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EXPRESSION ASSOCIATION OF *APOBEC3B* GENE AND LNCRNA-*GAS5* IN BREAST CANCER PATIENTS

M.Sc. THESIS

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Supervisor:
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APPROVAL

We certify that we have read the thesis submitted by (Omed Qadir Ibrahim) titled **"Expression Association of *APOBEC3B* Gene and lncRNA-*GAS5* in breast cancer patients"** and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Educational Sciences.

Thesis defense was held online. The Jury members declared their acceptance verbally which is recorded.

Tez savunması online (çevrim içi) yapılmıştır. Jüri üyeleri onaylarını sözlü olarak vermişlerdir. Tüm süreç kaydedilmiştir.

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DECLARATION

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

….. /….. /2021

Omed Qadir Ibrahim

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Omed Qadir Ibrahim

ÖZET

Meme Kanserli Hastalarda *APOBEC3B* Geni ve lncRNA-*GAS5*'in Ekspresyon İlişkisi

Omed Qadır Ibrahım

Yüksek Lisans Tezi, Tıbbi Biyoloji ve Genetik Programı

Danışman: Doç. Dr. Rasime Kalkan

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Kasım, 2021

115 Sayfa

Amaç: Çalışmamızda lnc- lncRNA-*GAS5* ve *miR-103* ve *miR-103* ve *APOBEC3B* arasındaki ilişkiyi göstermeyi hedefledik.

Genel Bilgiler: Son yıllardaki önemli ilerlemelere rağmen, meme kanseri kadınlarda en sık teşhis edilen kanserdir. Hücresel süreç ve tümör oluşumunda epigenetik değişikliklerin rolü pek çok çalışma ile gösterilmiştir. *APOBEC3B*, endojen bir immün DNA sitozin deaminazıdır. Birden fazla ana biyolojik süreci kontrol ettiği gösterilen kodlamayan RNA'lar, son zamanlarda daha fazla araştırılmaktadır. Artan çalışmalar, LncRNA-*GAS5* ve *miR-103* gibi lncRNA'ların gen ekspresyon farklılıklarının kanser biyolojisinde önemli bir rol oynayabileceğini göstermektedir.

Materyal ve Metod: Mecvut çalışmada Lnc-*GAS5*'in meme tümörü progresyonundaki etkisinin incelesi hedeflenmiştir. Ayrıca lncRNA-*GAS5* ve *APOBEC3B* genleri arasındaki ilişki araştırılmıştır. Bu araştırmada meme kanseri alttiplerine ve hastaların klinik özelliklerine göre ayrılmış 49 tümör ve sağlıklı örnek üzerinde gen ekspresyon analizi ve *APOBEC3B* mutasyon analizi gerçekleştirilmiştir.

Bulgular ve Sonuç: *APOBEC3B* ve *miR-103*'ün ifadesinde istatistiksel olarak önemli bir artış saptanmıştır. Öte yandan, lncRNA-*GAS5* ekspresyonu önemli ölçüde azaldığı saptanmıştır. lncRNA-*GAS5* ifadesinin *APOBEC3B* ve *miR-103* ifadesi ile negatif ilişkili olduğu gösterilmiştir. *APOBEC3B* geni üzerinde herhangi bir mutasyon bulunmamıştır. Özetle, *APOBEC3B* ve *miR-103* ekspresyonunun meme kanseri hastalarında arttığı ve lncRNA-*GAS5*'in azaldığı gösterilmiştir. Meme tümörleri ve biyobelirteçler arasındaki ilişkiyi daha iyi anlamak için *miR-103*'ün epigenetik etkisinin *miR-103*'ü susturarak *APOBEC3B* gen ekspresyonu üzerindeki etkisini ortaya çıkarmak için ek çalışmalara ihtiyaç duyulmaktadır.

Anahtar kelimeler: *APOBEC3B*, Meme kanseri, lncRNA-*GAS5*, *miR-103*, Sekanslama

ABSTRACT

Expression Association of *APOBEC3B* Gene And lncRNA-*GAS5* in Breast Cancer Patients

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Aim: The association between lncRNA-*GAS5* and *miR-103* has been investigated recently. Besides, we used statistical methods to find the relationship between *miR-103* and *APOBEC3B*.

Background: Despite significant advances in recent decades, breast cancer is the most diagnosed cancer in women. On a clinical and molecular basis, the factors that influence breast cancer heterogeneity are complex. It is highlighted the crucial role of epigenetic changes in the cellular process and tumorigenesis. *APOBEC3B* is an endogenous immune DNA cytosine deaminase. Non-coding RNAs, which have been shown to control multiple main biological processes, have recently gained further recognition. Growing evidence suggests that aberrant expression of certain lncRNAs, such as LncRNA-*GAS5* and *miR-103*, can play a key role in cancer biology.

Material and method: In this study, we examine the involvement of lncRNA-*GAS5* in breast tumor progression for the first time, we intended to discover the relationship between lncRNA-*GAS5* and *APOBEC3B* genes and found that the present results implicated them. Gene expression analysis and *APOBEC3B* gene sequencing have been used to examine 49 paired tumor and healthy samples divided by breast cancer types and patients' clinical characteristics in this research.

Findings and results: There were a statistically significant increase in the expression of *APOBEC3B* and *miR-103*. On the other hand, the amount of lncRNA-*GAS5* expression was significantly reduced. lncRNA-*GAS5* expression was shown to be negatively associated with *APOBEC3B* and *miR-103* expression. There were no mutations found when the *APOBEC3B* gene was screened. In summary, *APOBEC3B* and *miR-103* expression were shown to be upregulated in breast cancer patients, along with down-regulated expression level of lncRNA-*GAS5*. To better understand the correlation between breast tumors and biomarkers, additional studies are required to reveal the implication of epigenetic influence of *miR-103* on *APOBEC3B* gene expression by silencing *miR-103*.

Key Words: *APOBEC3B*, Breast cancer, lncRNA-*GAS5*, *miR-103*, Sequencing

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LIST OF ABBREVIATION

APOBEC:	Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-like
RNA-*GAS5*:	Ribonucleic Acid Growth Arrest-Specific 5
LncRNA:	Long non-coding Ribonucleic acid
LncRNA-*GAS5*:	Long Non-Coding Ribonucleic Acid Growth Arrest-Specific 5
***miR-103*:**	micro-Ribonucleic acid 103
ER:	Estrogen Receptor
PR:	Progesterone Receptor
HER-2:	Human Epidermal Growth Factor-2
FISH:	Fluorescent in Situ Hybridization
TNBC:	Triple Negative Breast Cancer
***BRCA1*:**	Breast Cancer Susceptibility Gene 1
***BRCA2*:**	Breast Cancer Susceptibility Gene 2
DCIS:	Ductal Carcinoma in Situ
LCIS:	Lobular Carcinoma in Situ
UVL:	Ultra Violet Ray
EtBr:	Ethidium Bromide
cDNA:	Complementary Deoxyribonucleic Acid
qPCR:	Quantitative Polymerase Chain Reaction
TNM:	Tumor Nodes Metastasis
APOB:	Apolipoprotein B
AID:	Activation-Inducing Deaminase
RT-qPCR:	Quantitative Reverse Transcription Polymerase Chain Reaction
***APOBEC3B*:**	Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 3B
POBEC1:	Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 1C
***APOBEC3A*-D:**	Apolipoprotein B mRNA Editing Enzyme Catalytic subunit 3 A-D
***APOBEC3C*:**	Apolipoprotein B mRNA Editing Enzyme Catalytic subunit 3C
***APOBEC3F*:**	Apolipoprotein B mRNA Editing Enzyme Catalytic subunit 3F
***APOBEC3G*:**	Apolipoprotein B mRNA Editing Enzyme Catalytic subunit 3G
***APOBEC3H*:**	Apolipoprotein B mRNA Editing Enzyme Catalytic subunit 3H
ALH:	Atypical Lobular Hyperplasia
IDC:	Invasive Ductal Carcinoma
***GAPDH*:**	Glyceraldehyde 3-Phosphate Dehydrogenase

WES:	Whole Exon Sequencing
WGS:	Whole Genome Sequencing
TIMP-3:	Inhibitor of Metalloproteinases-3
HOTAIR:	HOX Transcript Antisense RNA
mRNP:	Messenger Ribonucleoprotein
SHM:	Somatic Hypermutation
snoRNAs:	Small nucleolar RNAs
CSR:	Class Switch Recombination
C to U:	Conversion of Cytosine to Uracile
CpG:	Cytosines Followed by Guanine Residues
ncRNA	Non-Coding Ribonucleic Acid
5'TOP5'	Terminal Oligopyrimidine Tract

CHAPTER I

1. Introduction

Even though extensive considering worldwide to detect and establish modern treatment, breast cancer remains a significant cause of death in women among other forms of cancer. It placed the second most prevalent cause of cancer-related mortality in females (Ahmad, 2019; De Leeneer & Claes, 2015). Additionally, one million women are diagnosed with breast cancer per year, with a global occurrence of one every three minutes, comprising about 1 in 4 cases of cancer in women. There have been 2.1 million new breast cancer cases in 2018, which were 626679 deaths globally, as confirmed by IARC (Balekouzou *et al.*, 2017; Bray *et al.*, 2018). The American Cancer Society claims that breast cancer's malignancy originates naturally from the breast tissue and can enter the healthy cells (Malih *et al.*, 2016).

Metastasis is a nuanced multistep mechanism that essentially allows malignant epithelial cells to establishment secondary tumors at different places (Mar-Aguilar *et al.*, 2013). There have been comparatively higher patient survival records, though malignant cells reside inside the ducts or lobules. However, in the event of cells entering the duct-lobular zone, the prognosis deteriorates markedly (Lorusso & Rüegg, 2012). The transport of cancer cells to other locations, such as the brain, lungs, skeletal system, and liver, will occur through the bloodstream or lymphatic vessels (Ullah, 2019). The tumor generated contributes to restrictions and local homeostasis disturbance (Al-Mahmood *et al.*, 2018). This mechanism is categorised into three major components: territorial invasion, intravasation, and extravasation (Sambi *et al.*, 2019). The generation of metastatic lesions directly responsible for ~90 % of mortality associated with breast cancer and shortens the life expectancy (Gooding *et al.*, 2017; Yousefi *et al.*, 2020). While certain women's elevated predisposition to experience the disease, malignancy is not present in all breast cancer patients (Harbeck *et al.*, 2019). Significantly, distal metastasis rates and locations may differ according to age and stage of diagnosis (McGuire *et al.*, 2015).

Better prognosis is also correlated with early-stage diagnosis, contributing to an intensified concentration on timely and enhanced screening techniques (Ahmad, 2019). The histological examination of breast cancer, is based on Immunohistochemistry (IHC) (Criscitiello *et al.*, 2012). The vital molecular targets are sex hormone receptors

and epidermal growth factor 2 (ERBB-2, previously HER-2 or HER-2/neu) (Waks & Winer, 2019). The modern approach for forecasting prognosis and diagnosis is that tumor gene expression profiling tends to be a successful breast cancer approach (Mourtada-Maarabouni *et al.*, 2009). The latest findings indicate that ncRNA breast tumor profiles have contributed to remarkable insights into the diagnosis and variety of tumor forms (Heneghan *et al.*, 2009). It is recently reported that LncRNAs are linked to distinct types of cancer, and involved in multiple biological processes, and may be used as a candidate cancer detection biomarker (Malih *et al.,* 2016). The functions of miRNAs as emerging diagnostics, prognostic markers, and possible therapeutic goals have been highlighted in various reports (De Leeneer & Claes, 2015; Sohel, 2020; K. Zhang *et al.*, 2017). Using plasma miRNAs as predictive biomarkers to look at someone of a breast cancer diagnosis should be further explored (Aggarwal *et al.*, 2020; Hamam *et al.*, 2017). It is appropriate to use separate expression profiles of lncRNAs and miRNAs to differentiate different subtypes and use them in different subtypes as biomarkers for prognosis and diagnosis (Yousefi *et al.,* 2020).

Based on the conventional microscopic histological analysis and molecular features, different subtypes of breast cancer are identified (Penault-Llorca & Viale, 2012). Although breast cancer may exist in any breast section, the ducts and lobules are typically located. Depending on the capacity of the cancer cell to expand to other ducts or lobules. It is referred to as non (in situ) or invasive carcinoma (infiltrating) (Leong & Zhuang, 2011).

Furthermore, in situ breast tumors have been subcategorised as lobular or ductal. In full systematic name can be lobular cancer in situ (LCIS) or ductal carcinoma in situ (DCIS), respectively (Nounou *et al.*, 2015). On the other hand, breast cancer is divided into four main tumor subtypes based on the presence or lack of molecular indicators for estrogen or progesterone receptors and human epidermal growth factor-2 (HER-2) (Waks & Winer, 2019; Yeo & Guan, 2017). Luminal A that ER/PR-positive, and HER2-negative. Luminal B that ER/PR-positive, and HER2-negative. HER2-enriched that ER/PR-negative, and HER2-positive. Triple-negative breast cancer is known as Basal-like that ER/PR negative and HER2 negative (Garrido-Castro *et al.*, 2019). TNBC is thus more destructive and has a lower prognosis than the other subtypes (Beetch *et al.*, 2020; Le *et al.*, 2019). The histological and molecular features of breast cancer significantly impact systemic treatment choices (Harbeck *et al.*, 2019).

Selecting the appropriate therapeutic modality is an essential issue for treatment (Hamam *et al.*, 2017). Appropriate therapy to maintain a high-level survival rate relies on the exact diagnosis of subtypes of breast cancer. Chemicals/agents can cure both *HER-2* gene amplification and TNBC or may be cured with chemicals/agents, or surgery, chemotherapy, hormone therapy, and radiotherapy (Al-Mahmood *et al.,* 2018; Waks & Winer, 2019). Today, focusing on miRNAs, lncRNAs represent a comparatively recent approach to current treatment. They are increasingly becoming critical clinical goals (Huang *et al.*, 2018).

The possibility of mammary cancer induction is increased by numerous genetic, epigenetic, and environmental factors (Kolak *et al.*, 2017). A risk factor increases the likelihood that breast cancer will grow, including non-modifiable factors such as gender, age and race. Also, modifiable factors including, overweight, tobacco, excessive drinking wine, reproduction, radiation exposure, breast cancer background, depression, and physical inactivity (Balekouzou *et al.*, 2017; Gu, Wang, Wang, Zhou, Wang*, et al.*, 2018). Jeongeun *et al.* recently revealed that air pollution could directly induce genetic alterations or impact breast cancer prevalence by raising breast density (Hwang *et al.*, 2020). It is revealed that there is a different type of mutation in genes that related to breast cancer. The genes are categorised into two leading groups according to influences to rise cancer and frequency. High penetrance genes include *TP53*, *PTEN*, *BRCA1*, and *BRCA2*. Low penetrance genes include cytochrome *P450* genes and genes encoding cell signalling molecules estrogen/progesterone receptors. However, proteins that have been a concern as a growth factor, including HER-2/new antigen, are over-expressed with various cancers. In 20 to 30% of invasive breast cancer, the *HER 2/neu* proto-oncogene was amplified (Harbeck *et al.,* 2019; Nounou *et al.,* 2015; Shu *et al.*, 2020). Protein/pathway modifications, such as the estrogen receptor factor pathway and the growth receptor pathway, are strongly associated with breast cancer (Behl *et al.*, 2020; Jagsi, 2018). Besides, recent findings have indicated that copy number variants (CNVs) and single nucleotide polymorphisms (SNPs) could be at risk of breast cancer in specific particular genes (Kumaran *et al.*, 2018). Epigenetic modifications, such as DNA methylation, histone modifications, microRNAs, and long-noncoding RNAs, are generally recognised as contributors to breast cancer (Thakur *et al.*, 2018). Consequently, non-coding RNAs include; Long non-coding RNAs (LncRNAs) and microRNA (miRNA) associated

with many biological processes. A particular form of cancer, like breast cancer, is linked with the dysregulation of ncRNAs depending on their functions (Hajjari & Salavaty, 2015).

Long non-coding RNAs (lncRNAs), which have recently been found in different forms of cancer, are a significant and most extensive class of ncRNAs in many biological processes (Malih *et al.,* 2016). Mounting research suggests that in common cancer forms, including breast cancer, the aberrant expression of specific lncRNAs has emerged (Li *et al.*, 2015). Tumorigenesis coincides with the aberrant expression of lncRNAs because LncRNAs could behave as a tumor suppressor or oncogenic lncRNAs (Cheetham *et al.*, 2013; Yousefi *et al.*, 2020). Increased LncRNA growth arrest-specific transcript 5 (*GAS5*) expression, which occurs on chromosome 1q25, was significantly decreased in HER2-positive breast cancer (Gu, Wang, Wang, Zhou, Shao*, et al.*, 2018). Also, in previous research, the ability of lncRNA-*GAS5* to act as a sponge miR-23a was demonstrated. Furthermore, miR-23a expression in patients with breast cancer has been considerably higher (Gu, Wang, Wang, Zhou, Wang, et al., 2018). Several research and analyses have shown that lncRNA-*GAS5* is abnormally expressed in various cancers, including bladder cancer, breast cancer, gastric cancer, and hepatocellular carcinoma. However, in breast cancer, lnc-*GAS5*'s expression and mode of action were poorly known (Ji *et al.*, 2019).

A small class of endogenous, single-stranded non-coding RNA includes microRNAs (miRNAs) (Bahrami *et al.*, 2018). Importantly, again or lack of function of specific miRNAs theoretically capable of functioning as genes that inhibit tumors or oncogene (De Leeneer & Claes, 2015). Numerous miRNAs have recently been found to be essential regulators of gene expression (Suksangrat *et al.*, 2019). Dysregulated miRNAs promote tumorigenesis by preventing the expression of target genes. One of the significant findings is that *miR-103* stimulates the development of breast cancer. It is confirmed that lncRNA-*GAS5* as an inhibitor of *miR-103* expression (Guo *et al.*, 2015; Mar-Aguilar *et al.*, 2013).

The *APOBEC3B* gene belongs to the Cytidine Deaminase Genes group (Burns *et al.*, 2015). It is considered part of a cluster of seven gen or pseudogenes found on chromosome 22, thought to be the product of a previously duplicated chromosome. This protein has a structural and functional relationship to the C to U RNA-endonucleases in clusters. It is rumoured that the proteins could be responsible for

RNA editing and cell cycle controls (Y. Zhang *et al.*, 2015). Initially, it was stated that the APOBEC3 gene family played a crucial role in the enzymatic combination of virus restrictions to prevent the replication of distinct viruses (Verhalen *et al.*, 2016). On the other side, it seems to be closely linked to the expression of the *APOBEC* family of genes; the rising number of breast cancer cases has often appeared to be related to genetic mutation (Gooding *et al.,* 2017). A variety of recent studies have converged on a model of signal transduction pathways responsible for the upregulation of *APOBEC3B* (Brandon Leonard *et al.*, 2015).

In this thesis, lncRNA-*GAS5* expression and *APOBEC3B* mutations were investigated in 49 tumor samples (49 tumor, 49 control sample) using RT-qPCR. In addition, *APOBEC3B* gene's mutation was examined using Seqstudio genetic analyser (Sanger sequencing) (Applied Biosystem, USA). To analyse *APOBEC3B* and lncRNA-*GAS5* profiling's relative expression, we need to evaluate the *miR-103* expression quantification in breast cancer patients. We observe the relationship between *miR-103* and *APOBEC3B* in a computerised way. The first time in the literature, potential associations of the lncRNA-*GAS5*, *miR-103*, and *APOBEC3B* gene in breast cancer will be seen based on the presented data.

CHAPTER II

2. Literature Review

2.1. Cancer

Around the globe, cancer is a significant public health concern (Coleman & Tsongalis, 2017). Cancer remains an essential factor for morbidity and mortality in the country, following much improvement in detecting and preventing illness (Karpiński & Adamczak, 2018). In the year 2012, there were more than 14.1 million new cancer cases reported. The figures in 2018 show 18.1 million new cancer cases were recorded worldwide, with 9.6 million cancer deaths. By 2025, it is projected that 20 million recent cases would occur worldwide in less than a decade (Bens *et al.*, 2020; Ullah, 2019).

Cancer is a complicated disorder that requires multiple spatiotemporal shifts in cell physiology, contributing eventually to malignant tumors (Seyfried & Shelton, 2010). Tumor development also describes the process by which a normal cell transforms into life-threatening metastatic cancer (Welch & Hurst, 2017). Carcinogenesis is a complex mechanism where normal cells use standard regulatory and aberrant signals to increase spontaneously and unplanned, allowing them to progress through cancerous states (Irani, 2019).

Cancer is a hereditary disorder induced by the accumulation of somatic alterations and/or impacts of epigenetic changes on essential genes that regulate mechanisms such as cell replication and death (Pollack, 2017). The majority of cancers arise sporadically. For example, it is stated that over 90% of breast cancer cases are spontaneous; however, as with other forms of cancer, just 10% to 5% of these cases are passed on by inheritance (Tempfer & Reymond, 2017).

Tobacco, diet and obesity, diseases, radiation, lack of physical exercise, and environmental toxins are common ecological factors contributing to mutations (Wu *et al.*, 2018). Cancer is a dynamic evolutionary mechanism in which cancers are a phenotypically and genetically heterogeneous cell community that competes for finite resources (Levy-Lahad & Friedman, 2007).

Cancer was named according to the originated cell or tissue type (Seyfried & Huysentruyt, 2013). Carcinoma that develops in the skin or epithelial cells (internal or external organs) and tissues, such as the kidneys, lungs, and breasts. The cancers that emerge in the cells of the immune system are lymphoma and myeloma. Leukemia is a disease that starts in the bone marrow's blood-forming progenitor cells. It seems to evolve cancer in the central nervous, precisely in the spinal cord and brain (Almeida & Barry, 2011).

2.1.1. Hallmarks of cancer

Many essential breakthroughs regarding cancer development and cell behaviour patterns were revealed in Hanahan and Wein's previous work. Six notable physiological alterations and possible roles for tumor cell mutations have been offered (Seyfried & Shelton, 2010). The hallmarks are an organisational theory that rationalises the complications of neoplastic disorder (Hanahan & Weinberg, 2011).

These distinct and related hallmark capabilities include the maintenance of proliferative signalling, the avoidance of growth suppressors, regeneration, immortality, invasion and metastasis initiation, angiogenesis induction, and cell death tolerance (Gutschner & Diederichs, 2012). These characteristics can be developed through genetic instability, facilitating their development and increasing inflammation. Another extension, this theory applied to the concept that belief mutations do not have to be obtained in a particular order (Negrini *et al.*, 2010). Cancer in recent years has been characterised by a wide range of hallmarks. These hallmarks are: DNA damage and stress capabilities in the levels of DNA replication, mitotic cell division, metabolic, proteotoxic, and oxidation. Also, the ability to evading immune surveillance has been added (Kroemer & Pouyssegur, 2008). Tumors have evolved to the point that they have a repertoire of recruited, potentially normal cells that create the tumor's microenvironment, resulting in hallmark characteristics. Recognising broad acceptance of these concepts (Figure 2.1) would have a growing effect on the growth of alternative ways of managing human cancer (Hanahan & Coussens, 2012).

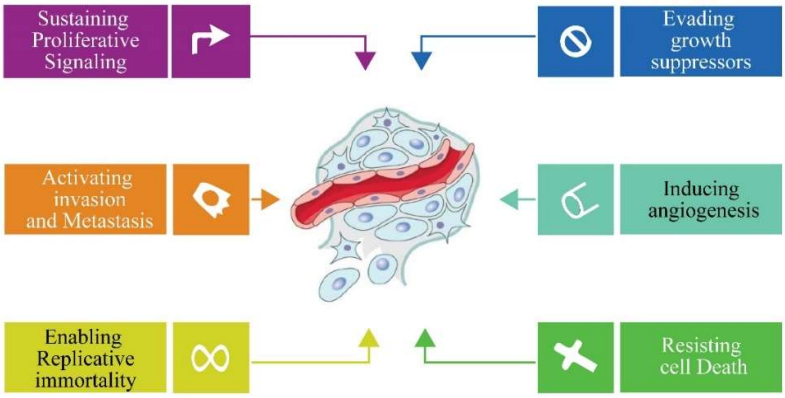

Figure 2.1. The illustrations provided combine the six essential characteristics of cancer (Hanahan & Weinberg, 2011).

2.1.2. Genetic Causes of Cancer

Mutations, epigenetic alterations, chromosomal alterations are the major genetic reason for cancer (Béroud, 2017). For several reasons, random mutations arise unexpectedly over a cell's lifespan: a result of an error created when a cell copies the DNA before dividing, an incomplete repair of a defective DNA molecule, or a chemical alteration of the DNA, any of which interferes with the genetic knowledge being expressed (Almeida & Barry, 2011; Tempfer & Reymond, 2017).

2.1.2.1. Cancer genes and their mutations

Many cancers emerge based on genetic alterations (mutations) to cellular genes typically classified into oncogenes and tumor suppressor genes (Sever & Brugge, 2015). Oncogene and tumor suppressor genes are critical regulatory genes that express proteins that regulate changes in and out of the cell cycle and play a role in the pathway to terminal differentiation (Weinberg, 2014).

Oncogenes encode proteins whose presence typically results in a proliferative phenotype and apoptosis inhibitions. Some oncogenes encode transcription factors active in pro-growth signalling processes or factors that increase cell survival. The RAS family is one of the more conventional oncogene groups (*HRAS, KRAS*, and *NRAS*). RAS family proteins, such as epidermal growth factor receptor (EGFR) signalling pathways, function downstream of growth factor signalling pathways (Cassidy *et al.*, 2015). Oncogenes encode proteins with the capacity to induce cellular transformation via either overexpression or causing mutations, these genes function

dominantly (Rivenbark, 2017). Many parameters characterise cellular transformation. These involve changes in morphology, lack of communication inhibition, development outside of anchorage, and the capacity to form tumors, including accelerated cell growth and proliferation (Hahn, 2014).

The realisation that oncogenes are altered or overexpressed forms of regular cellular genes named proto-oncogenes was an important finding in cancer. Oncogene mutations are almost missense, influencing only one allele, rendering them heterozygous. Their mutations may trigger either missing (inactivated) or enhanced (activated) gene activity (Vogelstein & Kinzler, 2004). Tumor suppressor genes are usually altered in the gene; several mutations will invert the encoding protein and typically impact all alleles, triggering loss of heterozygosity (LOH). Chromosomal rearrangements, minor insertions and deletions (indels), nucleotide substitutions, and copy number variations are necessary forms of mutations found in oncogenes (Jagsi, 2018).

Tumor suppressor genes functionally induce cell death, inhibit unplanned cell proliferation, or trigger persistent cell cycle arrest (Vogelstein & Kinzler, 2004). Thus, the genes that suppress tumors will act as negative oncogene regulators, as anticipated. In addition, they are responsible for organizing cells' control points, which guarantee the precise division into cells under normal or stress-induced circumstances (Hoeijmakers, 2009).

Tumor suppressor genes do not appear to play an essential role in tumor growth until any or more tumor suppressor genes have been inactivated, which typically allows each parental allele to be dysfunctional in a single cell. This heritable manner demonstrates that these genes' mutated alleles will be transmitted through the germline and enable human beings to inherit cancer predisposition types. Among the inactivating tumor suppressor mechanisms are nonsense and missense mutations, methylation-mediated gene silencing, and deletions (Hahn, 2014).

In general, tumor suppressor genes fall into three groups. The first set includes those genes which by inhibiting essential signalling pathways drive apoptosis or block proliferation. The second set contains the genes needed to repair DNA damage, the lack of which contributes to a phenotype of the 'mutator.' As negative regulators of major growth factor signalling pathways, tumor suppressors, such as *APC* and *PTEN*,

perform crucial roles. The cell proliferation process that occurs without the growth factor pathway's influence was significantly increased when these genes were eliminated; thus, activation of the growth factor-independent pathway (Brown, 2014). While *TP53* is the most frequently mutated tumor suppressor gene, the exact role of the p53 protein necessary to suppress tumors remains uncertain. Following increased genomic instability, DNA destruction, distorted homeostasis, impaired DNA repair, metabolism alterations, a transcription factor, loss of p53 leads to an inability to induce apoptosis and growth arrest, both of which could be necessary tumor growth (Cassidy *et al.*, 2015).

In the third class of carcinogenic genes, numbers of directly activated cell processes that maintain basal genomic or chromosomal stability. The ability to correctly deal with genomic damage (typically from single-strand DNA breaks or double-strand DNA breaks) is contingent on detection of such damage, and the ability to mobilising distinct repair enzyme complexes (Hoeijmakers, 2009).

Ultimately, in some tumors, the causes are infectious agents. Viruses cause a few human cancers. Virus encoding genes facilitate tumorigenesis by oncogene pathway activation or tumor suppressor inactivation. For example, human papillomavirus linked with cervical and head and neck cancers encode E6, promoting p53 degradation (Sever & Brugge, 2015).

2.1.2.2. Epigenetic alterations

Reversible, heritable variations in gene expression that arise without a mutation are pointed to in epigenetics. These changes include DNA methylation, histone modifications, and gene silencing induced by miRNA, all of which influence genes' expression. The central portion of the genome is not expressed in normal, differentiated cells. Cancer cells, on the other side, are distinguished by global hypomethylation of DNA (Kumar, 2018). Epigenetics plays a key role in a different physiological process, including growth, imprinting, X-chromosome inactivation, chromosomal stability, and gene transcription regulation. Gene expression changing by altering DNA methylation or adjusting chromatin structure has currently been linked with cancer. On the other hand, silencing gen by ncRNAs as a different epigenetic form has been confirmed in numerous studies (Martín-Subero & Esteller, 2017).

This mechanism relies on the methylation of cytidine residues present in CpG dinucleotide sequences in the vicinity of different gene promoters or by changing histones in chromatin (Moore & Chang, 2010). CpG dinucleotides are broadly present in the genome and gene promoter domains. It may be that silencing occurs by inhibiting the transcription factor's binding to the promoter regions through methylation. Hypomethylation around the genome and promoter-specific hypermethylation are two critical components of cancer. However, the first epigenetic modification found in cancers was the depletion of DNA methylation on CpG islands (Feinberg & Tycko, 2004). In addition to DNA methylation, alteration in histone acetylation plays another essential impact in the modulation of gene expression. Inactive histone deacetylation-mediated chromatin is a critical factor in silencing tumor suppressor genes (Ropero & Esteller, 2007).

Another influential characteristic of epigenetic change is the covalent modification of histones, which shapes the nucleosome's nucleus. The most well-known modifications are acetylation and methylation of lysine (K) residues at the N termini of histones H3 and H4. A combinational change of K residues of H3 and H4 with acetyl or methyl groups may serve as a "histone code dictation of chromatin condensation, which then becomes a mechanism for controlling transcription. Though global histone acetylation levels have not yet been established, many studies support the findings that deviations in histone patterns encourage tumorigenesis (Baylin, 2014).

Presently, in many pathological and physiological situations, non-coding RNAs have been determined to play a significant function. In the modulation of gene expression, the study of non-coding RNAs has revealed surprising complications. Via modulating classic oncogenic pathways, these modern regulating factors may play critical roles in cancer onset and development (Ji *et al.*, 2019). Given their wide variety of roles, it's not surprising that abnormal miRNA expression contributes to tumorigenesis. By profiling miRNA expression, it was concluded that cancer cells have lower miRNA levels than normal cells. The deregulation of individual miRNAs can cause cancerogenesis by disrupting traditional oncogenes and tumor suppressor gene control (Calin & Croce, 2006; Kumar, 2018).

2.2. Breast Cancer

The incidence of breast cancer is one of the more frequent human tumors and develops in mammary gland cells with various morphological characteristics. Several genetic modifications are implicated in the production of breast tumors (Lakhani *et al.*, 2012). Around 95% of malignant breasts emerge from the epithelium of the mammary gland. It takes decades from the start and end of tumor growth, depending on some epidemiological data (Kumar, 2018). The majority of the process has progressed from the slow-growing benign and solitary tumor to an invasive carcinoma that spreads to the various tissues through the blood and lymph vessels, metastases from the primary tumor forms (Makki, 2015).

2.2.1. Epidemiology and Etiology of Breast Cancer

Breast cancer is the second most often diagnosed type of cancer in females (Kolak *et al.*, 2017). Breast carcinoma constantly disrupts millions of women's lives. It was reported that in 2008 that about 1.3 million women were diagnosed with breast cancer worldwide (Fares *et al.*, 2019). In 2018, an estimated 2.1 million patients were freshly diagnosed with breast cancer, with one new woman diagnosed every 18 seconds. As a result, more than 20 million new cancer cases are expected globally by 2025 in less than a decade (Bray *et al.*, 2018). According to World Health Organisation (WHO) figures, breast cancer is the first or second leading cause of death before age 70 by 91 out of 172 states in 2019 (Coleman & Tsongalis, 2001). The scale of the prevalence of female breast cancer varies.

In the United States of America, women have the most often diagnosed breast cancer is the second-leading source of cancer mortality in women. An expected 266,120 people will be diagnosed in 2018, with an estimated 40,920 fatalities, with Western Europe becoming the largest (89.7 per 100,000 women) and Eastern Africa is the lowest (19.3 per 100,000 women) (Fares *et al.*, 2019; Ullah, 2019). With 51 cases per 100,000 people per year, Spain holds an intermediate place between Western and Eastern European countries, but breast cancer prevalence rises by around 2-3 percent per year (Baeyens-Fernández *et al.*, 2018).

In a woman of any age, breast cancer can be detected. While most malignancy cases begin in older women, there is a considerable risk of acquiring a tumor in younger females who have gotten mammary (Coughlin, 2019). The likelihood of

developing breast cancer in women is enhanced by several factors, including genetics, family background, age, hormonal and reproductive factors, alcohol, obesity, diet, physical inactivity (Balekouzou *et al*., 2017). Heritable influences are involved in breast cancer and responsible for about 5-10% in all cases. *BRCA1* and *BRCA2* are the two central genes most strongly related to breast and ovarian cancer (Nounou *et al.,* 2015). Associations with breast cancer, lifestyle aspects such as food, fat consumption, smoking, alcohol consumption, and physical activity levels were examined (Yedjou *et al.*, 2019). Environmental factors are substantially correlated with reproductive factors that influence women's sensitivity to circulating estrogen, who use hormone replacement therapy for five years, or more have a reasonable possibility of breast cancer. Other environmental conditions, including sensitivity to chemical carcinogens, radiation, and smoking, are complex risk factors attributed to more than 70% of breast cancers (Hamam *et al.,* 2017).

Besides, improved knowledge, better diagnostic services, and more successful care significantly improve breast cancer prevalence and survival risks. On the other hand, it is still the most often diagnosed type of all-cause cancer in both developing and developed regions, seventh among all forms of cancer in the world (Fares *et al.*, 2019).

2.2.2. Histopathology

Breast cancer has wide-ranging properties that include distinct anatomical and physiological components (Criscitiello *et al.*, 2012). Accumulated mutations in several cellular regulatory mechanisms are reported to induce excessive cell proliferation (Gong *et al.*, 2015). A tumor consists of a mass of irregularly structured cells. There may be benign (non-cancerous) or malignant (cancerous) properties of a tumor. The benign (or non-aggressive) tumors don't enter the other tissue and stay where they are raised. In comparison to benign tumors, malignant tumors can enter the adjacent healthy tissues (invasion) and travel through the lymphatic or circulatory processes to the other areas of the body (metastasis) (Almeida & Barry, 2011).

In breast cancer, the breast cells became the source of malignant tumors. Also, breast tumors are malignancies of mammary gland epithelial cells termed "carcinomas." Approximately carcinomas make up 90% of all human tumors (Guerini-Rocco & Fusco, 2017). Breast carcinomas are categorised according to the location where they develop in the breast into two classes. They are called "ductal carcinoma" if a tumor arises in the ductal tissue, and if it exists in the lobules, they are considered "lobular carcinoma." (Figure 2.2) (Guerini-Rocco & Fusco, 2017).

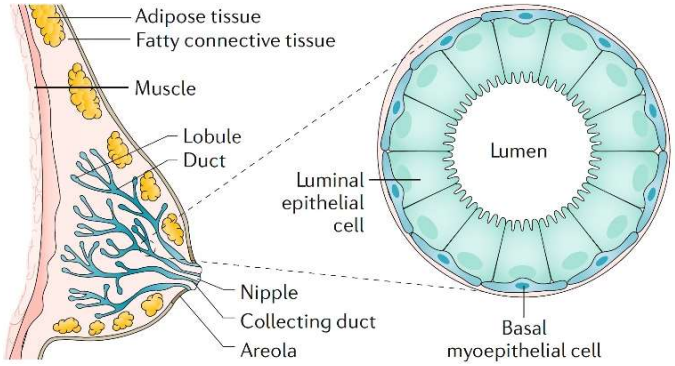

Figure 2.2. The ducts and lobules of the breast are seen in a cross-section of the breast and papilla. The terminal duct lobular units of the collecting duct are where all breast cancers originate (Harbeck et al., 2019).

2.2.3. Classification

2.2.3.1. Histological classification

Several research lines indicate that breast cancer is a heterogeneous disorder and more importance occurs within distinct subtypes. Histopathology is an appropriate diagnosis for determining breast cancer subtypes and then further examining tumor aggressiveness through tumor grade measurement (Sarkar & Mandal, 2011).

Based on their invasive properties, carcinomas are often categorised as "invasive carcinoma" if they penetrate the basement membrane. They are called "in situ carcinoma" if they are not (Ullah, 2019). Conclude, breast cancer has a broad spectrum of morphological characteristics, distinct immunohistochemical profiles, and histopathological subtypes that allow unique clinical courses and findings to be shown. Table 2.1 presents the histopathological classification of invasive breast cancer based on the guidelines made by Foote and Steward (Coleman & Tsongalis, 2001).

Table 2.1. Classification of invasive breast cancer.

No.	Types of breast disease	Incidence (%)
I	Paget's disease	
II	Invasive ductal carcinoma	
	A. Adenocarcinoma	80
	B. Medullary carcinoma	4
	C. Mucinous carcinoma	2
	D. Papillary carcinoma	2
	E. Tubular carcinoma	2
III	Invasive lobular carcinoma	10
IV	Rare cancers (adenoid cystic, squamous cell, apocrine)	

The breast cancer classification is crucial to obtain knowledge about the disease's clinical course and its effect and select the best treatment plan. In addition to the methods mentioned above to classification, breast cancer may be divided into sub-categories dependent on multiple parameters to assess the disease's level. For example, utilising criteria such as tumor size, presence of the lymph node, and metastasis, the standard method decides the categories. This classification scheme is called the classification of TNM (tumor, node, metastasis) and was first proposed by Pierre Denoix (Cadiz *et al.*, 2018). The TNM method has been generally recognised worldwide and has driven the treatment strategy, allowing the treatment and disease prognosis to follow (Almeida & Barry, 2011). While the TNM classification and the immunohistochemistry-determined hormone receptor expression status (IHC) offer certain advantages, breast cancer subtypes' fundamental biology and clinical actions remain mysterious (Cho, 2016).

2.2.3.2. Molecular classifications

Conventional classification methods can have some restrictions. Besides, tumor histology will not adequately assess the fundamental dynamic genetic changes and the biochemical events involved in cancer production and growth (Yersal & Barutca, 2014). Researchers also provided information on the value of gene expression based on global gene expression profiling (GEP) to classify breast cancer into distinct survival subgroups according to the patients (Eroles *et al.*, 2012; Rakha & Ellis, 2011). New molecular science offers a massive opportunity in cancer biology; also, it allows basic and translational researchers to look toward new goals. The analysis of breast cancer by new molecular approaches often promises to provide more accurate repeat assessment studies. Due to gene activity patterns being discovered, a protein production therapy that specifically influences cancer cells' growth and development has become essential (Wirapati *et al.*, 2008).

Molecular breast cancer detection causes further sub-classification of the main subtypes, such as IDC, to be discovered into subtypes with varying outcomes. Previously, the first biomarker discovered and studied more than 43 years ago was the estrogen receptor alpha (ER). The ER receptor-positive tumors may be further subdivided by estrogen-inducible gene levels with progesterone receptor (PR) loss, suggesting a lack of ER function and poor outcome (Sarkar & Mandal, 2011). Breast cancer can be immunohistochemically classified as positive and negative estrogen receptor (ER) tumors. However, fluorescent in situ hybridisation (FISH) is determined by detecting amplification of HER-2 gene (human epidermal growth factor (HER-2) receptor) on tumor samples (Yersal & Barutca, 2014). Around 20% of Invasive Ductal Carcinoma has HER2 degrees of amplification and over-expression. The triple-negative breast cancer (TNBC) concept has been provided as tumors without ER, PR, or HER2 expression with a worse prognosis. Thus, there are three subgroups in breast cancer: positive for ER/PR, negative for ER or positive for HER-2 receptor, and negative for every three receptors of the breast cancer cell (ER, PR, HER-2) as having been known triple-negative (Fragomeni *et al.*, 2018).

However, a new expression-based classification system for breast cancer is valuable, mainly because it aims to enhance the current classification standards for differentiating different outcomes. These attempts culminated in comprehensive DNA profiling, microRNA and eventually enabled subtypes of breast cancer to be formed (Network, 2012).

In 2000, researchers demonstrated that breast carcinomas' heterogeneity was attributed to differential gene expression patterns of breast cancer tumors. For breast cancer, a molecular proposed approach has been suggested utilising the differences in gene expression levels (Yersal & Barutca, 2014). It is assigned that breast cancer can be classified into subgroups based on their framework, including "Luminal," "HER2-enriched", "Basal-like," and 'Normal breast-like" subtypes (Table 2.2). Each subtype exhibits disparities in its prevalence rate, prognostic properties, treatment reaction, preferential organ metastasis, recurrence outcomes or disease-free survival (Sørlie *et al.*, 2001). Since 2011, the St. Gallen International Advisory Consensus panel for systematic breast cancer therapies has utilised this subtype-based classification system (Perou *et al.*, 2000). A robust, complete genetic study of breast cancer, however, requires a high budget and comprehensive tools. Therefore, surrogate subtype descriptions focused on semiquantitative IHC scoring of ER, PR, and in situ hybridisation studies have been suggested for *HER2* overexpression (Goldhirsch *et al.*, 2013). This system is regarded as "Molecular classification" and offers the foundation for modern approaches to be used in prognostic and predictive tests (Fragomeni et al., 2018).

Table 2.2. Surrogate definitions of the intrinsic subtypes of breast cancer.

	IHC status			
Intrinsic subtype	ER	PR	HER2	Ki-67 (%)
Luminal A	+	+	-	<15
	+	-	-	<15
	-	+	-	<15
Luminal B	+	+	-	≥15
	+	-	-	≥15
	-	+	-	≥15
Luminal B-like HER2+	+	+	+	≥15
	+	-	+	≥15
	-	+	+	≥15
TNBC (triple-negative breast cancer)	-	-	-	any
HER2-enriched	-	-	Overexpressed	any

(HER2-negative: score 1, score 2; HER2-positive: score 3).

2.2.4. Screening, Diagnosis, and Treatment Methods

Breast cancer is the second most common cause of death from cancer in women, but early diagnosis and care will enhance outcomes considerably (McKinney *et al.*, 2020). In people without any signs or symptoms of breast cancer, breast screening is performed so that the condition can be diagnosed as early as possible. Breast screening evaluation components focus on patient age and other considerations, such as personal and family background. They can involve breast awareness, clinical inspection, risk assessment, mammography, and MRI screening in selected situations (Bevers *et al.*, 2006).

Although detected in the early stages of cancer, breast cancer is a condition that has a high probability of recovery. According to the studies, 5-year survival for the whole patients diagnosed with the disease stage 0 condition was 90% not influenced by the disease (Woodward *et al.*, 2003). This average is 75% in stage II patients but only 13% in stage IV patients. Therefore, for the treatment of the disease, early diagnosis of breast cancer is hugely significant (Berg *et al.*, 2008; Elmore *et al.*, 2005).

Mammography tends to be the most successful approach for the diagnosis of breast cancer. Small lumps found in particular breast tissue might not be cancerous. Therefore, experimental analyses on these lumps are conducted to show the lumps' attributes and inform whether benign or malignant tumors. Statistics suggest that malignant tumors that cause breast cancer are one-quarter of breast tumors (Ferlay *et al.*, 2019; Harbeck et al., 2019). The phases of malignant tumors may be defined by evaluating their clinicopathological characteristics dependent on tumor size (T1-4), lymph node interaction (N1-3), and remote metastases (M0-1) (Brierley *et al.*, 2017).

Diagnostic reliability has a favourable association with treatment outcomes; therefore, the correct diagnosis is currently utilised for many interventions. For breast cancer detection, a breast biopsy is the most definitive type. To enhance diagnostic accuracy and remove false negatives as possible, a medical breast evaluation, breast scanning, and biopsy were performed concurrently (Nounou *et al.*, 2015). In comparison to the histological analysis of breast cancer, FISH is the most reliable and predictive technique that is concerned with HER-2 receptors as a diagnostic molecular goal(Criscitiello *et al.*, 2012; Waks & Winer, 2019). The new approach to diagnosis is that profiling of tumor gene expression appears to be an excellent approach to breast

cancer (Mourtada-Maarabouni *et al.*, 2009). The latest results suggest that ncRNA's breast tumor profiles have led to remarkable insights into the detection and diversity of tumor types (Heneghan *et al.*, 2009). Significantly, miRNAs are present in cancer metastasis, and miRNA signatures are being investigated as current therapeutic diagnostic objectives (McGuire *et al*., 2015).

Several treatment methods, such as surgery, radiation care (adjuvant or neoadjuvant), endocrine therapy, and chemotherapy, have been used to manage breast cancer. Chemotherapy has been the most commonly adopted route to breast cancer care for several years, but with virtually no accuracy, it was non-targeted (Ullah, 2019). The HER-2 receptor expression and the hormone receptor condition (ER, PR) of tumor tissue, specifically tailored therapy methods, have developed over the past twenty years. Mammary tumors that are positive for hormone-receptors such as ER and PR consist of 60 percent of all breast cancer cases being hormone receptor-positive (Tan *et al.*, 2008; Yanovich *et al.*, 2018). ER/PR+ tumors are called "Luminal" and are more convenient for their prognosis. Treatment this subtype, medications such as tamoxifen or raloxifene that target the endocrine system are recommended. In nearly 20 percent of all breast cancer instances, HER-2/neu is over-expressed. Trastuzumab (Herceptin), an inhibitory monoclonal antibody attacking HER2 receptors, reacts to HER2+ tumors (Goldhirsch *et al.*, 2013; Network, 2012).

One year of adjuvant trastuzumab was a standard treatment for women with HER2-positive breast cancer (Ahmed *et al.*, 2015). Bevacizumab, a monoclonal antibody to vascular endothelial growth factor A, has shown clinical effectiveness in patients with human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer(von Minckwitz *et al.*, 2012). Nearly 20 percent of breast cancer cases lack *ER/PR/HER-2/neu* expressions. These tumors are subtyped as triple-negative tumors (TN) (Foulkes *et al.*, 2010; Tan & Dent, 2018). Since TN tumors suffer from the lack of established drug targets, their prognosis is low, and chemotherapy is sometimes used to treat them (Tan *et al.*, 2008) (Yanovich *et al.*, 2018).

When genomic array studies were adequately considered, the subtyping of breast cancer was more detailed. Several distinct molecular subtypes of breast cancer were defined based on the study of array results. "Luminal A," "luminal B," "Basal," "Natural breast-like," and "HER-2-enriched" are certain sub kinds(Perou *et al.*, 2000; Sotiriou & Pusztai, 2009). There are still breast cancer subtypes, considering the

thorough study of molecular subtypes, that do not fulfil the existing subtyping requirements. Since breast cancer is a highly heterogeneous disease , Breast cancer care's future should be focused on designing more comprehensive treatment methods that will allow morbidity and mortality rates to decrease (Anjum *et al.*, 2017). The detection of novel target molecules is required for the production of novel drugs with reduced side effects. With Omics technology availability, the identification of novel tumor markers and drug target molecules is now accessible.

2.2.5. Genetics of breast cancer

Breast cancer is regarded as being exceptionally nuanced. The family background and aggregation of genetic aberration are the two crucial causes for raising the occurrence of breast cancer (Aydiner *et al.*, 2015). Molecular technologies developed especially next-generation DNA sequencing, significantly enhance our understanding of the genomic aberrations underlying malignant transformation. These modifications include coding mutations, epigenetic alterations, changes in the promoter and enhancer sequence, DNA insertions and deletions, variations in the copy number, and chromosomal translocations (Harris & McCormick, 2010). This disorder can be explained by susceptibility genes, which play an essential role in breast cancer growth. It can be divided into two forms: inherited and sporadic. Germline mutations related to some hereditary breast cancer syndromes are responsible for 10% of all breast cancers (Turnbull & Rahman, 2008).

The fundamental inherited genetic aberrations of breast cancer include variations in *BRCA1* and *BRCA2*. Nearly half of hereditary breast cancers are caused by these genes. The *BRCA1* and *BRCA2* genes encode substantial proteins with various functions. The *BRCA1* gene is located on the 17th chromosome and represents a zinc finger protein code with 1863 amino acids. During DNA replication and DNA repair, the *BRCA1* and *BRCA2* proteins repair double-stranded breaks to ensure the DNA molecule's proper rewinding, and importantly they serve as tumor suppressors. *BRCA2* is found in the 13th chromosome, and it is mutations the second predisposing factor in breast cancer. Any of these mutations will increase the occurrence of breast cancer to 25 percent (Harbeck *et al.*, 2019; Veronesi *et al.*, 2017). Tumor development was observed in patients with *BRCA1* or BRCA positive breast cancer versus non-BRCA tumors (Robson *et al.*, 2017).

Consequently, during replication, extra mutations accumulate, and carcinogenesis is encouraged. The *TP53* tumor suppressor gene mutation is another necessary mutation in breast cancer. *TP53* modification is also the primary symptom of Li-Fraumeni syndrome (LFS). Few cancers, like breast cancer, are more likely to occur in patients with LFS. Since the genes *TP53* and *HER2* are on the same chromosome (17th chromosome), the *TP53* mutation increases the risk of subtype HER2+ breast cancer. The loss of p53 functionality will influence HER2-related signal transduction pathways with sustainable activity (Melhem-Bertrandt *et al.*, 2012; Schon & Tischkowitz, 2018).

According to the probability of mutation and mutation frequency, hereditary mutations may be divided into three groups. The first group includes the *BRCA1/2*, *PTEN*, and *TP53* mutations, classified as high-penetrance, low-frequency predisposition genes. The second group, moderate-penetrant, low-frequency predisposition genes, consists of the *CHEK2, ATM,* and *PALB2* genes. Additionally, some mutations affect the genes *FGFR2, MAPK1K, TGF*; they have been identified as low-penetrant and high-frequency predisposition genes. Recent multicentre research proposed that a breast cancer risk gene may be the *PHIP* gene located at 6q141 (Behl *et al.*, 2020).

Relevant gene deletions or amplification, such as amplification of the *HER2* gene and deletions of the *PTEN* and *TP53* genes, arise as somatic alterations in breast cancer in addition to germline mutations. In certain situations, single-nucleotide polymorphisms (SNPs) may impact cancer pathogenesis. The *MDM2* gene, for instance, encodes a critical ubiquitin ligase that controls p53 negatively (Hirshfield *et al.*, 2010). The prevalence of breast cancer in *JAK2, ESR1, NOTCH3, MAP3K1, HCN1*, and *HIF1A* gene regions has been significantly increased with single nucleotide polymorphism (SNP)(Chan *et al.*, 2017; Kuo *et al.*, 2017). In the development of breast cancer, mitochondrial DNA polymorphism is implicated in tumor growth and changes in metastatic dissemination Variations in metabolic processes also have a vital part in developing several tumorigenic properties (Mishra *et al.*, 2018). Cancer cells grow in an abnormal form of the phosphoglycerate dehydrogenase (PHGDH) enzyme, which results in malfunctioning of the epigenetic regulatory mechanisms. The invasion and movement of cancer cells are enhanced by overexpression of methyltransferase (Yizhak *et al.*, 2014). In this case, metformin, which is used in the management of diabetes, can also be used to treat breast cancer (Athreya *et al.*, 2017; Davies *et al.*, 2017).

2.2.6. Breast Cancer Biomarkers in Clinic

The national health institutes define a biomarker as a molecular (including genetic or epigenetic) molecular, biochemical and/or cellular portion that can be scientifically measured and analysed as an indicator of normal biological processes, pathogens, or pharmacological responses to treatment (Scatena, 2015). Bio-markers are molecular indicators of illness progression or the presence of a physiological condition (Omenn *et al.*, 2012). Additionally, it has been identified that as well as before that a variety of biochemicals, including DNA, RNA, peptides, and hormones, biological processes such as apoptosis and angiogenesis, and development may be cancer biomarkers. It is revealed that cancer bio-marks may be found in secretions or circulating that include (stools, vomiting, sputum, nipple discharge, whole blood, saliva, and plasma) or other human-biological fluids (Scatena, 2015).

Biomarkers may be categorized into three major groups according to the place's usability: diagnostic, prognostic, and predictive (Goossens *et al.*, 2015). The markers used to identify the existence of disease are diagnostic biomarkers. The indicators used to detect improvements in cancer status, the risk of recurrence, and tumor hostility are prognostic biomarkers. In classifying patients who are more likely to respond to the care choices, predictive biomarkers are used. Thus, the medicine that reacts to the patient's body may be calculated or planned (Omenn *et al.*, 2012). Proteins (circulating plasma or serum proteins, tissue proteins), autoantibodies, miRNAs, methylated nucleic acids, lipids, and metabolites may be biomarkers (Dong *et al.*, 2013; Qiu *et al.*, 2018; Wang-Johanning *et al.*, 2014; L. Zhang *et al.*, 2015; Zhang *et al.*, 2014).

Serum tumor markers are soluble compounds that cancer cells or other cells in the tumor environment release into the bloodstream (Banegas *et al.*, 2012). A non-invasive and cost-effective solution is to measure the number of these biomarkers in the blood or serum of patients and compare the results with the diagnosis and prognosis of the disorder (Marrugo-Ramírez *et al.*, 2018). Besides, the optimal biomarker should have the following characteristics: (1) it should be precise and sensitive to a specific category of tumor (2) it should assist in the early identification of the disease (3) it should provide information on clinical options (4) it should provide information on the prognosis of the disease (5) it should help in evaluating the efficacy of the initial treatment (6) it should provide information on potential treatment options (Banegas et al., 2012; Kabel, 2017; Marić *et al.*, 2011).

Several serum markers have been used for breast cancer prognosis or prediction, but those used to date have not fulfilled the breast cancer monitoring, early warning, or diagnosis criteria (Mirabelli & Incoronato, 2013; Srivastava, 2017). Tissue polypeptide antigen, CEA, tissue polypeptide specific antigen, CA 27-29, MUC-1 protein, circulating cytokeratins (CKs), CK 19 fragment and CA 15-3, HER2, ER, PR, urokinase plasminogen activator, inhibitor plasminogen activator 1 is the most widely used breast cancer marker proteins (Duffy, 2006; Kabel, 2017; Mirabelli & Incoronato, 2013). P53, cathepsin D, cyclin E, and nestin are still used in breast cancer screening; however, due to their lack of accuracy and sensitivity, they may not provide enough data to be widely used in clinical practice (Loke & Lee, 2018; Marić *et al*., 2011).

2.3. The *APOBEC* family

Somatic mutations are critical in cancer development. Exogenous and endogenous factors are essential agents for DNA damage and cancer development, where the exogenous sources emerge from the environment and the endogenous sources arise from within the cell itself (Burns *et al.*, 2015). It is appropriate to further distinguish endogenous sources into passive and active DNA damage sources. After it has been activated, passive alteration is defined by an inability to restore the DNA damage. Agents that directly impair DNA, like hydrolytic cytosine deamination, are active endogenous causes of mutation (Zou *et al.*, 2017). The *APOBEC* family mutation is the second leading cause of DNA cytosine deaminase, which induces signature C-to-T transformation and C-to-G transversal mutations, breast, bowel, cervical, lung, and ovarian cancer (B. Leonard *et al.*, 2015). *APOBEC* mutation signature was present in various cancer types today, i.e., dC >Dt converts in a TCW motif (to *APOBEC3*) as apportion of approaches complete exome sequencing (WES) and whole-genome sequencing (WGS) in more than 7,000 human cancers (Rebhandl *et al.*, 2015).

In the human genome, the *APOBEC* gene family is made up of 11 sets of genes. The tandem distribution, on chromosome 22, enzymes editing apolipoprotein B mRNA, catalytic polypeptide 3 genes are seven of those 11 component genes consisting of *APOBEC3A*(A3A), *APOBEC3B*(A3B), *APOBEC3C*(A3C) and *APOBEC3D*(A3D), *APOBEC3F*(A3E), *APOBEC3G*(A3G) and *APOBEC3H*(A3H) (Soussi & Wiman, 2015; Y. Zhang *et al.*, 2015). The remaining four representatives are genes on other chromosomes, namely the Activation Induced Cytosine Deaminase

(*AID* or *AICDA*), *APOBEC1*(A1), and "*APOBEC4*" and "*APOBEC2*" genes are located on chromosome 12 and 6, respectively (Figure 2.3. A) (Jarmuz *et al.*, 2002; Moore & Chang, 2010). Several physiological functions in cell processes are possibly conducted by family members of cytidine deaminases, including antibody differentiation, DNA inhibition, and the innate immune system (Burns, Temiz*, et al.*, 2013a).

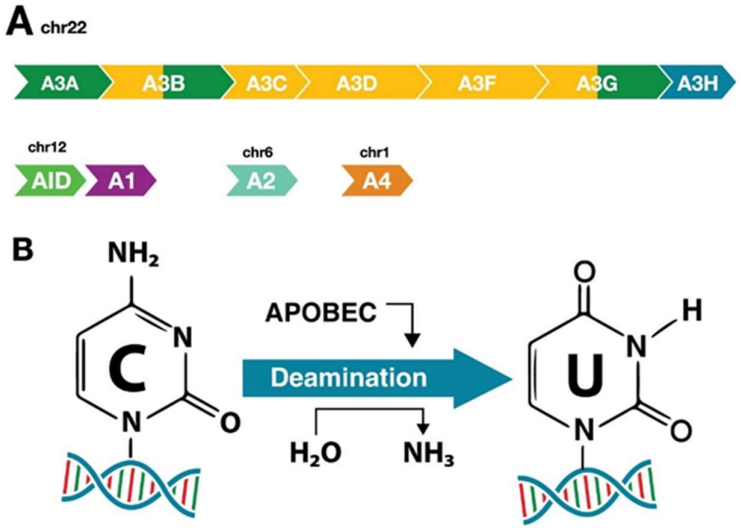

Figure 2.3. Introduction of Family *APOBEC*. (**A**) The chromosome-tandem location of the *APOBEC* family and APOBEC3 genes provides a representation of the spatial organisation of chromosome 22. The resides of "APOBEC1" and "*AID*" on chromosome 12. Besides, "*APOBEC2*" and "*APOBEC4*" are encoded on chromosomes 6 and 1, respectively. (**B**) The hydrolytic reaction of C to U in single-stranded DNA is catalysed by *APOBEC3* family enzymes (Swanton *et al.*, 2015).

Apolipoprotein B mRNA editing of the protein 1 (APOBEC1), the RNA editing enzyme and a popular member of the APOBEC cytidine deaminase family were the first enzymes identified in this family. In mammals, the small intestine is confined to the active control of the metabolism of cholesterol (Smith, 2017). By extracting the NH_2 group from the RNA or DNA base, APOBEC1 is responsible for deaminating cytidine (Figure 2.3. B). In this mechanism, APOBEC1 performs in a precise way in combination with the APOBEC complementing factor and usually deaminates only a single cytosine (C6666) on more than 14,000 nucleotides of long apolipoprotein B mRNA to establish a premature translational stop codon that generates a shorter ApoB48 protein that deaminates cytosine 6666 to uracil mRNA for apolipoprotein B. An in frame stop codon is produced by the deamination of cytidine 6666 and creates an abbreviated APOB protein (Goila-Gaur & Strebel, 2008; Neuberger *et al.*, 2003).

Activation-induced deaminase (AID) was discovered in 1999 as a significant similarity to *APOBEC1*, which is involved in the diversification of antibody genes (Conticello *et al.*, 2005). Petersen and colleagues showed, help only deaminated cytosines inside the single-stranded DNA and not RNA (Navaratnam & Sarwar, 2006). Deoxycytidine is deaminated and deoxyuridine produced in single-stranded DNA by the activation-induced deaminase (AID). This is the basis for recombination (CSR) and somatic hypermutation (SHM) to promote antibody diversification in B lymphocytes.

B lymphocytes are used by active induced deaminase (AID) to develop antibody diversity in the variable areas of expressed immunoglobulin genes, producing uracil lesions that are ultimately converted into the 6-basic substitution-mutations (somatic hypermutation).

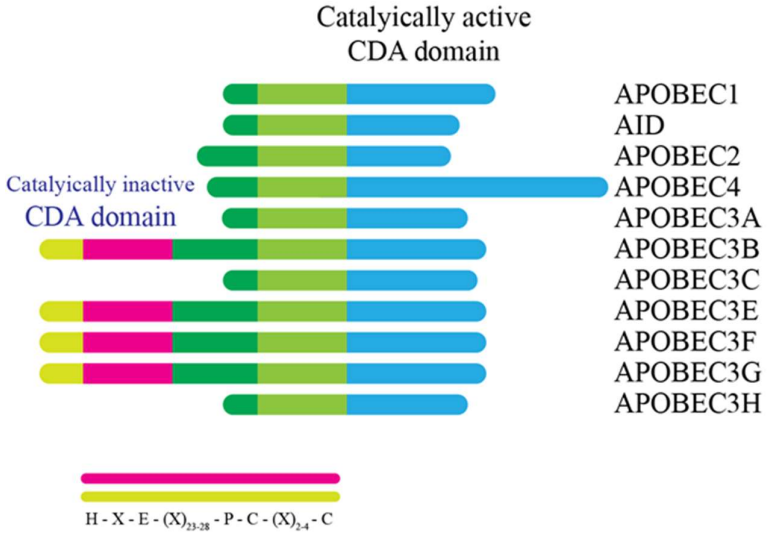

Figure 2.4. Members of the human family *APOBEC* comprise only one or two CDA domains found in green depending on their enzymatic active deaminase domain (CDA). The single-stranded RNA cytosine deaminase is *APOBEC1*. The other participants either have little activity on nucleic acids or are cytosine deaminases of single-stranded DNA. Both family members have at least one cytosine deamination sequence motif, as highlighted in red and green colors (Revathidevi *et al.*, 2021).

AID frequently produces uracil lesions that contribute to DNA breaking and the juxtaposition of the expressed and often mutated variable area with a new constant component of an antibody gene changing regions (recombination of the isotype switch) (Burns et al., 2015; Burns, Temiz, et al., 2013a).

2.3.1. Super Family of APOBEC3

The cytosine deamination activity of APOBEC proteins is dictated by their structural and conformational properties. At the cellular level, the main catalytic site of the cytidine deaminase (CDA) domain of all 11 APOBECs induce distinct function (Figure 2.4). Seven cytidine deaminases belong to the Apolipoprotein B Editing Complex (*APOBEC3* or A3) family members in the human genome (Stavrou & Ross, 2015). A tandem gene cluster is distributed linearly on chromosome 22 (Figure 2.3 A) (Rebhandl *et al.*, 2015; Warren *et al.*, 2017).

APOBEC3 is a class of enzymes that can eliminate cytidine in both DNA and RNA (Silvas & Schiffer, 2019). Their proteins transform cytidine into non-native uridine residues in single-stranded DNA polynucleotides. A random mechanism (hydrolytic deamination) occurs at a scale of 100-500/cell per day is the deamination of cytosine to uracil. Compared to double-stranded DNA deamination, the deamination of single-stranded DNA is 140-fold higher (Burns, Temiz, *et al.,* 2013a; Rebhandl *et al.*, 2015). It is considered that it provides the first line of protection against exogenous and endogenous retroviruses by the human cytidine deaminases subfamily of A3 (Silvas & Schiffer, 2019). *APOBEC*3 proteins play a role in the intrinsic/innate reaction to and subsequent regulation of early virus infection by inhibiting retroviral infection, replicating hepatitis B virus, and retro transposing endogenous DNA components (Stavrou & Ross, 2015). During a viral assembly in the host cell, APOBEC3 may associate with retroviral RNA and combine with retroviral particles. The recently delivered *APOBEC3* enzyme can deaminate cytidine residues after infecting new cells with a virus carrying APOBEC3 to form uracil in the single-stranded DNA synthesised through reverse transcription (Koning *et al.*, 2009).

Depending on zinc coordinating, the characteristic feature of APOBEC3 proteins can be grouped into three modified distinct "Z-domains" (Z1, Z2, Z3) (Refsland & Harris, 2013; W. K. Xu *et al.*, 2020). In amino acid compounds comprising the retained H-X-E- X25-31-P-C-X2 -4-C zinc-binding motif, all representatives of APOBEC3 differed, where X can use any amino acid (Warren *et al.*, 2017). The X25- 31-C-X2 -4-C-C, the Z domain-recognizing motifs vary from one to another (Harris & Dudley, 2015). The SW- S/T-C-X2 -4-C motif is distinguished by Z1 and Z2 domain deaminases, while the Z3 domain proteins produce the TW-S/T-C- X2-C motif '(Figure 2.5) (Münk *et al.*, 2012). Every APOBEC3 protein includes one or two copies

of the single "Z-domain" (A3A, A3C, and A3H) zinc-dependent cytidine deaminase domain. At the same time, catalyse the conversion of cytosine to uracil in polynucleotide substrates conducted by the presence of single or two zinc-coordinating domains in double "Z-domain" (A3B, A3DE, A3F, and A3G) proteins (Wang *et al.*, 2011). In APOBEC3 family members, the variety of Z-domain contributes to various biological roles and can be interchanged with different members of the APOBEC3 family (Refsland & Harris, 2013).

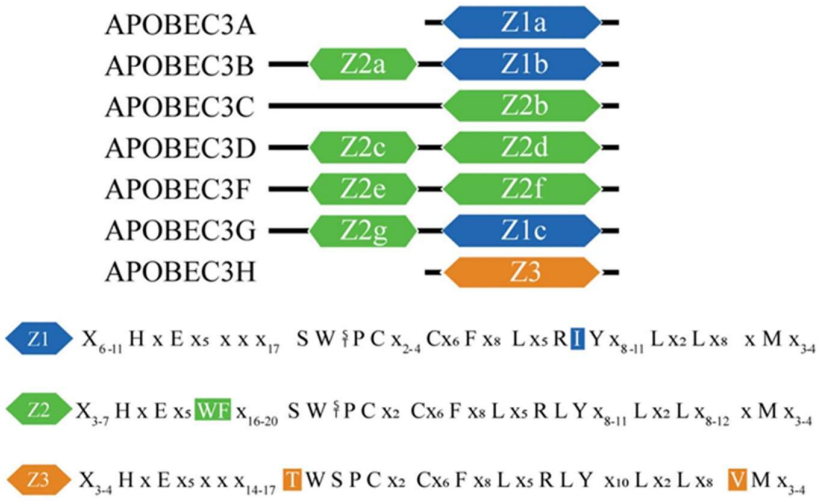

Figure 2.5. Illustrate the Z-Domain existence of the superfamily *APOBEC3*. The *APOBEC3* family Z domain (Top) structure indicates conserved and distinct amino acids in each subtype (bottom). Conserved residues are bold, and deviant amino acids are coloured in the zinc deaminase domain (Vasudevan *et al.*, 2013).

Additionally, the potential of these proteins in ssDNA to deaminate deoxy-cytidines allows APOBECs a double-edged sword. When *APOBEC3* is over-expressed, genomic instability and cancer are induced by misregulated deaminase activity (Silvas & Schiffer, 2019). After explored 7000 form of human cancers by complete exome sequencing (WES) and whole-genome sequencing (WGS) methods, it has emerged that an *APOBEC* mutation signature, i.e., dC>dT transforms inside a TCW (for *APOBEC3*) motif in several cancer types, including bladder, breast, B cell lymphomas, multiple myelomas (Rebhandl *et al.*, 2015). *APOBEC3* genes have recently been shown to have an oncogenic role in cancers with viral etiologies, according to several reports. It has been demonstrated that *APOBEC3* involvement is highly typical in cervical cancer caused by virally infected cell mutations. Infected cells by HPV produce cytokines such as interferons and interleukins, which trigger *APOBEC3* protein expression and initiate an innate immune response (Revathidevi *et al.*, 2021)

2.3.1.1. APOBEC3B Gene

2.3.1.1.1. *APOBEC3B* Structure and Function

The *APOBEC3B* gene belongs to the family of cytidine-deaminase complex. It is one of seven associated genes or pseudogenes in a cluster believed to be the product of gene-duplication on chromosome 22q13.1. (Figure 2.6), containing four exons (The human gene database, 2012,July 01). The overall structure of the A3 domain comprises six "alpha-helices" and five "beta-strand"s in the center of the zinc-binding region (Hou *et al.*, 2018). A3B is dual-domain favouring deaminating cytosine in single-stranded (ss) DNA at TC motifs, with weaker preference imposed on more bases upstream and downstream. Only the C-terminal domain (ctd) displays major catalytic activity in *APOBEC3B* with two deaminase domains. The position of the N-terminal domain (ntd) is considered to be regulatory and responsible for activities such as subcellular localisation in these two-domain A3B (Wagner *et al.*, 2018).

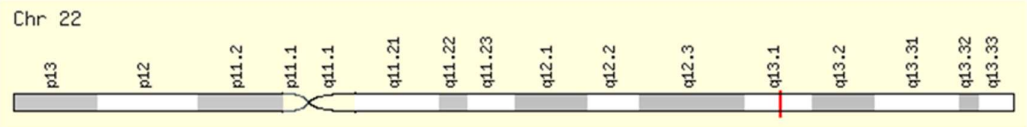

Figure 2.6. Position of the *APOBEC3B* gene on chromosome three (The human gene database, 2012,July 01).

Similarly, other cluster members encode *APOBE3B* proteins structurally and functionally aligned with cytidine deaminase editing C to U RNA. Proteins can indeed be RNA editing enzymes with roles of growth or cell cycle control and innate protection by protecting the host cell from exogenous viral infections and endogenous retro-components by introducing G to A hyper-mutations (Burns, Lackey*, et al.*, 2013).

Recently, it has been identified that A3B has a tumorigenesis role in several types of cancer as a part of having an enzymatic feature (Hirabayashi *et al.*, 2021). It is the only fundamental nuclear component of the family, overexpressed in several different forms of cancer and up-regulated by cancer-causing viruses such as HPV (Shi *et al.*, 2020). However, when overexpressed, the host genome may be mutated by A3B to cause cancer phenotypes. Increased expression of A3B in tumor cells is correlated with tumor protein 53 (p53) inactivation and the increased frequency of mutations on the cytidine and adverse patient results in cancer therapy, both scattered and clustered.

A3B will deliberately produce genetic mutations, unlike other cancer sources, which suggests an increasing abundance of DNA mutations would be generated in tumor cells (Radbruch, 2006). For instance, cancer evolution will gain much more to prevent immune monitored, outgrowth, metastasis, and finally, tolerance of clinical therapy. A3B is a suitable target to produce new anti-cancer medicines and their nonessential existence (McCann *et al.*, 2019).

2.3.1.1.2. *APOBEC3B* in Breast Cancer

APOBEC3B (A3B) overexpression has been identified in many tumor forms, for instance, ovarian carcinoma (Leonard *et al.*, 2013). A3B has also recently been implicated in primary breast tumors due to mutation and significant influences (McCann *et al.*, 2019). Burns et al. were the first to recognise *APOBEC3B* as one of the APOBEC groups acting on human cancer. The entire repertoire of *APOBEC* familial mRNA organisms has been quantified in human breast cancer tissues and cell lines. Surprisingly, data shows that only *APOBEC3B* was selectively and precisely up-regulated (Burns *et al*., 2015; Burns, Lackey, *et al.*, 2013). It has been demonstrated that *APOBEC3B* mRNA is overexpressed in most main breast tumors and cell lines of breast cancer. *APOBEC3B* expression and action have been attributed to the quantity of genomic uracil, the transformation dC>dT, and the mutation frequencies (Rebhandl et al., 2015).

The DNA cytosine deaminase *APOBEC3B* is the leading source of these mutations. Patients with increased *APOBEC3B* expression have a significantly higher mutation rate for *APOBEC3B* than patients without elevated *APOBEC3B* or possibly with *TP53* mutations (Nikkilä *et al.*, 2017). In breast cancer, *APOBEC3B* deamination establishes a cumulative source of DNA damage that could choose *TP53* inactivation and increase tumor heterogeneity. In particular, up-regulation of *APOBEC3B* coincides with the inactivation of the *TP53* tumor suppressor gene, which strongly implies that this could be an early tumor-initiating occurrence (Burns, Lackey, *et al.,* 2013). The latest results showed that *APOBEC3B* is a biomarker for poor prognosis and poor outcome with estrogen receptor (ER)+ cases, strongly suggesting that *APOBEC3B*-induced genetic aberrations lead to the development of breast cancer (Zou *et al.*, 2017).

2.4. Non-coding RNAs

The human genome is complicated; the developments in sequencing technology of the next decade show that around 70-80% of the genome is transcribed and the remainder functionally unknown. Code for the polypeptide (about 2 percent compared to the overall genome) in the transcribed proportion comprising between 20-30 percent of DNA sequences (Brooker, 2018; Dong *et al.*, 2018; Wong *et al.*, 2001; Yousefi et al., 2020; Zhang *et al.*, 2019). The rest of the transcripts are characterized as non-coding RNA that do not produce any protein. While evidence for their importance and functionality is mounting, many non-coding RNAs are vital regulators for many cellular processes (Yarmishyn & Kurochkin, 2015). ncRNAs may be categorised into three groups depending on their length: first, ncRNAs greater than 200 nucleotides, like lncRNAs. Second, the length of ncRNAs between 40 and 200 nucleotides. Third, is short of ncRNA than 40 nucleotides like microRNA (Aras *et al.*, 2020).

In recent years, it has become increasingly apparent that the non-protein-coding section of the genome has critical functional significance for normal development, physiology, and disease (Esteller, 2011). By taking part in the regulatory system of gene expression at the transcriptional and post-transcriptional level, ncRNAs perform housekeeping roles in many biological processes. On the other hand, in cellular infrastructure, ncRNAs perform primarily generic functions, like the tRNAs and rRNAs involved in translating mRNAs on ribosomes (Dahariya *et al.*, 2019).

A significant expansion of understanding about ncRNAs and their essential functions has been observed in recent years. Numerous data indicate that these ncRNAs are abnormally expressed in multiple forms of cancers and are widely implicated in tumor development and growth (Peng *et al.*, 2019; Yarmishyn & Kurochkin, 2015). Given the irregular expression of these ncRNAs in tumors induced by mutations in particular genes or epigenetic modifications, it is suspected that their dysregulation may impair one or more indicators of tumor development and growth. These ncRNAs may perform as either an oncogene or a tumor suppressor under some conditions based on their target genes (Peng *et al*., 2019). Concerning miRNAs, the function of ncRNAs in cancer has been most thoroughly examined. Also, distinct forms of human cancers are linked with lncRNAs. Expression amounts of various miRNAs and LncRNA vary between normal and cancer cells in almost all types of

human cancer, including breast cancer. Therefore, it might be more elusive behind the cancer causes to determine their quantity (Brooker, 2018).

2.4.1. LncRNA

LncRNAs are a group of RNA transcripts with a length exceeding two nucleotides, which, relative to mRNAs, are distinguished by more spatial and temporal precision, less interspecific conservation, and no evident protein-coding potential (Li *et al.*, 2017; Nie *et al.*, 2013). Often transcribed by RNA polymerase II, polyadenylated and they may present complicated splicing trends throughout the genome from various regions (Cheetham *et al*., 2013; Hajjari & Salavaty, 2015). LncRNAs are also widely recognised as active in the cell division development mechanism and the pathogenesis of many disorders, including cancer (Chi *et al.*, 2019). The function of lncRNA relies on its subcellular position. In the nucleus and cytoplasm, lncRNAs are located exclusively. LncRNAs are major transcriptional and epigenetic nuclear activity modulators in the nucleus, whereas cytoplasmic lncRNAs target mRNA transcripts and modulate the stability and translation of mRNA (Dong *et al*., 2018). By interacting with DNA regulatory elements and interacting with chromatin-altering complexes at the epigenetic stage, lncRNAs perform critical functions at the transcriptional level (Joensuu & Gligorov, 2012). Furthermore, by forming double-stranded RNAs with mRNAs or connecting protein stability to control their stability, LncRNAs regulate the translation and stability of messenger RNAs (mRNAs) (Zhang *et al.,* 2019). Latest studies have shown that lncRNAs are major cancer-related cell pathway regulators (Joensuu & Gligorov, 2012).

Mainly due to the broad size spectrum, different positions in the genome, and wide range of functions, there is no adequate classification for lncRNAs, novel defined classes of lncRNAs are classified as long intergenic ncRNAs, long intronic ncRNAs, dual-function lncRNAs, telomere-associated lncRNAs, pseudogene RNAs, and transcribed ultra-conserved regions (Malih *et al.*, 2016).

Dysregulation of lncRNAs is implicated in many illnesses, like tumors, including breast cancer, depending on their functions (Hajjari & Salavaty, 2015). Abnormal lncRNA expression leads significantly to the onset of cancer and development of breast cancer (Zhang *et al.*, 2019). In breast cancer, several lncRNAs have been found

to be aberrantly expressed. They can be separated into tumor suppressors and oncogene groups according to their roles and expression styles (Soudyab *et al.*, 2016).

LncRNAs and miRNAs can interact directly with each other through multiple processes, including miRNAs can decrease lncRNA abundance by reducing its stability, lncRNAs can serve as sponges or decoys for miRNAs to decrease the available miRNA levels, lncRNAs can compete with miRNAs to bind to mRNAs, and lncRNAs can provide miRNAs from their exons and introns. In tumorigenesis, such as breast cancer, any aberrant expression in any form may play a role (Dong *et al.*, 2018).

2.4.1.1. LncRNA-*GAS5*

A significant aspect of the human transcriptome is lncRNAs. Regulatory roles have emerged in recent years for different molecules in critical cellular processes (Ji *et al.*, 2019). It is progressively understood that significant pathologies, such as cancer, mark the dysregulated expression of lncRNAs. LncRN-*GAS5* is critically vital for both clinicopathological and patient prognosis, which is downregulated in many cancers in this respect (Li *et al.*, 2016). Moreover, lncRNA-*GAS5* small interfering RNA is induced apoptosis while limiting replication in several cell lines has been demonstrated, and their mode of action has been described as tumor suppressor function.

2.4.1.1.1. Structural characterisation and biological functions of *GAS5*

It is known that growth arrest-specific transcript 5 (*GAS5*) is localised at 1q25. In addition to having complexity, its gene expression is not fully understood, and there are various kinds of small non-coding RNAs encoded by *GAS5*, such as microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and small nucleolar RNAs (snoRNAs). The gene comprises 12 exons, which only have a small, open reading frame and are not known to encode a functional protein, and are split to produce two possible mature lncRNAs, named "*GAS5*a" or "*GAS5*b", through the addition of alternate 5'-splice donor sites inside Exon 7 (Pickard & Williams, 2015).

GAS5 is identified as a 5'-terminal oligopyrimidine (5'TOP) gene. 5'TOP genes are classified as a gene class that has an uncommon 5'-terminal sequence rich in pyrimidine. The mRNAs of the 5'TOP genes accumulate during cell growth arrest in

messenger ribonucleoprotein particles (mRNPs). The introns of *GAS5* encode ten nucleolar RNAs (snoRNAs) and two mature isoforms of lncRNA: *GAS5a* and *GAS5b*. At 77 nt in length, *GAS5a* is the key isoform, while *GAS5b* only has 45 nt, losing 32 nt. The prospective open reading frame, however, is limited and poorly maintained (Yu & Li, 2015). *GAS5* is non-protein-coding, but its RNA is spliced, polyadenylated, and ribosome-associated (McCann *et al.*, 2020). Introns, which encode several snoRNAs, mediate the critical biological function of *GAS5*.

The roles of *GAS5* are not yet well defined, but several snoRNAs that control the biosynthesis of ribosomal RNA are expressed (Kino *et al.*, 2010). *GAS5* is predominantly expressed in tissues, but in cells that proliferate, it is defective. RNA splicing is poor in rising cells but strongly expressed during growth arrest, cycloheximide inhibition, pactamycin, and rapamycin translation (Cerk *et al.*, 2016). The elevated *GAS5* transcript level was assumed to be induced by sustained rates of decay. *GAS5* has also shown to be essential in human T lymphocytes for normal growth arrest (Mourtada-Maarabouni *et al.*, 2008). A variety of experiments have been done to elucidate the human roles of *GAS5*. The cell cycle, cell progression, and essential to normal growth arrest are proven to be correlated with *GAS5*. Overexpression of *GAS5* prevents the cell cycle progression, while inhibition of *GAS5* reduces cell apoptosis and leads to faster progression of the cell cycle (Williams *et al.*, 2011; Yu & Li, 2015).

2.4.1.1.2. LncRNA-*GAS5* in Breast Cancer

Long non-coding RNAs (lncRNAs), which have been shown to control several critical biological processes, have recently gained further study. In tumorigenesis, lncRNAs may function as proto-oncogenes (e.g., *HOTAIR*) or tumor-suppressor genes (e.g., *GAS5*). Through it is capable of inhibiting apoptosis in a mouse thymoma cell line, lncRNA-*GAS5* was detected using a practical screen. It is revealed in many research that aberrant expression in lncRNA-*GAS5* has a significant impact on some different forms of cancer, such as endometrial carcinoma, hepatocellular carcinoma, gastric cancer, and bladder cancer (Tu *et al.*, 2014). In various human tumors, multiple lines of evidence have demonstrated aberrant development of lncRNA-*GAS5* (Alahari *et al.*, 2016). GAS expression is reduced across numerous cancer types, indicating that lncRNA-*GAS5* could suppress the tumor. Also, in various cancer lines, overexpression of lncRNA-*GAS5* is related to growth arrest in vitro.

Based on quantitative bioinformatics analysis, have argued the inhibition impact of lncRN-*GAS5* on *miR-103* expression, and lncRN-*GAS5* inhibits *miR-103* in endometrial tumor cell expressions, which promote *PTEN* expression. To investigate the relationship between *PTEN* and lncRN-*GAS5* in endometrial cancer, a correlation analysis between lncRN-*GAS5* and *PTEN* was conducted. The lncRN-*GAS5* expression was positively linked to the PTEN expression (Guo *et al.*, 2015).

Latest studies of lncRNA-*GAS5* expression in the class of human breast cancer show that lncRNA-*GAS5* transcript levels were dramatically decreased relative to nearby non-cancerous tissue in breast cancer cells, utilising reverse transcription-quantitative polymerase chain reaction study (Yu & Li, 2015). The lncRNA-*GAS5* over-expression may facilitate the demise of breast cells and accelerate various treatment processes; it has been observed in triple-negative and ER-positive breast tumor cells (Mourtada-Maarabouni *et al.*, 2009). Additionally, it was found that mTOR inhibitors could increase lncRNA-*GAS5* levels, thus leading to lower rates of cancer growth (Pickard & Williams, 2014). Several studies showed that lncRNA-*GAS5* changes levels in the plasma of patients who have breast cancer during surgery and that lncRNA-*GAS5* might constitute an active degree of proliferation before operation. Therefore, the plasma lncRNA-*GAS5* may also be a biomarker to determine the prognosis assessment following surgery (Han *et al.*, 2016). lnc-*GAS5*, breast cancer expression cells in patients treated with trastuzumab have been diminished and have been shown to lead to tolerance to trastuzumab (Li *et al.,* 2016). As a consequence, in the control of breast cancer cells, lncRNA-*GAS5* plays a significant function.

2.4.2. microRNAs

miRNAs are small, non-coding RNA sequences of 18-24 nucleotides, which regulated gene expression via translational modulation. However, miRNAs were not accepted until the early 2000s as another type of biological regulator with roles sustained (De Leeneer & Claes, 2015; Gebert & MacRae, 2019). miRNAs are now seen as involved in critical cellular processes such as cell cycle regulation, growth, neuronal patterning, aging, and metabolic processes, as evidenced by increased use in recent years (Chen *et al.*, 2015). Translational control and developmental timing are determined mainly by miRNAs, especially interactions with the mRNA 3'UTR (Zhao & Srivastava, 2007). Finally, miRNAs can bind to the specific mRNA sequences and control the target gene expression by corrupting and/or blocking the corresponding

mRNA (Yang *et al.*, 2011). They are generated in long ncRNA precursors or from introns of non-coding genes from incomplete hairpin structures. In the cell nucleus, they are processed and generated by interactions with the DICER and DROSHA enzymes. miRNAs interfere in the cytoplasm by pairing with mRNA, whose translation they interrupt. The creation combines the complex of miRNAs with ARGONAUTE and other proteins as part of a silencing complex caused by RNA (Gebert & MacRae, 2019). The mRNA is degraded or impaired by the microRNA / RISC complex, and the result of one of these pathways is a reduction of the protein level in the target gene (Ballantyne *et al.*, 2016).

Constitutionally, in many pathological and physiological circumstances, miRNAs have been determined to play a significant function. In specific, miRNA deregulation in various cancer forms has been extensively studied (De Leeneer & Claes, 2015). It is considered that deregulations in miRNA expression were to play a role in cancer (Chen et al., 2015). Also, miRNAs provide tremendous potential as biomarkers for cancer diagnosis due to their exceptional blood consistency and distinctive expression in various diseases (Mar-Aguilar et al., 2013).

2.4.2.1. miR-103

Several genes are negatively regulated by non-coding small RNA. Rising research suggests a connection between miRNAs and cancer. miRNAs function as tumor suppressors or oncogenes depending on the target genes (Kolak *et al*., 2017). Different mechanisms include chromosome aberration, genomic mutation, polymorphism, and epigenetic alternation of miRNAs in cancer, critical in tumor initiation (Yang *et al*., 2011). On the normal side of 50% of human-targeted miRNA genes are also in affected areas. Based on array genomic hybridisation, a significant prevalence of genomic alterations in miRNA loci has been found in many forms of human cancer. miRNA expression variations have recently been studied in humans to classify certain forms of human cancers, including prostate, bowel, cervical, breast, and lung cancers (Sundarbose *et al.*, 2013; Yang *et al.*, 2011).

Highly significant variations between cancer patients and regular volunteers have been observed in different trials, for instance, miR145 in breast carcinoma, miR29a in colorectal tumor, and *miR-141* in prostate cancer. Researchers suspect that miRNAs may influence the expression of some gene's products in tumors, with either up or

down-regulation. The down-regulated ones are suggested to have a tumor suppressor capacity in malignancies (Zhao & Srivastava, 2007).

In these functional miRNAs, *miR-103* has shown that they are active in multiple biological and pathological processes. Several studies indicate that the aberrant expression of *miR-103* in several cancers (Geng *et al.*, 2014). In endometrial cancer cell lines, post-transcriptional *miR-103* impaired tissue inhibitor metalloproteinase-3 (*TIMP-3*) expression that role as a tumor suppressor gene and induced development and invasion (Yu *et al.*, 2012). High *miR-103* expression was correlated with the metastasis capacity of colorectal cancer cells and worse prognosis subsequently. *PTEN* is a vital gene suppressor for tumors. It has been documented that up-regulated miR-130 will inhibit *PTEN* expression in colorectal cancer. *PTEN* has been established to be the explicit goal of *miR-103*, thus reducing the expression in colorectal cancer by *miR-103* at a post-transcriptional level considerably (Geng *et al.*, 2014).

All of the above studies indicate that the *miR-103* gene could be a central factor in cancer advancement as an oncogene. The association between *miR-103* and breast cancer, however, must be further studied.

2.5. Mechanism of lncRNA-miRNA interactions

Evidence has recently shown that functional crosstalk between lncRNA and miRNA via a double-negative feedback loop is involved in gene expression control (Bian *et al.*, 2019). LncRNAs, identical to miRNAs in composition and function, often contain recognition elements (MREs). Consequently, by competing with miRNAs for unique identification and binding at the 3'UTR of target mRNAs, lncRNAs can inhibit miRNAs' negative control on target mRNAs (Chen *et al.*, 2018; Ebert & Sharp, 2010).

It has been shown that a particular miRNA regulates an excessive level of ncRNA, and the mutually. Many of these controls significantly affect physiological processes, and each miRNA and lncRNA's abundance binding can specifically alter cell function. One of the characteristics of long non-coding RNA molecules is sequencing and competing with miRNA, allowing for repression and alleviation of expression of miRNA target sites by forming common target-binding areas with their mRNA targets (Ebert & Sharp, 2010). In the same way that microRNA (miRNA) sponges sequester miRNAs, competing for endogenous RNAs (ceRNAs), affect the rate of expression of other transcripts with identical miRNA response elements (MREs). It is found that any reduction in the amount of ln-cRNA access to free further miRNA molecules and then

attached to mRNA includes such MREs, making proteins assemble less efficiently. However, excessive levels of lncRNA molecules lead to lower amounts of miRNA binding and increasing mRNA expression (Zhao *et al.*, 2020).

Long non-coding RNAs (lncRNAs) have appeared, in addition to miRNAs, as essential factors leading to breast cancer growth and progression. LncRNAs and miRNAs interactions have been studied in human pathology, including hepatocellular carcinoma (Y. Zhang *et al.*, 2017). Numerous studies have documented the function of abnormally expressed lncRNAs and miRNAs in breast cancer (J. Xu *et al.*, 2020). Advances in sequencing methods have shown that lncRNAs, just like mRNAs and miRNAs, are deregulated in cancer. Over-expression of lncRNA *HOTAIR* is, for example, documented in people who suffer from breast tumors. An evolving role of lncRNAs is that they compete to bind to miRNAs, serving as a sponge to stabilise the gene's action. In several cancer types, including breast cancer, this regulatory relationship between lncRNAs, miRNAs and mRNAs is observed (Olgun *et al.*, 2018).

CHAPTER III

3. Materials and Methods

3.1. Sample collection:

The study group included a total of 98 samples from the Zheen International Hospital Erbil, Iraq. A paired sample that consists of normal and tumor were obtained from the same female breast cancer patient. Thus, 98 examples were grouped into 49 healthy and 49 tumor samples in this study. The clinical features, including patients' age, cancer type, and tumor grade, were also compared. The tissue samples were held at -80 ^{0}C for further examination. The informed consent form was obtained from all of the participants. This study was approved by the Near East University Scientific Review Board (YDU/2020/79-1075).

Both tumor and control in each pair of samples were collected from the same female patient. The characters of the specimens that were assessed include age, cancer type, and grade. Based on the patients age they were divided to four categories (Age: 25-40; no.=15/ 41-55; no.=19/ 56-70; no.10/ 71-85; 5). Moreover, four cancer types with different grades were observed in the total amount of tumor samples, including 36 samples of invasive ductal carcinoma (Grade: I; no.=8/ II; no.=15/ III; no.=13), three samples of invasive ductal carcinoma, medullary likes (Grade: I; no.=1/ III; no.=2), two samples of matrix producing metaplastic carcinoma(Grade: I; no.=1/ II; no.=1), four samples Metaplastic carcinoma, matrix producing type (Grade: I; no.=2 / II; no=1 / III; no=1) and four samples of invasive lobular carcinoma (Grade: I; no=1/ II; no.=2/ III; no.=1).

3.2. Instruments, Chemical Materials, and Solutions:

Instruments, chemicals, and solutions which were used in this study has been shown in Table 3.1, 3.2, and Table 3.3.

Table 3.1 Instruments, brands and manufacturers have been utilized.

Instruments	Brands	Manufacturers
RT. PCR Rotor Gene Q	Qiagen	Germany
Heating magnetic stirrer	Velp	Italy
Refrigerator	Bosch	Turkey
Incubator	Uniequip	Germany
Vortex	VELP	Italy
Centrifuge 5810R	Eppendorf	Germany
Micropipettes set	Eppendorf	Germany
Biological safety	Bilser	Turkey
Microcentrifuge 5425D	Eppendorf	Germany
Thermal cycler (PCR)	ABI	USA
IQ5 RT-qPCR	Biorad	USA
Horizontal gel electrophoresis	EC apparatus	USA
Microwave oven	Arcelik	Turkey
Autoclave	Hirayama	Japan
Horizontal shaker	J.P. SELECTA	Spain
Spectrophotometer	Biometrics	Taiwan
Ultraviolet transilluminator	UVPBioChemisystem	USA
Sensitive balance	Shimadzu	Japan
Nucleotide sequence analyzer	Applied Biosystems	Singapore

Table 3.2. Chemical materials, brands, and manufacturers that have been utilized.

Chemical materials	Brands	Manufacturers
RNA Stabilizer (RNALater)	Intirogen	USA
Ethidium bromide	Sigma	Germany
Safe stain	GenetBio	Korea
Isopropanol	Sigma	Germany
Binding Silane	Promega	Germany
Gelsave	Merck	Darmstadt,Germany
$MgCl_2$	Fermentase	Applied Biosystems
Tris	Fermentase	Applied Biosystems
dNTP	Fermentase	Applied Biosystems
Taq DNA polymerase	Fermentase	Applied Biosystems
Agarose	Prona	Madrid, Spain
EDTA	Merck	Darmstadt,Germany
100 bp marker	Fermentase	Applied Biosystems
Bromofenol Blue	Merck	Darmstadt,Germany
Orange G	Sigma	Germany
Ethyl alcohol	Sigma	Germany
Sephadex G-50	Sigma	Sweden
BigDye	Invitrogen	USA
Ribo-sol RNA extraction kit	AmplisSens	Russia
Revertal-L RT reagent kit	AmplisSens	Russia
Syber RT-PCR kit	Qiagen	Germany

Table 3.3. Solutions and their chemicals content that has been utilized.

Solutions	Content
10X TBE	108,9 g Tris
	55 g boric acid
	9,37 g EDTA
	1000 ml distilled water
6X Orange G loading dye	% 60 glyserol
	15%Orange G
	10 mM Tris - HCl PH 7,6
	60 mM EDTA
Ethidium bromide	0,5 Ethidium bromide
	1 ml distilled water
Exo-Sap	Exonuclease I (10 U/µl)
	Shrimp Alkaline Phosphatase (10 U/µl
	5x sequencing buffer
	Sterile water

3.3. RNA extraction

Ribo-sol RNA extraction kit (AmpliSens, Russia) was used to obtain the RNA from tissue samples depending on the manufacturer's instruction. The Spectrophotometer was used to measure and rate the overall RNA concentration (Biometrics,Taiwan) to quantify and qualify total RNA. Samples with (A260 – A280) ratios less than 1.7 and/or yields less than 0.5 μg total RNA were excluded from subsequent analysis.

3.4. Complementary DNA synthesis

Revertal-L RT reagent kit (AmpliSens, Russia) was used to synthesis cDNA to measure the level of expression of the *APOBEC3B* gene and lncRNA-*GAS5*. However, in obtaining cDNA from isolated *miR-103*, miScript II RT Kit (Qiagen GmbH, Hilden, Germany) has been utilized for expression analysis. In addition, the thermocycler machine (ABI, USA) has received cDNA in thermal cycling procedures. Because the quality and quantity of total RNA are not equal, a variable amount of total RNA was used for each sample.

3.5. Primer design:

Primers for *APOBEC3B*(Exp.) and *APOBEC3B*(Mut.), and LncRNA-GAS4(Exp.) have been designed. The online primary design program http://workbench.sdsc.edu was used. Table 3.4 provides the sequence of the primers, the temperature of the annealing, and the length of PCR products. Regarding *miR-103*, the miScript Primer Assay (Qiagen, Germany) was utilized.

In coding regions, the primers for gene expression could be anywhere to know whether the gene is being expressed. Due to the fact, the main objective is coding RNA products, one or two exon-exon junction sites should be included to prevent the increase in the copy number of any product other than the RNA product concerned. Otherwise, we could not determine whether DNA or any other contaminant generated the product whose number of copying increased. In this context, *APOBEC3B*/Exp and lncRNA-*GAS5* primers were designed. *APOBEC3B*/Mut was designed for mutation screening in coding sequence regions without UTRs.

Table 3.4. Represent of primer sequences, optimal annealing temperature, and PCR product size of three different target regions of *APOBEC3B*(Exp.) *APOBEC3B*(Mut.) and lncRNA-*GAS5* genes.

Primer Name	Sequence 5′ to 3′	Optimal Annealing temperature	PCR Product Size
APOBEC3B/Exp.			
Forward Primer (F)	GAGCGGGACAGGGACAAG	60.4 °C	546 bp
Reverse Primer (R)	GGCATGAATTGCTGACCTTCA		
LncRNA-*GAS5*/Exp.			
Forward Primer (F)	TGGTTCTGCTCCTGGTAACG	55.3 °C	185 bp
Reverse Primer (R)	AGGATAACAGGTCTGCCTGC		
APOBEC3B/Mut.			
Forward Primer (F)	CAGCAATTCATGCCTTGGTACA	61.2°C	405 bp
Reverse Primer (R)	CATTTGCAGCGCCTCCTTAT		

3.6. PCR Optimization

The PCR was run with a cDNA sample to conducting the gradient PCR for each primer pairs. Amplification was carried out in a thermocycler machine (ABI, USA). The outcome of agarose gel electrophoresis was relied on to determine the optimal melting temperature of all primers. The mixture utilized for the PCR gradient is shown in Table 3.5.

Table 3.5. The different components of PCR reactions, their respective quantities in 25µL.

Chemical Substances	Quantity (µL)
dH2O	14.875µL
10X PCR buffer Ammonium sulfate (NH4) 2SO4	2.5µL
25 mM MgCl2	2µL
2 mM dNTP	1.5µL
20 mM Forward primer	1µL
20 mM Revers primer	1µL
5 U/ML Taq DNA polymerase	0,125 µL
cDNA template	2µL
Mixture	25µL

The thermocycler program was configured to operate 35 cycles, and relevant specifications are shown in Table 3.6.

Table 3.6. Gradient PCR reaction conditions.

Step	Temperature	Time
Pre denaturation at	95°C	3 minutes
Denaturation at	95°C	30 seconds
Primer annealing	53.5°C – 64.5°C	30 seconds
Extension	72°C 35 cycles	30 seconds
Final extension	72°C for	2 minutes
Hold	4°C	0

The resulting components have been evaluated using electrophoresis of 2% agarose gel and bromide ethidium stained. For 45 minutes at 100 volts, the gel was handled. UV-light highlighted the cDNA pieces. The optimum temperature for annealing was calculated. The image findings obtained from gel electrophoresis for gradient PCR of all primers were 60.4°C for *APOBEC3B*/Exp, 55.3°C for lncRNA-*GAS5*/Exp, and 61.2°C for *APOBEC3B*/Mut.

3.7. *GAPDH* amplification

Housekeeping genes are important for basal cellular functions and critical for a cell's life, regardless of their particular position in the tissue or organism (Eisenberg & Levanon, 2013). Therefore, within normal circumstances, they are supposed to be extensively expressed in all cells of an organism, regardless of tissue type, stage of development, cell cycle condition, or external signal, and constitute the basal transcriptome for the conservation of basic cell functions (Zhu *et al.*, 2008). Housekeeping genes, widely used to normalize mRNA levels across various samples, are referred to a control gene (Silver *et al.*, 2006). In molecular assays, it is assumed that housekeeping genes' expression is highly constant in cells, samples, both within treatments and through patients, because the gene expression may change. While all methodology needs validation, it is not simple to determine if household gene expression has biological variation without a validated alternative way of normalizing results (Glare *et al.*, 2002). Many kinds of control genes, such as *ß-Actin, ACTB*, *GAPDH*, *HPRT1*, and *B2M*, were considerably used in RNA expression analysis. Studies have indicated that specific cytoskeletal proteins, such as ß-actin, and glycolytic enzymes, including *GAPDH*, are highly and consistently expressed. Therefore, they are widely used as control genes (De Jonge *et al.*, 2007). Whereas may vary the expression of those genes under different experimental conditions (Eisenberg & Levanon, 2003). The gene *GAPDH* is used as a reference gene to evaluate the *APOBEC3B*, ncRNA-*GAS5*, and *miR-103* gene expression levels in this research.

3.8. Agarose gel electrophoresis:

The most appropriate technique to distinguish DNA fragments in different sizes varying from 100 bp to 25 kb is agarose gel electrophoresis (Lee *et al.*, 2012). This technique uses various electronic charges to separate the molecules and purifies macromolecules, particularly proteins and nuclear acids, in a mixture (Gebert & MacRae, 2019). There are negative charges on DNA molecules. They are moved towards that have a positive charge in the electrical field. Depending on two factors, it migrated through the way its various shapes and mass charges had to differ (Gebert & MacRae, 2019; Lee *et al.*, 2012).

For primary optimization, agarose gel electrophoresis was used in this research. Staining the samples with a chemical that enables UV-visible detection of the DNA

also helped running in 2% agar gel. To dye DNA in agarose gel, ethidium bromide (EtBr) is routinely used. For up to 45 minutes, our cDNA samples have been electrophoresed under the electric field at 100 volts. The actual product size of the PCR may be somewhere from 179-662 base pairs.

3.9. Real-time PCR:

The capability for real-time PCR involves simultaneous amplifying with the support of binding DNA dyes. One of the dyes used extensively for this role is SYBR Green dye. It attaches and provides fluorescence to double-stranded DNA molecules. During the extending phase carried out after linking the primer, SYBR Green binds to DNA increases as the target DNA is double-stranded. Hence, increases in fluorescence emitted are observed. The value, which is called Ct or Cp, is the stage where the irradiation of the SYBR Green dye entered the limit value. DNA is elevated at this stage, and the radiation released from the DNA binding dye and reaches its limits.

When the radiation passes the threshold, amplification is achieved. Still, it is necessary to evaluate the melting curve to see whether the appropriate amplicon is followed because SYBR Green binds all double-stranded DNA molecules. At the same time, it is not a specific dye binding to a particular DNA molecule.

3.9.1. RT-PCR components

The real-time PCR reaction was carried out on the RT. PCR Rotor-Gene Q (Qiagen, Germany) and IQ5 RT-qPCR (Bio-Rad, USA). RT^2 SYBR Green ROX FAST Mastermix (Qiagen GmbH, Hilden, Germany) for *APOBEC3B*, lncRNA-*GAS5*, and miRNA 103 expressions were used as the master mix in the expression evaluation. The Real-Time PCR master mix was organized on ice. The volumes of reagents used for the RT-PCR amplification are presented in Table 3.7 below.

Table 3.7. Quantitation of *APOBEC3B* and lncRNA-*GAS5* expression has been performed in Real-Time PCR component-based evaluation.

Component	Quantity (ul)
RT² SYBR Green ROX FAST Mastermix	7.125 ul
Fw Primer (10 μM)	1 ul
Rv Primer (10 μM)	1 ul
RNAse/DNAse free water	12.875 ul
cDNA (50 ng)	3 ul
Total	25 ul

For each sample, cDNA was moved to tubes containing Real-Time PCR master mixes. The tubes were combined well to spin-downed momentarily and then left on the ice again. The program is saved in RT.PCR Rotor-Gene Q (Qiagen, Germany) and IQ5 RT-qPCR (Bio-Rad, USA) were run (Table 3.8).

Table 3.8. Configuration for Real-Time PCR to detect *APOBEC3B* and LncRNA-*GAS5* expression measurement.

Step	Temperature (°C)	Time (sec)	Cycle
Enzyme activation	95	10 min	
Denaturation	95	15	
Primer annealing/extension	60	60	40
Melting curve	65	1	
	95	Continuous	

3.10. Nucleotide Sequencing

3.10.1. PCR sequencing procedure

Separation for 40 cDNA fragments of *APOBEC3B*/Mut (20 tumor+ 20 normal) was performed using agarose gel electrophoresis procedure because of its need for PCR amplification cDNA template source. The proportion of other substrates is the same as the proportion shown in Table 3.7 above. For each sample, the total volume of the PCR reaction mix will be 25 μL and then run on a thermocycler machine (ABI, USA) with the same PCR reaction condition shown in Table 3.8 above.

3.10.2. Pre-Sequencing Preparation procedure

3.10.2.1. Protocol of PCR production cleanup with ExoSAP

Regarding the ExoSAP mixture combination, 10U/µl Exonuclease-I have been added to 1 U/µl shrimp alkaline phosphatase and then mix with sterile water. The exonuclease-I function for the degradation of primers, and Shrimp Alkaline Phosphatase degrades unincorporated nucleotides to prepare the template for sequencing. The given following preparation shows cleanup PCR products with an ExoSAP mixture.

5µl of PCR product + 2µl of ExoSAP

According to the specific protocol shown in Table 3.9, thermocyclers (ABI, USA) were used to purify cDNA template.

Table 3.9. Appropriate conditions for PCR product cleanup using ExoSAP ready-to-use mixture.

Step	Temperature	Time
Left over primers are degraded	37C	30 minutes
Enzyme is degraded	85C	15 minutes
Hold	4C	∞

3.10.2.2. Cycle sequencing reaction

BigDye is highly sensitive to light; all light sources must be turned off during the cycle sequence reaction mix. The protocol of cycle sequencing is illustrated in Table 3.10.

Table 3.10. Given requirements need to each cycle sequence according to a specific protocol.

Chemical Substances	Quantity
DNA	1µl
Forword primer (0.8 µM)	2µl
5X BigDye buffer	2µl
BigDye (v3. 0) Mix	1µl
ddH2O	4µl

Then amplification of the cDNA samples after it was put on the thermocycler machine and performed according to the instructions in Table 3.11.

Table 3.11. Thermal cycling conditions of the sequencing PCR reaction.

Step	Temperature	Time
Pre denaturation at	96°C	1 minutes
Denaturation at	96°C	10 seconds
Primer annealing	61.2°C	5 seconds
Extension	60°C	30 minutes
	25 cycles	
Hold	4° C	∞

3.10.2.3. Sephadex spin-column protocol for cleaning PCR products

The solution was put on a vortex for 45 minutes to mix it. The empty receiver column was placed in 1.5ml collecting tubes, and 850µl of Sephadex solution was added into each receiver column tube. The tubes were placed in a centrifuge, turned on at 3,800 rpm for two minutes. Then, the collection tube was substituted with a new one. After that, 10µl of the PCR sample was added to a prepared column. Then the PCR samples were transferred and placed in the receiver column matrix center without touching it. Then, the tubes were placed in the centrifuge at 3,800 rpm for 2 minutes. Table 3.12 revealed a Sephadex solution.

Table 3.12. Component of sephadex spin–column of cleaning PCR product.

Component	Quantity
Sephadex G-50 powder	4g
ddH2O	42ml

3.11. Statistical Analysis

Data analysis was carried out using GraphPad Prism software version 8.4.3. Statistical significance was assessed by T-tests (and nonparametric tests). Also, Microsoft office excel was used to find significant expression targets according to tumour properties. Variables that achieved a probability of p-value<0.05 was considered statistically significant.

Relative quantification RT-PCR was performed in triplicate. First, values were obtained as the threshold cycle (C_T) for *APOBEC3B*, lncRNA-*GAS5,* and *miR-103* and normalized using the housekeeping gene and internal control. ΔC_T method was used to calculate relative changes (gene expression concerning the housekeeping gene) in *APOBEC3B*, lncRNA-*GAS5,* and *miR-103* expressions in different tissues, separately. This value gave information about the expression level of related gene in a breast cancer tumor based on the expression level in the control group.

CHAPTER IV

4. Results

4.1. Expression results

4.1.1. RT-PCR Fluorescence Signals

Comparative RNA expression analysis in tumor versus normal cells (calibrator) was performed as follows:

ΔC_T (Normal) = C_T (Target) − C_T (*GAPDH*)

ΔC_T (Tumor) = C_T (Target) − C_T (*GAPDH*)

The examination has been based on the uses of Real-Time qPCR to assess the value of the *APOBEC3B* gene expression levels in these 98 of total normal and tumor samples. The normalization was done on the target expression, the *GAPDH*, which is also used as a housekeeping gene. Given figure 4.1. exhibits the *APOBEC3B* gene results.

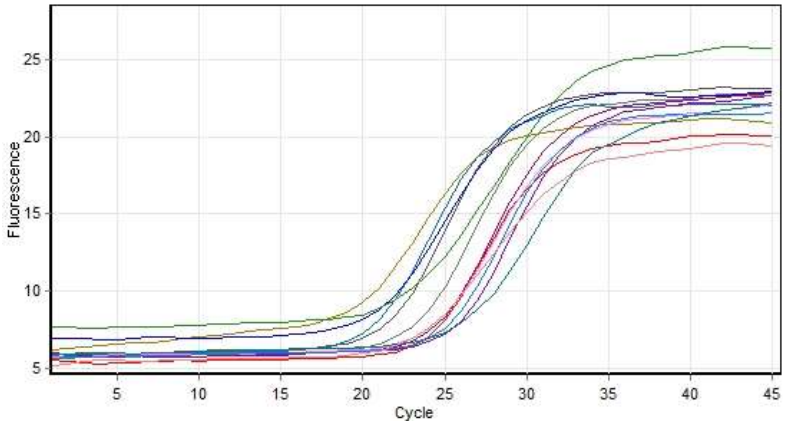

Figure 4.1. Expression results of *APOBEC3B* gene utilizing RT-PCR.

The *APOBEC3B* gene expression mRNA level was achieved from tumors in 49 tumor samples. Using Figure 4.2, we can examine the expression levels of each patient and identify those with differing expression values compared to 49 other normal samples.

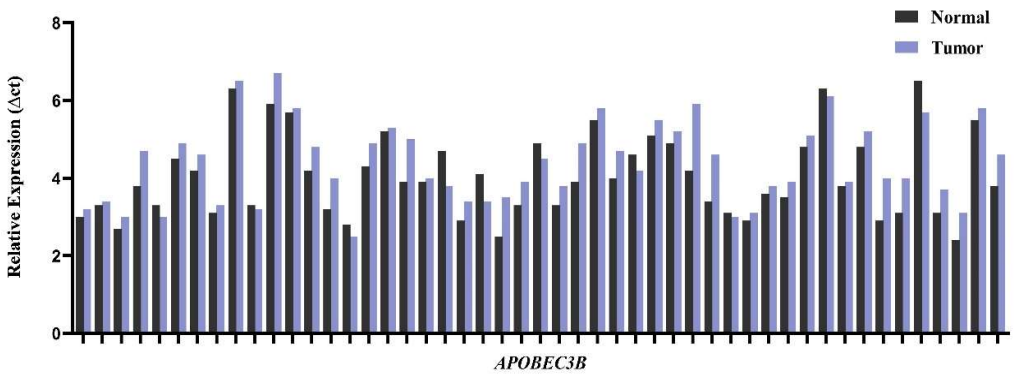

Figure 4.2. According to the ΔC_T of the *APOBEC3B* gene, the mRNA expression level of normal controls and tumors. Contrary to control measurements, the mRNA expression of cancer patients was found to be increased.

The lncRNA-*GAS5* expression results in 98 of samples were taken by RT- qPCR. The normalization process to the expression of the target has been provided by utilizing *GAPDH* as a housekeeping gene (Figure 4.3).

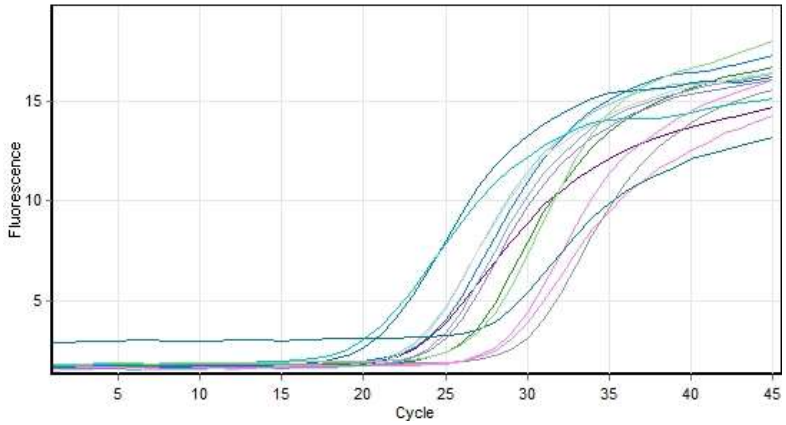

Figure 4.3. Expression results of lncRNA-*GAS5* gene utilizing RT-PCR.

The examination has been based on the uses of Real-Time qPCR to assess the value of the lncRNA-*GAS5* gene expression levels in these 49 tumor samples. Using Figure 4.4, the expression levels of each patient and compared to normal samples has been done.

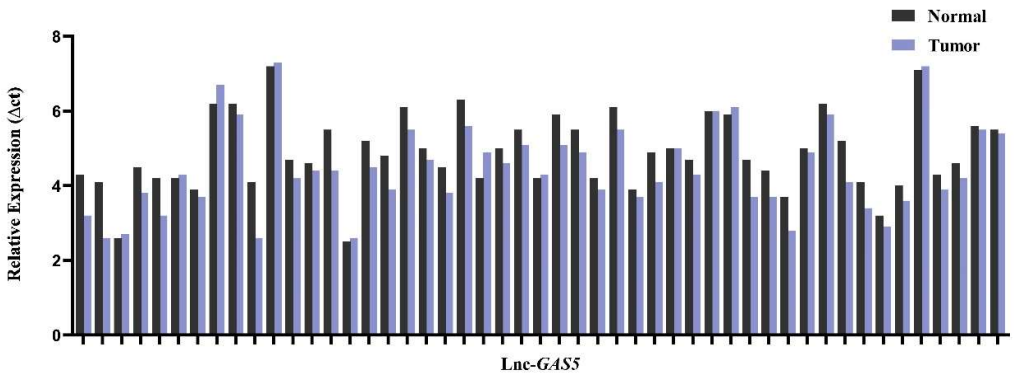

Figure 4.4. According to the ΔC_T of the lncRNA-*GAS5* gene, the expression level of normal controls and tumors. Contrary to control measurements, the RNA expression of cancer patients was found to be downregulated.

The *miR-103* expression results in 98 of the samples were conducted by RT- qPCR. Housekeeping *GAPDH* was used to normalize the reference expression (Figure 4.5).

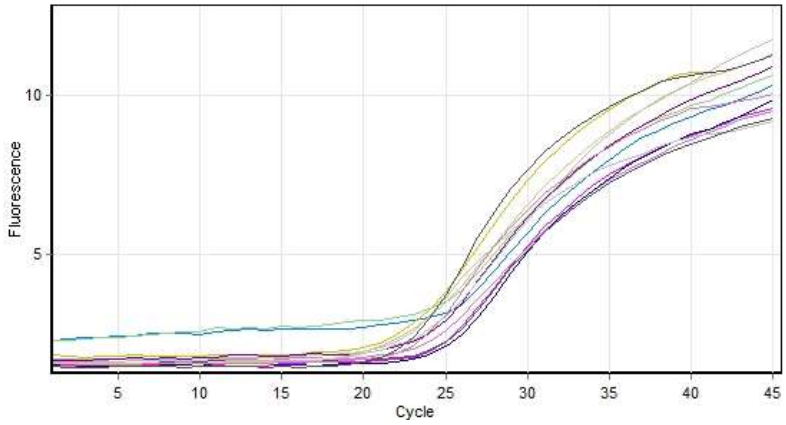

Figure 4.5. Expression results of *miR-103* gene utilizing RT-PCR.

The *miR-103* expression level was achieved from the breast tissues in 49 tumor samples. Using Figure 4.6, the expression levels of each patient and compared to normal samples has been done.

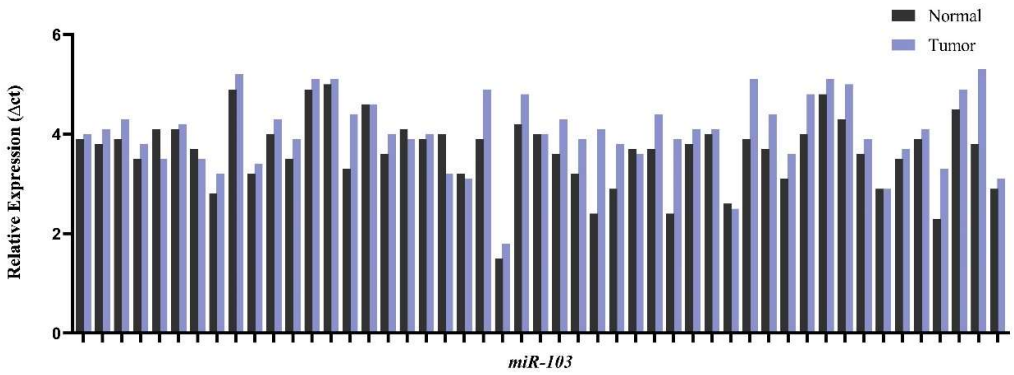

Figure 4.6. According to the ΔC_T of the *miR-103* gene, the expression level of normal controls and tumors. Contrary to control measurements, the RNA expression of cancer patients was found to be up-regulated.

Due to the exponential nature of PCR, fold change is calculated. It describes increasing or decreasing multiple between our targets in normal and tumor samples (Figure 4.7). The calculation was conducted as follows:

$\Delta\Delta C_T$ (Normal) = ΔC_T (target) − ΔC_T (average ΔCT Normal)

Fold change (Normal) = $2^{-\Delta\Delta CT\ (Normal)}$

Fold change (tumor) = $2^{-\Delta\Delta CT\ (Tumor)}$

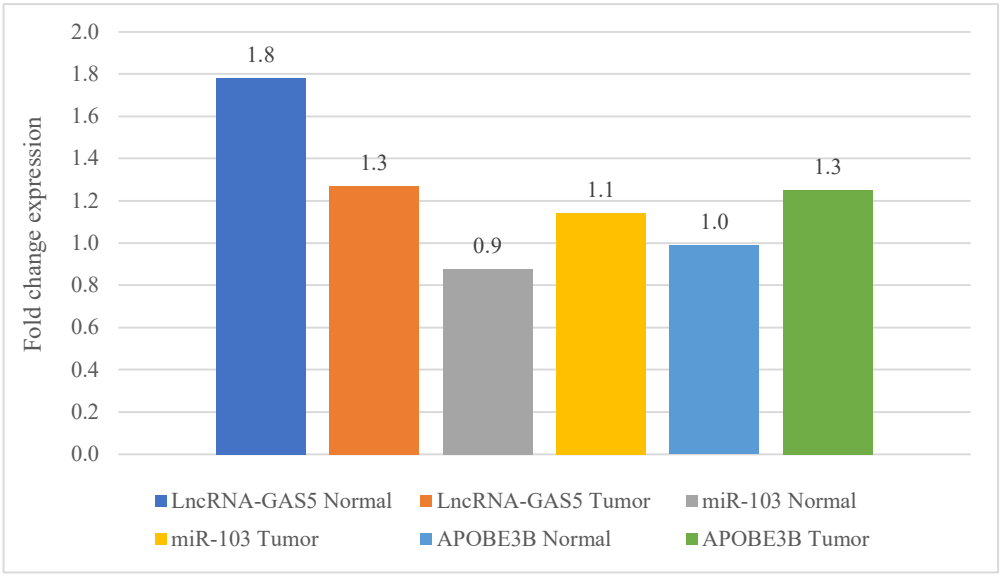

Figure 4.7. Expression results of lncRNA-*GAS5*, *miR-103* and *APOBE3B* in normal and tumor tissue.

4.2. Statistical Result

The expression levels of 98 samples were evaluated in female breast cancer patients. The control group was 49 samples, and the tumor group was 49 samples. Demographic characteristics of samples, and the statistical result of the targets; *APOBEC3B*, lncRNA-*GAS5* and *miR-103* according to patient's age (Table 4.1).

Table 4.1. Expression result of the targets.

Age	NO. (%)	lncRNA-*GAS5* Expression (Mean ± SD)		*miR-103* Expression (Mean ± SD)		*APOBEC3B* expression (Mean ± SD)		lncRNA-*GAS5* Expression (p-value)	*miR-103* Expression (p-value)	*APOBEC3B* Expression (p-value)
		Normal	Tumor	Normal	Tumor	Normal	Tumor			
25-40	15 (30.6)	4.4 ± 0.9	3.9 ± 09	3.6 ± 0.8	3.9 ± 0.8	3.6 ± 0.9	4.1 ± 0.9	0.00033	0.017	0.00037
41-55	19 (38.7)	4.9 ± 1.0	4.4 ± 1.3	3.5 ± 0.7	4.1 ± 0.5	4.1 ± 1.0	4.5 ± 1.1	0.0012	0.00015	0.00019
56-70	10 (20.4)	5.0 ± 0.7	4.6 ± 0.8	3.8 ± 0.5	4.0 ± 0.7	4.1 ± 0.8	4.2 ± 0.8	0.037	0.34	0.58
71-85	5 (10.2)	6.2 ± 0.6	6.0 ± 0.8	4.1 ± 0.9	4.3 ± 1.1	5.1 ± 1.7	5.0 ± 1.5	0.31	0.081	0.8

The results of RT- qPCR dependence expression quantities of *APOBEC3B* tumor samples were revealed that mRNA was increased proportionally to the expression level of normal samples. The findings are statistically significant (Age: 25-40; p=0.00037/ 41-55; p=0.00019, T-test; p<0.05). Expression quantities of lncRNA-*GAS5* tumor mammary samples were revealed that reduced proportionally to the lncRNA-*GAS5* expression level of normal samples. The findings are strongly significant (Age: 25-40; p=0.00033/ 41-55; p=0.0012/ 56-70; p=0.037, T-test; p<0.05). Expression quantities of *miR-103* mammary tumor samples were revealed that increased proportionally to the *miR-103* expression level of normal samples. The findings are statistically significant (Age: 25-40; p=0.017/ 41-55; p=0.00015, T-test; p<0.05) (Figure 4.8).

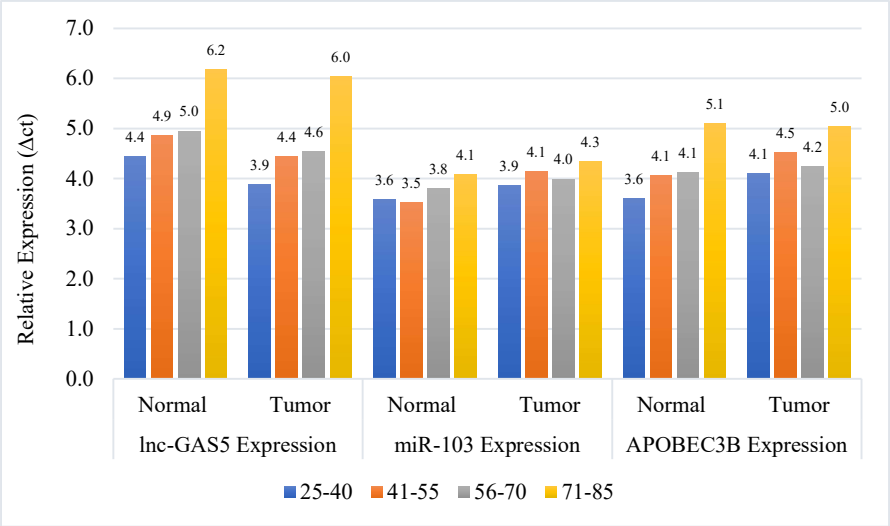

Figure 4.8. Expression result of the targets; *APOBEC3B*, lncRNA-*GAS5* and *miR-103* according to patient's age.

The expression levels of 98 samples were evaluated in female breast cancer patients. The control group was 49 samples, and the tumor group was 49 samples. Demographic characteristics of samples, and statistical result of the targets; *APOBEC3B*, lncRNA-*GAS5* and *miR-103* according to patient's grade (Table 4.2).

Table 4.2. Expression statistical result of targets according to sample grades.

Cancer Grades	NO. (%)	lncRNA-*GAS5* Expression (Mean ± SD)		*miR-103* Expression (Mean ± SD)		*APOBEC3B* expression (Mean ± SD)		lncRNA-*GAS5* Expression (p-value)	*miR-103* Expression (p-value)	*APOBEC3B* Expression (p-value)
		Normal	Tumor	Normal	Tumor	Normal	Tumor			
I	12 (26.5)	4.3 ± 0.9	3.9 ± 0.9	3.5 ± 0.8	3.9 ± 0.7	3.9 ± 1.0	4.0 ± 1.0	0.02	0.115	0.53
II	19 (38.7)	5.2 ± 0.8	4.7 ± 1.1	3.7 ± 0.9	4.0 ± 0.9	3.9 ± 1.0	4.3 ± 1.0	0.0002	0.0001	0.0007
II	17 (34.6)	4.9 ± 1.2	4.6 ± 1.3	3.7 ± 0.5	4.2 ± 0.5	4.4 ± 1.2	4.7 ± 1.0	0.002	0.0005	0.0029

The results of RT- qPCR dependence expression quantities of *APOBEC3B* tumor samples were showed that RNA was raised according to the expression level of normal samples. The findings are significant (Grade: II; p=0.0007/ III; p=0.0029, T-test; p<0.05). Expression quantities of lncRNA-*GAS5* tumor breast samples were revealed that reduced proportionally to the lncRNA-*GAS5* expression level of normal samples. The findings are statistically significant (Grade: I; p=0.02/ II; p=0.0002/ III; p=0.002, T- test; p<0.05). Expression quantities of *miR-103* mammary tumor samples were revealed that increased proportionally to the *miR-103* expression level of normal

samples. The findings are statistically significant (Grade: II; p=0.0001/ III; p=0.0005, T-test; p<0.05) (Figure 4.9).

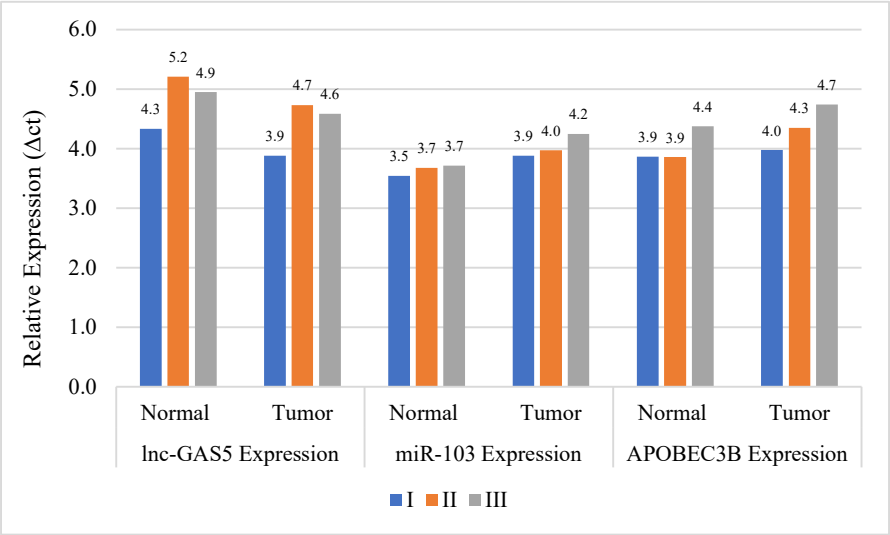

Figure 4.9.Expression result of the targets; *APOBEC3B*, lncRNA-*GAS5* and *miR-103* according to patient's cancer grade.

The expression levels of 98 samples were evaluated in female breast cancer patients. The group control was 49 samples, and the tumor group was 49 samples. Demographic characteristics of samples, and statistical result of the targets; *APOBEC3B*, lncRNA-*GAS5* and *miR-103* according to types of breast cancer. (Table 4.3).

Table 4.3. Expression statistical result of targets according to sample grades.

Cancer Types	NO. (%)	lncRNA-*GAS5* Expression (Mean ± SD)		*miR-103* Expression (Mean ± SD)		*APOBEC3B* expression (Mean ± SD)		lncRNA-*GAS5* Expression (p-value)	*miR-103* Expression (p-value)	*APOBEC3B* Expression (p-value)
		Normal	Tumor	Normal	Tumor	Normal	Tumor			
1	36 (73.4)	4.9 ± 0.9	4.5 ± 1.1	3.6 ± 0.7	4.1 ± 0.7	3.9 ± 0.9	3.9 ± 1.0	0.00001	0.000001	0.000001
2	3 (6.1)	4.4 ± 0.4	3.2 ± 0.7	3.6 ± 0.4	3.8 ± 0.3	3.8 ± 1.2	3.9 ± 1.2	0.022	0.11	0.52
3	2 (4)	4.4 ± 2.6	4.3 ± 2.3	4.1 ± 1.1	4.8 ± 0.6	4.6 ± 2.5	4.5 ± 2.8	0.7	0.33	0.87
4	4 (8.1)	4.2 ± 0.3	3.8 ± 0.4	3.8 ± 0.2	3.7 ± 0.4	4.8 ± 1.1	4.4 ± 0.6	0.15	0.56	0.31
5	4 (8.1)	5.9 ± 1.2	5.8 ± 1.4	3.4 ± 1.2	3.8 ± 1.1	4.8 ± 1.9	4.6 ± 1.5	0.54	0.22	0.69

1) Invasive ductal carcinoma 2) Invasive ductal carcinoma, medullary likes 3) Matrix producing metaplastic carcinoma
4) Metaplastic carcinoma, matrix producing 5) Invasive lobular carcinoma

The RT-qPCR expression quantities results of *APOBEC3B* tumor samples were revealed that RNA was raised according to the expression level of normal samples. The findings are statistically significant (Tumor type: Invasive ductal carcinoma; p=0.000001, T test; p<0.05). Expression quantities of lncRNA-*GAS5* tumor breast samples were revealed that reduced proportionally to the lncRNA-*GAS5* expression level of normal samples. The findings are statistically significant (Tumor type: Invasive ductal carcinoma; p=0.00001/ Invasive ductal carcinoma, medullary likes; p=0.022, T-test; p<0.05). Expression quantities of *miR-103* mammary tumor samples were revealed that increased proportionally to the *miR-103* expression level of normal samples (Tumor type: Invasive ductal carcinoma; p=0.000001, T test; p<0.05) (Figure 4.10).

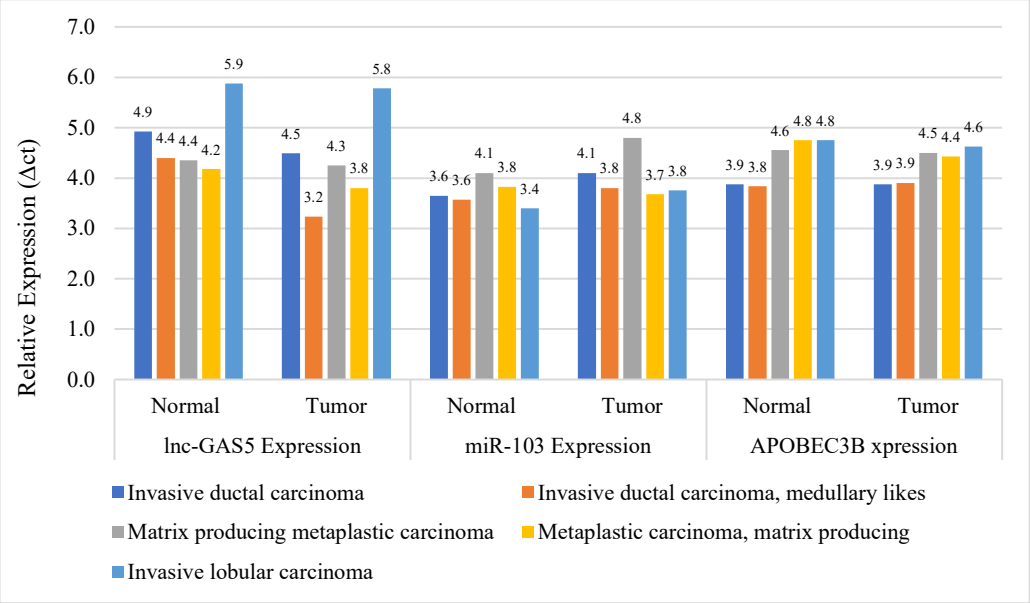

Figure 4.10. Expression result of the targets; *APOBEC3B*, lnc-*GAS5,* and *miR-103* according to patient's cancer type.

Statistically, we have evaluated the relationship between demographic characteristics of 49 cancer patients. Table (4.4) and figure (4.11) are shown a statistical comparison of breast cancer patients with the grade, cancer type, and age.

Table 4.4. The relationship between grade, type of patients who have breast cancer.

One Way ANOVA Multiple Comparison Test	Significant	Summary	Adjusted P-Value
Grade Vs. Cancer Type	No	NS	0.9957
Grade Vs. Age	Yes	****	<0.0001
Cancer Type Vs. Age	Yes	****	<0.0001

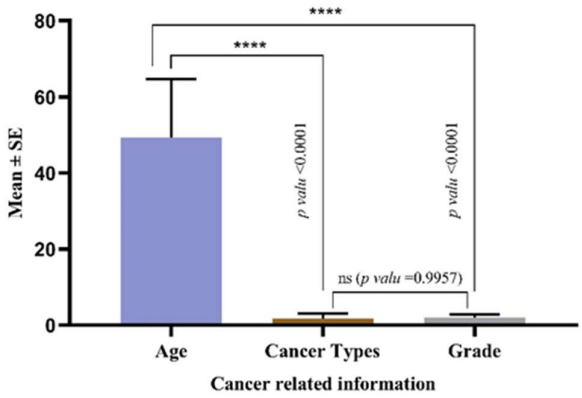

Figure 4.11. Statistical comparison of breast cancer patients with the grade, cancer type, and age.

It is showed in Figure 4.11 that a correlation between age and type of cancer and grade highly significant. On the other hand, the correlation between the type of cancer with a grade of cancer not significant.

4.3. Expression correlation of *APOBE3B*, lncRNA-*GAS5*, and miRNA 103

To determine the influences of lncRNA-*GAS5* in breast cancer cells, we studied the relative expression of lncRNA-*GAS5* by qRT-PCR techniques in tissue samples taken from breast cancer patients and tissue samples taken from healthy controls. In patients with breast cancer, lower lncRNA-*GAS5* expression was found (Figure 4.6). In addition, in the patient's cells and control cells, we identified *APOBEC3B* expression. The expression of *APOBE3B* in patients with breast cancer tissues was also up-regulated, as seen in figure 4.3. A correlation analysis was conducted between lncRNA-*GAS5* Expression and *APOBE3B* to explore the link between *APOBE3B* and lncRNA-*GAS5* in breast cancer. lncRNA-*GAS5* has a negative correlation with *APOBE3B* expression. Figure 4.12 demonstrated the relation between *APOBE3B* and lncRNA-*GAS5*.

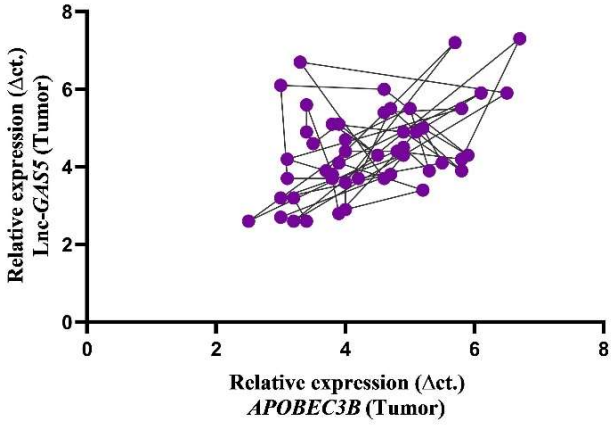

Figure 4.12. Evaluating the association between lncRNA-*GAS5* and *APOBE3B* gene expression in breast cancer patients.

Previously, according to the bioinformatics research online platform, lncRNA-*GAS5* was anticipated to be bound to *miR-103* (Guo et al., 2015). In this study, the expression of *miR-103* in breast cancer patients' tissue and health controls was further analyzed. Also, we found that in patients with breast cancer tissue, *miR-103* was significantly up-regulated (Figure 4.9). Moreover, the study also indicates that the expression of lncRNA-*GAS5* was negatively correlated to *miR-103* (Figure 4.13).

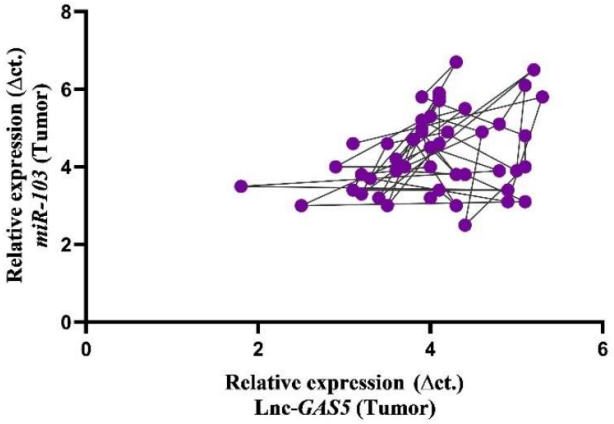

Figure 4.13. Exploring the relationship between lncRNA-*GAS5* and *miR-103* gene expression in patients with breast cancer.

4.4. Mutation screening results

APOBEC3B gene's mRNA sequence was screened. The investigation was carried to see whether distinct sorts of genotypes existed. Nucleotide sequencing was conducted to observe the coding sequence of the *APOBE3B* gene without an untranslated region. Without the untranslated region, nucleotide sequencing was performed to examine the coding sequence of the *APOBC3B* gene (Figure 4.14). The NCBI website has been used to find the sequences of DNA of the *APOBEC3B* gene to compare the generated sequences (Query sequence) to the referral sequence of *APOBEC3B* genes.

```
ORIGIN
        1 actttaagga gggctgtcca actgcaagga cgctgtaagc aggaagtgaa accacagagc
       61 ttcaaaaaaa gagcgggaca gggacaagcg tatctaagag gctgaacatg aatccacaga
      121 tcagaaatcc gatggagcgg atgtatcgag acacattcta cgacaacttt gaaaacgaac
      181 ccatcctcta tggtcggagc tacacttggc tgtgctatga agtgaaaata aagaggggcc
      241 gctcaaatct cctttgggac acaggggtct ttcgaggcca ggtgtatttc aagcctcagt
      301 accacgcaga aatgtgcttc ctctcttggt tctgtggcaa ccagctgcct gcttacaagt
      361 gtttccagat cacctggttt gtatcctgga ccccctgccc ggactgtgtg gcgaagctgg
      421 ccgaattcct gtctgagcac cccaatgtca ccctgaccat ctctgccgcc cgcctctact
      481 actactggga aagagattac cgaagggcgc tctgcaggct gagtcaggca ggagcccgcg
      541 tgacgatcat ggactatgaa gaatttgcat actgctggga aaactttgtg tacaatgaag
      601 gtcagcaatt catgccttgg tacaaattcg atgaaaatta tgcattcctg caccgcacgc
      661 taaaggagat tctcagatac ctgatggatc cagacacatt cactttcaac tttaataatg
      721 acccttggt ccttcgacgg cgccagacct acttgtgcta tgaggtggag cgcctggaca
      781 atggcacctg ggtcctgatg gaccagcaca tgggctttct atgcaacgag ttggacccgg
      841 cccagatcta cagggtcact tggttcatct cctggagccc ctgcttctcc tggggctgtg
      901 ccggggaagt gcgtgcgttc cttcaggaga acacacacgt gagactgcgc atcttcgctg
      961 cccgcatcta tgattacgac cccctatata aggaggcgct gcaaatgctg cgggatgctg
     1021 gggcccaagt ctccatcatg acctacgatg agtttgagta ctgctgggac acctttgtgt
     1081 accgccaggg atgtcccttc cagccctggg atggactaga ggagcacagc caagccctga
     1141 gtgggaggct gcgggccatt ctccagaatc agggaaactg aaggatgggc ctcagtctct
     1201 aaggaaggca gagacctggg ttgagcagca gaataaaaga tcttcttcca agaaatgcaa
     1261 acagaccgtt caccaccatc tccagctgct cacagacacc agcaaagcaa tgtgctcctg
     1321 atcaagtaga ttttttaaaa atcagagtca attaatttta attgaaaatt tctcttatgt
     1381 tccaagtgta caagagtaag attatgctca atattcccag aatagttttc aatgtattaa
     1441 tgaagtgatt aattggctcc atatttagac taataaaaca ttaagaatct tccataattg
     1501 tttccacaaa cacta
```

Figure 4.14. The *APOBE3B* gene mRNA sequence, a coding area shown in brown.

4.5. *APOBEC3B* mutation result

It is known that the National Institutes of Health (NIH) has provided the NCBI website. In the modern study, the researchers can use it to gain a particular sequence as a reference to find any variation. The DNA sequences of patients (Query Sequence) generated have been compared with the reference sequence. Furthermore, in comparison to the reference sequence, no alteration was identified in the PCR template sequence for 20 target areas (GenBank sequence). They are completely identical. The partial sequence findings were indicated and revealed in Figure 4.15 without any variance.

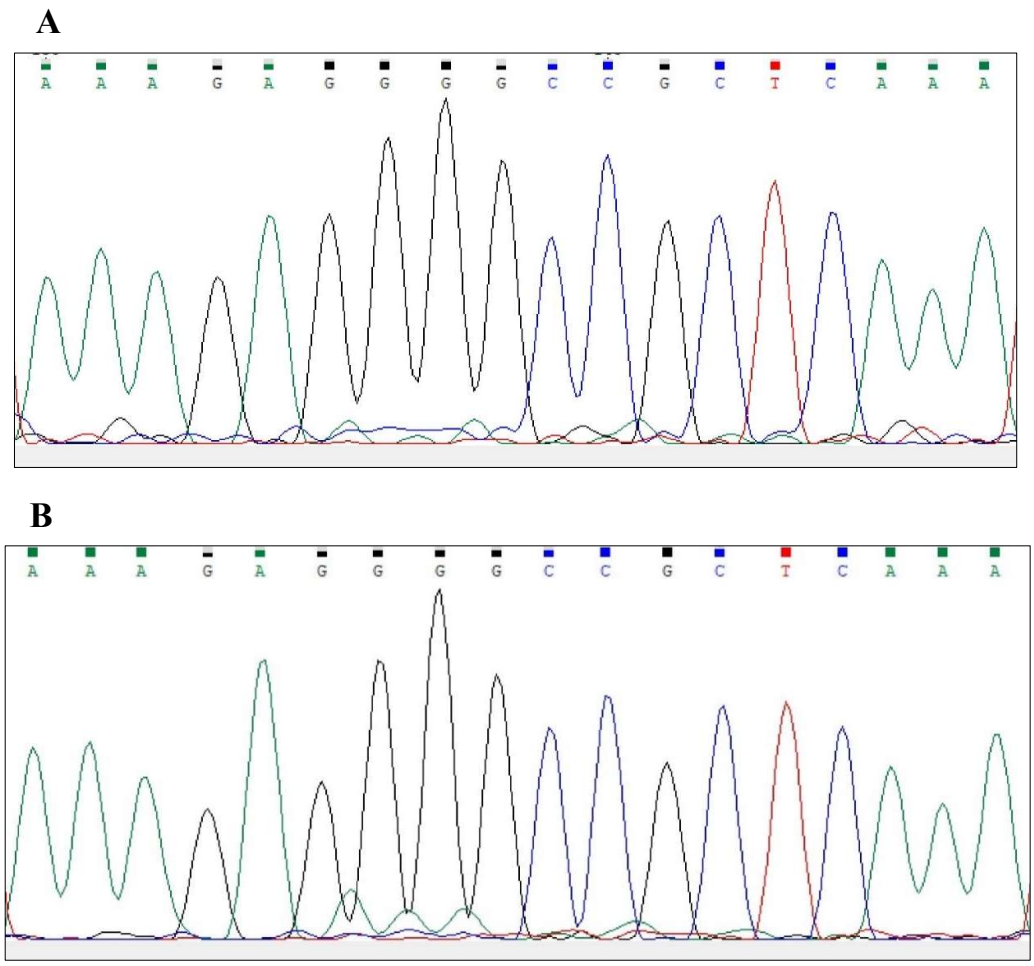

Figure 4.15. *APOBEC3B* Gene Selective Sequence Result**. A)** The normal *APOBEC3B* gene sample was analyzed through sequencing. **B)** The *APOBEC3B* tumor sample gene was examined through sequencing, and no mutation (variation) was detected.

CHAPTER V

5. Discussion

We performed an expression analysis for lncRNA-*GAS5* and *miR-103* to examine the expression levels of *APOBEC3B* in breast cancer patients utilizing RT-qPCR. Also, the relationship between lncRNA-*GAS5* and *miR-103* has been studied. The mRNA coding area of the *APOBEC3B* genome was screened using a genetic analyzer to identify potential mutations. After new molecular technology tools were discovered, clinically, breast cancer has been cleared more than single disease, rather than a group of disorder with different molecular frameworks that influence treatment responses and patient survival ability (Rivenbark *et al.*, 2013). Breast cancer originates in a particular breast region, with additional morphological features, such as the ducts, lobules, or the tissue between, which can become malignant (Feng *et al.*, 2018). There are two aspects of deep understanding sites in the molecular investigates of breast cancer, profiling mRNA expression or studies of DNA copy number, and most lately, massively parallel sequencing.

Regarding those, just one or both of them have concentrated (Koboldt *et al.*, 2012). A significant range of attempts has recently been introduced in breast cancer biology to establish novel parameters and principles. Since recognizing that only one aspect of the equation includes the protein-coding, non-coding RNAs have become a modern field to address numerous breast cancer concerns that have emerged. RNAs that are never translated into a protein have now been established as a potential key appearing in cancer pathogenesis (Cerk et al., 2016). In several types of cancer, including breast cancer, dramatic change of expression in non-coding RNAs has been reported in multiple investigations.

miRNAs have been defined as short ncRNAs that can modulate gene expression, which their roles after transcription can be observed. It is highlighted in breast carcinoma that miRNAs interfere with the gene target signaling pathway (Gebert & MacRae, 2019). The existence of miRNAs in many cellular processes has recently been confirmed. Deviant miRNA expression is also linked to diseases like cancer (Frankel *et al.*, 2008). By manipulating other genes, for instance, some miRNAs indirectly influenced particular target signaling. In this regard, miR-221/222 facilitated proliferation in breast cancer by restraining p53 increase apoptosis expression

modulator (Chen *et al.*, 2013). Depending on the ability to inhibit multiple gene activities, including p53 that suppress tumors and facilitate tumor cell development, invasion, and metastasis, miR-21 is also regarded as an oncomiR (Qian *et al.*, 2009). It has been considered that *TP53* expression is negatively regulated by another group of miRNAs, including miR-504 and miR-125b (Hu *et al.*, 2010; Le *et al.*, 2009).

As already stated, a considerable amount of literature has been published on deregulated non-coding RNAs in a different type of cancer. In comparing distinct patterns of expression between normal and patient breast tissue, multiple lncRNAs also have been reported (Cerk et al., 2016). For instance, trust in next-generation sequencing in the HER-2-enriched subtype of breast cancer, Yang *et al.* (2016) has recognized more than 1300 lncRNAs that display substantially aberrant expression patterns. On the other hand, Peng *et al.* (2017) screened out 2,178 differently expressed lncRNAs and were associated with ER-positive subtype breast cancer using the microarray technique. (Shen *et al.*, 2015) also observed that over 1750 lncRNAs were represented differently in triple-negative breast cancer (TNBC) (Shen *et al.*, 2015). These findings strongly suggest that aberrant expression patterns of lncRNAs in breast could play a significant role in carcinogenesis.

Humans have 11 *APOBEC* gene family members, namely A1, A2, A3A, A3B, A3C, A3D, A3F, A3G, A3H, A4, AID, or AICDA (W. K. Xu et al., 2020). They are encoded Zn^{2+}-dependent DNA cytosine deaminases that deaminate cytidine to uridine in DNA and RNA (Y. Zhang et al., 2015). Although the genes have been known clusters, they are located in a different location on chromosomes. For example, the A1, A4, and AID genes are present on chromosome 12, respectively. However, the A2 gene located on chromosome 6. Simultaneously, the rest of the others reside at the chromosomal loci 22q13 (Jarmuz *et al.*, 2002; Revathidevi *et al.,* 2021; Smith, 2017). One of the most significant contributions of the APOBEC family proteins, which have various types of DNA cytosine deaminase domains and different tissue expression profiles, suggests that they are controlling diverse biological process, including multiple actions to limit viral restriction and prevent retrotransposition of L1 and Alu elements in human cells (Burns *et al.,* 2015; Y. Zhang *et al.*, 2015).

The *APOBE*C family genes are frequently deregulated, and their overexpression induces genomic instability in cancer cells (Goila-Gaur & Strebel, 2008). It is observed that *APOBEC3A* expression levels in breast cancer tissues pretended to be higher than

in healthy breast tissues. Several expression studies have indicated the ability of gene expression models to differentiate between histologic subtypes of breast cancer (Kim *et al.*, 2020). However, distinct mutational forms and genomic modifications have also been documented in breast cancer subtypes, indicating that APOBEC-mediated mutagenesis and family expression of *APOBEC* may differ in cancer subtypes (Burns, Lackey, *et al.*, 2013; Olgun *et al.*, 2018; Petljak & Maciejowski, 2020).

In our study, consistent with the previous findings, the mRNA expression level of the *APOBEC3B* gene was elevated (up-regulated). The T-test was statistically considerable significant; $p<0.05$. According to a demographic study, the expression *APOBEC3B* statistically significant in the first two age categories (Table 4.1), Grade II, III (Table 4.2), and Invasive ductal carcinoma cancer type based (Table 4.3). *APOBEC3B* upregulation has also been proven in multiple forms of cancer (Burns, Temiz*, et al.*, 2013b; Gara *et al.*, 2020; Zou et al., 2017). Besides that, *APOBEC3B* has been implicated in the close association of somatic base-substitution mutation with *APOBEC3B* mRNA levels in cancer samples as an enzymatic trigger that induces C-to-T somatic mutations (Brandon Leonard et al., 2015). Many basic diagnostic indicators have been shown through gene expression research. Our expression analysis revealed that expression patterns of *APOBEC3B* were associated with tissues of breast cancer and clinical outcome, giving an additional inference that other genes of the *APOBEC* family member may lead to aberrant and clinical outcome expression in subtypes of breast cancer.

The key factors contributing to a shift in gene expression of mRNA are epigenetic influences, DNA hyper-methylation, histone alteration, and non-coding RNA. One of the vital factors contributing to low-expression gene expression is DNA hyper-methylation, especially in the promoter region (Moore & Chang, 2010). The epigenetic profile of the activation and inactivation of participants in the family of *APOBEC* in breast cell lines has been established according to Y. Zhang et al. (2015) (Y. Zhang et al. (2015). As their findings revealed, the *APOBEC3B* gene was poor relative to normal breast cell lines, whereas other members of *APOBEC* were not expressed or controlled in breast cancer cell lines (Moore & Chang, 2010). In their research, 74 epigenetic markers, including H3K4me3, H3K27ac, and H3K36me3, were triggered in the *APOBEC3* genes, both in the ER+ and ER-breast cancer cell lines (Moore & Chang, 2010).

Furthermore, all other *APOBEC* family members display DNA hypermethylation in the ER-cell line at their promoters, which could lead to their loss of expression or down-regulation (Tsuboi *et al.*, 2016). It was shown that DNA methylation and histone alteration might control gene expression in *APOBEC* family members in the breast cancer cell line (Cortez *et al.*, 2019). The microenvironment of cell lines also resulted in gene expression levels. It is found that *APOBEC3B* mRNA expression differed greatly in ovarian cancers. Even though, as a command, relative to normal ovarian tissue, it was substantially up-regulated. In contrast, lower levels of downregulated *APOBEC3B* in ovarian cancer than in normal ovaries have been found in the tumor or are improperly articulated in cells that eventually develop into tumors. This feature is further influenced by changes in the microenvironment that arise during tumor growth (Leonard *et al.,* 2013). Also, non-coding RNAs are considered to play a crucial part in cell cycle regulation and apoptosis; they may both act as genes that inhibit tumors when others are organized as oncogenesis (Ghafouri-Fard *et al.*, 2020; Slack & Chinnaiyan, 2019).

The expression level of lncRNA-*GAS5* was down-regulated in this study. Statistically, the T-test was strongly significant; $p<0.05$. However, according to a demographic study, the expression LncRNA-*GAS5* statistically significant in the first three age categories (Table 4.1), Grade I, II, III (Table 4.2), and invasive ductal carcinoma cancer; Invasive ductal carcinoma, medullary likes type based (Table 4.3). The role of lncRNA-*GAS5* in human cancers was first investigated in breast cancer reported that lncRNA-*GAS5* is down-regulated in breast cancer tissues and (Mourtada-Maarabouni *et al.*, 2009). LncRNA-*GAS5* is commonly known to be down-regulated in multiple cancers, and reduced expression levels also suggest poor prognosis in cancer patients (McCann *et al.*, 2020). In addition, in numerous cell types, particularly breast cancer cells, lncRNA-*GAS5* encourages cell replication and/or apoptosis, and its tumor suppressor function is suggested by its suppression of breast tumor development (Pickard & Williams, 2014).

As has been known, lncRNA-*GAS5* is a tumor suppressor gene, a highly expressed gene in growth-arrest cells. lncRNA-*GAS5* acts in various molecular mechanisms such as transcriptional regulation by working as a decoy and histone methylation/demethylation. Also, can act as sponge function in different type miRNAs (Ji *et al.*, 2019). The normal level of lncRNA-*GAS5* plays an essential role in the maintenance of cell proliferation,

apoptosis. Although the precise molecular mechanisms have not been fully known, lncRNA-*GAS5* plays an important role in carcinogenesis, tumor progression, and invasion. Recent studies have demonstrated that the lncRNA-*GAS5* was pervasively down expressed in most human cancers compared with adjacent non-cancerous tissues, including gastric, breast, lung, and prostate cancer (Yu & Li, 2015). The downregulation of LncRNA-*GAS5* in breast cancer tissue samples is associated with a large tumor volume, advanced tumor lymph node metastasis, and estrogen receptor negativity ((Yang *et al.*, 2020). In addition, it was also reported that lncRNA-*GAS5* is down-regulated in breast cancer and that it negatively impacts disease prognosis (Lambrou *et al.*, 2020). LncRNA-*GAS5* overexpression contributes to growth inhibition in cancer cells (Li *et al.*, 2019). It is observed that overexpressed lncRNA-*GAS5* could significantly increase the apoptosis rate of TNBC cells.

Furthermore, they have also found that the up-regulated lncRNA-*GAS5* may promote the chemosensitivity of TNBC cells, which could be an efficient treatment of TNBC and use as an anticancer. Morris and Mattick (2014) observed that amounts of lncRNA-*GAS5* were reduced in trastuzumab-resistant SKBR-3/Tr cells and trastuzumab-treated breast cancer tissue. lncRNA-*GAS5* knockdown improved in vivo cell increase and tumor development and low histological grade and progressed TNM stage associated levels of lncRNA-*GAS5*. It is suggested that trastuzumab lowers LncRNA-*GAS5* and that trastuzumab-resistant breast cancer can serve as a tumor suppressor (Cerk et al., 2016; Yang et al., 2020).

Although the vital role of miRNAs in various cellular processes and interactions with other molecules, Multiple microRNAs (miRNAs) have recently been shown to facilitate tumorigenesis or metabolic disorders by reducing gene-target expression, for instance, on the gene expression level, miR-103 was a contributor to colorectal cancer (Guo et al., 2015; Shandilya *et al.*, 2014). Strongly shown the abnormal over-expression of *miR-103* involves endometrial neoplasia first by Boren *et al.* (2008) and depends on valuable evidence in their study according to *miR-103* expression. After a thorough further examination of the matter, it was reported that in various forms of cancer, *miR-103* was greatly amplified. It has been observed that ten different miRNAs, including *miR-103*, were particularly elevated in bladder cancer (Yu et al., 2012). Besides this, another study shows that *miR-103* induced the development and invasion of endometrial cancer cell lines by downregulating the tumor suppressor

TIMP-3 expression post-transcriptionally (Qian *et al.*, 2016). In contrast, *miR-103* is down-regulated in prostate cancer, according to Fu *et al.* (2016) is related to tumor proliferation and migration. As a result, their findings show that *miR-103* is a proto-oncogene miRNA that can inhibit prostate cancer. As seen in Figure 4.7, the expression level of *miR-103* was up-regulated in this research. By T-test, it was statistically significant; $p<0.05$. However, according to a demographic study, the expression *miR-103* statistically significant in the first two age categories (Table 4.1), Grade II, III (Table 4.2), and Invasive ductal carcinoma cancer type based (Table 4.3). Our findings are close to those of Chang *et al.* (2016) who discovered that breast cancer patients had up-regulated expression of *miR-103a-3p*. According to previous research, *miR-103*'s function in cancer is more likely to be that of an oncomiR rather than a tumor suppressor (Chang et al., 2016). In breast cancer, high expression of *miR-103* has been linked to metastasis, tumor relapse, and poor prognosis (Xiong *et al.*, 2017).

In the present research, we found that in breast cancer cells, lncRNA-*GAS5* was downregulated. in addition, the expression of *miR-103* was significantly increased in tumors cells. Besides, the expression of *APOBEC3B* in patient samples was upregulated. Regarding demographic study for 49 samples, the level expression of targets is not significant in all categories. Still, it is most significant according to cancer grades. However, the expression level of targets significant just only in invasive ductal carcinoma. Several recent studies confirmed that lncRNA-*GAS5* regulates signaling pathways in the different models (Zhou & Chen, 2020). LncRNA-*GAS5* can act as a decoy, where it can behave as a molecular sponge that binds directly to the target, thereby blocking or inhibiting functions (Qian et al., 2016). For example, lncRNA-*GAS5* could bind to *miR-103* to prevent its inhibitory action on PTEN in endometrial cancer cells through inhibiting the expression of *miR-103*. Through transfecting lncRNA-*GAS5* plasmid or si-*GAS5* into cancer cells, the mRNA level of *miR-103* was significantly decreased or increased, respectively (Guo et al., 2015). We found that *miR-103* is not caused to decrease the mRNA levels of *APOBEC3B* during reduced expression of lncRNA-*GAS5* in breast cancer cells. Therefore, it is possible to present another factor that interferes with regulating *APOBEC3B* expression, which *miR-103* may control. In this context, also we predict that the high-level expression of *miR-103* indirectly factors to *APOBEC3B* up-regulation. It is necessary to more investigation in innovative approaches to clarify this mystery.

Nucleotide sequencing was used to investigate the coding sequence regions of the *APOBEC3B* gene in this research. However, no mutations were detected in this coding area (Figures 4.13 and 4.14), which is compatible with previous findings. Recently, it has been established that a significant positive correlation between the number of C>T/G>A mutations per tumor exome and *APOBEC3B* mRNA levels is observed in breast cancers. It is identified that the amount of C>T/G>A mutations per tumor exome is significantly positive with *APOBEC3B* mRNA levels in breast cancers (Y. Zhang et al., 2015).

CHAPTER VI

6. Conclusions, Recommendations

6.1. Conclusion

This research was conducted to determine the expression association of the APOBEC3B gene and lncRNA-*GAS5* through evaluation of *miR-103* expression level in both breast cancer patients and normal groups. The findings clearly indicate that *APOBEC3B* and *miR-103* expression levels were significantly elevated in breast cancer patients, whereas lncRNA-*GAS5* expression levels were significantly decreased. In breast cancer cells, a negative association between *APOBEC3B* and lncRNA-*GAS5* and a positive correlation between *APOBEC3B* and *miR-103* have been discovered. Also, this study investigated the expression levels of targets according to demographic characters. The statistical results show that the same significance was observed of the molecules (lncRNA-*GAS5*, *APOBEC3B*, *miR-103)* according to age, grade of cancer and cancer type. The coding sequence regions of the APOBEC3B gene was analyzed by nucleotide sequencing analysis. However, no mutation was observed in this coding region in the similarly of previous studies.

6.2. Recommendations

Manipulating the lncRNA-*GAS5* and/or *miR-103* expression may be a new therapeutic target for breast cancer treatment. Additional study is required further to understand the interaction between breast tumors and biomarkers. Also, it was found that *miR-103* is not caused to decrease the mRNA levels of *APOBEC3B* during reduced expression of lncRNA-*GAS5* in breast cancer cells as predicted in this study. Therefore, it is possible to present another factor that interferes with regulating *APOBEC3B* expression, which *miR-103* may control. It would be interesting to do further investigation to answer this question.

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APPENDICES

Appendix A

Letter of Coollaboration

Dr. Rozhgar A. KHAILANY
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----------*April 22th 2020*

Dear Prof. Dr. Rasime Kalkan,

I am pleased to write to you regarding the "Thesis Collaboration" of one of your students in the department of Medical Biology and Genetics. I would like to inform you that I will be helping (Omed Qadir Ibrahim) by providing my Genetics lab and supplying all the necessary chemical agents and materials which have been recommended by (Prof. Dr. Mustafa Saber Al-Attar). The list of items is prepared along with this letter.

His thesis topic is: **"The association of the expression of LncRNA-GAS5 and its Target APOBEC3B Gene through miR-103 in Breast Cancer Patients"** which is related to my specialty; therefore, my lab is enough equipped and suitable for his experiments and investigation.

As an academic integrity, I would to declare that I have no commercial or personal interest in this project. It is only an academic contribution that I would humbly offer to this student.

Sincerely
Dr. Rozhgar A. KHAILANY

Zheen International Hospital
Adminstration

List of Chemical Agents and materials

	Chemical materials	Brands	Manufacturers
1	Ethidium bromide	Sigma	Germany
2	Safe stain	GenetBio	Korea
3	Isopropanol	Sigma	Germany
4	Binding Silane	Promega	Germany
5	Gelsave	Merck	Darmstadt,Germany
6	MgCl2	Fermentase	AB
7	Tris	Fermentase	AB
8	dNTP	Fermentase	AB
9	Taq DNA polymerase	Fermentase	AB
10	Agarose	Prona	Madrid, Spain
11	EDTA	Merck	Darmstadt,Germany
12	100 bp marker	Fermentase	AB
13	Bromofenol Blue	Merck	Darmstadt,Germany
14	Orange G	Sigma	Germany
15	Ethyl alcohol	Sigma	Germany
16	Sephadex G-50	Sigma	Sweden
18	BigDye	Invitrogen	USA
19	RNA extraction kit	Qaigen	German
20	cDNA synthesis kit	Qaigen	German
21	Syber RT-PCR kit	Qaigen	German

Appendix B

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

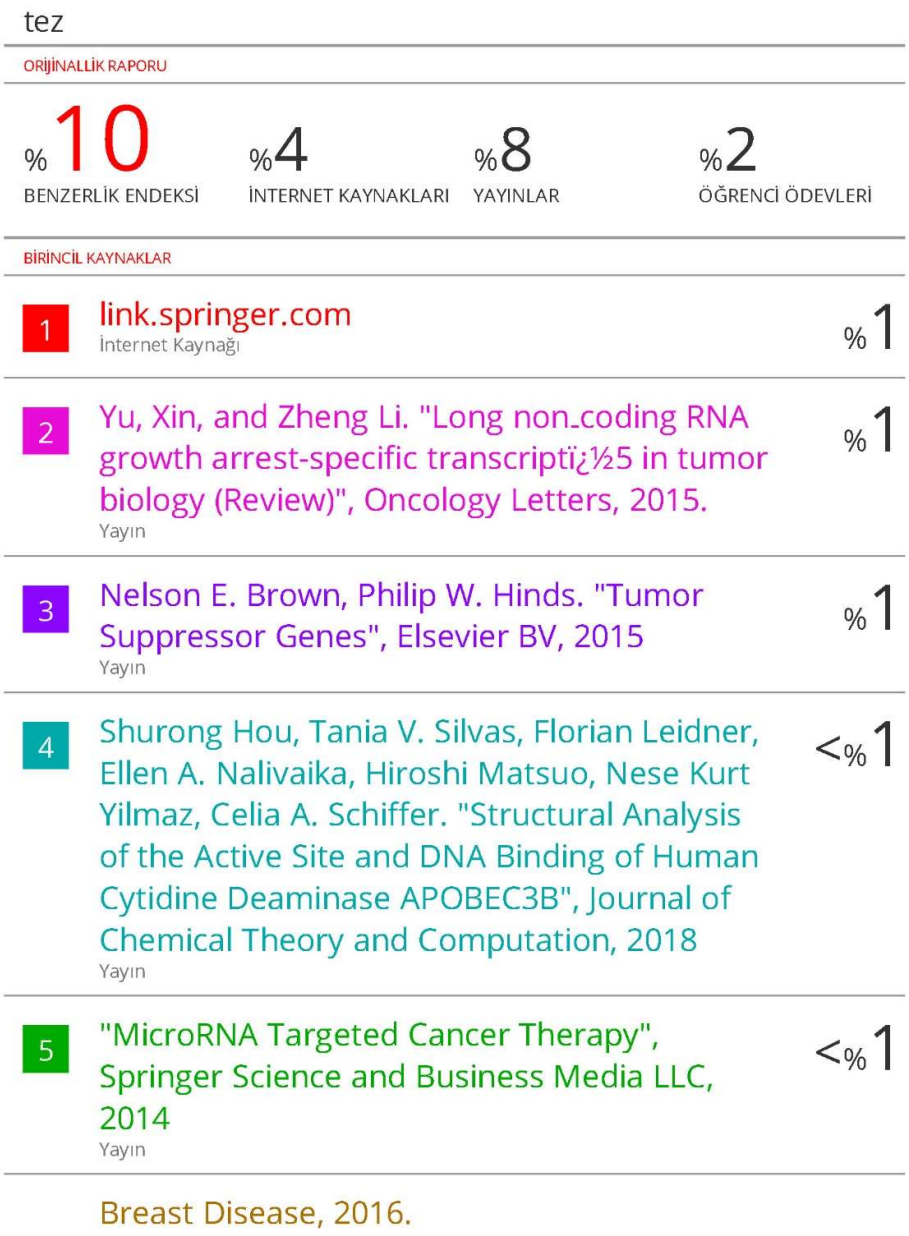
Toplantı Tarihi : 28.05.2020
Toplantı No **:** 2020/79
Proje No **:**1075

Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Rasime Kalkan'ın sorumlu araştırmacısı olduğu, YDU/2020/79-1075 proje numaralı ve **"The interaction between expression of LncRNA-GAS5 and its target gene APOBEC3B in Breast Cancer Patients."** başlıklı proje önerisi kurulumuzca online toplantıda değerlendirilmiş olup, etik olarak uygun bulunmuştur.

Prof. Dr. Rüştü Onur
Yakın Doğu Üniversitesi
Bilimsel Araştırmalar Etik Kurulu Başkanı

Appendix C

Turnitin Similarity Report



tez

ORİJİNALLİK RAPORU

%10	%4	%8	%2
BENZERLİK ENDEKSİ	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCİ ÖDEVLERİ

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CURRICULUM VITAE

1. PERSONAL INFORMATION

NAME, SURNAME: DATE of BIRTH and PLACE:	Omed Qadir Ibrahim 1/1/1984 Sulaymaneyah
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2. EDUCATION

YEAR	GRADE	UNIVERSITY	FIELD
2008/2009	%66.571	Salahaddin University-Erbil	Biology

3. ACADEMIC EXPERIENCE

PERIOD	TITLE	DEPARTMENT	UNIVERSITY

4. FIELD OF INTERESTS

FIELDS OF INTERESTS	KEYWORDS
Medical Genetics	Genetics, Molecular Genetics, Epigenetics, RNA Biology, Molecular Cytogenetics, Cancer, Biotechnology, Embryology, Prenatal Genetics, Bioinformatics

5. SELECTED PUBLICATIONS OF THE LAST 5 YEARS

- None