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RESEARCH ARTICLE

COMPARATIVE ANALYSIS OF EXON 11 MUTATIONS OF *BRCA1* GENE IN REGARD TO CIRCULATING TUMOR DNA (CTDNA) & GENOMIC DNA IN A COHORT OF BREAST CANCER PATIENTS IN PAKISTAN

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ABSTRACT

The aim of our study was to identify BRCA1 mutations in Pakistani population and direct comparison between circulating tumor DNA and genomic DNA of breast cancer patients. The complete screening of genomic DNA and ctDNA of 22 Patients for Exon 11 of 3426bp was costly and time consuming. Thus our method of choice was High Resolution Melt Curve Analysis (HRM). We analyzed and detected founder mutation (185delAG) in Exon 2 of BRCA 1 gene in genomic DNA of 30 patients with 2 negative controls by HRM. We found that a founder mutation was present in 15 patients, thus bringing the total contribution of BRCA 1, Exon 2 founder mutation to 50% in our study population. Contrary to one common mutation of BRCA 1 Exon 2, many variants are identified in exon 11. But interestingly we found that same variants were present in ctDNA of patients in 20 cases. Our data also showed that in 12 samples, ctDNA displayed variation that was not present in genomic DNA , which enable us to assume that ctDNA is a significant biomarker that can not only display mutations of genomic DNA but also evolution of tumor.

INTRODUCTION

Breast Cancer

Breast cancer is the heterogeneous disease which causes abnormal proliferation, mutation, invasion and metastasis. Even though breast cancer is very much common in women, it can also develop in men. In both genders, breast cancer normally develops in inner lining of milk ducts (ductal cancer) but in women, cancer can develops in lobules also known as lobular cancer. (Sharma *et al*., 2010)

Breast Cancer Epidemiology

According to Globocan 2012 online report, breast cancer ranks as the fifth cause of death in the world and is the second most commonly diagnosed cancer in women. Worldwide 1.7 million cases of female breast cancer (corresponding to a rate of 43 per 100,000) were diagnosed during 2012. America has the highest annual incidence rates of breast cancer in the world; corresponding to a rate of 112.6 per 100,000 among African Americans and 128.6 per 100,000 in white Americans. Incidence rates vary nearly four-fold across the world regions, with rates ranging from 27 per 100,000 in Middle Africa and Eastern Asia to 96 in Western Europe. In 2012, Asia-Pacific region has an incidence rate of 24% of breast carcinoma with greatest number in Chinese population (46%), high incidence rates (85 per 100,000) in New Zealand and Australia are noted as compare to the other major countries in the region (Abdulrahman, 2014). According to WHO 2014 Health Profile of Pakistan, Breast Cancer is among top 10 causes of death in Pakistan and its rate is highest among all the cancer types in Pakistan (WHO-2014; Pakistan Health Profile). According to the PUNJAB CANCER REGISTRY REPORT-2014, breast cancer is at the top of ten most frequently reported cancers in Females of Pakistan. The total count is 1,425 out of 5521 (25.8%) of frequently reported cancer cases.

Breast Cancer Diagnosis

Breast cancer is a genetically and clinically heterogeneous disease and it is classified on basis of histopathological classification and molecular subtyping.

Histopathological Classification

Histopathologic classification is based upon characteristics seen upon light microscopy of biopsy specimens. Breast cancer is generally classified into in situ carcinoma and invasive (infiltrating) carcinoma (Sakamoto, 1985). In situ

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carcinoma is further sub-classified as either ductal or lobular. Ductal carcinoma in situ (DCIS) account for 20% of all breast cancers and lobular carcinoma in situ (LCIS) account for up to 15%. DCIS has usually classified in five categories such as Micropapillary, Comedo, Solid, Cribiform and Papillary based on the tumor architectural features. Invasive (infiltering) carcinomas are group of tumors which are further classified into histological subtypes. The invasive tumor types include infiltrating ductal (55% of all breast cancers), invasive lobular (5% of all breast cancers), ductal/lobular, mucinous (colloid)(3% of all breast cancers), tubular (2% of all breast cancers), Medullary (3% of all breast cancers), papillary carcinomas (1-2% of all breast cancers).

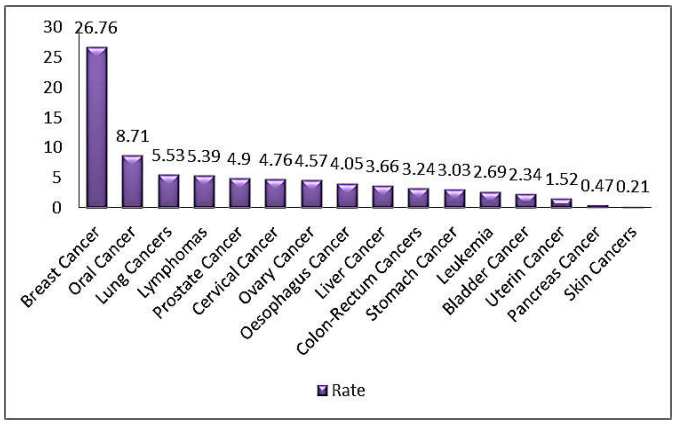

Figure 1. Cancer related mortality rate in Pakistani Population (WHO-2014: Pakistan Cancer Factsheet)

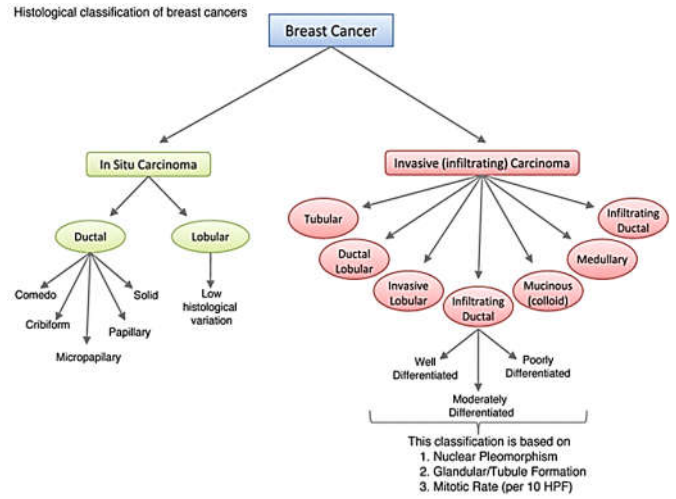

Figure 2. The histological classification of breast cancer subtypes (Malhotra *et al*., 2010)

Of these, infiltrating ductal carcinoma (IDC) is, by far, the most common subtype of breast cancers. IDC is further sub-classified as Grade 1 (well-differentiated), Grade 2 (moderately differentiated) and Grade 3 (poorly differentiated) based on the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index (Malhotra *et al*., 2010).

Molecular Subtyping of breast cancer

Breast Cancers are classified as Luminal, Her2 and Triple negative breast cancers on the basis on their molecular receptor status.

- Luminal Breast cancer is further divided as Luminal A and Luminal B subtypes. In Luminal A Breast Cancer hormone-receptor status of Estrogen and/or Progesterone is positive with negative HER2 and Ki67 protein status. Growth rate of such cancers is very slow and classified as low grade. Luminal A breast Cancer have best prognosis (Kim *et al*., 2012).

In Luminal B Breast Cancer status of Ki67, Estrogen and/or Progesterone is positive with HER2 protein status either positive or negative. Growth rate of such cancers is slightly faster than Luminal A cancers and have slightly worse prognosis (Tran and Bedard, 2011).

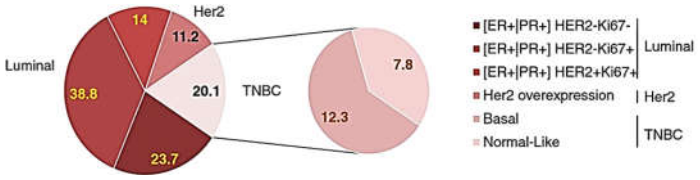

Figure 3. Molecular classification of breast cancer (Gajulapalli *et al*., 2016)

- HER2-enriched breast cancer has hormone-receptor status of Estrogen and Progesterone negative with high level of HER2 and Ki67 protein status. Growth rate of such cancers is much faster than Luminal cancers and have a worse prognosis (Carey *et al*., 2006).
- Triple-negative/basal-like breast cancer is further divided as Basal like and Normal like subtypes. In Triple-negative/basal-like breast cancer hormone-receptor status of Estrogen and Progesterone is negative with negative HER2 protein status. TNBC is more common in women with BRCA1 gene mutations (Badve *et al*., 2011).

In Normal-like breast cancer hormone-receptor is positive for either estrogen or progesterone-receptor with negative other receptor, negative HER2, and low levels Ki-67 protein (Lehmann *et al.,* 2011).

Molecular Genetics of Breast Cancer

Mutations in BRCA1 and BRCA2 genes are associated with breast cancers that inherit in families, with a high risk of developing ovarian and several other types of cancer in women. Men with mutations in these genes also have a very high risk of developing various forms of cancer, including breast cancer (Filippini *et al.,* 2013). The proteins produced from the *BRCA1* and *BRCA2* genes play an important role in fixing DNA damage, and in this way it maintain the stability of a cell's genetic information. BRCA 1 and BRCA 2 genes are described as tumor suppressors because they suppress the uncontrolled or too fast growth and division of cells. Mutations in these genes cause potentially damaging mutations to persist in DNA, allowing cells to divide and grow without control or order to form a tumor (Foulkes, 2014).

BRCA 1 Gene

The BRCA1 gene provides information for making a protein that acts as a tumor suppressor, which helps to prevent cells from dividing too fast or in an uncontrolled way. The BRCA1 protein is play a vital role in repairing damaged DNA.

Chromosomal Location

Cytogenetic Location: 17q21.31, which is the long (q) arm of chromosome 17 at position 21.31. Molecular Location: base pairs 43,044,295 to 43,125,483 on chromosome 17 (Homo sapiens Annotation Release 108, GRCh38.p7) (NCBI).

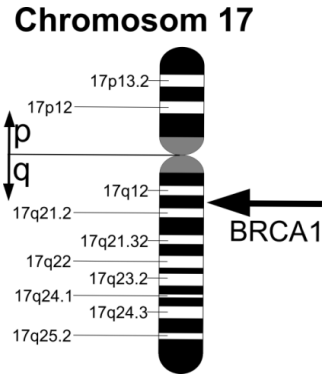

Figure 4. Location of the *BRCA1* gene on chromosome 17 (Hall *et al*., 1990)

Role of BRCA1 in DNA Repair

In response to DNA damage, BRCA1 gene undergoes hyperphosphorylation and moved to sites of replication forks specified by proliferating cell nuclear antigen also known as PCNA. In reaction to ionizing radiation, BRCA1 is activate and phosphorylated by an ataxia-telangiectasia mutated (ATM) kinase. BRCA1 is phosphorylated at several residues by various kinases after DNA damage. Phosphorylated BRCA1 triggers DNA repair through Homologous Repair, in cooperation with BRCA2 and Rad5 and form a complex together with its partners MRE11 and NBS1. MRE11 encodes nuclease activity, which resects flush ends of DSBs to generate ssDNA tracts. BRCA1 binds DNA directly and inhibits this MRE11 activity, regulating the length and the persistence of ssDNA generation at sites of DNA damage. (Scully *et al.,* 1997)

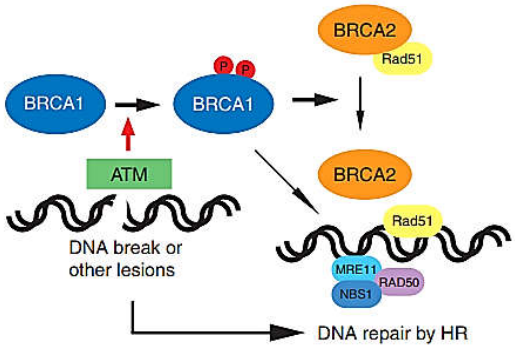

Figure 5. Role of BRCA proteins in repairing damaged DNA (Scully *et al*., 1997)

Role of *BRCA 1* in DNA Damage-Response and Cell Cycle Checkpoints

BRCA1 activation is attenuated by the CtIP-CtBP complex, which binds to the BRCT domain of BRCA1. Ionization radiation activates ATM which phosphorylates CtIP to disrupt the CtIP-CtBP-BRCA1 complexes. BRCA1 is then released and activates p21 and GADD45. Activation of cell cycle checkpoints induces replication arrest to allow repair of DNA damage. BRCA1 also functions as a co-activator of p53-mediated gene transcription (Cortez *et al.,* 1999).

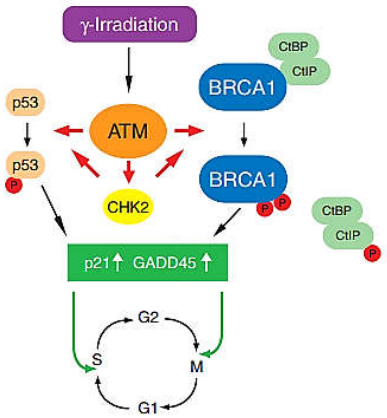

Figure 6. Role of BRCA1 in transcriptional regulation after exposure to ionizing radiation (IR) (Cortez *et al*., 1999)

Exon 11

BRCA1 gene consists of 22 exons but exon 11 stands out from the rest because it is the largest human exon, consisting of 3426 bases. It accounts for 60% of the gene. Exon 11 has been target for mutation detection due to its large size. As there are lots of repeated regions in this exon, alterations within this regions are most frequent and prevalent. Greater than 600 variants have been described in BRCA 1. Mutations in exon 11 of BRCA1 also have been associated with ovarian cancer. Most mutations of exon 11 lead to the production of an abnormally short version of the BRCA1 protein. As a result defected protein is not able to repair DNA damage which leads to the accumulation of such mutations that trigger uncontrolled multiplication and proliferation to form tumor (Jaure *et al*., 2015).

Diagnostic Modalities

Current breast diagnosis is carrying out by imaging modalities (breast magnetic resonance imaging, breast ultrasound, mammography and thermography) and by tissue Biopsy. No single imaging modality is completely useful in all areas of breast cancer management and tissue biopsy also has following limitations (Berg *et al*., 2004).

Limitations of Tissue Biopsy

First of all biopsy is invasive, costly, may not be feasible based on tumor accessibility or patient condition and are not without clinical complications. Secondly primary tumor biopsy may not reflect current disease condition because there are high chances that therapies may causes changes in tumor cells and periodic tissue biopsy is impractical for monitoring progression and reoccurrence. Furthermore, tumors are heterogeneous in nature showing different genetic profiles. The same is obvious at metastases of tumor in the same patient. A biopsy from a part of the tumor will not display the whole picture of molecular aspects or intermetastatic heterogeneity. Finally, and most importantly tissue biopsy by fine-needle aspirates or core-needle biopsies is inadequate for molecular analysis because of its limited amount (Diaz and Bardelli, 2014).

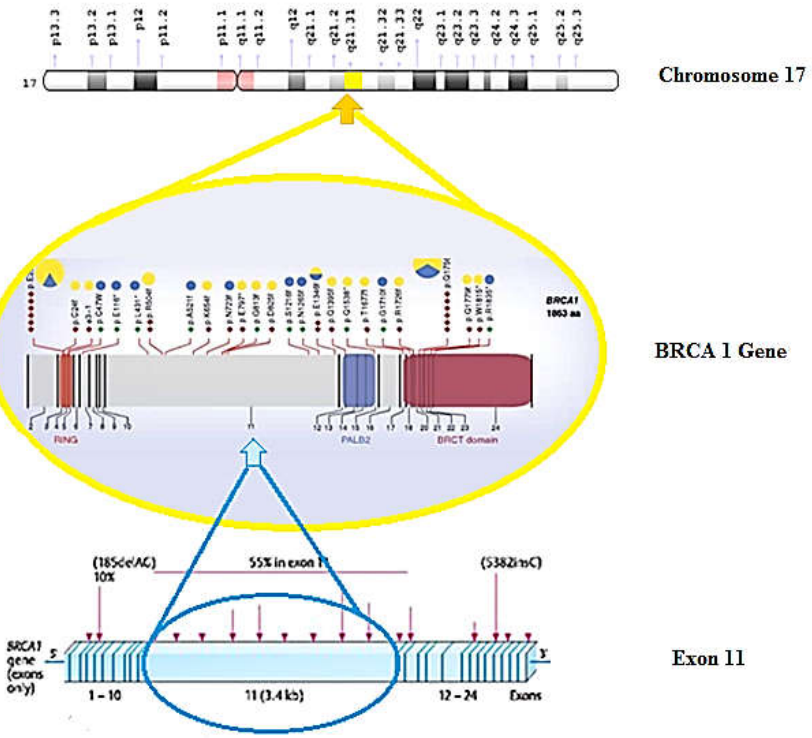

Figure 7. Exon 11 as the largest exon of BRCA1 gene (Jaure *et al*., 2015)

Liquid Biopsy

Liquid biopsy is a minimally invasive technology for detection of molecular biomarkers without the need for costly or invasive procedures. It is a simple and non-invasive alternative to surgical biopsies which enables medical doctors to discover a range of information about a disease or a tumor through a simple blood sample. Liquid Biopsy includes following biomarkers:-

- Circulating Free DNA (cfDNA)
- Circulating Small-RNA
- Circulating Tumor Cells (CTCs)
- Extracellular Mirco-vesicles (including exosomes) containing small-RNA, mRNA and DNA (Heitzer *et al*., 2015).

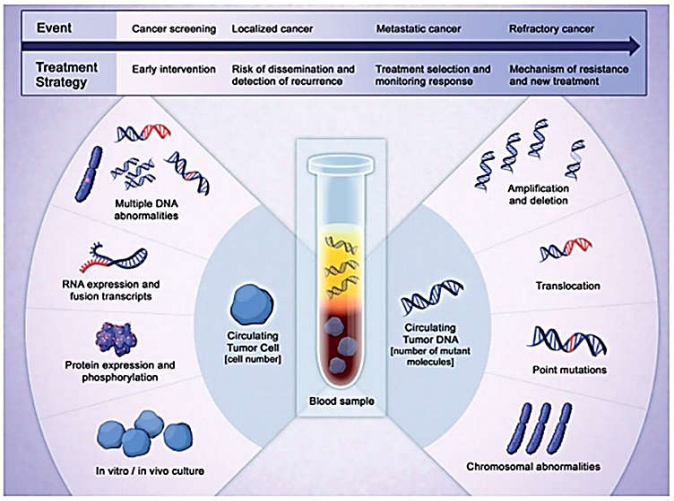

Figure 8. Liquid biopsy has several potential advantages (Pantel *et al.*, 2016)

Liquid Biopsy as a Game Changer

To overcome the limitations of tissue biopsies, less invasive techniques which allows early disease detection and is capable of assessment of tumor heterogeneity and monitoring of tumor dynamics when tumor is exposed to therapy. Circulating tumor DNA can in principle provide the same genetic information as a tissue biopsy and allows evaluation of metastasis, reoccurrence and monitoring of the actual treatment response through a very simple, non-invasive blood tests and it is a source of fresh DNA, unhampered by preservatives. It is much faster, cheaper and convenient than classical biopsy testing. Furthermore, blood can be drawn at any time during the course of therapy and allow for dynamic monitoring of molecular changes in the tumor rather than relying on a static time point (Caldas *et al.*, 2016).

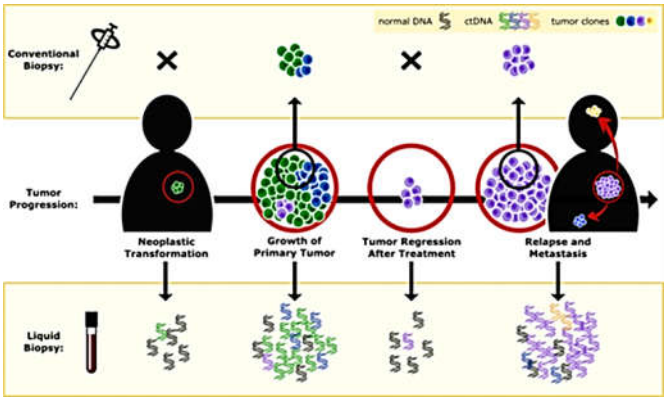

Figure 9. Tissue Biopsy vs. Liquid Biopsy (Ilie and Hofman, 2016)

Mechanisms of Tumor DNA Shedding

The level of circulating cfDNA is generally much higher as compare to healthy individuals. The number of apoptotic and

necrotic cells increase with increase in tumor volume and the phagocytosis process become insufficient to clear the cellular debris in such a large mass. As a result debris of tumor mass leads to accumulation and release into circulation of cancer patients. Genetic alterations which are associated with cancers such as chromosomal rearrangements, copy number variations, point mutations and methylation patterns can be detected in circulating cell-free DNA. The amount of ctDNA is associated to the tumor burden and its level varies significantly among cancer patients with different clinical settings (Hashad *et al*, 2012).

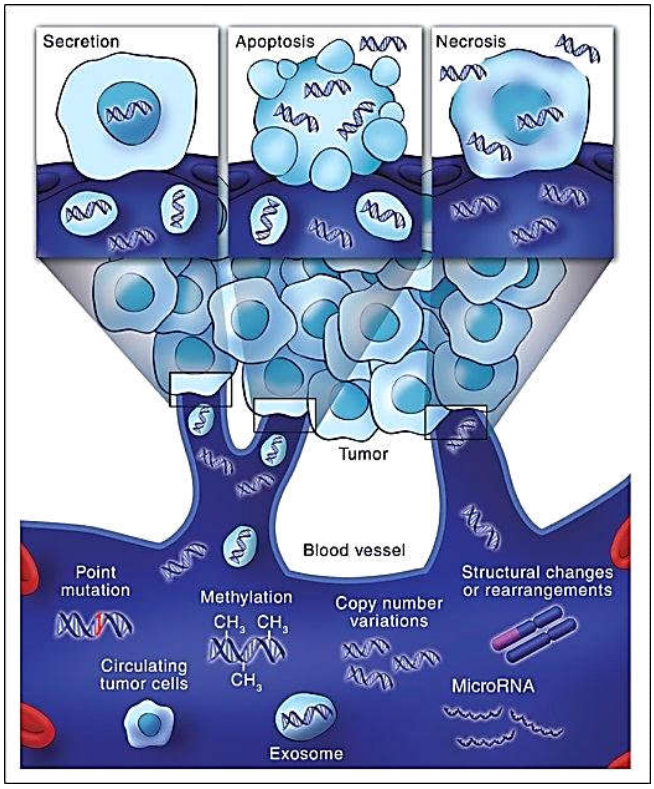

Figure 10. Tumor cells release small fragments of cell-free DNA into circulation by multiple mechanisms (Diaz and Bardelli, 2014)

Circulating tumor DNA and Its Significance

Monitoring Tumor Burden

Circulating tumor DNA is very significant for assessing disease burden and predicting treatment response. Various studies on breast, ovarian, melanoma and colon cancers shown that just like viral load changes, ctDNA levels correspond with course of therapy. As the level of ctDNA increase with disease progression, declines after treatment and corresponding increase with disease reoccurrence (Forshew *et al*., 2012).

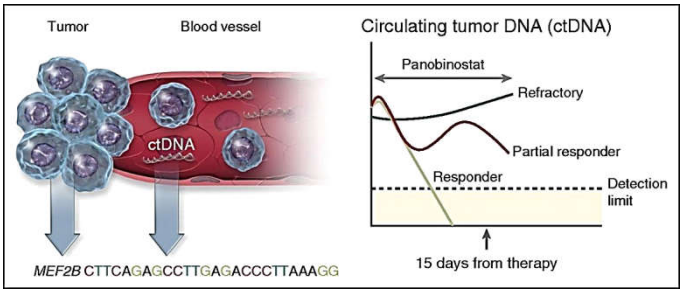

Figure 11. ctDNA as a potential biomarker (Forshew *et al*., 2012)

Monitoring of molecular resistance and heterogeneity

ctDNA fragments are released from tumor cells; thus they contain the similar genetic variation as exist in the primary tumor. This shows that ctDNA analysis has a potential to use as a significant alternative to typical tissue biopsies. Therefore, ctDNA analysis may be used to analyze intra-tumor genetic heterogeneity, that emerges as a result of therapeutic selective pressure as well as dynamic changes that occur during therapy (Murtaza *et al.*, 2013).

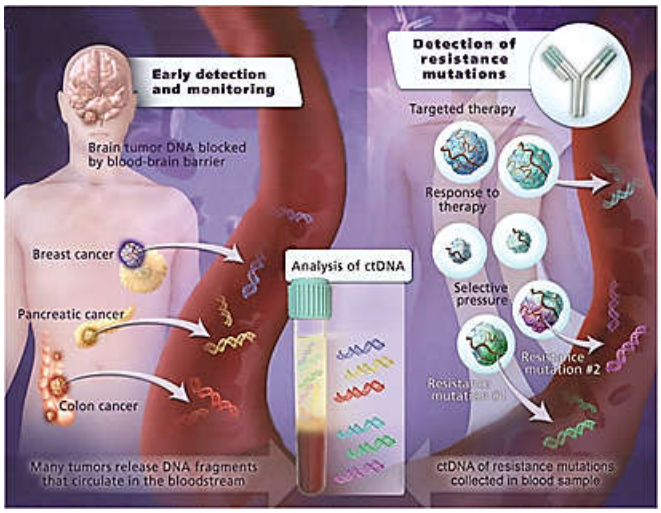

Figure 12. Application of ctDNA in monitoring resistance and heterogeneity (Umetani *et al*., 2006)

Objectives of study

- To Compare High Resolution Melt Curve Analysis (HRM) of Genomic and ctDNA in Breast Cancer patients.
- To evaluate the use of High Resolution Melt Curve Analysis (HRM) to screen for BRCA1 (Exon 11) gene mutations.

Literature review

Tazzite et al. detected five deleterious mutations in BRCA1 gene and four mutations in BRCA2 gene in ten unrelated Moroccan families. They also found 51 distinct polymorphisms and unclassified variants with one novel mutation in BRCA1 (c.2805delA/2924delA) and three in BRCA2 (c.3381delT/3609delT; c.7110delA/7338delA and c.7235insG/7463insG). They concluded that BRCA1 and BRCA2 mutations have significant role in familial breast cancer of Moroccan patients. Therefore full BRCA1/2 screening should be offered to patients with a family history of breast/ovarian cancer (Tazzite *et al*., 2012). Loman and coworkers conducted a study in women with early-onset breast cancer, in southern Sweden population in breast cancer patients with at least one first- or second-degree relative with ovarian or breast cancer. They concluded that 48% of their study population had mutation in BRCA1 AND BRCA2 genes. They also concluded that BRCA 1 and BRCA 2 mutation carrier patients had breast cancer at very young age (Loman *et al*., 2001). Backe group used the PCR primer mismatch assay to determine the frequency of 5382insC founder mutation in the BRCA1 gene in 248 breast cancer patients in German population based on a history of breast

and/or ovarian cancer in the families. They also enrolled 800 breast cancer patients without any information of family history of breast cancer. They concluded that in German breast cancer patients, 5382insC founder mutation is present very frequently (Backe *et al*., 1999). Casey et al investigated the occurrence of intra-abdominal carcinomatosis, following prophylactic oophorectomy in 72 hereditary breast–ovarian cancer syndrome (HBOC) families who carry cancer susceptibility mutations of BRCA1 (BRCA1 exon 11 1240 del C, BRCA1 1008 bp del-exon 17 and BRCA1 5382 ins C ter 1829) and BRCA2 (9132 del C-ter 2975). They concluded that intra-abdominal carcinomatosis was diagnosed only in BRCA1 mutation carriers, which tend to be small and localized (Casey *et al*., 2005).

Risch and coworkers screened cancer patients for germline mutations throughout the BRCA1 and BRCA2 genes in Ontario population. They showed an association of BRCA1 with ovarian, female breast, and testis cancers and BRCA2 particularly associated with ovarian, female and male breast, and pancreatic cancers. Cancer risks differed according to the mutation's position in the gene. They concluded that BRCA1 exon 2 and 5 (185delAG, 300T>G) and BRCA2 exon 10 and 11 (1538del4, 6872delACTC) mutations may be more frequent in general populations than previously thought and may be associated with various types of cancers (Risch *et al*., 2006). Jaure et al. investigated the prevalence of BRCA 1 gene polymorphism in 154 breast cancer patients from south region of Argentina by single strand conformational polymorphism technique. 15 primers were used to investigate BRCA 1 polymorphism in 2^{nd}, 3^{rd}, 5^{th}, 20^{th} and 11^{th} exons and direct sequencing was used to confirm mutations. They showed that 16 mutations were identified in the BRCA1 gene and 11 out of 16 mutations were present in different regions of exon 11.Only 1 patient was identified with a polymorphism in exon 2, and 4 in exon 20. They concluded that the most prevalent polymorphisms of the BRAC1 gene in Argentinian patients were located in exon 11 (Jaure *et al.*, 2015). Kwong and group conducted a study to characterize the spectra of BRCA mutations in ovarian and breast cancer patients in Chines population. Mutation screening was performed for all exons of BRCA1 and BRCA2 in 651 breast and/or ovarian cancer patients. They found that two deleterious mutations (c.981_982delATand c.3342_3345delAGAA) of Exon 11 accounts for 40% of all BRCA 1 mutations. Four mutations were found in BRCA2 gene (c.9097_9098insA, c.7436_ 7805del370, c.3109C.T and c.2808_2811delACAA). They suggest that BRCA mutations account for a considerable proportion of hereditary ovarian and breast cancer in Chinese population (Kwong *et al*., 2012). Takano and group evaluated the High Resolution Melt curve scanning method for founder mutations in exon 2 (185delAG) and exon 20 (5382insC) of BRCA1 and founder mutation in exon 11 (6174delT) of BRCA2 in 29 breast cancer patient. They showed that all the mutations were readily identified, showed distinctive melting curves and were visibly differentiated from the wild type and from each other. They concluded that HRM is economical and significant technique for mutational screening and testing (Takano *et al*., 2008).

El Khachibi et al investigated the significance of HRM analysis for SNPs detection in exon 11 of BRCA1 gene in 71 breast cancer patients. The evaluation of the presence of possible mutations was done by conventional Sanger sequencing. They showed that the mutations were readily detected by characteristic melting curves. All of the SNPs and mutations detected by the HRM approach were confirmed using Sanger sequencing method. They concluded that the HRM approach highly specific, very sensitive and a reliable method for mutation screening, especially in diagnosis (El Khachibi *et al.*, 2015). de Juan Jimenez group conducted mutational study of BRCAs genes and compared the obtained results obtained with conformation sensitive gel electrophoresis (CSGE) with high-resolution melting analysis (HRMA), for BRCAs. The study concluded that HRMA exhibited higher sensitivity than CSGE as it was able to detect wide mutational spectra of genetic variants in a larger number of samples. Furthermore, HRMA use less time, allowing the reduction of analysis time (Jimenez *et al*., 2011). De Leeneer and group evaluated 2 high-resolution melting instruments for screening BRCA1 and BRCA2 mutations. They screened the large exon 11 of BRCA1 by direct sequencing and all other coding exons were screened by denaturing gel gradient electrophoresis (DGGE). High-resolution melting analysis was performed on the 96-well Lightscanner (Idaho Technology Inc.) and the 96-well LightCycler 480 (Roche) instruments. They concluded that the run time of high-resolution melting has been 1/3 of that with direct sequencing and DGGE, as there is no post-PCR handling and the software allows fast analyses (Leeneer *et al*., 2008).

Circulating tumor DNA (ctDNA) is a new biomarker which has recently emerged as liquid biopsy tool in oncology with superior diagnostic and prognostic relevance. A study conducted by Madic et al showed high prevalence of TP53 mutations (84 percent) in triple negative breast cancer (TNBC) when compared ctDNA and CTC detection rates and prognostic value in 40 patients with metastatic TNBC patients (Madic *et al*., 2015). Beaver at el conducted a study, in which primary breast tumors and respective pre- and postsurgery blood samples were collected from 29 patients with early-stage breast cancer. Tumor DNA from tissue and plasma were then analyzed for PIK3CA mutations using ddPCR. Three PIK3CA mutations in exon 9 (E545K) and seven PIK3CA mutations in exon 20 (H1047R) were identified. They concluded that accurate mutation detection in tumor tissues using ddPCR, and that ptDNA can be detected in blood before and after surgery in patients with early-stage breast cancer (Beaver *et al*., 2014). Leaery and coworkers detected alterations comprised of chromosomal copy number changes and rearrangements, including amplification of cancer driver genes such as *ERBB2* and *CDK6* with parallel sequencing of DNA from the plasma of 10 breast and colorectal cancer patients with 10 healthy individuals. They conclude that chromosomal abnormalities can easily detected in circulating tumor DNA (Leary *et al.,* 2012).

Mouliere and coworkers used nude mice xenografted with SW620 or HT29 human colon cancer cells, and they correlated their results by examining plasma from metastatic *Colorectal cancer* patients. They observed that fragmentation and concentration of tumor-derived ctDNA is positively correlated with tumor weight (Mouliere *et al*., 2011). Similarly Dason and coworker compared the circulating tumor DNA with cancer antigen 15-3 (CA 15-3) by measuring them at identical time points in women with metastatic breast cancer who were receiving systemic therapy. Their results showed that as compare to CA 15-3 and circulating tumor cells, circulating

tumor DNA levels have greater correlation with changes in tumor burden and have a greater dynamic range (Dawson *et al*., 2013). Olsson et al investigated the significance of circulating tumor DNA (ctDNA) for earlier detection of metastasis. After long follow-up they quantify tumor-specific rearrangements in plasma by droplet digital PCR and whole-genome sequencing of primary tumors of 20 patients, they conclude that ctDNA monitoring is highly precise for postsurgical discrimination between patients with and without recurrence (Olsson *et al*., 2015). Kohler group investigated the levels of ccfnDNA and mtDNA in plasma samples from patients with malignant (n=52), benign breast tumors (n=26), and from 70 healthy controls. They showed that the level of ccfmtDNA was found to be significantly lower in both tumor-groups but the levels of ccfnDNA in the malignant cancer group were significantly higher in comparison with the other tumor group and the healthy control group, and. The level of ccfnDNA was also associated with tumor-size. They suggested that nuclear and mitochondrial ccf DNA are significant biomarkers in breast tumor management. However, ccfnDNA demonstrates more sensitivity and specificity (Kohler *et al*., 2009). Bettegowda and coworker found the significance of circulating tumor DNA (ctDNA) to detect tumors by using digital polymerase chain reaction-based technologies, in 640 patients with various cancer types. They found that ctDNA was detectable in > 75% patients with advanced cancers as well as in localized tumors in every type. They also investigate the sensitivity and specificity of ctDNA in 206 patients with metastatic colorectal cancers, for KRAS gene mutations. They showed that ctDNA have specificity of 99.2 % and its sensitivity was 87.2%. The suggested that ctDNA is highly specific and very sensitive biomarker, which is broadly applicable that can be used for various different types of cancer (Bettegowda *et al*., 2014). Jahr and coworkers conducted a study to differentiate circulating tumor DNA from cell free DNA in plasma of 30 cancer patients by quantitative methylation-specific PCR of the promoter region of the tumor suppressor gene (CDKN2A). They detected quantities of ctDNA from 3% to 93% of total cell free DNA. They also investigated possible origins of nontumor cell free DNA in the plasma. They performed studies with necrotic and apoptotic cells in vitro and with mice after induction of liver injury. Increasing amounts of DNA in the blood plasma samples of treated animals showed same characteristic patterns of DNA fragments in plasma derived from different cancer patients (Jahr *et al*., 2001).

MATERIALS AND METHODS

Sample Size

Whole blood samples of 30 clinically diagnosed female breast patients were collected from Oncology unit Jinnah Hospital Lahore and 15 healthy controls (5 males, 10 females) were obtained from Forman Christian College. An informed consent was obtained from each enrolled patient and record is kept along with relevant disease status.

Inclusion and Exclusion Criteria

Inclusion Criteria

- Female diagnosed with breast cancer.
- Patient who sign the informed consent.
- Age should be between 20-75 years.
- ECOG status of patient should be 0-2.

Exclusion Criteria

- Male patients with Breast Cancer.
- Female patient who didn't give informed consent.
- Patient below 20 and above 75 years of age.
- Patients with ECOG value greater than 2.
- Patients with other cancers or concomitant ovarian cancers.

Sample Collection and Storage

Whole blood samples of approximately 3ml were collected in vacutainers containing EDTA (Purple Top), with the help of Jinnah hospital staff. Samples were transported in Icebox to FCCU and aliquots of 750 µl were stored at -20°C for DNA extraction later on.

DNA Extraction

Genomic DNA Extraction

Genomic DNA was extracted from whole blood using manual method. Briefly stating the method consisted of the following steps of treatment with solution A, treatment with solution B, addition of proteinase K, treatment with solution C, treatment with Iso-Propanol, washing with Ethanol, drying the pallet and solubilization of extracted DNA in TE buffer for stability and long term storage. The extraction method proceeded by first shaking the whole blood vacutainer gently using a vortex. Blood samples (750 µl) were then transferred to 2ml microfuge tubes. To it 750 µl of Solution A was added. The tube was gently shaken to homogenize blood with the solution A and kept on room temperature for 10 minutes. After 10 minutes samples were subjected to centrifugation for 5 minutes at 13000 rpm.

Reagents

Table 1. List of reagents used for genomic DNA extraction

Solution/ Reagents	Manufacture name/Concentration
Solution A	0.32 M Sucrose, 10mM Tris-HCl(pH 7.5), 5mM Mgcl2, 1%v/v Triton X-100
Solution B	10Mm Tris-HCl(pH7.5), 400mM NaCl, 2 Mm EDTA (pH 8.0)
Solution C	Phenol-Chloroform with mercaptoethanol
Proteinase K	50 µg/mL
10%SDS	100g SDS in 800 ml D.H2O till 1 Liter, Adjusted pH to 7.2
TE Buffer	1M Tric-HCl (pH 7.6), 0.5M EDTA (pH 8.0) Adjusted to 8.0

The supernatant was discarded and the pellet was again treated with 400 µL of solution A. The pellet was broken down in solution A by gentle vortexing and was then subjected to centrifugation (5 min; 13000 rpm). The step was repeated thrice to wash out the hemoglobin and a clean white blood cell (WBC) pellet was obtained. To each tube 400 µl of solution B was added and the pellet was pipetted until dissolved. Next, 25 µl of 10% SDS was added to the tubes kept at room temperature for 10 minutes. Then 8 µL of Proteinase K added to each tube and incubated at 65ºC for three hours. After incubation 500 µL of phenol-chloroform in each sample and the sample were shaken properly. Samples were then

centrifuged at 13000 rpm for 10 minutes. After centrifugation the upper aqueous layer was separated carefully and transferred to new 1.5 ml microcentrifuge tube. To the aqueous phase from the previous step, 55 µL of 3 M sodium acetate was added along with 500 µL of chilled Iso-propanol. The tubes were then shaken gently in order to visualize the DNA thread. If transparent threads were visible then the samples were further proceeded to the next step otherwise the tubes were the tubes were placed overnight at -20ºC. After treatment with Iso-propanol samples were centrifuged at 13000 rpm for 15 minutes. The supernatant then discarded and 350 µL of 100% ethanol (stored at -20ºC) was added to wash the impurities present in the tube. After centrifugation, samples which were placed in incubator at 65ºC to dry the ethanol. 30 µL of TE buffer was then added to the microfuge tube to dissolve the DNA. Samples were then placed at 4ºC to dissolve DNA and later stored at -20ºC.

ctDNA Extraction

Blood plasma was obtained from fresh human EDTA blood samples by centrifugation for 10 min at 2000 ×g. These plasma samples were frozen and stored at −80◦C. Prior to ctDNA isolation, plasma samples were re-centrifuged for 3 min at 11,000 ×g to remove any particulate matter, and the cleared supernatant was further processed. ctDNA was extracted using the NucleoSpin Plasma XS kit (Macherey Nagel) according to the optimized manufacturer's protocols. Briefly, 240 µL plasma sample was mixed with 20 µL of Proteinase K and incubate at 37C for 10 min. After incubation sample was mixed with 360 µL of binding buffer. After vortex (3sec) and short spin, the mixture was applied to the spin column and spun for 30 sec at 11,000 ×g. After two subsequent washing steps (500 µL wash buffer; 30 sec. 11,000 ×g for the first wash and 250 µL wash buffer; 3min 11,000 ×g for the second wash), the ctDNA was eluted with 30µL elution buffer. At the end for removal of residual ethanol elute was heat-treated for 8 min at 90◦C and later stored at -20ºC.

DNA Quantification

Extracted genomic DNA was quantified at FCCU using Thermo Scientific Nano Drop ND-2000/2000c Spectrophotometer. The absorbance wavelength was set at 260 nm. TE buffer was used as a blank for DNA and Tris HCL (Elution Buffer) used as a blank for ctDNA. The absorbance ratios; A260/280 and A260/230 were also determined to check the purity level of DNA.

Primers Designing and Optimization

Eight primers for Exon 11 (3426bp), and one primer for founder mutation in Exon 2 of BRCA 1 gene were designed by CLC Genomic Workbench and USCS Genome Browser. Veriti 96-well thermal cycler (Applied Biosystems) was used to optimize primers for BRCA 1 Exon 11 and 2. On successful optimization, one annealing temperature was determined for both exons. Amplification profile for BRCA 1 Exon 11 and Exon 2 consisted of initial denaturation at 95◦C for 5 minutes, 35 cycles of denaturation at 94◦C for 30 seconds, amplification at 54◦C for 30 seconds, extension at 72◦C for 30 seconds and a final extension of 72◦C for 5 minutes. Concentration of template DNA was kept constant for each reaction at 100 ng. Primers having 10µM working concentration were added to the reaction to make a final concentration of 0.5µM. 2µl of 10x Taq buffer containing KCl, 25mM of MgCl2, 2mM dNTPs mix, 0.5µM of each primer, 100ng of template DNA, 1U Taq polymerase. In terms of reaction volumes the reaction prepared had 1µl of DNA, 2µl of the provided Taq buffer, 2.5µl MgCl2, 2.5µl of dNTPs mix, 2µl of each primer and 2µl of Thermo Scientific Taq DNA polymerase and Nuclease free water was used to raise the volume up to 25µl of the total reaction. The PCR product was analyzed by using 2% agarose gel made by 2g of agarose for six 600-700bp primers and 1% agarose gel made by 1g of agarose for three 200-300bp primers in 100ml of 1X TBE buffer. Before the gel was poured into the caster for solidification, 10µl of Gel Red Dye was dissolved into it. 6µl of each PCR product was stained with 1µl of loading dye (0.25% Bromophenol blue in 40% sucrose solution) and loaded into wells. For each gel a Thermo Scientific Gene Ruler 1000kb DNA ladder was loaded alongside the pcr products. Following were the primer used.

Table 2. Primers used for Exon 11 and Exon 2 of BRCA 1 Gene

Exon	Primer #	Primer Sequence	Product size
11	1	F1: 5'-TTGATTTCCACCTCCAAG-3' R1: 5'-AACGTCCAATACATCAGCTA-3'	664bp
11	2	F2: 5'-CATGATGGGGAGTCTGAA-3' R2: 5'-ACTACTAGTTCAAGCGCA-3'	686bp
11	3	F3: 5'-GAATAGGCTGAGGAGGAA-3' R3: 5'-GGTTAACTTCATGTCCCA-3'	692bp
11	4	F4: 5'-CCCCAAGGGACTAATTCA-3' R4: 5'-GTTCTCATTTCCCATTTCTC-3'	625bp
11	5	F5: 5'-CCACCACTTTTTCCCATCA-3' R5: 5'-ATTTCTTGGCCCCTCTTC-3'	685bp
11	6	F6: 5'-AGCCCTTTCACCCATACA-3' R6: 5'-CTCCCCAAAAGCATAAACA-3'	600bp
2	7	F9: 5'-GAAGTTGTCATTTTATAAACCTTT-3' R9: 5'-TGTCTTTTCTTCCCTAGTATGT-3'	258bp

Mutation Screening Methodology

High Resolution Melt Curve Analysis

For mutation Screening, *Luminaris Color HRM Master Mix kit (#K1031BID; Thermo Scientific)* reagents were used, which contained hot start taq DNA polymerase in an optimized buffer system with EvaGreen™ fluorescent dye. The final volume in the reaction mixture was 10 µl (1.5 µl of Nuclease-free water, 5 µl of Master Mix, 0.5 µl of each primer (10 µM) and 2.5 µl of 20 ng genomic DNA). The HRM protocol was set as follows on Thermo Scientific PikoReal Real-Time PCR System:

Table 3. Conditions used for HRM

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	10 min	1
Denaturation	95	10 sec	35-45
Annealing	54	30 sec	
Extension*	72	30 sec	
Heteroduplex formation (optional)**	95	30 sec	1
	50	30 sec	1
Melt curve/dissociation***	65-95	Increments of 0.2 °C/2s Data acquisition	

Same Tm of 54°C for all the primers was used and the results were analyzed With PikoReal Software 2.2.

DNA Sequencing

To validate the HRM method, 10 samples were reanalyzed using Next Generation sequencing. These DNA samples were first amplified by same primers used for HRM method. Sequencing tubes were loaded with 25µl of the amplified product and 50µl of each primer was freshly constituted from the stock and placed in 1.5ml microfuge tubes. All of the sequencing tubes and primer containing tubes were properly labeled and wrapped with Parafilm to avoid evaporation. Codes were assigned to each tube and recorded accordingly. Tubes were packaged and sent to Korea (MACROGEN) for DNA sequencing (sense strand only).

Sequencing Analysis

DNA sequencing results for each reaction was received in the form of a zip folder containing 4 files for each sample that was sequenced. Sequencing results were analyzed using Serial Cloner 2.6.1 and Finch TV software.

Statistical Analysis

Statistical analysis as performed by using SPSS software in collaboration with biostatistics.

RESULTS

Demographic Characteristics of Enrolled Patients

The age range of our patients was 27 years to 65 years. 10 % of patients were in range of 27-34 years . 26% of patients were in range of 35-42 years. 30% of patients were in range of 43-49 years. 23% patients were in range of 50-57 years.10% of patients were in range of 58-65 years.

Table 4.1: Demographics of the patients included in the study (n = 30)

Age Groups (Years)	No. of Patients	Percentage %
27-34	3	10%
35-42	8	26.67%
43-49	9	30%
50-57	7	23.33%
58-65	3	10%
Range	27-65	
Mean	45.03	
Standard Deviation	8.52	

According to available information of enrolled patients only few had the family history of breast cancer. 7% patients had first degree relative with breast cancer, 3% had second degree relative with breast cancer and rest of 90% enrolled patients had no family history of breast cancer.

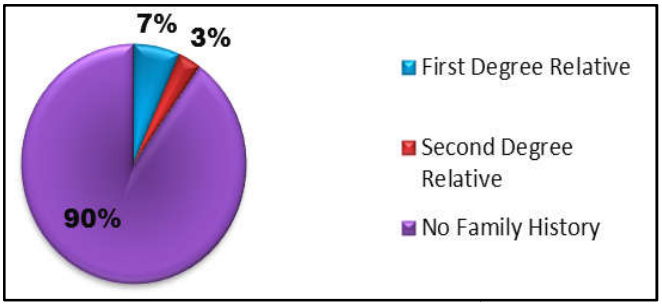

Figure 4.1. Distribution of patients according to family history with breast cancer (n=30)

Clinical Pathological Features

Histopathological Classification

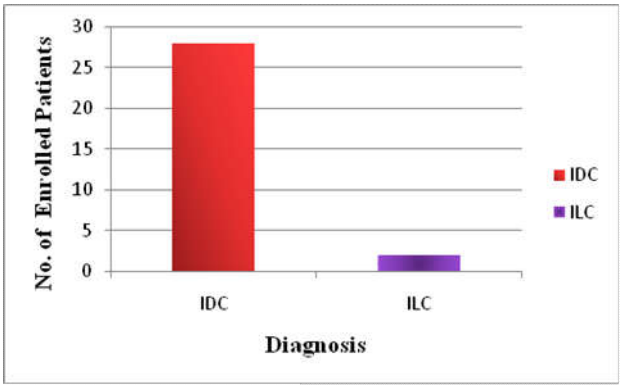

Figure 4.2. Histological classification showing types of breast cancer. (n=30)

Histological classification of breast cancer showed that out of 30 patients enrolled in study only 2 patients had invasive lobular carcinoma (ILC) represented by purple, while the rest of patients had Invasive ductal carcinoma (IDC) shown in red.

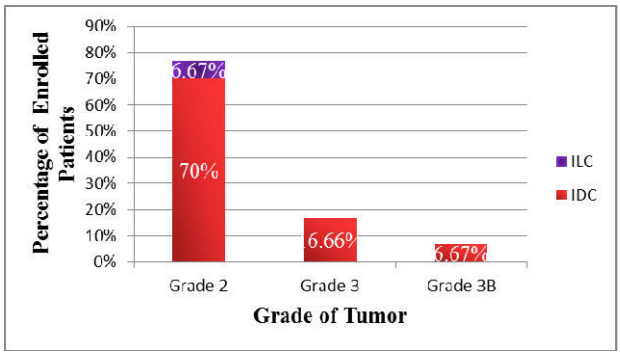

Figure 4.3. Histological classification showing grades of breast cancer. (n=30)

Grading of the tumor showed that 76.67% patients had grade 2 tumors, 16.6% patients had grade 3 and rest of the 6.67% had grade 3B tumor.

Molecular sub classification

Patients enrolled in the study were subdivided into four molecular subtypes and we found that out of 30 patients 50% were Triple Negative, 7% were of Luminal Type A in which Estrogen receptor was positive. From the rest Luminal B constituted 28% and 15% patients were Her 2/Neu enriched.

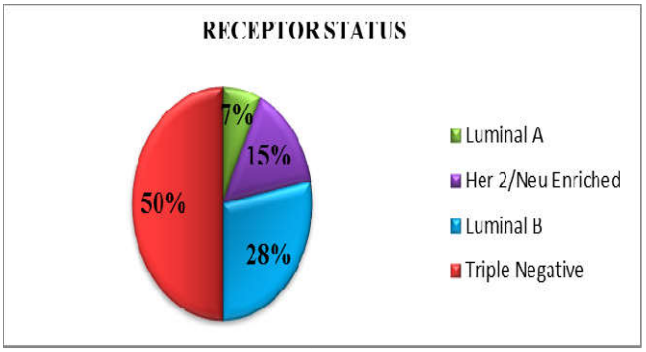

Figure 4.4. Molecular sub-classification of patients enrolled in study. (n=30)

Primers Optimization

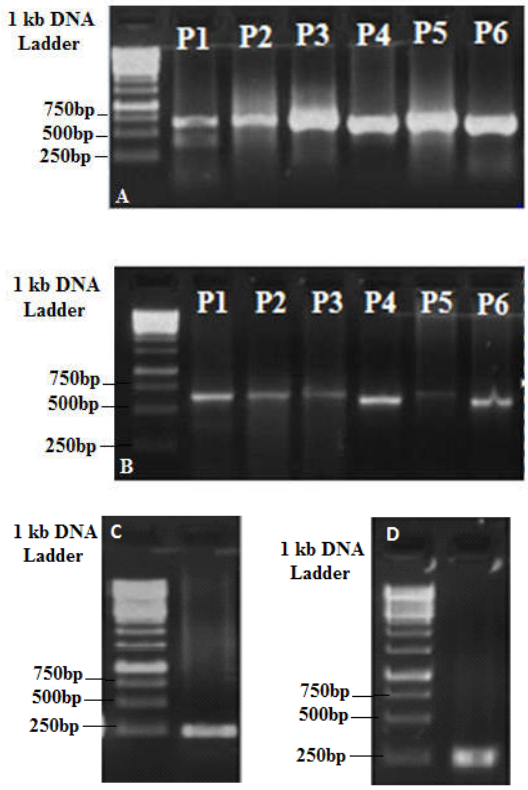

Figure: 4.5 Optimization of primers for Exon 11 and Exon 2 of BRCA 1 Gene

- Optimization of 6 primers of Exon 11, BRCA 1 Gene in genomic DNA.
- Optimization of 6 primers of Exon 11, BRCA 1 Gene in ctDNA.
- Optimization of primer of Exon 2, BRCA 1 Gene in genomic DNA.
- Optimization of primer of Exon 2, BRCA 1 Gene in ctDNA.

Veriti 96-well thermal cycler (Applied Biosystems) was used for optimization of primers having 10µM working concentration. 1.5% gel was used to analyze PCR product by Gel Doc-It gel documentation system (UVP). On successful optimization, same annealing temperature (54º) was determined for all of the 6 primers of Exon 11 and 1 primer for Exon 2.

High Resolution Melt Curve Analysis

HRM Analysis of Exon 2 of BRCA 1 Gene in Genomic DNA of 30 Neo adjuvant Breast Cancer Patients and 2 Healthy Controls

We have analyzed 30 DNA samples of breast cancer patients along with 2 negative controls of healthy persons. The melting curves shown (in purple) for Exon 2 represents founder mutation c.68_69delAG (BIC: 185delAG) in 15 samples. All the patient's samples whose melting curves follow the pattern of negative control are considered as wild type, represented in red. Each trace represents the amplicon from a different individual's DNA sample. All mutations were clearly distinct from the wild type sample and controls. From the 15 wild type patients and 2 negative controls, 3 samples were reanalyzed for confirmation of normality of sequence by Next Generation Sequencing (NGS) which showed normal sequence.

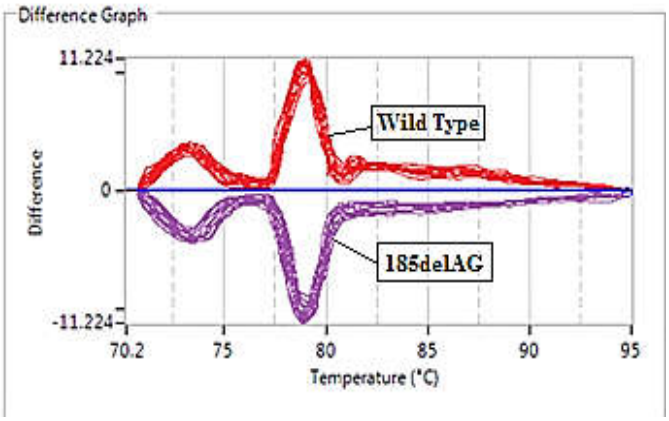

Figure 4.6: Difference plot showing the founder mutation (185delAG) in BRCA 1 exon 2 relative to the wild type controls [Controls (n=2), Patients (n=30)]

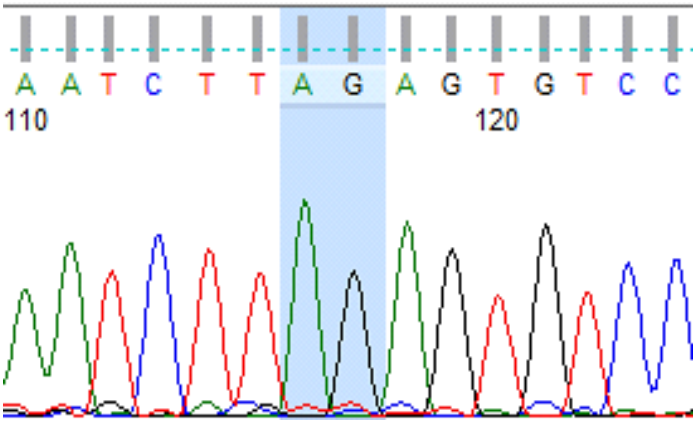

Figure 4.7: Sequencing profile of selected samples of Genomic DNA for Exon 2 (Founder Mutation)

HRM Analysis of Exon 11 of BRCA 1 Gene in Genomic and Circulating tumor DNA of 22 Neo adjuvant Breast Cancer Patients and 3 Healthy Controls

Primer 1

- Comparison of genomic DNA of 5 patients (N1,N2, N4,N12, N18) and 2 healthy controls (C16, C8) to ctDNA of the same patients. Wild type profile is shown in pink. The orange melting curves represents the mutation in DNA and ctDNA of same patient (N1) whereas, green represents variation only in ctDNA of a patient (N2).
- Melting curves shows the difference, where genomic DNA of 6 patients (N28, N30, N31, M2, M4, M11) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in red. The orange melting curves represents the mutation in DNA and ctDNA of same patient (N28) whereas, purple represents mutation in DNA and ctDNA of another patient (M4).
- Genomic DNA of 5 patients (N19, N22, N25, N26, N27) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. Wild type

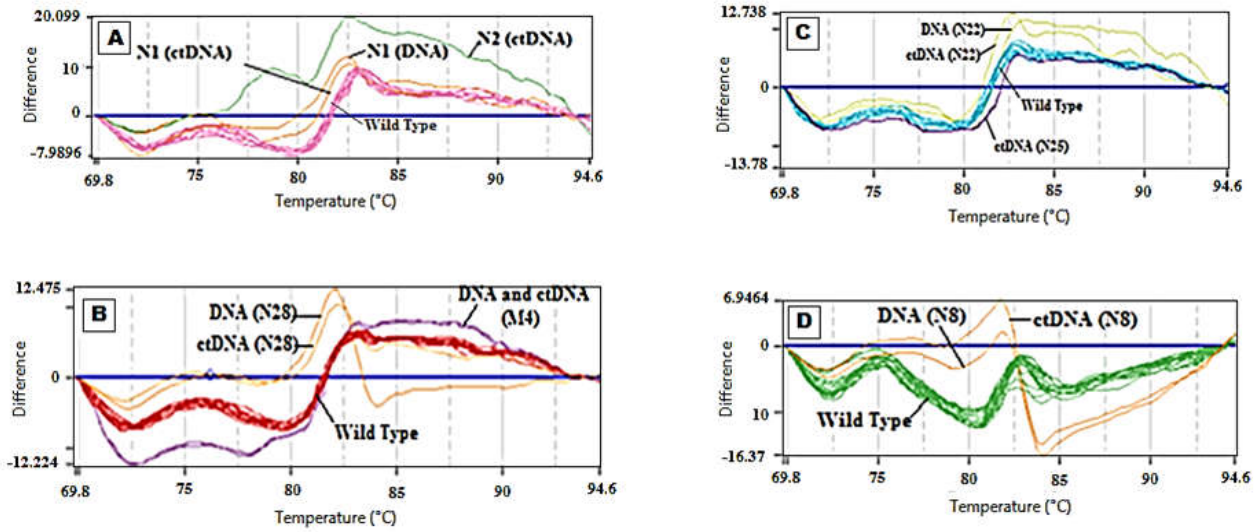

Figure 4.8: Difference plot of DNA and ctDNA samples showing the mutation relative to wild type melting profile in primer 1 of exon 11 [Controls (n=3), Patients (n=22)]

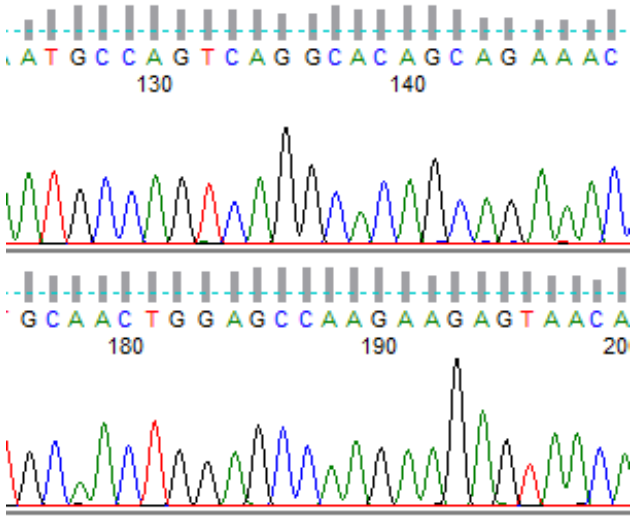

Figure 4.9: Sequencing profile of selected sample of genomic DNA (N2) for Exon 11 primer 1.

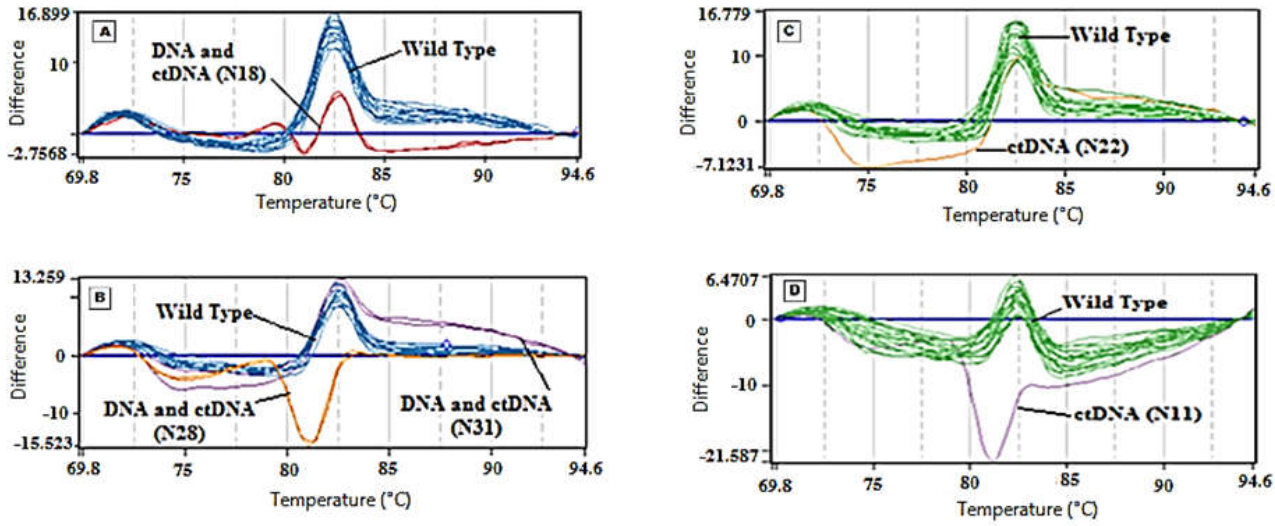

Figure 4.10: Difference plot of DNA and ctDNA samples showing the mutation relative to wild type melting profile in primer 2 of exon 11 [Controls (n=3), Patients (n=22)]

profile is shown in blue. The green melting curves represents the mutation in DNA and ctDNA of same patient (N22) whereas, and purple profile represent variation only in ctDNA of a patient (N25).

- Melting curves shows the difference, where genomic DNA of 6 patients (N6, N7, N8, N9, N10, N11) and 3 healthy controls (C16, C8, C10) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in green. The orange melting curves represent the mutation in DNA and ctDNA of same patient (N8).

In Primer 1, 7 variant has been identified. It has been noted that 5 variant profiles of DNA correspond to variant profile of ctDNA of same patient and it was also noted that in 2 cases that ctDNA showed variation but DNA of same patients correspond to wild type profile. DNA sample of these patient were selected for rescreening by sequencing to confirm whether it is false negative result or not. Sequencing results showed no mutation in a DNA sequences, proving accuracy of HRM results.

Primer 2

- Melting curves shows the difference, where genomic DNA of 5 patients (N1,N2,N4,N12,N18) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in blue. The red melting curves represents the mutation in DNA and ctDNA of same patient (N18).
- Melting curves shows the difference, where genomic DNA of 6 patients (N28, N30, N31, M2, M4, M11) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in blue. The orange melting curves represents the mutation in DNA and ctDNA of same patient (N28) whereas, purple represents mutation in DNA and ctDNA of another patient (N31).
- Melting curves shows the difference, where genomic DNA of 5 patients (N19, N22, N25, N26, N27) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. The mutant produce clearly distinct pattern from wild type shown in green. The orange melting curve represent the mutation in only ctDNA of patient (N22).
- Melting curves shows the difference, where genomic DNA of 6 patients (N6, N7, N8, N9, N10, N11) and 3 healthy controls (C16, C8, C10) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in green. The purple melting curves represent the mutation only in ctDNA of one patient (N11).

In Primer 2, 5 variant were present. 3 variant profiles of DNA correspond to variant profile of ctDNA of same patient and again in 2 cases, ctDNA showed variation but DNA of same patients correspond to wild type profile.

Primer 3

- Melting curves shows the difference, where genomic DNA of 5 patients (N1,N2,N4,N12,N18) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in red. The green melting curves represents the mutation in DNA and ctDNA of same patient (N12) whereas, blue represents variation only in ctDNA of a patient (N2).
- Melting curves shows the difference, where genomic DNA of 5 patients (N19, N22, N25, N26, N27) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in pink. The green melting curves represents the mutation in DNA and ctDNA of same patient (N26) whereas, and purple profile represents mutation in DNA and ctDNA of another patient (N25).
- Melting curves shows the difference, where genomic DNA of 6 patients (N28, N30, N31, M2, M4, M11) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. The mutant produce clearly distinct pattern from wild type shown in green. The blue melting curves represents the mutation in ctDNA of patient (N28).
- Melting curves shows the difference, where genomic DNA of 6 patients (N6, N7, N8, N9, N10, N11) and 3 healthy controls (C16, C8, C10) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in green. The orange melting curves represent the mutation in ctDNA of patient (N8) whereas, green melting curves represent the mutation in ctDNA of another patient (N6).

In Primer 3, 7 variant were noted. The melt curve graphs showed that 3 variant profiles of DNA correspond to variant profile of ctDNA of same patient and ctDNA of 4 patients showed variation but DNA of same patients correspond to wild type profile.

Primer 4

- Melting curves shows the difference, where genomic DNA of 5 patients (N1,N2,N4,N12,N18) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. The mutant produce clearly distinct pattern from wild type shown in orange. The blue melting curves represents the mutation in ctDNA of a patient (N18).
- Melting curves shows the difference, where genomic DNA of 6 patients (N28, N30, N31, M2, M4, M11) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in purple. The blue melting curves represents the mutation in DNA and ctDNA of same patient (M11).
- Melting curves shows the difference, where genomic DNA of 5 patients (N19, N22, N25, N26, and N27) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in pink. The brown melting curves represent the mutation in DNA and ctDNA of two patients (N22 and N25) whereas, and orange profile represents variation only in ctDNA of a patient (N27).
- Melting curves shows the difference, where genomic DNA of 6 patients (N6, N7, N8, N9, N10, N11) and 3

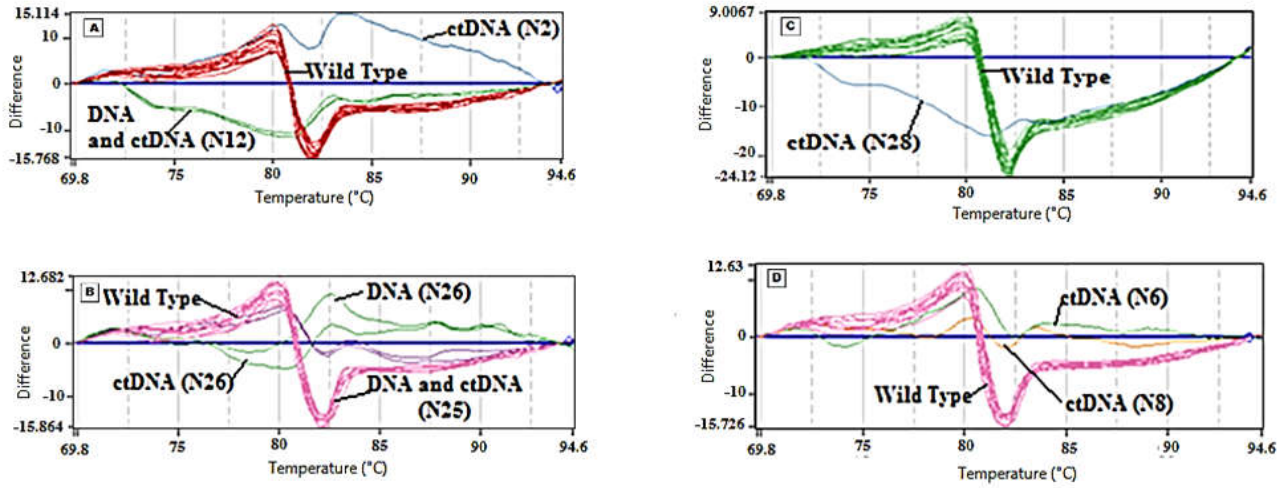

Figure 4.11: Difference plot of DNA and ctDNA samples showing the mutation relative to wild type melting profile in primer 3 of Exon 11 [Controls (n=3), Patients (n=22)]

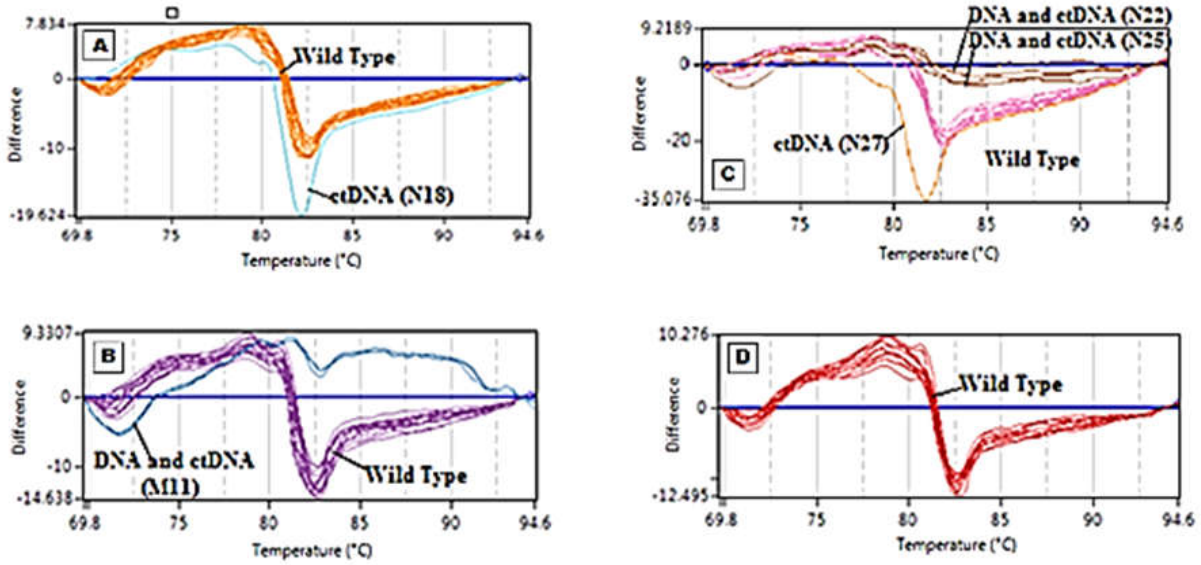

Figure 4.12: Difference plot of DNA and ctDNA samples showing the mutation relative to wild type melting profile in primer 4 of Exon 11 [Controls (n=3), Patients (n=22)]

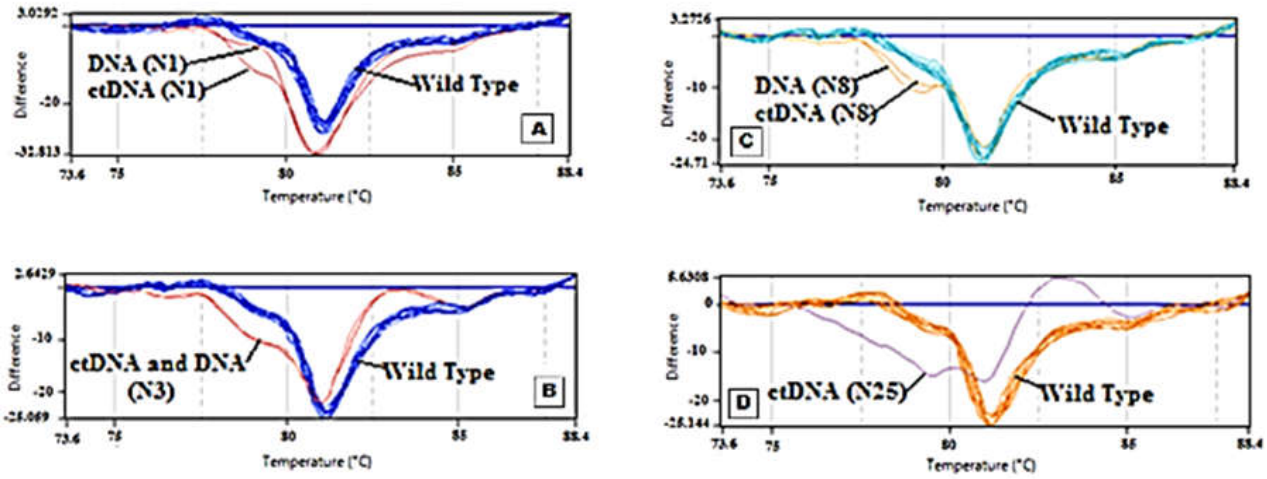

Figure 4.13: Difference plot of DNA and ctDNA samples showing the mutation relative to wild type melting profile in primer 5 of Exon 11 [Controls (n=3), Patients (n=22)]

healthy controls (C16, C8, C10) have been compared to ctDNA of the same patients. No melt curve shown any variation from wild type shown in red.

In region 4 (Primer 4) 5 variant has been identified. Results showed ctDNA variation in 2 samples with their DNA correspond to wild type profile. In 3 cases DNA variation correspond to ctDNA variation but interestingly in 2 samples DNA and ctDNA of same patient showed exactly same melt curve.

Primer 5

- Melting curves shows the difference, where genomic DNA of 5 patients (N1,N2,N4,N12,N18) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in blue. The red melting curves represents the mutation in DNA and ctDNA of same patient (N1).
- Melting curves shows the difference, where genomic DNA of 5 patients (N19, N22, N25, N26, N27) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in orange. The purple melting curve represents the mutation only in ctDNA of a patient (N25).
- Melting curves shows the difference, where genomic DNA of 6 patients (N28, N30, N31, M2, M4, M11) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in blue. The red melting curves represents the mutation in DNA and ctDNA of same patient (N31).
- Melting curves shows the difference, where genomic DNA of 6 patients (N6, N7, N8, N9, N10, N11) and 3 healthy controls (C16, C8, C10) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in blue. The orange melting curves represent the mutation in DNA and ctDNA of same patient (N8).

In region 5 (Primer 5) 4 variant were shown in results. 3 variant profiles of DNA correspond to variant profile of ctDNA of same patient and in one sample ctDNA showed variation but DNA of same patient correspond to wild type profile.

Primer 6

- Melting curves shows the difference, where genomic DNA of 5 patients (N1,N2,N4,N12,N18) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in purple. The orange melting curves represents the mutation in DNA and ctDNA of same patient (N2).
- Melting curves shows the difference, where genomic DNA of 5 patients (N19, N22, N25, N26, N27) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. All the mutants produce clearly distinct pattern from wild type shown in orange. The purple melting curves represent the mutation in DNA and ctDNA of same patient (N25).
- Melting curves shows the difference, where genomic DNA of 6 patients (N28, N30, N31, M2, M4, M11) and 2 healthy controls (C16, C8) have been compared to ctDNA of the same patients. No melt curve shown any variation from wild type shown in green.
- Melting curves shows the difference, where genomic DNA of 6 patients (N6, N7, N8, N9, N10, N11) and 3 healthy controls (C16, C8, C10) have been compared to ctDNA of the same patients. The blue melting curves represents the mutation in ctDNA of only one patient (N11).

In region 6 (Primer 6) results demonstrated 4 variations. In 3 cases variant profiles of DNA correspond to variant profile of ctDNA of same patient and only in 1 sample ctDNA showed variation but DNA of same patients correspond to wild type profile. Because of theoretical concerns that pathogenic mutations may coexist with polymorphisms in populations which may distort the HRM curve and make it appear like a normal sequence, DNA sample of those 2 patients were selected for re-screening by sequencing in which DNA and ctDNA showed no variation in High resolution melt curve graph, to confirm the normality of sequence. Sequencing results showed no significant variance.

DISCUSSION

Breast cancer (BC) is a significant health care problem worldwide, and approximately 1.7 million new cases were diagnosed in 2012, representing 25% of all cancers (Globocan). The three commonest cancers in the Pakistani population are breast cancer, oral cancer and lung cancer (WHO Cancer Fact Sheet, 2014). Breast cancer is currently the leading cause of cancer morbidity and mortality among Pakistani women. Our data showed that about in enrolled subjects, 66% of breast cancer cases occur in women less than 50 years old, and 34% of cases at more than 50 years. Because of the strong association between age of onset and genetic susceptibility, a high proportion of breast cancer cases in Pakistan may be attributable to BRCA1 and BRCA2 mutations. BRCA 1 (MIM#113705) is tumor suppressor gene which is most frequently mutated in breast and ovarian cancers but the number of mutations varies widely among populations in the world. To date, few molecular genetics studies of BRCA 1 have been reported in Pakistani population, consequently knowledge of prevalence and spectrum of BRCA1 mutation in our population is sparse. Among the 22 coding exons of BRCA 1, our area of interest was Exon 2 and Exon 11 because a founder mutation (185delAG) has been reported in many populations in Exon 2 (Risch *et al*., 2006) and as Exon 11 is largest human exon so it was the main target for mutation detection. Moreover it has been reported that most prevalent alteration in BRCA1 are located in exon 11 (El Khachibi *et al*., 2015). Our primary aim of study was to investigate that whether circulating tumor DNA can provide the same genetic mutation information as a genomic DNA or they are carrying some new mutations as cancer evolves with passage of time. For that purpose we provide a direct comparison between circulating tumor DNA and genomic DNA of breast cancer patients. Out of 30 patients only 22 patients had measurable levels of circulating tumor DNA. The complete screening of genomic DNA and ctDNA of 22 Patients for Exon 11 of 3426 bp was costly and time consuming. Thus our method of choice was High Resolution Melt Curve Analysis (HRM).

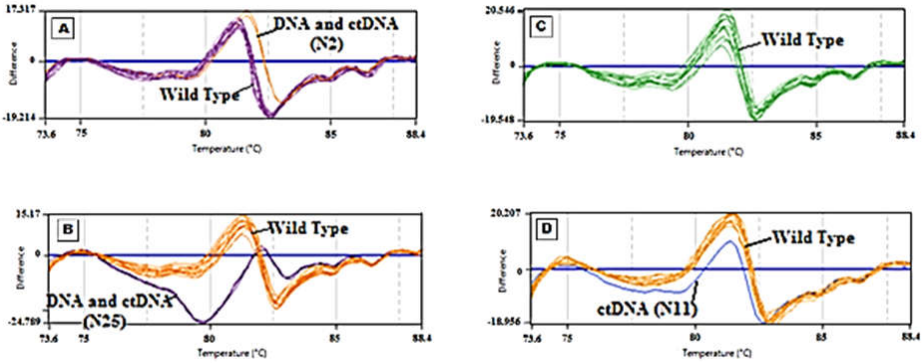

Figure 4.14: Difference plot of DNA and ctDNA samples showing the mutation relative to wild type melting profile in primer 6 of Exon 11 [Controls (n=3), Patients (n=22)]

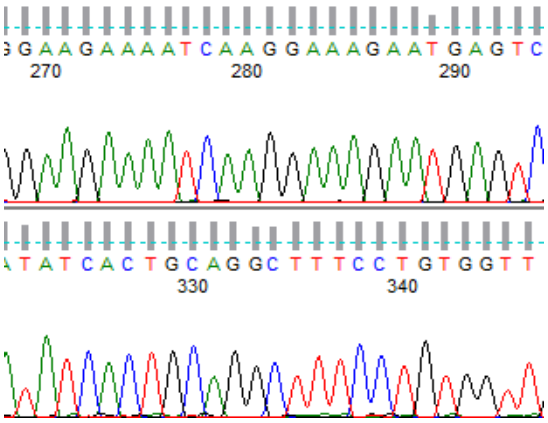

Figure 4.15: Sequencing profile of selected sample of genomic DNA (N28) for Exon 11

It is a post-PCR method which enables the detection of sequence variations within an amplified region of DNA. In first part of study we compared DNA of 22 patients and 3 healthy controls with ctDNA of same patients (22) for mutational screening in Exon 11 of BRCA 1 with 6 primers. Contrary to one common mutation of BRCA 1 Exon 2, many variants are identified in exon 11. But interestingly we found that same variants were present in ctDNA of patients in 20 cases. Our data also showed that in 12 samples, ctDNA displayed variation that was not present in genomic DNA , which enable us to assume that ctDNA is a significant biomarker that can not only display mutations of genomic DNA but can also be followed also be over a time for studing evolution of tumor. Rebbeck and group conducted a study on 381 breast cancer patients. 18/381 (4.7%) patients had mutations in BRCA1 gene and 11 novel variants (2361insC, 2354AtoC, 2026TtoA, 2025insC, 1961delG, 1614insC, 1611delC, 1415insA, 1273insG, 1238delA) were identified in exon 11 of BRCA1 gene. This study suggested the association of mutations in exon 11 of BRCA1 with breast cancer inheritance in families (Rebbeck *et al*., 2015). El Khachibi and group validate HRM analysis approach to screen entire exon 11 of BRCA 1 gene for 71 breast cancer patients with 12 primers, in order to detect different variants. Their results showed that 69 samples had same wild type profile while two samples showed atypical curves. They confirmed the presence of same SNP in both samples (c.2612C > T) after re-analyzation of samples by Sanger sequencing (El Khachibi *et al*., 2015). Takano and group evaluated the High Resolution Melt curve scanning method for founder mutations in exon 2 (185delAG) and exon 20 (5382insC) of BRCA1 and founder mutation in exon 11 (6174delT) of BRCA2 in 29 breast cancer patient. They showed that all the mutations were readily identified, showed distinctive melting curves and were visibly differentiated from the wild type and from each other. They concluded that HRM is economical and significant technique for mutational screening and testing (Takano *et al*., 2008). De Leeneer and group evaluated 2 high-resolution melting instruments for screening BRCA1 and BRCA2 mutations. They screened the large exon 11 of BRCA1 by direct sequencing and all other coding exons were screened by denaturing gel gradient electrophoresis (DGGE). High-resolution melting analysis was performed on the 96-well Light scanner (Idaho Technology Inc.) and the 96-well Light Cycler 480 (Roche) instruments. They concluded that the run time of high-resolution melting has been 1/3 of that with direct sequencing and DGGE, as there is no post-PCR handling and the software allows fast analyses (De Leeneer *et al*., 2008). In the second part of this study we analyzed and detected founder mutation (185delAG) in Exon 2 of BRCA 1 gene in genomic DNA of 30 patients with 2 negative controls by HRM. We found that founder mutations is present in 15 patients, thus bringing the total contribution of BRCA 1, Exon 2 founder mutation to 50% in our study population. Dillenburg et al. conducted a study to estimate the carrier frequency of founder mutations in the BRCA1 gene (185delAG and 5382insC) and founder mutation of BRCA2 gene (6174delT) in Brazil for 225 breasr cancer patients. They found a carrier frequency of for 185delAG (0.78%), no mutations for 5382insC and 0.4% carrier frequency for 6174delT mutation in BRCA 2 (Dillenburg *et al*. 2012). Abeliovich and group analyzed the frequency of 5382insC, 188del11 and 185delAG in the BRCA1 gene and 6174delT in the BRCA2 gene in 199 Ashkenazi patients with breast and/or ovarian cancer.

Individual mutation frequencies among breast cancer Ashkenazi patients were 185delAG (6.7%), 5382insC (2.2%), and 6174delT (4.5%), among ovarian cancer patients; 6174delT and 185delAG were approximately equally common (29% and 33% and, respectively), but no 5382insC mutation was present in any ovarian cancer patient (Abeliovich *et al*., 1997). Our aim was to investigate the whether BRCA 1 gene (Exon 11 and Exon 2) have any contribution in breast cancer in Pakistani population and secondly whether ctDNA which is tumor derived DNA that is circulating freely in the blood of a cancer patient is a significant biomarker to display mutations in genomic DNA or whether it carries some new mutations. Our results reinforced the idea that ctDNA show a high concordance rate with genomic mutations and can be used for real-time monitoring in vivo, such as prediction of clinical treatments. Moreover it seems that penetrance of BRCA 1 mutations specifically founder mutation in exon 2 is about 50% in population which is parallel with the literature. But based on this preliminary research, it seems that there might be some other genes that contribute more significantly to familial breast carcinoma in Pakistani population. Our results also suggest that HRM has many advantages over other sequencing scanning methods. Firstly it is performed in a "closed tube" system where the PCR amplification and HRM are performed within the one instrument thus reduce the risk of contamination in less processing time. Secondly multiple samples can screen at the same time in order to detect mutant sequence relative to multiple wild type sequences. Thirdly only 25ng/µl DNA is required for effective screening of variant in sequence. There are certain limitations of our study that can be rectified in future. Firstly the small sample size due to limited of resources. Initially 60 blood samples were collected before and after chemotherapy of 30 patients. But due to limited number of columns for ctDNA extraction and limited quantity of master mix for HRM analysis number of samples reduced to 30. Another potential concern is that due to very less amount of ctDNA, we were not able to re analyze ctDNA by sequencing to confirm the status of mutation. All efforts will be made to further characterize SNPs in BRCA1 gene of breast cancer patients.

Regarding future directions ctDNA have provided evidence that the use of ctDNA as a liquid biopsy can improve cancer diagnosis and treatment via genotyping, disease monitoring, treatment evaluation, and so on. ctDNA is proved to be a non-invasive and easily accessible way for cancer diagnosis. Moreover the changing rate ctDNA during systemic treatments allows to consider the use of ctDNA in monitoring patients to identify early recurrence or progression. The field is now in an exciting transitional period in which ctDNA analysis is beginning to be applied clinically, although there is still much to learn about the biology of cell-free DNA.

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