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Correlation Between TNRC9 Polymorphisms and BRCA1 Gene Promoter Methylation Status in Palestinian Breast Cancer Patients

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Thesis Approval

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Declaration

I declare that, except where explicit reference is made to the contribution of others, this dissertation is substantially my own work and has not been submitted for any other degree at the Arab American University or any other institution.

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Dedication

﴿قُلْ إِنَّ صَلَاتِي وَنُسُكِي وَمَحْيَايَ وَمَمَاتِي لِلَّهِ رَبِّ الْعَالَمِينَ﴾

To my beloved husband, whose unwavering support, patience and encouragement have been my anchor on this journey.

To my family, whose love, sacrifice and constant faith in me have been my greatest source of strength and inspiration.

This work is dedicated to all of you, with deepest gratitude and infinite love

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Abstract

Breast cancer continues to be a major public health problem worldwide, with genetic and epigenetic factors playing a major role in its pathogenesis. Inactivation of the BRCA1 gene, mainly due to germline mutations, is a major risk factor for breast cancer. However, BRCA1 mutations are less common in the Palestinian population, suggesting that other mechanisms, such as epigenetic alterations, may play a role.

This study investigated the association between single nucleotide polymorphisms (SNPs) in the TNRC9 (TOX3) gene (rs3803662, rs8051542 and rs12443621) and BRCA1 promoter methylation in a cohort of Palestinian breast cancer patients. Genotyping was performed by PCR-RFLP, ARMS and sanger sequencing.

Our analysis revealed that rs3803662 is significantly associated with increased BRCA1 promoter methylation, suggesting a possible epigenetic mechanism by which this SNP influences BRCA1 silencing and breast cancer risk. This association was particularly pronounced in younger patients (<50 years), who had a higher frequency of the C allele of rs3803662. In contrast, SNPs rs8051542 and rs12443621 showed no significant correlation with BRCA1 methylation, highlighting the specific role of rs3803662 in epigenetic regulation. Linkage disequilibrium (LD) analysis showed minimal non-random association between the SNPs, supporting their independent effects on BRCA1 regulation.

Haplotype analysis revealed that haplotype 4 (T-C-G) was significantly associated with reduced BRCA1 methylation, suggesting a possible protective effect. However, other haplotypes showed limited or no significant associations. The observed high prevalence of BRCA1 promoter methylation in the Palestinian cohort compared to other

populations underscores the critical importance of considering population-specific epigenetic landscapes in breast cancer research.

Our results suggest that the rs3803662 SNP in TOX3 may contribute to increased BRCA1 promoter methylation and breast cancer susceptibility in the Palestinian population. This study highlights the importance of considering both genetic and epigenetic factors to understand breast cancer risk in this population. Further research is needed to clarify the underlying mechanisms and their clinical implications. A deeper understanding of these mechanisms has the potential to improve risk assessment strategies and support the development of personalized therapeutic interventions for breast cancer.

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List of Abbreviations

Abbreviation	Definition
APC	Adenomatous polyposis coli
ARH1	ADP-ribose-acceptor hydrolase
ATM	Ataxia-Telangiectasia Mutated
ATR.	Ataxia telangiectasia and Rad related protein
BACH1	Brain Acyl CoA Hydrolase
BARD1	BRCA1 Associated RING Domain 1
BC	Breast Cancer
BCL2	B-cell lymphoma 2
BLBC	Basal- like breast cancers
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
BRCT.	Domain BRCA1C-Terminus Domain
BRIP1	BRCA1 Interacting Protein C-Terminal Helicase 1
CCDC98.	Coiled-Coil Domain Containing 98
CCND2.	Cyclin D2
CGIs	CpG islands
cMYC	Cellular Myc
CpG	Cytocine Guanine (linear dinucleotide)
CREB.	cAMP response element-binding protein
CSCs	Cancer stem cells

CtIP	C-terminal–binding protein–interacting protein
Cyclin D2	G1/S-specific cyclin-D2
CYP1B1.	Cytochrome P450 1B1
DCIS	Ductal Carcinoma In Situ
DNMTs.	DNA methyltransferases
DSB.	Double Strand Break
DSBs	DNA double-strand breaks
E3	Ubiquitin Ligase Enzyme
ER	Estrogen Receptor
ESR1	Estrogen Receptor 1 gene
FGFR2	Fibroblast growth factor receptor 2
FFPE	Formalin fixed paraffin embedded
GEP	Ggene expression profiling
GSTP1.	Glutathione S-Transferase Pi 1
GWAS.	Ggenome-wide association studies
HDACs.	Histone Deacetylases
HER2	Human Epidermal Growth Factor Receptor 2
hMLH1.	Human mutL Homolog 1
HR	Homologous Recombination
HWE:	Hardy–Weinberg Equilibrium
ILC	Invasive lobular Carcinoma
LCIS.	Lobular Carcinoma In Situ
LOH	Loss Of Heterozygosity
LSP1	Leukocyte-specific protein 1

m5C	5methylcytocine
MAP3K1	Mitogen-activated protein kinase 1
MBD	Methylated CpG Binding domain
MBD4	Methyl-CpG-binding domain protein 4
MreII.	Meiotic Recombination 11 Homolog A
Myc.	Myelocytomatosis oncogene
NES	Nuclear Export Sequence
NHEJ	Non-Homologous End joining
NLS	Nuclear Localization sequence
NST	Invasive Carcinoma of no Special Type
P16INK4A	cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1
p16INK4α.	Cyclin-dependent kinase inhibitor 2A
PALB2	partner and localizer of BRCA2
PCR	Polymarase Chain Reaction
PI3K	phosphoinositide 3-kinase
PI3KC3	Phosphatidylinositol 3-Kinase catalytic subunit
PR	Progestrone Receptor
PRB	Phosphorylated retinoblastoma
pSXXF	phosphorylated protein sequencemotif
PTEN	phosphatase and tensin homolog
Rad 50	Radiation sensitive 50
Rad 51	Radiation sensitive 51
RARβ.	Retinoic Acid Receptor Beta

RAS.	Rat Sarcoma virus oncogene
RASSF1A.	Ras association domain family member 1
RB	Retinoblastoma
RFLP	Restriction Fragment Length Polymorphism
RON	Receptor tyrosine kinase
SCD	Serine-cluster domain
SNP	Single Nucleotide Polymorphisms
TNBC	Triple Negative Breast Cancer
TNRC9	Trinucleotide-repeat-containing 9
TSG	Tumor suppressor genes
Ub	Ubiquitin
UbcH5	Ubiquitin-Conjugating Enzyme H5
WHO	World Health Organization

Chapter 1: Introduction

1.1 Breast Cancer & Epidemiology

Breast cancer (BC) is a major global health problem, as it is the number one cancer in terms of both incidence and mortality among women worldwide (Sung et al., 2021a). In 2023, approximately 2.3 million women diagnosed with breast cancer, accounting for 12% of all new cancer cases worldwide(WHO, 2024). This disease can affect women in all countries at any age after puberty, with incidence rates rising particularly sharply in the older age groups. According to forecasts, the prevalence of breast cancer will increase and reach around 3 million new cases annually by 2040, accompanied by an estimated one million deaths. This escalation is primarily due to population growth and aging of the population(Arnold et al., 2022).

The incidence of breast cancer is generally lower in Arab countries than in Europe and the USA(Mokdad et al., 2014; Salim et al., 2010). The age-standardized breast cancer rate for women in Arab countries is 37 cases per 100,000, in contrast to the global rate of 56 cases per 100,000(Zahedi et al., 2020). Statistics also show that women in their thirties in this region are more likely to develop breast cancer than their counterparts in the western world. Egypt, Lebanon and Tunisia have the highest rates of the disease, with 58,44 and 39 cases per 100,000 women respectively(Karim et al., 2015; Lakkis et al., 2010; Mehdi et al., 2014; Najjar & Easson, 2010; Tas & Keskin, 2012).

The available data show that the incidence of breast cancer in the Arab world has increased over the last two decades and the mortality rate is higher compared to the Western world(Hashim et al., 2018a). Furthermore, the average age of breast cancer diagnosis among women in the Arab world is 50 years, with one third of women being

diagnosed at the age of 40 or younger(El Chediak et al., 2017; El Saghir et al., 2007; Najjar & Easson, 2010). However, accurate knowledge of the prevalence and incidence of breast cancer in the Arab world is not available as most countries do not have comprehensive cancer registries. The lack of death registries and specific records of breast cancer-related deaths also limit the overall understanding of the disease in the region. Information on breast cancer in the Arab world comes primarily from literature reviews of published data from individual countries, including retrospective data reports and records from various institutions, many of which are high-quality reports published in peer-reviewed journals. In addition, the rate of increase in breast cancer incidence in the Arab countries appears to be consistent with the global trend (Hashim et al., 2018b)with the incidence of breast cancer is predicted to double by 2030(Taha & Eltom, 2018).

In Palestine, breast cancer is the fifth overall cause of death for females with a weighted age rate of 10/100000(PHIC, 2023). Ten percent of the newly diagnosed cancer cases each year are breast cancer(PHIC, 2023). Unfortunately, most cases of BC are diagnosed in later stages. Current data revealed only 40% of cases were diagnosed at an early stage which is much lower than the World Health Organization (WHO) target of 60%(PHIC, 2023). The average age of breast cancer diagnosis between 2017 and 2021 was 53 years, with most cases occurring between the ages of 40 and 64 (63.4%) of cases(PHIC, 2023). Survival statistics for breast cancer in Palestine have not been updated since 2011, and is predicted to be around 40%, significantly lower compared to 90% in other countries (Joan Jubran, 2019). It is interesting to indicate the five-year survival rate for breast cancer patients in Gaza was 65.1 %between 2005 and 2014 (Panato et al., 2018). The lack of effective programs for early diagnoses with restricted movement of patients combined with the limited availability of essential medicines and deficiencies in the

healthcare system in the Occupied Palestinian Territories, poses a significant barrier for quality treatment and care for Palestinian women diagnosed with breast cancer (MAP, 2017).

1.2 Classification of Breast Cancer Subtypes

1.2.1 Histologic classification of breast cancer

Breast cancer is characterized by its heterogeneity and manifests itself in different histopathological subtypes. It is considered a complex disease with various biological and morphological features(Abramson et al., 2015). According to the categorization of WHO, there are numerous histopathological subtypes of breast cancer, which are distinguished by differences in cell morphology, growth characteristics and architectural arrangement(Lakhani et al., 2012). Recently, the understanding of the molecular biology of breast cancer has improved significantly. Various strategies have been used to categorize breast cancer in order to develop more precise biological profiles of breast tumors(Gannon et al., 2013). Nevertheless, the conventional morphological classification continues to serve as the primary defined classification and has undergone revisions by integrating molecular and genetic information into the morphologically defined framework(Cserni, 2020; Sinn & Kreipe, 2013).

The predominant histologic subtype is invasive carcinoma of no special type (NST), commonly known as invasive ductal carcinoma, which accounts for approximately 75% of all cases of invasive breast cancer(Weigelt, Geyer, et al., 2010). In addition, the most recent version of the WHO classification identified at least 17 unique histologic specialty types, including invasive lobular carcinoma (ILC). These special types account for up to 25% of all breast cancer cases (Eroles et al., 2012). Only a small

percentage of breast cancers are classified as rare subtypes, making it difficult to collect comprehensive data on their clinical behavior and prognosis. As a result, there is uncertainty and conflicting evidence about the outcomes associated with these particular histologic subtypes(Hoda & Kaplan, 2013).

Only morphological categorization (including nuclear grade, tubular grade, mitotic index, histological grade and architectural characteristics) and clinico-pathological factors (including tumor size, lymph node involvement, metastasis) prove to be insufficient in predicting the authentic behavior of breast tumor pathophysiology(Fragomeni et al., 2018; Masood, 2016).

Breast cancer classification is evolving from traditional morphological assessment to more sophisticated systems for appropriate patient management, which requires precise and meaningful tumor classification. Molecular classification of breast cancer refers to the specific phenotype of cancer cells and the characteristics and actions exhibited by cancer cells. The improved understanding of the different classifications of breast cancer has enabled researchers to develop precise therapeutic strategies which allowed identification the specific type of cancer that responds to certain treatments in order to improve outcomes.

1.2.2 Molecular classification of breast cancer

High-throughput methods have highlighted the molecular diversity in breast cancer and have led to change in the basic principles of breast cancer research. The use of microarrays has facilitated the incorporation of gene expression profiling (GEP) into breast cancer prognostic testing(Weigelt, Baehner, et al., 2010). Using 456 cDNA clones, a unique "molecular profile" of breast cancer was established, leading to

classification of tumors into five intrinsic subtypes with different clinical prognosis, namely luminal A, luminal B, human epidermal growth factor receptor 2(HER2-) overexpressing, basal-like, and normal-like tumors(Perou et al., 2000; Sørlie et al., 2001). According to St. Gallen Consensus 2011, breast cancer can be divided into several molecular subtypes based on certain characteristics: Luminal A tumors are estrogen receptor-positive (ER+), progesterone receptor-positive (PR+), HER2-negative (HER2-) with low Ki-67 levels; Luminal B tumors are also ER+ and PR+, but may be HER2-positive (HER2+) or have high Ki-67 levels; HER2-overexpressing tumors are ER-negative (ER-) and PR-negative (PR-) with HER2-positivity (HER2+); and triple-negative breast cancers (TNBC) with ER-, PR- and HER2-negative (HER2-)(Goldhirsch et al., 2011). The basal-like subtype of breast cancer, commonly referred to as TNBC, is characterised by positive expression of basal markers such as CK5/6.(Elesawy et al., 2014; M. Zhang et al., 2012)These studies have further clarified the importance of immunophenotypic classification based on hormone receptor expression and HER2 status.

ER expression divides breast cancer cases into two different groups: ER+ and ER−. The Luminal A and B subcategories consisted predominantly of ER-positive tumors, while the HER2- overexpressing, basal- like breast cancers (BLBC) and normal-like tumors are ER−. In addition to ER and ER-related genes, each subtype exhibited different levels of gene expression related to proliferation, HER2 amplicon, and myoepithelial cells. These intrinsic subtypes differed in their frequency of occurrence as well as in their clinical and pathologic features and are largely consistent with established clinical and histopathologic classifications(Perou et al., 2000).

Detection of ER, PR and HER2 is usually carried for consideration in the treatment of breast cancer. These markers help predict how cancer will behave and respond to certain treatments such as hormone therapy and targeted therapies. The nuclear sex steroid receptors ER and PR are responsible for the growth of normal and neoplastic breast epithelial cells(Hammond et al., 2010). Most cancers that are positive for ER and the PR are usually less aggressive and have lower grade. Most ER-positive cancers are usually also PR-positive. However, there is a small subset of breast cancers that are only positive for one hormone receptor. These cancers are thought to be more aggressive and less responsive to hormone therapy compared to ER/PR-positive cancers(Cui et al., 2005; Ethier et al., 2018).

Around 15% of breast cancer cases show Overexpression of HER2, (Slamon et al., 1987). Which is associated with severe disease progression and unfavorable prognosis, but with potentially favorable response to therapies targeting HER2(Bedard et al., 2009). Approximately 10% to 15% of breast cancer cases are TNBC. TNBC is a subset of breast cancer with no receptors for estrogen, progesterone or HER2. TNBC is usually high grade and has poor prognosis since current targeted therapies are not effective for this type of breast cancer(Yam et al., 2017).

1.3 Hereditary Breast Cancer

The main reason for familial breast cancer is the inheritance of an abnormal gene. If this gene is inherited from one parent, there is a 50% chance that it will be passed on to the child, also known as germline mutations or variants(Brody & Biesecker, 1998). If a woman inherits one of these genes, the likelihood that she will develop breast cancer at younger age, usually under 40, increases significantly(Huber-Keener, 2022). In addition,

the risk of developing ovarian cancer also increases. The most common cases are caused by mutations in the Breast cancer 1(BRCA1) and Breast cancer 2 (BRCA2) genes. If a woman inherits a mutation in one of these genes, her risk of developing breast cancer can be as high as 80%(King et al., 2003; Lindor et al., 2008). The prevalence of germline mutations in breast cancer depends on several factors, including the population being studied, the practice of genetic testing and the specific genes being analyzed. A mutation in these genes is clinically responsible for 5–10% of all breast malignancies and a significant proportion of familial breast and ovarian cancers(Armaou et al., 2009). In addition, carriers of the mutation in the BRCA1 or BRCA2 genes not only have an increased risk of developing breast cancer, but also an increased susceptibility to various other cancers such as colorectal, prostate, pancreatic, melanoma and stomach cancer(Dutil et al., 2012; Jancarkova et al., 2003).

Understanding the incidence of hereditary breast cancer in different racial and geographic contexts worldwide is critical to evaluate its public health significance. Figuring out how genetic and environmental factors interact to cause differences in the prevalence of this genetic disease will aid in research on factors that can be modified to reduce the likelihood of breast cancer in individuals with the gene. An appreciation of how genetic and environmental factors interact to cause differences in the prevalence of this hereditary disease will facilitate studies on modifiable factors that could reduce the incidence of breast cancer in gene carriers(Hu et al., 2021; Kashyap et al., 2022; Yoshida, 2021).

BRCA1 and BRCA2 mutations associated with breast cancer are observed in different populations. Research suggests that the Ashkenazi, a group of Eastern European

Jews, may have a higher prevalence of BRCA1 and BRCA2 mutations than the broader U.S. population(Levy-Lahad et al., 1997; Struewing et al., 1997).

The West Bank and the Gaza Strip are the two areas at the center of the Palestinian conflict. The women living in these areas suffer from great poverty, constant violence, disruption of daily life and permanent violations. This has led to high levels of depression, fear and anxiety among the population. In addition, early reproductive age and high fertility rates are characteristic of the Palestinian population(Dhair & Abed, 2020; El Sharif & Khatib, 2021). All of this can lead to significant stress on the family and is a prime setting for the occurrence of family-related diseases. This includes breast cancer(Jin et al., 2022).

In Palestine, several studies have been conducted to investigate the genetic analysis of familial cases of breast and ovarian cancer. Between 2006 and 2013, the researchers recruited 200 patients with breast or ovarian cancer from hospitals in the West Bank and Gaza Strip. In these studies, various analytical methods were used to investigate the genetic aspects of the disease(Kariri et al., 2017; Lolas Hamameh et al., 2017). The most recent study deals with several Palestinian-Arab families in which several patients with breast and ovarian cancer patients. For this study, an initial attempt was made to identify the presence of founder mutations in the BRCA gene and to derive a likely candidate for a founder mutation to be studied in additional families with breast/ovarian cancer(Abdel-Razeq et al., 2021; Abu-Helalah et al., 2020; Ahmad et al., 2023; Alhuqail et al., 2018). Analyses of mutation frequency conducted in breast cancer patients in Palestine showed a significant disparity between the prevalence of BRCA1 and BRCA2 mutations. In the study, which involved 875 patients recruited from Augusta Victoria Hospital in East Jerusalem and other Palestinian hospitals between 2008 and 2016,

epidemiologic and clinical data were collected through interviews and medical record review(Lolas Hamameh et al., 2017). Blood samples were taken from all 875 patients. The results showed that only 6.8% of breast cancer patients carried BRCA1/BRCA2 germline mutations. The carrier frequency was highest for BRCA1 (11 patients) and BRCA2 (20)(Lolas Hamameh et al., 2017). A recent study on the methylation status of the BRCA1 gene in breast cancer patients found that about 34% of the samples showed methylation of the gene, which was associated with a near absence of protein production(Ahlam I. Mujahed, 2016). These results reconfirm that BRCA1 and BRCA2 mutations significantly increase the risk of developing breast cancer(Armaou et al., 2009; D. Thompson & Easton, 2002).

1.4 BRCA1 structure and function

The BRCA1 gene, which is susceptible to breast and ovarian cancer, was cloned in 1994 by two independent research groups(Friedman et al., 1994; Miki et al., 1994). Using genetic linkage analysis in families with multiple cases of breast and ovarian cancer and positional cloning, both groups were able to identify the same gene in the long arm of chromosome 17 at band 17q21. responsible for the early onset of breast and ovarian cancer(Friedman et al., 1994; Miki et al., 1994). This work provided invaluable tool for determining individual risk for breast and ovarian cancer and for developing preventive therapies(Easton et al., 1993). BRCA1 spans 100 kilobases and consists of 24 exons that code for a protein with 1863 amino acids. This protein comprises several domains, each associated with one or more specific functions (Figure 1)(Rosen et al., 2003). Three crucial components critical to its functionality are present; an N-terminal RING finger domain spanning exon 2 to 7, a set of nuclear localization signals from exon 11 to 13, and

a duo of BRCA1 C-terminal (BRCT) domains spanning exons 16 to 24 (Bonavida & Baritaki, 2020). The gene is expressed in many tissues, particularly in thymus, spleen and testes(Gardner & Liu, 2000). Its expression is highest in the S and G2 phases of the cell cycle, which is consistent with its role in DNA repair(Dasika et al., 1999; Deng & Wang, 2003; Jasin, 2002; Venkitaraman, 2004).

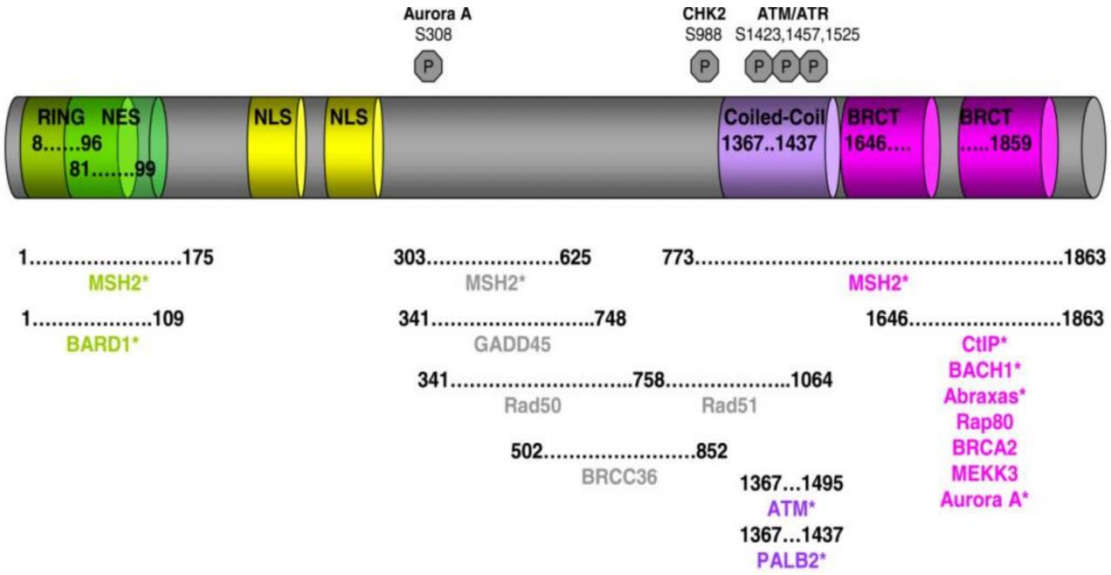

Figure 1.1 The functional domains of BRCA1 and its partners are listed, with asterisks indicating a direct interaction with BRCA1(Christou & Kyriacou, 2012).

BRCA1 is involved in various biological activities ranging from control of gene transcription to involvement in DNA repair mechanisms(Jiang & Greenberg, 2015; Lord & Ashworth, 2012). Furthermore, it shares protein homology sequence with other known proteins(Miki et al., 1994). BRCA1 contains putative nuclear localization signals and has structural similarities to a group of proteins known as RING finger proteins(Bienstock et al., 1996). Within BRCA1 there is a RING domain, a structural motif that coordinates two zinc ions to form a distinctive cross-brace structure(Hashizume et al., 2001a; Meza et al., 1999). These multi-element zinc- motifs are often involved in interactions with nucleic acids and other proteins (Wu et al., 1996a). Any mutation affecting the eight

cysteine or histidine residues involved in the coordination of zinc ions can disrupt the cross-brace structure, resulting in a loss of DNA-binding activity(Johnson & Kruk, 2002).

While there are numerous reports suggesting the RING domain possesses ubiquitin E3 ligase activity(Brown et al., 2015; Budhidarmo et al., 2012; Deshaies & Joazeiro, 2009; Lorick, 1999), there is no definitive consensus on whether this activity is intrinsic to the RING domain itself or whether it requires the cooperation of other proteins, such as BRCA1-associated RING domain 1 (BARD1) (Meza et al., 1999). The RING finger motifs of BRCA1 and BARD1 interact to form a heterodimeric complex that enhances the ubiquitin ligase activity of BRCA1(Bonavida & Baritaki, 2020). The ubiquitin ligase activity of BRCA1 is significantly increased when it partners with BARD1 (Hashizume et al., 2001b). As with all other E3 ubiquitin ligases, the process of labeling a substrate with ubiquitin can only occur through interaction with an E2 ubiquitin-conjugating enzyme. The Ubiquitin-Conjugating Enzyme H5 (UbcH5), together with other E2 enzymes, attaches to the surface of BRCA1 opposite BARD1 binding (Brzovic et al., 2003) .The large number of cancer-associated mutations affecting the interaction between BRCA1/BARD1 or BRCA1/UbcH5 and the function of RING-E3 ligase suggest that the ubiquitin ligase activity of BRCA1 is critical for its role as a tumor suppressor(Porollo & Meller, 2007). In addition, the C-terminal sequence of the RING domain contains a nuclear export sequence (NES). The association between BRCA1 and BARD1 means that this NES remains hidden and the protein therefore remains in the cell nucleus(Rodríguez & Henderson, 2000).

The C-terminus of BRCA1 comprises two BRCT domains (BRCA1 C-terminus), which extend from exon 16 to exon 24(S. L. Clark et al., 2012b). Each domain consists of approximately 95 amino acids and has a sequence motif known for its interactions with

proteins, as well as a conserved sequence(Wu et al., 1996b). In addition to its involvement in DNA damage response and cell cycle regulation, it has a central function in mediating protein-protein interactions. In particular, it contains a phosphoprotein binding module capable of recognizing the phosphorylated protein sequence motif (pSXXF), which is characteristic of substrates targeted by the kinases Ataxia-Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3-related protein (ATR), —both of which are activated in response to DNA damage(S. L. Clark et al., 2012b). Its binding partners include the BRCA1-Associated C-terminal Helicase 1(BACH1) helicase, also known as BRCA1-interacting protein C-terminal helicase (BRIP1), C-Terminal Binding Protein Interacting Protein (CtIP) and Coiled-Coil Domain Containing 98 (CCDC98), which are commonly referred to as Abraxas(S. L. Clark et al., 2012b). The interaction between Abraxas and BRCA1 is essential for the repair of DNA double-strand breaks (DSBs) and the maintenance of genome stability. Abraxas is necessary for the recruitment of BRCA1 to sites of DNA damage, where together they play important roles in the DNA damage response and contribute to tumor suppression(Castillo et al., 2014). Furthermore, studies have shown the BRCT domain of BRCA1 has the ability to bind directly to DNA and also interact with non-phosphorylated proteins(Yamane et al., 2000).

Exons 11–13, which span 65% of the BRCA1 sequence, encode two nuclear localization sequences (NLS) and binding sites for several proteins related to transcription, DNA repair and cell cycle progression, such as Retinoblastoma Protein (RB), Cellular Myc (c Myc), Radiation Sensitive 50 (Rad50) and Radiation Sensitive 51(Rad51). The amino acids produced by these exons also include regions of a coiled-coil domain that facilitate interaction with Partner and Localizer of BRCA2 (PALB2) and a portion of a serine-containing domain (SCD) that is subject to phosphorylation by

ATM(S. L. Clark et al., 2012a). Furthermore, the proteins that interact with BRCA1 exons 11-13 play crucial role in cellular processes: Myc acts as a transcription factor, while Rad50, Rad51 and PALB2 are involved in DNA repair and RB regulates cell cycle progression. The high frequency of mutations in this region, which often lead to significant functional loss, underlines its importance for the tumor suppressor function of BRCA1(S. L. Clark et al., 2012a). NLS1 is determined by amino acids 501–507, while NLS2 is encoded by amino acids 607–614. Both sequences are recognized by importin-alpha, which binds to them and transports the protein into the cell nucleus(C.-F. Chen et al., 1996). Mutations within the nuclear localization signals NLSs result in a repositioning of BRCA1 within the cytosol, thereby causing a rise in uncorrected mutations and chromosomal abnormalities in cancerous conditions.

BRCA1 and the Phosphorylated Retinoblastoma gene (pRB) are among the most important and most intensively researched tumor suppressor proteins. Both proteins are known to control the progression of the cell cycle and the repair of DNA damage. The interaction of BRCA1 with RB is involved in the control of cell proliferation and differentiation(Aprelikova et al., 1999). BRCA1 has been shown to be present at sites of DNA double-strand breaks (DSBs) and to forms nuclear foci together with RAD51, a crucial factor in homologous recombination (HR)(Scully et al., 1997).

Moreover, BRCA1 interacts with the Meiotic Recombination 11 Homolog A (MRE11A)-RAD50- Nijmegen Breakage Syndrome 1(NBS1) (MRN complex), which acts as a DNA breakage sensor and monitors the repair of DSBs by both HR and non-homologous end joining (NHEJ)(Fu et al., 2003; Greenberg et al., 2006).BRCA1 interacts also with c-Myc (a potent oncogene) both (in vitro) and (in vivo) by utilizing its Myc-binding regions between amino acids 175 to 303 and 433 to 511. In addition, BRCA1 was

found to repress the transcription of c-Myc(Q. Wang et al., 1998). In addition, in BRCA1, a potential coiled-coil domain spanning exons 11-13 (amino acids 1364-1437), harbors the binding site for PALB2. At this site, PALB2 acts as a scaffold that helps BRCA1 and BRCA2 to form a group with all three proteins. This interaction is important for DNA repair, particularly in a process called homologous recombination (HR)(Sy et al., 2009).

BRCA1 has a segment known as the serine cluster domain (SCD), part of which is located in exons 11-13 and extends from amino acids 1280 to 1524(Traven & Heierhorst, 2005a). These domains are frequently present in transcription factors and serve as targets for various kinases(C. Chen et al., 1999). This region is rich in potential phosphorylation sites and is phosphorylated by ATM/ATR kinases both in the laboratory and in living organisms. ATM and ATR are kinases that are activated in response to DNA damage(Bakkenist & Kastan, 2003). Phosphorylation of BRCA1 results in its recruitment to sites of double-strand breaks. SCDs are widely distributed among the ATM/ATR targets and include various DNA damage response proteins(Traven & Heierhorst, 2005b). In vivo, serines 1189, 1457, 1524 and 1542 can be phosphorylated in BRCA1, and additional serines can be phosphorylated in vitro (Cortez et al., 1999a). Mutations in these serine residues, as observed in clinical settings, might influence BRCA1's capability to localize to DNA damage sites and its role in responding to DNA damage(S. L. Clark et al., 2012a). Studies have shown that these regions are phosphorylated by ATM kinase in response to ionizing radiation and DNA damage(Bakkenist & Kastan, 2003). ATM-mediated phosphorylation of BRCA1 is thought to modulate its interactions with the RAD50/MRE11/NBS1 complex during DNA damage, affecting the intra-S phase and G2/M checkpoints(Lavin et al., 2015; Xu et al., 1999). The enhanced binding of BRCA1 to RAD50 and Mre11 after ionizing radiation exposure suggests its involvement in DNA

repair and cell cycle regulation(B. Wang et al., 2007). The association of the serine-rich region and the N-terminal ATM phosphorylation site of BRCA1 (around residue 69) with the DNA damage response establishes a link between BRCA1 mutations and the incidence of breast and ovarian cancer(Cortez et al., 1999b).

The discovery of a unique form of DNA damage-induced G2/M cell cycle arrest by the BRCA1 protein has been recognized as a critical mechanism for tumor suppression(C.-X. Deng, 2006; J. Zhang & Powell, 2005). Disruption of this arrest increases the risk of genetic instability in this particular phase of the cell cycle, as damaged cells continue to grow before the repair process is complete. This genetic instability is thought to be hereditary and contributes to an increased susceptibility to early breast cancer(C.-X. Deng, 2001, 2006; Kaufmann & Kaufman, 1993). In addition, BRCA1 responds to DNA damage by enhancing the transcription of p21, a universal inhibitor of cyclin-dependent kinases. Inhibition of these kinases is another method to stop cell cycle progression, with p21 serving as a backup for the G2/M checkpoint(C. Deng et al., 1995; Harper et al., 1993). Failure to increase the expression of p21 increases the likelihood of accumulation of unrepaired damage. This underscores the primary function of BRCA1 to prevent transcription and progression of damaged cells, a role that distinguishes it from other repair genes that do not pose as great a cancer risk when lost(Chai et al., 1999; Krishnan et al., 2021; Tarsounas & Sung, 2020). The BRCA1 protein is essential for maintaining genomic stability by facilitating accurate DNA repair through homologous recombination(Jiang & Greenberg, 2015; Lord & Ashworth, 2012). When BRCA function is lost, this leads to genomic instability, which ultimately results in oncogenic transformation of non-tumorigenic cells that turn into tumor-initiating cells

or cancer stem cells (CSCs) and promote further tumor development(Kreso & Dick, 2014; Peitzsch et al., 2017).

Mutations within the BRCA1 gene show considerable diversity in terms of type and frequency and include deletions, insertions and numerous single nucleotide polymorphisms in both coding and non-coding regions. Over 1600 different mutations have been discovered in the BRCA1 gene, with a wide range of deleterious effects. These mutations are classified as either deleterious or non-deleterious(Godet & Gilkes, 2017). For example, the specific mutation 185delAG, located in the RING and BRCT domains, has been identified among Ashkenazi Jews(Laitman et al., 2013), while the frameshift mutation 5382insC probably originates from Scandinavia or northern Russia(Hamel et al., 2011). Furthermore, mutations in exons 11-13 of the BRCA1 gene have been associated with breast and ovarian cancer(Gayther et al., 1995; D. Thompson et al., 2002). Most mutations in the BRCA1 gene involve insertions/deletions that cause frameshifts, non-synonymous truncations and disruption of splice sites, ultimately leading to missense mutations or synthesis of dysfunctional proteins(Karami & Mehdipour, 2013; D. Thompson & Easton, 2004).

BRCA1 in breast and ovarian tumors has been shown to be associated with loss of heterozygosity (LOH), a second hit leading to inactivation of the gene in somatic tissue (Maxwell et al., 2017). This suggests that BRCA1 inactivation is critical in the development of these cancers.

In breast cancer, around 5-10 % of cases are associated with a BRCA1 germline mutation(Daly et al., 2010). Of these cases, approximately 10 % exhibit loss of heterozygosity (LOH) (Maxwell et al., 2017). In addition, alterations of the BRCA1 gene can also result from mechanisms other than germline mutations, e.g. somatic mutations

or epigenetic silencing, especially in sporadic cases that are not hereditary(Kenemans et al., 2004).

Mutations in BRCA1 exhibit lower prevalence in sporadic cases when contrasted with hereditary cases; nevertheless, they may manifest, albeit with low frequency(Janatova et al., 2005). Epigenetic modification; mainly hypermethylation of the BRCA1 gene was found in 9.1% of sporadic breast tumors(Birgisdottir et al., 2006a). Promoter hypermethylation is known to be a mechanism responsible for many tumor suppressor genes inactivation including BRCA1 gene(Esteller et al., 1999).

1.5 Epigenetic and DNA methylation

Epigenetics refers to a heritable molecular process that is influenced by external factors and controls gene expression without directly altering the underlying DNA sequence(Holliday, 1994). During the development of breast cancer, there is an accumulation of abnormal changes in both the genetic and epigenetic makeup of the cell. Epigenetic modifications including DNA methylation, histone modification, nucleosome remodeling and RNA-mediated gene targeting play an important role in modulating various molecular, cellular and biological pathways associated with the development and progression of breast cancer(Dawson & Kouzarides, 2012). Recent discoveries highlight the involvement of epigenetic disorders in important features of breast cancer, such as drug resistance and the presence of stem cell-like properties(Pasculli et al., 2018).

DNA methylation indicates a methyl group is covalently bound to cytosine or adenine within the DNA sequence. In this process, the cytosine ring at the 5' position of a CpG dinucleotide is chemically modified by attaching a methyl group to the fifth carbon, using S-adenosylmethionine as the supplier of the methyl group(Dong et al.,

2014; Robertson, 2005). This is made possible by a group of enzymes known as DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B(Robertson, 2005). DNMT1 is responsible for the maintenance of DNA methylation in methylated regions, while DNMT3A and DNMT3B are involved in de novo methylation and concentrate on unmethylated or partially methylated CpG sites(Lyko, 2018). DNA methylation plays a central role in the regulation of various processes, including transcription, post-transcriptional modifications, chromatin remodeling, genomic imprinting, X chromosome inactivation and suppression of repetitive DNA elements(Kurihara et al., 2008; Robertson, 2001). Methylation blocks binding of specific gene regulatory proteins to DNA, which hinders the access of transcription factors to chromatin and thus influences gene expression. Modification in the regulatory sequences of genes due to methylation make DNA binding site recognition by transcription factors more difficult. Moreover, DNA methylation leads promotes condensed and less accessible chromatin structure, which is less susceptible to nuclease digestion activities which results in reduced histone acetylation that makes the DNA more accessible and transcriptionally active. Interestingly, intragenic regions associated with transcription elongation and alternative splicing exhibit increased DNA methylation(Jones, 2012).

In the human genome, CpG-rich regions, located in promoter regions, transcription start sites and repetitive sequences, are generally unmethylated. In contrast, the majority of the genome that is not GC-rich is highly methylated, which contributes to the stability of the chromosome(Deaton & Bird, 2011). Consequently, depending on the genome region, both hypomethylation and hypermethylation can occur simultaneously, affecting disease outcomes. Genome-wide hypomethylation, characterized by loss of DNA methylation, is observed in various tumors and is associated with effects on genome

stability, DNA damage, and retrovirus/transposon activation(Brothman et al., 2005; Hon et al., 2012; Robertson, 2001).

Abnormal DNA methylation, triggered by both internal and external factors that induce mutations, usually affects CpG-rich regions in gene promoters. This phenomenon can either activate proto-oncogenes or deactivate tumor suppressor genes(Sharma et al., 2010), thus promoting the development of cancer and its spread to other parts of the body. Essentially, carcinogenesis and metastasis are associated with hypomethylation in proto-oncogenes and the activation of transposable elements(Hur et al., 2014; Zelic et al., 2015). In addition, in many cancer cells, downregulation of tumor suppressor genes such as B-Cell Lymphoma 2 (BCL2), BRCA1 and Rat Sarcoma Virus Oncogene (RAS) together with increased levels of hypermethylation actively promote the process of malignant transformation(Bennett & Licht, 2018).

Recent evidence suggests that proteins from the methyl-CpG-binding domain (MBD) family play a crucial role in the interpretation of DNA methylation signals by facilitating the recruitment of enzyme machinery to produce inactive chromatin states(Bogdanović & Veenstra, 2009; Du et al., 2015). Methyl-CpG-binding domain protein 4 (MBD4), a member of the MBD family, selectively binds to methylated DNA via a conserved MBD domain and suppresses the transcription of genes with methylated promoters(Hendrich & Bird, 1998; Kondo et al., 2005). Studies have shown, that MBD4 binds to hypermethylated promoters of tumor suppressor genes such as p16INK4a and hMLH1, leading to a repression of their transcription(Kondo et al., 2005).

1.6 DNA Methylation in Breast Cancer

DNA hypermethylation usually leads to gene repression, resulting in the inactivation of DNA repair mechanisms, tumor suppressor genes and other cancer-related genes. These genes are involved in critical cellular processes such as apoptosis (programmed cell death), cell cycle regulation, cell adhesion, invasion and other essential biological functions(Esteller, 2005; Jovanovic et al., 2010; F. Wang et al., 2014a). While both global DNA hypomethylation and promoter hypermethylation have been observed as separate processes in the development of breast cancer and at different stages of the disease simultaneously, global hypomethylation has been shown to occur primarily at later stages. This is evidenced by the fact that the degree of hypomethylation in genomic DNA increases as the lesion progresses. In contrast, promoter hypermethylation tends to be an early event that is directly associated with the development and progression of breast tumors(Esteller, 2005; Tan et al., 2013).

Given the importance of DNA methylation changes in regulating gene expression in breast cancer cells, it's reasonable to expect that different subtypes of breast cancer would exhibit unique patterns of DNA methylation. In a previous study, Roll and colleagues categorized approximately 950 primary breast tumors according to their DNA hypermethylation status. They found a robust correlation between abnormal DNA hypermethylation patterns and the basal-like and claudin-poor (Triple-Negative) subtypes of breast cancer(Roll et al., 2013). The results of earlier analysis of the methylation status of the five molecular subtypes showed that only luminal A, luminal B and basal-like tumors had different methylation patterns. In contrast, tumors classified as normal-like and HER2-amplified molecular subtypes showed no discernible differences in methylation profile(Holm et al., 2010).

DNA methylation is increasingly recognized as an important area of biomarker research in various tumor types, including breast cancer. A significant number of genes have been found to be inactivated by promoter methylation in breast cancer. These genes play a central role in regulating various biological processes, including cell cycle regulation, apoptosis, tissue invasion and metastasis, DNA repair and hormone receptor signaling (Esteller, 2008; Jovanovic et al., 2010; Roll et al., 2013; Szyf, 2012). many of them are establish methylation profiles in breast tumors that are significantly related to known prognostic factors and biomarkers for potential therapeutic response(Klajic et al., 2014; Shinozaki et al., 2005).

As shown with different types of cancer, aberrant DNA methylation patterns, such as hypermethylation of tumor suppressor genes (TSG) and global hypomethylation, have been detected in human breast cancer too. Suppression of TSG expression by DNA hypermethylation represents a molecular mechanism by which this methylation aberration could trigger tumor formation by interfering with the binding of transcription factors to the promoters of TSG genes (Baylin et al., 2001; Dworkin et al., 2009; Esteller, 2007; Hanahan & Weinberg, 2000). Consequently, numerous studies aimed to investigate the role of TSG gene promoter hypermethylation in breast cancer as well as the correlation between methylation of specific CpG islands (CGIs) within TSGs and various clinical conditions of breast cancer. Cyclin D2, Adenomatous Polyposis Coli (APC), Ras Association Domain Family Member (RASSF1A), Retinoic Acid Receptor Beta (RARβ), Glutathione S-Transferase Pi 1 (GSTP1), Phosphatase and Tensin Homolog (PTEN), p16INK4α and BRCA1 are the most notable hypermethylated genes associated with various functions in breast cancer to date(Dworkin et al., 2009; Hinshelwood & Clark, 2008; Radpour et al., 2011). Methylation of these TSG promoters is associated with the

complete loss of TSG protein products in cancer cells and the development of a malignant phenotype(Ito et al., 2000; Strahl & Allis, 2000).

DNA methylation in breast cancer affects complex gene networks rather than individual genes and influences various biological processes(Szyf, 2012). An illustrative example of gene dysregulation is the phosphatidylinositol 3-kinase (PI3K) signaling pathway. Mutations in components of this pathway are found in almost 70 % of breast cancer cases(Morgensztern & McLeod, 2005). The PI3K pathway controls several cell functions that are critical for tumorigenesis, including cell proliferation, growth, survival and motility. These functions include autophagy, which plays a central role in cell differentiation, apoptosis and the maintenance of cellular homeostasis(Klionsky & Emr, 2000). Some studies have demonstrated hypermethylation of the Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3 (PIK3C3) gene in breast cancer cells. Improper inhibition of PIK3C3 disrupts autophagy in breast tissue and thereby promoting tumorigenesis(F. Wang et al., 2014b).

Methylation of BRCA1 gene promoter is detected in 5–31% of breast cancer cases(Bal et al., 2012a; Buyru et al., 2009). Some studies suggest the pattern of gene expression in BRCA1-mutated breast cancer is similar to somatic breast tumors in which BRCA1 inactivation is due to methylation of the promoter(Esteller, 2000; Wei, Grushko, Dignam, Hagos, Nanda, Sveen, Xu, Fackenthal, Tretiakova, Das, et al., 2005). This suggests that methylation alteration could represent a hereditary factor in the development of breast cancer. Moreover, sporadic breast tumors with BRCA1 methylation status show similar pattern of DNA copy number alterations as observed in breast cancer patients with BRCA1 mutations(Stefansson et al., 2009). Recent studies have shown that BRCA1 promoter methylation can be detected in normal breast tissue from individuals with

BRCA1-methylated tumors, but not in breast tissue with unmethylated BRCA1 in tumor cells(Otani et al., 2014).

Development and progression of breast cancer are complex processes that are influenced by both genetic and epigenetic factors. Although progress has been made in understanding the genetic predisposition to breast cancer, known susceptibility genes explain only a fraction of the familial risk of breast cancer. Subsequently, case-control or cohort studies were conducted to further investigate the association between single nucleotide polymorphisms (SNPs) and breast cancer. However, the results of these studies are not entirely consistent. Significant differences in breast cancer risk were found between different ethnic groups(Sung et al., 2021). In addition, various environmental influences may affect the associations between SNPs and breast cancer risk through influencing the intricate interactions between multiple genes(Shariff-Marco et al., 2015; Travis et al., 2010). Therefore, further research is needed to clarify the association between these SNPs and breast cancer risk.

To uncover additional susceptibility alleles, researchers have conducted genome-wide association studies (GWAS). The aim of these studies was to identify new genetic variants that are associated with breast cancer risk. One study, Click or tap here to enter text. identified four novel loci, including trinucleotide-repeat-containing 9 (TNRC9(TOX3)), fibroblast growth factor receptor 2 (FGFR2), mitogen-activated protein kinase 1 (MAP3K1), and leukocyte-specific protein 1 (LSP1), which contain trinucleotide repeats and show consistent evidence of association with breast cancer risk(Easton, Pooley, Dunning, Pharoah, Thompson, et al., 2007a).

Although the involvement of TOX3 gene in breast cancer risk has been confirmed by similar GWAS studies in different ethnic populations, the exact mechanism underlying

its contribution to breast cancer susceptibility remains uncertain(M.-B. Chen et al., 2011; Easton, Pooley, Dunning, Pharoah, Ponder, et al., 2007; Ruiz-Narváez et al., 2010a; Stacey, Manolescu, Sulem, Rafnar, Stefansson, et al., 2007). This uncertainty prompted us to investigate its role in breast cancer. TOX3 is a nuclear protein that possesses nuclear localization signal and high mobility group box domain that enables it to modify chromatin structure. In neurons, TOX3 regulates Ca^{2+}dependent transcription by interacting with cAMP-responsive element-binding protein (CREB)(Yuan et al., 2009). In addition, studies have shown the introduction of TOX3 forms a complex with CREB that protects neuronal cells from apoptosis by activating BCL-2(Dittmer et al., 2011). Previous studies have suggested that transcriptional components involving CREB may influence the continuous expression of BRCA1(Hockings et al., 2008). As mentioned above, the regulation of BRCA1 transcription depends on dynamic balance between transcriptional coactivators and corepressors. These factors monitor histone acetylation and BRCA1 promoter accessibility(Di et al., 2010).The possible involvement of TOX3 in chromatin structure, combined with its transcriptional control of BRCA1 may involve epigenetic mechanisms(Di et al., 2010). This led us to hypothesize that selected SNPs in TOX3 gene that affect the expression of TOX3 may play a role in breast cancer biology by influencing the activity of BRCA1.

Recent work provided substantial evidence of a notable association between breast cancer and rs3803662, a tag SNP in TOX3 gene, among Eastern Asian, British and European ethnic groups(Garcia-Closas et al., 2008; Stacey, Manolescu, Sulem, Rafnar, Stefansson, et al., 2007). However, this tag SNP did not show a similarly strong correlation with breast cancer among African American or Hispanic women(Ruiz-Narváez et al., 2010b). In a study with Egyptian women, the investigation of the

relationship between TOX3 rs3803662 and the risk of breast cancer revealed no significant correlation(El-Shafie et al., 2021). In another study in the same population, the rs12443621 polymorphism of the TOX3 gene was genotyped in 100 breast cancer patients and 80 healthy controls. The results showed a remarkable association between the GG genotype of TOX3 rs12443621 and increased risk of breast cancer, suggesting the G allele as a potential risk factor(Safan & El-Sisi, 2016). A study conducted in the Tunisian population revealed a significant association between rs8051542 of TOX3 and breast cancer risk. In particular, the association for rs8051542 was more pronounced in high-grade SBR (Scarff, Bloom and Richardson) tumors(Shan, Mahfoudh, Dsouza, Hassen, Bouaouina, Abdelhak, Benhadjayed, Memmi, Mathew, Aigha, et al., 2012). In another study in the Jordanian population, rs1420546 of TOX3 was shown to be significantly associated with increased risk of breast cancer(Al-Eitan et al., 2017).

In a recent report, 37 studies with 97,275 cases and 128,686 controls were analyzed(). and showed that the genetic variations rs3803662 C > T, rs12443621 A > G and rs8051542 C > T were all associated with increased risk of breast cancer L. Zhang & Long, 2015. When the different ethnic groups were considered separately, Caucasians were found to have significantly increased risk of breast cancer in all genetic models with these three SNPs. In Asian populations, a significant association was found between breast cancer risk and rs3803662 and rs8051542. In Africans, however, no statistically significant association was found between these three SNPs and breast cancer risk(L. Zhang & Long, 2015).

A previous investigation conducted earlier by our group (unpublished data), on BRCA1 promoter methylation in a Palestinian breast cancer women cohort. showed that 34% of breast cancer cases among Palestinians had methylation in the BRCA1 gene

promoter. Based on these findings, and on findings that correlate between breast cancer susceptibility and TOX3 gene SNPs, we hypothesized a possible association between selected variants (SNPs), namely rs3803662 C > T, rs12443621 A > G and rs8051542 C > T in the TOX3 gene and the observed increase in BRCA1 methylation. This hypothesis suggests a mechanism by which genetic variations in TOX3 may influence breast cancer risk by affecting BRCA1 methylation in Palestinian women.

1.7 Statement of the Problem

TOX3 gene appears to reduce the expression of BRCA1 by enhancing methylation of its promoter, a mechanism that increases the aggressiveness of breast cancer (Shan et al., 2013). Moreover, TOX3 polymorphisms were shown to be associated with early onset of breast cancer in Arab women (Shan, Mahfoudh, 2012). Therefore, we propose to investigate the association between different TOX3 gene polymorphisms and BRCA1 promoter methylation in Palestinian breast cancer patients.

1.8 Significance of the research

Breast cancer is the most common malignancy among women in Palestine and often appears at a young age with aggressive features. Research into the molecular mechanisms underlying this disease could therefore reveal biomarkers that are crucial for early detection and prevention and could mitigate the devastating effects of cancer.

1.9 Research Aims

Investigate the correlation between TOX3 gene SNPs and BRCA1 promoter methylation among Palestinian breast cancer patients.

1.10 Research hypothesis

TOX3 overexpression was shown to induce BRCA1 promoter methylation. Moreover, specific TOX3 variants were found to be associated with increased susceptibility to breast cancer. Based on these findings, we hypothesized that **TOX3 SNPs could affect BRCA1 promoter methylation.** This hypothesis suggests a

mechanism by which genetic variations in the TOX3 gene may influence breast cancer risk by affecting BRCA1 methylation in Palestinian women.

1.11 Objective

1. Examine the association between specific genetic variants, including rs3803662, rs12443621 and rs8051542, and breast cancer risk among Palestinian patients.
2. Investigate the association between the indicated genetic variants in the TOX3 gene, associated with increased breast cancer susceptibility, and the degree of methylation in the BRCA1 gene in the Palestinian breast cancer patients.
3. Explore the underlying mechanisms by which genetic variations in TOX3 may affect breast cancer risk by impacting BRCA1 methylation levels.

Chapter 2: Materials and Methods

2.1 Study population and Specimens

Formalin-fixed, paraffin-embedded (FFPE) core or surgical biopsies of breast tissue were randomly collected between 2012 and 2015 from a cohort of 112 patients at Augusta Victoria Hospital in Jerusalem and the CAP laboratory in Bethlehem. These samples represented different geographical regions in the West Bank and Gaza Strip. In addition, blood samples were taken from the patients. All sampling procedures were carried out with the appropriate authorizations.

Information on the hormone receptor status (estrogen ER, progesterone PR and Her2 new), cancer type, age, stage and grade of each patient was taken from the pathology reports, as shown in Table1.

Table 2. 1 Sample Characteristics

Character	Description	No. of cases	Percentage
Age	**<40 years**	56	58.30%
	>40 years	40	41.60%
Grade	**I**	4	4%
	II	24	26%
	III	65	70%
	N/A	19	
Stage	**I**	14	15%
	II	32	34.40%
	III	42	45%
	IV	5	5%
	N/A	19	
Receptors	**Luminal A**	45	49%
	Luminal B	17	18.50%
	HER2 type	18	19.50%
	TNBC	12	13%
	N/A	20	

2.2 Samples

The DNA samples were obtained from previous studies. DNA samples were extracted from FFPE tissue and then purified using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's guidelines. The DNA samples were then prepared for genotyping analysis using PCR-RFLP, ARMS and Sanger sequencing .

2.3 SNP Selection and Genotyping

Three selected TOX3 gene variants from the NCBI SNPs database were analyzed for their association with breast cancer SNPs (Table 2.2). The genotyping of these SNPs was performed using RFLP-PCR and ARMS-PCR. The selected SNPs were chosen based on previous studies indication their association with increased susceptibility to breast cancer in different ethnic populations(Garcia-Closas et al., 2008; Ruiz-Narváez et al., 2010b; Safan & El-Sisi, 2016; Stacey, Manolescu, Sulem, Rafnar, Gudmundsson, et al., 2007c; Stacey, Manolescu, Sulem, Rafnar, Stefansson, et al., 2007; L. Zhang & Long, 2015).

2.4 Genotyping Using PCR-RFLP

Primers were designed to amplify specific regions of the TOX3 gene encompassing the genetic variants of interest using Primer3 online tool covering three specific regions corresponding to SNPs rs3803662, rs12443621 and rs8051542. For each amplified segment, restriction enzymes were selected to recognize and cut at the SNP site. The details of the primers and restriction enzymes used are listed in table 2.2. To evaluate the efficacy of our designed primers and to ensure precise PCR conditions, we performed PCR with gradient temperature to determine the optimal temperature for

amplification. Each PCR reaction tube contained 10 µl of mix (Thermo Scientific dreamtaq green PCR Master MIX (2X)), 8 µl of distilled water, 1 µl of genomic template DNA (approximately 200 ng/µL), 0.5 µl of reverse primer (From 10uM stock concentration) and 0.5 µl of forward primer (From 10uM stock concentration), giving a total reaction volume of 20 µl. PCR was performed using a FlexCycler2 thermocycler (Analytik Jena, Germany). The cycling program consisted of 32 cycles that included a denaturation step for 20sec at 94C, an annealing step at 57C and an extension step at 72C. After the thermocycler, the PCR products were analyzed on a 2% agarose gel, with electrophoresis performed at 100 V for 40 minutes in TAE buffer. The thermocycler conditions for each SNP are listed in Table3.

For RFLP analysis PCR amplicons flanking the SNPs (rs3803662), (rs8051542) and (rs12443621) were digested with the allele-specific restriction enzymes BcoDI, FokI, and BsrDI, respectively. Each reaction mixture consisted of 10 µl PCR product, 1.5 µl 10x enzyme buffer, 0.2 µl of the enzyme (54 units) and 3.3 µl distilled water. Incubation was carried out at 37°C for 24 hours for rs3803662 and (rs8051542), while for (rs12443621) the incubation temperature was set to 65°C for 24 hours. After incubation, the resulting DNA fragments were separated on a 2.5% agarose gel. Electrophoresis was performed at 100 V for 40 minutes in TAE buffer. Gel analysis was then performed using the ChemiDoc Imaging System (BIO-RAD, USA), and alleles and genotypes were interpreted based on specific digestion patterns.

Table 2. 2 SNPs details, sequences of primers and method of investigation

Gene	SNP Reference	Position (GRCh38.p14)	Gene Region	Variation	Primer	Product Size	Method used
TOX3	rs3803662	chr16:52552429	Non Coding Transcript Variant	NC_000016.10:g.52552429A>C	TOX3_rs3803662_Seq_71_For_5'-gctgctagtccttggctgtt-3' TOX3_rs3803662_Seq_523_Rev_5'-gggaggggtttatacagga-3'	472bp	RFLP using BcoDI restriction enzyme

TOX3	rs8051542	chr16:52500255	Intron Variant	NC_000016.10:g.52500255T>C	T3_B_R_F_5'-gatgtgcatgggttgtacctg-3' T3_B_R_R_5'-ttagtctgtaaaggaagtcacatcg-3'	160bp	RFLP using FokI restriction enzyme
TOX3	rs12443621	chr16:52514125	Intron Variant	NC_000016.10:g.52514125A>G	T3_C_R_F_5'-tggagcctagtaagccagga-3' T3_C_R_R_5'-ccactgcagaaaagggagag-3'	182bp	RFLP using BsrDII restriction enzyme
TOX3	rs3803662	chr16:52552429	Non Coding Transcript Variant	NC_000016.10:g.52552429A>C	T3_A_N_F_5'- ccttaatgcctctatagctgtct-3' T3_A_M_R_5'- ccacagttttattcttcgctaagg -3' T3_A_C_R_5'- gggaggggtttatacagga-3'	190bp	ARMS
TOX3	rs8051542	chr16:52500255	Intron Variant	NC_000016.10:g.52500255T>C	T3_B_N_F_5'- gctccaatcatagtgctgca -3' T3_B_M_R_5'- catttaggttattagaggac -3' T3_B_C_R_5'- catggttttctcctccaaaca -3'	181bp	ARMS
TOX3	rs12443621	chr16:52514125	Intron Variant	NC_000016.10:g.52514125A>G	Normal and mutant with mismatch T3-C-N-F 5' – gtaatacctacctcaagttaat - 3' (normal forward) T3-C-M-F 5' – gtaatacctacctcaagttaac - 3' (mutant forward) T3-C-C-R 5' – cagaaagaagtattctgggtttca - 3' (common reverse)	136bp	ARMS
TOX3	rs8051542	chr16:52500255	Intron Variant	NC_000016.10:g.52500255T>C	T3_B_R_R_5'-ttagtctgtaaaggaagtcacatcg-3'	160bp	Sanger Sequencing

2.5 Genotyping Using ARMS (Amplification Refractory Mutation System)

The ARMS primers were designed using NCBI website and the Primer3.0 online tool. The primer sequences used in the study are listed in Table 2.2. Two primer sets were designed for each SNP; one for each allele. Of note, we mutated the primers in the base that falls 2bases upstream to the 3' end of the genotyping primer. For PCR, each reaction tube contained 10 µl of ready mix (a lyophilized mixture of Taq polymerase, MgCl2, dNTP and buffer), 7 µl of distilled water 2.0 µL of genomic DNA and 0.5 µL of allele-specific forward primers (10µM stock solution) and 0.5 µL of common reverse primers (10µM stock solution). The ARMS-PCR products were then separated by electrophoresis on 2% agarose gels. Electrophoresis was performed at 100 V for 40 minutes in TAE buffer. Gel analysis was then performed using the ChemiDoc Imaging

System (BIO-RAD, USA), and alleles and genotypes were interpreted based on specific digestion patterns. The thermocycler conditions for each SNP are listed in Table 2.4.

Table 2. 3 PCR Protocols for ARMS

SNP	**Initial denaturation (2 min)**	**Denaturation 20 sec**	**Cycling Annealing 30 sec**	**Elongation 30 sec**	**Final extension (5 min)**
A (rs3803662)	94 ◦C	94 ◦C	60 ◦C	72 ◦C	72 ◦C
B(rs8051542)	94 ◦C	94 ◦C	54 ◦C	72 ◦C	72 ◦C
C (rs12443621)	94 ◦C	94 ◦C	54 ◦C	72 ◦C	72 ◦C

2.6 Sanger sequencing

The PCR products were initially cleaned using the EPPIC-Fast kit (A&A Biotechnology, Cat# 1021-500F) by adding 1 µL of the EPPIC-Fast enzymatic solution to 5 µL of the PCR product. This mixture was then placed in a thermal cycler for 10 minutes at 37°C, followed by 1 minute at 80°C. Sanger sequencing was then performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Cat# 4336774) according to the manufacturer's instructions.

2.7 Statistical analysis

To investigate the correlation between TOX3 polymorphisms and BRCA1 promoter methylation, we used a robust statistical analysis framework. Specifically, we used Student's t-tests to assess whether genetic variations in TOX3 are associated with differences in BRCA1 promoter methylation levels. Using this method, we were able to

compare mean methylation levels between different genotypic groups of TOX3 single nucleotide polymorphisms (SNPs) to assess the significance of these associations. To ensure a thorough examination of the data, we also performed multivariate analyses, accounting for potential confounding variables such as age, cancer subtype and disease stage. This approach allowed us to isolate the effects of each SNP on BRCA1 methylation, which provided a better understanding of the genetic and epigenetic interactions involved. By integrating these analytical techniques, we aimed to provide a comprehensive and robust assessment of the relationship between TOX3 polymorphisms and BRCA1 promoter methylation, increasing the reliability of our results and their impact on breast cancer susceptibility.

2.8 Bioinformatics analysis

SNPStats online software (https://www.snpstats.net) used to analyze multiple SNPs within the same gene to gain insight into the combined effect of genetic variations on breast cancer risk and potentially identify haplotypes associated with increased or decreased susceptibility to the disease. SNPStats is a virtual toolbox tailored to genetic association studies, focusing in particular on single nucleotide polymorphisms (SNPs). SNPStats helps researchers explore the links between genetic variations and traits or diseases and supports them in identifying potential genetic factors behind complex traits. Ultimately, it improves the understanding and interpretation of genetic data, facilitates efficient research into the relationships between genetic variants and traits, and uncovers the underlying genetic mechanisms.

2.9 Linkage Disequilibrium Analysis

In our study, we used linkage disequilibrium (LD) analysis to investigate the degree of non-random association of alleles between three specific single nucleotide polymorphisms (SNPs) located within the same gene. First, we determined the observed allele frequencies for each polymorphism by coding the genotypes at each locus as 0, 1, or 2 and then tabulating the occurrence of specific alleles (e.g., C and T for rs3803662, C and T for rs8051542, G and A for rs12443621). We then calculated the allele frequencies at the respective loci and delineated the alleles based on the expected haplotypes.

Linkage disequilibrium (LD) analysis:

1. LD analysis evaluates the non-random association of alleles at different genetic loci. Here, statistics such as D, D' and r are commonly used to quantify the degree of LD between pairs of SNPs.
2. D statistic: ** Measures the degree of LD between pairs of SNPs. Negative values indicate inverse LD.
3. D' statistic:** A normalized measure of LD that ranges from 0 to 1, with 1 indicating complete LD.
4. r statistic:** Represents the correlation coefficient between pairs of alleles at different loci. Values range from -1 to 1.
5. P-values: These indicate the significance of the observed associations between SNP pairs. Lower p-values indicate stronger evidence against the null hypothesis of no LD.

2.10 Haplotype Analysis

Estimation of haplotype frequencies:

Haplotypes are combinations of alleles at multiple loci on a single chromosome. Here the frequencies of the different haplotypes formed by alleles at three specific SNPs (rs3803662, rs8051542, rs12443621) are estimated.

Each row represents a haplotype identified by the combination of alleles at the three SNPs. The frequencies of these haplotypes are given to determine how often each haplotype occurs in the sample.

Haplotype association with Response:

After estimating haplotype frequencies, an association analysis is performed to assess the relationship between haplotypes and a response variable.

For each haplotype, the analysis presents the frequency, the odds ratio (OR) along with a 95% confidence interval (CI), and the p-value. The odds ratio serves as an indicator of the likelihood of the response variable manifesting in individuals possessing a specific haplotype when compared to a designated reference haplotype (often haplotype 1).

The p-value denotes the level of significance regarding the relationship between each haplotype and the response variable. A decrease in p-values signifies more compelling evidence contradicting the null hypothesis of no association.

Chapter 3: Results

In this study, we extend and built on unpublished findings (Ahlam I. Mujahed, 2016), which first characterized the methylation status of the BRCA1 gene promoter in these patient samples, by re-analyzing the same patient samples to further investigated possible association between specific single nucleotide polymorphisms (SNPs) in the *TOX3* gene with BRCA1 promoter methylation and susceptibility to breast cancer. We focused on three SNPs: rs3803662, rs8051542 and rs12443621. We included both methylated and unmethylated samples to investigate this relationship.

For three SNPs, two genotyping methods, RFLP and ARMS-PCR, were used to ensure accuracy and reliability. Initially, the RFLP method was used, but due to difficulties in obtaining clear and consistent banding patterns for certain samples, ARMS-PCR was used as a secondary method for confirmation. This approach allowed validation of the RFLP results and ensured accurate genotyping of the SNPs.Additionally, Sanger sequencing was used to validate rs8051542

3.1 Analysis of rs3803662 in BC samples using RFLP

To study the correlation between rs3803662 and BRCA1 methylation, we used RFLP method to genotype samples. We first amplified DNA sequences using primers that flank rs3803662 site. As shown in figure 3.1 (lane 2) we were able to amplify the expected 472 bp DNA sequence of interest. Most of the samples showed fairly strong amplification acceptable for further analysis. For weak samples, we repeated PCR after increasing the DNA amount used in the reaction.

After amplification, samples were genotyped using BcoDI restriction enzyme. As shown in figure 3, samples that were homozygous for the T allele gave two distinct fragments of 306 bp and 166 bp (sample 3A), while heterozygous samples (C/T genotype) gave three distinct fragments of 472bp, 306bp and 166bp size (Sample 5A). For C allele homozygous samples, no enzyme cut is expected and thus, only one 472bp fragment was observed (Samples 1A, 2A and 4A). The frequencies of the different alleles and their correlation with BRCA1 methylation is described below in the statistical analysis section.

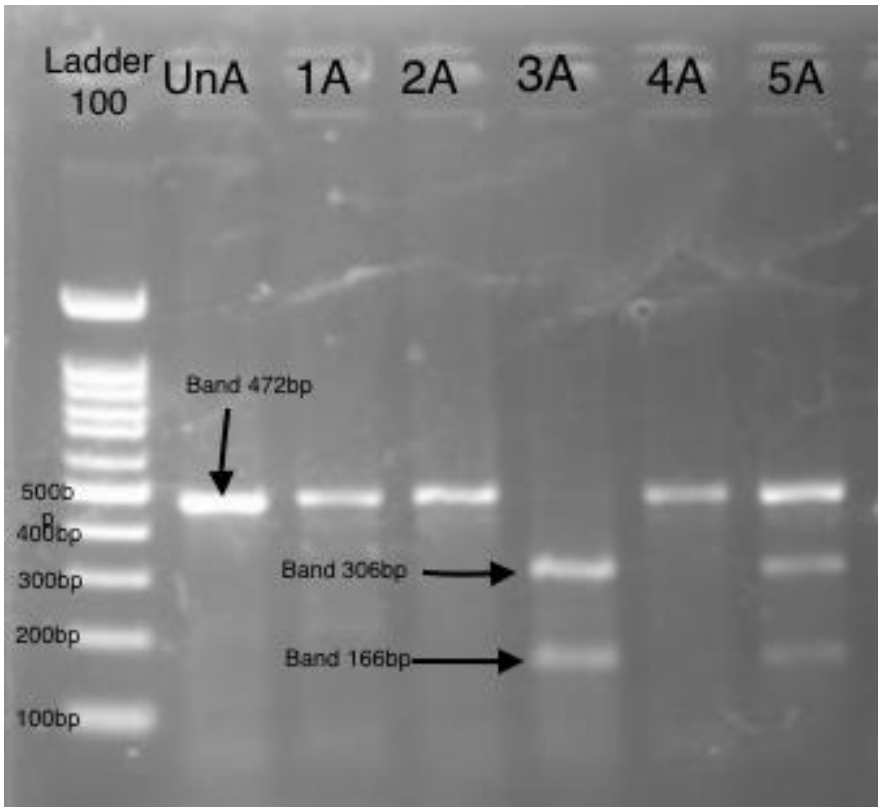

Figure 3. 1 RFLP analysis of the rs3803662 SNP. Agarose gel electrophoresis showing results of RFLP analysis of BC samples. Lane1; 100bp ladder. Samples homozygous for the T allele show 306 bp and 166 bp fragments. Heterozygous samples show 472bp, 306bp and 166bp fragments. Samples homozygous for thC allele show 472bp fragment.

3.2Analysis of rs8051542 in BC samples using RFLP

To study the correlation between rs8051542 and BRCA1 methylation, we used RFLP method to genotype samples. We first amplified DNA sequences using primers that flank rs8051542 site. As shown in figure 3.2, we were able to amplify the expected 160bp DNA sequence of interest. Most of the samples showed fairly strong amplification acceptable for further analysis. For the weak samples (like samples 8, 24 and 32), we repeated the PCR after increasing the DNA amount used in the reaction. After amplification, samples were further analyzed using FokI restriction enzyme. As shown in figure 3.3, samples that were homozygous for the T allele gave two distinct fragments of 108 bp and 52 bp (sample1,2,3 and 4 while heterozygous samples (C/T genotype) gave three distinct fragments of 160bp, 108bp and 52bp size (Samples 5,6,7,8 and 9). For C allele homozygous samples, no enzyme cut is expected and thus, only one 160bp fragment was observed (Samples 10,11, and 12). The frequencies of the different alleles and their correlation with BRCA1 methylation is described below in the statistical analysis section.

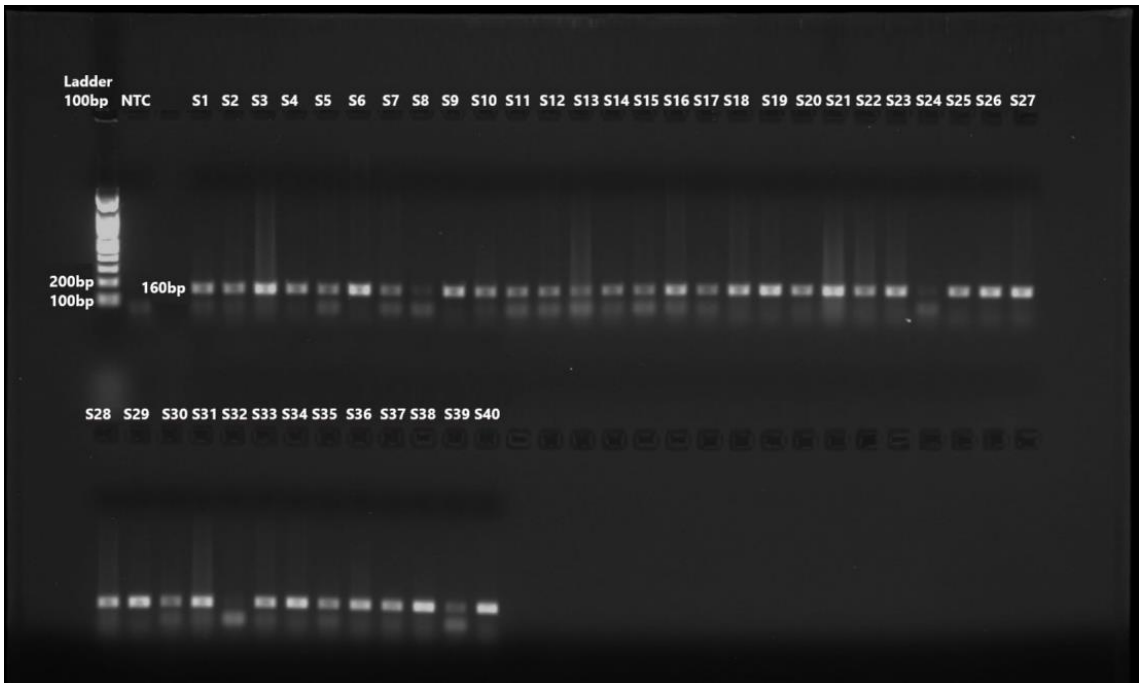

Figure 3. 2 PCR amplification of DNA sequences flanking the rs8051542 SNP. Agarose gel electrophoresis showing results of PCR amplification of DNA sequence flanking

rs8051542 SNP. Lane1; 100 ladder, lane 2; No DNA template control (NTC), PCR product expected size is 160bp.

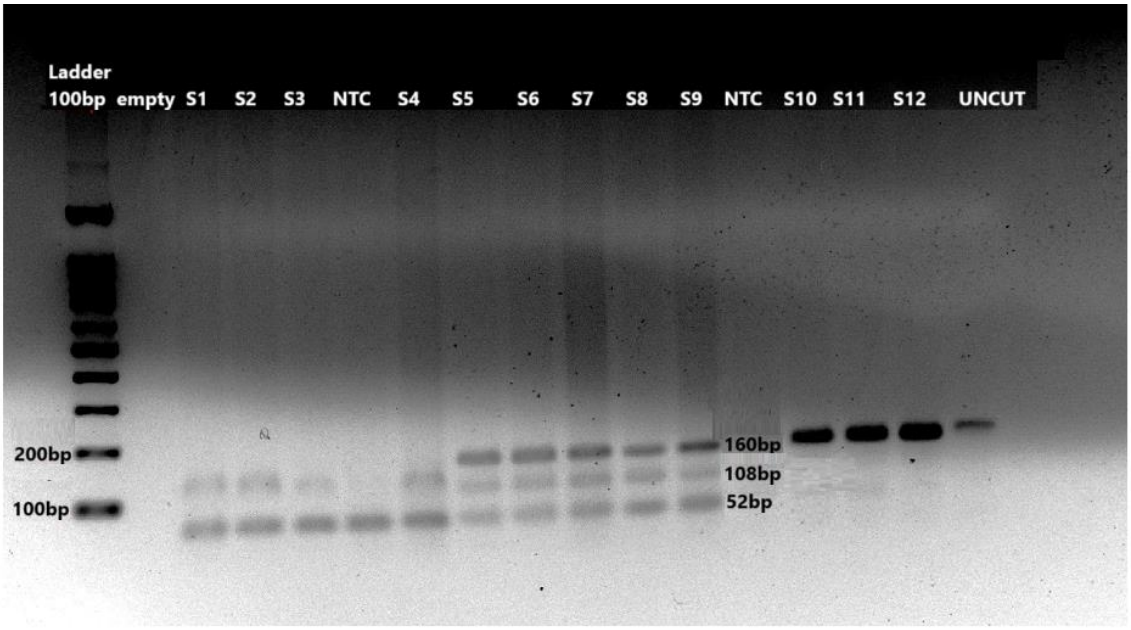

Figure 3. 3 RFLP analysis of the rs8051542 SNP. Agarose gel electrophoresis showing results of RFLP analysis of BC samples. Lane1; 100bp ladder. Samples homozygous for the T allele show 108 bp and 52 bp fragments. Heterozygous samples show 160bp, 108bp and 52bp fragments. Samples homozygous for C allele show 160bp fragment.

3.3Analysis of rs12443621 in BC samples using ARMS

To study the correlation between rs12443621 and BRCA1 methylation, we used ARMS technique to genotype samples. We used common reverse primer with SNP specific forward primer that either amplify A allele or G allele. As shown in figure 3.4, samples with heterozygous allele (G/A genotype) amplified by both primers (samples10,12.), while the homozygous for A allele (A/A genotype) samples only amplified by A allele specific forward primer (samples 49,75), and the samples with homozygous for G allele (G/G genotype) only amplified by G allele specific forward primer (samples 58,36.).

The experimental results of the ARMS-PCR confirmed the results of the PCR-RFLP analysis. The successful amplification and clear differentiation of SNP genotypes underscores the effectiveness of the ARMS technique in elucidating genetic variation and contributes to valuable insights into the role of the TOX3 gene in disease susceptibility and other phenotypic traits.

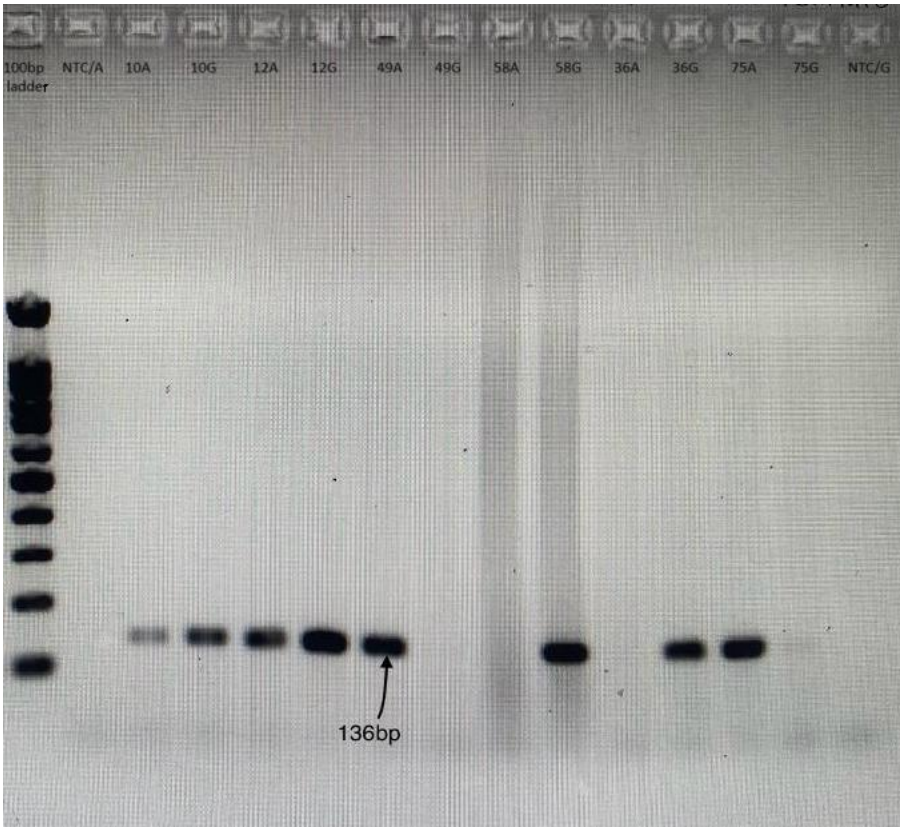

Figure 3. 4 ARMS analysis of the rs12443621 SNP (A>G). Agarose gel electrophoresis showing results of ARMS analysis of BC samples. Lane1; 100 ladders. lane2; No DNA template for allele A(NTC/A). lanes 3 to 14; Samples homozygous for the A allele show 136 bp product only on A labeled lane. Heterozygous samples show 136bp on A and G labeled lane fragments. Samples homozygous for G allele show 136bp product only on G labeled lane. Lane 15; No DNA template for allele G (NTC/G)

3.4Data analysis results

Using SNP stat online tool (https://www.snpstats.net/) all SNPs data from methylated and unmethylated samples were analyzed to search for association between each SNP individually and methylation status of BRCA1 gene promotor. Also, multiple SNP analysis for linkage disequilibrium and Haplotype analysis was performed using same software. The following results contain allele and genotype frequencies, Hardy-Weinberg equilibrium (HWE) tests, and association with methylation status of BRCA1 gene for different genotype models for each SNP.

3.4.1 Rs 3803662 correlates with increased methylation on BRCA1 gene

The analysis of SNP (rs3803662) in relation to the methylation status of the BRCA1 gene, performed with the SNPSTAT software (table 3.1), shows that the C allele is the most frequent overall and is also more frequent in samples with methylation. Furthermore, the frequency of each genotype remains relatively the same across the different methylation statuses (figure 3.5 and 3.6). However, C/T heterozygotes are observed more frequently in the methylated group than in the unmethylated group. The SNP appears to be in equilibrium in both the methylated and unmethylated states, suggesting that the observed genotype frequencies are likely due to random genetic variation (figure 3.7 and 3.8).

A significant association was found in the dominant model (TT/TC vs. CC) ($P = 0.037$), suggesting that the T allele may be associated with a lower methylation probability (M). Other models did not show significant associations, although the over dominant model approached significance (Figure 3.9). Analysis of genotype frequencies and Hardy-Weinberg equilibrium revealed no major deviations. Overall, the association

analysis shows a significant relationship in the dominant model, suggesting a possible influence of the T allele on methylation status.

Genetic analysis of rs3803662 revealed a potential association between higher C allele frequency and younger age of onset of breast cancer. In patients in the <50 years age group, the frequency of the C allele was 69%, while the frequency in those aged ≥50 years was 62. Although this difference was not statistically significant (p=0.508), the trend suggests a possible association between the C allele and an earlier onset of the disease. In addition, the analysis examined the interaction of methylation and SNP, but no significant interactions were found. Further studies with a considerably larger number of samples are needed to validate these results and clarify the potential role of this SNP and methylation in breast cancer susceptibility and early onset. (figure 3.10)

Table 3. 1 SNPSTAT tool analysis result for TOX3 RS3803662 rs3803662 correlation with BRCA1 gene methylation status

Single-SNP analysis

SNP: rs3803662

Percentage of typed samples: 111/111 (100%)

SNIP rs3803662..sense. allele frequencies (n=111)						
	All subjects		Methylation=M		Methylation=U	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
C	147	0.66	45	0.59	102	0.7
T	75	0.34	31	0.41	44	0.3

SNIP rs3803662..sense. genotype frequencies (n=111)						
	All subjects		Methylation=M		Methylation=U	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
C/C	47	0.42	11	0.29	36	0.49
C/T	53	0.48	23	0.61	30	0.41
T/T	11	0.1	4	0.11	7	0.1

SNIP rs3803662..sense. exact test for Hardy-Weinberg equilibrium (n=111)						
	N11	N12	N22	N1	N2	P-value
All subjects	47	53	11	147	75	0.53
Methylation=M	11	23	4	45	31	0.18
Methylation=U	36	30	7	102	44	0.79

SNIP rs3803662..sense. association with response Methylation (n=111, crude analysis)							
Model	Genotype	Methylation=M	Methylation=U	OR (95% CI)	P-value	AIC	BIC
Codominant	C/C	11 (28.9%)	36 (49.3%)	1.00			
	T/C	23 (60.5%)	30 (41.1%)	**0.40 (0.17-0.95)**	0.1	144.1	152.2
	T/T	4 (10.5%)	7 (9.6%)	0.53 (0.13-2.17)			
Dominant	C/C	11 (28.9%)	36 (49.3%)	1.00	0.037	142.3	147.7
	T/C-T/T	27 (71%)	37 (50.7%)	**0.42 (0.18-0.97)**			
Recessive	C/C-T/C	34 (89.5%)	66 (90.4%)	1.00	0.88	146.6	152
	T/T	4 (10.5%)	7 (9.6%)	0.90 (0.25-3.30)			
Overdominant	C/C-T/T	15 (39.5%)	43 (58.9%)	1.00	0.051	142.9	148.3
	T/C	23 (60.5%)	30 (41.1%)	0.46 (0.20-1.01)			
Log-additive	---	---	---	0.60 (0.33-1.11)	0.1	143.9	149.4

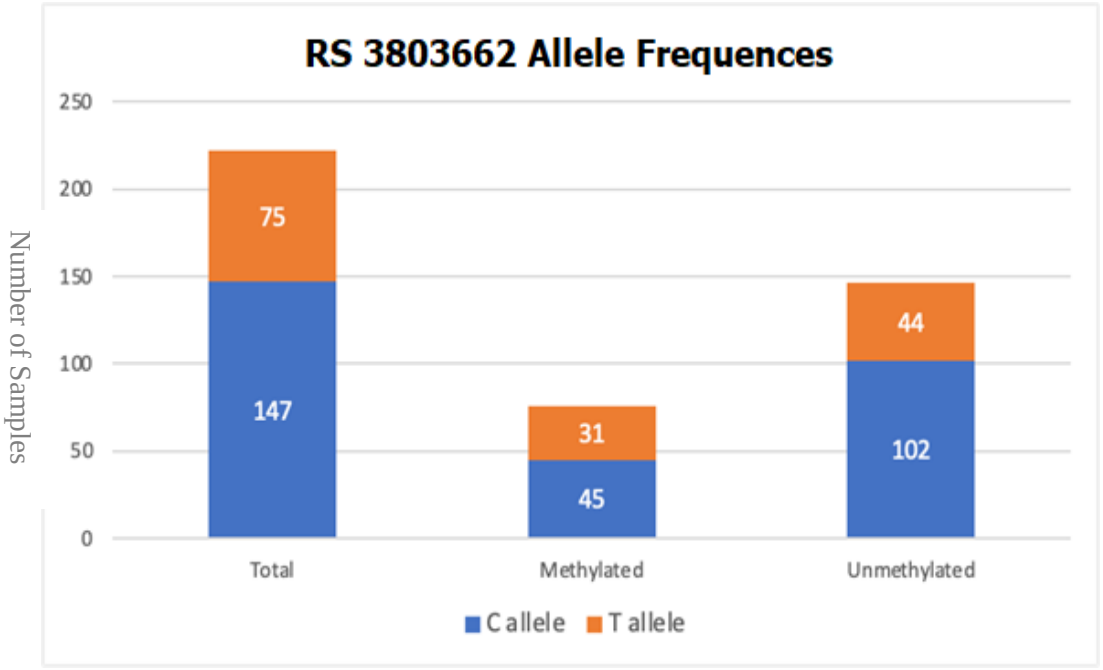

Figure 3. 5 allele frequencies for TOX3 RS3803662 in BRCA1 samples. Orange color for T allele and blue color for C allele. 1st column represents total allele frequencies, 2nd column represents allele frequencies for BRCA1 methylated samples, and 3rd column represents allele frequencies for BRCA1 unmethylated samples

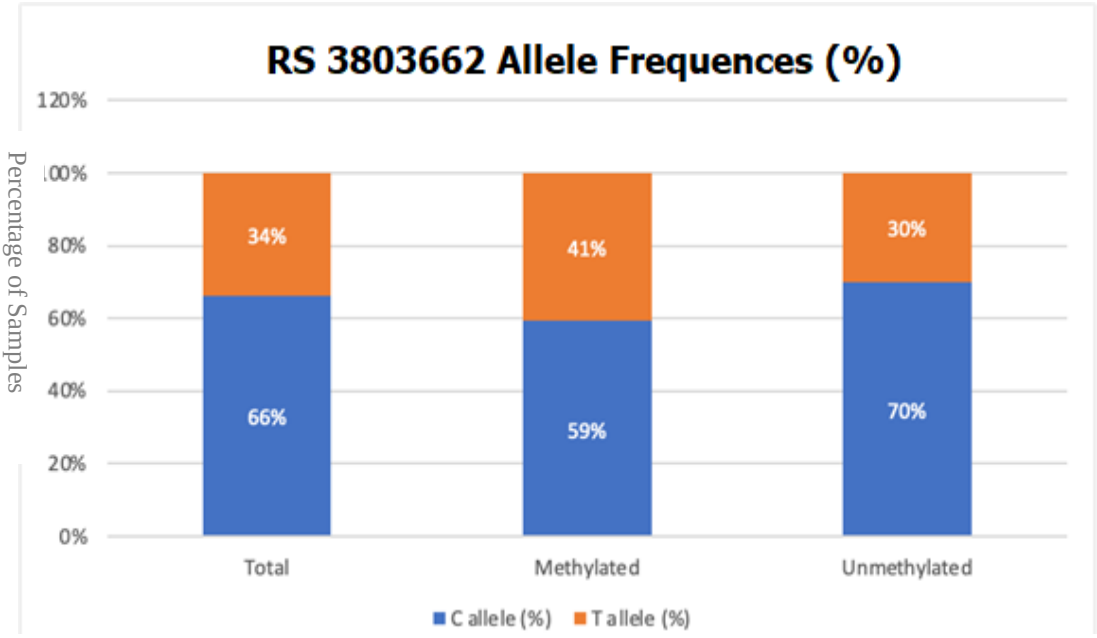

Figure 3. 6 allele frequencies for TOX3 RS3803662 in BRCA1 samples represented in percentage (%). Orange color for T allele and blue color for C allele. 1st column represents total allele frequencies, 2nd column represents allele frequencies for BRCA1

methylated samples, and 3rd column represents allele frequencies for BRCA1 unmethylated samples.

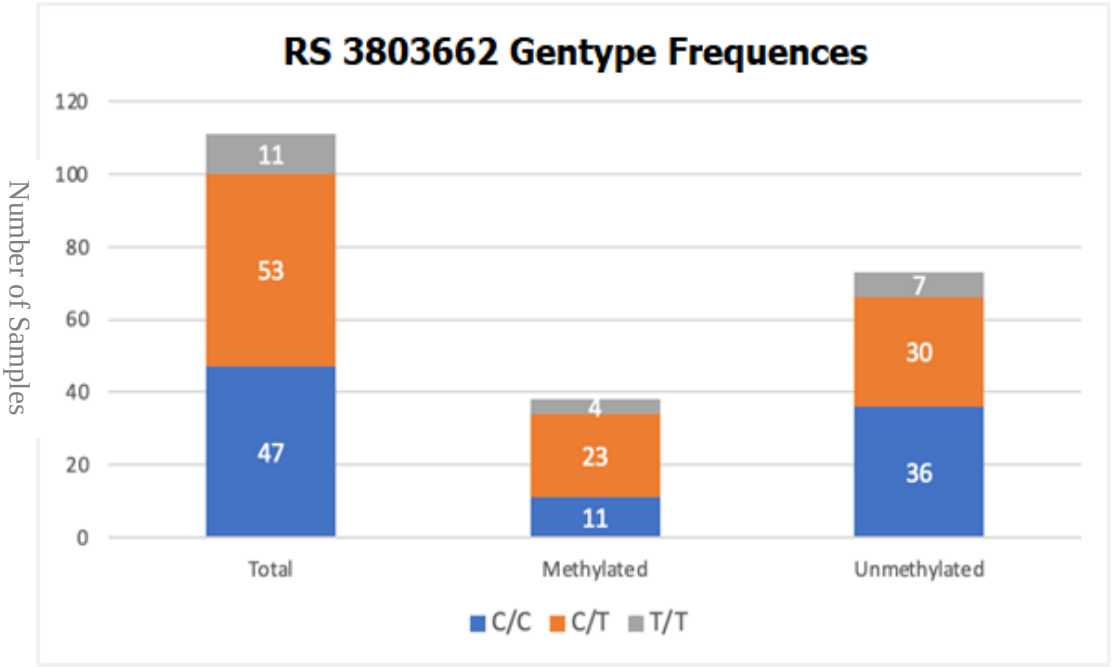

Figure 3. 7 genotype frequencies for TOX3 RS3803662 in BRCA1 samples. Gray color for T/T genotype, orange color for C/T genotype and blue color for C/C genotype. 1st column represents total genotype frequencies, 2nd column represents genotype frequencies for BRCA1 methylated samples, and 3rd column represents genotype frequences for BRCA1 unmethylated samples

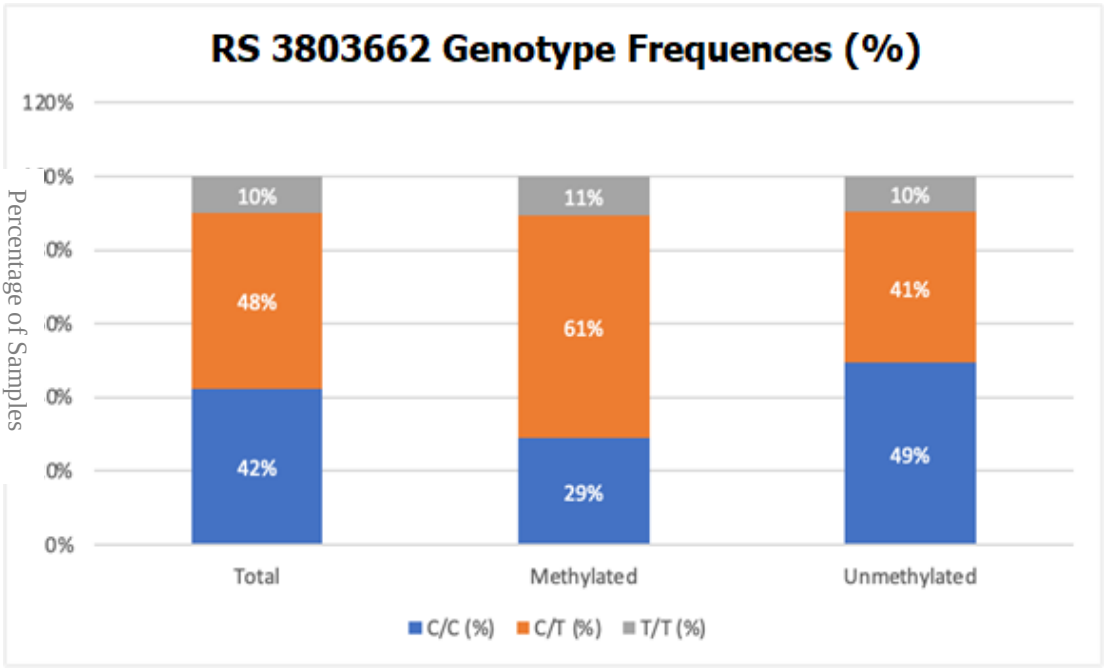

Figure 3. 8 Genotype frequencies for TOX3 RS3803662 in BRCA1 samples represented in percentage (%). Gray color for T/T genotype, orange color for C/T genotype and blue color for C/C genotype. 1st column represents total genotype frequencies, 2nd column represents genotype frequencies for BRCA1 methylated samples, and 3rd column represents genotype frequences for BRCA1 unmethylated samples.

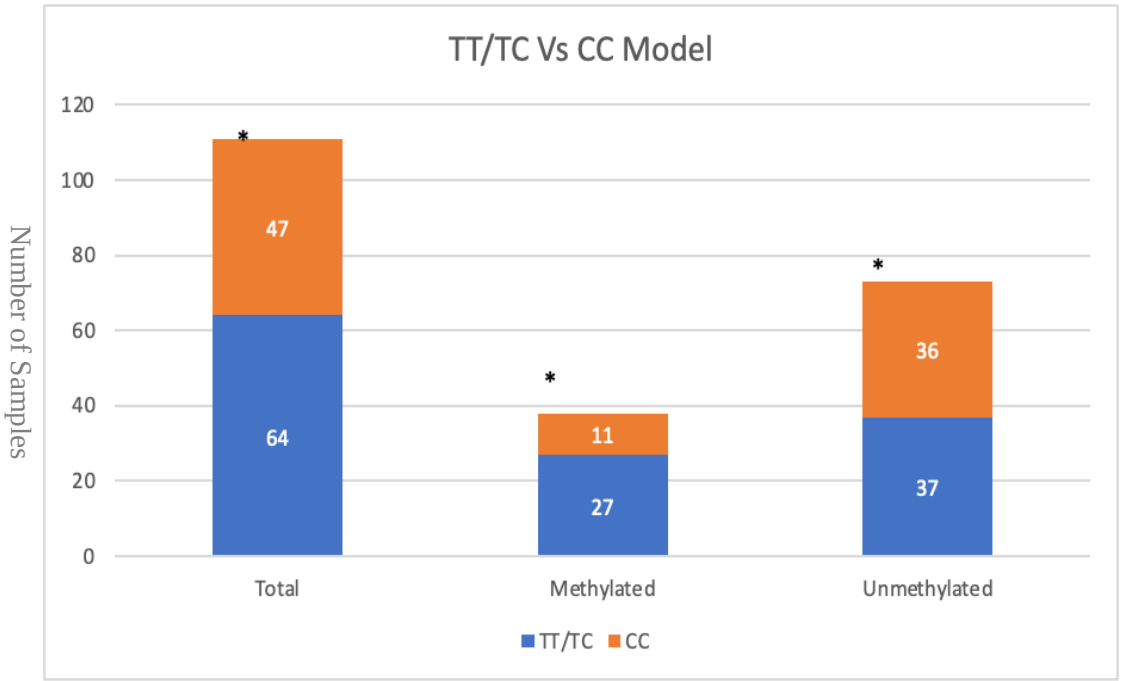

Figure 3. 9 Genotype frequencies for TOX3 RS3803662 in BRCA1 samples in TT/TC vs CC Model. Orange color represent CC genotype, blue color represents TT and TC genotype. 1st column represents total genotype frequencies, 2nd column represents genotype frequencies for BRCA1 methylated samples, and 3rd column represents

genotype frequences for BRCA1 unmethylated samples. The star (*) symbol indicate that P value is <0.05

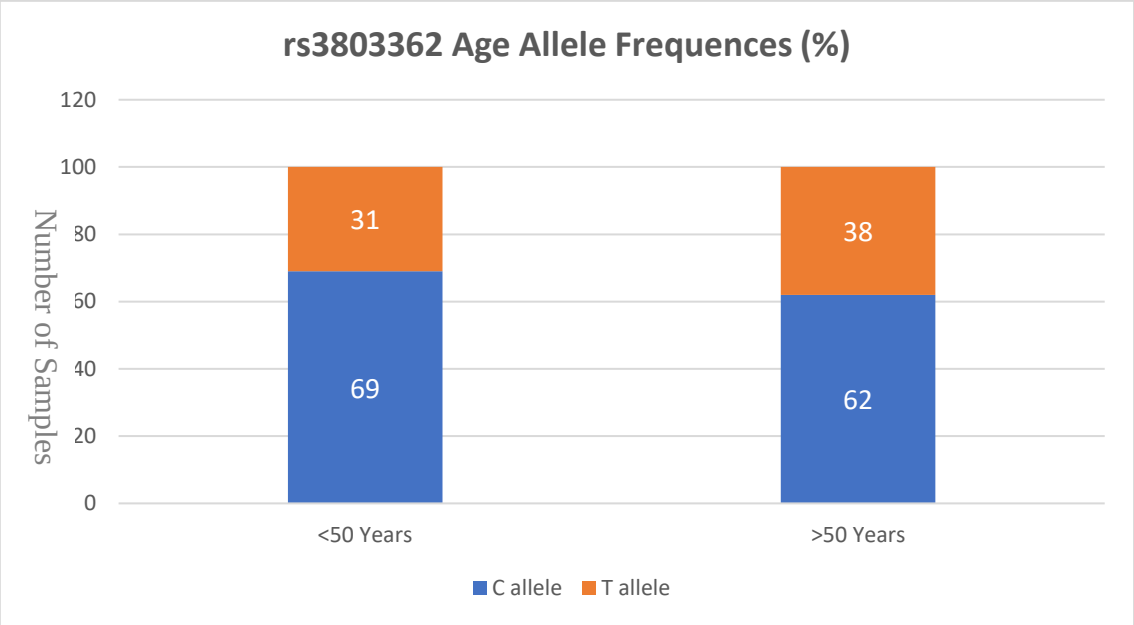

Figure 3. 10 Allele frequencies for TOX3 RS3803662 in BRCA1 samples based on age of patients represented in percentage (%). Orange color for T allele and blue color for C allele. 1st column represents allele frequencies in samples from women aged <50 years, and 2nd column represents allele frequencies fin samples from women aged >50 years

3.4.2 SNP rs8051542 does not correlate with methylation status on BRCA1 gene.

The analysis results of (rs8051542) with methylation status of BRCA1 gene promoter using SNP stat software (table 3.2) shows that the allele frequencies are relatively similar for the different methylation and unmethylation statuses with a slightly higher, but not significant, proportion of the C allele is observed in the methylated samples than in the unmethylated samples (figure 3.11 and 3.12). The genotype frequencies are consistent across the different methylation statuses and show no major deviations (figure 3.13 and 3.14). The SNP appears to be in Hardy-Weinberg equilibrium across both methylation statuses, indicating that the observed genotype frequencies are consistent with the expected genetic variation. None of the models showed a significant

association with methylation status. The odd ratios and p-values indicate that there is no strong evidence of an association between SNP genotype and methylation status in the data set. The log-additive model also shows no significant association, suggesting that the effect of this SNP on methylation may be minimal or absent in this sample. Overall, Analysis suggests that this SNP may not have a strong association with methylation status in the sample.

We encountered a major challenge in the PCR analysis of rs8051542 due to DNA degradation, a common problem when using DNA from formalin-fixed paraffin-embedded (FFPE) tissue. This degradation affected 28 samples, so we were unable to obtain reliable results for this SNP. Preliminary observations indicate that the results of rs8051542 show a similar trend to those of rs12443621, with no correlation found with rs3803662.

Table 3. 2 SNPSTAT tool analysis result for TOX3 rs8051542 correlation with BRCA1 gene methylation status

SNP: SNIP..rs8051542..antisense.
Percentage of typed samples: 84/111 (75.68%)

SNIP..rs8051542..antisense. allele frequencies (n=84)						
	All subjects		Methylation=M		Methylation=U	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
c	107	0.64	42	0.64	65	0.64
t	61	0.36	24	0.36	37	0.36

SNIP..rs8051542..antisense. genotype frequencies (n=111)						
	All subjects		Methylation=M		Methylation=U	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
c/c	38	0.45	15	0.45	23	0.45
c/t	31	0.37	12	0.36	19	0.37
t/t	15	0.18	6	0.18	9	0.18
NA	27	---	5	---	22	---

SNIP..rs8051542..antisense. exact test for Hardy-Weinberg equilibrium (n=84)						
	N11	N12	N22	N1	N2	P-value
All subjects	38	31	15	107	61	0.063
Methylation=M	15	12	6	42	24	0.26
Methylation=U	23	19	9	65	37	0.22

SNIP..rs8051542..antisense. association with response Methylation (n=84, crude analysis)							
Model	Genotype	Methylation=M	Methylation=U	OR (95% CI)	P-value	AIC	BIC
Codominant	c/c	15 (45.5%)	23 (45.1%)	1.00			
	t/c	12 (36.4%)	19 (37.2%)	1.03 (0.39-2.73)	1	118.6	125.8
	t/t	6 (18.2%)	9 (17.6%)	0.98 (0.29-3.32)			
Dominant	c/c	15 (45.5%)	23 (45.1%)	1.00			
	t/c-t/t	18 (54.5%)	28 (54.9%)	1.01 (0.42-2.44)	0.97	116.6	121.4
Recessive	c/c-t/c	27 (81.8%)	42 (82.3%)	1.00			
	t/t	6 (18.2%)	9 (17.6%)	0.96 (0.31-3.02)	0.95	116.6	121.4
Overdominant	c/c-t/t	21 (63.6%)	32 (62.8%)	1.00			
	t/c	12 (36.4%)	19 (37.2%)	1.04 (0.42-2.58)	0.93	116.6	121.4
Log-additive	---	---	---	1.00 (0.55-1.79)	0.99	116.6	121.4

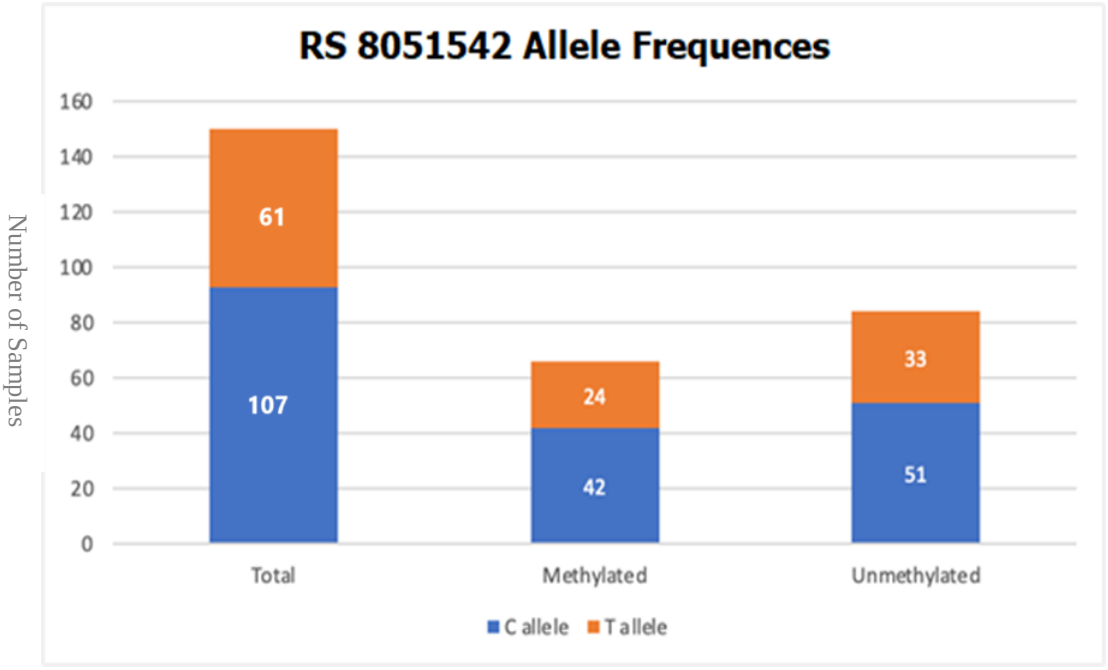

Figure 3. 11 Allele frequencies for TOX3 RS8051542 in BRCA1 samples. Orange color for T allele and blue color for C allele. 1st column represents total allele frequencies, 2nd column represents allele frequencies for BRCA1 methylated samples, and 3rd column represents allele frequencies for BRCA1 unmethylated samples

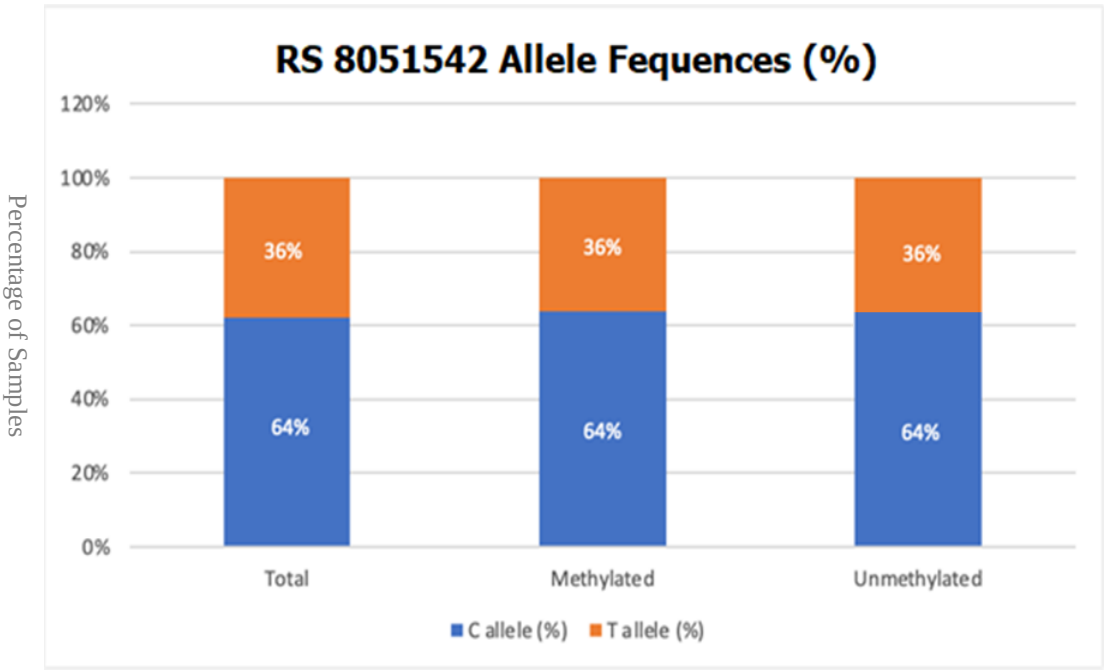

Figure 3. 12 Allele frequencies for TOX3 RS8051542 in BRCA1 samples represented in percentage (%). Orange color for T allele and blue color for C allele. 1st column represents total allele frequencies, 2nd column represents allele frequencies for BRCA1 methylated samples, and 3rd column represents allele frequencies for BRCA1 unmethylated samples.

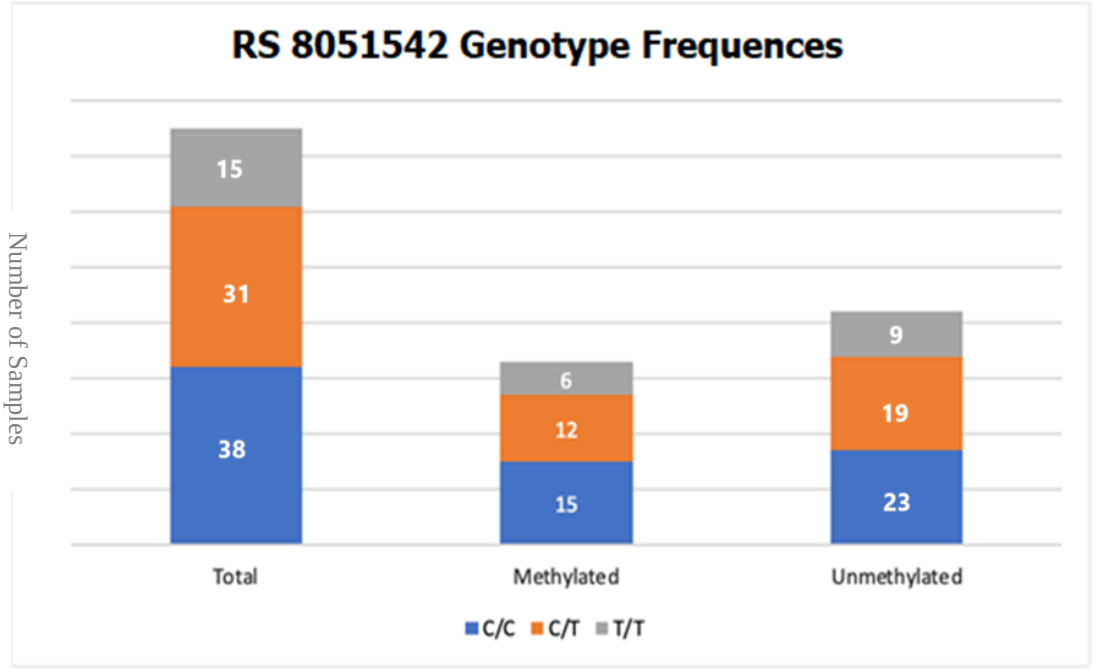

Figure 3. 13 Genotype frequencies for TOX3 RS8051542 in BRCA1 samples. Gray color for T/T genotype, orange color for C/T genotype and blue color for C/C genotype. 1st column represents total genotype frequencies, 2nd column represents genotype frequencies for BRCA1 methylated samples, and 3rd column represents genotype frequences for BRCA1 unmethylated samples

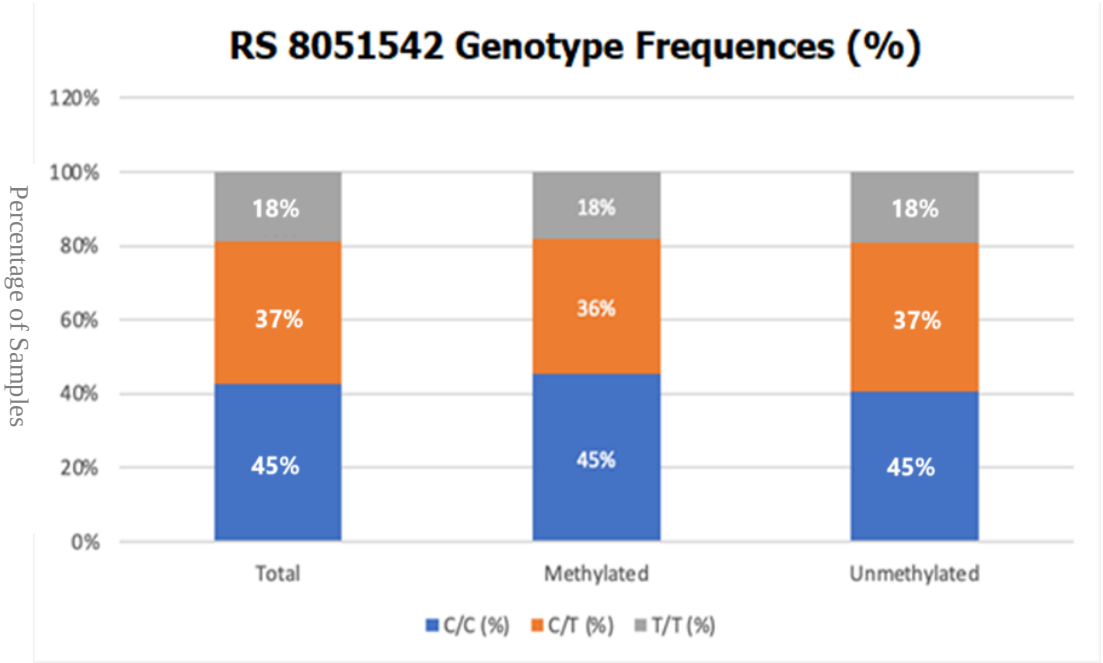

Figure 3. 14 Genotype frequencies for TOX3 RS8051542 in BRCA1 samples represented in percentage (%). Gray color for T/T genotype, orange color for C/T genotype and blue color for C/C genotype. 1st column represents total genotype frequencies, 2nd column represents genotype frequencies for BRCA1 methylated

samples, and 3rd column represents genotype frequences for BRCA1 unmethylated sample

3.4.3 SNP rs12443621 does not correlate with methylation status on BRCA1 gene

The (rs12443621) was analyzed in 108 samples (Table 3.3). Allele frequencies were found to be comparable between methylated and unmethylated samples, with a slightly higher proportion of G alleles observed in the unmethylated samples. This finding suggests that the G allele is slightly more common in unmethylated samples than in methylated samples, although the observed difference is minimal (figure 3.15 and 3.16). In addition, genotype frequencies remained relatively constant in the different methylation statuses. In particular, the proportion of A/A genotypes was slightly higher in the methylated group, while G/A genotypes were more frequent in the unmethylated group (figure 3.17 and 3.18). The SNP was in Hardy-Weinberg equilibrium in both methylation states, indicating that the observed genotype frequencies were consistent with expectations of random genetic variation, again suggesting that there are no significant problems with population structure. Furthermore, none of the genetic models showed a significant association between rs12443621 and methylation status, which is reflected in the odd ratios and p-values that do not indicate a strong association within the data set. The log-additive model also showed no significant association, suggesting that rs12443621 may not exert a significant influence on methylation status in this samples.

Table 3. 3 SNP STAT tool analysis result for TOX3 RS12443621 correlation with BRCA1 gene methylation status

SNP: rs12443621.
Percentage of typed samples: 108/111 (97.3%)

SNIP rs12443621..antisense. allele frequencies (n=108)						
	All subjects		Methylation=M		Methylation=U	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
G	124	0.57	40	0.56	84	0.58
A	92	0.43	32	0.44	60	0.42

SNIP rs12443621..antisense. genotype frequencies (n=111)						
	All subjects		Methylation=M		Methylation=U	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	19	0.18	8	0.22	11	0.15
G/A	54	0.5	16	0.44	38	0.53
G/G	35	0.32	12	0.33	23	0.32
NA	3	---	2	---	1	---

SNIP rs12443621..antisense. exact test for Hardy-Weinberg equilibrium (n=108)						
	N11	N12	N22	N1	N2	P-value
All subjects	35	54	19	124	92	1
Methylation=M	12	16	8	40	32	0.52
Methylation=U	23	38	11	84	60	0.63

SNIP rs12443621..antisense. association with response Methylation (n=108, crude analysis)							
Model	Genotype	Methylation=M	Methylation=U	OR (95% CI)	P-value	AIC	BIC
Codominant	G/G	12 (33.3%)	23 (31.9%)	1.00			
	A/G	16 (44.4%)	38 (52.8%)	1.24 (0.50-3.08)	0.61	142.5	150.5
	A/A	8 (22.2%)	11 (15.3%)	0.72 (0.23-2.26)			
Dominant	G/G	12 (33.3%)	23 (31.9%)	1.00	0.88	141.5	146.8
	A/G-A/A	24 (66.7%)	49 (68.1%)	1.07 (0.45-2.50)			
Recessive	G/G-A/G	28 (77.8%)	61 (84.7%)	1.00	0.38	140.7	146.1
	A/A	8 (22.2%)	11 (15.3%)	0.63 (0.23-1.74)			
Overdominant	G/G-A/A	20 (55.6%)	34 (47.2%)	1.00	0.41	140.8	146.2
	A/G	16 (44.4%)	38 (52.8%)	1.40 (0.63-3.12)			
Log-additive	---	---	---	0.89 (0.50-1.59)	0.69	141.3	146.7

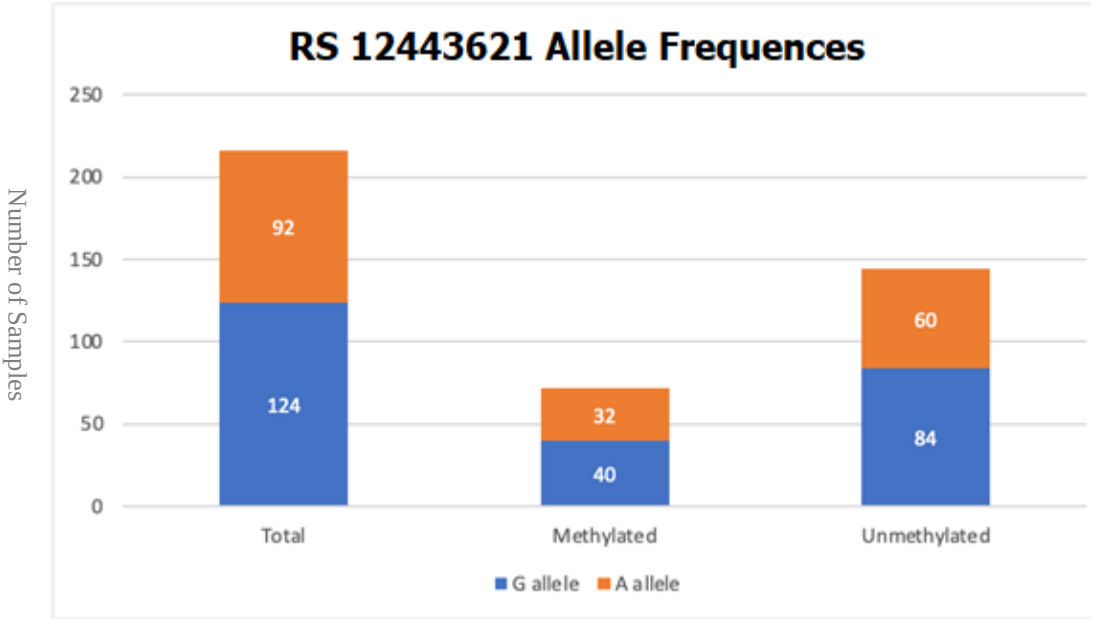

Figure 3. 15 Allele frequencies for TOX3 RS12443621 in BRCA1 samples. Orange color for A allele and blue color for G allele. 1st column represents total allele frequencies, 2nd column represents allele frequencies for BRCA1 methylated samples, and 3rd column represents allele frequencies for BRCA1 unmethylated samples

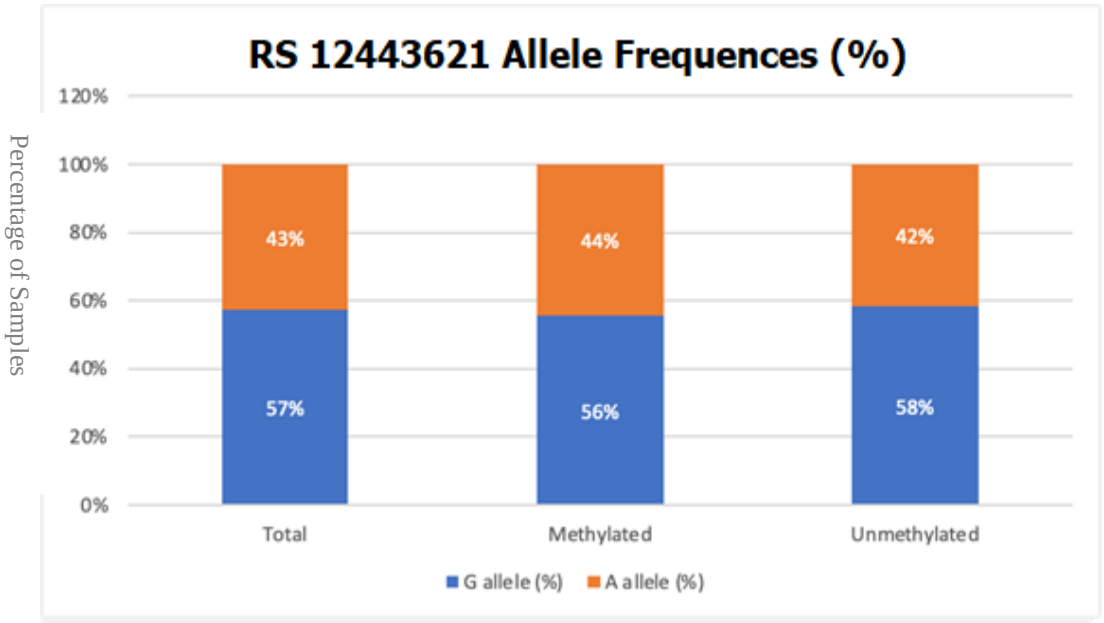

Figure 3. 16 Allele frequencies for TOX3 RS12443621 in BRCA1 samples represented in percentage (%). Orange color for A allele and blue color for G allele. 1st column represents total allele frequencies, 2nd column represents allele frequencies for BRCA1 methylated samples, and 3rd column represents allele frequencies for BRCA1 unmethylated samples.

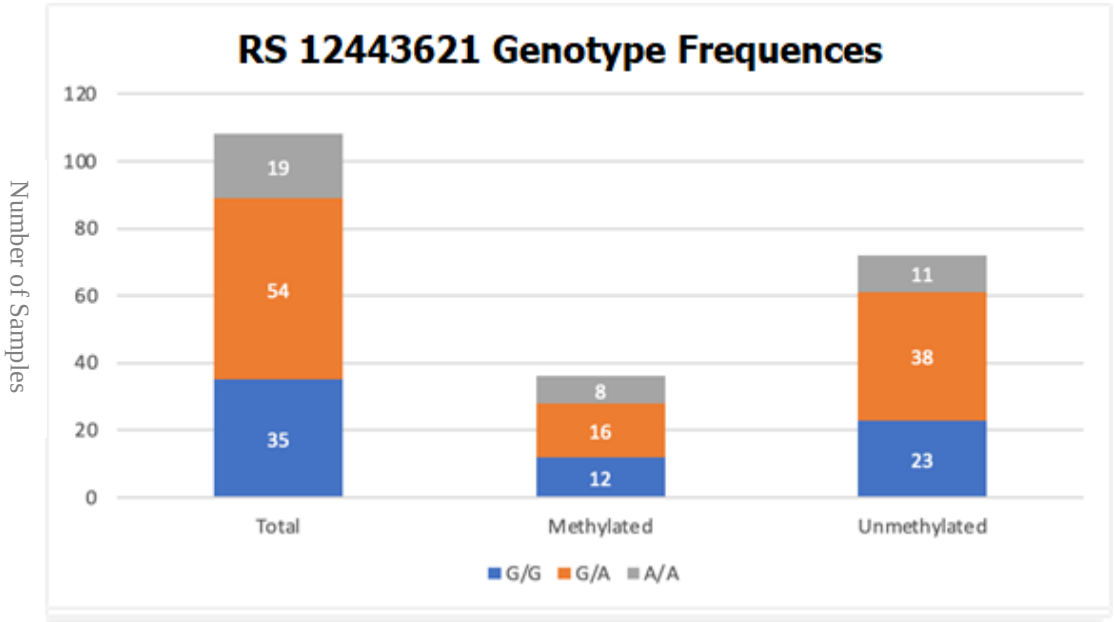

Figure 3. 17 Genotype frequencies for TOX3 RS12443621 in BRCA1 samples. Gray color for A/A genotype, orange color for G/A genotype and blue color for G/G genotype. 1st column represents total genotype frequencies, 2nd column represents genotype frequencies for BRCA1 methylated samples, and 3rd column represents genotype frequences for BRCA1 unmethylated samples

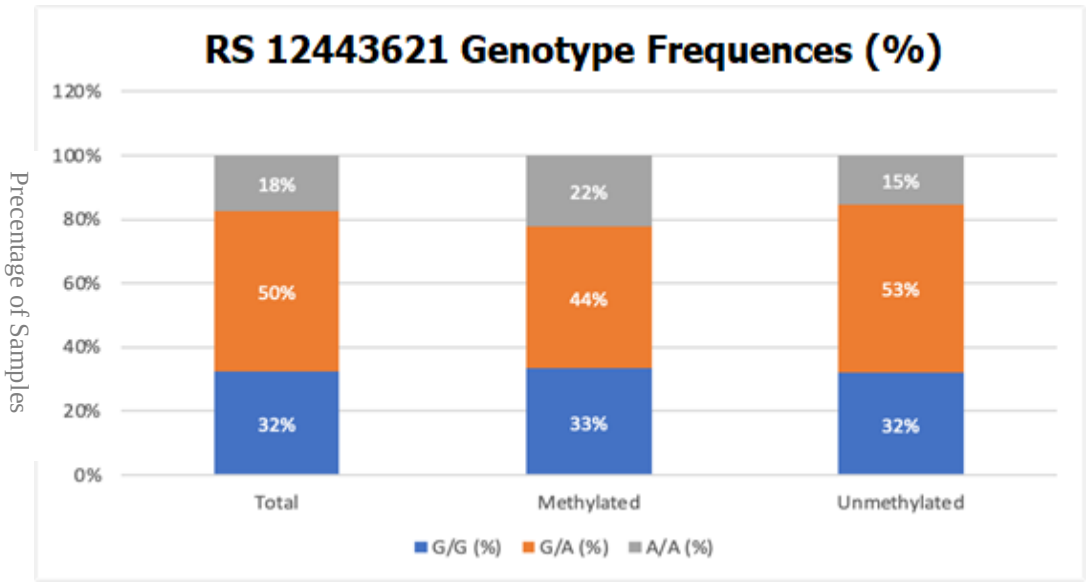

Figure 3. 18 Genotype frequencies for TOX3 RS12443621 in BRCA1 samples represented in percentage (%). Gray color for A/A genotype, orange color for G/A genotype and blue color for G/G genotype. 1st column represents total genotype frequencies, 2nd column represents genotype frequencies for BRCA1 methylated samples, and 3rd column represents genotype frequences for BRCA1 unmethylated samples.

3.5 Multiple SNP analysis

We performed multiple SNP analysis to detect the link between the different SNPs, in addition to haplotype analysis.

3.6 Linkage Disequilibrium Analysis

Linkage disequilibrium (LD) measures the non-random association of alleles at different loci. Here we performed analysis for the three SNPs. To understand the degree of LD between SNPs we reviewed D, D', and r statistics, and the corresponding P value (table 3.4).

The D statistic measures the deviation from the expected allele frequencies assuming no LD. Here the D values are very close to zero, indicating minimal linkage disequilibrium between these SNPs. The D' statistic standardizes the D value to a value between 0 and 1, providing a clearer picture of the strength of linkage disequilibrium. The D' values are all relatively low, indicating weak linkage disequilibrium between these SNPs. The low values indicate that there is little to no correlation between the SNPs in terms of allelic association (table 3.4).

The r-statistic or Pearson's correlation coefficient measures the linear relationship between the SNPs. The r values are all close to zero, indicating a very weak or no linear correlation between these SNPs. The negative values are minor and indicate that if there is a correlation, it is very weak. Finally, all p-values are high (above 0.05), indicating that there is no statistically significant linkage disequilibrium between any pair of SNPs. The

high p-values support the conclusion that the SNPs are not significantly associated with each other (Table 3.4).

Table 3. 4 SNPStat linkage disequilibrium results for TOX3 gene multiple SNP analysis

Multiple-SNP analysis

Linkage disequilibrium analysis

D statistic

	SNIP.rs3803662..sense.	SNIP.rs8051542..antisense.	SNIP.rs12443621..antisense.
SNIP.rs3803662..sense.	.	-0.0122	-0.0064
SNIP.rs8051542..antisense.	.	.	-0.0084
SNIP.rs12443621..antisense.	.	.	.

D' statistic

	SNIP.rs3803662..sense.	SNIP.rs8051542..antisense.	SNIP.rs12443621.antisense.
SNIP.rs3803662..sense.	.	0.0953	0.0446
SNIP.rs8051542..antisense.	.	.	0.0516
SNIP.rs12443621..antisense.	.	.	.

r statistic

	SNIP..rs3803662..sense.	SNIP..rs8051542..antisense.	SNIP.rs12443621..antisense.
SNIP.rs3803662..sense.	.	-0.0533	-0.0274
SNIP.rs8051542..antisense.	.	.	-0.0348
SNIP.rs12443621..antisense.	.	.	.

P-values

	SNIP.rs3803662..sense.	SNIP.rs8051542..antisense.	SNIP.rs12443621..antisense.
SNIP.rs3803662..sense.	.	0.5138	0.687
SNIP.rs8051542..antisense.	.	.	0.6699
SNIP.rs12443621..antisense	.	.	.

3.7 Haplotype Analysis

The provided haplotype frequencies for different combinations of SNPs (rs3803662, rs8051542 and rs12443621) were divided in two groups (group M and group U). The association analysis examines how each haplotype correlates with the response variable, with odds ratios (OR), 95% confidence intervals (CI) and p-values (table 3.5).

Haplotype frequencies varied between groups, with some haplotypes being more common in one group than the other. Haplotype 1 (C-T-G) and haplotype 2 (C-C-A) are the most common overall, while haplotype 8 (T-T-G) is the least common. Haplotype 4

(T-C-G) shows significant association with lower probability of BRCA1 gene methylation with a p-value of 0.048. However, other haplotypes have p-values above 0.05, indicating that there is no significant association with the response variable. Finally, the global p-value of 0.41 indicates that there is no significant overall association with the response variable when all haplotypes are considered together (table 3.6 table 3.7).

Most haplotypes have odds ratios close to 1, indicating no strong association with response. Confidence intervals for several haplotypes are wide, indicating high variability and limited precision of estimates (table 3.8).

Table 3. 5 SNP stat Haplotype result analysis for TOX3 gene RS3803662, rs8051542, and rs12443621 with BRCA1 gene methylation

Haplotype analysis

Haplotype frequencies estimation (n=111)

	..rs3803662..sense.	..rs8051542..antisense.	..rs12443621..antisense.	Total	group.M	group.U	Cumulative frequency
1	C	T	G	0.2211	0.0612	0.2561	0.2211
2	C	C	A	0.2098	0.1773	0.2071	0.4309
3	C	C	G	0.173	0.1724	0.19	0.604
4	T	C	G	0.1563	0.2034	0.1188	0.7603
5	T	T	A	0.0875	0	0.0888	0.8478
6	T	C	A	0.0705	0.0838	0.0746	0.9182
7	C	T	A	0.0582	0.1813	0.0454	0.9764
8	T	T	G	0.0236	0.1207	0.0191	1

Haplotype association with response (n=111, crude analysis)

	.rs3803662..sense.	rs8051542..antisense.	rs12443621..antisense.	Freq	OR (95% CI)	P-value
1	C	T	G	0.2183	1.00	---
2	C	C	A	0.2097	0.39 (0.10 - 1.57)	0.19
3	C	C	G	0.1738	0.47 (0.09 - 2.43)	0.37
4	T	C	G	0.1513	0.20 (0.04 - 0.97)	0.048
5	T	T	A	0.0825	0.28 (0.04 - 2.14)	0.22
6	T	C	A	0.0731	0.57 (0.08 - 4.05)	0.57
7	C	T	A	0.0603	0.11 (0.01 - 2.06)	0.14
8	T	T	G	0.0309	0.14 (0.00 - 5.03)	0.29

Global haplotype association p-value: 0.41

Table 3. 6 TOX3 gene SNPs Haplotype frequencies for rs3803662, rs8051542, and rs12443621 in methylated and unmethylated BRCA1 gene

Haplotype	rs3803662	rs8051542	rs12443621	Group M Frequency	Group U Frequency	Cumulative Frequency
1	C	T	G	0.2211	0.0612	0.2211
2	C	C	A	0.2098	0.1773	0.4309
3	C	C	G	0.1730	0.1724	0.6040
4	T	C	G	0.1563	0.2034	0.7603
5	T	T	A	0.0875	0.0000	0.8478
6	T	C	A	0.0705	0.0838	0.9182
7	C	T	A	0.0582	0.1813	0.9764
8	T	T	G	0.0236	0.1207	1.0000

Table 3. 7 Haplotype frequencies response

Haplotype	Frequency	OR (95% CI)	P-value
1	0.2183	1.00	---
2	0.2097	0.39 (0.10 - 1.57)	0.19
3	0.1738	0.47 (0.09 - 2.43)	0.37
4	0.1513	0.20 (0.04 - 0.97)	0.048
5	0.0825	0.28 (0.04 - 2.14)	0.22
6	0.0731	0.57 (0.08 - 4.05)	0.57
7	0.0603	0.11 (0.01 - 2.06)	0.14
8	0.0309	0.14 (0.00 - 5.03)	0.29

Chapter 4: Discussion

The relationship between the tumor suppressor gene BRCA1 and breast cancer is a much-discussed topic in genetic research. However, in the Palestinian population, pathogenic BRCA1 mutations are observed at a significantly lower frequency compared to other populations(Birgisdottir et al., 2006b). |Immunohistochemical analysis from a previous study in Palestine reported that 75.4% of cases had no nuclear expression of BRCA1 protein, a finding consistent with the results of several other studies (Ahlam I. Mujahed, 2016; M. E. Thompson et al., 1995; Turner et al., 2004). In addition, as reported in a previous study on Palestinian women with breast cancer this study found that 34% of breast cancer cases of Palestinian women cohort exhibited BRCA1 gene promoter methylation(Ahlam I. Mujahed, 2016), which is higher than the proportions reported in European, Taiwanese and Indian populations, ranging with a considerably larger number of samples from 11% to 32%(Ahlam I. Mujahed, 2016; Bal et al., 2012b; Birgisdottir et al., 2006c; Catteau et al., 1999; Esteller et al., 2000; Hsu et al., 2013; Krop et al., 2003; Wei, Grushko, Dignam, Hagos, Nanda, Sveen, Xu, Fackenthal, Tretiakova, & Das, 2005).

The increased methylation in the BRCA1 promoter observed in the Palestinian cohort suggests that epigenetic modifications play an important role in BRCA1 expression regulation and thus breast carcinogenesis among Palestinian women. It is known that methylation of the BRCA1 promoter silences gene expression and may serve as an alternative pathway for BRCA1 gene inactivation, especially in populations with fewer gene mutations (Agarwal et al., 2022; Jagtap & Jagtap, 2023; Lopez-Serra & Esteller, 2012). The increased methylation may reflect a particular epigenetic landscape in the Palestinian population that influences BRCA1 gene expression and susceptibility to breast cancer. Therefore, the aim of our study was to investigate the correlation between

TOX3 gene SNPs and BRCA1 promoter methylation in Palestinian breast cancer patients to elucidate the interaction between genetic and epigenetic factors in our population. In particular, TOX3 appears to reduce the expression of BRCA1 by enhancing methylation of its promoter, which may contribute to the increased aggressiveness of breast cancer (Shan et al., 2013).

Our results show that rs3803662 in the TOX3 gene correlates with increased BRCA1 gene promoter methylation, suggesting that this SNP may influence gene silencing through epigenetic mechanisms. This finding is consistent with studies from other populations, e.g. from Europe, where associations between these SNPs and breast cancer risk have been reported (Easton, Pooley, Dunning, Pharoah, Thompson, et al., 2007b; Stacey, Manolescu, Sulem, Rafnar, Gudmundsson, et al., 2007a), In contrast, neither (rs8051542) nor (rs12443621) showed any correlation with BRCA1 methylation status. The lack of association underscores a distinct epigenetic landscape in the Palestinian population that may influence BRCA1 gene expression and susceptibility to breast cancer in a manner distinct from other populations.

SNP rs3803662, located 8 kilobases upstream of the TOX3 gene, is located in the promoter region, where it can influence gene expression by interfering with the binding of transcription factors or interacting with enhancer or silencer elements, thereby altering the gene's response to various signals. In contrast, SNPs rs12443621 and rs8051542 are located within a linkage disequilibrium (LD) block, which is a region of DNA where alleles are inherited together more frequently than expected by chance, at the 5′-end of TOX3 (Easton, Pooley, Dunning, Pharoah, Thompson, et al., 2007c). These SNPs near the 5′ untranslated region (UTR) or promoter may affect transcription initiation, mRNA stability or translation efficiency (Wieder et al., 2024) and could interact with other

functional variants in the LD block that affect gene expression (Araujo et al., 2012; Kochetov et al., 1998; Leppek et al., 2018; Pesole et al., 2001). While rs3803662 likely exerts a more direct effect on gene expression through transcriptional regulation and promoter activity, rs12443621 and rs8051542 may potentially affect gene expression indirectly through association with other functional variants (Easton, Pooley, Dunning, Pharoah, Thompson, et al., 2007c). However, further research is needed to confirm these indirect effects and to determine the specific functional variants within the LD block that may affect TOX3 expression.

Our analysis of the rs3803662 in a Palestinian population revealed a potential association between higher C allele frequency and younger age of breast cancer onset. Specifically, patients diagnosed before the age of 50 had a C allele frequency of 69% compared to 62% for those diagnosed at age 50 or later. Although this difference did not reach statistical significance ($p=0.508$), it suggests a possible correlation between the presence of the C allele and an earlier age of onset of breast cancer. This observation is consistent with previous research suggesting that rs3803662, located in a genomic region associated with susceptibility to breast cancer, may contribute to the development of the disease(Easton, Pooley, Dunning, Pharoah, Thompson, et al., 2007d; Stacey, Manolescu, Sulem, Rafnar, Gudmundsson, et al., 2007b). However, we concede that this p-value is far from significant and that this trend could be due to chance or confounding factors. Further research with a larger sample size is required to confirm this association. A higher frequency of the C allele in younger patients could indicate its contribution to signalling pathways that predispose individuals to earlier tumorigenesis. However, carcinogenesis is complex and multifactorial, and this SNP is likely only one of many contributing factors. The lack of statistical significance in our study suggests that this association may

be subtle or influenced by other genetic, epigenetic or environmental factors. In addition, functional tests are required to further investigate this association. Future research needs to use cell line models to assess the effects of this variant on gene expression and relevant metabolic pathways.

We performed comprehensive analysis of multiple SNPs in the TOX3 gene to investigate their association individually or through their interaction and breast cancer development. This included performing linkage disequilibrium (LD) and haplotype analyses to investigate how these SNPs might relate to each other and to BRCA1 methylation. Our data revealed minimal non-random association, as evidenced by D and D' values close to zero and Pearson's r statistic with values close to zero. The calculated p-values confirmed the lack of statistically significant LD between these SNPs suggesting that there is no interaction or co-inheritance among them in the investigated Palestinian cohort. These results are consistent with previous studies indicating weak or the absence of linkage disequilibrium (LD) between these SNPs in different genetic contexts. Shifman et al. showed that SNPs within certain gene regions may have minimal LD, reflecting the genetic variations in different populations (Shifman et al., 2003). This observation is also consistent with the findings of A. G. Clark et al, who investigated how LD varies with allele frequencies within different gene regions and emphasized that LD can vary considerably between genes and populations (A. G. Clark et al., 1998). Similarly, (Einarsdóttir et al., 2006) observed weak LD between SNPs in their study of CHEK2 gene variations and breast cancer risk, further illustrating the variability of SNP interactions based on genetic background and population differences. In contrast, other studies have found strong LD between SNPs within specific genes or loci, which may influence the effectiveness of genetic association studies. For example, (Cargill et al., 1999; Halushka

et al., 1999) reported significant LD between SNPs within specific genomic regions , In contrast to our study, which found only minimal non-random association, these studies reported significant LD, demonstrating that strong LD can affect the interpretation of genetic associations and complicate the identification of disease-related variants. This difference in results underscores the complexity of genetic research and highlights the importance of considering LD patterns, which can vary considerably between genes and populations, when analyzing SNP associations.

The lack of significant LD between the SNPs analyzed highlights the importance of assessing the individual impact of each SNP on gene regulation and disease susceptibility, especially in diverse populations such as the Palestinian population, where LD patterns may vary. For example, the different epigenetic landscape observed in the Palestinian cohort, reflected by the association of rs3803662 with BRCA1 methylation and not with SNPs rs8051542 and rs12443621, emphasizes the need for population-specific studies to accurately assess genetic risk factors.

Haplotype analysis of SNPs rs3803662, rs8051542 and rs12443621 in two groups provided important insights into their association with BRCA1 gene methylation. The analysis was performed on the same samples for which BRCA1 methylation status was determined, as shown in the association analysis of rs3803662 genotypes and methylation status shown in Table 3.1. The results of our haplotype analysis, as shown in Table 3.5, haplotype 4 (T-C-G) was found to be significantly associated with lower probability of BRCA1 gene methylation with p-value 0.048. Haplotype 4 (T-C-G) contains the T allele of the rs3803662 SNP. This finding suggests that haplotype 4 (T-C-G) may exert a protective effect against BRCA1 methylation. This result is consistent with our analysis of rs3803662, which showed that the T allele is associated with lower BRCA1 gene

methylation. Notably, haplotype 4 contains the T allele of rs3803662, and as shown in Table 3.1, the T allele is associated with an increase in the percentage of methylated samples (60.5% in the T/C genotype) and a decrease in the percentage of unmethylated samples (28.9% in the C/C genotype) in the codominant model. This indicates a correlation between the T allele and reduced methylation. Haplotype 4 exhibited an odds ratio of 0.20 (95% CI: 0.04-0.97), indicating a lower probability of BRCA1 methylation associated with this haplotype. The confidence interval indicates a range of uncertainty in this estimate. In contrast, other haplotypes did not show statistically significant associations, as indicated by p-values greater than 0.05, and the global p-value of 0.41 suggests that there is no significant overall association with BRCA1 methylation when all haplotypes are considered together. The global p-value indicates that the overall haplotype analysis is not significant, but the p-value of haplotype 4 shows that this specific haplotype has a significant association. Most haplotypes showed odds ratios close to 1, indicating a lack of significant correlation with BRCA1 methylation. The wide confidence intervals for several haplotypes emphasize the high variability and limited precision of the estimates. These results illustrate that although haplotype 4 is significantly associated with BRCA1 gene methylation, the impact of haplotype variations on BRCA1 methylation is generally not substantial. The observed significance of haplotype 4, despite the non-significant global p-value, could be due to specific genetic interactions within this haplotype or to other factors unique to the Palestinian population. Further studies with larger samples and refined methods are needed to clarify the role of haplotypes in the regulation and methylation of the BRCA1 gene and cancer susceptibility in the Palestinian population.

In the haplotype analysis and the individual analysis, rs3803662 showed that the C allele is the most common allele in the samples analyzed. However, in the normal population in our region, T allele is the most prevalent (Ref Allele T = 57,98%) (NCBI, 2024). This comparison serves to highlight possible differences in allele frequencies between our breast cancer patient cohort and the general population in the region, which may provide insights into the role of the C allele in breast cancer susceptibility. Since all the samples analyzed were breast cancer samples, the presence of the C allele may reflect that the C allele correlates more with breast cancer cases. The presence of the C allele in homozygous form appears to be the most genotype that correlates with BRCA1 methylation, as the only significant association was found with rs3803662 with dominant model (TT/TC vs. CC). Since other SNPs (rs8051542 and rs12443621) do not show significant association with the methylation status of the BRCA1 gene, this is consistent with other studies showing that rs3803662 is the major TOX3 gene SNP that correlates with breast cancer cases (Han et al., 2011; Long et al., 2010; Stacey, Manolescu, Sulem, Rafnar, Gudmundsson, et al., 2007b). It is important to be aware that the comparison of our patient cohort with a general population database may have limitations. However, further studies need to be conducted to investigate the mechanism of action of this SNP in increasing methylation of the BRCA1 gene in the Palestinian population.

In conclusion, our study is the first to establish an association between TOX3 SNPs and BRCA1 gene methylation status and to include epigenetic events in the context of breast cancer research in the Palestinian population. We found that rs3803662 in the TOX3 gene, specifically in the dominant model, was significantly ($p = 0.037$) associated with increased BRCA1 promoter methylation, suggesting a possible epigenetic mechanism influencing susceptibility to breast cancer in this cohort. This result is

particularly noteworthy considering that BRCA1 methylation has been observed in our study found BRCA1 methylation in our Palestinian cohort, and that there are no significant associations with SNPs rs8051542 and rs12443621. Our study highlights the need for further research to elucidate the mechanisms involved in BRCA1 methylation and their contribution to breast cancer risk assessment and management in the Palestinian population.

4.1 Recommendations for future research

To further elucidate the role of the TOX3 gene in BRCA methylation, future work should focus on investigating gene expression of TOX3 in both FFPE and fresh tissue samples, with special emphasis on samples with the C allele of rs3803662 using quantitative PCR analysis. Simultaneously the assessment of the impact of TOX3 expression on BRCA methylation using methylation-specific PCR, bisulfite sequencing or methylation arrays will provide valuable insights. Moreover, the use of gene editing techniques such as CRISPR/Cas9 to create TOX3 knockdown or knockout models could help to assess the direct its direct effect on BRCA methylation. In addition, the use of immunohistochemistry to visualize TOX3 protein expression in tissue samples will complement these results by revealing the presence and localization of the TOX3 protein. By integrating these methods, the generated data will significantly contribute to the understanding of the functional links between TOX3 expression and BRCA methylation that could reveal novel therapeutic targets or interventions.

4.2 Conclusion

This study represents a significant advance in understanding the interplay between genetic and epigenetic factors in susceptibility to breast cancer in the Palestinian

population. Our analysis of TOX3 polymorphisms revealed that the SNP rs3803662 is significantly associated with increased BRCA1 promoter methylation. Specifically, under a codominant model, individuals with the T/C genotype were significantly lower odds of methylation (OR=0.40, 95% CI=0.17-0.95, p=0.037) compared to the C/C genotype. In a dominant model, individuals with the T/C-T/T genotypes together also showed a lower probability of methylation (OR=0.42, 95% CI=0.18-0.97, p=0.037) compared to the C/C genotype. These results suggest that the presence of the T allele in rs3803662 may be associated with a lower probability of BRCA1 promoter methylation. The association result indicates that rs3803662 may contribute to BRCA1 gene silencing via epigenetic mechanisms, potentially influencing breast cancer risk in this cohort. In contrast, SNPs rs8051542 and rs12443621 did not show significant correlations with BRCA1 methylation, emphasizing the specificity of the role of rs3803662. The high prevalence of BRCA1 promoter methylation observed in the Palestinian cohort compared to other global cohorts emphasizes the importance of considering population-specific epigenetic landscapes when studying breast cancer. The observed lack of significant linkage disequilibrium between the SNPs analyzed also suggests that these genetic variations act independently on BRCA1 regulation rather than through complex interactions.

Our findings provide valuable insights into the genetic and epigenetic mechanisms underlying breast cancer susceptibility in the Palestinian population. For example, the association between the SNP rs3803662 and BRCA1 promoter methylation suggests that this SNP may affect the expression of BRCA1, an important tumor suppressor gene involved in DNA repair. Disruption of BRCA1 function can increase the risk of breast cancer. The lack of association between the other SNPs and BRCA1 methylation also suggests that these SNPs may contribute breast cancer risk through other mechanisms that

are independent of BRCA1 methylation. These findings emphasize the need for further research to directly investigate these associations at the functional level. Future studies should aim to elucidate the detailed mechanisms by which the SNP rs3803662 affects BRCA1 promoter methylation and explore the implications of these findings on breast cancer risk assessment and personalized management strategies. Extending this research to different populations will be critical for validating these findings and improving our understanding of the genetic and epigenetic factors that contribute to breast cancer development.

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الملخص

يُعد سرطان الثدي مشكلة صحية عامة رئيسية في جميع أنحاء العالم، حيث تلعب العوامل الوراثية واللاجينية دورًا مهمًا في حدوثه. يُعد تعطيل جين BRCA1، وخاصةً بسبب الطفرات الجينية الوراثية، عامل خطر رئيسي للإصابة بسرطان الثدي. ومع ذلك، فإن طفرات BRCA1 أقل شيوعًا في المجتمع الفلسطيني، مما يشير إلى أن آليات أخرى، مثل التغيرات اللاجينية، قد تلعب دورًا في ذلك.

استهدفت هذه الدراسة التحقيق في العلاقة بين تعدد أشكال النوكليوتيدات المفردة (SNPs) في جين TOX3 (rs3803662, rs8051542, rs12443621) وميثيلة محفز BRCA1 لدى مجموعة من مريضات سرطان الثدي الفلسطينيات. تم إجراء التنميط الجيني باستخدام تقنيات PCR-RFLP وARMS وSanger Sequencing.

كشفت تحليلاتنا أن rs3803662 مرتبط بشكل كبير بزيادة ميثيلة محفز BRCA1، مما يشير إلى وجود آلية لاجينية محتملة من خلالها يؤثر هذا SNP على تثبيط BRCA1 وزيادة خطر الإصابة بسرطان الثدي. وكانت هذه العلاقة بارزة بشكل خاص لدى المريضات الأصغر سنًا (<50 عامًا)، حيث كان لديهن تردد أعلى للأليل C الخاص بـ rs3803662. وعلى النقيض من ذلك، لم تُظهر SNPs rs8051542 وrs12443621 أي ارتباط كبير بميثيلة BRCA1، مما يبرز الدور المحدد لـ rs3803662 في التنظيم الوراثي-البيئي. أظهرت تحليلات توازن الارتباط (LD) ارتباطًا غير عشوائي ضئيلًا بين هذه SNPs، مما يدعم تأثيراتها المستقلة على تنظيم BRCA1. كشفت تحليلات الأنماط الوراثية أن النمط الوراثي الرابع (T-C-G) يرتبط بشكل كبير بانخفاض ميثيلة BRCA1، مما يشير إلى تأثير وقائي محتمل. ومع ذلك، أظهرت الأنماط الوراثية الأخرى

ارتباطات محدودة أو غير ذات دلالة إحصائية. وتؤكد النسبة المرتفعة الملحوظة لميثيلة محفز BRCA1 في المجتمع الفلسطيني مقارنةً بغيرهم من المجتمعات الأخرى على الأهمية البالغة لأخذ الخصائص الوراثية-البيئية (اللاجينية) الخاصة بكل مجموعة سكانية بعين الاعتبار في أبحاث سرطان الثدي.

تشير نتائجنا إلى أن تعدد الأشكال الجيني rs3803662 في جين TOX3 قد يساهم في زيادة ميثيلة محفز BRCA1 وزيادة القابلية للإصابة بسرطان الثدي في المجتمع الفلسطيني. تسلط هذه الدراسة الضوء على أهمية مراعاة العوامل الجينية والوراثية-البيئية (اللاجينية) لفهم مخاطر الإصابة بسرطان الثدي في هذه الفئة السكانية. هناك حاجة إلى مزيد من الأبحاث لتوضيح الآليات الأساسية وآثارها السريرية. يمكن أن يؤدي فهم أعمق لهذه الآليات إلى تحسين استراتيجيات تقييم المخاطر ودعم تطوير تدخلات علاجية مخصصة لسرطان الثدي.