportion of people with a particular genetic variant (or gene mutation) who exhibit signs and symptoms of a genetic disorder. Those genes can be classified into three main classes based on their level of penetrance and the estimated risk for developing breast cancer during women lifetime, genes that develop risks estimated at 50% and above considered

at high penetrance, from 25% to 50% are moderate, while below 25% considered at low penetrance genes (Valentini *et al.*, 2024).

BReast CAncer (*BRCA*) genes perform several important functions. The *BRCA1* gene contributes to different cellular processes, including DNA repair, transcriptional activation, cell cycle regulation, and chromatin remodeling. While the *BRCA2* gene has a role in cell cycle and transcriptional regulation, DNA repair, mitophagy, and stabilization of replication fork. Both *BRCA1/2* genes normally act as tumor suppressors, helping to prevent cells from growing and dividing too rapidly or in an uncontrolled way (Sadeghi *et al.*, 2020). Mutations *BRCA1* and *BRCA2* will increase the risk for developing breast cancer which is estimated to be up to 70% to 90% by the age of 70 (Hassan and Mustafa, 2024).

Several genetic tests are available using different molecular techniques to allow the identification of the genetic bases of any condition or disease, including breast cancer. Most genetic tests aim to identify genes with high penetrance, like *BRCA1* and *BRCA2*, if they are not diagnosed, the second class of moderate genes will be investigated, while identification of low penetrance genes is not among routine tests as they may contribute to a minority of cases (Petrova *et al.*, 2022).

The most effective and used genetic test is next generation sequencing (NGS) that enables the detection of the mutations (variants) all over the gene regions. Different strategies can be applied based on the purpose of the test, including whole exome sequencing (WES), to whole genome sequencing (WGS). Through advances in recent years, NGS can be performed in a single day with relatively low cost compared to the previous years. Performing NGS, data analysis and interpretations requires a skilled technician to perform it, nowadays, several websites and databases are available that include huge data regarding all the genes, mutations, and interpretation of the variants clinical significance. Unfortunately,

there are some important differences among those databases which may cause confusion for the physicians and genetic counselors (Kanzi *et al.*, 2020, Brlek *et al.*, 2024).

Age of the women considered as one of the risk factors for developing breast cancer. The stage of the cancer at the time of the diagnosis play an important role in determining the therapeutic strategies for the patients. Unfortunately, women in Low- and Middle-income countries diagnosed at lower ages and higher stages of breast cancer. Among those countries, the level of awareness and screening practices for the early diagnosis of breast cancer is very low compared to the High-income countries (Zhu *et al.*, 2023, Eremici *et al.*, 2024).

Breast cancer, beside health consequences, has several negative psychological impacts, and it reduces the quality of the sleeping among the women who diagnosed with it (Lim *et al.*, 2022, Kashyap *et al.*, 2022). Family support play an important role in reducing the negative impacts and consequences of the disease (Yang *et al.*, 2022). Several treatments are available, like chemotherapy, mastectomy, radiation, hormonal therapy, and tablets, in most cases, more than one treatment is given which mainly depends on the stage of the cancer and the status of the tumor at the time of the diagnosis (Amjad *et al.*, 2024).

Finally, although breast cancer cannot be prevented totally, but several preventive steps, as well as regular screening test enables the early detection of the disease that minimize its consequences (Ginsburg *et al.*, 2020). Health care providers, ministry of health, and non-governmental organizations NGO have responsibility to raise the level of awareness about different aspects of the breast cancer and encourage women to undergo regular screening tests.

The present study aimed to:

1- Detecting the hereditary breast cancer caused by *BRCA1* and *BRCA2* genes mutation using next generation sequencing technique among Kurdish women with breast cancer in Erbil city.
2- Investigating ages of the women and stages of the cancer at time of diagnosis with breast cancer.
3- Determining family history, mastectomy, level of awareness, screening practices, and methods used for the pre-test purposes.
4- Investigating important epidemiological parameters as well as, influences of breast cancer on the patient's life, psychology, sleeping quality, and other parameters.

2. LITERATURE REVIEW AND THEOROTICAL BACKGROUND

2.1. Breast Cancer

Breast cancer (BCa) is a type of cancer that forms in the cells and tissues of the breasts and/or the surrounding tissue. It is considered the most common cancer in women around the world; it affects one in every eight to ten women during their lifetime. Men are also susceptible to developing it, but it is approximately 1% compared to women (Momenimovahed and Salehiniya, 2019, Łukasiewicz *et al.*, 2021).

The earliest discovery of familial breast cancer was a long time ago, about 100 AD, when clustering of breast cancer in families was described and recorded in Roman literature. In the recent era, a French surgeon, Paul Broca, in the mid-1800s documented the first obvious details about hereditary breast cancer that clusters in families. Later, the British Government Ministry of Health, in 1926 declared that women who have first-degree relatives affected with breast cancer are at greater risk to get BC (de Moulin, 2013, Hurst, 2014). All these descriptions were based on observations only, while since the 1970s, a significant understanding of familial breast cancer and its genetic bases has been obtained based on the major advances in screening technologies (de Moulin, 2013, Baum, 2019).

2.2. Epidemiology of Breast Cancer

Breast cancer is a major global problem; it is among the three most common cancers worldwide. It is the main cause of death from cancer among women, and it causes death in about 30% of the cases. According to recent statistics, breast cancer became the most frequent cancer, followed by lung cancer. Every year, approximately 2.5 million new cases are detected, and in

2020, about 11.7% of new cancer cases were BC (Arnold *et al.*, 2022, Xu and Xu, 2023). In Kurdistan region-Iraq, breast cancer is the most common type of cancer and number of cases has been duplicated in the last decade. According to previous reports, cancer incidence rates were 50.0 cases/100,000 individuals for Erbil and 61.5 for Duhok cases/100,000 individuals. While the incidence rate showed an increasing trend in Sulaymaniyah from 38.5 cases/100,000 individuals in 2006 to 61.7 cases/100,000 individuals by 2013. According to the analysis and predictions, the number of the cases of breast cancer in the Erbil governorate is predicted to increase by >2x in the current decade (M. Amen *et al.*, 2022). In Iraq, breast cancer had the highest percentage and incidence rate of the top ten cancers in 2019, and it was the main cause of mortality among Iraqi women, accounting for about one-third of all cancer cases recorded in 2019 (Al-Hashimi, 2021).

According to statistics worldwide, incidences of breast cancer vary among different countries, regions, populations, and ethnicities. Statistics revealed that in high-income countries, the incidence of BC was higher compared to low- and middle-income countries. Higer incidences among those countries could be attributed to risk factors like lifestyle, hormonal factors, and higher detection percentages due to the health care system and regular screenings (Mullooly *et al.*, 2017, Kashyap *et al.*, 2022). While the mortality rates are controversial among those countries, low- and middle-income countries have higher mortality rates than high-income ones.

2.3. Types of Breast Cancer

Generally, there are two main classifications that can be listed as:

2.3.1. Invasive and Non-invasive BC

Based on the tumor situation and location, it is classified into invasive and non-invasive breast cancer.

2.3.1.1. Invasive BC

Also called infiltrating, it means that the tumor has spread into the surrounding breast tissues. It includes two main types: invasive ductal carcinoma (IDC), which is considered the most common type and contributes to 80% of all types of BC. It refers to the type that the cancer initiated from the milk ducts. The second one is invasive lobular carcinoma (ILC), which is less common and contributes 10% of all breast cancer. It refers to the type of cancer initiated by lobules (milk-producing glands) (Feng *et al.*, 2018, Wang *et al.*, 2024).

Beside these two main invasive types, there are several other invasive types that are classified based on their development and treatment, including triple-negative BC, triple-positive BC, Inflammatory BC, micrometastasis, metastatic BC, recurrent BC, male BC, and paget BC (Orrantia-Borunda *et al.*, 2022).

2.3.1.2. Non-invasive BC

Also called precancer (or *in situ*), includes those types in which the tumor hasn’t spread into the surrounding breast tissues. It includes two types: Ductal carcinoma *in situ* (DCIS) and Lobular carcinoma *in situ* (LCIS) (Tomlinson-Hansen *et al.*, 2024).

2.3.2. Sporadic (non-inherited) and Germline mutations (inherited) BC

2.3.2.1. Sporadic BC

It is also called somatic BC, refers to those types that are acquired through the lifetime, not through inheritance or germline mutations. It occurs from

damaging genes in an individual cell and is distinguished by the meaning that the mutated genes are restricted to the tumor cells only, not all cells of the body. Somatic BC contributes to most of the BC cases, more than 80% of the cases belong to this type (De Silva *et al.*, 2019, Miles and Tadi, 2024).

2.3.2.2. Germline Mutations (inherited) BC

This type occurs when a mutated gene(s) is inherited from one or both parents, usually. Inherited type means that the mutated genes, when inherited, are present in all body cells of that person. This type contributes to fewer cases of breast cancer, about (or at least) 20% of all cases (Feng *et al.*, 2018, Hu *et al.*, 2020).

2.4. Types and Inheritance Patterns of Genetic BC (Hereditary vs. Familial)

Before studying the inheritance pattern, the two types of genetic breast cancers, hereditary and familial breast cancer, must be differentiated. However, both types have genetic bases, but they are totally different from each other, including their pattern of inheritance. Hereditary Breast Cancer (HBC) refers to the inheritance of an abnormal gene that follows the autosomal dominant pattern of transmission (Mendelian inheritance). For example, *BRCA* mutations are inherited in an autosomal dominant fashion, but act recessively on the cellular level as tumor suppressor genes involved in double-stranded DNA (dsDNA) break repair (Shiovitz and Korde, 2015). On the other hand, familial BC resulted from interactions of genetic mutations with environmental factors; the inheritance pattern of familial BC is still not specified as its mechanism, and genetic bases are not clear until now (Meaney-Delman and Bellcross, 2013, AlHarthi *et al.*, 2020, Barili *et al.*, 2024). Differences between the two types are listed in Table (2-1).

Table 2-1 Main differences between hereditary and familial breast cancers (Meaney-Delman and Bellcross, 2013).

Differences	Hereditary	Familial
Cause	Single gene mutation	Multiple genetic factors interacting with environmental factors
Inheritance pattern	Autosomal dominant	Unspecific pattern
Frequency	Responsible for 5% to 10% of all cases	Responsible for 15% to 20% of all cases
Prevalence	Usually affects multiple individuals in all generations	Affects two or more members of the first- or second-degree relatives, tends to skip generations.
Appearance	In early age, before 50	In later age, after 50 years
Type	Bilateral/multifocal	Unilateral in most cases, or late-onset bilateral in some cases

2.5. Mutation Types of BC

Different types of mutations contribute to breast cancer; they could be at the chromosomal level or a point mutation. Chromosomal mutations may be structural or numerical; structural abnormalities include duplication and amplification, inversion, deletion, and translocation, while numerical abnormalities occur through an imbalance in the number of chromosomes (aneuploidy and polyploidy). Mutations at the DNA level generally include substitution of a single nucleotide, deletion or insertion of 1-10 nucleotides. DNA mutations which create oncogenes or turn off tumor suppressor genes or DNA repair genes may lead to cancer, even though, typically it takes several gene changes before a cell becomes a cancer cell. (Richardson *et al.*, 2006, Desmedt *et al.*, 2016, Cosenza *et al.*, 2022). Types of mutations are shown in (Figure 2-1).

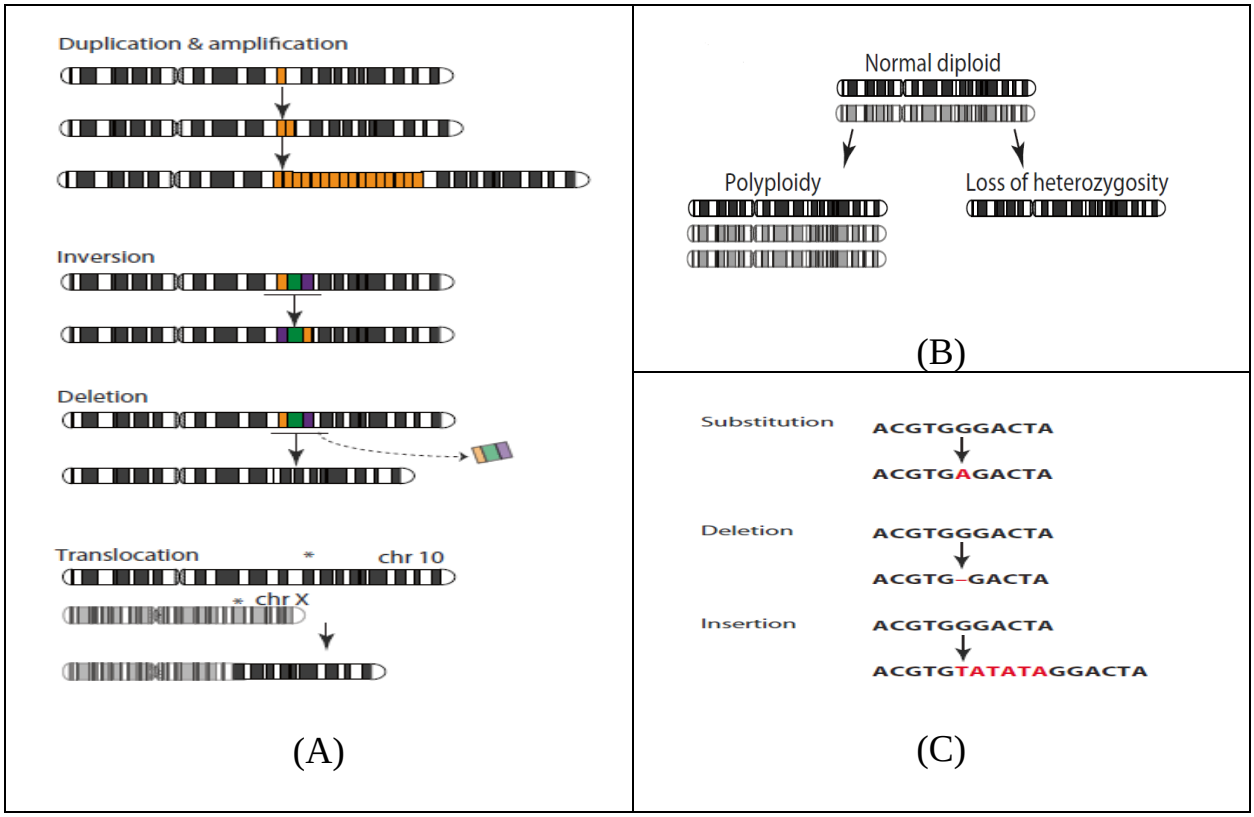

Fig. 2-1 Different mutation types of breast cancer. A: structural variants of the chromosomes; duplication and amplification, inversion, deletion, and translocation. B: the numerical imbalances of the chromosomes. C: point mutations; single nucleotide substitutions, or the deletion or insertion (Desmedt *et al.*, 2016).

2.6. Risk Factors of BC

Breast cancer can originate from several genetic or non-genetic factors; most of the cases resulted from non-hereditary (environmental) factors, while fewer cases were caused by genetic factors. Although environmental and genetic factors differ, but they are related to each other, and the process of breast cancer development is affected by a complex interaction between these two factors (Sun *et al.*, 2017a, Mbemi *et al.*, 2020).

Risk factors like age, lifestyle, weight, smoking, diet, and environmental factors such as exposure to mutagenic substances are among the main causes of

the acquired (somatic) form of this cancer (Wu *et al.*, 2018). BC is mainly caused by non-genetic factors, while hereditary factors contribute to about 20% of the cases (Catana *et al.*, 2019). Most cases of hereditary type result from mutations in those genes that are linked to the breast; those genes can be classified into 3 classes depending on their penetrance. *BRCA1*/2 are the main two genes that are considered high-penetrant genes, and they are responsible for the most cases of inherited BC. Beside *BRCA1/2*, there are several other genes for inherited breast cancer, but with lower penetrance, like *PALB2, ATM, CHEK2, BARD1, BRIP1, PARP4, CASP8,* and *TOX3* (Cornejo-Moreno *et al.*, 2014, Godet and Gilkes, 2017a, Bedrosian *et al.*, 2024).

2.6.1. Genetic risk factors, penetrance, and level of penetrance

Genetic factors of BC refer to the inheritance of an abnormal (mutated) susceptible gene that is linked to the breast. Inherited BC is responsible for 5% to 10% of total cases of the disease, while some studies indicated that about 20% of the cases are hereditary (Catana *et al.*, 2019, Shen *et al.*, 2021). The most important risk factor and indicator related to genetic BC is family history. It is true that the main causes of inherited BC are caused by mutations in *BRCA* genes, which are considered high-penetrance genes, but, there are several other genes identified as susceptible genes whose mutation are linked to breast cancer (Valentini *et al.*, 2024).

Hereditary BCs are caused by mutations in one or more of the susceptible genes related to BC. The number of susceptible genes varies according to different studies, in 2018, a group of researchers, using a genetic analysis technique, identified 110 genes associated with a higher risk of BC. Until now, up to 34 genes are expected to be linked to this disease. Some genes are proven to have a direct link to the disease, while others have no clear evidence and are

only suggested by studies to be linked with increasing the risk of developing BC (Sierra-Díaz *et al.*, 2024).

Penetrance refers to the proportion of people with a particular genetic variant (or gene mutation) who exhibit signs and symptoms of a genetic disorder. Those genes were classified into three main classes depending on their level of penetrance and the estimated risk of developing BC during a woman's lifetime. Genes that develop risks estimated at 50% and above are considered to have high penetrance, and those from 25% to 50% are moderate, while those below 25% are considered to have low penetrance genes (Barili *et al.*, 2024). Research showed different categories regarding the classification of these genes, they may be classified into two, three, or even four groups. Also, regarding groups of some genes, differences can be seen, for instance, the *PTEN* and *CDH1* genes are considered high-penetrance genes, while in other research, they have been classified as other genes (Slavin *et al.*, 2017, Mares-Quiñones *et al.*, 2024).

Below are the three classes of breast cancer susceptibility genes; only those genes are included that are inherited. It should be noted that there may be some differences regarding the class of certain genes that differ among different studies and research (Wang *et al.*, 2021).

2.6.2. Class 1: High penetrance breast cancer susceptibility genes

2.6.2.1. BReast CAncer genes (*BRCA)*

BRCA genes, *BRCA1* and *BRCA2*, are responsible for cell growth, division, the repair of damaged DNA, and tumor suppressor. They function to keep the normal growth of breast, ovarian, and other cells. Mutated forms of these two genes are unable to function normally, leading to an increased risk of developing breast, ovarian, and other types of cancers. *BRCA1/2* are the most

common genes for hereditary breast cancer; they account for up to 10% of all cases of genetic BC (Mehrgou and Akouchekian, 2016).

1- BReast CAncer gene 1 (*BRCA1*)

The *BRCA1* gene is located on chromosome 17q21 and contributes to different cellular processes, including DNA repair, transcriptional activation, cell cycle regulation, chromatin remodeling, and works as a tumor suppressor gene. Mutated form of it causes early-onset hereditary BC with an estimated risk of 57% to 81%, while it causes hereditary OC with an estimated risk of 90% in families with a high incidence of breast and ovarian cancers (Barili *et al.*, 2024). The *BRCA1* gene considered as the most aggressive gene related to breast cancer, because *BRCA1* gene has a higher rate of mitosis and greater lymphatic permeability, it is more related to breast cancer than other types of cancers, and it is often linked to triple negative, estrogen receptor negative, progesterone receptor breast cancers (Loboda *et al.*, 2023).

The *BRCA1* gene has 24 exons that spread over 81 kb of DNA, among those exons, 22 of them are coding exons. Exon number one is named exon number 2 for historical reasons, and exon 4 is missing due to an initial oversight during BRCA1 protein characterization; all following exons have a number increased by one. Those exons are varied in their length and coding for amino acids. Also, the numbers of coding sequences vary among the exons; exon 10 (11) has the highest number of coding sequences (61.3%) (Barili *et al.*, 2024). All exons, percentage of total coding DNA, and functional domain are shown in (Figure 2-2) below:

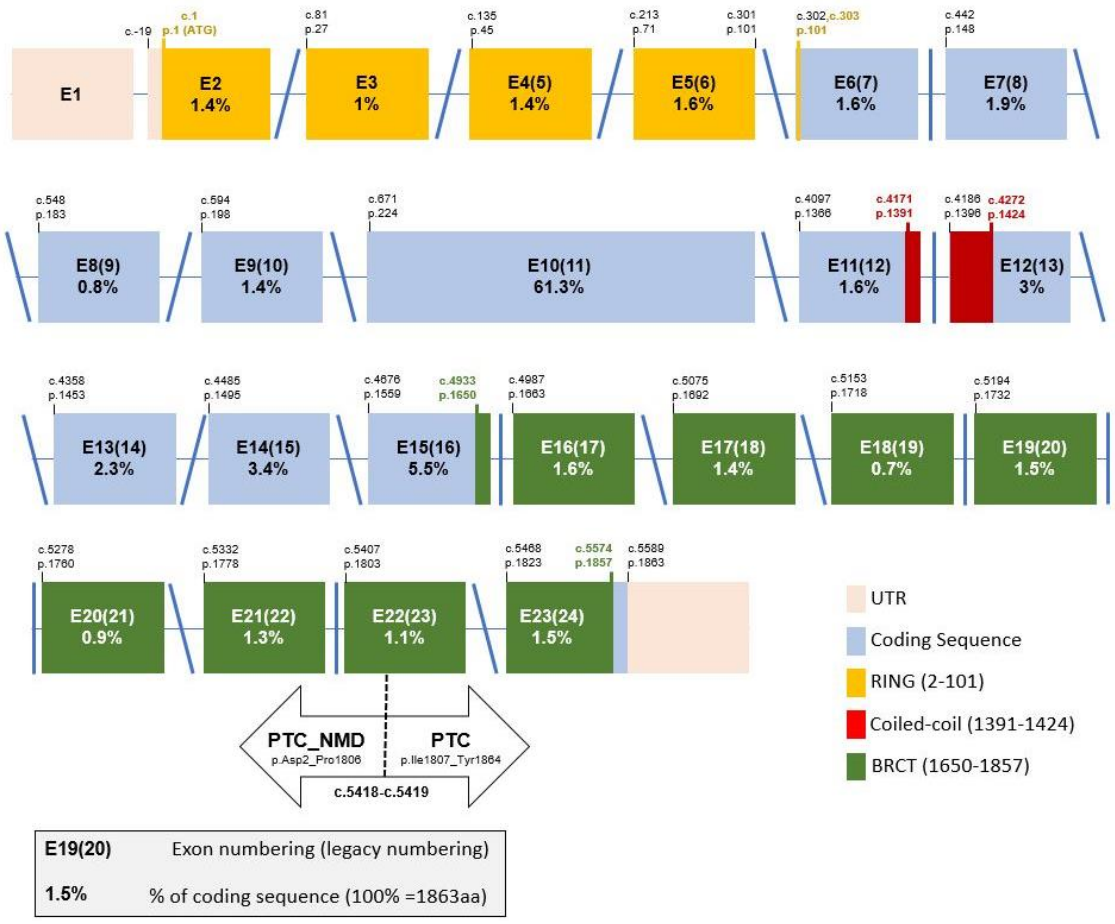

Fig. 2-2 *BRCA1* gene map, exons, and its functional domains (O'Donnell *et al.*, 2018).

The *BRCA1* gene interacts with so many other genes, at least 20 other genes, mainly the *BRCA1* Associated Ring Domain 1 gene (*BRAD1*) gene (Hawsawi *et al.*, 2022). It's physical interaction, co-expression, predicted, co-localization, genetic interaction, pathway, and shared protein domains are shown in order as in (Figure 2-3).

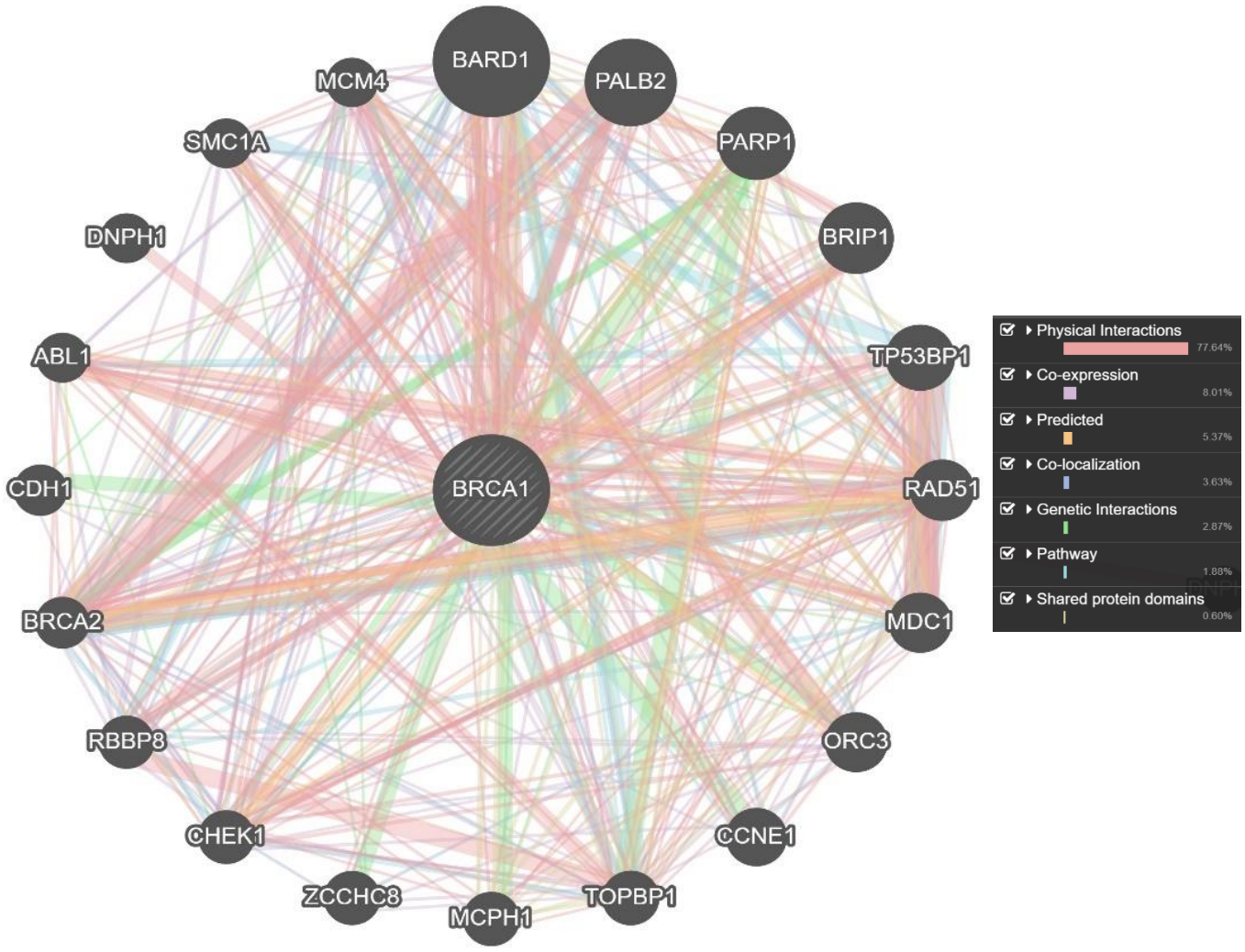

Fig.2-3 *BRCA1* and its interaction with other genes (GeneMANIA, 2024).

2- BReast CAncer gene 2 (*BRCA2*)

The *BRCA2* gene is located on chromosome 13q12.3 and has a role in cell cycle and transcriptional regulation, DNA repair, mitophagy, and stabilization of replication fork. Mutations in *BRCA2* cause an increase in the lifetime risk of 45% to 85%, while they cause hereditary ovarian cancer with a lower risk than BC. Mutations in *BRCA1* and *BRCA2* together led to an increased risk of

developing cancer that was estimated to be up to 70% to 90% by the 70 the age of 70 (Madar *et al.*, 2023).

BRCA2 gene has 27 exons, like *BRCA1*, those exons are varied in their length and coding DNA sequencing, exon number one codes for no cDNA sequences, while most of the cDNA (48.1%) occur on exon 11 (Madar *et al.*, 2023). All exons, percentage of cDNA and functional domain are shown in (Figure 2-4) below:

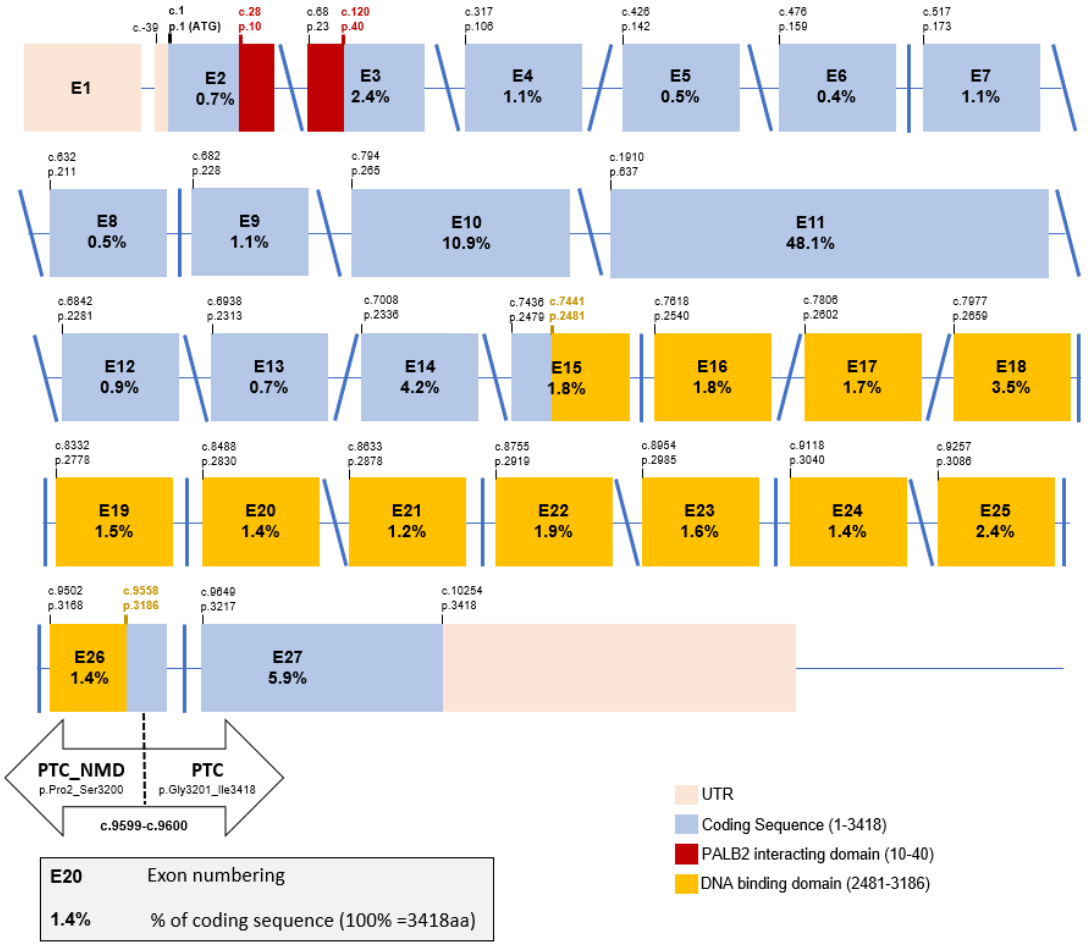

Fig. 2-4 *BRCA2* gene map, exons, and their functional domains (O'Donnell *et al.*, 2018).

The *BRCA2* gene interacts with so many other genes, at least 20 other genes, mainly with the Partner and Localizer of the *BRCA2* gene (*PALB2*) (Lehrer and Rheinstein, 2022). Its physical interaction, co-expression, predicted, co-localization, genetic interaction, pathway, and shared protein domains are shown in order as shown in (Figure 2-5) below.

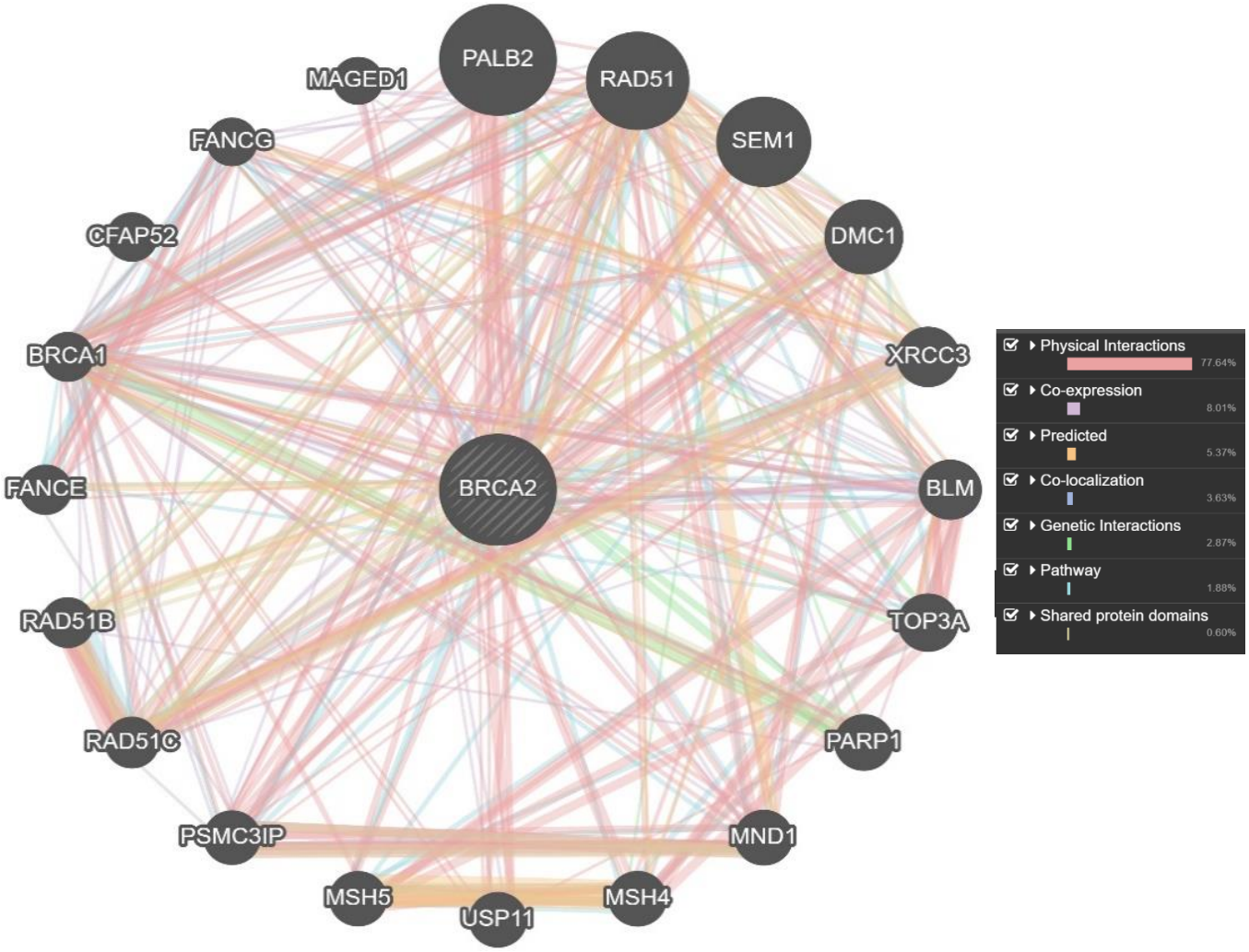

Fig. 2-5 *BRCA2* and its interaction with other genes (GeneMANIA, 2024).

2.6.2.1.1. *BRCA1* and *BRCA2* encoded proteins

These two genes are encoded for different and unrelated proteins in different tissues, including breast tissue. Proteins produced by these 2 genes carry different important functions; BRCA1 protein has a role in checkpoint activations and DNA repair, while BRCA2 protein works as a mediator of homologous recombination. Repairing of DNA damage, destroying cells whose DNA cannot be repaired, repairing of chromosomal damages, and error-free repair breaks in the DNA double strands are among the functions of these two proteins. They are also combining with other tumor suppressors and subunits to perform different other functions (Godet and Gilkes, 2017b, Divya Bhargavi *et al.*, 2022).

The *BRCA1* gene includes 22 exons that encode for 1863 different amino acids, BRCA1 protein includes domains like the N-terminus zinc-finger binding domain RING, the C-terminus domain (BRCT), nuclear localization signals (NLS) domains at the core region, and a coiled-coil domain. There are more than 1700 mutations on *BRCA1*, and most of them are related to these domains (Divya Bhargavi *et al.*, 2022).

The *BRCA2* gene, with 27 exons that encode for more than 3418 amino acids, BRCA2 proteins include 67 domains and features, like the transcriptional activation domain (TAD), RAD51-binding BRC repeats, DNA-binding domain, 3 oligonucleotide binding (OB) folds, conserved helical dominion, two NLS domains, and TR2 domain. More than 1800 mutations were recorded on *BRCA2* (Divya Bhargavi et al., 2022).

2.6.2.1.2. Types of Mutations of *BRCA1/2* genes

BRCA1/2 genes and their proteins are susceptible to different types of mutations. Mutations at the gene (DNA) level include more than nine different types: nonsense substitution, missense substitution, synonymous substitution, inframe insertion, frameshift insertion, inframe deletion, frameshift deletion, complex mutation, and others (Clark *et al.*, 2022). Types of mutation and their percentages on *BRCA1* and *BRCA2* are determined according to the Catalogue of Somatic Mutations in Cancer (COSMIC), as shown in Table (2-2) below:

Table 2-2 Types of mutations on *BRCA1* and *BRCA2* genes and their frequencies (COSMIC).

Types of Mutations	***BRCA1* (%)**	***BRCA2* (%)**
Nonsense substitution	7.86%	9.52%
Missense substitution	55.2%	57.76%
Synonymous substitution	10.4%	13.41%
Inframe insertion	0.13%	0.23%
frameshift insertion	1.98%	3.48%
Inframe deletion	0.52%	0.85%
frameshift deletion	5.28%	8.68%
complex mutation	0.04%	0.23%
Others	4.68%	2.6%

Even though *BRCA1/2* genes are responsible for the most cases of hereditary BC, it is important to know that having those mutations doesn't mean that women will be indefinitely diagnosed with this disease. Certain factors may

play a role, including the age at the time of developing breast cancer, the number and ages of relatives in the same parental lineage, and environmental factors (Mehrgou and Akouchekian, 2016).

3- Partner and localizer of the *BRCA2* gene (*PALB2*)

Located on chromosome 16p12.2, responsible for making a protein that contributes to DNA repair with the BRCA2 protein as well as suppressing tumors. The risk of developing BC with the *PALB2* gene depends on age. Women with a faulty *PALB2* gene have about a 55% chance of developing breast cancer and about a 5% chance of developing ovarian cancer over their lifetime, while it reaches its maximum after age 70 at 58% (Toss *et al.*, 2023, Maioru *et al.*, 2023).

4- Phosphatase and tensin homolog gene (*PTEN*)

Located on chromosome 10q23.3, is responsible for phosphatase and the tensin homolog protein that works as a tumor suppressor and helps in cell growth regulation (He *et al.*, 2021). Women with a mutant *PTEN* gene have a risk estimated at 25% to 50% of developing breast cancer during their lifetime, while other research have detected a higher risk, at 77% to 85% for this gene (Ngeow *et al.*, 2017, Li *et al.*, 2018).

5- Tumor protein p53 gene (*TP53*)

It is responsible for tumor protein 53, that has a crucial role in DNA repair and tumor suppression located on chromosome 17p13.1. Mutations in this gene contribute to several types of cancer (Wang *et al.*, 2023). Women who have this syndrome will have a higher risk of developing BC, estimated at 56% to 90%,

and other types of cancer in their lifetime (Silwal-Pandit *et al.*, 2017, Duffy *et al.*, 2018).

6- Cadherin 1 gene (*CDH1*)

Located on chromosome 16q22.1 is responsible for the production of the cadherin-1 protein that works as a tumor suppressor and binds cells to form tissue. Women with the mutant *CDH1* gene have a higher risk of developing breast cancer, estimated at 39% to 52% during their lifetime, while other studies detected a higher risk that may reach 60% (Shenoy, 2019).

7- Serine/Threonine Kinase 11 gene (*STK11*)

Located on chromosome 19p13.3. It is responsible for making serine and threonine kinase11 proteins that work as tumor suppressors and help regulate cell growth (Khanabadi *et al.*, 2023). Women who have this syndrome are at higher risk that estimated at 32% to 54% for developing BC, especially at the age of 70 (Alkaf *et al.*, 2017, Wendt and Margolin, 2019).

2.6.1.2. Class 2: Moderate penetrance breast cancer susceptibility genes

1- Checkpoint kinase2 gene (*CHEK2*)

Located on chromosome 22q12.1. It is responsible for making a protein that involves DNA repair and suppresses tumors (Stolarova *et al.*, 2020). Women that have mutated *CHEK2* gene have a risk of 28% to 37% of developing BC during their lifetime, while in those families that have more members with breast cancer, the risk will be increased (Wendt and Margolin, 2019, van Jaarsveld *et al.*, 2020).

2- Ataxia telangiectasia mutated gene (*ATM*)

Located on chromosome 11q22.3 and is responsible for making a protein involved in DNA repair (O'Donnell *et al.*, 2018). Women with a mutated form of this gene have a risk estimated at 33% to 38% of developing breast cancer in their late ages (Jerzak *et al.*, 2018, Renault *et al.*, 2018).

2.6.1.3. Class 3: Low penetrance breast cancer susceptibility genes

The third class involves those genes that have low or an uncertain risk; they may or may not increase the risk and susceptibility of breast cancer (Lindor *et al.*, 2016). Low-penetrance genes include a long list of genes, and still, new genes are added to this group as studies candidate new genes to be linked to the development of BC. Some have evidence, while others are waiting for clear evidence through new research. Studies have suggested other genes to be included in this class, like; *NBN*, *NF1*, *BARD1*, *CASP*, *TGFβ1*, *FGFR2*, *MAP3K1*, *LSP1*, and *TNRC9* (Wendt and Margolin, 2019, Mahdavi and Nassiri, 2019).

2.6.2. Non-genetic risk factors of BC

Breast cancer is caused by non-hereditary factors without the involvement of germline mutations called sporadic breast cancer; these factors are responsible for about 80% of the cases, usually referred to as environmental factors. Non-genetic factors include numerous factors, including age and healthy states like hormonal state, early menarche, late menopause, lifestyle choices like exercise, body weight, diet, smoking, socioeconomic condition, and environmental factors like exposure to toxic materials, radiation, and air pollution (Wu *et al.*, 2018, Shi *et al.*, 2020).

2.7. Diagnosis methods of BC

There are several methods used to diagnose breast cancer, below are the methods and techniques used:

2.7.1. Mammography

This method is considered the gold standard for breast imaging. It is a type of X-ray-based technique that produces an x-ray image of the breast and surrounding tissues to detect any abnormalities or tumors in the breast. Perhaps the only limitations are the defects in detecting dense breast tissue. To overcome these limitations, ultrasound is used with mammography, which can be used for diagnosis purposes as well as screening purposes. According to the guidelines of the American Cancer Society (ACS), mammography is recommended for women aged 40 and older every year as a regular screening (Forrai *et al.*, 2022, Nicosia *et al.*, 2023).

2.7.2. Magnetic Resonance Imaging (MRI)

This method is used to get an image that shows the detailed structure of the breast by using low-energy radio waves in combination with a magnetic field. It is useful to detect the tumor's size and the metastatic status among those who are already diagnosed with BC; however, the American College of Radiology recommended MRI as a screening method for those who are at high risk for BC (Bhushan *et al.*, 2021). The MRI is helpful for identifying tumors of 2 cm or less (Azhdeh *et al.*, 2021).

2.7.3. Positron Emission Tomography (PET) Scanning

In the field of oncology, PET scans are considered an important scanning method for different types of cancer. It depends on the metabolic and/or biochemical function of the cells when using a radioactive drug (tracer). Several

types of PET scans have been developed, but the most widely used one is fluoro-D-glucose (FDG), which depends on the metabolism of glucose in cancer cells, which are characterized by a highly glucose-sensitive metabolism compared to normal cells (Kapoor and Kasi, 2024).

2.7.4. Computer aided Tomography (CT) scanning

A computed tomography scan (CT), which is also known as computed axial tomography scan CAT that developed in the 1970s. It is a technique that provides high-quality medical imaging, especially when contrast agent is used, for the detailed internal of the body. This technique produces images through X-ray in combination with computer technology that provides excellent details for all parts and tissue of the body. The limitation of this technique includes high radiation dose, use of radioactive substance, high cost, needs a professional technician to perform and interpretation of the results (Kauffman *et al.*, 2014, Schulz *et al.*, 2021).

2.7.5. Ultrasound

Ultrasound is considered a supplementary tool that combines with mammography to overcome its limitations for screening dense breast tissue and suspicious areas that are not seen by mammography. The advantages of ultrasound are its availability and the fact that no radiation is used, while disadvantages include failure in detecting microcalcifications and cases with early stages (Sood *et al.*, 2019, Dan *et al.*, 2023).

2.7.6. Breast Self-Examination (BSE)

This examination is not based on a device or instrument; it is based on self-observation of the breast through the naked eye and touching the breast to

observe any abnormalities of the breast shape, color, or any mass or tumor in the breast or the surrounding areas. The advantages include being costless, non-invasive, no physician or technician is needed; it can be carried anywhere and everywhere, while the disadvantage is the failure to detect the early stages of the cancer. BSE is recommended for all women regularly, but it doesn't replace the need for other trusted methods like mammography (Getu *et al.*, 2022, B and Kaphle, 2023).

2.8. Genetic Testing for Inherited BC

Genetic testing refers to those tests that aim to detect the genetic basis of breast cancer by detecting abnormalities in those genes that are related to this disease. Those tests vary among them in their techniques, numbers of detected genes, and even type of sampling. In most tests, blood is the first choice, while oral rinse or saliva also can be used depending on the test (Piccinin *et al.*, 2019, Litton *et al.*, 2019). Genetic tests may identify only 2 genes, 5 or 6 genes, 25 to 30 genes, hundreds of genes, or even thousands by one run through gene panel by next-generation gene sequencing (NGS) (Zelli *et al.*, 2020). Most genetic tests aim to identify genes with high penetrance, like *BRCA1* and *BRCA2*. If they are not diagnosed, the second class of moderate genes will be investigated, while identification of low-penetrance genes is not among routine tests as they may contribute to a minority of cases (De Silva *et al.*, 2019, Barili *et al.*, 2024).

Different techniques and tests are available for the detection of inherited breast cancer, including DNA sequencing, next-generation sequencing (NGS), DNA rearrangement by quantitative PCR, fluorescent PCR, deletion/duplication analysis, exon array CGH, and multiplex ligation-dependent probe (MLPA). These are the scientific names of the techniques, while different corresponding trade names differ according to the manufacturer companies. Among those tests,

NGS allows sequencing of many genes at once, that's why it is preferred (Lynch *et al.*, 2015, Kamps *et al.*, 2017).

Genetic tests can be divided into two stages, stage one, which includes diagnostic tests and stage two, which includes predictive tests. Stage one; if an individual has been already diagnosed with BC, a diagnostic test can be done through a full screening to find out the specific type of the mutation. Stage two depends on the result of stage one. If the altered gene is diagnosed by an individual, all family members can perform a predictive test to find whether they have the same type of mutation or not. Several factors play a role in selecting the suitable test, including personal history, family history, susceptibility to breast and/or other cancers, and ethnicity. Ethnic background affects the choice of the test; for instance, there are tests designed only for Ashkenazi Jews or Hispanics. Choices from many different options can be offered by a genetic counselor and/or physician (Manahan *et al.*, 2019).

2.9. Sequencing and Generations of Sequencing

Despite those efforts for understanding the sequences of the DNA that started in the fifties of the past century, the practical and real sequencing of the DNA dates back to 1977, when Frederick Sanger developed the first type named Sanger sequencing. Since that time, several developments and new sequencing techniques have appeared; although the aim and usage of these techniques differ, the main difference is the read length per run (Heather and Chain, 2016, Satam *et al.*, 2023). Generally sequencing is classified into four generations, they are:

1- **First generation**: Sanger sequencing.
2- **Second generation sequencing**: Pyrosequencing, Sequencing by Reversible Terminator Chemistry, and Sequencing by Ligation.

3- **Third generation sequencing**: Single Molecule Fluorescent Sequencing, Single Molecule Real Time Sequencing, Semiconductor Sequencing, Nanopore Sequencing.

4- **Fourth generation sequencing**: Conducting genomic analysis directly in the cell.

Types of the sequencing, year of evolutions, and their data output per read in Gigabytes (GB) are shown in the (Figure 2-6) below:

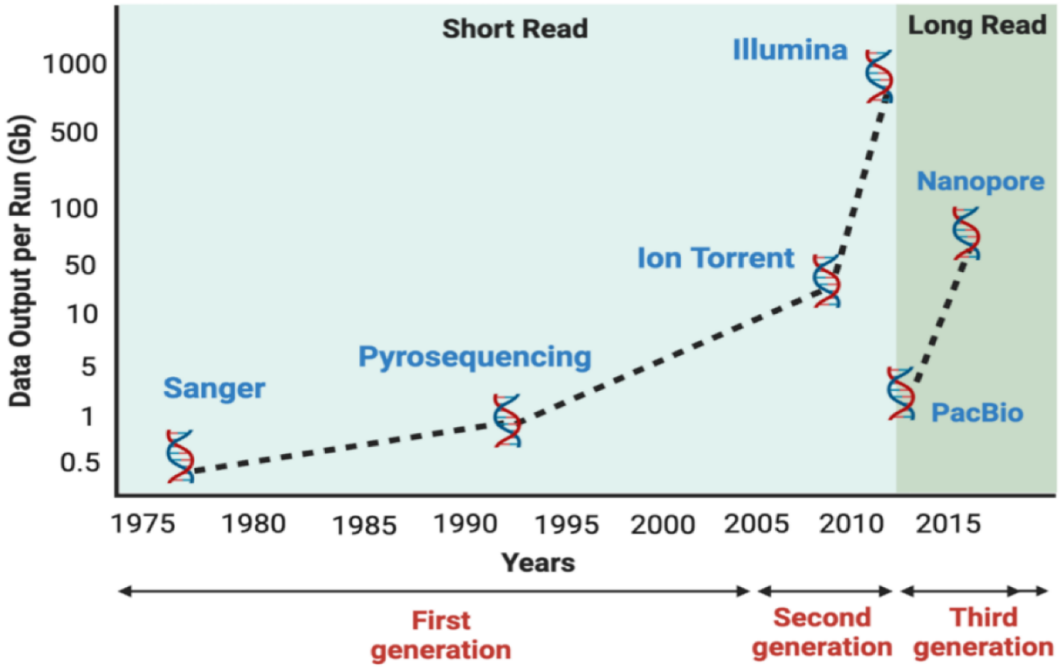

Fig. 2-6 The development of different Sequencing generations and their data output (Satam *et al.*, 2023).

2.9.1. Next Generation Sequencing (NGS)

After finishing the human genome project at the beginning of this century through sanger sequencing, soon after, approaches for the development of second-generation sequencing appeared by the related companies. Lynix Therapeutic Company, in 2000, launched the first NGS technology. Since that

time, several companies have developed other types of it by adding their own new methodologies (Durmaz *et al.*, 2015, Qin, 2019).

NGS is used for sequencing DNA and/or RNA and the detection of mutations (variants). Within a short period of time, NGS has the ability to sequence thousands of genes or even the whole genome, and its results are used for different purposes like the diagnosis of diseases, prognosis, therapeutic purposes, and research (Qin, 2019, Satam *et al.*, 2023).

2.9.1.1. Types of NGS

Since 2000 and the development of the first NGS technique, several other types of NGS have developed and become available, they are:

1- Lynx therapeutics' massively parallel signature sequencing (MPSS).
2- Polony sequencing.
3- Pyrosequencing.
4- Illumina (Solexa) Sequencing.
5- SOLiD sequencing.
6- DNA nanoball sequencing.
7- Helioscope single molecule sequencing.
8- Single molecule SMRT sequencing.
9- Single molecule real time (RNAP) sequencing.

Illumina (Solexa) Sequencing is the most popular NGS platform developed by Solexa and became available in 2006 as the Solexa Genome Analyzer, acquired later by Illumina. It uses sequence-by-syntheses approach (SBS), a special flow cell with an optical transparent slide provided with lanes, and a method based on reversible dye terminators on bridge amplification, in which the

primers are attached to the molecules of DNA and amplified on a specific slide using four different fluorescently labeled reversible terminators. Each time, one fluorescently labeled nucleotide is added to the DNA, and an image is taken before removing the dye from the DNA sequence to start the next cycle (Slatko *et al.*, 2018). Thousands of copies will be generated by each template, leading to millions of unique clusters on the flow cell. Also, it is a fast technique, MiSeq, which is one platform of illumina sequencing, can be carried out within 4 hours for bacterial samples (Gupta and Verma, 2019, Pervez *et al.*, 2022).

2.9.1.2. Different NGS (Illumina) Techniques, Platforms, and Subtypes

NGS could be used for different purposes, depending on the aim of the sequencing. There are three main techniques: whole genome sequencing (WGS), whole exome sequencing (WES), and targeted sequencing (gene panel) that covers hundreds (tens or thousands) of genes. It is worth mentioning that due to the development of the instruments and techniques in the last few years, the time and cost of NGS have reduced dramatically, fortunately, the WGS can be carried out within one to two days and for less than 1000 US dollars (Pei *et al.*, 2023, Satam *et al.*, 2023). Differences among those techniques are shown in the Table (2-3) below:

Table 2-3 Different aspects of NGS techniques (Satam *et al.*, 2023).

	Genome Sequencing	**Exome Sequencing**	**Targeted Gene Panel**
Coverage	All genes and non-coding DNA	Entire exome (20 to 25k genes)	10 to 500 or more genes
Accuracy	Low	Good	High
Cost	Most expensive	Cost-effective	Most cost-effective
Read depth	>30X	>50-100X	>500X

NGS Illumina sequencing offers different types of platforms and models, the use of these platforms depends on the purpose of the test. The main platforms are genomic sequencing, MiniSeq, MiSeq, NextSeq, HiSeq, HiSeqX, and other types. These models vary in their accuracy, read length, output/run, and applications (Cheng *et al.*, 2023). A comparison of the characteristics of these platforms is shown in Table (2-4).

Table 2-4 Characteristics of different NGS platforms (Cheng *et al.*, 2023).

	NGS platforms				
Characteristics	**MiniSeq**	**MiSeq**	**NextSeq**	**HiSeq**	**HiSeqX**
Read length	2 x 150bp	2 x 300bp	2 x 150bp	2 x 150bp	2 x 150bp
Maximum output/run (Gb)	7.5	15	120	1500	1800
Accuracy (%)	99.2	99.2	99.2	99.74	99.74

Regarding the applications of these platforms, MiniSeq is used for low-throughput targeted DNA or RNA sequencing, MiSeq is used for amplicon sequencing besides targeted DNA or RNA sequencing, NextSeq used for exome and transcriptome sequencing, HiSeq is used for large scale genome sequencing besides exome and transcriptome sequencing, and HiSeqX is used for large scale whole genome sequencing (WGS) (Cheng *et al.*, 2023).

2.9.1.3. The Workflow of NGS (Illumina)

The procedure of the NGS workflow differs depending on the type of NGS, the workflow for Illumina (Solexa) sequencing as an example includes four main steps: library preparation starting with DNA or RNA extraction, DNA library bridge amplification (library hybridizations and amplified clusters), DNA library sequencing (fluorescent labeling of the nucleotides, repeating the cycles

of the sequencing, and data collection), and finally, alignment and data analysis (Aastha Shrestha, 2024). All steps are shown in the diagram (Figure 2-7).

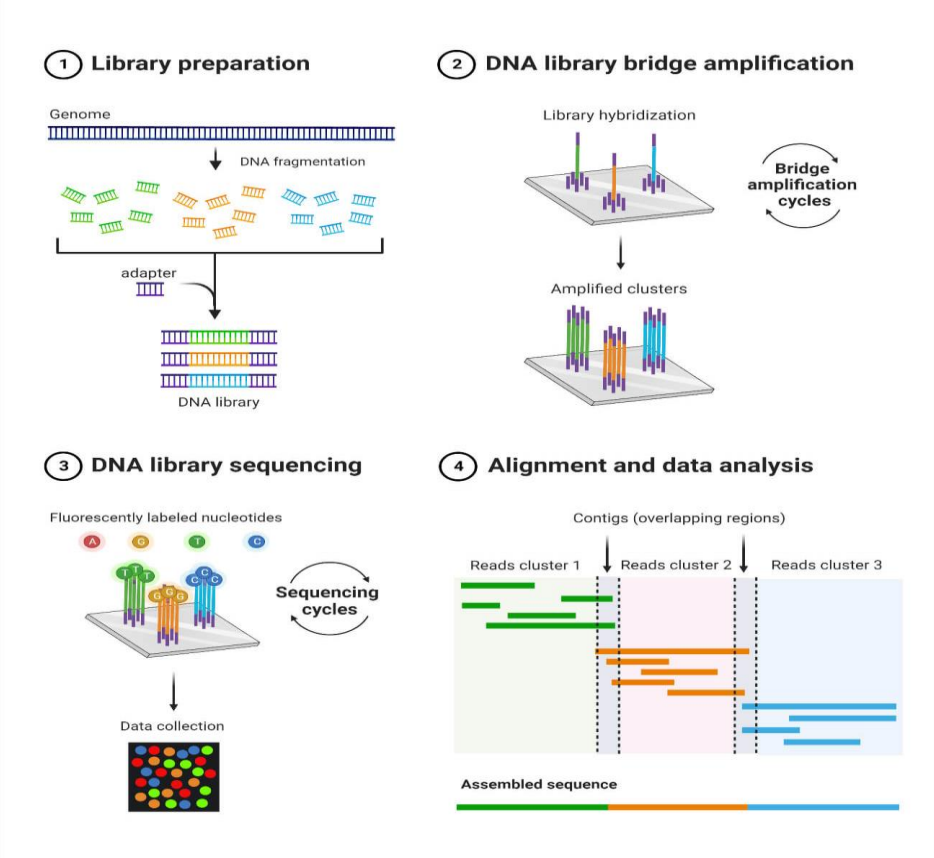

Fig. 2-7 The workflow and steps of NGS by Illumina sequencing (Aastha Shrestha, 2024).

2.9.1.4. Bioinformatic Approaches for Data Analysis of NGS

Different tools are used for the analysis of the NGS data depending on the sequence based omics and types of common analysis and genomics (WGS, WES, Targeted Panel). These tools are used for sequencing, adapting, quality contril, trimming, ,alignment, visualizing, removing duplicated reads, variant calling, filtering, annotating, and other functions (Kanzi *et al.*, 2020). The common tools are listed in the Table (2-5) below:

Table 2-5 Bioinformatic steps and commonly used tools for data analysis of NGS (Kanzi *et al.*, 2020).

Bioinformatic steps (Analysis)	Tools
Sequences quality checking	FastQC, FASTX-toolkit, MultiQC.
Adaptors trimming and low qualtiy bases	Trimmomatic, Cutadapt, fastp.
Sequence read alignment to the refrence genome	BWA, Bowtie, dragMAP.
Visualization of the reports	MultiQC.
Duplicated reads removal	Picard, Sambamba.
Variant Calling (SNP)	GATK, freeBayes, Platypus, VarScan, DeepVariant, Illumina Dragen.
Variants filtering and merging	Bcftools.
Variant annotation	ANNOVAR, ensembleVEP, snpEff, NIRVANA.
Structural Variant Calling	DELLY, Lumpy, Manta, GRIDDS, Wham, Pindel.
Copy Number Variation Calling (CNV)	CNVnator, GATK gCNV, cn.MOPS, cnvCaoppSeq, ExomeDepth.

Abbreviations: ANNOVAR—ANNOtate VARiation; BWA—Burrows Wheeler Aligner; cn.mops Copy Number Estimation by a Mixture Of PoissonS; Ensembl VEP—Ensembl Variant Effect Predictor; Fastp—Fsatq Preprocessor; GATK—Genome Analysis Tool Kit.

2.9.1.5. Results of the NGS

Cluster generation and sequencing by synthesis (SBS), used by Illumina sequencing technology for sequencing the clusters on the flow cell, sequences a huge number of clusters that could be millions or even billions. For each cycle of sequencing, a base call will be produced and stored by the RTA software. The base call data will be stored as BCL or individual base call. Later, at the end of the sequencing, these BCL file formats converted FASTQ sequence data. For each sample, a specific FASTQ file must be created with \. fastq.gz. extension.

After creating the FASTQ files, they will be generated through MiSeq Reporter on Miseq and local run manager on MiniSeq, and at the end of the analysis, the generated files could be obtained in \Data\intensities\BaseCalls on the Miseq, \alignment_#<subfolder>\Fastq on the MiniSeq (Aastha Shrestha, 2024).

The sequencing outcomes of the NGS are obtained as FastQ files, a text file that contains the sequence data from the clusters that pass filter on a flow cell. These files then undergo alignment reads through converting tools like (Samtools) to get Binary Alignment Map (BAM) files. Finally, the BAM files will converted through variant calling by using tools like GATK to the simplest file type called Variant Call Format (VCF) files that identify variants with colors, as shown in (Figure 2-8) (Torri *et al.*, 2012, Lan *et al.*, 2023).

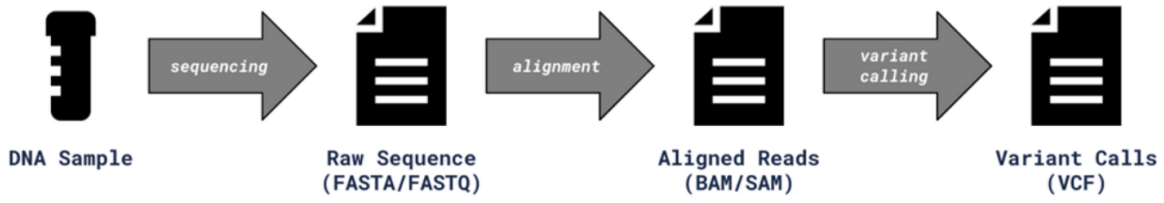

Fig. 2-8 How to obtain results of NGS (Ian Maurer, 2020).

2.9.1.6. Viewing the results of NGS and detection of the variants

Different software and tools are used for visualizing the outcomes of the NGS test, the used software differs depending on the sample and NGS type. Integrative Genomics Viewer (IGV), Next-Generation Clustered Heat Map (NG-CHM) Viewer are examples for visualizing the sequenced samples (Robinson *et al.*, 2017). Variant detection can be carried out manually through IGV or automatically detecting all the changes (variants) through using applications and software like MutationTaster, Franklin by genoox, and QIAGEN QCI Interpret

Translational. Manual interpretation is carried out by viewing the variant, what changed, and the exact location of the variant, then going to databases like; NCBI/ClinVar, gnomAD, COSMIC, and Ensemble, then finding the exact variant in the database that matches the detected variant with the exact location and amino acid change. Automated variant detection and analysis are carried out by uploading the VCF or BAM files depending on the software and selecting some options that match your exact work and information, then clicking on the analyze or submit button. It will automatically detect all the variants with their interpretation of the clinical significance (Robinson *et al.*, 2017, Gall *et al.*, 2022, Rodrigues *et al.*, 2022).

2.9.1.7. Variants classifications and interpretations

There are different categories and criteria for the variant classification regarding their clinical significance. The most well-known guidelines used are those provided by the American College of Medical Genetics and Genomics (ACMG), the Association for Molecular Pathology (AMP), GeneDX General Variant Classification Assertion Criteria, Ambry Genetics Variant Classification Scheme, Sherloc, and the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA consortium), but, in 2024, ENIGMA no more working for variant classifications. Most databases, like NCBI/ClinVar, Ensemble, Franklin by Genoox, Brcaexchange, Genome Aggregation Database (gnomAD), and Catalogue of Somatic Mutations in Cancer (COSMIC), depend on these guidelines (Stecker *et al.*, 2020, Masson *et al.*, 2022).

Regarding the variant nomenclature, the Human Genome Variation Society (HGVS) established the standards for the nomenclature of the gene variants (Richards *et al.*, 2015). Generally, it depends on the type of mutation and the effect of that mutation (variant consequences) carried out by in silico

bioinformatic tools like: Sorting Intolerant From Tolerant and Polymorphism Phenotyping (SIFT/PolyPhen) tools to predict aminoacidic and protein changes (Garcia *et al.*, 2022). The clinical significance of the variants is classified into the following types: benign, likely benign, conflict interpretation of pathogenicity, variants of uncertain significance (VUS), likely pathogenic, and pathogenic variants (Walsh *et al.*, 2024).

It is worth mentioning that there are differences among the databases regarding variant interpretations. Regarding *BRCA1*, for example, during the past decade, the interpretations of its variants were 70% similar among the different laboratories, most of these differences belonged to conflict variants (variants of conflict interpretation). Recently due to collaborations between different databases and laboratories, the differences have been reduced to about 10%. Differences among different databases for variant interpretation are problematic and confusing for physicians and genetic counselors (Hovland *et al.*, 2022, Schmid *et al.*, 2022, Ahmad *et al.*, 2023).

2.9.1.8. Advantages and Disadvantages of NGS (Illumina)

Next generation sequencing has several great advantages in different fields of biology, medicine, and research. Recently, due to the huge progress, it became possible to sequence one terabase of the data within one day. Having a high accuracy, the Illumina platform, as an example, exceeded 99% and enabled the running of 96 per time. While the main disadvantages include, substitution errors occur resulting from the noise background in each cycle of sequencing, scars remain on the nucleotide structure after the cleavage of the blocking groups that interact with proteins, leading to decreased the efficiency of sequencing reactions (Ari and Arıkan, 2016, Satam *et al.*, 2023).

2.10. Age and Stage of the Women at Time of Diagnosis with BC

Age is considered one of the risk factors for acquiring breast cancer, and by increasing the age, the risk of getting this cancer increases (Łukasiewicz *et al.*, 2021). Women's age at the time of diagnosis with this cancer varies among different populations, ethnicities, and countries. In low- and middle-income countries, women are diagnosed at earlier ages in comparison with Western and high-income countries (Bidoli *et al.*, 2019, Lemij *et al.*, 2022). Such differences resulted from different factors like exposure to environmental risk factors, the mean age of the population, lifestyle, health awareness, and genetic factors related to mutations in high-penetrance genes related to breast cancer (Francies *et al.*, 2020, Kashyap *et al.*, 2022).

Cancer stage refers to the state of the cancer based on the tumor size and status; smaller tumors indicate early stages, while larger and more spread tumors indicate more advanced stages. Cancer statues are categorized into stages or grades; for the staging category, there are four stages: I, II, III, and IV (Berek *et al.*, 2023). Cancer's stage at the time of diagnosis is very important; it plays a crucial role in the survival rate, prognostic factor, and minimizing the consequences of the disease. Treating strategies in the early stages is easier than in the advanced stages (Roche *et al.*, 2017, Ding *et al.*, 2022, Alkazaz *et al.*, 2024). In low- and middle-income countries, women are at higher risk of being diagnosed with advanced stages of this cancer compared to high-income countries. Routine screening, health awareness, socioeconomic status, and lifestyle are among the main causes of such differences (Gutnik *et al.*, 2016, Lim *et al.*, 2022, Koçak and Çiçek Gümüş, 2023).

2.11. Level of Awareness and Screening Practices among BC Patients

Having previous knowledge regarding breast cancer is very important and has a key role in the early diagnosis of the disease (Almeshari *et al.*, 2023). Generally, levels of awareness and sufficient knowledge differ among different countries and societies. Women in low- and middle-income countries have less awareness and knowledge regarding this issue (Liu *et al.*, 2018, Manson and Achel, 2023). Several factors, like education, socioeconomic status, health care levels, and geographical distribution, contribute to the level of awareness among women in different countries and populations (Liu *et al.*, 2018).

There are different methods that can be used for screening breast cancer, like breast self-examination (BSE), sonar, and mammography. The simplest way is BSE that women can perform it at home at any time and it costs nothing, women should be trained well to be able to do it regularly (Lera *et al.*, 2020, Apatić and Lovrić, 2023). But it should be mentioned that BSE is not able to detect cancer at its preliminary stages. That's why sonar and mammography techniques are highly recommended for their ability to detect this cancer even at its first stage (Huang *et al.*, 2022, Steyerova and Burgetova, 2021). Detecting breast cancer for the first time could be done either by the patient or by physicians and health care workers. Self-detection of cancer is popular, especially in low- and middle-income countries where there are poor screening practices for early detection (Albeshan *et al.*, 2020).

2.12. Psychological Impacts of BC

Up to 50% of the patients with BC suffer from different psychological consequences, including short-term and long-term impacts. Perhaps stress and depression are the main side effects. Several reasons may contribute to having psychological problems among BC patients, like having a disease named cancer,

side effects of the treatments (especially chemotherapy), physical symptoms among mastectomies, becoming worried about the recurrence (Álvarez-Pardo *et al.*, 2023). Emotional support from family members, partners, and friends plays an important role in reducing the psychological impacts of BC among the patients (Calhoun *et al.*, 2022).

2.13. Treatment of BC

Treatment strategies and the selection of treatment types depend mainly on the type of breast cancer, stage, and status of the tumor, in which advanced stages and metastatic tumors require more advanced treatment strategies (Moo *et al.*, 2018). Different medications and drugs available for breast cancer, some of them aim to prevent, like: Evista and Soltamox, while other used for the treatment, among them; Taxol (Paclitaxel), Cisplatin, Epirubicin, Xeloda (Capecitabine), Cyclophosphamide, Carboplatin, Abemaciclib, Abraxane, Epirubicin, and several others. The American Cancer Society (ACS) classified the treatment into local and systematic treatments. Local treatment refers to treating the tumor without affecting other parts of the body, which is carried out by surgery or radiation, while systematic treatment is carried out by using drugs including chemotherapy, hormonal therapy, targeted drug therapy, and immunotherapy (Miller *et al.*, 2022).

2.14. Preventive Steps and Strategies

If an individual has been detected with an altered gene that is linked to breast cancer, the individual and even the same family members can take some steps to minimize the risk of developing it. Preventive steps can be divided into primary and secondary ones. Steps like a healthy lifestyle and environmental factors are among the primary preventive measures that every woman may also

consider, while the secondary ones include more advanced steps that women who are at high risk must take. Both stages, steps, and their descriptions are mentioned in Table (2-6) (Kolak *et al.*, 2017, Costa and Saldanha, 2017).

Table 2-6 Protective steps to minimize breast cancer for women with abnormal breast cancer gene (Sun *et al.*, 2017b).

Prevention stage	**steps**	**Description**
Primary	lifestyle choices	Healthy weight and healthy food, physical exercise, reducing alcohol consumption and never smoking.
	Environmental factors	Environmental carcinogens like exposure to pesticides, radiation, and toxic materials.
	screening	Every woman must perform it, even by self-exam.
Secondary	More frequent screening	Women who are at higher risk should perform screening regularly, before 30 years or even younger. Screening plan, besides self-examining monthly, a digital mammogram and an MRI scan must be done every year.
	chemoprevention	Hormonal therapy medicines can help women at high risk through reducing the risk of developing hormone-receptor-positive breast cancer.
	Protective surgery	Also called prophylactic surgery, it is the process of removing all the tissues of the healthy breasts and ovaries from women with mutated *BRCA genes* through a protective surgery that may reduce the risk as much as 97%.

Prophylactic surgery offers a better survival rate for those women who have abnormal genes, but it is a very aggressive, difficult-to-decide, and

irreversible risk-reduction option. Taking such a decision is very complex, because it requires a great deal of thought, patience, and a full discussion with experts and their families over time (Macadam *et al.*, 2021). It's important to know that nothing will eliminate the risk of BC; even after prophylactic surgery, there will still be a small risk that cancer can arise in the areas where the breasts used to be. That's why close follow-up is necessary. Women, especially those with high-risk factors and abnormal inherited genes, must take preventive steps that help in minimize or avoid breast cancer (Alaofi *et al.*, 2018).

Finally, even though breast cancer cannot be eliminated totally, there are strategies that can reduce or prevent its occurrence. Prevention steps and early diagnosis may have a key role in fighting against BC and significantly contribute to reducing its incidence. Having greater awareness and modifying behavior are among the primary steps that every woman must take into consideration. While women who have a higher susceptibility to the disease must undergo more advanced steps, like more frequent screening, chemoprevention, and sometimes prophylactic surgery for those who have a very high risk (Mina *et al.*, 2016, Kolak *et al.*, 2017).

3. MATERIALS AND METHODS

3.1. Equipment and Devices

Table 3-1 List of the Equipment and Tools Used in the Present Study.

Item	Company	Origin
Autoclave	Daikyo	Japan
Bio-Imaging System	ER Biyotek	Mexico
BIO-RAD T100 Thermal Cycler	BIO-RAD	U.S.A.
Centrifuge	biosan	Latvia
Class 2 Safety Cabinet	metisafe	Türkiye
Electrophoresis System (PowerPac Basic)	BIO-RAD	U.S.A.
Electrophoresis Tank	Thermoscientific	U.S.A.
ETDA Tube K2 (vol.3ml)	VACUTEST, Kima	Italy
Fast Thermal Cycler	LongGene	China
Freezer	UGUR	Türkiye
Gloves	Medline	Spain
Heating/Cooling Dry Block	biosan	Latvia
Ice box	Tank	Egypt
Ice pack	O'meara camping	Ireland
Micropipette	Eppendorf	U.S.A.
Micropipette (AXYPET)	Cultek	Spain
Micropipette (BioPette Plus)	Labnet	U.S.A.
Micropipette Tips	NITRILO	Spain
Micropipette Tips	Skgmed	China
Mini Centrifuge	Hettich GmbH & Co. KG	Germany
MiSeq	illumina	U.S.A.
Miseq Flow Cell	illumina	U.S.A.
NanoDrop Spectrophotometer	Thermoscientific	Singapore
NucleoFast® 96 PCR kit	Macherey-Nagel GmbH & Co.	Germany
PCR-tube 0.2 ml	Axygen	U.S.A.
PCR-tube 2ml	Axygen	U.S.A.
Qubit Flex Fluorometer	Thermo Fisher Scientific	U.S.A.
Refrigerator	Delcon	Italy
Sensitive Electronic Balance	VWR life science	Italy
Thermocycler	BIO-RAD	U.S.A.
Vortex	biosan	Latvia

3.2. Kits and Reagents

Table 3-2 List of the Chemicals, Kits and Reagents Used in the Present Study

Item	Company	Origin
5X Phire Reaction Buffer	ThermoFisher Scientific	U.S.A.
Absolute Ethanol	VWR life science	Italy
Agarose	VWR life science	Italy
Bead-Linked Transposome (BLT)	illumina	U.S.A.
Deionize Sterile Distilled Water (dH_2O)	VWR life science	Italy
Dimethyl sulfoxide (DMSO) 100%	ThermoFisher Scientific	U.S.A.
DNA Ladder 100 bp	Thermoscientific	Lithuania
DNA Polymerase (Hot FIREPol)	SOLIS BIODYNE	Estonia
DNA Polymerase MyTaq	Bioline-Meridian	Germany
dNTP buffer	Bioline-Meridian	Germany
HiPure Blood DNA Mini Kit	Megan Biotech Co., Ltd.	China
High prepTM PCR	MAGBIO	U.S.A.
High Sensitivity Dye (HS)	ThermoFisher Scientific	U.S.A.
Hot Start II DNA Polymerase	ThermoFisher Scientific	U.S.A.
Hyb Buffer	illumina	U.S.A.
Loading Dye	VWR life science	Italy
PCR Master Mix	ThermoFisher Scientific	U.S.A.
Primers	INTERGEN	Türkiye
Proteinase k Solution	Megan Biotech Co., Ltd.	China
Qubit dsDNA BR Buffer	ThermoFisher Scientific	U.S.A.
Qubit dsDNA Reagent 200X	ThermoFisher Scientific	U.S.A.
Reaction Buffer (MyTaq 5X)	BIOLINE	Germany
Safe Stain	Sentebiolab	Türkiye
Sterile Distilled Water	POLIFARMA	Türkiye
Tagmentation Buffer 1X (B1)	Illumina, Inc	U.S.A.
Tris/Borate/EDTA	VWR life science	Italy
Water, nuclease-free	ThermoFisher Scientific	U.S.A.

3.3. Methodology

3.3.1. Study Design

The present study is based on a cross-sectional study that based on blood sample collection and filling a structured questionnaire to investigate parameters related to the aim of the study. The overall study design of the present research is summarized in the following diagram (Figure 3-1):

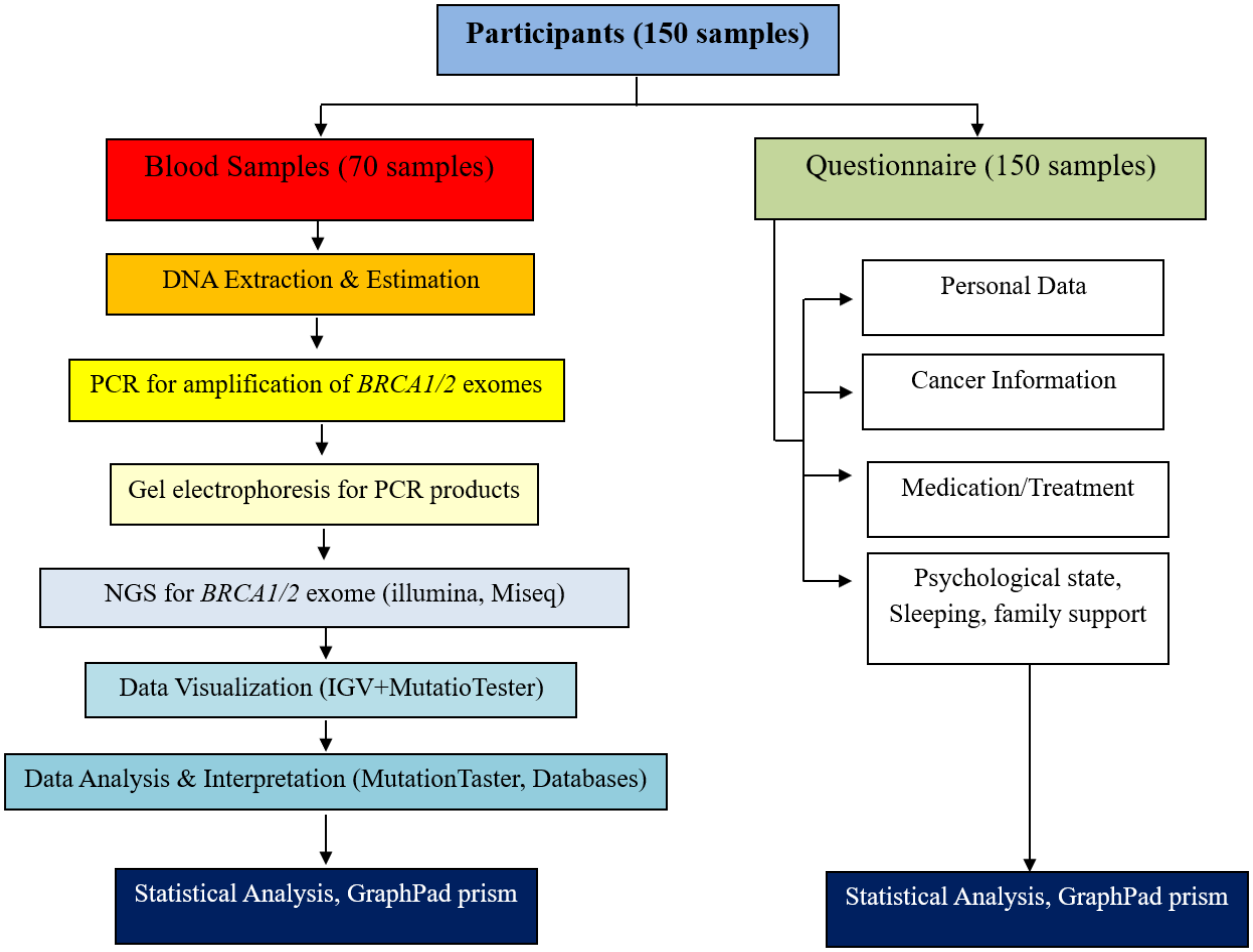

Fig. 3-1 Diagram showing the study design of the present study.

3.3.2. Questionnaire Form

A special questionnaire was designed for this research; it included so many questions that were relevant to the study. The questionnaire was divided into four sections: personal information, cancer information, medications and treatments, and finally psychological and sleep habits, as shown in Appendix 1. Before taking blood samples, the patients, during their periodical visit for

clinical examination, were asked to fill out the questionnaire form. The questionnaire was filled out through a direct interview with the patients.

3.3.3. Patients and Sample Collection

A total of 150 samples that were diagnosed with breast cancer and registered at Nanakali Hospital for Blood Diseases and Cancer, Erbil, Iraq, were included. Sample collection and practical work carried out from March 2022 to September 2023. For NGS, 70 samples were selected, about 3 milliliters of their blood samples were collected in a new sterile ethylenediaminetetraacetic acid (EDTA) tube. The whole blood samples were preserved by freezing until further analysis. All participants were given informed consent, and after achieving their agreements, they were included as samples in accordance with the Helsinki Declaration.

3.3.4. Genomic DNA Extraction

Genomic DNAs were obtained from their blood samples with the isolation of 200 µl blood samples from each participant by using the HiPure Blood DNA Mini Kit (Magen, China) and following the manufacturer's instructions according to the following steps:

3.3.4.1. Protocol for Blood

1. Pipet 20 µl of Proteinase K into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 µl of sample to the microcentrifuge tube. Use up to 200 µl of whole blood, plasma, serum, buffy coat, or body fluids, or up to 5 x 10^6 lymphocytes in 200 µl of PBS.
3. Add 200 µl of buffer AL (lysis solution) to the sample. Mix by pulse-vortexing for 15 seconds.
4. Incubate at 70 °C for 10 min.

5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 µl of ethanol (96–100%) to the sample and mix again by pulse-vortexing for 15 seconds.

7. Insert a HiPure DNA Mini Column I into a 2 mL collection tube (provided).

8. Carefully apply the mixture from step 6 to the column without wetting the rim. Close the cap, and centrifuge at 10,000 x g for 1 min.

9. Add 500 µl of Buffer DW1 without wetting the rim. Close the cap and centrifuge at 10000 x g for 1 min.

10. Discard the flow through and reuse the collection tubes. Add 650 µl of buffer GW2 without wetting the rim. Close the cap and centrifuge at 10000 x g for 1 min.

11. Discard the flow-through and reuse the collection tubes. Centrifuge at 10000 x g for 1 min. This step helps to eliminate the chance of possible buffer GW2 carryover.

12. Place the column in a clean 1.5 ml microcentrifuge tube (not provided) and discard the collection tube containing the filtrate. Add 50~200 µl of buffer AE or distilled water. Incubate at room temperature for 2 min, and then centrifuge at 10000 x g for 1 min.

3.3.5. Estimation of the Extracted Genetic Materials

Qualification and quantification of the extracted DNA were performed using NanoDrop (Thermo Scientific, Multiskan Sky-1530, Singapore), following the manufacturer's instructions. Samples of genomic DNA with (A260/A280) ratios greater than 1.7 and outputs greater than 40 ng/µl were obtained.

3.3.6. *BRCA1* and *BRCA2* protocol

Primers were used for the coding regions (exons and the boundary intronic regions) of these two genes. There were 22 primers for the amplification of the *BRCA1* gene and 28 primers for the *BRCA2* gene at INTERGEN (Genetics and Rare Diseases Diagnosis Research & Application Center), Ankara, Türkiye. Sequences of the primers weren't mentioned due to copyright issues at the INTERGEN Center. The PCR reaction mixture and PCR conditions and cycles are shown in Table (3-3) and (3-4) below.

Table 3-3 The PCR reaction mixture.

Contents	Volume (µl)
dH_20	18,3
dNTP containing 10X Buffer (Bioline-Meridian)	2,5
Forward Primer (5 µM)	1
Reverse Primer (5 µM)	1
DNA Polymerase (MyTaq Bioline-Meridian)	0,2
DNA	2
Total	**25 µl**

Table 3-4 Thermocycler program of PCR reactions.

Step	Temp. (^{0}C)	Time (min)	Cycle
Initial denaturation	95	10:00	1
Denaturation	95	00:45	45
Annealing	60	00:45	
Extension	72	00:45	
Final extension	72	10:00	1
Holding	12	∞	1

PCRs were carried out on isolated DNA samples by using designed primers, and the reactions (amplicons) were checked by using (2%) agarose gel electrophoresis. PCRs belonging to each participant were mixed to obtain PCR pools, which have all the amplicons of each participant in one tube. While mixing, the amplification efficiency and length of the amplicons were taken into consideration; the volume and time for each PCR is directly proportional to the length of the amplicon and inversely proportional to the efficiency of the reaction, which was estimated with the help of gel electrophoresis.

The PCR pools for each participant were purified using the NucleoFast® 96 PCR kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The purified pools were quantified and standardized to 0.2 ng/ul, which was needed for the sample preparation step. The samples were prepared ready for next-gen sequencing by using the NexteraXT sample preparation kit (Illumina Inc.). Next-gen sequencing of the samples was carried out using the Miseq system (Illumina Inc., San Diego, CA).

3.3.6.1. Nextera DNA sample preparation workflow for Miseq sequencing

3.3.6.1.1. Tagmentation Mixture (volume 1X)

Preparation needs the following reagents: Tagmentation Buffer 1X (B1.3), deionized water (dH_2O), and Bead-Linked Transposome (BLT).

- In a 0.2 Eppendorf tube, add 12.5 µl of B1.3 buffer.
- Add 7.4 µl of dH_2O.
- Add 0.1 µl of the BLT enzyme.
- The total volume is 20 µl/sample; vortex for 5 seconds (optional).

3.3.6.1.2. Nextera PCR Mix (NPM Mix)

Preparations need the following: 5X Buffer, dNTP, Dimethyl Sulfoxide DMSO, dH2O, Phinell enzyme.

- Add 10 µl of 5X Buffer. (added for activation of the Phinell enzyme).
- Add 0.5 µl of dNTP.
- Add 2 µl of DMSO. (used as Boxer).
- Add 6.5 µl of dH_2O.
- Add 1 µl of Phinell enzyme. (acts as Taq polymerase)
- The total volume will be 20 µl, then vortex for a few seconds to ensure well mixing.

After NPM Mix preparation, add 5 µl of the primers (2.5 µl of the forward primer and 2.5 µl of the reverse primers). These primers are not used to amplify any gene or sequence; they are used as signals or indicators for analyzing the alignment for Miseq.

Until the sample is processed, the Tagmentation mixture and NPM Mix should be stored at (4°C) in the refrigerator for the preservation of the enzymes.

3.3.6.1.3. Concentration measurement of the amplified samples

Amplified samples were measured using the Qubit™ Flex fluorometer (Invitrogen by ThermoFisher Science) following the below steps:

- For each sample, add 200 µl of Qubit dsDNA broad range buffer (BR).
- Add 1 µl of broad-range dye (Qubit dye).
- Add the X200 Qubit dsDNA reagent.
- 189 µl of the above mixture was added to a new 0.5 ml Eppendorf tube.
- Add 2 µl of the sample to the new tube.
- Vortex the tube for a few seconds.

Note: The above tube and mixture will be discharged after measuring the concentration by Qubit Flex because dye and buffers used cannot be used for the next steps.

Now, the tube became ready to be measured:

1. Blank the device.
2. Transfer the sample to its specific place in the device.
3. Select the right options: (dsDNA: Broad range).
4. Make calculations by tapping the (Export) bottom.

The obtained results from Qubit Flex were used for calculating the amount of dH_2O (or elusion buffer) that must be added and required for the next step, with calculations made as follows Table (3-5):

Table 3-5 Calculation of the dH_2O amount (or elusion buffer).

Obtained results	**Amount of the sample**	**dH_2O amount (or elusion buffer)**	**Final Equation**
Enter the result obtained from Qubit Flex here	Already known (5 µl)	This value calculated	m1v1=m2v2

m1: First calculated

v1: already obtained (5µl)

m2: 0.3 ng/µl

v2: obtained from the previous calculation

3.3.6.1.4. Sample preparation for limited-cycle PCR (second PCR)

After calculations, the following steps were carried out to perform the second PCR before sequencing:

- The obtained result (v2) represents the amount of dH_2O (or elusion buffer) that will be added to a new 0.2 ml Eppendorf tube.
- Add 5 µl of the sample (PCR product).
- Vortex for a few seconds.
- Perform a dilution of 10X by adding 45 µl of dH_2O. (Dilution carried out two times, first by adding the water amount obtained from previous calculations and second by diluting with 10X to make each sample 0.3 ng/µl.).
- Vortex for a few seconds.
- Transfer 5 µl of each sample to the tube of the tagmentation mixture that has already been prepared.
- Incubate for 10 minutes at 55°C. (Incubation can be carried out in an incubator or using the thermocycler as incubator).
- Mix the obtained solution with the NPM mixture tube. The obtained mixture that contains the tagmentation mixture, NPM Mix, and DNA sample, is ready for the next PCR.
- Transfer the 0.2 Eppendorf tube into the thermocycler (Biorad T100 thermocycler). By applying 13 cycles of the following conditions: initial denaturation 95°C for 1:30 minutes, denaturation 95°C for 45 seconds, annealing 67°C for 45 seconds, extension 75°C for 45 seconds, and final extension 72°C for 5 minutes.
- The amplified product is ready to be processed for Miseq sequencing.

3.3.6.1.5. PCR clean-up

The obtained products of the second PCR must be washed and purified by using the washing machine NucleoFast® 96 PCR kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The tubes are transferred into the machine, then

dH2O is added to the wells of the machine, starting the machine, which took 60 minutes to finish.

3.3.6.1.6. Concentration measurement for the second time

After obtaining the second PCR product, measurements of the concentrations for the PCR product were carried out again using the Qubit™ Flex fluorometer (Invitrogen by ThermoFisher Science) following the below steps:

- Add 2 µl of the second PCR product to a new 0.2 Eppendorf tube.
- Add 190 µl of Qubit™ dsH_2O.
- Add 1 µl of the highly sensitive dye (HS).
- Vortex for a few seconds.
- Centrifuge for a while.
- Transfer the tube to the Qubit Flex fluorometer to measure the concentration of the amplified samples using the High Sensitivity (HS) program option. And obtain the results.

3.3.6.1.7. Sample processing for Miseq sequencing

The Miseq kit that was preserved at -20°C transferred to a water bath at room temperature for 1-2 hours for thawing.

The concentrations obtained from the second measurement by Qubit Flex were entered into an Excel sheet to calculate how many microliters (µl) should be taken to the kit (usually 3 µl used). At this point, several samples can be mixed and added to the same Miseq well to be run together, then they can be separated again. Usually, up to 24 samples run together to minimize the cost and time.

Before running the samples on Miseq, they must be purified. The purification is carried out by magnetic beads for DNA purification through the following steps:

- Add 30 µl of (High prep^{TM} PCR 250 ml, MAGBIO) to the tube that contained the second PCR product.
- Calculate the volume of samples used: total amount of the sample multiplied by 1.8 magnetic beads, 30/samples x 1.8 = 54 µl.
- Wait for 1-2 minutes.
- Transfer the tube into the magnetic spreader rack and wait for 1 minute.
- After this period, the samples stick to the sides of the Eppendorf tube.
- Discharge the excess water that remained in the center of the tube.
- Add 200 µl of 80% ethanol to each sample.
- Remove the excess ethanol by using a micropipette.
- Repeat, adding 200 µl of 80% ethanol.
- Remove the excess ethanol by using a micropipette.
- Leave the tube open for 20 minutes to let the remaining ethanol evaporate.
- Add 30 µl of elusion buffer (or dH_2O).
- Mix gently using a micropipette tip, then wait for 2–3 minutes.
- Transfer the tube to a magnetic spreader for 30 seconds, until the supernatant appears precipitate unwanted.
- Transfer the supernatant to a new 0.2 ml Eppendorf tube.
- Measure the DNA concentration again using the Qubit Flex fluorometer, to calculate the µl of the samples. (Note: if the DNA concentration is low, free-RNase water can be added to increase the DNA concentration, then calculate the dilution percentage that must take into consideration).

3.3.6.1.8. Loading the samples on the flow cell and running the Miseq illumina (library pooling for Miseq sequencing)

Before loading the samples, they must be treated with the following steps:

- Add the calculated volume (from the previous step) to a new Eppendorf tube.
- Add 1000 µl of hybridization solution (Hyb buffer) illumina.
- Vortex for a few seconds.
- Add 2 µl of sodium hydroxide (NaOH).
- Incubate the tube at 96°C for 5 minutes using the heater block.
- Transfer the tube into an ice container to enable ice shock for 5 minutes or more.
- Now, the samples are ready to be loaded on the Miseq Kit.

Then, prepare the flow cell that was preserved at 4°C in the refrigerator and transfer the whole tube containing the samples into well number 21 in the illumina kit. Change the dH_2O bottle and remove the washing kit that was used prior to the run. Put the new flow cell in its place in the Miseq Illumina device. Select the appropriate running program and enter the required details. Wait till the device becomes ready, and then start by tapping the run button, the running time took about 26 hours.

3.3.7. Base calling, Quality Control and Trimming

The sequencer output (zipped-bcl files) underwent demultiplexing through bcl2fastq to obtain raw fastq files. The raw fastq files quality controlled, the quality checking of the raw sequencing carried out with FastQC tool. Adaptors trimming, and low-quality bases removed by trimmomatic tool.

3.3.8. Miseq Alignment and Read (pipeline step)

Raw reads were aligned to hg19/hg37 using the Burrow-Wheeler Aligner (BWA-mem 0.7.17) (Li and Durbin, 2010). Sorting, duplicate marking, and base recalibration steps were performed subsequently by Genome Analysis Toolkit 4 (GATK4) (Van der Auwera *et al.*, 2013). Variant Calls were made using two separate algorithms. GATK UnifiedGenotyper and GATK HaplotypeCaller were both used to complement each other (Van der Auwera *et al.*, 2013). Low-quality variants from both sets were eliminated based on strand bias, read depth, and call quality parameters using the GATK SelectVariants option (Van der Auwera *et al.*, 2013).

3.3.9. Mutation Visualization, Interpretation and Analysis

The data were visualized and read using Integrative Genomics Viewer (IGV 2.3 software, Broad Institute), the whole exome was analyzed, and each detected change (variant), was interpreted for its clinical significance using the following databases: the National Center for Biotechnology Information / public archive of interpretations of clinically relevant variants (NCBI/ClinVar) (https://www.ncbi.nlm.nih.gov/clinvar/), and the website of BRCA Exchange (https://brcaexchange.org/), which integrated with an international expert panel, the Evidence-Based Network for the Interpretation of Germline Mutant Allele (ENIGMA) consortium. Mutations with pathogenic, conflict interpretations of pathogenicity, and uncertain significance were assessed for the prediction of possible damaging effects using the MutationTaster changelog 2021 (https://www.genecascade.org/MutationTaster2021/).

For the *BRCA1* gene (NM_007294.4) and for the *BRCA2* gene (NM_000059.3, NM_000059.4) were used as reference sequences from the NCBI database (http://www.ncbi.nlm.nih.gov).

3.3.10. Other Data by Questionnaire

Several other data were collected through a specially designed questionnaire; the form was filled out through a direct interview with the 150 participants after obtaining their agreement to participate in the present study.

3.3.10.1. Personal information

This section included questions about gender, age, marriage status, having children and number of children, inhabitant, education, economic status, job, and workplace.

- Age groups were divided into age at the time of data collection and age at the time of diagnosis with breast cancer. Each category is subdivided into six classes, each class includes a ten-years interval starting from age 20 to 70 and above.
- Level of education was divided into 4 different categories, illiterate, primary school, secondary school, and college or institute.

3.3.10.2. Cancer information

This section included several questions targeting different aspects of breast cancer. The questions included how and when diagnosed, stage at diagnosis time, having any signs or symptoms, family history, and other relevant questions as in (Appendix1).

3.3.10.3. Medications and/or Treatment

This section of the questionnaire was designed to collect information about receiving treatment and medications or not? If yes, the questions included the type of the treatment, breast removing surgery, and other relevant questions as in (Appendix1).

3.3.10.4. Psychological status, sleeping category, and family support

This section included several questions targeting the psychological impact of breast cancer, the sleeping category, and family support (Appendix1).

3.3.11. Inclusion and Exclusion Criteria

Women who were diagnosed with breast cancer and registered at Nanakali Hospital, belonged to the Iraqi Kurdish ethnicity, and agreed to participate were included in the present study. Women who did not meet these criteria have been excluded from the study.

3.3.12. Statistical Analysis

Clinical and pathological characteristics of *BRCA1/2* mutations were compared using the Fisher exact test, other parameters were compared using the Chi-square test and the Fisher exact test. Data analysis was carried out using GraphPad Prism 9.0.0 (121) (GraphPad Software LLC, San Diego, CA). A probability value of less than 0.05 was considered to indicate significance.

3.3.13. Ethical Consideration and Statement of Patient's Consent

The research project was reviewed by the Medical Ethics Committee of Erbil Polytechnic University (Approval No. 23-0011). The participants were fully informed about the research details through a written concept form and information in accordance with the Declaration of Helsinki; after obtaining their agreement, they were included as samples (Appendix-2).

4. RESULTS

4.1. Results of NanoDrop Spectrophotometry

The results of DNA concentration were 140.7 ng/µl on average, with the lowest concentration of 65, while the highest concentration was 261 ng/µl. A260/A280 ratio 1.76 on average. As shown in Appendix (4).

4.2. Results of Gel electrophoresis

Results of the gel electrophoresis for the PCR product for the amplified *BRCA1*/*BRCA2* exome are shown in (Figure 4-1) and (Figure 4-2) below.

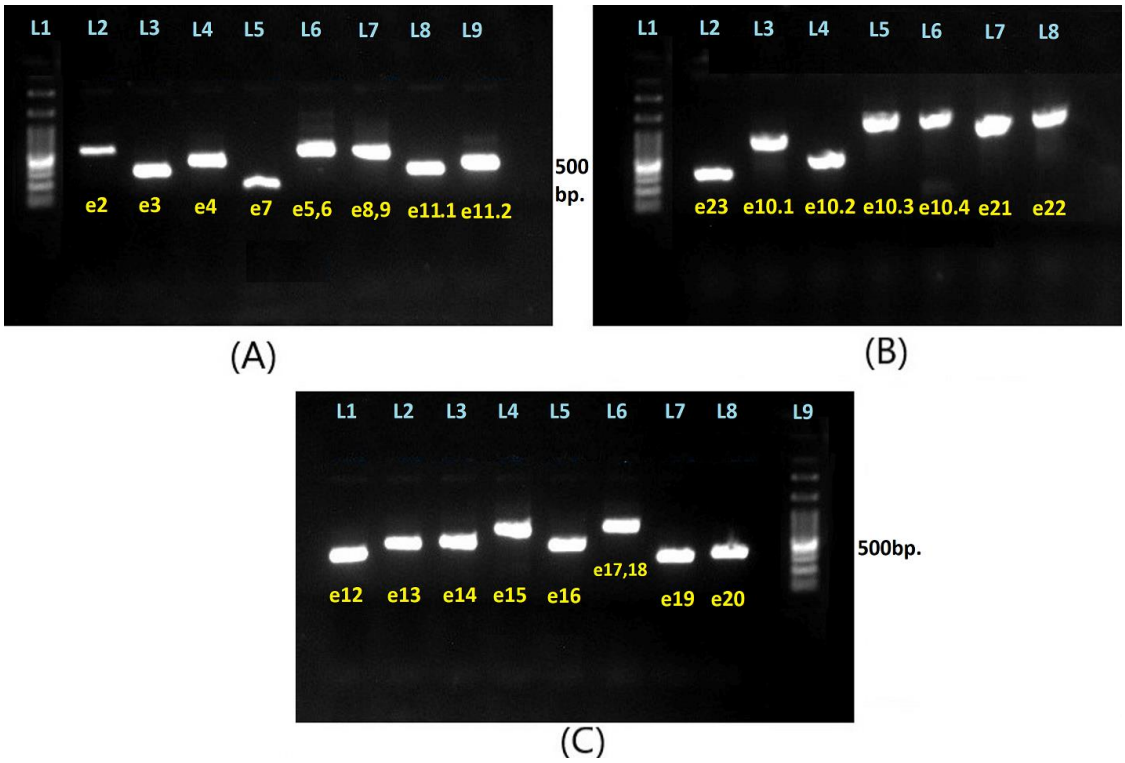

Fig. 4-1 (2%) Gel electrophoresis for the PCR products of *BRCA1* exons. (A): Lane 1: DNA marker of 100 bp. Lanes 2 to 5: exon 2 to 4, and 7. Lane 6: exons 5 and 6, Lane 7: exons 8 and 9, lane 8: exon 11.1, Lane 9: exon 11.2. (B): Lane1: DNA marker 100 bp. Lanes 2: exon 23, lanes 3 to 7: exon 10.1,10.2, 10.3, and 10.4. Lane 7: exon 21, Lane 8: exon 22. (C): Lanes 1 to 5: exons 12 to 16. Lane 6: exons 17 and 18, Lane 7: exon 19, Lane 8: exon 20, lane 9: DNA marker 100 bp. (The sizes of the PCR products are shown in Appendix 5).

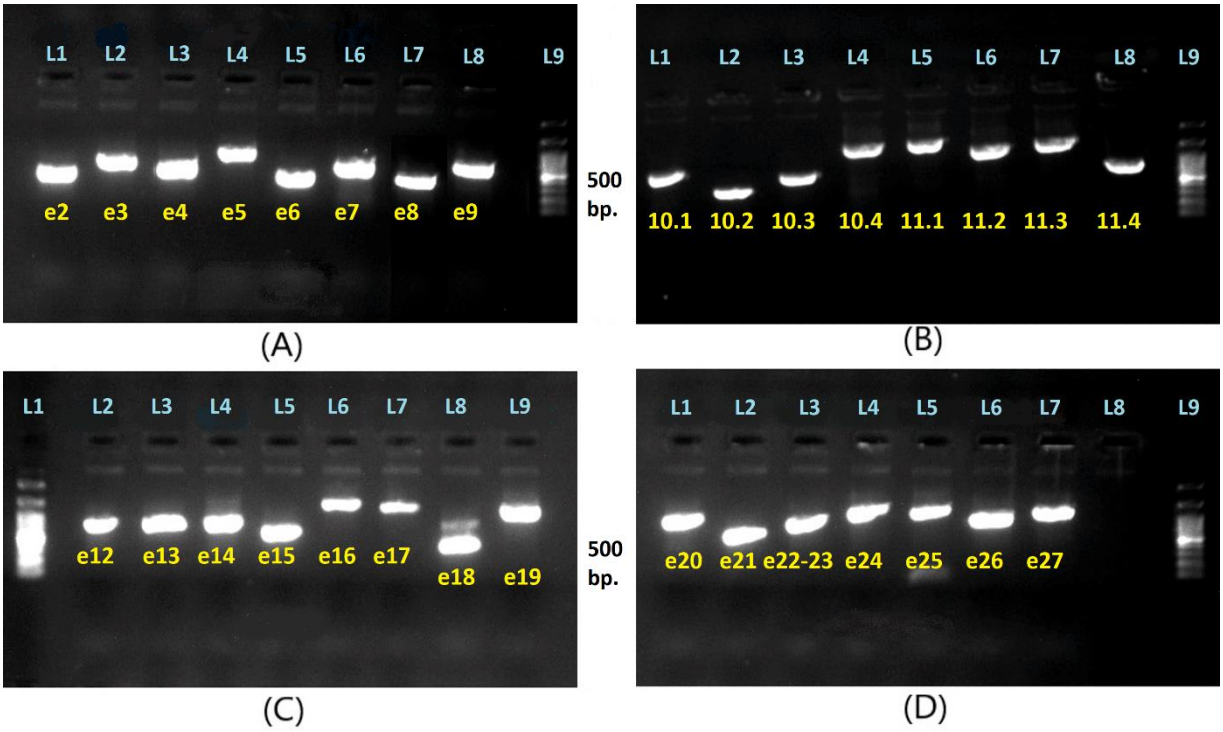

Fig. 4-2 (2%) Gel electrophoresis for the PCR products of *BRCA2* exons. (A): Lanes 1 to 8: exons 2 to 9. Lane 9: DNA marker 100 bp. (B): Lanes 1 to 4: exon 10.1 to 10.4. Lanes 5 to 8: exon 11.1 to 11.4. Lane 9: DNA marker 100 bp. (C) Lane 1: DNA marker 100 bp. Lanes: 2 to 9: exons 12 to 19. (D) Lane 1 and 2: exons 20 and 21, Lane 3: exons 22, 23, Lanes 4 to 7: exons 24 to 27. Lane 9: DNA marker of 100 bp. (The sizes of the PCR products are shown in Appendix 5).

4.3. Results Variants and Variant Analyses

Among the 70 samples that were included for NGS, 42 distinct variants were detected. Classification of these variants based on their types were as the following: on *BRCA1*: 10 missense, 3 synonymous, 2 frameshift, and 2 nonsense variants were observed, plus three new variants. On *BRCA2*: 9 missense variants, 8 synonymous, 2 nonsense, 1 frameshift, and one intronic variant, plus 1 new variant.

The classification of the variants based on their clinical significance were as the following: among 42 variants, nine of them had clinical significances, in which 6 (14.3%) of them were pathogenic, 4 of them on the *BRCA1* gene, which

were: c.3607C>T, c.3544C>T, c.68_69del, and c.224_227delAAAG. The other 2 pathogenic variants were on the *BRCA2* gene: c.100G>T and c.1813delA, as shown in Table (4-1). Regarding variants of conflicting interpretations of pathogenicity, there were 2 (4.76%) variants, and both were on the *BRCA2* gene: c.1909+12delT and c.3318C>G. Also, 1 (2.38%) variant of uncertain significance was detected on the *BRCA2* gene: c.6966G>T. The exact picture of the variants is shown in Appendix-6 to 14. All variants of *BRCA1/BRCA2* are shown in (Figure 4-3).

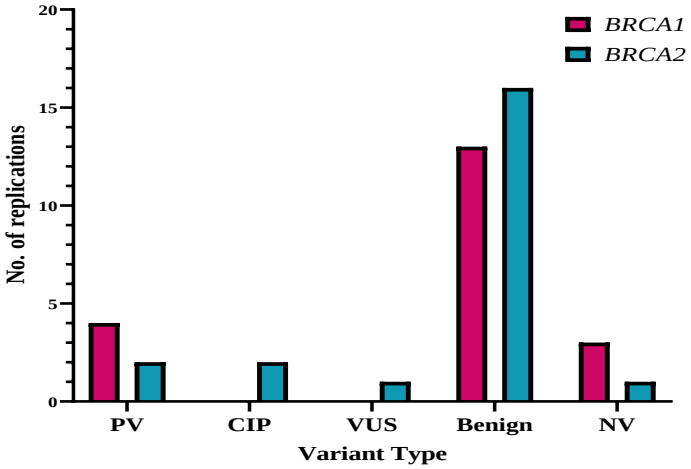

Fig. 4-3 All variants detected on *BRCA1* and *BRCA2* genes. (PV: pathogenic variants, CIP: conflict interpretation of pathogenicity, VUS: variants of uncertain significance, NV: new variants).

Also, among the 42 detected variants, 29 (69%) of them were benign variants, of which 13 (30.9%) were on the *BRCA1* gene and 16 (38%) were on the *BRCA2* gene, as shown in the Tables (4-3, 4-4).

Finally, 4 (9.52%) new variants were detected, 3 of them on the *BRCA1* gene and 1 on the *BRCA2* gene, table (4-5). Their details are available in the following link: https://www.ncbi.nlm.nih.gov/clinvar/?term=erbil+polytechnic+university.

Table 4-1 List of pathogenic, conflicting interpretations of pathogenicity, and uncertain significance variants on *BRCA1*/*BRCA2* genes according to the present study.

Variant				Case Freq/Zygosity	Mutation database		db SNP ID	MAF (min)	MAF (max)
Exon / Intron	cDNA	AA	Variant Effect		ClinVar	BRCA Exchange/ENIGMA			
BRCA1: pathogenic variants									
E10	c.3607C>T	p.Arg1203Ter	Nonsense	1 (1.43%)	Pathogenic	Pathogenic	rs62625308	N/A	< 0.01
E10	c.3544C>T	p.Gln1182Ter	Nonsense	1 (1.43%)	Pathogenic	Pathogenic	rs80357296	N/A	< 0.01
E4	c.224_227delAAAG	p.Glu75fs	Frameshift	1 (1.43%)	Pathogenic	Pathogenic	rs80357697	N/A	N/A
E10	c.68_69del	p.Glu23fs	Frameshift	1 (1.43%)	Pathogenic	Pathogenic	rs80357914	N/A	0.01
BRCA2: pathogenic, conflict interpretation of pathogenicity, and VUS variants									
E3	c.100G>T	p.Glu34Ter	Nonsense	1 (1.43%)	Pathogenic	Pathogenic	rs80358391	N/A	< 0.01
E1	c.1813delA	p.Ile605TyrfsTer9	Frameshift	1 (1.43%)	Pathogenic	Pathogenic	rs80359306	N/A	0.01
Intronic	c.1909+12delT	-	Frameshift	44 (62.8%)	Conflicting interpretations of pathogenicity	Not Yet Reviewed	rs276174816	N/A	0.15
E11	c.3318C>G	p.Ser1106Arg	Missense	1 (1.43%)	Conflicting interpretations of pathogenicity	Not Yet Reviewed	rs1298550035	N/A	< 0.01
E13	c.6966G>T	p.Met2322Ile	Missense	1 (1.43%)	Uncertain significance	Not Yet Reviewed	rs80358924	N/A	< 0.01

Table 4-2 Clinical significance according to different databases

Variant			**Databases**				
Exon / Intron	**cDNA**	**RS number**	**ClinVar**	**BRCA Exchange/ENIGMA**	**gnoomAD**	**Ensembl**	**COSMIC**
BRCA1:							
E10	c.3607C>T	rs62625308	Pathogenic	Pathogenic	Pathogenic	Pathogenic, Uncertain significance	-
E10	c.3544C>T	rs80357296	Pathogenic	Pathogenic	-	Pathogenic	-
E4	c.224_227delAAAG	rs80357697	Pathogenic	Pathogenic	-	Pathogenic	-
E10	c.68_69del	rs80357914	Pathogenic	Pathogenic	Pathogenic	Pathogenic	-
E10	c.3190A>C	-	-	-	-	-	-
E10	c.981del	-	-	-	-	-	-
E7	c.463dupC	-	-	-	-	-	-
BRCA2:							
E3	c.100G>T	rs80358391	Pathogenic	Pathogenic	-	Pathogenic	-
E10	c.1813delA	rs80359306	Pathogenic	Pathogenic	Pathogenic	Pathogenic, Likely pathogenic, Uncertain significance, not provided	-
Intronic	c.1909+12delT	rs276174816	Conflicting interpretations of pathogenicity	Not Yet Reviewed	Benign/Likely benign	Benign/Likely benign, Uncertain significance	-
E11	c.3318C>G	rs1298550035	Conflicting interpretations of pathogenicity	Not Yet Reviewed	Conflicting interpretations of pathogenicity	Uncertain significance	-
E13	c.6966G>T	rs80358924	Uncertain significance	Not Yet Reviewed	-	Uncertain significance	-
E11	c.3787A>G	-	Uncertain significance	-	-	-	-

Table 4-3 List of benign variants on *BRCA1* gene according to the present study.

Variant				Case Freq/Zygosity	Mutation database		db SNP ID	MAF (min)	MAF (max)
Exon / Intron	cDNA	AA	Variant Effect		ClinVar	BRCA Exchange/ENIGMA			
BRCA1: Benign variants									
E6	c.536A>G	p.Tyr179Cys	missense	3 (4.28%)/Het	Benign	Benign / Little Clinical Significance	rs56187033	N/A	0.03
E10	c.1067A>G	p.Gln356Arg	missense	10 (14.28%)/Het	Benign	Benign / Little Clinical Significance	rs1799950	N/A	0.08
E10	c.2077G>A	p.Asp693Asn	missense	3 (4.28%)/Het	Benign	Benign / Little Clinical Significance	rs4986850	N/A	0.11
E10	c.2612C>T	p.Pro871Leu	missense	38 (54.28%)/Het	Benign	Benign / Little Clinical Significance	rs799917	N/A	0.50
E10	c.2311T>C	p.Leu771=	synonymous	38 (54.28%)/Het	Benign	Benign / Little Clinical Significance	rs16940	N/A	0.50
E10	c.3113A>G	p.Glu1038Gly	missense	38 (54.28%)/Het	Benign	Benign / Little Clinical Significance	rs16941	N/A	0.50
E10	c.3548A>G	p.Lys1183Arg	missense	38 (54.28%)/Het	Benign	Benign / Little Clinical Significance	rs16942	N/A	0.50
E10	c.2082C>T	p.Ser694=	synonymous	38 (54.28%)/Het	Benign	Benign / Little Clinical Significance	rs1799949	N/A	0.50
E10	c.1648A>C	p.Asn550His	missense	2 (2.85%)/Het	Benign	Benign / Little Clinical Significance	rs56012641	N/A	0.03
E11	c.4308T>C	p.Ser1436=	synonymous	38 (54.28%)/Het	Benign	Benign / Little Clinical Significance	rs1060915	N/A	0.50
E15	c.4837A>G	p.Ser1613Gly	missense	38 (54.28%)/Het/Hom	Benign	Benign / Little Clinical Significance	rs1799966	N/A	0.50
E15	c.4883T>C	p.Met1628Thr	missense	1 (1.43%)/Het	Benign	Benign / Little Clinical Significance	rs4986854	N/A	0.05
E15	c.4956G>A	p.Met1652Ile	missense	3 (4.28%)/Het	Benign	Benign / Little Clinical Significance	rs1799967	N/A	0.06

Table 4-4 List of benign variants on *BRCA2* gene according to the present study.

Variant				Case Freq/Zygosity	Mutation database		db SNP ID	MAF (min)	MAF (max)
Exon / Intron	cDNA	AA	Variant Effect		ClinVar	BRCA Exchange/ENIGMA			
BRCA2: Benign variants									
E10	c.865A>C	p.Asn289His	missense	7 (10%)/Het	Benign	Benign / Little Clinical Significance	rs766173	N/A	0.17
E10	c.1365A>G	p.Ser455=	synonymous	7 (10%)/Het	Benign	Benign / Little Clinical Significance	rs1801439	N/A	0.17
E10	c.1114A>C	p.Asn372His	missense	35 (50%)/Het/Hom	Benign	Benign / Little Clinical Significance	rs144848	N/A	0.40
E11	c.2971A>G	p.Asn991Asp	missense	7 (10%)/Het	Benign	Benign / Little Clinical Significance	rs1799944	N/A	0.17
E11	c.3807T>C	p.Val1269=	synonymous	25 (35.7%)/Het	Benign	Benign / Little Clinical Significance	rs543304	N/A	0.28
E11	c.3055C>G	p.Leu1019Val	missense	1 (1.43%)/Het	Benign	Benign / Little Clinical Significance	rs55638633	N/A	< 0.01
E11	c.5199C>T	p.Ser1733=	synonymous	1 (1.43%)/Het	Benign	Benign / Little Clinical Significance	rs28897734	N/A	0.01
E11	c.4563A>G	p.Leu1521=	synonymous	70 (100%)/Hom	Benign	Benign / Little Clinical Significance	rs206075	N/A	0.13
E11	c.6513G>C	p.Val2171=	synonymous	70 (100%)/Hom	Benign	Benign / Little Clinical Significance	rs206076	N/A	0.13
E11	c.3396A>G	p.Lys1132=	synonymous	25 (35.7%)/Het	Benign	Benign / Little Clinical Significance	rs1801406	N/A	0.48
E11	c.2229T>C	p.His743=	synonymous	7 (10%)/Het	Benign	Benign / Little Clinical Significance	rs1801499	N/A	0.17
E14	c.7397T>C	p.Val2466Ala	missense	70 (100%)/Hom	Benign	Not Yet Reviewed	rs169547	N/A	0.12
E14	c.7242A>G	p.Ser2414=	synonymous	14 (20%)/Het	Benign	Benign / Little Clinical Significance	rs1799955	N/A	0.48
E18	c.8187G>T	p.Lys2729Asn	missense	1 (1.43%)/Het	Benign	Benign / Little Clinical Significance	rs80359065	N/A	0.02
E22	c.8851G>A	p.Ala2951Thr	missense	1 (1.43%)/Het	Benign	Benign / Little Clinical Significance	rs11571769	N/A	0.06
E27	c.9976A>T	p.Lys3326Ter	nonsense	3 (4.28%)/Het	Benign	Benign / Little Clinical Significance	rs11571833	N/A	0.04

Table 4-5 List of the new detected variants on *BRCA1/2* genes and their accession numbers on NCBI/ClinVar according to the present study.

Gene	Exon	Codon	Wild t.	mut.	cDNA	Change	Accession number
BRCA1	7	155	CAA	CCAA	c.463dupC	duplication/insertion	SCV005196609
BRCA1	10	1064	AGT	CGT	c.3190A>C	substitution	SCV005199865
BRCA1	10	327	ACA	AC-	c.981del	deletion	SCV005199845
BRCA2	11	1263	AGT	GGT	c.3787A>G	substitution	SCV005196610

In the present study, 9 clinically significant variants were detected, 8 of them were in the coding regions (exons), of which 4 were on *BRCA1*: p.Arg1203Ter, p.Gln1182Ter, p.Glu75fs, p.Glu23fs, and 5 were on *BRCA2*: p.Glu34Ter, p.Ile605TyrfsTer9, p.Ser1106Arg, and p.Met2322Ile, in the exotic regions and (c.1909+12delT) that located in the noncoding (intronic) region of the *BRCA2* gene. The protein changes of the clinically significant variants and their locations are illustrated in (Figure 4-4):

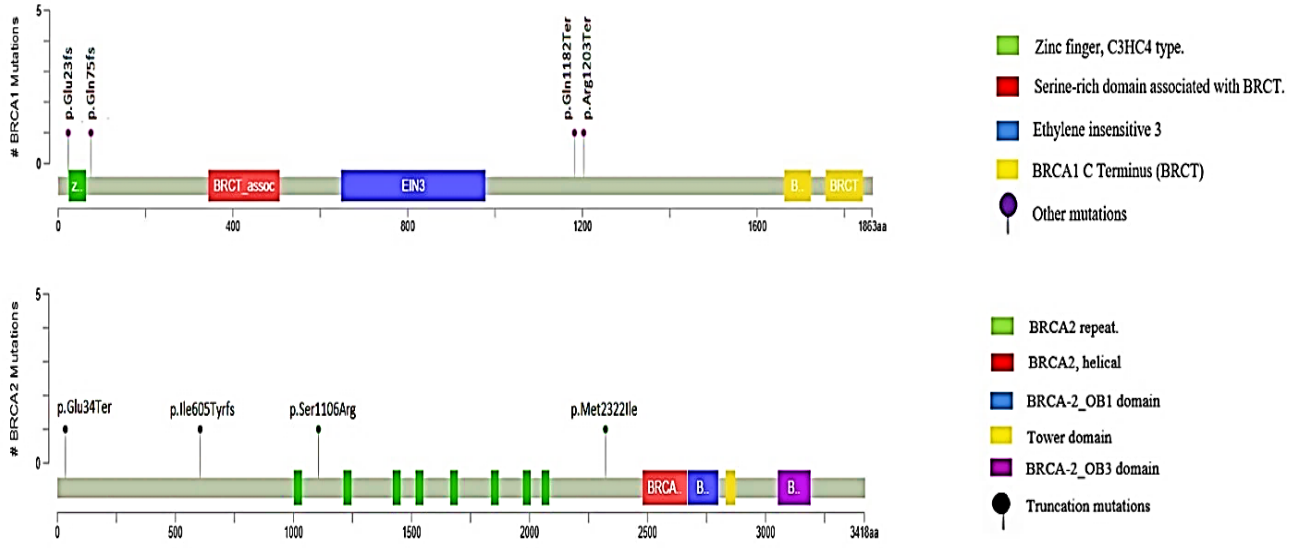

Fig. 4-4 The schematic diagram of BRCA1 and BRCA2 proteins changes with their positions according to the present study. The diagram is drawn using (www.cbioportal.org/mutation_mapper).

4.4. Educational Level

Regarding the educational level, 64 (42.7%) were illiterate (not attending any school), 37 (24.7%) attended primary school, 8 (5.3%) attended secondary school, and 41 (27.3%) attended colleges or institutions. There were highly significant differences regarding the level of education with a *p*-value <0.0001, as shown in Table (4-6).

4.5. Economical Level

Economical states have been classified into three categories, good, average, and bad. 34 (22.7%) stated a good economic state, 79 (52.7%) stated an average economy, and 37 (24.7%) stated a bad economic state. There were highly significant differences regarding the economic status with a *p*-value <0.0001, as shown in Table (4-6).

4.6. Rural/Urban

Among the 150 participants, the majority, 105 (70%), lived in urban areas, while 45 (30%) lived in rural areas. There were highly significant differences regarding the places of residence (*p*-value <0.0001), as shown in Table (4-6).

4.7. Marital Status

Among the participants, 131 (87.3%) were married, 8 of them were widows, 4 were divorced, and 19 (12.7%) were single. There were highly significant differences regarding marital status with a *p*-value <0.0001, as shown in Table (4-6).

Table 4-6 Parameter of level of education, economical level, rural/urban, and marital status according to the present study. (n=150).

Parameters	**Classes**	**N**	**%**	***p*-value**
Level of Education:	*Illiterate*	64	42.7%	<0.0001
	primary school	37	24.7%	
	secondary school	8	5.3%	
	colleges or institutions	41	27.3%	
Economical level	*good*	34	22.7%	<0.0001
	average	79	52.7%	
	bad	37	24.7%	
Rural/Urban	*Rural*	45	30%	<0.0001
	Urban	105	70%	
Marital status	*Married*	119	79.3%	<0.0001
	Widows	8	5.3%	
	Divorced	4	2.7%	
	Single	19	12.7%	

4.8. Age of the Participants at Time of Data Collection

The age of the participants at the time of data collection was as the follows: 2 cases were between (20-29) years, 10 cases were between (30-36), 23 cases were between (40-49), 73 cases were between (50-59), 36 cases were between (60-69), and finally 6 cases were between (70-79) years of age, as shown in (Figure 4-5). There were highly significant differences among the age groups with a *p*-value <0.0001.

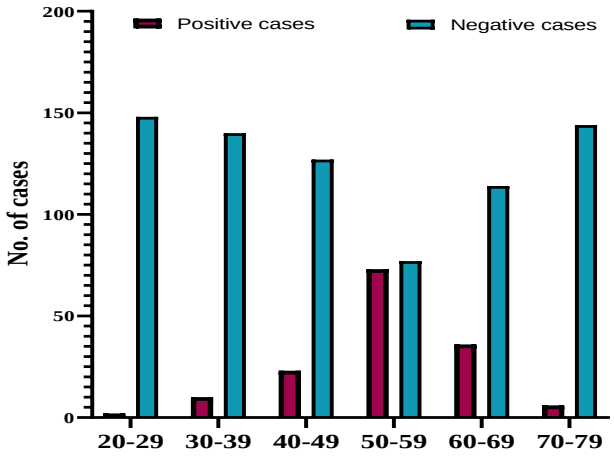

Fig. 4-5 The age groups of the participants at the time of data collection.

4.9. Age of the Participants at Time of the Diagnosis with BC

Ages at the time of diagnosis were as follows, 62 cases (41.3%) were between 50-59 years of age, 48 cases (32%) were between 40 and 49, 19 (12.7%) were between 30 and 39, 15 cases (10%) were between 60 and 69, 4 (2.7%) between 20 and 29, and finally, only 2 cases (1.3%) were above 70 years of age. The mean age was (49.5) and the ages ranged from 27 to 70 years of age. There were highly significant differences among the age groups (p-value <0.0001)., as shown in (Figure 4-6).

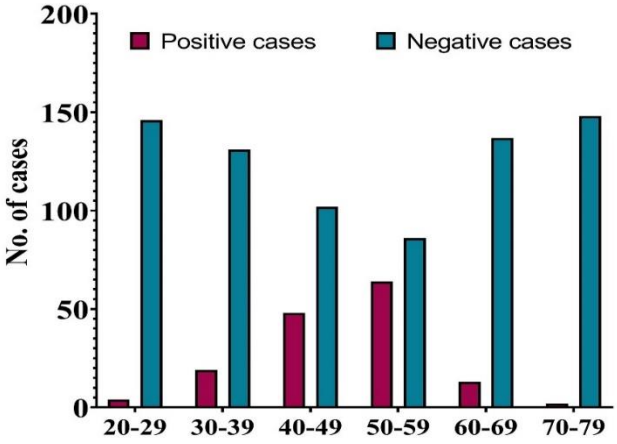

Fig. 4-6 The age groups of the participants at the time of diagnosis with BC.

4.10. Having Previous Knowledge about Breast Cancer.

Among 150 participants, only 30 (20%) had some previous knowledge about some aspects of breast cancer, while 120 (80%) had no previous knowledge about breast cancer. With a *p*-value <0.0001 between them. The results of level of awareness and having previous knowledge are shown in (Figure 4-7).

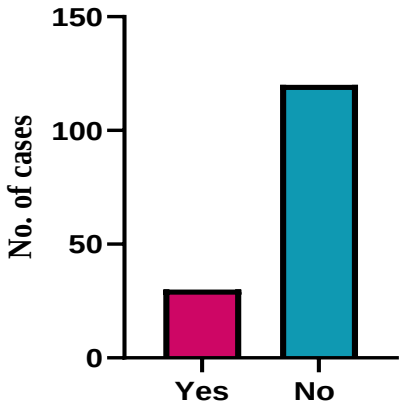

Fig. 4-7 Having previous knowledge about BC prior to the time of diagnosis.

4.11. Performing Any Test Prior Detection (Pre-tests)

Among 150 participants, only 19 (12.7%) of the participants declared that they had undergone a pre-test at least once before being diagnosed with breast cancer, while the majority, 131 (87.3%), did not undergo any pre-tests before the time of diagnosis. A highly significant difference was found among the two groups (with a *p*-value <0.0001). The results of screening practices are shown in (Figure 4-8).

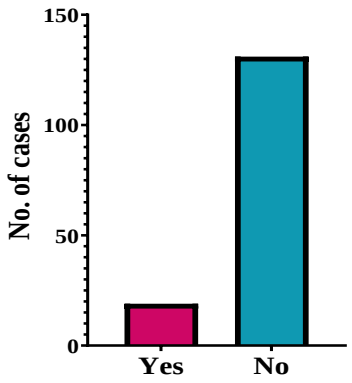

Fig. 4-8 Performing any screening practices before the time of diagnosis.

4.12. Type of the Screening Method Used

Among those 19 participants who did the pre-test, the screening methods were as follows: 9 participants performed breast self-examination (BSE), 8 participants performed (sonar), and 2 participants underwent (mammography). (with a *p*-value <0.03), results are shown in (Figure 4-9).

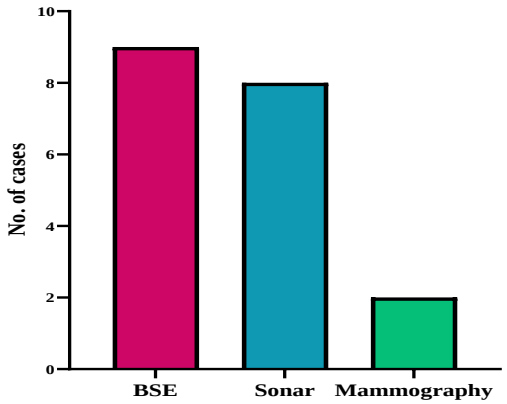

Fig. 4-9 Types of screening practices prior to the time of diagnosis.

4.13. Stage of the Cancer at Time of the Diagnosis

The stage of the cancer at the time of diagnosis was as the follows: 68 (45.3%) were of stage III, 38 (25.3%) of the cases were of stage I, 34 (22.7%) were diagnosed with stage II, 4 (2.7%) were of stage IV, and finally, 6 (4%) of the cases were unknown regarding their stage of the cancer. With a *p-value* <0.0001, the results of the stage of the cancer are shown in (Figure 4-10) below:

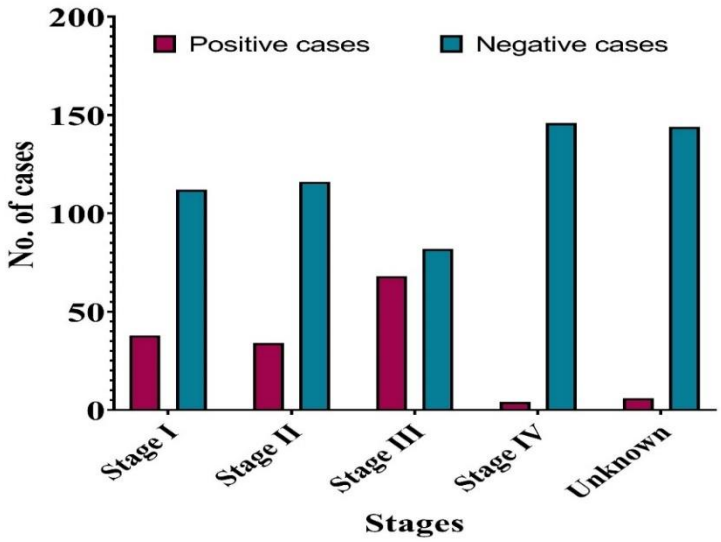

Fig. 4-10 The stages of the cancer at the time of diagnosis.

4.14. How was The Case Observed for the First Time? Self-detection vs. Physicians or Health Care Worker (HCW).

In most of the cases, 103 (68.7%) declared that the case was observed at first through self-observation without interfering with any second party, while 47 (31.3%) of the cases were detected by physicians and/or health care workers (HCW), as shown in (Figure 4-11). There were highly significant differences according to statistical analysis (with a *p-value* <0.0001).

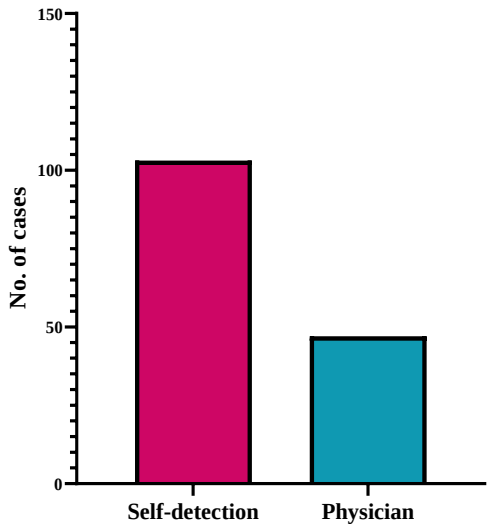

Fig. 4-11 Who observed the case for the first time.

4.15. Having Any Signs or Symptoms Prior Detection?

Among the points that were investigated and discussed with the participants was having any signs and symptoms before being diagnosed or close to the time of diagnosis. Of 150 participants, 89 (59.3%) experienced some signs and symptoms prior to detection, while 61 (40.7%) of the participants revealed that they had no signs.

Most observed signs before the time of diagnosis included the following: 50 (33.3%) cases of swelling of the breast or under the armpit, 24 (16%) cases of pain, 6 (4%) cases of vomiting, 3 (2%) cases of stiffness of the breast, 3 (2%) cases of shortness of breath, 2 (1.3%) cases of abnormal stuns in the breast, and finally, 1 (0.7%) case of discharge from the breast. There were highly significant differences according to statistical analysis (with a *p-value* <0.0001). As in (Figure 4-12).

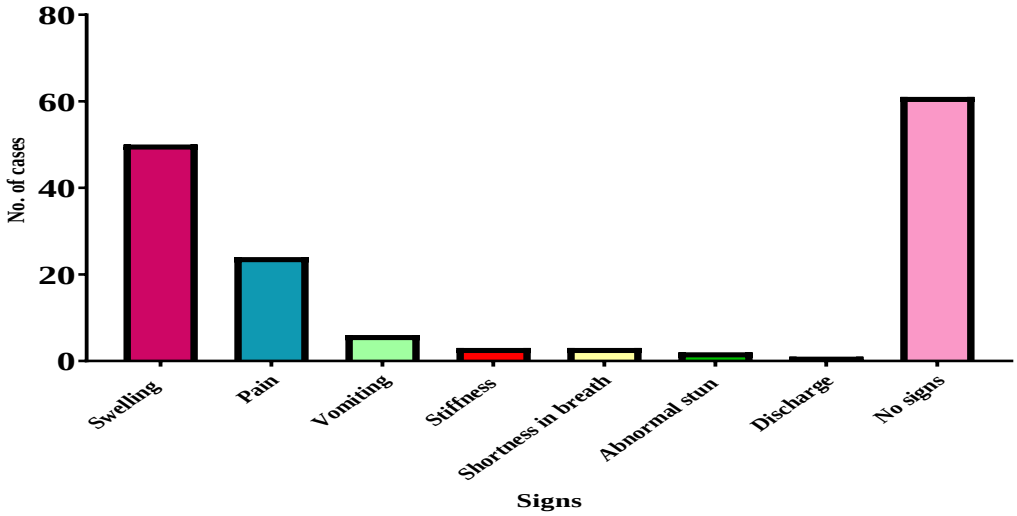

Fig. 4-12 Having any signs or symptoms prior detection.

4.16. Family History

Among those 150 participants, 101 (67.3%) had no relatives with breast cancer, while 49 (32.7%) of them had relatives, of whom 28 (18.7%) had first-degree relatives, as shown in (Figure 4-13). There were highly significant differences according to statistical analysis with a *p-value* <0.0001.

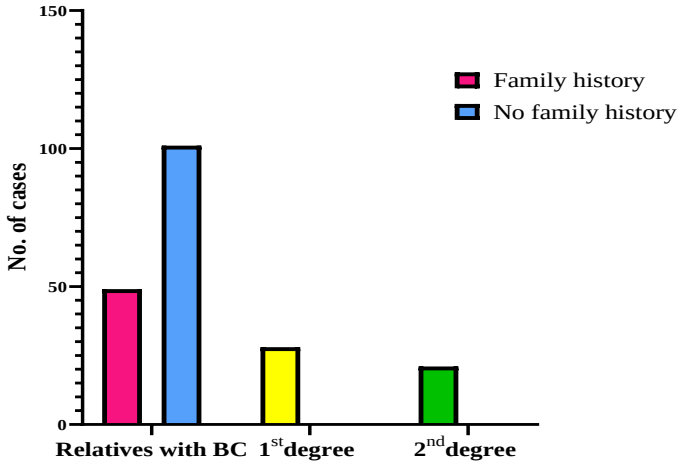

Fig. 4-13 Family history of breast cancer.

4.17. Breast Removing Surgery (Mastectomy)

In the present study, among 150 cases, 62 (41.3%) underwent mastectomy, and 88 (58.7%) didn't undergo mastectomy surgery. There was a slightly significant difference according to statistical analysis (with a *p-value* <0.04). As in (Figure 4-14).

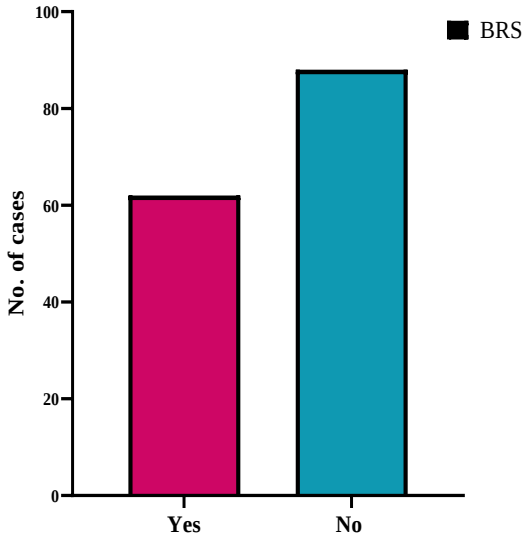

Fig. 4-14 Breast removing surgery (Mastectomy).

4.18. Ready to Undergo Mastectomy if Necessary?

Among the participants, 88 didn't undergo mastectomy. When they asked if they were ready to undergo mastectomy in the future if it was needed, 73 (82.9%) were ready to perform it, while 15 (17.1%) refused to do mastectomy (with a *p-value* < 0.0000), as shown in (Figure 4-15).

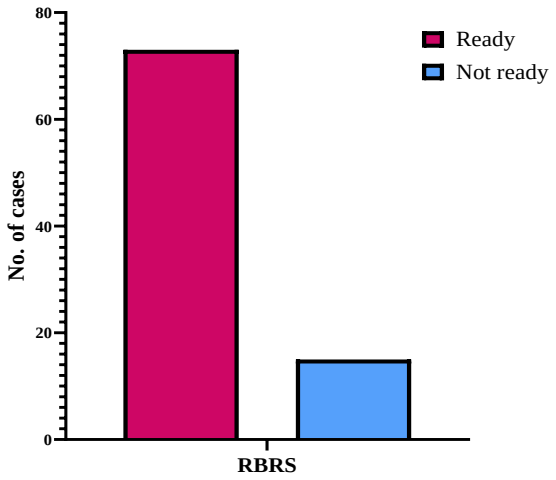

Fig. 4-15 Ready to undergo mastectomy if necessary.

4.19. Did Breast Cancer Affect or Have Influence Your Life?

Among the 150 participants in the current study, 118 (78.7%) stated that breast cancer had influenced their lives, and the rest, 32 (21.3%), answered no. There were highly significant differences according to statistical analysis with a *p*-value <0.0000.), as shown in (Figure 4-16).

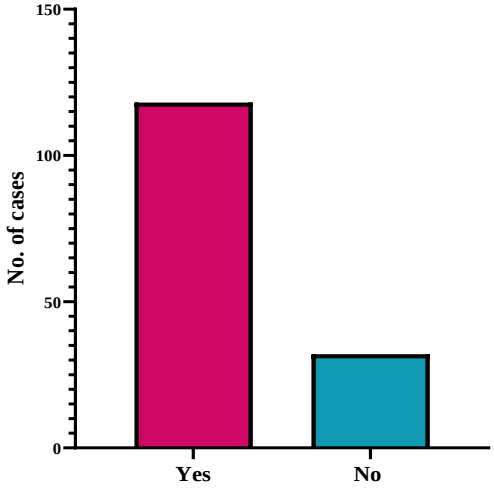

Fig. 4-16 Did Breast Cancer affect or have influence on your life.

4.20. How BC Affected the Life of the Participants?

The consequences of breast cancer have been categorized into five categories: depression, weakness or sadness, stress, headache, and hopelessness. Among the 118 participants who stated that the disease affected their lives, 56 (47.4%) felt depression, 30 (25.4%) felt weakness and sadness, 24 (20.3%) had stress, 4 (3.4%) felt headaches, and 4 (3.4%) felt hopelessness (with a *p*-value <0.0001), as shown in (Figure 4-17).

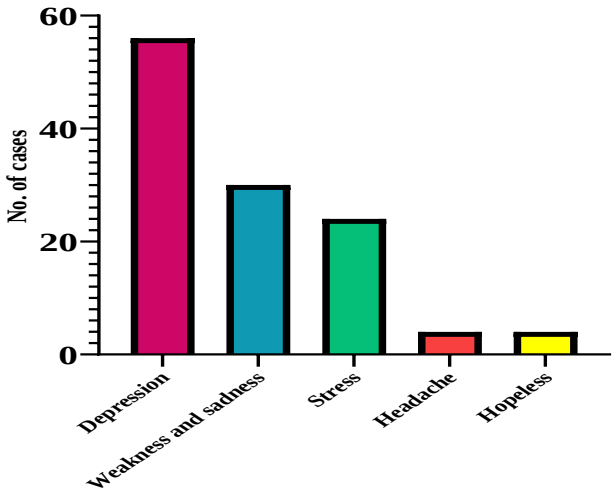

Fig. 4-17 How BC affected the life of the participants.

4.21. The Sleeping Quality Before and After breast cancer

Sleeping quality and characteristics were categorized into three categories (good, average, and bad), and classified before and after being diagnosed with breast cancer. Before being diagnosed with the disease, sleeping quality was as follows: 86 (57.33%) good, 38 (25.33%) average, and 26 (17.33%) bad. After being diagnosed with the disease, the results dramatically changed: 15 (10%) were good, 52 (34.7%) were average, and 83 (55.3%) were bad. There were highly significant differences according to statistical analysis with a *p*-value <0.0001, as shown in (Figure 4-18).

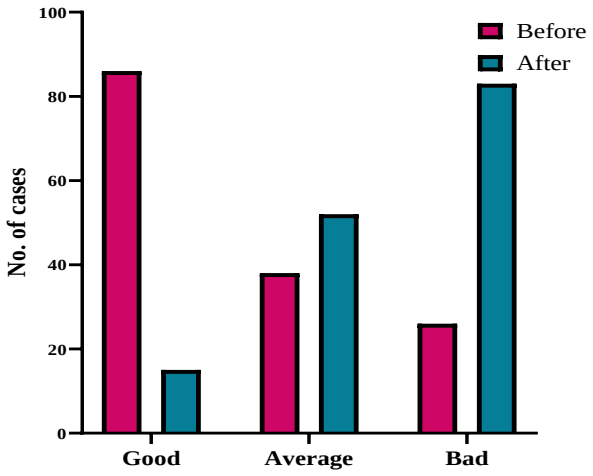

Fig. 4-18 The sleeping quality before and after breast cancer.

4.22. Being well informed and receiving sufficient information about the cancer state and the case

Among the participants in the present study, 97 (64.7%) declared that they were well informed and got sufficient information regarding their situation; 38 (25.3%) answered no, while 15 (10%) declared that they got a little information about their situation (with a *p-value* <0.0001), as shown in (Figure 4-19).

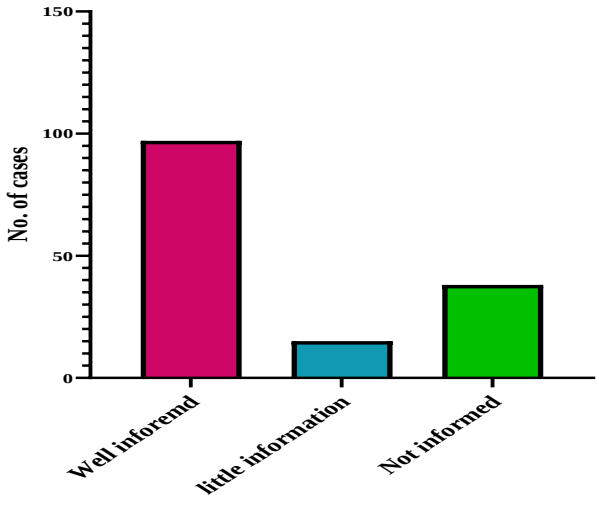

Fig. 4-19 Being informed about the cancer state and the case.

4.23. Receiving support from family members and/or partner

The family support and understanding included two questions: whether your partner or family member had a positive response to the case or not. And the response or the support itself was classified into three types: good, average, and bad. Regarding the type of response, 140 (93.3%) of them stated that the response was good, 10 (6.7%) said it was average, and none of the participants indicated a bad response (with a *p-value* <0.0001), as shown in (Figure 4-20).

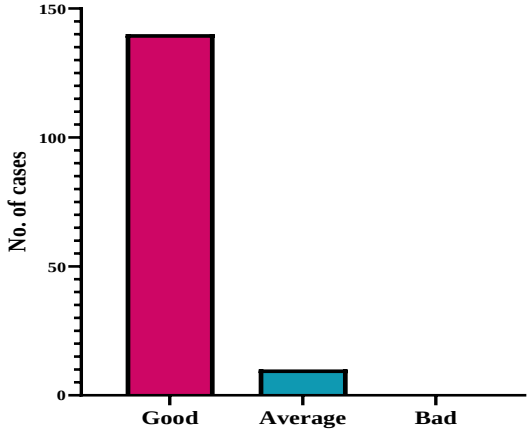

Fig. 4-20 Receiving support from family members and/or partner.

4.24. Medications and treatments

Regarding taking any types of medications or treatments, most of the participants, 148 (98.7%), were taking one or more types of medications, while only 2 (1.3%) were not taking any types of medications. Types of medications: 129 (86%) took chemotherapy, 62 (41.3%) underwent breast-removal surgery, 43 (28.7%) underwent radiation, and 61 (40.7%) were taking tablets (with a *p*-value <0.0001), as shown in (Figure 4-21). Detecting the type of medication accurately and separately is not applicable, as many participants took more than one type of medication, or they started with a medication and continued with tablets later.

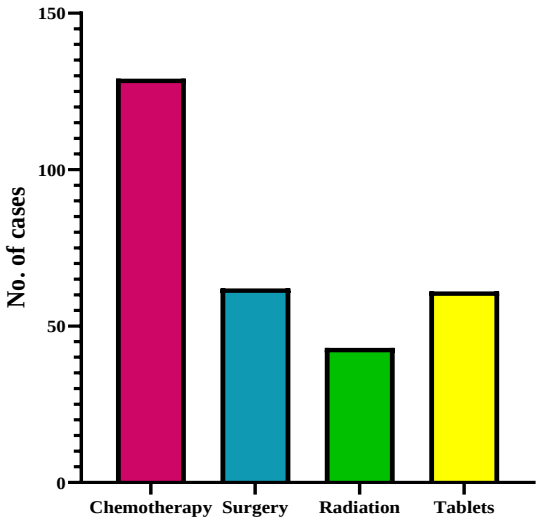

Fig. 4-21 Medications and treatments.

Regarding having problems and complications with taking the medications, among those 148 patients who were took medications, 26 (17.6%) had one or more complications, while 122 (82.4%) had no problem with them (with a *p-value* <0.0001), as shown in (Figure 4-22).

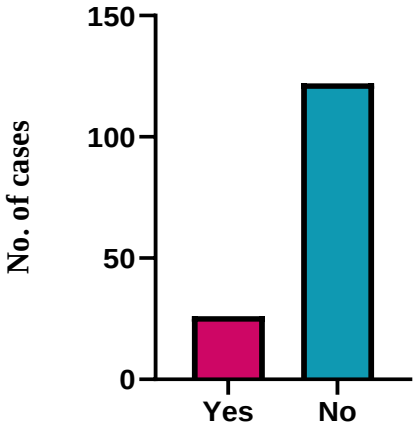

Fig. 4-22 Having complications and side effects with taking the medications.

5. DISCUSSIONS

5.1. Variants

Genetic testing for germline mutations in *BRCA1/2* provides important information for those who have been diagnosed with breast cancer and for those who are at risk of developing breast cancer. Those who are at risk will have a better imagination if they know that they hold pathogenic or clinically significant variants to do a pretest from time to time to enable them to prediagnosis the cancer when it is initiated. The current study applied NGS to the whole exomes' of *BRCA1/2*, as they are contributing to most cases of hereditary breast cancer.

The present study detected nine variants with clinical significance, among them, six variants (8.57%) among 70 participants were pathogenic, of which four pathogenic variants (5.71%) were on the *BRCA1* gene and two pathogenic variants (2.85%) were detected on the *BRCA2* gene. The detection of pathogenic variants in *BRCA1* more than *BRCA2* has been proven by previous studies. A study of Geredeli *et al.* (2019) in Turkey, detected 11 germline mutations in *BRCA1* and eight in *BRCA2*. In Italy, (Concolino *et al.*, 2019) detected 24 deleterious variants on *BRCA1* and 13 on *BRCA2*. In Pakistan, a study of Tariq *et al.* (2021) detected seven variants on *BRCA1*, four pathogenic, and three VUS, while on *BRCA2*, only three VUS were detected.

The reason why more mutations detected on *BRCA1* gene compared to *BRCA2* could be related to the differences in the contribution of these two genes in breast cancer. *BRCA1* gene is worse by age of 70, women who carries mutated forms of this gene have a higher risk for developing breast cancer than *BRCA2* gene. *BRCA1* mutations are seen in about 7% of families with multiple breast cancers and in about 40% of families with ovarian and breast cancer. While *BRCA2* mutations are found in 20% of families at high risk for ovarian and breast

cancers but in less than 3% of women with early onset breast cancer. Also, *BRCA1* mutations are linked to triple negative breast cancer which is more aggressive and harder to be treated than other genes related to breast cancer (Chang and Kwong, 2022, Pavese *et al.*, 2022). Another reason why *BRCA1* gene is considered more aggressive is that mutation in the *BRCA1* gene has a higher rate of mitosis and greater lymphatic permeability. Generally, about two-thirds of the *BRCA1* mutations found in breast cancer are germline, and the remaining proportion relates to somatic mutations (Loboda *et al.*, 2023).

The detection of six pathogenic variants that relate to *BRCA1*/*BRCA2* genes among 70 participants is considered to be within a normal percentage compared to previous studies that included other populations, and according to the standards, the percentage of breast cancer that results from mutations in high-penetrance genes usually ranges from 5% to 10%, and the proportion that these two genes contribute to the HBOC that attributes to pathogenic variants in gene in 66% for *BRCA1* and 34% for *BRCA2* (De Silva *et al.*, 2019, Petrucelli *et al.*, 2022, Doraczynska-Kowalik *et al.*, 2022). It is true that our findings are within the normal range, but we should note that the present study included *BRCA1/2* genes only; it is true that these two genes are responsible for most of the germline breast cancer cases, but we should not forget that there are other genes that contribute to hereditary breast cancer, and if they are investigated, this percentage may increase more.

According to the present study, the percentage of germline breast cancer somehow goes along with the normal range worldwide, but unfortunately, cases of breast cancer in Erbil city increased dramatically. Only between 2013 and 2019, the number of cases increased about three times, from 675 to 1884 in 2019 (M. Amen *et al.*, 2022). And according to the same research, they revealed that the

percentage of cases is predicted to increase during the present decade from >2x in the current decade, from 3,457 cases to 4,547 and 4,449 cases in the Erbil governorate. Based on these statistics, it can be concluded that breast cancer is a main issue in this region, and sporadic breast cancer contributes to the main percentage of the cases. This is somehow logic if we know that the Kurdistan region and Iraq are among the polluted regions around the world. Unfortunately, previous wars in this region that used unconventional weapons, the oil industry, the existence of a high number of illegal oil refineries, and many other kinds of environmental factors like air, water, and soil pollution with carcinogenic pollutants can explain the growing percentage of cancer cases in our country (Hama-Aziz, 2022, Fattah Ali *et al.*, 2023).

The other three variants that were neither pathogenic nor benign detected on the *BRCA2* gene, in which two of these variants were of conflicting interpretations of pathogenicity (c.3318C>G, c.1909+12delT) and one variant was of uncertain significance (c.6966G>T), have been detected and reported previously on the ClinVar database. Variants of conflict interpretation and uncertain significance are somehow problematic and cause confusion for decision making by physicians and genetic counselors; two of them (c.3318C>G and c.6966G>T) are not even yet reviewed by some databases like BRCAexchange and ENIGMA. For understanding that, it is important to know that the classification of the variants regarding their clinical significance is changeable, and they depend on the submitted research to the databases and the tools used for the analyses. In the future, Artificial Intelligence (AI) will be used more efficiently for making more precise decisions (Caputo *et al.*, 2021, Favalli *et al.*, 2021). An example of that is the variant of (c.5199 C>T), which was reported as (not yet reviewed) until 2017, then classified as (benign/little clinical significance)

according to the Brcaexchange website https://brcaexchange.org/variant/833751, despite having some differences between different databases.

5.1.1. New Detected Variants (Novel Variants)

Four new variants were detected in the current study that were never reported on *BRCA* genes in any databases before, so they can be reported as novel variants. Three of these new variants were detected in the *BRCA1* gene: (c.3190A>C, c.463dupC, and c.981del), while in the *BRCA2* gene, one new variant was detected (c.3787A>G). These variants were submitted to NCBI/ClinVar, and accession number obtained to them as shown in the Table (4-5), and they are available online.

Detection of new variants is normal because mutations and types of variants of these two genes vary depending on geographical origin, population, and ethnicity, as has been proven previously by other studies (Hirotsu *et al.*, 2015, Ava *et al.*, 2016). Concolino *et al.* (2019) reported seven novel variants on *BRCA1/2* genes in Italy; Abu-Helalah *et al.* (2020) carried out a similar study in Jordan, they detected several novel variants on *BRCA1/2* genes, and some of them were pathogenic according to their analysis. Further analysis and investigations using bioinformatics tools and family history are required to estimate the clinical significance of these novel variants.

The variant of (c.3190A>C), which occurred in the genomic location (17:41,244,358) of the *BRCA1* gene with the amino acid coding number (1064), which was coded originally for Serine, we detected changing of first amino acid, AGT to CGT, that coded for Arginine. Regarding (c.463dupC) variant that occurred on the *BRCA1* gene in the genomic location of (17:41251875-41251876) with the amino acid coding number (155) which is coded for making Glutamine (Gln), we couldn't find an exact variant at this location, either in the ClinVar

database or any other database. On this genomic location, there are other variants like c.463C>T (p.Gln155Ter) and c.463C>G (p.Gln155Glu). Also, the variant was reported on the ClinVar database (NM_001407587.1:c.463dup), but on a different location (17:41251872-41251873 GRCh37), a few base pairs previous to our finding, and considered pathogenic with rs397507236. Our frameshift variant can be considered pathogenic according to the ENIGMA *BRCA1/2* Gene Variant Classification Criteria.

The other new variant on the *BRCA1* gene that was detected in the present study is c.981del, also not reported on this gene before. This variant with a deletion of 1 bp has been reported in different locations, like chromosome 19 [NM_173483.4(CYP4F22):c.981del (p.Glu328fs)] with rs1568362644 and has been related to autosomal recessive Congenital Ichthyosis 5 (https://www.ncbi.nlm.nih.gov/clinvar/variation/560327/). Also, it has been reported on chromosome 5 (*GHR*): growth hormone receptor gene (NM_000163.5:c.981del) with (p.Ile328fs) protein change (https://www.ncbi.nlm.nih.gov/clinvar/RCV000009181/). In all reported cases, it was determined to be pathogenic, and it is logical as it is a deletion in the coding region (exon), and according to the ENIGMA definition for variant classification, they were considered pathogenic.

The new variant that was detected on the *BRCA2* gene (c. 3787A>G) was never reported previously in relation to breast cancer in any databases like NCBI (ClinVar), BRCAexchange, ENIGMA, gnomAD, and Ensemble. This variant, with a protein change of p. Thr1263Ala, has been related to Cystic Fibrosis (CF). The mutation was detected by DHPLC analysis and characterized by direct sequencing; it has been seen only once in over 3000 control chromosomes of the Italian population. The mutation was identified in one patient with azoospermia,

the husband of a CF carrier, from North-East Italy, Information related to this study and all details about the variant is found at this link: http://www.genet.sickkids.on.ca/cftr/MutationDetailPage.external?sp=1582.

Making a final decision regarding those four new mutations requires further investigation by Sanger sequencing and an accurate evaluation of family history analysis by performing genetic testing on all consenting family members to be sure about them (Mu *et al.*, 2016, Bozsik *et al.*, 2022).

5.1.2. Benign Variants

Regarding benign variants, more than 29 variants were detected; those were in the coding regions. On *BRCA1*, 13 variants were detected, while on *BRCA2*, 16 variants. There was a big difference regarding the frequencies of these benign variants. Variants of (c.4563A>G, c.6513G>C, and c.7397T>C) were detected on all samples with 100% frequency; other variants of (c.4883T>C, c.3055C>G, c.5199C>T, c.8187G>T, and c.8851G>A) were detected once among the seventy samples. Other variants ranged in their frequencies. When those variants were compared to the highest population (MAF) from the Ensembl database (https://www.ensembl.org/Homo_sapiens/Variation/Explore?r=17:43099286-43100286;v=rs), most of them are within the normal range with some exceptions.

The classification of these variants as benign depends on the NCBI/ClinVar database. It's worth mentioning that most of these variants have been classified as having (benign/little clinical significance) on the ENIGMA database. One of the reasons for that is that those databases are based on submitted data from clinical studies, which could lead to different interpretations based on their results, the software used by them, and the guidelines for the variant classification (Sharo *et al.*, 2023).

Even within the same databases, searching by RS number gives different information than searching by the coding DNA. For example, (c.536A>G) with (rs56187033), when the search carried out by the coding DNA, it is considered benign (https://www.ncbi.nlm.nih.gov/clinvar/variation/37661/), while when the search is carried by the RS number, it gives a result of (not reported on ClinVar) and asks for submission (please consider submitting your interpretation of this variant to ClinVar) (https://www.ncbi.nlm.nih.gov/clinvar/?term=rs56187033). Such differences are confusing and should be fixed.

It could be concluded from these facts, that the clinical significance of the variants is not fixed; they could be changed from one database to another and even within the same database from time to time as they are updating their information from time to time. Having differences among different databases is a problematic issue that causes confusion for genetic counselors, physicians, carriers of genetic laboratories, and researchers (Gudmundsson *et al.*, 2022, Walsh *et al.*, 2024).

Finally, many variants have been detected on *BRCA1/2* genes, and there is huge data regarding these two genes around the world, especially in western and high-income countries. But, unfortunately, very little is known regarding these two genes among low- and middle-income countries, including the Kurdish population. To our knowledge, the present study is the first one carried out among Kurdish women using NGS to look for germline breast cancer in blood samples. It is true that this is a strong point for the current study, and the findings can be used as a reference for the next studies, but we cannot compare it to previous data and research in our population as there are not any previous studies.

5.2. Age of the Participants at the Time of Diagnosis

A hundred fifty participants who were already diagnosed with breast cancer were included in this study with a mean age of 49.5. The statistical analysis showed a highly significant difference regarding the age groups with a *p-value* <0.0001. The mean age at diagnosis with breast cancer differs between different countries and populations. Generally, in Western countries like the United States and Europe, people are about 62 to 63 years old. While this mean is lower in developing countries to about 50 or even less (Alizadeh *et al.*, 2021). According to a study carried out in Sulaymaniyah Governorate, northern Iraq in 2015, the mean age was 49.42 years, which is very close and approves our results (Molah Karim *et al.*, 2015).

Detecting breast cancer at a younger age in Middle Eastern countries was approved by several previous studies. In Arab countries, according to research carried out by Najjar and Easson in 2010, they did a meta-analysis study that included 28 previous studies from seventeen different Arabic countries, including Iraq. They found that the average age for diagnosing breast cancer was 48 years, ranging from 43 to 52 years, their mean age is close and approves our results (Najjar and Easson, 2010). In Iran, according to a meta-analysis study carried out by Alizadeh between 2008 and 2017 including 92 studies and 15000 patients, their result mean age was about 46.76±1.19 (Alizadeh *et al.*, 2021). In Turkey, according to research that included 10,149 patients, the age at the time of diagnosis was 50 years (Tas and Keskin, 2012).

In western countries, the situation is different; in the United States, for example, the mean age for diagnosing breast cancer is 59.8 years, and according to another study, was 58.4 years (Lund *et al.*, 2008, Franco-Marina *et al.*, 2015). In Canada, the mean age is 60.1 years (Franco-Marina *et al.*, 2015). While among

British women, the mean age is 67, which is considered the highest mean age among different countries (Bowen *et al.*, 2008).

In general, women in Middle Eastern countries are diagnosed with this cancer at a younger age than in western countries, which is about 10 years younger. One of the reasons is that the population of these countries is younger than of western countries (Molah Karim *et al.*, 2015, Francies *et al.*, 2020). Also, other factors contribute to this, like exposure to environmental risk factors, general health, lifestyle, and genetics (Francies *et al.*, 2020). Unfortunately, Iraq is among the most polluted countries; toxic and carcinogenic materials are distributed widely in this country, and women are exposed to more risk factors than women in western countries. All these factors will contribute together and affect getting breast cancer at earlier ages in these countries and populations compared to western countries.

5.3. Having previous knowledge about Breast Cancer

Among the participants in the current study, 80% of them had no previous knowledge regarding breast cancer. Only 20% answered with (yes), and those 30-participant had preliminary knowledge about this type of cancer without having sufficient knowledge about causes, risk factors, diagnosis, or screening methods. Having poor knowledge among women in developing countries has been proven by other researchers as well. According to research carried out in Bangladesh, 61.5% of the women were unaware of the causes and risk factors of this type of cancer (Mehejabin and Rahman, 2022). In India, specifically in Delhi, research carried out by Dey and his colleagues revealed that 53.4% of the women had awareness at different levels about different aspects of breast cancer; their results differed from ours, indicating that geographical distribution plays a role in the level of awareness (Dey *et al.*, 2015).

Several factors may play a role in having low knowledge about breast cancer, like education, age, health care services, socio-demographic characteristics, community, religion, etc. (Prusty *et al.*, 2020b, Afaya *et al.*, 2023). An explanation for having such a high number of participants without having sufficient information about the disease is the relationship between age and education, as most of the participants are of old age, and most of those women are either non-educated or just admitted to primary school, which is popular in our society. Based on the obtained results, an intensive awareness program for the community by the Ministry of Health and NGOs is required to increase the level of awareness among women in developing countries.

5.4. Doing any test prior detection

Regarding the results of performing routine screening for breast cancer before being diagnosed with the disease, among participants in this study, 87.3% of them did not undergo any pre-tests, while only the rest did pretest at least once before being diagnosed with breast cancer. The pre-test methods for examination among those 19 participants were breast self-examination (BSE), sonar, and mammography. BSE is among the easiest and cost-effectives ways that women can perform it at home by themselves. Unfortunately, women in Middle Eastern countries don't perform this examination (Apatić and Lovrić, 2023). According to a study carried out in Turkey, among 103 participants, 26.2% had knowledge about BSE, and only 4.3% of the participants performed BSE, which approves the results of the present study (Avci, 2008). In Delhi, India, BSE was higher among Indian women; 34.9% of their participants performed BSE, while only 6.9% of their participants underwent clinical breast examination through mammography (Dey *et al.*, 2015). Another study carried out in Bangladesh in 2022 declared that

only 14% of the participating women had information about screening tests for breast cancer, which supports our findings (Mehejabin and Rahman, 2022).

Doing pre-tests among the 12.7% of participants in the present study is a dangerous sign that should be noticed. Unfortunately, the majority of women in developing countries are not undergoing any pre-tests or screenings for breast cancer (Koçak and Çiçek Gümüş, 2023). The explanation for that is the low level of awareness and feeling of shame regarding this issue among women in these countries, along with the negligence of the competent authorities in the related ministries and directorates. Thankfully, NGOs have tried to increase awareness and the importance of screening for breast cancer among healthy women in recent years, but still not enough.

5.5. Stage of the cancer at time of diagnosis

Regarding the cancer stage at the diagnosis time in the current study, most of the cases (45.3%) were detected at stage III. According to the result of the statistical analysis, a highly significant difference was found regarding the stage of the cancer, with a *p-value* <0.0003. The stage of the cancer at diagnosis has a very important role in its treatment and recovery.

The diagnosis of cancer at stage III in the current study is problematic as it increases its consequences. This is very different compared to other countries. A study carried out in Iraq by Alwan, in 2016 revealed that 46% of the cases were diagnosed in the late stages, which supports the findings of the present study (Alwan, 2016). According to research carried out in Brazil, most of the cases (58.9%) were diagnosed with advanced clinical stages, which confirms our results (de Mello Ramirez Medina *et al.*, 2019).

The detection of breast cancer at late stages in relation to the demographic, educational, and socioeconomic status of the area even within the same country was approved by research carried out in China in 2012, in which cases with late stages were 25.5% in areas with low socioeconomic status compared to 14.8% in areas with the highest socioeconomic status (Wang *et al.*, 2012). In Western countries and the United States, for instance, the diagnosis of breast cancer at the metastatic stage is very low, ranging from 0% to 6% (Benitez Fuentes *et al.*, 2024).

Perhaps one of the most scientific explanations for detecting women with higher stages at the time of diagnosis resulted from not performing screening for pre-tests among women, which enables the detection of the disease at early stages (Elgendi *et al.*, 2024). A study carried out in Korea included 17,689 women who were recently diagnosed with breast cancer. Their results revealed that late stages of the cancer were detected among women who had never been screened for this type of cancer, while women who underwent screening using mammography were diagnosed with earlier stages (Choi *et al.*, 2018). The association between regular screening and stage of cancer is proven by another study by Ding and his colleagues in 2022, who found that women who never undergo pre-tests had a higher risk of being diagnosed with later stages about six times than those who did regular screening (Ding *et al.*, 2022).

The present study detected 4% of the cases with an unknown stage of cancer, which was considered an interesting result. An unknown stage of the cancer at diagnostic time has been detected by another study carried out in Sulaymaniyah, northern Iraq, that included 539 cases from 2006 to 2008 and declared that 18.2% of the cases were of the unknown stage, which proves our results with a much higher percentage (Majid *et al.*, 2009). Although it is neither logical nor scientifically proper that a woman diagnosed with breast cancer doesn’t

know her stage of the cancer, this may be related to poor diagnosis approaches or the woman not being informed about her exact status, especially if the cancer reached late stages, considering her psychological state and avoid being shocked. Despite that, it contradicts one of the main ethical principles, which is autonomy and the patient's right to be fully informed about his or her case.

Finally, different factors contribute to the stage at which the cancer will be detected at the time of diagnosis, including routine screening tests, geographical distribution, socioeconomic status, age, health services and insurance, level of awareness, and education. Breast cancer is usually diagnosed at lower stages in Western countries in comparison to low- and middle-income ones (Wang *et al.*, 2012, Benitez Fuentes *et al.*, 2024). The results of the present study for the cancer stage at diagnosis time show the importance of doing routine tests for breast cancer to diagnose it earlier and minimize its consequences. Increasing the awareness level among women through an intensive screening program by related authorities is recommended.

5.6. Who observed/detected the disease? And how detected for the first time?

Regarding the detection of a tumor in the breast and who detected it for the first time, among participants, 68.7% of them observed the tumor by themselves and, mainly accidentally or by chance. While the rest mentioned that a physician diagnosed the tumor, self-observation is done by observing the following changes or abnormalities immediately before being diagnosed: 59 (39.3%) tumors under the armpit or tumor in the breast, 41 (27.3%) pain under arm, 13 (8.7%) abnormal breast shape, 7 (4.7%) repetitive vomiting, 2 (1.3%) changing of the breast color, and finally, 28 (18.7%) detected accidentally or by chance. Tumor under the armpit, breast lump, and accidentally together constitute most of the cases in which 41 (58.57%) detected breast cancer through a feeling of abnormal mass

(tumor). The results of the present study regarding this issue have been proven by a previous study carried out by Khoshnaw and his colleagues in Erbil, Iraq, in which most of the cases were diagnosed after the observation of a tumor (Khoshnaw *et al.*, 2023).

The self-observation of breast cancer is popular among women, according to research carried out in the United States of America that included 361 participants, 43% of the participants detected breast cancer by themselves; among them 18% detected it accidentally, and 25% detected it through BSE (Roth *et al.*, 2011). On the other hand, according to another study carried out also in America, the situation is vice versa; 88% of the new cancer cases were diagnosed in hospitals (Lund *et al.*, 2008). This has definitely resulted from pre-test and screening policies because usually stage I cancer cannot be detected by women themselves, while it can be detected through screening and mammography, and this shows the importance of routine screening for early detection of the disease. The detection by women themselves rather than physicians or health care workers in the present study is logical, as most of them did not take any pre-tests or screenings for having breast cancer before being diagnosed with the disease. These results again show the significance of performing pre-tests periodically.

5.7. Having any signs or symptoms prior detection?

Among the points that were investigated and discussed with the participants was having any signs and symptoms before being diagnosed or close to the time of diagnosis. Of 150 participants, 89 (59.3%) experienced some signs and symptoms prior to detection, while the rest of the participants revealed that they had no signs. Most observed signs before the time of diagnosis included the following: 50 (33.3%) cases of swelling of the breast or under the armpit.

According to a study carried out in Iraq by Alwan (2016), declared that most of the cases (94%) showed palpable breast cancer, which means having solid bodies in the breast, consequently leading to breast swelling, which confirms the results of the present study. Also, he indicated that (4.7%) experienced discharge from their nipples, while the present study indicated (2%) for the same sign; such differences could be related to the sample size and the percentage of the stages of the cancer. Unfortunately, there were no studies covering this issue among breast cancer patients, there were few studies that included women without breast cancer investigated their general knowledge, not their real experience (Prusty *et al.*, 2020a, Elshami *et al.*, 2022). Despite the fact that most cancers are silent, especially at their primary stages, there are some signs and symptoms that help women and physicians be considered as signs of having breast cancer. Those signs may be used as indicators for requesting further investigations and not neglecting them.

According to the Centers for Disease Control and Prevention (CDC), in 2024, women may experience a variety of symptoms when it comes to breast cancer. It's possible for some patients to not show any signs or symptoms at all. Generally, signs that could indicate breast cancer include a new lump in the breast or underarm (armpit), swelling or thickness in an area of the breast, skin irritation or dimpling on the breast, redness or flakiness around the nipple, unusual nipple discharge (such as blood), changes in breast size or shape, and localized pain within the breast. Finally, it’s important to remember that these symptoms can also be associated with other conditions.

5.8. Having family history?

Regarding having relatives with breast cancer, among the participants in the present study, 32.7% of them had relatives, of whom 18.7% had first-degree

relatives, 14% had second-degree relatives. These results were approved by a previous study carried out in Iraq by Alwan (2016), which showed 35% of women with breast cancer had a family history of cancer and among them 18.5% had relatives with breast cancer. Their results are very close and approve our results.

A study carried out in the United Kingdom (UK) in 2017 declared that 15% of the participants confirmed that they had one or more family members (first-degree relatives) with breast cancer, which confirms our findings (Brewer et al., 2017). Another study that was carried out in the United States of America included statistics on 306147 cases from 1996 to 2016. According to their results, 11% of the participants had one or more relatives with breast cancer (Durham *et al.*, 2022). This percentage is lower than ours, which may be related to sample size and ethnicity. Also, they only included cases within specific ages (30 to 59), years which affects the results as older people are more likely to have relatives with this cancer.

Having first-degree relatives with breast cancer, like a sister, mother, or daughter, is considered a risk factor in that family because it duplicates the risk. In such cases, other family members are advised to undergo pre-tests at earlier ages than families without first-degree relatives with breast cancer to allow early diagnosis of the disease (Durham *et al.*, 2022).

Finally, it is worth mentioning that not all cases that have relatives with breast cancer mean that they have inherited breast cancer among their family, because usually the percentage of inherited breast cancer is about 15-20%. It could be by chance that this occurs. To ensure this point, those who have relatives with breast cancer are encouraged to have genetic tests to make sure they have inherited or somatic breast cancer.

5.9. Breast removing surgery (Mastectomy)

In the present study, among 150 cases, 62 (41.3%) underwent mastectomy. According to a study carried out in the United States of America that included data from 2004 to 2014, 49.9% of the cases underwent total simple mastectomy, in which the percentage increased from 35% in 2004-2005 to 61.8% in 2012-2014 (Lu *et al.*, 2022). The overall percentage approves the results of the present study. According to a study carried out in China that included 1787 participants between 2009 and 2017, 61.3% underwent breast-removing surgery (Huang *et al.*, 2023). This percentage is higher than the present study; maybe time distribution played a role in such differences, as nowadays more choices and protocols like chemotherapy and radiology are applied before the decision is made for mastectomy. The explanation for why mastectomy in these studies is higher than in the present study is the age at which breast cancer was diagnosed. Mastectomies are higher than older women, and the age of diagnosis among Kurdish women, like other neighboring and Middle East countries, is about 10 years younger than in Western and high-income countries. This will explain why the mastectomy rate in this study is lower than in those studies (Morgan *et al.*, 2020).

Undergoing mastectomy is not an easy decision. Women who are targeted to undergo breast removal should be fully informed about this surgery because, at the end, they will take the final decision regarding this procedure. Unfortunately, women are not fully informed about the choices of treatment before taking them as options. According to a study carried out in California, United States of America, in 2017, 67% of the participant declared that they were completely informed regarding the treatment options before underwent mastectomy. If this were the situation in the United States of America, despite a lack of data, the

percentage would definitely be extremely lower in middle- and low-income countries (Mitchell *et al.*, 2018).

Removing breasts is related to the stage at diagnosis of breast cancer; in stage I, for example, there is no need for breast removing surgery. While, when the advanced stages are detected, it means metastatic happened within breast tissue and the breast will be removed, a study carried out in the UK in 2013 and including 106,952 women showed that mastectomy is more popular among older ages and with higher tumor stages (Miller *et al.*, 2023). To avoid mastectomy and its psychological consequences for women, early diagnosis should be considered, and women are encouraged to do pre-tests regularly (Huang *et al.*, 2023).

5.10. Ready to undergo breast removal if necessary?

Among the participants, 88 didn't undergo a mastectomy. When they asked if they were ready to undergo mastectomy in the future if it was needed, 73 (82.9%) were ready to perform it, while 15 (17.1%) refused to do mastectomy. It is logical that most women who have already been diagnosed with BC are ready to undergo mastectomy if it is needed in the future. Also, women refusing to remove their breasts is understood as it is not an easy decision, especially among younger women. This surgery has many short-term and long-term consequences. It is something about a woman's femininity, and it has psychological consequences for their life in the future besides their sexual life (Taze and Kanan, 2020, Lundberg and Phoosuwan, 2022).

The decision to remove breasts as a prophylactic step is not an easy one. It is neither the physician's decision nor the patient's decision alone, even though the final decision should be taken by the patient herself. Genetic testing and genetic counseling play an important role in taking such a decision. Having a

family history and/or detecting an inherited mutation like *BRCA1/2, PALB2, CDH1, PTEN, or TP53* in the patient will increase the possibilities of taking such a decision to avoid the recurrence possibilities of the cancer in the future (Griffin *et al.*, 2024). The mastectomy will reduce the risk of recurrence of breast cancer by more than 90% among those who have family history or inherited high penetrance genes, but it should be noted that there is no guarantee for total elimination of the risk because the cancer can originate in the remaining breast tissues or even in the breast tissue that sometimes extends to the collarbone and armpit. Also, it should be mentioned that mastectomy for women who did not inherit high penetrance genes is not considered standard care or a step (Jerome-D'Emilia *et al.*, 2015).

5.11. Did Breast Cancer affect or have influence on your life?

Among the participants in the current study, 78.7% stated that breast cancer had influenced their lives. The name (cancer) alone is enough to influence the lives of any person, so breast cancer will affect the life of those who are diagnosed with the disease. Among the consequences are feelings of fear, being shocked, being worried, and anxiety. According to a recent study carried out in Sweden in 2022, all participants were affected after being diagnosed with the disease (Lundberg and Phoosuwan, 2022).

About 21.4% of the participants stated that breast cancer has not affected their lives too much. Positive thinking and coping with disease are very important among those suffering from any disease, especially cancer. Although it is illogical to say that the disease does not affect the affected patient, it is more correct to say that we accept the disease and live with it. One of the reasons that explain the acceptance of the disease among those infected can be a strong belief in God and in destiny.

5.12. How BC affected the life of the participants?

The consequences of breast cancer have been categorized into five categories: depression, weakness or sadness, stress, headache, and hopelessness. Among the 118 participants who stated that the disease affected their lives, 47.4% felt depression, 25.4% felt weakness and sadness, 20.3% had stress, 3.4% felt headaches and the same for hopelessness.

Being diagnosed with breast cancer has several psychological and mental impacts on the patients, which have been proven by several previous studies (Mertz *et al.*, 2012, Villar *et al.*, 2017, Wu *et al.*, 2016, Cordova *et al.*, 2017, Taze and Kanan, 2020). According to a meta-analysis study that included thirty-nine quantitative studies carried out by Fortin and his colleagues in 2021, according to their results, 39% suffered from distress, 34% suffered from anxiety, 31% experienced stress for post-traumatic stress, and 20% felt depression (Fortin *et al.*, 2021). There may be some differences regarding their percentages compared to our percentages; this may be caused by differences in the categories as they did not include weakness, sadness, headache, and hopelessness which affects the percentages between the present study and their study.

The psychological effects of being diagnosed with breast cancer have higher effects at the beginning of the diagnosis, or early after being diagnosed (Mertz *et al.*, 2012, Ivanauskienė *et al.*, 2014). This is logic, as the patients will be shocked at first, but then, step by step, they become somehow better when they survive and learn how to deal with the disease. Here, social support plays an important role in improving the psychological state of the patients (Drageset *et al.*, 2012, Ivanauskienė *et al.*, 2014, Cordova *et al.*, 2017, Taze and Kanan, 2020).

One of the main reasons for feeling depression among women with breast cancer could be mastectomy, A study carried out by Lundberg and Phoosuwan in

2022 that investigated several parameters among women who underwent mastectomy revealed that 100% of the participants were affected psychologically by breast removal (Lundberg and Phoosuwan, 2022).

Despite that, the consequences were classified into five categories, but perhaps all the consequences are related to each other, and they could be listed under one type, which is depression, that is related to the psychology of the patients. Hence, psychological support is highly recommended to overcome the consequences of the disease (Ivanauskienė *et al.*, 2014, Taze and Kanan, 2020). Besides social support, psychological support by psychiatric physicians or social workers is also recommended.

5.13. The sleeping quality before and after breast cancer

Sleeping quality and characteristics were categorized into three categories (good, average, and bad) and classified before and after being diagnosed with breast cancer. The good sleeping group was 57.33% before diagnosis, which dramatically lowered to 10% with a significant difference, indicating the high influence of the disease on the sleeping quality of the patients.

The negative impact of breast cancer on sleep quality has been proven by previous studies as well (Weng *et al.*, 2021, Fortner *et al.*, 2002, Zhu *et al.*, 2023). Several factors contribute to decreasing the quality of sleep among breast cancer patients, including psychological states and treatment methods. One of the main causes is psychological impact, according to a study carried out by Fortner and his colleagues, 61% of the patients had sleeping disorders resulting from psychological impact (Fortner *et al.*, 2002). Another cause of sleeping disturbance among breast cancer patients is the treatment method; more than half of the breast cancer patients suffer from sleeping disturbance (Cheng *et al.*, 2023). Sleep disturbance is higher among those who receive therapies compared to those who

are not receiving them (Cheng *et al.*, 2023). According to a study carried out in Saudi Arabia in 2021, sleeping disturbance was higher among those who received radiation therapy, followed by chemotherapy (Grayson *et al.*, 2022). This disturbance resulted from the severity of the pain, nausea, anxiety, depression, and other factors related to the disease, as well as losing self-confidence.

To improve the sleeping quality of patients with breast cancer, several factors can play an important role. It has been proven that improving the psychological state of patients will improve the quality of their sleep. Reducing anxiety, treating depression, providing social and medical support are among the main factors contributing to this issue (Zhu et al., 2023). Another way to increase the quality of sleep is to minimize the side effects of the therapeutic strategies of chemotherapy and radiation.

5.14. Being well informed about the cancer state and the case

Among participants in the present study, 53 (35.3%) either answered with (no) to receiving sufficient information or got little information about their situation. Patients who feel they are not fully informed about different aspects of their cancer are confirmed by other studies as well. A study carried out by Herbert and his colleagues in Germany, included women with breast cancer after five years of follow-up. Their results indicated that (78.5%) felt well informed about the medical tests, while a lower percentage (69.3%) felt well informed about the disease itself. Their findings approve the results of the present study (Herbert *et al.*, 2021). They went deeper by asking more questions about what they needed to know, but they did not get sufficient information. The participants declared that they should receive better knowledge about the side effects and consequences of the therapies in their long-term actions, how to deal better after being diagnosed

with this cancer, the prognosis, recurrence opportunity, complementary medicine and therapies, and more general information.

Generally, breast cancer patients want to be fully informed about their status to be able to deal better with their situation. Also, women prefer to have one close person inform them about their cases. A study carried out by Osei-Tutu *et al.* (2023) in Ghana revealed that most participants in their study prefer full, gradual disclosure of diagnosis in a conducive environment in the presence of loved ones in a humane manner. This will improve their psychological state, which is a very important factor when facing any disease, especially cancer. Finally, informing patients is one of the basic principles of medical ethics. Every breast cancer patient should be fully informed about the case, stage of the cancer, tumor size and location, what to do and what not to do in the future, and any other information that helps them deal with the situation.

5.15. Receiving support from family members and/or partner

The family support and understanding included two questions, whether your partner or family member had a positive response to the case or not? And the response or the support itself was classified into three types: good, average, and bad. Among the participants, all of them stated that their partner and/or family members had a positive response and a good understanding of the situation. Regarding the type of responses, (93.3%) of them stated that the response was good.

One of the aims of the present study was to investigate family support for the patients. Fortunately, cancer patients stated that their husbands and/or family members had a good understanding of the case, and they supported the participants in their dealing with the cancer. According to a study carried out by Aprilianto and his colleagues, aimed at investigating the types and frequencies of family support,

they classified the support into three categories, like our study. Their results showed that 64.3% was good, 19.6% was average, and 16.1% was less (Aprilianto *et al.*, 2021).

Unfortunately, breast cancer has several psychological and physical consequences for the women who are diagnosed with it. Stress, depression, and feeling hopeless are among the main consequences. These consequences have a negative impact on the overall immunity of the patients (Antoni and Dhabhar, 2019). Family support has a direct impact on lowering stress and depression among breast cancer patients (Su *et al.*, 2017).

The psychological and emotional status of patients with any disease is very important, especially for cancer patients. Psychology has a direct impact on the immunity and the overall wellbeing of patients (Wang and Feng, 2022). Receiving support from surrounding people like family members, partners, and friends will have a positive effect on the psychology of the women who are diagnosed with breast cancer and help them cope better with the disease (Lundberg and Phoosuwan, 2022). Based on these facts, it is very important to improve the quality of life for those patients, and this became part of the treatment as it will affect the overall life quality and health status, which has a direct impact on the recovery and progression of the disease. It is worth mentioning that, considering the psychology of the patient, physicians and health care workers must consider this issue while they are dealing with the patient.

5.16. Medications and treatments

Regarding taking any types of medications or treatments, most of the participants, 148 (98.7%), were taking one or more types of medications, while only 2 (1.3%) were not taking any types of medications. Detecting the type of medication accurately and separately is not applicable, as many participants took

more than one type of medication, or they started with a medication and continued with tablets later.

According to the results of the present study, most participants were taking one or two therapies. About half of the participants took two or three therapies. Taking combinations of therapies depends on the stage of the cancer, and as most of the participants were detected at later stages, it is logical that they received combinations of therapies. It has been proven that at the higher stages in which the tumor has spread, more than one treatment is required (Bayat Mokhtari *et al.*, 2017).

Regarding having problems and complications with taking the medications, 26 (17.6%) had one or more complications, while 122 (82.4%) had no problem with them. All of those who stated that they had problems with the complications and consequences of taking medications were those who were taking chemotherapy. They stated that the complications are pain, feeling weak, and other physical side effects. Side effects and complications of chemotherapy have been approved by other studies. In general, the side effects of chemotherapy can be classified into short term and long-term side effects. The short-term side effects include fatigue, nausea, vomiting, dental problems, skin changes, hair loss, and others. While long-term side effects include: pulmonary embolism, lung damage, heart damage, permanent infertility, and even developing other types of cancer (Dhara PI, 2022). A recent study carried out by Katta and his colleagues on the side effects of chemotherapy indicated that fatigue, loss of appetite, and diarrhea are among the main side effects (Katta *et al.*, 2023). Finally, as lower stages are easier to treat and higher stages are more complicated and require more than one therapy, it is very important to diagnose the disease at an early stage to minimize the consequences of the therapies.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. CONCLUSIONS

1. Molecular screening using NGS, and bioinformatics tools provides important information about hereditary types of breast cancer. Having information for those who inherited pathogenic variants is helpful in the prediagnosis of BC among relatives and those who are at risk for getting the disease. The percentage of pathogenic variants among Kurdish women is lower compared to other populations.

2- The prevalence and types of variants differ among different populations and ethnic groups. Also, new and novel variants could be detected among various ethnicities.

3- Epidemiological and other different parameters differ between low- and middle-income countries compared to high-income ones. Generally, women are diagnosed at earlier ages and in advanced stages in those countries.

4- Women in our region, like other low- and middle-income countries, have a very low awareness level and very poor screening practices.

5- As most of the participants were detected at advanced stages of their cancer, they experienced and observed some signs prior to detection, including swelling of the breast and pain.

6- Like other types of cancer, breast cancer has several influences on the lives of the participants, including psychological effects and reducing the quality of sleep. Unfortunately, not all breast cancer patients are properly informed regarding their situation and status with the cancer. Fortunately, most of the women in our region received good family and social support.

7- The majority of cancer patients receive treatments, in which most of them receive one, two, or even combinations of different treatments. The stage of the cancer and the status of the tumor play a crucial role in determining therapeutic strategies.

8- At the end, the strengths of the present study include being the first to investigate hereditary breast cancer (from blood samples) caused by *BRCA1*/*BRCA2* genes targeting all exomes using NGS among Iraqi Kurdish women and detecting four new variants that were not recorded in any other databases or previous studies. As well as investigating several other epidemiological and other important factors among those patients that, some of them, had never previously been investigated.

6.2. RECOMMENDATIONS

1- All women are recommended to undergo tests for breast cancer, including self-examination and mammography. Women of any age, especially those above 40 years of age, should undergo breast examinations every six months. A pre-diagnosis of the case will minimize the consequences.

2- Genetic testing of NGS for all related genes to this cancer is recommended for women, especially for those who have relatives with breast cancer. Having modern tests like NGS in related hospitals will become a routine test in Erbil city.

3- Advanced scientific courses and training are needed for specialized physicians, laboratory managers, and geneticists regarding NGS, using different bioinformatic tools and programs for the right interpretation of the results, especially when new variants are detected.

4- More awareness is recommended among women, and related authorities and NGOs should play a more important role.

5- More studies are needed among Kurdish women, including a larger sample size and other related genes (gene panel) to breast cancer, to better understand hereditary breast cancer among Kurdish women in Erbil city.

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Questionnaire Form Date:

Sample no:			
Personal Information			
Ethnicity/nationality:			
Gender		Job	
Age		Place of working	
Mob. No.		Marital status	
Place of inhabitant		Economic status	
Level of Education		No. of children	

Comments	**Cancer Information**	
Types of Cancer	Yes: No:	
If yes, when and how known?		
At which stage diagnosed?		
Having any signs?	Yes: No:	
Having previous knowledge about BC screenings?	Yes: No:	
Performing any pre-test?	Yes: No:	
Having relatives with BC	Yes: No:	
Having inherited BC among the family?	Yes: No:	
Having other types of cancer among the family?	Yes: No:	
Having knowledge about BC where and how you inherited it?	Yes: No:	
Having knowledge about reasons of BC?	Yes: No:	
Having knowledge about signs of BC?	Yes: No:	
Did you get sufficient info. About your cancer?	Yes: No:	

Medication or Treatments		
Taking any medications or therapy?	Yes: No:	
Which type?	Yes: No:	
Was it useful?	Yes: No:	
Mastectomy?	Yes: No:	
Having problems or complications with taking treatments?	Yes: No:	
Ready to undergo mastectomy if it was necessary?	Yes: No: Already Removed:	

	Personal and Psychological state	
Did BC affect your life? How?	Yes: No: How:	
Did you accept it?	Yes: No:	
Do you fel stress or disagreement	Yes: No:	
Do you feel depression?	Yes: No:	
How was your sleep before BC?	Good: Normal: Bad: How many hours:	
How is your sleep after BC?	Good: Normal: Bad: How many hours:	
Did your family members/husband supported you?	Yes: No: Good: Normal: Bad:	
Other notes:		

(Consent form and information)

This research conducted through a blood sample collection as well as a questionnaire form

Introduction: you are invited to participate in the current research that looks for hereditary breast cancer among women. As it known, research is a way to investigate and search facts and finding answers that may help other people as well as the population. This consent form answers your questions regarding this research. You are free to participate or reject the participation.

Aims of the study: The current research looks for mutations in two main genes that are responsible for hereditary breast cancer. And detecting the specific types and frequencies of the mutations that enables the patients to know wither their cancer is hereditary or not.

Who can participate: any women diagnosed with breast cancer can participate and get the results of the genetic test for free.

Note: Personal information and names of the participants remain secure and hidden, all participants' information kept securely and will not be used against them under any circumstances.

Approval of Scientific Research Ethics

Scientific Ethics Review

Approval No.: 23-0011 Date:2023/ 10 / 30

Project Name	Molecular Detection and Frequency of Breast Cancer High Penetrance Genes Mutations among Kurdish Women in Erbil Province		
Project Purpose	PhD project	Project Source	Human Genetics
Project Leader	Prof. Dr. Mustafa S. Mustafa	Major Researcher	Ahmed Nawzad Hassan
Other Researchers	Non		
Review Materials	● Experimental protocol (essential) ● Informed consent ● Material description ● Other documents (Include)		
Review Comments	① The person in charge of the project has sufficient years of clinical and scientific research experience; has sufficient time to participate in clinical research; the project team will be reasonably contributed and the equipment conditions met the requirements. ② The project will be fully considered the health and rights of the subjects. Ensure that each subject (his or her legal guardian) will fully understand the purpose and process of the clinical research. In addition, the prolonged treatment cycle, and informed consent were prepared before the clinical trial, the experimental protocol clearly identifies the test under different circumstances. Subjects which can withdraw from the study trial will not have an adverse impact on the research efficacy; the study protocol is scientific and appropriate. The ethical principles will be fully considered and followed. ③ The method of subjects' enrollment, providing relevant information relating to the experimental procedure and results to the subjects (or its legal guardian) are sufficient and understandable; the method of achieving informed consent is appropriate.		

The research project was reviewed by the Medical Ethics Committee of Erbil Polytechnic University and complied with the requirements of the Helsinki declaration and related medical ethics

Signature

The Head of Scientific Ethics Committee

Date: 2023 / 10 / 30

(Official Stamp)

Erbil Polytechnic University EPU Scientific Research Committee

Address: Committee Ethics Section, Erbil Polytechnic University, Hadichawshil Street, Kurdistan Region, Erbil 44001, Iraq

e-mail: ethicscommittee@epu.edu.iq

حكومة اقليم كوردستان/ العراق
وزارة الصحة
المديرية العامة لصحة اربيل
قسم الامور الفنية

حکومەتی هەرێمی کوردستان / عێراق
وەزارەتی تەندروستی
بەڕێوەبەرایەتی گشتی تەندروستی هەولێر
بەشی کاروباری هونەری

Kurdistan Region Government/ Iraq
Ministry of Health
General Directorate of Health – Erbil

العدد: ٢٦٩٢
التاريخ: / / 2021

ژمارە:
ڕێکەوت: / / 2720 ی کوردی

بۆ/ نەخۆشخانەی نانە کەلی

بابەت/ئاسانکاری

ئاماژە بە نووسراومان ژمارە(١٦٣٥٣)لە(٢٠٢١/٨/٤)وە نوسراوی زانکۆی پۆلیتەکنیکی هەولیر//کۆلێژی تەکنیکی تەندروستی وپزیشکی ژمارە (١٢١٠) لە (٢٠٢١/١٠/١٤)
ئاسانکاری بکرێت بۆ قوتابیانی خوێندنی باڵا (أحمد نوزاد حسن) دکتۆرا / لە بەشی (شیکاری نەخۆشیەکان) بۆ سالی خوێندنی (٢٠٢١- ٢٠٢٢) بەمەبەستی ئەنجام دانی پرۆژەی توێژینەوەی زانستی وە وەرگرتنی داتا سامپلی خوێن بەمەرجێك نابێت موادی تاقیگە بەکاربهێندرێت.
لەگەڵ ریزماندا .....

دکتۆر
دلۆڤان محمدفاتح جلال
بەڕێوەبەری گشتی

وێنەیەك بۆ /
- نووسینگەی بەڕێز بەڕێوەبەری گشتی // بۆ زانیین لە گەڵ ڕێزماندا. ..
- بەشی کاروباری هونەری // هۆبەی تاقیگەکان...
- زانکۆی پۆلۆتەکنیکی هەولێر//کۆلێژی تەکنیکی تەندروستی وپزیشکی//بۆزانین لەگەڵ ڕێزماندا....

A3

Table. Results of the NanoDrop for the 70 samples in the present study.

Sample No.	Sample Code	DNA Concentration ng/μL	260/280
1	D435	105	1.71
2	D436	201	1.77
3	D437	76	1.81
4	D438	106	1.72
5	D439	132	1.69
6	D440	83	1.72
7	D441	211	1.7
8	D442	96	1.68
9	D443	87	1.73
10	D444	106	1.82
11	D515	109	1.71
12	D516	261	1.69
13	D517	201	1.72
14	D518	208	1.7
15	D519	201	1.79
16	D520	153	1.81
17	D521	126	1.73
18	D522	174	1.78
19	D523	128	1.7
20	D524	98	1.69
23	D525	65	1.73
24	D526	109	1.78
25	D527	204	1.72
28	D528	106	1.8
30	D529	123	1.72
31	D530	261	1.73
32	D531	148	1.78
33	D532	129	1.7
34	D533	112	1.72
35	D534	109	1.72
36	Zh01	201	1.72
37	Zh02	109	1.78
38	Zh03	174	1.78
39	Zh04	211	1.7
40	Zh05	109	1.71
41	Zh06	105	1.78
42	Zh07	120	1.71
43	Zh08	150	1.8
44	Zh09	102	1.73
45	Zh10	202	1.81
46	Zh11	113	1.74
47	Zh12	89	1.76

49	Zh13	208	1.83
50	Zh14	254	1.73
51	Zh15	91	1.7
52	Zh16	212	1.82
53	Zh17	85	1.71
54	Zh18	206	1.78
56	Zh19	135	1.9
57	Zh20	160	1.72
58	Zh21	174	1.78
59	Zh22	151	1.9
60	Zh23	112	1.74
61	Zh24	149	1.86
62	Zh25	88	1.72
63	Zh26	156	1.8
64	Zh27	91	1.69
65	Zh28	135	1.76
66	Zh29	111	1.74
67	Zh30	95	1.8
68	Zh31	102	1.74
69	Zh32	79	1.72
70	Zh33	201	1.86
71	Zh34	105	1.72
73	Zh35	126	1.72
74	Zh36	113	1.8
75	Zh37	98	1.76
76	Zh38	220	1.9
77	Zh39	105	1.74
78	Zh40	176	1.82

Note: sample number: 21, 22, 26, 27, 29, 48, 55, and 72 were excluded and replaced by other samples, that's why the order is 78 but the total is 70 samples.

			exon	target	PCR product
Exon	1	100			
Intron	1	1.155			
Exon	2	99	99	683	
Intron	2	8.237			
Exon	3	54	54	399	
Intron	3	9.192			
Exon	4	78		524	
Intron	4	1.499			
Exon	5	89			
Intron	5	606	730	832	
Exon	6	140			
Intron	6	4.241			
Exon	7	105	105	281	
Intron	7	2.485			
Exon	8	46			
Intron	8	605	728	804	
Exon	9	77			
Intron	9	985			
Exon	10	3.426	3.426	3.55	
Intron	10	402			
Exon	11	890	890	1.940	
Intron	11	8.368			
Exon	12	172	172	470	
Intron	12	5.789			
Exon	13	127	127	622	
Intron	13	1.966			
Exon	14	191	191	694	
Intron	14	3.092			
Exon	15	311	311	783	
Intron	15	3.232			
Exon	16	88	88	593	
Intron	16	3.656			
Exon	17	78			
Intron	17	500	619	904	
Exon	18	41			
Intron	18	6.197			
Exon	19	84	84	448	
Intron	19	5.934			
Exon	20	55	55	480	
Intron	20	1.868			
Exon	21	74			
Intron	21	1.417	1.552	1.740	
Exon	22	61			
Intron	22	1.157	1.157	1.357	
Exon	23	1.508	1.508	456	

(A)

		exon	target	PCR product
Exon	1	194		
Intron	1	754		
Exon	2	105	105	483
Intron	2	2.549		
Exon	3	249	249	706
Intron	3	5.750		
Exon	4	109	109	602
Intron	4	916		
Exon	5	50	50	820
Intron	5	91		
Exon	6	41	41	422
Intron	6	216		
Exon	7	115	115	644
Intron	7	2.829		
Exon	8	50	50	390
Intron	8	1.426		
Exon	9	112	112	630
Intron	9	1.241		
Exon	10	1.116	1.116	1.861
Intron	10	2.877		
Exon	11	4.932	4.932	5.947
Intron	11	3.361		
Exon	12	96	96	639
Intron	12	2.173		
Exon	13	70	70	650
Intron	13	7.964		
Exon	14	428	428	689
Intron	14	1.139		
Exon	15	182	182	483
Intron	15	1.132		
Exon	16	188	188	976
Intron	16	4.593		
Exon	17	171	171	904
Intron	17	485		
Exon	18	355	355	420
Intron	18	6.868		
Exon	19	156	156	844
Intron	19	398		
Exon	20	143	143	604
Intron	20	5.569		
Exon	21	122	122	537
Intron	21	2.525		
Exon	22	199		
Intron	22	234	597	748
Exon	23	164		
Intron	23	93		
Exon	24	139	139	944
Intron	24	14.54		
Exon	25	243	243	930
Intron	25	1.964		
Exon	26	147	147	841
Intron	26	1.117		
Exon	27	1.049	1.049	1.170

(B)

Fig.: (A): *BRCA1* exons and their sizes of PCR product, (B): *BRCA2* exons and their sizes of PCR products. As provided by the supplier (INTERGEN).

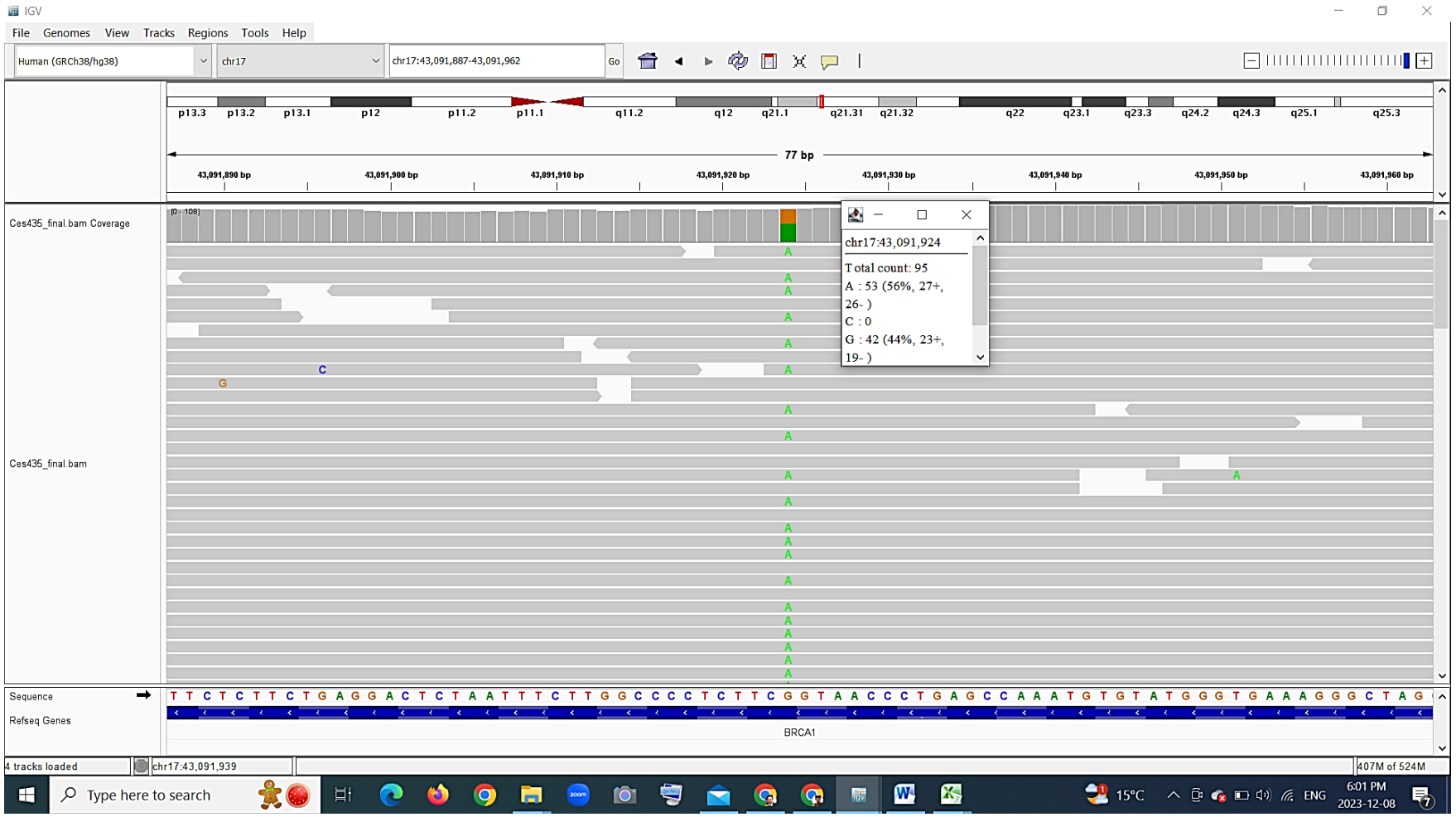

Fig. Pathogenic variant of (c.3607C>T) on *BRCA1* gene, located at chr17:43,091,924 by hg38, viewed by Integrative Genomic Viewer (IGV), C is substituted by T. As the sequence is reverse, G on the reference sequence is (C), and A is (T).

A6

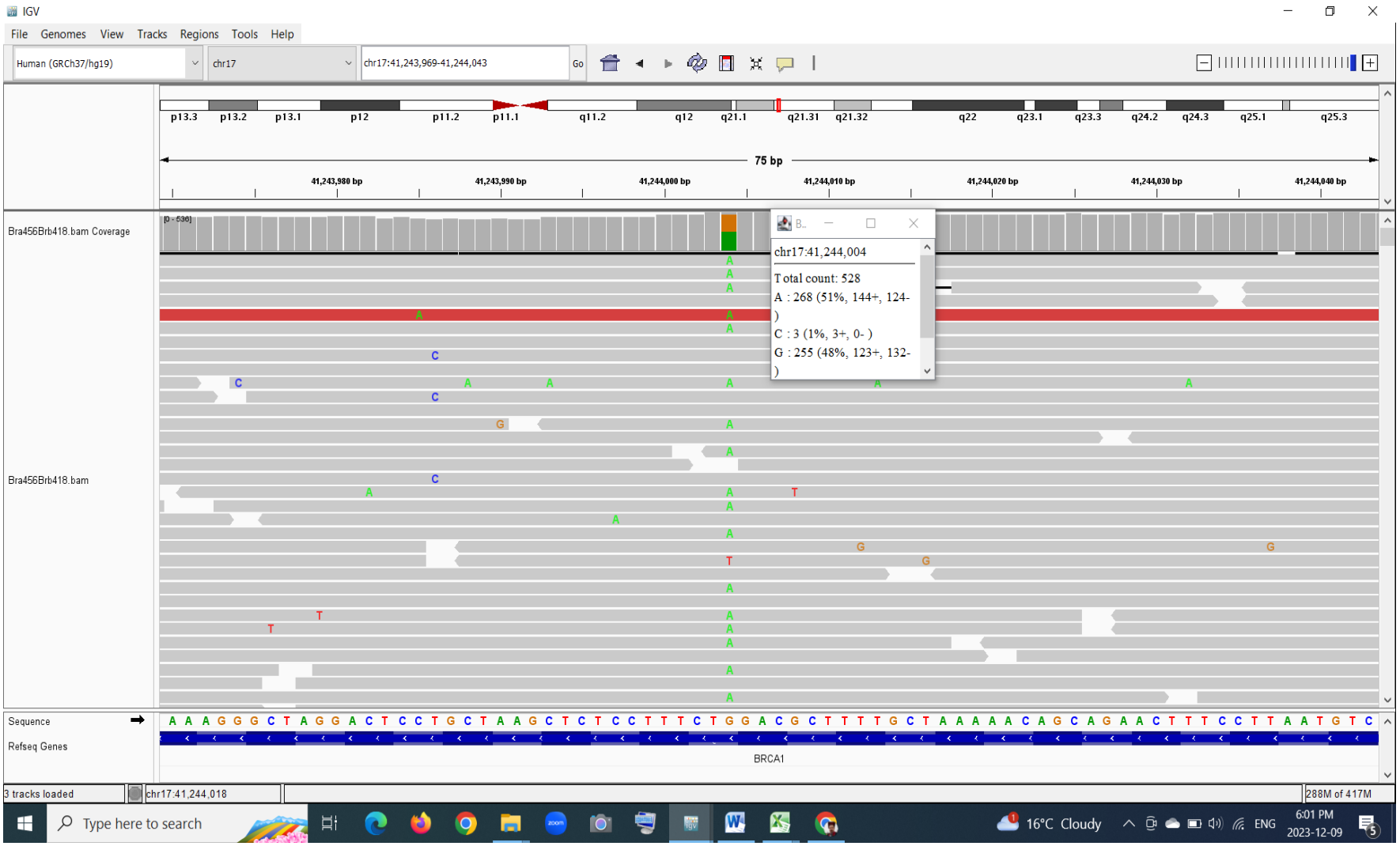

Fig. Pathogenic variant of (c.3544C>T) on *BRCA1* gene, located at chr17:41,244,004 by hg37, viewed by Integrative Genomic Viewer (IGV), nucleotide C substituted with T. As the sequence is reversed, G on the reference sequence is (C), and A is (T).

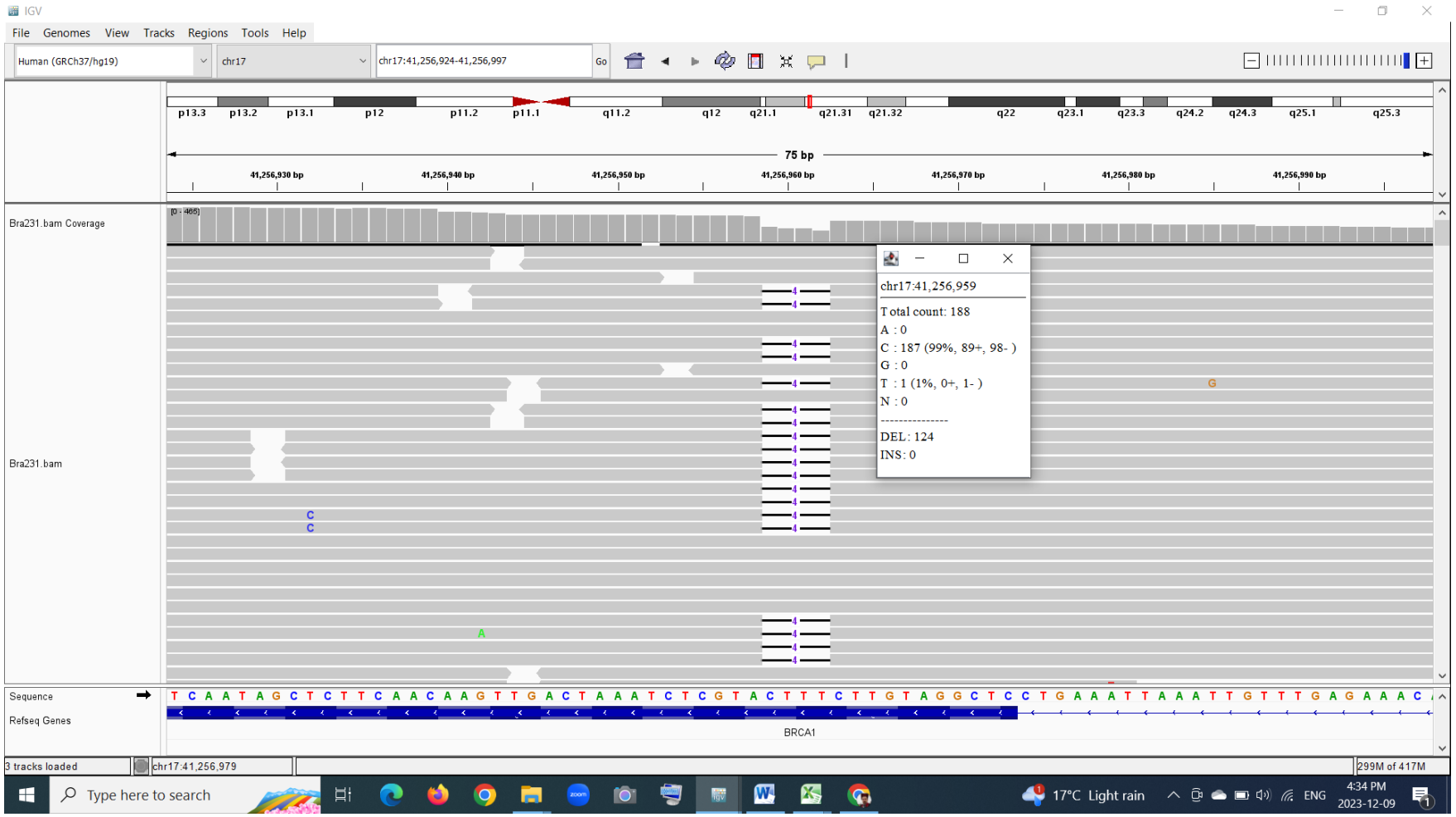

Fig. Pathogenic variant of (c.224_227del) on *BRCA1* gene, deletion of 4 base pairs located at chr17:41,256,959-41256962 by hg37, viewed by Integrative Genomic Viewer (IGV), as the sequence is reversed, the deleted nucleotides are: AAAG.

A8

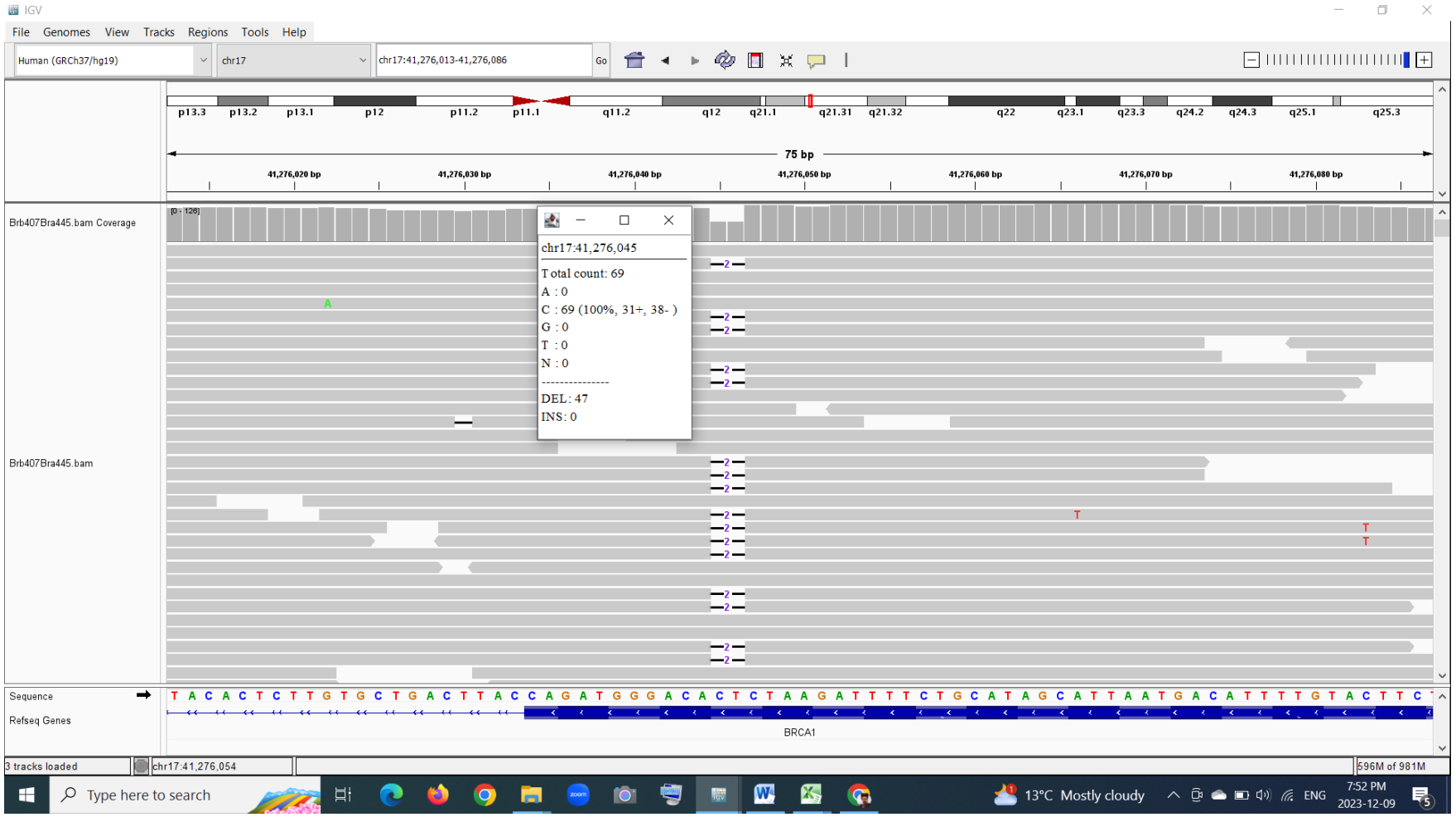

Fig. Pathogenic variant of (c.68_69del) on *BRCA1* gene, deletion of 2 base pairs located at chr17:41,276,045-41,276,047 by hg37, viewed by Integrative Genomic Viewer (IGV), as the sequence is reverse, the deleted nucleotides are: AG.

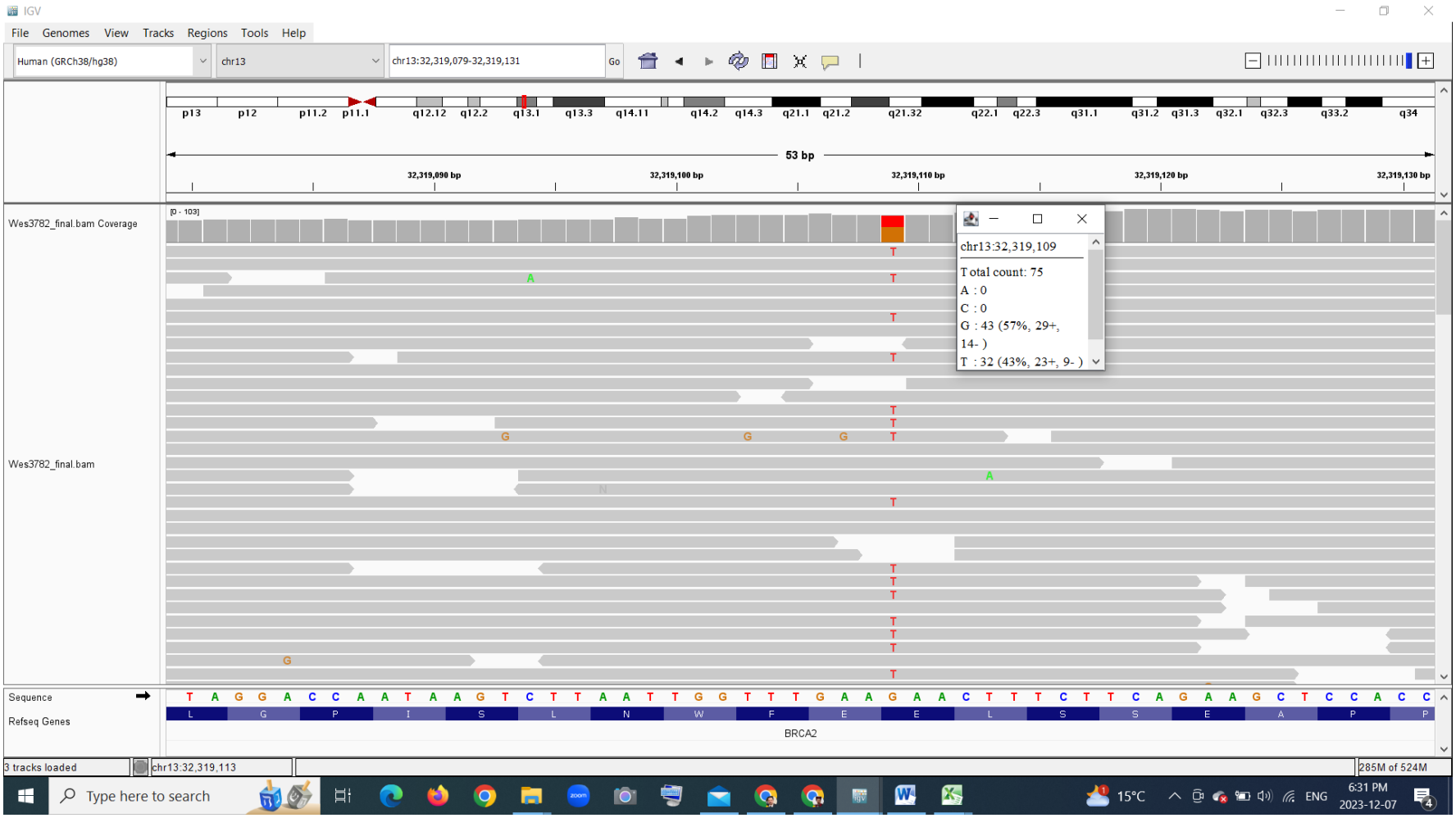

Fig. Pathogenic variant of (c.100G>T) on *BRCA2* gene, substitution on one bp. located at chr13:32,319,109 by hg38, viewed by Integrative Genomic Viewer (IGV), nucleotide G substituted by T.

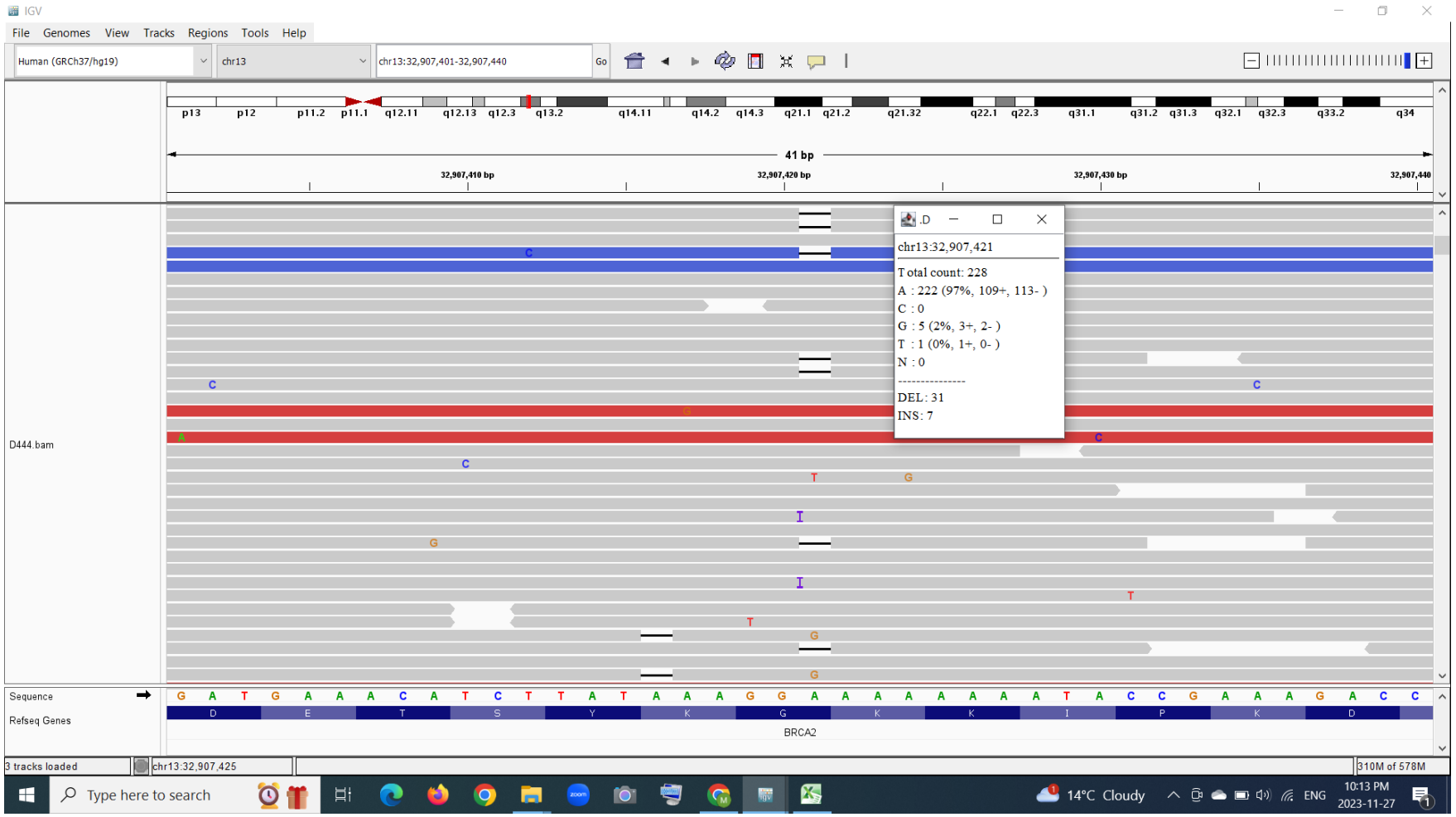

Fig. Pathogenic variant of (c.1813delA) on *BRCA2* gene, deletion of 1 base pair located at chr13:32,907 by hg37, viewed by Integrative Genomic Viewer (IGV), the deleted nucleotide is: A.

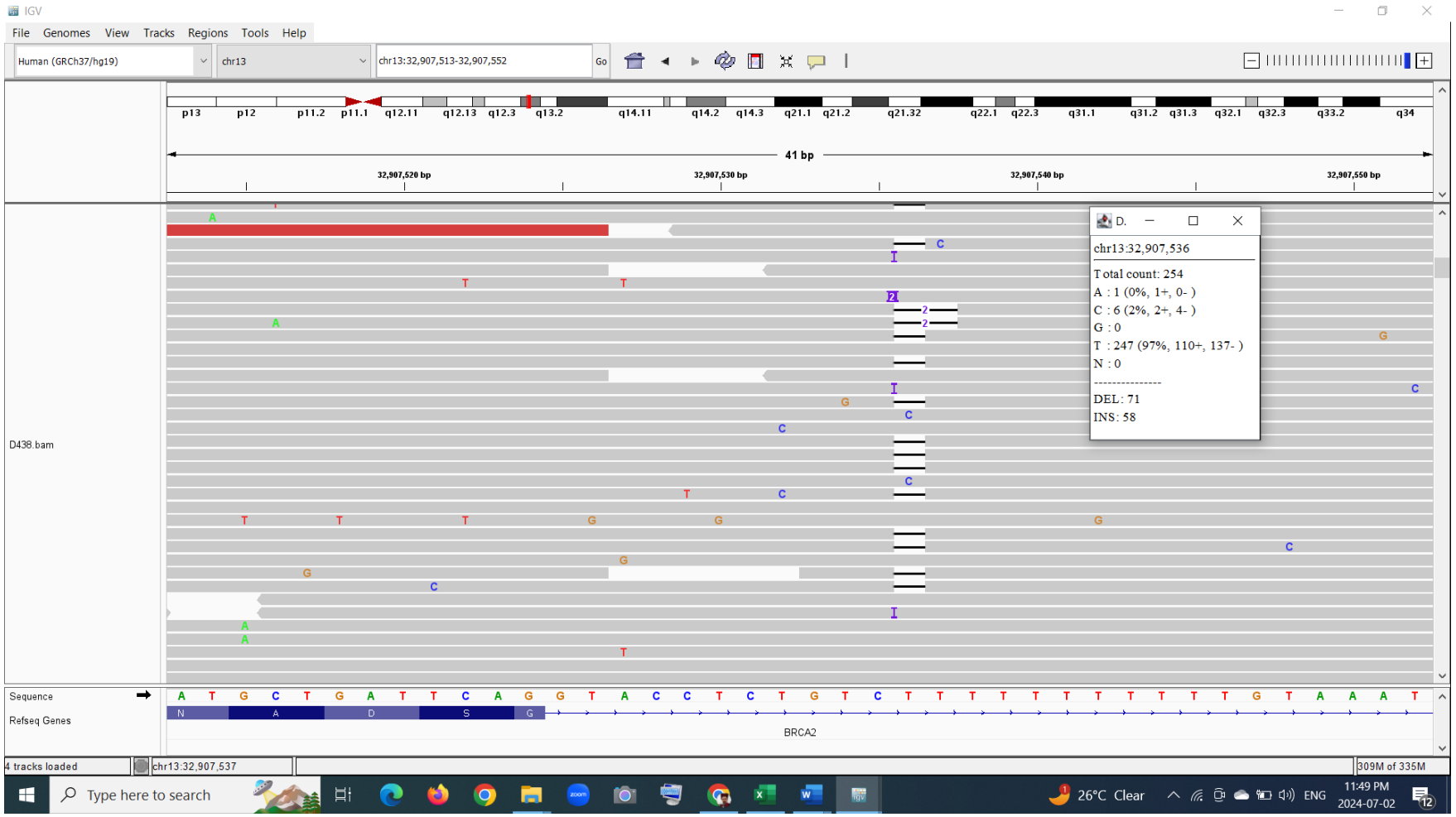

Fig. Variant of conflict interpretation of pathogenicity, (c.1909+22del) on *BRCA2* gene, deletion of 1 base pair located at chr13:32,907,535-32907536 by hg37, viewed by Integrative Genomic Viewer (IGV), the deleted nucleotide is: T.

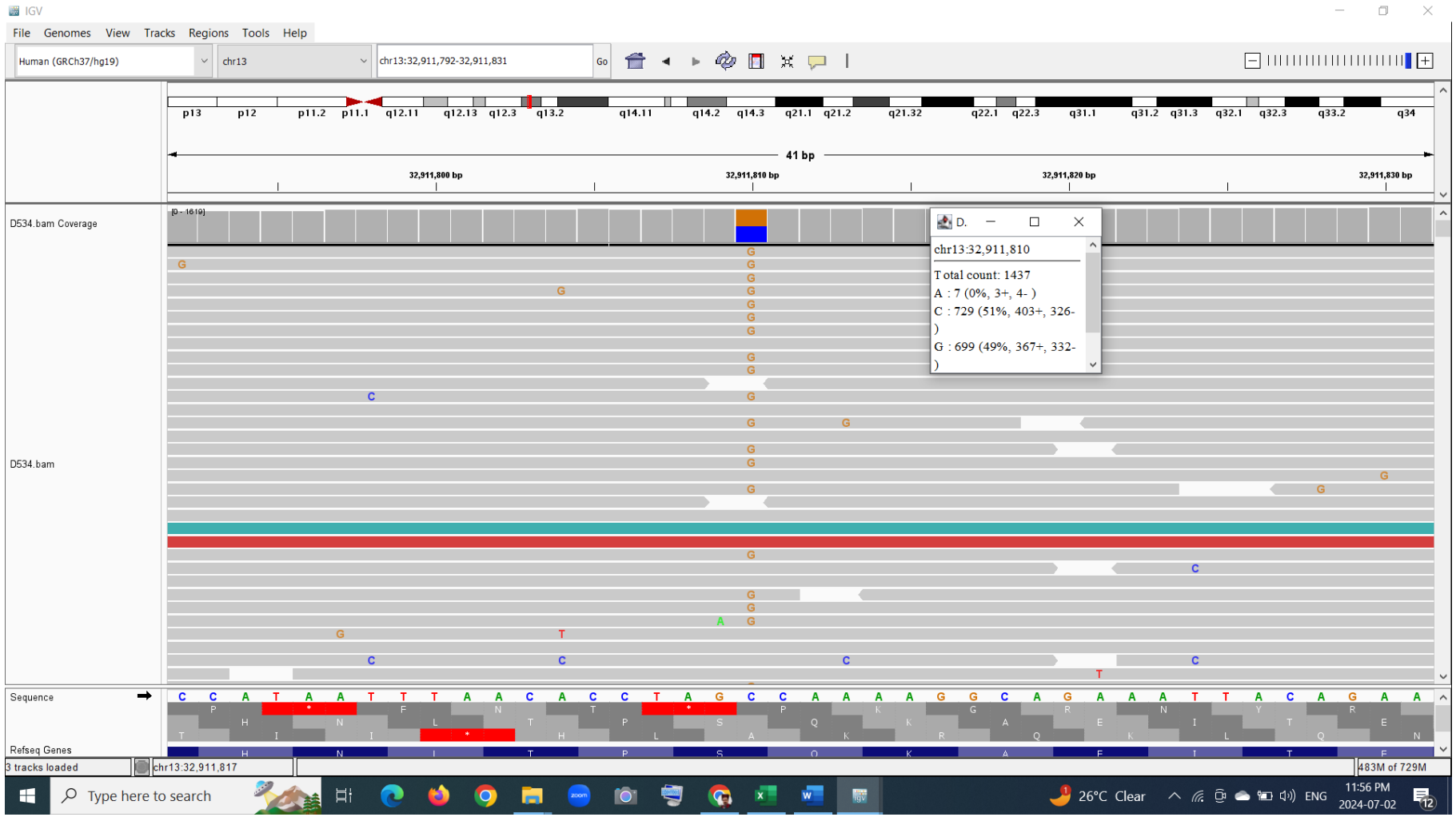

Fig. variant of conflict interpretation of pathogenicity, (c.3318C>G) on *BRCA2* gene, substitution on one bp. located at chr13:32,911,810 by hg37, viewed by Integrative Genomic Viewer (IGV), nucleotide C substituted with G.

A13

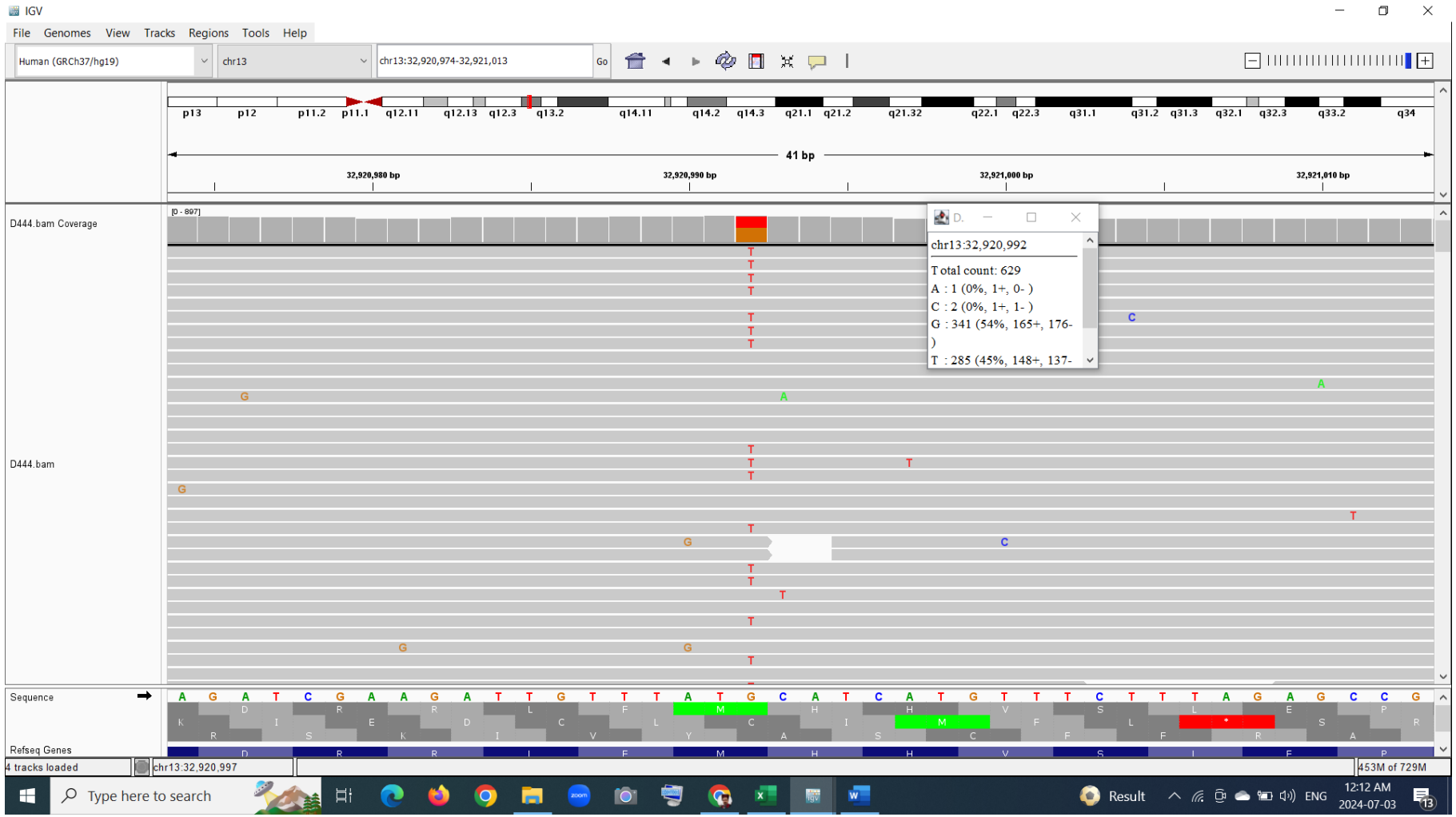

Fig. variant of uncertain significant of (c.6966G>T) on *BRCA2* gene, substitution of 1 base pair located at chr13:32,920,992 by hg37, viewed by Integrative Genomic Viewer (IGV), the G nucleotide substituted to T.

Cureus Journal of Medical Science

Jun 11, 2024

Hi Ahmad,

On behalf of the Cureus Journal of Medical Science, I hereby confirm the acceptance and publication of your article entitled Breast Cancer High-Penetrance Genes BRCA1 and BRCA2 Mutations Using Next-Generation Sequencing Among Iraqi Kurdish Women. This article has been peer-reviewed and will be indexed in PubMed Central after publication.

Title: Breast Cancer High-Penetrance Genes BRCA1 and BRCA2 Mutations Using Next-Generation Sequencing Among Iraqi Kurdish Women

Authors: Ahmad N. Hassan, Mustafa S. Mustafa

Publication date: June 11, 2024

DOI: 10.7759/cureus.62160

Best regards,

Graham Parker-Finger
Director of Editorial Operations

Cureus
Part of SPRINGER NATURE

Open Access Original Article

DOI: 10.7759/cureus.62160

Breast Cancer High-Penetrance Genes BRCA1 and BRCA2 Mutations Using Next-Generation Sequencing Among Iraqi Kurdish Women

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Review ended 06/01/2024
Published 06/11/2024

Abstract

Background

BRCA1 and *BRCA2* genes are the main high-penetrance genes that are responsible for most cases of inherited breast cancer. The present study aimed to detect the frequencies of inherited breast cancer caused by *BRCA1* and *BRCA2* genes among Kurdish breast cancer patients, including all the exome of these two genes, using next-generation sequencing (NGS).

Methodology

Seventy women who were diagnosed with breast cancer and registered at Nanakali Hospital in Erbil, Iraq, were included. Blood samples were collected for molecular testing (polymerase chain reaction (PCR)) targeting all exomes of *BRCA1* and *BRCA2* genes. All exome regions are sequenced by NGS using the Miseq system (Illumina Inc., San Diego, CA). Obtained data were visualized using Integrative Genomics Viewer (IGV 2.3 Software, Broad Institute, Cambridge, MA). Data were interpreted based on the National Center for Biotechnology Information (NCBI), Clinically Relevant Variation (ClinVar) archives, and other databases.

Results

Among 70 samples, more than forty-two variants have been detected, 20 on *BRCA1* and 22 on *BRCA2*. Regarding clinical significance, six (14.28%) variants were pathogenic, four of them on the *BRCA1* gene, which were: c.3607C>T, c.3544C>T, c.68_69del, and c.224_227delAAAG, and two pathogenic variants were on *BRCA2* gene: c.100G>T, and c.1813delA. Also, two (4.76%) variants were conflict interpretations of pathogenicity, one (2.38%) was a variant of uncertain significant VUS, and the rest 29 (69%) variants were benign. In addition, four new variants (three in *BRCA1* and one in *BRCA2* gene), never previously reported, were identified.

Conclusions

In conclusion, analyzing the *BRCA1/2* genes provide a better prediction for the risk of developing breast cancer in the future. Variant types and frequencies differ among different populations and ethnicities, the common mutations worldwide may not be prevalent in the Kurdish population. The current research findings will be useful for future screening studies of these two genes in the Kurdish population.

Categories: Genetics, Obstetrics/Gynecology, Oncology
Keywords: kurdish population, variants, ngs, brca2, brca1, breast cancer

Introduction

Breast cancer is a type of cancer that forms in the cells and tissues of the breasts. It is the most common type of cancer among women, and it affects one in every eight to 10 women during their lifetime [1,2]. Breast cancer is caused mainly by non-genetic factors, while hereditary factors contribute to 5%-10% of the cases. Genetic factors refer to the inheritance of an abnormal (mutated) form of a susceptible gene; most inherited cases of this cancer result from mutations in genes that are linked to the breast [3,4].

BRCA1/2 genes have expanded the knowledge of familial breast cancer, and *BRCA* genes are responsible for cell growth, division, and repair of damaged DNA. Their function is to keep the normal growth of breast, ovarian, and other cells. Altered forms of these genes cannot function normally and subsequently may lead to breast, ovarian, prostate, and colon cancer. In inherited breast cancer, these two genes are the most common causes; they may account for up to 10% of all cases [4-6].

Mutations in the *BRCA1* gene cause early-onset hereditary breast cancers with an estimated risk of 57% to 81% and cause hereditary ovarian cancers with an estimated risk of 90% in high-incidence families of breast