Investigation of Breast Cancer Causing Gene(s) in Pakistani Patients

By

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Investigation of Breast Cancer Causing Gene(s) in Pakistani Patients

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"In the name of ALLAH The Most Gracious and The Most Beneficial"

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Dated: _____

FINAL APPROVAL

It is certified that we have read the final thesis entitled **"Investigation of Breast Cancer Causing Gene(s) in Pakistani Patients"** submitted by **Mr. Shamsul Ghani** and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

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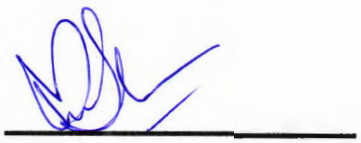

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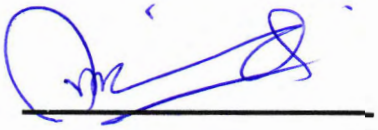

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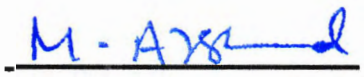

A thesis submitted to Department of Bioinformatics and Biotechnology,
International Islamic University, Islamabad as a partial Fulfillment of requirement of the award of the
Master in Sciences of Biotechnology
(MSBT)

This humble effort is

Dedicated

To

My beloved

Parents,

Brothers, Sisters

And

Teachers

Who inspired me for higher ideals of Life

DECLARATION

I hereby solemnly declare that the work **"Investigation of Breast Cancer Causing Gene(s) in Pakistani Patients"** presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

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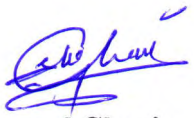

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Shamsul Ghani

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ABBREVIATIONS

AA	Amino Acids
ADH	Atypical ductal hyperplasia
BER	Base Excision Repair
bp	Basepair
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
Ca LB	Carcinoma Left Breast
Ca RB	Carcinoma Right Breast
DCIS	Ductal carcinoma in-situ
EDTA	Ethyline diamine tetra Acetic acid
HER2	Human Epidermal Growth Factor 2
HRT	Hormone replacement therapy
IDC	Invasive Ductal carcinoma
ILC	Invasive lobular carcinoma
INFDC	Infiltrating ductal carcinoma
LCIS	Lobular carcinoma in-situ
mM	Millimolar
PAGE	Polyacrylamide Gel Electrophoresis
PIN	Patient Identification Number
PR	Progesterone
SDS	Sodium Dodecyl Sulphate
Ser	Serine
TDLUs	terminal ductal lobular units
TNM	tumor-node- metastasis

ABSTRACT

Breast cancer in women is a lethal disease of developed and developing countries of the world. Breast cancer arise when multiple mutations occur in a single gene. Many genes are involved of arising breast tumor. Our study focus was on BRCA1 gene as it has main role of arising this disease. BRCA1 gene is tumor suppressor gene but when multiple mutation(s) occur, it causes tumor. BRCA1 gene have total 24 exons of which 22 are coding. Exon 10,11 was selected for In-silico analysis as it has the largest sequence and codes for many amino acid, that's why chances of mutations are too much. For this study, blood samples were collected from females with sporadic breast cancer from research institute of Nuclear Medicine Oncology and Radiotherapy Institute (NORI) Islamabad. These samples were tested for the detection of in silico analysis as well as to check pathogenic variant. DNA were extracted from these collected samples following non-organic method and then formulated on PCR with designed primer. Further checked on gel electrophoresis for bands confirmation and after that direct sequencing approach was used to find any mutation. Three mutations were found through Sanger sequencing in the desired exon. Two were found to be dinucleotide substitute mutation of which one mutation alter the amino acid (Serine which change to Phenylalanine). Serine has polar side chain while Phenylalanine has nonpolar side chain. Both amino acid has different nature, Serine is classified as non-essential in nature while Phenylalanine are essential amino acid. The second change were synonym in which TCC in wild type change to TCA in mutant but both codon codes same amino acid Serine. The third change were found in intron 10 of BRCA1 and is SNP mutation. Further study is required of this gene to find any other mutations as it has 21 other coding exons.

INTRODUCTION

1. INTRODUCTION

Breast cancer is a heterogeneous disease. It can be described via confrontation, increased angiogenesis, limitless replicative potential, activating invasion and in certain cases, metastasis (Wang *et al.*,2000). Breast cancer usually starts when a single cell acquires a series of mutations, which collectively leads to abnormal gene expression, then in combination with other epigenetic alterations drives the development of cancer. Breast tissue comprises of connective tissues, blood vessels, lymph vessels and mammary ducts both consist of the basement membrane, a layer of luminal epithelial and myoepithelial cells. The basement membrane acts as anchorage for epithelial cells, separating the epithelial layer from the loose connective tissue beneath and functions as a barrier to prevent metastasized cell from invading tissues. During lactation, the luminal epithelial layer synthesizes milk proteins and the myoepithelial layer contracts under the stimulation of oxytocin to secrete milk (Sopel *et al.*, 2010) However, in cases of in situ carcinomas, myoepithelial cell number decreases due to epigenetic and phenotypic alterations and the basement membrane starts to degrade (Polyak K. 2007a). Simultaneously, stromal fibroblasts, lymphocytes, myofibroblasts and endothelial cell numbers increase drastically (Polyak *et al.*, 2010b). In invasive carcinomas, loss of the basement membrane and myoepithelial cells occurs (Pandey *et al.*, 2010). If left untreated, a single or multiple cells from the tumor can break off and travel to other parts of the body via the lymphatic system or the blood stream. Once mobile, these breast cancer cells can penetrate the walls of the lymph and/or blood vessels of other organs to form new tumors (Zhu *et al.*, 2008). The translocation of tumor cells to other portions of the body is known as metastasis.

Breast cancer affects the lives of millions of women worldwide. Over the past several decades, the incidence of breast cancer has increased, while the death rate has steadily decreased. This observation may be explained by increased mammographic screening and early detection of the pre-invasive stages of breast cancer. The disease is most frequent among women older than 50 years. The prognosis of the patients is mostly dependent on tumor stage at the time of diagnosis. Without spread to axillary lymph nodes, five years' survival rate is reported to be 95 %. The survival rate is decreasing to 18 % if distant metastasis is present. Cancer is thought to be the most lethal cause of death worldwide which is reported that nearly

82 lacks people face death because of cancer in the year twenty twelve 2012 in which different types of cancer were studied and lung cancer was the ever highest contributor to the deaths, liver and stomach cancer were following seconding and third position on the death list. Lungs cancer with highiest number of death which was 1.59 million livers (745 000 deaths) stomach cancer deaths were 723,000 (Boskovic, M., and Baltic, M. Z. 2016). Literature shows that in every 9 women 1 woman come with the breast cancer in Pakistan (Riedemann., *et al.,* 2007).

Breast cancers are separated after origin of disease. The mammary gland consists of lobules (milk producing glands) and branching ducts (milk channels). The ends of the ducts are termed the terminal ductal lobular units (TDLUs) (Dimri *et al.,* 2005). Most breast cancers are thought to arise in the TDLU. Tumors that arise in the ducts or the lobules are termed ductal carcinoma and lobular carcinoma, respectively. The TDLUs consist of two types of epithelial cells: the inner luminal epithelial cells and the outer myoepithelial cells. Luminal epithelial cells line the normal breast duct and have secretory properties. Myoepithelial cells have both contractile muscle and epithelial properties. The two cell types are distinct and the precursors to various forms of breast cancer. Cancers from luminal epithelial cells are most common. The basement membrane surrounds the epithelial cells and works as a mechanical barrier. Its function is to anchor the epithelial layer to the connective tissue underneath.

Breast cancers can be either non-invasive or invasive. Invasive breast cancer cells have penetrated the basement membrane and invaded surrounding breast tissue. At this point, the cancer cells have the ability to spread to the lymph nodes and blood stream and metastasize to all organs of the body. Among the invasive forms of breast cancer, invasive ductal carcinoma (IDC) accounts for 70-80% of all breast carcinomas, while invasive lobular carcinoma (ILC) is the second most common type and accounts for 10-20% of all breast cancer cases. Other less common invasive types make up the remaining percent and include mucinous carcinoma, papillary carcinoma and tubular carcinoma among others. Preinvasive breast cancer possesses some malignant properties, but is still connected to its original site and has not broken through the basement membrane. The term in situ means in place, which characterize both ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). The another important form of cancer which affect the ductal portion is Ductal carcinoma which are found in almost 20-25 individuals out of hundred and it can be detected by using the screening method of mammography (Mork 2012b). Progression from normal epithelial cells in the duct wall of the

breast to metastatic cancer cells is thought to develop through multiple stages. Atypical ductal hyperplasia (ADH) is the precursor to DCIS and is used to describe increased proliferation of the epithelial cells (Silver *et al.,* 2010) Progression from ADH to DCIS marks the transition from benign stage to malignant disease. Some DCIS lesions are believed to rapidly transit to invasive ductal carcinoma, while others remain unchanged or disappear (Collins *et al.,* 2005). Breast cancer is a complex and heterogeneous disease with distinct histopathological features (Vargo-Gogola *et al.,* 2007). Markers for classifying the different types of breast cancer involve tumor type, tumor grade, tumor stage, expression of hormone receptors (estrogen and progesterone) and HER2 receptor status.

Histological grade describes proliferation and differentiation of breast cancer cells and is considered an important prognostic factor. The grading system is based on three morphological features: 1) mitotic count (rate of cell division), 2) tubule formation and 3) nuclear pleomorphism (change in cell size and uniformity) (Ignatiadis *et al.,* 2008). Each feature is assigned a score from 1 to 3, indicating slow and fast cell growth, respectively. All invasive forms of breast cancer are graded after the same criteria.

Tumor staging is useful to estimate breast cancer prognosis. The TNM (tumor-node-metastasis) system was developed by Pierre Denoix in 1942 and is based on size of the primary tumor (T), spread to axillary lymph nodes (N) and presence of distant metastases (M) (Mork *et al.,* 2012c). There are five tumor stages. Stage 0 represents non-invasive breast cancer (DCIS). Stage I describes small tumors that are localized to the breast. Stage II describes larger tumors with possible spread to the axillary lymph nodes. Large tumors that have invaded tissues around the breast fall into Stage III and Stage IV represents disease with metastases throughout the body. Stage 0/I/II patients have a significantly better prognosis than Stage III/IV patients (Singletary *et al.,* 2006).

BRCA1 is a large 1,863 amino acid phosphoproteins, with a molecular weight of 220 kDa, and contains domains that exhibit both enzymatic activity and protein binding sites (Figure 3). BRCA1 contains an N-terminal ring domain which exhibits E3 ubiquitin ligase activity and two nuclear export signals recognised by nuclear export receptor export in 1 (Rosen *et al.,* 2005). BRCA1 is involved in many crucial pathways such as cell cycle regulation, DNA repair, apoptosis, spindle formation and telomere regulation. BRCA1 controls G1 arrest through coupling with hyper phosphorylated RB (Aprelikova *et al.,* 1999). BRCA1 also plays an

important role in DNA repair pathways for both chromosomal and mitochondrial DNA repair. BRCA1 and MRE11/RAD50/NBS1 (MRN) complex are part of the BRCA1-associated genome surveillance complex. Besides MRN, the BRCT domain of BRCA1 can also bind double stranded breaks in DNA, which suggests that BRCA1 itself might recognize these types of breaks (Zhang *et al.,* 1998). Recent data suggests that BRCA1 is also involved in another DNA repair pathway called Base Excision Repair (BER) (Saha *et al.,* 2010). Furthermore, BRCA1 directly inhibits centrosome-defendant microtubule nucleation. In addition, BRCA1 inhibits telomerase activity, regulating telomere length. More recent studies also suggest that BRCA1 inhibits cell motility and promotes detoxification. Loss or reduction of functional BRCA1 levels observed in breast cancer can be due to absence of full length BRCA1 through protein truncation, loss or decreased BRCA1 expression or elevated protein degradation (Wu *et al.,* 2010). BRCA1 consists of 24 exons and spans a length of 81,189 bp on chromosome 17q21. The largest exon of BRCA1 is exon 11. BRCA1 is positioned between NBR2 (Neighbour of BRCA1 gene 2) and RND2 (Rho family GTPase 2). Detection of BRCA1 with different protein sizes, 210, 184, 160, 135 and 85 kDa suggests that different isoforms might have different functions, however, the function of these isoforms are still unclear (Gudas *et al.,* 1996). BRCA1 mutations can confer risks of up to 71.4% to breast cancer by the age of 70. BRCA1 mutations such as splice sites mutations, nonsense mutations, frame-shift mutations or missense mutations can result in abnormal length of mRNA, premature termination during RNA synthesis, loss/decreased transcription or change of codons that ultimately lead to production of non-functional BRCA1 or down-regulation of BRCA1 (Saxena *et al.,* 2006). In contrast, there are also some silent mutations that do not result in a change of codons therefore such mutations do not affect BRCA1 functionality (Syamala *et al.,* 2008). Tumors from patients with a germline mutation of BRCA1 have a distinct pathology. BRCA1 mutation type tumors arise at a younger age and commonly display pushing margins, lymphocytic infiltration and are usually of higher grade than other tumors types. In addition, they often display aneuploidy have a higher S-phase fraction, higher mitotic counts and exhibit a higher degree of nuclear pleomorphism and are frequently associated with the axillary lymph node. BRCA1 type tumors are also usually triple negative for progesterone (PR), ER- and human epidermal growth factor 2 (HER2) (Lakhani *et al.,* 2002).

1.1 AIMS AND OBJECTIVES

- Identification and enrollment of Patients with breast cancer
- Screening of collected sample for selected breast cancer gene
- In-silico analysis of observed pathogenic variants

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Breast Cancer Biology

The body of human is programmed to do function at normal and specific intervals but some time these abilities are affect due to certain reasons like alteration in genome because of the process like mutagenesis which is caused by mutation. Cell cycle is also one of such processes which highly regulated to control body weight and height. Sometimes due to the process of mutagenesis body of an organism loses its property of normal division and such abnormal division of cells of body is termed as a cancer. Cancer is one of the alarming disease which affect large human population and it affect each and every part of the body like nerve cells, muscles cell and sometimes even bone. Cancer can harm each and every part of the body and all systems such as immune the defense system of the body also. The symptoms of cancer are highly variable according to the cell type and location. Like symptoms the treatment of the cancer also varies from type to type and site to site. Cancer management and chemo protocols also depend on the progression and site where it develops. Cancers like breast, lung, liver, colorectal, prostate, head and neck carcinoma are most commonly diagnosed in Pakistan. (Tariq *et al.,* 2015).

2.1.1 Epidemiology

Breast cancer is the most commonly occurring cancer in women. According to a case report in the year 2008 1400000 women were investigated with breast cancer which caused approximately 460000 deaths. The interesting part of case study was that of all these 450000 women were investigated in Europe out of which 140000 were died. The same case study reported that in Africa 68000 women were identified with breast cancer which caused 37000 deaths (Abdulrahman and Rahman 2012). Breast cancer is the second most common cancer overall (1.4 million cases, 10.9%) after lung cancer, but ranks 5th in death causing diseases (458,000, 6.1%) (Ferlay *et al.,* 2010). Breast cancer is going to the alarming conditions in some of the country like in United States of America majority of women dies because of breast cancer which normally takes place in the middle ages of life that is from 40-50 years. Breast cancer can be a risky condition for any woman for the whole life and approximately every 12 or 13 th woman gets effected with breast cancer in every 100 female individuals. So it is very much known that 1/18 woman will have the chances of cancer (Richie and Swanson 2003a). All though recent survey shows that the

number of deaths because of cancer is little looking controlled as compared to the precious two decades because the death numbers due to breast cancer is decreased up to certain level after 1988. In USA it is the major cause of female deaths and it contribute about 30-35 out 100 deaths which means 1/3 of female population dies because of breast cancer. It the same time the other part of the statistical survey shows that the remaining 70-72/ 100 women dies because of other causes or natural death. The rate of deaths is high in those women who are under 35 and also some old women as will who are in the old age of their life. It also shows that the reason behind high level of deaths may be because of cell division rates which very much high in young age than compared to old age so breast cancer taking place in young age with more aggression as compared to the old ages because the level of cell division gets lowered after 25 years so the lethalness of breast cancer increases in early life (Richie and Swanson 2003b).

2.1.2 Pathology of breast cancer

There are several things involved in breast cancer causes so many research scientist reveals that 95 individuals out of 100 who are suffering with breast cancer have been identified the early onset to their epithelial cells of breast (Spiliopoulos *et al.,* 2009). There are two broad types of breast cancer which is classified on the bases of their ability of infiltration. These types include invasive cancer and another one is in situ cancer. In situ cancer uses lobular or ductal cells as its early onset site however it lacks the ability to effect nearby cells of the surrounding area which means these cancerous cells are localized and biologically termed as malignant and it also does not have the capability of metastasis. In some cases, the malignancy of a particular cell took place further and near the membrane base which intern forming the clear border between epithelial cells so the malannacy of such cell converts into the invasive type of carcinomas and it is a clear cause of death because in this case cells have the ability of metastasis so cells lose their identity which ultimately causing death (Bateman 2006).

2.1.3 Breast cancer etiology and risk factors

The etiology of human breast cancer remains largely unknown. However, the risk factors associated with breast cancer can be classified into three major classes: family history (hereditary) factors, hormonal (reproductive) factors and environmental (including lifestyle) factors. An epidemiological study by (Lichtenstein *et al.,* 2000) reported that

more than 70% of breast cancers are attributable to environmental factors. The common risk factors are as follows.

2.1.3.1 Gender

Gender is the greatest risk factor for breast cancer. That occurs 100 times more frequently in women than men (Block and Murad 2013). They also pointed out that women's breast cells are constantly changing and growing, mainly due to the activity of the female hormones estrogen and progesterone. This activity puts them at much greater risk for breast cancer.

2.1.3.2 Age

The prevalence and occurrence of breast cancer is directly proportional to increase in age up to certain limits mainly from 45 to 50 years. So after this there occurs gradual decrease in breast cancer occurrence because of certain hormonal changes in women especially at stoppage of sexual cycles (menopause). It chances decreases even more than previous at the ages of 75 to 80 years, incidence decreases sharply (Cady *et al.,* 1998).

2.1.3.3 Genetics

Beyond other factor that causes breast cancer genetic factors have a very little but important contribution in causing breast cancer. According to some of the researchers' genetic cancer have a very little prevalence and gene alteration can contribute 5-6/100 of the breast cancers that occurs in a society which means that in 100 only 5-6 female individuals out of 100 would have the hereditary breast cancer (Malone *et al.,* 1998). Two of the most studied contributors in approximately all type of breast cancer are BRCA1 and BRCA2 which are involved in 80% of hereditary cancers (Haber 2002).

Polymorphisms in genes associated with metabolism of estrogens and/or carcinogens (CYP1A1, CYP1B1, CYP19, COMT, NAT2, GSTM1 etc.), estrogen, androgen and vitamin D, co-activation of gene transcription (AIB1) and DNA damage response pathways (CHEK2, HRAS1, XRCC1, XRCC3, XRCC5 etc.) have also been found to be the risk factors for breast cancer in different populations (Lacroix and Leclercq 2005).

2.1.3.4 Familial history

Familial history of breast and other cancer is considered to be a risk factor if a first degree relative develops a premenopausal breast cancer and sometime breast may occur in correlation with other type of cancers especially in some cases with ovarian cancer. The

risks of occurring cancer in women increases with degree of relationship, if a women have any first degree relative having breast cancer than chances of her infection with cancer of breast increases by two fold of others who have second degree relatives (Greene 1997).

2.1.3.5 Hormones

The hormonal environment plays a pivotal role in the breast cancer development. Oophorectomy reduces the risk consecutively, the risk increases with higher levels of endogenous and pharmaceutical estrogen exposure (Henderson and Feigelson 2000). (Toniolo *et al.,* 2007) suggested that estrogens are promoters of mammary tumors which act over a long period of time by causing cell proliferation and clonal expansion of initiated cells. Depending upon concentration of estrogen and ERs and PRs estrogens bind with high affinity to estrogen receptor alpha (ER-α) and progesterone receptors (PR). This binding induces DNA synthesis, cell division and production of growth factors and progesterone receptor proteins. Estrogens and progesterone are essential for normal mammary gland development and function, but their stimulation of breast cell proliferation may be procarcinogenic (Rogan *et al.,* 2003).

2.1.3.6 Hormone replacement therapy (HRT)

To relieve the symptoms of menopause and osteoporosis in postmenopausal hormone therapy (PHT) has been used for many years (Miller *et al.,* 2002). There are two main types of HRT. For women who have undergone a hysterectomy, estrogen alone can be prescribed. This is commonly known as estrogen replacement therapy (ERT). But, generally estrogen and progesterone both are prescribed for women who still have a uterus (womb) (combined HRT). Because estrogen alone can increase the risk of cancer of the uterus, to prevent this progesterone is added (Kohn and Liotta 2006). Use of combined HRT therapy increases the risk of getting breast cancer. This increase in risk can be seen with a use of 2 years. Combined PHT also increases the likelihood that the cancer may be found at a more advanced stage, because it reduces the effectiveness of mammograms (Ross *et al.,* 2008).

2.1.3.7 First child birth and full time pregnancy

Women had their first child, full time pregnancy after 30 years of age or have no children are having a little more risks of cancer of breast as compare to those who are below 30 years. The chances of breast cancer also minimize with the early age marriage and those

who marries at early age have a very little chances of the breast cancer. Pregnancies reduce total number of lifetime menstrual cycle sand reduce the continuous and prolonged exposure of estrogen and progesterone which may be the reason for this effect (Maughan *et al.,* 2010).

2.1.3.8 Breast feeding

Breast feeding reduces a total number of lifetime menstrual cycles in women (Ross *et al.,* 2008). Especially if breast feeding is continued for 1½ to 2 years it may slightly lower breast cancer risk. However, this is a difficult area to study particularly in countries like United States and Europe where breast feeding is uncommon (Fisher *et al.,* 2005).

2.1.3.9 Other factors

In addition of above reviewed factors for the breast cancer there are some other factors which may contribute to the incidence of breast cancer. Such factors are use of contraceptive pills to prevent the child birth, smoking (McKenzie *et al.,* 2013), higher body weight and lack of physical activities (Huang *et al.,* 1997).

2.2 Types of breast cancer

There are several types of breast cancer, but some of them are quite rare. In some cases, a single breast tumor can be a combination of many types.

2.2.1 Ductal carcinoma in situ

Some of the breast cancers lack their ability to invades other cells of the tissue which means that those are localized cancer cells. All those cancers which effect the ductal cells of breast are noninvasive and are broadly known as ductal carcinoma in situ and abbreviated as (DCIS) which is also given another name of intra ductal carcinoma which means that it has only the ability to affect those cells which are lying within the duct not outside duct. The ratio of ductal carcinoma is 1 out of every 5 breast cancer. Breast cancer when identified early onset can be cured. The techniques used to determine breast cancer is known as mammogram (Welch *et al.,* 2008). When DCIS is diagnosed an area of dead or dying cancer cells called tumor necrosis within the tissue sample is found that the tumor is likely to be more aggressive. The term comedo-carcinoma is often used to describe DCIS with large areas of necrosis.

2.2.2 Lobular carcinoma in situ

Lobular carcinoma in situ (LCIS) is not as common as DCIS, LCIS incidence rates also increased 4-fold during late 1970s to early 2000s. Highest increase observed among women having 50 years and older LCIS was first defined as a separate entity in 1941 to differentiate it from invasive carcinoma involving the lobular structures of the breast. LCIS is generally considered as a risk factor rather than a precursor for invasive lobular and ductal carcinomas (Li *et al.*, 2005).

2.2.3 Invasive (or infiltrating) ductal carcinoma

Breast cancer has also been classified upon its ability of division. Because there are two types of tumor some are localized and some tumor is not localized which have the capability of effecting the other adjacent cells. Those breast cancers which are not specific or localized to their site of occurrence are also known as invasive or infiltrating. Some of the breast cancers are not localized mostly those which are affecting duct cells of mammary glands. A duct cell helps in milk production. So it may be capable of spreading to the other near tissues or cells through the process of metastasis via blood vessels and it is investigated that out of 10 approximately 8 breast cancers are infiltrating type which are particularly known as invasive or infiltrating ductal carcinomas (Eheman *et al.*, 2009).

2.2.4 Invasive (or infiltrating) lobular carcinoma

There is another most important type of cancerous cells which effect that part of memory glands that are actually involved in milk product and that particular type is called Invasive lobular carcinoma (ILC). It has also the ability of affecting nearby cells so called invasive because it has the capability of invasion to the nearby cells. The property of affecting other nearest cells by any abnormal cell is known as metastasis. The detection of ductal carcinoma is easy through mammography as compared to invasive lobular carcinoma because of high level cell division and due to which cells loses its identity (Lopez and Bassett 2009).

2.2.5 Less common types of breast cancer

2.2.5.1 Inflammatory breast cancer

This uncommon type of invasive breast cancer accounts for about 1% to 3% of all breast cancers. Usually there is no single lump or tumor. Inflammatory breast cancer (IBC) makes the skin on the breast look red and feels warm. These changes are not caused by

inflammation or infection, rather due to blockage of lymph vessels by cancer cells in skin. This type of breast cancer tends to have a higher chance of spreading and a worse outlook (prognosis) than typical invasive ductal or lobular cancer (Alitalo and Detmar 2012).

2.2.5.2 Paget disease of the nipple

This is another most serious and damaging type of breast carcinoma that is normally acting nipples of the breast. This at early stages starts at breast ductal cell but because of having property of invasion and continuous its penetration to our cutaneous skin layer and comes up to the surface of the breast and attack the dark circles around the nipple. Although this is rare form of breast cancer and accounts for approximately affecting 1 female out of 100 but still very dangerous. cancer. (Spiliopoulos *et al.,* 2009b).

2.2.5.3 Phyllodes tumor

As cancer occurred when cell loses its capability of regulation of cell division due to some external agent that causes alteration in those genes which are involved in cell proper division so the phyllodes type of cancer is also known as phyllodes tumor because cancerous cell converts into tumor cells. Although such tumors which is caused because of this particular type of cancer are usually localized and do not have the invasive ability so it can easily be treated and control to some extent (Mishra *et al.,* 2013).

2.2.5.4 Angiosarcoma

Angiosarcoma starts in cells that line blood vessels or lymph vessels. It rarely occurs in the breasts. It usually develops as a complication of previous radiation treatments. Angiosarcoma can also occur in the arms of women who develop lymphedema as a result of lymph node surgery or radiation therapy to treat breast cancer (Vuille-Dit-Bille *et al.,* 2013).

2.2.6 Molecular sub-types of breast cancer

There are many types of the cancer that particularly effect breast and it is grouped to mainly four classes which are, Luminal B, Triple negative/basal like , Luminal A and Her-2 type. Beside these some other types have4 also been identified on the bases of their molecular characteristics similarities claudin type and apocrine molecular type. There are some other types of cancer which are not been listed in these types because of it lacks the similarity on the bases of which these are classified so they are given the name unclassified. All these types of cancer have been studied using certain techniques for their classification

like information from tumor cell including both genetic and molecular information. The grouping to four subtypes are roughly done on the bases of their hormonal receptor status (HER2/neu status and proliferation rate) (Pusztai *et al.,* 2006).

2.2.6.1 Luminal A

One of the most important property for classification of cancer is their physiology and that is why luminal A is a type of cancer that has given a name because in this cells looks like luminal because the early site of cancer cells are inner luminal cells. In this cancer the cancerous cells must be HER2/neu-negative (HER2-), progesterone receptor positive (PR+) and estrogen receptor positive (ER+). Luminal A subtype is identified with highest survival rate and low death rate (Fung-Kee-Fung M *et al.,* 2006a).

2.2.6.2 Luminal B

Tumors type are based on certain receptor on which they are dependent which are involved in cell divisions one way or the other and luminal B type tumor is dependent on progesterone receptor-positive (PR+), estrogen receptor-positive (ER+) and Ki67 is observed with high level of cell division and it is also HER2/neu-positive (HER2+) dependent. Breast cancer early age onset then the women have been detected with luminal B type tumor so it is effecting young women. While no women or relatively few women are identified with luminal A tumor and luminal A have been identified with some specific properties like lymph node positive, large tumor size, Poorer tumor grade, and p53 mutation (about 30%) (Dawood *et al.,* 2011a).

2.2.6.3 Triple negative/basal like

It is not necessary for all basal like tumor to be triplet negative and for all triplet negative to be like basal like and vice versa. It is known that all cancers which are caused because estrogen receptor negative (ER-) triplet negative. \and HER2/neu negative (HER2-). Some of the tumor cells are having homogeneous properties like in some case there occurs similarities in cells of outer cells to that of ductal mammary cells. The expression of p53 mutation and expression of HER1/or cytokeratin 5/6 proteins. (Turner and Reis-Filho 2006).

2.2.6.4 HER2 type

This tumor type is named because it is HER2 dependent which is a receptor and it does not depend upon estrogen receptor so it is (ER-) and it is not dependent on

progesterone receptor so it is also (PR^-). Although it is dependent on lymph node with fairly weak tumor causing ability and it is prone to early and frequent ability of effecting nearby cells particularly known as metastasis (Hartman *et al.,* 2011). All those women who are identified with breast cancer at younger age are having HER2 type tumors mostly than the other two type which are, luminal A and luminal B tumors (Voduc *et al.,* 2010).

2.2.6.5 Normal basal like

This is a less common type of breast cancer. About 6 to 10 percent of all breast cancers fall into the normal breast like category. These tumors are usually small and tend to have a good prognosis (Fung-Kee-Fung M *et al.,* 2006b).

2.2.6.6 Race/ethnicity and subtypes of breast cancer

The prevalence rates of the four major molecular subtypes of breast cancer are different among races. However, Luminal B and HER2 type tumors do not appear to differ by race (Dawood *et al.,* 2011).

2.3 Breast cancer types on the basis of heredity

Breast cancer may arise due to inheritance or without involvement of heredity. On the basis of inheritance breast cancer can be classified into two broad classes; namely, familial (hereditary) breast cancer and sporadic (spontaneous) breast cancer.

2.3.1 Familial breast cancer

A breast cancer is regarded as familial if patient have a family history. In other words, if breast cancer has been diagnosed at least once either in last two generation or up to second cousins of the patient, the patient will be regarded as familial breast cancer patient (Hasan *et al.,* 2013). When breast cancer was properly studied by various researcher it was found and they came to know that not all type of breast cancers is familial but only 15-20 cases of breast cancer is familial out of 100 while rest is not hereditary cancer. While some other reasons were also identified like germ line mutation have also been reported in 10 out 100 individuals with breast cancer which is caused by alteration in BRCA 1 and BRCA2 gene mutation, so it means it's not always because of genetic history. Familial breast cancers attributable to mutations in BRCA1 exhibit higher grade tumor, elevated mitotic count, presence of a lymphocytic infiltrate and the presence of a smooth non infiltrative border to the cancer. Familial Breast cancers attributable to BRCA2 mutations are also of higher overall grade, predominantly as a result of less tubule formation (Lakhani

et al., 2000). In addition to BRCA1 and BRCA2 genes mutations in the PTEN, TP53, and ATM etc. are known or suspected to be associated with an increased risk of breast cancer. But none of these genes fail to explain an important fraction of familial aggregation of breast cancer (Liaw *et al.,* 1997).

2.3.2 Sporadic breast cancer

Sporadic breast cancers arise spontaneously/sporadically rather through inheritance of certain genes. This may occur due to the epigenetic effects (DNA methylation) or the defects of DNA repair mechanisms that allow environmentally triggered mutations. Approximately 80% of the breast cancers are sporadic without any familial history of the disease. Primary tumors are of high grade. They show lower expression of estrogen and progesterone receptors (mostly ER- and PR-). EGFR is over expressed in sporadic tumors with BRCA mutations. However, they are mostly HER2/neu positive (HER-2+) (Van der Groep *et al.,* 2006).

2.4 Diagnosis of breast cancer

Sometimes breast cancer found to be appeared after symptoms. But in many cases women with early breast cancer have no symptoms.

2.4.1 Symptoms of breast cancer

Formation of new painless, hard and irregular lump or mass in the breast is the most common symptom of breast cancer. But breast cancers can be tender, soft or rounded. They can even be painful in some cases. Other possible signs of breast cancer include swelling of all or part of a breast (even if no distinct lump is felt), skin irritation or dimpling, breast or nipple pain, nipple retraction (turning inward), redness, scaliness, or thickening of the nipple or breast skin and nipple discharge (other than breast milk) (Barber *et al.,* 2004).

2.4.2 Staging of breast cancer

Number of techniques are used for curing breast cancer and some of which are currently used is known as staging of breast cancer which is dependent upon two main properties one is their clinical size and the second one which is most important is the way or ability of its cell division and its property to effect other cells. Some other properties which are also considered most important are capability of invasion of cells means the property of presence or absence of invading nearby cells which is represented by alphabet N and another property is that of its capability of cell invasions which is biologically known

as metastasis and is denoted by word M. All these stages are combinely showed by TNM which are further subdivided into different stages.

Stage 0 This is the early stage of cancer start up that's why it is named as stage 0 which have two main types one is known as carcinoma in situ (lobular carcinoma in situ (LCIS) and other one ductal carcinoma in situ (DCIS) because it effects ductal portion of cells.

Four broad categories are distinguished by the Union International Centre Cancer (UICC) which are;

Stage I – the starting stage in which the size of mass is of about or less than 2 cm across but still localized and it is not being spread to the other parts.

Stage II – the size of tumor remains less than 2 cm but it has invaded the cells of lymph nodes which are found under arm and tumor continuous to 5 cm (it may or may not be spread to the lymph nodes).

Stage III – in this case the cancerous cells start spreading to the lymph nodes under the arm and cover more than 5 cm area of size or the tumor have reached to the main bone of the chest and near lymph nodes and other tissues in near vicinity.

Stage IV –the breast cancer loses its identity and become sporadic and stars invading the other organs and nearst cells which are only adjacent to mammary gland but belongs to another organ like hand cells etc. (Singletary *et al.*, 2002).

2.5 Breast cancer susceptibility genes

Approximately 5–10% of all breast cancers are caused by germ line mutations in well recognized breast cancer susceptibility genes. These genes can be roughly divided into high risk and low to moderate risk breast cancer susceptibility genes. The high risk breast cancer susceptibility genes include BRCA1, BRCA2, PTEN, TP53, LKB1/STK11 and CDH1 with relative lifetime risks higher than 4. The CHEK2, TGFβ1, CASP8 and ATM genes belong to the low to moderate risk breast cancer susceptibility genes (Oldenburg *et al.*, 2007).

2.5.1 High risk susceptibility genes

2.5.1.1 BRCA1 and BRCA2

The BRCA1 gene is located on chromosome 17q21 and the BRCA2 gene is located on chromosome13q12.BRCA1 and BRCA2 do not share any distinct sequence homology; the similarities between them are interesting. Both genes are larger genes: BRCA1 has 22

exons, spans approximately 100 kb of genomic DNA and encodes an 1863 amino acid protein, while BRCA2 has 27 exons, spans around 70 kb and encodes a protein of 3418 amino acids (Tavtigian *et al.,* 1996). They are both characterized by the presence of an extremely large exon 11. Both genes are the caretakers as these genes behave like sensors of DNA damage and participate in the repair process. Their inactivation allows other genetic defects to accumulate and leads to genetic instability (Breivik 2005). During the past two decades many of the cellular and biochemical functions of the BRCA1 and BRCA2 proteins have been discovered. Together these suggest how BRCA1 and BRCA2 might play a role in carcinogenesis. For BRCA1 these roles include DNA repair, protein ubiquitylation, chromatin remodeling and cell cycle checkpoint control. BRCA2 is involved in double strand break DNA repair through homologous recombination (Narod and Foulkes 2004). (Antoniou *et al.,* (2002) estimated that in a Caucasian population the prevalence of heterozygous carriers of high risk mutations for BRCA1 is one in 1000 whereas for BRCA2 is one in 750. However, this frequency may be higher in certain populations due to founder mutations. Germ line mutations in BRCA1 or BRCA2 have lifetime higher risks of breast cancer and ovarian cancer as well as smaller risks to some other cancer types. The cumulative risk of breast cancer at the age of 70 in BRCA1 and BRCA2 mutation carriers had been estimated as 85% and 84%, ovarian cancer 63% and 27% respectively in multiple case families. Meta-analysis on 22 populations based and hospital based studies showed that the average cumulative risks in BRCA1 and BRCA2 mutation carriers at 70 years were 65% and 45% for breast cancer and 39% and 11% for ovarian cancer. The relative risks of breast cancer declined significantly with age for BRCA1 mutation carriers (Antoniou *et al.,* 2003). There are other genes which may be the risk modifier of breast and ovarian cancer in BRCA1 and BRCA2 mutation carriers. For example, a C/G polymorphism in the 5'untranslated region of RAD51 was found to modify both breast and ovarian cancer risk in carriers of a germ line BRCA2 mutation (OR, 3.2; 95% CL, 1.4–40; p = 0.01) (Wang *et al.,* 2001). A length variation of the poly glutamine repeats in the estrogen receptor co-activator NCO3A influences breast cancer risk in carriers of BRCA1 and BRCA2 (OR, 1.96; 95% CI, 1.25–3.08; p for trend = 0.0036). For both BRCA1 and BRCA2 it is also investigated by researchers that the frequency of occurrence of cancer is also have some other factors involved and the position of mutation

in a particular transcriptional unit play the most important role also which means that the fate of cancer is concerned or dependent on the site it which alteration do occurs in the genes (Thompson and Easton 2002). As most of the researcher reported that chances of cancer of breast are minimum when the alteration occurs the middle part of the transcriptional unit of BRCA1 gene in contrast to those women in which the alteration in BRCA1 occurs it the outside ages of the transcriptional unit which mostly includes the regulatory sequences of the upstream. However, the case in the BRCA2 gene is a bit different from that of BRCA1. When the alteration occurs in between upstream and downstream including full string of the coding region then it results cancer of ovary than cancer of breast and this region is also known as (OCCR; ovarian cancer cluster region), whereas mutations in the OCCR were associated with a lower breast cancer risk than mutations outside the OCCR. In addition to a predominantly high increased risk to female breast cancer and ovarian cancer, BRCA1or BRCA2 mutation carriers are at increased risk to other cancers as well. An increased relative risk to colon cancer, cervix cancer, uterus, pancreas and prostate has been suggested in BRCA1 mutation carriers. In BRCA2 mutation carriers an increased relative risk to male breast cancer, gall bladder and bile ducts cancer, gastric cancer, malignant melanoma, pancreas, prostate, bone and pharynx cancer has been observed (Van Asperen *et al.*, 2005).

2.5.1.2 TP53: Li-Fraumeni syndrome

The TP53 gene is located on chromosome 17p13.1 which encodes a protein involved in many overlapping cellular pathways that control cell proliferation and homeostasis such as cell cycle, apoptosis and DNA repair. The expression of the TP53 gene is activated in response to various stress signals, including DNA damage. Loss of TP53 function is thought to be as a tumor suppressor (Pluquet and Hainaut 2001). Germ line mutations in TP53 are very rare: fewer than 400 families with germ line mutations have been reported worldwide. Mutations in the TP53 gene account for roughly 70% of families fulfilling the classical criteria for Li-Fraumeni syndrome (e.g. one patient with a sarcoma diagnosed <45 years with a first degree relative with any cancer diagnosed <45 years and an additional first or second degree relative diagnosed with cancer <45 years or a sarcoma at any age). Mutations in TP53 are less common in breast cancer/sarcoma families not fulfilling these classical criteria. Susceptibility to cancer in Li-Fraumeni

families follows an autosomal dominant pattern of inheritance. One of the most frequently occurring cancers in Li-Fraumeni families is breast cancer with an estimated penetrance in TP53 mutation carriers of 28–56% at 45 years (Evans *et al.,* 2002).

2.5.1.3 PTEN

The chromosomal region containing the Cowden syndrome gene was known to contain a tumor suppressor gene (PTEN) that had been found to be mutated in sporadic brain, breast and prostate cancer. In study germline mutations in the PTEN gene were found in four of five families with Cowden syndrome (CS). About 80% of CS families Mutations in the PTEN gene are present (Liaw *et al.,* 1997b). Women carrying a PTEN mutation have 25–50% (2–4 folds) lifetime breast cancer risk. The majority of Cowden syndrome related breast cancers occur after the age of 30–35 years. Breast cancer at young age has been observed in male carriers of a germ line PTEN mutation with the classical CS phenotype suggesting an increased risk for males as well (Fackenthal *et al.,* 2001). However, no mutations in the PTEN gene have been detected in breast cancer families without features of CS. Also in sporadic breast cancer patient's germ line and somatic mutations in the PTEN gene are rare. Although LOH at the PTEN locus is found in 11–41% of sporadic breast cancers, no somatic mutations have been observed in the remaining allele (Feilotter *et al.,* 1999).

2.5.1.4 LKB1/STK11

The LKB1/STK11 gene is located on chromosome 19p13.3 and encodes a transcript of ~1.3 kb, which acts as a tumor suppressor. Germ line mutations in the serine/threonine kinase gene (LKB1/STK11) causes Peutz-Jeghers syndrome (PJS). An elevated risk of gastrointestinal malignancies, breast cancer, pancreas, ovary, uterus, cervix, lung and testicular cancers is recognized in patients with PJS (Boardman *et al.,* 1998). It is suggested that LKB1/STK11 can play the role of a tumor suppressor gene in sporadic breast cancer and low expression of the LKB1/STK11 protein is significantly associated with a shorter survival (Shen *et al.,* 2002).

2.5.1.5 CDH1/E-cadherin

The E-cadherin gene (CDH1) is located on chromosome 16q22.1. The mature protein product belongs to the family of cell–cell adhesion molecules and plays a fundamental role in the maintenance of cell differentiation and the normal architecture of

epithelial tissues. Germ line CDH1 truncating mutations are associated with hereditary diffuse gastric cancer syndrome (HDGC syndrome). The lifetime risk of developing breast cancer was estimated at 20–40%. Somatic CDH1 mutations are frequently found in infiltrating lobular breast cancer and in situ lobular breast cancer (LCIS) (Mastracci *et al.*, 2005).

2.5.2 Low to moderate risk breast cancer susceptibility genes

2.5.2.1 ATM

The ATM gene is located on chromosome 11q22–23. The ATM protein plays a central role in sensing and signaling the presence of DNA double strand breaks. In the irradiated cell nucleus, ATM is held inactive which is dissociated by rapid intermolecular auto phosphorylation. This initiates cellular ATM kinase activity which has many substrates including the protein products of TP53, BRCA1 and CHEK2. Carriers of homozygous or compound heterozygous mutations in the ATM gene suffer from the rare recessive disorder ataxia-telangiectasia. Female heterozygous carriers AT patients are at increased risk of breast cancer. The estimated relative risk of breast cancer in obligate AT heterozygotes range between 1.3 and 13 in the different studies conducted (Hall 2005). Studies of sporadic and familial breast cancer have failed to demonstrate an elevated prevalence of germ line ATM gene variants among breast cancer cases relative to controls (FitzGerald *et al.*, 1997).

2.5.2.2 TGFβ1

The TGFβ gene is located on chromosome 19q13.1 which contains seven exons and very large introns. TGFβ is a multifunctional peptide that controls proliferation, differentiation and other functions in many cell types. Dysregulation of TGFβ activation and signaling may result in apoptosis. For most normal cell types, TGFβ acts as a potent inhibitor of proliferation, migration and promotes apoptosis, properties associated with tumor suppression (Janda *et al.*, 2002). However, studies support a model in which TGFβ inhibits the development of early benign lesions but promotes invasion and metastasis when the tumor suppressor activity is overridden by oncogenic mutations in other pathways. To date several somatic mutations that disrupt the TGFβ-signaling pathway have been reported in human breast tumors (Xie *et al.*, 2002).

2.5.2.3 Caspase 8 (CASP8)

The CASP8 gene is located on chromosome 2q33–34 that contains 13 exons and spans 51.2 kb. Caspases are important mediators of the apoptotic process. Death receptor mediated apoptosis provokes the formation of death inducing signaling complex (DISC)which comprises the death receptors, adaptor proteins, initiator caspase 10 (CASP10) and caspase 8 (CASP8). Because of the involvement in initiation of apoptosis, it was hypothesized that CASP8 and CASP10 might act as low penetrance familial breast cancer susceptibility genes. Combined analysis of two different studies surprisingly showed that one missense variant (D302H) in CASP8 was associated with a reduced risk of breast cancer in a dose dependent manner (Frank *et al.,* 2006).

2.6 Single Nucleotide Polymorphism

This is the biological phenomena due to which changes or variation occurs in those organism which are belonging to same biological group or specie. In this phenomenon there occurs a 1 base pair change occurs which may be occring coding or noncoding sequences of DNA. These changes are random it have no spefic place on the genome but it occurs in bases pairs of the organism which are T,G,A and C and this particular changes in bases cost for the wide differences which are found all the organism of the same species. There are certain other mechanisms are involved to scrutinize some of the allele of SNP which can give the most favored combination for the best adaptation to the environmental conditions (Barreiro *et al.,* 2008). However, the process of selecting single nucleotide change for the environment favored combination some other mechanism is also playing an important role like alteration rate at which changes in a sequence occurs, genetic recombination etc. Rate of Single nucleotide change can be measure by the presence of some special sequences known as microsatellite and those sequences having AT microsatellites are having high rate of single nucleotide changes and the low GC contents (Varela and Amos 2010).

Yet now, there are 62,676,337 SNPs in human genome were reported and Out of them 44,278,189 have validated. This data base reports for 27,608,151SNPs in coding regions (dbSNP,2013). The differences among similar population in any of the living species are because of SNP. Approximately 99% of the human genome is identical but the

rest 1% difference is because of single nucleotide change (Dobrovic and Simpfendorfer 1997).

2.6.1 Types of SNPs

Single nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes (3'and 5'untranslated regions) or in the intergenic regions (regions between genes). Due to degeneracy of the genetic code SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein produced. Single nucleotide changes in a sequence which is biologically known as SNPs are having two types on the bases of their property on end product the protein structure and function and these are, non-synonymous and synonymous nucleotides. Those single nucleotide changes which alter the protein product are known as synonymous while those which have no effect on the final protein product are called non-synonymous. It is not necessary that those single nucleotides which is alter have no affect at all because it may affect other process like mRNA degradation, process of splicing, transcription factor binging and also effect on the sequence of non-coding RNA. When the single nucleotide change affects the gene expression process then it is called eSNPs which be location anywhere at transcription unit either upstream r downstream. (Alshatwi *et al.*, 2012).

2.6.2 SNPs in BRCA1 and breast cancer

Mutations in the cancer susceptibility gene BRCA1 greatly increase the risk of breast and ovarian cancer (Miki *et al.*, 1994). At present most mutations in the BRCA1 gene have been identified to be point mutations or small insertions and deletions. Literature survey showed that there is a wide choice of literature on the BRCA1 gene related to breast cancer. However, a computational study revealed that a total of 477 SNPs in the BRCA1 gene, 65 were found to be unsynonymous and 4 and 10 SNPs were found to be in the 5′ and 3′ untranslated regions (UTR). Of 65 nsSNPs, 12 nsSNPs were found to be deleterious. Two SNPs in the 5′ UTR and 3 SNPs in the 3′ UTR were found to be of functional significance. It was found that the major mutation in the native protein of the BRCA1 gene was from proline to serine. It concluded that rs1800751 with a mutation of proline to serine at position 1812 in the native protein could be the main target mutation for the breast cancer caused by the BRCA1 gene (Rajasekaran *et al.*, 2007).

2.6.3 DNA Methylation

Modern aspects of epigenetics refer to the modification of DNA and related proteins without variation in nucleotide sequences which passes the contained information to next generation. DNA methylation is the most studied and the best understood epigenetic modification. On the basis of many studies on a battery of housekeeping genes and growth regulator genes, DNA methylation is established as an additional mechanism for gene inactivation in different cell types including cancer cells (Lehmann *et al.,* 2002). DNA methylation plays an essential role in maintaining cellular function. Its role in carcinogenesis has been a topic of considerable interest in recent years. Changes in DNA methylation patterns may contribute to the development of cancer. Aberrant global methylation of DNA is frequently found in tumor cells. Global hypo methylation can result in chromosome instability. Hyper methylation has been associated with the inactivation of tumor suppressor genes (Whitman *et al.,* 2008).

The methylation of DNA is an outcome of the activities in a family of enzymes known as DNA methyl transferases (DNMTs) which take up the methyl group from co-factor SAM (Jeltsch *et al.,* 2007) and mediate the transfer to a nucleotide particularly cytosine. Three families of mammalian DNMTs have been found and are DNMT1, DNMT2, and DNMT3. Among these DNMT2 has little involvement in methyl group transfer to DNA. Recently it has been found to be involved in the methylation of a cytosine residue in the anticodon loop of tRNA-Asp (Goll *et al.,* 2006).

In normal cells actively transcribing chromatin is hypo methylated whereas non transcribing chromatin is methylated in such a way that it makes compact structures which hinder RNA Pol II activities (Lorincz *et al.,* 2004). The methylation of DNA is found to be involved in the stabilization of chromatin structure in an inactive conformation and inhibits gene transcription. Therefore, it is believed that the mechanism of gene regulation through DNA methylation involve essential genetic events including differentiation, genomic imprinting and X-chromosome inactivation (Bernardino-Sgherri *et al.,* 2002).

2.6.4 Types of DNA methylation

Methylation or demethylation of DNA is an essential feature of a normal genome. But whenever DNA becomes abnormally methylated it leads to many abnormalities. Abnormal DNA methylation accounts for (a) the excess of methylation (hyper methylation)

of genes that must be transcriptionally active in normal conditions and (b) the excess of genome wide demethylation (global hypo methylation). A major abnormality associated with hyper methylation of the promoter regions of tumor suppressor genes and DNA repair genes (p15, p16, p57, p53, SLC5A8 and BRCA1) is an onset of different kind of cancers, e.g. skin, blood, breast and ovary (Hayslip and Montero 2006). Hypo methylation of DNA causes instability of the genome and is also related with certain cancers. For example, a case control study from Spain reports that leukocyte genomic DNA hypo methylation is associated with increased risk of developing bladder cancer. Hyper methylation takes place mostly at CpG islands of promoter region of a gene, but hypo methylation usually is concerned with repeated DNA sequences such as Long Interspersed Nuclear Elements (Ehrlich 2002). Global hypo methylation also activates transposable elements (TE) to transcribe in both sense and antisense directions (Roman-Gomez *et al.,* 2005).

2.6.5 CpG island in gene promoters and whole genome

The main target of DNA methylation is cytosine. The majority of 5'methylcytosine in mammalian DNA is present in cytosine–guanine (CpG) dinucleotides which are present in promoter regions of many genes. Among all human promoters 72% contain high CpG content and 28% of promoters with low CpG content (Saxonov *et al.,* 2006). As far as the whole human genome is concerned, CpGs are present approximately once per 80 dinucleotides in 98% of the genome; however, 1–2% of the human genome, the frequency is five times higher (Bird 1986).

2.6.6 Methylation of cancer related genes in breast cancer

It has been found from number of evidences that hyper methylation of Breast cancer causing genes which causes Breast cancer can be minimize by CpG island methylation due to which expression of gene. There are many broad categories like tumor susceptibility, carcinogen detoxification, steroid receptor and cell cycle regulation. P16 is a gene which is encodes for a cyclin dependent kinase inhibitor, p16 INK4A. The process of methylation of upper regulative sequences that is 5′ promoter and exon 1 regions of p16 INK4A and which is a cause of more than 20-30% of cancer of female breast (Woodcock *et al.,* 1999). There are some genes which are involved in DNA damage and repair mechanism in human and 14–3–3σ gene (also known as HME1) is also a member of this family and he function of this gene is to place proper checkpoints for cell division in response of DNA damage

and repair mechanism (Chan *et al.,* 1999). There are many researchers who have tried to introduce a mechanism for reduce expression of certain genes that are involved expression. CpG methylation in extreme level is known as hyper methylation and this process takes place at exon number 1 of the gene which also brings about some other alteration like loss of heterozygosity (LOH) and intragenic mutations in breast cancer. Some alteration like epigenetics alteration play in inactivating role or down regulatory role of estrogen receptor (ER) gene. There are some other genes that are called nuclear receptors superfamily and RAR gene is one of the most important member of the receptor genes and receptor genes are usually those genes which have in indirect function in regulating certain processes at cellular level. The cytogenetic location of RAR gene is at chromosomes number 3 long arm p band 24 which have been known for controlling cellular process specially cell division which have dominant role in breast cancer, lung and others. However, some processes are also known which actually down regulated cancerous cells the phenomenon is known as DNA methylation (Widschwendter *et al.,* 2000).

Hyper methylation of regulatory sequences like the starter sequences which is specifically called promoter region which in turn found in CpG Island of breast tissues which is having inhibiting properties of metalloproteinase 3 (TIMP3) which normally digests protein which is founded in the cancer of stomach or associated. Another regulatory gene which is known as IGFBP7 belongs to growth factors and it have regulatory functions. The hyper methylation of IGFBP7 is concerned with reverse regulation of various carcinomas and particularly in prostate cancer (Sullivan *et al.,* 2012).

2.6.7 Promoter methylation of BRCA1 and decreased gene expression

Number of genes ae involved in breast cancer like BRCA1 gene. This gene is having cytogenetic location 17q21 which scientifically means that at is situated on chromosome number 17 of human and band 21 of short arm which is symbolized as q. It is studied that it has been found that approximately 50 out of 100 individuals who are affected with breast cancer because of BRCA1. Whenever there occurs some alteration in the gene due to mutagens which are of broad range like UV light, Gamma radiations and some other are also involved and all such cancer types are inherited. In comparison all those genes which are involved in tumor suppresser are not very susceptible to alteration.

In other words, gene mutation that is involved breast cancer and ovarian cancer causes sporadic breast cancer (Janatova *et al.,* 2005).

But according to some recent studies which were carried out by (Zhang *et al.,* 2012) showed that the frequency of occurring these cancer ranges from 0.05-0.032. However there some remedies have been thought to overcome the cancer causing property of BRCA1 gene and that particular remedy is controlling the gene product by early methylation of DNA. There were some techniques which were used to identify methylation process which was identified in almost 11% sporadic cancer which was indirectly proportional to ER and PR expression. In one of the study which was composed up of 194 patients who were diagnosed with primary breast cancer and which revealed that BRCA1 methylation took place in almost 13 out 100 patients who were selected in primary breast cancer (Esteller *et al.,* 2000). In comparison to the affected individuals' normal individuals were identified in no methylation in those tissues were having normal function but it was also reported in 21 patients of breast. Methylation with high frequency of those genes which are called BRCA1 in some of regulatory regions like promoter which are involved in all cellular machinery. BRCA1 as the most known candidate for causing breast cancer with known percentage of 7–31 in all familial cases (Catteau and Morris 2002). Methylation of gene that is called BRCA1 which is known for invasive tumors display which have the similarities with BRCA1 in cause's pathogenicity of cancer specified breast. The feature of both of the breast cancer either sporadic or not sporadic are caused in women with inherited BRCA1 germ line mutations because of similarities in their global gene expression profiles (Hedenfalk *et al.,* 2001). Methylation of BRCA1 was only identified in breast cancer and ovarian cancer as a cause but not found inother cancer like cancer of colon liver cancer or leukemia which is showing that it supports specific processes at tissue level. There are different techniques which are chromatin accessibility assays and chromatin immunoprecipitation endonuclease, transcriptional repression of the gene so called BRCA1 is linked to histone mechanically which works in activation and deactivation of chromatin (Fiegl H *et al.,* 2001).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.0 Materials

The materials used are categorized into two main sections and are as follows.

3.1 Patients identification and collection of samples

Diagnosed females with breast cancer were ascertained from oncology ward from research institute of Nuclear Medicine Oncology and Radiotherapy Institute (NORI) were source of sampling. Patient's blood samples (5 ml/individual) were collected in 5 ml vacutainers with EDTA after informed consent. A copy of the consent form is attached in the annexure at the end of this thesis. The drawn blood put immediately in ice cooler and shifted to Human and Molecular Genetics laboratory department of Bioinformatics and Biotechnology IIUI and stored in refrigerator on -40°C.

3.2 Lab Work

Lab work includes DNA isolation from blood and quantification, selection of candidate genes for sequencing, primer designing and synthesis for DNA sequencing, PCR amplification of sequencing primers, DNA sequencing and DNA sequence analysis.

3.2.1 DNA Isolation

Genomic DNA was isolated from the blood samples following non-organic method (Grimberg *et al.,* 1989). Blood samples were thawed for the RBCs lysis. Washing/T.E buffer (10mM Tris HCl pH8, 2mM EDTA) was added to each sample by raising the volume up to 20ml in falcon tubes. These were centrifuged at 2700 rpm in the Beckman TJ-6 Centrifuge for 20 minutes at 20°C. Supernatant was discarded and pellet was broken by gentle tapping. Washing was repeated for three to four times till the WBCs' pellet got free of hemoglobin (RBCs). The pellets were re-suspended in proteins' digestion mixture containing 6ml of TNE/A1 buffer (10 mM Tris HCl pH 8, 2 mM EDTA, 400 mM NaCl), 13 µl of Proteinase K (10 ug/µl) and 50 µl of 10% SDS (each for 2.5ml of blood volume). The samples were left overnight in an incubating shaker at a temperature of 37 °C and a speed of ~250 rpm.

Proteins were precipitated by adding 250µl of 6M NaCl per ml of blood volume, followed by vigorous shaking and chilling on ice for 15 minutes. The samples were centrifuged at ~2700 rpm for 15 minutes at 20°C. Supernatant was taken out in a separate tube and centrifuged as in previous step. Supernatant was taken out in a separate tube and the protein pellet was discarded. DNA was precipitated in the form of white threads from the supernatant by gently inverting the tubes after adding equal volume of isopropanol. After 10 minutes' samples were centrifuged at ~2700 rpm for 10 minutes at 20°C. Supernatant was discarded and 10ml volume of 70 % ethanol was added to wash the pellet. Samples were again centrifuged as in previous step. Supernatant was discarded carefully and pellet was dried at room temperature. DNA was dissolved in 375 µl (for each 2.5 ml of blood) of autoclaved TE for DNA (Tris 10 mM, EDTA 0.1 mM). The samples were left overnight in an incubating shaker at a temperature of 37 °C and a speed of ~250 rpm to dissolve the DNA. Heat shock was given at 70 °C in a shaking water bath for 1 hour to inactivate any remaining nucleases. All the samples were transferred into properly labeled small screw capped tubes.

3.2.2 Quantification of Isolated DNA Samples

DNA samples were quantified by agarose gel electrophoresis and Optical Density (OD) measurement and NanoDrop Spectrophotometer.

3.2.2.1 Agarose Gel Electrophoresis

This method uses the UV-induced fluorescence of Ethidium Bromide dye intercalated into the nucleic acid. The amount of fluorescence is proportional to the amount of nucleic acid present. Fluorescence of the test DNA and that of a standard DNA was compared. 1.0 % Agarose gel slab (stained with 6µl (10µg/µl) Ethidium Bromide) was prepared. Gel was loaded by 2µl of each DNA sample mixed with 4 µl of loading dye (Bromophenol blue 2X) and was run on 120 V for 35 minutes. The gel slab was observed in gel documentation apparatus (UV trans-illuminator) by using Grab IT software. The samples quantity was estimated and recorded in DNA extraction sheets. All the DNA samples were stored in IIUI repository at -20°C until processed further.

3.2.2.2 Optical Density Measurement (Spectrophotometry)

Following steps were used for measuring concentration of DNA in the sample tubes.

Step1: Diluted DNA stock solution with water in a ratio of 1:100 in a 1.5ml centrifuge tube was made.

Step2: Dispensed 400µl autoclaved distilled water into the cuvette as a reference.

Step3: DNA sample was poured in the cuvette for spectrophotometry and quantification. DNA was scanned for a range of UV radiation (260nm-280nm).

Step4: UV absorbency values at 260nm and 280nm of radiation were noted and A260/A280 was calculated as follows Amount of DNA (ng/µl) = Absorption at 260 x 50 x DF Dilution Factor (DF) =Total vol. of dilution/Vol. of stock DNA in the dilution

Step 5: After calculations 5ng/µl dilutions were prepared in 100µl volume and stored at 4°C for further use. Dilutions were prepared by using the following calculations

V1= (C2xV2)/C1 • C1 = Amount of DNA in stock solution.

• V1 = Volume of DNA of stock solution to be diluted.

• C2 = Amount of DNA in dilution to be prepared (5ng/µl).

• V2 = Volume of DNA dilution to be prepared (100ml).

3.2.2.3 NanoDrop Spectrophotometer

Nano Drop test is done for the conformation that either DNA is present in the stock sample. If it is present, then it will show the concentration of DNA.

Start the program on the computer if you want to calculate concentration of DNA or RNA sample select the Nucleic Acid tab. First of all, make sure that the Nano Drop sensor is free of any type of debris. Open the Nano Drop arm and clean the sensor. Before measuring the sample first measure the blank concentration by 1.5 µl PCR Water once you loaded the blanking solvent onto lower sensor then lower the another arm and click the blank tab on the computer. Once the blank is measure then measure then sample, add 1-2

µl sample on the lower arm and close the arm after that click on the measure button it will show the result in the form of unit ng/µl.

3.3 Candidate Gene Selection

Instead of linkage analysis, direct sequencing approach has been used during this study. For this purpose, selection of the candidate genes was based on four approaches:

(a) **Previous research work** was used to identify the relationship of clinical analysis and mode of inheritance with the possibility of finding causative genes in a certain pedigree.
(b) **Exons with most mutations** were searched from the reported mutations from different world populations for BRCA1 genes. Only the exons which are having most of mutations reported for a certain type of BRCA1 confirmed by clinical analysis and mode of inheritance with the help of reported literature.

3.4 Designing of Sequencing Primers

Sequencing primers were designed after selection of candidate gene and their most plausible candidate exon selection. Primers were designed using Primer3 web server (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) for selected gene. The virtual performance of designed primers against human genome were checked by In-Silico PCR utility (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start) and BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat?hgsid=345100591&command=start) tools of UCSC
Genome Browser. Finally, the selected primers were synthesized by Macrogen, Korea.

Overview of the primer sequences used to identify the mutations of BRCA1

GENE	EXON	Forward primer	Reverse primer
BRCA1	10-a	5'-ATATAGCCAGTTGGTTGATTTC-3'	5'-ACGTCCAATACATCAGCTACTT-3'
	10-b	5'-AGCAGCATTCAGAAAGTTAATG-3'	5'-ACAATTAGGTGGGCTTAGATTT-3'
	10-c	5'-AATATCCACAATTCAAAAGCAC-3'	5'-ATTCCTCTTCTGCATTTCCT-3'
	10-d	5'-AAGCATAGAAATGGAAGAAAGT-3'	5'-TGGAGAGAAATCTGTATTAACAGT-3'

10-e	5'-GCTATGCTTAGATTAGGGGTTT-3'	5'-TGCTTTGCTCTTCTTGATTATT-3'
10-f	5'-TCCTTTCTTGATTGGTTCTTC-3'	5'-CGTGTGTGTGAAATAAAAGGTA-3'

3.5 DNA Sequencing (by Sanger Dideoxy Chain Termination Method)

3.5.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed in 0.2 ml tubes (Axygen, USA) containing 20µl total reaction mixture. Following recipe were used for PCR mixture shown in (Table 2.1).

Table 2.1 PCR Reaction Mixture

Ingredients	Final Conc.	Stock Conc.	Required Volume
Genomic DNA	50 ng	25 ng/µl	2 µl
Primer Forward	0.5µM (5pmol)	10µM	1 µl
Primer Reverse	0.5µM (5pmol)	10µM	1 µl
dNTPs (dATP,dTTP,dCTP,	0.2mM (200µM)	2.5 mM	1.6 µl
PCR Buffer	1x	10x*	2 µl
Magnesium Chloride	2.0mM	50mM	0.8 µl
Taq Polymerase	1 units	5 units/µl	0.5 µl
dH_2O	-	-	**q.s to 20 µl

3.6 Optimization for amplified products

Optimization of PCR conditions for BRCA1 gene exon ten primers was carried out using various reagents and temperature conditions. A wide range of annealing temperatures (50°C-58°C) along with variable concentrations of MgCl2 conditions (2.5mM- 3.5mM) were used to remove non- specific primer binding. Individual exons for the respective

genes were optimized and verified after running the samples on 2% agarose gel. A list of the reagents used for amplified products is provided in the table 2.4. After optimization, amplification conditions set for each exon were as 95°C for 4min; 95°Cfor 30s, 55°C for 30s, 72°C for 1min for 30cycles with a final extension of 72°C for 45 min.

3.6.1 Agarose Gel Electrophoresis

After amplification of desired region, PCR products were subjected to agarose gel electrophoresis. Agarose gel electrophoresis is the utmost convenient way for separating DNA fragments depending on size in range from 100 bp to 25 kb (Sambrook *et al.,* 2001). Ethidium bromide was used for staining as it illuminates the molecules under ultra violet (UV) light.

1. Agarose gels 2% was prepared according to w/v percentage solution. 6gm agarose was weighed in an Erlenmeyer flask and 30ml of 10x TBE gel buffer was added in the flask.
2. Agarose gel solution was heated in oven for short intervals 60 sec and swirled until all agarose crystals disappear.
3. Hot gel solution left for few minutes then ethidium bromide (EtBr) was added to a concentration of 0.5 µg/ml.
4. Gel tray was placed into the casting apparatus. Appropriate comb was placed in gel mold to create wells.
5. Molten agarose was poured into the gel mold and left at room temperature for gelation around 45-60 minutes.
6. Later the comb was swiftly removed from the agarose gel.
7. The gel caster was shifted into electrophoresis tank containing 1X TBE buffer sufficient to cover the gel surface to 2mm, waited for 15 min to allow the gel to be equilibrated with buffer.
8. A 3 µl cDNA was mixed with 3 µl loading dye [0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol] at para film or inside a PCR tube, this mixture was loaded into a well within gel alongside a standard DNA marker.

9. The electric supply was attached to the electrophoresis tank and set for 45min run at 120 Volts and 500mA.
10. The separated DNA was visualized under UV light using trans-illuminator and gel documentation.

3.6.1.1 10X TBE preparation

To prepare 1L of 10X TBE buffer, 108gm of Tris, 55gm of Boric acid and 7.44gm of EDTA were weighed in a glass beaker, at the end, dH_2O was added to make up final volume. The buffer was stored at room temperature for further use.

3.6.1.2 Gel Visualization

1. The power supply turned off and the lid was removed from the electrophoresis tank at completion of electrophoresis.
2. Gel caster was removed from the electrophoresis tank and excessive buffer was drained off.
3. The gel was removed from gel caster and placed on the UV trans-illuminator and after adjustment the picture of DNA bands was taken by gel documentation system and saved for record.

The gel was properly disposed-off as per instruction of the department.

RESULTS

4. RESULTS

4.1 Cohort study

Phenotype of the collected blood sample patient are described in following Table no. 4.1

Table 4.1 Patients Phenotype(s)

Patient ID	Mammary	Stage	Grade	Type	Age
PIN 1	Ca LB	III	0	Mammary carcinoma	36
PIN 2	Ca RB	IV	0	INFDC	50
PIN 3	Ca RB	II	II	IDC	70
PIN 4	Ca RB	II	0	IDC	50
PIN 5	Ca RB	II	0	IDC	40
PIN 6	Ca LB	II	II	IDC	80
PIN 7	Ca LB	II		IDC	35
PIN 8	Ca RB	IV	$T_2N_2M_1$	IDC	45
PIN 9	Ca LB	III		ILC	49
PIN 10	Ca RB	III		INFDC	28
PIN 11	Ca RB	III		IDC	56
PIN 12	Ca RB	IV		INFDC	39
PIN 13	Ca LB	II	T_2	IDC	36
PIN 14	Ca RB		$T_{2b}N_2M_0X$	IDC	63
PIN 15	Ca LB	III		IDC	41
PIN 16	Ca LB	III	III	INFDC	48
PIN 17	Ca RB	III	$T_{4C}N_3$ III	IDC	55
PIN 18	Ca LB	III		IDC	50
PIN 19	Ca RB	III	III	IDC	45
PIN 20	Ca LB		$T_2N_0M_0$	IDC	50
PIN 21	Ca RB	II	II	IDC	60

PIN 22	Ca LB	IV	II BONE METASTASIS	ILC	55
PIN 23	Ca LB	IV	LUNG METASTASIS	IDC	46
PIN 24	Ca LB	II	II T_2N_1	IDC	42
PIN 25	Ca LB	IV	BONE METASTASIS	IDC	27
PIN 26	Ca LB	II	$T_3N_0M_0$	ILC	53
PIN 27	Ca LB	IV	BONE METASTASIS	IDC	31
PIN 28	Ca RB	IV	III LUNG METASTASIS	IDC	45
PIN 29	Ca LB	IV	BONE METASTASIS	IDC	47
PIN 30	Ca RB	II	III ER PR-ve	IDC	60
PIN 31	Ca LB	IV		IDC	60
PIN 32	Ca RB	II	II $T_2N_0M_0$	IDC	43
PIN 33	Ca RB	IV	$T_0N_0M_1$ LIVER METASTASIS	ILC	40
PIN 34	Ca RB	IV	$T_2N_1M_1$ BONE METASTASIS	IDC	50
PIN 35	Ca LB	IV	LUNG METASTASIS	IDC	50
PIN 36	Ca LB	II	II $T_2N_0M_0$	IDC	38
PIN 37	Ca RB	II	T_2N_1	IDC	25
PIN 38	Ca RB	III	$T_{4b}N_1$	IMC	56
PIN 39	Both	III	$T_{4b}N_1$ M_0	IDC	62

Table No. 4.2 History of Patients

Patient id	Familial disease CA Breast	Other familial disease	Marital status	Consanguineous marriage
PIN 1	No	Diabetes	Married	Non
PIN 2	Yes	No	Unmarried	Non
PIN 3	No	No	Married	Non
PIN 4	No	No	Married	Yes
PIN 5	No	No	Married	Non
PIN 6	No	No	Married	Non
PIN 7	No	Throat cancer	Married	Non
PIN 8	No	No	Married	Yes
PIN 9	No	No	Married	Non
PIN 10	No	Blood pressure	Married	Yes
PIN 11	Yes	No	Married	Non
PIN 12	No	No	Married	Non
PIN 13	No	No	Married	Non
PIN 14	No	No	Married	Non

(Table 4.2 shows familial disease of patients, marital status, non-familial disease as well.

4.2 DNA Extraction

DNA was extracted from the collected samples through non-organic method. Washing buffer, NaCl, Iso-propanol, proteinase K, T.E, Ethanol 70%, TNE buffer were used in extraction. The extracted DNA was confirmed by Gel Electrophoresis.

4.2.1 DNA Confirmation on Gel Electrophoresis

Extracted DNA was also run on 2% agarose gel electrophoresis for confirmation.

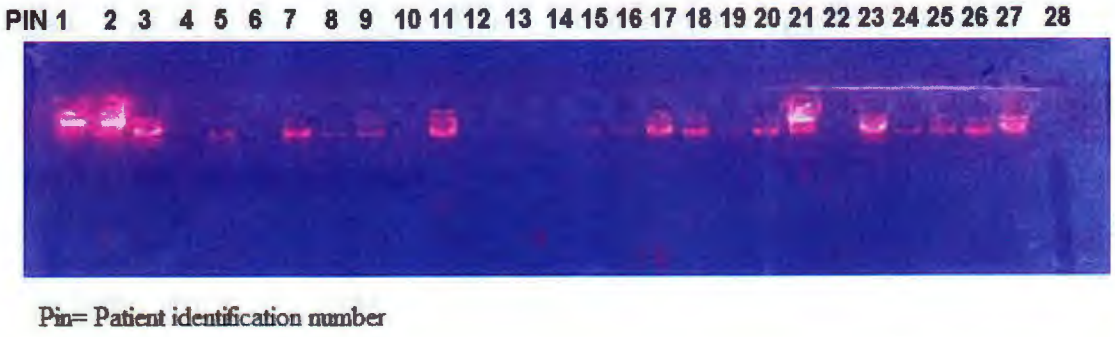

Figure 4.1 DNA Quantification on Agarose Gel electrophoresis of sample 1-28

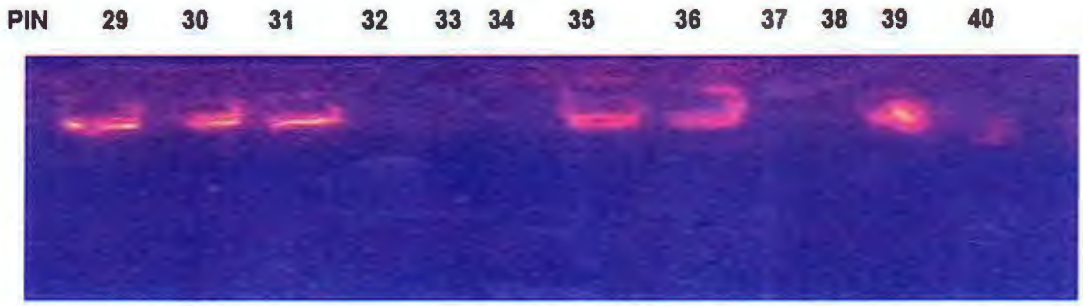

Figure 4.2 DNA confirmation on Agarose Gel Electrophoresis, Sample 29 to 40

4.3 Primer Designing

Primer were designed using Primer 3 software, UCSC genome browser and blat were used in designing of the primer for BRCA1 gene exon eleven. Six primer designed for it as it is the longest exon of BRCA1 and chances of mutation are too high.

Below are the sequences of designed primers of BRCA1 gene Exon 11.

Table No 4.3 Primer Designing

Exon No.	Primer Sequence	T.M
11a-F	5'ATATAGCCAGTTGGTTGATTTC-3'	55.06
11a-R	5'-ACGTCCAATACATCAGCTACTT-3'	55.61
11b-F	5'AGCAGCATTCAGAAAGTTAATG-3'	56.43
11b-R	5'-ACAATTAGGTGGGCTTAGATTT-3'	56.12
11c-F	5'AATATCCACAATTCAAAAGCAC-3'	55.89
11c-R	5'-ATTCCTCTTCTGCATTTCCT-3'	55.53
11d-F	5'AAGCATAGAAATGGAAGAAAGT-3'	54.17
11d-R	5'TGGAGAGAAATCTGTATTAACAGT 3'	54.13
11e-F	5'GCTATGCTTAGATTAGGGGTTT-3'	55.96
11e-R	5'TGCTTTGCTCTTCTTGATTATT-3'	56.08
11f-F	5'TCCTTTCTTGATTGGTTCTTC-3'	56.00
11f-R	5'CGTGTGTGTGAAATAAAAGGTA-3'	55.45

4.4 PCR amplification

4.4.1 Standardization of primers

All the included primers of gene BRCA1 were standardized by using gradient PCR technology. For this purpose, BIO RAD T100™ thermal cycler was used. Primers were standardized at different temperatures between 51°C to 58°C. Most of the primers were optimized at 52.1°C. The samples were amplified using first denaturation step at 95°C for 60 seconds, annealed with the primers for 45 seconds at 52.1°C and extended at 72°C for 2 minutes for 35 cycles. The initial denaturation step at the beginning of the PCR was for 5 minutes at 95°C. A post PCR step for 10 minutes at 72°C was added to extend all unfinished products (Fig 4.). PCR reaction mixture contained 1X Taq Buffer (10 mM Tris Cl-pH 8.4, 50 mM KCl, 0.1X Triton,), 200 uMdNTPs, 1 unit of Taq DNA polymerase, and 5ρmol of each primer (0.5mM) (Table 4.5).

4.4.2 STANDARD Protocol for PCR

Following recipes used for PCR master mix

Table No 4.4 PCR Master Mix

Target DNA	PCR buffer	Ddntps	$MgCl_2$	Forward primer	Reverse primer	Taq polymerase	Ddh_2O
1µl	2µl	2µl	0.8µl	1µl	1µl	2µl	10.2µl

4.4.3 PCR Cycling Steps for one step PCR

Table No. 4.5 PCR Cycle

Steps	Time	Temperature	Cycles
Initial PCR activation step	15 min	95°C	1
Denaturation	45 second	95°C	1
Annealing	45 second	52.1°C	30
Extension	45second	72°C	30
Final Extension	10min	72°C	1

4.5 Gel Electrophoresis

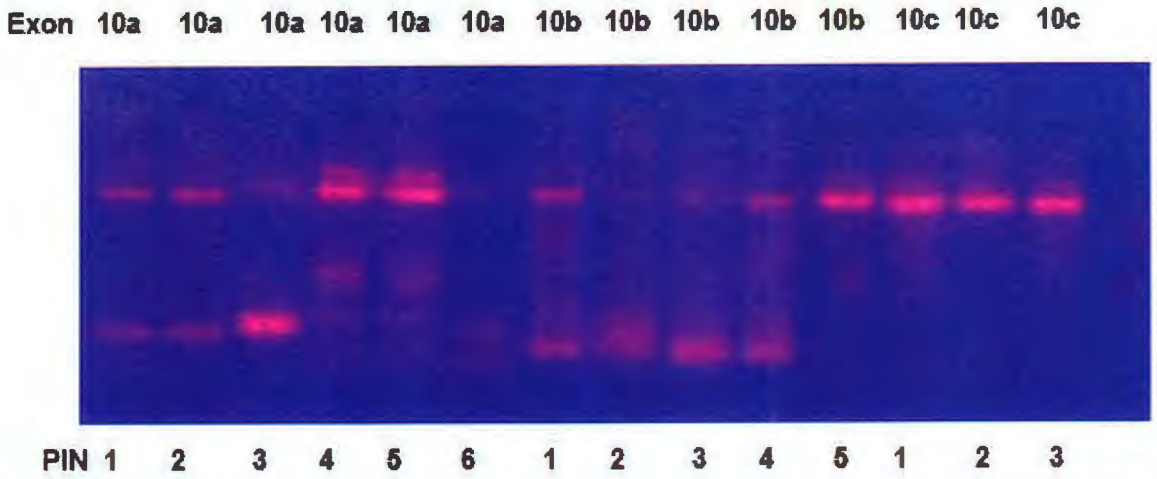

Figure 4.3 PCR product bands on Gel Electrophoresis

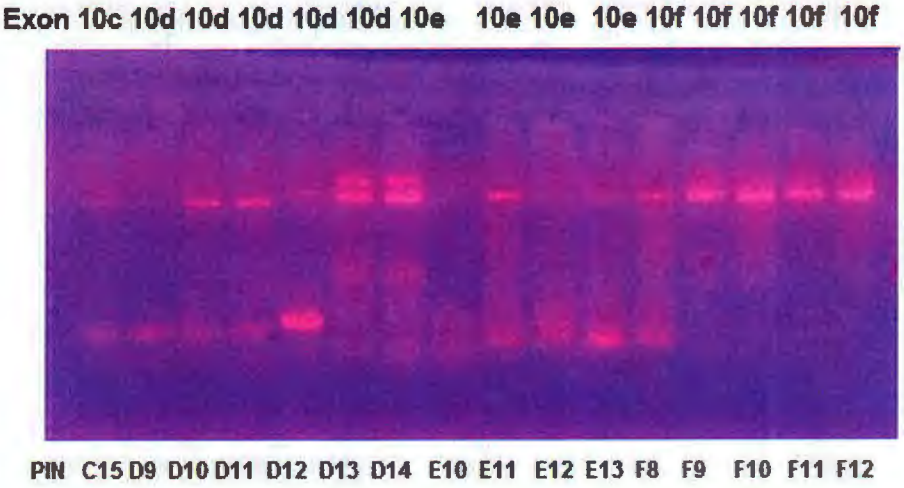

Figure 4.4 PCR product bands on Gel Electrophoresis

4.6 DNA Purification through Gel Extract Method

DNA was purified followed Qiagen gel extraction protocol. Cut pieces shown in (Figure 4.5) that desire band of PCR product isolated and put in labeled Eppendorf tube. And further send to Macrogen for Sanger sequencing.

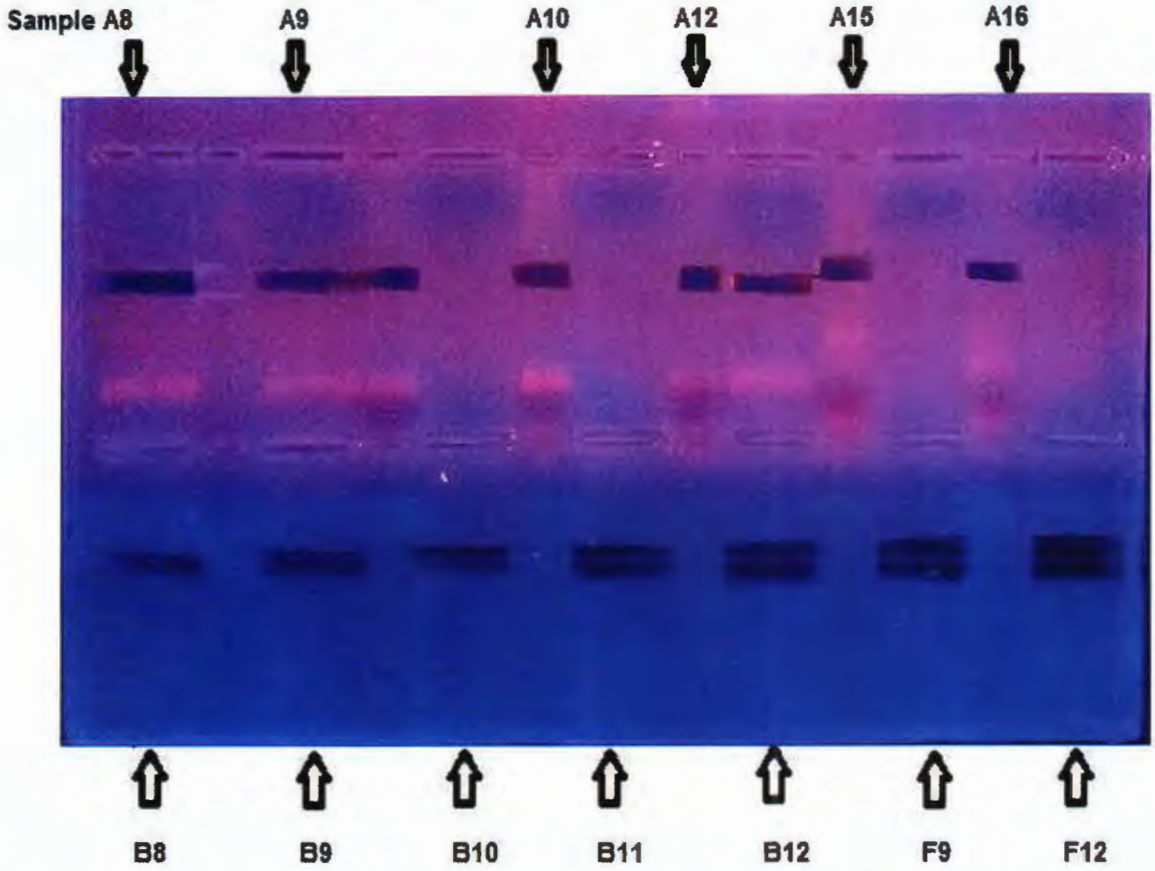

Figure 4.5 Gel Extract method for purification of amplified PCR product for sequencing.

4.7 DNA Sequencing

Exon 11 of BRCA1 gene were selected for analysis as it is the largest exon of the desire gene and was found most mutation in sporadic breast cancer patients. Therefore, six pairs of primers were designed for it and were sequenced by Sanger dideoxy chain termination method (Figure 4.). Sequence was analyzed manually by using Chromas software version (v1.45). The sequence was also blast against normal sequence by using either Nucleotide-nucleotide BLAST (blastn) or BLAST 2 Sequences on the NCBI web.

4.7.1 B10 Exon 11b

Pathogenic variant or mutation has been observed in this sequenced exon of BRCA1 gene of sample id B10 nucleotide number 1301 and 1302. There wild type or cDNA sequenced code AGT which translate serine which is polar side chain and the mutant alter to make Phenylalanine TTT which has non polar side chain Shown in (Figure 4.6).

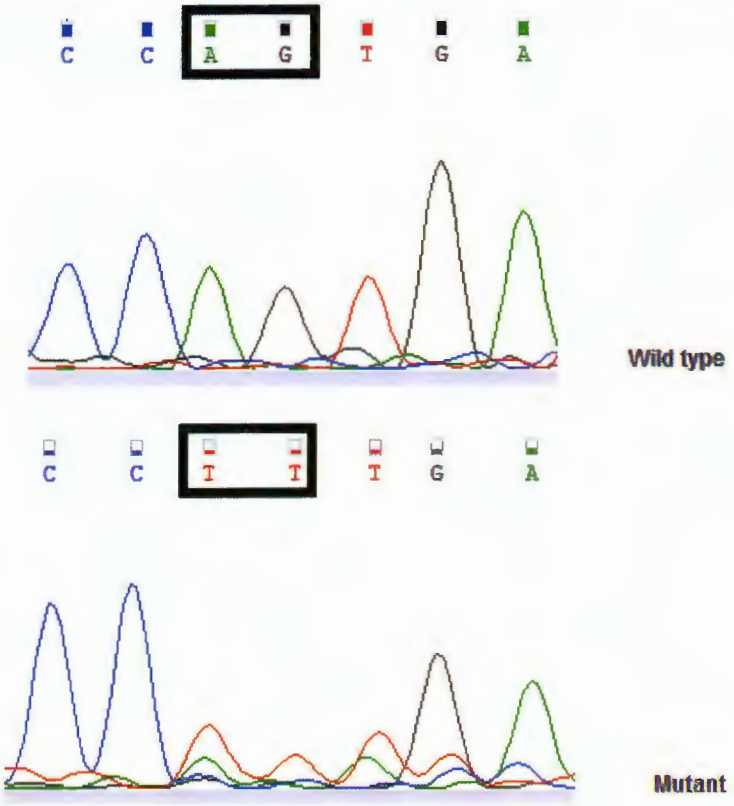

Figure 4.6 Representative electropherogram of DNA sequence of sample B10.

Figure 4.7 Sequence alignment of peptide sequence coded by Exon 11.

This image was taken from ENSEMBL genome browser (http://asia.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g). Red color represent stop gained and top lose, while the yellow color represents missense mutation. The blue color represents untranslated region at 3 and 5 end. The line below the nucleotide is amino acid line.

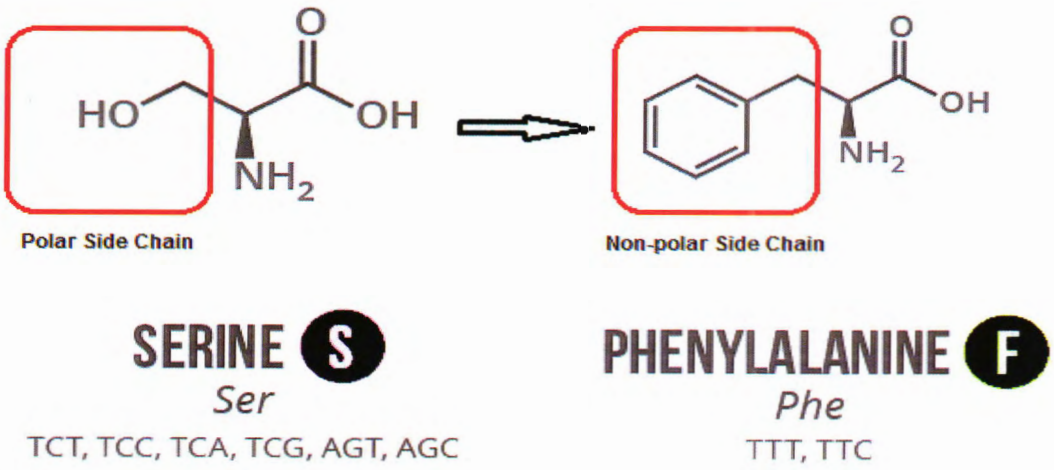

Figure 4.8 Conversion of Serine Amino Acid to Phenylalanine

4.7.2 F12 Exon11f

Pathogenic variant was found in Exon11f of sample F12 in which the codon TCA change to TCC. Nucleotide A were replacing by C but the alter codon also code for same amino acid Serine to Serine. Below in (figure 4.9 and 4.10) show the Electropherogram as well Multiple sequence alignment respectively.

Mutation in sample 12 found shown in figure 4.8

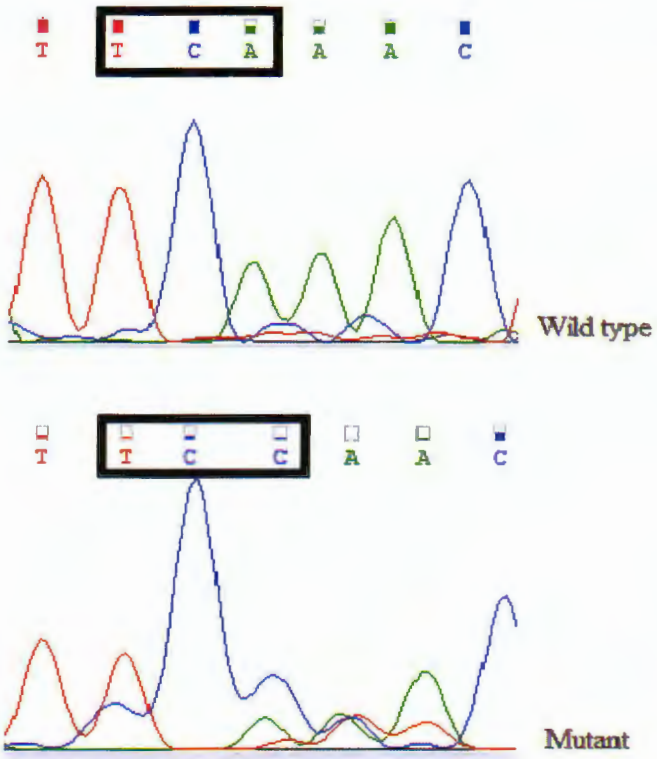

Figure 4.9 Representative Electropherogram of Exon11 sequence sample F12.

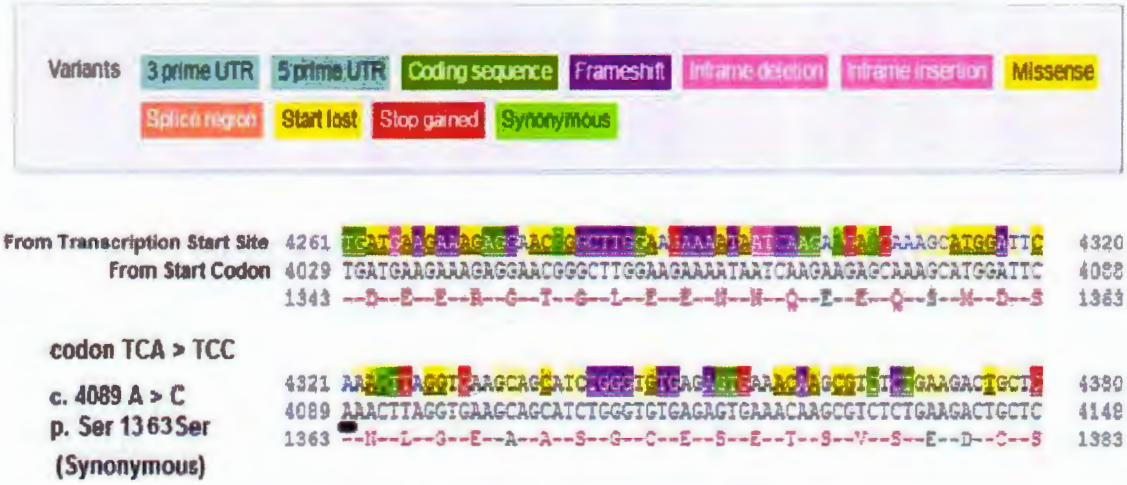

Figure 4.10 Multiple alignment sequence of exon 11 sample F12.

4.7.3 F9 Exon11f

SNP or single nucleotide polymorphism mutation were found in Intron eleven of BRCA1 gene in which nucleotide A substitute by C. Below the Figure (4.11) show mutation insertion of C instead of A.

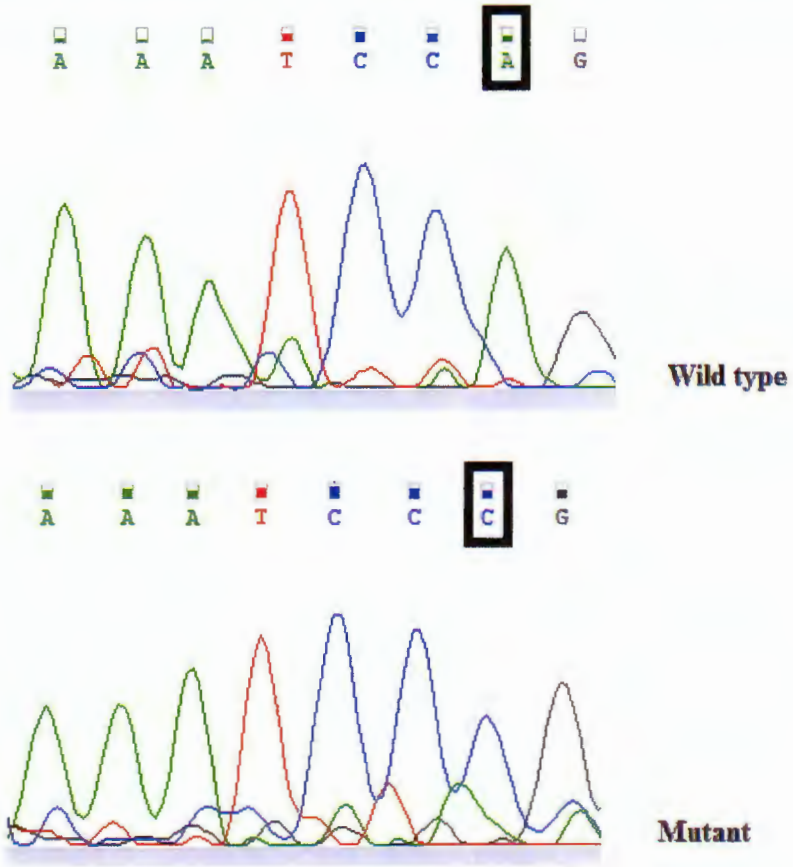

Figure 4.11 Mutation show in Sample F9 Multiple sequence alignment of Intron 11 sample F9.

4.8 In-silico Analysis of Pathogenicity

Different online bio-informatics tools were used for prediction of pathogenicity of the mutant codon. These include Polyphen-2, SIFT and PROVEAN. These tools have their own predicting scoring parameter which are shown in fig (4.12, 4.13, 4,14) respectively.

4.8.1 Polyphen-2 tool for pathogenicity

With Polyphen-2 prediction tool, S434F variant from exon 11 of BRCA1 gene was predicted to as "PROBABLY DAMAGING" with score of 0.998 (figure 4.12, table 4.6).

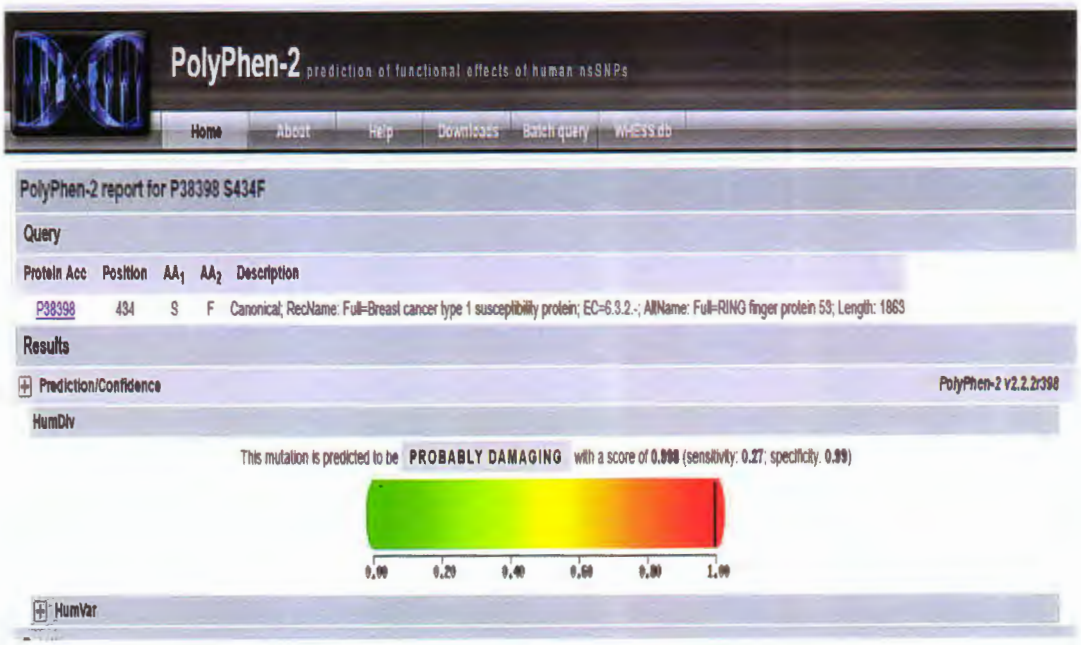

Figure 4.12 PolyPhen-2 prediction for S434F

4.8.2 SIFT online tool result for pathogenicity prediction

With SIFT prediction tool, S434F variant from exon 11 of BRCA1 gene was predicted to as "PROBABLY DAMAGING" with score of 0.02 (figure 4.13, table 4.6).

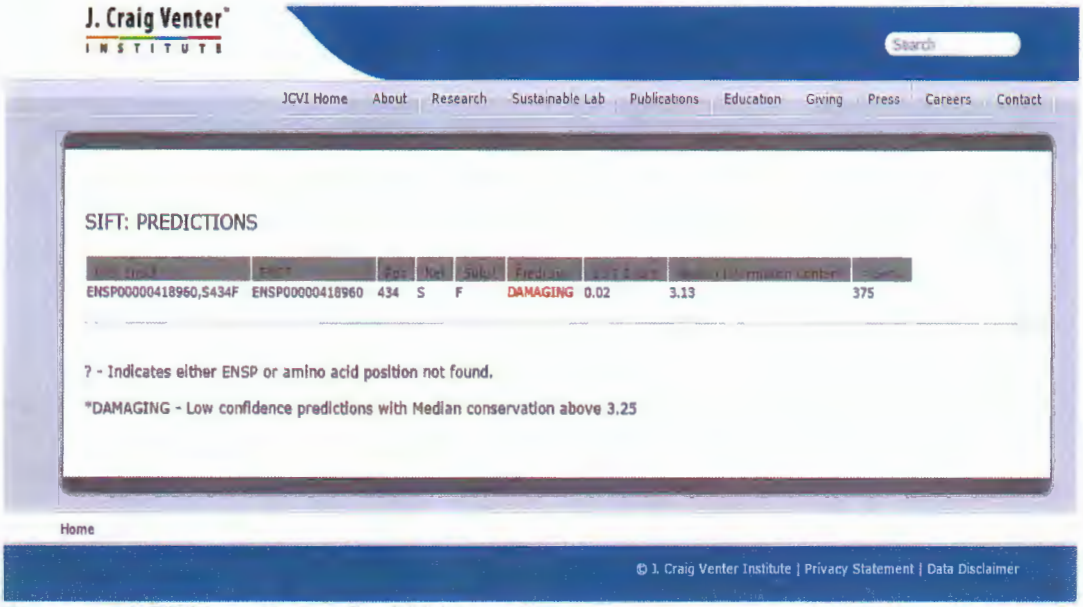

Figure 4.13 SIFT prediction for S434F

4.8.3 Provean prediction tool

With Polyphen-2 prediction tool, S434F variant from exon 11 of BRCA1 gene was predicted to as "DELETERIOUS" with score of -5.041 (figure 4.14, table 4.6).

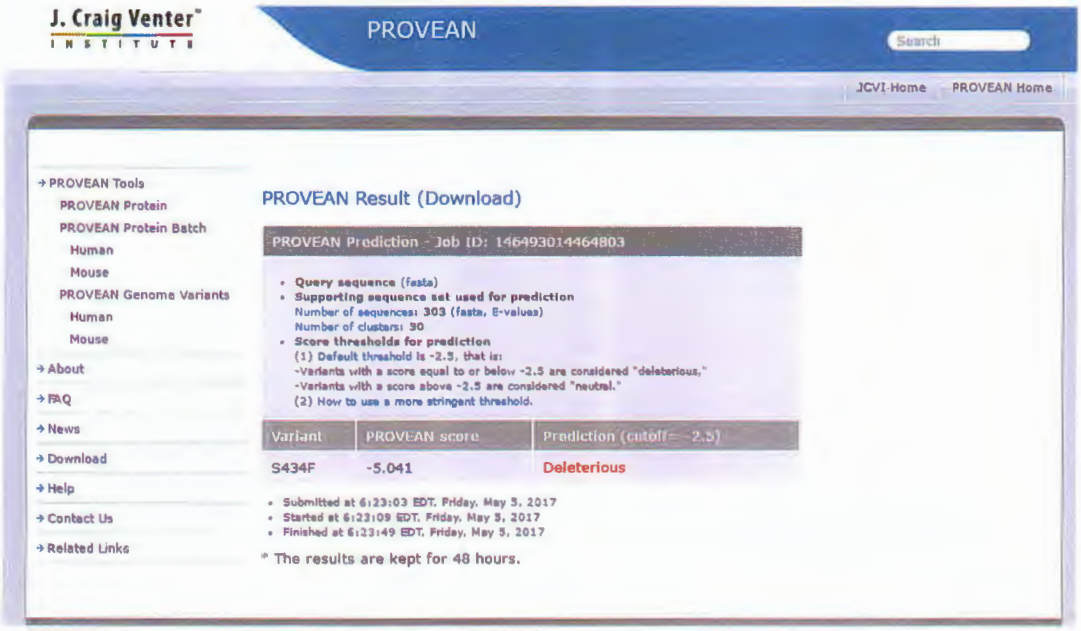

Figure 4.14 Provean prediction for S434F

Table 4.6 B10 Exon 11b Mutation result testing summary of online tool

Individual	**B10**
Nucleotide variant	c. AG1301-1302TT
Protein variant	p. S434F
Types of mutation	Missense
Previously reported	Yes
Polyphen	PD (0.998)
SIFT	Dam (0.02)
Provean	Del (-5.041)

DISCUSSION

5. DISCUSSION

In the current era cancer is one of the most health problem and second leading cause of death among the people after cardiovascular disease (Siegel *et al.* 2012). Among the cancer, Breast cancer after lung cancer is second leading causing type for women worldwide. In women account 29 % (226,870) is expected to be breast cancer of all new cancer cases. Mostly new cases are diagnosed seen abnormality on a mammogram or felt a lump with the fingertips can also be a warning symptom of the disease (Jorgensen and Gotzsche 2006). Lack of awareness in women, breast cancer is the most prevalent cause of fatality between the ages of 45 and 55 (Ismail and Bui 2010).

Key genes involved in causing of breast cancer is BRCA1 and BRCA2. These both are tumor suppressor gene but when mutation occurs in any one of them lead to breast cancer. In this study our focus is on BRCA1 gene as its mainly highlighted in sporadic breast cancer. BRCA1 gene located on chromosome 17q21 and span approximately 100 kb of genomic DNA. BRCA1 gene have total 24 exons of which ist and 4^{th} are non-coding while the rest 22 are coding exons. They code for 1863 amino acid protein. Exon 11 is the hotspot region, so the chances of mutation is high as compared to other coding exons of this gene (Futtreal *et al.,* 1994).

Total 1168 variant type have been observed in BRCA1 gene up to date. Out of them 501 deletions, 171 insertions, 28 indeleltion, 113 missenses, 243 nonsenses, 112 splice site mutation were found. Ninety-two percent out of these is pathogenic and seven percent is nonpathogenic.
(www.arup.utah.edu/database/BRCA/database/Home/BRCA1_Landing).

Thirty-nine breast cancer patients blood samples of 5 ml in EDTA tube along with consent form were collected from Nuclear Oncology Medicine and Radiotherapy Institute (NORI) hospital Islamabad and shifted to Human Molecular and Genetics Lab 127 I.I.U.I for further study. DNA was extracted following non-organic method protocol and then primers designed for desired gene BRCA1 exon 11 as it was study objective. Out of forty amplified samples, 24 samples were send to Korea Macrogen for Sanger dideoxy chain termination method sequencing. Three pathogenic variants were observed.

In present study, two pathogenic variants were found in exon 11 and one non-pathogenic variant in intron 11, both the variant were dinucleotide substitution but one of these two mutations were pathogenic variant as the wild type codon No 1301 and 1302 AG along with T Code Serine amino acid while the mutant substitute AG by TT along with T which code Phenylalanine. Serine has polar side chain and the mutant Phenylalanine has non polar side chain as mention above in (figure 4.7). The second mutation in cDNA 4089 codon TCA code amino acid Serine which substitute by C the nucleotide A become TCC but it also codes same amino acid Serine. The third mutation observed in intron 11 nucleotide No 262 from start codon of BRCA1 gene exon 11 SNP (Single Nucleotide Polymorphism). Nucleotide A replaced by C.

Out of these three one mutation found pathogenic and novel as compared to previous study.

Mutations in the cancer susceptibility gene BRCA1 greatly increase the risk of breast and ovarian cancer (Gene 1994). At present most mutations in the BRCA1 gene have been identified to be point mutations or small insertions and deletions. Literature survey showed that there is a wide choice of literature on the BRCA1 gene related to breast cancer. However, a computational study revealed that a total of 477 SNPs in the BRCA1 gene, 65 were found to be unsynonymous and 4 and 10 SNPs were found to be in the 5′ and 3′ untranslated regions (UTR). Of 65 nsSNPs, 12 nsSNPs were found to be deleterious. Two SNPs in the 5′ UTR and 3 SNPs in the 3′ UTR were found to be of functional significance. It was found that the major mutation in the native protein of the BRCA1 gene was from proline to serine. It concluded that rs1800751 with a mutation of proline to serine at position 1812 in the native protein could be the main target mutation for the breast cancer caused by the BRCA1 gene (Rajasekaran *et al.*, 2007).

In present study three mutation were observed in twenty-four samples which were sequenced hence two were dinucleotide substitute and the third one was SNPs in cDNA of intron 11. Many other genetic mutations rather than BRCA1 are also involved like BRCA2, pT53, HER- and many more which can cause mutation. Our study focus was on BRCA1 gene exon 11 as it is the largest exon spanning 3624 bases so the chances of mutations were high and also mutation were find out in previous work in Pakistani patients. The found mutations are novel as compared to previous work and also check on online BRCA1

database of which the link is mention above but the present study observed mutation are new as compared to previous one.

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