# MUTATIONAL ANALYSIS OF *PTEN* GENE IN PAKISTANI BREAST CANCER PATIENTS

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# MUTATIONAL ANALYSIS OF *PTEN* GENE IN PAKISTANI BREAST CANCER PATIENTS

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By

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**2023**

بسم الله الرحمن الرحيم

# DECLERATION

I certify that research work titled "Mutational analysis of *PTEN* gene in Pakistani breast cancer patients" is my own work. The work has not been presented elsewhere for assessment. Where material has been used from other sources it has been properly acknowledge/ referred.

Rumaisa Asif

**CERTIFICATE**

This report, submitted by Ms. Rumaisa Asif to the Department of Microbiology (M-phil program), Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the requirement of Research thesis (MIC -699) for the degree of M-phil Microbiology.

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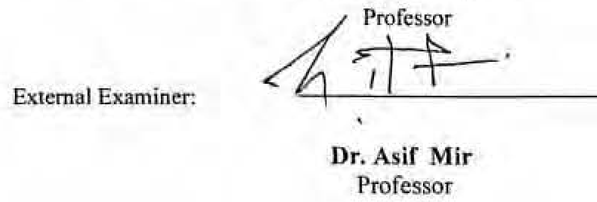

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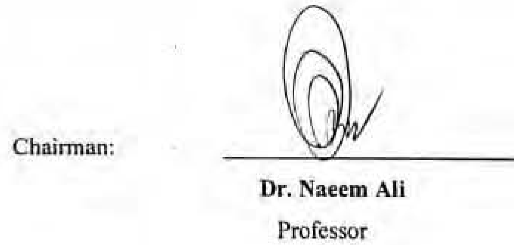

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| GLOBOCAN | Global Cancer Observatory |
| DCIS | Ductal carcinoma *in-situ* |
| ASMR | Age Standardized Mortality Rate |
| ACA | American Cancer Society |
| CDC | Center of disease control |
| ER | Oestrogen Receptor |
| PR | Progesterone receptors |
| HER2 | Human epidermal growth factor receptor-2 |
| EGFR | Epidermal growth factor receptor family |
| LCIS | lobular carcinoma *in-situ* |
| TNBC | Triple negative or Basal like breast cancer |
| TNM | Tumour node metastasis |
| *STK11* | Serine/Threonine Kinase 11 |
| *NF2* | Neurofibromin 2 |
| *APC* | Adenomatous Polyposis Coli |
| *CHEK2* | Checkpoint Kinase 2 |
| DSPs | Dual specificity protein phosphatases |
| PTP | Protein tyrosine phosphatases |
| MAGI3 | Membrane Associated Guanylate Kinase |
| PIP3 | Phosphatidylinositol (3,4,5) triphosphate |
| PKB | Protein Kinase B |
| BAD | BCL2 associated agonist of cell death |
| CREB1 | cAMP-responsive element-binding protein 1 |
| IRS1 | Insulin receptor substrate 1 |
| MAPK | Mitogen-activated protein kinase |
| JAK | Janus kinase |
| STAT | Signal transducer and activator of transcription |
| mTOR | Mammalian target of rapamycin |
| PHTS | *PTEN* hamartoma tumour syndrome |
| CS | Cowden syndrome |
| CML | Chronic myeloid leukaemia |
| FOXO | Forkhead family of transcription factors |
| WMA | World Medical Association |
| EDTA | Ethylene di amine tetra acetate |
| TBE | Tris Borate-EDTA buffer |
| SSCP | Single stranded conformation polymorphism |
| SIFT | Sorting Intolerant From Tolerant |

| PAGE | Polyacrylamide gel |
|---|---|

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## ABSTRACT

Breast cancer is defined as the uncontrolled cell division in breast tissue. Breast cancer can originate in any part of the breast and is categorized into different types. Breast cancer is the most prevalent type of cancer among females globally and every eighth female is at risk of breast cancer. Breast cancer is a multifactorial disorder with genetic factors being major risk factor for disease development. Different genes including tumour suppressor genes are involved in breast cancer development. Phosphatase and Tensin homolog (PTEN) acts as a phosphatase, which dephosphorylate phosphor-peptides and phospholipids. As phosphatase, it regulates cell cycle which prevents rapid growth and division of cells. The aim of the study was detection of single nucleotide polymorphisms (SNPs) in *PTEN* gene among breast cancer Pakistani population. For this purpose, DNA was extracted from blood samples and was electrophoresed on 1% gel, later amplified by polymerase chain reaction. Variations in Exon 4, Exon 5 of the *PTEN* gene were observed by performing single stranded conformational polymorphism(SSCP) followed by Sanger sequencing. BIOEDIT and Mutation Taster were used to analyse the sequencing results by aligning the sequences with reference sequence NG_007466.2 from NCBI. Two novel disease causing mutation were detected in Exon 4 of *PTEN* at chromosome position chr10:89690839_89690839delT and chr10:89690825A>TN/A. Both mutations were non-synonymous, hence might have alteration in PTEN. Such SNPs in tumour suppressor protein due to *PTEN* gene might affect the cell cycle. Based on risk factors, the age groups above 40 years; 58-72 years (OR: 2.8615 [1.5668 to 5.2259] p value = 0.0006) and 73-87 years (OR: 3.4561 [0.9367 to 12.7519] p value = 0.0626) and a positive family history of the breast cancer (OR: 5.7486 [1.9361 to 17.0689] p value = 0.0016) were significantly associated with the risk of breast cancer development. Beside known *BRAC1* and *BRAC2* pathogenic genes, novel SNPs identified in local breast cancer patients indicates that other genes like *PTEN* should be investigated for its role in oncogenesis. Such novel variants are addition to the knowledge of breast cancer as predictive marker to assess the risk of breast cancer development.

# Introduction

### 1.1 Cancer

Cancer is a category of illness involving abnormal cell proliferation with an ability to spread or invade other body regions. Cancer is a leading cause of death globally and has resulted in approximately 10 million deaths (a ratio of 1 in 6) in 2020 (WHO, 2022). The risk of cancer development in males and females at some point in their lives is 20% and 17% respectively (WHO, 2022). Cancer cells develops a neoplasm or tumour, which is characterized as a group of uncontrollably dividing cells, forming a lump or mass of cells. The tumour can be benign or of malignant type (Patel, 2020). Some common types of cancer include lung cancer, prostate cancer, colorectal cancer, stomach cancer, cervical cancer and breast cancer.

#### 1.1.2 General Classification of Cancer

Cancer cells are generally named or classified on the basis of the type of cells where these tumours originate. Table 1.1 shows different categories of cancer.

**Table 1.1** : Different types of cancer on basis of tumour origin adapted from Cancer research UK, (2020)

| Sr. no | Cancer type | Originating site (Type of cell) | Type of cancer |
|---|---|---|---|
| 1. | Germ cell tumour | Pluripotent cells | Testicles ,ovaries (seminoma, dysgerminoma) |
| 2. | Sarcoma | Connective tissue (bone fat, muscles, cartilage, nerve) | Osteosarcomas |
| 3. | Lymphoma | Originates in immune system cells like Hematopoietic cells (blood forming cells) maturing in lymph nodes | Diffuse large B-cell lymphoma |
| 4. | Blastoma | Immature precursor cells/embryonic cells | Hepatoblastoma, Medulloblastoma, Nephroblastoma |
| 5. | Carcinoma | Epithelial cells | Breast, prostate, lung, pancreas, colon cancer |

## 1.2 Breast Cancer

Breast cancer is defined as the uncontrolled cell division in breast tissue. Breast cancer is one of the most prevalent type of cancers and a leading cause of mortality among women (Momenimovahed & Salehiniya , 2019). Breast cancer can originate in any part of the breast and is categorized into different types (WHO, 2021). Breast cancer can be benign or metastatic as it can spread from breast to other body regions through blood or lymph vessels (Bertucci *et al.*, 2019).

### 1.2.1 Global Prevalence of Breast Cancer

Breast cancer is the most common type of cancer among females globally, accounting for 1 in 8 women being at risk of breast cancer diagnosis. In 2020, around 2.3 million new breast cancer cases and 685, 000 deaths of breast cancer patients were reported worldwide (WHO,2021). According to GLOBOCAN (2020) the estimated new cases of breast cancer among females in 2020 worldwide were 2,261,419 (24.5%) The incidence of new cases is higher in developing countries (Francies *et al.*, 2020) and the future burden of breast cancer by year 2040 is estimated to be 3 million (new cases) with a number of deaths up to 1 million (Arnold *et al.*, 2022).

The incidence and mortality rates of breast cancer among females vary among continents. Highest incidence rates of breast cancer were reported in Western Europe and North America whereas the lowest was reported in Africa and Asia. Breast cancer is prevalent in over-populated areas of South Asian low income countries (Akram *et al.*, 2017). Mortality rates are high in most of low income / low-middle income countries ( lacking preventive screening for early detection of disease and the adequate treatment resources) including regions of Africa and Oceania (Lima *et al.*, 2021). Lowest mortality rates were recorded among the Korean women (Lukong *et al.*, 2017). An estimate calculated in 2020, indicated that the incidence rates of breast cancer were highest in Belgium and Netherlands (Global Cancer Observatory ,2022).

According to American Cancer Society (2022), the estimates of breast cancer in USA shows 287,850 new cases for invasive breast cancer, 51,400 new cases of ductal carcinoma *in-situ* (DICS) and 43,250 women deaths from breast cancer. According to these estimates, women with age range of 50 years and older account for more than two-thirds of breast cancer diagnoses among the developed nations accounting for the majority of these cases (Coughlin, 2019). Asia-Pacific is home to over 24% of all breast cancer cases, with China, Japan, and Indonesia having the highest rates. Breast cancer is becoming more common among Asian and American women. Breast cancer

is now considered the main cause of mortality in Asian countries as most of these countries belongs to low - middle income status (Momenimovahed & Salehinya, 2019).

### 1.2.2 Breast Cancer Prevalence in Pakistan

Pakistan, being the 6th most populous Asian country, also has high rate of breast cancer incidence and mortality. The aetiology and biological behaviour of breast cancer is diverse in Pakistani population (Arshad *et al.*, 2019). Being a lower middle class country, Pakistan is facing many plights including financial and health constraints (Khan, 2017). As an alarming rise of breast cancer in Pakistan it is effecting both genders and becoming a serious health burden. According to estimate new cases of breast cancer in Pakistani population were 25,928 (GLOBOCAN, 2020). The diagnosis of cancer particularly breast cancer is delayed due to multiple factors as 60% of patients are diagnosed at an advanced stage of the disease (Soomro *et al.*, 2018). Breast cancer data obtained from Karachi shows that 69.1 per 1 million breast cancer cases were of either stage III or IV (Arshad *et al.*, 2019).

Pakistan is reported with high Age Standardized Mortality Rate(ASMR) among South Asian nations. The primary cause of high ASMR is advanced illness stage, which is due to lack of knowledge and access to care, delay in clinical evaluation, diagnosis and staging, delay in receiving the best possible treatment (Naseem, 2018). According to reports of Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH & RC), Lahore, and Pakistan Atomic Energy Commission Cancer Registry (PAECCR), breast cancer is one of the most prevalent cancer type in Punjab province (Shamsi, 2020).

## 1.3 Breast Anatomy

Human breast are the exocrine ( in females only) glands present on the anterior chest region of the body lying between the region of sternum and the mid axillary line (McGhee & Steele, 2020). Human breast are bilateral organs and its size increases particularly in females at puberty because the epithelial and connective tissue of breast proliferates under the hormonal influence (including progesterone, oestrogen) and development of mammary glands along with an increased deposition of the fatty tissue. The stratified epithelium of the typical breast is made up of two distinct cell groups; myo-epithelial and epithelial cells. The anatomy of breast shows three major regions; the lobules ( the milk producing glands) , ducts (carrying milk from the lobules to the breast nipples) and the connective tissue with a function of holding the breast tissue together and is comprised of fibrous and fatty tissue.

Breast architecture has stratified epithelial cells surrounded by a basement membrane. The general anatomy of the breast shows two major type of tissues including the glandular and stromal supporting tissue (Torre *et al.*, 2016). The milk passages or the ducts and milk producing glands called lobules, are composed of the glandular tissue while the fatty and fibrous tissue of the breast are present in the stromal tissue (Sharma *et al.*, 2010). There are 10-15 lobes present in the breast which further divide into lobules made up of tubule-alveolar glands. The lobes empty into lactiferous ducts, which dilates into lactiferous sinus lying under the areola and further open via a narrow opening into the nipple of the breast. The space between the lobes of breast is filled up by the adipose tissue. The other major structures in the breast incudes the nipple and areola (a circular pigmented area) forming nipple areola complex. At puberty, hormones brings changes in the breast structure including the darkening of the circular pigmented areolar region and elevation of the nipple from the surface. Moreover, the areolar region contain sebaceous and apocrine sweat glands(Figure 1.1). The openings of the Montgomery glands at the periphery of the areola form nodular elevations called the tubercle of Morgagni (Pandya & Moore, 2011). Understanding of breast structure is important as it can help an individual in early detection of the breast cancer via clinical breast examination.

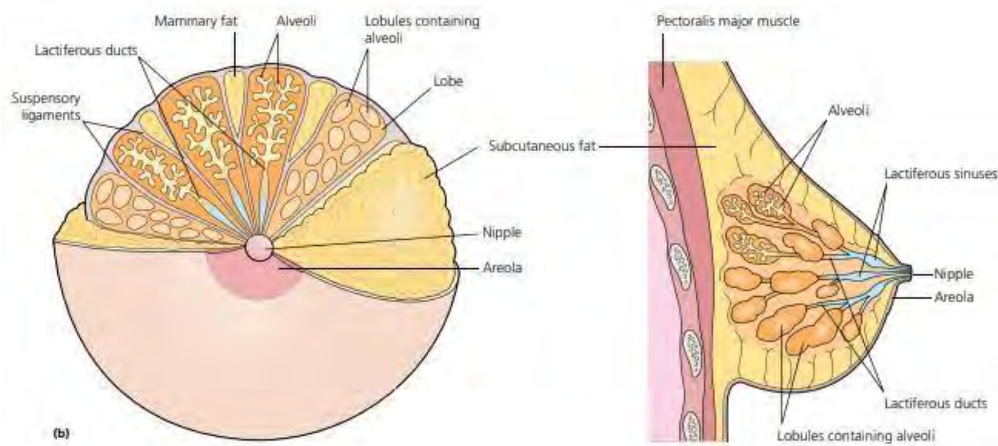


**Figure 1.1** : Anatomy of breast adapted from Bistoni & Farhadi, (2015)

### 1.4 Pathophysiology of Breast Cancer

The normal breast development contributes in the establishment of cellular heterogeneity which can lead to diseases of breast including breast cancer. This breast cancers' heterogeneity can arise from the neoplastic transformation of either myo-epithelial or epithelial cells, or even from the stem cells of breast which can differentiate into different kind of cells (McGhee & Steele, 2020).

Despite presence of suppressor genes neoplastic cells grow and even increase their proliferative ability. Injury to deoxyribonucleic acid (DNA) and hereditary alteration in various genes (*P53, BRCA1 and BRCA2*) are involved in the breast cancer development risk. Mutations in genes (tumour suppressor) are involved in protective pathways for regulating cell growth like RAS/MEK/ERK pathway and PI3K/AKT pathway. In case of these mutated tumour suppressor genes, cells loss control which led to continuous cell division and development of cancer. Lack of an effective immune defence and surveillance is another mechanism for breast cancer development (Akram *et al.*, 2017).

### 1.5 Symptoms of Breast Cancer

The structure of a normal breast varies from person to person. The appearance of the breast also depends upon conditions including monthly period, parity, body weight and various medications. Similarly, the symptoms of breast cancer also vary from individual to individual. These symptoms include the formation of lump in breast or underarm region, swelled breast or a part of breast along with irritation or dimpling of the breast skin, change in nipple structure, pain in nipple region, nipple discharge other than breastmilk (ACA, 2022) like blood discharge or redness in the nipple area, sore breast or armpit region and any marked change of size and shape of the breast (CDC, 2018).

### 1.6 Hormone Receptors on Breast Cells

Breast cells have different receptors which are triggered by hormones, these are oestrogen receptor and progesterone receptor which promote the growth and proliferation of tumour cells (Scabia *et al.*, 2022). These hormone receptors, in case of cancer cells act as molecular markers which help in cancer diagnosis and predicting the response to a certain therapy (Mohanty *et al.*, 2022).

#### 1.6.1 Oestrogen Receptor

Oestrogen, a sex steroid hormone secreted from ovaries, affects the growth and differentiation of mammary glands of breast by binding to the oestrogen receptors (ER) which includes Oestrogen Receptor-α and Oestrogen Receptor-β receptors (Mills *et al.*, 2018). ER mediates the impact of endogenous hormones and therapeutic agents, and also acts as a predictive marker for checking responsiveness of endocrine therapy. ER targets the expression of various signalling elements of the insulin-like growth factor system, promoting the growth of breast cancer cells. In clinical

oncology, analysing the ER-status of tumour samples is a common diagnostic tool (Jafari *et al.*, 2018).

### 1.6.2 Progesterone Receptors

Progesterone receptors (PR), an oestrogen-regulated protein and a highly structured transcription factor, controls a variety of physiological processes in breast cancer cells. Oestrogens is responsible for activation of PR gene (PR-A and PR-B), which are expressed in about one-third of the luminal epithelial cells in a normal breast. PR was the first prognostic and predictive indicator of endocrine therapy response (Horwitz & Sartorius, 2020).

### 1.6.3 Human Epidermal Growth Factor Receptor-2

Human epidermal growth factor receptor-2 (HER2-receptors) is a member of epidermal growth factor receptor family (EGFR). It is composed of four transmembrane receptors that are involved

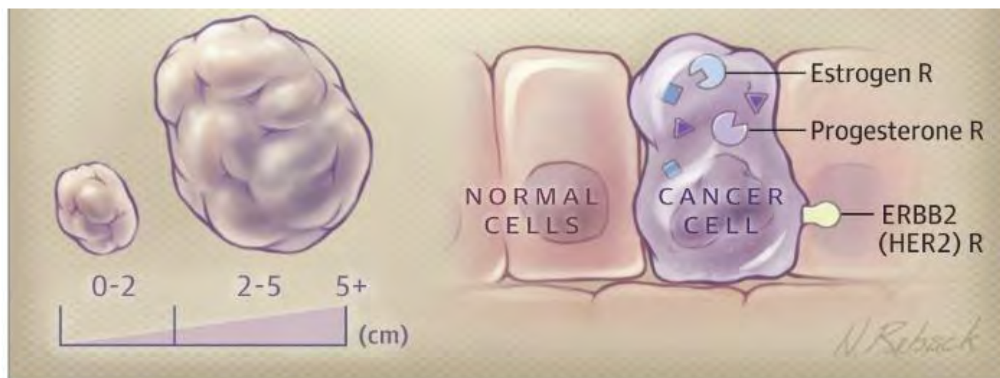


**Figure 1.2** Hormone receptors on breast cells adapted from Waks & Winer, (2019)

in signal transduction pathway which regulate cell growth and differentiation causing the cell proliferation. Approximately in 15-30% of breast cancer patients there is an overexpression of HER2 (Poturnayová *et al.*, 2019) which leads to poor prognosis of the cancer (Kunte *et al.*, 2020). Figure 1.2 shows different types of hormone receptors.

## 1.7 Breast Cancer Classification

Breast cancer refers to different malignancies of the breast or mammary glands among which carcinomas predominates. Other rare malignancies includes sarcomas such as phyllodes tumours

and angiosarcomas (Feng *et al.*, 2018). Breast cancer is divided into different types on the basis of different factors including the anatomical origin of cancer and expression of hormone receptors. Different types of breast cancer are mentioned below:

### 1.7.1 Histological Classification of Breast Cancer

#### 1.7.1.1 Non-Invasive Breast Carcinoma

The non-invasive type of breast cancer, based on the origin of carcinoma is further categorized as ductal carcinoma *in-situ* (DCIS) and lobular carcinoma *in-situ* (LCIS). These types originate in the milk ducts (DCIS) and lobules (LCIS) of breast (Tsuda, 2020). The term *in-situ* refers to the early cancer stage and can be treated but may develop into an invasive form of cancer if left untreated as it may spread to surrounding breast tissues.

#### 1.7.1.2 Invasive Breast Carcinoma

On the basis of anatomical origin breast cancer is classified majorly as invasive ductal carcinoma and invasive lobular carcinoma. Invasive ductal carcinoma originates in ducts of the breast while invasive lobular carcinoma in lobules (Agarwal *et al.*, 2022). It is different from non-invasive carcinoma as it is initiated in the milk ducts and spread to the surrounding breast tissue.

##### 1.7.1.2.1 Invasive Lobular Carcinoma

Invasive lobular carcinoma (ILC) originates in the lobules of the breast and spread from its originating sites to rest of the breast tissues (CDC, 2022). This can spread to other body parts through blood and lymph system. ILC is termed as the special type of breast cancer and possess a different morphology which have monotonous, small, round and dis-cohesive cells gathered into clusters and have an ability to invade other tissues (Chen *et al.*, 2017). This subtype comprises of 15% of all the breast cancer cases. ILC originates from a family of non-obligate precursor lesions termed as lobular neoplasia (LN) and includes atypical lobular hyperplasia (ALH) (Reed *et al.,* 2021). This subtype shows a good response to the endocrine therapy and has good prognostic phenotype. These cancer cells have a low mitotic index and are PR/ER+, HER2- with no p53 or basal marker (Reed *et al.*, 2015). Diagnosis of ILC is complicated as mammography and ultrasound results of ILC patients are ambiguous. ILC in comparison to IDC are larger in size at detection which are prevalent in older patients (Cosar *et al.*, 2023).

##### 1.7.1.2.2 Invasive Ductal Carcinoma

Invasive ductal carcinoma (IDC) is also termed as invasive carcinoma of no special type or infiltrating ductal carcinoma (Cserni, 2020). Such carcinoma accounts for the majority of breast

cancer types and is sub classified on the basis of histological structure of the invasive carcinoma component into; tubule forming type, solid type, scirrhous type, and others (Tsuda, 2020). Invasive ductal carcinoma differs from ILC on the basis of clinic pathological characteristics and respond differently to systemic therapy (Barroso-Sousa & Metzger-Filho, 2016).

### 1.7.2 Molecular Subtypes of Breast Cancer

Hormone receptors positive breast cancers are highly heterogeneous subgroup, which grow slowly as compare to hormone negative breast cancer and have risks of relapse. Based on molecular subtyping, there are various categories which includes Luminal A, luminal B and HER2 enriched subtypes (Pellegrino *et al.*, 2021).

#### 1.7.2.1 Luminal A

Luminal A breast cancer has a good prognosis, slower growth than other malignancies, and is of lower grade subtype. Such tumour types have a low levels of proliferative marker *i.e* expression of Ki-67 expression (≤ 14%). Luminal A is ER/PR + and HER2 - (Gao & Swain, 2018).

#### 1.7.2.2 Luminal B

Luminal B type is further categorized into two sub-types based on expression of HER2. Luminal B have one subtype, which is ER+, PR+/- and HER2-, with high expression of Ki67 ≥ 14 %. A Luminal B like subtype has ER+, PR+/-and HER2+ with varied Ki-67 expression. (He *et al.*, 2019). This Luminal B subtype is comparatively more aggressive subtype than Luminal A and shows a poor response to hormone therapy (Pellegrino *et al.*, 2021).

#### 1.7.2.3 HER-2 Enriched Breast Cancer

HER-2 enriched sub type are both ER and PR negative. This type has a worse prognosis as compared to luminal subtypes but fortunately a targeted therapy is available for this subtype (Swain *et al.*, 2023).

#### 1.7.2.4 Triple Negative or Basal like Breast Cancer

In Triple negative or Basal like breast cancer (TNBC) cancer, the tumour cells lack ER and PR and either lack HER2 protein or has a low expression. The ER and PR in breast tissue are responsible for the enhancement of cancer progression by binding to the respective hormones (Yin *et al.*, 2020). TNBC is an aggressive type of breast cancer as it possess a higher early recurrence rate, greater metastatic potential to other organs (liver ,CNS) and a poor prognosis with availability of limited treatment options (Garrido-Castro *et al.*, 2019). Patients diagnosed with TNBC have a low survival rate. Molecular targeted therapy or the endocrine therapy are not

effective against TNBC owing to its special molecular phenotype being ER-, PR-, HER2 - (Yin *et al.*, 2020). TNBC shows diverse clinical outcomes due to tumour heterogeneity and ultimately shows a varied responses to traditional and new targeted therapies (da Silva *et al.*, 2020).

### 1.7.3 Inflammatory Breast Cancer

The blockage of the lymph vessels in the breast skin leads to development of inflammatory breast cancer and the breast looks swollen (edema) and red (erythema) along with appearance of ridges skin. Cancer cells result in blockade of lymph vessels and prevent a normal flow of lymph through the breast tissue. Most of the inflammatory breast cancer are categorized as invasive carcinomas in nature and the cancer majorly originates in the cells, making up the lining of milk ducts which can further spread beyond the ducts (NIH, 2016).

### 1.7.4 Other Rare Types of Breast Cancer

Paget's disease of the nipple and phylloides tumour are some of the rare forms of breast cancer. Paget's disease of nipple originates in milk ducts, spread to the areolar and nipple skin of the breast whereas the phylloides tumour develop in the connective tissue of the breast and can be removed surgically (Sharma *et al.*, 2010).

## 1.8 Stages of Breast Cancer

Breast cancer stages are categorized on the basis of size of tumour and the extent of the spread or penetration of tumour. Tumour node metastasis (TNM) staging of breast cancer is assigned by American Joint Committee on cancer (AJCC,2022) and is based on size of tumour, presence of tumour in lymph nodes, or either the metastatic nature of tumour.

A broader staging system categorize breast cancer into stage 0 to IV in which the non-invasive cancers (confined to the site of origin) are categorized as stage 0 and the invasive breast cancers are categorized from stage I- IV (Akram *et al.*, 2017). The extensive breast cancer staging system includes the following stages (Polo, 2022).

### 1.8.1 Stage 0

Stage 0 includes non-invasive breast cancer. Ductal carcinoma *in-situ* is categorized as stage 0 cancer where cancer cells are confined to the site of origin and do not spread to the surrounding tissues.

### 1.8.2 Stage I

Stage I includes invasive breast cancer as the cancer cells possess micro invasion property. Stage I is further categorized into two stages; IA and IB. In stage IA, tumour remains confined to the

breast and do not spread to lymph nodes. The tumour approximately measures up to 2cm. Moreover the cancer cells with hormone receptors ER+/PR+ and HER2 + status and tumour size of 2-5cm that can spread to axillary lymph nodes, is categorized in stage I cancer. On the other hand, small groups of cancer cells in lymph nodes or small tumours less than 2mm in size are categorized in stage IB.

### 1.8.2 Stage II

It is the invasive form of cancer having tumour size greater than 2mm. It is further subcategorized as follows. Stage IIA tumours are small in size. The condition of not having tumour in breast but in one of the three axillary lymph nodes lymph nodes under arm, lymph nodes near breast bone) is categorized as stage IIA. The tumours ≤ 2cm, metastasized to axillary lymph nodes or the tumour in between 2-5cm but not spread to axillary lymph nodes are also included in stage IIA. Stage IIB have tumour size comparatively bigger than that of stage IIA which can be of the size of a walnut or lime (ranging in between 2-5cm). The small groups of tumour (0.2mm-2mm) found in axillary lymph nodes or in lymph nodes present near breast bone are also categorized as stage IIB.

### 1.8.3 Stage III

Stage III cancer is harder to fight but the cancers cells do not spread to the bones or organs. In stage IIIA the tumour can occur in nine lymph nodes (in region of underarms to the collarbone) forming a chain. Stage III tumour can also spread to lymph nodes present deep in the breast. Stage IIIB tumour spread to skin around the breast or to the chest wall or may spreads to lymph nodes. If the cancer cells spread to greater number of lymph nodes or spread to areas below the collarbone, then it is categorized as stage IIIC.

### 1.8.4 Stage IV

In the stage IV, breast cancer cells spread to distant areas including lungs, distant lymph nodes, bones, brain or liver.

## 1.9 Comorbidities and Breast Cancer

Breast cancer patients (approximately 20–35%) have chances of developing chronic comorbidities. The prevalence of such conditions in patients of age ranging 65 years or above is approximately 86%. Such comorbid conditions include dementia and pulmonary disease (Wu *et al.*, 2019). Chemotherapy used in breast cancer patients effects patient's health negatively and results in development of various comorbid conditions like renal failure (Tanveer *et al.*, 2019). The risk of development of recurrent metastatic disease is higher in post-menopausal women having multiple

comorbidities. Over 10% of breast cancer patients have obesity and other metabolic comorbidities, which are strongly associated with negative outcomes (Anwar *et al.*, 2021).

### 1.10 Genetic Risk Factor of Breast Cancer

Genetic predisposition is a major factor of breast cancer (Momenimovahed & Salehinya, 2019). Over an average 5-10% of the breast cancer development is associated with the genetic mutations either inherited or sporadic (Coughlin, 2019). The high penetrant genes and their mutations, which have been associated with increased risk of breast cancer development are *BRAC1* and *BRCA2* genes. Mutations in other genes including *ATM, TP53, CHEK2, PTEN, CDH1, STK11*and *PALB2* can also leads to breast cancer development. Inheritance of such gene mutations increases the risk of cancer development in individuals. Some of these genes are mentioned in Table 1.2.

**Table 1.2:** Various genes contributing to breast cancer development adapted from Feng *et al.*, (2018)

| Sr. no | Gene | Contributing toward breast cancer development |
|---|---|---|
| 1. | ***ATM*** | Two abnormal copies of *ATM* gene results in development of ataxia-telangiectasia disease. |
| 2. | ***TP53*** | Development of Li-Fraumeni syndrome by the inherited mutations of this gene which increases risk of cancer including breast cancer. |
| 3. | ***CHEK2*** | Mutation in this gene amplify the risk of breast cancer about 2-fold |
| 4. | ***CDH1*** | Inherited mutations of *CDH1* majorly results in hereditary diffuse gastric cancer causing an increased risk of invasive lobular breast cancer development in inherited individuals. |
| 5. | ***STK11*** | Mutations in this gene results in development of Peutz-Jeghers syndrome putting the individual at a higher risk of many types of cancer, including breast cancer |
| 6. | ***PALB2*** | *A protein of PALB2* gene after interacting with a protein subunit of BRCA2 , resulting in this gene to mutate and causing a higher risk of breast cancer development. |
| 7. | ***PTEN*** | Any Inherited mutations in this gene can result in development of Cowden syndrome which is associated with an increased risk for tumour development (non-cancerous and cancerous ) in the breasts. |

Mutations in various genes including oncogenes and tumour suppressor genes lead to cancer development. Among tumour suppressor genes , variants of *PTEN* are also reported to be involved in a variety of tumour types, primarily glioblastoma, endometrial, and prostate cancer, and to a lesser extent in breast, lung, and colon(Vidotto *et al.*, 2020) .Understanding of the role of the *PTEN* gene in the development of cancer has been a topic of extensive research. Numerous studies have shown that alterations in the *PTEN* gene plays a significant role in the initiation and progression of cancer. These alterations can occur through various mechanisms, such as gene mutations or epigenetic modifications, which results in either loss of PTEN function or altered expression (Christian *et al.*, 2022).

Breast cancer is a multifactorial condition that is influenced by a variety of genetic and environmental factors in distinct ways (Faramarzi *et al.*, 2021). Abnormal amplification and mutations in both oncogenes and tumour suppressor genes are major causes of breast cancer emergence. Breast cancer incidence varies among low to high income countries. There is a high prevalence of disease and low rates of early diagnosis of breast cancer among Pakistani population. Few studies have examined the variants of *PTEN* gene contribution towards the risk breast cancer among Pakistani population. Still, there is further need to investigate the mutations or single nucleotide polymorphisms in *PTEN* gene for breast cancer development, considering the high burden of breast cancer in Pakistan and significance of *PTEN* gene. This study was designed for the purpose to detect genetic variants among *PTEN* gene in breast cancer patients and healthy individuals in Pakistani population.

## Aim and Objectives

The aim of the current case control study was to detect mutations and single nucleotide polymorphism (SNPs) in *PTEN* gene among breast cancer patients in Pakistani population.

The objectives of the study were:

- Collection of blood samples of breast cancer patients and healthy individuals from Pakistan Institute of Medical Sciences (PIMS) and Swat Institute of Nuclear Medicine Oncology and Radiology (SENIOR) hospital Swat.
- Collection of demographic and clinical data of both breast cancer patients and healthy individuals through designed structured questionnaire.
- To determine prevalence of distinct types of breast cancer among study individuals (patients) and their association with multiple risk factors of disease.
- Determining mutations or SNPs in *PTEN* gene among study individuals and their association with breast cancer susceptibility.

# LITERATURE REVIEW

## 2.1 Risk factors for Development of Breast Cancer

A number of factors contributes towards development of breast cancer among individuals. These factors include the non- modifiable factors like age gender, race, and the modifiable factors including diet, physical inactivity, lifestyle factors, hormonal and female reproductive factors (Momenimovahed & Salehiniya, 2019). Approximately 5-10 % of breast cancer is caused by the inherited genes (Islami *et al.*, 2018). Some important risk factors for breast cancer are mentioned below:

### 2.1.1 Gender and Age

A major uncontrollable risk factor for the development of breast cancer is being a woman. Breast cancer is more common among females than males. Age is another factor contributing towards an increased risk of breast cancer. Women of older age of above 55 years are at higher risk of breast cancer development (Feng *et al.*, 2018).

### 2.1.2 Reproductive Factors

Reproductive factors of an individual particularly females contribute towards breast cancer development. Conditions like early menarche, late menopause (Thakur *et al.*, 2017) or late age for first pregnancy can increase breast cancer risk (Ozsoy *et al.*, 2017). Delay in menopause increases the breast cancer risk by 3% and an year delay in menarche decreases the risk of breast cancer by 5% (Sun *et al.*, 2017).

### 2.1.3 Lifestyle Factors

Various lifestyle factors including, diet, being overweight, lack of physical activity, consumption of alcohol and caffeine, smoking, and increased sleep duration are linked with breast cancer. Obese women have excessive body fats and oestrogen is produced by fat cells. After menopause, ovaries stop synthesizing oestrogen. In elder obese women, due to more body fat there is more oestrogen production, which increases the breast cancer risk among such women. Higher blood insulin levels due to excessive body fats also increases breast cancer risk. An increased physical activity is linked with normal weight maintenance thus, decreases the risk of breast cancer development in women. Women with a longer sleep duration are at more risk of developing breast cancer (Feng *et al.*, 2018). Alcohol consumption increases the level of oestrogen like hormones in blood and result in an increased risk for breast cancer (Naeem *et al.*, 2019).

### 2.1.4 Obesity

Breast cancer is associated with different anthropometric factors that includes body weight, height and amount of body fats (adiposity). The generation of oestrogen from androgen in adipose tissue uses body fat as a substrate. Due to these facts, breast cancer is linked to obesity. The risk of hyperinsulinemia and insulin resistance is significantly influenced by obesity and physical inactivity. Thus, insulin resistance and hyperinsulinemia are risk factors for breast cancer too (Coughlin, 2019) .

### 2.1.5 Hormonal Factors

In females the intake of hormonal contraceptives like oral-contraceptives increases the risk of breast cancer development. Hormonal contraceptives pills contain combined oestrogen and progestogen, while more advanced contraceptives like some oral contraceptives have progestogen as a major component with a reduced oestrogen level. These hormonal drugs have biological impact on the breast epithelium of women (Zolfaroli *et al.*, 2018). The use of other hormonal drugs by females, like ovulation-stimulation drugs or getting a postmenopausal therapy in older age also make such females at a greater risk of developing breast cancer (Taheripanah *et al.*, 2018).

### 2.1.6 Socioeconomic Factors

Some important socioeconomic variables like educational level and the employment status affect the incidence of breast cancer development in individuals. Individuals having low or middle income status makes a major portion of cancer associated deaths worldwide as they lack access to essential palliative care (Stoltenberg *et al.*, 2020). Women with low socioeconomic status are at higher risk of developing breast cancer (Coughlin, 2019). Women with a high social status have more income and spend money on medical care or use health insurance more profoundly. Whereas those having a low income or social status due to use unbalanced vitamins and higher intake of fats are at risk (Momenimovahed & Salehiniya, 2019).

### 2.1.7 Family History

In women having a first-degree relative diagnosed with breast cancer, the risk of breast cancer development is double (Sun *et al.*, 2017). Women with mothers having breast cancer have significantly high levels of progesterone and oestrogen in them and thus, are more prone to risk of breast cancer development (Trabert *et al.*, 2020). According to an estimate, a quarter of all breast cancer cases have a strong family history of breast cancer (Brewer *et al.*, 2017). Women with

unilateral breast cancer are at a risk of developing cancer in other breast as well (Ozsoy *et al.*, 2017).

### 2.1.8 Diabetes and Dyslipidaemia

Diabetic women, approximately 20 % are more prone to breast cancer development then non-diabetic women (Bronsveld *et al.*, 2017). Postmenopausal elder women with a high body mass index and hyperglycaemic index are more prone to cancer. Cancer cells uptake more glucose as they rely on aerobic glycolysis because of their altered metabolism for energy generation (Kang *et al.*, 2018).

In dyslipidaemia condition , there is an imbalance of lipids that develops in obese and type 2 diabetic patients (Zhao *et al.*, 2020). Cholesterol, being an important component of cellular membrane is required in large amount by the rapidly dividing cancer cells. Elevated cholesterol in such patients increases the risk of breast cancer development because an excessive cholesterol promotes an increased tumour cells proliferation (Kang *et al.*, 2018).

### 2.1.9 Mutations in Tumour Suppressor Genes

Beside these factor, many genetic factors are also involved in cancer formation. Tumour suppressor genes are one such genetic player as these genes have crucial role in regulating cell growth and preventing the formation of cancerous cells. When these genes are functioning properly, they help in controlled cell division, repair damaged DNA, and promote cell death when necessary (Kamel *et al.*, 2019). However, when tumour suppressor genes undergo mutations or are inactivated, they are unable to perform their normal functions, which can lead to the initiation and progression of cancer.

*P53,* a tumour suppressor gene is a vital gene in preventing cancer initiation and growth. Along with cell cycle regulation. *P53* is involved in apoptosis induction, a process of programmed cell death that eliminates damaged or abnormal cells. Furthermore, *P53* is responsible for DNA repair, ensuring that damaged DNA is fixed before it can lead to the formation of cancerous cells (Boutelle & Attardi, 2021). *BRCA1*, is another tumour suppressor gene, which is associated with breast and ovarian cancers as it is involved in repairing damaged DNA and maintaining the stability of the genome (Li & Engebrecht, 2021). *P16* is involved in controlling the cell cycle by inhibiting the activity of cyclin-dependent kinases. Along with some well-known and extensively studied genes, there are many less common tumour suppressor genes like *PTEN* (Phosphatase and Tensin

Homolog), *STK11* (Serine/Threonine Kinase 11), *NF2* (Neurofibromin 2), *APC* (Adenomatous Polyposis Coli), *CHEK2* (Checkpoint Kinase 2) *etc.* which contribute to maintaining cellular homeostasis and preventing cancer (Feng *et al.*, 2018).

## 2.2 Phosphatase and Tensin Homolog

Phosphatase and Tensin homolog (PTEN) is a type of interfacial enzyme also called as hooping enzyme due to its functioning at the interface of aqueous and membrane phase of the cells (Csolle *et al.*, 2020). It acts as a negative regulatory protein that is involved in tumour suppression owing to its lipid phosphatase-dependent or scaffold-dependent properties (Lee *et al.*, 2018). PTEN has dual phosphatase activity as it act on both lipid and proteins. PTEN acts as a growth survival and regulatory protein due to its ability to act on Phosphatidylinositol 3-phosphate ($PIP_3$). Along with its tumour suppresser activity, it functions as a metabolic regulator of both lipids and glucose as well as regulates mitochondrial functioning (Chen *et al.*, 2018). *PTEN* is encoded on chromosome number 10q23 and is comprised of 9 exons. The protein acts as a phosphatase and dephosphorylate phospho-peptides and phospholipids. The protein is comprised of 2 major parts with a C- tail. i) N-terminal domain or catalytic domain, ii) C-terminal domain, iii)C-terminal tail is made up of 50 amino acids long chain having a PDZ motif (important for the protein- protein interaction) and casein kinase II (CK2) site for phosphorylation. The N terminal phosphatase domain and C2 domain make a smaller unit of the protein having the enzymatic function while the C terminal tail has a role in PTEN regulation (Masson & Williams, 2020)

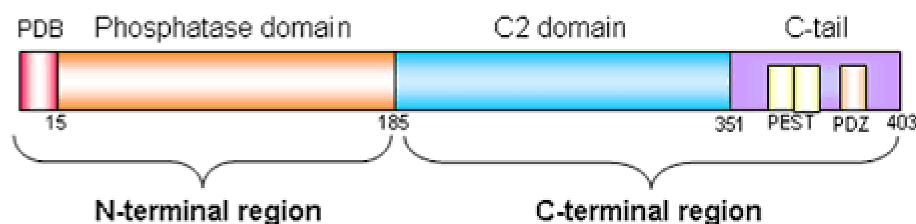


**Figure 2.1 :** Structure of PTEN protein adapted from Molinari & Frattini, (2014)

### 2.2.3 Domains of PTEN Protein

PTEN protein has a multiple domain structure and is comprised of five functional domains including; N-terminal domain (phosphatidylinositol (4,5) P2-binding domain), phosphatase

domain, C2 lipid/membrane-binding domain, a C-terminal tail containing Pro, Glu, Ser and Thr (PEST) sequences and a PDZ-binding (PDZ-BD) motif (Lee *et al.*, 2018).

The catalytic domain or phosphatase domain is homologous to auxilin and tensin and structurally wide in order to incorporate large substrates. This domain is comprised of five-stranded β-sheet which are flanked by two α-helices on one side and four helices on the other side. The active site of this domain binds with phosphoinositide as a substrate. Active site of PTEN protein is composed of three loops including WPD loop, T1 loop, and P loop generating a deep, wide and a positively charged pocket for the binding of the substrate. The protein structure possess a signature motif His-Cys-X-X-Gly-X-X-Arg (where X is any amino acid) in the active sites of dual specificity protein phosphatases (DSPs) and protein tyrosine phosphatases (PTPs) (Hopkins *et al.*, 2014). An arginine loop is also present in the phosphatase domain forming a positively charged patch on the domain which is important for its membrane binding function (Irvine *et al.*, 2019).

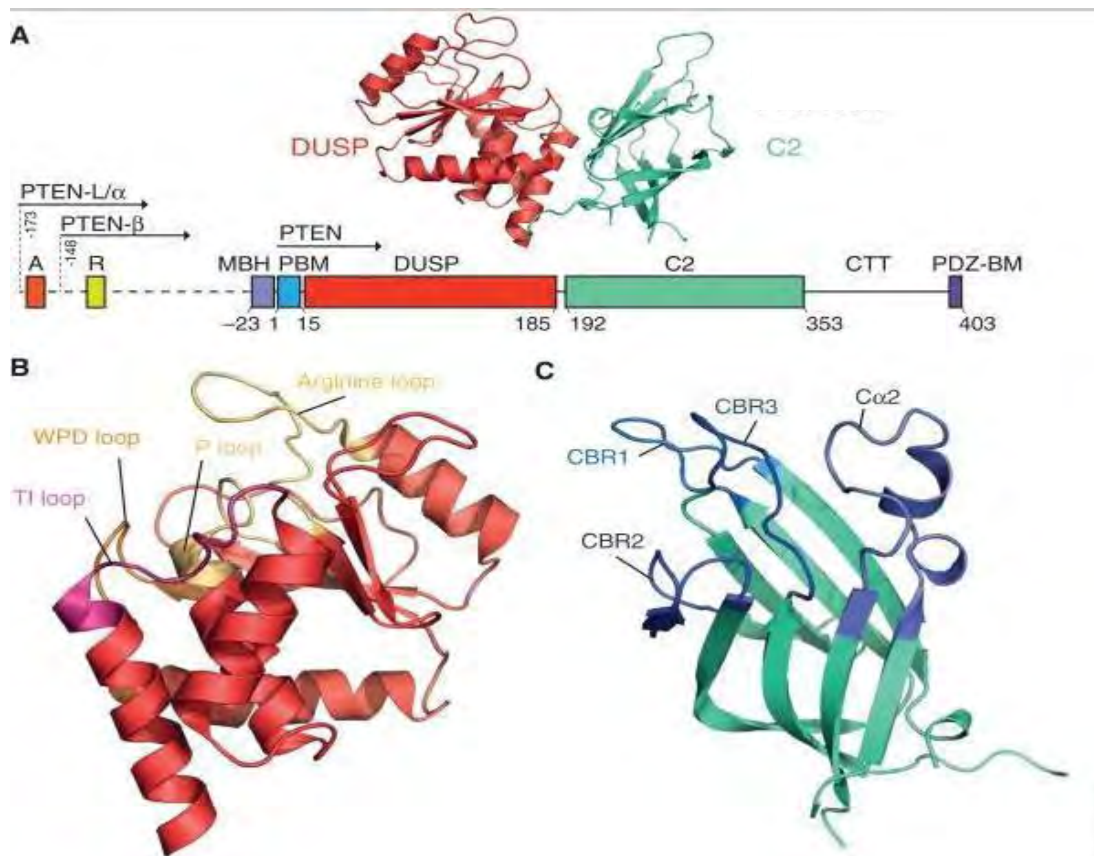


**Figure 2.2:** A detailed structure of various domains of PTEN protein adapted from Masson & Williams, (2020)

The $C_2$ domain is part of C- terminal domain which binds the protein with the phospholipid membrane. This domain is devoid of canonical $Ca^{2+}$ chelating residues and binds independent of calcium ions to the phospholipid membrane. The domain has loops having two antiparallel β-sheets and two small α-helical segments which connects the β-strands (Smith *et al.*, 2019). The association of C2 and phosphatase domain is assisted by the inter domain regions (Gericke *et al.*, 2006) of the PTEN and are important for the phosphatase activity of the protein (Brito *et al.,* 2015). The carboxyl terminal domain of PTEN regulates it. Two PEST sequences (Pro-Glu-Ser-Thr ) also called as degradation signals, are present in the tail region of PTEN. PDZ domain-binding motif (PDZ-BM) is comprised of Thr-Val-Lys residues and associates with second PDZ domain of specific scaffolding protein including S-SCAM/MAGI-2 and MAGI-3 resulting in enhancement of inhibitory effect of PTEN signalling (Chu & Tarnawski, 2004). A flexible loop of 166 residues comprising of some basic subdomains of protein (common to other signalling molecules) connect the C-terminal and N-terminal domain (Sharma, 2022).

PTEN has specificity for the phosphate group of phosphatidylinositol (3,4,5) triphosphate and thus, can dephosphorylate $PIP_3$, which further activates downstream effectors including the AKT/Protein Kinase B (PKB) pathway. AKT pathway has multiple roles including anti-apoptotic roles and growth stimulation. The PI3K phosphorylates $PIP_2$ at its OH group of third position of the inositol ring, converting it into $PIP_3$. The amino terminal of PTEN, which is 190 amino acids long region and is comprised of signature motif, having sequence homology with actin filament capping protein tensin and auxilin ( both proteins are involved in uncapping of calthrin coated vesicles).

## 2.3 Function of PTEN

*PTEN* is a tumour suppressor gene and its translated form performs a phosphatase activity regulating cell cycle and thus, preventing rapid growth and division of cells. *PTEN* gene translate into a dual protein-phosphatase activity. It acts on different substrates including phosphor-serine/threonine and phospho-tyrosine (Luongo *et al.*, 2019). It modulates various cellular functions including cell migration, invasion, cell survival and cell arrest by regulating the activity of a number of target proteins present in cell. These target proteins include glycogen synthase kinase-3 (GSK3), BCL2 associated agonist of cell death (BAD), Caspase 9, IκB, focal adhesion kinase 1, cAMP-responsive element-binding protein 1 (CREB1), insulin receptor substrate 1 (IRS1) and proto-oncogene tyrosine-protein kinase SRC (Lee *et al.*, 2018). Translocation of PTEN

into nucleus also occurs in various cells and in nucleus PTEN influences a number of cellular functions which involves genomic stability, pre-mRNA alternative splicing, apoptosis, cell cycle arrest and senescence. Other functions performed by PTEN involves inhibition of ribosomal biogenesis (in nucleolus), regulation of mitochondrial function and energy production. According to studies, PTEN act at various levels on cancer cells, stromal compartments and on immune response of an individual. It is also involved in modulation of the tumour microenvironment, which in turn control disease initiation, progression and its metastases (Luongo *et al.*, 2019).

## 2.4 Regulation of PTEN

The expression of *PTEN* is regulated at both transcriptional and post transcriptional level. Epigenetic inhibitory mechanisms (hyper-methylation of *PTEN* promoter and histone acetylation), various transcription factors and some micro RNAs (miRNAs acts as *PTEN* suppressors) are found to be involved in *PTEN* regulation (Luongo *et al.*, 2019).

PTEN regulation at post translational level includes ubiquitination of lysine residues of PDB and C2 domain along with oxidation and SUMOylation in C2 regions. Also acetylation event occurring on phosphatase and PDZ-binding domains, Phosphorylation of some serine and threonine residues present in the C2 domain and C-tail region are involved in *PTEN* regulation (Figure 2.3).

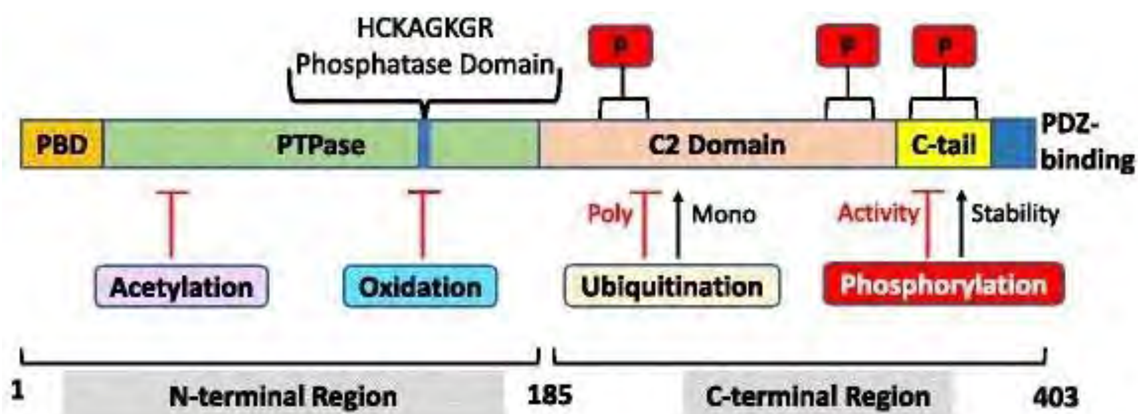


**Figure 2.3** : Post translational modification sites on PTEN adapted from Haddadi et al., (2018)

## 2.5 PTEN Signalling Pathways

PTEN signalling network is linked with other tumour suppressor and oncogenic signalling pathways (Nathan *et al.*, 2017), signals from growth factor receptors present on the plasma membrane and transcription factors that act in the nucleus(Keniry & Parsons, 2008). Cellular

levels of phosphatidylinositol-3,4,5-trisphosphate is negatively regulated by PTEN and it also acts negatively on AKT/PKB signalling pathway and receptor tyrosine kinase signalling pathway (NCBI, 2022).

### 2.5.1 Receptor Tyrosine Kinase pathway

Receptor Tyrosine Kinase (RTK) pathway, a signal transduction pathway is a crucial pathway in cancer development as this pathway is important in various cellular processes including cell division, cell growth, survival and angiogenesis(Figure 2.4). RTKs present in plasma membrane, having an inherent phospho-tyrosine kinase activity are activated by the binding of ligand to it, leading to activation of downstream signalling molecules. Mitogen-activated protein kinase (MAPK), Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) and phosphoinositide 3-kinase (PI3K)/Akt, are important pathways activated by RTKs (Butti *et al.*, 2018). PTEN in normal cells, inhibits mTOR and RTK signalling but in some breast cancer cells mutated PTEN fails to regulate RTK/mTOR pathway and thus, halting the ability of the cancer cells to destroy themselves (Rahimi *et al.*, 2022).

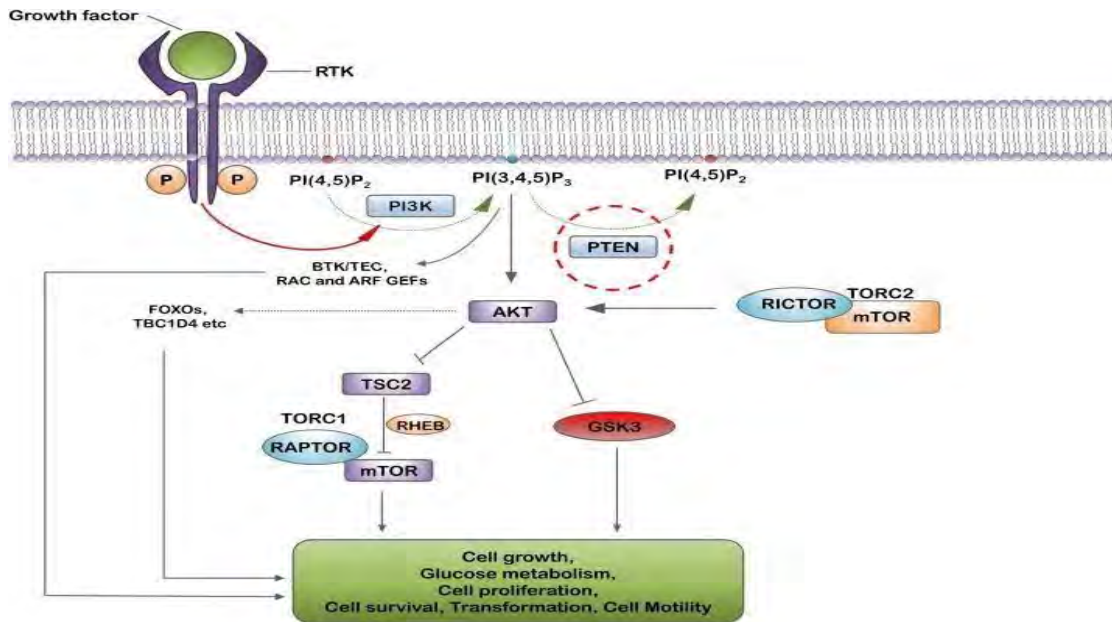


**Figure 2.4:** Receptor tyrosine kinase pathway involving PTEN as effectors molecule for downstream signalling adapted from Álvarez-Garcia *et al.*, (2019)

### 2.5.2 PI3K/AKT/mTOR Signalling Pathway

A number of cellular pathways associated with cell growth and survival are triggered by PI3-kinase and PTEN works antagonistically to Phosphatidylinositol 3- kinase (PI3K)/in order to generate cellular arrest signals (Chen *et al.*, 2018). PI3K/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signalling pathway is important for various cellular activities important for cell proliferation, survival, invasion, migration, differentiation, apoptosis, DNA repair and glucose metabolism (Lim *et al.*, 2014). Ligand specific receptors present on cell membrane after binding to extracellular ligands results in PI3K activation, followed by phosphorylation of Phosphatidylinositol 4,5-bisphosphate (PIP2) at 3 position of its inositol ring forming Phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Two downstream proteins; including AKT (protein kinase B/ PKB), a serine/threonine kinase and PDK1 (phosphoinositide-dependent protein kinase 1), are recruited to plasma membrane by binding of activated PIP3 to the pleckstrin homology (PH) domain of these proteins (Dahia, 2000). Recruitment of AKT to plasma membrane, activates another protein complex *i.e* mTOR complex 2 to the plasma membrane which phosphorylates the AKT protein on its serr473 position. Phosphorylation of AKT by mTORC changes its conformation allowing it to be phosphorylated by PDK1 at Thr308 position. Activated or phosphorylated AKT phosphorylates other target proteins in plasma membrane and in cytosol in order to perform the targeted functions, Figure 2.5 shows the PI3K/AKT/mTOR pathway.

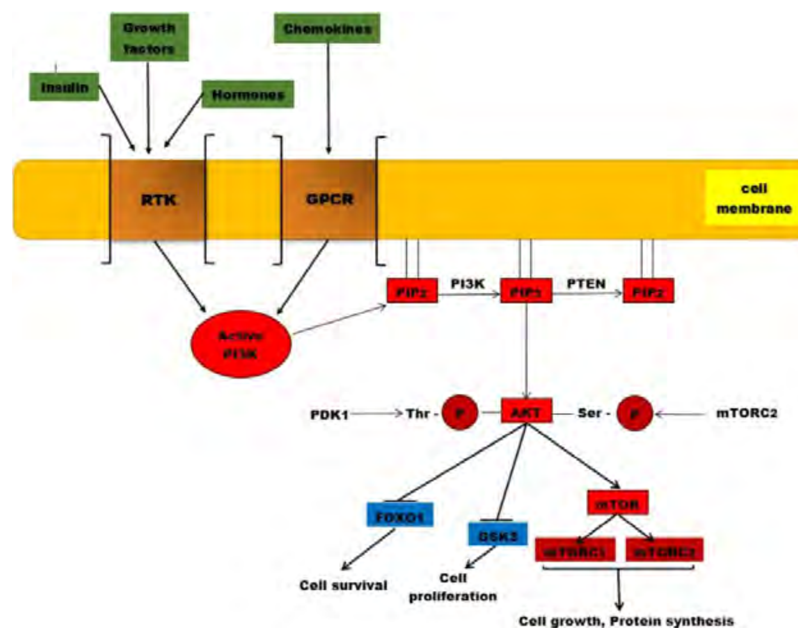


**Figure 2.5 :** PI3K/AKT/mTOR pathway adapted from Miricescu *et al.*, (2020)

## 2.6 Role of *PTEN* Mutations in Development of Various Cancers

The loss of *PTEN* function can result in the dysregulation of various signalling pathways involved in cell growth, survival, and proliferation. This dysregulation can promote the unchecked growth and division of cancer cells, ultimately leading to the development and progression of cancer(Kazim *et al.*, 2019). Moreover, Germline mutations in *PTEN* causes development of various malignancies including those of breast, thyroid, renal and also neurodevelopmental disorders when function of PTEN is altered (Ngeow *et al.*, 2017).

*PTEN* hamartoma tumour syndrome (PHTS) is a hereditary cancer predisposition syndrome occurring as a result of mutation in *PTEN* gene. PHTS includes Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba Syndrome, Proteus syndrome, and Proteus like syndrome. The disease is inherited in an autosomal dominant pattern and patients with these syndromes develop benign hamartomas in various organs. Individuals with PHTs have a greater risk of developing cancers including thyroid and breast cancer (Hopkins *et al.*, 2014). The mutation in *PTEN* gene results in development of benign tumours as cells lost control over cell division and growth (Pilarski, 2019). The most common malignancy associated with CS is breast cancer (Brewer *et al.*, 2022). Furthermore, *PTEN* gene mutations have been found in a significant percentage among breast cancer cases, with approximately 60-80% of CS and Bannayan-Riley-Ruvalcaba Syndrome cases (Liu *et al.*, 2015).

*PTEN* gene tend to have a fairly even distribution of somatic inactivating mutations, which is a characteristic of many tumour suppressor genes. The codons that encode arginine residues at position 130, 173, and 233, however, are reported with a significant number of mutations. The *PTEN* coding sequence has undergone numerous genetic changes, such as deletions, insertions, and frameshift, nonsense, and missense mutations.(Álvarez-Garcia *et al.*, 2019). The frequency of mutations in the exon 9 region, which codes for the *PTEN* protein's C-terminal tail, is lower than in other regions necessary for its catalytic activity. Additionally, these C-terminal mutations are more likely to only affect the protein's stability and post-translational regulation rather than result in a total loss of function (Mingo *et al.*, 2019).

In exons 5, 7, and 8, three typical nonsense mutations (R130X, R233X, and R335X) have been thoroughly studied. It has been demonstrated that some pathogenic promoter mutations alter the

RNA secondary structure, which in turn affects translation and transcription of the *PTEN* gene. Exon skipping, alternative splicing, or the use of cryptic splice sites can be caused by specific pathogenic *PTEN* intronic variants (Yehia *et al.*, 2020). According to literature, the loss of single *PTEN* allele is enough to initiate tumorigenesis (Berger *et al.*, 2011). PTEN-long (PTEN-L), a PTEN variant, is discovered recently and is generated due to the presence of a different translation initiation site resulting in formation of a 567 amino acid protein instead of normal 403 amino acid protein (Ferri *et al.*, 2019). According to a study, more of the *PTEN* mutations are present in exon 5,7 and 8. In patients with Cowden syndrome, the *PTEN* mutations (approximately 40%) are localized in exon 5 which encodes majorly the phosphatase-coding domain of the protein (Yang *et al.*, 2010).

**2.9 Single Nucleotide Polymorphism**

SNPs are the most common type of genetic variation in humans in which there is a change of single nucleotide in the genetic sequence. This type of variation may occur at various locations in a gene including promoter region, exons, introns and the 5’ and 3’ UTR regions. The effect of the SNPs on any disease condition depends on the position of variation present in gene. SNPs at promoter region effects the promoter activity and the transcription factor binding sites. It causes methylation of DNA and has role in histone modification. SNPs in intronic region causes generation of variants of transcripts which effects the further transcription and translation processes. SNPs in 5’UTR and 3’UTR affects the translation and miRNA binding respectively. SNPs in regions far from the actual gene affects the transcription of gene through long-range cis effect.

Numerous SNPs in *PTEN* gene have been linked to a number of tumour types, including oesophageal squamous cell carcinoma, breast cancer, prostate cancer, and endometrial cancer. Various mutations are reported in multiple exons of *PTEN* resulting in loss of its function. According to a study on cervical cancer risk in Chinese women, it was seen that an increased risk was due to SNP (rs34140758) present in 3′UTR of the *PTEN* gene (Yu *et al.*, 2020). In Argentine population, the allele A of *PTEN*-L rs1257378 is a risk factor for the emergence of chronic myeloid leukaemia (Ferri *et al.*, 2019). A *PTEN* c. 697C>T (R233*) SNP in exon 7 results in addition of a premature stop codon, which effects the C2 domain part of the protein and this mutation is found to be associated with breast cancer (https://www.mycancergenome.org/content/disease/).

## 2.10 Role of *PTEN* mutations in Breast Cancer

Breast cancer is the most prevalent malignancy with a high mortality rate among women. For the cell cycle and the preservation of genome integrity, tumour suppressor genes like *PTEN* are crucial. Germline mutations in *PTEN* results in development of rare autosomal syndromes (Dahia, 2000) known as *PTEN* hamartoma tumour syndromes (PHTS) and Cowden syndrome (an autosomal dominant disorder) which increases the risk of development of specific malignancies like breast cancer (Luongo *et al.*, 2019). In a number of malignant tumours including breast cancer, the abnormal expression of *PTEN,* due to mutation in *PTEN* gene alters, its ability to exert a negative regulatory effect on PIP3/AKT mTOR pathway, which leads to an increased activation of AKT and PI3K/AKT/mTOR signalling pathway. PI3K/AKT/mTOR signalling pathway is involved in growth, proliferation, survival, motility, metabolism, and immune response regulation (Ortega *et al.*, 2020) . Mutations in *PTEN* effects its tumour suppressor function, thus promoting enhanced cellular proliferation (Miricescu *et al.*, 2020). Moreover, an uncontrolled transduction of PI3K signal causes an increased activation of downstream signalling molecules such as AKT. These enhanced downstream signalling molecules further regulates cytosolic and nuclear targets including GSK3β, PRAS40, FOXO, mTORC1 and p27 (Csolle *et al.*, 2020) contributing in generation of primary and metastatic breast tumours (Carbognin *et al.*, 2019).

According to a study , multiple mutations were found in exon 5 (encoding phosphatase domain), sporadic mutation in exon 7 (responsible for calcium binding region) and in exon 8 ( Chen *et al.*, 2018). According to another study , in the important regions of *PTEN* mutation are detected among breast cancer patients, there were located in regions of exon 3,4,5,and 7 where exon 5 is the hottest region with missense mutation (Yang *et al.*, 2010). According to a research from Karachi (Pakistan), five novel SNPs were observed in *PTEN* gene by sequencing analysis among breast cancer individuals which were predicted to be pathogenic in nature (Azim *et al.*, 2021). Another study indicated that the presence of a novel *PTEN* variant which was a homozygous frameshift substitution (GCGCCG > CCGCCGC) was detected in exon 2 (Malik *et al.*, 2021)

# MATERIAL AND METHODS

### 3.1 Study design

The current retrospective, case control study was designed to identify mutations and single nucleotide polymorphisms in *PTEN* gene among breast cancer patients and healthy individuals among local Pakistani population.

### 3.2 Collection of Blood Samples

Blood samples of breast cancer patients were collected from Pakistan Institute of Medical Sciences (PIMS) and Swat Institute of Nuclear Medicine Oncology & Radiology (SINOR), whereas, blood samples of healthy individuals were collected from local population in . A consent was taken from patients and healthy controls as per World Medical Association (WMA) Declaration of Helsinki (Appendix I). A total of 3mL blood sample was collected from breast cancer patients and healthy controls by using 5mL sterile syringes, in vials containing potassium ethylene di amine tetra acetate (EDTA) as an anticoagulant agent. These samples were kept in an icebox and brought directly to the Molecular Medicine Laboratory at the Department of Microbiology (Quaid-i-Azam University Islamabad). By using molecular techniques these samples were then analysed for *PTEN* gene variants.

### 3.3 Sample Size

A total of 200 blood samples from breast cancer patients and 200 blood samples from healthy individuals were included in present study to find out variants of *PTEN* gene. Demographic data (information including name, age, marital status, residence) was obtained through designed structured questionnaire (Appendix II) while, clinical data was obtained through histopathology reports, immunohistochemistry (IHC), mammography and ultrasound reports.

### 3.4 Inclusion Criteria

Individuals diagnosed with breast cancer only, were considered as breast cancer patients in the current study while individuals who did not suffer from any disease were considered as healthy individuals.

### 3.5 Exclusion Criteria

Individuals with severe malignant disorder other than breast cancer or individual with other immunological, metabolic and allergic diseases were not included in the current study.

### 3.6 Extraction of DNA from Blood Samples

DNA extraction from the collected blood samples was performed through phenol-chloroform method in two consecutive days. The solutions used in this process include Solution A, Solution B, Solution C, Solution D, Proteinase K, Sodium Acetate, Ethanol, Chloroform and Iso-amyl alcohol. All the recipes for DNA extraction are mentioned in Table 3.1 and Table 3.2. The various solutions used in phenol-chloroform method extracted pure DNA as sodium dodecyl sulphate (SDS) as strong detergent disintegrated cell membrane whereas ethanol precipitated extracted DNA. The solution A improved the permeability of cell membrane and solution B helped in precipitation of DNA. However, solution C removed DNA associated proteins and solution D disrupted cell membrane. $MgCl_2$ protected DNA from DNase proteins where Proteinase K degraded proteins.

**Table 3.1:** Preparation of stock solution for DNA Extraction

| Sr. No. | Solution | Chemicals | Amount | Distilled Water (mL) | Final Volume (mL) |
|---|---|---|---|---|---|
| 1 | Tris HCl (1M) | Tris HCl | 14.532 mg | 120 | 150 |
| 2 | EDTA (0.5M and pH 8.8) | EDTA | 73 | 450 | 500 |
| | | NaOH | Few drops to adjust pH | | |
| 3 | Sodium dodecyl Sulphate (20%) | SDS | 20g | 50 | 100 |
| 4 | Ethanol (70%) | Absolute Ethanol | 70mL | 30 | 100 |
| 5 | Sodium Acetate | $CH_3COONa$ | 9.84g | 25 | 40 |
| 6 | TE Buffer | EDTA (0.2M) | 10mL | 70 | 100 |
| | | Tris HCL(1M) | 20mL | | |

**Table 3.2: Preparation of working solution for DNA Extraction**

| Sr. No. | Solutions | Chemicals | Amount | Distilled water (mL) | Final volume (mL) |
|---|---|---|---|---|---|
| 1 | Solution A | Sucrose (0.32M) | 27.36g | 150 | 250 |
| | | Tris (10 mM) | 0.303g | | |
| | | $MgCl_2$ (5 mM) | 0.254g | | |
| | | Triton X 100 | 2.5mL | | |
| 2 | Solution B | Tris (10 mM) | 0.303g | 200 | 300 |
| | | NaCl (400 mM) | 5.85g | | |
| | | EDTA | 0.146g | | |
| 3 | Solution C | Tris HCL (10Mm) | 0.605g | ______ | 500 |
| | | Phenol | 500 mL | | |
| 4 | Solution D | Chloroform | 20 mL | ______ | 500 |
| | | Iso-amyl Alcohol | 480 mL | | |

**Procedure of DNA Extraction**

**Day 1**

- In Eppendorf tube (1.5µL), 750µL blood of sample was taken from EDTA tube.
- Solution A (500 µL) was added into the eppendorf tubes and the tubes were suspended in inverted position for 15 minutes at room temperature.
- The tubes were centrifuged at 13000 rpm for 5 minutes to separate pellet and supernatant.
- After segregation of supernatant and pellet, the supernatant was discarded and 500 µL of solution A was added to the pellet.
- The tubes were vortexed till the pellet was dissolved.
- The dissolved pellet was again centrifuged for 5 minutes at 13,000 rpm.
- After the separation of supernatant from pellet, 400 µL of Solution B was added in the tubes.

- Additionally, 15µL of 20% SDS solution and 4µL of proteinase K was added in the Eppendorf tubes.
- The tubes were then kept in the incubator for 24 hours at 37 $^0$C.

**Day 2**

- A mixture of solution C and D was prepared by taking equal amount of both solutions and was kept at room temperature till the formation of two layers.
- From the lower layer, 500µL of solution C+D was added to the incubated tubes.
- The labelled tubes were then centrifuged at 13000 rpm for 10 minutes.
- Supernatant was collected in a new tube after separation from pellet.
- Then solution D was added in equal volume (equal to that of the upper layer or supernatant separated) in the tubes.
- The tubes were then centrifuged at 13000 rpm for 10 minutes.
- Supernatant was collected again in newly labelled eppendorf tubes.
- Sodium Acetate (55 µL) and cooled isopropyl alcohol (500 µL) were added in the tubes and then rest them for few minutes.
- The tubes were centrifuged at 13000 rpm for 4 minutes.
- After centrifugation, supernatant and pellet were obtained from which supernatant was discarded.
- The obtained pellet containing DNA was washed with 400 µL of 70% ethanol and centrifuged for 3 minutes at 13000 rpm.
- After centrifugation, ethanol was discarded and pellet was left to dry at room temperature.
- Lastly, Tris EDTA (100 µL) was added to preserve the extracted DNA and the eppendorf tubes containing the extracted DNA were stored at -20$^0$C in freezer.

### 3.7 Qualitative Analysis of Extracted DNA via Horizontal Gel Electrophoresis

The quality of DNA was checked via gel electrophoresis using 1% gel. The protocol for running DNA samples on 1% gel is mentioned below:

1. To prepare 1% agarose gel, 1g of agarose powder was mixed in 100mL of 1X Tris Borate-EDTA buffer (TBE Buffer). TBE (1X) buffer was prepared by mixing 90mL distil water and 10mL 10X TBE buffer.
2. To dissolve the measured agarose powder, the mixture was microwaved for 3-4 minutes in order to get a clear gel.
3. After cooling the gel below 40ºC, 18µL of ethidium bromide (working 1µg/mL from 10mg/mL stock) was added for the purpose of DNA staining after loading.
4. The gel was poured in the gel caster and allowed to solidify for 15-20 minutes.
5. After removing the solidified gel from the casting tray, the gel was then placed in gel tank filled with 1X TBE buffer. Table 3.3 showed the recipe followed to prepare the TBE stock solution.
6. After proper mixing of 2µL DNA sample with 2-3µL of bromophenol blue (loading dye), the sample was loaded carefully into the wells with the help of micropipette. The composition of bromophenol is shown in Table 3.4
7. The gel was run at 120V for 30 minutes.
8. For observation of DNA bands on gel, the gel was visualized by using Ultraviolet transilluminator (UV) (Syngene InGenius3).

**Table 3.3**: Chemical composition of 10X TBE solution

| Sr.no | Chemicals | Amount (g) | Distilled $H_20$(mL) | Final volume(mL) |
|---|---|---|---|---|
| 1. | Tris base | 27 | | |
| 2. | EDTA | 2.325 | 220 | 250 |
| 3. | Boric Acid | 14.5 | | |

**Table 3.4:** Chemical composition of bromophenol blue

| Sr.no | Chemicals | Amount (g) | Distilled $H_2O$ (mL) | Final volume (mL) |
|---|---|---|---|---|
| 1. | Bromophenol blue | 0.05 | 20 | 50 |
| 2. | Sucrose | 8.0 | | |

**Primer Designing**

Primers were designed specifically against the exon 4 and 5 of *PTEN* gene. The sequence of *PTEN* gene ( NG_007466.2 ) was retrieved from National Center for biotechnology information (NCBI). For the purpose of primer designing,, multiple bioinformatics tools including Primer 3 web (https://primer3.ut.ee/) . These primers were then further verified by using online tools such as Ensemble(https://asia.ensembl.org/index.html),Oligocalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) to check for hairpin loop formation and self-priming issues while, *In-sillico* PCR was carried out by UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgPcr) The primer sequence of exon 4 and 5 of *PTEN* gene obtained are mentioned in Table 3.5.

**Table 3. 5:** Primer sequences for exon 4 and 5 of *PTEN*

| **Sr.no** | **Primers** | **Sequence** | **Amplicon size (bps)** |
|---|---|---|---|
| 1. | **Exon 4** | | |
| | Forward primer | CATTATAAAGATTCAGGCAATG | 205 |
| | Reverse primer | GACAGTAAGATACAGTCTATC | |
| 2. | **Exon 5** | | 379 |
| | Forward primer | ACCTGTTAAGTTTGTATGCAAC | |
| | Reverse primer | TCCAGGAAGAGGAAAGGAAA | |

### 3.7.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed to amplify exon 4 and exon 5 of the *PTEN* gene. PCR conditions with these designed primers of exon 4 and 5 of *PTEN* gene were optimized. The concentrations and volumes of the chemicals used in PCR for the DNA amplification of the target region were optimized. The volumes of the reagents used in PCR amplification are mentioned in Table. 3.6.

**Table 3.6*:*** Optimized concentrations of PCR reaction mixture for 10 µL (total volume)

<table>
<tr><th>Sr.no</th><th>Reagents</th><th>Volumes (µL)</th><th>Final volume (µL)</th></tr>
<tr><td>1</td><td>Master mix (Thermoscientific, UK)</td><td>5</td><td rowspan="4">9.0</td></tr>
<tr><td>2</td><td>Forward primer</td><td>0.2</td></tr>
<tr><td>3</td><td>Reverse primer</td><td>0.2</td></tr>
<tr><td>4</td><td>PCR water</td><td>3.6</td></tr>
<tr><td>5</td><td>DNA sample</td><td>1.0</td><td>1.0</td></tr>
</table>

The optimization of primers was carried out by changing the annealing temperature time and the concentration of primers. Primers of *PTEN* exon 4 and exon 5 were both optimized at 57 ºC. The primer the annealing time range applied was 30-50 seconds. The total volume of the PCR reaction mixture for each sample was 10 µL. To avoid degradation of the chemical reagents, all the contents of a polymerase chain reaction were kept on an ice block. The optimized conditions for the amplification of *PTEN* gene are mentioned in Table 3.7.

**Table 3.7:** Optimized thermocycler conditions for Amplification of Exon 4 and 5

<table>
<tr><th>Sr.no</th><th>Stages</th><th></th><th>Temperatures (ºC)</th><th>Time</th><th>No. of cycles</th></tr>
<tr><td>1</td><td>Initial denaturation</td><td></td><td>95</td><td>5 min</td><td>1X</td></tr>
<tr><td>2</td><td>Denaturation</td><td></td><td>95</td><td>45 sec</td><td rowspan="4">35X</td></tr>
<tr><td rowspan="2">3</td><td rowspan="2">Annealing</td><td>Primer E4</td><td>57</td><td rowspan="2">45 sec</td></tr>
<tr><td>Primer E5</td><td>57</td></tr>
<tr><td>4</td><td>Extension</td><td></td><td>72</td><td>45 sec</td></tr>
<tr><td>5</td><td>Final extension</td><td></td><td>72</td><td>7 min</td><td>1X</td></tr>
</table>

### 3.7.2. Gel Electrophoresis of Amplified Products

Amplified products were visualized through horizontal gel electrophoresis. Agarose gel (2%) was prepared by adding 2g of agarose powder in 100mL of 1X-TBE buffer. Mixture was microwaved for 1-2 min until the solution became completely transparent. Gel was cooled at room temperature and 18µL (10mg/mL) of ethidium bromide was added for staining of DNA. Gel was poured in the gel casting tray and the combs were firmly placed and the gel was left to solidify. Solidified gel became translucent, which was then removed from the casting tray and placed in the buffer tank filled with 1X-TBE. As the gel apparatus was set, 2µL of the PCR product, was loaded in the wells

of the gel. Gel electrophoresis was performed at 90Volts for 40min. After completion, the electrophoresed gel was visualized by UV documentation system (Syngene InGenius3).

### 3.7 Single Stranded Conformation Polymorphism

Single stranded conformation polymorphism (SSCP) analysis is used to detect and identify different genomic variants. It can detect changes in gene sequence including single point mutations or small scale changes due to the differences in the electrophoretic mobility of the loaded samples. Single-stranded DNA can move differentially under non-denaturing electrophoresis conditions due to altered conformation caused by a single base mutation in the sequence. As a result, the band patterns of wild-type and mutant DNA samples differs (Haidong, 2005). The presence of any kind of mutation causes the change in mobility of the single stranded DNA as compared to the wild. PCR coupled with SSCP provides a powerful tool to screen proficient genetic variability.

#### 3.7.1 Denaturation of PCR Products

The amplified products were denatured by adding 2.5 µL formamide solution in 8 µL of PCR product and then placed in the thermocycler at 96°C for 8 min. These products were then immediately placed into the ice to provide the heat shock treatment.

#### 3.7.2 Preparation of 8% Polyacrylamide Gel

Polyacrylamide gel (PAGE) can be prepared of various percentages according to the PCR product size. In current study, 8% polyacrylamide gel were used for detection of mutation. The chemicals used in the preparation of gels is mentioned in Table 3.8

**Table 3. 8:** Chemical composition of polyacrylamide gel to prepare 8% vertical gel for SSCP analysis

| Sr.no | Reagents | Amount | Final volume(mL) |
|---|---|---|---|
| 1. | 30% acrylamide-Bisacrylamide Solution | 13.5mL | 50mL |
| 2. | 10X TBE | 5mL | |
| 3. | 10% Ammonium per sulphate | 350µL | |
| 4. | TEMED ( Thermo Scientific Pierce Tetramethylethylenediamine) | 25µL | |
| 5. | Distil Water (adjusted up to 50mL) | 31.125mL | |

Polyacrylamide (30%) was prepared by mixing acrylamide and bisacrylamide in a specific ratio according to the volume required. Ratio of both acrylamide and bis-acrylamide for preparing 250mL of 30% polyacrylamide is mentioned in Table 3.9. Ammonium per sulphate solution (10%) composition is mentioned in Table 3.10.

**Table 3.9:** Composition of 30% acrylamide-bis acrylamide gel

| Sr.no | **Chemicals** | **Amount (g)** |
|---|---|---|
| 1. | Acrylamide | 72.5 |
| 2. | Bis-acrylamide | 2.5 |
| Dissolved them in 100mL of distilled water and raised the total volume up to 250mL | | |

**Table 3.10:** Ammonium persulphate composition

| Sr.no | **Chemicals** | **Amount** |
|---|---|---|
| 1. | Ammonium persulphate | 5g |
| 2. | Distilled water | 45mL |

### 3.7.3 Vertical Gel Electrophoresis Protocol

SSCP was used for the separation of banding patterns and variation analysis in exon 4 and 5 of *PTEN* gene on vertical gel electrophoresis system using 8% poly acrylamide gel.

1. The plates of the apparatus were cleaned with 70% ethanol, air dried and held together with spacers between them.
2. Plates were clamped tightly to prevent the leakage of gel from in between the spacers.
3. The composition for preparation of 8% gel and reagents used for its preparation are mentioned in the Table 3.8.
4. The prepared 8% gel was poured in between the arranged glass plates with care to avoid any bubble formation and combs were also placed promptly into their position for the formation of the wells.
5. The gel was left to get polymerize for 45-50 minutes at room temperature.
6. The tank apparatus for vertical gel was set up having two tanks. First tank is the vertical one and the second one is the horizontal one. The tanks were arranged by placing the vertical tank in the horizontal one such that the two electrodes were in opposite directions. Tanks were filled with 1XTBE (1500mL).
7. Lower clamps spacers and combs from the solidified gel were removed and the wells formed were washed with 1x TBE.
8. Plates were arranged in such a position that the smaller plate face the inner side towards the tank so the wells remained filled with buffer.
9. Denatured products were then mixed with 5 µL bromophenol blue also called as loading dye and the total 15µ L of the product was loaded into the wells. The polyacrylamide gel electrophoresis was performed at 120V for 3 to 5hrs.

After 3 hours, the clamps and spacers were removed to separate the glass plates and the gel was then placed in 10 µg/mL ethidium bromide solution for staining. UV Gel documentation system was used for the visualization of the gel. Alterations in the banding pattern of amplicons was observed and these samples were selected for sequencing.

### 3.8: Sequencing Analysis

BIOEDIIT (version 7.00.5.3) was used for alignment and analysing variations in nucleotide sequence. The mutations were then analysed by using various online tools such as mutation taster (https://www.mutationtaster.org/). The non-synonyms mutations were further analysed by online servers like SIFT (https://sift.bii.a-star.edu.sg/), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/ . UniProt (https://www.uniprot.org/) was used to identify the site/region of interaction on PTEN after inclusion of identified mutations.

### 3.9 : Data Analysis

Microsoft Excel, Microsoft word and SPSS 16.0 were used for the analysis of demographic as well as clinical data. By using Microsoft excel, percentages and mean of different variables were evaluated. Data was arranged in the form of graphs and tables by using Microsoft excel and MS word.

# RESULTS

### 4.1 Study Population

The current study was a case control study. The main focus of the study was to detect variants in the *PTEN* gene, an important tumour suppress of cell growth and cycle which if mutated can be potentially oncogenic. For this purpose, 200 breast cancer patients and 200 healthy individuals (controls) of local Pakistani population were included in the current study

### 4.2 Demographic and Clinical Features of Subjects

During sampling, data from study individuals were collected through designed questionnaires. Information regarding demographic and some key risk factors including age, gender, living area (urban/rural), number of children, marital status, breastfeeding status along with type of breast cancer and disease grade of the cancer patient were obtained. The clinical data regarding the disease including the diagnostic reports (mammogram, ultrasound, histopathology, immunohistochemistry reports) was also obtained from the patients.

Considering age factor, age range categories were made as: category 1 (28-42 years), category II (43-57 year), category III (58-72 years) and category IV (73-87 years). According to the data, majority of breast cancer patients (46%) were of category II; 43-57 years. However, age groups above 40 years; 58-72 years (OR: 2.8615 [1.5668 to 5.2259] p value = 0.0006) and 73-87 years (OR: 3.4561 [0.9367 to 12.7519] p value = 0.0626) were significantly associated with the risk of breast cancer development. Figure 4.1 and Table 4.1 shows the age distribution of the study individuals.

Similarly, categorization based on the area of residence shows that greater percentage (59.4 % patients and 40.52% controls) of the study individuals was from urban areas (Figure 4.2). The study individuals of the present case controls study were mostly married and the greater percentage of both patients (97.5%) and controls (98.5%) were married out of family. Moreover, the parity status of the study individuals shows that 93% of the patients were parous and only 7 % controls were nulliparous. Breast feeding was found common among study individuals > 90% of patients and controls (Table 4.2).

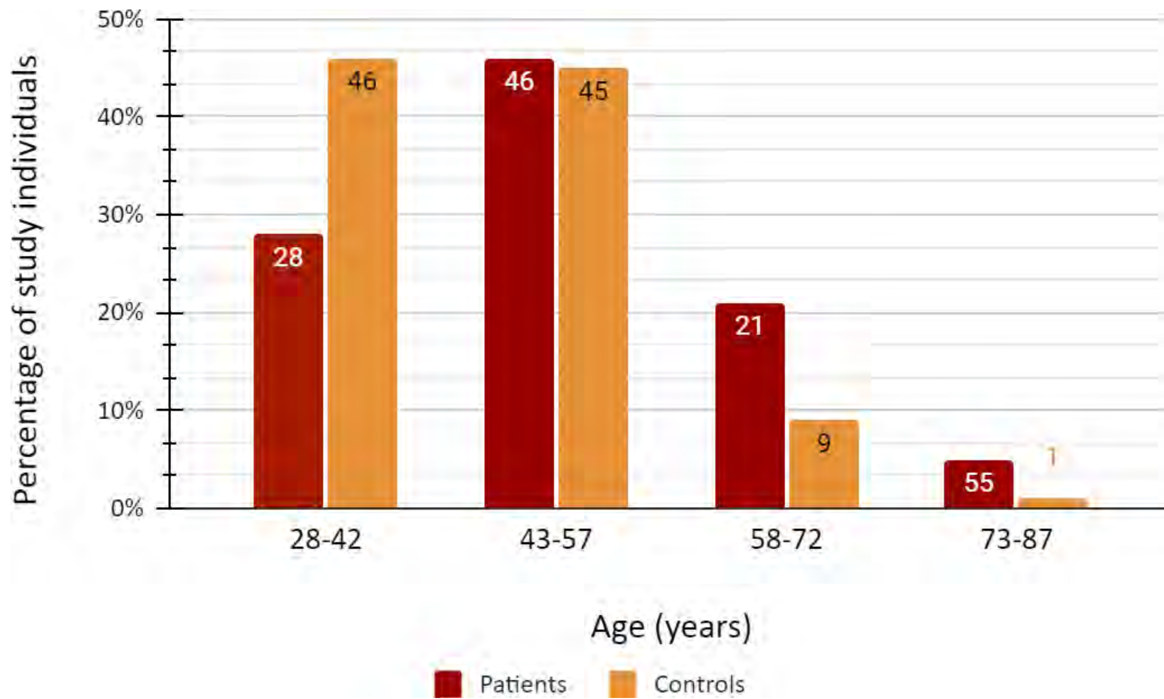


**Figure 4. 1**: Percentage distribution of breast cancer patients and healthy individuals on based on age factor

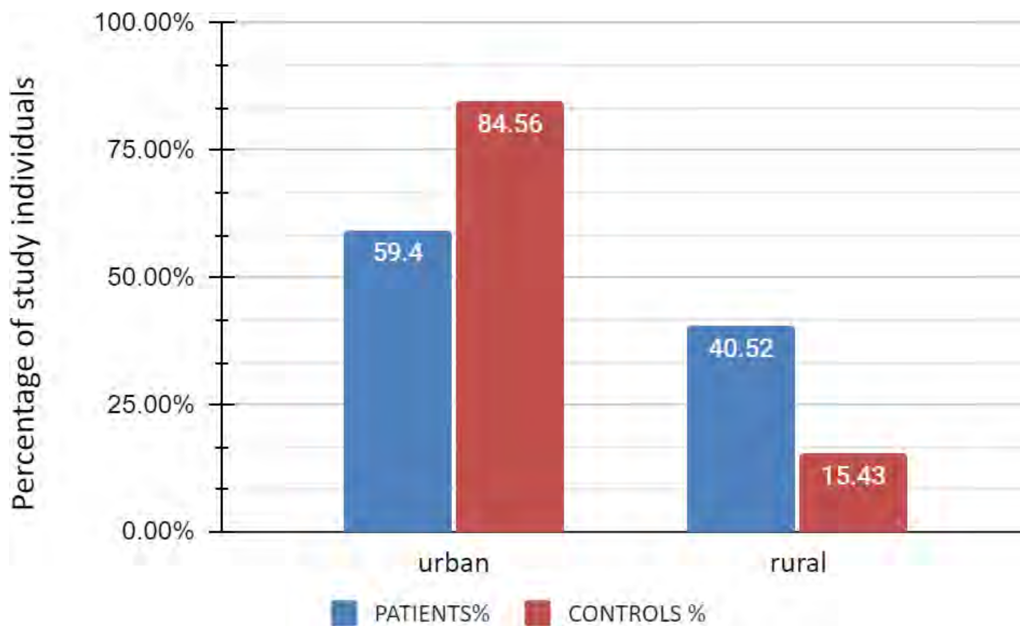


**Figure 4. 2** Percentage distribution of breast cancer patients and healthy individuals on the basis of area of residence

**Table 4.1:** Distribution of age groups of breast cancer patients and healthy individuals

| Sr.no | **Age group (years )** | **Patient (n)** | **Control (n)** | **Odd Ratio** | **Confidence Interval 95% (CI)** | ***p* value** |
|---|---|---|---|---|---|---|
| 1. | 28-42 | 56 | 91 | 0.4658 | 0.3074 to 0.7058 | 0.0003 |
| 2. | 43-57 | 92 | 89 | 1.0624 | 0.7166 to 1.5752 | 0.7631 |
| 3. | 58-72 | 42 | 17 | 2.8615 | 1.5668 to 5.2259 | 0.0006 |
| 4. | 73-87 | 10 | 3 | 3.4561 | 0.9367 to 12.7519 | 0.0626 |

**Table 4.2 :** Percentage distribution of breast cancer and healthy individuals on the basis of marital status, breast feeding and parity status

| **Sr. no** | **Features** | **Number of Patients (%)** | **Number of Controls (%)** | **Odd Ratio** | **Confidence Interval (ci)** | ***p* value** |
|---|---|---|---|---|---|---|
| **1.** | **Marital Status** | | | | | |
| | Married | 195 (97.5) | 197 (98.5) | 0.7959 | 0.2106-3.008 | 0.7365 |
| | Unmarried | 5 (2.5) | 3(1.5) | | | |
| **2.** | **Breast Feeding** | | | | | |
| | Yes | 171(90) | 179 (94) | 0.5732 | 0.2719 to 1.2083 | 0.1435 |
| | No | 20(10) | 12 (6) | | | |
| **3.** | **Parity status** | | | | | |
| | Parous | 180(93) | 185 (96) | 0.5988 | 0.2424 to 1.4791 | 0.2663 |
| | Nulliparous | 13 (7) | 8 (4) | | | |

The current study shows that 10.55% of the patients have family history of breast cancer, which is significantly associated with breast cancer risk [OR: 5.7486 (1.9361 to 17.0689) *p* value = 0.0016] in shown in Figure 4.3. As cancer patients belonged to different province, the distribution of study individuals based on provincial residence shows that Punjab had maximum (57.9%) number of cancer patients following Khyber Pakhtunkhwa 34.03% (Figure 4.4**).**

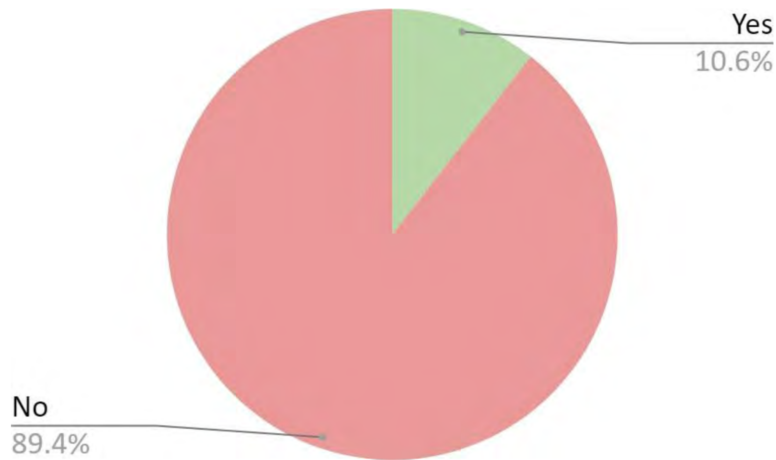


**Figure 4. 3** Percentage distribution of breast cancer patients on the basis of family history

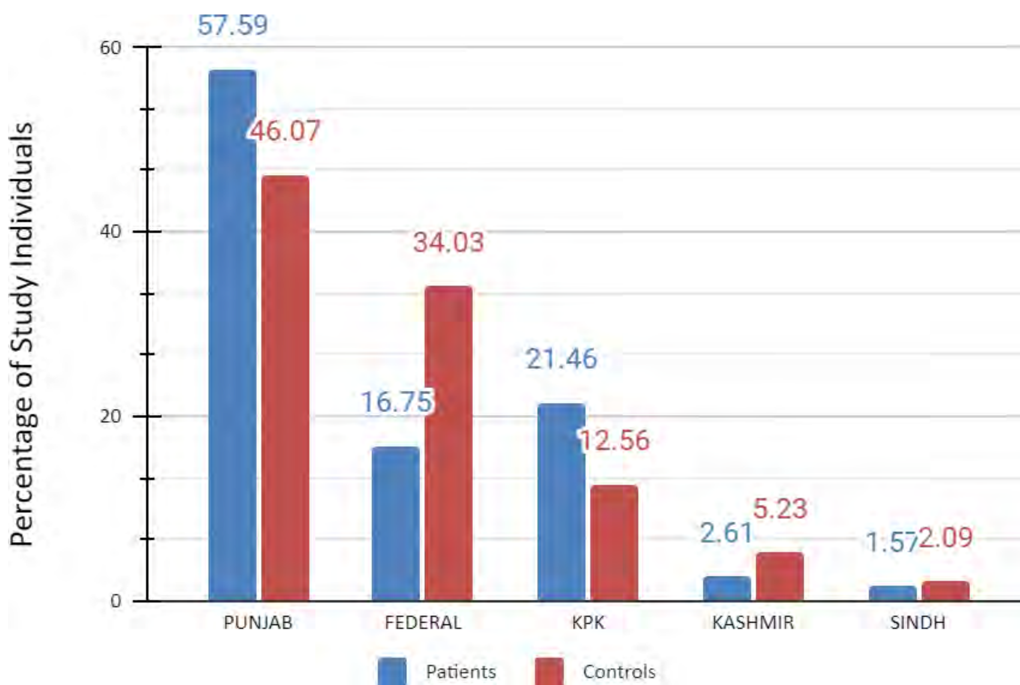


**Figure 4. 4:** Percentage distribution of breast cancer and healthy individuals on the basis of provincial residence

Based on the type of tumour, the patients were categorized into three grades including grade I, grade II and grade III based on the Nottingham classification. On the basis of grades, 74.7% of patients had grade II breast cancer (Figure 4.5). Considering the affected breast with lump as a symptom, it was found that patients with right breast lump were higher in number as compared to those having left breast lump (45%). Moreover 1.5% of patients also had bilateral lumps in breasts (Figur 4.6).

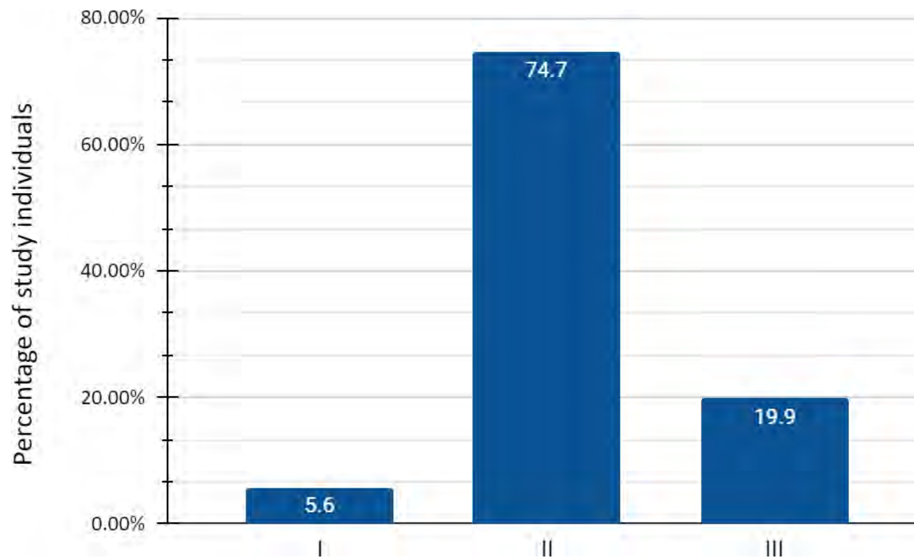


**Figure 4. 5** Percentage distribution of breast cancer patients on the basis of Nottingham classification for cancer grading

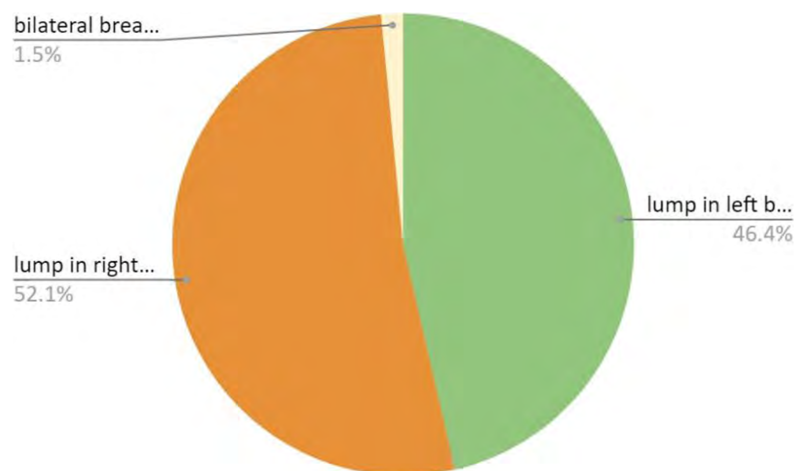


**Figure 4. 6 :** Percentage distribution of breast cancer patients on basis of affected breast with lump

In the current study, invasive ductal carcinoma was the most prevalent type of breast cancer as 74% of the study patients were diagnosed with invasive ductal carcinoma followed by 18% with invasive mammary carcinoma and 4% with ductal carcinoma *in-situ* (Figure 4.7). On the basis of expression of biomarkers ER, PR, HER2 and Ki67, breast cancer cases were categorized into subtypes. In the current study 12.5% of the patients have Luminal A breast cancer, 9% had triple negative subtype and only 5% had HER-2+ subtype (Figure 4.8).

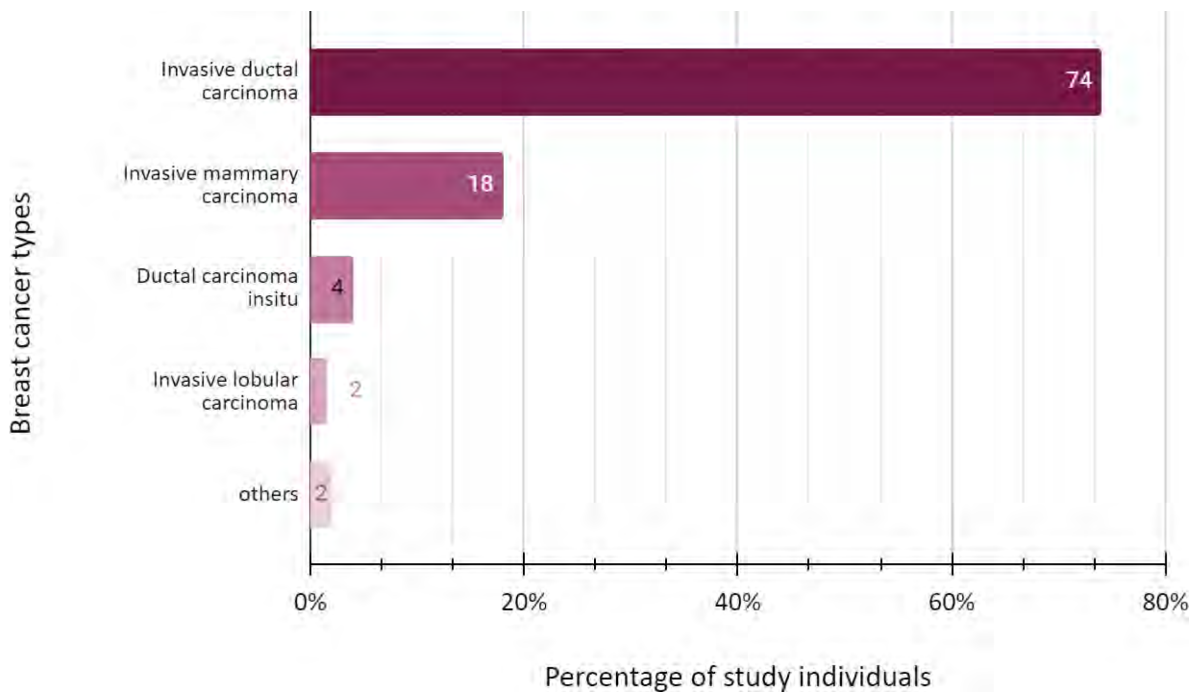


**Figure 4. 7 :** Percentage distribution of breast cancer patients on the basis of types of cancer

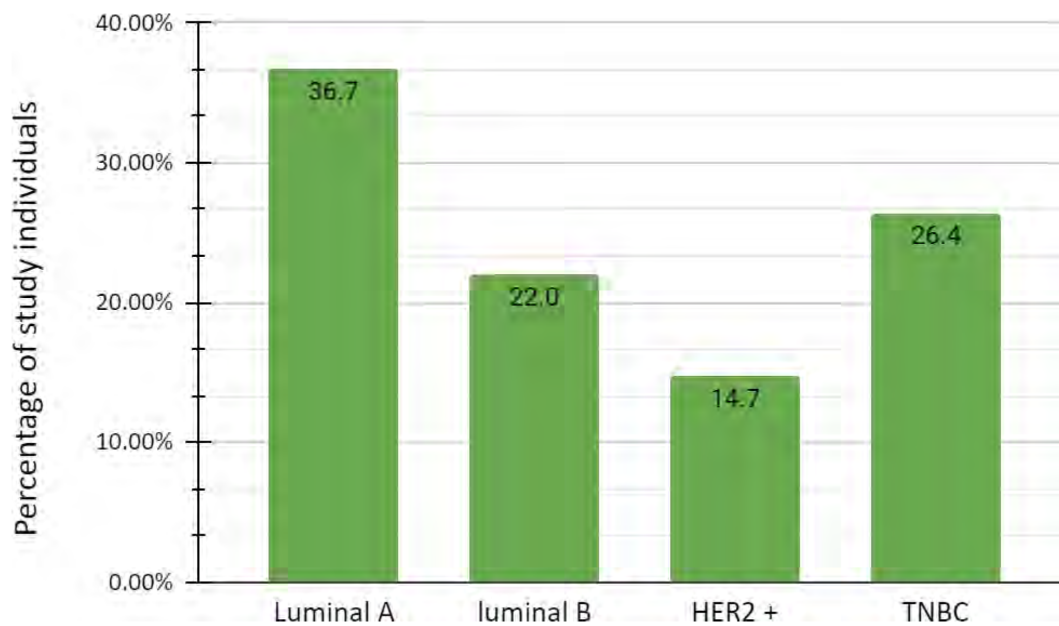


**Figure 4. 8 :** Percentage distribution of breast cancer patients on the basis of molecular classification

### 4.3 Extraction DNA from Blood of Patients and Healthy Individuals Blood

DNA extraction was carried out by the phenol-chloroform method from the blood samples of both patients and healthy individuals. The extracted DNA samples were visualized on 1% agarose gel (electrophoresed at 120 volts for 30 minutes) as shown in Figure 4.9.

**Figure 4. 9:** Extracted DNA visualized on 1% agarose gel, a. breast cancer patients (B170, B177, B178, B179, B181-B187) b. controls (C1-C5).

The exon 4 and 5 of the gene were amplified by PCR. Specific primers for the target exons were designed by using bioinformatics tools including primer 3, NCBI, UCSC and OLIGOCALC. Amplified target regions of the exon 4 was 205bps long and exon 5 was 397bps. All amplicons were analysed on 2% agarose gel (Figure 4.10- 4.13).

**Figure 4. 10 :** Amplicons (size 205bp) of Exon 4 showing control lane 1-2 (C69, C70) and breast cancer patients in Lane 3-13 (B58,B92, B93, B117, B95, B126, B124, B113, B101, B106, B121) whereas Line14 has 100bps ladder (thermos- scientific)

**Figure 4. 11 :** Amplicons (size 205bp) of Exon 4 showing control lane 1-10 (C107, C150, C151, C152, C39, C36, C18, C14, C26, C23) and breast cancer patients in lane 11-20 ( B22, B137, B34, B33, B05, B53, B25, B24, B140, B138)

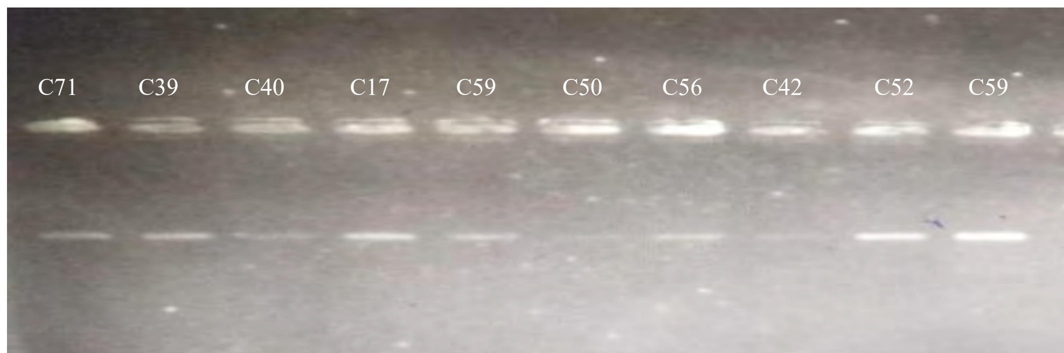


**Figure 4. 12 :** Amplicons (size 397bp) of Exon 5 showing controls starting from lane 1-10 (C71, C39, C410, C17, C59, C56, C42, C52, C59)

**Figure 4. 13** Amplicons (size 205bps) of Exon 4 showing patients starting from lane 1-20 (B92, B105, B89, B90, B113, B95, B99, B115, B112, B100, B118, B91, B88, B87, B86, B82, B103, B104, B76, B78)

### 4.5 Single Stranded Conformational Polymorphism (SSCP)

PCR products were analysed for detecting variants via SSCP. After amplification of exons, 8% PAGE was done for SSCP analysis of exon 4 and 5 of *PTEN* gene. Formamide was added in the amplified PCR products and the products were denatured into single strands. The denatured products were the run on 8% PAGE and change in banding pattern of the single strands was checked. After electrophoresis, the stained gel was visualized under trans illuminator (Figure 4.14-4.16)

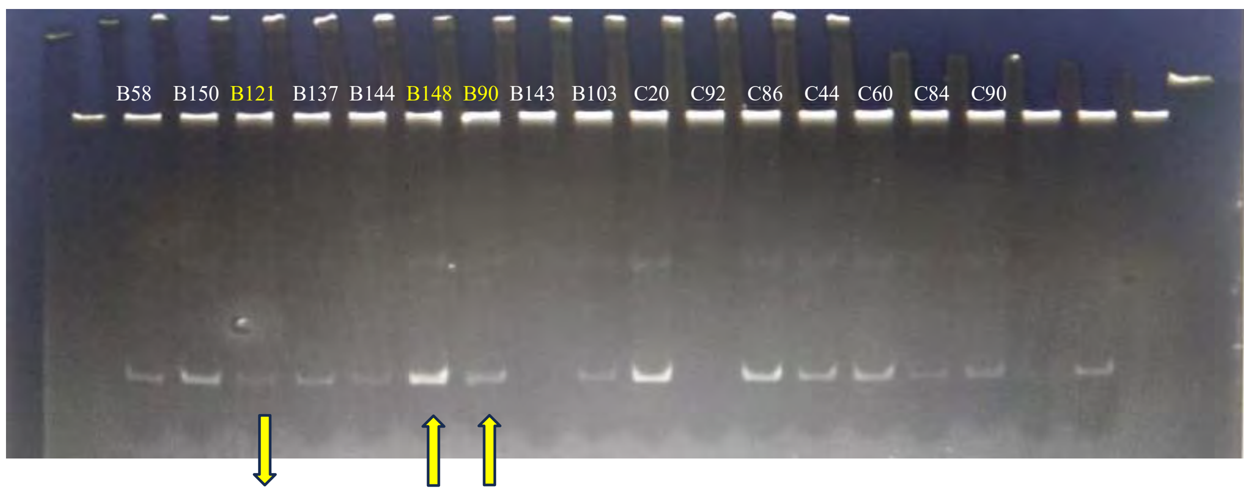


**Figure 4. 14:** PAGE (8%) showing denatured amplicons of exon 4. Lane 1-9 shows patients (B58, B150, B121, B137, B144, B148, B90, B143, B103). Lane 10-16 shows control samples (C20, C92, C86, C44, C60, C84, C90).

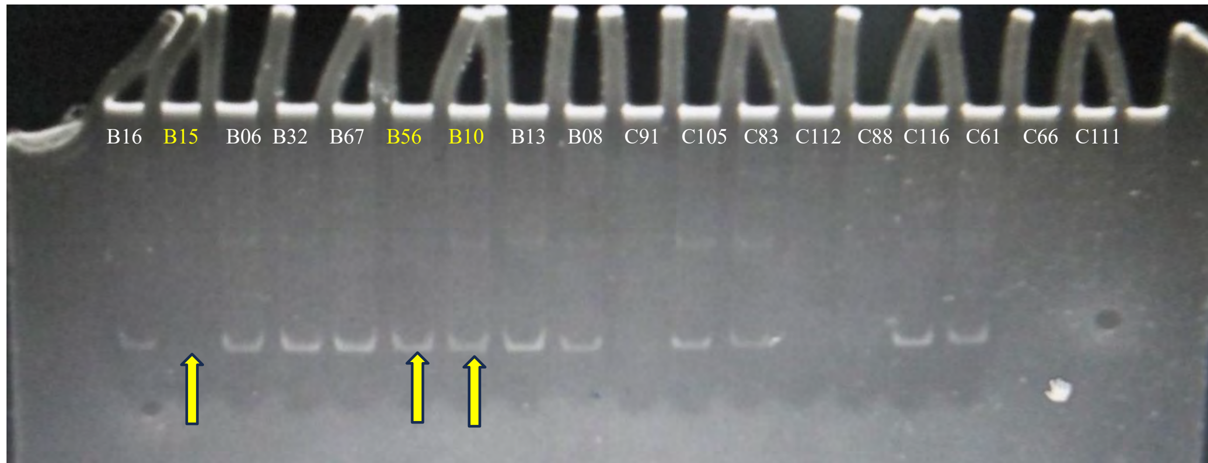


**Figure 4. 15** PAGE (8%) showing denatured amplicons of exon 4: Lane 1-10 shows patients (B16, B15, B6, B32, B67, B56, B10, B13, B8). Lane 11-20 shows control samples(C91,C105,C83, C112, C88, C116, C61, C66, C111).

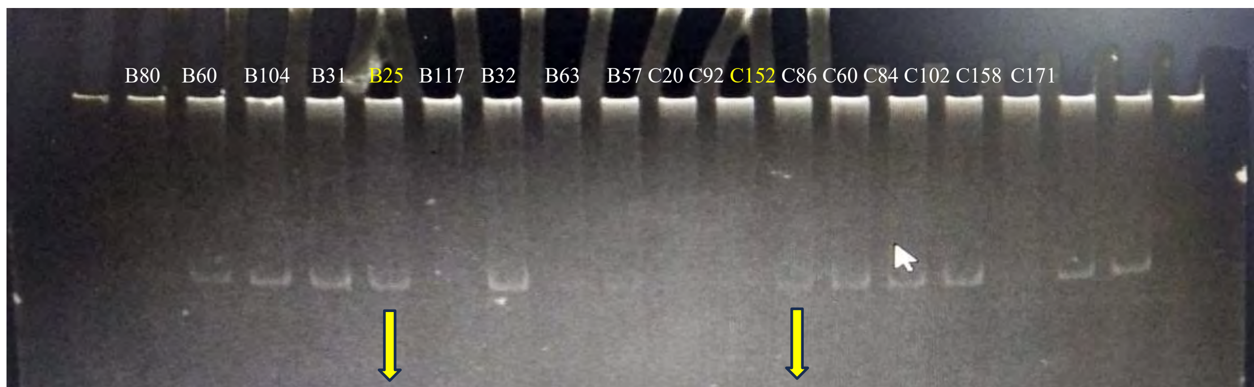


**Figure 4. 16**: PAGE (8%) showing denatured amplicons of exon 5 Lane 1-10 shows patients (B80, B60, B104, B31, B25, B117, B32, B63, B57). Lane 11-20 shows control samples (C20, C92,C152, C86, C60, C84, C102, C158, C171)

### 4.6 Sequencing Analysis

The PCR products of exon 4 and 5 of *PTEN* gene were sent for Sanger sequencing to Eurofins, USA. The results of Sanger sequencing were aligned with the reference sequence NG_007466.2 accessed from NCBI. The sequences were further analysed through bioinformatics software BIOEDIT. For the furthers analysis of variants, Mutation Taster Software version of 2021 was also used.

In the current study, a total of 2 disease causing mutations were detected in exon 4 of *PTEN* ,among which 1 was novel and the other one was reported mutation. The novel mutation was a frameshift mutation (C83Afs*16) with a single base deletion (T/-) at chromosome position chr10:89690839_89690839delT and it was detected in 4/200 patients with invasive carcinoma (Figure 4.17).

The second mutation at chr10:89690825A>TN/A was reported in Human Genome Mutation Database (HGMD-ID CM1110051), however, it was not reported in breast cancer but was reported in autism. This mutation was detected in exon 4 of *PTEN* gene in only one patient diagnosed with invasive ductal carcinoma. This non-synonym mutation had an amino acid change as T78S, Figure 4.18 and Table 4.2 shows the detail of these mutations.

**Table 4.3:** Sequence analysis of breast cancer patients for the identification of mutations and SNPs

| Sr. no | SNP | Physical location | DNA changes | Patients (n) | Patients' age (years) | Type of polymorphism | Reported/Novel |
|---|---|---|---|---|---|---|---|
| 1. | T/- | chr10:89690839_89690839delT | c.246_246delT cDNA.1603_1603delT g.67970_67970delT | 4 | 48, 67, 61, 56 | frame shift | Novel |
| 2. | A/T | chr10:89690825A>TN/A | c.232A>T cDNA.1589A>T g.67956A>T | 1 | 50 | Non-synonyms | Reported (HGMD-ID CM1110051) |

c

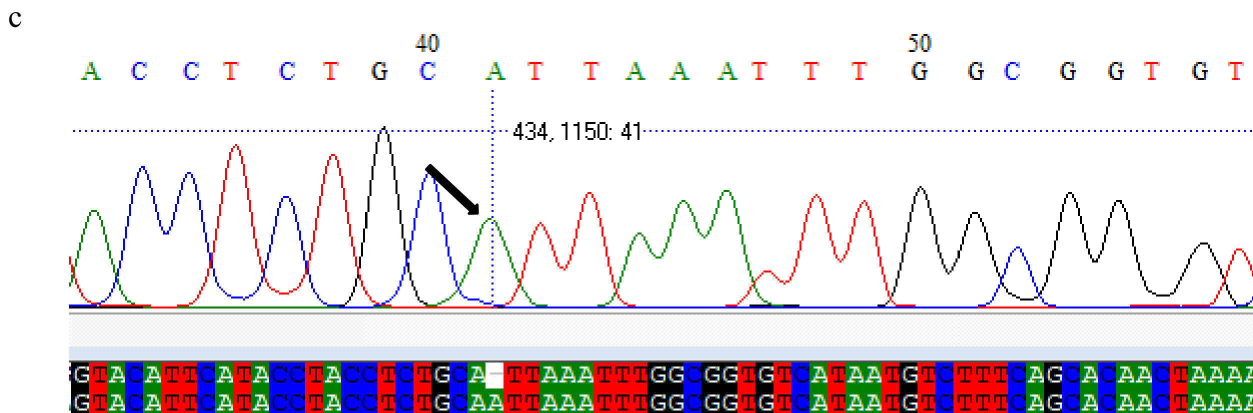


**Figure 4. 17:** Disease causing mutation (T/-) at nucleotide position 41 in exon 4 of PTEN detected in breast cancer patients (B04, B86, B159, B24)

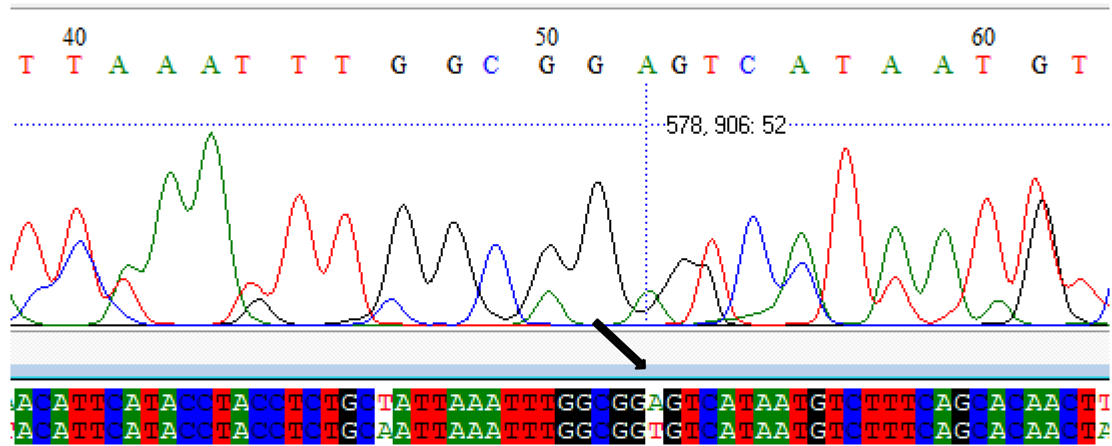


**Figure 4. 17:** Disease causing mutation (A/T) at nucleotide position 52 in exon 4 of *PTEN* detected in breast cancer patient (B115).

# DISCUSSION

Breast cancer is the most common cancer among women worldwide (Behravan *et al.*, 2020). According to WHO (2022) around 2.3 million new breast cancer cases were reported globally. In term of population, Pakistan is the 6th most populated country. In such scenario, a very high rates of breast cancer incidence and mortality is a serious concern. Pakistan has high breast cancer prevalence of 14.5% which is effecting both genders differentially (GLOBOCAN, 2020). Numerous epidemiological studies have identified several risk factors associated with breast cancer. The main risk factors identified in these studies are various susceptibility genes, familial history of breast cancer or any cancer, ovary or endometrium, and an individual history of reproductive function and breast diseases (Sun *et al.*, 2022). Moreover, various lifestyle attributes along with hormonal and malfunction of reproductive system also contributes towards breast cancer development (Momenimovahed & Salehiniya, 2019). Among the modifiable risk factors diet, obesity, diabetes and body mass index are most important factors in the development and progression of breast cancer (Fader Kaiser *et al.*, 2022).

In the present study, one of the objective was to analyse the impact of demographic factors for their role in development of breast cancer. The another main objective was to study genetic alterations in *PTEN* gene in breast cancer patients and healthy individuals in Pakistani local population to their possible involvement in cancer. The primary function of *PTEN* gene is in the tumour suppression. Hence it was hypothesized that any kind of variation in the gene can result loss of phosphatase activity of the protein and leads to the development of various types of cancers.

In the current study, majority of individuals were females and two male cases were found. But male cases were excluded from the analysis of both demographic and molecular investigation. Being women is the major risk factor of breast cancer development (Niell *et al.*, 2021). In a study from Karachi , breast cancer was the most frequently recorded malignancy (53.2%) among adult females (Qureshi *et al.*, 2020) In the current study greater percentage (46%) of patients were of age range of 43-57 years. In the current study, the age groups above 40 years was found to be most venerable age group as in range of 58-72 years (OR: 2.8615) and 73-87 years (OR: 3.4561) had more odds of acquiring breast cancer as it was found to be significantly associated with the risk of breast cancer. According to a review of 142 published articles on breast cancer globally, they estimated that incidence rate of breast cancer was high among individuals above 50 years (Momenimovahed & Salehiniya, 2019). In study from Pakistan, greater number of breast cancer

individuals were reported in an age range of 27-46 years with a mean age of 35 years (Malik *et al.*, 2021). A study conducted in Peshawar (Pakistan), found greater number of breast cancer patients in a range of 18-35 years (Ullah *et al.*, 2021). According to another study conducted in Karachi hospitals, the mean age of breast cancer patients was 48 years (Shamsi *et al.*, 2020). The findings of the current study are in accordance with those of previous studies from Pakistan as well worldwide, also substantiating that increasing age enhances the incidence rate of breast cancer. In Pakistan, it could be associated with lack of knowledge and awareness about self-examination, early detection, complexity of breast cancer in older individuals compared to young individuals

In the current study, approximately half of the patients belonged to urban areas. A cross-sectional study conducted in United States supports the finding of the current study showing that women of both urban and rural areas were equally adherent to breast cancer screening (Shete *et al.*, 2021). On the other hand, contrary to the finding of the current research, a study conducted in China showed that the incidence and mortality of breast cancer continue to increase in China, particularly in rural areas (Lei *et al.*, 2021). A study from USA suggested that living in large metropolitan areas was associated with a lower overall mortality compared to rural areas (Obeng-Gyasi *et al.*, 2020). A study conducted in Pakistan showed that the rural residents have significant delay in diagnosis and treatment (Majeed *et al.*, 2021). In the current work, breast cancer patients were from both urban and rural area, this might be due to the migration of rural population to urban area due to presence of facilities, a better access to cancer services (eg, screening, detection, and treatment) and awareness regarding the disease (self-examination of breast, leads to a higher early detection of disease), hence have placed both urban and rural residents at an equal risk of breast cancer development (Zaheer & Fatima, 2022).

The prevalence of breast cancer in different provinces of Pakistan varies, reflecting the diverse population and regional disparities in healthcare resources. The greater percentage of breast cancer patients in the current study were from Punjab (57.59%) followed by Khyber Pakhtunkhwa (21.46%) and least (0.52%) were from Sindh. These results were in accordance with a study showing a high incidence (76.6%) of breast cancers among females in Punjab (Badar *et al.*, 2022). A study based on prevalence of breast cancer in Lahore, (Punjab) also reported high breast cancer incidence (Arshad *et al.*, 2019).To reduce such high breast cancer burden in Punjab, there is need for public awareness and an easy access to health care centres for early detection of breast cancer.

Also there is need for proper inventory for these cases cancer cases in mentioned provinces for health authorities to properly deal with cancer cases and making more diagnostic facility centres.

In Pakistan, breast cancer is a significant health concern, and it has been suggested that marital status might play a role in the development of this disease (Qureshi *et al.*, 2020). Several studies have indicated that marital status consistently correlate with breast cancer and its screening behaviour (Al-Naggar & Osman, 2015) . In a recent study conducted in Iran, found that family history and marital status impact the incidence of breast cancer in Iranian women (Khazaee-Pool *et al.*, 2017). The results of the current study in accordance to this previous studies as 97.5% of the patients were married. However, in unmarried females the cancer might be linked to lack of breast tissue differentiation along with more exposure to non-oestrogenic mutagens and genotoxicity by oestrogen hormone (Kashyap *et al.*, 2022).

Breastfeeding is linked to hormonal changes and changes in the molecular histology of the breast, which can lower a person's risk of developing breast cancer. Breast feeding contributes in reducing breast cancer risk and also proved beneficial to Infants (Anstey *et al.*, 2017). Among the study individuals, greater percentage of mother patients (90%) and controls (94%) breastfed their children. According to a study in 2022 from UK, breast feeding was seen to reduces the risk of breast cancer among mothers by 4.3% (Stordal, 2023). According to a study in Pakistani population, majority (75%) of the patients have a positive breast feeding history similar to current work (Malik *et al.*, 2021).

Moreover, the parity status of females is an important risk factor for breast cancer development. According to previous case control study parous females have a decreased risk of breast cancer development and also indicates that on every child birth, the risk of PR+ and ER+ cancers decreases by 10% (Momenimovahed & Salehiniya, 2019). According to another study, parity was associated with lower risk of ER + breast cancer (Fortner *et al.*, 2019). The results of current study are in accordance to previous studies as in current study, 93% of the breast cancer patients were parous.

In the current study, only 10.6% of the breast cancer patients have a positive family history of breast cancer and was found to be significant risk of breast cancer development (OR: 5.7486).

According to a study on Southern Punjab population, positive family history of breast cancer was significantly associated with breast cancer risk (Ahmad *et al.*, 2021). However, there can be discrepancies due to various factors such as cultural differences, sample size, and study design.

Classification on histological basis of the current study showed that 74% of the patients were diagnosed with invasive ductal carcinoma and only 4% patients were diagnosed with ductal carcinoma *in-situ*. A study conducted in tertiary care setting in Pakistan presented 95% of the breast cancer patients diagnosed with invasive ductal carcinoma. (Baig *et al.*, 2019). Another study from Karachi, Pakistan reported invasive ductal carcinoma (91.3%) as the most prevalent type of breast cancer among study individuals (Beg *et al.*, 2020). Overall, present study has same type of cancer earlier reported from Pakistan. The prevalence of invasive breast cancer among individuals might be due to fear of cancer treatment, misdiagnosis at proper time, taboo, non- availability of health care services, lack of knowledge and late detection of breast cancer among individuals, where cancer status changes from benign to invasive.

Moreover, in the current study 74.7% of the breast cancer patients were classified in Grade II category and 5.6% were found in Grade I category. These results are in accordance with a study conducted in Pakistan in which 85% of the patients were presented with Invasive Ductal Carcinoma among which 55% have Grade III carcinoma (Malik *et al.*, 2021). Another study performed in tertiary care presented 59% of the patients with grade III/IV (Gulzar *et al.*, 2019). The findings of the current study are contradictory to the previous studies. This can be explained by the presence of awareness campaigns among local population and concept of self-breast examination which leads to detection of cancer at an early stage.

Breast cancer symptoms common among the study individuals of the present study were lumps in breasts (51.4% in right breast and 45.7% in left breast). According to a study in Khyber Pakhtunkhwa, (Pakistan), 17% of the study individuals have lump or thickness in the breasts as a warning sign of the disease ( Ullah *et al.*, 2021). Another study conducted in Karachi, (Pakistan) showed 55.2% of women were detected with a breast lump (Shamsi *et al.*, 2020). The findings of another study supports findings of current study as 60% of the patients had unilateral cancer of right breast (Malik *et al.*, 2021). These findings show that the major symptom of the breast cancer is a lump in breast in Pakistani females.

Based on the molecular subtypes, 36.7% of the patients in the current study were diagnosed with Luminal A type and rest with Luminal B 14.7%with HER2+ type and 26.4% with TNBC. According to a study conducted on Black women (USA), 66.1% had breast cancer with ER/PR+HER2− (Luminal A) and 18.1% TNBC (Friebel-Klingner *et al.*, 2021). A study from Pakistan reported a higher percentage of patients with luminal A type of breast cancer (Shakeel *et al.*, 2021). According to another study from Karachi,(Pakistan), invasive lobular carcinoma was found significantly associated with luminal A type disease (Beg *et al.*, 2020). The current findings are according to the previous studies from Pakistan showing luminal A as the most prevalent molecular subtype among Pakistani population.

Sequencing analysis revealed 2 disease causing mutations in exon 4 of *PTEN* gene, among which 1 was novel and the other was reported mutation. The novel mutation was a non-synonyms frameshift mutation (C83Afs*16) in the CDSS region, with a single base deletion (T/-) at chromosome position chr10:89690839_89690839delT. Here, cysteine amino acid was replaced by alanine amino acid at position 83, which encodes for the phosphatase tensin domain of the *PTEN* proteins. The second mutation was also a non-synonyms mutation detected in exon 4 of *PTEN* gene in patient diagnosed with invasive ductal carcinoma. In this mutation, threonine amino acid at position 78 is predicted to be replaced by serine amino acid. The threonine at 78 position is involved in phosphatase-tensin domain of the *PTEN*. This mutation was earlier reported in Human Genome Mutation Database (HGMD-ID CM1110051), however, it was not reported in breast cancer except it was reported in autism.

Both of the detected mutations are non-synonymous mutations and were found to be present in the region which encodes for the phosphatase-tensin type domain of *PTEN.* The catalytic function of *PTEN* is due to its phosphatase domain that is involved in the dephosphorylation of the inositol ring of phosphatydilinositol-3-4-5-triphosphate (PIP3),a lipid second messenger which further activates the downstream signalling molecules including AKT, which regulates various functions of the cell including cellular proliferation. There is presence of arginine loops in this domain important for the membrane binding function of protein (Irvine *et al.*, 2019). Thus, mutation in the region encoding for phosphatase domain compromises the dephosphorylation of the inositol ring of PIP3, which downregulates the downstream signalling pathways. thus there might be loss

of regulation of cellular proliferation increasing chances of uncontrolled cellular proliferation or tumour development.

# CONCLUSION

The current retrospective study was designed to perform mutational analysis of *PTEN* gene in Pakistani breast cancer patients. The important findings of the present study are:

- Majority of cancer patients were females, within age range of 43-57 years and advancing age is significantly associated with risk of breast cancer.
- The frequency of patients from urban and rural settings was almost equal according to recent study suggesting that females of both settings were equally prone to breast cancer development.
- Married, parous women with a positive breast feeding status were at a higher risk of developing breast cancer.
- Family history is significantly associated with increased risk of breast cancer
- The maximum of the breast cancer cases were of sporadic origin.
- Invasive ductal carcinoma was the most prevalent type of breast cancer and prevalent molecular subtype was Luminal A among patients.
- Majority of the cancer patients were diagnosed with stage/grade II cancer.
- Two novel mutations (non-synonymous) were detected in *PTEN* gene of breast cancer patients. These novel SNPs were detected in total of 5 patients diagnosed with invasive ductal carcinoma and invasive mammary carcinoma with ages ranging from 48-67 years.

# FUTURE PROSPECTS

Based on the current study following future recommendations are suggested.

- The association of *PTEN* gene with other tumour suppressor genes should be assessed in order to get a better understanding of breast cancer.
- Along with detection of DNA changes, epigenetic changes should also be assessed in *PTEN* gene.
- For an early detection of the disease further studies should be performed with a large study population for getting a better understanding of the risk factors crucial for breast cancer development.
- Awareness campaigns should be conducted in order to educate local population in both urban and rural settings.
- Counselling of the female population should be done and they should be encouraged for regular physical and clinical examination.
- The susceptibility pattern of breast cancer among different ethnic groups in Pakistani population should be evaluated.

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## APPENDIIX I

عہد نامہ

میں نے اس معلوماتی پرچے میں موجود تمام معلومات کو بغور پڑھ اور سمجھ لیا ہے اس حوالے سے
مجھے اپنے خدشات کے اظہار کا مکمل موقع اور سوالات کا تسلی بخش جواب دیا گیا میں بغیر کسی دباؤ
کے اس تحقیقی مطالعے کا حصہ بننا چاہتی ہوں۔

مریضہ / شریک کی معلومات

نام ________________ شناختی کارڈ نمبر ________________

دستخط / انگوٹھے کا نشان ________________ تاریخ ________________

| | | | |
|---|---|---|---|
| تاریخ پیدائش | | عمر | |
| والد/ شوہر کا نام | | | |
| مکمل پتہ | | گلی نمبر / محلہ | |
| قصبہ/ گاؤں | | تحصیل/ ڈسٹرکٹ | |
| فون نمبر 01 : | | فون نمبر 02 | |
| خون کا گروپ | | شادی شدہ غیر شادی شدہ : | |
| شادی کی نوعیت : | (خاندان یا خاندان سے باہر) | بچوں کی تعداد | |
| ماں کا دودھ پلایا | ( ہاں/ نہیں) | | |
| تشخیص کی نوعیت | | | |
| موجود میڈیکل ٹیسٹ ۔ رپورٹس | | | |
| چھاتی کے سرطان سے متعلق کوئی اور طبی مسئلہ | | | |
| خاندان کا اور کوئی متاثرہ فرد یا افراد : | | | |
| آبائی علاقہ | | | |
| میں نے خون کا سیمپل دیا ہے (3ml) | | | |

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