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CROSSTALK BETWEEN COLLAGEN XII AND COLLAGEN I TO CREATE A PRO-INVASIVE MICROENVIRONMENT FOR METASTASIS

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Abstract

Several studies have shown breast cancer BC metastasis is the second leading cause of cancer-related deaths among women. To prevent cancer metastasis, it is crucial to understand how the metastasis process unfolds and to pinpoint suitable targets for effective treatments. This study focuses on COL1A1 (Collagen I) and COL12A1 (Collagen XII) which are major players of tumor microenvironment and how their expression levels can serve as an early metastatic marker to save the lives of BC patients. Rationale of the study is to analyze the gene expression of COL1A1, COL12A1, Lnc SNHG5, TWIST1 and LOXL2 in blood samples of BC patients, to identify their relation to the pre-metastatic niche formation and metastatic potential of tumors. This research uses primary data. A total of 32 blood samples collected from BC patients as well as 15 healthy donors as control. We performed RNA extraction from the tumor samples by using Trizol and quantification of RNA was done by Nanodrop and gel electrophoresis. The cDNA was synthesized from 1 µg of total RNA which was diluted at 1:4. RT-qPCR analysis was utilized to quantify the expression of COL1A1, COL12A1, Lnc SNHG5, TWIST1 and LOXL2 and to measure the mRNA level of these genes. The results revealed that the expression of COL1A1, Lnc SNHG5, TWIST1 and LOXL2 were upregulated in BC patients whereases mRNA level of COL12A1 is decreased in patient sample. Kaplan Meier analysis showed that increase in COL1A1 is associated with decreased distant metastatic free survival of the patients which is vice versa in the case of COL12A1. Further it is observed that high expression of COL1A1 is particularly found in patients with high grade tumor/ metastasis. Since COL1A1 and COL12A1 are a significant contributor in development of metastatic niche leading to metastasis therefore breast cancer patients with high tumor grade are exhibiting higher expression of these genes. Levels of COL1A1, and COL12A1 can be utilized as an early diagnostic marker for breast cancer metastasis, and with enhanced analysis methods, a rapid blood-based test can be designed for early diagnosis. Studies have also revealed that the development of pre-metastatic niches (PMNs) in BC plays a vital role in cancer metastasis, leading to high mortality rates. Targeting and understanding the multifaceted mechanisms of the COL1A1, COL12A1, Lnc SNHG5,

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TWIST1 and LOXL2 that contribute to PMN formation offer new opportunities for potential diagnostic and therapeutic strategies, aiming to prevent cancer metastasis at an early stage and improve outcomes for breast cancer patients.

INTRODUCTION

Breast cancer BC is a leading cause of mortality, resulting in the death of over 2.1 million women annually on a global scale, constituting an estimation of 15% of all cancer-related deaths. (Bray *et al.,* 2018; Terceiro *et al.,* 2021). Breast cancer develops when breast cells expand out of control. Doctors often detect it through mammograms (special X-ray scans of breast tissue) or by finding abnormal lumps in the breast. Sometimes, changes in the skin or nipple can also indicate breast cancer. When diagnosed, breast cancer may be found only in the breast, in nearby lymph nodes under the arm, or in distant body's parts. The severity of the cancer is categorized into stages I through IV, with stage IV being metastatic breast cancer, which means it has migrated outside of the breast and lymph nodes. all these subtypes greatly vary in prognosis and treatment. BC can be categorized into three distinct subtypes based on specific proteins' expressions within the cancerous cells. The initial category is hormone receptor- positive, which constitutes around 70% of instances and is characterized by the absence or presence of either estrogen receptor (ER) or progesterone receptor (PR) proteins within cancer cells. The second subtype is characterized as ERBB2-positive, accounting for around 15% to 20% of patients. This subtype has elevated amounts of ERBB2 protein, previously referred to as HER2, on the cancerous cells, which were previously identified as HER2-positive. The third subtype, known as triple-negative breast cancer (TNBC) accounts for around 15% of cases, characterized by the absence of three key proteins: estrogen receptor (ER), progesterone receptor (PR), and ERBB2. (Waks & Winer, 2019).

The phenomenon known as epithelial-to-mesenchymal transition (EMT) is analyzed in cancer cells and represents an initial stage in the progression of metastasis. EMT is generated by the loss of intercellular adhesion and the ability to migrate. Cancer cells experiencing Epithelial-Mesenchymal Transition (EMT) exhibit reactivation of developmental processes, leading to the activation of the transcription factors associated with EMT, like *SNAI1, SLUG, TWIST1,* and *ZEB1/2* (Nieto, 2013).

These transcription factors modify the adhesion molecules generated by cancer cells. As part of the reprogramming process that occurs during organogenesis, E-cadherin molecules are replaced by N-cadherin molecules. These transcription factors modify the adhesion molecules generated by cancer cells. Allowing cancer cells to invade neighboring cells are alterations such as increased expression of matrix-degrading proteases, enhanced motility, and are resistance to apoptosis. Subsequently, these cells enter in blood and/or in lymphatic vessels through a process known as intravasation, are leached out to reach distant tissues via extravasation, and establish micro metastatic lesions that eventually become visible secondary tumors during the colonization process (Boudhraa *et al.,* 2020).

In the event of a positive screening mammography (X-ray imaging of the breasts), an indication of breast cancer problems, or breast modifications (lump, targeted pain, or skin variations), a proper diagnostic diagnosis is required. While a complete characterization of the cancer malignancy can be confirmed by the cancer grade, ER, PR and HER2 receptor status, its size, lymph node involvement, level of vascularization and surgical margin state. However medical diagnosis is verified by a "triple test," which includes needle biopsy, imaging, clinical examination. are all necessary for the complete characterization of the malignancy, these steps can be beneficial in the context of management, prevention, and control of BC ("Breast cancer," 2019).

In clinical practice, tumor metastasis is a significant issue and the prime reason of cancer-related mortality. However, only 5–10% of individuals who have recently been diagnosed with BC exhibit initial signs of cancer spreading to distant body parts (Cardoso *et al.,* 2012; Gupta & Massague, 2006).

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Despite primary tumor removal and treatment, patients with localized cancer still face significant risk of metastasis. Despite receiving standard treatment, approximately 30% of BC patients with no lymph node involvement, even greater proportion of patients with lymph node involvement are estimated to develop metastatic disease (Tang *et al.*, 2021).

Both tumor cells as well surrounding stroma undergo genetic and epigenetic alterations during tumor development, which increases the likelihood of distant metastasis. The complicated and poor metastatic colonization process contributes to the low probability of circulating tumor cells forming distant colonies. The majority of the cells die once they get separated from the original tumor while attempting to invade distant organs. Less than 0.02% of circulating tumor cells (CTCs) have observed to survive and survive in preclinical studies. (Chambers *et al.*, 2002).

For more than a century, research on metastasis has been ongoing and metastasis has been a focal point of research, as well as recent findings reveal how metastatic cells originate from primary tumors and why certain tumor types preferentially spread to specific organs. Stephen Paget's 1889 theory on cancer cell interaction with organ microenvironments (the 'soil') is nonetheless relevant today. We now know that a tumor cell's capacity to spread to other body's parts depends on how it interacts with factors that help it grow, survive, create new blood vessels, invade nearby tissues, and form metastases. Over the past century, the field has made significant progress in identifying genetic and molecular mechanisms, tumor microenvironment influences, angiogenesis's role, and the impact of immune cells. Promising therapeutic approaches include targeting molecular pathways involved in metastasis and manipulating tumor cell- microenvironment interactions. In order for metastases to occur, original tumor cells must overcome physical obstacles, enter either circulatory system or lymphatic system, move to distant organs, then continuously proliferate as well as expand to produce metastatic lesions. (Chambers *et al.*, 2002).

Tumor cell's chances to metastasize is influenced by its origin and oncogenic alterations. Cells at various stages or lineages may have varied metastatic capabilities, despite possessing identical oncogenic alterations (Fidler, 1973; Marusyk & Polyak, 2010). Furthermore, the oncogenic driver mutation could possibly influence the tumor's potential for metastasis to distant sites in body(Kimbung *et al.*, 2015; Wan *et al.*, 2013).

Concept of "seed-and-soil" hypothesis, firstly proposed in the 19th century by Stephen Paget and it suggests that cancer cells can initiate metastasis if they encounter a compatible tissue microenvironment. Subsequent evidence has partially supported these early ideas (Fidler, 2003).

In the tumor microenvironment (TME), cancer-associated fibroblasts (CAFs) are known to exert a significant effect on several aspects for cancer progression. These include metabolic alterations, angiogenesis, extracellular matrix (ECM) remodeling, inflammation, cellular proliferation, invasion, and metastasis (Kalluri, 2016; Wu *et al.*, 2020).

New studies have brought attention to the involvement of CAFs in immune suppression and their contribution to chemotherapy resistance. making them attractive targets for advanced breast cancer treatments (Brechbuhl *et al.*, 2017).

Despite being well-studied, CAFs still pose mysteries, with limited evidence suggesting diverse characteristics, adaptability, and functional variety, exhibiting both tumor- promoting and tumor-suppressive properties. The diverse nature of CAFs implies the existence of distinct subpopulations and gaining a better understanding of their heterogeneity provide insight on how Cancer-associated fibroblasts (CAFs) contribute to dynamic complexity and adaptability of the tumor ecosystem (Su *et al.*, 2018).

Collagen XII organizes collagens and has a significant influence on the 3D structure of the extracellular matrix. In a study using mouse models, researchers examined tumors from early to late stages and observed changes in matrix molecules, particularly an increase in collagen XII levels. Elevated level of Collagen XII altered the tumor properties, making it more aggressive by reorganizing collagens to facilitate cancer cells to leave tumor and metastasize distant locations like the lungs. To investigate further, the team manipulated collagen XII production through

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genetic engineering and studied its effects on metastasis to other organs. The results showed that higher levels of collagen XII corresponded to increased metastasis. These findings were corroborated by human tumor biopsies, which demonstrated a correlation between high collagen XII levels, heightened metastasis rates, and poorer overall survival. Future research will
focus on studying additional human samples and exploring potential therapeutic pathways related to collagen XII. In short, collagen XII, mainly produced by CAFs, controls the arrangement of collagen I fibers, stimulating cancer cell invasion and metastasis in BC.

Extracellular matrix changes during breast tumor growth, researchers proposed investigating collagen XII as a possible marker for BC patients who might face a higher risk of cancer coming back. Additionally, when collagen XII is reduced in CAFs, the structure of collagen I and the rigidity of the tumor are transformed, which in turn helps in preventing the activation of the surrounding tissue, creating a microenvironment less favorable for cancer cells to invade and grow within the developing tumor. Recent findings show that collagen XII mutations cause connective tissue disorders in humans. Moreover, a clear link exists between collagen XII's unique structure and molecular
interactions, vital for preserving structural integrity in load bearing connective tissues of the musculoskeletal system (Chiquet *et al.,* 2014). Whereas a detailed signaling pathway of tumor microenvironment is also shown in figure 1.2.

Similarly, *SNHG5*, which is also known as U50HG, *SNHG5* is associated with B-cell lymphoma, and it is very much popular as it plays its role in a chromosomal translocation breakpoint. This non-coding RNA has six exons and two nucleolar RNAs (snoRNAs), and it is 524 base pairs long in length. *SNHG5* is present on chromosome 6q14.4 and is a long non-coding RNA (lncRNA) because it cannot produce proteins on its own, *SNHG5* has been observed to express abnormally in various cancer types, like BC, colon cancer, bladder cancer etc., showing abnormal levels of expression in these diseases (Li *et al.,* 2020).

SNHG5, which is known as a cancer-causing gene (an oncogene) in breast cancer, can be used as a separate indicator to analyze outcomes in BC patients. When *SNHG5* is reduced, it can effectively slow down the growth and proliferation of BC cells and causes cell-cycle arrest, especially in G1 phase (Quinn & Chang, 2016).

Moreover, *SNHG5* functions as a miRNA sponge, for small molecule called miR-154- 5p. By doing this, it reduces the miR-154-5p's ability to suppress a protein called PCNA, which leads to increased cell growth. Using these findings, researchers created a model illustrating *SNHG5*'s influence on BC cell growth. Multiple studies suggest the vital role of *SNHG5*-miR-154-5p-PCNA combination in BC growth, potentially offering a target treatment (Chi *et al.,* 2019).

MATERIALS and METHODS

Ethical Approval and Compliance

Before data collection or participant participation began, the Ethical Board Committee (ERC) and IRB reviewed and approved the studyprotocol. This thesis elucidates a study that adhered rigorously to ethical protocols and carried out according to the standards set forth by the ERC and the principles outlined by the IRB.

Informed Consent

Before including any participants in the study, consent forms were requested from each one of them. Participants provided comprehensive descriptions of the research's objectives, methods, potential risks, advantages, and confidentiality. They were given a sufficient amount of time to think about participation and they were given the assurance that they may leave at any moment without experiencing penalties or losing benefits.

Anonymity and Confidentiality

The study's participants' privacy and confidentiality were always upheld. Personal identifying information was maintained aside from any study data and each component of information gathered was handled with the strictest discretion. To prevent individual participants from being recognized in any publications or reports resulting from this research, anonymized coding techniques were implemented.

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Publication and Reporting

The results of this investigation shall be accurately, unbiasedly, and publicly disclosed. Disclosure of any possible conflicts of interest was made. The right recognition and reference of appropriate articles and earlier research were made ensured.

We appreciate the participants for their willingness to participate and acknowledge their contributions to the study. The main goal of this research project is to enhance patient care and elevate clinical outcomes. while simultaneously contributing to the advancement of knowledge within the domain of breast cancer.

Sample collection and size

Between November 2022 and March 2023, research participants were recruited from Jinnah Hospital and Mayo Hospital in Lahore. 32 blood samples gathered from female BC patients undergoing chemotherapy as well as surgery at Jinnah Hospital and Mayo Hospital in Lahore.

The form for collecting patient data.

The document for collecting patient data is also preserved.

Participant recruitment

The recruiting procedure involved working with health professionals with expertise in breast cancer and qualifying volunteers were chosen in accordance with certain criteria.

Inclusion standards

The following inclusion criteria were applied while choosing participants:

1. Breast cancer has been confirmed with certainty as a result of histological analysis.
2. Female and male patients
3. To ensure the appropriate demographic representation, the age range was from 25 to 78.
4. Patients undergoing surgery, radiotherapy, hormone therapy, and chemotherapy.
5. Breast cancer that is triple-negative (TNBC)
6. ER+, PR+, Her 2 -, and Her 2 + for Luminal A and B, respectively.

Exclusion criteria

1.The age range for male and female patients is 25 to 78.

Process for informed consent:

The Ethical Committee or Institutional Review Board of Jinnah Hospital and Mayo Hospital Lahore provided their permission in accordance with ethical standards. All participants received thorough information regarding the study's goals, methods, potential hazards, rewards, and confidentiality prior to giving their signed consent for sample collection. Before giving their permission, participants had plenty of time to clarify any doubts and ask questions.

Sample Collection Procedure:

In the context of an invasive procedure, healthcare professionals who have received specialized training employ standardized methodologies and techniques to perform blood extraction from patients diagnosed with metastatic breast cancer. The following steps were followed:

Participants were seated comfortably, and aseptic procedures were used to reach the antecubital vein.

An alcohol swab was used to disinfect the venipuncture site before a sterile needle was used to draw blood into the proper collecting tubes.

In order to ensure an adequate sample size for analysis, approximately 4 milliliters of blood were extracted from each participant.

2ml of blood was drawn and placed in an EDTA vial for specific downstream uses, including RNA extraction.

To check the cortisol level of patients, 2 ml of blood samples were taken and placed in serum separator vials.

RNA Extraction

The following stepwise protocol was employed for RNA extraction:

Homogenization:

Following the manufacturer's guidelines, RNA extraction performed using Trizol reagent (Invitrogen, USA). To begin, 750µL of Trizol was added to a 1.5ml Eppendorf tube, while 500µL of blood was collected in a separate 1.5ml Eppendorf

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tube using a pipette. These Eppendorf tubes subsequently incubated at a designated temperature for 10 minutes.

Phase separation:

Following this, 200µl of chloroform (Merck, Germany) added to separate mixture in upper aqueous (RNA), intermediate white (DNA), and lower red (proteins) layers. After a 15-second gentle shake, the mixture incubated at room temperature for 10 minutes, followed by centrifugation at 12,000 g and 4°C for 15 minutes. This enabled the precise transfer of the RNA-rich upper aqueous phase to a new 1.5 ml microfuge tube.

Washing:

After removing and discarding the pellet, the supernatant was quickly vortexed or tapped in 1 ml of 75% ethanol (prepared in DEPC water) by briefly vortex or tapping the tube. The mixture was centrifuged at 7,500 g and 4°C for 5 minutes. The resulting pellet was dried, then dissolved in 15-20 ml of RNase-free DEPC water. Refer to Table 3.1 for the necessary RNA extraction reagents.

RNA Quantification:

The determination of purification and concentration for each RNA sample was conducted using a Nanodrop instrument (ND-1000, USA) by evaluating the A260/280 ratio of the purified RNA. The RNA that undergoes purification was kept at a temperature of -20°C until it was ready to be used, or alternatively, it was maintained at a temperature of -80°C for extended periods of storage. Important considerations for this step are: In order to retain the RNA in the entire sample, it is essential to quickly add TRIzol to fresh tissue samples. To obtain the highest possible RNA quality, RNA extraction should be done on the same day as blood collection. Due to the great susceptibility of RNA to destruction at high temperatures, the extraction procedure must be carried out on ice. RNA has to be stored at -80°C in order to avoid degradation and increase storage duration. A schematic diagram for experimental protocol of RNA extraction, created from biorender software is also mentioned in the Figure 3.1.

cDNA Synthesis from RNA

Thermo Scientific RevertAid First Strand cDNA Synthesis Kit from Invitrogen (USA), and sterile nuclease-free PCR tubes on ice received sequential addition of components. After quick centrifugation, a 5-minute heat shock at 65°C eliminated RNA secondary structures. Following heat shock, components were added, mixed, and swiftly centrifuged. Thermo-cycler was used for DNA synthesis. cDNA samples were stored at -40°C for later use. Important Considerations:

Prior to initiating cDNA synthesis, it is essential to normalize the primary concentrations derived from Nanodrop analysis of RNA samples. The cDNA reaction should be accelerated by abruptly increasing the temperature from 42 to 70 degrees Celsius. Any delay in temperature variation can reduce the reaction's efficacy. The following table 3.2 outlines the reagents utilized in cDNA synthesis:

Normalization of cDNA

The normalization of complementary DNA (cDNA) samples was performed using primers specific to *β-ACTIN* and *GAPDH* genes. It is essential for housekeeping genes such as *β-ACTIN* and *GAPDH* to exhibit similar expression levels across every individual. However, the synthesized cDNA may vary in terms of cell quantity and subsequently the presence of mRNA transcripts per sample. Initially, PCR amplification of cDNA was performed using *β-ACTIN* or *GAPDH* primers, followed by gel electrophoresis.

Primers Designing

The designed primers target *COL12A1, COL1A1, SNHG5, LOXL2,* and *TWIST1* genes for expression analysis. Gene mRNA sequences obtained from the National Center for Biotechnology Information (NCBI). Process of manual primer design was conducted by utilizing Consensus DNA sequences (CDS) of the genes, with the aid of Serial Cloner and SnapGene software. The primer specificity, annealing temperatures, product size, and self-complementarity were confirmed using NCBI Primer-BLAST, UCSC In Silico PCR, Gear Genomics Insilco PCR, and Kun's Oligonucleotide tools. SnapGene software was employed to choose primer sequences with specific binding, and NCBI

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Primer-BLAST software was used to validate the primers, ensuring specific product generation for the desired genes and organism. Gear Genomics In-silico PCR was specifically employed to verify primer products, specificity, and amplicons, while UCSC In Silico PCR was used to further confirm product specificity and size. Finally, the primer sequences for the genes were prepared for ordering.

Primer Sequences of Genes:

Forward and reverse primers were designed on alternating exons to facilitate PCR amplification, ensuring accurate expression analysis of the particular genes. This step is important because the extracted RNA may contain genomic contamination. Genomic DNA also has introns between exons, thus if primers are designed on the same exon, both the PCR product from genomic DNA and the PCR product from RNA may have the same size, which might result in false-positive expression. Therefore, to accomplish precise and reliable expression analyses, it is essential to design primers on alternative exons. The primers were subsequently optimized for determining the optimal annealing temperature. The annealing temperatures for corresponding primers are as follows (table 3.3.)

PCR Amplification using Gene-Specific Primers:

In sterile PCR tubes, polymerase chain reaction (PCR) experiments were conducted. A premix was prepared with sufficient volume for the intended reactions. The reaction mixture's ultimate volume and composition are already mentioned below in table 3.4.

Conventional PCR:

PCR was conducted in 0.2 ml PCR tubes, utilizing cDNA as the template. Optimization of PCR parameters was performed for each primer under consideration, including cDNA concentration, annealing temperatures, MgCl2 concentration, dNTPs, and the number of PCR cycles. After thawing, the PCR components mentioned in the table below were subjected to a brief vortexing and centrifugation process. To avoid any errors associated with pipetting, a master mix was carefully created within a 1.5ml microfuge tube. This was achieved by cautiously combining the necessary components for each 10 µL reaction, while maintaining a low-temperature environment on ice. The tube was quickly centrifuged to ensure thorough mixing. The resulting mixture was evenly distributed into PCR tubes. Each tube received 1 µg/µL concentration of template cDNA, followed by brief centrifugation. Tubes were placed in the thermocycler. The cycling protocol includes initial denaturation at 95°C for 3 minutes, followed by 35 cycles: 95°C denaturation for 30 seconds, primer-specific annealing at optimized temperature for 30 seconds, and 72°C extension for 20 seconds. Final 72°C extension for 10 minutes concluded procedure, as mentioned in Figure 3.2.

Detection of PCR Amplification

The PCR results were subjected to amplification and subsequently separated using a 1.5% agarose gel. The resultant bands were seen by staining with ethidium bromide.

Agarose Gel Electrophoresis

Gelelectrophoresis was done according to following steps:

Agarose Gel Preparation:

Measurements complete, 1.5g of agarose powder was taken in 150 ml conical flask, filled with 90 ml distilled water and 10 ml 10X TAE buffer, totaling 100 ml. The agarose powder was heated using a microwave until complete dissolution occurred, resulting in a clear solution. Following the process of cooling to a temperature range of 50-55°C, with frequent swirling to promote uniform cooling, a volume of 7µL of ethidium bromide solution (10 µg/ µL) was introduced into the gel and thoroughly mixed. The agarose solution in a molten state was carefully transferred into a casting tray and allowed to undergo the process of solidification. The gel was positioned within an electrophoresis chamber, and an adequate amount of 1X TAE buffer was introduced to ensure a buffer height of approximately 1 cm above the gel.

Loading the Gel:

The wells of the gel were carefully loaded with 7-8µ1 of DNA mixed with 2.0 µL of loading dye. Additionally, 7-8 µL of the 1kb DNA ladder and

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50bp ladder standard, along with loading dye, were run as reference markers.

Running the Gel:

The gelelectrophoresis procedure consisted of an initial run lasting 20 minutes, during which a constant voltage of 40 volts was applied. This was followed by a subsequent run of 40 minutes, during which the voltage was increased to 80 volts. The electrophoresis chamber's cover was taken off when the electrophoresis was finished, and the gel tray was removed. Gel was observed through UV transilluminator as well as Gel Doc system for band visualization.

RESULTS

Table 4.1: Patient Demographics and Clinical Characteristic

Sample No.	Patient ID	Age	Cancer Type and Grade	Cancer stage	ER status	Her2 status	PR status	Family history of cancer
1	RS-JHL-3101- 0-57-01	57	CA metastatic (invasive ductal) grade 2	Stage 4	Positive	Negative	Positive	N/A
2	RS-JHL-3101- 1-73-02	73	CA metastatic (invasive ductal) grade 2	Stage 4	Positive	Negative	Positive	N/A
3	RS-JHL-3101- 0-30-03	30	CA metastatic (invasive ductal) grade 2	Stage 3 B	Positive	Negative	Positive	N/A
4	RS-JHL-3101- 0-40-04	40	CA metastatic (invasive ductal) grade 2	Stage 4	Negative	Positive	Negative	N/A
5	RS-MH-0302-0-50-05	50	Grade II (malignant)	Stage 3	Positive	Negative	Positive	No
6	RS-MH-0302-0-31-06	31	Grade II	Stage 3	Negative	Positive	Negative	Yes
7	RS-MH-0302-0-35-07	35	CA metastatic (invasive ductal) grade 3	Stage 2 B	Negative	Positive	Positive	N/A
8	RS-MH-0302-0-36-08	36	CA metastatic (invasive ductal) grade 2	Stage 3	Positive	Equivocal	Positive	No
9	RS-MH-0302-0-40-09	40	CA metastatic (invasive ductal) grade 2	Stage 3A	Negative	Positive	Negative	N/A
10	RS-MH-0302-0-42-10	42	CA metastatic (invasive ductal) grade 2	Stage 4	Positive	Negative	Positive	No

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11	RS-MH-0802-0-42-11	42	CA metastatic (invasive ductal) grade 3	Stage 2 B	Negative	Positive	Negative	N/A
12	RS-MH-0802-0-55-12	55	Grade 2	Stage	Negative	Positive	Negative	N/A
13	RS-MH-0802-0-45-13	45	Grade 3	Stage 3A	Positive	Negative	Positive	No
14	RS-MH-0802-0-28-14	28	Grade 3 (malignant)	Stage 3	Negative	Positive	Negative	N/A
15	RS-MH-0802-0-42-15	42	CA metastatic (invasive ductal) grade 3	Stage 4	Positive	Negative	Positive	N/A
16	RS-MH-0802-0-55-16	55	CA metastatic (invasive ductal) grade 3	Stage 4	Negative	Positive	Negative	Yes
17	RS-MH-0902-0-50-17	50	CA metastatic (invasive ductal)	Stage 3B	Negative	Positive	Negative	N/A
18	RS-MH-0902-0-46-18	46	CA metastatic (invasive ductal) grade 2	Stage 3	Negative	Positive	negative	N/A
19	RS-MH-0902-0-42-19	42	CA metastatic (invasive ductal) grade 3	Stage 4A	Negative	Negative	Negative	N/A
20	RS-MH-0902-0-55-20	55	CA metastatic (invasive ductal) grade 2	Stage 3	Positive	Negative	Positive	N/A
21	RS-MH-0902-0-47-21	47	CA metastatic (invasive ductal) grade 2	Stage 3	Negative	Positive	Negative	No
22	RS-MH-0902-0-47-22	47	TNBC	Stage 4	Negative	Negative	Negative	N/A
23	RS-MH-0902-0-45-23	45	CA metastatic (invasive ductal) grade 2	Stage 2	Positive	Positive	Negative	N/A
24	RS-MH-0902-0-25-24	25	TNBC/CA meta static (invasive ductal) grade 3	Stage 2	Negative	Negative	Negative	N/A
2	RS-MH-1002-0-	44	TNBC	Stage 3	Negative	Negative	Negative	N/A

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5	44-25							
26	RS-MH-1002-0-49-26	49	TNBC	Stage 4	Negative	Negative	Negative	N/A
27	RS-MH-1002-0-41-27	41	Invasive breast cancer	Stage 3	Negative	Positive	Negative	No
28	RS-MH-1002-0-42-28	42	CA metastatic (invasive ductal) grade 3	Stage 2	Negative	Positive	Negative	N/A
29	RS-MH-1002-0-35-29	35	CA metastatic (invasive ductal) grade 2	Stage 3	Negative	Positive	Negative	No
30	RS-MH-1002-0-41-30	41	CA metastatic (invasive ductal) grade 3	Stage 3	Positive	Negative	Negative	N/A
31	RS-MH-1002-0-45-31	45	CA metastatic (invasive ductal) grade 2	Stage 3	Positive	Negative	Positive	N/A
32	RS-MH-1002-0-45-32	45	TNBC	Stage 4	Negative	Negative	Negative	No

Table 4.1 addressing the BC patient's details, which were involved in this research, their age, cancer type and stage is also mentioned in table, furthermore ER, PR and Her2 status and their family history is also mentioned.

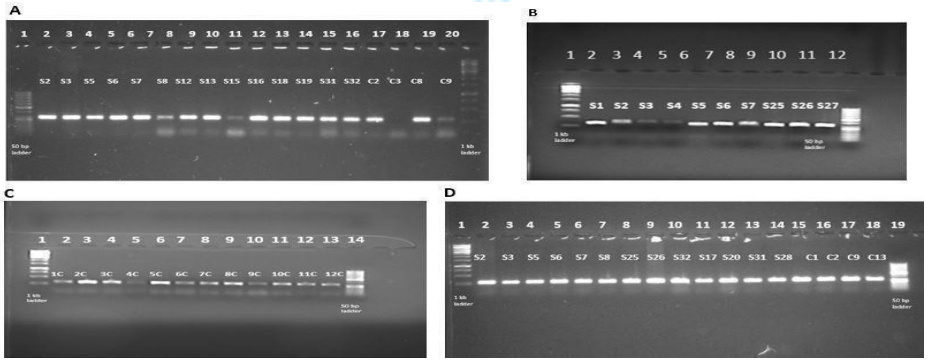

Figure 4.1: Gel Electrophoresis and qRT-PCR Verification of *β-ACTIN* and

***SNHG5* Amplification in Patient and Control Samples**Above mentioned Fig 4.1 explains of PCR-amplified *β-ACTIN* and *SNHG5* on gel Electrophoresis using cDNA templates from both patients and controls. Fig. 4.1(A) demonstrates q RT PCR verification of *β-ACTIN* in patients and controls, as well as the visualize 250bp amplified PCR product of *β-ACTIN* on a 1.5% agarose gel. Lane 1 (A) showing a 50bp DNA ladder and lane 20 (A) showing a 1 kb DNA ladder, with patients and control PCR products in the middle portion.

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Similarly, Fig. 4.1(B) depicts *β-ACTIN* PCR product amplified from patient samples on a 1.5% agarose gel. Lane 1 of (B) depicts a 1 kb DNA ladder, while lane 12 depicts a 50 bp DNA ladder. Fig. 4.1(C) displays *β-ACTIN* amplified by PCR in control samples on 1.5% agarose gel. Lane 1 of
(C) depicts a 1 kb DNA ladder, while lane 12 depicts a 50 bp DNA ladder. Fig 4.1(D) demonstrates the q RT-PCR verification of *SNHG5* in patients and controls, as well as the visualization of the 285bp amplified q RT-PCR product of *SNHG5*, lane 1 of (D) highlighting a 1kb DNA ladder, and lane 12 depicting a 50bp DNA ladder.

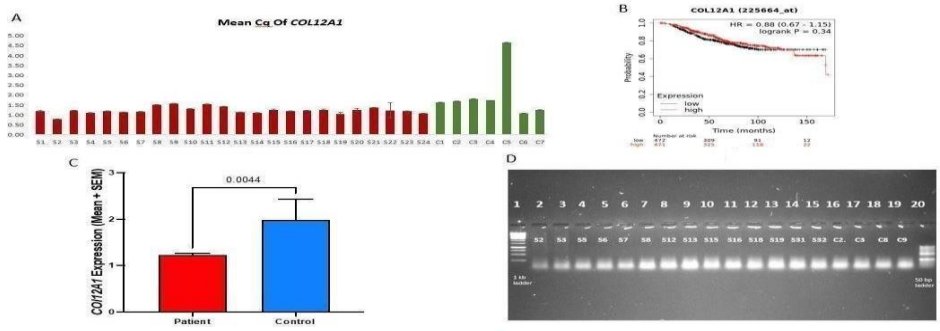

Fig. 4.2 COL12A1 Expression Analysis

While Fig. 4.2 explaining *COL12A1* expression analysis, In Fig 4.2 A and C we used Cq mean values obtained from q RT PCR and after normalizing these values we created graph from graph pad prism software, while Fig 4.2 B and D representing KM plotter analysis and gel visualization of q RT PCR.

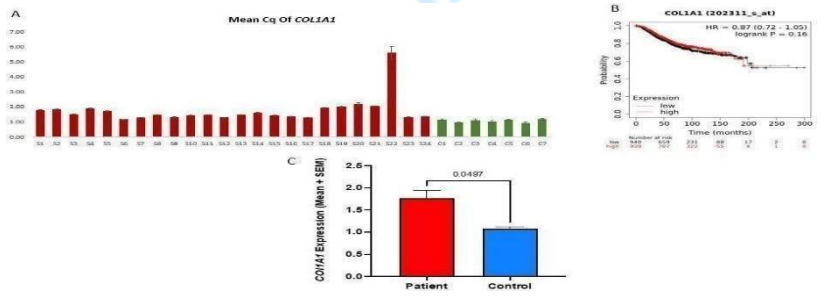

Fig. 4.3 *COL1A1* Expression Analysis Results

Similarly, Fig 4.3 A depicting Cq mean values of *COL1A1* expression analysis through a graph, and we compared it with control involved in this research and drawn a graph as seen in Fig 4.3 C through GraphPad prism, while B is showing KM plotter analysis for *COL1A1* expression.

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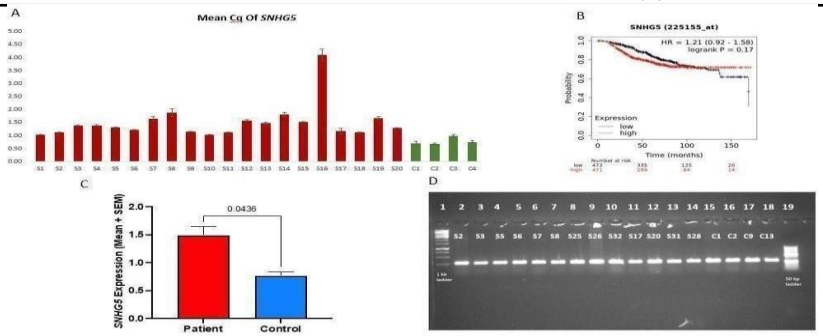

Fig. 4.4 *SNHG5* Expression Analysis Results

Next the Fig 4.4 A, B, C showing the same strategy and protocol that we have used for *COL12A1* and *COL1A1* gene, but one more interesting thing about this figure is in part D, which showcasing the gel visualization of *SNHG5* gene expression in addition to its q RT PCR analysis.

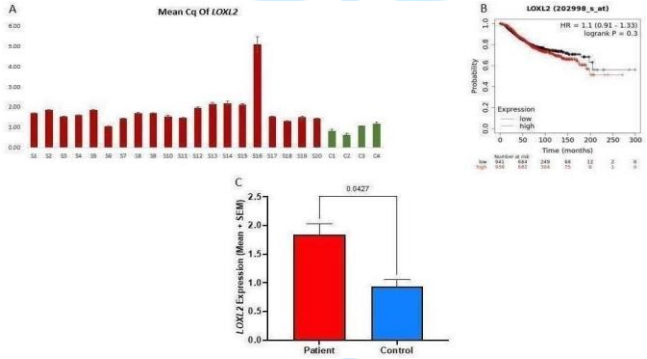

Fig. 4.5 LOXL2 Expression Analysis Results

In Fig.4.5, we have explained expression analysis for *LOXL2* gene, in part A and compared it with the control one in part C while in part B we have a KM plotter for this gene.

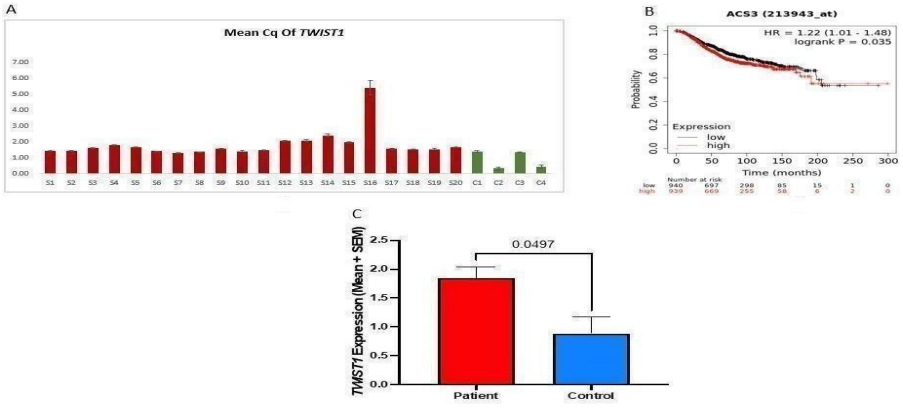

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Fig. 4.6 *TWIST1* Expression Analysis Results

Also Fig.4.6 depicting *TWIST1* expression, we obtained Cq mean values for the samples and control from the results of q RT PCR, so after normalizing their expression with *β-ACTIN* we have generated this graph, similarly in part C we used the same thing but this time we have average expression of all samples and control ones, while part B representing KM plotter for this gene expression.

DISSCUSSION

Table 4.1 highlighting the demographic details of BC patients with 37.5% ER-positive, 62.5% ER-negative, we have also TNBC patients with 15.625%, similarly the findings of our study, as depicted in Figure 4.2, suggest a notable association between the expression of *COL12A1* and the observed results. Figure 4.2 (A), displays the mean Cq value of *COL12A*, including a sample size of 24 patients and 7 control subjects. For further verification of results, Kaplan-Meier analysis was performed to assess OS in BC patients, keeping in view the *COL12A1* gene expression levels using a KM-plotter. It was revealed that patients with a high *COL12A1* gene expression had a considerably greater overall survival than those with low *COL12A1* gene expression. Additionally, *COL12A1* downregulation was observed to be prevalent in most cases, as depicted in Figure 4.2 (B). When an examination was conducted to assess the levels of *COL12A1* expression across various groups of BC, interesting results were revealed as shown in figure 4.2 (C). Here Graph Pad Prism software illustrates the average expression level of *COL12A1*. It is evident from the graph that BC patients exhibit comparatively lower levels of *COL12A1* expression in contrast to control cases. Furthermore, a decreased *COL12A1* gene expression was observed in BC patients having TNBC status in contrast to BC patients with other stages and grades. Figure 4.2 (D) shows a verification of *COL12A1* in patients and control samples, obtained by using q RT-PCR, followed by visualized on a 1.5% agarose gel. Lane 1 of the gel (D) had a 1 kilobase (kb) DNA ladder, whereas lane 12 of the same gel (D) contained a 50-base pair (bp) DNA ladder. Therefore, our findings about *COL12A1* expression contradict with those from the literature as we observed its high expression in controls compared to patients. However, our data relates well with the KM plotter data.

The *COL12A1* gene, which codes for a member of the fibril-associated collagen family, is found on human chromosome 6 at position 6q12-q13 (Gerecke *et al.* 1997). The significance of its involvement in facilitating connections between fibrils and the stroma is noteworthy, and there is evidence linking mutations in this protein to disease of myopathy (Hicks *et al.* 2014). The role of *COL12A1* in cancer is of significant interest, as its overexpression is observed in various cancer types, such as gastric, colorectal and

breast cancers. While its upregulation is related to enhanced migration of tumor metastasis and invasiveness (Duan *et al.*, 2018). Additionally, a study suggested the presence of elevated levels of *COL12A1* in ovarian cancer cell lines resistant to chemotherapy procedures (Januchowski *et al.*, 2016). The gene *COL12A1* is responsible for encoding the α-chain of *COL12A1*, as well as having a role in encouraging the growth of tumor due to which it has gained much importance from research point of view. It also exerts an influence on the proliferation and motility of cancer cells. Moreover, an excessive expression of *COL12A1* counteracts the inhibitory effects, exerting a significant impact on various types of malignancies, including colorectal cancer and esophageal squamous cancers. A notable correlation has been observed between bad prognosis with *COL12A1* expression and different cancer types, including gastric, pancreatic and breast cancer (Wu & Xu, 2020). Numerous research studies provided understanding about oncogenic characteristics of *COL12A1* and its potential value as a prognostic marker in BC. In addition, the researchers have devised an innovative predictive tool centered around *COL12A1*, which combines *COL12A1* levels

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with clinical variables to enhance the evaluation of breast cancer prognosis. This approach considers the potential influence of *COL12A1* on tumor immunity and its potential as a valuable biomarker for forecasting prognosis and assessing how they react to immunotherapy in patients (Yan *et al.*, 2023).

The findings from the *COL1A1* analysis in Figure 4.3 indicate the average Cq value of *COL1A1*. This analysis involved a total of 24 patient samples and 7 control samples, as depicted in Figure 4.2 (A). The study utilized a Kaplan-Meier (KM)-plotter to assess the OS of BC patients based on varying *COL1A1* expression levels. The findings revealed that patients exhibiting high *COL1A1* expression experienced significantly poorer overall survival outcomes compared to those with low *COL1A1* expression. Furthermore, the study observed an overall upregulation of *COL1A1* in most of the cases, as depicted in Figure 4.2 (B). In addition, the present study involved an examination of *COL1A1* expression levels across several breast cancer cohorts. The diagram presented in Figure 4.2 C of the Graph Pad Prism software illustrates the average expression level of *COL1A1*. It is evident from the diagram that BC patients exhibit considerably higher *COL1A1* expression levels than the control group. Furthermore, within the breast cancer patient population, individuals with TNBC display relatively higher *COL1A1* expression compared to patients with other stages and grades of BC. The expression of *COL1A1* in BC patients is related to aggressive cellular behavior and has the potential to promote metastasis. Furthermore, the study revealed a significant association between *COL1A1* expression and ER/PR status, indicating the potential utility of a combined biomarker for individuals diagnosed with BC.

One research article explained the stiffness of ECM consisting of a major role in driving prolactin signals. It also explains the stiff matrices and prolactin signals in BC containing a feed forward loop (Barcus *et al.*, 2013). Hence, there exists an association between the *COL1A1* level and PR status, indicating a prospective therapeutic strategy. In conclusion, individuals exhibiting elevated levels of *COL1A1* demonstrated a heightened response rate to cisplatin-based chemotherapy. This finding suggests that *COL1A1* could potentially serve as a novel biomarker for evaluating patients with advanced breast cancer who may benefit from chemotherapy treatment. There exists a hypothesis suggesting that the human gene responsible for promoting metastasis in the extracellular matrix is *COL1A1*, located at chromosomal region 17q21.33. The application of invasive carcinoma 3D culture has been employed as an experimental framework incorporating type I collagen-rich matrices (Dudley *et al.*, 2014). The relationship between rigid matrices and prolactin is observed in a feed-forward loop throughout BC progression, as explained by a recent study that highlights significant influence of ECM stiffness on prolactin signaling (Barcus *et al.*, 2013). Consequently, the correlation between the level of *COL1A1* and the PR status indicates a potential therapeutic approach. It has been observed that individuals exhibiting elevated levels of *COL1A1* demonstrated a greater likelihood of responding positively to cisplatin-based chemotherapy (Liu *et al.*, 2018). This finding implies that *COL1A1* may function as a potential biomarker for patients undergoing chemotherapy and help treat advanced BC.

The expression of *COL1A1* has been predominantly observed within the cytoplasmic compartment of breast cancer cells. In addition, inhibition of metastasis in BC cells has been shown through downregulation of *COL1A1*. Therefore, metastatic potential of BC cells could be inhibited through the downregulation of *COL1A1*. In vitro experiments have explained that the inhibition of *COL1A1* results in a suppression of BC cell invasion and migration. This suggests that the expression of *COL1A1* in cells has a contributory role in promoting BC metastasis. Nevertheless, suppression of *COL1A1* did not exhibit any impact onthe indicators related to EMT, implying that intracellular

COL1A1 might facilitate the progression as well as metastasis of BC via other signaling pathways. Willis and Kluppel have demonstrated *COL1A1* as target gene of WNT/beta- catenin pathway, favorably regulated in BC cells. (Willis & Klüppel, 2014)

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Various studies postulated the EMT activation by TGF-β has the potential to improve the survival of cholangiocytes by modulating gene expression that facilitates programmed cell death. Additionally, it has been observed that the synthesis of *COL1A1*, stimulated by TGF-β, does not seem to be associated with an EMT-like transition. Therefore, results of our study offer verification in favor of the idea that there exist other mechanisms having importance in regulation of *COL1A1* as well as EMT (Liu *et al.*, 2012).

The prognosis for *SNHG5* is poor and we confirmed *SNHG5* expression in the progression of BC by q RT-PCR, further comparing its expression between 20 BC samples and control group. Importantly, a Kaplan-Meier (KM)-plotter has been used to compare the OS in BC patients having various *SHNG5* expression levels. It was found that patients having a high *SNHG5* expression showed significantly lower OS than those with decreased *SNHG5* expression. Moreover, an upregulation of *SNHG5* was observed in most cases.

Fig. 4.4 (A) highlights the findings of *SNHG5* expression and displays the mean Cq value of *SNHG5* for 20 patient samples and 4 control samples. Using a Kaplan-Meier (KM)-plotter, we compared OS of BC patients with various expression levels of *SNHG5* and found patients with its increased expression, significantly lesser OS than with low *SNHG5*, and results confirmed upregulation of *SNHG5* in most cases (figure 4.4 B). Moreover, *SNHG5* expression levels in various BC groups were analyzed. Graph Pad prism diagram (in figure 4.4 C) demonstrates the average *SNHG5* expression level, revealing a relatively higher *SNHG5* expression levels in TNBC patients compared to patients with other stages and grades. Fig 4.4 (D) shows PCR validation of *SNHG5* in both patients and controls, with a clear display 285bp amplified product. In Fig 4.4 (D), lane 1 exhibits a 1kb DNA ladder, while lane 12 displays a 50bp DNA ladder.

The oncogene *SNHG5* has been identified to play role in BC, having potential to function as a separate prognostic marker. It has been observed that a decreased *SNHG5* level slows the progression of BC r and promotes a G1-phase cell cycle arrest. Moreover, *SNHG5* serves as an RNA molecule that sequesters miR-154-5p, thereby reducing its suppressive effect on PCNA and facilitates cellular proliferation. Prior research has emphasized *SNHG5*'s importance in regulating cell cycle progression in BC development. These studies have relied on experimental findings and indicated that irregular cell-cycle control contributes to uncontrolled cell growth seen in tumors. Cell cycle progression is facilitated by cyclin-dependent kinase (CDK) complexes and their cyclin partners. CDK4/6-Cyclin D1 and CDK1-Cyclin B1 activation is vital for transitioning from G1 to S phase and G2 to M phase, respectively (Yu *et al.*, 2014). When *SNHG5* is inhibited, the proportion of cells in G1 phase increases. Cyclin B1 expression is unaffected by *SNHG5* depletion after cell synchronization. Whereas Cyclin D1 expression decreases upon *SNHG5* depletion. Hence, the investigation uncovers how *SNHG5* drives oncogenesis in BC by speeding up G1 to S phase cell cycle transition. Thus, *SNHG5*-miR-154-5p-PCNA pathway significantly advances BC progression and holds promise as a therapeutic target (Chi *et al.*, 2019).

LOXL2 protein has a low expression in normal breast tissue, particularly in the stroma and epithelial cell luminal layer. Conversely, BC tissues show elevated *LOXL2* levels in ECM and within cells, encompassing cytoplasm as well as cell nuclei (Moreno- Bueno *et al.*, 2011). Based on findings from immunohistochemical analyses, it has been observed that there is a correlation between mRNA overexpression and an elevation in intracellular *LOXL2*, which exhibits a perinuclear distribution, in approximately 60% of basal breast carcinomas. Moreover, elevated *LOXL2* expression in metastatic tissues versus tumor on primary (Cano et al., 2012). *LOXL2* has shown independent prognostic value in patients with BC, indicating its relevance for metastasis and mortality prediction. It serves as a unique indicator for OS and MFS in BC cases. Specifically, a retrospective study noted that patients having ER-negative tumors status and higher *LOXL2* mRNA levels had a poorer prognosis. The study demonstrated a strong link between *LOXL2* expression and decreased OS and MFS (Barker *et al.*, 2011). TNBC accounts for an estimated 15% of all BC cases and is notably associated with the

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occurrence of metastases. Elevated *LOX* and *LOXL2* expression have been observed in TNBC patients, suggesting a potential importance of these proteins as targets for TNBC's systemic treatment. Furthermore, there have been suggestions proposing that the inhibition of *LOXL2* could potentially enhance the susceptibility of TNBC cells to conventional treatment. (Leo et al., 2018) Some studies indicated the pro-metastatic effects of *LOXL2* are inherent to breast tumor cells and are predominantly unaffected by the protein's extracellular activities on the extracellular matrix (ECM). Hence, the efficacy of future therapeutic interventions targeting *LOXL2* inhibition in breast cancer should be enhanced by preventing intracellular *LOXL2* (Salvador *et al.,* 2017). A wide range of *LOXL2* inhibitors have been developed in response to the potential therapeutic benefits associated with the inhibition of this protein in the context of cancer treatment (Ferreira *et al.,* 2021).

Our current research explored how *LOXL2* expression impacts BC progression. We analyzed *LOXL2* expression in 20 BC patient samples with controls using q RT-PCR. In addition, we conducted a comparative analysis of OS among BC patients with varying *LOXL2* levels by KM plotter. The OS of patients with high versus low *LOXL2* expression, varied significantly. Increased *LOXL2* expression was found to relate with significantly decreased OS rates in Figure 4.5 (B). In most cases, *LOXL2* was found to be increased, as depicted in Figure 4.5A, which shows the average Cq value of *LOXL2* expression for a sample of 20 patients and 4 control subjects.

Furthermore, our study examined *LOXL2* expression across several BC subgroups. The average *LOXL2* expression level, as illustrated in Figure 4.5 (C) using GraphPad Prism software, distinctly showcased higher *LOXL2* levels in BC patients and control group. Moreover, individuals with TNBC exhibited relatively elevated *LOXL2* expression within the BC patient population, than other stages and grades.

Hence, the results of our study described the role of *TWIST1* expression in BC development. Using q RT-PCR, we analyzed *TWIST1* expression in 20 BC patient samples and controls and compared their OS rates. The Kaplan-Meier plotter revealed a significant finding. It was found that patients with higher *TWIST1* expression had poor OS than those with lesser expression.

Figure 4.6 illustrates the effects of *TWIST1* expression. In Figure 4.6 (A), the mean Cq value of *TWIST1* is shown, incorporating data from 20 patients and 4 controls. Kaplan- Meier plotting was used to assess OS rates based on *TWIST1* expression, revealing poorer survival for high expressors. Figure 4.6 (B) depicts the prevalent upregulation of *TWIST1* in most cases. Additionally, Figure 4.6 (C) displays the average *TWIST1* expression, indicating higher levels in breast cancer patients than controls. TNBC patients also exhibited higher *TWIST1* expression compared to different stages and grades of BC, their demographic details are also mentioned in Table 4.1.

The transcription factor *TWIST1* exhibits upregulation in various carcinomas, exerting a major influence on diverse cancer progression factors like tumor initiation, stemness, invasion, metastasis, angiogenesis as well as treatment resistance. While the connection between epithelial-mesenchymal transition (EMT) and several cancers is well established, further investigation is required to understand its involvement in tumor development, stem cell properties, angiogenesis, and resistance to therapies. Furthermore, there is a growing trend towards examining the interconnectedness of various functions, rather than considering them separately. For example, certain studies suggest that the influence of *TWIST1* is associated with its degree of expression. It has been proposed that the low expression levels of *TWIST1* may affect the beginning of tumors and the maintenance of stemness, whereas greater expression levels may potentially induce epithelial-mesenchymal transition (EMT). Further investigation is warranted to have a more comprehensive knowledge of the association between *TWIST1* expression and its associated consequences (Zhao *et al.,* 2017).

Conclusion:

In summary, both *COL1A1* and *COL12A1* emerge as innovative biomarkers with diagnostic, prognostic, and chemoresistance potential in breast cancer. These findings offer new avenues for delving

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into these genes' precise molecular mechanisms and developing novel anti-breast cancer drugs tied to *COL1A1*. Notably, *COL1A1*'s cellular presence can drive breast cancer metastasis, making it a significant prognostic marker and a plausible treatment focus, especially in ER+ patients, the inhibition of *COL1A1* curbs metastasis independently of the epithelial-mesenchymal transition (EMT) process. Elevated *COL1A1* levels correlate with poor survival, particularly in ER+ and TNBC patients. Likewise, *LOXL2*'s expression fosters EMT and invasiveness in basal- like breast cancer cell lines, aligning with prior in vitro research, marking *LOXL2* as a potential survival-improving target in breast cancer. Despite study limitations like a small retrospective sample size, the connection between *LOXL2*'s enzymatic activity, experimental metastasis, and patient survival underscores its significance. Additionally, while *TWIST1*'s gene expression and historical literature underscore its role in PMN of BC, exploring these mechanisms holds promise for early BC metastasis diagnosis and treatment. Furthermore, *SNHG5* stands out as an oncogene and an independent predictor in breast cancer patients. Reducing *SNHG5* suppresses breast cancer proliferation, inducing G1 phase cell-cycle arrest. This highlights *SNHG5*'s pivotal role in breast cancer proliferation and positions it as potential therapeutic target for BC.

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