TURKISH REPUBLIC OF NORTHERN CYPRUS

NEAR EAST UNIVERSITY

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APPROVAL

We certify that we have read the thesis submitted by Malik Suleman Naseem titled "**THE EFFECT OF LIQUID OZONE ON THE WNT/BETA-CATENIN PATHWAY GENES IN MCF-7 BREAST CANCER CELL LINE**" and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Health Sciences.

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STATEMENT (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.

Malik Suleman Naseem

26/07/2022

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ABSTRACT

Aim: Aim of this study was to analyze the effect of different concentrations of nanobubble ozone stored niosomes (NOSN) on the expression of genes involved in the destruction complex of WNT/β-catenin signal pathway (*AXIN*, *APC* and *GSK3B*) and the *β-catenin* itself.

Background: Cancer is a disease defined by abnormal cell development that invades and spreads from one spot to other parts of the body. Benign and malignant tumors are the two types of tumors, with benign tumors being those that do not spread throughout the body; however, some benign tumors may be life threatening due to their location. Cancer cells have a stretched, round morphology and lack contact inhibition, which results in piles of cells (foci). Hanahan and Weinberg (2000) identified six cancer hallmarks. One of the primary causes of tumor development is oncogenes that are formed when proto-oncogenes are activated.

Materials and methods: Our target was to treat MCF-7 breast cancer cells with nanobubble ozone stored niosomes (NOSN) (Sonofarma Pharmaceuticals Chemical Industry Trade Ltd Sti, Patent No PCT/TR2022/050177) and analyze gene expression patterns of *AXIN, APC, GSK3B* and the *β-catenin* genes. Different concentrations of NOSN were added to the growth media of MCF7 cells. After 24 hours treatment, RNA isolation and cDNA synthesis was performed using commercially available kits. Then using the cDNA, gene expression analysis was performed with gene specific primers using quantitative real time PCR method. Expression results from each NOSN concentration was compared to the control (non-treated) cells.

Results: A low concentration of NOSN increased *AXIN* gene expression by 15 folds, *GSK3B* gene expression by 5 folds, *APC* gene expression by 4 folds, and

β-*catenin* gene expression by 3 folds.

Conclusion: The study has revealed a role for the NOSN in *AXIN, APC, GSK3B* and the *β-catenin* gene expression patterns in MCF7 cell line, suggesting a potential role for the use for this chemical in selective destruction of cancer cells by manipulating key signaling pathways in the cells.

Keywords: WNT/β-catenin signal pathway, NOSN, MCF7, cell culture

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LIST OF ABBREVIATIONS

µl.: Microliter

mL: Milliliter

µM: Micromolar

nM: Nanomolar

WHO: World Health Organization

BMI: Body Mass Index

ERBBS: Erb-B2 Receptor Tyrosine Kinase 2

MDM2: Proto-Oncogene

Bax: BCL2 associated x, apoptosis regulator

FasR: Fas Receptor

Rb: Retinoblastoma protein

P53: Tumor protein

GDP: Guanosine Diphosphate

GTP: Guanosine Triphosphate

MMPs: Matrix Metalloproteinases

EMMPRIN: Extracellular Matrix Metalloprotease Inducer

CSF1: Colony-Stimulating Factor 1

VEGF: Vascular Endothelial Growth Factor

FGF: Fibroblast Growth Factor

HGF: Hepatocyte Growth Factor

HIF-1: Hypoxia-Inducible Factor 1

IAPs: Inhibitors of Apoptosis Proteins

BER: Base Excision Repair

NER: Nucleotide Excision Repair

DAPK: Death-Associated Protein Kinase

APC: Professional Antigen Presenting Cells

HRT: Hormone Replacement Therapy

PTEN: Phosphatase and Tensin Homolog

PALB2: Partner and Localizer of *BRCA2* (tumor suppressor gene)

TP53: (*TP53* gene provides instructions for making a protein called tumor protein p53)

MBC: Minimum Bactericidal Concentration

STK11: Serine/ Threonine Kinase 11 (tumor suppressor gene)

CDH1: Cadherin-1 or Epithelial Cadherin (E-cadherin)

ER: Estrogen Receptors

RAC3: Ras-Related C3 botulinum toxin substrate 3

GHRHR: Growth Hormone (GH)-Releasing Hormone (GHRH) Receptor (GHRHR)

ZO-1: Zonula Occludens-1

RAS: Rat Sarcoma Virus

RBD: Receptor-Binding Domain

MAP: Mitogen-Activated Protein kinase

ERK-MAP: Extracellular-Signal-Regulated Kinase- Mitogen-Activated Protein kinase

MYC: Master Regulator of Cell Cycle Entry and Proliferative Metabolism

PTEN: Phosphatase and Tensin Homolog

mTORC1: mammalian Target Of Rapamycin Complex 1

KRAS: Kirsten Rat Sarcoma Virus

NRAS: Neuroblastoma Ras Viral Oncogene Homolog

NOTCH: Neurogenic Locus Notch Homolog

NICD: NOTCH Intracellular Domain

ADAM: A Disintegrin and Metalloproteinase

PTCH: Drosophila Segment Polarity Gene Patched (PTCH), a tumor suppressor gene

SHH: Sonic Hedgehog

SUFU: Suppressor of Fused

WNT: Wingless-Related Integration Site

TCF/LEF: T-Cell Factor/Lymphoid Enhancer Factor

DVL: Disheveled Proteins

FAP: Familial Adenomatous Polyposis

DIX: DIX (Dishevelled/Axin) Domain

CKI: Cyclin-Dependent Kinase Inhibitor

GSK3: Glycogen Synthase Kinase *(GSK)3*

HCC: Hepatocellular Carcinoma

SOX7: SOX7 is a Transcription Factor and acts as a tumor suppressor

SMAD7: Mothers Against Decapentaplegic Homolog 7

FZD: Frizzled (FZD) Proteins

LGR4: Leucine Rich Repeat Containing G Protein-Coupled Receptor 4

AKT: Protein Kinase B, also known as Akt

HIV: Human Immunodeficiency Virus

SARS: Severe Acute Respiratory Syndrome

ROS: Reactive Oxygen Species

OIS: Oncogene-Induced Senescence

PDH: Pyruvate Dehydrogenase

BRCA-1 gene: Breast Cancer type 1 gene

BRCA-2 gene: Breast Cancer type 2 gene

caspase-3: Protein product of *CASP3* gene., member of cysteine-aspartic acid protease (caspase) family.

DMSO: Dethyl Sulfoxide

E-cadherin: Cell adhesion molecule E

EGFR gene: Epidermal Growth Factor Receptor

ERα: Estrogen Receptor alpha

Erβ: Estrogen receptor beta

EDTA: Ethylenediaminetetraacetic Acid

FBS: Fetal Bovine Serum

GSH: Reduced Glutathione

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER-2/Neu: Human Epidermal Growth Factor Receptor 2/ proto-oncogene Neu

HIPK2: Homeodomain Interacting Protein Kinase 2

MCF-7: Breast cancer cell line isolated by Michigan Cancer Foundation

MDA-MB-231: Breast cancer cell line isolated by M. D. Anderson

Mg: Magnesium

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

p53: *TP53*, Type of tumor suppressor gene

P450: Cytochrome *P450* gene

PC12: Cell line from a pheochromocytoma of the rat adrenal medulla

PR: Progesterone Receptor

RCF: Revolutions per minute in a centrifuge

ROS: Reactive Oxygen Species

SH-SY5Y: Neuroblastoma cell line

Th 1, Th 2: Helper cells

CHAPTER ONE: INTRODUCTION

1.1.INTRODUCTION

Cancer is defined as uncontrolled cell division that is capable of invading and spreading from one spot to the other sections of the body. As a lethal disease, it is responsible for one out of every six deaths among the 10 million deaths in 2020. Breast, lung, colon, rectum, and prostate cancers are the most common cancers and one-third of their fatalities include the usage of tobacco, a high body mass index (BMI), consumption of alcoholic beverages, less use of fruit and veggies, and a secondary lifestyle (Chandraprasad, Dey et al. 2022). According to the World Health Organization (WHO), the human papillomavirus and hepatitis contribute 30 percent as cancer-causing agents in the low- and middle-income countries.

They are more than 100 types of cancer, and each type of the cancer have its own feature and characteristics, these features depend upon the roots of the origin of cancer. A tumor can be formed by the alteration of just one of the bodies 10^{14} cells. Basically, they are two main types of the tumor, one is called malignant and other one is called benign tumor. Malignant tumor spread all the body, as it has metastasis capability while benign tumor do not have metastasis capability (Cooper and Hausman, 2000). On the basis of classification, the cancer is classified according to their locations from where they originated, for example if cancer originated from glandular tissue such as breast tissues than its called adenocarcinomas, if the origin of cancer is mesodermal cells such as bone and muscles than its called sarcomas, and if cancer originated from epithelial cells than we called this cancer is carcinomas (Cooper, Hausman et al. 2007).

Normal cells also show distinct characteristics in culture, same as the cancer cells but normal cells form monolayers because contact with neighboring cells limits development, a process known as contact inhibition. Cancer cells, on the other hand, have a stretched, round morphology and lack contact inhibition which results in piles of cells (foci). Cancer cells proliferate in low serum environments and do not adhere to a substrate, such as with the petri dish surface (Cooper, Hausman et al. 2007).

Hanahan and Weinberg (2000) identified six hallmarks of the cancer. According to them carcinogenesis requires the invasion-metastasis, evasion of growth inhibitory

signals, infinite replication capability, growth signal autonomy, evasion of cell death and angiogenesis. They identified two enabling traits in 2011 which are particularly important for acquiring six cancer hallmarks. These are inflammation that promotes tumor growth and genomic instability. Avoiding immune breakdown and reprogramming energy metabolism are also gaining importance (Fouad and Aanei 2017).

One of the mechanisms that contributes to genomic instability is faulty DNA repair pathways. This results in the development of cancer's essential characteristics. One of the primary causes of tumor development is oncogene that are formed when proto-oncogenes are altered and activated. Cancer causing gene is a mutated gene that produces a significant amount of protein or has increased activity. This means that a single allele mutation makes a big impact, like a deletion and point mutation in the coding sequence of the gene, which may be leads to the alteration in the proto-oncogene's product. Basically, overexpression is the process of increasing gene expression which enhance the production of specific protein, and overexpression of the gene located in the regulatory region. This overexpression is caused by the deletion and point mutation. Mainly expressional activity is altered by the fusion proteins and chromosomal translocations. Out of many overexpression examples in the breast cancer, the one of them is *erythroblastic oncogene B* (*ERBB2)*. In the other hand tumor suppressor genes produce proteins that prevent tumor development and its progression. If tumor suppressor genes lose their function due to a mutation, then the growth of the tumor can no longer be stopped. Tumor suppressor gene mutations are recessive (Hanahan and Weinberg 2011). According to the Knudsons two-hit hypothesis, both alleles must be mutated to initiate the carcinogenesis mechanism. A person can inherit a mutated version of tumor suppressive allele, and still have a possibility of 2nd somatic mutation in the future. Recent research reveals that haploinsufficiency (just one mutated allele) causes cancer phenotype. Just one of the typical alleles is present in this situation. Haploinsufficiency linked with the genes which regulates the DNA repair and DNA damage respond, it causes genetic instability (Hino and Kobayashi 2017). Gene dosage may influence the tumor spectrum. One of most prominent mutation that is responsible for cancer is the mutation of *p53* pathway. Other include missense mutation, which results in single amino acid substitutions, account for 75 percent of these mutations. They are referred to as hotspots because 90 percent of them

are involved in the binding domain of the DNA, which encodes the amino acid ranging from 102 to 292 and 30 percent are in six codons. In the cells, many mutant *p53* molecules aggregate and demonstrate gain of function. The overexpression of the Proto-Oncogene (MDM2) protein disrupts *p53* control. The apoptotic response is disrupted when BCL2 associated x, apoptosis regulator (Bax) and Fas Receptor (FASR) (downstream effectors) are inactivated. Another tumor suppressor gene which is known as *Retinoblastoma (Rb*) is involved in cell cycle regulation. Many cancer forms have mutations in this gene. Cell growth is generally inhibited by the protein product of the *Rb* gene. *Retinoblastoma* gene's activity is regulated by the phosphorylation of cyclin-dependent kinases (4/6) and cyclin D. Cancer biology is also regulated by a protein family known as the kinase family. This family of enzymes is in charge of adding a phosphate group to the hydroxyl terminus of a specific amino acid in a protein. The tyrosine kinase phosphorylates tyrosine residues, while the serine threonine kinase phosphorylates serine threonine residues, causing structural changes in the protein. Generally, kinases play a role as transmembrane receptors on the cell surface and in the nucleus, while they also act as transducers in the cell.

Kinases have important roles in transcription, cell cycle progression, and signal transmission, that's why they are molecular targets for cancer therapy development. Ras participates in the transmission of a signal from the receptor through the cell by binding a growth factor to its receptor. G proteins are found on the plasma membrane's intracellular side. They become active when the cells exchange Guanosine Diphosphate (GDP) for Guanosine Triphosphate (GTP). Ras family mutations are identified in over 50 percent of certain malignancies (Hanahan and Weinberg 2011).

Inflammatory immune cells are found in all cancers. Inflammation aids the acquisition of cancer's basic characteristics. Because these cells produce enzymes and growth factors, that stimulate angiogenesis and invasion. Inflammatory immune cells also generate mutations and enhance tumor inflammation by producing oxygen species. Growth factors are required by normal cells as external signals to proliferate. Contrary to it, cancer cells do not require growth factors because short-circuiting of growth factor pathways, as well as acquired mutations, result in uncontrolled proliferation (a phenomenon known as growth signal autonomy). Cancerous cells do not respond to inhibitory signals that keep homeostasis in check. "Evasion of growth inhibitory

signal" occurs when gene silence and acquired mutations interfere with inhibitory pathways. To avoid being destroyed by the immune system, successful cancer cells do not elicit an immunological response. Before becoming old, normal cells can cycle 50 times because chromosomal ends and telomeres shrink with each round of DNA replication, whereas cancer cells' telomeres do not shorten, resulting in a limitless replicative capacity (Grivennikov, Greten et al. 2010, Lazebnik 2010).

Cancer cells migrate to different parts of the body whereas normal cells do not migrate from their origin to other parts of the body. This difference could be due to changes in the genome of cancer cells. This has an effect on the functional activity of the invasion-related enzymes, same as also impact on the cell-to-cell adhesion molecules (Invasion and metastasis) or cellular–extracellular. Tumor cells infiltrate, transfer, intravasate, extravasate, and colonize metastatic sites. The modification of the membrane, methylation of the E-cadherin gene's promoter section, changes in integrin receptor expression and mutations in the extracellular domain in tumor cells aid mobility and invasion of metastasizing cells. E-cadherin (epithelial marker) is downregulated, but N-cadherin is increased, as are other mesenchymal proteins. MMPs (matrix metalloproteinases) and serine proteases are secreted by cancer cells and migratory tumor cells. EMMPRIN so called "extracellular matrix metalloprotease inducer" number increased at the cell membrane of the tumor, causing MMP synthesis in nearby stromal cells. When tumor cells enter a lymphatic or blood vessel and attach to the stromal face of the channel, it is known as Intravasation. MMPs are used by tumor cells to breakdown the basement membrane (lymphatic vessels do not have). Serine proteases then migrate into the bloodstream via endothelial cells so calked trans endothelial migration. When tumor cells stimulate new blood vessels, they become tortuous and leaky (Hanahan and Weinberg 2011) .

Colony-stimulating factor 1 (CSF1) is produced by tumor cells, which causes chemotaxis-mediated co-migration. Tumor cells travel through the bloodstream alone or in clumps with platelets (called emboli). Emboli protect tumor cells from the bloodstream's extreme stresses. The liver and the lungs are first-pass organs. Tumor cells are able to escape from a lymphatic or blood artery (extravasation). Cancer cells connect to the endothelium via an adhesion molecule (E-selection), allowing them to move across the endothelium. When a tumor gradually spreads to a different section

of the body, with new blood vessels forming from the old ones (angiogenesis), the process is then termed as Metastatic colonization. When mature vessels become unstable, endothelial cells expand and migrate, resulting in the formation of new blood vessels (sprouting). Pro-angiogenic factors includes vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and epidermal growth factor (EGF). HIF-1 so called "Hypoxia inducible factor-1" helps tumor cells grow and establish hypoxic conditions, which leads to angiogenesis. It can also hit the *VEGF* genes because its promoter contains a hypoxia response element. The loss of tumor suppressors and oncogenic proteins aids in the angiogenic switch's alteration. Endothelial cells proliferate because abnormal growth factors are created. Oncogenic proteins such as intracellular transducers like Ras, receptor tyrosine kinases like epidermal growth factor receptor (EGFR), intracellular tyrosine kinases like Src, and transcription factors all upregulate VEGF. Angiogenic inhibitors are generally upregulated/increased by some tumor suppressor proteins. *P53* interacts to the promoter region of the thrombospondin-1 gene and activates it. However, if the *p53* gene is altered, angiogenic inhibitors are reduced, resulting in angiogenesis. Tumor cells, like endothelial cells, can develop vascular-like structures (Hanahan and Weinberg 2011, Fujiwara, Yakoub et al. 2021).

Procaspases are dormant in normal cells. Apoptotic cues activate these dormant procaspases. Because of mutations, tumor cells have a faulty apoptotic mechanism. Caspases are activated in cancer cells, but increased inhibitors of apoptosis (IAPs) prevent them from doing so. IAPs inhibition of active caspases is stopped by apoptosis signals. Cancer cells evade apoptosis in this way, and more mutations accumulate (Evasion of cell death). Glycolysis occurs in cancer cells even when oxygen is present. In contrast to normal cells, glycolysis intermediates are employed in biosynthetic pathways (McIlwain, Berger et al. 2015).

Base excision repair (BER), nucleotide excision repair (NER), one step repair, recombinational repair, and mismatch repair are some of the mechanisms involved in DNA repair. Before cell replication the DNA damages are generally repaired but If not then this adds to the development of cancer (Pecorino 2021). Epigenetics, which affects chromatin conformation and transcription control, is heritable information encoded via alterations of chromatin components and the genome. Epigenetic

alterations are not mutations since they do not change the sequence of nucleotides in DNA. Histone modifying gene mutations and DNA methylation are both possibilities. DNA methyltransferase mutation has been found in various cancer forms. The unmethylated CpG islands of gene promoters undergo hypermethylation. Methylation-induced gene silencing has been linked to cancer. In non-inherited breast cancer, for example, *BRCA1* inactivation is caused by hypermethylation. Increased mutation rates have been seen in methylated CpG islands as a result of CT transitions. Many genes are affected by methylation, including *DAPK*, $p16^{INK4a}$, *APC*, *Rb* and the estrogen receptor gene. In cancer cells, hypomethylation in repetitive DNA sequences and hypermethylation in certain genes or coding areas may occur at the same time. The activation of genes that are not ordinarily expressed in cells has an impact on transcriptional activity. Some enzymes that are critical for epigenetic control may be disabled by mutations. Epigenetic changes can lead to more epigenetic changes, resulting in genome-wide changes and genomic instability, which leads to the cancer initiation and progression (Kanwal and Gupta 2012, Nishiyama and Nakanishi 2021).

1.1.2. CANCER BIOLOGY

Cancer is described as an uncontrol cell division. Basically, our body cells are genetically programmed and control all the basic structural and functional activity of the body throughout our life. As our body makes new cell in the replacement of old or damaged cell through the process called cell division. In our body, there is proper cell division control system that keep everything in check and regulates all the mechanisms of the cell division and maintain proper check and balance during cell division. If there is any mutation that inhibit the normal check and promote the oncogenesis properties including initial activation of oncogenes, deactivation of tumor suppressor genes, and inactivation of apoptotic mechanism leads normal cell division towards cancerous. Unlike to the benign tumor, malignant malignancies acquire metastasis which is facilitated by the down regulation of the cell adhesion receptors required for tissue specific cell-cell attachment and the upregulation of receptors that promote cell mobility. Aside from that, there are different epigenetic phenomena such as DNA methylation and Histone modification that alter these features and induce cancer and promote metastasis (Krieghoff-Henning, Folkerts et al. 2017).

1.1.3. TYPES OF CANCER

Because cancer can arise from the aberrant multiplication of any of the body's cells, there are more than a hundred different varieties of cancer, each with its own unique behavior and therapeutic response. The difference between benign and malignant tumors is the most critical topic in cancer pathology. Any aberrant multiplication of cells, whether benign or malignant, is referred to as a tumor. A benign tumor, such as a common skin wart, remains limited to its original spot, not infecting nearby normal tissue or spreading to various parts of the body. On the other hand, malignant tumor has the ability to invade normal tissues which are present in the surroundings as well as it spreads throughout the body via the circulatory and lymphatic systems (metastasis). Cancer is only appropriately referred to as malignant tumors, and it is cancer's propensity to penetrate and spread that makes it so hazardous whereas benign tumors can be surgically removed. The spread of malignant tumors to other parts of the body makes them resistant to such therapy (Cooper, Hausman et al. 2007).Tumors are classified as benign or malignant based on the type of cell that gives rise to them. Carcinomas, sarcomas, and leukemias and lymphomas are the four main types of cancer.

Carcinomas

Carcinomas are often solid tumors and the cancers of this type are more common than others. It grew on the outer surface like tissue or skin of the glands and organ. Breast cancer, prostate cancer, lung cancer, colorectal cancer is some of the main examples of the of this carcinoma.

Sarcomas

Sarcomas develops in the body's supporting and connecting tissues of the body. Some specific region of this sarcomas include nerve, tendon, cartilage of the bone, lymph vessel, and joints.

Leukemias

Leukemia occurs in blood so leukemias is a type of blood cancer. It starts to grow when there was some kind of mutation has been happed in the healthy cell than they tend to start to grow irrepressibly. Basically, they have 4 main types including acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia, and

chronic myeloid leukemia.

Lymphomas

The lymphatic system consists of a network of tubes and glands that aid in the fight against infection. Lymphoma is a cancer that starts in the lymphatic system and spreads to other parts of the body. There are two forms of Hodgkin's lymphoma: Hodgkin's lymphoma and non-lymphoma Hodgkin's.

1.1.4. HALLMARKS OF CANCER

The hallmarks of cancer include six biological characteristics that have developed during the multistep evolution of human malignancies. The hallmarks provide a foundation for understanding the intricacies of neoplastic disease. Maintaining proliferative signals, resisting cell death, evading growth suppressors, enabling replicative immortality, initiating angiogenesis, and activating invasion and metastasis are only a few of them. Genome instability creates the genetic diversity that speeds up their inflammation and acquisition, which supports many hallmark functions and serves as the root of these hallmarks. The two key features of potential universality that have emerged in the previous decade are reprogramming of energy metabolism and escape from immune destruction. Tumors have an additional layer of complexity in addition to cancer cells: they comprise a collection of recruited, presumably normal cells that aid in the acquisition of hallmark features of cancer cells (Fouad and Aanei 2017).

1.1.5. BREAST CANCER

Breast cancer is the most common and lethal cancer among women, with approximately one out of every ten women being diagnosed every year. Generally, in a woman's body, the gland that is responsible for producing milk is present in front of the chest wall. In the pectoralis major muscle, there is a ligament that helps connect the breast to the chest wall. Basically, there were 15 to 20 lobes managing themselves in a circular manner in the breast of a woman. The amount of fat that covers the lobes determines the size of the breasts of a woman. However, each lobe is made up of lobules that have milk producing glands that are responsible for the hormonal stimulus.

Breast cancer grows unnoticed at all times, so routine screenings is required through which the majority of people learn about their diseases. Others may experience a change in breast shape or size, an unintentional breast lump, or nipple discharge (Krieghoff-Henning, Folkerts et al. 2017). Mastalgia, on the other hand, is a rather frequent affliction. A physical examination, tissue biopsy, and imaging, particularly mammography, are all required to detect breast cancer. The odds of survival increase when cancer is detected early. The tumor has a tendency to spread lymphatically and hematologically, resulting in a poor prognosis and distant metastases. This emphasizes the importance of breast cancer screening programs (Arslan, Küçükerdem et al. 2016).

1.1.6. PREVALENCE OF BREAST CANCER

According to the world health organization, the breast cancer is one of most common Cancer diagnosed in the woman. Every year their diagnosed cases rises 12 percent overall. Specifically in united states 1 out of every 8 woman is diagnosed with invasive breast cancer. According to the statistic provided by the United States there was an 287,850 new cases of invasive breast cancer has been diagnosed in 2022 only in united states, meanwhile more than fifty thousand new cases will be expected till the end of 2022. The overall breast cancer risk for a male is approximately 1 in 833. In addition, 2,710 new instances of metastatic breast cancer are predicted to be discovered in 2022. Breast cancer is estimated to kill 43,500 people in the United States by the year 2022. Every year from 2013 to 2018, the total mortality rate of women from breast cancer decreased by one percent. This decrease is assumed to be the result of breakthroughs in breast cancer treatment and earlier diagnosis of the disease through screening. Breast cancer incidence began to decline in the United States in the early 2000s as a result of the use of hormone replacement therapy (HRT), with younger women experiencing a bigger reduction than older women.

In 2008, 1.38 million new cases of breast cancer were projected to have been detected worldwide. Breast cancer rates in different parts of Western Europe ranged from 89.9 per 100,000.

It varied as 19.3 / 100,000 in Eastern Africa. For the past 25 years, the death rate from breast cancer has been declining in North America and some other areas of Europe. The therapy deserves all of the credit.

The American Cancer Society (ACS) reports that the prevalence of breast cancer differs by racial and ethnic group. As, the incidence rate of female breast cancer between African Americans was 14.3 per 100,000, but it was 91.0 per 100,000 in the Hispanic/Latina region. The Asian American/Pacific Islander population has an incidence of female breast cancer that is 83.3 percent of the world population, which is considered very high. Breast cancer fatality rates, but at the other hand, are increasing year after year. However, it's worth noting that the incidence rate of breast cancer rises with age. Breast cancer incidence rates among women aged 20 to 24 years old, for example, ranging from 1.5 to 2.5 incidences per 100,000. As a woman's age ranges from 75 to 79, the incidence rate climbs, accounting for 421.3 cases / 100,000. In general, the incidence rate of breast cancer rises with a woman's age.

Her odds of acquiring breast cancer increase if she has a first-degree relative with breast cancer, such as a mother, sister, or daughter. Around 15 percent of individuals with breast cancer have a first-degree relative who has been diagnosed with the disease. In fact, five to ten percent of known inherited breast cancer genetic abnormalities are inherited from the parents, with *BRCA1* and *BRCA2* gene mutations inherited from either the father or mother being the most frequent. While the frequency of mutations varies depending on the type, a woman with a *BRCA1* gene mutation has a 72 percent probability of developing breast cancer during her lifetime. A woman with a *BRCA2* gene mutation, on the other hand, has a 69 percent chance of developing breast cancer. To summarize, a woman with alterations specific to these genes (*BRCA1* and *BRCA2*) has a significant risk of developing breast cancer, particularly in young women. While these mutations are linked to the genetic changes linked to ovarian cancer, men who possess the *BRCA1* mutation have a low risk of acquiring breast cancer throughout their lifetime, men who carry the mutant *BRCA2* have a 6.8 percent risk of developing breast cancer over their lifetime (Brewer, Jones et al. 2017) (Fouad and Aanei 2017).

Breast cancer is not usually inherited; additional variables like sex and age account for about 85 percent of the female breast cancer population. Sex (being a woman) and age are the two major risk factors that contribute to the development of breast cancer in women, aside from inherited mutation (getting older). These are the main factors that cause women to get breast cancer (Schneider, Zainer et al. 2014).

1.1.7. ETIOLOGY OF BREAST CANCER

1.1.7.1 ETIOLOGY IN WOMAN

Identifying variables connected to an increased risk of breast cancer development is crucial in routine health screening for women. Age, obesity, alcohol consumption, a family or personal history of breast cancer, hormone replacement therapy (HRT), reproductive and hormonal factors, physical inactivity, ionizing radiation exposure, and genetic predisposition have all been linked to an increase in breast cancer incidence in women. There are list of risk factors that contribute to the onset and progression of cancer (Hvidtfeldt, Tjønneland et al. 2015, Dydjow-Bendek and Zagożdżon 2021).

Risk of breast cancer factors are divided into seven groups in general:

• Age and sex are two of the most critical factors linked to breast cancer in women; as women's ages rise, so does their risk of developing breast cancer.

• Men are at a reduced risk for breast cancer than women.

• Having a family history of breast cancer, there are more likely to get a secondary tumor in the other breast.

• Breast cancer in the family and its genetic risk factors: First-degree relatives of breast cancer patients had a 2 to 3 times higher risk of having the disease. While genetic variables were responsible for five to ten percent of all breast cancer cases, genetic factors were responsible for just five percent to ten percent of all breast cancer cases. Surprisingly, women under the age of 30 are more likely to acquire breast cancer at a rate of 25 percent however the woman older than 45 having more and more chance of developing breast cancer. *BRCA1* and *BRCA2* are two of the most critical genes linked to an elevated risk of breast cancer.

• Exogenous hormone administration: Hormone replacement therapy in postmenopausal and premenopausal women has used exogenous hormones such as supplementary and therapeutic progesterone and estrogen for a variety of purposes, the most prevalent of which is contraception.

• Histologic risk factors: Histologic abnormalities discovered after a breast biopsy comprise a large number of breast cancer incidence. These abnormalities include LCIS

(lobular carcinoma in situ) and proliferative changes with atypia.

• Reproductive risk factors: Reproductive milestones that enhance lifetime estrogen exposure are thought to raise women's breast cancer risk. These include menarche before the age of twelve, first live births after the age of thirty, nulliparity shortly after the age of 55, and menopause after the age of 55 (Hvidtfeldt, Tjønneland et al. 2015, Dydjow-Bendek and Zagożdżon 2021).

1.1.7.2. ETIOLOGY IN MALE

When investigating the origins of male breast cancer, it's critical to consider both genetic and environmental risk factors. It's also important to realize that the majority of men with Male breast cancer (MBC) have no recognized risk factors other than getting older (average age of diagnosis of 71 years)

Males, like females, are more likely to acquire breast cancer if their first- and second-degree relatives have been diagnosed with the disease. According to study, having a breast cancer-prone brother or father of either gender increases the chance of breast cancer in both males and females in the family. In a study in 2012, Bevier and colleagues discovered that when the father or mother had breast cancer, the relative risk (RR) was equal in both offspring (RR = 1.73 and 1.74, respectively), while the risk of having breast cancer in females was slightly greater (RR=2.48 and 1.39, respectively) (Campos, Rouleau et al. 2021).

In addition to having a parental history of breast cancer, men with the specific *BRCA* mutation appear to have an increased risk of breast cancer. Although a specific *BRCA* mutation in a man is uncommon, for example *BRCA2* carriers have a six percent increased risk of developing the disease in man, while *BRCA1* carriers have a four percent increased risk. Male breast cancer (MBC) has also been associated to Klinefelter syndrome (47, XXY), Cowden syndrome (*PTEN* tumor suppressor gene), Li-Fraumeni syndrome (*TP53*), and Lynch syndrome (*TP53*) (*PALB2* and mismatch repair genes).

It's critical to consider variations in estrogen to androgen ratios when considering the etiology of male breast cancer. Estrogen, which stimulates ductal growth in the breasts, has also been suggested as a possible risk factor for breast cancer in women. As

previously mentioned, excessive estrogen stimulation may raise the risk of MBC in Klinefelter syndrome. Obesity, hepatic dysfunction, marijuana use, thyroid disorders, and estrogen-based medicines are all thought to be contributing factors. Orchitis, cryptorchidism, and orchiectomy, for example, may result in an increase in estrogen levels due to a decrease in circulating androgens (Campos, Rouleau et al. 2021, Zheng and Leone 2022).

Furthermore, a surge in the prevalence of men breast cancer cases has been attributed to environmental variables. Radiotherapy has been identified as a possible risk factor in the past, just as it has been in the case of women. Gynecomastia is treated with radiotherapy, but it has been revealed that these patients have a seven-fold greater risk of MBC. Several occupations have also been associated with a higher risk of MBC, including those that use organic compounds such as trichloroethylene, as well as those who work in steel and rolling mills (Campos, Rouleau et al. 2021, Zheng and Leone 2022).

1.2. RISK FACTORS

1.2.1. GENETIC RISK FACTORS (MAJOR)

A risk factor is something that increases a person's chances of contracting a disease. There are several risk factors for breast cancer, some of which are genetic and others which are hormonal, but the key risk factors that are associated to the majority of breast cancer incidences include sex, age, and family history. Breast cancer, for example, is 100 times more common in women than it is in men. In fact, older women, those over the age of 55, have a higher risk of breast cancer than younger women. It means that being a woman and the aging process are obviously linked to the development of breast cancer, and that the risk of acquiring breast cancer is twice if a first-degree relative has been diagnosed with the disease. In the United States, it is believed that 15 percent of breast cancer patients come from a family history of the disease.

In most cases, those who inherit the *BRCA* gene mutation have a five to ten percent probability of developing breast cancer. *BRCA1* and *BRCA2* are the two most frequent breast cancer mutations that are passed down through generations. According to the findings, women who are carriers of the *BRCA1* genetic mutation had a 72 percent chance of having breast cancer. Women who have inherited the *BRCA2* genetic

mutation have a 69 percent lifetime risk of developing breast cancer. In fact, anyone who receives either of these has a 50 percent chance of having breast cancer by the time they reach the age of 80. Gender, age, and family history all have a role in the impact of the mutation and the chance. The *BRCA* mutation can affect anyone from any ethnic group, but it is more common in Ashkenazi (Eastern European) Jews than in any other racial group in the United States. Those who have either of the two mutations are more likely to get breast cancer later in life. Ovarian cancer is also increased by the *BRCA1* and *BRCA2* mutations. When it comes to men, *BRCA2* has a greater impact on them than *BRCA1*. According to various studies, men with *BRCA2* mutations have a six percent probability of acquiring breast cancer, whereas men with *BRCA1* mutations do not have this risk as much (Feng, Spezia et al. 2018).

Inherited mutations in many other genes can also cause breast cancer, albeit they are less prevalent and have a lesser effect than *BRCA* gene mutations. For example, a mutation in the *CHEK2* gene doubles the risk of breast cancer, whereas a mutation in the *PTEN* gene is connected to cowden syndrome, as well as other syndromes involving the digestive system, uterus, thyroid, and ovaries. Despite all of the above, another gene, *TP53*, has been related to an increased risk of breast cancer double fold. Mutations in this gene have also been connected to a greater risk of leukemia, brain tumors, and other sarcomas. Another gene, *STK11*, has a mutation that can cause Peutz-Jeghers syndrome, which increases the risk of numerous cancers, including breast cancer. While mutations in the *PALB2* gene increase the risk of breast cancer because this gene interacts with the protein produced by the *BRCA* gene, any mutation disrupts this interaction and increases the risk of breast cancer. A mutation in the *CDH1* gene causes hereditary diffuse gastric cancer with an increased risk of invasive lobular breast cancer.

Genetic testing for the *BRCA1* and *BRCA2* genes, as well as other less prevalent gene mutations including *TP53* and *PTEN*, can aid in the early detection and prevention of breast cancer in high-risk women. Because breast cancer genetic testing is pricey, the person who performed it should be fully aware of its handling and limitations. While genetic testing may be advantageous in some cases, it is not required for every woman (Lynch, Venne et al. 2015, Feng, Spezia et al. 2018).

1.2.2. NON-GENETIC RISK FACTOR (MINOR)

Breast cancer is caused by more than just inherited genetic factors. Other factors, known as non-genetic factors, play a role. These factors' cancers are associated with somatic mutations in breast cells acquired during person's lifetime (Feng, Spezia et al. 2018).

• Factor that isn't genetic.

• Breast cancer in the family.

• Race and ethnicity

• There are a few benign breast problems.

• Breast Cancer Risk Factors Associated with Livelihood and Personal Behavior

• Contraception and pregnancy prevention.

• Obesity or being significantly overweight.

• Insufficient physical activity.

• Lobular neoplasia or lobular carcinoma in situ (LCIS).

After menopause, hormone replacement therapy (HRT) is used.

• Diethylstilbestrol exposure (DES).

• Radiation therapy to the chest.

• Breast lesions that are proliferative

1.3. CURRENT TREATMENT TECHNIQUES AND APPROACHES TO NEW TECHNOLOGIES

Cancer is a worldwide health problem that is widely regarded as the leading cause of mortality. Treatment for cancer has always been challenging. Chemotherapy and Radiotherapy all been employed in the past, but there have been some important advancements recently. Stem cell therapy, targeted therapy, ablation therapy, nanoparticles, radionics, natural antioxidants, sonodynamic therapy, ferroptosis-based therapy, and chemodynamic therapy are all new breakthroughs in breast cancer research. Current oncology techniques are centered on the creation of safe and

effective cancer nanomedicines. Stem cell treatment has shown potential usefulness in regenerating and repairing diseased or damaged tissues in both metastatic and primary cancer foci, and nanoparticles have brought new diagnostic and therapeutic options (Debela, Muzazu et al. 2021).

By inhibiting the spread and reproduction of select cancer cells while causing minimum harm to healthy cells, targeted therapy has the potential to be a game-changer. Ablation is a less intrusive method of burning or freezing malignancies that does not require open surgery. Antioxidants found in nature have showed promise in reducing free radical damage, detecting them, and possibly treating or preventing cancer. Several cutting-edge technologies are now being tested in clinical trials, while others have already been approved.

Meanwhile, we're working on a nanobubble ozone stored noisome (NOSN) treatment for breast cancer, which we'll test in MCF-7 cell lines using the *APC*, *AXIN*, *β-catenin* ,and *GSK3B* genes (Singh, Bhori et al. 2018, Debela, Muzazu et al. 2021).

1.4. MOLECULAR CLASSIFICATION OF BREAST CANCER

Breast carcinoma refers to a group of diseases with different histopathologic, clinical, and molecular features. Cancerous cells have traditionally been categorized into several types based on their form, which differ in behavior and prognosis. Traditional classification procedures, on the other hand, have their limitations, and new molecular technologies will almost certainly improve classification systems. Breast cancer molecular subtypes have been found and studied extensively over the last 11 years. Much of the information has only recently become available, and molecular taxonomy appears to be shifting and evolving (Eliyatkın, Yalçın et al. 2015).

Breast cancer is classified only on the basis of its characterization:

- Histological Classification
- Basal like or triple negative breast cancer
- Claudin-Low breast cancer
- Luminal breast cancer
- HER2-Enriched breast cancer
- Surrogate Markers Classification

- American Joint Committee on Cancer Classification

1.5. BREAST CANCER CELL LINE

The American type cell culture collection has numerous cell lines of breast cancer derived from the breast cancer specimens. A most common cell line that we used in our experiment is MCF-7 which is an estrogen receptor (ER-Positive Cell Line) obtained from a pleural effusion in a breast cancer patient. Others are ZR-75-1 and BT-474 are two more ER-Positive cell lines. Other ER-Negative but positive for epidermal growth factor receptor (EGFR) are MDA-MB-486 amd MDA-MB-231 breast cancer cell line (Hahn, Weinberg et al. 2015).

1.5.1. MCF-7 BREAST CANCER CELL LINE

In 1973, the MCF-7 Breast Cancer Cell Line was named by Dr. Soule and his co-workers from the Michigan Cancer Foundation. This cell line was initially isolated from a 69-year-old Caucasian woman with breast cancer. Origin of this sample was the mammary gland of this breast cancer lady. This old lady having adenocarcinoma metastatic by the pleural effusion. Anti-estrogen named as Tamoxifen, which have ability to inhibit the development of MCF-7 cell, while different research has been reported that this effect can be rolled back through estrogen (Comşa, Cimpean et al. 2015).

1.5.2. CHARACTERIZATION OF MCF-7 BREAST CANCER CELL LINE

MCF-7 cells form three-dimensional multicellular aggregations that form lumen filled spheroids.

MCF-7 p1 forms a densely packed colony that is essentially a lock-like polygonal shape that is close to one another. But few of the cells located at the border of the colony have a fibroblastic shape, and these cells migrate away from the colony.

Basically, the protein named E-Cadherin protein, present in spheroids, is responsible for cell-cell adhesion. On the other hand, the MCF-7 colonies are not tightly packed and these colonies migrate away from their primary colonies. It is well known that the

MCF-7 cell line is basically a breast cancer cell line and has an estrogen receptor, but many sub colonies of the MCF-7 cell line reflect various kinds of ER-Positive cancers with varying amounts of nuclear receptor expression. Such cells are progesterone receptor positive and belong to the lumina A. Basically, they are a molecular sub-type of low aggressive, non-invasive cell lines (do Amaral, Rezende-Teixeira et al. 2011).

MCF-7 Breast cancer cells exhibit substantial aneuploidy and have a low spreading capability. They have cytogenetic variation, such as a presence or absence of certain chromosomal markers. They also exhibit high amount of genetic instability. MCF-7 cell line having stem cells that can capable to generate the clonal diversity, with distinct variant diverging at the RNA expression or in genomic levels. As MCF-7 breast cancer cells keep ER expression while culturing, they are considered as ideal for study the anti-hormonal therapy resistance (Vargas-Rondón, Pérez-Mora et al. 2020)(Comşa, Cimpean et al. 2015).

1.5.3. MOLECULAR PROFILE OF MCF-7 BREAST CANCER CELL LINE

Human E2 is required for the proliferation of the MCF-7 breast cancer cells. MCF-7 have a high amount of HER alpha and a low level of ER β expression. while they also have 17 β-estradiol receptors. Progesterone is highly expressed in the paternal line, whereas it is missing or shows weak expression in tamoxifen-resistant sublines. It is also being analyzed that the rate of proliferation has been reduced and lasts around a month after the estrogen has been withdrawn (Leung, Lee et al. 2012).

MCF-7 breast cancer cells are responsive to HE2 because they are dependent on an autocrine factor, as this factor triggers the IGF-IR. IGF-1 signaling is involved in mRNA regulation. Basically, MCF-7 cell growth is regulated by the Human epidermal growth factor receptor-2, ER, PR, and EGFR, which are all activated by EGF. While the ER-positive MCF-7 cell line is the source of triple negative sublines, MCF-7 cells have characteristics of a developed mammary epithelium. Different epithelial markers such as E-cadherin, cytokeratin 18, and *β-catenin* are positive in them. MCF-7 cells, on the other hand, show a negative correlation for mesenchymal markers such as vimentin and smooth muscle actin (Felice, El-Shennawy et al. 2013).

Claudins, which are considered as a unique molecular marker of epithelial cells, are expressed in MCF-7 parental cells. They produce intercellular connections by expressing "Zona occulden protein 1" (ZO-1)(Kwon 2013). MCF-7 cells are CD44 deficient and do not express *GHRHR*. The other factor known as *RAC3*, which also shows a minimum effect on MCF-7 cells, is known as *RAC1*. MCF-7 cells have high levels of VEGF receptor 1 and neuropilin-1, but low levels of VEGFR2. VEGF-A, VEGF-C, and VEGF-D are secreted in small amounts.

Another study reveals that the angiogenic capacity of the MCF-7 breast cancer cells is also low. Surprisingly, the deprivation of serum induces a significant decrease in the proliferation activity but does not cause apoptosis (Pinto, Badtke et al. 2010) (Felice, El-Shennawy et al. 2013).

1.6. MAIN SIGNAL TRANSDUCTION PATHWAYS IN BREAST CANCERS

Cells have the ability to receive and digest data (or "signals") from their environment in order to respond and adjust to their surroundings. Cell signaling, also known as transmembrane signaling or signal transduction, is a complex mechanism that controls cellular functions. Signaling pathways aid in cell surface, nucleus, and extracellular matrix coordination.

A single pathway's abnormal signaling can have a major impact on larger signaling networks, promoting cancer growth and spread. Many of the particular properties of tumor cells that distinguish them from "normal" cells in cancer are due to dysregulated cell signaling. The term "disease hallmarks" refers to these traits. (Juliano 2020).

These sections provide simple summaries of several of the most important cancer signaling pathways.

1.6.1. RAS RELATED SIGNALING

The dysregulation of RAS GTPases has a critical role in a variety of cancers; it acts as a molecular switch, activating or deactivating the cancer-regulating cascades of events. In normal cells, RAS is activated by attracting guanine nucleotide exchange factors such as SOS (Son of Sevenless) to the plasma membrane, where they can interact with

membrane-bound RAS and convert it to its active GTP-bound state. GTPase-activating proteins like neurofibromin 1 restore RAS to its GDP-bound inactive state (Simanshu, Nissley et al. 2017). RAS activation triggers the activation of a slew of downstream effectors, triggering signaling cascades that regulate cellular functions like growth and division, survival, metabolic activity, and cytoskeletal architecture. RAS effectors with weakly homologous RAS-binding domains (RBDs) interact with RAS and cause RAS to change conformation, which activates the effector (Ferro and Trabalzini 2010).

The two RAS signaling pathways most strongly associated to cancer are the MAP kinase pathway, which regulates cellular proliferation, and the phosphoinositide 3-kinase (PI3K) system, which governs cell metabolism and survival.

The RBD of a serine–threonine kinase from the RAF family binds to activated RAS, allowing the kinase to be activated in the MAP kinase pathway and assisting auto-inhibition. MEK, a dual-specificity kinase, is then activated by RAF, causing ERK MAP kinase to become active. Many transcription factors in the nucleus, including ELK-1 and MYC, can be phosphorylated by ERK, causing the transcription of genes that favorably control the cell cycle to be initiated (Terrell and Morrison 2019).

PIP2 is converted to PIP3, which is used as a secondary messenger in a variety of processes by PI3Ks. There are several PI3Ks in mammals, but only a handful of them are activated when active RAS binds with an RBD in the p110 kinase subunit. PI3K activity is reduced by inositol lipid phosphatases, such as PTEN, a tumor suppressor. AKT, a serine/threonine kinase that affects cell survival and metabolism, is a prominent PI3K downstream effector. As a result of AKT-mediated phosphorylation, FOXO transcription factors are still unable to activate pro-apoptotic genes, allowing cells to live longer. AKT has an effect on the mTORC1 complex, which regulates metabolism and protein synthesis as well as maintaining food levels (He, Sun et al. 2021).

RAS and its downstream effectors have been found to have genetic abnormalities in a number of malignancies. RAS mutations are common in pancreatic, colorectal, and lung malignancies, especially in the *KRAS* and *NRAS* isoforms, while *B-RAF* mutations are common in melanoma and colorectal cancer. Cancers of the breast, colon, stomach, cervix, prostate, and lung have all been linked to genetic changes in PI3K, notably in the p110 subunit. One of the most important aspects of PI3K pathway

participation in cancer is the loss or deactivation of the PTEN tumor suppressor, which results in PI3K pathway dysregulation (Simanshu, Nissley et al. 2017)(He, Sun et al. 2021).

1.6.2. NOTCH AND HEDGEHOG SIGNALING

NOTCH ligands in mammals are made up of three delta-type and two jagged transmembrane proteins (Jag1 and Jag2). Cell-to-cell communication via the NOTCH pathway is critical for cell survival during developmental stages and is also important for tumor advancement. When NOTCH ligands from one cell engage with NOTCH from another, the NOTCH intracellular domain (NICD) is secreted through proteolysis. The protein subsequently travels to the nucleus, where it interacts with CSL transcription factors. The four NOTCH receptors in mammals are also transmembrane glycoproteins. Conformational changes occur whenever the ligand makes contact with the receptor, allowing NOTCH to be cleaved by an ADAM protease and then by -secretase, releasing the NICD. The signaling consequences of this seemingly basic system are complex and context-dependent, reflecting different outputs from different ligand–receptor combinations as well as epigenetic alterations among cells. NOTCH may play a primary oncogenic function in some malignancies, such as T-cell lymphomas. It has a bigger effect on the microenvironment of the tumor. As a result, tumor cells and neighboring stromal cells, as well as tumor cell lineages, can interact with NOTCH ligand–receptors. NOTCH signaling, for example, can help cancer stem cells select a suitable niche, especially when the Jag1 ligand is present(Andersson, Sandberg et al. 2011).

Hedgehog signaling is helpful at many phases of development. Mutations in this system have been related to cancers such as basal cell carcinoma, breast, prostate, lung, medulloblastoma, and possibly pancreatic cancer. Primary cilia, which are microtubule-based projections on the cell surface, are associated to hedgehog signaling in mammals. Three hedgehog ligands cause signaling, the most powerful of which is Sonic hedgehog (SHH). The GPCR-like protein SMO is inhibited by PTCH and signaling is suppressed due to the lack of SHH binding to its transmembrane receptor PTCH. Gli1–3 transcription factors are kept in a repressive state by SUFU proteins and cytosolic kinases. SMO migrates to the primary cilium in the presence of SHH and

begins downstream signal transductions, lowering inhibition. Gli1–3 is activated as a result and goes to the nucleus (Sever and Brugge 2015, Riobo-Del Galdo, Lara Montero et al. 2019).

1.6.3. WNT PATHWAY SIGNALING

Since the discovery of changes in adenomatous polyposis coli (*APC*), the importance of the WNT pathway in cancer has been recognized. Key components of the pathway are detected in 80–90 percent of colon cancers. WNT signaling, which consists of a multimolecular degradation complex, regulates the intracellular contents of the dual function protein -catenin. Cytosolic -catenin is essential for the formation of cadherin adherens junctions in epithelial cells, whereas this protein binds with TCF/LEF transcription factor in the nucleus to promote the activation of genes involved in cell cycle progression. *AXIN* 1 and *APC* are structural proteins that regulate -catenin levels, as do casein kinase 1, glycogen synthase kinase 3 (GSK3), and *β*-catenin itself (Abreu de Oliveira, El Laithy et al. 2022).

The cytosolic -catenin degradation complex is destroyed when WNT interacts to its cell surface receptors FZD and LRP5/6. The cytosolic protein DVL is activated by WNT-bound receptors, allowing *AXIN* 1 and its related kinases to connect to the membrane. As a result, -catenin accumulates in the nucleus and is less broken down. A second significant WNT-related regulation mechanism has just been discovered, including enzymes known as tankyrases (TNKS). The TNKS bind to *AXIN* 1 and accelerate the poly (ADP-ribose) moiety inclusion. The mutant *AXIN* 1 is ubiquitinated by the RNF146 E3 ligase, resulting in proteosomal degradation and disruption of the -catenin degradation complex (Pohl, Brook et al. 2017, Abreu de Oliveira, El Laithy et al. 2022).

1.6.3.1. SMALL MOLECULE INHIBITORS OF THE WNT PATHWAY

Despite the relevance of the WNT pathway in cancer, little progress has been made in identifying inhibitors. A number of TNKS medications have been developed, however they have all been found to be detrimental to the digestive system. GSK3 inhibitors were described, as well as FZD and DVL binding inhibitors. They are, nevertheless,

in the early stages of development. As a result, novel therapeutic techniques to alter the WNT pathway appear to be on the horizon(Tran and Zheng 2017).

1.7. B-*CATENIN* DESTRUCTION COMPLEX

The regulation of a β-*catenin* signaling pathway is highly regulated by the group of genes that tightly control the activity of *β-catenin* at the right time in the right place. This group of genes is called the destruction complex. This destruction complex is made up of a group of versatile multi-proteins (Figure 1.1), including *β-Catenin* itself, *GSK3*, *AXIN*, *APC*, and *CK-1*, which plays important role in the degradation of *β-catenin* (Stamos and Weis 2013).

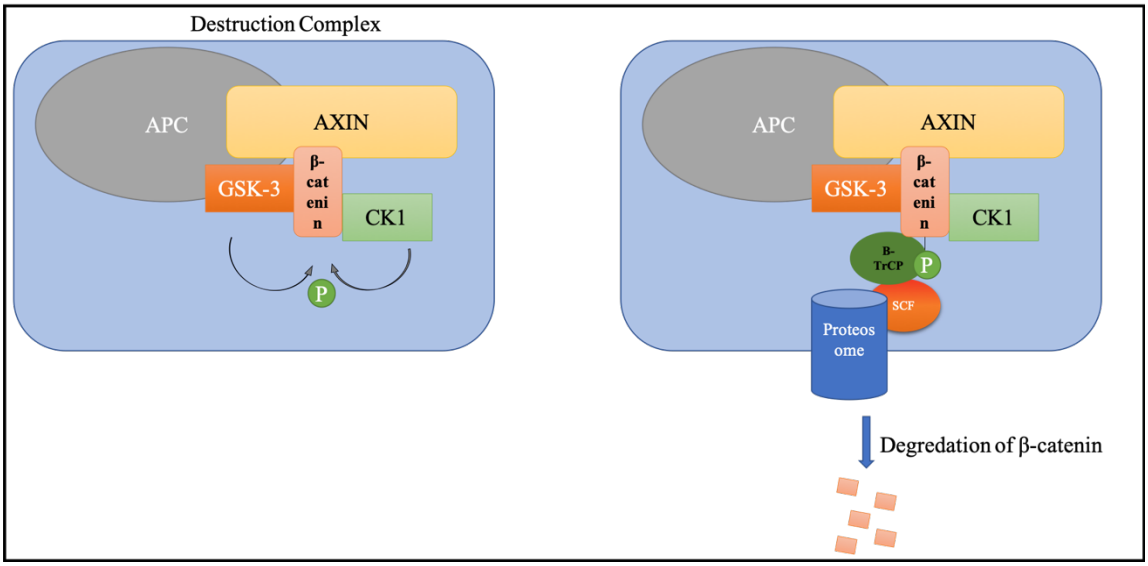

Figure 1.1. Illustrates the key component of destruction complex and shows the destruction of *β-catenin* through proteasome by interaction with the different adapter proteins in the presence of destruction complex (Adapted from Stamos, 2013).

Some other associated components include E3-ubiquitin ligase and protein phosphatase 2A (PP2A), which are also a part of this complex. Alteration or mutation in the component of the destruction complex has been reported in a variety of cancers as the destruction complex regulates the activity of *β-catenin*, and *β-catenin* is a key regulator in the WNT signaling pathway (Stamos and Weis 2013).

In a simple conclusion, this destruction complex is made up of a group of versatile multi-proteins (Figure 1.1), including *β-Catenin* itself, *GSK3*, *AXIN*, *APC*, and *CK-1*,

which play an important role in the degradation of *β-catenin* (Stamos and Weis 2013). In their paper, Stamos et al illustrate the key components of the destruction complex and show the destruction of β-catenin through the proteasome by interaction with the different adapter proteins in the presence of the destruction complex (Stamos et al 2013). In this model, two components, GSK-3 and CK-1, of the destruction complexes (APC+GSK-3+beta-Catenin+Axin+CK1), can phosphorylate beta-catenin through its serine (by GSK-3 and CK-1) or threonin (by GSK-3) side chains, the interaction of the phosphorylated beta-catenin with the beta-TrCP which binds to SCF ubiquitin ligase, leads to the destruction of β-catenin through the proteasome (Stamos and Weis 2013).

1.8. TARGET GENES AND ITS CORELATE PROPERTIES

If a person's DNA repair gene is defective, mistakes are not fixed. The errors then become mutations. These mutations, particularly those in tumor suppressor genes or oncogenes, may eventually lead to cancer. DNA repair gene mutations can be inherited or acquired. There are four distinct sets of genes (*APC*, *AXIN*, *β-catenin*, and *GSK3B* genes) that express in different cancers, but their research has been limited to breast cancer. We believe that these four genes might play a role in breast cancer under expression or overexpression via dealing with different signaling pathways, which is why we targeted them. From *APC* to *GSK3B*, here are the details of these genes.

1.8.1. *APC* GENE, WNT SIGNALING PATHWAY REGULATOR

The *APC* gene directs the production of the *APC* protein, which is involved in various cellular activities. The *APC* protein functions as a tumor suppressor and prohibiting cells from quickly growing and dividing uncontrollably. It aids in the regulation of cell division, cell attachment to other cells within a tissue, and cell movement within or away from a tissue. This protein also ensures that the number of chromosomes in a cell after cell division is accurate. The *APC* protein performs these functions mostly through interactions with other proteins, particularly those involved in cell attachment and signaling, and it is considered a major modulator in the WNT signaling cascade (Jin, Tamura et al. 2001).

β-catenin is one protein that *APC* binds with *β-catenin* aids in the regulation of gene activity (expression) and stimulates cell growth and division (proliferation), as well as the maturation of cells to perform certain roles (differentiation). *β-catenin* is also vital for tissue formation and helps cells adhere to one another. When *APC* and *β-catenin* are linked, *β-catenin* is broken down when it is no longer needed (Jin, Tamura et al. 2001, Parker and Neufeld 2020).

1.8.1.2. MOLECULAR ANALYSIS OF *APC* AND ITS LINK WITH CARCINOMAS

A nearly 9.5 kilobyte mRNA that encodes the massive 2,843 amino acid protein is basically translated by this *APC* gene present in the 5q21 region of chromosome. The coding region is made up of 15 exons, with the last one accounting for more than three-quarters of the total. Mutations in the *APC* gene, which is a tumor suppressor gene, have been linked to the both familial and sporadic colorectal cancer. A somatic mutation in this gene also shows a extracolonic malignancies in esophagus, pancreas and in stomach cancer. However, unlike to the gastrointestinal tract carcinomas, a primary breast cancer in human also shows a very few mutations in *APC* gene. In fact, an experiment analysis of female mice shows a nonsense mutation in the codon 850 of this *APC* gene which is basically responsible for develop the mammary carcinomas, so that it clarifies the suggestion of further analysis of the role of this *APC* gene in primary breast cancer is strongly recommended. However, they are more than 60 percent region of this gene show somatic mutation at exon 15 in a tiny region called mutation cluster region (MCR) which is basically accounts for ten percent of the coding region. Same like in familial adenomatous polyposis, the *APC* gene also has a truncation mutation in its N-terminal region (FAP). As a resultant, the molecular genetic diagnosis of this *APC* gene become complicated because of wide-ranging of many mutations in this gene.

Detect the *APC* mutations by using nucleic acid-based approaches is time consuming, because more than 95 percent of the *APC* gene mutation produce shorten APC protein due to vast variety of mutations including frameshift, nonsense or splice site mutations. The protein truncation assay, which detects protein truncating mutations in vitro or in yeast, is seems another option for detect the *APC* mutations (Furuuchi, K., et al. 2000).

It is hypothesis that the disturbance in the adenomatous polyposis coli (*APC*)/*β*-catenin pathway has been linked with the development of breast cancer, and this same of type of disruption has been hypothesized for colorectal tumor. In contrast to the colorectal malignancies, the somatic mutation of *APC* and catenin is uncommon in breast cancer. To understand more about the role of *APC*/*β*-catenin gene in breast cancer, the researcher evaluated the role of *APC* gene promoter methylation and the different *APC* and *β*-catenin gene mutation to understand their role in primary breast cancer and in non-cancerous breast tissues. As a resultant they found 18 out of 50 hypermethylation of the *APC* promoter CpG island in primary breast tumor but found none in 21 non-cancerous sample of breast tissue. Despite the role of *APC* and catenin mutations, there was no significant connection has been identified between *APC* promoter hypermethylation with respect to different prospects including patient age, metastases, lymph node, presence or absence of estrogen and progesterone receptor, size, stage, or histological type of the tumor. These data imply that the hypermethylation of the CpG island in the *APC* promoter is a cancer specific change considered as a general mechanism in the deactivation of the *APC* gene in primary breast cancer than previously thought (Jin, Tamura et al. 2001).

1.8.2. THE *AXIN* Gene

AXIN1 (also known as *AXIN*) and *AXIN*2 (also known as conduction or Axil), which encode isoforms a and b, share 45 percent nucleotide identity and function. In the mouse model analysis of *AXIN*1 and 2 shows, *AXIN*1 is expressed throughout while *AXIN*2 is expressed specifically. *AXIN*1 plays an important role as a transcriptional factor component, which is a component of catenin degradation, responsible for the stabilization of low signaling of WNT signaling activity. On the other hand, *AXIN* 2 is increased when the level of *β*-catenin is high, so that it can serve to limit the WNT signals' length and amplitude. In a WNT-dependent manner, *AXIN*1 is dephosphorylated and downregulated. The *AXIN*s that receive WNT ligand signals show less in their concentration in the cell as compared to the normal *AXIN* level in the cell. According to the biochemical research, the *AXIN*'s intracellular concentration is 1000 times lower than those of other destruction complex components, showing that the *AXIN* is the pathway limiting factor (Salahshor and Woodgett 2005).

1.8.2.1. THE *AXIN 1*

Axin1 was first discovered as the result of the murine fused locus. Human equivalent on chromosome 16p13.3 that is 87 percent similar to the mouse protein. *AXIN*1 "isoform a" (GenBank NP 003493) encodes a polypeptide of 862 amino acids (aa), although "isoform b" (GenBank NP 851393) is a shorter version of *AXIN* that lacks 36 aa in the N-terminal domain encoded by exon 8. A exon 8-encoded polypeptide segment's function is unknown. This splice variation is seen in all species, showing that it has a similar function. The polypeptide encoded by exon 8 is located between both the *AXIN* Binding Domain (DIX) and catenin binding and disheveled domains. A proposed CKI phosphorylation site, as well as the *AXIN* oligomerization site, in which *AXIN* binds to itself, are both adjacent. In cells, *AXIN* dimerization is assumed to be necessary for *AXIN* stability and function. A putative nuclear export signal for *AXIN* is wedged between the spliced exon and two other probable nuclear export signals (Salahshor and Woodgett 2005).

1.8.2.2. THE *AXIN 2*

Because of its interaction with catenin, *AXIN*2 was recognized as a protein family member of the *AXIN*. Mutation of the *AXIN*2 gene increases the level of catenin in colorectal cancers with a malfunctioning mismatch repair system. According to the different research, there are 11 exons that encompass more than .5kb. As with *AXIN* 1, *AXIN* also has two isoforms of *AXIN*2 (A and B). According to a fish analysis, *AXIN* 2 was located on human chromosome 17q24, a site where heterozygosity loss is common in breast cancer, neuroblastoma, and other cancers. *AXIN*2 also has certain domains like *APC*, *GSK3* and a catenin binding domain like Aixn1. Downregulation of *AXIN*2 has been associated with a decreased overall survival rate in BC patients. BC vulnerability is conferred by the rs11079571 and rs3923087 polymorphisms. (Salahshor and Woodgett 2005).

1.8.2.3. *AXIN* VARIANT LINKED WITH CARCINOMAS

There are two variants of *AXIN*, *AXIN*1 and *AXIN*2. whose mutation has been found to cause a variety of *AXIN*s, including breast cancer. Specific to the domains, the

mutation of these genes is also found in the variety of domains, including catenin binding domain and *APC* (RGS). All of these mutations or any of them disturb the GSK3 binding and alter the linkage between *AXIN* and two upstream TCF-dependent transcription activators, Frat 1 and DVF. Several studies have been published on the role of *AXIN*1 and *AXIN*2 mutations in cancers such as breast cancer, ovarian cancer, and the HCC cell line. Breast cancer, in particular. *AXIN*2 has been linked to the rs151279728, *AXIN*2 rs2240308 C> T, rs1133683 C> T, and mrs7224837 A> polymorphisms in breast cancer samples. A significant increase in *AXIN*2 expression was detected in breast cancer patients. *APC*, *β*-catenin, *CK1*, *GSK3*, and *PP2A* gene expression were found to be linked to clinic-pathological features in further research (Sayad, Abdi-Gamsae et al. 2021).

Another study discovered that *AXIN*2 downregulation is linked to lower overall survival in BC patients. The rs11079571 and rs3923087 polymorphisms confer vulnerability to BC (Salahshor and Woodgett 2005)(Sayad, Abdi-Gamsae et al. 2021).

1.8.2.4. WNT/ B-CATENIN SIGNALING LINED WITH *AXIN*

WNT/β-catenin signaling control the expression of SOX7 and *AXIN*2, in breast cancer these *SOX7* and *AXIN2* express at the low level (Liu, Mastriani et al. 2016).

SOX7, which is a tumor suppressor gene, is a part of the *SOXF* gene family. This gene has been linked with a variety of human cancers, including breast cancer. Despite the fact that their mechanism of action is still unknown, Previous research has been conducted to analyse the linkage between *AXIN*2 and *SOX7* and their co-regulatory role on the WNT/β-catenin signal pathway is the best can be done by using clinical specimens and microarray gene expression data. This analysis was also done to investigate the label of *SOX7* and other WNT/β-catenin pathway co-expression genes and found that *SOX7*, *SOX17*, and *SOX18* expression were prominently lower in breast cancer tissue as compared to the normal controls. The underlying research results also indicated positive relations between *AXIN*2 and *SOX7* in the WNT/β-catenin signaling pathway. Downregulation of *SOX7* has been associated with late stage and poorly differentiated breast cancer in clinicopathological studies. Sox7 shows a positive correlation with *AXIN* while it shows a negative correlation with catenin, indicating that *SOX7* and *AXIN*2 play a co-regulatory role in the WNT/β-catenin signaling

pathway in breast tissue to modify the carcinogenesis process. Analyzed through the bio informatics tools, *SMAD7* has been recognized as a target of *SOX7* and *AXIN*2 in the WNT/β-*Catenin* signaling pathway, which influences breast cancer cell growth (Liu, Mastriani et al. 2016, Dai, Gao et al. 2019)**.**

1.8.3. THE *GSK3B* GENE

In 1980, the serine threonine kinase glycogen synthase kinase 3 (*GSK3*) was discovered in rat skeletal muscle. Despite the fact that it has the number 3, it is the only enzyme known to phosphorylate glycogen-synthase, however enzymes formerly known as *GSK1* and *GSK2* do not. *GSK3* has been associated to a variety of cellular functions, including embryogenesis, immune responses, inflammatory responses, apoptotic activities, autophagy, wound repair, neurodegeneration, and carcinogenesis. *GSK3* is made up of two different isoforms: *GSK3* and *GSK3*. The isoforms and share 85 percent of their DNA. Both genes are found on chromosomes 19q13.2 (*GSK3*) and 3q13.3 (*GSK3*) in humans. Both of which have seven antiparallel β-plates, a short connecting segment, and an alpha helix, but they are encoded by distinct genes and expressed in human tissues in different ways. These isoforms are neither functionally comparable or redundant, despite their close similarity and functional overlap. *GSK3* has a much better understood signaling mechanism and protein function. *GSK3* is generated in natural killer (NK) cells, bone marrow granulocytes, and the ovaries by the *GSK3B* gene on chromosome 3's long arm. Because it lacks the glycine-rich N-terminal domain, it has a somewhat lower molecular mass than *GSK3*. It's found in both the nucleus and the cytoplasm. The expression of another isoform cannot compensate for the loss of one, which is especially important during embryonic development when the *GSK3B* gene is lethal (Rayasam, Tulasi et al. 2009, Glibo, Serman et al. 2021).

GSK3B dysregulation has been associated to cancer development and carcinogenesis. *GSK3B* appears to have a deleterious impact on carcinogenesis in mammary cancers. Demonstrate that tissue-specific production of a kinase-inactive *GSK3* (dominant negative) promotes breast cancer via lowering *GSK3's* endogenous activity. The buildup of *β*-catenin and cyclin D1 is accompanied by the acceleration of mammary carcinogenesis by this kinase-inactive *GSK3*, showing that the stimulation is carried

out by the dysregulation of the WNT/β -catenin pathway. *GSK3* activation, on the other hand, decreases the growth of breast cancer. In MDA-MB-231 human breast cancer cells, *GSK3* regulation by adiponectin causes apoptosis and cell cycle arrest, which is related with lower cyclin D1 levels and suppressed intracellular accumulation of -catenin and its nuclear activities. In addition, in nude mice, in vivo activation of *GSK3* through recombinant adiponectin supplementation or adenovirus-mediated adiponectin overexpression significantly lowers MDA-MB-231 cell mammary tumorigenicity. *GSK3* activation by rapamycin causes cyclin D1 expression to be downregulated, cell cycle arrest to occur, and anchorage-dependent growth to be inhibited in breast cancer cells. Constitutively active *GSK3* (S9A mutant) promotes apoptosis in human breast cancer cells, and injecting the liposome complex with *GSK3* into tumor-bearing animals greatly lowers mammary tumor growth. *GSK3* activity antagonism is carcinogenic in mammary epithelial cells, but tumor suppressor in mammary tumors (Luo 2009, Glibo, Serman et al. 2021)**.**

1.8.4. THE *B*-CATENIN GENE

Canonical WNT signaling pathway which is commonly known as WNT/β-catenin signaling pathway is well maintained signaling pathway that control the growth, division, differentiation, migration, invasion, and tissue homeostasis. According to research evidence, disruption of the WNT/β-catenin cascade is associated to the formation and progression of several solid tumor and blood cancers. (Zhang and Wang 2020).

During the initial tumorgenesis activities in the WNT/β-catenin signaling pathway, it was observed that the transcription factor catenin, which is a critical component of the WNT signaling system, is incorrectly controlled. While casein kinase 1 (GK1) and glycogen synthase kinase 3 (GSK3) promote catenin phosphorylation inside the degradation complex, allowing it to be ubiquitinated and eliminated by proteasomes. The initiation of catenin dependent signaling cascade take place, when secrated cysteine- rich glycoprotein WNT bind to the LRP-5/6 receptors and FZD receptors. In the presence of WNT ligand, Disheveled (DVL) is formed when WNT ligand interacts with receptors on the cell surface, resulting in the buildup of the complex's including GSK3, CK1, *APC* and *AXIN* mostly on receptor. The amount of cytosolic -catenin

increases as a result of GSK3 phosphorylation and inhibition. While the overexpression of the TCF/LEF target genes linked with cascade of events linked by the cytoplasmic migration of the unphosphorylated-catenin to the nucleus and then accumulate, to activate the WNT target gene like c-Myc, cyclin D1, and CDKN1A, the interacting with TCF/LEF and co-activators like pygopus and Bcl-9 take place, which results in the overexpression of the TCF/LEF target genes.

More ever through the degradation complex, the ubiquitination and phosphorylation of catenin has also been connected to various regulatory mechanisms. By eliminating palmitoleate, Notum blocks WNT proteins from secreting extracellularly. When the dickkopf (DKK) interacts with the LRP5/6 receptor, it totally suppresses the beginning of WNT protein-mediated signaling. Released FZD related protein (sFRPs) interacts to the FZD receptor and suppresses WNT protein-mediated signaling. WNT inhibition factor, on the other hand, inhibits signaling by directly interacting with the WNT protein. LGR4, LGR5, and LGR6 are seven transmembrane receptors that bind to R-spondins (RSPO) with high affinity, enhancing WNT signaling even at low WNT ligand concentration. (Zhang and Wang 2020).

WNT/β-catenin signaling dealing with the number of cell signaling pathways including epidermal growth factor receptor, nuclear factor kappa-B, hippo/yap, PI3K/Akt pathway and sonic hedgehog are involved in cancer development. EGFR and -catenin may create a complex, boosting cancer cell proliferation and metastasis. In the WNT/β-catenin signaling pathways, the hippo/yap pathways also has been found to reduce the Dvl phosphorylation, transcript of catenin/TCF target genes and nucleus accumulation of catenin. In glioblastomas cells also interact with the PI3K, AKT and GSK3 cascade to stimulate the WNT/β-catenin pathway, laying a molecular foundation for temozolomide resistance. -catenin has also been demonstrated to be activated by the AKT kinase. As a result, researchers have discovered that crosstalk among PI3K/AKT pathway and

WNT/ β-catenin pathway to promotes carcinogenesis and resistance to cancer therapies.

Targeted medicines have been found to have promise therapeutic potential in preclinical investigations and clinical testing of a variety of cancer types, highlighting the physiological relevance of the WNT/β-catenin signaling pathway underlying

tumorigenesis. The goal of many studies is to provide the rationales and insights into developing cancer therapeutic options by elaborating on the advantages and disadvantages of WNT/β-catenin signaling cascade targeted therapeutics in cancer. (Jang, Kim et al. 2015, Zhang and Wang 2020).

1.8.4.1. INTERVENTION IN THE WNT/B-CATENIN SIGNALING PATHWAY IN BREAST CANCER

Catenin is involved in a number of signaling pathways, such as the WNT signaling pathway. The purpose of this involvement is to maintain cell polarity, it also interacts with E-cadherin, which play a central role in the regulation of cell-cell adhesion in the cell membrane. Breast cancer is caused by an increase of -catenin in the nucleus as a result of abnormal WNT signaling or a mutation in the -catenin gene. Nuclear -catenin transactivates the gene producing cyclin D1 when combined with Tcf/lymphoid enhancer factor (Lef), inducing hyperplasia of the mammary glands Nuclear-catenin increases the synthesis of pro-invasive proteins as well. As a result, the subcellular distribution of -catenin has a significant impact on tumor cell phenotype and behavior. Because of a link between cytoplasmic and nuclear *β*-catenin in adenocarcinoma, excessive cytoplasmic -catenin could be a malignant sign for breast cancer (Wang, Zhang et al. 2015, Zhang and Wang 2020).

The following conclusion has been reached on the link between WNT/β-catenin and breast cancer based on various study analyses.

- WNT/β-catenin signaling activity is higher in malignant breast cancer tissues than in normal breast cancer tissues.
- *In vitro*, WNT/β-catenin signaling regulates breast cancer cell proliferation and death, and inhibiting it lowers breast cancer invasion and migration.
- In a mouse xenograft model, blocking WNT/β-catenin signaling reduces tumor growth.
- In a mouse xenograft model, inhibiting WNT/β-catenin signaling inhibits tumor spread.

1.9. THE OZONE

The ozone is a colorless gas and made up of three oxygen atoms. A layer of ozone gas protects the planet from the sun's UV rays in the upper atmosphere. However, ozone is a dangerous air contaminant at ground level. When a person inhales ozone gas, it causes irritation of the lungs and throat, coughing, and increased asthma symptoms. High levels of exposure can cause lung damage and even death (American Lungs Association).

However, researchers believe that ozone has medicinal properties and might be used to cure arthritis, viral infections like HIV and SARS, sterilize wounds, activate the immune system, heal ischemic heart disease, and even cancer.

Over several decades, ozone (O3) and other ionization radiation have been utilized to create oxidative stress, which causes molecular mechanisms to change, inhibiting tumor cell growth without harming normal cells. The concentration of ROS specific to ozone is closely related to tumor cell proliferation, which indicates that increasing ozone concentration suppresses tumor cell growth more effectively. In this regard, an experiment was conducted to examine the human cancer cell line of the breast, uterine, and lungs tumor during 8 days of culture by treating the human cancer cell line with various concentrations of ozone ranging from 0.3 to 0.8 parts per million, with results showing that 0.3 to 0.5 parts per million inhibit cancer cell growth from 40 to 60 percent, while exposure to 0.8 parts per million inhibits cancer cell growth from 40 to 90 percent (Sweet, Kao et al. 1980, Clavo, Rodríguez-Esparragón et al. 2019).

1.9.1. TARGETED TUMOR THERAPIES AND RECENT USE OF OZONE IN THERAPY

Traditional surgical removal techniques such as radiation (RT) and chemotherapeutic techniques (CT) are still effective for many cancer types; however, innovative treatments such as immunotherapy or treatment with Reactive Oxygen Species (ROS) are showing strong potential in the way of cancer treatment.

RT as well as some CT drugs attack cancerous cells in general by producing reactive oxygen species (ROS) and free radicals within cancer cells. Because of the well-known but still unknown Warburg effect, cancer cells have a delicate tolerance to increased

ROS production (aerobic glycolysis or aerobic fermentation of glucose). Externally induced ROS could throw the equilibrium off. As a result, apoptosis occurs in the cell. The Warburg effect and higher glucose consumption are associated with increased ROS formation in cancer cells. (Liou and Storz 2010).

Adaptation, on the other hand, occurs when the body produces more antioxidant enzymes as well as improves some metabolic processes (for instance, the pentose phosphate pathway from glycolysis produces NADPH), which assists to balance excess ROS. According to the recent studies conducted, a secondary consequence of the Warburg effect could play a significant role in oncogene-induced senescence (OIS). OIS has a tumor-suppressive biological function, and pyruvate dehydrogenase (PDH)-mediated glucose oxidation has been found to modulate OIS.

Because ROS enhance cellular proliferation, survival, and motility, they have been associated to cancer. Inactivating tyrosine phosphatases, phosphatase, and tensin homolog produce chromosomal damage that leads to tumorigenicity and tumor development. ROS, from the other hand, can cause cellular damage by damaging cell membranes and causing other harmful effects (Liou and Storz 2010, Clavo, Rodríguez-Esparragón et al. 2019).

The anti-cancer actions of cisplatin and rapamycin are limited by Reactive oxygen species suppression, whereas photodynamic therapy with specific ROS creation seems to have a focused anti - tumor effect.

Ozone can produce rapid injury by oxidizing the fatty acids which constitute the cell membrane (lipid peroxidation). Criegee's reaction produces hydroperoxides, primarily hydrogen peroxide (H2O2), aldehydes such as malonyldialdehyde (MDA), alkenes like 4-hydroxynonenal (4-HNE), and lipoperoxides (primarily 9 alfa-hydroxy-hydroperoxide), that are slightly tinkered by antioxidative enzymes found in the inner layer of the cell membrane. NADPH and other cytoplasmic molecules react with non-reduced substances. When NADPH levels have dropped, as they would be in cancer cells, oxidative stress occurs, causing cell damage. In non-cancer cells, these chemicals activate nuclear factor NFR2, leading to an increase an antioxidant molecule synthesis and a change in nuclear factor NFK. As cancerous cells' antioxidant systems are already overworked as a consequence of high level of reactive oxygen species, they have limited capacity to boost antioxidant synthesis even more. As a result, non-

cancerous cells may survive ozone amounts that are harmful to cancer cells. Moreover, there is a simultaneous increase in membrane permeability, which leads to a change in the cytoplasmic ion concentration, resulting in cell death. (Liou and Storz 2010, Clavo, Rodríguez-Esparragón et al. 2019).

During the last six decades, high-impact journals has been published fascinating research that shows the in vivo and in vitro role of ozone (O3) in cancer cell killing while being harmless for non-cancerous cells. However, there is very less evidence-based support for its therapeutic usage in cancer patients, according to a few published clinical investigations.

In this study, nanobubble ozone stored niosomes (NOSN) produced and patented by Sonofarma Pharmaceuticals Chemical Industry Trade Ltd Sti (Patent No PCT/TR2022/050177) was used.

CHAPTER 2: MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. CHEMICAL REAGENTS AND KITS

Dulbecco modified eagle medium (DMEM) F-12, HEPES (Ref31330038, ThermoFischer Scientific, Pittsburg, USA), Fetal bovine serum (FBS) (Ref10500064, ThermoFischer Scientific, Pittsburg, USA) and penicillin streptomycin (Ref 15140122, ThermoFischer Scientific, Pittsburg, USA) were used for the preparation of cell culture media. Trypsin / EDTA (0.25%) (Ref25200056, ThermoFischer Scientific, Pittsburg USA) was used to detach the cells from flask surface during cell culture experiments. DMEM, FBS, Penicillin-Streptomycin and Trypsin/EDTA were kindly provided by Dr. Ümit Sabancı from Sonofarma Pharmaceuticals Chemical Industry Trade Ltd Sti. TRIZOL reagent (Hibrizol, Hibrigen, Istanbul, Turkey) was used for RNA isolation. ABM OneScript plus cDNA-synthesis kit (Applied Biological Materials Inc. (ABM), Richmond, Canada) was then used for cDNA synthesis. Q-PCR experiments were performed using 2X SYBR Green qPCR Mix (Hibrigen, Istanbul, Turkey).

2.1.2. INSTRUMENTS

- MetiSafe® Laminar Air Flow Cabinet (Ankara, Turkey)
- Sanyo MCO-5AC Incusafe Compact CO2 Incubator (Osaka, Japan)
- MetiSafe® PCR Cabinet (Ankara, Turkey)
- Nano-drop™ 2000/2000c Spectrophotometer (Thermo-scientific, Pittsburg, USA)
- RotorGene Real-Time PCR (Qiagen, Hilden, Germany)

2.1.3. OLIGONUCLEOTIDES

Primers that used in this project were from Oligomer Company (Turkey).

2.1.3. CELL LINE

MCF7 breast cancer cell line (ATCC #HTB-22), which were kindly provided by Prof. Dr. Pınar Tulay, was used in the cell-culture experiments. MCF7 cells are epithelial cells that were isolated from the breast tissue of a white, 69-year-old, female patient with metastatic adenocarcinoma. Detailed information about the characteristics of cell line is provided in the introduction section (1.5.1) of this thesis.

2.2. METHODS

2.2.1. PREPARING MCF7 CULTURE MEDIA

MCF7 culture media, or growth media, was prepared by adding ten percent filtered fetal bovine serum and one percent penicillin and streptomycin into DMEM / F-12 (1.1) (1X) (Dulbecco modified eagle medium F-12, + L-glutamate, +15Mm HEPES).

2.2.2. THAWING MCF7 CELLS

MCF7 cells were obtained frozen at -80^0 Celsius in cryotubes containing five percent DMSO (Dimethyl Sulfoxide). This DMSO helps to inhibit the production of ice crystals. After thawing the frozen cells in a 37°Celsius water bath, they were transferred to a centrifuge tube containing 15 mL of culture media. The tubes' contents were then centrifuged (speed 1000, RCF 192, time 8 min, temperature 22^0Celsius). After being removed from the tube, the supernatant was discarded.

2.2.3. CELL CULTURE

After thawing, cells were mixed gently with 5ml of culture media and transferred to a T-25 flask. Then they were incubated at $C0_2$ incubator having five percent $C0_2$. The culture media was changed every day with the new one of same concentration until cells reach 90 percent confluency.

2.2.4. SUB-CULTURING

The T-25 flasks were removed from the incubator and examined under a low-power inverted microscope. If there were no floating cells and the cells were 80-100 percent confluent, sub-culture was undertaken. For the subculturing, first the culture media was removed from the T-25 flask, then added 1 mL of trypsin so that the cells were detached from the bottom, and then incubated for three minutes. After incubation, samples were controlled under a microscope to make sure that the cells were detached from the surface properly after being treated with trypsin. 6 ml growth medium was added to the flask and mixed t gently. After thoroughly mixing, 3 mL of culture was transferred to the new T-25 flask. The new flask was then placed in a five percent CO_2 incubator at 37^0 C. Four T-25 flasks were prepared for NOSN treatment and were used when 80 percent confluent.

2.2.5. NOSN TREATMENT

Nanobubble ozone stored niosomes (NOSN) were kindly provided by Dr. Ümit Sabancı from Sonofarma Pharmaceuticals Chemical Industry Trade Ltd Sti (Patent No PCT/TR2022/050177). Varying concentrations of NOSN (stock 100ppm) was added to three T-25 flasks (**Table 2.1)**. One flask was used as a non-treated control. Then these samples were incubated in a five percent CO_2 incubator at 37°C for 24 hours.

Table 2.1. Shows the different concentration of NOSN applied on MCF7 cells

Flask	**NOSN Concentration(ppm)**
1	6.25 ppm
2	3.125 ppm
3	1.5625ppm
Control	No NOSN

2.2.6. HARVESTING CELLS

In the initial step of cell harvesting, culture medium was removed in the first step. After removing the culture medium, 1 ml of trypsin was added to all four T-25 flasks. Then flasks were placed into the CO_2 incubator for five to ten minutes. After incubation and confirmation of the detachment of cells from the flask surface, 3 mL of fresh growth medium was added and mixed gently. Then transfer the T-25 flask media containing cells or culture to the 13ml falcon tube and centrifuge them for 10 min at 13000rpm. After centrifugation, remove the supernatant and store the pellet for onward RNA extraction at -80C.

2.2.7. RNA ISOLATION

RNA extraction was performed using the TRIZOL reagent (Hibrizol, Hibrigen, Istanbul, Turkey). The first step in the RNA extraction protocol is to add 550ul of TRIZOL reagent to break the cell wall chemically. Then add 100µlof chloroform in the next step. Cap the sample tube tightly and then vortex for 15 seconds at room temperature for two to three minutes. Then, centrifuge the sample at 14000rpm for 15 minutes at 4^0C. After centrifugation three different colors has been appeared having different phases and component in them. A lower red having phenol chloroform phase, a colorless upper aqueous having interphase and RNAs remain in the aqueous phase. Then transfer the upper aqueous solution in to the fresh tube carefully without disturb the interphase. Isopropyl was used for the precipitation of the RNA present in the aqeous phase. In general, for the initial homogenization we used 250µl isopropyl alcohol per 0.5 ml of trizol reagent than incubate the samples for 10 minutes at 15 to 30 degrees. After incubation has been done, a sample were centrifuged at 3-degree Celsius for 10 minutes at 14000 rpm. The precipitated RNA often invisible before centrifugation, form a gel like pellet at the bottom or sides of the tube. Discard the supernatant and add 1ml of 75 percent ethanol per 1ml of trizol reagent for initial homogenization, then vortex the solution and centrifuge it at 6 degrees Celsius for 5 minutes at 10000 rpm. Repeat the above cleaning process one of two time to get the

pure product. To get the pure RNA, remove the ethanol by vacuum or air dry for five to ten minutes. Please be sure that do not try to dry the RNA pellet by using centrifugation under vacuum. At last, to elute the RNA add 50ul of distilled water (DNAase and RNAase free water). The purity of RNA was evaluated by the nano drop spectrometer.

2.2.8. RNA QUANTIFICATION

Quantification and purity satisfaction of RNA has been done by Nano-drop™ 2000/2000c Spectrophotometer (Thermo-scientific, Pittsburg, USA). The Thermo Scientific nanodrop are full spectrum UV-vis spectrophotometers, which can not only be used for RNA quality or purity analysis, but can also be used for DNA, protein, and many more compounds. The benefits of this instrument include the ability to measure volume samples as small as 0–5L. Our results for the Nanodrops are shown in the results section, and after ensuring the adequate quantity and quality of our RNA, we processed our experiment onward.

2.2.9. COMPLEMENTARY DNA (cDNA) SYNTHESIS

The abm One Script plus cDNA-synthesis kit (abm business, Richmond, Canada) was used to synthesize cDNA. One Script Plus reverse transcriptase, One Script Plus RT reaction buffer, Oligo(dT) primer, dNTPs mix, and anchored oligo dt primer were all included in this package, which was kept at -15 to - 25°C. For each of the sample, a 10µl of RNA, 1µl of oligo(dt) primers, 1µl of reverse transcriptase enzyme, 2µl of deoxyribonucleotides triphosphate(dNTPs)**,** 4µl of buffer solution and 2µl of nucleases free water was used. After the addition of these components in reaction tubes, the tubes were placed in incubation at 55° C for 15 minutes by using the conventional PCR to synthesize the cDNA from RNA through a reverse transcription mechanism.

2.2.10. REAL TIME (QUANTITATIVE) PCR

The RotarGene Real Time PCR apparatus was used to perform real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR). By adjusting the

temperatures in accordance with the procedure, enabling reactions to occur, and collecting data, this device produces accurate results. Basically, this device was used according to the manufacturer's guidelines to analyze the expression pattern of *APC*, *AXIN*, *β-catenin,* and *GSK3B* genes in the MCF-7 cell line of breast cancer after applying NOSN. Calculation of the RT-qPCR Master Mixture for each gene is listed in Table 2.2. As we used different concentrations of GSK3 B, its condition is listed in a separate Table 2.3. While the concentration of cDNA of the *APC*, *AXIN*, *β-catenin,* and *GSK3B* genes used is 2µl. The primer sequences, melting temperatures, GC content and expected product sizes for all genes are listed in Table 2.4 below.

In this process, the Real-time PCR Rotor-Gene Q (Qiagen) was used to observe the expression of the targeted genes via the use of cDNA samples. Real-time PCR was set up according to the conditions listed in Table 2.5 below, as we didn't get any results for *GSK3B* on these conditions, so we applied different conditions for *GSK3B* that are mentioned in table 2.6. While for *β-Actin*, we used different annealing temperatures and different times for initial denaturing, which are shown in table 2.7. A no template control was included in every RT-qPCR reaction to control any possible contamination.

After real time PCR reaction was completed, cycle threshold was automatically adjusted and ΔCt were calculated.

Table 2.2: RT-qPCR Master mixture calculation for *APC, AXIN, β-catenin* and *ACTB*

Components	**Final Concentration**	**Mixture Volume for 1X**
SYBR Mix	1X	10 µl
Forward Primer	0.9 µM	2 µl
Reverse Primer	0.9 µM	2 µl
dH_20	-	5 µl
cDNA	-	2 µl
Glycerol (For *GSK3B* and *β-catenin*)	4.5%	1 µl

Table 2.3: RT-qPCR Master Mixture calculation for *GSK3B*

Components	**Final Concentration**	**Mixture Volume for 1X**
SYBR Mix	1X	10 µl
Forward Primer	0.8 uM	2 µl
Reverse Primer	0.8 uM	2 µl
dH_20	-	6 µl
cDNA	-	2 µl
Glycerol (For *GSK3* and *β-catenin*)	4.3%	1 µl

Table 2.4: List of gene-specific primer sequences, melting temperature, GC content and product sizes.

Gene Name and Accesion Number	**Primer name**	**Sequence (5'-3')**	**Primer Tm**	**GC%**	**Product size**
β-CATENIN (NM_001904.4)	Forward	AGACGGAGGAAGGTCTGAGG	60.32	60.00	112bp
	Reverse	TTCAAATACCCTCAGGGGAACA	59.01	55.01	
GSK3B (NM_001146156.1)	Forward	ACAGCAGCGTCAGATGCTAA	59.75	50.00	151bp
	Reverse	TGACCAGTGTTGCTGAGTGA	59.17	50.00	
APC (NM_000038.6)	Forward	ACGCGCTTACTGTGAAACCT	60.25	50.00	183bp
	Reverse	GCCTGTAGTCCCCCTAGTTC	58.88	59.10	
AXIN (NM_004655.4)	Forward	CCCGAGAGCCGGGAAATAAA	59.82	55.00	101bp
	Reverse	CTCCTCTCTTTTACAGCAGGGC	60.68	54.55	
β-ACTIN (NM_001101.5)	Forward	GCACTCTTCCAGCCTTCCTT	59.96	55.0	111bp
	Reverse	GTTGGCGTACAGGTCTTTGC	59.76	55.0	

Table 2.5: Real-Time PCR Conditions for *APC, AXIN*.

	PCR Steps	**Temperature ^{0}C/Time**	**Cycles**
Steps	Initial Denaturation	95 ^{0}C / 2 minutes	1
	Denaturation	95 ^{0}C / 0.30 seconds	30
	Annealing	58 ^{0}C / 0.30 seconds	
	Elongation	72 ^{0}C / 0.45 seconds	
	Termination	72 ^{0}C / 7 minute	1

Table 2.6: Real-Time PCR Conditions for *β-catenin* and, *GSK3B*.

	PCR Steps	**Temperature ^{0}C/Time**	**Cycles**
Steps	Initial Denaturation	95 ^{0}C / 2 minutes	1
	Denaturation	95 ^{0}C / 0.30 seconds	30
	Annealing	59^0 C / 0.30 seconds	
	Elongation	72 ^{0}C / 0.45 seconds	
	Termination	72 ^{0}C / 10 minute	1

Table 2.7: Real-Time PCR Conditions for *β-Actin*.

	PCR Steps	**Temperature ^{0}C/Time**	**Cycles**
Steps	Initial Denaturation	95 ^{0}C / 5 minutes	1
	Denaturation	95 ^{0}C / 0.30 seconds	30
	Annealing	60 ^{0}C / 0.30 seconds	
	Elongation	72 ^{0}C / 0.45 seconds	
	Termination	72 ^{0}C / 7 minute	1

2.2.11. STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS software (Statistical Package for the Social Sciences 25.0, SPSS Inc, Chicago, IL, USA). The data was expressed as mean ± standard error (SE). The gene expression data was obtained as Cycle Threshold (CT) values (CT = cycle number at which logarithmic PCR plots cross a calculated threshold line). The expression of each gene was compared between depots using the 2ΔΔCT method (ΔΔCT = CT of the target gene-CT of the housekeeping gene), respectively. The differences between normal and abnormal distributed continuous variables were compared using Student's t-test and Mann-Whitney U test respectively. Statistical significance will be taken as $p<0.05$.

CHAPTER THREE: RESULTS

3.1. RESULTS

This chapter includes the results of the conducted experiment. The results that are mentioned in this chapter are obtained from the nano-drop spectrophotometer and real time PCR. Moreover, these results were statistically analyzed using the one-way ANOVA student's T-test. This method was helpful in creating the following numerical and graphical results that are presented in this chapter.

Three different concentrations of NOSN was applied along with one sample having no NOSN in the breast cancer cell line culture. These samples were divided into tubes. The concentration of NOSN used in each sample is given in Table2.1. RNA extraction was successfully performed from these samples.

3.2. NUCLEIC ACID CONCENTRATION

The purity of RNA was evaluated by using the Nano-Drop Spectrophotometer. The results are shown in the table 3.1 below.

Table 3.1 shows the nucleic acid concentration and purity of our sample

Sample Number	**Nucleic Acid Concentration ng/ul.**	**Absorbance at 260/280**
1	33.9	1.67
2	48.1	1.68
3	21.1	1.68
Control Group	32.6	1.67

The RNA purity was determined by measuring the ratio of their absorbance at 260 and 280 nm (A260/280). The A260/280 ratio of pure RNA is 2.0, and the A260/280 ratio

obtained for all the RNA samples. In this experiment, the mean ratio was approximately 1.67. The range of concentration of extracted RNA during this experiment in ng/µl is shown in (Table 3.1). The level of expression of *APC*, *AXIN*, *β-catenin*, and *GSK3B* after NOSN treatment were determined by real time PCR analysis and the results were evaluated using the one-way ANOVA statistical analysis. The Ct values were obtained from the real time PCR equipment. These values were used to obtain the log ΔΔCT (fold change) values for comparative ΔΔCT analysis (Table 3.2).

Table 3.2 represent the Ct values of the *APC, AXIN, β-catenin, GSK3B gene*

APC	1st Ct Values	2nd Ct Values
1 (6.25 ppm)	22.71	23.64
2 (3.125 ppm)	23.09	22.22
3 (1.5625 ppm)	23.21	22.08
Control (No NOSN)	24.57	22.62

AXIN	1st Ct Values	2nd Ct Values
1 (6.25 ppm)	20.34	19.34
2 (3.125 ppm)	19.81	19.26
3 (1.5625 ppm)	17.63	20.52
Control (No NOSN)	21.27	22.16

β-catenin	1st Ct Values	2nd Ct Values
1 (6.25 ppm)	18.49	17.55
2 (3.125 ppm)	17.9	16.85
3 (1.5625 ppm)	17.57	16.61
Control (No NOSN)	20.8	17.43

GSK3B	1st Ct Values	2nd Ct Values
1 (6.25 ppm)	24.08	24.06
2 (3.125 ppm)	24.39	24.48
3 (1.5625 ppm)	24.9	Nill
Control (No NOSN)	24.74	24.4

3.3. *APC* GENE EXPRESSION ANALYSIS

The expression levels of *APC* were investigated in four groups. The average Ct value of group 1 was 22.71, group 2 was 23.09, group was 23.21 and control group 4 was 24.57 respectively. The results of Ct values showed that the fold change of *APC* is low in high concenteration while high in low concenteration of NOSN, showing that gene is down-regulated in high concenteraion and up regulated in low concentearion of NOSN. Student's T-test was performed between each group and the control group. The decrease in expressionlevels at 6.25ppm and 3.125ppm NOSN concentrations was not significantly significant compared to the control group, however the increase in 1.5625ppm NOSN-treated cells was found to be statistically significant (Figure 3.1).

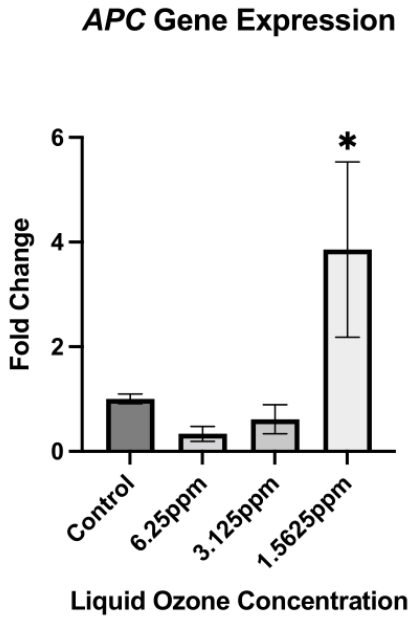

Figure 3.1: The statistical analysis of the log ΔΔCTof value *APC* in different concentration of NOSN.

3.4. *AXIN* GENE EXPRESSION ANALYSIS

The expression levels of *AXIN* were investigated in four groups. The average Ct value of group 1 was 20.34, group 2 was 19.81, group was 17.63 and control group 4 was 21.27 respectively. The results of Ct values showed that the fold change of *AXIN* is low in high concenteration while it gradually increases by descreasing the concenteration of NOSN and showing drastically increase at the (1.625 low concentraion of NOSN), showing that gene is down-regulated in high concenteraion and up regulated in low concentearion of NOSN. The decrease in expressionlevels at 6.25ppm and 3.125ppm NOSN concentrations was not significantly significant compared to the control group, however the increase in 1.5625ppm NOSN-treated cells was found to be statistically significant (Figure 3.2).

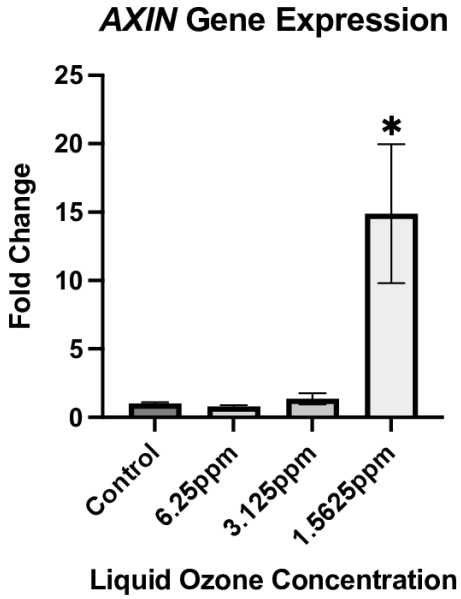

Figure 3.2: The statistical analysis of the log ΔΔCT of value *AXIN* in different concentration of NOSN.

3.5.*GSK3B* GENE EXPRESSION ANALYSIS

The expression levels of *GSK3B* were investigated in four groups. The average Ct value of group 1 was 24.08, group 2 was 24.39, group 3 was 24.9 and control group 4 was 24.74 respectively. The results of Ct values showed that the fold change of *GSK3B* is low in high concenteration while it gradually increases by descreasing the concenteration of NOSN and showing drastically increase at the (1.625 low concentraion of NOSN), showing that gene is down-regulated in high concenteraion and up regulated in low concentearion of NOSN. Thedecrease in expression levels at 6.25ppm and 3.125ppm NOSN concentrations was not significantly significant compared to the control group, however the increase in 1.5625ppm NOSN-treated cells was found to be statistically significant (Figure 3.3).

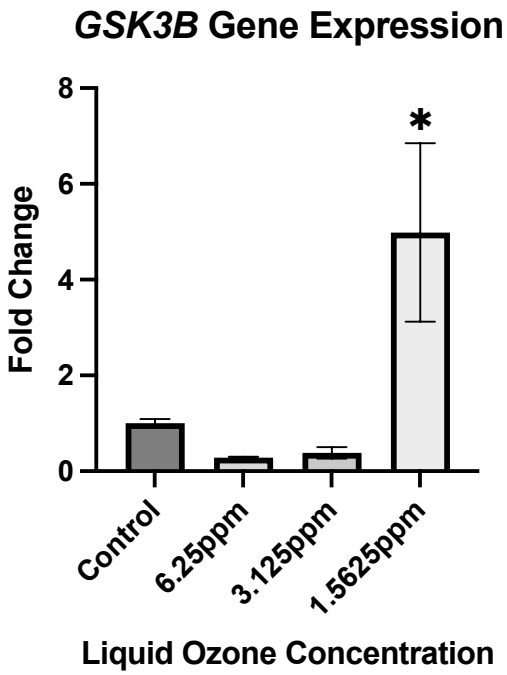

Figure 3.3: The statistical analysis of the log ΔΔCTof value *GSK3B* in different concentration of NOSN.

3.6. *β-CATENIN* GENE EXPRESSION ANALYSIS

The expression levels of *β-catenin* were investigated in four groups. The average Ct value of group 1 was 18.49, group 2 was 17.9, group 3 was 17.57 and control group 4 was 20.80 respectively. The results of Ct values showed that the fold change of *β-catenin* is low in highconcenterationwhile it gradually increases by descreasing the concenteration of NOSN and showing drastically increase at the (1.625 low concentraion of NOSN), showing that gene is down-regulated in high concenteraion and upregulated in low concenteration of NOSN. Interestingly, like in othergenes, the decrease in expression levels at 6.25ppm and 3.125ppm NOSN concentrations was not significantly significant compared to the control group, however the increase in 1.5625ppm NOSN-treated cells was found to be statistically significant (Figure 3.4).

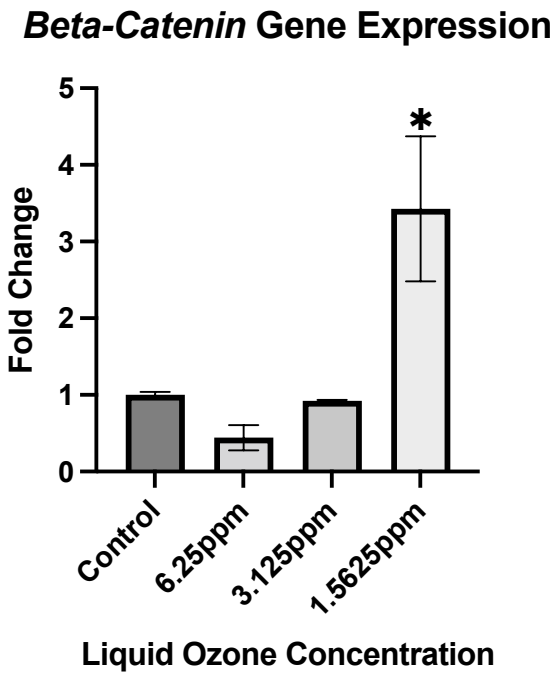

Figure 3.4 The statistical analysis of the log ΔΔCTof value *B-catenin* in different concentration of NOSN.

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1. DISCUSSION

Cancer is defined as uncontrolled cell division that invades and spreads from one spot to other sections of the body. One-third of their fatalities include the usage of tobacco, a high BMI, consumption of alcoholic beverages, less use of fruit and veggies, and a secondary lifestyle. Oncogene are formed when proto-oncogenes are altered and activated. Overexpression can be caused by deletions and point mutations in regulatory regions. Haploinsufficiency linked with the genes which regulates the DNA repair and damage respond, it causes genetic instability. Tumor cells infiltrate, transfer, intravasate, extravasate, and colonize metastatic sites. MMPs (matrix metalloproteinases) and serine proteases are secreted by cancer cells and migratory tumor cells. Emboli protect tumor cells from the bloodstream's extreme stresses. Endothelial cells proliferate because abnormal growth factors are created. Tumor cells can develop vascular-like structures. Angiogenic inhibitors are generally upregulated/increased by some tumor suppressor proteins. Caspases are activated in cancer cells, but increased IAPs prevent them from doing so. DNA methyltransferase mutation has been found in various cancer forms. Methylation-induced gene silencing has been linked to cancer. Activation of genes that are not ordinarily expressed in cells has an impact on transcriptional activity. Some enzymes that are critical for epigenetic control may be disabled by mutations(Hanahan and Weinberg 2011, Fujiwara, Yakoub et al. 2021).

Breast cancer is estimated to kill 43,500 people in the United States by the year 2022. Routine screenings are how the majority of people learn about their diseases. Mastalgia, on the other hand, is a rather frequent affliction. The odds of survival increase when cancer is detected early(Arslan, Küçükerdem et al. 2016). According to the WHO, In 2008, 1.38 million new cases of breast cancer were projected to have been detected worldwide. The death rate from breast cancer has been declining in North America and some other areas of Europe. In general, the incidence rate of female breast cancer rises with a woman's age. Breast cancer is not usually inherited. Sex and age are the two major risk factors that contribute to the development of breast cancer in women. A woman with alterations specific to this gene (BRCA1 and BRCA2) has

a significant risk of developing breast cancer, particularly in young women. Identifying variables connected to an increased risk of breast cancer development is crucial in routine health screening for women. Age, obesity, alcohol consumption, a family or personal history, hormone replacement therapy (HRT), and genetic predisposition have all been linked to an increase in breast cancer incidence in women(Kamińska, Ciszewski et al. 2015)**.**

MBC has been associated to Klinefelter syndrome (47, XXY), Cowden syndrome (PTEN tumor suppressor gene), Li-Fraumeni syndrome (TP53) and mismatch repair genes. Obesity, hepatic dysfunction, marijuana use, thyroid disorders, and estrogen-based medicines are thought to be contributing factors. Breast cancer is 100 times more common in women than it is in men. Gynecomastia patients have a seven-fold greater risk of developing breast cancer. Occupations such as steel mill workers and organic chemist are associated with a higher risk of MBC (Campos, Rouleau et al. 2021, Zheng and Leone 2022). In most cases, those who inherit the BRCA gene mutation have a five to ten percent probability of developing breast cancer. Those who have either of the two mutations are more likely to get breast cancer later in life. Gender, age, and family history all have a role in the impact of the mutation. Breast cancer is caused by more than just inherited genetic factors. Other factors, known as non-genetic factors also play a significant role(Kamińska, Ciszewski et al. 2015). Genetic testing can aid in the early detection and prevention of breast cancer in high-risk women. However, it is not required for every woman to undergo such testing(Lynch, Venne et al. 2015, Feng, Spezia et al. 2018).

Stem cell therapy, targeted therapy, ablation therapy, nanoparticles, radionics, natural antioxidants, sonodynamic therapy, and chemodynamic therapy are all new breakthroughs in breast cancer research. Several cutting-edge technologies are now being tested in clinical trials, while others have already been approved(Debela, Muzazu et al. 2021). MCF-7 is an estrogen receptor (ER-Positive Cell Line) obtained from a pleural effusion in a breast cancer patient. Other ER-Negative but positive for epidermal growth factor receptor (EGFR) are MDA-MB-486 and MB-231 breast cancer cell lines (Hahn, Weinberg et al. 2015). Breast cancer cells exhibit substantial aneuploidy and have a low spreading capability. They have cytogenetic variation, such as a presence or absence of certain chromosomal markers. The rate of proliferation has

been reduced and lasts around a month after the estrogen has been withdrawn. MCF-7 cells have characteristics of a developed mammary epithelium. Different markers such as E-cadherin, cytokeratin 18, and β catenin are positive in them. They produce intercellular connections by expressing "Zona occulden protein 1" (ZO-1) (Pinto, Badtke et al. 2010)(Felice, El-Shennawy et al. 2013).The *APC* gene directs the production of the *APC* protein, which is involved in various cellular activities. *β*-catenin is one protein that *APC* binds with and aids in the regulation of gene activity (expression) and stimulates cell growth and division (proliferation). The *APC* gene is a tumor suppressor gene present in the 5q21 region of chromosome. Mutations in this gene have been linked to both familial and sporadic colorectal cancer(Zhang and Shay 2017). An experiment analysis of female mice shows a nonsense mutation in the codon 850. It is hypothesis that the disturbance in the adenomatous polyposis coli (*APC*)/-catenin pathway has been linked with the development of breast cancer, and this same of type of disruption has been hypothesized for colorectal tumor(Zhang and Shay 2017) (Furuuchi, K., et al. 2000). The somatic mutation of *APC* and catenin is uncommon in breast cancer. *AXIN*1 was first discovered as the result of the murine fused locus. Human equivalent on chromosome 16p13.3 that is 87 percent similar to the mouse protein. *AXIN*'s intracellular concentration 1000 times lower than those of other destruction complex components (Salahshor and Woodgett 2005).

Evidence shows disruption of the WNT/β-catenin signaling pathway is associated to the formation and progression of several solid tumor and blood cancer. Catenin is a critical component of the WNT signaling system that control the growth, division, differentiation, migration, invasion, and tissue homeostasis. Catenin is involved in a number of signaling pathways, such as the WNT signaling pathway. In glioblastomas cells also interact with PI3K, AKT and GSK3 cascade. catenin has also been demonstrated to be activated by the AKT kinase. The regulation of a WNT/B-catenin signaling pathway is highly regulated by the group of genes that tightly control the activity of *β-catenin*. Alteration or mutation in the component of the destruction complex has been reported in a variety of cancers (Duchartre, Kim et al. 2016). Figure 1 illustrates the key component of destruction complex.

The dysregulation of RAS GTPases has a critical role in a variety of cancers. Dysregulation acts as a molecular switch, activating or deactivating the cancer-

regulating cascades of events. The two RAS signaling pathways most strongly associated to cancer are the MAP kinase pathway and PI3K system(Terrell and Morrison 2019). RAS and its downstream effectors have been found to have genetic abnormalities in a number of malignancies. PI3K activity is reduced by inositol lipid phosphatases, such as PTEN, a tumor suppressor(He, Sun et al. 2021). WNT signaling consists of a multimolecular degradation complex that regulates the intracellular contents of -catenin. The -catenin degradation complex is destroyed when WNT interacts to its cell surface receptors FZD and LRP5/6. The cytosolic protein DVL is activated by WNT-bound receptors, allowing *AXIN* 1 and related kinases to connect to the membrane(Pohl, Brook et al. 2017, Abreu de Oliveira, El Laithy et al. 2022). When NOTCH ligands from one cell engage with NOTCH from another, the NOTCH intracellular domain (NICD) is secreted through proteolysis. NOTCH may play a primary oncogenic function in some malignancies, such as T-cell lymphomas(Andersson, Sandberg et al. 2011).

The ozone is a colorless gas and made up of three oxygen atoms. High levels of exposure can cause lung damage and even death. Researchers believe that ozone might be used to cure arthritis, viral infections, sterilize wounds, activate the immune system, heal ischemic heart disease, and even cancer. Photodynamic therapy with specific ROS creation seems to have a focused anti-tumor effect. Warburg effect could play a significant role in oncogene-induced senescence. As cancerous cells' antioxidant systems are already overworked, they have limited capacity to boost antioxidant synthesis even more (Liou and Storz 2010, Clavo, Rodríguez-Esparragón et al. 2019). In this study, ozone in the form of nanobubble ozone stored niosomes (NOSN) was used.

In the conclusion of the discussion, a low concentration of NOSN increased *AXIN* expression by 15 folds, *GSK3B* expression by 5 folds, *APC* gene expression by 4 folds, and *β-catenin mRNA* expression by 3 folds. High levels of NOSN suppress β catenin expression, which suppresses the WNT signaling pathway. We studied the expression of four genes, *AXIN*, *GSK3B*, *APC* and *β-catenin.* Graph of all 4 genes shows that their mRNA expression has been suppressed in the presence of NOSN. However, they show high expression in the low concentration of NOSN. NOSN suppresses *β-catenin* expression, which suppresses the WNT signaling pathway, which is important in the

initiation and progression of cancer.

4.2. CONCLUSION

The purpose of this research is to analyze the effect of NOSN on the beta-catenin pathway genes in the MCF-7 breast cancer cell line. As these four distinct sets of genes (*APC, AXIN, β-catenin*, and *GSK3B* genes) are also known as beta-catenin pathways genes that plays as a very important role along with CK1 gene in the regulation of WNT/β-catenin signaling pathway. *APC*, *AXIN*, *GSK3* and *CK1* combinedly form the complex called the destruction complex. This destruction complex tightly regulated the amount of *beta-catenin*, as the high concentration of this destruction complex degraded the *beta-catenin*. Which means that the less availability of *beta-catenin*, as *beta-catenin* is considered a regulatory gene in the activation of the WNT/ β-catenin signaling pathway. As activation of the WNT/β-catenin pathway promotes cancer stem cell (CSC) progression and thus leads to the deterioration and metastasis of cancer. So, in our research, we basically used different concentrations of NOSN to check whether at which concentration of NOSN the expression of these destruction complex genes will be expressed more or less as compared to their general expression, as if destruction complex genes will express more, it means they more degrade the beta-catenin. As a result, the beta-catenin isn't activating the WNT signaling pathway, so that the metastatic of cancer could be ceased to some extent.

Our applying one-way ANOVA student's T-test, the results shows that the high concentration of NOSN suppressed the expression of all of these destruction complex genes, but the low concentration of NOSN massively enhanced the expression of these destruction complex genes, which means that by use the low NOSN we can degrade the more amount of beta-catenin, as the less availability of beta-catenin means WNT/ β-catenin signaling pathway activity will be restricted to some extent in the metastasis of cancer.

As we analyzed the genes' expression at the mRNA level and we have limited variability of NOSN, so we can't surely conclude that the low concentration of NOSN will be used for the treatment of breast cancer, but the conformation will be done in the future by using the more variability of NOSN. Analysis of the other genes in different cell lines and analysis of the result will be done at protein level by using

western blotting will surely confirm the usage and role of NOSN in the treatment of breast cancer.

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