A Case Control Candidate Gene Study on Identification of *P53* Polymorphisms/Mutations in Breast Cancer Patients

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By

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بسم الله الرحمن الرحيم

DECLERATION

I certify that research work titled “A Case Control Candidate Gene Study on Identification of *P53* Polymorphism/Mutations in 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.

Raja Haziq Hasnat

CERTIFICATE

This dissertation, submitted by Mr. Raja Haziq Hasnat to the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the requirement for the degree of Master of Philosophy in Microbiology.

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ABSTRACT

Breast cancer is the most predominant disease and leading cause of death in women worldwide. This disease is responsible for 30% malignancy in females and around 2.3 million new cases are reported globally. In Pakistan, the incidence rate of breast cancer is very high as every 1 in 8 women are at risk of getting this disease. Inherited mutations are responsible for 10% cause of breast cancer specially in tumour suppressor genes like *P53*. Therefore, the aim of the current study was to identify single nucleotide polymorphisms(SNPs)/mutations in *P53* gene and find the association of these mutations with breast cancer. A questionnaire was developed to collect demographic data of cancer patients. Blood samples of cancer patients (n=200) and controls (n=200) were collected for the detection of polymorphisms in *P53* gene. DNA was extracted via phenol chloroform method and amplified by designed primers for the hotspot region of exon 4, 5 and 6 of *P53* gene. Single strand conformation polymorphism detection was done on the variants identified by banding pattern followed by Sanger sequencing. The Sanger sequencing results were aligned with reference sequence (NG_017013.2) retrieved from NCBI, and analysed by using BIOEDIT software and Mutation Taster. Sequencing analysis revealed 2 novel SNPs at chromosomal position chr17:7578423C>GN/A, chr17:7578539T>GN/A and 1 reported SNP at chromosomal position chr17:7578645C>TN/A. In these two novel SNPs, both were non- synonymous in exon 4 and upon *in silico* analysis indicated that these mutations may have interactions with several other genetic factors like CCR2, HIPK1 and AXN1. These novel mutations can change interaction of P53 protein and may have role in breast cancer development and progression. These findings will contribute not only towards research on variations in *P53* gene (tumour suppressor gene) but also possible candidate for development of diagnostic biomarker. Additionally, our findings provide data for breast cancer awareness, campaigns and further research on local population to address the high prevalence that causing a national disease burden.

1.1 Breast Cancer

Breast cancer is defined as the abnormal growth of the breast cells. This abnormal cell growth leads to production of an undifferentiated cell mass or tumour (Hussain *et al.*, 2022). Tumour formation is very common in breast. The disease is characterized by swelling of all parts of breast and lymph nodes, nipples discharging, lump formation on breast surface and breast pain (Koo *et al.*, 2017). Incidence of breast cancer has increased day by day and is marked as the most common cancer in the world (WHO, 2020). Breast cancer incidence varies greatly in different parts of the world, a report from 2018 recorded that the rate of breast cancer incidence is greater in developed countries as compared to low income countries, which was supported by the fact that metastatic breast cancer is more common in European countries as compare to Asian and Western African countries (Wyld *et al.*, 2018). In addition to the incidence rate, the mortality rate is also increasing as study of 2019 from America indicated that the mortality rate is increased about 70% in 2015 as compared to 1995 (Azamjah *et al.*, 2019). This is an alarming situation for the future and hence, timely diagnosis and treatment of breast cancer is utmost important.

1.2 Prevalence

The new cases of breast cancer are increasing globally. According to American Society of Breast Cancer, breast cancer accounts for 12.5% of the total cancer cases(Lehman *et al.*, 2007). Similarly, the rate of acquiring the breast cancer is 30% in females per year. A study of USA estimated the incidence rate was increased by 0.5% in 2017 from 2008. On the other hands in United Kingdom (UK), a study estimated 55,920 cases annually with survival rate of 79% (CDC,2017). Up to year 2020, the overall abundance of breast cancer was highest in Belgium in term of cases per year and lowest in Iran (Lei *et al.*, 2021).

Similarly, the mortality rate due to breast cancer is also increased in the recent times. According to WHO (2020), 68, 500 deaths were recorded globally. A study from USA estimated about 43,250 females with breast cancer will die by end of 2023 (breastcancer.org/facts-statistics, 2023). Similarly, in UK 11,500 women died in a year 2019 with breast cancer (Lei *et al.*, 2021).

Pakistan is hotspot region in Asia with highest incidence of breast cancer disease. According to studies and data, one out of every eight females is at risk of being diagnosed of breast cancer at some stage of life (Ahmed *et al.*, 2021). Additionally, about 34,066 deaths were reported in 2018 which give alarming situation of breast cancer trend in Pakistan. Similarly, in the year 2021 about 2 million cases with 62,700 deaths annually were reported (Khan *et*

al., 2021).Women in Pakistan has high prevalence due to lack of facilities and lack of literacy, which ultimately leads to the late diagnosis of the disease (Shamsi *et al.*, 2020). A report of 2015 reveals that Pakistani females take different alternative medicines, ignore lump pain and have several misconceptions, which lead to increased incidence of breast cancer (Khan *et al.*, 2015). In addition, there are several other causes of breast cancer in Pakistan, which include lack of financial resources, poor medical resources feminine sensitivity, stigmatization and lack of social support making breast cancer treatment in early stage of life difficult (Saeed *et al.*, 2021).

1.3 Anatomy and Physiology of Breast

The pectoralis muscle helps in the development of the base of the breast. The muscle extension is up to the sixth rib of the thoracic region, which is dominant in the early life stages but it can range below the sixth rib in later stages. The nexus of pectoralis major fascia and cooper ligament help in the movement of the breast. The poles of the breast are divided on the basis of gravity which makes the lower pole a slightly fuller. Breast receive the blood supply from subdermal plexus which are dividedly in deeper tissues (Rivard *et al.*, 2018).

Primarily, there are two types of tissues in the breast. Glandular tissues are responsible for the production, storage and passaging of milk via lobules and ducts respectively while, stromal tissues have fatty acids and connective tissues. Additionally, there is a network of lymphatic system (tissues for immunity) for the filtration of breast by removing wastes. Overall breast is composed of several parts which include 15-20 lobes and are surrounded by the nipple. Lobes contain lobules which are tiny and fine tissues particularly for the transfer of milk via ducts. The final collection area is areola, which is supported by nipples and has nine milk ducts. Areola is circular dark coloured area which has additional glands that release oil to protect nipples and skin. There is different networking of lymph vessels, nerves and blood vessel (Susan G. Komen. Breast Anatomy 2020, National Breast Cancer Foundation 2020). Additionally, the organ has with the oestrogen hormone receptor which perform different functions.

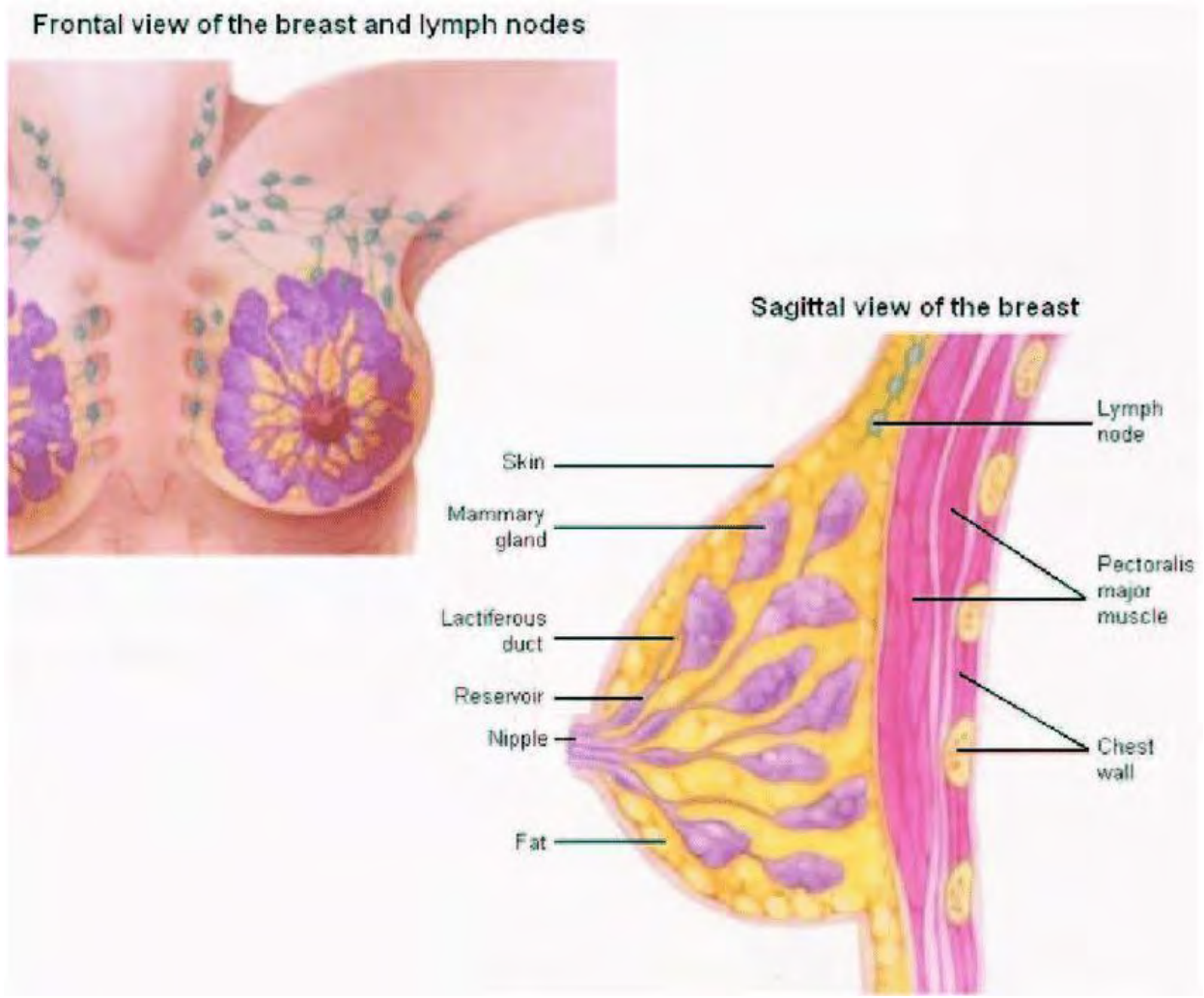

Figure 1.1: Anatomy of Breast (Adapted from Conceicao *et al*., 2011)

1.4 Classification of Breast cancer

Classically, most cancers are benign tumours which are regarded as harmless cancers in the breast. For instance, development of cysts or fibrosis are the example of non-cancerous stage. Most of the carcinomas develop in either duct cells or in lobular cells (Makki, 2015). On the basis of sites, there are several types of breast cancers.

1.4.1 Non-Invasive Breast Cancer

This type of cancer is only confined to ducts and lobules. Non- invasive breast cancer has following two types

1.4.1.1 *In Situ* Lobular Carcinoma

This carcinoma is confined to the milk glands characterized by the abnormal cell growth in the (*in situ*) glands or lobules (Wen *et al.*, 2018).

1.4.1.2 *In Situ* Ductal Carcinoma

This type of cancer is restricted to the milk ducts of the breast tissue(Tomlinson-Hansen *et al.*, 2021).

1.4.2 Invasive Breast Cancer

This type of cancer spread to the fats and tissues through ducts and lobules. Cancer in this case may or may not be spreading to the other body organs and cells (Sharma *et al.*, 2010). Ductal invasive carcinoma is the most common type of invasive carcinoma. On the other hand, lobular carcinoma is less common but effective marker for the diagnosis (Collins *et al.*, 2018).

1.4.2.1 Infiltrating Ductal Carcinoma

This cancer is also known as invasive ductal carcinoma, which is diagnosed in 80% cases of breast cancer cases. This type is characterized with abnormal cell growth in the milk ducts and penetrates in the wall. Another aspect of this cancer is that there is higher probability of growth to other parts of body as well.

1.4.2.2 Invasive Lobular Carcinoma

Invasive lobular carcinoma accounts for 10-15% of total breast cancer cases globally and is characterized by uncontrolled cell division in the lobules (milk glands) and it metastasize to other body parts (Wilson *et al.*, 2021).

1.4.3 Rare types of breast cancer

Additionally, there are also rare types of breast cancer which are as follow:

1.4.3.1 Medullary Carcinoma

This carcinoma is invasive breast cancer that forms a clear boundary between tumour and normal cells (Limaiem & Mlika, 2019).

1.4.3.2 Mutinous Carcinoma

It is a rare type of breast cancer which is characterized by the mucus production in cancer cells (Limaiem *et al.*, 2023).

1.4.3.2 Tubular Carcinoma

This is a special type of infiltrating breast cancer with better prognosis in patients. It accounts for 2% of the total breast cancer cases (Limaiem & Mlika, 2019).

1.4.4 Inflammatory Breast Cancer

In inflammatory breast cancer, there is appearance of inflammation and redness of breasts. This is due to the blockage of lymph vessels in outer part of the breast. It is not common type but is very fast-growing cancer type (Chippa *et al.*, 2020).

1.4.5 Paget Disease of Nipples

This cancer is initiated in the milk ducts and then spread to the skin of the nipples, which is characterized by the pain in the nipples (Ooi *et al.*, 2019).

1.4.6 Phyllotides Tumour

This tumour is developed in the connective tissues of the breast. It can be benign or malignant. It can be treated with help of surgical removal. It is the least common type of the breast cancer, but it is highly studied in the United States (Limaiem & Kashyap, 2019).

1.5 Classification of Breast Cancer on Basis of Molecular Profiling

Breast Cancer can be classification on the basis of different receptor status. These receptors include oestrogen (E), progesterone (P), Human Epidermal Growth Factor-2 (HER-2) and Ki index.

1.5.1 Luminal A

In this type of breast cancer, there is increase expression of oestrogen receptors (ER) and their associated genes, low expression of HER-2 with low Ki-67 (< 14%) or with intermediate Ki-67 (14-19%) and high levels of progesterone genes.

1.5.2 Luminal B

These tumours have high expression of ER gene, low expression of HER-2 gene with intermediate level Ki-67 (14–19%) and low levels of PR with high Ki-67 (≥ 20%). This type of breast cancer has poor progression as compared to Luminal A (Viale *et al.*, 2019).

1.5.3 HER-2 positive

In HER-2 positive, only HER-2 gene is expressed while there is low levels of ER and ER genes expression (Hashmi *et al.*, 2018).

1.5.4 Triple Negative Breast Cancer (TNBC)

The most aggressive type of breast cancer is triple negative breast cancer. It is called triple negative due to absence of the expression of the receptors of three major hormones i.e. ER, PR and HER-2 (Palma *et al.*, 2015). Oestrogen plays an important role in reproduction, reproductive cycle and development of secondary sex characters. There are three major types of oestrogen (E1, E2 and E3), each performing specific functions when these bind with specific receptors (Delgado *et al.*, 2019). These receptors are important in the development of the mammary glands, hence are

important in the breast cancer progression (Bhardwaj *et al.*, 2019). On the other hand, progesterone hormone is responsible for maintaining menstrual cycle and innate immunity (Cable *et al.*, 2020). The PR with ER specifically as ER- alpha is also responsible for mammary gland development. If there is upregulation of the hormone receptor, it causes abnormal cell growth in breast cells and hence, cause breast cancer (Lange *et al.*, 2008). Similarly, HER-2 hormone plays an important role in epidermal cell growth. In case of over expression of the hormone receptor, imbalance of hormone expression results in breast cancer (Albagoush *et al.*, 2019). TNBC accounts for the 15-20% of the total breast cancer with aggressive biology as compared to other types of the breast cancer (Zagami *et al.*, 2022). There is also limited treatment options in this case and is difficult to treat as the receptors for the treatment is blocked. The activity of these hormones varies with the age specially in females, therefore different outcomes can take place.

1.6 Stages of Breast cancer

The American Joint Committee on Cancer (AJCC) has published a staging system based on anatomic findings: tumour size (T), nodal status (N), and metastases (M), which is termed as TNM staging. The eighth edition of the AJCC staging for breast cancer include classification of biomarkers to define prognosis and determine therapy (Giuliano *et al.*, 2018). The Table 1.1 Shows the various stages and their survival rate.

1.6.1 Stage 0

This stage refers to ductal carcinoma *in situ* (DCIS) and lobular carcinoma in situ (LCIS). This stage is an early form of breast cancer that originates in the milk ducts or lobules of the breast.

1.6.2 Stage 1

When cancer ell become activated and invasive, it is termed as stage 1 breast cancer. The size of lump size is 2cm in this stage. If the size is less than 2cm, it is not malignant and regraded as stage 0 but if the size is greater than 2cm then it is termed as invasive (Katz *et al.*, 2001).

1.6.3 Stage 2

In this stage the cells are dividing very fast and enter the lymph nodes under the arms. In this stage, the cancer cell is about 5cm but it cannot penetrate the auxiliary lymph nodes. Stage 2 breast cancer has two subcategories. In stage 2a, the breast tumour

size is between 2 and 5cm without involvement of lymph nodes. But in stage 2b, size of breast tumour is between 2 and 5 cm and involved 4 or fewer lymph nodes

1.6.4 Stage 3

In this stage, the cells continue to divide in the auxiliary nodes and in the breast bone. Additionally, these cells may have the capability to established in other body parts as well.

1.6.5 Stage 4

This stage is metastatic cancer stage. The cells are dividing abruptly and then goes to the surrounding organs as well which are very hard to treat (Mariotto *et al.*, 2017).

Table 1.1 Five years' survival rate based on breast cancer stages (Adapted from National Cancer Institute)

Stages	Five year survival Rate (%)
Localized	99
Regional	86
Distant	30
All stages combined	91

1.7 Grades of Breast Cancer

Grading system of the breast cancer quantitatively shows that how much the abnormal cells are different form normal cells under microscope (Kalli *et al.*, 2018). Additionally, Nottingham histologic score is also used to assess the "grade" of breast cancers. Score giving is based on 3 characteristics, which include tubule formation (tumour structure examination), nuclear pleomorphism (to what extend tumour cells look different from normal cells) and mitosis. On this basis, some scores from 3 to 9 are assigned. On the basis of these scores there are 3 grades of the breast cancers are as fellows;

1.7.1 Grade 1

In this stage, there is not much difference between normal cells and cancerous cell, score of 3 to 5 is given.

1.7.2 Grade 2

In this stage, the cancerous cell and the normal cells are well differentiated with accelerated growth and the score is 6 to 7.

1.7.3 Grade 3

In this stage the abnormal cells and the normal cells are clearly differentiated with increase rate of growth, where cells spread to other site, the score given is 8-9 (Elston, 2005).

1.8 Diagnosis of breast cancer

Advance diagnoses of breast cancer is done by mammogram, Positron Emission Tomography (PET), ultra-sonography and Magnetic Resonance Imaging (MRI) (Bhushan *et al.*, 2021). In mammogram special X ray images are taken to detect uncontrolled cell growth (Sharma *et al.*, 2010). Ultrasonography is also helpful in detection of tumour with help of sound waves to make image of inner side of breast. In PET, the dye is used to detect the lump in the breast. In this technique the dye is injected in the blood of patient, which has affinity for the specific site (Srivastav *et al.*, 2018). In MRI, the inner anatomy of the breast is detected with help of radio-waves (Radhakrishna *et al.*, 2018). However, it is important to note that every person should self-examine to check lump formation in any part of the breast (Pippin *et al.*, 2020).

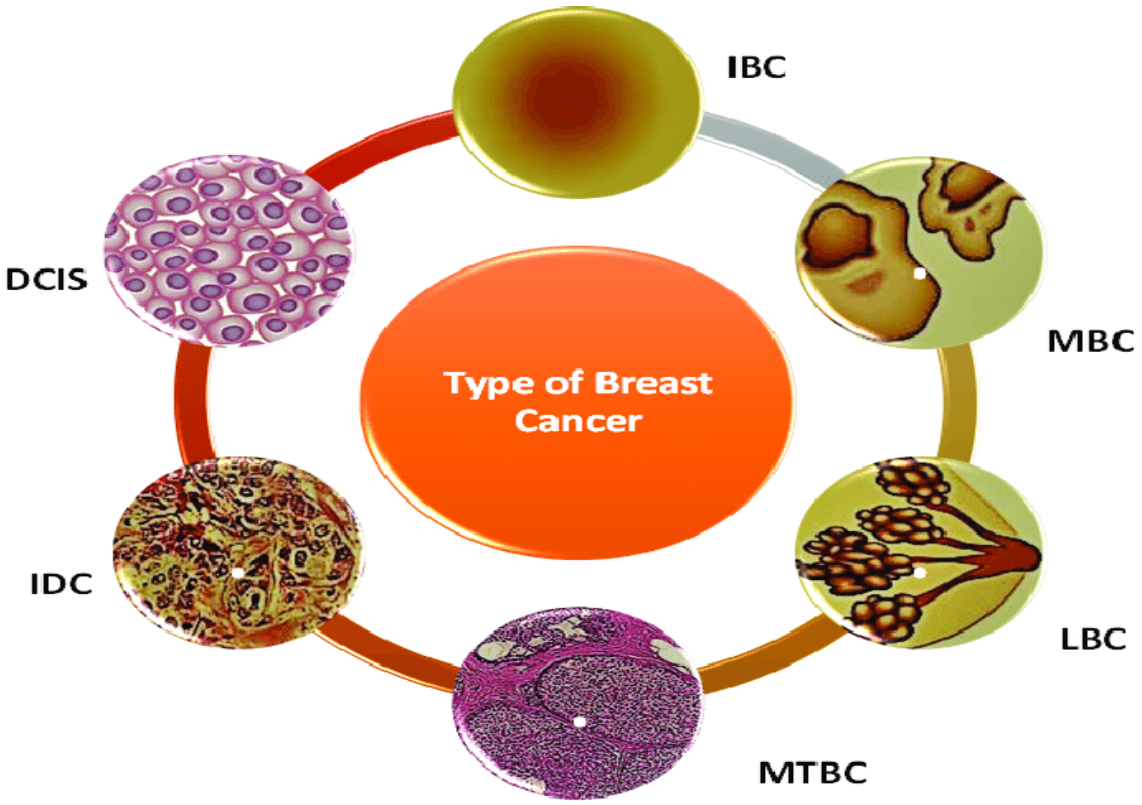

Figure 1.2 Types of breast cancer (Adapted from Fatima *et al.*, 2015)

1.9 Treatment of breast cancer

Commonly, mastectomy (surgical removal of breast), lumpectomy (removal of tumour) and radical mastectomy are used to treat breast cancer in addition to chemotherapy and radiotherapy (Goethals *et al.*, 2022). Chemotherapy is the use of the drugs to treat the breast cancer, which are in addition to surgical methods for better treatment. TNBC cancer is difficult to treat with chemotherapy because all the receptors for the treatment are blocked in this case. Common chemotherapeutic drugs used for the treatment of the breast cancer include trastuzumab, anthracyclines and cyclophosphamide (Hassan *et al.*, 2010). In Western countries, due to advance technologies, breast cancer is early detected. While in low –income countries like Pakistan, mortality rate is very high as compare to incidence due to late diagnosis and treatment (Khan *et al.*, 2021).

1.10 Risk factors of Breast Cancer

Breast cancer is a complex disease and several factors contributes to its aetiology, which include:

1.10.1 Gender

Although, the anatomy of both male and female breasts is similar, the disease is much more common in females as compare to males (Muir *et al.*, 2003). The main physiological change between male and female breast is the presence of lobules and ducts which are well developed in females (Javed *et al.*, 2013) . Additionally, the age of menopause, pregnancy and hormonal changes may be important causative factors for breast cancer in females. Moreover, oestrogen and progesterone play very important role in development of female reproductive cycle, which ultimately increase the risk of breast cancer in females due to their higher production (DeSantis *et al.*, 2015). Thus, females are at higher risk of acquiring breast cancer. According to WHO 2021, 1% males acquire breast cancer while in females, the rate of getting breast cancer is about 14% globally.

1.10.2 Family history

Patients with family history of the breast cancer and other comorbidity are at higher risk of getting breast cancer. It is an risk as once the genetic makeup is changed, it can be transmitted to progeny and family (Braithwaite *et al.*, 2018).

1.10.3 Hormonal activity

Change in menstrual cycle, early pregnancy or hormonal replacement therapy, can cause breast cancer (Khalis *et al.*, 2018). Oestrogen is the key hormone, which can play an

important role in the progression of the breast cancer. Additionally, progesterone and other hormones can also cause cancer both in healthy and old age women (Jerry, 2007).

1.10.4 Life style and dietary causes

Lack of exercise, high intake of fats and obesity specifically in post-menopausal women are other high risk of getting breast cancer. Alcohol consumption is also a major cause of the breast cancer. Breast feeding can reduce the probability of this cancer (Kamińska *et al.*, 2015).

1.10.5 Environmental factors

It has been studied that specific area may have specific rate of breast cancer cases. High radiation area people have higher risk of getting the breast cancer (Singletary, 2003). Some studies have revealed that organochlorides as high risk factor for those cancer as are organic pollutants. Metal ions like cadmium, copper, cobalt, tin *etc.* can increase the oestrogen receptors which activate robustly breast cell division. Similarly, there is high risk of getting breast cancer if any person is exposed to strong electromagnetic radiations. Additionally, solar radiation and sun exposure time can also cause increase in breast cancer development (Strumylaitė *et al.*, 2010).

1.10.6 Genetic Factors

Additionally, genes also play a major role in the disease development as any mutation in gene can lead to uncontrollable cell divisions. A very extensively studied *BRCA1* and *BRCA2* genes over years show that mutations in *BRCA1* and *BRCA2* have role in breast cancer development (Mehrgou *et al.*, 2016). Main role of these genes is to help in repairing of DNA but are prone to alteration at very fast rate. Moreover, *PTEN, ATM, CHEK2*, *PALB2* and *P53* mutations are observed extensively in male and females breast cancer.

1.11 Role of variant *P53* gene in Breast Cancer

P53 gene is the tumour suppressor gene, which make *P53* protein. This protein function as tumour suppressor in developing tumour cells. This gene is responsible for mitosis, apoptosis and growth of the cells under normal conditions. Mutations in *P53* are very common and can lead to development of several cancers for instance, cervical cancer, lung cancer and myeloma progression (Rivlin *et al.*, 2011). However, role of *P53* in breast cancer development and progression is of great interest as any mutation in this gene can cause the abnormal cell growth. Breast cancer is a physiological disease and mutations in gene so *P53* mutations can trigger the breast cancer formation (Talib *et al.*, 2018). Globally, *P53* is

extensively studied in breast cancer development but in Pakistan limited research is conducted to-date regarding the role of mutations in *P53* in breast cancer, where breast cancer is highly prevalent. Therefore, the purpose of the study is to decipher the role of mutated *P53* gene in breast cancer development among Pakistani population.

Aim and Objectives

The aim of the current case-control study was to detect variants in *P53* gene in breast cancer patients and healthy individuals with objectives mentioned below:

- Collection of blood samples from breast cancer patients visiting Pakistan Institute of Medical Sciences (PIMS) and Swat Institute of Nuclear Medicine Oncology and Radiology (SINOR) along with samples from healthy individuals as controls from local population of Pakistan.
- Collection of demographic and clinical data of patients and healthy controls to determine the prevalence rate of distinct types of breast cancer and their association with different risk factors.
- Detection of Single Nucleotide polymorphisms or mutations in *P53* gene in breast cancer patients and healthy controls to determine its susceptibility of breast cancer.

2.1 Genetic factors Associated with Breast Cancer

Breast cancer is a complex disease which is dependent on many factors especially the genetic factors, genetic alterations in different genes have been documented in the development and proliferation of breast cancer. There are several oncogenes which promote breast cancer, for example Proline rich 14 (PRR14) protein is a component of the nuclear envelope which contributes to breast cancer by its overexpression. This over expression lead to increase in level of transcription and gene amplification (Ren *et al*., 2020).

The oncogene-induced replication stress is phenomenon which induces aggressive breast cancer especially the TNBC by aberrant expression of oncogenes and tumour suppressor genes (Llobet *et al*., 2022). Beside the oncogenes, mutations in tumour suppressor genes also play a key role in the activation of breast cancer tumorigenesis. *BRCA 1* and *BRCA 2* genes are well known breast cancer causing genes, which are highly penetrated genes of breast cancer. Similarly, *CDH1*, *PTEN*, and *STK11* are the other genes involved in the aggressive breast cancer development. There are several genes like *ATM*, *PALB2*, *BRIP1*, or *CHEK2,* which interact with tumour suppresser genes to cause moderate level of breast cancer. Moreover, DNA repair gene like *XRCC2* is associated with high risk of breast cancer (Łukasiewicz *et al*., 2021). Out of these genes, *P53* is a very important gene as it is a tumour suppressor and mutations in it can lead to development of breast cancer (Figure 1).

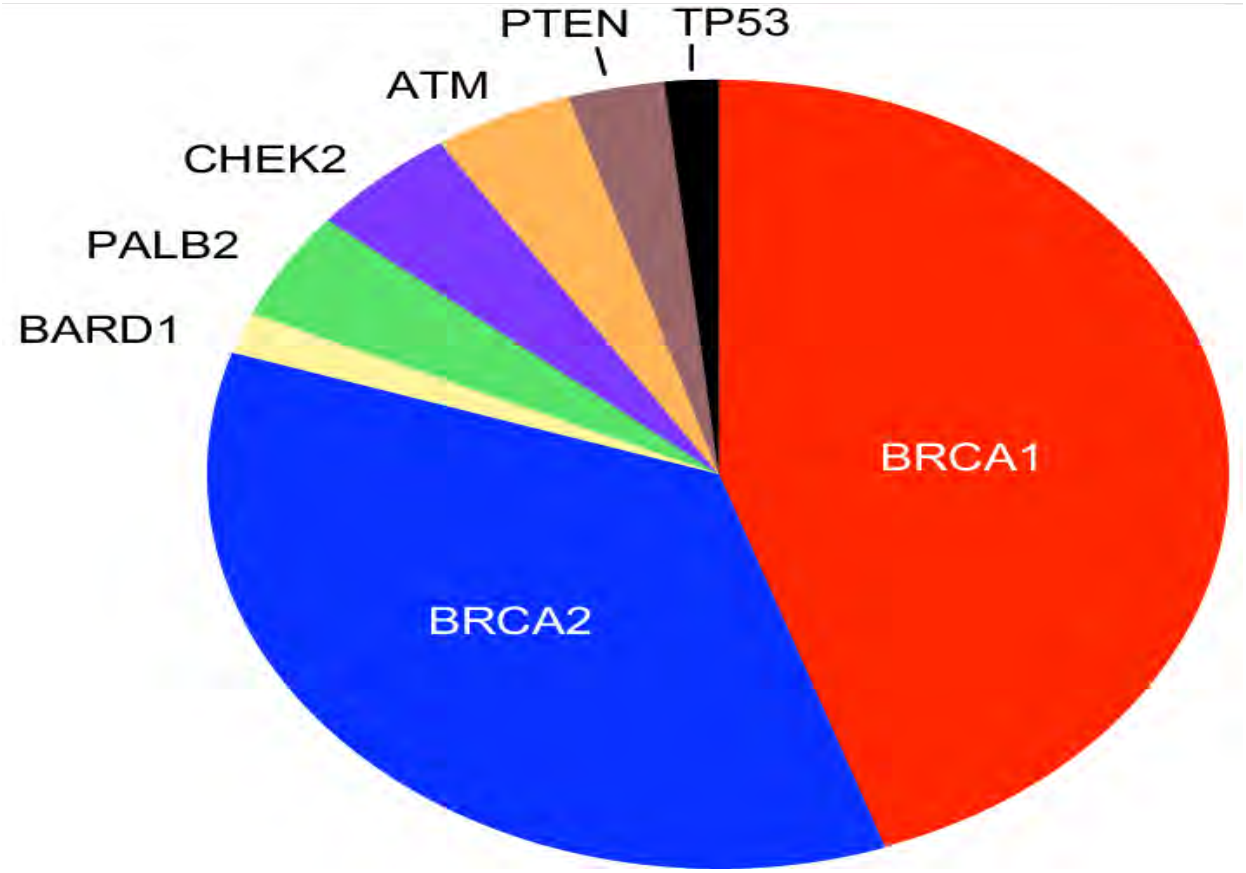

Figure 2.1*:* Association of genetic mutation in different genes with breast cancer (Adapted from Churpek *et al.*, 2015)

2.2 *P53* gene

P53 (or *Tp53*) gene is called tumour suppressor gene and is located at chromosome 17 typically at the short arm (Kern *et al.*, 1991). Overall, there are 11 exons and 10 introns in *P53* gene, which transcribe tumour suppressor protein P53 (Hamzehloie *et al.*, 2012). This protein is 53 kDa in size with 393 amino acids which is composed of different domains (Choi *et al.*, 2009). This gene is important in several biological processes like regulating cell division and cell cycling, any change in any of its domain or amino acids can alter the function or structure of the protein (Ozaki *et al.*, 2011). The expression of the *P53* gene plays a crucial role in cellular maintenance. When a cell encounters stress or sustains injury, the *P53* gene expression makes a protective response by triggering apoptosis – a tightly regulated process of self-destruction (Aubrey *et al.*, 2018). Hence, the insight details of *P53*gene and mutation are very important to study in various diseases and cancers.

2.3 Biological Functions of *P53* gene

2.3.1 Role as a Transcription Factor

In some studies, *P53* is regarded as transcription factor which maintain the integrity of a dividing cells primarily as a tumour suppressor (Beckerman *et al.*, 2010). *P53* is activated via phosphorylation cascade, where several post translation modifications occurs under some cellular or oncogenic stresses which ultimately trigger activation of many target genes involved in DNA repair (Joerger *et al.*, 2016). One of the important function of *P53* is its role in senescence, hence it can be said to be aging factor. *P53* induce senescence by activating expression of P21 or senescence associated beta galactosidase (SA-ß-gal) (Mijit *et al.*, 2020). *P53* can switch off senescence if DNA is damaged and repair it, so *P53* has dual function which is very helpful in maintaining the normal functioning of the cell (Serrano, 2010).

2.3.2 Role in Angiogenesis and Autophagy

P53 also have pivotal role in angiogenesis and autophagy. *P53* play dual role in both activation and deactivation of these processes depending on the cell conditions and situations (Rahman *et al.*, 2022). For instance, for the growth and survival of the tissues, cell angiogenesis (process of formation of the blood vessel) is very important. In some case, it can activate several angiogenetic factors like vascular endothelial growth factor (VEGF) which promote the angiogenesis process (Farhang *et al.*, 2013). While in cell repairing, *P53* promote the activation of anti-angiogenetic factors which switch off this process (Rivlin *et al.*, 2011). Such dual role is present in the autophagy as well (White, 2016).

2.3.2 Role in Activation of Signalling pathways

The pathway followed by P53 can also play very important role in the activation of several genetic pathways as well. For example, P53 can induce the expression of E2F Transcription Factor (E2F1) which help in the cell cycling and damage repairing (Hershko *et al.*, 2005). Similarly, P53 regulates many important transcription factors like Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells (*NF-κB*) and Signal Transducer and Activator of Transcription 3 (STAT-3) of the inflammation and immune response (Shi *et al.*, 2021). Moreover, P53 is very helpful in activating several other signalling cascades like Mechanistic Target of Rapamycin (mTOR-2) pathway which regulates the normal metabolism of the cell (Soliman, 2013). Overall, TP53 is very vital in maintaining the cell homeostasis and balancing (Soliman, 2013) .

2.4 Structures and Functions of Domains

P53 as a transcription activator has a functional configuration of homo tetramer. Generally, the domains of this form are intrinsically disordered N terminal transactivation domain 1 and

2 (TAD 1 and 2) along with proline rich domain. With the help of linker, DNA Binding Domain and tetramerisation domains are present. At the extreme terminal is an intrinsically disordered Carboxy Terminus Domain (CTD) is present, this genomic region encoding this domain has hotspot region for mutations. Due the intrinsic disordered structure, the signalling is easy and effective via conformational adaptability with ease of interactions (Joerger *et al.*, 2010) . P53 exists in several isoforms, so the overall structure of the domains varies different functions (Figure 2.2), some of the important domains are mentioned as follow:

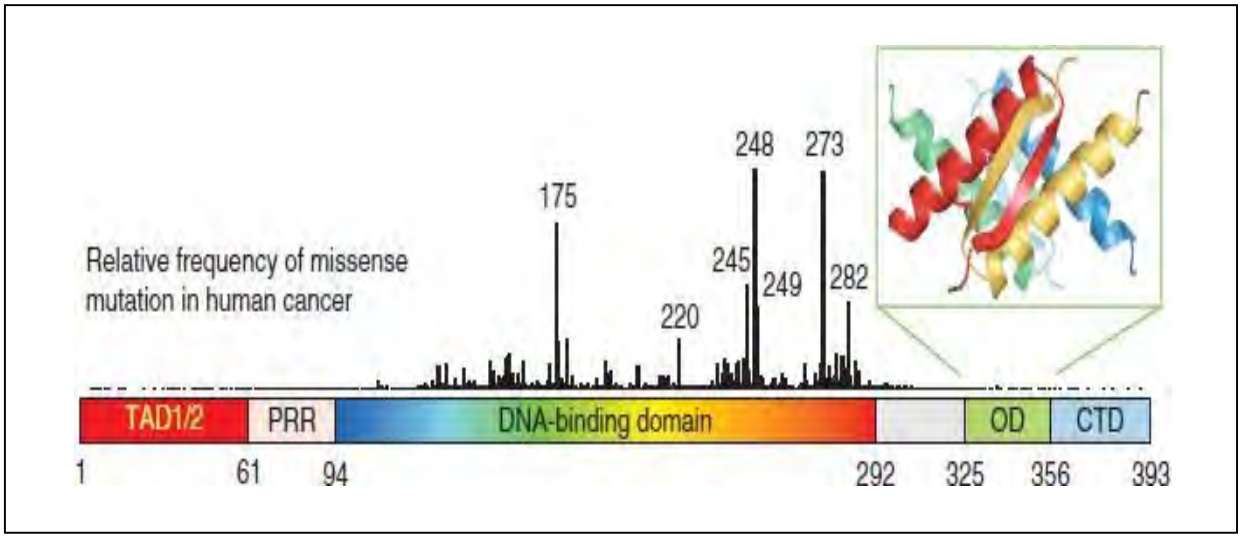

Figure 2.2 Various domains of P53 (Adapted from Joerger *et al*., 2005)

2.4.1 DNA Binding Domain (DBD)

The role of T*P53* is highly dependent on the DNA binding activity. DNA binding domain is used to anchor *P53* protein on specific region and maintain DNA RNA interaction. P53 proteins as tetramer complex bind to the double helix DNA via specific binding sites that consists of two motifs with specific sequence of 13 base pairs (Cho *et al.*, 1994). The tetramer configuration of P53 is maintained by tetramerisation domain. The structure of DNA binding domain consists of 94-292 amino acids and is composed of central immunoglobulin domain and essential elements that make DNA binding surface. DNA binding surface consists of loop sheet helix motifs covered by two large loops (Cañadillas *et al.*, 2006). Zinc ion is mainly present in these loops that is used for the stabilization of the loops and also helps in tetrahedral interaction of the cysteine and lysine amino acids (Wang *et al.*, 2007). With the help of these loops the DBD easily bind to the major groove of the DNA which help in the correct binding to specific sequences. Some studies reveal that P53 might also have another loop (L3) which help in the binding of the protein with minor groove of the DNA via

arginine motif. The binding of DBD with the specific DNA sequence requires the electrostatic interaction (Joerger *et al.*, 2010).

DBD is thermodynamically very unstable and unfolds rapidly at body or higher temperature. This rapid unfolding can be very helpful in controlling gene expression binding to specific region and regulation of the gene with aid of ubiquitination (Friedler *et al.*, 2003) . However, if DBD get mutated , it can lead to cancer development (Niskanen *et al.*, 2013).

2.4.2 Tetramerisation Domain

The oligomerisation and normal configuration of the P53 protein is retained due to tetramerisation domain, which spans from amino acids 325 to 355 (Joerger *et al.*, 2010). With help of crystallography technique, the structure of this domain was determined. It consists of beta strands and alpha helix in configuration. Glycine act as a bridge between these two structural elements (Mora *et al.*, 2008). When P53 form a tetramer configuration, it can undergoes a transformation into dimeric form first which is proved via several *in vitro* experiments (Nicholls *et al.*, 2002).

Thermodynamically, this domain is relatively more stable than DNA binding domain and this this region is less susceptible to mutation (Mora *et al.*, 2008). However, the complex architecture of this domain can up or down regulate the overall *P53* activity. For instance, in case of pre-apoptosis, genes may bind to *P53* and inhibit the tetramerisation of the protein which promotes its nuclear export. Hence, these domains are very helpful in the signalling of *P53* (Borrero *et al.,* 2021). This signalling regulates the network of proteins for the cell structure and physiology. Therefore, any alteration in this pathway can be very dangerous in terms of cancer development of other diseases.

2.5 *P53* Signalling Pathway

Normally, *P53* gene is activated in various cellular stress like DNA damage and repairing even in oncogenesis. P53 protein play role in various cellular functions including cell cycling, apoptosis and DNA repairing by regulating the transcription (Vousden *et al.*, 2009).

Normally, the gene is active due to negative feedback mechanism by Mouse Double Minute 2 protein (MDM-2). The P53 is regulated and stabilized with help of ubiquitination which takes place in different steps involving E1, E2 and E3 proteins/enzymes. E3 step of ubiquitination is the most important step in which MDM-2 serves as an ubiquitin ligase. The function of this ligase is to recognize the specific sequence in of P53 and form a poly-ubiquitin chain, hence it is later degraded by specific proteases. The protease destruction of the protein helps in the negative regulation of P53 protein because it leads to the transcriptional repression of *P53* (Chène, 2003) (Brooks *et al.*, 2011).

Under normal condition in cell, the MDM-2 keep tight regulation of P53 and keep it inactivated but under several emergency and drastic responses in the cells like cell death or DNA damage, MDM-2 gets deactivated (Li *et al.*, 2015). On the activation of P53, the P53 binds to the specific promotors of the target genes, which can lead to the activation or repression of the gene depending on the cellular environment and scenario. During DNA repairing, it is important to stop the cell cycle, where P53 induce the expression of a regulator gene cyclin-dependent kinase inhibitor (*CDKN1A)* which further activate the cell cycle inhibitor *p21 (*Li *et al.,* 1994*).* On the other hand, the *P53* gene act as pro-apoptotic genes which upregulate modulator of apoptosis (*PUMA*) for apoptosis (Li, 2021) as shown in (Figure 2.3).

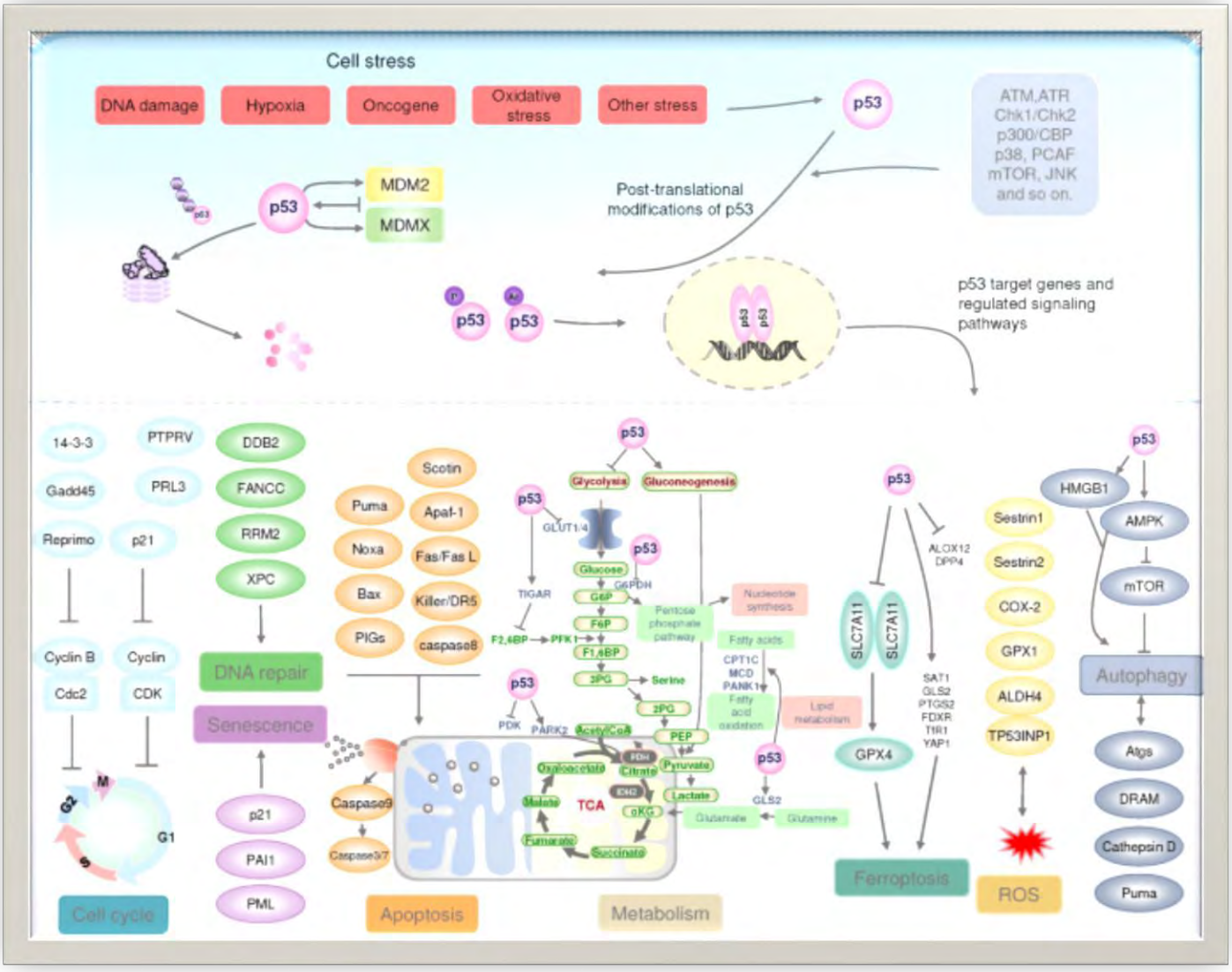

Figure 2.3: *P53* Signalling Pathway (Adapted from Wang *et al.,* 2023)

Overall, *P53* gene is an important gene which maintain integrity and normal functioning of cell. However, if there are any mutations in gene or the protein, it may lead to downregulation or upregulation of the gene or the protein expressions, which ultimately lead to the development and progression of the cancer.

2.6 Role of *P53* in Development of Different Cancers

P53 gene being the "guardian of the genome", is very helpful in maintaining the check and balance of cells. However, altered gene or protein is very risky as it can cause the development of different cancer in humans (Olivier *et al.*, 2010). Mutations in *P53* can cause loss of tumour suppression activity and can make it unable to rectify the uncontrolled cell division. *P53* inactivation can be caused by various genetic alterations like single-base substitution or loss of alleles for proper functioning. Additionally, it can be inactivated by viral or cellular proteins as they perform key role in development of specific cancers (Tommasino *et al.*, 2003). Additionally, *P53* gene is involved in the viral life cycle once they entered the host for instance it involves in the life cycle of Influenza virus, Smallpox Virus, Zika virus, Human Immunodeficiency Virus Type 1, Human Herpes Simplex virus-1 *etc*. These viruses require activity of *P53* for efficient viral replication while some virus require inactivation of *P53* activity (Aloni-Grinstein *et al.,* 2018). If the mutations in *P53* are inherited or its activity is reduced, it causes early-onset of tumours like breast cancer, sarcomas, brain tumours or different syndromes like the Li-Fraumeni (LFS) and Li-Fraumeni-like (LFL) syndromes (Olivier *et al.*, 2003). *P53* has highly variable coding and noncoding regions which can increase the cancer susceptibility or modifications in different cancer phenotypes (Whibley *et al.*, 2009). Altered *P53* expression can even cause cancer progression due to resistance to chemotherapy (Hientz *et al.*, 2017). It is also very important to note that mutant *P53* can be involved in the development of different cancer by enhancing signalling through Transforming Growth Factor (TGF-ß) or Epidermal Growth Factor (EGF) as shown in Figure 2.4(Elston *et al.*, 2012). In addition, mutant *P53* activate many other factors like integrin cycling and growth receptors to increase the cell proliferation and uncontrolled division (Alvarado-Ortiz *et al.*, 2021). Similarly, mutant *P53* interactions with other important pathways deactivate apoptosis and autophagy. For instance, *P53* may involve with the activity of several proteins like MRE11- Rad51-NSB complex, p73, and SP-1 to induce genetic instability, chemo resistance, and metastasis. Moreover, mutant form of this gene effects the expression of hematopoietic and mesenchymal stem cell progenitors resulting in tumours (Muller *et al*., 2014).

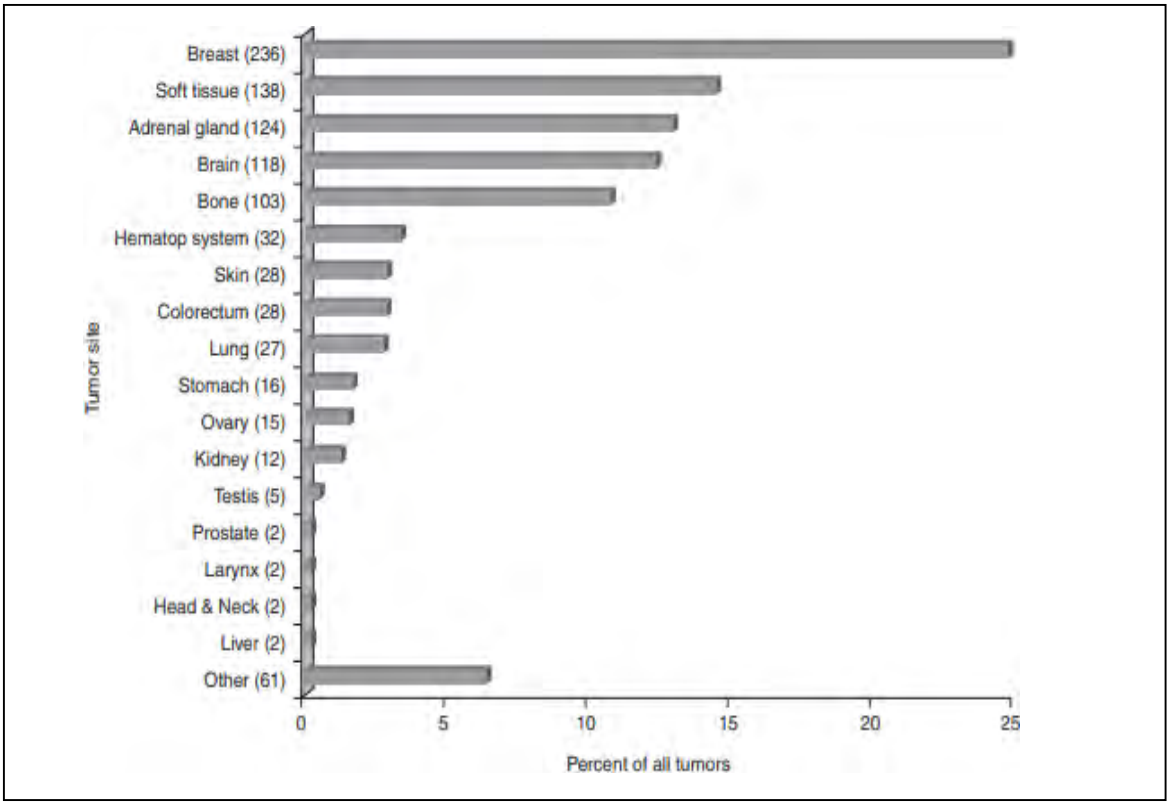

Figure 2.*4:* Different types of cancer associated with *P53* Mutations (Adapted from Olivier *et al*., 2010)

2.7 Impact of Mutations on *P53* Gene

DBD encoding genetic region is usually prone to different mutations and this domain play a central role in the interaction of P53 protein with the double helical DNA. Most mutations are found in the amino acids, which are involved in making interaction with the DNA or making support for binding surface. Altered proteins after mutations are classified as contact proteins (R248 and R273) and structural proteins (R175, G245, R249, and R282). This classification is on the basis of NMR spectroscopy and X- ray crystallography. Notably, these mutant proteins have the same structure of the domain but the its activity may change. For instance, if any hydrophobic side chain is introduced in the base pair of the protein, it may prevent the DNA binding. Additionally, proteins responsible for the zinc binding may affect the central zinc motif and thus may destabilize the whole domain. Moreover, some conformational changes are also observed in the domain after the mutations in protein scaffold (Olivier *et al.*, 2010; Joerger *et al.*, 2005; Cho *et al.*, 1994).

P53 normally regulates expression of target genes after binding to specific sequence of response elements in the regulatory regions of the target genes. This targeted sequence is somehow similar across various genes to which *P53* binds. Mutations may alter the *P53*

binding to these specific regions and *P53* may bind to other sites, causing alteration in targeted gene expression. Due to this feature, the targeted genes may be up or down regulated. Similarly, mutant *P53* proteins may get aggregated in the nucleus of cells, particularly in metastatic cancer cells where these cause an oncogenic effect. This process of altered functioning after mutation is called "gain of the functioning" where the mutated gene gains new functions and activities. Such mutations in gene may cause change in the overall functioning of the cell which have drastic effect on the cells and cellular environment making it more conducive for oncogenesis (Inga *et al.*, 2002 ; El-Deiry *et al.*, 1992).

Cancers caused by *P53* are mainly due different types of mutations which includes frameshift or nonsense mutations or missense mutations. The specific types of *P53* mutations, along with their sequence, position, and structural changes, contribute to the development of different types of cancer *e.g*. lung cancer, colon cancer, ovarian cancer and breast cancer. However, *P53* mutations have key role in the development of breast cancer with higher ratio as compared to the other cancers as mentioned in (Figure 2.4) (Hinds *et al.*, 1990; Brachmann *et al.*, 1996).

2.8 SNPs of *P53* and Cancers

There are different SNPs reported in different regions of *P53* which leads to the tumour generation and proliferation. One of the important SNP in *P53* gene is reported in the codon 72 (rs1042522) of exon 4 of *P53* which is common Caucasian Americans and African Americans (Huszno *et al.,* 2018). This SNP alters activity and expressions of *P53* gene (Pim *et al.*, 2004). Moreover, some studies reveal that some non-coding variants may have role in the progression of cancer and its susceptibility (Huang *et al.*, 2020). One of the major non coding SNP is rs78378222 which was reported in 2011, specifically in population of Iceland. This SNP is cause conversion of polyadenylation signal of *TP53,* and is primarily located in the 3-UTR region. The overall impact of this mutation was found to be the reduction of P53 expression (Wang *et al.*, 2016). Additionally, later in 2013, 128 SNPs was reported in UTR region of lymphoma patients which showed that *P53* mRNA level reduction (Li *et al.*, 2013). Moreover, another reported polymorphism was intron 3 duplication (rs17878362) which was associated with high risk of cancer susceptibility. Also, some major SNPs were reported in intron 4 were rs1794287 and P47S (rs1800371) that are involved in apoptosis (Huszno *et al.,* 2018).

P53 stability is also effected due interaction with different other gene. MDM-2 in this regard is very important as MDM-2 promoter is important in the activation of *P53* gene and any mutation in this region cause disruption of transcription in *P53*. In this regard, an important

SNP in the promoter of MDM-2 is rs2279744, which is also involved in different cancers and most importantly in breast cancer (Huszno *et al.,* 2018). Additionally, another important SNP was reported in African population, which is primarily change 47th codon of *P53* (rs1800371). This SNP was found to be involved in the disruption of phosphorylation of serine via proline kinases (Doffe *et al.*, 2021). This mutation is further involved in the hepatocellular carcinoma which was confirmed from different mouse models (Jennis *et al.*, 2016). Additionally, this variant is associated with increased risk of breast cancer in African women as well (Felley-Bosco *et al.*, 1993). Several demographic factors as well as some ethnic diversity contributes to the risk of specific *P53* mutations, which can then increase the susceptibility to various types of cancer like human like breast cancer, basal cell carcinoma, prostate cancer, glioma and colorectal adenoma etc. (Doffe *et al.*, 2021)

2.9 Role of *P53* in Breast Cancer Progression

P53 gene can play important role in breast tumours and is responsible for the aggressive type tumour progression typically triple negative breast cancer. once the gene gets altered, the whole pathway of gene gets change. For instance, *P53* is responsible for the DNA damage and repair. If it gets mutated, it loses function of DNA damage and repair, ultimately lead to the accumulation of faulty DNA. Alteration in *P53* cause increased cell proliferation which is normally arrested in case of DNA repair by *P53*. The cell proliferation can increase the risk of getting breast cancer. Another reason for the breast cancer development and aggressiveness is metastasis and invasiveness. Mutated *P53* facilitate invasiveness and metastasis in breast cancer. Additionally, chemotherapy resistance can play important role in the progression of breast cancer. Mutated *P53* can promote drug resistance by changing drug delivery pathway and transport. Therefore, mutations in *P53* gene have role in breast cancer progression (Lehmann *et al.*, 2014; Freed-Pastor *et al.*, 2012; Olivier *et al.*, 2010).

2.10 SNPs reported in *P53* in Breast cancer

As discussed earlier, *P53* play a key role in the prognosis and increase risk of breast cancer in humans. Besides somatic mutations, there are also junk line mutations which can be causative of breast cancer in humans (Shahbandi *et al.*, 2020). More than 200 natural SNPs cause *P53* dysfunction either in DNA binding site or transactivation domains (Hafner *et al.*, 2019).

In Russian population, breast cancer patients have variant (rs1625895), which is involved in a *P53* dysregulation. Another population based case control study from USA showed that rs1625895 polymorphism has huge impact on invasive breast carcinoma among women aged 50 or in younger ones (Assad *et al.*, 2019).

In Pakistan, different germ line mutations are detected in *P53* in case of breast cancer and Li-Fraumeni syndrome. It has been studied that these mutations are more common in female as compared to males (Rashid *et al.*, 2012). Additionally, inactivation of *P53* gene is trigged by polymorphism in exon 4 and intron 6 which is common in northern areas of Pakistan and promote breast cancer (Khaliq *et al.,* 2000). Another important SNP in *P53* gene is rs1042522 polymorphism which was found to be associated with breast cancer risk in population of Khyber Pakhtunkhwa, Pakistan (Khan *et al.,* 2023). Moreover, in exon 5,8 and 3' UTR region of *P53* gene, frame shifts and silent mutations and various SNPs have been reported in breast cancer patients from different areas of Pakistan (Sadia *et al.*, 2022). Both germ line and somatic mutations have been detected from the Pakistani population.

2.11 Hotspot Regions of *P53* gene in Breast Cancer

P53 plays a vital role in the progression and development of the breast cancer and several mutations are reported in it. However, there are several hotspot regions which are very significant in the development of breast cancer. For instance, several studies have reported that majority of SNPs in case of breast and ovarian cancer are majorly composed of missense mutations primarily located in exons 5–8, which are located in DNA-binding domain of the protein *P53*. Another study on *P53* reported mutations in the central DNA-Binding Domain having several hotspots such as R175, G245, R248, R249, R273 and R282 (Marei *et al.*, 2021). Additionally, another study reveals that most of the *P53* mutations are located at exons 5–8, that code for DBD. Mostly these mutations are missense mutations. Moreover, different hotspot codons responsible for DBD are 175, 213, 245, 248, 273, and 282 which are responsible for at least 2% of all mutations (Bai *et al.*, 2021). Similarly, a study shows that *P53* mutations are located predominantly in the DBD encoded by exon 4-9, which results in the inactivation of *P53* gene and protein (Rivlin *et al.,* 2011). These studies highlight the important mutations in hotspot regions which are primarily located in the DBD located in exons 5-8.

3.1 Study Design

A retrospective, case control study was designed to identify mutations and single nucleotide polymorphisms (SNPs) in *P53* gene among breast cancer patients and healthy Pakistani local population.

3.2 Collection of Blood Samples

In current study, blood samples (3mL) of breast cancer patients were collected from Pakistan Institute of Medical Sciences and Swat Institute of Nuclear Medicine Oncology and Radiology. Whereas, control samples from healthy individuals were collected from local population. A consent was taken from patients and controls as per World Medical Association (WMA), Declaration of Helsinki (Appendix I). The samples were collected in ethylene diamine tetra acetic acid (EDTA) tubes via sterile syringes and then transported in ice box to Molecular Medicine Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad.

3.2.1 Sample Size

The present study recruited 200 breast cancer patients and 200 healthy individuals to detect *P53* genetic variations. The information about the history and medical status of the breast cancer was analysed via pre designed structured questionnaire. The demographic information included name, age, occupation, breast feeding and marital status, number of children, family history. Clinical data was collected through mammography, ultrasound and immunohistochemistry (IHC) reports.

3.3 Inclusion Criteria

Individuals with diagnosed breast cancer were considered as patients while individuals having without any disease history were taken as controls.

3.4 Exclusion Criteria

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

3.5 Extraction of DNA from Blood Samples

DNA was extracted from blood of both patients and controls samples via phenol-chloroform method in two consecutive days. The solutions used are enlisted in Table 3.1 and Table 3.2.

Solutions used in this organic method had following functions:

1. Sodium Dodecyl Sulphate (SDS) is a strong detergent used for the cell membrane disintegration
2. Ethanol is used for precipitation and washing of extracted DNA
3. Solution A is used to improve the permeability of cell membrane
4. Solution B is used for the precipitation of DNA
5. Solution C is used for removal of DNA associated proteins
6. Solution D is used for disruption of the cell membrane
7. $MgCl_2$ provides the protection of DNA from the DNase proteins
8. Proteinase K is used for general protein degradation in cell

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 (0.1M and pH 8.8)	EDTA	73 mg	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)	27.63g	150	250
		Tris (10 mM)	0.303g		
		$MgCl_2$ (5 mM)	0.254g		
		Triton X 100	2.5mL		
2	Solution B	Tris (10 mM)	0.364g	200	300
		NaCl (400 mM)	7.02g		
		EDTA	0.1752g		
3	Solution C	Tris HCL (10Mm)	0.605g	_____	500
		Phenol	500 µL		
4	Solution D	Chloroform	20 µL	_____	500
		Iso-amyl Alcohol	480 µL		

Procedure

Day 1

- In 1.5mL Eppendorf tube, 750µL of blood sample was collected.
- Solution A (500 µL) was added into Eppendorf tubes and was suspended in inverted position for 15 minutes at room temperature.
- The tubes were centrifuged at 13,000 rpm for 5 minutes to separate pallet and supernatant.
- Supernatant was discarded and then pellet was re-dissolved with solution A (500 µL via vortex.
- These tubes were centrifuged again for 5 minutes at 13,000 rpm.
- After the removal of supernatant from pallet, 400 µL of Solution B was added.
- Additionally, 15µL of 20% SDS solution and 4µL of proteinase K were added in the Eppendorf tubes.
- The samples were then kept in the incubator for 24 hours at 37 ^{0}C.

Day 2

- Equal amount of Solution C+D (250 µL each) were mixed.
- Solution C+D (500µL) was added in the incubated tube.
- The labelled tubes were then centrifuged at 13,000 rpm for 10 minutes.
- Supernatant was collected in new tube.
- Afterwards, solution D was added (500uL) and then the tubes were centrifuged at 13,000 rpm for 10 minutes.
- Supernatant was again added in a new tube after separation from pellet
- Then 55 µL of 20 % SDS and 500 µL of cooled isopropyl alcohol was added in the tubes and placed in inverted position for few minutes.
- The tubes were centrifuged at 13,000 rpm for 4 minutes.
- After centrifugation, supernatant was discarded and 400 µL of 70% ethanol was added to the pellet.
- The tubes were washed via centrifugation for 3 minutes at 13000 rpm.
- After centrifugation, ethanol was discarded and pellet was left for air dry at room temperature.
- Then, Tris EDTA (100 µL) was added to preserve the extracted DNA.
- Finally, Eppendorf tubes having extracted DNA were stored at -20^{0}C in freezer (Table 3.1).

Horizontal Gel Electrophoresis for Extracted DNA

After DNA extraction, the extracted DNA was checked via horizontal gel electrophoresis. For this purpose, 1 % agarose gel was used (Table 3.2). For making 1%, following steps were performed:

1. Agarose power (0.5 g) was weighed using mini electronic balance which was then poured in the flask.
2. TBE buffer of strength 1X (50mL) was added up to in the flask. For proper mixing and to obtain clear solution it was heated in microwave.
3. The clear solution was kept for cooling at room temperature for 20-25 minutes.
4. Ethidium bromide (2 µL) was added in the solution.
5. The gel was poured in the gel tray for solidification and combs were inserted at the appropriate positions.

6. When the gel was solidified, it was then transferred to gel tank which contained 1X TBE as a running buffer.
7. Extracted DNA samples (2µL) were mixed with bromophenol blue and loaded in the wells of gel.
8. Gel was run for 30 minutes at 120V.
9. At the end, gel was visualized under UV gel documentation system.

Table 3.3: Preparation of Bromophenol Blue

Sr. No.	Chemicals	Amount (g)	Distilled water (mL)	Final volume (mL)
1	Bromophenol Blue	0.05	20	50
2	Sucrose	8.0		

Table 3.4: Protocol for 10X TBE for Gel Electrophoresis

Sr. No.	Chemicals	Amount (g)	Distilled water (mL)	Final volume (mL)
1	Tris Base	27	220	225
2	EDTA	2.325		
3	Boric Acid	14.5		

3.6 Primer Designing

The reference sequence of *P53* gene, NG_017013.2 was retrieved from National Centre for Biotechnology Information (NCBI). Primers for exons (4,5,6) of *P53* gene were designed by using primer 3 web (version 4.1.0). These primers were cross checked with Ensemble (https://asia.ensembl.org/), UCSC genome browser (https://gnome.ucsc.edu/) and Oligocalc software (Kibbe,2007) for specific binding with exon region, hairpin formation and annealing temperature.

3.7 Polymerase Chain Reaction

For the detection of the genetic variation in *P53* gene, Polymerase Chain Reaction (PCR) was performed to amplify the exons 4,5 and 6. The amplification of extracted DNA samples was done with the help of designed primers (Table 3.5).

Table 3.5: List of primers for the amplification of various exons of *P53*

Primers	Sequences	Base pairs	Tm (°C)	Amplicon size (bp)
E4&,5 F	GCAATCCCAGCACTCTCAAA	20	58.46	864
E4 &5 R	ACAACCACCCTTAACCCCTC	20	59.22	
E6 F	AGGAAGGAGAATGGCGTGAA	20	59.02	510
E6 R	CAGGTAGGTGGAGGAGAAGC	20	59.17	

The condition of PCR reaction mixture and thermal cycler were optimized by varying annealing temperature, time and primers concentrations. The temperature range applied for primer *P53* exon 4 and5 was kept at 58-63°C and 57-62°C for exon 6. The annealing time was 45 seconds. The volume range of primer was 0.2-0.5 µL. The total reaction mixture for each sample was 10 µL. Ice box was used for maintenance of temperature. The optimized volume of the reagents used for the preparation of PCR reaction mixture are mentioned in Table 3.6. The reaction mixture was prepared by using the solutions mentioned in Table 3.6. The amplification of the target region was performed under the optimized PCR thermal cycler conditions (Table 3.7)

Table 3.6: Optimized composition of PCR reaction mixture used for the amplification of Exon 4,5 and 6 of *P53*

Sr.no.	Reagents	Volume (µL)	Final Volume (µL)
1	Master Mix (2X)	5.0	9.0
2	Forward primer	0.2	
3	Reverse primer	0.2	
4	PCR water	3.6	
5	DNA Sample	01	1

Table 3.7: Optimized conditions of thermal cycler for the amplification of Exon 4,5 and 6 of *P53*

Sr no.	Conditions	Temperatures (°C)	Time duration	No. of cycles
1	Initial Denaturation	95	5 min	1X
2	Final Denaturation	95	45 sec	35X
3	Annealing for E4&5 Annealing for E6	62.5 59	45 sec	
4	Extension	72	45 sec	
5	Final Extension	72	7 min	1X

3.8 Horizontal Gel Electrophoresis of Amplified Products

Horizontal gel electrophoresis was performed using 2% agarose gel. Following steps were followed:

1. For making 2 % agarose gel, 01 g agarose powder was weighed on electronic balance and poured into the flask.
2. TBE buffer of strength 1X was added to the flask up to 50 mL and heated in the microwave for 2 minutes.
3. Ethidium Bromide (8µL) was added in the solution.
4. The solution was then poured in the gel casting tray and was allowed to solidify

   and was transferred in the gel tank containing 1X TBE buffer.
5. DNA samples of 2 µL were mixed with 2 µL of bromophenol blue and were loaded.
6. Gel electrophoresis was done at 90 volts for 40 minutes.
7. The gel was then visualized under UV gel documentation system.

3.9 Single stranded chain polymorphism

For detection of genetic variations of *P53* gene among breast cancer patients and healthy individuals, the amplified PCR products were analysed by Single stranded chain

polymorphism (SSCP). It was performed by using 6% poly acrylamide gel (PAGE). SSCP technique is used for mutation detection based on the principle that alteration in single base change cause change in band pattern.

Procedure

1. Poly acrylamide gel of 6% was prepared and immediately transferred between the pre-assembled glass plates carefully to avoid any bubble formation. The composition of 6% PAGE is mentioned in Table 3.8.
2. The combs were inserted between the plates for formation of the wells.
3. The gel was then allowed to solidify at room temperature for 40-60 minutes.
4. After polymerization, the spacers were removed and glass plates were placed vertically along the buffer tank.
5. TBE buffer (1 X) was then carefully poured in to gel tank, the formation of air bubbles along the glass plates was avoided.
6. Amplified DNA products were subjected to denaturation using 2 µL formamide solution and providing denaturing condition of 95°C at 8 minutes in thermal cycler.
7. After that, the denatured samples were placed in ice for 10 minutes.
8. Bromophenol (5 µL) was mixed with 8 µL of denatured product and were loaded in the wells.
9. Electrophoresis was performed at 120 volts for 180 minutes.

The composition of the reagents used for the preparation of 6% PAGE are mentioned in Table 3.9 and 3.10.

Table 3.8: Poly Acrylamide Gel composition (6%)

Sr. no.	**Reagents**	**Volume**	**Final Volume (mL)**
1	30% Acryl- Bisacrylamide Solution	10mL	50
2	10X TBE buffer	5mL	
3	10% APS	350µL	
4	TEMED	25 µL	
5	Distilled Water	34.625mL	

Table 3.9: Composition of 30% Acryl- Bisacrylamide Solution for SSCP

Sr. No.	Chemicals	Amount (g)
1	Acryl Amide	72.5
2	Bisacrylamide	2.5

Table 3.10: Composition of 10% Ammonium per Sulphate Solution (APS)

Sr. No.	Chemicals	Amount (g)
1	Ammonium per Sulphate	5 g
2	Distilled Water	45mL

3.10 Staining and Visualization of gel

After electrophoresis, the gel was treated with ethidium bromide solution for 10 minutes. The stained gel was visualized in UV Gel Documentation system. The banding pattern variations among the patients and control samples were then analysed for varied banding pattern.

3.11 Sanger Sequencing

The banding pattern of the variants were visualized and selected variants were amplified through PCR. About 8µl of the sample was added in the sequencing plate along with 4µL reverse primer for *P53* exon 4&5 primer and exon 6. The selected samples were loaded properly on the sequencing plate and were sent to Eurofins (USA) for Sanger sequencing.

3.12 Data analysis

Different online bioinformatics software was used for 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/). UniProt (https://www.uniprot.org/) was used to identify the site/region of interaction of the mutated amino acid. Microsoft Excel, Microsoft Word and SPSS 16.0 were used for the analysis of demographic as well as clinical data.

In order to screen the mutations/polymorphisms in *P53*, a case control candidate gene study was conducted that included breast cancer patients and healthy individuals from Pakistani local population. For present study, a total of 400 blood samples were processed, out of which 200 were patients and 200 were healthy individuals. A structures questionnaire was designed to analyse demographic factors from both controls and patients. This information included was age, gender, residence, family history *etc.* The clinical data included medical test reports (mammography, histopathology, ultrasound report and IHC). Microsoft Excel sheet was used to arrange and analyse the clinical data as well as the demographic data of the patients and healthy controls.

4.1 Demographic and Clinical Data of Patients and Controls

Based on area of residence, most of the breast cancer patients were of urban residence (59.4%) as compared to rural (40.5%) as shown in Figure 4.1. On the basis of province of residence, most patients were from Punjab (57.59%) followed by KPK (Figure 4.2) Based on the age factor, breast cancer patients and controls were categorized into four age groups: age group one (28-42 years), age group two (43-57 years), age group three (58-72 years), and age group four (73-87 years). On the basis of age groups, maximum number of patients belonged to age group 43-57 years (46%), while most of healthy individuals (46%) were from age group one (28-42 years). However, the age group 58-72 years (OR: 2.8615 [1.5668-5.2259] *p* value = 0.0006) and age group 73-87 years (OR: 10.4737 [1.3279-82.6093] *p* value = 0.0258) were significantly associated with breast cancer risk (Table 4.1). Most of the patients (97.5%) were married (Table 4.1) and among breast cancer patients 51% were married out of family while 49% were married within family (Figure 4.3). On the basis of parity status, it was observed that 93.2% of breast cancer females and 96.8% of controls were parous (Table 4.1; Figure 4.4). Based on family history of breast cancer, it was found that 10.5% patients had family history while in case of controls only 2% had positive family history of breast cancer (Figure 4.5). It was found that family history was significantly associated with risk of breast cancer (OR: 5.7486 [1.9361-17.0689] p value = 0.0016].

Table 4.1: Percentage distribution of patients and controls based on demographic factors

Characteristics	**Patients n (%)**	**Controls (%)**	**Odd Ratio**	**Confidence Interval (CI) 95%**	**P value**
Age					
28-42	56 (28)	91 (46)	0.4658	0.3074-0.7058	0.0003
43-57	92 (46)	89 (45)	1.0624	0.7166-1.5752	0.7631
58-72	42 (21)	17 (9)	2.8615	1.5668-5.2259	0.0006
73-87	10 (5)	1 (1)	10.4737	1.3279-82.6093	0.0258
Marriage Status					
Married	195 (97.5)	196 (98)	0.7959	0.2106-3.0084	0.7365
Unmarried	5 (2.5)	4 (2)			
Parity Status					
Parous	180 (93.2)	185 (96.8)	0.5988	0.2424 to 1.4791	0.2663
Nulliparous	13 (6.7)	6 (3.14)			

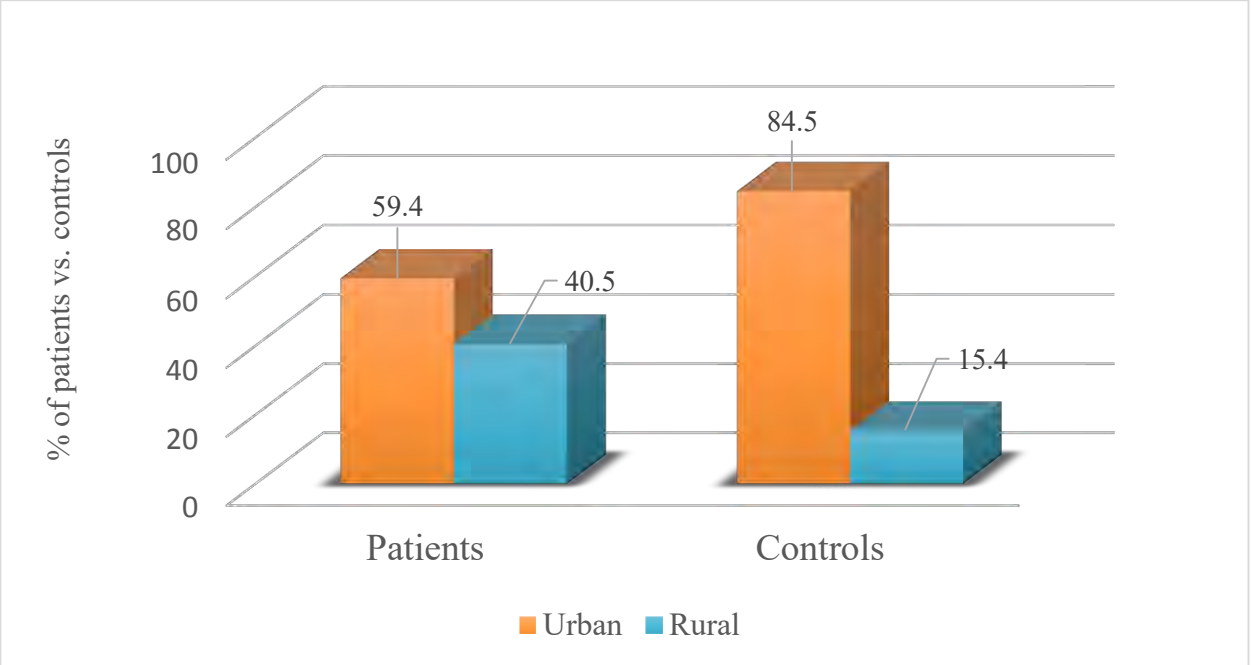

Figure 4.1: Percentage distribution of breast cancer Patients and Controls on the basis of area of residence

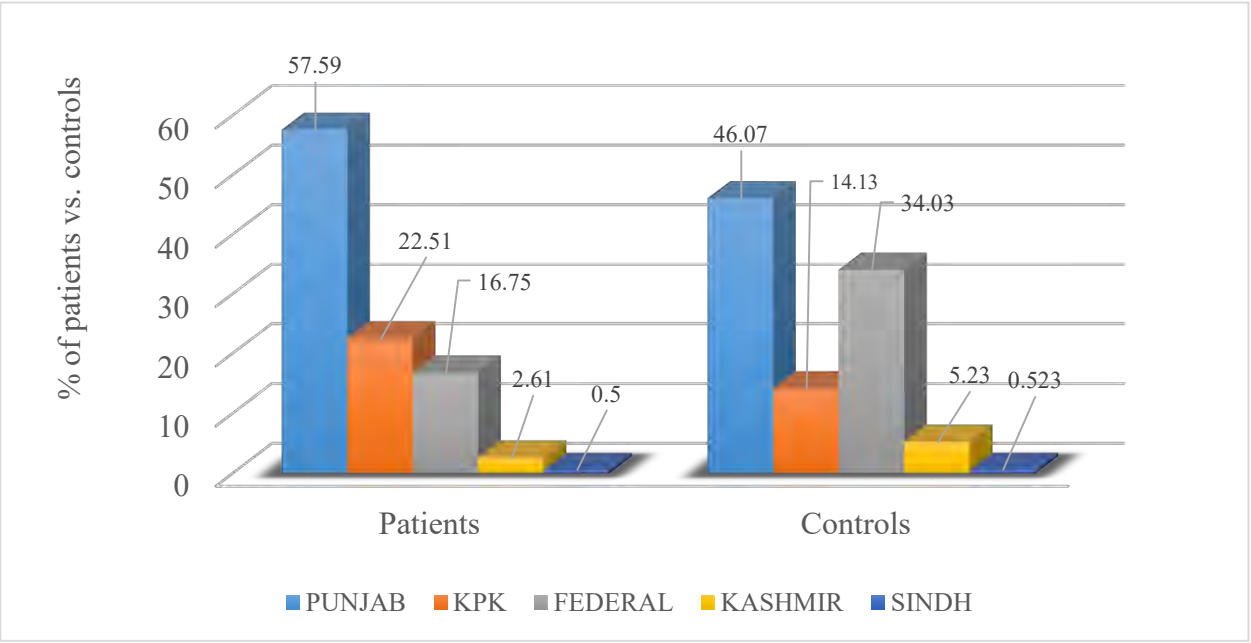

Figure 4.2: Percentage distribution of breast cancer Patients and Controls on the basis of provinces

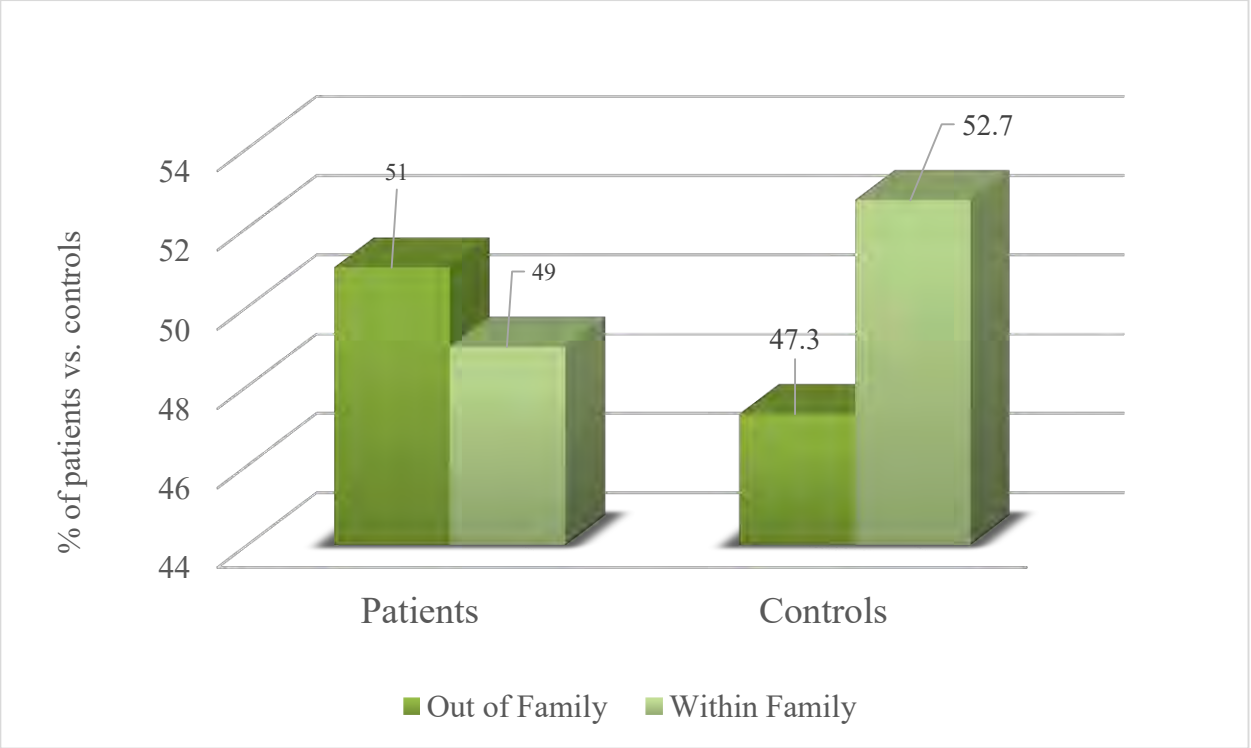

Figure 4.3 Percentage distribution of breast cancer Patients and Controls on the basis of their marriage

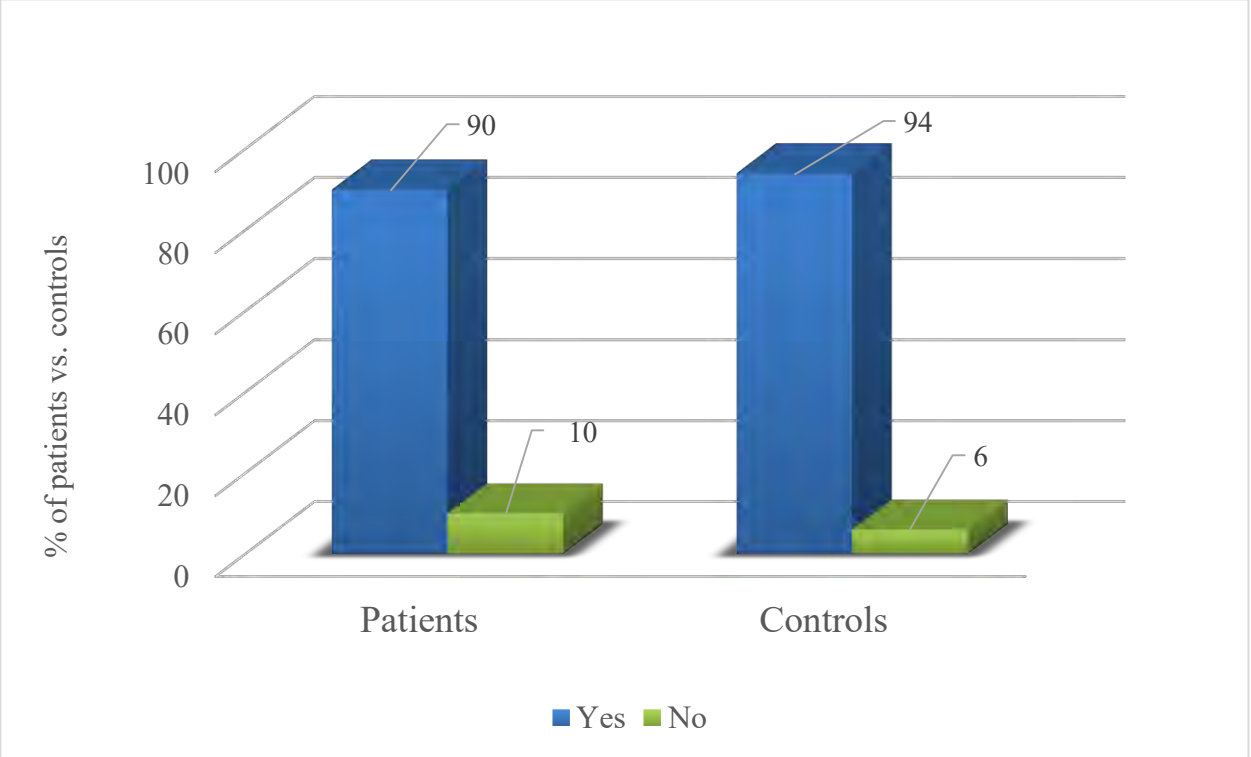

Figure 4.4: Percentage distribution of breast cancer Patients and Controls on the basis of breast feeding

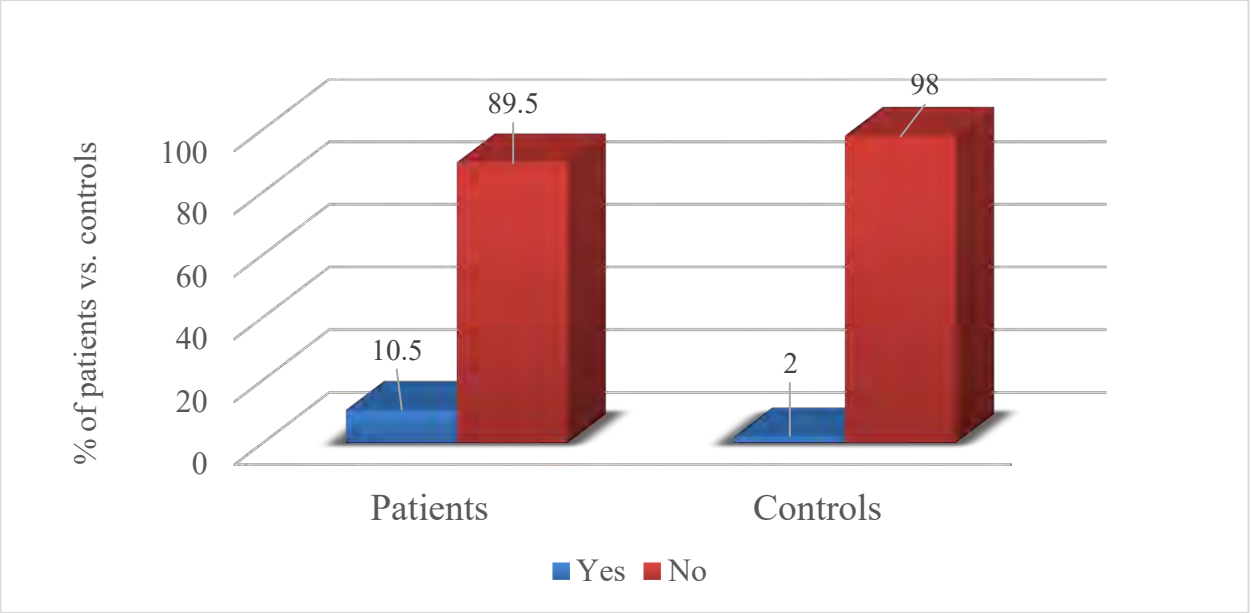

Figure 4.5 Percentage distribution of breast cancer Patients and Controls on the basis of family history

On the basis of grades, 74.44% of patients had grade 2 as shown in Figure 4.6. On the basis of symptoms in breast cancer, it was found that patients with right breast lump were higher (52.79%) as compare to left breast lump (45.68%), while some were detected with bilateral lumps (Figure 4.7). On the basis of breast cancer type invasive ductal carcinoma (44%) was the most prevalent type followed by invasive ductal carcinoma non special type (Table 4.2). On the basis of molecular subtypes, 36% of patients were diagnosed with luminal A, which is highest percentage as compared to rest of types in local patients (Figure 4.8)

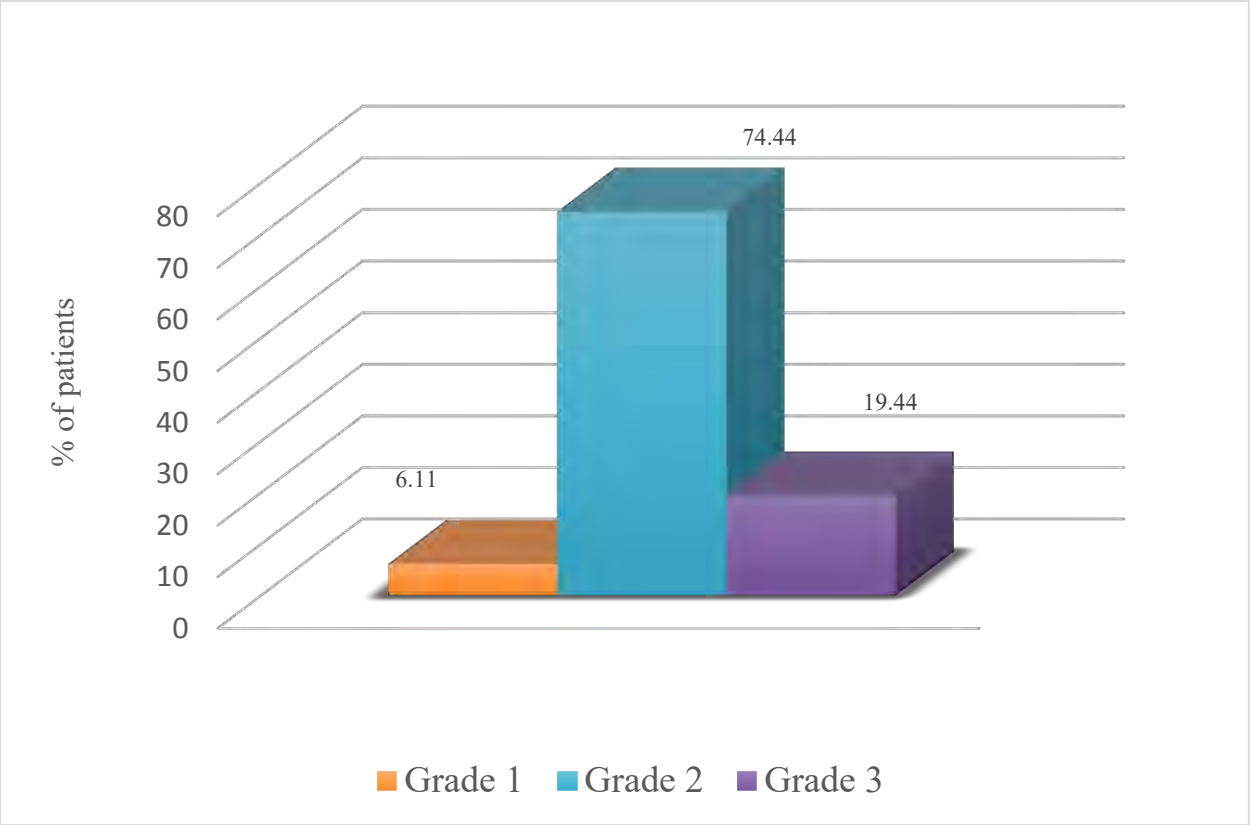

Figure 4.6: Percentage distribution of breast cancer patients on the basis of grading system

Figure 4.7 Percentage distribution of breast cancer patients on the basis of symptoms

Table 4.2: Percentage distribution of breast cancer patients on the basis of types of cancer

Sr. no	Types of Breast cancer	Patients (%)
1	Ductal Carcinoma *in situ*	4
2	Invasive Ductal Carcinoma	74
3	Invasive Mammary Carcinoma	18
4	Invasive Lobular Carcinoma	2
5	Others	2

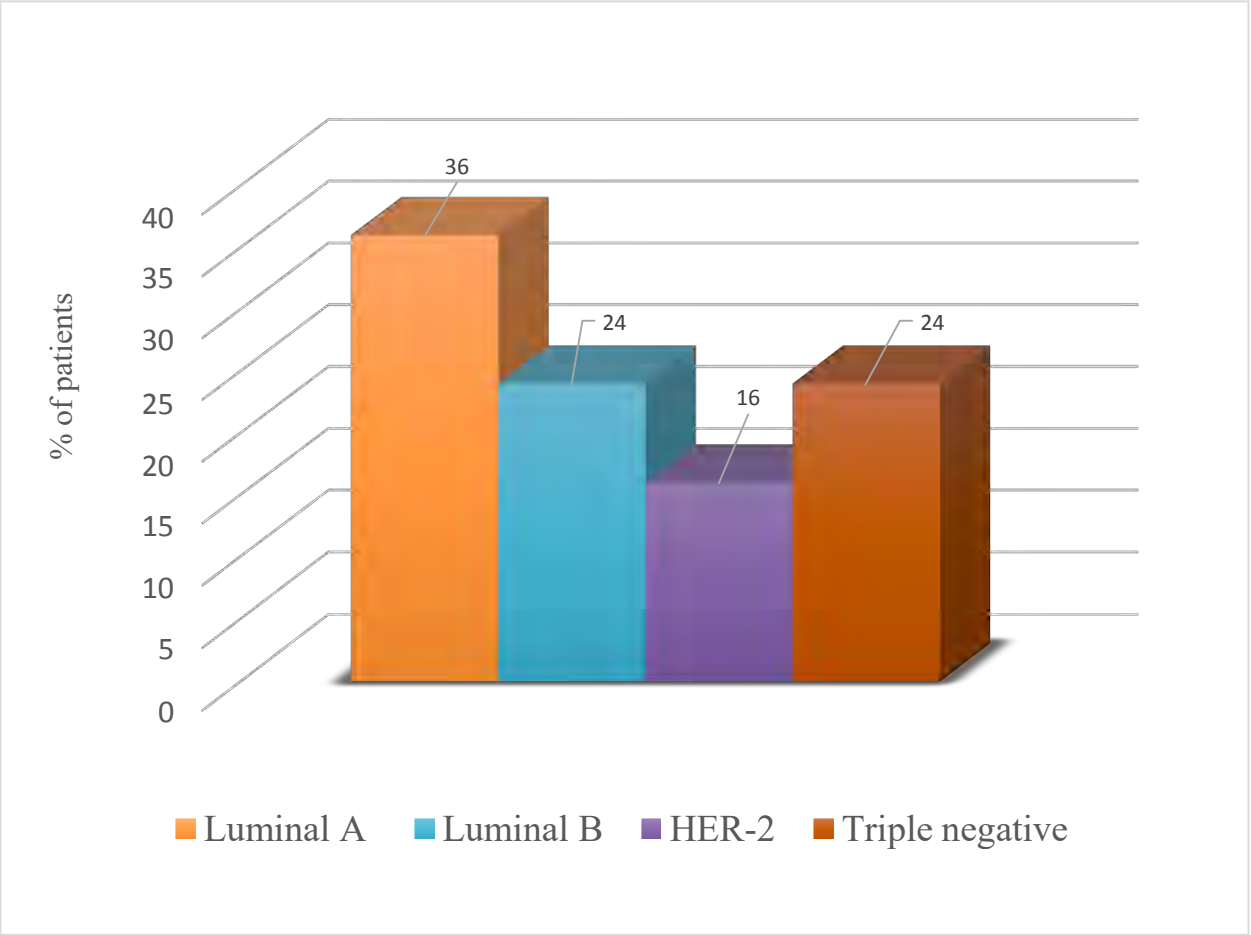

Figure 4.8: Percentage distribution of breast cancer patients on the basis of molecular subtypes

4.2 Extracted DNA from Blood of Patients and Controls

For the detection of polymorphism or mutations in the hotspot exons of *P53* gene, DNA was extracted from the blood of both healthy and breast cancer patients via phenol chloroform method. After extraction, these were visualized on 1% agarose gel (Figure 4.9 a & b).

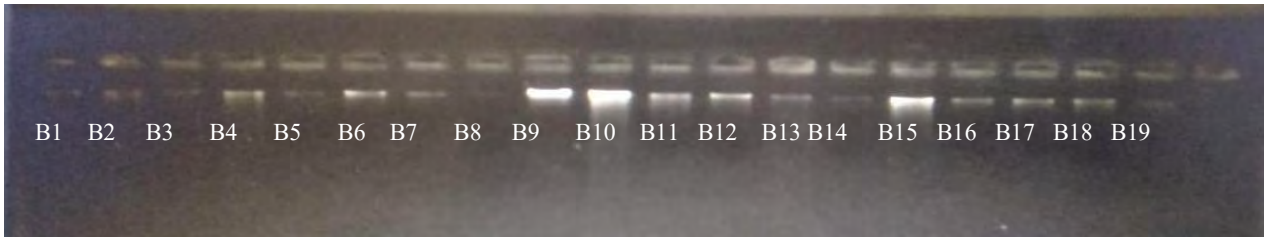

Figure 4.9 (a): DNA Extraction by phenol chloroform method visualized on 1% agarose gel, Breast Cancer patients (B1-B19)

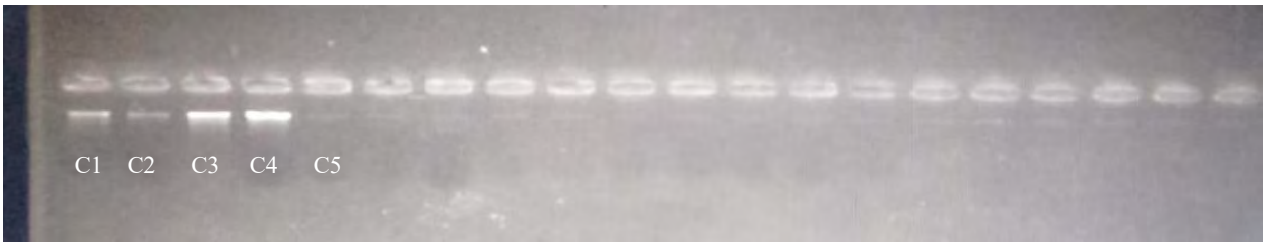

Figure 4.9 (b) DNA Extraction by phenol chloroform method visualized on 1% agarose gel, Controls (C1-C5)

4.3 Polymerase Chain Reaction

Polymerase Chain Reaction was done for the amplification of hotspot exons in *P53* gene by using 2 set of primers, where only one primer was designed for exon 4 and 5 as their size was small. The amplification was done after the optimization of the results at specific annealing temperature for each exon by using specifically designed primers via primer 3. These primers were cross checked by UCSC, Oligo calc and NCBI. As hotspot regions anticipated in exon 4,5 and 6, only amplification was done for these exons and compare with the ladder of 100bps (Thermo-scientific). The optimum annealing temperature for exon 4 and 5 was 62^{o}C and optimum annealing temperature for exon 6 was 55^{o}C. The optimum PCR conditionswere determined using 10µL of reaction mixture in gradient PCR and optimum annealing time was found to be 45 secs/cycle (Figure 4.10-4.11).

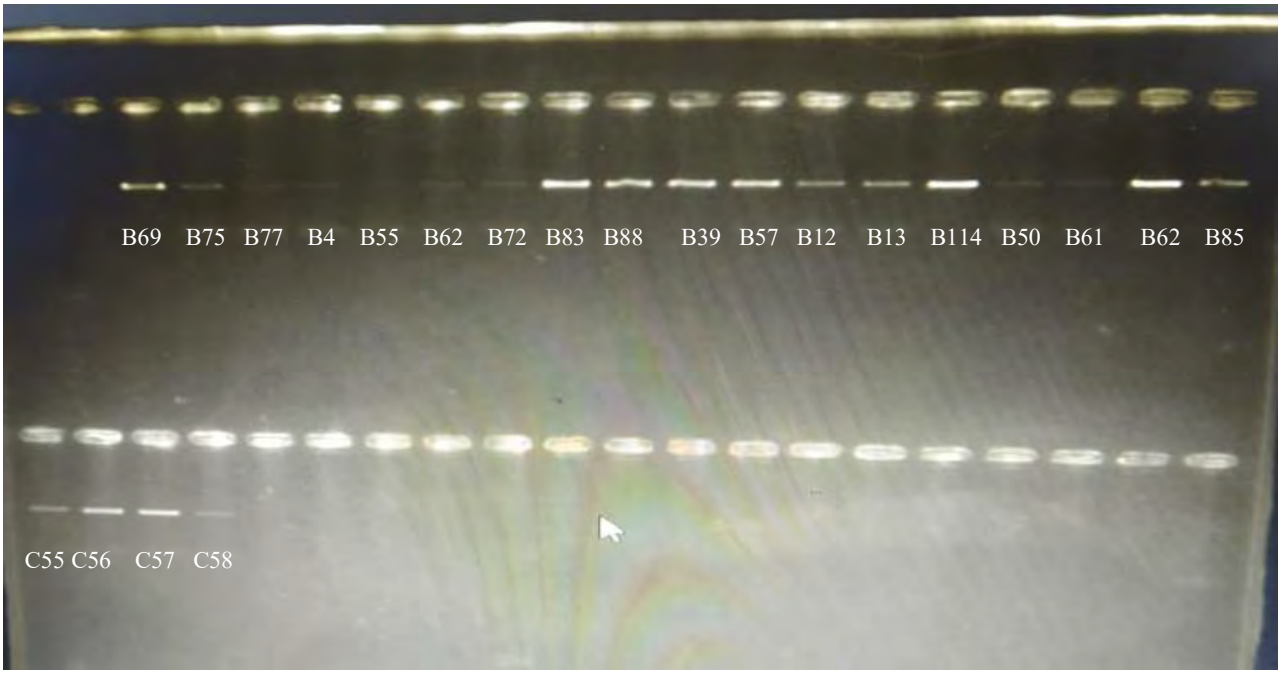

Figure 4.10: Amplified PCR products of Exon 4 and 5 visualized on 2% agarose gel. Upper well have patients (B69-B85) while lower well have controls (C55-C58).

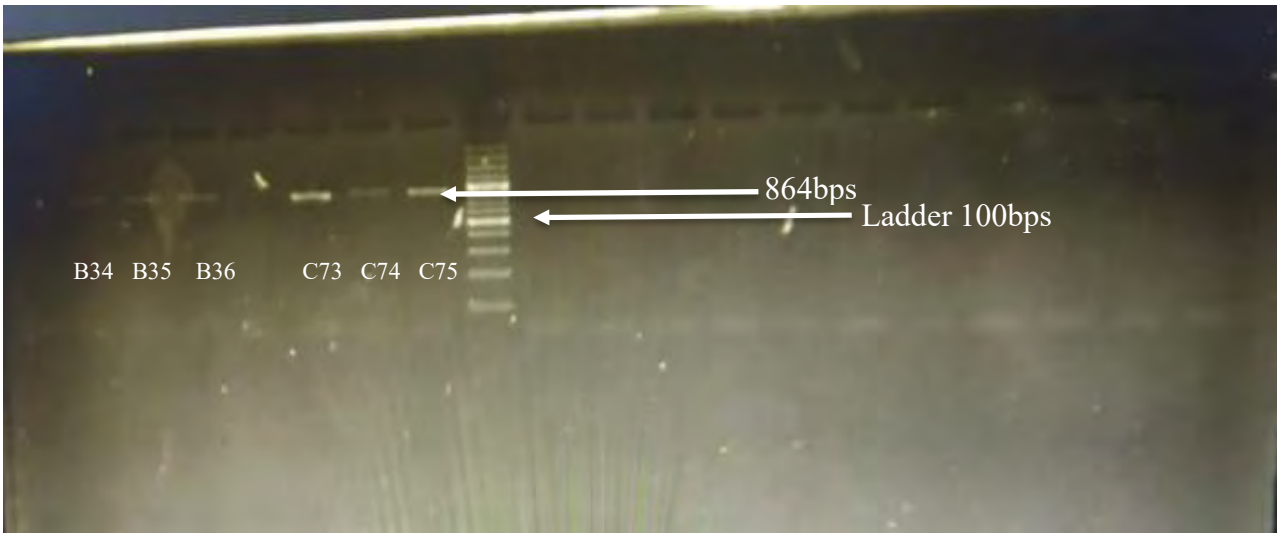

Figure 4.11: Amplified PCR products Exon 4 and 5 visualized on 2% agarose gel. Wells 2-4 shows patients (B34-B36) while wells 6-8 shows healthy samples (C73-C75).

4.4 Single stranded conformational polymorphism

After PCR amplification, the products were analysed for polymorphisms via SSCP. SSCP is useful for mutation identification identical nucleotide sequence with help of variation in

banding pattern. After amplification of hotspot exons, 8% PAGE was used for exon 4 and 5 while 6% PAGE was used for exon 6. Firstly, amplified products were then run on 6% and 8% PAGE to detect change in banding pattern. After electrophoresis, the gel was stained with ethidium bromide and visualized under trans illuminator (Fig 4.12-4.13).

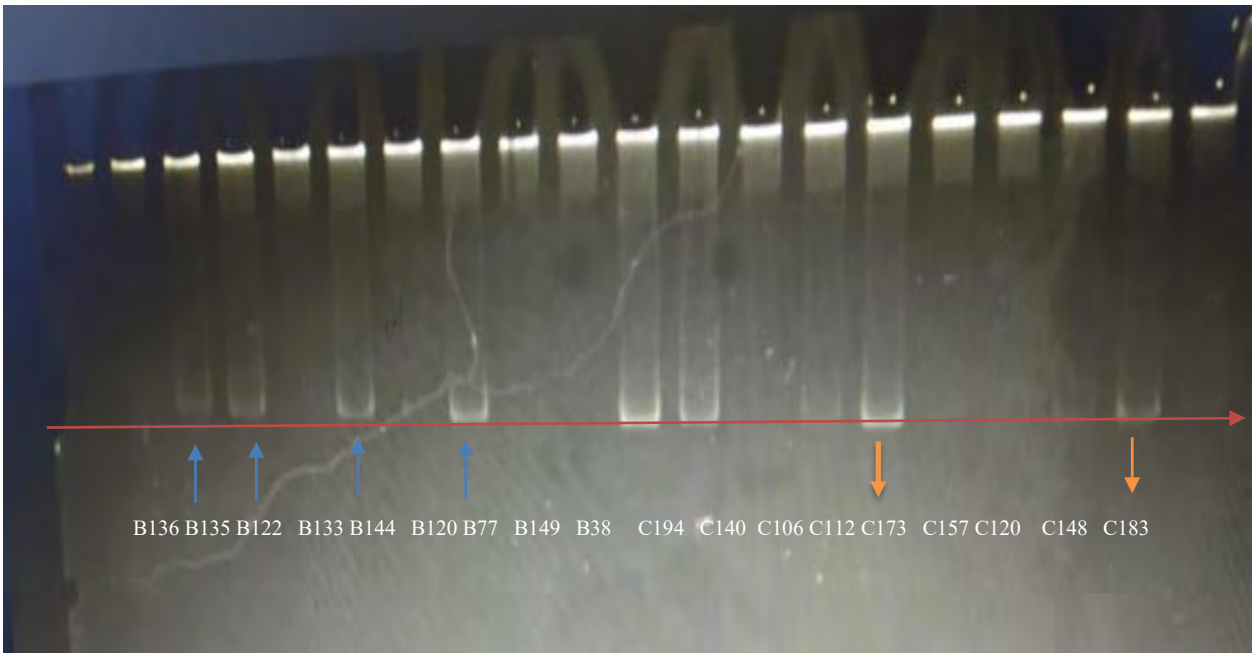

Figure 4.12: 8% PAGE of exon 4 and 5 banding pattern for breast cancer patients (B136-B38) in wells 1-9 and Control (C194-C183) in wells 10-18

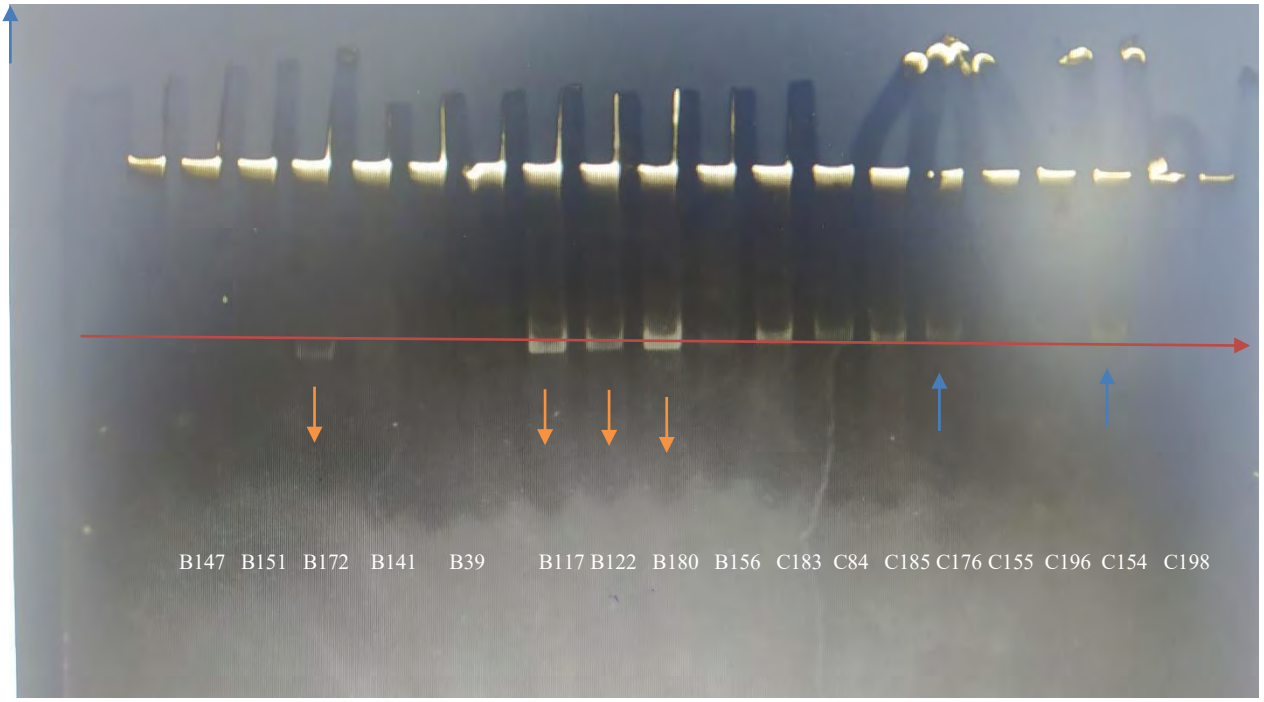

Figure 4.13: 6% PAGE of exon 6 banding pattern for breast cancer patients (B147-B156) in wells 1-9 and Control (C183-C198) in wells 10-18

4.5 Sequencing Analysis

The amplicons of exon 4 and 5 of *P53* gene, which showed difference in mobility on gel were sent with reverse primes for Sanger sequencing to Eurofins, USA. The results after the Sanger sequencing were aligned with the reference sequence NG_017013.2 from National Centre for Biotechnology Information. After alignment, it then further analysed through bioinformatics software BIOEDIT. For the analysis of SNPs in these hotspot mutations Mutation Taster Software was also used.

A total of 3 different polymorphisms were detected in Exon 4 and 5 that included two novel and one reported mutations. No SNP was detected in exon 6. Both reported and novel mutation were detected in exon 4 of *P53*, however, no SNPS was detected in exon 5 region. The reported mutation (chr17:7578645C>TN/A, rs2909430), was detected in patients (n=2) diagnosed with invasive ductal carcinoma. (Table 4.3).

Similarly, 1 novel mutation (chr17:7578423C>GN/A) was detected in a patient (n=1) diagnosed with invasive ductal carcinoma. Moreover, another novel mutation

(chr17:7578539T>GN/A) was detected in a patient (n=1) diagnosed with invasive ductal carcinoma.

Table 4.3: Sequence analysis of breast cancer patients on the basis of SNPs in Exon 4

Sr. No.	SNPs	Physical Location	DNA Change	Patients (n)	Patients Age (years)	Type of polymorphism	Reported/Novel
1	C>T	chr17:7578645C>TN/A	g.12212G>A	2	53 79	Intron	rs2909430
2	G>C	chr17:7578423C>GN/A	g.12434G>C	1	52	Non Synonymous	Novel
3	A>C	chr17:7578539T>GN/A	g.12318A>C	1	52	Non Synonymous	Novel

Chromatograms of Identified Polymorphisms

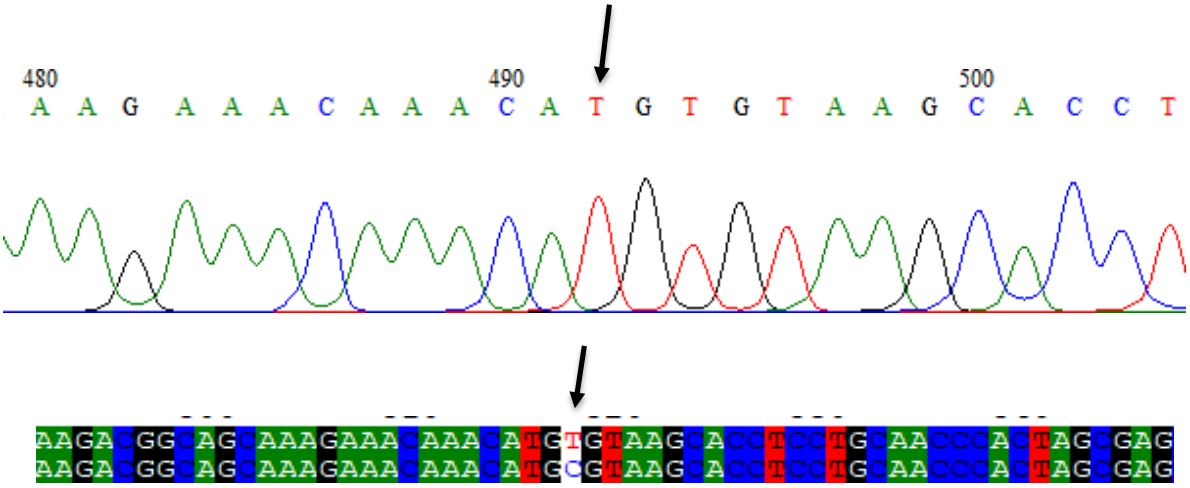

Fig 4.14 SNP C>T in intron of *P53* detected in breast cancer patients (n=2**)**

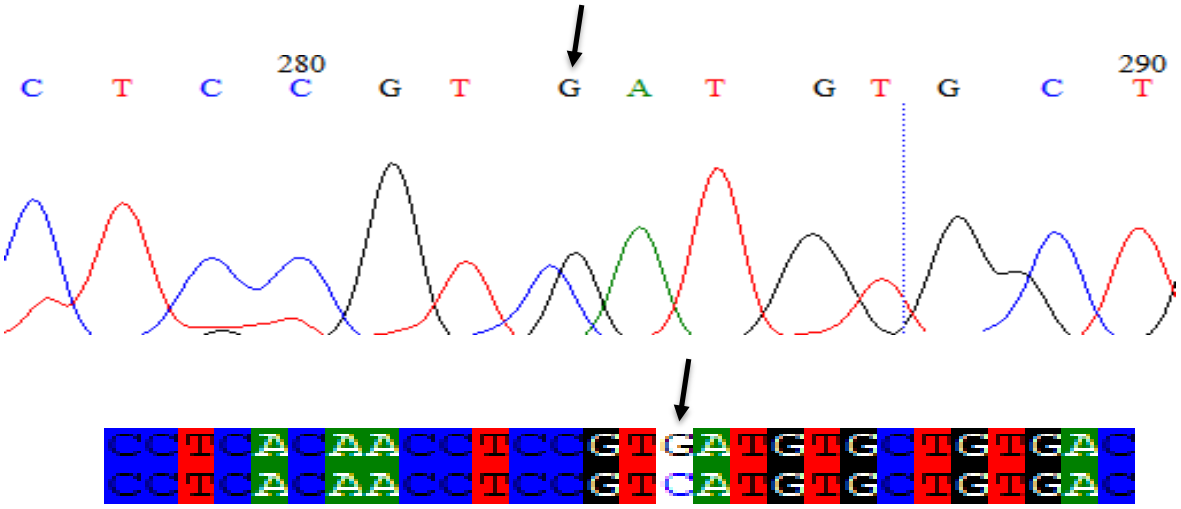

Fig 4.15 SNP G>C in exon 4,5 of *P53* detected in breast cancer patients (n=1)

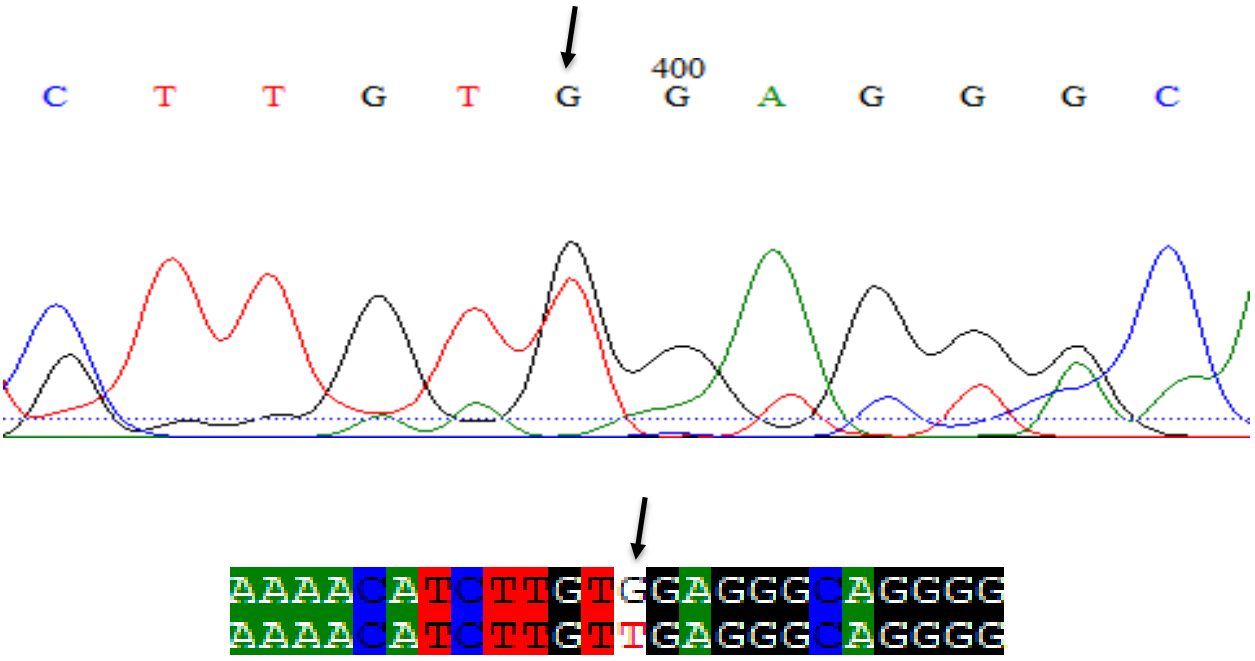

Fig 4.16 SNP A>C in exon 4,5 of *P53* detected in breast cancer patients (n=1)

Breast cancer is considered as the most diagnosed cancer worldwide with higher prevalence and death rates as compare to other cancers (Iacopetta *et al.*, 2023). It is a multifactorial disease and has been estimated that approximately that 2.3 million cases of the breast cancer are reported each year (Łukasiewicz *et al.*, 2021). As per WHO (2023) statistics, breast cancer with its high incidence rate accounts for the 30% of all cancer cases. Moreover, the trends, incident and death rates of breast cancer can vary from country to country. The rate of breast cancer incidents is more common in high income countries as compare to low income countries (Kashyap *et al.*, 2022). For instance, China, being the most populist country, had highest number of breast cancer cases in 2021 which accounted for the 18.4% of the all breast cancer incidence globally. After China, increased incidence of breast cancer was reported from USA, Belgium, Iran and South Korea. Additionally, some African and Asian countries have low breast cancer cases as compared to the developed countries but due to globalization and rapid change in trends in socio-economic and lifestyle, there is increase in probability of more cases in these regions now too (Lei *et al.*, 2021). Moreover, on the basis of the number of case reporting, it has been estimating that the number of cases will reach 4.4 million by the end of year 2070 (Soerjomataram *et al.*, 2021).

In Asia, Pakistan has the most significant number of breast cancer rate and it is believed that every one out of every 8 women is at risk of breast cancer development (Rashid *et al.*, 2021; Zaheer *et al.*, 2019). Additionally, approximately 25, 928 breast cancer cases were reported in Pakistan in 2020, which accounted for 14.5% of all types of cancer (Hussain *et al.*, 2022). Age, family history, reproductive factors, hormonal status and multiple genetic factors like *P53* altogether increase the risk of breast cancer (Hussain *et al.*, 2022; Rashid *et al.*, 2012). Irrespective of sporadic nature of breast cancer, 5-10% of breast cancer is familial in nature and different genetic factors like *BRCA 1,2*, *PTEN*, *ATM*, *CHEK2*, *PALB2*, *RAD51C*, *RAD51D* and *P53* play a key role in the familial breast cancer (Sokolova *et al.*, 2023). However, germ like mutations in *P53* play a key role in the development of the breast cancer (Grill *et al.*, 2021). Thus, considering the burden of breast cancer and role of variants in *P53* gene, the present case control study was designed to correlate the demographic and clinical features of the breast cancer patients and healthy individuals to screen the patients for *P53* gene variants.

The incidence of breast cancer cases is highly dependent on the age factor (Hussain *et al.*, 2022). Similarly, in Pakistan, the rate of age dependent breast cancer is very high as compared to other Asian countries (Zaheer *et al.*, 2019). In the current study, majority of the breast cancer patients (46%) were of age group 43-57 years, however the age group 58-72

years (OR: 2.8615 [1.5668-5.2259] *p* value = 0.0006) and age group 73-87 years (OR: 10.4737 [1.3279-82.6093] *p* value = 0.0258) were also significantly associated with risk of breast cancer. Soomro *et al.*, (2018) reported that in Pakistan, most frequent age of breast cancer diagnosis is the forth decade (28.51%). Another study, conducted by Gulzar *et al.* (2019) reported that in Pakistan breast cancer patients were of age greater than 40 years. However, Badar *et al*. (2022), reported most of the patients in their study were of age between 60-70 years. So overall findings indicate that age is one of the most important risk factor in breast cancer and population affected from this disease is in range between 40-50 years and onwards. Globally, mortality rate of breast cancer has been seen to increase among women with age from 50-70 years (Lima *et al.*, 2021).

In current study, majority of the breast cancer patients were married. A study conducted by Azam *et al.* (2021) correlates with present study were also 98% of breast cancer patients were married. The current study also found that 93.2% of Pakistani population were parous. Similar results were reports by Ahmad *et al.* (2021) in which 92% of breast cancer patients in their study were parous and 8% were nulliparous. However, a study conducted in Germany reported the association of parity status with oestrogen receptor status and reveals that parous and nulliparous have separate effect on oestrogen receptor positive breast cancer disease (Fortner *et al.*, 2019). Parous women have reduced risk of developing luminal A or B breast cancer as compare to nulliparous women but this trend is independent of triple negative breast cancer (Lei *et al.*, 2021). So the status of parity can play a significant role in the development of different subtypes of breast cancer.

The current study found 90% of the patients had breastfeeding history. A study conducted by Majeed *et al.*, 2020 is also in accordance with this study, as 96% of patients had breast fed their children in Majeed et al., study too. However, according to Centre of Disease Control and Prevention (2021), breast feeding has positive impact on both mother and child, where breast feeding leads to reduce breast cancer development and provides protections against breast cancer due to less exposure to hormones. Thus , the finding of current study are contradictory as majority of patients had history of breast feeding however, it might be due to interaction of different environmental factors, late marriages, ethnicity or hormonal unbalancing in later stages of pregnancy as breast cancer is a multifactorial disease (Saeed *et al.*, 2021). Beside these, there are also combination of other modified and non-modified risk factors for the progression and development of breast cancer.

In current current study, area of residence was also found to be a major risk factor as urban population had more cases (59.4%) as compared to the rural population. This trend is in

accordance with the study conducted by Azam *et al.*, (2021), where also most patients were of urban areas. However, study of Ahmad *et al.* (2021) indicated that the number of breast cancer patients were independent of area of residence, both rural and urban areas are equally prone to breast cancer. The urbanization and availability of advance facilities for screening of breast cancer might be the reason for increased breast cancer cases as compared to the rural areas in this study. Most of the patients (57.59%) in current study belonged to the Punjab province while 21.46% from KPK. Similar trend was reported by another study that reported majority of breast cancer patients were from Punjab (Badar *et al.*, 2022). However, these findings are similar to study of Qureshi *et al.* (2020) reported that breast cancer most common type of cancer diagnosed reported in Karachi. This could be due to reason that people in Karachi have more access to hospital in their districts for diagnosis and treatment.

In hereditary or familial cases, genetic changes are risk for initiation and progression of breast cancer (Francies *et al*., 2020). According to current study, 10.5% of the patients had positive family history of breast cancer and which was significantly associated with breast cancer risk (OR: 5.7486 [1.9361-17.0689] *p* value = 0.0016). Similarly, a study conducted by De Silva *et al.* (2019) reported 10% of breast cancer patients with a hereditary background. Thus, these results support the previous findings of 10% of the breast cancer cases being familial and it run from generation to generation.

Based on histological type of breast cancer, majority of the breast cancer patients were diagnosed with invasive ductal carcinoma (74%). The current findings are in accordance with the study conducted by Ahmad *et al.* (2021), whoreported 87.1% patients of invasive ductal carcinoma, while rest were diagnosed with other types of cancer. Similarly, Ahtzaz *et al.* (2017) reported 93.33% of the patients had invasive ductal carcinoma. Invasive breast carcinoma is the most frequently reported type of breast cancer according to American cancer society (Giaquinto *et al.*, 2022). The current study also found that 74.44% of breast cancer were diagnosed cases were of invasive ductal carcinoma with grade II, followed by 19.44% of grade III and 6.1% of grade I. A study conducted by Soomro *et al.* (2018) also reported 47.26% of the patients diagnosed with grade II followed by grade I patients (4%). Similarly, in another study from Pakistan shows that 73% of the patients were diagnosed with grade II of breast cancer (Ahmad *et al.*, 2021). In contradiction to these studies, a study conducted by Shafi *et al.*, (2019) showed that majority of the patientswere of grade III (43%). This study confirm the previous studies, however grade III patients found in later study might be due lack of knowledge, social taboos and poor treatment in that study area (Saeed *et al.*, 2021). However, in this study grade II patients have higher

prevalence which shows that due to social media campaign and knowledge about breast cancer, there is a reduction in the cases of advance stage.

According American Cancer Society, luminal A is the most prevalent molecular subtype of breast carcinoma (Yin *et al*., 2020). Similarly, on the basis of molecular subtypes, in current study, 36% of the patients were diagnosed with Luminal A type, 24% with Luminal B type, 16% with HER-2 type and 26% with triple negative breast cancer. Another study from Pakistan also reported most of the cancer patients diagnosed with luminal A type of breast cancer and had positive impacts of chemotherapy (Shakeel *et al.*, 2021). However, current findings study are in contradiction to another study from (Hashmi *et al.*, 2018) who reported that 69% cases of breast cancer were of Luminal B followed by 31% cases of luminal A. This contradiction might be due to the fact that previously people were not aware of breast cancer, which might have led to high metastatic potential i.e. Luminal B cases.

Sequencing analysis revealed 3 types of variants in exon 4 of *P53* gene in breast cancer patients, among which2 were mutations (chr17:7578539T>GN/A and chr17:7578423C>GN/A) and 1 was polymorphism 1 (chr17:7578645C>TN/A). Both the mutations were novel while polymorphism was reported previously (rs2909430). A novel substitution of G/C at position chr17:7578423C>GN/A was found in patient (n=1) diagnosed with Invasive Ductal Carcinoma. This is non-synonymous mutation in which methionine is replaced by isoleucine after mutation (M169I). According to the UniProt Software, this region has interaction with different genes like CCAR2, HIPK1 and AXN1. Moreover, this region is required for the conformational interaction of several other genes like ZNF385A and FBXO42. The current mutation lies in one of the most important region of *P53* gene i.e. site of interaction with cell cycle and apoptosis regulator 2 (CCAR2). This gene was previously known as tumour suppressor gene and its main role was to stabilize and activate *P53* gene in for regulation of apoptosis, DNA repair, metabolism and tumourigenesis. Additionally, CCAR2 may play a dual role in cancer development by acting as a tumour suppressor and tumour promoter (Kim *et al.*, 2023). Therefore, mutations in regions of interaction site of *P53* might change P53 hence can change interaction with CCR2. Such changes can affect the activity of both *CCR2* and *P53,* hence, can be crucial in development of cause breast cancer.

Another novel mutation was found in exon 4 of *P53*gene due to substitution of A/C at position chr17:7578539T>GN/A in patient (n=1) diagnosed with invasive ductal carcinoma. This mutation is a non-synonymous mutation where asparagine is predicted to be replaced by

is histidine(N131H). According to the UniProt Software, this region has interaction of different protein like CCAR2, HIPK1 and AXN1 with P53.Among these Homeodomain interacting protein kinase 1 (HIPK1) is also very important regulator of P53. It's role is still not known whether it activate or suppress tumour but it helps in the upregulation of P53 (Rey *et al.*, 2013). Hence, due to mutation in this region, P53 expression may alter and result in tumour activation.

One polymorphism (chr17:7578645C>TN/A), reported previously (rs2909430) was detected in intron region of P53 gene in patients (n=2) diagnosed with invasive ductal carcinoma. According to ClinVar (NCBI), this mutation is benign in nature. However, this mutation is reported in malignant pulmonary nodules (Changdan *et al.*, 2023). Moreover, this mutation is also reported in by Eiholzer *et al.* (2020) in different cancer, it was shown that this intronic polymorphism of *P53* gene can increase the risk of getting cancer. Another study conducted by Zhang *et al.* (2019) reported that this mutation along with 4 other SNPs in *P53* gene have role in rectal cancer of Chinese population. These findings show that this mutation has a significant role in the development of other cancers, hence, can also have role in breast cancer development.

The current study, identified 2 novel mutations and 1 polymorphism among local Pakistani population. These novel mutations have not been reported previously from Pakistan as well as globally. Therefore, expressional studies are needed in order to find the association of identified novel mutations with development of breast cancer.

Conclusion

The current case control candidate based study was designed to detect polymorphisms/mutations in *P53* gene is breast cancer patients of Pakistani Population. The major findings of the current study are

- Most of the prevalent cases of breast cancer were observed in urban setting may suggest that females are more prone to increased risk of breast cancer development.
- Majority of the cases of breast cancer were sporadic in nature.
- High prevalence was observed among females of age group 43-57 years suggesting older age is significantly associated with risk of breast cancer.
- In our study, parous women with history of breast feeding were more prone to develop breast cancer.
- Positive family history was significantly associated with risk of breast cancer.
- Invasive ductal carcinoma with grade II patients were most prevalent type of breast cancer among all diagnosed cases suggesting late referral to doctors.
- On the basis of molecular subtypes, luminal A subtype was most prevalent.
- In *P53* gene, three different polymorphisms (1 intron variant and 2 exon variants) were detected specifically in exon 4. The detected mutations included 2 novel SNPS and 1 reported. These mutations were detected in 3 patients diagnosed with invasive ductal carcinoma.

Future recommendations

Based on current findings, following future recommendation are proposed:

- *P53* being an important tumour suppressor with significant role in different biological processes undergoes drastic alteration and it is recommended that *P53* gene should be examined based on its expression.
- As *P53* gene is associated with several oncogenes and other tumour suppressor which play role in breast cancer, more molecular based studies should be conducted for in depth study.
- In addition to genetic variants detection, studies should be conducting for the epigenetic changes in *P53* gene.
- The functional analysis of the novel SNPs should be done in order to assess their role on breast cancer development and progression.
- Further studies on the genetic profiling of *P53* should be conducted for early detection of breast cancer.
- Public awareness programs and seminars on breast cancer awareness should be conducted especially in rural areas.
- In Pakistan, females should consult experts to get screened for breast cancer once in every 6 months.
- Clinical and physical examination of breast cancer should be recommended by higher authorities for Pakistani women.

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Appendix I

Questionare used for the retrospecific study

عہد نامہ

میں نے اس معلوماتی پرچے میں موجود تمام معلومات کو بغور پڑھا 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/ ٹھانہ کٹ
فون نمبر 01 :		فون نمبر 02
خون کا گروپ		شادی شدہ خیر 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 ۔ رپورٹ		
چھاتی کے سرطان سے متعلق کوئی اور طبی مسئلہ		
خاندان کا اور کوئی متاثرہ فرد یا افراد :		
ایاتی علاقہ		
میں نے خون کا سیمپل دیا ہے (3ml)		