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Research Article

Study to Identify the Signalling Cascade behind Expression Level of *PTEN* and *RB1* gene in Breast Cancer

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Abstract

Breast cancer is the second most prevalent cancer among Pakistani women and the disease burden of breast cancer is continuously uprising. There are several genes involved in breast cancer incidence. The current study was designed to analyze the gene expression level of hereditary onco-suppressive *PTEN* and *RB1*, proto-oncogenes i.e. *Src* and *KRAS*, and microRNA-140/145/238in breast cancer patients performed through qRT-PCR.Results showed significant up-regulation of proto-oncoSrc and *KRAS* genes ($p<0.05$). On the other hand, a significant down-regulation of hereditary onco-suppressive *PTEN* and *RB1* genes while as, the micro RNA signalling cascade involvement through higher expression levels of MI-140, Mi-145, and Mi-238 was also observed ($p<0.05$). Biopsy samples were preserved in 10% formalin for histopathology as well as in Trizol for mRNA extraction. Histopathlogical examination showed multilayering, hyperplasia, and a complete distortion of ductal and glandular epithelium of breast gland in breast cancer patients.

Keywords: Breast cancer; Histopathology; Proto-oncogenes; Down regulation; mRNA signalling; Expression level

Introduction

Pakistan is ranked highest for mortality and incidence of breast cancer[1-3]. According to 2020 statistics, 2.3 million women were reported with an incidence of breast cancer with 685,000 deaths [4].In 2020, the detailed breast cancer cases in Pakistan were 25,928 which represented 14.5% of a wide range of diseases [4].

Genes associated with breast cancer incidence include *BRCA1, BRCA2, PALB2* (Partner And Localizer of BRCA2), *CHEK2* (Checkpoint Kinase 2), *CDH1* (CaDHerin 1), *PTEN* (Phosphatase and TENsin homolog), *STK11* (Serine/Threonine Kinase 11), *Tp53* (Tumor Protein p53), *RB1, src, KRAS, ATM, BARD1, BRIP1, CASP8, CTLA4, CYP19A1, FGFR2, H19, LSP1, MAP3K1, MRE11A, NBN, RAD51,* and *TERT*[5,6]. Oncogenesis mainly contributed to mutations in two types of genes: tumor suppressor genes and proto-oncogenes[7]. *PTEN* and *RB1* are examples of tumor suppressor genes while *Src* and *KRAS* belong to a class of proto-oncogenes.

PTEN is a tensin and phosphatase homolog that on chromosomal deletion on chromosome number 10.*PTEN* plays an important part not only in apoptosis induction and arrest of the cell cycle but also in many other physiological functions including migration, differentiation, and cellular adhesion.*PTEN* is an important tumor-suppressing gene in breast cancer and normally suppresses cellular proliferation by down-regulating the PI3K/AKT signalling pathway.mTOR/PI3K/AKT is the most commonly deregulated pathway in a different type of carcinogenesis including breast cancer[8]. *PTEN* is one of the most commonly mutated tumor suppressor genes in human malignancies. Many different types of mutations that occur at the genetic level have been found to occur at phosphatase and tensin homolog including insertions, deletions, frameshift mutations, splice site variants, nonsense, and missense mutations that are linked with the cancers associated with complete inhibition or reduction in phosphatase activity of the phosphatase and tens in homolog deleted on chromosome ten.

RB1 is retinoblastoma which is normally controlling many important physiological functions of the cell including the formation of retinoblastoma protein, cell survival, cell cycle progression, control of apoptosis, or programmed cell death. The transcription of early growth factor-2 receptor is inhibited by the canonical type retinoblastoma protein. The retinoblastoma protein normally functions in signaling pathways that are controlled by its most important regulators working in the upstream direction *including CDK4, p16,* and *cyclin D1* which controls the activation of retinoblastoma protein via its phosphorylation and also controls the ability of retinoblastoma protein to inhibit the normal functioning of early growth factor 2 receptors [8]. Many different types of mutations are reported at various steps of retinoblastoma signalling pathways in various types of cancers reported to date. *RB1* is normally a tumor suppressor gene, loss of expression of *RB1* results in the development of basal-like breast cancer[9].

Src, the gene product of avian Rous sarcoma virus is reported for progression, development, and maintenance of various forms of cancer including breast cancer is a proto-oncogene belonging to *Src* family kinases (SFKs)[10]. *Src* is an oncogenic steroid receptor coactivator. It is reported that alterations in type enzymes controlling two types of functions including transcriptional programming, and cellular metabolism are one of the most important hallmarks of cancer resulting in uncontrolled proliferation and metastasis[11]. According to one study, the metabolic enzyme(PFKB4) regulates the transcriptional factor responsible by activating the *Src*, and any type of mutation in that enzyme results in a mutational change in *Src*, and as a result, there will be initiation and progression of carcinogenesis including breast cancer. Studies report the decrease of invasion and proliferation of cancer cells when *Src* was disrupted genetically[10].

KRAS (Kirsten rat sarcoma virus) proto-oncogene located on 12p12.1 belongs to the *RAS* superfamily of small GTPases and is involved in the RAS/MAPK pathway to relay signals for cellular proliferation and growth[12]. The key feature of the *RAS* family is the presence of catalytic G- domain. The most frequently studied proteins in the *RAS* subfamily include K-RAS (KRAS4A and KRAS4B, N-RAS, and H-RAS[13]. *KRAS* is usually associated with the progression of the cell cycle while in cases of increased levels can also induce apoptosis and growth arrest[14]. Wild-type *KRAS* is usually associated with tumor suppression while the mutated version of genes induces oncogenic properties. *KRAS* is one of the most commonly mutated proto-oncogenes in human breast cancers most frequently in triple-negative breast cancer[15]. Most of the studies indicated that functions of *KRAS* in normal cells are controlled mainly by the miRNAs. In the case of triple-negative breast cancer one of the mutations in miRNA-873 results in loss of the normal functional ability of *KRAS* and as a result, there will be initiation and progression of carcinogenesis in various parts of the body including breast tissue causing breast carcinogenesis. Oncogenic *KRAS* mutations are seen in a round of about 15% of all tumors and deregulation of the MEK/RAF/ERK pathway by extracellular signal-regulated kinase (*KRAS*) hyperactivation is found in roughly 30% of all tumors.

MicroRNAs are a group of small endogenous non-coding RNAs that impart a significant role in controlling the gene expression level in normal body cells. Many pieces of evidence indicated that disorganized expression of microRNAs occurs in various types of cancers through different types of mechanisms including microRNA genes amplification or deletion, dysregulated

control of microRNA at the transcriptional level, unorganized changes at the epigenetic level, and defects at different levels of biogenesis networking of microRNAs.miRNAs play a crucial part in the development and proliferation of pathogenesis of solid tumors and assist in their role as tumor suppressors and proto-oncogenes[16]. In the diffused form of B cell large lymphoma elevated expression of unorganized and abnormal microRNAs was identified in serum samples. Some non-coding large microRNAs also play a crucial part in the progression and development of breast cancer by altering its regulatory functions through various mechanisms including interaction with different types of proteins including modifiers of the epigenetic system, and transcriptional co-activators [17].

In this study, we analyzed the expression of miRNA-140, mi-RNA145, miRNA-238, *PTEN, RBA1, KRAS*, and *Src* in breast cancer patients and control along with the Histopathological Examination of adenocarcinoma, ductal carcinoma, fibrous carcinoma, and lobular carcinoma in breast tissues.

Methodology

Sample Collection

The clinical study was conducted on breast cancer patients of Allied Hospital Faisalabad with the permission of the ethical review committee of Faisalabad Medical University (FMU). Biopsy breast tissue samples were collected from patients who suffered from breast cancer. After collection biopsy samples were preserved in 10% formalin and 0.9% normal saline (NS) solution. Tissue samples were collected for RNA extraction and histopathology irrespectively.

Sample Processing for Expression analysis

Biopsy samples were further processed for mRNA isolation, performed manually by this standard protocol[18].cDNA was synthesized by the following standard protocol [19].qRT-PCR was performed followed by conventional PCR amplification of first strand cDNA[20]using specifically designed primers.

Histopathological Examination

For routine examination of histopathology, biopsy samples from breast cancer patients were collected in 10% formalin solution. Tissue specimens were embedded in paraffin before sectioning. Tissue was stained by using Haematoxylin and Eosin (H & E) stain for staining and analyzed under a microscope [21].

Statistical Analysis

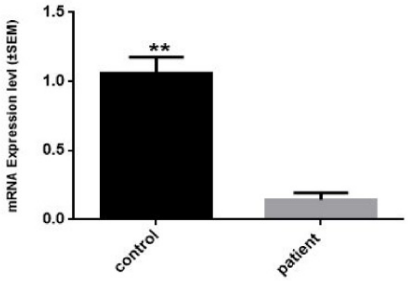
Two-way ANOVA and DMR tests were used to examine graphical data statistically[22]. Graph pad prism 6 was used to draw graphs.

Results

Relative gene expression T-Test for *PTEN* and *RB1*gene

The graph obtained from data analysis showed the relative gene expression of the *PTEN* gene and *RB1* gene in control and patient samples as 1.363±0.085 and 0.17±0.065 for the *PTEN* gene respectively and 1.060±0.1153 and 0.1433±0.04933for RB1 gene respectively. Mean, standard deviation, and standard error mean were calculated using Two way ANOVA and DMR tests. Results showed that the *PTEN* and *RB1* gene expression level is significantly high expression level in the control samples.

(a)



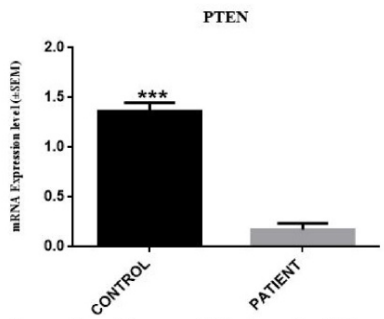
(b)

	N	Mean	St Dev	SE Mean
Control	3	1.06	0.115	0.066
Patient	3	0.143	0.049	0.028
Difference	3	0.9167	0.1332	0.0768

P-Value = 0.0070(<0.05)

Figure (a): Control sample of normal breast tissue and Patient sample with breast cancer. Graph illustrates values of RB1 gene expression levels in control and sample as 1.060±0.1153 and 0.1433±0.04933 respectively. RB1 gene is downregulated in patients with breast cancer. **Figure (b):** Table represents the different values of RB1 gene, mean, standard deviation, standard error means in control as well as in patients. Control have significantly higher gene expression level i.e. Mean=1.06, St Dev=0.115, SE Mean=0.066 as comapred to patients i.e. Mean= 0.143, St Dev=0.049, SE Mean= 0.028.

(a)



(b)

	N	Mean	St Dev	SE Mean
Control	3	1.363	0.085	0.049
Patient	3	0.17	0.065	0.037
Difference	3	1.193	0.0208	0.012

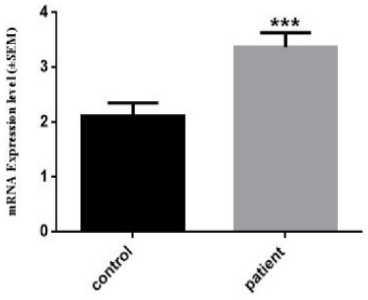
P-Value = 0.0001(<0.05)

Figure (a): Control sample of normal breast tissue and Patient sample with breast cancer. Graph illustrates values of PTEN gene expression levels in control and sample as 1.363±0.085 and 0.17±0.065 respectively. PTEN gene is downregulated in patients with breast cancer. **Figure (b):** Table represents the different values of PTEN, mean, standard deviation, standard error means in control as well as in patients. Control have significantly higher gene expression level i.e. Mean=1.363, St Dev=0.085, SE Mean=0.049 as comapred to patients i.e. Mean= 0.17, St Dev=0.065, SE Mean= 0.037.

Relative gene expression T-Test for*KRAS* and *Src*gene

The graph obtained from data analysis showed the relative gene expression of *KRAS*gene and *Src* gene in control and patient samples as2.120±0.2352 and 3.377±0.25725 for KRAS gene respectively and1.283±0.1026 and 3.467±0.2857 for*Src1* gene. Mean, standard deviation, and standard error mean were calculated using two-way ANOVA and DMR tests. Results showed that *KRAS* and *Src* gene expression level is significantly high expression level in the patient's samples.

(a)



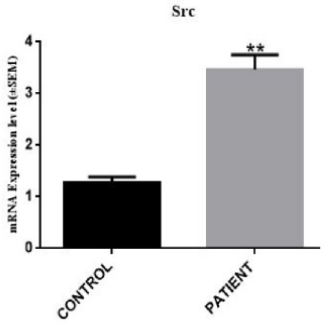
(b)

	N	Mean	St Dev	SE Mean
Control	3	2.12	0.235	0.165
Patient	3	3.37	0.257	0.148
Difference	3	1.257	0.0251	0.0145

P-Value = 0.0001(<0.05)

Figure (a): Control sample of normal breast tissue and Patient sample with breast cancer. Graph illustrates values of KRAS gene expression levels in control and sample as 2.120±0.2352 and 3.377±0.2572 respectively. KRAS gene is upregulated in patients with breast cancer. **Figure (b):** Table represents the different values of KRAS gene, mean, standard deviation, standard error means in control as well as in patients. Patients having breast cancer have significantly higher gene expression level i.e. Mean=3.37 and St Dev=0.257 as comapred to control i.e. Mean= 2.12, and St Dev=0.235.

(a)



(b)

	N	Mean	St Dev	SE Mean
Control	3	1.283	0.102	0.059
Patient	3	3.467	0.285	0.164
Difference	3	2.183	0.1976	0.1141

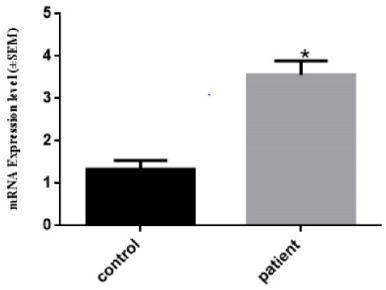
P-Value = 0.0027(<0.05)

Figure (a): Control sample of normal breast tissue and Patient sample with breast cancer. Graph illustrates values of Src gene expression levels in control and sample as 1.283±0.1026 and 3.467±0.2857 respectively. Src gene is upregulated in patients with breast cancer. **Figure (b):** Table represents the different values of Src gene, mean, standard deviation, standard error means in control as well as in patients. Patients having breast cancer have significantly higher gene expression level i.e. Mean=3.46 and St Dev=0.285 as comapred to control i.e. Mean= 1.28, and St Dev=0.102.

Relative gene expression T-Test for MIR-140, MIR-145, MIR-238

The relative gene expression analysis of MIR-140, MIR-145, and MIR-238 genes showed an up-regulation in breast cancer patients. The values obtained from graphical analysis are 1.330±0.2107 (control) and 3.560±0.33 (patient) for MIR140, 1.263±0.08737 (control) and 3.510±0.4762 (patient) for MIR145, and 1.087±0.1422 (control) and 3.487±0.2577 (patient)for MIR238.Mean, standard deviation, and standard error mean was calculated using two-way ANOVA and DMR tests showed that MIR-140, MIR-145-, and MIR-238 gene expression level is significantly high expression level in patients' samples.

(a)



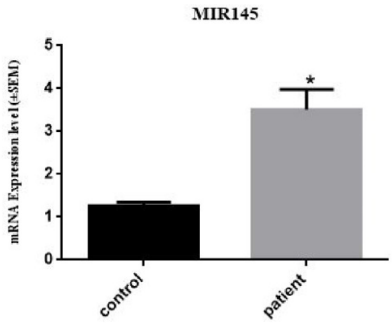
(b)

	N	Mean	St Dev	SE Mean
Control	3	1.330	0.2107	0.1217
Patients	3	3.560	0.3300	0.1905
Difference	3	2.230	0.5403	0.3119

P-Value = 0.0190(<0.0.5)

Figure (a): Control sample of normal breast tissue and Patient sample with breast cancer. Graph illustrates values of Mi-140 gene expression levels in control and sample as 1.330±0.2107 and 3.560±0.33 respectively. Mi-140 gene is upregulated in patients with breast cancer. **Figure (b):** Table represents the different values of Mi-140 gene, mean, standard deviation, standard error means in control as well as in patients. Patients having breast cancer have significantly higher gene expression level i.e. Mean=3.560 and St Dev=0.3300, and SE Mean= 0.1905 as comapred to control i.e. Mean= 1.330, and St Dev=0.2107, SE Mean= 0.121.

(a)



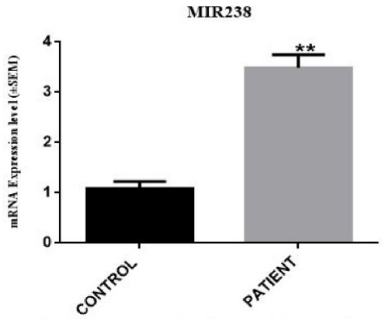
(b)

	N	Mean	St Dev	SE Mean
Control	3	1.263	0.0873	0.0504
Patients	3	3.51	0.476	0.2749
Difference	3	2.247	0.4805	0.2744

P-Value = 0.0149(<0.05)

Figure (a): Control sample of normal breast tissue and Patient sample with breast cancer. Graph illustrates values of Mi-145 gene expression levels in control and sample as 1.263±0.08737 and 3.510±0.4762 respectively. Mi-145 gene is upregulated in patients with breast cancer. **Figure (b):** Table represents the different values of Mi-145 gene, mean, standard deviation, standard error means in control as well as in patients. Patients having breast cancer have significantly higher gene expression level i.e. Mean=3.51, St Dev=0.476, and SE Mean= 0.2749 as comapred to control i.e. Mean= 1.263, St Dev=0.0873, and SE Mean= 0.0504.

(a)



(b)

	N	Mean	St Dev	SE Mean
Control	3	1.0867	0.1422	0.0821
Patients	3	3.4867	0.2577	0.1488
Difference	3	2.400	0.2893	0.1670

P-Value = 0.048(<0.05)

Figure (a): Control sample of normal breast tissue and Patient sample with breast cancer. Graph illustrates values of Mi-238 gene expression levels in control and sample as 1.087±0.1422 and 3.487±0.2577 respectively. Mi-238 gene is upregulated in patients with breast cancer. **Figure (b):** Table represents the different values of Mi-238 gene, mean, standard deviation, standard error means in control as well as in patients. Patients having breast cancer have significantly higher gene expression level i.e. Mean=3.4867 and St Dev=0.2577, and SE Mean= 0.1488 as comapred to control i.e. Mean= 1.0867, St Dev=0.1422, and SE Mean=0.0821.

Histopathological examination of adenocarcinoma, ductal carcinoma, fibrous carcinoma, and lobular carcinoma in breast tissues

Histopathological examination of adenocarcinoma: Image A, shows normal breast tissue with the proper symmetrical shape of glandular tissue the lumen of the gland, lobules, lobular epithelium, and lobular ducts are present in normal histological confirmation while images B, C, and D indicates the breast tissue with cancerous growth of cells. The normal structure of glands, ducts, lobules, and the epithelium is lost and there are pycnotic nuclei and hypertrophy of epithelial cells is also evident in the lobular cuboidal epithelium.

Histopathological examination of ductal carcinoma: Image E is taken from normal breast tissues which indicates the normal functional histology of breast tissues with a single layer of simple cuboidal epithelium and myoepithelial cells with normal lobular and glandular tissue while images F, G, and H are taken from cancerous tissue of breast cancer affected patients where the structure of gland is completely lost with hypertrophy of glandular cell cuboidal epithelium and multilayering of glandular epithelial cells. In this patient ductal carcinoma was diagnosed because the ductal structure of the gland is destroyed and ducts of glands are blocked with the growth of fibrous connective tissue inside the lumen of ducts and as a result, there will be a complete loss of glandular secretion.

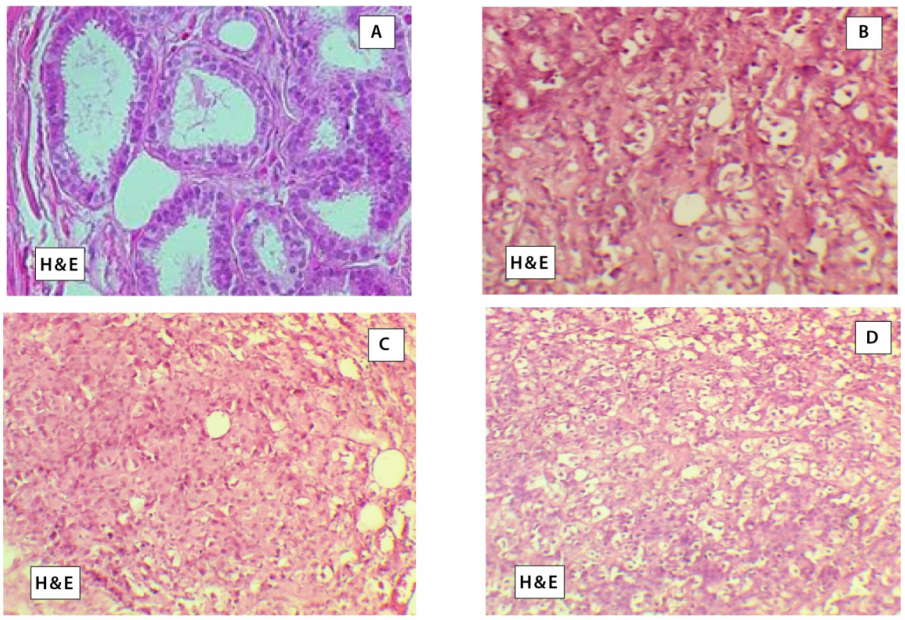


Figure. Histopthaological examination of adenocarcinoma. **A)** Histopathological image of the Control sample of normal breast tissue in symmetrical shape **B), C)** and **D)** is the histopathological image of the patient sample with breast cancer at 10X magnification with lost of normal structure for glands, ducts, lobules, epithelium ,pycnotic nuclei and hypertrophy of epithelial cells .

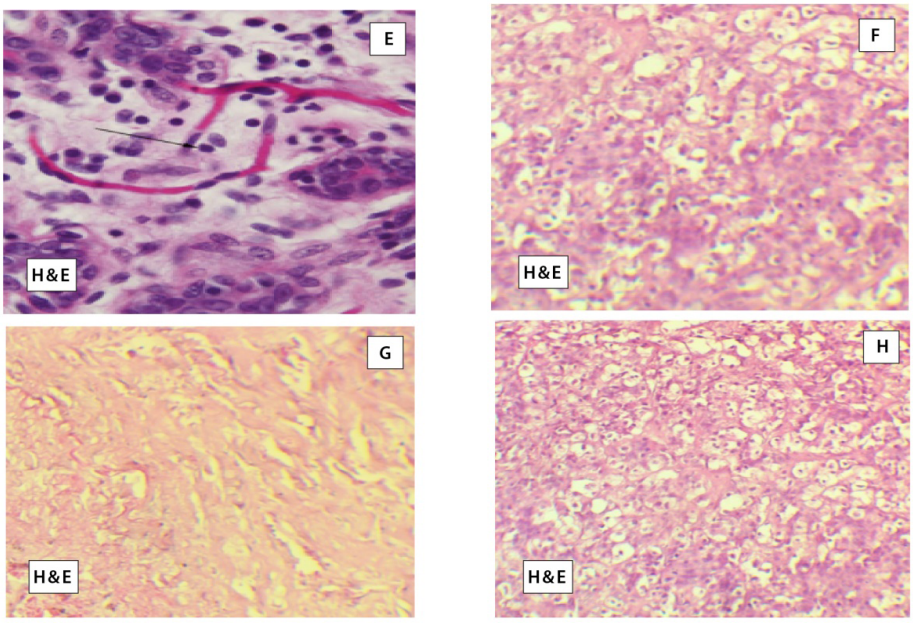


Figure . **E)** is the histopathological image of control sample with single layer of simple cuboidal epithelium and myoepithelial cells with normal lobular and glandular tissue while as, **F), G)** and **H)** is the histopathological image of patient sample with breast tissue at 10X magnification showing lost of normal structure with hypertrophy of glandular cell cuboidal epithelium and multilayering of glandular epithelial cells.

Histopathological Examination of lobular carcinoma: Image I, shows the histology of normal breast tissue in the parous female after menopause which indicates the normal structure of gland lobules and ducts with enormous amounts of lymphocytes present along the ducts lobules. Image J, K, and L is the histology of breast tissue from breast cancer patients which typically indicates lobular carcinoma in which the normal structure of lobules is destroyed and there is hyperplasia of glandular epithelium.

Histopathological examination of fibrous carcinoma: Image M, indicates the normal glandular structure of normal breast tissue with the normal cuboidal epithelium of glandular lobules and ducts along with the presence of myoepithelial cells. The lumen of ducts is normal where secretion of the gland is poured.

Images N, O, and P indicate the histology of fibrous carcinoma where the fibrous tissue growth is uncontrolled and fibrous tissue starts growing inside the lobules and ducts of glands.

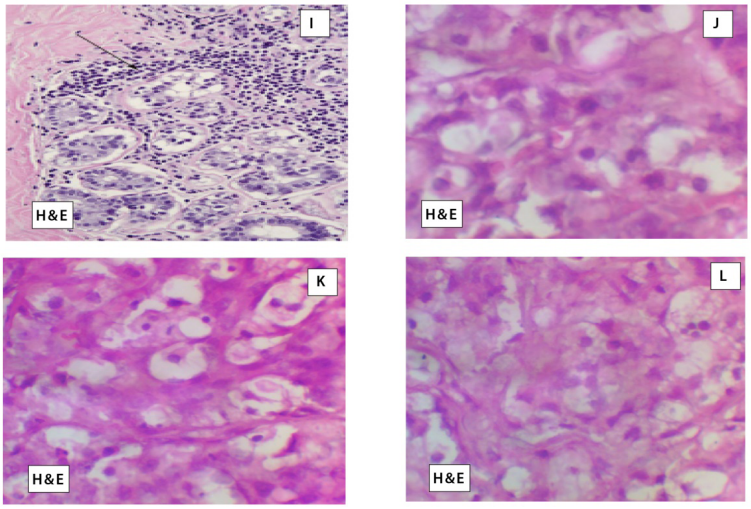


Figure . I) is the histopathological image of normal breast tissue at 40X magnification showing normal structure of gland lobules and ducts with enormous amounts of lymphocytes present along the ducts of lobules. while as, **J), K)** and **L)** is the histopathological image of patient tissue sections with breast cancer showing destroyed structure of lobules with hyperplasia of glandular epithelium

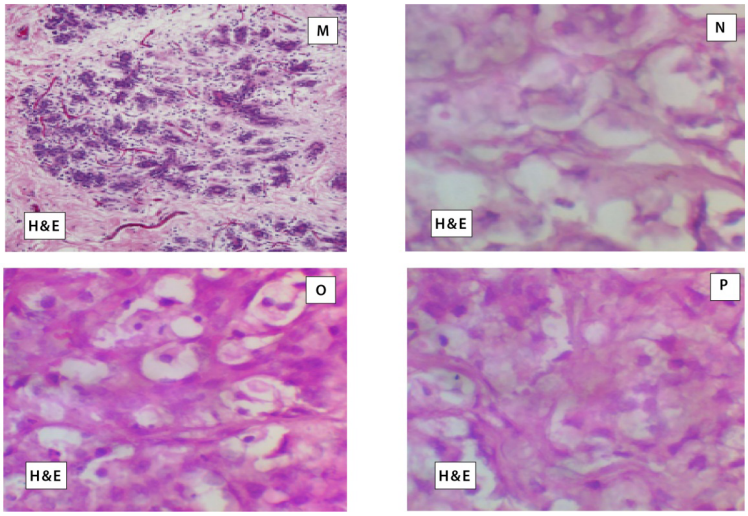


Figure . M) is the image of histopathological section of normal breast tissue showing normal cuboidal epithelium of glandular lobules and ducts along with the presence of myeoepithelial cells while as, **N), O)** and **P)** is the histopathological image of the patient with breast cancer at 40X magnification showing abnormal growth of fibrous tissues growing inside the lobules and ducts of glands.

Discussion

Breast cancer is the most commonly identified malignancy and the major contributor to cancer-associated deaths in females worldwide[23]. There is an upward trend in breast cancer burden in Pakistan, with one in every nine women having a lifetime chance of developing breast cancer [24]. The purpose of this study was to analyze the expression of tumor suppressor gene (*PTEN and RB1*), proto-oncogene (*Src and KRAS*), and micro RNA-140/145-238 in control and breast cancer patients along with the histopathological examination of adenocarcinoma, ductal carcinoma, fibrous carcinoma, and lobular carcinoma in breast tissues breast cancer patients.

Inside a normal cell, the microRNA-145 controls the growth and regulation of cells acting as a tumor suppressor and controlling the growth of normal cells but mutations and disorganized expressions can result in variable cancers. Previous studies report a significant increase in microRNA-140 expression levels in breast cancer patients with later stages[25]. The results of our study showed that there exists a direct relationship between the last breast cancer stage, metastasis, and the up-regulation of microRNA-140. In addition, a progressive increase in microRNA-140 was found in metastatic breast cancer compared to primary tumors[26]. Similarly, another study also reported elevated levels of miRNA-140 in cancers for promoting cell invasion [27,28].

The Preceding research on microRNA-145 revealed an important role played by this miRNA in the development and progression of breast carcinogenesis in human females. Our study reported that microRNA-145 is highly up-regulated in breast cancer patients. It is observed that the Rho factor which is encoded by the *RTKN* gene and inside the normal cell is present in low concentration but if miRNA-145 suppresses this *RTKN* gene it results in the origination of cancer cell lines. It is reported that over-expression of MicroRNA-145 results in resistance to apoptosis and the development of carcinogenesis [29].A mutational change in miRNA-145 results in the loss of its growth-promoting function resulting in the initiation and development of breast carcinogenesis[30]. A study reported the over-expression of micro RNA 145 in late stages of cancer growth i.e. stage III and stage IV similar to our results [31].

Similarly, miRNA-238 acts as a proto-oncogene in breast cancer-positive patients indicating its upregulation which results in carcinogenic growth in various tissues of the body including breast tissue. Our study showed that up-regulation of miRNA-328 is responsible for the progression of breast cancer in females which accept the previous findings.

Upregulation of the Src gene results in an increased level of expression of various growth factors including growth factors for epidermis, growth factors present on the surface of blood vessels growth factors for fibroblasts may be due to increased expression of the *Src* gene in breast cancer [32]. Results of our study indicated the upregulation of the *Src* gene in breast cancer patients as compared to normal patients which indicate that the role of *Src* in a normal cell is as proto-oncogenic. A similar study depicted the up-regulation of *Src* in breast cancer while studying the importance of *Src* as a potential target for treatment [33].

KRAS plays a significant role in signaling for cellular proliferation and growth and mutations in *KRAS* have been directly associated with breast cancer. Our study showed high levels of *KRAS* expression in breast cancer patients as compared to control patients. A study reported a higher level of mRNA expression of the *KRAS* gene with a worse prognosis in breast cancer patients which relates to our findings[34]. A similar study was conducted to show the up-regulation of *KRAS* genes in variable cancers due to mutation[35].

It has been revealed that *PTEN* is concerned with many processes of cells such as cell growth [36] apoptosis[37], angiogenesis [38], migration, and invasion through interfering with various signalling pathways especially PIP3, PTEN/PI3K/AKT signalling pathways are greatly involved in oncogenesis. *PTEN* is rarely mutated in NSCLC but the loss of its proteins is the most common event that takes place in breast cancer [39]. Our results showed that *PTEN* is a hereditary gene that is up-regulated in breast cancer and our findings accepts sthe role of *PTEN* from previous studies [40-43].

RB1 is a tumor suppressor gene and plays its role in controlling the cell cycle and apoptosis. Significant loss of *RB1* leading to triple-negative breast cancer has been reported in previous studies due to mutation [44]. The results obtained for our study also shows the down-regulation of *RBA1* genes in breast cancer patients which is also confirmed by the results of another study by Bondhopadhyay in 2021[45]. Similarly, frequent loss of the *RB1* gene in metastatic cancers like breast and prostate cancers has also been confirmed by another study which agrees with our findings [46].

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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