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DNA METHYLATION PROFILLING of ING1 GENE in TRIPLE NEGATIVE BREAST CANCER

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Prof. Dr. Işık Didem KARAGÖZ

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ABSTRACT

DNA METHYLATION PROFILLING of ING1 GENE in TRIPLE NEGATIVE BREAST CANCER

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Due to the high incidence of triple-negative breast cancer in women globally, which is characterized by poor diagnosis and treatment, there was a need to search for new and innovative ways to diagnose and investigate this type of breast cancer at the epigenetic level. Epigenetic inheritance is the key to understanding diseases and the physiological changes in the organs and tissues of the body, and global and normal precancerous breast cancer in particular. DNA methylation plays an essential role in the pathogenesis of diseases in the human body. Understanding gene expression, epigenetics, and DNA methylation and their relationship to the ING1 tumor suppressor gene family can contribute to the development of treatment for triple-negative breast cancer. Through our research, we investigated the relationship between methylation of the ING 1 promoter site and triple-negative breast cancer. As a result of this study, we found the relative methylation rate was 93.5% in our triple-negative breast cancer samples. We noticed an increase in DNA methylation in triple breast cancer cells, which may be related to its expression decreasing in this aggressive breast cancer subtype. We suggested the ING-1 gene may be a potential marker for the early diagnosis of triple-negative breast cancer. We used 92 samples taken from women diagnosed with triple breast cancer with different stages of cancer. We found a close relationship between DNA methylation and this type of breast cancer. We also found a decrease in the expression of the ING-1 gene in the cells. As 93.5% of the samples showed the presence of DNA methylation, and only 6% showed un-methylation.

Key Words: Breast Cancer, TNBC, Biomarkers, Epigenetics, DNA Methylation, ING1.

ÖZET

ÜÇLÜ NEGATIF MEME KANSERINDE (ÜNMK) ING1 GENININ DNA METILASYON PROFILININ BELIRLENMESI

EZZAT, Alaa
Yüksek LisansTezi, Biyoloji Bölümü
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Teşhisi ve tedavisi zor olarak karakterize edilen üçlü negatif meme kanseri dünya genelinde kadınlarda insidansının yüksek olması nedeniyle, bu tür meme kanserini epigenetik düzeyde teşhis etmek ve araştırmak için yeni ve yenilikçi yollar aramaya ihtiyaç vardır. Epigenetik kalıtım, hastalıkları ve vücudun organ ve dokularındaki fizyolojik değişimi ve özellikle genel ve normal kanser öncesi meme kanseri oluşumunu anlamanın anahtarıdır. DNA metilasyonu, en kararlı epigenetik olaylara katkıda bulunur. DNA metilasyonu insan vücudundaki hastalıkların patogenezinde önemli bir rol oynar. Gen ifadesinin, epigenetiğin ve DNA metilasyonunun ve bunların ING1 tümör baskılayıcı gen ailesiyle ilişkilerinin anlaşılması, üçlü negatif meme kanseri tedavisinin geliştirilmesine katkıda bulunabilir.. Araştırmamız boyunca, ING 1 promoter bölgesinin metilasyonu ile üçlü negatif meme kanseri arasındaki ilişkiyi araştırdık. Bu çalışma sonucunda üçlü negatif meme kanseri örneklerimizde göreceli metilasyon oranını %93,5 olarak bulduk. Üçlü meme kanseri hücrelerinde DNA metilasyonundaki artışın, bu agresif meme kanseri alt tipinde ekspresyonunun azalmasıyla ilişkili olabileceğini fark ettik. ING 1 geninin üçlü negatif meme kanserinin erken teşhisi için potansiyel bir belirteç olabileceğini düşündük. Üçlü negatif meme kanseri teşhis alan ve kanserin farklı evrelerindeki kadınlardan alınan 92 örneği kullandık. DNA metilasyonu ile bu tür meme kanseri arasında yakın bir ilişki bulduk. Ayrıca hücrelerde ING-1 geninin ekspresyonunda da düşmek tespit ettik. Örneklerin %93,5'i DNA metilasyonunun varlığını gösterdi ve yalnızca %6'sı metilasyondan sadace unmatilasyon gösterdi.

Anahtar Kelimeler: Meme Kanseri, ÜNMK, Biyobelirteçler, Epigenetik, DNA Metilasyonu, ING1.

"Dedicated to my family"

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OFABBREVIATIONS

AR	Androgen Receptor
ASPM	Active-State Power Management/ Assembly Factor For Spindle Microtubules
BC	Breast Cancer
BCL2	B-Cell Lymphoma 2
BDDs	Benign Breast Disorders
BL1, 2	Basal-Like 1, 2
BMI	Body Mass Index
BRCA1, 2	Breast Cancer Type 1 Susceptibility Protein
CCND1, E1	Cyclin D1
CDH1	Cadherin 1
CENPK	Centromere Protein K
CHK1/2	Checkpoint Kinase 1/2
CNAs	Copy-Number Alterations
CSPG4	Chondroitin Sulfate Proteoglycan 4
DCC	Deleted in Colorectal Cancer
DDT	Dichloro Diphenyl Trichloroethane
DHh	Destert Hedgehog Signaling Molecule
DLL1	Delta Like Canonical Notch Ligand 1
ECD	Ecdysoneless Cell Cycle Regulator
EGFR	Epidermal Growth Factor Receptor
ELF-EMFs	Extremely Low-Frequency Electromagnetic Fields
ER	Estrogen Receptor
ERBB	Receptor Tyrosine Kinases
ERK	Extracellular Signal-Regulated Kinase
FGFR1, 2	Fibroblast Growth Factor Receptor 1, 2

FK506	Tacrolimus
FRAP1	Recombinant Human Protein
GLI1	Glı Family Zinc Finger 1
HER1 or ERBB1	Receptor Tyrosine-Protein Kinase Erbb-1
HER2	Human Epidermal Growth Factor Receptor
HES	Hes Family Bhlh Transcription Factor
Hh	Hedgehog Signaling Molecule
HRT	Hormone Replacement Therapy
IBC	Inflammatory Breast Cancer
IGF-I	Insulin-Like Growth Factor I
IHh	Indian Hedgehog Signaling Molecule
IncRNA	Long Non-Coding RNAs
INPP4B	Inositol Polyphosphate-4-Phosphatase Type B
KLF6	The Kruppel-Like Factor 6
LKB1	Liver kinase B1
mAb	Monoclonal Antibodies
MAPK	Mitogen-Activated Protein Kinases
MELK	Maternal Embryonic Leucine Zipper Kinase
MES	Manufacturing Execution System
MLH1	DNA Mismatch Repair Protein Mlh1
MSH2	MutS Homolog 2
MSH2	Recombinant Human MSH2 Protein
mTOR	Mammalian Target Of Rapamycin
mTORC1, 2	Mammalian Target Of Rapamycin Complex 1, 2
MYO3A	Myosin IIIA
NEK2	NIMA Related Kinase 2/Serine/Threonine-Protein Kinase
NISBD2	Inflammatory Skin And Bowel Disease, Neonatal, Type 2
NLS	Nuclear Localization Signal or Sequence
NNK	Nicotine-Derived Nitrosamine Ketone
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs

p14 (ARF)	Also Called ARF Tumor Suppressor, ARF, p14ARF
p16 (INK4a)	Anti-CDKN2A (Isoform 1)/P16INK4A Antibody Produced In Goat
p53	Tumor Protein p53
p54	Inner Membrane Protein p54
PAH	Polycyclic Aromatics Hydrocarbons
PARPs	Poly (ADP-Ribose) Polymerases
PBK	PDZ Binding Kinase
PCB	Poly chlorinated Biphenyl
PCR	Pathologic Complete Response
PGD	Preimplantation Genetic Diagnosis
PR	Progesterone Receptor
PRS	Prieto X-Linked Mental Retardation Syndrome
PTEN	Phosphatase and Tensin Homolog
RAS	Ras GTOPase
RB1	Transcriptional Corepressor 1
RTKs	Receptor Tyrosine Kinases
SHH	Human Sonic Hedgehog
SMAD2	SMAD Family Member 2
TGF-RI	TGF-β RI Kinase Inhibitor IV
TKI	Tyrosine Kinase Inhibitors
TNBC	Triple Negative Breast Cancer
TP53	Tumor Suppressor Protein 53 or p53
TSC	Tuberous Sclerosis Complex
TSGs	Tumor Suppressor Genes
VEGF	Vascular Endothelial Growth Factor Human
VEGF165	Vascular Endothelial Growth Factor 165 Human
VEGFRA	Vascular Endothelial Growth Factor A Human
VMAT	Volumetric Modulated Arc Therapy

CHAPTER 1
INTRODUCTION

1.1 Breast Cancer

Women are now exposed to the risk of breast cancer at an increasing rate compared to other types, and this type of cancer leads to death in some cases. This type of cancer does not differentiate between races, as it affects all females without any difference (Bou Zerdan et al., 2022). According to statistics, breast cancer ranks second in the number of deaths among women, preceded by lung cancer, despite the steady decline in the mortality rate since 1990, according to statistics conducted in the United States of America (Youlden et al., 2014). This disease does not have a preferred age for its occurrence in women's breasts, but the rates of incidence rise with age and affect number of factors on its occurrence, including age, family history of the disease, and a variety of other factors (Azamjah et al., 2019). The breast tissue is the site of occurrence of this disease, where it grows there into heterogeneous malignant tissue linked to many genetic mutations (Ibragimova et al., 2021). Breast cancer has a group of types, the most important of which is triple-negative breast cancer, where the danger of this type lies in its recurrence in a limited period between one and five years (Nunnery et al., 2021). The triple negative breast cancer recurrence rate ranges between 20 and 30%, and this percentage depends mainly on the early stages of the disease (Macías-García et al., 2020). To prolong the patient's life, chemotherapy is widely used, and in order to prevent a recurrence, a treatment that includes biomarker tracking is currently used (Yu et al., 2019).

1.1.1. Definition and Epidemiology

Breast cancer is a malignant tumor that forms in abnormal cells of the breast in particular, rapidly and out of control. It begins to grow (invade) and spread in the breast tissue at an early stage, then invades and spreads (metastasize) to other and distant organs in the body (Ely & Vioral, 2007; Girish et al., 2014; Jayashree & Velraj, 2017; Feng et al., 2018; Jaikrishnan et al., 2019; Aroef et al., 2020). Breast

cancer is a type of cancer called “carcinoma” a tumor derived from epithelial cells (Varricchio, 2004). The incidence of women with this type is 36% of the total affected resulting from carcinogenesis, compared to carcinogenesis of the rest of the body, as this type of cancer affects all women of each race around the world and, despite that, the highest rates of were observed in developed countries (Smolarz et al., 2022), this disease is detected by mammography and specialized X-rays (Waks & Winer, 2019).

1.1.2. History of Breast Cancer

The first description of breast cancer in history was mentioned (The Edwin Smith Surgical Papyrus) in dating back to 3,000-2,500 BC attributed to the Egyptian physician-architect Imhotep, as it was considered an incurable disease and was described as a cold mass spread throughout the breast (Breasted, 1930). As for the origin of the word cancer, it goes back to the origins of the cancerous tumor (carcinoma/ karkinoma/ scirrous) and cacoethes (malignant disease) to the medical lexicon in the Hellenistic writings (Homer, 1966; Lakhtakia, 2014). Hippocrates developed a theory in the year 400 BC to describe breast cancer, its symptoms, and the progressive stages of breast cancer.And the first to remove a malignant tumor from the breast without affecting healthy tissue was Leonides of Alexandria in the first century AD. Galen coined the word "crab/ cancer " to describe the dilated veins emerging from a tumor (Sambon, 1924; Lewison, 1953; Nounou et al., 2015).

1.1.3. Breast Cancer Etiology and Risk Factors

Although the causes of cancer are not yet fully known, there are many potential factors that play a role in carcinogenesis. Many risk factors with potential for breast cancer can be divided into two main groups (Figure 1.1) as non-modifiable and modifiable risk factors (Nindrea et al., 2017; Almansour, 2022; Cohen et al., 2023).

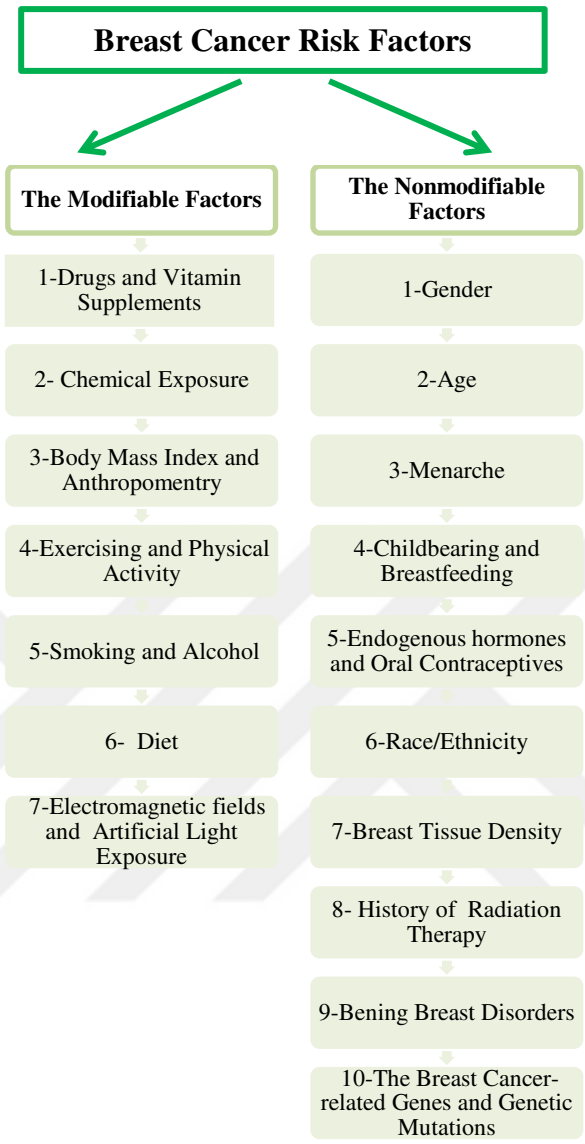


Figure 1.1. Breast Cancer Risk Factors (Nindrea et al., 2017; Almansour, 2022; Cohen et al., 2023).

1.1.3.1 The Non-Modifiable Factors:

1.1.3.1.1 Gender

Breast cancer has a very low prevalence in men and this cancer type is almost unknown among men, with less than 1% affected (Giordano, 2018). According to the American Society of Clinical Oncology (ASCO) publication of 2019, 50% of cases in women especially triple negative breast cancer (TNBC), do not have any specific factors other than sex. This is due to the stimulation of female hormones and their different roles in both sexes (Marino et al., 2019).

1.1.3.1.2 Age

Females under the age of 65 have higher rates of breast cancer (McGuire et al., 2015). The incidence is significantly increased in young women over the age of 40 (Berger et al., 2021). Age is not a determining factor for a particular type of cancer, such as triple-negative breast cancer or others (Cao et al., 2020; Aine et al., 2021).

1.1.3.1.3 Menarche

The age of onset of menstruation affects carcinogenesis. Detected at the beginning of the menstrual cycle before the age of 12, tumor development increases compared to the menstrual cycle at older ages. Missed periods for a year reduce the risk of cancer by 4% (Rojas & Stuckey, 2016). At each menopausal age, the risks go away. The risk is 3% each year. That is, the risk of developing breast cancer in menopause increases, so the rate for premenopausal and postmenopausal women is reported to be higher among women of the same age and a similar number of children (Key et al., 2001). It was shown that hormonal therapy for menopause was significantly associated with an increased incidence of breast cancer, but there was no difference in mortality from breast cancer (Beral et al., 2019; Chlebowski et al., 2020).

1.1.3.1.4 Childbearing and Breastfeeding

Pregnancy, childbirth, and lactation reduce the risk of breast cancer and have a preventive effect, and it increases when pregnancy is delayed in older women (Ma et al., 2006; Laamiri et al., 2015; Jeong et al., 2017; Lindsay et al., 2017), because during pregnancy, breast tissue swells (Tamakoshi et al., 2005). And female infertility is a cause of cancer (Lindsay et al., 2017).

1.1.3.1.5 Endogenous Hormones and Oral Contraceptives

Menopause, estrogen, androgen, and prolactin hormones (Tworoger et al., 2013), plasma hormones, density mammography (MD), and breast cancer risk score (PRS) can increase the risk of BC (Zhang et al., 2018). Hormone replacement therapy (HRT) risk increased once progesterone was added (Green et al., 2019). The usage of oral contraceptives increased recently, which led to an increase in bc proportionately (Westhoff & Pike, 2018; Barriga et al., 2019) and decreases when women stop using the treatment gradually (Zolfaroli et al., 2018). 30-year-old women who use pill

treatment have a lower chance of developing BC compared to older women (Hunter, 2017).

1.1.3.1.6 Race/ Ethnicity

White women have a higher rate of breast cancer. In addition, black women have a higher mortality rate and have lower chances of survival. (Weigelt et al., 2010; Howard & Olopade, 2021).

1.1.3.1.7 Breast Tissue Density

Breast density refers to the total volume of dense tissue in breast cancer and is divided into 3 categories: low-density, fatty, and high-density (Castello et al., 2016). Women receiving hormone replacement therapy were observed to have larger breasts during early puberty, pregnancy, and lactation. Breast density before and after menopause affects cancer risk in women (Checka et al., 2012). Breast density has been associated with an increased risk of both estrogen receptor positive (ER-positive) and estrogen receptor negative (ER-negative) invasive BC (Eriksson et al., 2017; Kerlikowske et al., 2017).

1.1.3.1.8 History of Radiation Therapy

As a result of radiation, secondary tumors in patients aged over 30 years with a family history of a patient receiving antenatal radiotherapy are at greater risk (Łukasiewicz et al., 2021). Radiological procedures such as multifield modulated radiotherapy (6F-IMRT) and volumetric modulated partial arc therapy (VMAT) are more likely than transverse field intensity modulated radiotherapy (2F-IMRT) (Ng & Travis 2009; Zhang et al., 2020; Łukasiewicz et al., 2021).

1.1.3.1.9 Benign Breast Disorders

Benign breast disorders (BBDs) can cause breast tissue carcinogenesis and classified into three main categories: non-reproductive disorders, reproductive disorders with atypia, and reproductive disorders with atypia (Worsham et al., 2007; Tamimi et al., 2010). The non-proliferative tumors do not rise BC (Zendehdel et al., 2018). But atypical proliferative lesions and a history of the disease in the family both raise the risk of cancer by up to 11 times (Hartmann et al., 2005).

1.1.3.1.10 Breast Disease History

The incidence of breast cancer in women is linked to family history, due to defect and mutations in Breast cancer type 1 susceptibility protein (BRCA1) and Breast cancer type 2 susceptibility protein (BRCA2) genes (Ozsoy et al., 2017). In the presence of a number of familial conditions with first-degree relatives, the risk of developing BC increases (Ban & Godellas, 2014).

1.1.3.1.11 Breast Cancer-related Genes and Genetic Mutations

Genetics may cause BC. 10-15% of the total causes of BC are genetic disorders and mutant genes, such as tumor suppressor protein 53 or p53 (TP53), phosphatase and tensin homolog (PTEN), and cadherin 1 (CDH1) (Shen et al., 2021). Tumor growth and development are aided by aberrant amplification, oncogene and anti-oncogene and gene (mutations) (Shiovitz & Korde, 2015).

1.1.3.2 Modifiable Risk Factors

According to WHO research, 35% of deaths caused by cancer worldwide are due to potentially modifiable risk factors (Lewandowska et al., 2018).

1.1.3.2.1 Drugs and Vitamin Supplements

When taking diethylstilbestrol during pregnancy, the risk of cancer affects the fetus, and the risk also increases with increasing doses of the drug. It doubles in women under the age of 40 (Hilakivi-Clark, 2014). Though the absorption of diethylstilbestrol even in the absence of estrogen and progesterone receptors (Palmer et al., 2006). Such as taking tricyclic antidepressants, paroxetine, tetracycline, and serotonin (Lawlor, 2003). Treatments taken to stimulate ovulation can cause BC (Lerner-Geva et al., 2006). Folic acid, multivitamins, and vitamins B, C, E, and D cause carcinogenesis (Cui & Rohan, 2006), and the use of vitamin D supplementation is resistant to breast cancer during pre- and post-menopause (Atoum & El-Zoghoul, 2017). When the level of 25-hydroxychloride is increased in serum vitamin D, the incidence of BC decreases in pre- and post-menopausal ages (Estébanez et al., 2018).

1.1.3.2.2 Chemical Exposure

Carcinogens are more susceptible to infection, and breast cancer is exposed to genetic changes and mutations (Casey et al., 2015), and the risk increases when the

mammary glands are exposed to chemicals such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCB), organic solvents, oil mist PAHs, pesticides, polycyclic aromatic hydrocarbons (PAHs), and synthetic fibers, nonsteroidal anti-inflammatory drugs (including aspirin, and ibuprofen) (Velicer et al., 2003; Leso et al., 2019; Videnros et al., 2020).

1.1.3.2.3 Body Mass Index and Anthropometry

Postmenopausal obese women have more estrogen receptor-positive breast cancer (Kolb & Zhang, 2020). BC risks are higher for women over the age of 50 and with a higher BMI (Wang et al., 2019). These women may develop tumors of the lymph nodes, particularly with a family history (James et al., 2015). Obesity increases mortality rates, and an increased level of inflammation in the body raises the level of circulating hormones, which cause carcinogenesis (Sun et al., 2018). Obesity is associated with insulin resistance and hyperinsulinemia since insulin is a mitogenic hormone with a high concentration in the blood. It stimulates cell proliferation, can prevent apoptosis, and stimulates the formation of insulin-like growth factor (IGF-1), which stimulates cancer formation (Krupa-Kotara & Dakowska, 2021). Van den Brandt linked the risk of breast cancer to a woman's height. An increase in a woman's height by 5 centimeters may increase the risk by 1.02 in premenopausal women and increase the proportion of women with tumors to 1.07 after menopause (Van den Brandt et al., 2000). The average height has a low risk of infection (His et al., 2020).

1.1.3.2.4 Exercise and Physical Activity

Lack of physical activity increases the risk of breast cancer, and physical activity can reduce the risk of BC and non-recurrence (de Boer et al., 2017), especially in women between the ages of 50 and 79 (McTiernan et al., 2003). The reduce exposure to endogenous sex hormones and has significant effects on the immune response and insulin-like growth factor I (IGF-I) (Key et al., 2013).

1.1.3.2.5 Smoking and Alcohol

Smoking causes cancer, depending on the duration of smoking and smoking before the first pregnancy (Misotti & Gnagnarella, 2013). Smoking causes p53 and oncogenes due to cigarette metabolically active chemicals (polycyclic aromatic

hydrocarbons and tobacco-specific nitrosamines) (Terry et al., 2016; Kispert & McHowat, 2017). It produces DNA-like compounds that are not amenable to cellular repair, leading to permanent cancer-causing mutations (Coronado et al., 2011; Kispert et al., 2017; Zeinomar et al., 2019). Alcohol usage causes cancer, and causes morphological changes (Liu et al., 2015), which affect breast tissue. Alcohol consumption increases the level of estrogen hormone due to hormone imbalance in the female reproductive system, causing disease, especially in positive estrogen subtype, and reducing DNA efficiency (Erol et al., 2019). Drinking alcohol at an early premenopausal age causes BC (Chen et al., 2011; Brooks & Zakhari, 2013; Daly et al., 2021).

1.1.3.2.6 Diet

Breast cancer is associated with food and has a significant impact on the patient's health. Women should eat low-fat foods because it contains few calories. Eating plant proteins, reducing animal protein, eating whole grains, vegetables and fruits, drinking plenty of water, reduce caffeine, and eating beneficial fish can reduce the incidence of cancer (Dunneram et al., 2019; Marzbani et al., 2019; Ubago-Guisado et al., 2021). The disease increases during adolescence and menopause and is related to the intake of saturated fats, processed foods, and foods high in sugar and salt (Harris et al., 2017; Fiolet et al., 2018).

1.1.3.2.7 Electromagnetic Fields and Artificial Light Exposure

Extremely low-frequency electromagnetic fields (ELF-EMFs) refer to 0-300 Hz electromagnetic fields. Exposure to ELF-EMFs and artificial light at night (ALAN) causes reduced nocturnal melatonin release, which in turn raises the risk of BC (Zhao et al., 2014). The disruption of melatonin's rhythm and the modification and significant reduction in melatonin levels caused by exposure to ALAN raise the risk of breast cancer (Al-Naggar & Anil, 2016). Whereas the function of melatonin is to prevent the promotion and growth of spontaneous and chemical inducers of the tumor, as well as inhibit the incursion and proliferation of breast cancer cells (González-González et al., 2018).

1.1.4 Early Detection of BC

Early diagnosis of BC in younger women can reduce it risks and speed up treatment such as surgical intervention and chemotherapy (Terry et al., 2016). The infection in (35–55) age groups raise the risk rate and develops a visceral tumor in their bodies, and the risk of re-infection also increases after 1–3 years of curative surgery, raises the prevalence of antigen Ki-67 (Ki-67) to more than 40% (Ibragimova et al., 2021).

1.1.5 Molecular Classification of Breast Cancer and Types

1.1.5.1 Molecular Classification Based on Gene Expression Profilling

Clinical and molecular heterogeneity can be found in breast cancer (Turkoz et al., 2013; Zhang et al., 2019). The molecular classification of BC is used to identify carcinogenic tissue. Breast cancer is divided into a group of subtypes (Zhou et al., 2020). There are four various molecular subtypes of BC: two subgroups with positive expression of estrogen and/or progesterone receptors (ER+/PR+)- luminal A and B, which account for approximately 70% of all breast cancer cases; one subgroup with overexpression or amplification of the HER-2-neu gene and negative expression of hormonal receptors (HER-2-positive subgroup); and the last one is triple-negative breast cancer (TNBC) (Tamimi et al., 2012).

1.1.5.2 Molecular Classification Based on Immunohistochemistry

Scientists were able to classify breast cancer based on immunohistochemistry, as this classification proved effective in providing diagnostic, prognostic, and predictive information on breast cancer and was easily performed. This classification is based on the use of immunostaining for cytokeratin (CK) and epidermal growth factor receptors (EGFR [HER1]), along with the use of the three typical receptors, ER, PR, and HER2. Depending on the immunohistochemistry classification, breast cancer can be divided into four types that appear in Figure 1.2 (Adani-Ifè et al., 2020; Zheng et al., 2020).

Molecular subtype	Immunohistochemical profile	Frequency
Luminal A	ER+ and/or PR+, HER2–	50–60%
Luminal B	ER+ and/or PR+, HER2+ or HER2–/Ki67 ≥14%	10–20%
HER2-enriched	ER–, PR–, HER2+	10–15%
Triple-negative or Basal-like	ER–, PR–, HER2–, EGFR+ and/or CK5/6+	10–20%

Figure 1.2. Subtypes of Breast Cancer Based on Immunohistochemical Markers (Millikan et al., 2008).

1.2 Triple Negative Breast Cancer

1.2.1 Definition and Prognosis

Triple-negative breast cancer (TNBC), or basal-like breast cancer, is one of the four types of breast cancer. This type lacks the presence of receptors that are normally present in cells, which are estrogen (ER) and progesterone (PR) hormone receptors. It also lacks overexpression of HER2 and gene amplification (Foulkes et al., 2010; Sharma et al., 2018; Luo et al., 2020; Bou Zerdan et al., 2022). As compared to luminal A breast cancer and luminal B breast cancer, TNBC is the most aggressive of those kinds (Eliyatkin et al., 2015). Luminal breast cancers A and B are TNBC, which are more aggressive than other types (Eliyatkin et al., 2015). This high aggressiveness results in a lower median survival time of less than 6 months and is characterized by a higher recurrence rate within the first three years and a higher mortality rate for five years after treatment (Chang-Qing et al., 2020). The basal-like and breast-like subtypes of BC have recently been recognized as TNBC based on a new classification of BC that follows the gene expression pattern for classification (Borri & Granaglia, 2021). Other distinguishing features of TNBC are the large size of the tumor it causes, the higher degree of nuclear division, and the more aggressive expression profile (high Inner membrane protein p54 of p54 and Ki67 and low B-cell lymphoma 2 (Bcl-2 expression)) (Rhee et al., 2008). The molecular characteristics of this type of cancer make it difficult to predict and diagnose (Irvin & Carey, 2008). Early TNBC is linked to a varied prognosis (Trapani et al., 2022). According to some statistics, the recurrence rate in TNBC might be as high as 25% (Chaudhary et al., 2018). Pathologic complete response (pCR) is regarded as one of the strongest prognostic indicators for patients with early TNBC (Trapani et al., 2022).

1.2.2 TNBC Heterogeneity

TNBC is characterized by unparalleled clinical, histological, and molecular heterogeneity (Berger et al., 2021). Due to its high intensity and heterogeneity, this type of breast cancer presents the worst clinical outcome. Chemotherapy is still the standard of care for this type of cancer, and many patients develop resistance to

treatment and metastatic disease (Mahmoud et al., 2022). The BC microenvironment consists of the following: extracellular matrix and multiple types of stromal cells, including immune cells, tumor-related macrophages, fibroblasts, and adipocytes, as well as cancer-related and endothelial cells. The microenvironment substantially impacts how a disease develops, progresses, and responds to treatment (Place et al., 2011). Research findings in tumor microenvironment heterogeneity (TME) are translated into clinical diagnostic and therapeutic applications. Cancer cytology evaluation is used in the classical classification and type of breast cancer on an approved basis, and the pathologic evaluation of TME is used in how to treat BC (Li et al., 2021).

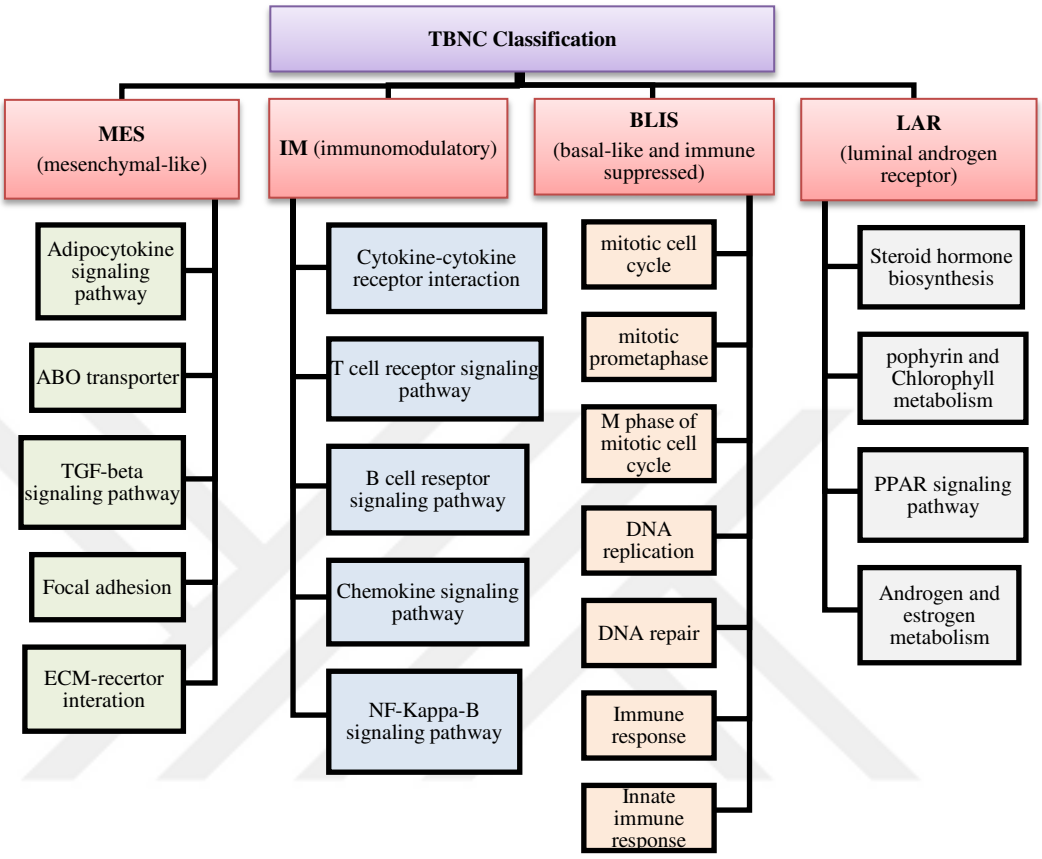
1.2.3 TNBC metastasis

During the first five years, patients with TNBC have a survival rate of about 77%, compared to 93% for other types of breast cancer, according to the American Cancer Society (2018). The rate of recurrence often increases during the first 1-3 years after the initial injury. The recurrence of cancer causes an excessive burden that reduces the quality of health and life. The effects of TNBC involve the lungs, brain, pleura, viscera, liver, and bones and can occur separately or simultaneously (Borri & Granaglia, 2021). TNBC causes more lung metastases than other organs, followed by bone, liver, pleura, and brain. Patients with lung metastases have a low survival rate, while metastases to the brain, liver, and pleura have a relatively high rate of OS (MacDonald et al., 2022). Patients experience tumor recurrence in the form of micrometastases, particularly after chemotherapy in TNBC (Chaudhary et al., 2018). The growth of metastatic cells in a foreign tissue environment causes a poor prognosis for breast cancer (Yin et al., 2020). Where it is difficult to detect breast cancer cells, which leads to resistance to chemotherapy due to the lack of spread of malignant tumors in the breast. For treatment to work, circulating cancer cells must undergo further molecular modifications and engage in beneficial interactions with the tumor (Fatima et al., 2018). Cancer cells are formed as a result of the evolution of pathways resulting from the genetic instability of primary tumor cells. The property of evading the immune system also plays an important role (Borri & Granaglia, 2021).

1.2.4 Triple-Negative Breast Cancer Molecular Subtyping

The identification of groupings that are molecularly homogenous and that can undergo genetic alteration is a benefit of subclassification (Table 1.1).

Table 1.1. TNBC subtypes based on the FUSCC (Lin et al., 2008; Dass et al., 2021).



This contributes to opening new horizons for effective drug manufacturing techniques and their development, which are linked to conducting specific clinical research specialized in a specific type (Bou Zerdan et al., 2022). And according to the latest classification, TNBC can be classified based on the following:

1.2.4.1 Genomic Alterations

A small number of somatic mutations can lead to the occurrence of breast cancer, as these mutations occur in the genomes of cancer cells (Watson et al., 2013). These somatic mutations are characterized by their contribution to carcinogenesis and the selective growth of cancer cells. Genomic profiling studies include for TNBC: Whole exome and whole genome analyses. These studies have identified a set of frequent changes in the so-called driver cancer genes. Genetic alterations in TNBC are mostly somatic mutations and copy number alterations (CNAs). As for the most

mutated gene, it is TP53, followed by phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA), PTEN, lysine methyltransferase 2C (KMT2C), and transcriptional corepressor 1 (RB1) (Nik-Zainal et al., 2016; Bareche et al., 2018; Jiang et al., 2019). It was also found that MYC amplification is the most prevalent CNA event in TNBC. It was also observed that the following genes were affected at a higher rate by somatic CNAs: EGFR, PTEN, cyclin D1 (CCND1), RB1, and cyclin E1 (CCNE1) (Zhao et al., 2020).

1.2.4.2 Gene Expression Profiling

TNBCs are now subdivided into further molecular subtypes using advanced technologies (Vanderbilt, Baylor, and Fodan). These techniques are used alongside gene expression analysis, clinical sequencing, and genetic analysis and profiling. These techniques also use several transcriptome-based approaches to classify TNBC (Shao et al., 2017).

1.2.4.2.1 Vanderbilt Subtyping

Lehmann et al., (2011) divided TNBC into six subtypes using molecular profiling of PAM50 gene expression: (BL1), BL2, (IM), (M), (MSL), and (LAR). The identification of cell lines compatible with different TNBC subtypes using pharmacokinetics is of paramount importance (Shao et al., 2017). Lehman et al., (2016) published a new approach that identified six molecular subtypes of TNBC according to response to chemotherapy. Then they reduced the subtypes from six to only four (BL1, BL2, M, and LAR) using gene expression data for breast cancer chemotherapy for more than 300 TNBC patients with a confidence rate of 95%. The study in which Masuda et al. used data from 130 patients who received chemotherapy to classify TNBC tumor subtypes (anthracycline and taxane). Using pCR, the highest pCR (52%) was found for BL1, while the lowest was for BL2, LAR, and MSL subtypes (0%, 10%, and 23%, respectively) (Masuda et al., 2013).

1.2.4.2.2 Baylor Subtyping

Using the genotyping of PAM50 gene expression, Burstein et al., with a team from Baylor College of Medicine classified TNBCs into four subtypes: LAR, M, basal immunosuppressant (BLIS), and basal-like immune activator (BLIA). Other studies have shown a close relationship between Vanderbilt BL1/BL2 and Baylor BLIA/BLIS subtypes. And by targeting specific targets, they determined the species

as follows: There are four stable subtypes of TNBC, each of which expresses unique molecular characteristics. A unique target-specific therapy can also be used to focus on each TNBC branch (Burstein et al., 2015).

1.2.4.2.3 Fudan Subtyping

At Fudan University Shanghai Cancer Center (FUSCC), a study done by Liu et al. in 2016 classified triple-negative breast cancer into four sections: IM, LAR, mesenchymal (MES), and basal-like immunostaining. suppressed subtype (BLIS). This team used a new classification system in which transcriptomes were combined. profiling of mRNA and lncRNA (Liu et al., 2016). In the same center for cancer research, Jiang et al. conducted a wider search on a larger group of patients—less than 500 patients. What distinguishes this study from its predecessor is that patients are of different ethnic origins from different places in East Asia, where the results of the research showed a remarkable variation in the rates of recurrence of mutations, especially PIK3CA, as well. The prevalence of the LAR subtype of triple-negative breast cancer in the population was observed to be higher (Jiang et al., 2019).

1.2.5 Genetic markers in TNBC

The complexity and high variety of the triple-negative breast cancer tumor, both molecularly and genetically, as well as the lack of hormone receptors in its cells, are what make it unique. Also, this sort of tumor lacks the variety of molecular markers that may be employed as therapeutic targets to improve how it is treated. The genetic indicators that influence prognosis and/or the best course of treatment are summarized in Figure 1.3 (Sporikova et al., 2018). There are 104 genes frequently expressed in TNBC, including cancer-specific kinases and genes involved in mitosis, which can be used as molecular targets that may contribute to the treatment of TNBC. A group of genes, including assembly factor for spindle microtubules (ASPM), Centromere Protein F (CENPK), maternal embryonic leucine zipper kinase (MELK), nima related kinase 2 (NEK2), and PDZ Binding Kinase (PBK), can also be targeted and used as therapeutic targets for tumors, due to their contribution to tumorigenesis and programmed cell death (Komatsu et al., 2013). A study conducted on a group of cases with TNBC reported that the most frequently mutated genes are as follows: More than half of cases had TP53, which was been found in instances with PIK3CA, myosin IIIA (MYO3A), PTEN, and RB1. However, most of these

mutations are not transmitted to mRNA, as the percentage of genes that are transmitted to mRNA reached 36% of the total mutations (Shah et al., 2012).

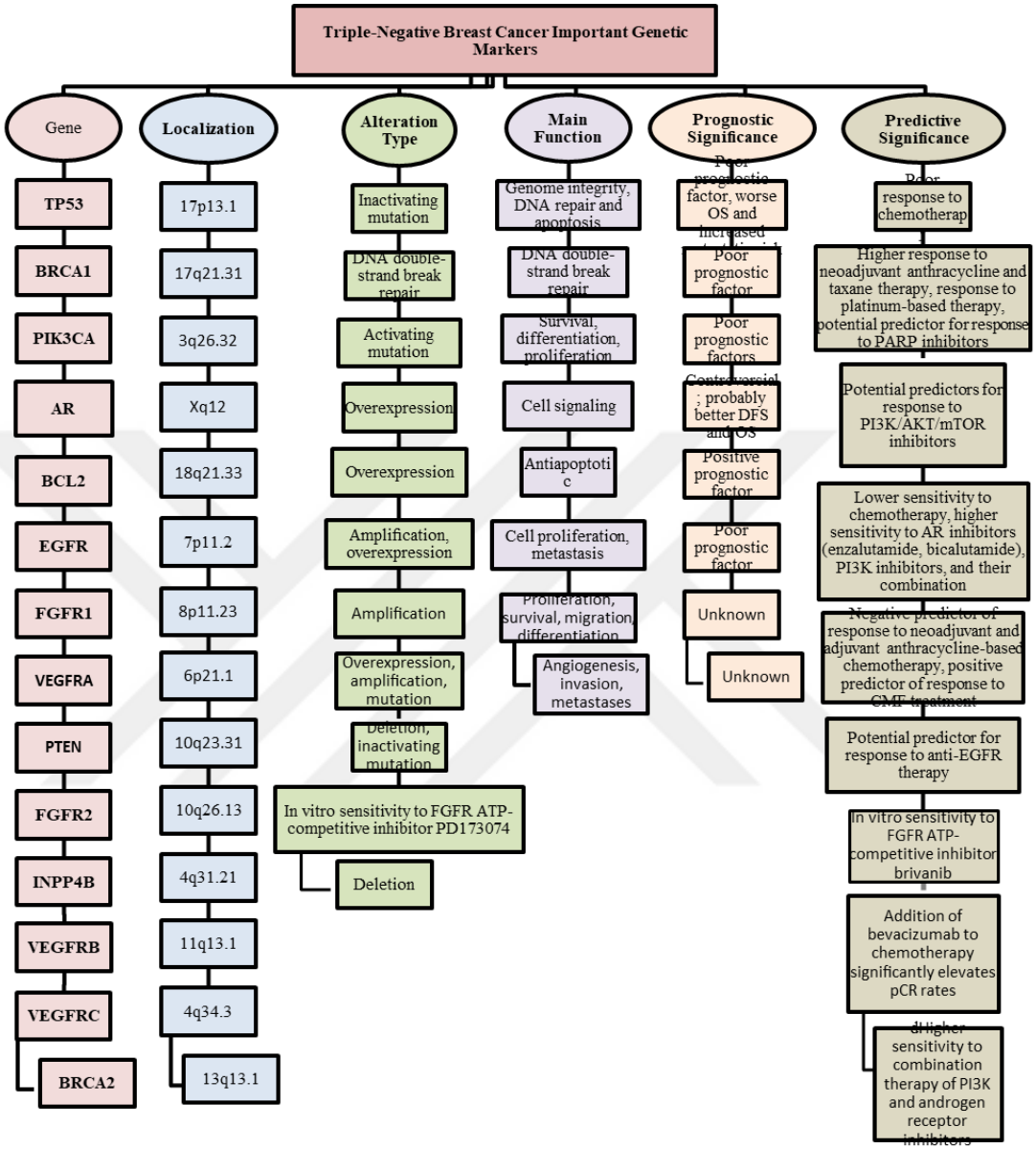


Figure 1.3. Summary of Triple-Negative Breast Cancer Important Genetic Markers (Sporikova et al., 2018).

1.2.6 TNBC Signaling Pathways

TNBC tumors are characterized by rather simple molecular patterns, and at the same time, they are heterogeneous tumors that show diversity in shape, gene expression, and signaling pathways (Lehmann et al., 2011) (Figure 1.4). There aren't many studies on particular molecular targets or how medical interventions affect signaling

pathways. It has been noticed that the presence of unusual signaling pathway activation may lead to tumor spread and recurrence and also play a crucial role in the diagnosis of TNBC. Where several signaling channels specifically interfere with various stages of a cancer cell's life cycle (Yang et al., 2021). Sever & Brugge studied signaling pathways that control tumor growth to monitor genetic alterations in cancer cells. They discovered many pervasive and misdirected signals that support the development of cancer. They concluded that the systems of the body cannot handle the uncontrolled messages (Sever & Brugge, 2015).

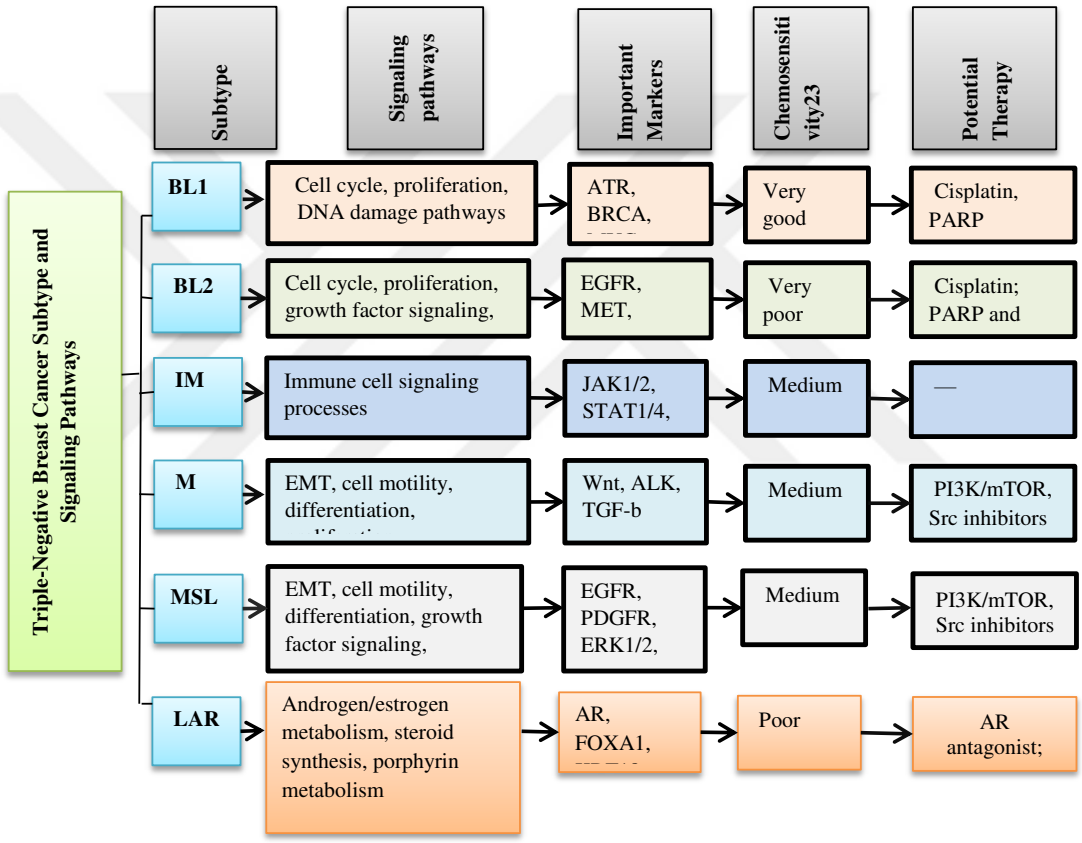


Figure 1.4. Triple-Negative Breast Cancer Subtype and Signaling Pathways (Lehmann et al., 2011; Sporikova et al 2018; Dass et al., 2021).

1.2.6.1 Notch Signaling Pathway

Thomas Hunt Morgan first used the term 'notch' in 1917 referring to transmembrane receptors and ligands (Gu et al., 2016). The Notch pathway is associated with pre-cancer genes including BC (Miele, 2008). Delta Like Canonical Notch Ligand 1 (Dll1), Delta Like Canonical Notch Ligand 3(Dll3), and Delta Like Canonical Notch

Ligand 4 (Dll4) are Notch ligands varieties (D'souza et al., 2008). BC cells grow and develop under the influence of Notch signaling (Giuli et al., 2019). This pathway is expressed by cell proliferation, differentiation (Palomero et al., 2006), and specific cell fate (Louvi & Artavanis-Tsakonas, 2012). This pathway supports the growth and persistence of tumors, particularly TNBC, and is conserved on stem cells and embryonic development (Speiser et al., 2013). Notch activates the HES and HEY families genes (Maier & Gessler, 2000). It has four types: Notch-1, Notch-2, Notch-3, and Notch-4 (Kumar et al., 2016). Notch-1 causes pancreatic and leukemia, while Notch-3 and 4 play a role in cancer survival and progression. When Notch-2 overexpression in TNBC was investigated, it was found to have a protective action (Weijzen et al., 2002). TNBC has connected with Notch receptors and ligand overexpression, which makes it possible to target a monoclonal antibody (mAb) (Espinoza & Miele, 2013). mAbs contribute to the reduction of expression of the HES and HEY-L families, which reduce cell proliferation and programmed cell death (Sharma et al., 2012). The DLL4 mAb can be used for TNBC curing (Benedito et al., 2009). Most of the drugs that block the Notch pathway were not granted authorization by the FDA (Food and Drug Administration) (Medina et al., 2020).

1.2.6.2 Hedgehog Signaling Pathway

Hedgehog (Hh) pathway has a role in stem cell programming and embryonic development (Bhateja et al., 2019). Deregulation of the Hh pathway leads to TNBC (Di Mauro et al., 2017). The Hedgehog signaling pathway causes drug resistance and is associated with tumor recurrence after treatment (Li et al., 2012). In mammals, there are three Hedgehog pathways: destert hedgehog signaling molecule (DHh), Indian hedgehog signaling molecule (IHh), and human sonic hedgehog (SHh) (Sasaki et al., 1999). SHh has an important role during embryonic development (Odent et al., 1999). While IHh has a role in chondrocyte differentiation, chondrocyte proliferation stimulation, and features of osteoblast cells that participate in bone production (Ohba, 2020). And DHh has a role in peripheral nerve formation (Mirsky et al., 1999), and pure gonadal dysgenesis (PGD) is caused by mutations in the DHh gene (Wei et al., 2022). Overexpression of SHh, DHh, and GLI Family Zinc Finger 1 (GLI1) has been observed in patients with early-stage breast cancer and premenopausal in carcinogenic tissue when compared to healthy breast tissue (Riaz et al., 2018).

1.2.6.3 Wnt/β-Catenin Pathway

There are three types of signaling pathways: the non-canonical Wnt planar cell Polar pathway, the non-canonical Wnt/Ca pathway, and the canonical Wnt/b-catenin pathway. This pathway is responsible for transferring signals and messages to cells by cell surface receptors (Katoh & Katoh, 2007). This pathway contributes to embryonic development and tissue homeostasis in adults, its dysregulation causes a variety of malignant and non-cancerous disorders (Liu et al., 2022), including TNBC. The Wnt receptor can be targeted to inhibit the proliferation of BC cells (King et al., 2012). WNT3A, WNT11, and WNT5A are among a set of Wnt ligands that participate in the migration and invasion of cancer cells (Zhu et al., 2012). Wnt ligands interact with Frizzled receptors leading to carcinogenesis. Induces movement and progression of resistance cells, invasion and proliferation in TNBC, and stability and accumulation of cytoplasmic β-catenin protein induced by this pathway (Yang et al., 2021). Wnt inhibitors help eliminate CSCs and drug-resistant cells (Dean et al., 2005). That is useful in anti-cancer therapies (Pai et al., 2017).

1.2.6.4 PI3K/Akt/mTOR Pathway

The most common signaling pathway in cells that promotes cell growth and survival is the (PI3K/AKT/mTOR). Excessive activity of this pathway causes ER+ mammary carcinogenesis and elevated drug resistance in endocrine therapy. Blocking this pathway also enhances the efficacy of endocrine therapy in ER + BC (Ciruelos Gil, 2014). This system controls the inflammatory reactions of cells as well as the survival, proliferation, migration and metabolism of cells (Yang et al., 2021). TNBC often have abnormalities in the PI3K/AKT/mTOR pathway. That can predict TNBC suppression (Costa et al., 2018). TNBC can be treated with rapamycin and paclitaxel due to the way they affect the PI3K/AKT/mTOR pathway (Ali & Wendt, 2017). Activating the Akt/mTOR pathway induces resistance to carcinogenesis treatment, when combining mutations for the PTEN and p53 tumor suppressors (Liu et al., 2014). PIK3CA mutations can be found in TNBC (Cossu-Rocca et al., 2015).

1.2.6.5 Mammalian Target of Rapamycin (mTOR) Inhibitors

Mammalian target of rapamycin (mTOR) is a protein complex that has two variants: mammalian target of rapamycin complex 1 (mTORC1), and mammalian target of rapamycin complex 2 (mTORC2). Controlling anti-inflammatory, anti-proliferative

activity and induces autophagy and apoptosis, it may contribute to cancer treatment (Chen & Zhou, 2020). And blocking the mTOR pathway acts as an effective anticancer therapy for a variety of human malignancies (Xie et al., 2016). This pathway controls cell death, autophagy, and cell proliferation, and may cause cancer and other diseases (Zou et al., 2020). The mTOR pathway causes a poor prognosis for cancer and tissue invasion (Xie et al., 2016). mTOR get activated when nutrients are available, which stimulates metabolism. The opposite happens when nutrients are deficient, which maintains cell integrity and energy. A large proportion of proteins, lipids, and nucleotides must be available for the growth and division of cancer cells (Liu et al., 2019). An error in this pathway causes carcinogenesis (Fruman & Rommel 2014).

1.2.6.6 Epidermal Growth Factor Receptor (EGFR)

The receptor tyrosine kinases (ERBB) family of tyrosine kinase receptors includes the epidermal growth factor receptor (EGFR), and three additional receptors called EGFR Receptor tyrosine-protein kinase erbB-1 (HER1 or ERBB1), receptor tyrosine-protein kinase erbB-2 (HER2 or ERBB2), receptor tyrosine-protein kinase erbB-3 (HER3 or ERBB3), and receptor tyrosine-protein kinase erbB-4 (HER4 or ERBB4) (London & Gallo, 2020). EGFR controls cell proliferation, differentiation, mitosis, survival, and cancer progression. Also plays a role in drug resistance and treatments (Sabbah et al., 2020). EGFR is located on the short arm p11.2 of chromosome 7 (7p11.2), (O'Leary et al., 2016). Overexpression of EGFR has been observed in approximately 50% of inflammatory breast cancer (IBC) patients (Masuda et al., 2012). Activation for this gene promotes DNA repair, invasion, adhesion, motility, differentiation, and metastasis (Sigismund et al., 2018). EGFR is targeted by small molecules that inhibit tyrosine kinase activity in the intracellular domain (ECD) for cancer therapy (Gala & Chandarlapaty, 2014). TNBC is being explored with a variety of EGFR inhibitors, including monoclonal antibodies (mAbs) (Hsiao et al., 2015). However, reports have shown that combination therapy with mAbs and chemotherapy has a more potent effect (Carey et al., 2012).

1.2.6.7 TGF- Signaling Pathway

Transforming growth factor (TGF) is a secreted cytokinin that has functions like; maintenance and early development of adult tissues (Miyazono et al., 2018),

inflammation, metastasis, and embryogenesis (Kim & Baek, 2019). TGF-β has two different roles during carcinogenesis, as it inhibits tumors in their early stages but stimulates tumors in their late stages. TGF-β pathway signals orchestrate the tumor microenvironment at the initiation of cancer cells, orchestrating the immunosuppressive tumor microenvironment (TME), and contributing to tumor growth, invasion, other organs' carcinogenesis, recurrence, and drug resistance. TGF-β signaling contributes to cancer metabolic reprogramming (Yang et al., 2021; Shi et al., 2022); demethylzeylasteral (T-96) is a compound that blocks the TGF-β pathway (Li et al., 2019). TGF signaling plays an active role in the growth of malignant tumors (Vishnubalaji & Alajez, 2021).

1.2.6.8 CSPG4 Protein Signaling Pathway

The non-glial antigen, also known as melanoma chondroitin sulfate proteoglycan or CSPG4, is a cell-surface proteoglycan that basal breast cancer cells express (Almansour, 2022). It is closely associated with malignancy progression and a bad prognosis for cancer. It functions as a co-receptor with certain receptor tyrosine kinases when several important pathways are activated (RTKs) (Nicolosi et al., 2015). Although this pathway has been overexpressed in cancer cells, it is moderately expressed in healthy cells. CSPG4 may cause the development, proliferation, motility, and metastasis in cancer. Expression of this pathway may predict survival and relapse risk in treatment-resistant cancers, including TNBC (Jordaan et al., 2017). For the treatment of TNBC, the chondroitin sulfate proteoglycan 4 (CSPG4) pathways have been classified as a very potential target antigen (Amoury et al., 2016).

1.2.6.9 MAPK/ERK Pathway

The phosphorylation of mitogen-activated protein kinases/ extracellular-signal-regulated protein kinase (MAPK/ERK) signaling is related to a range of substrates in the cytoplasm and the nucleus and is engaged in several biological processes. The MAPK/ERK pathway when it is activated, aids in the regeneration of organs, cell viability, cell fate, translocation, proliferation, growth, transcription, and translation (Wen et al., 2022). A set of proteins known as the MAPK/ERK pathway is in charge of transferring signals from a cell surface receptor to DNA. Gene expression results in cellular changes such as cell division, survival, motility, and invasion. The signal

leaves the cell's surface and eventually forms a protein. Several of the pathway's proteins function as "on/off" switches. Moreover, carcinogenesis happens when a flaw in the mechanism for stopping or initiating it arises (Zhou et al., 2020).

1.2.6.10 Poly (ADP-Ribose) Polymerase (PARP) Inhibitor

Poly (ADP-ribose) polymerase-1 (PARP1) is a DNA repair enzyme that is expressed in the nucleolus (Jannetti et al., 2020). A variety of biological activities, including DNA repair, gene transcription, signal transmission, cell cycle control, cell cleavage, and the antioxidant response, are aided by the poly (ADP-ribose) polymerase (PARP) pathway (Sargazi et al., 2021). Poly (ADP-ribose) polymerase is a group of 18 proteins. A triple-negative phenotype is present in about 70% of breast cancer cases (Medina et al., 2020). DNA repair mechanisms are present in normal cells. DNA restoration processes, however, are deficient in cancer cells. The BRCA gene performs DNA repair. Cancer cells are terminated when the DNA repair pathway is inhibited by the inhibitor PARPi, stopping cancer cells from repairing DNA (Tattersall et al., 2022).

1.2.7 TNBC Biomarkers

According to Duffy et al. (2015), biomarkers can be used to determine who is most at risk for developing breast cancer in families with a history of the disease, to estimate risk at the time of the initial diagnosis, to select the best course of treatment, to follow up after surgery, and to monitor in the late stages. TNBC Biomarkers are biomarkers that can be repeatedly measured quantitatively. According to the National Institutes of Health, they can be used as clinical criteria to predict survival or even response to treatment. Where the elements utilized for early diagnosis and follow-up therapy can be included because they give "personal" information about the treatment. To use vital signs in early diagnosis, it is possible to integrate them with laboratory and clinical data (da Silva et al., 2020).

1.2.7.1 Molecular Biomarkers in TNBC

1.2.7.1.1 Cytokeratins (CKs)

Cytokeratins are proteins that help epithelial cells form intermediate filaments (IF). They are found in human chromosomes 17q21.2 and 12q13.1 on 27 genes (Namagerdi et al., 2020). Cellular proliferation, apoptosis, migration, adhesion, and

molecular signaling are among the essential functions performed by CKs (Vasilevska et al., 2022). CK expression is associated with ER-negative and high-grade cancers (Schweizer et al., 2006). In the past, CKs have been used to distinguish breast cancer from benign tumors. TNBC treatment and prognosis were affected by CK, especially CK5/6 (Wang et al., 2021). TNBC predictors include CK5 and CK6 (Zhu et al., 2021).

1.2.7.1.2 Epidermal Growth Factor Receptor (EGFR)

On the epidermal growth factor receptor, the tyrosine kinase domain of the transmembrane glycoprotein family stimulates signaling pathways (da Silva et al., 2020). The relative molecular mass of the EGFR, also known as HER1, is 170 kDa. EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) are the four varieties that make up the EGFR family (Wells, 1999). It is located on chromosome 7p11.2 and is the type of modification that gets amplified and overexpressed. Its function is the proliferation of metastasis cells, and its prognostic significance is a weak prognostic factor when it has a predictive significance when EGFR is a negative indicator of response to neoadjuvant anthracycline-based chemotherapy and a positive indicator of response to CMF treatment (Kim et al., 2013; Sporikova et al., 2018).

1.2.7.1.3 Vascular Endothelial Growth Factor (VEGF)

The vascular Endothelial Growth Factor is a signaling protein that is produced by ECs, tumors, and myeloid cells. The VEGF gene in humans may be found on chromosome 6p21.3 (Zhang et al., 2021). VEGFA is the prototype member of the VEGF protein group, which also includes the placental growth factor and the mammalian counterparts of Vascular Endothelial Growth Factor B (VEGFB), Vascular Endothelial Growth Factor C (VEGFC), and Vascular Endothelial Growth Factor D (VEGFD) (PLGF). The three endothelial receptor kinases (VEGFR1, VEGFR2, and VEGFR3 tyrosine) are activated by VEGFs, which promote angiogenesis and lymphangiogenesis (Karaman et al., 2018). VEGF, particularly in tumors bigger than 2 mm, which lack oxygen and nutrients, is one of the reasons for tumor formation and spread and leads to angiogenesis. Other isoforms are less common than VEGF165 (Gerwins et al., 2000). Hypoxia, nitric oxide, growth regulators, tumorigenesis, tumor suppressor genes, and HER-2 are a few of the

factors that affect how this gene is expressed (Gupta & Qin, 2003). Thirty to sixty percent of TNBC patients express VEGFs, and a higher VEGF level is associated with a poorer clinical outcome (Taha et al., 2009; Sukumar et al., 2021).

1.2.7.1.4 The Breast Cancer Susceptibility (BRCA) Proteins, BRCA1 and BRCA2

The most frequent genetic risk factors for breast cancer are BRCA1 and BRCA2 (HBOC). This gene, which may be found on chromosome 17, was identified in 1994. DNA damage and cell survival during the cell cycle are examined by BRCA1 and BRCA2. Important functions of BRCA1 and BRCA2 include safeguarding the DNA replication fork and eliminating abnormal Topoisomerase II-DNA complexes caused by estrogen (Yoshimura et al., 2022). Genomic instability, loss of tumor suppressor capabilities, and carcinogenesis are caused by the BRCA biallelic gene being inactivated. A BRCA1 mutation raises the risk of breast cancer by 72% up to the age of 80, whereas a BRCA2 mutation raises the risk of cancer by 69% (Venkitaraman, 2019). Cell cycle regulation, DNA damage sensitization, E3 ubiquitin ligase activity, and other activities are among the roles played by BRCA1. whereas HR-related proteins are connected to BRCA2's activity. This indicates that BRCA1 and BRCA2 mutations and the malignancies they lead to differing due to the different molecular properties of the BRCA proteins (Hatano et al., 2020). TNBCs with BRCA 1/2 mutations make up almost 50% of cases (Choi et al., 2023).

1.2.7.1.5 Poly (ADP-ribose) Polymerases (PARPs)

Poly (ADPribosylated) DNA-binding are enzyme proteins, which are involved in cell signaling, are catalyzed by PARPs which is a character for the eukaryotes. The most common PARP isoform among the 18 known PARP isoenzymes is PARP1 (Fong et al., 2009). DNA repair, genomic stability, and programmed cell death are just a few of the biological activities that PARPs are engaged in (Herceg & Wang, 2001). The types of PARPs include PARP1, PARP2, PARP3, PARP4, PARP5a, and PARP5b. The PARP structure is composed of DNA-binding, auto-modification, and catalytic domains. In healthy cells, PARPs are less active, but when DNA is broken, their activity increases. Inhibiting PARP activity results in the death of cancer cells (Przybycinski et al., 2019). Overexpression of PARP-1 in the nucleus is more common than in the cytoplasm. Nuclear overexpression of PARP-1 has been

observed in glioblastoma multiforme (GBM) (Kumar et al., 2020). Lately, PARPi, such as olaparib, veliparib, and niraparib, have been chosen for the targeted treatment of TNBC (Sonnenblick et al., 2015).

1.2.7.1.6 Androgen Receptor (AR)

The nuclear receptor group for the steroid hormone AR includes the steroid hormone receptor, which has a molecular weight of 110 kDa (also known as NR3C4). The gene that codes for AR is found on the X chromosome (Xq11–Xq12) (You et al., 2022). It functions as a DNA-binding transcription factor to regulate gene expression. Breast cancer development and AR are linked. In benign breast tissue, luminal epithelial cells co-express 5–30% of AR together with ER and PR. Apocrine metaplastic cells that impact the breast's fibrocystic metamorphosis also express AR. Moreover, it is known that AR affects between 60 and 90% of all breast cancers (Astvatsaturyan et al., 2018).

1.2.7.1.7 Ki67

Ki67 is known as a cellular proliferation marker (Bertozzi et al., 2018). Human gene MKI67 (MKI67) is a gene that makes a protein called Ki-67 antigen, or proliferation marker Ki-67. The protein exists in two spongy forms with corresponding molecular weights of 345 and 395 kDa. Gerdes and Schultzer originally found Ki-67 in the nucleus of a Hodgkin lymphoma cell in 1983 (Davey et al., 2021). Except for the quiescent state, cells express the protein ki-67 in their nuclei at various times during the cell cycle. Breast cancer sample cell proliferation is measured using the Ki-67 metric. Breast cancer is linked to higher levels of Ki-67 expression, more tenacious spread, and a worse prognosis. Today, Ki-67 is utilized to determine prognosis, guide adjuvant therapy, and forecast therapeutic response (Zhu et al., 2020). Increased Ki-67 values have a high correlation with the TNBC phenotype and may be an independent risk factor for survival with or without illness as well as overall survival. The median value for individuals with TNBC was 44.7%, while the median value for patients without TNBC was 22.2%. When the circumstance develop, the level of danger rises (da Silva et al., 2020).

1.2.7.1.8 p53 Gene

The p53 gene, which is located on chromosome 17p13.1, generates a nuclear phosphoprotein with a molecular weight of 53 kDa. From a carcinogenesis biological perspective, the tumor suppressor p53 plays a significant role. p53 regulates the cell cycle arrest, DNA repair, and death in healthy cells as well as the inhibition of tumor cell growth; As more proteins that cause cancer build up as a result of the p53 protein's mutation, cancer develops (Moxley & Reisman, 2021). That's why It calls the "guardian of the genome" (Kern et al., 1991). p53 activates genes that check the cell cycle or lead to cell death in response to DNA damage. Yet, because of its abnormal position in the cell p53 inactivates several malignancies, including breast cancer. This situation has no bearing on it. A nuclear import/localization signal (NLS) and a nuclear export signal (NES) in p53 allow it to move to or from the nucleus (Wang et al., 2018). IHC (immunohistochemistry) techniques can be used to examine the increase of oncogenic p53 proteins brought on by missense mutations in the cytoplasm. Its frequency is as high as 30% in BC, and it rises to 70% in the TNBC phenotype and to 95% in the basal-like subtype (Ilie et al., 2018).

1.2.7.1.9 Tyrosine Kinases (TKs)

The human genome contains about 90 protein tyrosine kinases (TKs), which have a role in cellular growth, survival, differentiation, function, and mobility among more than 500 protein kinases. The receptor tyrosine kinase (RTK), a transmembrane protein, is found in the cytosol along with the receptor tyrosine kinase. (NRTK), according to the genome, transcriptome, and proteomic analyses, TKs may be useful as prognostic or therapeutic targets for TNBC (Limsakul et al., 2023). TK is frequently activated in cancer. Observed in many types of BC with ERBB2 amplification, TK activation is oncogenic. However, it is not known what the abnormal TK signal in TNBC means clinically (de Nonneville et al., 2019). There is a large family of enzymes called protein kinases. Tyrosine kinase inhibitors (TKIs) have made it possible to use active kinases as therapeutic targets for cancer (Poliaková et al., 2018).According to study by Duan et al., prolyl carboxypeptidase (PRCP) is a protein that inhibits the activity of a number of receptor tyrosine kinases as well as the proliferation of TNBC cells and tumors (Duan et al., 2022).

1.2.7.1.10 Mammalian Target of Rapamycin (mTOR)

Mammalian target of rapamycin (mTOR) is a member of the PI3K-associated protein family, also referred to as mechanistic target of rapamycin and 12-rapamycin-related protein 1 FK506, a kinase encoded by the human gene, regulates gene expression, cell growth, and proliferation (Lu et al., 2021), metabolism, and epigenesis at the cell level (Sabatini, 2017). mTOR regulates signaling networks that govern apoptosis, autophagy, and cell division. mTOR is active in malignancies and is associated with a variety of diseases, including cancer. This makes it a potential research target for cancer therapy (Zou et al., 2020). Inhibition of the PI3K/mTOR pathway is associated with a TNBC therapeutic strategy (Singh et al., 2014).

1.3 Epigenetics

The study of phenotypic variations that are inherited without changes to the DNA sequence is known as epigenetics (Zhou et al., 2020). Specifically, it is the study of how genes that go through genetic alteration, frequently during mitosis and meiosis, operate (Darwiche, 2020). Events involving structural modification of chromosomal regions, signaling, or maintaining altered levels of activity are epigenetic events that last for many generations of cells and are similar to transient modifications that repair DNA or the cell cycle (Bird, 2007). BC is started, promoted, and spread through epigenetic variations (Wu et al., 2015).

1.3.1 Epigenetic Changes and Epigenetic Alterations' Role in Genetics

Epigenetic modifications" refers to DNA or histone modifications that do not affect the DNA sequence but interpret the genome. Gene expression is suppressed through changes on the histone tail, especially acetylation, and depending on what happens in the amino acid in the body either it’s activated or inactivated, or the changes get inherited, and are essential for maintaining cellular growth and spatial control of gene expression during cellular proliferation (Mohammad et al., 2019). Epigenetic changes can be the main cause of cancer development (Byler et al., 2014), and can affect metastatic BC, treatment type caused by genetic variants dominate the mutations, cancer progression, and spread aided by demethylation at one site and hypermethylation at another site, and the oncogene family is closely monitored due to their activation promotes cancer growth (Sambi et al., 2019). Healthy growth and embryonic development are regulated by epigenetics. The epigenetic modifications of BC cells act as a regulatory layer for the chromosomes, coordinating biological

activities to keep the cells in homeostasis and the pathological conditions resulting from their deterioration (Shukla et al., 2019). Aging, carcinogens, radiation, ultraviolet (UV) radiation, acetaldehyde produced by ethanol, aflatoxins produced by fungi, benzo (a) pyrene (B[a]P) in incomplete combustion of organic matter, 4-(methylnitrosamino)-1-(3-pyridyl) radiation, oxygen radicals, and -1-butanone (NNK), among others, to alter epigenetic patterns. On the other hand, age, chronic inflammation, ulcerative colitis, hepatitis caused by hepatitis C or B, gastritis caused by *H. pylori* infection, and estrogens caused by smoking can all lead to genetic changes, as Figure 1.5 illustrating (Takeshima & Ushijima, 2019). Long non-coding RNAs, microRNA expression, DNA methylation, and histone modifications are the epigenetic alterations in TNBC (Zolota et al., 2021).

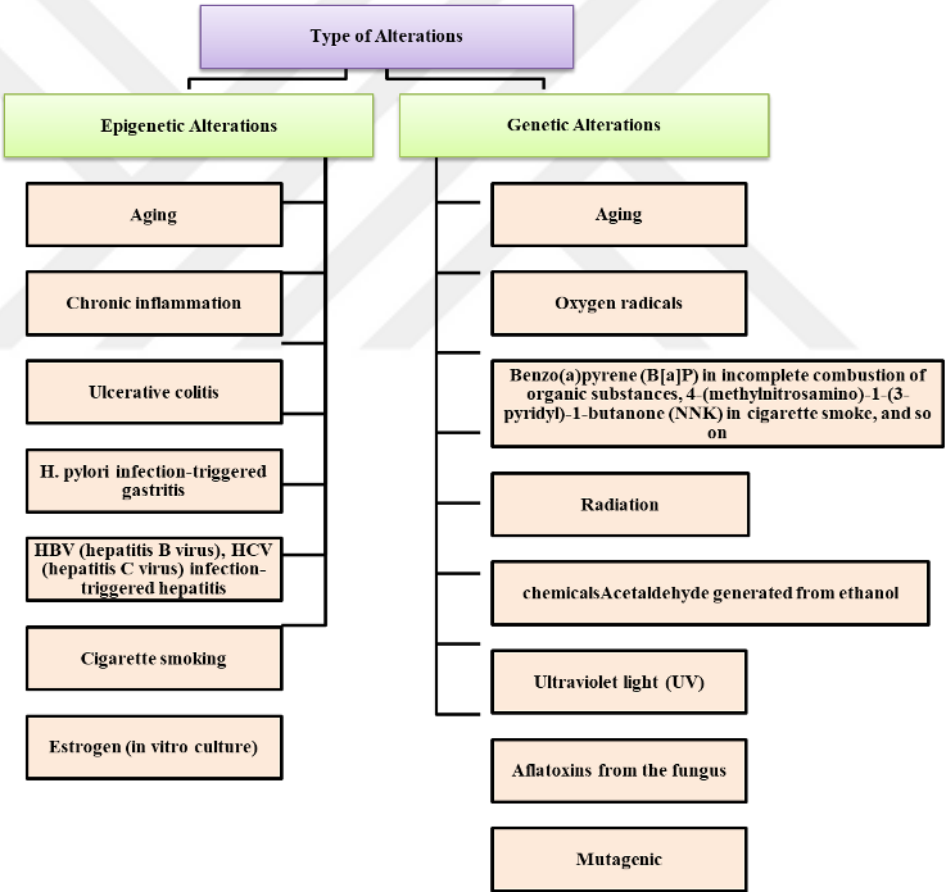


Figure 1.5. Genetic and Epigenetic Alterations Inducers (Takeshima & Ushijima 2019).

1.3.2 Epigenetic Mechanisms

Without altering the underlying nucleotide sequence, significant gene expression regulators called epigenetic processes influence the probability of heritable changes

in gene expression (Clark & Rager, 2020). Examples of epigenetic processes include DNA methylation, histone post-translational modifications (PTM), non-coding RNAs, nucleosome remodeling, and histone variants (Izquierdo-Torres et al., 2022).The mechanisms of embryonic inheritance influenced by the chromosome, which also controls gene expression. It regulates healthy adulthood and appropriate embryonic development, and its dysregulation aids in the development of cancer. Complex tumor formation leads to the emergence of the "characteristics of cancer" (Darwiche, 2020).

1.3.2.1 DNA methylation

DNA methylation take place when adding a group of methyl covalently to cytosine or adenine in the DNA sequence though the chemical process of DNA methylation, that take place by an enzyme (Figure 1.6) (Zicola et al., 2019). The first genetic alteration discovered in humans was DNA methylation in the early 1980s (Park & Han, 2019). DNA methylation suppresses the transcription of genes (Miller & Grant, 2013). Inherited methylation (genetic) alternation in DNA methylation is the root cause of several illnesses and ailments, including cancer, cardiovascular disease, atherosis, and neurological problems (Heyn et al., 2012; Kandi & Vadakedath, 2015). DNA methyltransferases (DNMTs), which receive the methyl from S-adenyl methionine (SAM), catalyze the process of DNA methylation in cytosines to produce 5-methylcytosine (5mC), this process happens in the CpG (cytosine-phosphate-guanine) dinucleotide sequence, DNA-Methyl Transferases, a class of five different enzymes, regulate this enzymatic process (Pasculli et al., 2018). These modifications affect the distribution of 5mC, which distinguishes cancer cells from normal cells. Ones of the CpG methylation-based phenotypic markers of the presence of cancer include the occurrence of hypomethylation in the cancer genome, TSG inducing localized hypermethylation, and the frequency of 5mC sequence-containing direct mutagenesis which is induced by deamination (Ilango et al., 2020). High stability and its role as a unique genetic memory for certain cells throughout different phases of the cell cycle are two characteristics of DNA methylation. It helps control the production and activity of histone codons (Moosavi & Motivalizadeh-Ardakani, 2016). DNA methylation contributes to repressing gene expression and is one of the epigenetic mechanisms as well as the other two mechanisms: histone modification and non-coding RNA (Киселев et al., 2021).

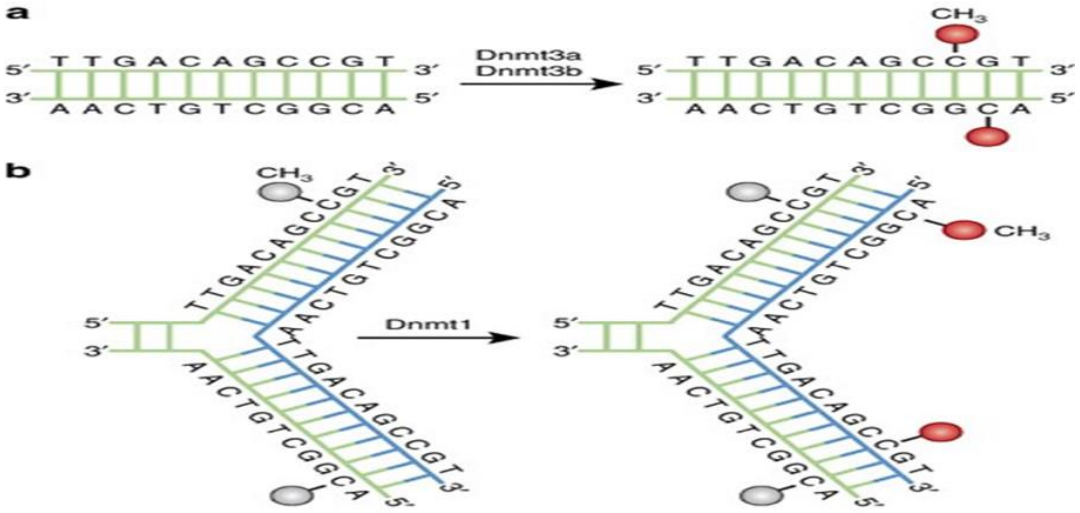


Figure 1.6. DNA Methylation (Normal DNA to Methylated DNA) (Goto et al, 1994; Feng et al, 2005; Moore et al., 2013).

1.3.2.1.1 DNA methyltransferases

Three types of DNMTs, DNMT1, DNMT2, and DNMT3A, 3B, and 3L, are responsible for processing DNA methylation (Figurte 1.7) (Hervouet et al., 2018). They are a class of proteins called DNA methyltransferases that catalyze DNA methylation. *De novo* methylation is the addition of methyl groups to cytosines that are not already methylated. DNMT3 enzymes are responsible for its catalysis and DNMT3L is an additional cofactor in *de novo* methylation in Bacteroides strains despite its minimal catalytic activity. DNMT3C is a tandem enzyme. DNMT3B was only discovered in rodents. DNMT3C is a tandem enzyme. DNMT3B has only been detected in rodents (Chen & Zhang, 2020). DNMT3A and DNMT3B are two important enzymes involved in the formation of DNA methylation during embryonic development (Handy et al., 2011). Whereas DNMT3A and DNMT3B establish dynamic methylation patterns, DNMT1 functions to maintain current methylation. Because altering their expression leads to aberrant methylation, these enzymes are tightly regulated, and aberrant methylation deregulates their production (Loaeza-Loaeza et al., 2020).DNMT1 has a role in DNA replication (Unterberger et al., 2006). The DNMT1 enzyme contributes to epigenetic stability due to its active role in the somatic inheritance of DNA methylation. A methylation abnormality causes cancer in humans (Peters et al., 2013).

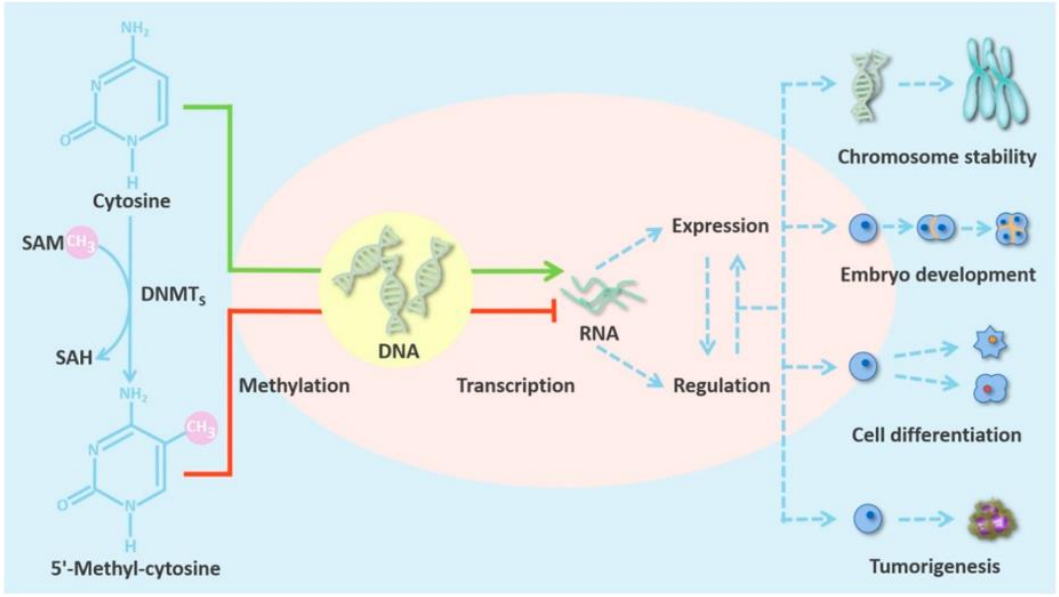


Figure 1.7. Characteristics of DNMTs in Mammalian Cells. The induction of DNA methylation by DNMTs influences chromosomal stability and patterns of gene regulation and expression, and abnormal DNMT may lead to cancer (Zhang et al., 2020).

1.3.2.1.2 DNA Methylation in Cancer

DNA methylation regulates cell type and lineage. It is a chemical alteration that helps keep the genome accurate and manage gene expression. Any problem in the control of DNA methylation leads to diseases like cancer (Figure 1.8) (Nishiyama & Nakanishi, 2021). This process accelerates the development of cancers as it works to silence tumor-causing genes within the regions that stimulate tumor suppressor genes, and the methylation process can contribute to the occurrence of mutations inside the gene (Wajed et al., 2001). Changes in the epigenome occur with the onset and spread of cancer. There are other areas with few CpG sites and repetitive elements where cancer cells lose CpG methylation. Concomitant site-specific hypermethylation of CpG islands and their shores occurs along with this process (Weisenberger & Liang, 2015).

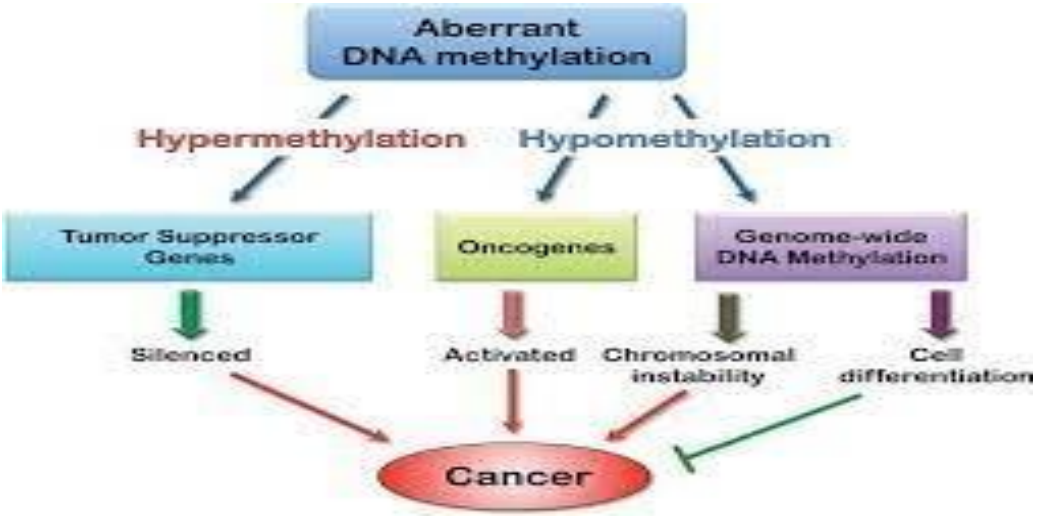


Figure 1.8. DNA Methylation in Cancer (Wang et al., 2017).

1.3.2.1.3 DNA Methylation in Normal Mammalian Tissue

The methylation of DNA does not occur during replication and transcription, which preserves the integrity of the genome in normal physiological conditions and does not produce specific genes in the tissues and helps in the expression of alleles through the genetic code, or deactivation of the additional copy of the X chromosome in females (DNA methylation is involved in the formation of heterochromatin and imprinting in mammals besides X chromosome inactivation) (Suzuki et al., 2014). Throughout evolution, the mammalian genome has collected a significant number of transposable elements, including parasites and retroviruses, which make up more than 30% of the human genome. CpG methylation silences transposable elements and prevents their replication, and DNA methylation helps to keep genes' integrity intact (Lakshminarasimhan & Liang, 2016).

1.3.2.1.3.1 Hypermethylation and Hypomethylation

Hypomethylation is the removal of a methyl group from the nucleotide 5-methylcytosine results in hypomethylation of DNA (Ehrlich, 2009). Frigola et al., (2005) in a study observed hypomethylation in the early stages of tumor formation, while hypermethylation was observed in more advanced stages. Hypermethylation is an increase in the methylation of DNA's epigenetic indicators (Rauluseviciute et al., 2019). Overrepresentation of hypermethylated CpGs is observed in tumor tissues (de

Almeida et al., 2019). DNA methylation in the cell changes in conjunction with the development of cancer. The genome contains many CpG islands that are focally hypomethylated (Figure 1.9) (Locke et al., 2019). While hypomethylation was discovered in other malignant tissues, induced hypermethylation and reduced gene expression were seen in precancerous breast tissues. This suggests that epigenetic modifications have a role in the development of cancer (Győrffy et al., 2016; Vietri et al., 2021). Both global hypomethylation and global hypermethylation of tumor suppressor genes are present in tumor cells (Tang et al., 2016; Lee & Kim, 2022). While hypomethylation causes the activation of cancer-related genes, hypermethylation contributes to the silence of genes involved in development, leading to uncontrolled growth. This means that DNA methylation inhibitors can be considered anti-cancer medications since they activate genes that hinder the development of tumors (Szyf et al., 2004).

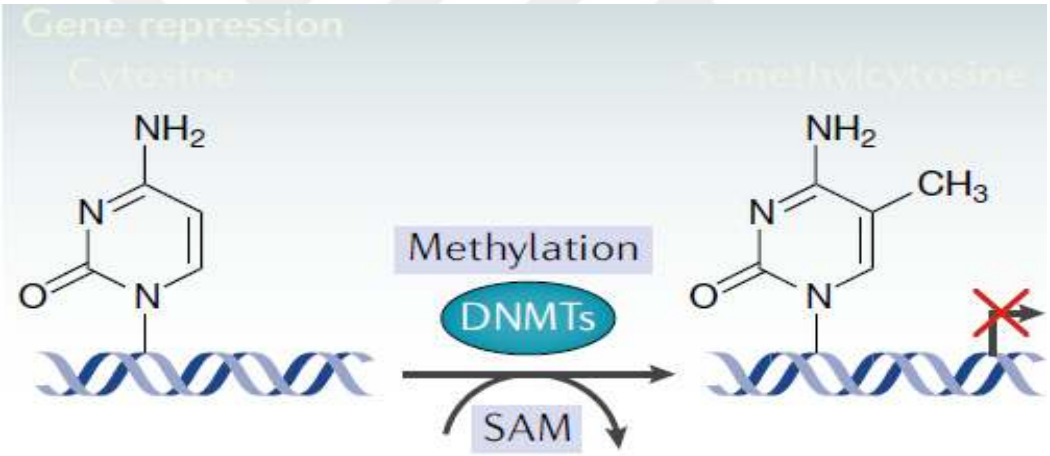


Figure 1.9. DNA Hypomethylation (Pfeifer, 2018)

1.3.2.1.4 DNA Methylation as a Therapeutic Target

Early neoplastic changes in DNA methylation make them useful early predictors of malignancy (Locke et al., 2019). Since DNMT is overexpressed in cancer it is beneficial to treat it with drugs that target DNMT. It enables scientists to restore normal levels of whole-genome methylation in malignant cells. Similarly, hypermethylation of TSG promoter regions, which been observed in malignancies, can also be reversed by targeting DNMTs. Any type of cancer can be treated with this type of customized therapy (Figure 1.10) (Zhang et al., 2020).

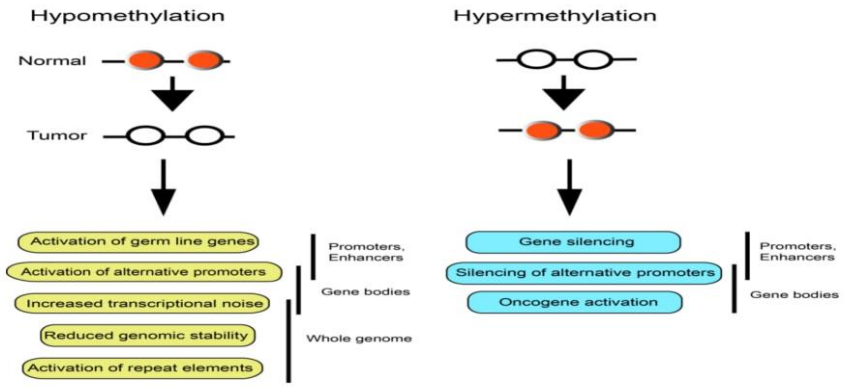


Figure 1.10. Mechanism of DNA Methylation (Ions et al., 2013).

1.4 ING1

1.4.1 ING1 Definition

ING1 3D structure was shown in Figure 1.11. In 1996, Garkavtsev et al., found that TSG known as ING1 is a protein that helps stop cancer growth and subsequent studies revealed that majority of ING family members are involved in cellular proliferation, a common feature of cancer cells (Garkavtsev et al., 1996; Jacquet & Binda, 2021). In addition, the type II tumor suppressor growth inhibitor 1 (ING1) is not downregulated in a large variety of human tumors (Thalappilly et al., 2011). Trimethyl-lysine 4 of histone H3 (H3K4Me3) is selected by ING proteins via an epigenetic readout when it controls both HAT and HDAC (histone acetyltransferase and histone deacetylase complexes) to chromatin (Thakur et al., 2012). The core histone proteins that interact with PHDs in INGs are sensitive to histone methylation, and the epigenetic histone code is translated by ING proteins as well (Pena et al., 2006). Over the past two decades, ING1 has undergone several comparison studies with other genes of the same family after its discovery in BC cells as a potential TSG (Taheri et al., 2022).

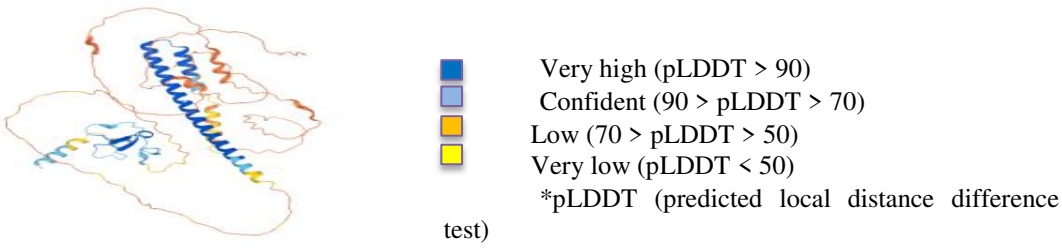


Figure 1.11. ING1 Gene 3D Structure (Safran et al., 2010).

1.4.2 INGs Family

ING1 through ING5 are the five types who make up the ING (growth inhibitor) protein family (Figure 1.12). The first member of this family, ING1, was found in 1996 as a result of a subtractive hybridization assay between normal breast epithelium and seven different BC cell lines, and ING2 to ING5 was discovered by sequence homology (Garkavtsev et al., 1996). ING proteins are important in cellular functions (Heliez et al., 2023). The ING family of proteins also participates in chromatin remodeling and binds to it. It affects the activity of the histone acetyltransferase (Feng et al., 2002). These genes persist in a variety of species, such as frogs, mice, yeast, and humans (Nouman et al., 2003).

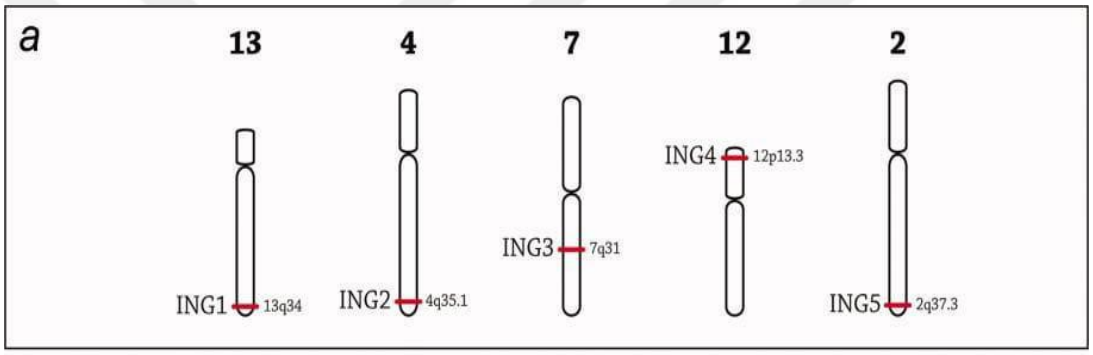


Figure 1.12. ING family's Chromosomal Locations of Genes (Ythier et al., 2008).

1.4.3 ING1 Function and Relationship with p53

After DNA damage, hypoxia, and oncogene activation, ING interacts with PCNA and p53, causes proliferative arrest and promotes replicative immortality, downregulates cellular energetics, avoids growth suppressors, prevents immune system destruction, and promotes inflammation that promotes tumor growth (Bertschmann, 2020). Aging and chemical sensibility are all influenced by ING1 (Cheung & Li, 2001). In many death checkpoints, p53-mediated apoptosis might face blocking due to direct p53 activity inhibition (Shen & White, 2001). The ING1 and ING2 interact physically with AR and work together to suppress it (Bartsch et al., 2021). According to clinical findings, INGs reduce the prevalence of specific cancer types and have been demonstrated in studies to impact how sensitive cancer cells are to radiotherapy and chemotherapy (Zhang et al., 2017).

1.4.4 ING1 Location

ING1 location on chromosome 13 was shown in Figure 1.13. ING1 is situated at the telomeric and subtelomeric areas on the arm of chromosome 13q34. In ING1 and ING2, 70% of the sequences are similar. Likewise, ING1 and ING2 are located inside the same HAT or HDAC complex (He et al., 2005).

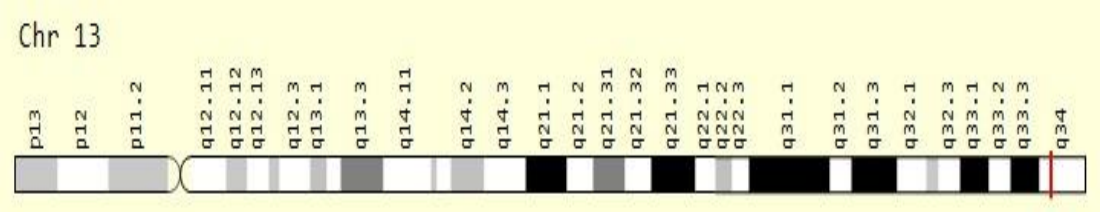


Figure 1.13. ING1 location on chromosome 13 (GeneCaRNA: The Human ncRNA Database)

1.4.5 ING1 Structure

All ING proteins have a plant homeodomain (Ph.D.) and an NLS (nuclear localization signal) at the C-terminus. The Ph.D. model is composed of about 60 amino acids, and the C4HC3 signature shows the connection between two Zn2+ ions. Ph.D (Aasland et al., 1995; Bienz, 2006). Domains integrate or enhance the distinct chromatin-binding activity of the same protein or a closely related protein (Figure 1.14). They are often found in proteins that make up bigger chromatin remodeling complexes and function in chromatin remodeling. The protein builds up in the cytoplasm as a result of the NLS deletion. Both the absence of nuclear ING1 staining in certain carcinomas and the presence of ING proteins in the nucleus are essential for their activity (Coles & Jones, 2009).

Figure 1.14. Structure and Interacting Domains of ING Family Proteins (Gunduz et al., 2011).

1.4.6 ING1 Proteins

Due to the NLS (nuclear localization sequence), ING proteins are mostly found in the nucleus (Archambeau et al., 2019). The ING family of proteins alters chromatin

by acting as epigenetic scanners for the transcriptionally active H3K4Me3 histone mark (Dantas et al., 2019).

1.4.7 ING 1 role in cancer

A study by Toyama et al., (1999) presented a list of details on INGl. It is improbable that INGl gene mutations will be present in primary breast, or ovarian cancer cell lines. All breast cancer cell lines were examined as well, and a sizable percentage of breast cancers (44%) displayed inadequate gene expression. Additionally, a link between metastasis and INGl expression levels was found. Furthermore, it was established that whereas ING1 mutations are frequently linked to tumor growth in breast cancer, they are relatively infrequent in breast or ovarian malignancies.

Thakur et al., (2014) observed that ING1 levels were lower in precancerous tissues than in healthy nearby tissues. Additionally, ING1 was employed to forecast disease-specific survival without metastasis. Moreover, ING1 overexpression removes tumor-induced mortality in mice models and stops the spread of tumor cells in vivo. His findings confirmed the therapeutic significance of ING1 in preventing metastasis, including that of breast cancer, and showed for the first time that modifying its levels routinely affects metastasis in vitro and in vivo. His findings also showed that ING1 protein levels are lowered in breast cancer (Bose et al., 2013; Thakur et al., 2014).

CHAPTER 2

AIM OF THE STUDY

The purpose of this study was to track the methylation status of the ING1 gene, where it is in TNBC tissues and as it is used as a useful marker in predicting the occurrence of TNBC. Therefore, the purpose of the proposed study is to evaluate the utility of the ING1 gene promoter methylation pattern as a biomarker in TNBC with an aggressive course.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Used Materials	Brand
Nanodrop2000/2000c Spectrophotometers	Thermo Scientific™
PCR, Fast Thermal Cyler	LongGene A300
Centrifuge	Neofuge 13R-Refrigerated Centrifuge-Heal Force Bio
Water Bath	Lab Companion
Vortex	Lab Companion
Microwave	Vestel
Gel Electrophoresis	Thermo
Beta Mercaptoethanol	M3148 Sigma-Aldrich
Proteinase K, recombinant, PCR grade	Thermo Scientific™
Nuclease Free, Ultra Distilled Water, Sterile	Biochrom AG
PCR HS Master Mix	Procomcure Hs Gold Master Mix
DNA Isolation Kit	MSTLAB DNA Isolation Kit
Agorose Gel Poster	BluPad
Xylene	Sigma
Etil Alcohol 100%-70%-50%	Isolab
1XTAE	Sigma

3.2 Tissue Samples

In our study, DNA samples from paraffin-embedded tissue samples from patients diagnosed with TNBC were from the Department of Pathology, Faculty of Medicine,

University of Gaziantep, which were isolated from 110 non-metastatic local patients. The ages of the patients were between (25- 91) years old. Breast biopsy tissue taken from the patient at the stage of diagnosis, the tumor was diagnosed in the breast as a result of the examinations conducted in the pathology department. Paraffin-embedded samples were obtained in tubes of different sizes ranging from 1-5 cm^3 tissue sections embedded in the block. Inform consent was obtained from patients or first degree relative for dead in written ethical form and permission was obtained from the local ethical committee in Gaziantep University (2022/328).

3.3 Gene Selection Criteria

Based on data from the Human Protein Atlas, the National Center for Biotechnology Information, and the Cancer Genome Atlas Program, the ING1 gene was chosen for the investigation. One or little methylation in healthy breast tissue (10%) and high methylation in primary breast tumor tissue (>50%) are the two basic characteristics that are sought. Class II tumor suppressor genes, which are often silenced by inducible hypermethylation, were analyzed, and their methylation was triggered in these potential cancer cells to identify biomarkers with functional relevance to breast cancer (Sharma et al., 2018).

3.4 DNA Isolation from Paraffin Embedded Tumor Tissue Samples Isolation using the CpG Genome™ Universal DNA Modification Kit

3.4.1 Deparaffinization

1- The samples were numbered sequentially first.

2- Placing the samples in a hot water bath (50-56°C) to melt the paraffin for 45 minutes.

3- 1000 µL of xylene was added to each tube, and the tubes are re-placed in a hot water bath for 10 minutes (if the paraffin does not melt within 45 minutes).

4- The tubes were centrifuged 10,000 X g for 1 minute, the liquid was withdrawn, and this process was repeated three times.

5- 1000 µL of alcohol were added to the samples with a concentration of 100%, the tubes are shaken by a vortex device, and centrifuged again at 10,000 rpm for 1 minute.

6- The alcohol was withdrawn and 1000 µL of alcohol were added at a concentration of 70% and shaken with vortex.

7- The tube is centrifuged at 10,000 rpm for 1 minute, the alcohol was withdrawn, then 1000 microliters of alcohol was added at a concentration of 50%, the tube is shaken with a vortex and centrifuged at 10,000 rpm for 1 minute, and the alcohol or supernatant has withdrawn.

3.4.2 DNA Isolation

1- The tubes were changed and beads were added, (1 large white, and 5-6 small sized brown beads) to each tube. 10 microliters of Proteinase K was added to each tube.
2- 500 µm extraction buffer was added, then placed in a Homogenizer device and mixed. The samples were cut to the same size.
3- Transferred to a water bath at 56 ºC for 30 minutes, and centrifuged for a short time and a supernatant is taken from it.
4- A banding buffer was added and then placed on the spin colon.
5- Add 300 µL of banding buffer to each tube and put it on a spin colon and shake it with a vortex.
6- The samples were centrifuged at 8000 rpm for one minute, and then the liquid under the tube is emptied.
7- Add 650 µL of Wash Buffer 1 to spin colon and centrifuge at 8000 rpm for one minute and empty the liquid under the tube.
8- Adding Wash Buffer 2 in two stages, the first stage includes adding 500 µL and centrifuging at 8000 rpm for one minute, after which the liquid under the tube is emptied. And 250 µL wash Buffer 2 has added in the second stage, centrifuged at 14000 rpm for 3 minutes, placed in a new tube, and then placed on a spin colon.
9- Add 30 µL of Elution buffer and centrifuge at 8000rpm for one minute. Spin colon is discarded and remains DNAX20.

3.5 Determination of DNA Purification by Nanodrop

1- A nanodrop measuring device has used, which is cleaned with water before use in the amount of 2 µL (for the small size of the samples).
2- Add 1 µL of Elution buffer to filter the device, and then adding the sample to the device by 1.5 microliters.
3- Select the device icon (Nanodrop) on the desktop.
4- Choose Nucleic acid from the list.
5- Choose the type of DNA,

6- Choose the standard unit for nucleic acid which is ng/µL.
7- Choose the appropriate wavelength for the solution to be tested, the wavelength 260-280 is suitable for measuring and quantifying DNA.
8- Choose the icon (add to the report) to add all the measurements of all your samples and save them for reference when needed.
9- Zero the device using (Blank DNA solvent). This solution must be a good solvent for the nucleic acid, in addition to that the Blank used to clear the device must be of the same pH and the same ionic degree as the solution used for the DNA solvent.
10- Put 1-2 l of the Blank solution on the lens of the device, and then lower the arm of the device. Then press the word (blank).
11- Clean the lens with special cleaning paper, then place the sample and press the word (Measure) to start the measurement.

3.6 Biosulfite Modification

The CpGenome™ DNA Modification Kit contains reagents need to perform a bisulfite modification on a DNA sample. Figure 3.1 shows the bisulfite reaction, the unmethylated cytosines are slowed down and sulfonated, turning them into uracils, while 5-methylcytosines remain unaltered. Thus, the sequence of the modified DNA will be different depending on if the DNA is originally methylated or unmethylated. Also, the initially complementary DNA strands will no longer be complementary after cytosine conversion. Primers for use in MSP can be designed to specifically amplify either a bisulfite-sensitive, unmethylated strand or a bisulfite-resistant, methylated strand, based on these chemically-induced differences.

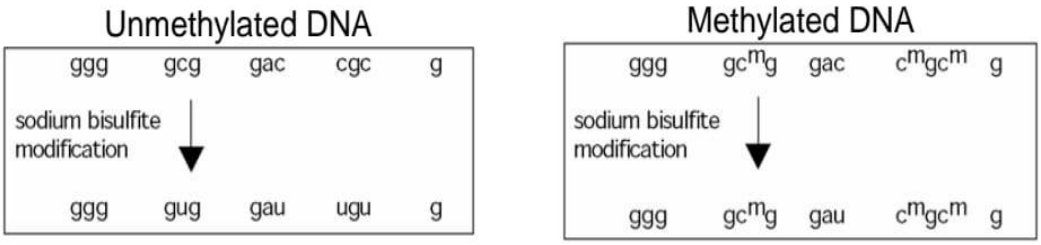


Figure 3.1. DNA treatment with Sodium Bisulfite

3.6.1 Reagent Preparation

1. NaOH (3 M stock), newly made for each use 1 g of dry NaOH pellets should be dissolved in 8.3 mL of water (When utilizing this caustic base, taking the necessary caution and good laboratory techniques).
2. 20 mM NaOH/90% EtOH (freshly made before usage) To make 1 mL of this solution, mixing 900 l of 100% EtOH, 93.4 l of HO, and 6.6 l of 3 M NaOH.
3. Dissolving the freshly prepared Reagent I before usage. Warming the vial to room temperature (before opening).
4. Adding 0.571 mL of water and 0.227 g of DNA Modification Reagent I to each sample that needs to be altered.
5. By vortexing, thoroughly combined. (Handling with care when using this reagent for its irritancy to the respiratory system and skin).

For Reagent II

1. The bottle has to be warmed at room temperature before opening.
2. 20 mL of deionized water should be mixed with 1 L of ß-mercaptoethanol.
3. Mixing 1.35 g of DNA Modification II with 750 L of this solution for each sample that needs to be changed.
4. To guarantee total disintegration, combining it thoroughly. In a foil-wrapped container, extra chemicals can be kept at 2 to 8°C in the dark for up to 6 weeks.

3.6.2 3.6.2. DNA Modification Procedure

1. In screw-cap 1.5-2.0 mL micro centrifuge tubes, combining 1.0 g of DNA with 100 mL of water (10 ng/mL), 7.0 l of 3M NaOH, and stir.

Note: Adding 2 L of DNA Modification reagent IV if the sample contains less than 1.0 g of DNA. Bring the whole capacity to 100 L with water before adding the sample DNA. Then mix in 7.0 mL of 3M NaOH.

2. Using a heat block or water bath to incubate the DNA at 50°C for 10 minutes.

3. Pouring 550 mL of freshly made DNA Modification Reagent I into the mixture, then vortex.

4. Incubate in a heat block or water bath shielded from light for 4–16 hours at 50 °C.

3.6.3 Initial Desalting

1. In the second stage of this process, 5 microliters of Regent 3 liquid was added to each tube, then 750 microliters of Regent 2 was added, which is in the form of granules, which leads to a change in color to yellow by vortex, centrifuged after that 5000 g for one minute and the supernatant was emptied.

2. Add 1.0 mL of 70% EtOH, vortex, and centrifuge at 5,000 X g for 10 seconds and discard the supernatant. Perform this step a total of 3 times.

3. After removing the supernatant from the third wash, centrifuge the tube at high speed for 2 minutes and remove the remaining supernatant with a plastic pipette tip.

3.6.4 Completion of DNA Modification (Desulfonation), Second Desalting, and Elution

1- To the appropriate samples, adding 50 L of the 20 mM NaOH/90% EtOH solution.
2- Vortex the pellet briefly to resuscitate it, then incubating it for 5 minutes at room temperature.
3- Spinning the tube at 5,000 X g for 10 seconds to transport all of the contents to the tip. To wash the pellet, add 1.0 mL of 90% EtOH and vortex. Reverse the spin, and then remove the supernatant. Repeat this action one more time.
4- Centrifuging the sample at high speed for 3 minutes after removing the supernatant from the second wash.
5- Using a plastic pipette tip, eliminate all of the residual supernatants. Dry the tube at room temperature for 10 to 20 minutes; the alcohol odor should fade.
6- Include TE Buffer.

Note: The amount of starting DNA and the necessary input concentration for the intended use determine the volume of TE that must be added. For instance, the ultimate concentration based on total recovery of 1 g of DNA would be 40 ng/L if 30 L of TE were added. Rapidly and vigorously vortex until the pellet is fully resuspended. Flick or tap the contents manually into the tube tip (do not centrifuge).

7- For eluting the DNA, the sample was incubated for 15 minutes at 50–60°C.

8- Centrifuging the samples for 2-3 minutes at high speed and taking the sample (supernatant) to a new tube by a plastic pipette tip.

3.7 Primer Design

For the amplification of bisulfite-converted DNA, site-specific primer pairs in the 24-32 base range were designed. The sequences are given below at Table 3.1.

Table 3.1. Primers for ING1

Primer	Sequence	Bp
ING1 Left M Primer	GAAAGGTGAAAATTGATAAGGTATC	25
ING1 Right M Primer	CTAACTCTCTAATTCCGAAACGAC	24
ING1 Left U Primer	AGGTGAAAATTGATAAGGTATTGT	24
ING1 Right U Primer	CCTAACTCTCTAATTCCAAAACAAC	25

3.8 MSP-PCR

Methylation specific PCR based on two primer sets used for methylation sequence. Unmethylated primer amplifies only bisulfite converted DNA in unmethylation sites and methylated primers are for bisulfite converted methylated DNA. MSP-PCR is a quick, sensitive, and cost-effective test for analyze methylation status CpG dinucleotides in CpG islands (Derks et al., 2004).

In order to stock the primers, which are dried materials, with a concentration of 100 µM, dissolving the primers by pipetting with nuclease free water or TE buffer according to the specified amount in the column titled "100 µM stock-µl TE" without using vortex. PCR Reaction conditions are as follows for 1 sample:

1. Placing the supernatant only in a new PCR tube strip.
2. Taking (499 - 611 - 863 - 636) microliters of stock and mixed with the primer.
3. On each PCR we took 10 mill moles of methyl and unmethyl (Reverse and Forward) and adding 80 microliters of water to it and adding 10 microliters of Forward and 10 microliters of Reverse and writing M (methyl) and UnM (unmethyl) on tube Strip
4. The mix found in the strip tube contains the following:
5. We took two tubes, wrote on one of them methyl and the other unmethyl, and addig 5 microliters of mix to 0.7 microliters of Forward and Reverse taken from the third step, 2.8 microliters of water and 1.5 microliters of DNA, and microcentrifuged it.
6. Placing in the PCR device for 90 minutes.

MSP-PCR mix was shown at Table 3.2. and PCR conditions at Table 3.3.

Table 3.2. MSP-PCR mix

Mix Content	Amount
Master Mix	10 µL
Primer	2 µL
ddH_20	6 µL
DNA	2 µL
Total	20 µL

Table 3.3. MSP-PCR Conditions

Temperature	Heat Time	Loop
95 º	5 dk	**1 Cycle**
95º	15 sn	
56 º	30 sn	**40 Cycle**
72 º	30 sn	
4 º	∞	

3.9 Agarose Gel Electrophoresis

1- The first material was prepared by mixing 1XTAE 50 mL with 1% agarose.
2- The mixture is placed in the microwave until the solid is completely dissolved.
3- After the mixture has completely cooled in the tank, 2.5 microliters of red safe is added and waited for a while.
4- It is read by the white light transilluminator within twenty seconds.
5- We get a reading in the form of an image.

CHAPTER 4
RESULTS

4.1 DNA Purification by Nanodrop

This assay investigates the purity of DNA samples from protein and salts. The determination of the DNA in the sample is an important process to detect the amount of DNA concentration. The measurement of DNA in this device is easy and fast. This process also reveals the percentage of DNA contamination with proteins and salts. It compares the wavelength and absorption rate. So, the pure DNA sample is read between 260-280 nm, provided that the absorption rate is between 1.70- 2.00. 110 DNA samples were measured as ng/µl and their purification rate were calculated. Our results were shown at Table 4.1.

Table 4.1. DNA Value and Absorbation Rate at 260/280 nm

Sample No	ng/µL	260/280 nm	Sample No	ng/µL	260/280 nm
1	255	1.84	**56**	141.4	1.82
2	124.6	1.88	**57**	131.9	1.9
3	161.1	1.84	**58**	62	1.79
4	112.1	1.91	**59**	44.5	1.81
5	192.2	1.86	**60**	143.7	1.84
6	171.1	1.76	**61**	98.2	1.78
7	18.2	1.65	**62**	68.8	1.84
8	245.7	1.84	**63**	33.5	1.84
9	120	1.81	**64**	4.2	1.33
10	101	1.65	**65**	134.1	1.86
11	100	1.87	**66**	4.9	1.33
12	222	1.82	**67**	42.9	1.76
13	368	1.87	**68**	100.5	1.83
14	66	1.49	**69**	180.1	1.92
15	112	1.53	**70**	9.2	1.56
16	84	1.89	**71**	39.6	1.77
17	147	1.84	**72**	108.5	1.7
18	52.3	1.87	**73**	53.3	1.59

19	42.2	2	**74**	39.3	1.66
20	78.9	1.92	**75**	43.9	1.72
21	230.8	1.82	**76**	41.4	1.73
22	82.1	1.97	**77**	229.5	1.86
23	151.4	1.58	**78**	88.4	1.88
24	143.5	1.84	**79**	121.7	1.74
25	89.5	1.69	**80**	115.3	1.79
26	33.5	1.92	**81**	92.8	1.81
27	77.3	1.71	**82**	65.2	1.75
28	132.1	1.85	**83**	29.6	1.57
29	103.1	1.58	**84**	28.2	1.89
30	14.3	1.93	**85**	143.7	1.89
31	22.6	1.86	**86**	32.7	1.57
32	11.4	1.96	**87**	92.8	1.87
33	85.7	1.79	**88**	35.6	1.81
34	3.8	2.06	**89**	64.9	1.92
35	58.4	1.76	**90**	117.8	1.8
36	75.9	1.91	**91**	223.6	1.9
37	109.5	1.86	**92**	48.9	1.51
38	240.6	1.85	**93**	54.9	1.87
39	136.6	1.85	**94**	18.5	1.64
40	102.7	1.84	**95**	37.5	1.64
41	108.7	1.67	**96**	25.8	1.63
42	119	1.89	**97**	100.6	1.86
43	138.7	1.88	**98**	100.4	1.75
44	229.7	1.85	**99**	28.7	1.29
45	136.7	1.91	**100**	48.9	1.79
46	56.2	1.7	**101**	84.4	1.78
47	113.3	1.91	**102**	62.1	1.48
48	68.1	1.86	**103**	91.1	1.91
49	105.5	1.86	**104**	40.1	1.88
50	54	1.77	**105**	48.9	1.84
51	186.5	1.91	**106**	24	1.63
52	65.2	1.85	**107**	7	3.69
53	89.2	1.78	**108**	27.12	1.72
54	9.9	1.33	**109**	28.2	1.95
55	36.8	1.66	**110**	24.2	1.83

4.2 MSP-PCR Results

Eighteen DNA samples were not visible on agarose gel. The rest samples were seen on gel and could be determined as methylated status (Figure 4.1- 4.9). As results,

total 92 samples could be distinguished due to both alleles methylated or not or also partial methylated as one allel methylated (Table 4.2).

Table 4.2. Methylation Status of Total 92 DNA Samples

Methylated (M) DNA	Un-Methylated (UM) DNA	Partial Methylated DNA (One allel methylated, one allel unmethylated
10	6	76

The samples (1-8, 10-14) are showing double band results. These samples are one allel methylated and one allel unmethylated. The sample (9) is unmethylated (Figure 4.1).

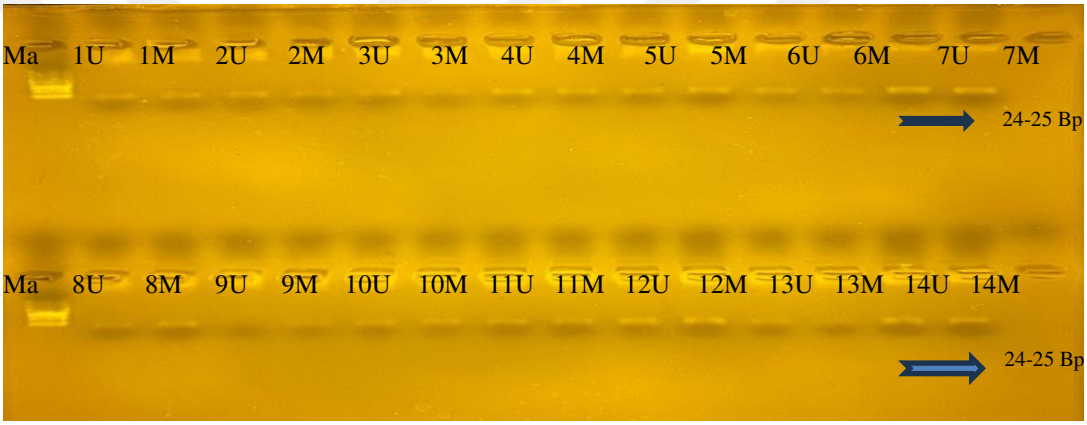


Figure 4.1. The Agarose Gel Electrophoresis DNA Analysis Results (1-14). The samples (15-21) are showing partial methylated as double band results (Figure 4.2).

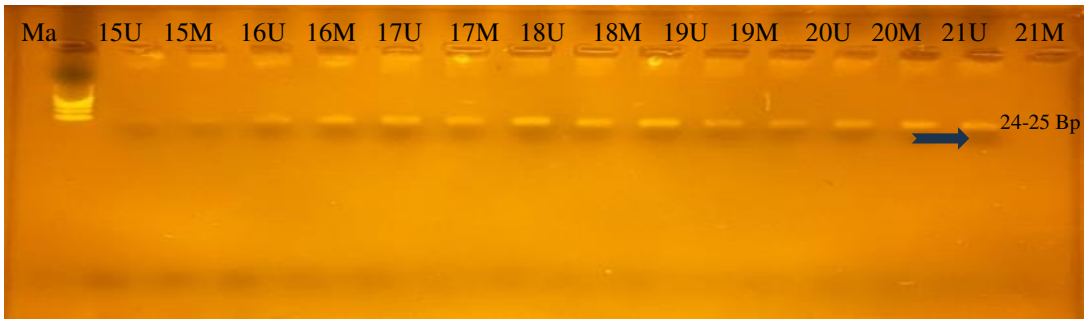


Figure 4.2. The Agarose Gel Electrophoresis DNA Analysis Results (15-21).

The samples (22-26, 28-31, 33-35) are showing one allel methylated status (Figure 4.3).

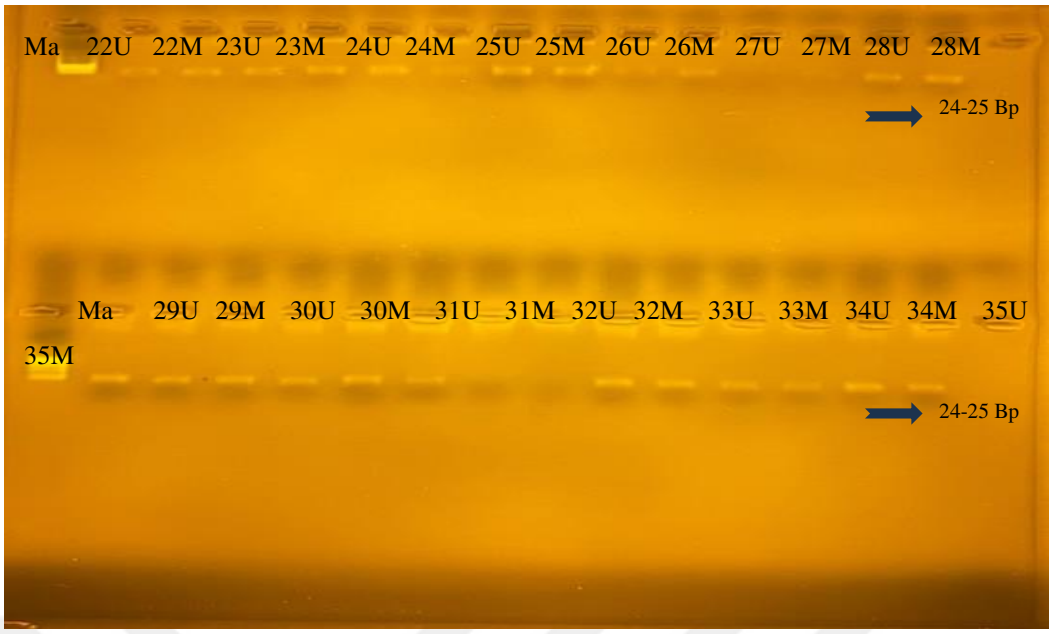


Figure 4.3. The Agarose Gel Electrophoresis DNA Analysis Results (22-35).

The samples (36-39, 45-49) are showing double band results meaning as one allel methylated. The sample (42) is showing only methylated result. The sample (44) is showing only DNA un-methylated result (Figure 4.4).

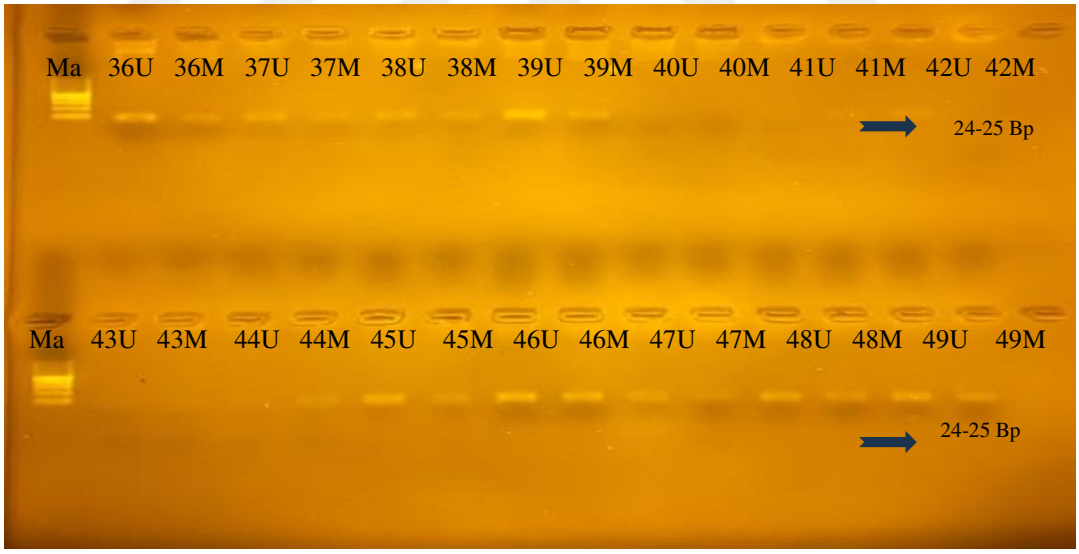


Figure 4.4. The Agarose Gel Electrophoresis DNA Analysis Results (36-49).

The samples (50, 59, 63) are showing DNA methylated double band results as a partial methylated DNA. The sample (54, 57, 61, 62) are methylated of all allelles. The samples (56, 58) are un-methylated (Figure 4.5).

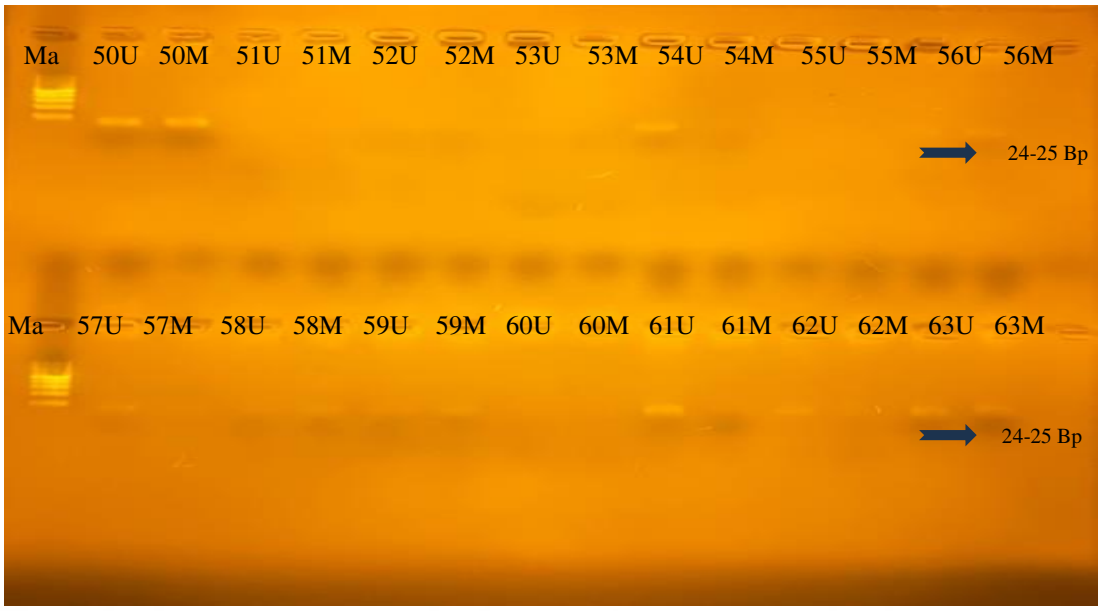


Figure 4.5. The Agarose Gel Electrophoresis DNA Analysis Results (50-63).

The samples (67 and 70) have un-methylated DNA. The samples (68, 71, 74-77) have partial methylated DNA showing as double band results. The sample (72) has strong methylated DNA (Figure 4.6).

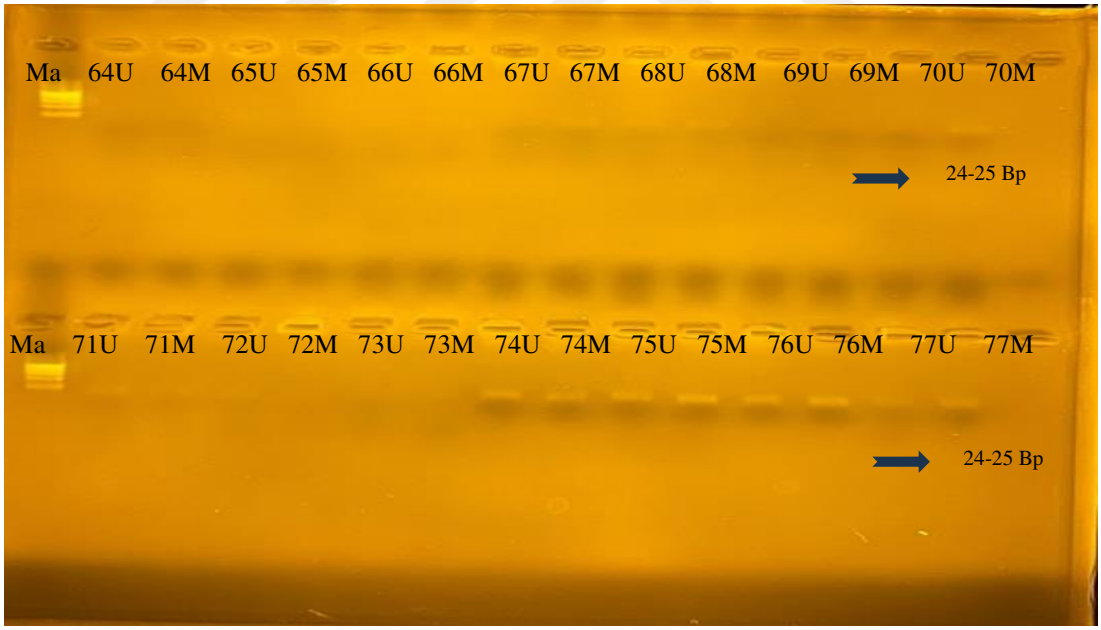


Figure 4.6. The Agarose Gel Electrophoresis DNA Analysis Results (64-77).

The samples (78-81, 86-90) have methylated one allel DNA and one allel unmethylated DNA. The sample (83) has strong methylated DNA (Figure 4.7).

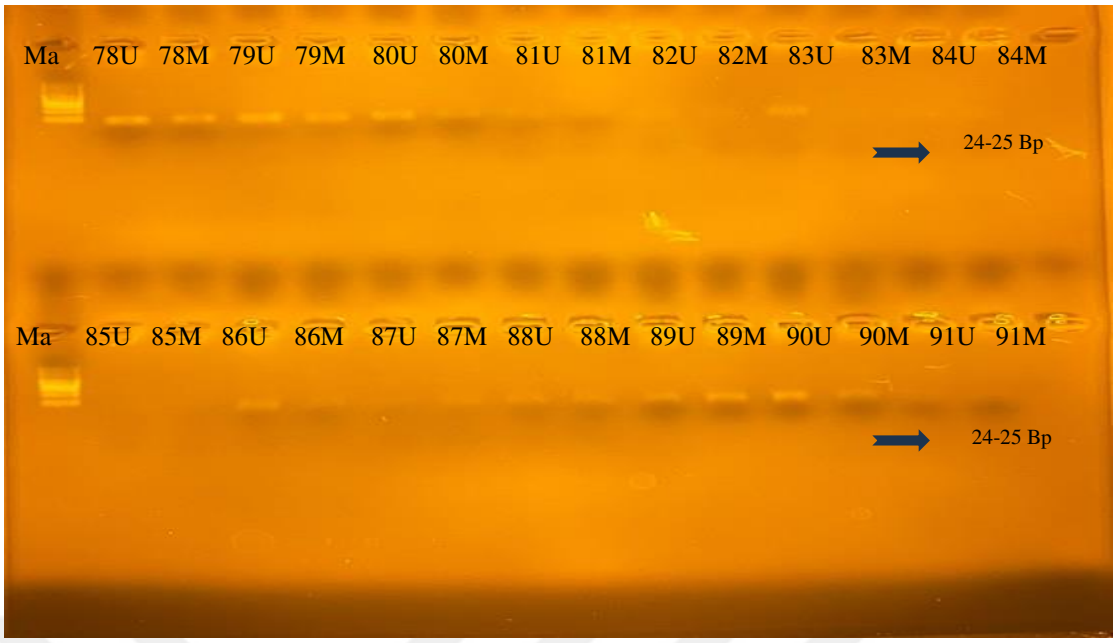


Figure 4.7. The Agarose Gel Electrophoresis DNA Analysis Results (78-91).

The samples (92-100, 102-104) are showing DNA double band results on methylated and unmethylated wells meaning as partial methylation. The sample (101) has strong methylated DNA as both alleles (Figure 4.8).

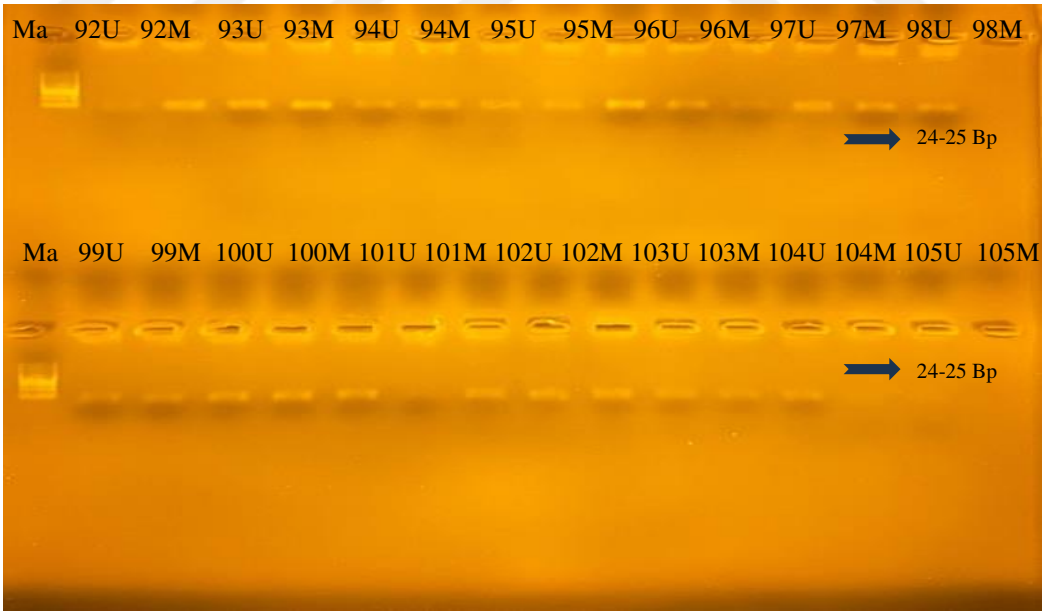


Figure 4.8. The Agarose Gel Electrophoresis DNA Analysis Results (92-105).

The samples (106-110) are one allel methylated and one allel unmethylated DNA (Figure 4.9).

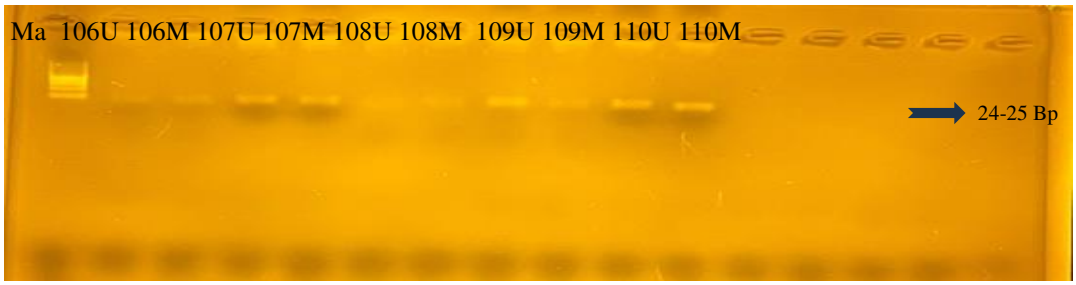


Figure 4.9. The Agarose Gel Electrophoresis DNA Analysis Results (106-110).

The relative methylation level determined by MSP-PCR band results and 93,5% methylation rate was significant for promoter site of ING1 in TNBC samples ($p<0.001$), indicating that promoter hypermethylation could be related with the silencing of ING1.

4.3 The patiants ages relationship with TNBC

The results that we reached during this research showed that the incidence of triple-negative breast cancer increased significantly between the ages of 40-50 years by 35%, while the percentage of infections between the ages of 50-60 increased by 25%, and between the ages of 30-40 the percentage reached 18%. The percentage decreased to less than 8% for the rest of the ages

Table 4.3. The patiants ages relationship with TNBC

No.	Patiant age	Cases' numbers
1	>30 (25-30)	2
2	30-40	17
3	40-50	33
4	50-60	24
5	60-70	8
6	70-80	5
7	80-90	3
8	90<	1

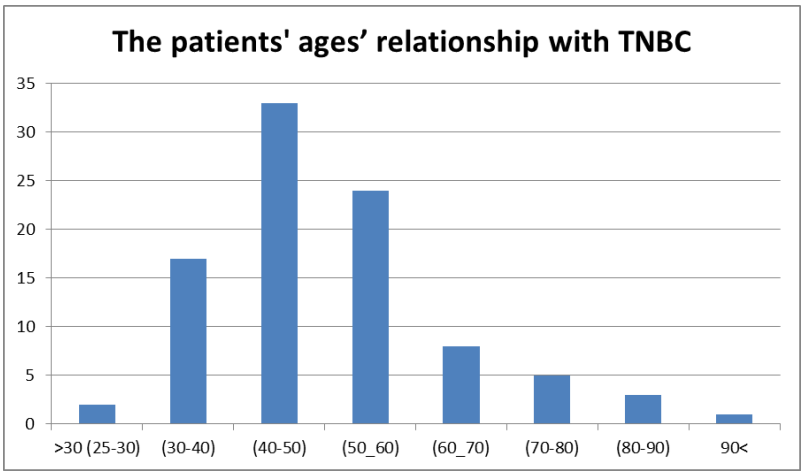


Figure 4.10. The patiants ages relationship with TNBC.

4.4 The relationship between the stage of triple-negative breast cancer and DNA methylation

We also compared the invasive stage and the severity of the cancer, in each type of triple-negative breast cancer with the DNA methylated in 93.5% of the samples. We reached the following results: 60% of the samples that suffered from DNA methylation were of the type of triple-negative breast cancer, stage III, while 20% of the samples were of the type of triple-negative breast cancer, stage II. Other types of breast cancer also suffered from DNA methylation by 19%, and we also found a rate of 1% of triple-negative breast cancer, stage one.

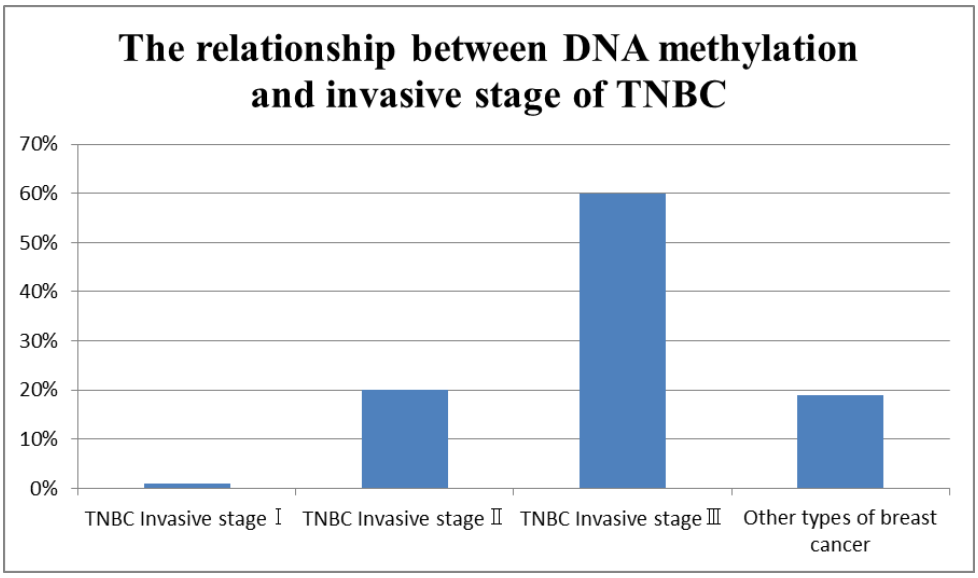


Figure 4.11. The relationship between DNA methylation and invasive stage of TNBC.

CHAPTER 5

DISCUSSION

Breast cancer, which is the second most common disease in women worldwide after lung cancer and is mainly restricted to females, affects females of all ethnicities and ages. Breast cancer rarely affects men; in fact, it accounts for just 1% of all occurrences. Garkavtsev et al., (1996) concluded in a study, they conducted that ING1 is an important gene in the growth of normal cells and a tumor suppressor gene, and if it is disrupted, it leads to the development of cancers such as breast cancer.

Triple-negative breast cancer is characterized by a poor prognosis by conventional methods and is characterized by the possibility of metastasis and other symptoms, as well as the occurrence of recurrence. Furthermore, traditional treatments harm both healthy and carcinogenic tissues, such as chemotherapy. Moreover, because they only attack cancer cells and leave healthy breast cells alone, contemporary therapeutic approaches that focus on genetic or non-genetic targets are thought to be more effective.

The gene belongs to a family of 5 genes, namely: ING 1 to ING 5. Described for the first time in 1996, this gen can be found in the nucleus and this protein contributes to cell functions, also has a role in in chromatin remodeling and promotes inflammation that promotes tumor growth (Heliez et al., 2023; Feng et al., 2002; Bertschmann, 2020). INGs family of different malignancies can be effectively eliminated by miRNA. Consequently, this family's genes might be a gene, increasing evidence supports the treatment of cancer (Zhang et al., 2017).

We selected one of the tumor suppressor genes which is ING 1 (growth inhibitor) gene to show the occurrence of DNA methylation coincides with a decrease in the expression of this gene. Where ING1 gene prevents the growth of cancer and the development of the original tumor and prevents the spread of cancer without affecting it (Garkavtsev et al., 1996; Jacquet & Binda, 2021). Ultimately, invasion

and metastatic colonization occur due to overlapping of these genes in proteins and gene pathways according to both researchers (Chen et al., 2020; Fares et al., 2020).

A study by Toyama et al., (1999) showed that INGl gene mutations could not be found in primary breast or ovarian cancer. It did not show sufficient gene expression when examining breast cancer cell lines (44%). At the breast tumor location, ING1 levels were shown to be lower. We used this study's findings to examine the similar result in triple-negative breast cancer.

Low expression of ING1 gene is a feature of many cancers. Esmaeili et al., in 2016, have shown a deficiency in ING1 was shown in prostate cancer. That proves that the low level of the ING1 gene contributes to the increase in the presence of cancer, as we found in our research. DNA methylation is elevated in the presence of prostate cancer when compared to normal prostate tissue (Esmaeili et al., 2016; Melekhova, & Baniahmad, 2021).

In an overview by Zhao et al. (2022), they reported the involvement of DNA methylation in the occurrence and development of pancreatic cancer as an epigenetic event at the level of regulatory molecules. Also, He et al. (2014) confirmed the critical role of ING1 in pancreatic cancer and the possibility of using it as a good therapeutic target against cancer. Sharma et al., (2020) also mentioned that DNA methylation is an important component of bladder cancer. While a study conducted by Zhou et al., in 2018, confirmed the close relationship between DNA methylation and head and neck cancer. While another study conducted by Kalinkova et al., in 2022, linked leukemia to DNA methylation. The ING1 gene contributes to the pathogenesis of stomach cancer and the development and subsequent events of cancer (Ding et al., 2003). Qu et al., (2013) confirmed that the three epigenetic events, including methylation, cause stomach cancer. Tahara & Arisawa, (2015) mentioned the close relationship between gastric carcinogenesis and the occurrence of DNA methylation. As for the study conducted by Peng and others in 2023 on 340 samples of patients with stomach cancer, they proved the relationship between DNA methylation in 10 genes and the cause of stomach cancer.

According to all the findings of the researchers that we mentioned above. The types of cancers mentioned, namely breast cancer, lung, prostate, pancreas, bladder, head and neck, stomach, skin and blood. All of these results agreed that DNA methylation causes various cancers and decreased gene expression of the ING1 gene causes cancer proliferation. Therefore, methylation and the ing1 gene can be challenged as a

potential therapeutic target for triple-negative breast cancer, as we demonstrated in our research that there is a relationship between DNA methylation, the ING1 gene, and TNBC.

Zhou et al., 2020 defined epigenetic changes as phenotypic differences that are inherited without changes in the DNA sequence. It is the epigenetic changes that occur in healthy living cells in vivo that cause cancer, as described by different researchers (Byler et al., 2014; Sambi et al., 2019; Mohammad et al., 2019). Wu et al., (2015) showed that BC initiation, development and proliferation is mediated by epigenetic variations. We based our work on a group of studies that confirmed the close relationship between breast cancer and epigenetic events.

Zolota et al., (2021) stated that a combination of epigenetic events including DNA methylation, and histone modifications are the epigenetic modifications in TNBC. This is what we investigated in our research and linked DNA methylation to TNBC.

Garcia-Martinez et al., (2021) stated that rather than fixing permanent genetic mutations, researchers focus on small, disorganized epigenetic circuits. Which exposes cancer cells to immune system attacks and treatment. It also occurs when the epigenetic molecular mechanisms that build healthy mammary glands are disrupted by BC. Epigenetic pathways induce cell death pathways. Accordingly, our research will contribute to finding a suitable treatment for the epigenetic changes that cause TNBC.

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

For genomic function, the methylation of deoxycytidine nucleotides in CpG islands is an epigenetic regulation mechanism. Alteration of the DNA methylation pattern has been closely associated with carcinogenesis. DNA hypermethylation at promoter sites of the tumor suppressors leads to silencing these critical genes and thus contributes with cancer development. Lots of research have focused on DNA methylation patterns as biomarkers for cancer.

In this thesis, we investigated the association of DNA methylation of ING1 suppressor gene promoter site with TNBC. Our research is considered original because we are the first to link DNA methylation of ING1 with TNBC. ING1 suppresses malignancy when it is active. We chose to work on investigating DNA methylation in TNBC to prove a relationship between DNA methylation and cancer, meaning that adding a methylation group on nitrogenous bases in DNA from ING1 contributes to the occurrence of cancer.

This gene is active in normal cells and suffers from regulation downregulation in cancer cells. To biomarker definitions, two major criteria are searched for in genes to be selected using the Cancer Genome Atlas (TCGA). These are: (1) no or low methylation in normal breast tissue (<10%), and (2) high methylation in primary breast tumor tissue (>50%). In this context, during the identification of biomarker candidates with functional relevance in breast cancer, class II tumor suppressor genes, which are normally silenced by inducible hypermethylation, were analyzed, and analysis of CpGs in promoter regions aimed to discover regions with the highest differentiation.

Where we expected this research to obtain a result that supports what we mentioned earlier in a similar increase by 50%. ING1 is an active gene in normal cells that helps in the reproduction of normal living cells when it is in an active state. It also performs the programmed death of cancer cells. If it loses its normal state, it loses its ability to suppress and suppress cancer cells, which leads to the spread of cancer. Addition of a methyl group to the nitrogenous bases, which makes it lose its function

of cancer suppression. The ING1 gene loses its primary function in tumorigenesis when it suffers from epigenetic events such as DNA methylation or when mutations occur. A group of epigenetic events causes the addition of a group of methyl nitrogenous bases to a gene, and the occurrence of mutations in it can lead to the same effect. Genes may suffer changes that cause them to transform from normal genes into oncogenes, which leads to cancer.

We chose this gene in particular because there was no previous research on this gene, despite the availability of research on breast cancer, which proved the occurrence of downregulation in ING1 gene in cancer research, and triple-negative cancer is a violent type and is difficult to diagnose and does not respond to treatment with conventional therapies, so we chose to investigate this gene especially to find new therapeutic methods.

We investigated the possibility of linking DNA methylation to the ING gene in TNBC, as no research or study had previously linked this tumor suppressor gene to this type of cancer and using DNA methylation as an evident. The appearance of DNA methylation of ING1 promoter in rate of 93,5% in TNBC samples could be meaning as the expression of this tumor suppressor gene (ING1 gene) has decreased and epigenetic changes have occurred to this gene, causing methylation. Based on the results, we obtained from 92 DNA samples from women with (25-91) ages diagnosed with TNBC, we found that 86 samples contained high and partial DNA methylation. This is a high rate for methylation of a gene related with cancer prognosis.

It should also be taken into account that 18 samples did not show DNA methylation or DNA non-methylation, as this is due to several reasons that lead to the absence of a reading, including: contamination of the samples with protein or contamination with salt, or that the sample is not completely sufficient for the purpose of being read by the device.

The results positively confirmed the subject of our research in the close relationship between the occurrence of a defect in the ING 1 gene and carcinogenesis, which led to the addition of a methylation group to the DNA of breast cells (TNBC). Targeting DNA methylation as a potential therapeutic target in TNBC, which is difficult to treat with traditional and currently common methods, such as chemotherapy. DNA methylation can be linked to other types of breast cancer. Investigating for the epigenetics of the ING1 gene in other types of breast cancer. Investigating for the

epigenetics of the ING1 gene in other types of other organs'' types of cancer. Relationship DNA methylation with age, as it is possible to investigate the incidence of DNA methylation at early ages to predict cancers. Making and developing new and innovative therapies to eradicate TNBC. Since breast cancer is the second most common cancer, it affects women around the world of all races, and it may also lead to death. According to all the findings of the researchers that we mentioned above. The types of cancers mentioned, namely breast cancer, lung, prostate, pancreas, bladder, head and neck, stomach, skin and blood. All of these results agreed that DNA methylation causes various cancers and decreased gene expression of the ING1 gene causes cancer proliferation. Therefore, methylation and the ING1 gene can be challenged as a potential therapeutic target for TNBC, as we demonstrated in our research that there is a relationship between DNA methylation, the ING1 gene, and triple-negative breast cancer.

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