BRCA1, BRCA2, HER2 and RAD51 gene polymorphisms in breast cancer patients of Bangladesh

A thesis submitted by
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Abstract

Purpose: Breast cancer is the most common malignancy among females in both developed and developing region of the world. Genetic factors are implicated in the etiology of breast cancer**.** Altered expression of some genes due to polymorphisms increases the risk of breast cancer incidence. This study was aimed to evaluate the role of BRCA1 (rs80357713), BRCA1 (rs76171189), BRCA2 (rs11571653), RAD51 (rs1801320), HER2 (rs1136201) polymorphisms as risk factors for breast cancer as well as their effects on breast cancer subtypes in Bangladeshi population.

Patients and Methods: For breast cancer risk determination, total three hundred and ten (310) patients with invasive breast cancers were recruited from different public and private hospitals of Bangladesh and as controls two hundred and fifty (250) Bangladeshi women were recruited matching age with patients coming from different region of the country where most of them were with benign tumor disease getting treatments in the surgical wards of the hospitals. Genetic polymorphisms of the mentioned genes were detected by using Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR RFLP).

Results: Patients carrying BRCA1 (rs80357713), BRCA1 (rs76171189) and BRCA2 (rs11571653) polymorphisms were collectively associated with breast cancer risk. Patients with GC and GC plus CC genotype of RAD51 (rs1801320) and AG plus GG genotype of HER2 (rs1136201) gene were found to be significantly associated with breast cancer. In sub group analysis, AG plus GG genotype of HER2 (rs1136201) was found to be associated with breast cancer risk in patient younger than 45 years of age in compared to patients older than 45 years of age. RAD51 (rs1801320) was related with tumor aggressiveness (higher graded tumor). No other significant association was found in this study.

Conclusion: Our results indicate that BRCA1 (rs80357713), BRCA1 (rs76171189), BRCA2 (rs11571653), RAD51 (rs1801320), HER2 (rs1136201) polymorphisms are associated with breast cancer in different ways.

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Salma Parvin

DECLARATION

Not any portion of this work referred to in this thesis paper has been submitted for another degree or qualification of the University of Dhaka or any other University or any other institute of learning.

DEDICATION

Dedicated to my parents and teachers who always inspire me in every steps of my life

List of Abbreviation

Name	Details
A	Adenine (where referring to a nucleotide)
AAs	Arylamine
ADH	Atypical ductal hyperplasia
ADRs	Adverse drug reactions
AFRO	WHO Africa region
ASCO	American Society of Clinical Oncology
Asp	Aspartic acid
ATM	Ataxia telangiectasia mutated
BMI	Body-mass index
BP	Benzo[a]pyrene
BRCA1	Breast Cancer susceptibility gene 1
BRCA2	Breast Cancer susceptibility gene 2
bp	Base pair
C	Cytosine (where referring to a nucleotide)
CEP17	Chromosome enumeration probe 17
cDNA	Complementary DNA
CI	confidence interval (where genotype, haplotype and allelic data compared)
Cl^-	Chloride ion
CYP	Cytochrome P450
Cys	Cysteine
D’	normalized disequilibrium constant
DCIS	Ductal carcinoma in situ
DF	Dilution factor
DMEs	Drug metabolizing enzymes
DNA	Deoxyribonucleic acid
dNTP	De-oxy nucleotide triphosphate
EGFR	Epidermal growth factor receptor
EMRO	WHO East Mediterranean region
EtBr	Ethidium Bromide
EURO	WHO Europe region
EU-28	European Union
FP	Forward Primer
G	Guanine (where referring to a nucleotide)
GWAS	Genome-wide association studies
HapMap	Haplotype map
HE	heterozygote
HER2	Human Epidermal growth factor receptor2
HR	Homologous recombination

HPLC	High-performance liquid chromatography
HRT	Hormone replacement therapy
IARC	International Agency for Research on Cancer
IDC	Invasive ductal carcinoma
IHC	Immuno-histochemistry
Ile	Isoleucine
INCC	International Lung Cancer Consortium
ISH	In situ hybridization
K^+	Potassium ion
L	Liter
LCIS	Lobular carcinoma in situ
Met	Methionine
MEX	Mexican ancestry in Los Angeles
mg	Miligram
MgCl2	Magnesium Chloride
MH	mutant homozygote
ml	Milliliter
mM	Millimolar
MPI	Mannose phosphate isomerase
mRNA	Messenger Ribonucleic acid
Na^+	Sodium ion
NCBI	National Centre for Biotechnological Information
NCI	National Cancer Institute
NE buffer	New England Biolab buffer
NEB	New England Biolab
NHEJ	Non-homologous end joining (NHEJ)
ng	Nanogram
NH	normal homozygote
OCs	Oral contraceptives
OD	Optical Density
OR	Odds Ratio (where genotype, haplotype and allelic data compared)
PAHO	WHO Americas region
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
Pre-mRNA	preliminary-mRNA
Pro	Proline
REase	Restriction endonuclease
RefSeq	NCBI Reference Sequences
REs	Restriction Enzymes
RFLP	Restriction Fragment Length Polymorphism

RNA	Ribonucleic acid
RP	Reverse Primer
rs	DbSNP record ID number
SEARO	WHO South-East Asia region
Ser	Serine
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Product and Service Solutions
T	Thymine (where referring to a nucleotide)
TAE	Tris-acetate-EDT
TE	Tris-EDTA
Thr	Threonine
TNM	tumor, node and metastases
Tris-HCL	Tris-Hydrochloride
U	unit
USA	United States of America
UTR	3´-untranslated region
UV	Ultraviolet
Val	Valine
WCRF	World Cancer Research Fund
WHO	World Health Organization
WMADH	World Medical Association Declaration of Helsinki
WPRO	WHO Western Pacific region
α	Alpha
β	Beta
γ	Gamma
δ	Delta
µg	Microgram
µl	Microlitre

Contents

CHAPTER ONE

INTRODUCTION

1.0 Introduction

1.1 Breast Cancer

Breast cancer is a type of malignancy caused by the abnormal growth and uncontrolled cell division within the terminal duct and lobular units of the breast that can invade and destroy surrounding normal tissue, and spread throughout the body via blood or lymph fluid to at new sites (Liu, 2007). It is the most frequent malignant disease and the leading cause of cancer death among women in both economically developed and developing regions of the world. Globally, 1.4 million new breast cancer cases are estimated each year and approximately one-third of the diagnosed patients are reported to die of the disease (Jemal et al., 2011). The incidence rates are highest in the Western world, where the life-time risk of developing breast cancer is found to be one in nine. Due to increased awareness, early detection, and availability of better treatment options, breast cancer mortality rates have declined in recent years (Coleman et al., 2011). Despite the common occurrence, the exact aetiology of the disease is still under investigations. Breast cancer is believed to be a multifactorial disease which is a result of the interaction of different genetic and environmental factors (Ponder, 2001). Over the past decade, significant progress has been done in defining risk factors, determining susceptibility of individuals to developing breast cancer as well as the genetic factors that contribute to this risk. Despite this improvement of the knowledge, the unravelling of the complex genetic and environmental influences on the disease is still at an initial stage. An even better understanding of the genetic mechanisms underlying the development and progression of breast cancer would be a major advance for improved prevention, detection and treatment strategies (Loizidou, 2009).

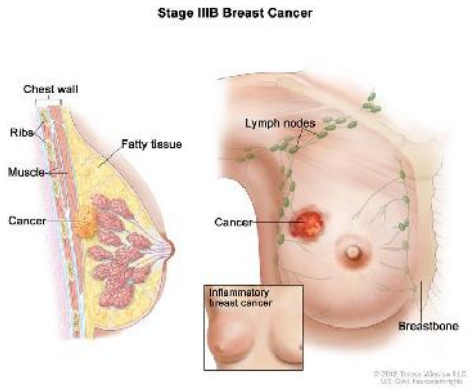


Fig 1.1: Breast cancer

1.2 Breast morphology

Development of the breast found about 7-8 weeks after conception, and the formation of nipples, areola and ducts begin at the later stages of gestation. Complete morphologic maturation of the breast tissue is formed following pregnancy. In brief, the mammary gland is found to consist of around twenty lobes, each of which has a branching duct system ending in terminal ducts. At the beginning of menarche, and with the influence of estrogen and progesterone, lobules are formed replacing pre-existing terminal ducts. Lobules are clusters of epithelial cells that radiate from the nipple terminating in dozens of tiny bulbs which get the capability to produce milk (alveoli). (Boron and Boulpaep, 2003; Loizidou, 2009).

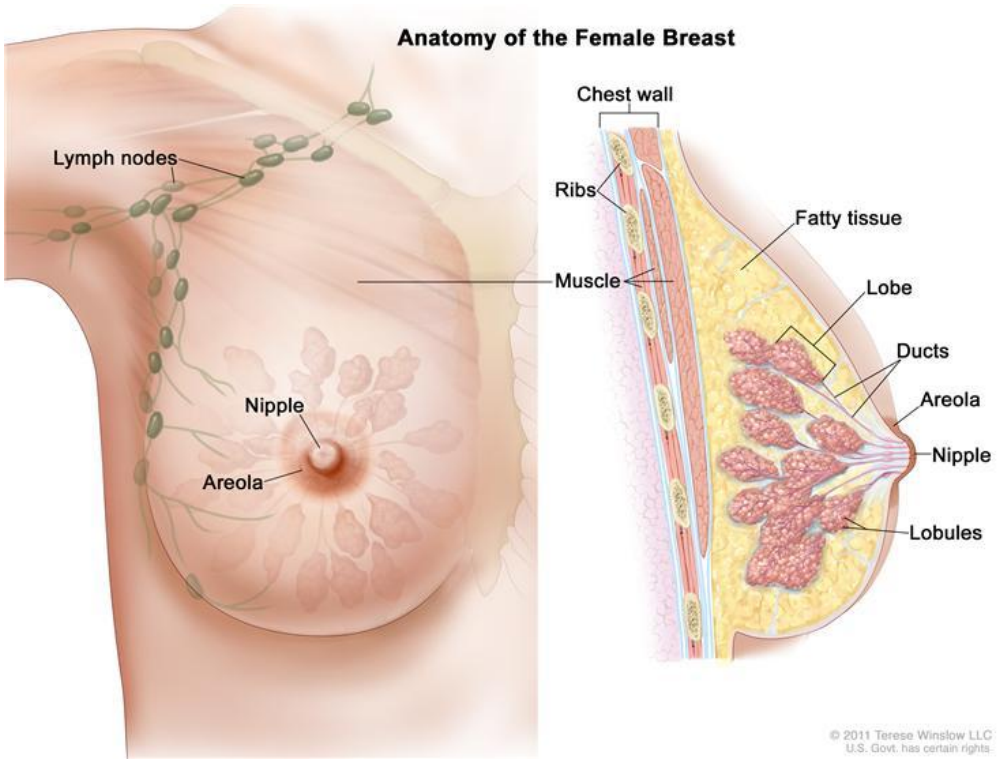


Fig 1.2a: Anatomy of female breast

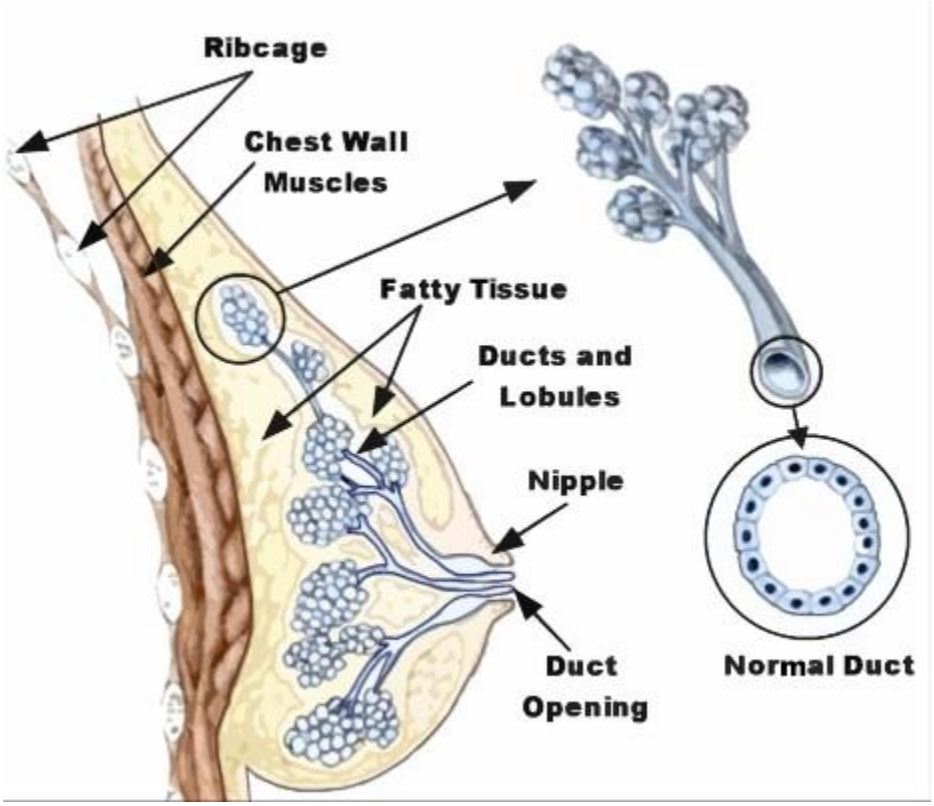


Fig1.2b: Anatomy of female breast

1.3 Pathology of Breast cancer

Breast cancer is the result of an interaction between an environmental (external) factor and a genetically susceptible host. Normal cells found to be divided as many times as it is needed and it is stopped. Cells become cancerous when their ability to stop dividing, to attach to other cells, to stay where they belong, and to die at the proper time are lost. Normal cells will die when they are no longer needed. Until then, they are found to be protected from cell suicide by several protein clusters and pathways. The important protective pathway is PI3K/AKT pathway and another one is the RAS/MEK/ERK pathway. Sometimes, it is found that the genes along these protective pathways get mutation in a way that turns them permanently "on", which makes the cell incapable of committing suicide when it is no longer needed. This is one of the major steps causing cancer in combination with other mutations. Normally, the PTEN protein is reported to be turned off the PI3K/AKT pathway when the cell is ready for cell suicide. In some breast cancers, the gene for the PTEN protein is found to be mutated, so the PI3K/AKT pathway is

stuck in the "on" position, and the cancer cell loses committing death (Adrian Lee & Carlos Arteaga, 2009).

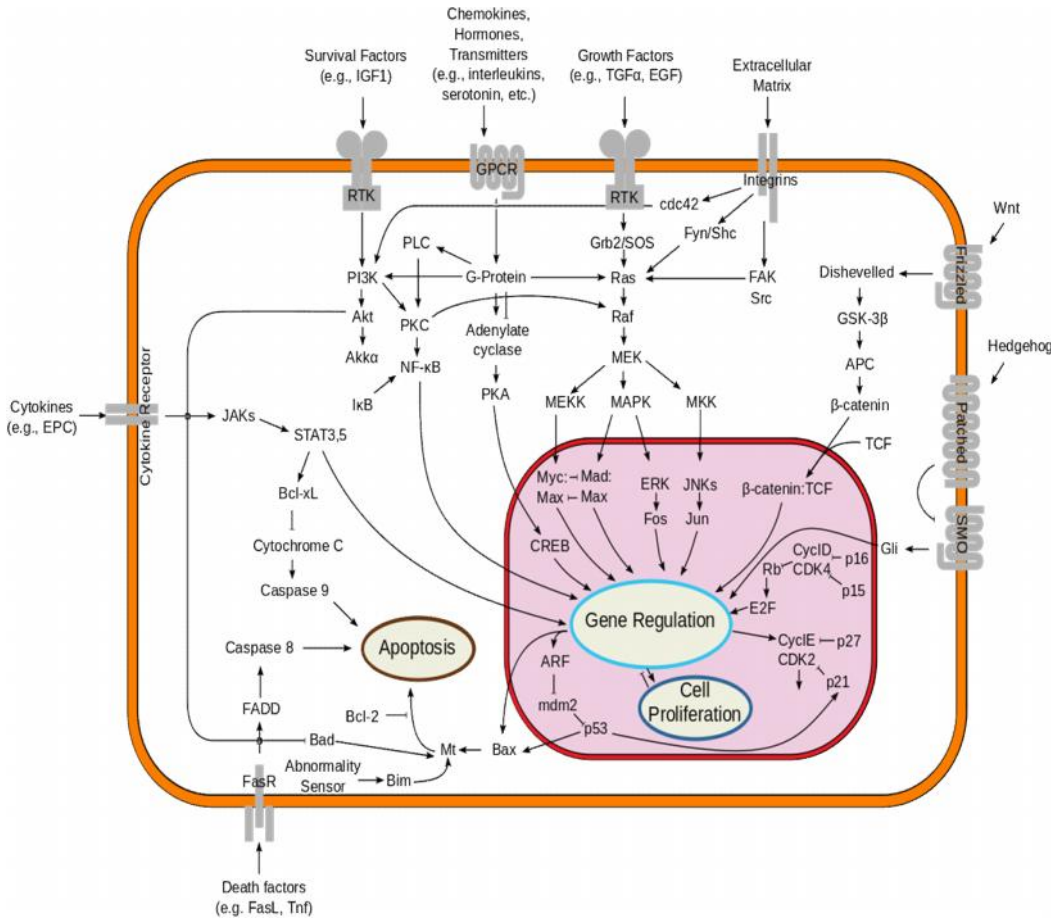


Fig1.3: Overview of signal transduction pathways involved in apoptosis. Mutations leading to loss of apoptosis can lead to tumorigenesis.

1.4 Signs and symptoms of breast cancer

The first noticeable symptom of breast cancer is typically a lump that feels different from the rest of the breast tissue. More than 80% of breast cancer cases are discovered when the woman feels a lump (Merck Manual of Diagnosis and Therapy, 2003). The earliest breast cancers are detected by a mammogram (American Cancer Society, 2007). Lumps found in lymph nodes located in the armpits (Merck Manual of Diagnosis and Therapy 2003) can also indicate breast cancer.

Indications of breast cancer other than a lump may include thickening different from the other breast tissue, one breast becoming larger or lower, a nipple changing position or shape or

becoming inverted, skin puckering or dimpling, a rash on or around a nipple, discharge from nipple/s, constant pain in part of the breast or armpit, and swelling beneath the armpit or around the collarbone (Watson et al., 2008).

Inflammatory breast cancer is a particular type of breast cancer which can pose a substantial diagnostic challenge. Symptoms may resemble a breast inflammation and may include itching, pain, swelling, nipple inversion, warmth and redness throughout the breast, as well as an orange-peel texture to the skin referred to as peau d'orange; (Merck Manual of Diagnosis and Therapy , 2003) as inflammatory breast cancer doesn't show as a lump there's sometimes a delay in diagnosis.

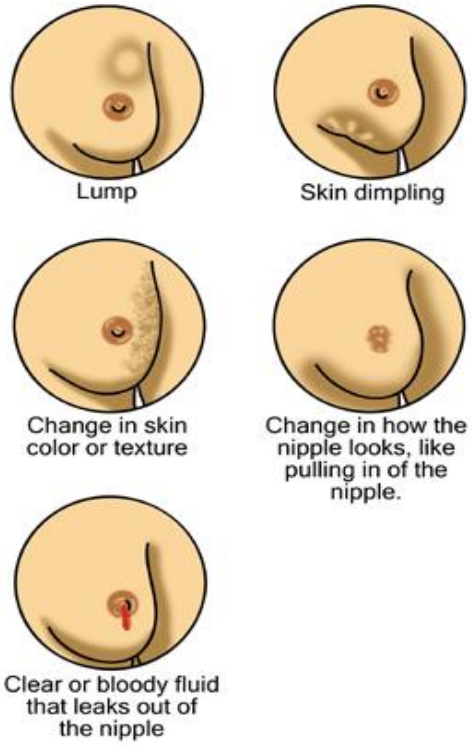


Fig 1.4: Early signs of possible breast cancer

Another reported symptom complex of breast cancer is Paget's disease of the breast. This syndrome presents as skin changes resembling eczema, such as redness, discoloration, or mild flaking of the nipple skin. As Paget's disease of the breast advances, symptoms may include tingling, itching, increased sensitivity, burning, and pain. There may also be discharge from the nipple. Approximately half of women diagnosed with Paget's disease of the breast also have a lump in the breast (National Cancer Institute , 2005). Occasionally, breast cancer presents as metastatic disease—that is, cancer that has spread beyond the original organ. The symptoms caused by metastatic breast cancer will depend on the location of metastasis. Common sites of metastasis include bone, liver, lung and brain (Lacroix, 2006). Unexplained weight loss can occasionally herald an occult breast cancer, as can symptoms of fevers or chills. Bone or joint pains can sometimes be manifestations of metastatic breast cancer, as can jaundice or neurological symptoms. These symptoms are called non-specific, meaning they could be manifestations of many other illnesses (National Cancer Institute , 2004).

Most symptoms of breast disorders, including the lumps, do not turn out to represent underlying breast cancer. Fewer than 20% of lumps, for example, are cancerous, ((Judith, 2008), and benign breast diseases such as mastitis and fibroadenoma of the breast are more common causes of breast disorder symptoms. Nevertheless, the appearance of a new symptom should be taken seriously by both patients and their doctors, because of the possibility of an underlying breast cancer at almost any age (Merck Manual of Diagnosis and Therapy , 2003).

1.5 Risk factors for breast cancer

Breast cancer is a life threatening disease with different risk factors that have a complicated role in its pathogenesis. These risk factors broadly can be classified into hormone, non-hormone factors and the genetic background of the susceptibility genes.

1.6 Hormone factors

1.6.1 Endogenous hormones

It has been observed from decades of studies that the increasing rate of breast cancer with age slows dramatically after menopause (McPherson et al., 2000). The pattern of this indicates the role of reproductive hormones in breast cancer risk (Pike et al., 1993), as hormone independent cancers should not exhibit a dramatic change in incidence. Oestradiol has been found to stimulate breast cell mitosis in the athymic nude mice model (McManus and Welsch, 1984). High oestrogen levels are reported to significantly increase breast cancer risk (Hankinson et al., 1998b) and the serum oestrogen levels can be a predictive factor of increased breast cancer risk (Key et al., 2002). Conversely, reducing exposure was thought to be protective (Hulka, 1997). The relative risk estimated was to be 2.0 for postmenopausal breast cancer development comparing the highest with the lowest quintile of serum estradiol concentration from nine prospective studies (Key et al., 2002). Furthermore, the risk for breast cancer increases while the risk substantially reduced by adjustment for serum oestrogen concentrations, showing that the higher oestrogen levels among heavier women account for breast cancer risk in obese women with increasing body-mass index (BMI) (Key et al., 2003). In the same way, factors that increase the number of menstrual cycles were found to be associated with an increased breast cancer risk, like early age at menarche, and late onset of menopause (Kampert et al., 1988). Statistically significant positive association was observed between plasma level of prolactin and breast cancer risk among postmenopausal women: women in the top quartile of levels had 2-fold risk of breast cancer relative to women in the bottom quartile (Hankinson et al., 1999; Tworoger et al., 2004). Increased levels of serum concentrations of testosterone, androstenedione, and dehydroepiandrosterone were also reported to be associated with increased risks of breast cancer with OR (highest versus lowest quartile) of 1.73, 1.56 and 1.48 respectively. In premenopausal women, the increased levels of blood concentrations of androgens are found to be associated with an increased risk of breast cancer (Kaaks et al., 2005). Plasma testosterone concentration levels were found to be associated with increased risk of breast cancer among postmenopausal women, (Key et al., 2002).

1.6.2 Exogenous hormones

1.6.2.1 Hormone replacement therapy

To treat the menopausal symptoms such as hot flushes and insomnia and to reduce the risk of chronic diseases such as osteoporosis, postmenopausal hormones have been prescribed for several decades. However, the duration of postmenopausal hormone use was found to be associated with increased breast cancer risk in some population. Hormone replacement therapy (HRT) was previously reported to be linked with a 10% higher breast cancer risk for each 5 years of use and the relative risk was 1.06 for oestrogen alone and 1.24 for oestrogen plus progestin (Ross et al., 2000). From the four studies, it is indicated that addition of a progestin to estrogen regimens increases breast cancer risk after 5 years of use from 10% (estrogen alone) to 30% (combined HRT) (Rossouw et al., 2002; Ross et al., 2000; Schairer et al., 2000). In contrast, tamoxifen (a drug with function of antiestrogen) have the capacity to reduce the breast cancer incidence (Fisher et al., 1998). In addition to this, the increase in risk associated with hormone use was most frequent for oestrogen-receptor positive tumours (Chen et al., 2004).

1.6.3 Oral contraceptives (OCs)

A lot of investigations have been done on the association between the use of OCs and breast cancer risk. In women taking combined OCs, independent of dose, age of first use, length of use, age of diagnosis or family history of breast cancer, a statistically significant increased risk of breast cancer has been observed (Collaborative Group on Hormonal Factors in Breast Cancer, 1996a; Collaborative Group on Hormonal Factors in Breast Cancer, 1996b). In parous women who used OCs before their first full-term pregnancy, the risk is significantly higher (OR=1.52; 95% CI=1.26-1.82) (Kahlenborn et al., 2006). The risk was reported to have 24% increased risk for breast cancer development, for the current users of oral contraceptive, although the risk decreases with the years after stopping of taken OCs. However, there was no significant excess risk of breast cancer after 10 or more years of stopping OCs (relative risk: 1.01) (Collaborative Group on Hormonal Factors in Breast Cancer, 1996a). In another phase of the study, women who began use OCs before age 20 has been found are resulting in higher relative risks of breast cancer comparing with those who began at older ages (Collaborative Group on Hormonal Factors in Breast Cancer, 1996a).

1.7 Non-hormone factors

It is indicated from some previous studies on migrants that breast cancer incidence rates in migrants moving from low-risk to high-risk countries tend to be increased to the rates of the host countries within the migrating generation itself, living for the duration of 10 years or less in the host country. This underscores the vital role of the environmental and sociocultural factors which are estimated to play more crucial role than the genetic factors (Parkin, 2004). In the carcinogenesis of breast cancer, the role of dietary or other life style changes has been confirmed in some studies (Robert et al., 2004).

1.8 Lifestyle

1.8.1 Alcohol and folate intake

Some epidemiological studies reported an increased risk of breast cancer with alcohol consumption, with an avarage risk of 1.6 fold compared to those nondrinkers (Singletary and Gapstur, 2001). Women with non proliferative breast disease consuming ≥ 15 g/day of alcohol had a 1.67 fold risk of breast cancer in the Nurses' Health Study (Tamimi et al., 2005). Acetaldehyde and free radicals coming from the metabolism of ethanol are known potential carcinogenic compounds. The acetaldehyde is found to bind with DNA and proteins and destroy folate resulting in secondary hyperproliferation (Poschl and Seitz, 2004). There may have some other mechanisms that are involved with increased serum estrogen levels in both premenopausal (Coutelle et al., 2004) and postmenopausal (Onland-Moret et al., 2005) women with long term alcohol consumption. Additionally, alcohol was reported to cause increased endogenous androgens, which may be converted to estrogens by peripheral aromatization pathway (Singletary and Gapstur, 2001). Besides, alcohol can impair the immune system and make nutritional deficiencies, like folate, vitamin E, vitamin B12, vitamin D, zinc and selenium, thus impair the ability to fight carcinogen (Poschl and Seitz, 2004). An increased risk for developing estrogen receptor-negative tumors has been reported in postmenopausal women with a higher-alcohol and low-folate intake (Sellers et al., 2002). Conversely, increased folate intake has been reported to protect chronic alcohol using women against cancer (Zhang, 2004).

1.8.2 Smoking

Cigarette smoking is associated with breast cancer risk. Pierre Band et al found that cigarette smoke can exert a dual action on the breast, with different effects in both premenopausal and postmenopausal women. Significant increased risk for breast cancer was observed in women who had been pregnant and started to smoke within 5 years of menarche in premenopausal participants (adjusted OR=1.69) and higher risk was observed in nulliparous women who smoked 20 cigarettes daily or more (adjusted OR=7.08) and nulliparous women who had smoked for 20 cumulative pack-years or more (adjusted OR=7.48). The difference was not significant in both ever pregnant and nulliparous postmenopausal women. Thediscrepancy of this result may be due to the postulated "antiestrogenic" effect of cigarette smoking (Baron et al., 1990, Clemons and Goss, 2001), incomparison with non-smokers, women who have an earlier age at natural menopause (Baron et al., 1990), reduced urinary concentrations of oestrogens during the luteal phase (MacMahon et al., 1982) and attenuated effects of hormone replacement therapy (Jensen and Christiansen, 1988). In another way, investigations have proposed that increased risk of breast cancer might be due to the reason that the breast is exposed to carcinogens in smoke (Petrakis, 1993). It is demonstrated from some studies that tobacco constituents can reach breast tissue. Carcinogens in tobacco smoke have the capacity to pass through the alveolar membrane (Yamasaki and Ames, 1977) and into the blood stream. These carcinogens are reported to be fat-soluble and can be stored in breast adipose tissue (Morris and Seifter, 1992) and these are metabolized and activated by human mammary epithelial cells (MacNicoll et al., 1980).

1.8.3 Diet

Various natural and chemical carcinogens and anti-carcinogens are found in our daily consumption (Sugimura, 2000). The carcinogens generating free oxygen radicals lead to DNA damage, or other deleterious components. For example, consumption of well done meat have been found to be associated with increased breast cancer risk (Zheng et al., 1998) due to the production of heterocyclic aromatic hydrocarbons and other harmful compounds in the process of preparation of meat at high temperatures. Fat is postulated to be a key breast cancer risk factor from the diet and increased consumption of saturated fatty acids were reported to be associated with an increased risk of developing breast cancer (Favero et al., 1999). Conversely, unsaturated

and polyunsaturated fatty acids, such as olive oil and fish oil, were observed to decrease the risk of breast cancer by up to 30% (Favero et al., 1998). But no significant association were found between breast cancer and total fat consumption, the relationship between saturated versus unsaturated fatty acids in other two large studies (Velie et al., 2000). Derivatives of the vitamins A, B and E, and selenium are reported to play an important role for protection against cancer. It has been identified that intake of fruits and vegetables, rich sources of natural vitamins, have the decreased breast cancer risk in numerous studies (Van Duyn and Pivonka, 2000), and in postmenopausal women, more significant protective effects were found (Gaudet et al., 2004). Soy foods, which are a rich source of fiber and phytoestrogen, have been reported to protective against breast cancer development (Yuan et al., 1995). It was postulated based on rat models, that genistein in soy promotes more differentiated tissue in the breast causeing less sensitive to later proliferative stimuli (Lamartiniere, 2000).

1.8.4 Obesity

It is observed from some previous studies that excess body mass has been implicated in approximately 5% of all cancers (Bergstrom et al., 2001). Obesity has little effect on the serum concentration of estrogen probably due to reduced ovarian estrogen by a negative feedback, hence contributes little change to the risk of breast cancer in premenopausal women, although obesity in these group women even has been associated with a decrease of breast cancer risk before menopause, yet the mechanism remains unclear (Lahmann et al., 2004). In another way, different large studies concluded that obesity and weight gain increase breast cancer risk in postmenopausal women. The risk is was more frequent among obese women who do not use hormone replacement therapy (HRT) with the relative risks up to 2 (Lahmann et al., 2004). In another study it is recorded that every 5 kg of weight gain since the lowest adult weight increased the breast cancer risk by 8% (Trentham-Dietz et al., 2000). In other study, women older than 55 with an increase in body mass of 10 kg have been found to be associated with 7% increase in breast cancer risk (Tryggvadottir et al., 2002). The mechanism by which obesity increases the risk for developing breast cancer in postmenopausal may due to the unregulated estrogen level by negative feedback, and obesity is found to instigate an increase in the serum concentration of bioavailable estradiol (McTiernan et al., 2003). Sex hormone-binding globulin

is also found to be decreased with increasing body mass index (BMI) which may contribute to the increased breast cancer risk. (Verkasalo et al., 2001)

1.8.5 Physical activity

Physical activity is a preventive factor for breast cancer due to the non-specific immune stimulation and decreased estrogen levels during recovery (Hardman, 2001) as well as delayed onset of menarche (Hankinson et al., 2004). Reduced insulin resistance and hyperinsulinaemia were linked with increased physical activity (Stoll, 2000), which has been proposed to be related to breast cancer (Kaaks, 1996). Additionally, increasedphysical activities have the potentiality to control weight gain lead to reduced breast cancer risk. It has been found from the Nurses' Health Study that decreased risk for breast cancer was associated with women with 7 or more hours per week of moderate exercises (relative risk: 0.82). This association had the similarity in both pre- and postmenopausal women (Rockhill et al., 1999). Women performing physical activity during adolescence, have the reduced breast cancer risk with 3% for each one-hour increase in recreational physical activity per week (Lagerros et al., 2004).

1.9 Other risk factors of breast cancer

1.9.1 Reproductive factors

From some different investigations, early pregnancy has been identified to have a protective effect against breast cancer risk (Pathak et al., 2000). Each full term pregnancy has been reported to cause a 3% reduction in breast cancer risk diagnosed early or before menopause and the reduction attained 12% for breast cancers diagnosed later (Clavel-Chapelon and Gerber, 2002). Another study found the result that the risk of breast cancer in women who have their first full-term pregnancy after the age of 30 is about twice of women who have their first child before the age of 20. Further reduced the risk of breast cancer has been found in women second pregnancy at early age (McPherson et al., 2000). On the other hand, women having a first child after the age of 35 have a higher risk than nulliparous women (Bernstein, 2002). Conversely, the immediate effect of pregnancy temporarily increases the risk, despite of having the long-term protection effect against breast cancer risk,

1.9.2 Mammographic density

Mammographic density has been reported to be a consistent marker for breast cancer risk both in pre and postmenopausal women. A variation in mammographic density has been considered to be associated with ovarian function. In women with dense breasts compared with those having low breast density, a significantly increased risk for breast cancer was identified. Breast density has been identified for a long time as a strong and independent risk factor for breast cancer in several epidemiological studies. The odds ratio and relative risks in different studies ranging between 4 and 6 greater risks in women with dense tissue in more than 60-75% of the breast, in comparison with those with no densities. It is suggested from Estimation of attributable risk that the breast density may responsible for as many as 30% of breast cancer cases (Boyd et al., 1998). As nulliparous and thinner women have an increased breast density in general, they may have increased risk for breast cancer, (Biglia et al., 2004). Nulliparity and high breast density are postulated to act synergistically and the breast cancer risk may be 7.1 times higher (van Gils et al., 2000).

1.9.3 History of benign breast disease

Women with benign breast disease have been proposed to get higher risk for breast cancer. The risk of developing breast cancer was reported to be determined by different groups of histologic lesions. Non proliferative lesions were not found to be associated with increased risk for breast cancer (London et al., 1992). The risk has been found to increase further to nine fold if the woman with atypical hyperplasias with a family history of breast cancer (first degree relative) (McPherson et al., 2000). The atypical ductal hyperplasia (ADH) was suggested as a precursor lesion to different molecular phenotypes of invasive breast cancer (Boecker et al., 2002). Chromosome copy number alterations were identified in ADH by sing comparative genomic hybridization analysis (Gong et al., 2001). ADH appeared to be a genetically advanced lesion with an expression profile that resembled ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) within the same specimen in comparison to the normal epithelium,. The ADH, DCIS, and IDC are reported to be highly similar to each other at the level of the transcriptome (Ma et al., 2003). The risks of breast cancer among women with atypical hyperplasia have been proposed to be influenced by the patient's menopausal status. In the Nurses' Health Study,

women with atypical hyperplasia were found to be a higher relative risk of premenopausal breast cancer (OR=5.3) compared to postmenopausal breast cancer (OR=3.7) (Marshall et al., 1997). The breast cancer risks among women with proliferative lesions without atypia were not found to vary according to menopausal status (Byrne et al., 2000). In addition to this, further increases of breast cancer for women with proliferative benign breast disease were not associated with postmenopausal exogenous female hormone use (Byrne et al., 2000).

1.9.4 Genetic risk factors for breast cancer

It has been demonstrated that up to 10% of breast cancer cases in developed countries are due to the genetic predisposition (McPherson et al., 2000). Women with family history of breast cancer have been found to have increased risk for developing breast cancer. A meta-analysis of 52 epidemiological studies came to the conclusion that 12% of women with breast cancer had one affected relative and 1% had two or more comparing with those who had no affected relatives, the risk ratios were 1.80, 2.93, and 3.90, respectively, for women with one, two, and three or more affected first-degree relatives (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). It was also found that women of a given age, the younger the relative diagnosed with breast cancer the greater the risk (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; McPherson et al., 2000). Germline mutations of high-penetrance breast cancer susceptibility genes like BRCA1, BRCA2, p53 and ATM, CHEK 2 and PTEN have been reported to have a high cumulative risk for developing hereditary breast cancer. However, the mutation frequencies of these genes are found to be very low in the general population. Mutations in these genes are reported to be responsible for only 5-10% of all breast cancers (Oesterreich and Fuqua, 1999, Liu, 2007, Loizidou, 2009).

1.10 Classifications of breast cancer

Breast cancers are classified by several grading systems. Each of these influences the prognosis and can affect treatment response. Description of a breast cancer optimally includes all of these factors.

Histopathology: Breast cancer is usually classified primarily by its histological appearance. Most breast cancers are derived from the epithelium lining the ducts or lobules, and these cancers are classified as ductal or lobular carcinoma. Carcinoma in situ is growth of low grade cancerous

or precancerous cells within a particular tissue compartment such as the mammary duct without invasion of the surrounding tissue. In contrast, invasive carcinoma does not confine itself to the initial tissue compartment.(Merck Manual, Professional Edition, Ch. 253, Breast Cancer.)

Grade: Grading compares the appearance of the breast cancer cells to the appearance of normal breast tissue. Normal cells in an organ like the breast become differentiated, meaning that they take on specific shapes and forms that reflect their function as part of that organ. Cancerous cells lose that differentiation. In cancer, the cells that would normally line up in an orderly way to make up the milk ducts become disorganized. Cell division becomes uncontrolled. Cell nuclei become less uniform. Pathologists describe cells as well differentiated (low grade), moderately differentiated (intermediate grade), and poorly differentiated (high grade) as the cells progressively lose the features seen in normal breast cells. Poorly differentiated cancers (the ones whose tissue is least like normal breast tissue) have a worse prognosis.

Stage: Breast cancer staging using the TNM system is based on the size of the tumor (**T**), whether or not the tumor has spread to the lymph nodes (**N**) in the armpits, and whether the tumor has metastasized (**M**) (i.e. spread to a more distant part of the body). Larger size, nodal spread, and metastasis have a larger stage number and a worse prognosis.
The main stages are:

- Stage 0 is a pre-cancerous or marker condition, either ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS).
- Stages 1–3 are within the breast or regional lymph nodes.
- Stage 4 is 'metastatic' cancer that has a less favorable prognosis. (Carlson et al., 2009; American Society of Clinical Oncology, 2012)

It is reported that at initial diagnosis, over 50% of breast cancers are stages 0 or I, (Fremgen et al., 1999) and 75% are Stage 0, I, or II. (Moore and Kinne, 1995) The quantity of lymph node involvement has a great impact on survival. Patients with stage IIA cancer (T0-T1, N1) with only 1 involved lymph node is found to have a 10-year disease-free survival of 71% and a 20-year disease-free survival of 66%. If 2 to 4 lymph nodes are involved, the 10-year disease-free survival is 62% and the 20-year disease-free survival is 56% (Moore and Kinne, 1995)

Table 1.1: TNM Definitions

Tis	Carcinoma in situ
T1	Tumor 2 cm or less in greatest dimension
T1a	0.5 cm or less
T1b	0.5 cm but ≤1 cm
T1c	1 cm but ≤2 cm
T2	Tumor 2 cm but ≤ 5 cm
T3	Tumor 5 cm
T4	Tumor of any size with direct extension to
N0	No regional lymph node metastases
N1	Metastases to moveable ipsilateral axillary
N2	Metastases to fixed ipsilateral axillary lymph nodes
N3	Metastases to ipsilateral internal mammary lymph node
M0	No distant metastases
M1	Distant metastases (including supraclavicular

Table1.2: TNM Stage

Stage	Description
0	Tis, N0, M0
I	T1, N0, M0
IIA	T0, N1, M0 or T1, N1, M0 or T2, N0, M0
IIB	T2, N1, M0 or T3, N0, M0

IIIA	T0–T2, N2, M0 or T3, N1, M0
IIIB	T4, any N, M0, or any T, N3, M0
IV	any T, any N, M1

(Islam, 2014)

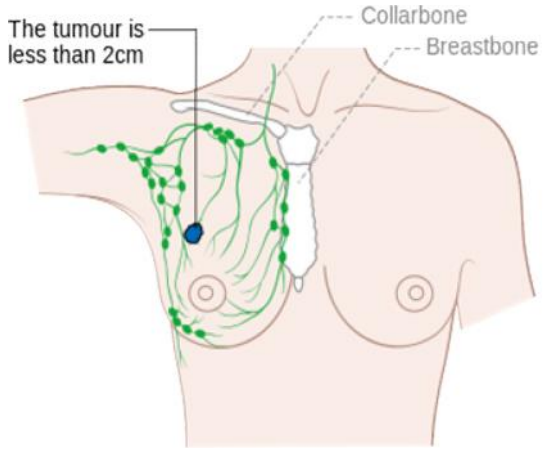


Fig1.5 Stage 1A breast cancer

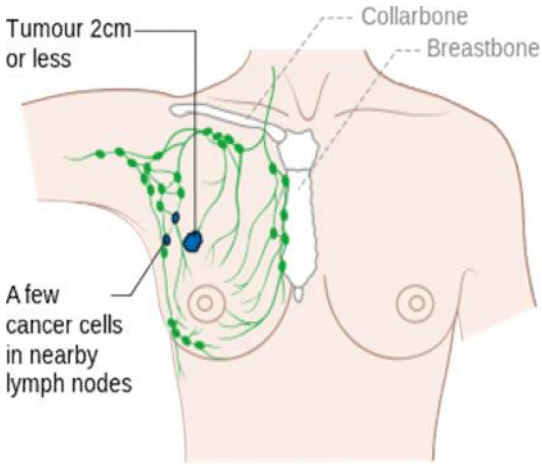


Fig 1.6: Stage 1B breast cancer

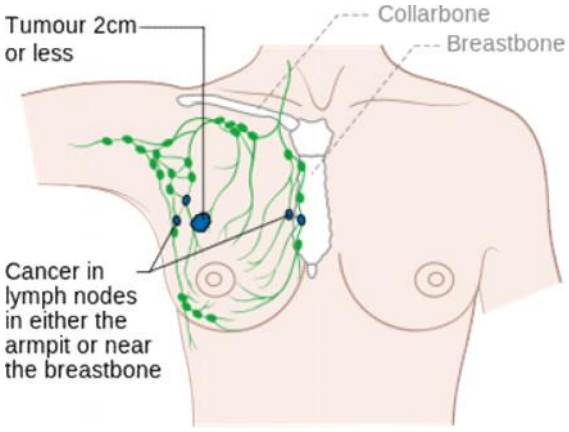


Fig1.7a: Stage 2A breast cancer

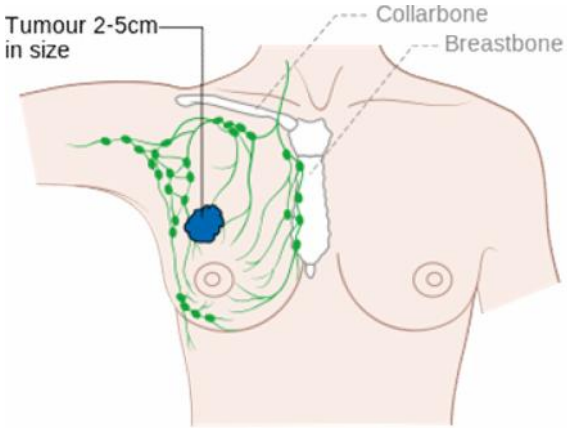


Fig 1.7b: Stage 2A breast cancer

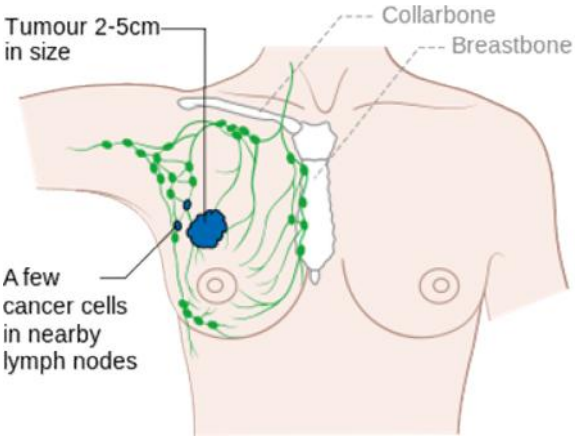


Fig 1.8a: Stage 2B breast cancer

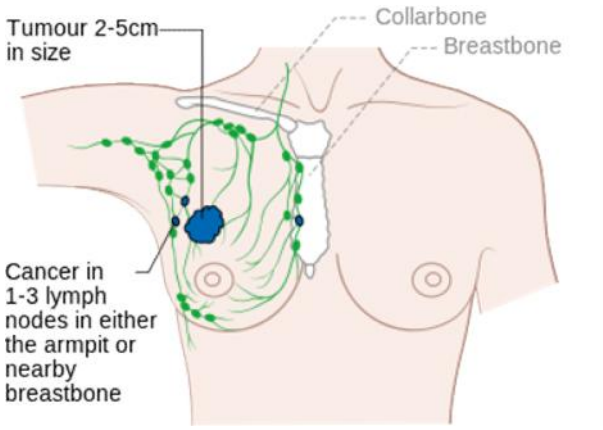


Fig 1.8b: Stage 2B breast cancer

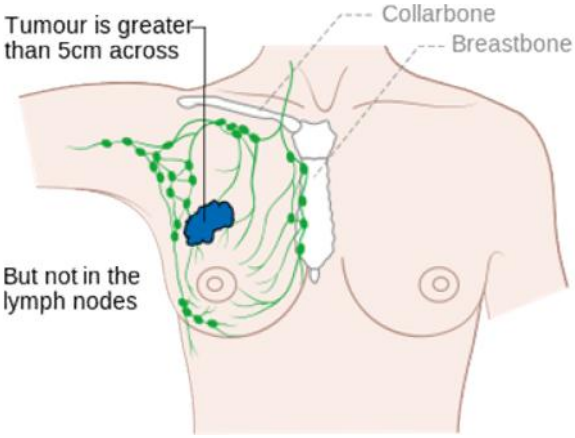


Fig 1.8c: Stage 2B breast cancer

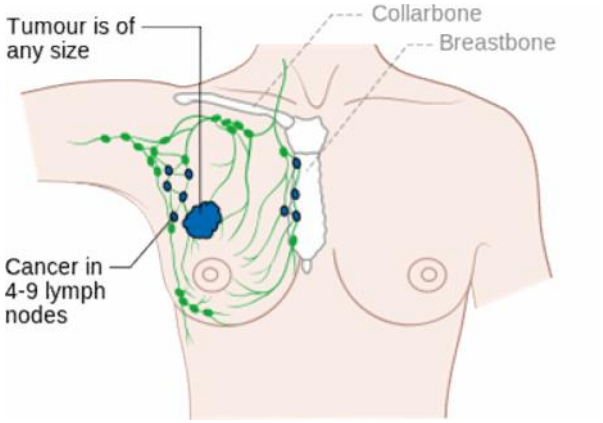


Fig 1.9a: Stage 3A breast cancer

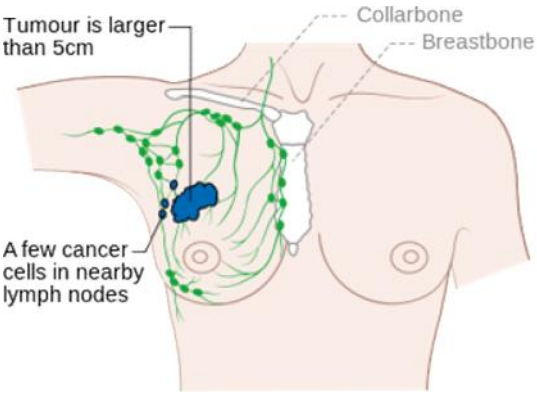


Fig 1.9b: Stage 3A breast cancer

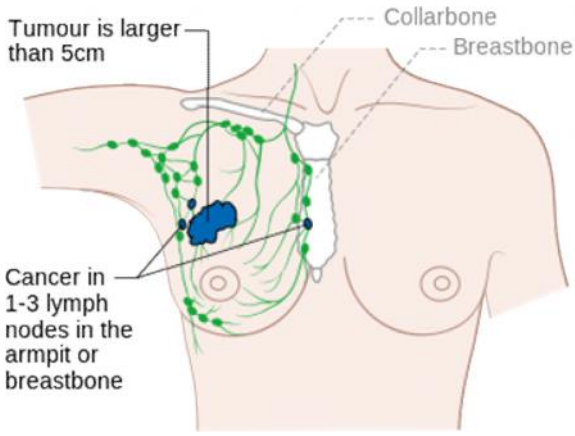


Fig 1.9c: Stage 3A breast cancer

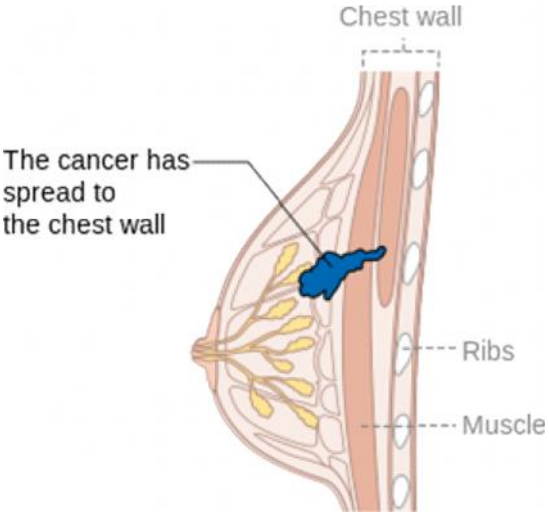


Fig 1.10a: Stage 3B breast cancer

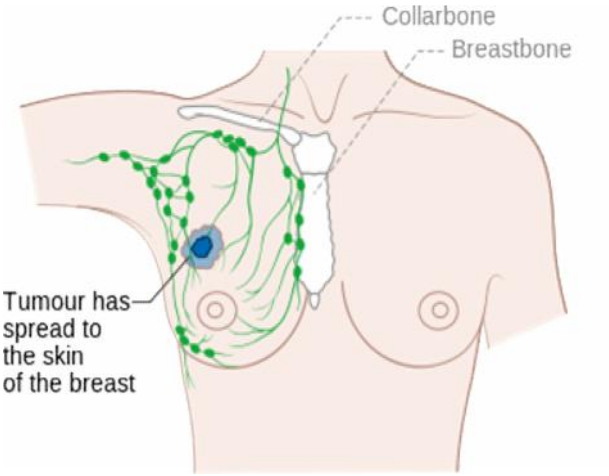


Fig 1.10b:Stage 3B breast cancer

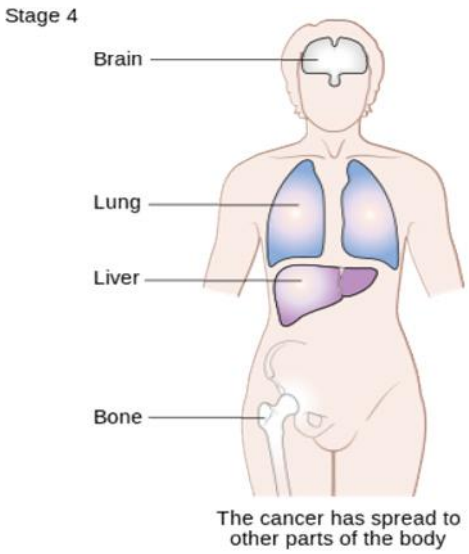


Fig 1.11: Stage 4 breast cancer

1.11 Prevalence of breast cancer

Incidence of breast cancer is higher in the developed countries of the world than the developing countries but the death rate is higher in the less developed regions and it is highest in the IARC membership (24 countries) (Fearly et al., 2012).

Table-1.3: Breast Cancer Estimated Incidence, Mortality and Prevalence Worldwide in 2012

Estimated numbers (thousands)	Cases	Deaths	5-year prev.
World	1677	522	6255
More developed regions	794	198	3224
Less developed regions	883	324	3032
WHO Africa region (AFRO)	100	49	318
WHO Americas region (PAHO)	408	92	1618
WHO East Mediterranean region (EMRO)	99	42	348
WHO Europe region (EURO)	500	143	1960
WHO South-East Asia region (SEARO)	240	110	735
WHO Western Pacific region (WPRO)	330	86	1276
IARC membership (24 countries)	940	257	3614
United States of America	233	44	971
China	187	48	697
India	145	70	397
European Union (EU-28)	367	91	1467

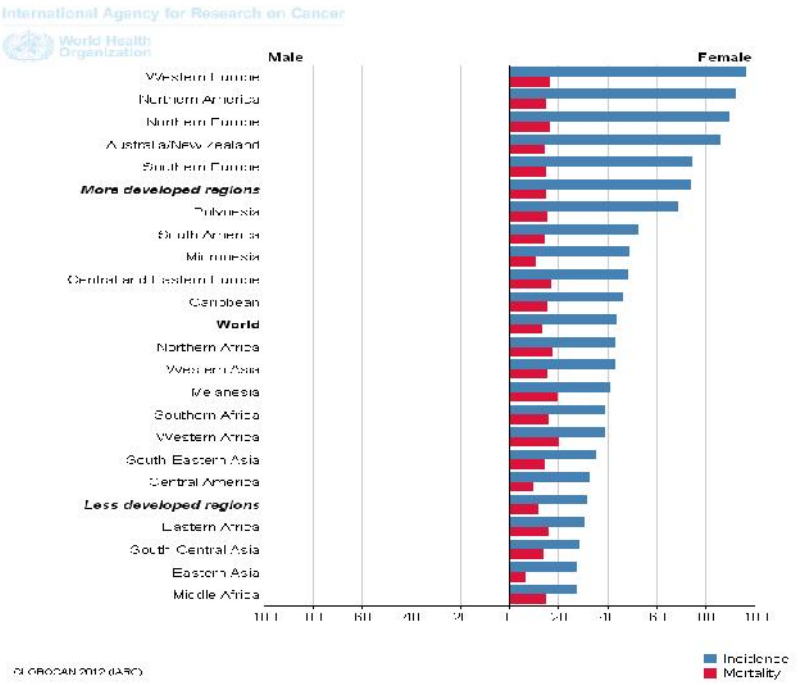


Fig 1.12: Breast Cancer Estimated Incidence Worldwide in 2012

1.12 Breast cancer in Bangladesh

In Bangladesh, the prevalence of breast cancer is very high and now it has become second in number and next to the lung cancer.

Table 1.4: Estimated incidence, mortality and 5-year prevalence: women

Cancer	Incidence			Mortality			5-year prevalence		
	Number	(%)	ASR (W)	Number	(%)	ASR (W)	Number	(%)	Prop.
Lip, oral cavity	3430	5.5	5.9	1977	4.7	3.5	8174	5.4	15.4
Nasopharynx	112	0.2	0.2	74	0.2	0.1	136	0.1	0.3
Other pharynx	1858	3.0	3.1	1590	3.8	2.7	4557	3.0	8.6

Oesophagus	5342	8.6	9.5	4984	11.8	8.9	5264	3.5	9.9
Stomach	2528	4.1	4.1	2354	5.6	3.9	3686	2.5	7.0
Colorectum	1754	2.8	2.9	1295	3.1	2.2	4178	2.8	7.9
Liver	1185	1.9	2.1	1134	2.7	2.0	730	0.5	1.4
Gallbladder	3495	5.6	6.2	3259	7.7	5.9	4286	2.9	8.1
Pancreas	287	0.5	0.5	291	0.7	0.5	209	0.1	0.4
Larynx	436	0.7	0.8	264	0.6	0.5	1160	0.8	2.2
Lung	2123	3.4	3.6	1929	4.6	3.3	1846	1.2	3.5
Melanoma of skin	71	0.1	0.1	44	0.1	0.1	110	0.1	0.2
Kaposi sarcoma	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0
Breast	**14836**	**23.9**	**21.7**	**7142**	**16.9**	**11.1**	**53476**	**35.6**	**100.9**
Cervix uteri	11956	19.3	19.2	6582	15.6	11.5	34439	22.9	65.0
Corpus uteri	944	1.5	1.5	357	0.8	0.6	3567	2.4	6.7
Ovary	2912	4.7	4.4	2166	5.1	3.7	7625	5.1	14.4
Kidney	280	0.5	0.4	263	0.6	0.4	604	0.4	1.1
Bladder	319	0.5	0.6	196	0.5	0.4	822	0.5	1.5
Brain, nervous	461	0.7	0.7	376	0.9	0.6	621	0.4	1.2

system									
Thyroid	532	0.9	0.9	260	0.6	0.5	1731	1.2	3.3
Hodgkin lymphoma	187	0.3	0.3	131	0.3	0.2	585	0.4	1.1
Non-Hodgkin lymphoma	868	1.4	1.4	650	1.5	1.1	1363	0.9	2.6
Multiple myeloma	347	0.6	0.6	311	0.7	0.6	573	0.4	1.1
Leukaemia	862	1.4	1.3	810	1.9	1.2	775	0.5	1.5
All cancers excl. non-melanoma skin cancer	62019	100.0	100.0	42220	100.0	71.9	150080	100.0	283.3

Incidence and mortality data for all ages. 5-year prevalence for adult population only.

ASR (W) and proportions per 100,000.

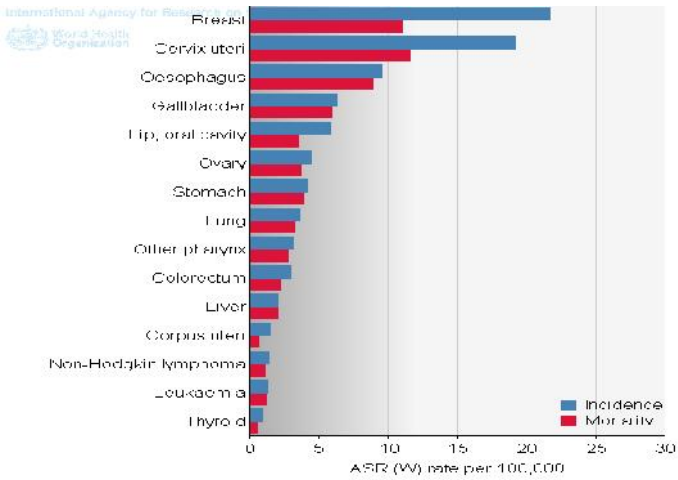


Fig1.13: Estimated age-standardized incidence and mortality rates: women

In Bangladesh, the incidence of breast cancer is the highest (23.9%) among women of all cancers (19.3%) (Fearly et al., 2012).

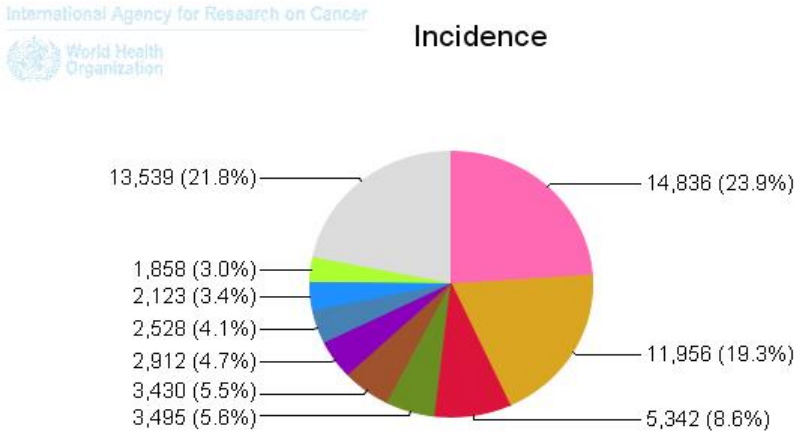


Fig 1.14: Incidence of breast cancer in Bangladesh (2012)

The mortality rate of breast cancer is highly frequent in Bangladeshi women (16.9%) than cervical (15.6%), oesophagal (11.8%), gallblader cancer etc. (Fearly et al., 2012).

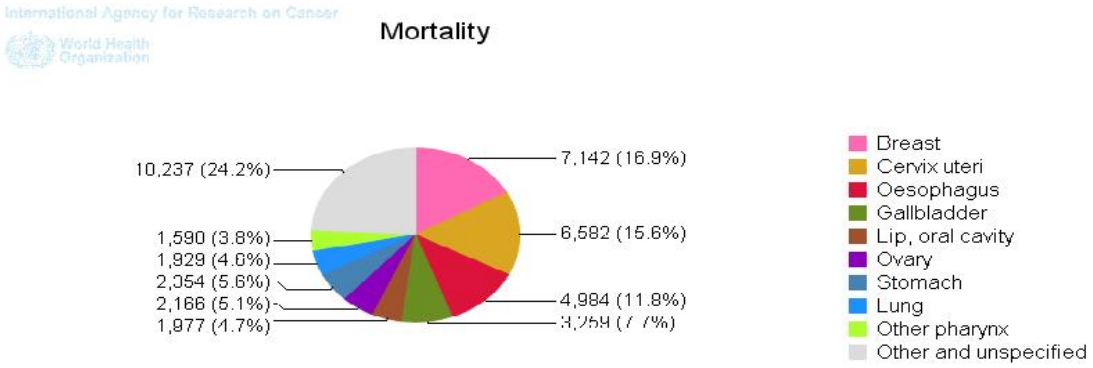


Fig 1.15: Mortality rate of breast cancer in Bangladesh (2012)

5-year prevalence rate of breast cancer in Bangladeshi women is observed as 35.6% in 2012 which is higher than any other type's cancer (Fearly et al., 2012).

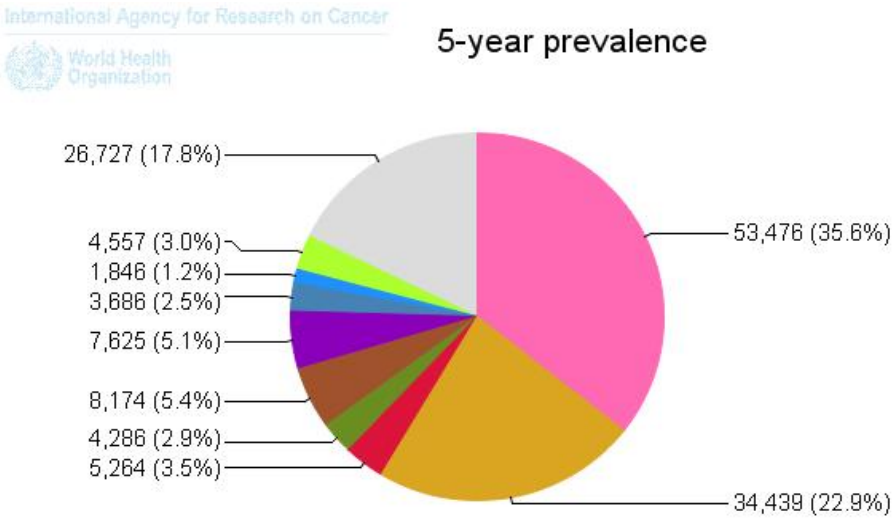


Fig1.16: 5-year prevalence of breast cancer in Bangladesh (2012)

1.13 Pharmacogenetics

Pharmacogenetics is the study of influence of genetic variations on a person's response to drugs. These variations are underlined to the response to therapy, including possible adverse effects. It also found to deal with the assessment of clinical efficacy and the pharmacological phenotype. From the view of some health care leaders it is proposed that pharmacogenetics have the potential to create personalized prescriptions; with the opportunity to improve patient compliance, reduce adverse events, and reduce the cost of managing chronic disease. Up to 90% of the variability in drug response between individuals can be explained by variations of genetics. For this reason, pharmacogenetic information is now included in the labeling of about 10% of drugs approved by the FDA. Inherited variants in the cytochrome P450 drug metabolism genes found to contribute significantly to an individual's drug response. The variation existing in all genes causes different members of a population to express different forms of proteins, including those that metabolise drugs or are the sites of drug action, leading to different responses to these drugs. Measuring the DNA differences we can predict the variation in response to the medicine (Roses, 2000). Pharmacogenetics targets at understanding how genetic

variation contributes to variations in response to medicines. “Pharmacogenetics” is defined as the study of the heritable basis of individual differences in response to pharmaceutical agents (Nebert and Dieter, 2000). “Pharmacogenomics” is a novel concept recently creating a great deal of excitement, and is the field of research that applies our pharmacogenetics knowledge to information gained from The Human Genome Project – principally in the pursuit of new drug design and discovery (Nebert and Dieter, 2000). Genetic diversity has the potentiality to provide a good contribution to both disease susceptibility and variability in response to drug therapy. Pharmacogenomics is a discipline which focuses on examining the genetic basis for individual variations in response to therapeutics (Dinneen et. al., 1992). Pharmacogenomics is already starting to influence how physicians / scientists design clinical trials and its impact on the practice of medicine is forthcoming, lthough the task of developing individualized medicines tailored to patient’s genotypes poses a major scientific challenge (Weyer et. al., 1999). It is suggested from the recent evidence that most prescribed medications are effective in no more than 60% of the individuals in whom they are used, and a significant number of patients also develop major adverse effects. Better understanding of the genetic factors regulating patient’s responsiveness to drugs is therefore needed to elucidate the molecular mechanisms involved and allow for development of new therapeutic strategies that match each patient and the most suitable drug (Medici et. al., 1999). Pharmacogenetics and Pharmacogenomics leads us to an especial young field of research in the domain of pharmacology. Both of the field deals with genetic variations which occur in individuals resulting reduced drug efficacy and more adverse drug reactions. Pharmacogenetics, mainly focuses the diversity of patients and their genetic background, set their response to a given drug therapy, making understood the biological variability whereas pharmacogenomic deals with the effects they cause in an individual (patient) different medications. The differences are examined on gene expression induction and repression of genes.

1.14 Genetic Polymorphism

Polymorphism is a term which literally can be defined as the variability of form, shape, size, structure and composition. It has a currency in a wide variety of disciplines in science and art. Genetic polymorphism is now a more specific term describing frequent variation at a specific locus in a genome. A useful practical definition indicates that a locus is polymorphic when there

are two or more allelic forms in the same population and the commonest allele has a frequency of 0.99 or less (Harris, 1980). A genetic polymorphism occurs if, within a population, a single gene accountable for producing a metabolising enzyme has a variant allele with the arbitrary frequency of 1% (Meyer, 2000). Single nucleotide polymorphisms (SNP) exist and an allelic site may have more than one SNP for many such genes. Genotype gives us the detailed gene structure of an individual whereas the more commonly measured phenotype provides the outcome of metabolism of a drug in an individual. Genetic Polymorphism is termed as difference in DNA sequence among individuals, groups, or populations. Genetic polymorphisms are proposed to be the result of chance processes, or may have been induced by external agents (such as viruses or radiation). If a difference in DNA sequence among individuals has been shown to be associated with disease, it will usually be termed as a genetic mutation. Changes in DNA sequence which have been evident to be caused by external agents are also generally called "mutations" rather than "polymorphisms". Genetic Mutation is defined as alteration in the nucleotide sequence of a DNA molecule. Genetic mutations are proposed to be a kind of genetic polymorphism. The term "mutation," as opposed to "polymorphism," is generally used to refer to changes in DNA sequence which are not present in most individuals of a species and either have been associated with disease (or risk of disease) or have resulted from damage indicted by external agents (such as viruses or radiation). Recent studies have demonstrated that the presence of sequence variants, such as pSNPs, within intronic regions could affect basic preliminary-mRNA (pre-mRNA) splicing mechanisms and thereby cause altered levels of normal transcripts (Pagani et al., 2003). A pSNP within the 3′-untranslated region (UTR) following the coding sequence is postulated to affect the intracellular stability of the mRNA gene transcript (Quirk et al., 2004).

1.15 Single Nucleotide Polymorphism

Single Nucleotide Polymorphisms (SNPs) within the coding regions of a gene which has been reported to changes in the amino acid sequence of the encoded protein are known as coding SNPs (cSNPs) which, because of greater selective pressures against changes at positions dictating amino acid sequence, are generally less common than SNPs or synonymous changes in coding sequence (Gray et al., 2000). A Single Nucleotide Polymorphism is defined as single base

mutation in DNA and a source variance in a genome. SNPs are found to be the most simple form and most common source of genetic polymorphism in the human genome (90% of all human DNA polymorphisms). There are two types of nucleotide base substitutions resulting in SNPs:
Type I- A transition substitution occurs between purines (A, G) or between pyrimidines (C, T). This type of substitution constitutes two thirds of all SNPs.
Type II- A transversion substitution occurs between a purine and a pyrimidine.
The different types of SNPs are found as multiple, as are their effects. SNPs can alter expression levels of a gene as well as the functionality of the encoded protein product or its affinity for its intended substrates, depending on their location within the genome and their patterns of co-occurrence (i.e. haplotypes). These effects of SNPs are found to cause veriable effects on many other phenotypic characteristics, greatly affect the manner in which a patient responds to drug therapy.

1.16 BRCA1 and BRCA2 genes

1.16.1 BRCA1 and BRCA2 penetrance

Germline mutations in the two breast cancer susceptibility genes have been found to have strong lifetime risks of breast and ovarian cancer (Thompson and Easton, 2002b; van Asperen et al., 2005). BRCA1 mutations have been reported to be strongly associated with families with ovarian cancer and BRCA2 mutations are strongly associated with families with male breast cancer cases. The calculated breast cancer risks for individuals' harbouring a BRCA1/BRCA2 mutation found to be dependend on the method of ascertainment of the families studied. The cumulative risk of breast cancer at age 70 years was 85% for BRCA1 and 84% for BRCA2 mutation carriers in high-risk families with multiple cases of breast and/or ovarian cancer. In addition, BRCA1 and BRCA2 mutations were previously estimated to have a cumulative lifetime risk of ovarian cancer at age 70 years of 63% and 27% respectively (Ford et al., 1998). However, the average estimates of breast cancer risks at age 70 were 65% and 45% respectively found on of a more recent meta-analysis of 22 population-based and hospital-based studies between BRCA1 and BRCA2 mutation carriers. Additionally, the average cumulative risks for ovarian cancer by age 70 years were 39% for BRCA1 mutation carriers and 11% for BRCA2 mutation carriers (Antoniou et al., 2003). Another observation forwarded in studies that their relative risks for

breast cancer declined significantly with age among BRCA1 mutation carriers (Chen et al., 2006). The same trend was reported for BRCA2 mutation carriers by Chen et al. (2006) but not by Antoniou et al. (2003). From the above information, it is quite evident that there is conflicting concepts regarding the penetrance of BRCA1 and BRCA2 mutations. The estimation of cancer risks in individuals who test positive for a mutation is still an area of intense research, since the identification of the two genes more than two decade ago. It should be kept in mind that penetrance estimates are based on multiple-case families and vary between countries because of the influence of nongenetic factors. It has been estimated that the lifetime breast cancer risk for BRCA1 mutation carrier's ranges between 46% and 85% and for BRCA2 mutation carriers between 43% and 84%, depending on the population studied (Oldenburg et al., 2007). The penetrance of these genes will decline because of preventive measures that will be undertaken such as prophylactic mastectomies and oophorectomies due to interest in genetic testing increases and more women become aware that they are BRCA1/BRCA2 mutation carriers, (Narod and Foulkes, 2004).

1.16.2 BRCA1 and BRCA2 structure and expression

The BRCA1 and BRCA2 genes have been reported not to share any obvious sequence homology despite of having common features. Both genes have been found to have an extremely large exon 11 which comprises 61% and 48% of the whole coding sequence of BRCA1 and BRCA2 respectively. In addition, both genes are found to have translational start sites at exon 2 and in humans, the highest levels of expression are observed in testis, thymus and ovaries (Tavtigian et al., 1996). BRCA1 and BRCA2 genes are found to be relatively poorly conserved between species with the exception of a few small domains.

1.16.3 BRCA1 structure

The BRCA1 gene is reported to be located on chromosome 17q21 and spans approximately 100 kb of genomic DNA which consists of 24 exons of which 22 are encoding a 1863 amino acid protein. BRCA1 exon 1 is found as non-coding, and the region originally identified as exon 4 is an Alu repeat which is not generally included in the transcript (Smith et al., 1996).

1.16.4 BRCA1 protein

BRCA1 is a 220 kDa protein demonstrating a predominantly nuclear localization forms nuclear "dots," or foci, during S phase of the cell cycle and following DNA damage (Chen et al., 1995; Scully et al., 1997). The BRCA1 protein has been reported to contain important functional domains that interact with a range of proteins. In some studies it is found that BRCA1 contains a zinc-binding RING finger motif in its amino terminal region (Miki et al., 1994), 2 nuclear export signals near its N terminus (Thompson et al., 2005), 2 nuclear localization signals (NLS) (Chen et al., 1996), a DNA binding domain in the central region of the protein (Paull et al., 2001), an SQ-cluster domain (SCD) between amino acids 1280 and 1524 (Cortez et al., 1999) and two carboxy-terminal BRCT domains (Bork et al., 1997).

1.16.5 BRCA2 structure

The BRCA2 gene has been reported to be located on chromosome 13q12 and spans approximately 70 kb of genomic DNA. It is found to be consists of 27 exons of which 26 encode a 3418 amino acid protein (Tavtigian et al., 1996).

1.16.6 BRCA2 protein

BRCA2 protein has been reported to contain two known functional domains, the BRC-repeats motifs and the DBD binding domain. The middle region of the protein, which is encoded by exon 11, is found to contain eight BRC-repeat motifs that are conserved among mammalian species suggesting an important function. It has been reported that the BRC repeats are essential for BRCA2 function in DNA repair by mediating direct binding to the DNA recombinase RAD51, a protein that is essential for DNA repair and genetic recombination. It is now postulated that in human BRCA2, six of the eight motifs can bind directly to RAD51 (Chen et al., 1998). Mutations in BRCA2 BRC repeats are found to be associated with cancer predisposition. Studies in mice have showed that deletions of all BRCA2 BRC domains are embryonically lethal whereas deletions of several BRC repeats lead to cancer (Donoho et al., 2003). The BRCA2 C-terminal region has been found to contains the DBD binding domain, which interacts with DSS1 (deleted in split-hand/split-foot 1), a highly conserved 70 amino-acid protein. DSS1 binding protein is essential for BRCA2 function (Gudmundsdottir et al., 2004).

Studies in mammalian cells have revealed that DSS1 is required for the formation of DNA damage-induced.

1.16.7 BRCA1 protein functions

BRCA1 has been reported to plays a key role in DNA double strand break repair and in the maintenance of genomic integrity. BRCA1 facilitates DNA repair through its involvement in homologous recombination (HR), non-homologous end joining (NHEJ) and nucleotide excision repair (NER). BRCA1 protein has been reported to serves as a scaffold that organizes and coordinates a number of proteins that are involved in maintaining genomic integrity (Deng and Brodie, 2000). The most deleterious form of DNA damage is found to be double strand breaks. Two main pathways have been postulated for repairing these breaks: HR and NHEJ. There is substantial evidence that BRCA1 is implicated in both these pathways. It is now proposed that BRCA1 protein is involved in NHEJ via its interaction with the MRN [MRE11A [meiotic recombination 11 homolog A (S. cerevisiae)] - RAD50 [RAD50 homolog (S. cerevisiae)] - NBS1 [Nijmegen breakage syndrome 1 (nibrin)] complex. The MRN complex has been found to plays an important role in both HR and NHEJ. The exact mechanism of BRCA1 interaction with the MRN complex and its involvement in NHEJ is yet under investigation. There is conflicting evidence on the role of BRCA1 in NHEJ, which is summarized in a study conducted by Bau et al. (2006). Many studies showed the evidence that BRCA1 deficient cells have decreased NHEJ fidelity. Furthermore, BRCA1-deficient mouse embryonic fibroblasts were reported to have significantly reduced NHEJ activity whereas, other studies have demonstrated that BRCA1 can promote only specific subtypes of NHEJ and has no effect on others. This may be a due to the different roles of BRCA1 in sub-pathways of NHEJ (Bau et al., 2006). BRCA1 is also found to be involved in DNA repair by homologous recombination. The first indication that BRCA1 participates in DNA repair forwarded the observation that it is associated and co localized with RAD51 in subnuclear clusters (Scully et al., 1997). RAD51 is thought to be the major component of the HR pathway. The nature of interaction between BRCA1 and RAD51 is still unknown but it is postulated that the association is likely to be indirect and possibly mediated by BRCA2. In the event of DNA damage, both RAD51 and BRCA1 localize to the region of damage. The subnuclear localization and the phosphorylation of BRCA1 protein indicate that it is involved in

DNA-damage dependent replication checkpoint response (Scully et al., 1997b; Thomas et al., 1997). It is also further evidence that BRCA1 is involved in DNA repair by homologous recombination comes from the observation that BRCA1 deficiency results in decreased RAD51 foci formation in cultured cells after γ-irradiation (Huber et al., 2001). Based on the fact that BRCA2 also interacts with RAD51, it was suggested that a complex consisting of BRCA1, BRCA2 and RAD51 is formed and functions to repair damaged DNA (Chen et al., 1999). There is an indication that this complex functions during or after DNA replication, since the levels of BRCA1, BRCA2 and RAD51 expression increase in cells when they enter the S phase of the cell cycle (Venkitaraman, 2002). BRCA1 has also been linked to a number of other DNA repair processes due to its interaction with other proteins that are involved in response to and in the repair of DNA damage. BRCA1 together with BRCA2, RAD51, BARD1 and other proteins is part of the BRCC (BRCA1-BRCA2-Containing Complex) that constitutes an E3 ubiquitin ligase that enhances cellular survival following DNA damage (Dong et al., 2003). Additionally, BRCA1 forms part of the BASC (BRCA1-Associated Genome Surveillance Complex) super complex. The BASC complex includes the DNA mismatch repair proteins MLH1 [mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)], MSH2 [mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)] and MSH6 [mutS homolog 6 (E. coli)], the MRN complex proteins MRE11ARAD50- NBS1, the Bloom syndrome helicase BLM, ATM (ataxia telangiectasia mutated) kinase, DNA replication factor C, RFC (replication factor C) and PCNA (proliferating cell nuclear antigen). It is believed that this complex acts as a sensor for DNA damage and is also directly involved in repairing DNA damage by DNA replication associated repair (Wang et al., 2000). The involvement of BRCA1 in repairing double strand breaks is supported by its participation in the BASC complex and its interaction with the MRN complex. The MRN complex plays a critical role in DNA damage sensing, signalling and repair mechanism, as well as in the maintenance of chromosomal integrity of the cell (Assenmacher and Hopfner, 2004). BRCA1 also functions in signaling the response to DNA damage. Following DNA damage, ATM and ATR (ataxia telangiectasia and Rad3 related) protein kinasesphosphorylate BRCA1 in response to different stimuli (Okada and Ouchi, 2003). Furthermore, in response to γ-irradiation ATM phosphorylates and activates CHEK2 [CHEK2 checkpoint homolog (S. pombe)], which in turn phosphorylates BRCA1 (Lee et al., 2000).

Hence, ATM, ATR and CHEK2 kinases regulate BRCA1 function and in turn cell cycle regulation and DNA repair by phosphorylation. There has been recent progress in elucidating the mechanism by which BRCA1 recognizes double-strand breaks. BRCA1, through its C-terminal domains forms three distinct complexes with Abraxas, BACH1 and CtIP proteins. RAP80 (receptor associated protein 80) recruits BRCA1-Abraxas and BRCA1-CtIP complexes to damaged DNA (Kim et al., 2007; Sobhian et al., 2007). Furthermore, the BRCA1-CtIP complex interacts with the MRN complex to facilitate double-strand break resection and to activate homologous recombination mediated repair of DNA (Chen et al., 2008). BRCA1 also plays a role in DNA repair by NER and is involved in both transcription coupled repair and global genome repair. In detail, it has been reported that BRCA1 deficiency leads to blockage of RNA polymerase II transcription machinery at the site of repair of oxidative 8-oxoguanine residues (Le Page et al., 2000).

1.16.8 BRCA2 protein functions

Although the exact role of the BRCA2 protein still unknown, it has been demonstrated that BRCA2 plays an important role in homologous recombination, both in meiosis and in the repair of double-strand breaks. The major role of the BRCA2 protein is found to assist in organizing RAD51 function and facilitate homologous recombination. BRCA2 is reported to bind RAD51 recombinase directly and regulates recombination-mediated double strand break repair. BRCA2 is required for the efficient nuclear localization of RAD51 and mediates the recruitment of RAD51 to the sites of double strand breaks. Hence it is essential for the cellular function of RAD51 (Davies et al., 2001). BRCA2-deficient cell lines are very sensitive to DNA damaging agents and exhibit a genomic instability phenotype that includes accumulation of double-strand breaks and in turn chromosomal breaks (Kraakmanvan der Zwet et al., 2002). Furthermore, BRCA2 has been identified as the FANCD1 (Fanconi Anemia complementation group D1) gene. When both BRCA2 alleles are inactivated, a Fanconi anemia phenotype can occur (Offit et al., 2003). Not long ago, a nuclear partner of BRCA2, namely PALB2 was identified. PALB2 (partner and localizer of BRCA2) provides stability to the BRCA2 protein to perform its cellular functions namely DNA repair by homologous recombination and checkpoint control. PALB2 is also required for BRCA2 intranuclear localization (Xia et al., 2006). In recent studies, it has been

demonstrated that BRCA2 plays a critical role in meiotic recombination through its direct interaction with DMC1 recombinase (Thorslund et al., 2007). Additionally, BRCA2 controls mitotic checkpoint activity (Yu et al., 2000), maintains normal centrosome number and function and has been implicated in regulation of cytokinesis in the final stages of cell division (Daniels et al., 2004). BRCA2 has been shown to contribute to activation of transcription (Shin and Verma, 2003), G2/M checkpoint control (Yuan et al., 1999), suppression of tumour development by inhibition of cancer cell proliferation (Wang et al., 2002) and mammalian gametogenesis (Sharan et al., 2004).

1.16.9 Ethnic differences in BRCA1 and BRCA2 mutation spectra

Germline disease-causing BRCA1 and BRCA2 mutations have been reported in individuals from all over the world, originating from different races and ethnic groups in many studies for decades long. It has been demonstrated in some studies that certain BRCA1 and BRCA2 mutations are restricted to certain ethnic populations and geographical areas. This observation is partly due to the presence of founder mutations within these genes (Neuhausen, 1999; Szabo and King, 1997) and most common and well-characterized mutations are those detected in individuals of Ashkenazic Jewish descent. It is has been reported that around 2.5% of Ashkenazi Jews carry one of the three founder mutations (185delAG and 5382insC in BRCA1 and 6174delT in BRCA2) (Fodor et al., 1998; Struewing et al., 1997). BRCA1 and BRCA2 mutational spectrum is also well characterized due to the availability of samples in Iceland. It has been found that around 0.4- 0.6% of the Icelandic population carry a single BRCA2 founder mutation (999delTCAAA) (Johannesdottir et al., 1996; Thorlacius et al., 1997). Several other recurrent mutations have been reported in a number of ethnic groups and populations and are summarized in a recent review conducted by Fackenthal and Olopade (2007). The small number of detected BRCA1 and BRCA2 pathogenic mutations characterizes some populations. On the other hand, others have a broader and more diverse mutational spectrum. It should be kept in mind that some of the recurrent mutations were found in a number of different populations and ethnic groups. It is now believed that the number of distinct recurrent mutations in a population depends on the population history and more specifically on influences of migration, population structure, geographical and cultural isolation (Szabo and King, 1997).

1.16.10 BRCA1 and BRCA2 in early onset breast cancer

Familial breast cancer has been found to occur at a considerably younger age compared to the typical age of onset in the general population (Claus et al., 1991). The occurrence of early onset breast cancer has been found to be associated with mutations in the BRCA1 and BRCA2 genes (Krainer et al., 1997). In the general population, women with an early age of breast cancer are more likely than others. Studies on the contribution of BRCA1 and BRCA2 mutations to the incidence of breast cancer were primarily focused on individuals with high-risk families and large founder effect populations. In contrast to this, few population based studies examining the prevalence of BRCA1 and BRCA2 mutations in women who were diagnosed with breast cancer at a young age, have been carried out. On the basis of time, the first population-based studies that were performed had concentrated on selected populations with highly recurrent founder mutations i.e. the Icelanders and the Ashkenazi Jews. Data from these studies revealed that the prevalence rates of BRCA1 and BRCA2 mutations amongst early onset breast cancer patients can be as high as 30% for Ashkenazi Jews (Abeliovich et al., 1997) and around 25% for Icelanders (Thorlacius et al., 1997). This high percentage is a result of the presence of founder mutations in these two ethnic populations. In detail, 25% of women in Iceland diagnosed with breast cancer below the age of 40 carried a single BRCA2 mutation (999delTCAAA) while among unselected Jewish breast cancer cases diagnosed below the age of 40, 30% were found to carry one of the three founder BRCA1/2 mutations (BRCA1 185delAG, 5382insC and BRCA2 6174delT). Furthermore, the contribution of BRCA1 and BRCA2 mutations to the population incidence of early-onset breast cancer has been examined in various other geographical regions and ethnic groups. In detail, studies were carried out in North America, United Kingdom, Spain, the Netherlands, Sweden, Germany, Philippines, Korea and Australia (Southey et al., 1999; Anglian Breast Cancer Study Group, 2000; Papelard et al., 2000; Loman et al., 2001; De Leon Matsuda et al., 2002; de Sanjose et al., 2003; Hamann et al., 2003; Choi et al., 2004). In these geographical regions, the prevalence of BRCA1 and BRCA2 mutations among early onset breast cancer patients' ranges between 5% and 10%. Mutations in the two cancer predisposition genes make approximately equal contributions to the incidence of early onset breast cancer, with the exception of the Philippines, where BRCA2 plays a more significant role compared to BRCA1

(De Leon Matsuda et al., 2002). The differences observed in the mutation frequencies between various populations proposed to be explained by the different study selection criteria as well as by differences in the sensitivity of the genotyping methods.

1.17. HER2 gene

1.17.1 HER family and HER2

The HER2 (Human Epidermal growth factor Receptor 2) protein has been found to be a part of four transmembrane growth factor receptors (ErbB protein family, or Epidermal Growth Factor Receptor - EGFR - family, or HER family) which function to activate intracellular signaling pathways in response to extracellular signals. HER2 (Human epidermal growth factor receptor 2, ERBB2/neu/EGFP2) gene is found to be located on chromosome 17q12-21q, spans 38kb, a 1255 amino acid glycoprotein of 185 kDa, comprises 27 coding exons, and over expressed in 20%-30% of breast cancer (Tommasi et al., 2004). HER proteins are reported to be widely expressed and important for the development and function of many organs and systems, including brain, skin, lung, gastrointestinal tract and heart. Their structure is found to consists of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. The binding of a ligand induces a conformational change in the extracellular domain of ErbB proteins that promotes their dimerization and consequent transphosphorylation. Unlike the other members of the family, the extracellular domain of HER2 exists in a constitutively active conformation. HER2 in fact lacks ligand-binding activity and its signalling function is engaged by its ligand-bound heterodimeric partners HER1, HER3 and HER4 (Moasser, 2007). HER2 plays a key role in many physiological processes such as cell growth, cell death, differentiation and tissue development, but it is also involved in carcinogenesis and metastasis. The transforming potential of the rodent cellular homologue of HER2 - neu - was first demonstrated in the 80s, when a point mutation - a valine residue to a glutamic acid residue at position 664 - in the transmembrane domain (Bargmann et al., 1986) was found to be essential for tumour formation. Subsequently, several other deletion mutations within the extracellular juxtamembrane region that promote dimerization and enhanced kinase activity (Siegel and Muller, 1996) were described. Differently from mice, human tumours appear to show over-expression of the receptor as the main or the only tumorigenic mechanism. The diverse and

interacting downstream pathways involved in HER2-induced tumorigenesis have been reviewed by Moasser (2007). Sequence analysis identified a common genetic variant at codon 655 in the transmembrane coding region of HER2 gene, an Ile-to-Val single-nucleotide polymorphism was found, resulting in the substitution of isoleucine (Ile: ATC) with valine (Val: GTC) (Cooke et al., 2001; Uzan et al., 2009). Isoleucine to valine changes might alter the hydrophobicity of the protein which affects the conformational stability of the domains (Papewalis etal., 1991). Meanwhile, there is evidence suggesting that changing the existing isoleucine (Ile: ATC) to valine (Val: GTC) at codon 655 suggests an increased dimerization, autophosphorylation of HER2, and tyrosine kinase activity, which may cause the transformation of cells (Takano et al., 1995). Clinically, HER2 is an important biomarker and target of therapy of breast cancer (Nakajima et al., 1999).

1.17.2 HER2 and breast cancer

HER2 has been reported to be abnormal in approximately 20% of breast cancers (Choritz et al., 2011) and this abnormality is found to consist in the amplification of the region where the gene is located (chromosome 17, long arm - 17q12-q21). In the plethora of cases, amplification leads to an over-expression of the protein on the tumour cell membrane. It has been suggested from a number of studies that amplification of HER2 is a very early event in the development of invasive carcinomas and it is usually maintained throughout the natural history of the tumour (Park et al., 2006). In addition to this, gene expression analyses have shown that HER2-amplified breast cancers are likely to represent one of the four or five distinct molecular subtypes of the disease (Perou et al., 2000). These tumours have been found to be clearly demonstrated to have an overall poorer prognosis in comparison with the HER2-negative group (Andrulis et al., 1998). They also are reported to have particular biological and clinical characteristics, including poor differentiation, high histologic grade, high proliferation rate (Prati et al., 2005) and a typical pattern of metastases to the brain, as reviewed by Leyland-Jones (2009). The potential influence of HER2 status on the response to various anticancer agents - anthracyclines, taxanes, tamoxifen, aromatase inhibitors - has been extensively studied, but with conflicting results (Pritchard et al., 2006; Hayes et al., 2007; Rasmussen et al., 2008). In particular, the potential role of HER2 in sensitivity to anthracyclines has been attributed by several authors to the occurrence of co-

amplification of the TOP2A gene (Di Leo et al., 2002; Press et al., 2011) encoding for the topoisomerase IIα enzyme (target of anthracycline therapy), located on 17q12-q21 and genetic aberrations of this region described in a significant proportion of HER2-amplified tumours (Jarvinen et al., 1999). However, data do not seem to be conclusive (Romero et al., 2012) and a recent meta-10 analysis of individual data conducted on more than 3,000 patients from five randomised adjuvant trials does not support the use of HER2 and TOP2A as molecular markers to predict anthracycline activity (Di Leo et al., 2011). HER2 is currently tested on all newly diagnosed breast cancers using Immuno-HistoChemistry (IHC) and/or In Situ Hybridization (ISH), according to the recommendations issued by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) (Wolff et al., 2007). Despite of the ASCO/CAP guidelines certainly provided an important framework for the standardization of HER2 testing, a number of issues are currently being debated. These include, among others, the assessment and the potential role of the chromosome enumeration probe 17 (CEP17) and chromosome 17 polysomy (Moelans et al., 2011; Tse et al., 2011) and the evaluation of varying degrees of HER2 amplification/overexpression within the same tumour (HER2 intra-tumoral heterogeneity) (Ohlschlegel et al., 2011).

1.17.3 Single Nucleotide Polymorphisms in HER2

Since the most important paper (Xie et al., 2000) indicating an increased risk of breast cancer among carriers of the Val allele in a Chinese case-control study, many epidemiological studies have evaluated this potential association in different populations, resulting in inconsistent or even contradictory data ((Xie et al., 2000; Mutluhan et al., 2008; Naidu et al., 2008; Tao et al., 2009; Kallel et al., 2010; Kara et al., 2010; Zhang et al., 2011; AbdRaboh et al., 2013; Lemieux et al., 2013; Ozturk et al., 2013; Roca et al., 2013;Wang et al., 2013).. From Kallel et al. (2010) Several recent meta-analyses (Tao et al., 2009; Lu et al., 2010; Dahabreh and Murray, 2011; Ma et al., 2011) collecting data from the numerous published reports (ranging from 20 to 33 reports per analysis), with total numbers ranging from 9,209 cases and 10,132 controls in Ma et al.

(2011) to 20,461 cases and 23,832 controls in Dahabreh and Murray (2011) were unable to consistently demonstrate an association between the Ile655Val Val allele and an increased risk of breast cancer. A second common coding SNP (Reference SNP Cluster Report rs1058808) encodes either alanine (Ala; GCC) or proline (Pro; CCC) at residue 1170, within a C-terminal, intracellular regulatory domain (Xu et al., 2000). The frequency of the proline allele varies from more than 50% in European series to approximately 10-20% in African populations (NCBI_b, 2009). This polymorphism has not been evaluated for its potential biologic function so far, but two studies have assessed its influence on breast cancer risk, both focusing on Caucasian cases of familial tumours. In particular, in the first paper (Frank et al., 2005) the authors did not find any association of the Ala1170Pro SNP with occurrence of familial breast cancer. Likewise, the second group (Tommasi et al., 2007) observed no difference in allele frequencies between cases and controls, but the proline variant was associated with the presence of oestrogen receptors and with two missense polymorphisms in the BRCA1 gene. These two SNPs15 have been examined in several studies which reported inconclusive results on their role in breast or ovarian cancer risk (Dombernowsky et al., 2009). After the discovery of the 'Haplotype Blocks' structure of the human genome (Gabriel et al., 2002), a few groups have applied a 'tagging SNP' approach in order to study linkage disequilibrium and haplotype patterns of different HER2 genetic variants in relation to breast cancer risk (Han et al., 2005; Benusiglio et al., 2006; Einarsdottir et al., 2006). In particular, Han and co-workers (2005) showed that in a Korean population the two Ile655Val and Ala1170Pro SNPs could be used as haplotype-tagging variants and the three haplotypes defined by their combinations would account for 94% of the total predicted haplotype variation (it should be noticed, however, that this finding is not generalizable to all ethnicities). None of these studies showed any significant association between HER2 haplotype patterns and risk of breast cancer (Nicola Cresti, 2012).

1.18 RAD51 gene

Human RAD51, one of the key proteins for homologous recombination, essential to meiotic and mitotic recombination have been found to plays a crucial role in homologous recombination repair of DNA double-strand breaks (Richardson, 2005). It is reported to function by forming nucleoprotein filaments on single stranded DNA, inducing homologous pairing and mediating

strand exchange reactions between single and double stranded DNA during repair (Vispe & Defais, 1997). The RAD51 gene is found to be located on chromosome 15q15.1 in humans and thought to participate in a common double-strand break repair pathway (Zhao et al., 2014). The RAD51 gene, a homologue of recA in Escherichia coli, has been mapped to chromosome 15q15.1 in humans (Shinohara et al., 1993) which span >39 kb, contains 10 exons and encodes a 339 amino acid protein (genomic accession no: NM_133487). The RAD51 gene is found to makes a protein also called RAD51, which is essential for the repair of damaged DNA. The protein made by the BRCA2 gene binds to and regulates the RAD51 protein to fix breaks in DNA (Lo et al., 2003). These breaks can be caused by natural or medical radiation. They also occur when chromosomes exchange genetic material (when pieces of chromosomes trade places) in preparation for cell division. The BRCA2 protein transports the RAD51 protein to sites of DNA dam age in the cell nucleus. RAD51 then binds to the damaged DNA and encases it in a protein sheath, which is an essential first step in the repair process. In addition to its association with BRCA2, the RAD51 protein also interacts with the protein made by the BRCA1 gene. By repairing DNA, these three proteins play a role in maintaining the stability of the human genome. Changes in RAD51 biosynthesis are usually preceded by changes in its gene transcription and mRNA level. Gene variability could contribute to the level of the RAD51 biosynthesis. A single nucleotide polymorphism in the 5′-untranslated region (5′-UTR) of RAD51 (a G to C substitution at position 135, the G/C polymorphism) can influence cancer risk among BRCA1/BRCA2 mutation carriers (Levy-Lahad et al., 2001; Wang et al., 2001). In view of the potential significant role of RAD51 for tumor development, it is important to know, whether this polymorphism can account for the development and/or progression of cancer. It is well reported that double-strand break damage is the most dangerous lesion observed in eukaryotic cells because it may cause cell death or constitute a serious threat to cell viability and genome stability. It has the potentiality to permanently arrest cell cycle progression and endanger cell survival (Suwaki et al., 2011). Since DNA repair mechanisms are essential to preserve genomic stability and functionality, defects in DNA repair can result in the development of chromosomal aberrations which may lead to an increased susceptibility to cancer (Dixon & Kopras, 2004; Wood et al., 2005; Berwick & Vineis, 2000). Homologous recombination and non-homologous end joining have been extensively studied as two distinct pathways in the repair of double-strand

breaks in mammalian cells. Homologous recombination is a high-fidelity process that utilizes DNA sequence, a sister chromatid or homologous chromosome in close proximity to the break as a template (West, 2003; Yano et al., 2009; Lisby M, Rothstein, 2009). In this repair process, an early procedure is the resection of the 3′ends of the DSBs to form single stranded tails that invade the intact homologous DNA double helix forming a Holliday junction (Khanna & Jackson, 2001; O'Driscoll & Jeggo, 2006). RAD51, a kind of ubiquitous strand exchange protein, is known to be a central enzyme involved in DNA double-strand break repair by homologous recombination. It could polymerize onto single-stranded DNA and searches for homology in a duplex donor DNA molecule, usually the sister chromatid (Karpenshif & Bernstein, 2012). Recent researches have suggested common polymorphism (G135C) located in the 5′ untranslated region seems to be of functional relevance. Furthermore, many functional studies revealed that these polymorphisms could affect mRNA stability or translational efficiency, resulting in changes in both polypeptide product levels and the function of encoding RAD51 protein, and thus influenced the DNA repair capacity to some extent (Hasselbach et al., 2005; Thacker, 2005). To date, a number of molecular epidemiological studies have been done to evaluate the association between RAD51 135G>C polymorphism and different types of cancer risk in diverse populations (Blasiak et al., 2003; Sliwinski et al., 2010). However, the results were inconsistent or even contradictory. Some recent meta-analysis only analyzed RAD51 135G>C polymorphism with breast cancer risk (Gao et al., 2011; Zhou et al. 2011), but the results were also inconsistent. (Gao et al. 2011) found that the CC genotype was associated with a significantly increased risk of breast cancer when compared with the GG, CG, and CG/GG genotypes. Subgroup analyses showed that individuals carrying the CC genotype were associated with an elevated tumor risk in European populations and in sporadic breast cancer. (Wang et al. 2010) observed an overall significant increased breast cancer risk (for the recessive model CC vs. GG/CG: OR = 1.35, 95% CI = 1.05–1.74, P (heterogeneity) = 0.06). (Yu et al. 2010) found that there was no evidence for a significant association between 135G>C and breast cancer risk in non-BRCA1/2 mutation. The study of (Sun et al. 2011) had 17 studies, with significantly decreased breast cancer risk being observed in the additive model (OR = 0.995, 95% CI = 0.991–0.998) and recessive model (OR = 0.994, 95% CI = 0.991–0.998) (Zhou et al. 2011) suggested that RAD51 variant 135C homozygote was associated with elevated breast cancer risk among BRCA2 mutation carriers.

Since then, additional several studies with a large sample size about RAD51 135G>C polymorphism with cancer risk have been reported with conflicting findings. Therefore, we selected this polymorphism to identify statistical evidence of the association between RAD51 135G>C polymorphism and risk of breast cancer.

1.19 The aim of the study

1. As no previous study has been done on Bangladeshi population, we would like to estimate the genetic susceptibility of breast cancer in this country.
2. Our second target is to measure the frequency of BRCA1rs80357713, BRCA1rs76171189, BRCA2rs11571653, RAD51rs1801320, HER2rs1136201 polymorphisms in Bangladeshi population.
3. We would like to correlate the BRCA1rs80357713, BRCA1rs76171189, BRCA2rs11571653, RAD51rs1801320, and HER2rs1136201 polymorphisms with breast cancer risk (Comparing the frequency of BRCA1rs80357713, BRCA1rs76171189, BRCA2rs11571653, RAD51rs1801320, HER2rs1136201 polymorphisms of breast cancer patients with those of the disease free volunteers [Controls]).
4. Another aim is to assess the impact of BRCA1rs80357713, BRCA1rs76171189, BRCA2rs11571653, RAD51rs1801320, HER2rs1136201 polymorphisms on the different clinicopathological parameters of breast cancer patients.

CHAPTER TWO

MATERIALS AND METHODS

2. 0 Materials and Methods

2.1 Subject Selection

Total 310 patients with invasive breast cancer were recruited from Bangabandhu Sheikh Mujub Medical University Hospital, Ahsani Mission Cancer Hospital, Dhaka Medical College Hospital, Delta Medical College Hospital and National Institute of Cancer Research and Hospital, Bangladesh from early 2009 to late 2014. Patients were informed about the experimental procedures and study aim before giving written informed consents. For breast cancer risk determination, 250 Bangladeshi women were recruited matching age with patients as controls coming from different region of the country where most of them were with benign tumor disease staying surgical wards of the hospitals. The study was conducted in accordance with Declaration of Helsinki and its further amendments (adopted by the 18th WMA general assembly, Helsinki, Finland, June 1964 and last amendment in Seoul, South Korea on October 2008). Ethical permission was taken to approve the protocol and consent form the ethical review committee of respective hospitals. The clinicopathological characteristics were recorder in a prescribed questionnaire. The genetic study was conducted in the laboratory of pharmacogenetics and pharmacokinetics of the Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.2 Materials

2.2.1 Instruments

Instruments	**Sources**
UNIVERSAL 240V 50i60Hz Refrigerated Bench-Top Centrifuge	Hettich GmbH & Co., Germany
MJ Mini Gradient Thermal Cycler	Bio-Rad Laboratories, USA
Alpha Imager® HP (Gel Doc. System)	Alpha Innotech Corporation, USA
Gel Electrophoresis Machine (Elite)	Wealtech, Germany
UV Probe V.2.1 Spectrophotometer	Shimadzu, USA
P^H Meter (Cyber Scan 500)	Eutech ,Singapore
Water Bath	Siemens, USA

Micropipette	Bio-Rad Laboratories, USA
Distillation Plant (Distinction D4000)	Bibby Sterlin Ltd., UK
Ultrapure Water System (Arium® 611)	Sartorius, Germany
Microcentrifuge Machine (Mikro 20)	Hettich GmbH & Co., Germany
Freeze (− 40^0 C)	Siemens, USA
Vortex Mixer Machine (Rotamixer-9	Hook & Tucker Instruments Ltd., UK
Autoclave Machine	Yongfeng Enterprise Co., UK
Heidolph Unimax-2010 Incubator	Wolf Laboratories Ltd., UK

2.2.2 Consumable materials

Materials	**Sources**
Reagent Bottle (250, 500, 1000 ml)	Schott GL-45, Germany
Conical Flasks	Schott GL-45, Germany
Pipettes (Precicolor)	HBG, Germany
Eppendorf Tube (1.5 ml)	Hamburg, Germany
Pipette Tips	ALA, USA
PCR Tubes (0.2/0.5 ml)	Bio-Rad Laboratories, USA
Falcon Tubes (50 ml)	Hamburg, Germany
Polypropylene Tubes (15 ml)	Hamburg, Germany

2.2.3 Chemicals and reagents

Reagents	**Sources**
Triton-X 100	Sigma Chemical Company, USA
Sodium Lauryl Sulphate	Sigma Chemical Company, USA
Ethanol	Sigma Chemical Company, USA
Chloroform	Sigma Chemical Company, USA
Sodium Perchlorate	Sigma Chemical Company, USA
Glacial Acetic Acid	Sigma Chemical Company, USA
Sodium Chloride	Sigma Chemical Company, USA
Sucrose	Sigma Chemical Company, USA
Magnesium Chloride	Sigma Chemical Company, USA

Tris-HCl	Sigma Chemical Company, USA
EDTA-Na_2	Sigma Chemical Company, USA
Nuclease Free Water	Promega Corporation, USA
Ethidium Bromide Sample Loading Dye,6x	Wako Pure Chemicals Ltd., Japan Promega Corporation, USA
Agarose	Wako Pure Chemicals Ltd., Japan
Go Taq® Flexi DNA Polymerase	Promega Corporation, USA
5X Green Gotaq® Flexi Buffer	Promega Corporation, USA
$MgCl_2$ Solution	Promega Corporation, USA
dNTP Mix	Promega Corporation, USA
EZ Load™ Molecular Rulers-100 bp and 2.5 Kbp	Bio-Rad Laboratories, USA
Acryl Amide	Sigma Chemical Company, USA
Bisacrylamide (N,N'-Methylene bisacrylamide)	Sigma Chemical Company, USA
Ammonium Persulfate (APS)	Sigma Chemical Company, USA
TEMED (N, N, N', N'-tetramethyle thylenediamine)	Sigma Chemical Company, USA

2.2.4 Restriction Enzymes (REs)

Genes	SNP	RE	Recognition sites	Source
BRCA1	BRCA1 185delAG (rs80357713)	DdeI (5000 U/ml)	5′...C▼TNAG...3′ 3′...GANT▲C...5′	New England BioLabs® Inc., USA
BRCA1	BRCA1-5382insC (rs76171189)	BstNI (10000 U/ml)	5′...CC▼WGG...3′ 3′...GGW▲CC...5′	New England BioLabs® Inc., USA.
BRCA2	BRCA2-A/G transition (rs11571653)	BspHI (5000 U/ml)	5′...T▼CATGA...3′ 3′...AGTAC▲T...5′	New England BioLabs® Inc., USA
RAD51	RAD51-135G/C (rs1801320)	MvaI (5000 U/ml)	5′...CC▼WGG...3′ 3′...GGW▲CC...5′	New England BioLabs® Inc., USA
HER2	HER2 Val655Ile (rs1136201)	*Bsm*AI (5000 U/ml)	5′...GTCTC(N)$_1$▼...3′ 3′...CAGAG(N)$_5$▲...5′	New England BioLabs® Inc., USA

▼ ▲ Cutting site

2.2.5 Buffers: (Supplied with REs)

Buffer name	Composition	Applicable for enzymes
1X NE Buffer 3	50 mM Tris-HCl 100 mM NaCl 10 mM $MgCl_2$ 1 mM Dithiothreitol (p^H 7.9)	*BsmA*I
1X NE Buffer 4	20 mM Tris-acetate 50 mM Potassium acetate 10 mM Magnesium Acetate 1 mM Dithiothreitol (p^H 7.9)	*HpA*II
1X NE buffer	50 mM Potassium Acetate 20 mM Tris-acetate 10 mM Magnesium Acetate 100 µg/ml BSA pH 7.9 @ 25°C	*DdeI*
1X NE Buffer 3	50 mM Tris-HCl 100 mM NaCl 10 mM $MgCl_2$ 1 mM Dithiothreitol (p^H 7.9)	*BstNI*
1X NE Buffer 4	20 mM Tris-acetate 50 mM Potassium acetate 10 mM Magnesium Acetate 1 mM Dithiothreitol (p^H 7.9)	*BspHI*
Sigma Buffer SL	100 mM NaCl 10 mM Tris-HCl 10 mM MgCl2 1 mM DTE (**pH:** 7.5)	*MvAI*

2.2.6 Solutions

Name	Composition
TAE Buffer (10x)	0.4 M Tris -(Hydroxymethyl)-amino methane, 11.4 %(v/v)/0.2 M Glacial acetic acid, 0.01 M EDTA-Na_2, pH adjusted to 7.6.
TE Buffer(1x)	10 mM Tris -(Hydroxymethyl)-amino methane, 0.001M EDTA-Na_2, pH adjusted to 8.

2.3 Genomic DNA isolation

2.3.1 Venous blood collection

Approximately 3 ml of venous blood was collected from each patient and volunteer in a sterile eppendorf tube containing ethylenediaminetetraacetic acid disodium (EDTA-Na_2) after proper explanation and appropriate counseling about the study. Then samples were stored at -80ºC until DNA extraction.

2.3.2 Genomic DNA Isolation

There are many available protocols for DNA isolation. A large number of commercial kits are also used for the extraction of genomic DNA from whole blood. In this study we have performed our DNA isolation with the help of Daly's Method (Daly *et al.*, 1998). As this procedure is cheap and robust, it is routinely used in both research and clinical service in our laboratory.

2.3.2.1 Preparation of DNA isolation reagents

Reagent name	Composition and preparation procedure	Storage condition
Cell Lysis Buffer (1L)	10 mM Tris-HCl, 320 mM Sucrose and 5 mM $MgCl_2$ was added to 850 ml of distilled water. p^H was adjusted to 8.0 by adding NaOH. Then it was autoclaved. 1% Triton X-100 was added to it and the total solution was made up to 1L by adding distilled water.	4°C

Nuclear Lysis Buffer (1L)	400 mM Tris-HCl, 60 mM EDTA-Na_2 and 150 mM Sodium chloride was added to 850 ml of distilled water. p^H was adjusted to 8.0 by adding NaOH. Then it was autoclaved. 1% Sodium lauryl sulphate was added to it and the total solution was made up to 1L by adding distilled water.	Room Temperature
5 M Sodium Perchlorate (100 ml)	61.22 gm of Sodium Perchlorate was dissolved in 100 ml distilled water	4°C
5 mM Tris HCl Buffer (250 ml)	0.197 gm of Tris HCl was added in 150ml of distilled water. p^H was adjusted to 8.0 by adding NaOH. The total solution was made up to 250 ml by adding distilled water. Then it was autoclaved.	4°C

2.3.2.2 Genomic DNA isolation procedure

1. 3 ml blood was transferred to a 50 ml Falcon centrifuge tube that contains 2 mg of EDTA.
2. Adding 20 ml of Cell Lysis Buffer it was mixed gently for 2 minutes by inversion. The content was then centrifuged for 10 minutes at 3000 rpm at 4°C by using UNIVERSAL 240V 50i60Hz Refrigerated Bench-Top Centrifuge Machine (Hettich GmbH & Co., Germany).
3. The pellet was collected after discarding the supernatant.
4. After adding 2 ml Nuclear Lysis Buffer and 0.5 ml of 5 M Sodium Perchlorate were the content was mixed in a rotary mixture at room temperature for about 15 min so that pellet was dissolved completely.
5. Incubating the sample containing tube was at 65°C for 30 min. (Heidolph Unimax-2010 Incubator, Wolf Laboratories Limited, UK), 2.5 ml of chilled Chloroform was added to it.
6. It was placed in a rotary mixture for 10 min at room temperature and centrifuged at 1500 rpm for 5 min. (37°C).
7. The DNA containing phase (uppermost phase) was taken to a fresh autoclaved 15 ml polypropylene tube with the help of a disposable Pasteur pipette.

8. Around two volumes of Ethanol (double that of DNA phase) was added to it.
9. The content was then mixed immediately by slow gentle inversion until all cloudiness was disappeared.
10. DNA was found to be come out of the solution as a white 'cotton-wool' pellet.
11. The white 'cotton-wool pellet' was collected with a disposable microbiology loop and it was then air dried.
12. The DNA was dissolved in 5 mM Tris-HCl Buffer contained in a 1.5 ml screw cap tube and was kept at 65°C overnight.
13. Then it was taken back and was stored in Freezer.(-20°C)

2.3.2.3 Quantification of genomic DNA

The quantity and purity of isolated DNA were determined with the help of a UV Spectrophotometer (UV Prove v2.1) at 260 nm. The samples were very gently shaken on a vortex shaker for approximately 30 minutes before measurements were taken to ensure complete sample homogeneity. A sample volume of 1.5 to 2 µl was pipetted onto the surface of the fiber optic measurement.

For calculation of DNA solution free from RNA, the following conversion factor is used:

1 OD260 = 50 µg of DNA/ml.

DNA concentration in µg/µl was calculated as follows:

$$\text{DNA Concentration (µg/µl)} = \frac{\text{OD 260} \times 50 \text{ (dilution factor)} \times 50 \text{ µg/ml}}{1000}$$

OD260/OD280 should be=1.7 -1.9. (OD= Optical density).

A value beyond this range is not acceptable due to the contamination (i.e., protein) in the DNA sample that may affect subsequent reactions. The purity and integrity of isolated genomic DNA were also determined by the help of Agarose Gel Electrophoresis. A sample volume of 5 µl (50-70 ng/µl) was resolved on a 1% (w/v) agarose gel.

2.3.3 Genotyping of single nucleotide polymorphisms (SNPs) of BRCA1, BRCA2, RAD51 and HER2

For genotyping of the DNA samples for the selected SNPs, PCR-RFLP was employed because of its affordability, ease of use and reliability. This method of genotyping goes through the restriction enzyme (REase) digestion of polymerase chain reaction (PCR) amplification product. The subsequent cutting or lack of cutting of PCR amplified product due to the presence or absence of an SNP within the REase recognition site allows proper genotyping. The grouping of an SNP genotype as 'wild-type' or 'variant' was done according to accepted nomenclature and the relevant reference sequences that are available from the National Centre for Biotechnological Information (NCBI).

Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

2.3.4 DNA amplification using PCR

The genomic target regions containing the SNPs of interest were amplified by the help of primer-directed PCR using thermostable DNA polymerase. This primer-directed PCR method is very helpful to the in vitro amplification of single-copy genomic DNA sequences with a factor of more than ten million.

2.3.5 Primer design

There are some guidelines for primer design:

• PCR primers should be between 15 to 30 nucleotides long.

• The GC content of the primer is better to be 40-60% and C and G nucleotides should be distributed uniformly along the primer sequence.

• More than three G or C nucleotides at the 3'-end placing should be avoided to reduce the risk of non-specific priming.

• Primer self-complementarity or complementarity between the primers should be avoided to avoid hairpin formation and primer dimerization.

• Possible sites of non-desirable complementarity between primers and the template DNA should be examined.

• Differences in melting temperatures (Tm) of the two primers should not be allowed to exceed more than 5°C.

By considering all the factors mentioned above, the primers for the study were designed. The sequences of the primers used and their sizes are presented in table 2.1

Table 2.1: Name of the allele, sequence of the designed primer with their size

No.	SNP	Allele	Primer sequence	Size(bp)
1	BRCA1 185delAG (rs80357713)	BRCA1 FP	5- AAAATGAAGTTGTCATTTTATAAACC-3	26
2		BRCA1 RP	5- CTGACTTACCAGATGGGACACT -3	22
3	BRCA1-5382insC (rs76171189)	BRCA1 FP	5- CCAAAGCGAGCAAGAGAATCAC3’	22
4		BRCA1 RP	5'- GACGGGAATCCAAATTACACAG3’	22
5	BRCA2-A/G transition (rs11571653)	BRCA2 FP	5’-TGGAATACAGTGATACTGAC-3’	20
6		BRCA2 RP	5’-TTGGATTACTCTTAGATTTG-3’	20
7	HER2-Val655Ile rs1136201	HER2 FP	5’-ATCCCTGACCCTGGCTTCC-3’	19
8		HER2 RP	5’-CGCTTGATGAGGATCCCAAA-3’	20
9	RAD51-135G/C rs1801320	RAD51 FP	5’- TGGGAACTGCAACTCATCTGG-3’	21
10		RAD51 RP	5’- GCGCTCCTCTCTCCAGCAG-3’	19

FP=Forward Primer; RP=Reverse Primer; M.T=Melting Temperature

Primers were procured from Bio Basic Inc, USA.

2.3.6 PCR parameters and Conditions

Taq® DNA polymerase, reaction buffer, dNTPs and MgCl2 were used for the PCR amplification of the relevant genomic target regions, containing the SNPs of interest.. PCR was carried out in total volume of 25 µl containing 1 µL genomic DNA samples (50-70 ng/µl), 2.5 µl of 10x standard Taq reaction buffer (with MgCl2), 0.5 µl dNTPs (10 mM), 0.5 µl of each primer (10 mM), 0.13 µl Taq DNA polymerase (5U/ µl) (NEB, USA) and 20 µl nuclease free water. PCR conditions to synthesize various BRCA1, BRCA2, HER2 and RAD51 alleles with their respective lengths are showed in Table-2.2.

Table 2.2: PCR conditions to synthesize various BRCA1, BRCA2, HER2 and RAD51 alleles and their respective lengths.

Allele	**PCR conditions**	**Size of PCR products (bp)**
BRCA1 185delAG (rs80357713)	95^0C 45 sec 56^0C 30 sec 72^0C 30 sec (40 cycles)	176
BRCA1-5382insC (rs76171189)	95^0C 45 sec 56^0C 30 sec 72^0C 30 sec (40 cycles)	273
BRCA2-A/G transition (rs11571653)	95^0C 30 sec 55^0C 30 sec 72^0C 30 sec (30 cycles)	346
HER2-Val655Ile rs1136201	94^0C 30 sec 54^0C 30 sec 72^0C 30 sec (35 cycles)	111
RAD51-135G/C rs1801320	94^0C 30 sec 56^0C 30 sec 72^0C 40 sec (35 cycles)	157

2.3.7 Restriction enzyme digestion

After PCR amplification, 10 µl of the PCR products of various BRCA1, BRCA2, HER2 and RAD51 were digested with approximately 2 units of respective engymes that were obtained from New England Biolabs®, USA. Incubation conditions are given in Table 2.3. Electrophoreses was done for the digested products using 3% agarose gel.

Table 2.3: The restriction enzymes, digestion condition and length of the expected fragments on digestion to diagnose various BRCA1, BRCA2, HER2 and RAD51 alleles

Allele	**REs**	**Digestion conditions**	**Expected fragments (bp)**
BRCA1 185delAG (rs80357713)	DdeI (5000 U/ml)	Incubation at 37° C overnight	**NH 150, 26** **HE 174, 150, 26** **MH 174**
BRCA1-5382insC (rs76171189)	BstNI (10000 U/ml)	Incubation at 60° C overnight	**NH 273** **HE 273, 250, 23** **MH 250, 23**
BRCA2-A/G transition (rs11571653)	BspHI (5000 U/ml)	Incubation at 37° C overnight	**NH 296, 50** **HE 296, 235, 61, 50** **MH 235, 61, 50**
HER2-Val655Ile rs1136201	*Bsm*AI (5000 U/ml)	Incubation at 55° C overnight	**NH 111** **HE 111, 60, 51** **MH** 60, 51
RAD51-135G/C rs1801320	MvaI (5000 U/ml)	Incubation at 37° C overnight	**NH 86, 71** **HE 157, 86, 71** **MH 157**

NH: Normal Homozygote; HE: Heterozygote; MH: Mutant Homozygote

2.3.8 Visualization of PCR products and REase digestion fragments

Amplified products of PCR were visualized with the help of agarose gel electrophoresis for estimation of size. REase cut fragments of sufficient size (>50 bp) and size differential between fragments (>30 bp) were also properly visualized on agarose gel. EZ Load™ Molecular ruler (50 and 100 bp) was used for size estimation of PCR amplified products to confirm the amplification of the desired genomic target region and to quantify PCR product prior to REase digestion reactions. EZ Load™ 50 and 100 bp DNA ladder was also used to estimate the size of all REase digestion fragments facilitating the accurate and reliable genotyping of samples. All the produced agarose gels were visualized on the UV transilluminator (Alpha Innotech Corporation, San Leandro, California).

2.3.9 Gel electrophoresis

Electrophoresis is a method that separate substances on the basis of the rate of movement with the help of an electric field. Agarose gel electrophoresis is used to estimate the presence and distinguish the types of nucleic acids obtained through DNA isolation procedure as well as to evaluate digestion of DNA products.

Agarose is a polysaccharide purified from seaweed. An agarose gel is produced by suspending dry agarose in a buffer solution which undergoes boiling until the solution to be clear, then pouring it into a casting tray allowing it to cool which resulted in a flexible gelatin-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and having a positive and negative electrode. The DNA materials are passed through the pores of the gel by the electrical current for analysis.

Under the influence of an electrical field, DNA material is moved to the positive electrode (red) and away from the negative electrode (black). Several factors influence the rate of the movement, like strength of the electrical field, the concentration of agarose in the gel and the size of the DNA molecules. The smaller DNA molecules move faster through the agarose than larger molecules. DNA in the gel is visualized with the help of Ethidium Bromide previously added to the gel. Ethidium bromide binds to DNA illuminating in the ultraviolet light that cause the DNA to 'glow'. All PCR products and REase digestion fragments were analyzed with the help of electrophoresis in 2% and 3% (w/v) agarose gel respectively at 80 volts (V).

2.3.10 Agarose gel electrophoresis procedure

All agarose gels were made with 1X tris acetate ethylenediaminetetraacetic acid (TAE) buffer which were stored as a 10X stock solution. The 10X stock solution was diluted to the required working concentration. 1 µg of ethidium bromide (EtBr) per ml agarose solution was added -i.e. 0.01% (v/v) EtBr stock solution (10 mg/ml) for visualization of DNA within the agarose gel under UV light.

2.3.10.1 Casting a gel

1. 50ml of 1X Tris-acetate-EDTA (TAE) buffer with 1-1.5 mg of agarose was mix in a conical flask that was swirling to evenly distribute the agarose.
2. The solution was heated in the microwave oven for 1 minute then removed from the oven (before it boiled over), swirled, and reheated while keeping constant watch to be sure not to boil over.
3. The flask was cooled and poured when the temperature of the solution fall to 55-65^0 C.
4. The gel apparatus was prepared for casting the gel during cooling.
5. Ethidium bromide was added to the gel and swirled to mix and the gel was allowed to be cooled and hardened (20-30 minutes) before use.
6. The gel was poured into the casting tray with the adjustment of the comb keeping the wells perpendicular.

2.3.10.2 Preparing the gel for electrophoresis

1. A few ml of 1X TAE buffer was firstly added to the well area of the gel and the comb was carefully removed.
2. Buffer solution (1X TAE) was poured in the electrophoresis tank to be filled with after properly placing the gel (In the casting tray) on the tank platform

2.3.10.3 Preparing samples for loading/running the gel

1. An appropriate volume of loading dye (6X) was applied to the sample (1 µl of 6X sample dye for every 5 µl of sample) with the help of a 1-10 µl micropipette. The marker was loaded at a suitable Lane.
2. The cover was gently placed on the apparatus after loading of the gel. The power leads were hooked up and adjusted to 80 volts (constant voltage). The gel was run until the first dye front (bromophenol blue) moves about two-thirds the length of the gel and the second dye front (xylene cyanol) moves about one-third of the length of the gel.
3. The gel was then placed on the UV transilluminator for visualization the DNA.

2.4. PCR-RFLP for BRCA1 185delAG (rs80357713)

AAAATGAAGTTGTCATTTTATAAACCTTTTAAAAAGATATATATATATGTTTTTCTAA
TGTGTTAAAGTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTTCGCGTTGAAG
AAGTACAAAATGTCATTAATGCTATGCAGAAAATCTTXAGTGTCCCATCTGGTAAGT
CAG

Red----------->Primer sequence Green ----------> SNP of interest

After completing PCR amplification with appropriate reagents a PCR product of BRCA1 was obtained. The PCR product size was 176 bp and this was visualized in 2% (w/v) agarose gel.

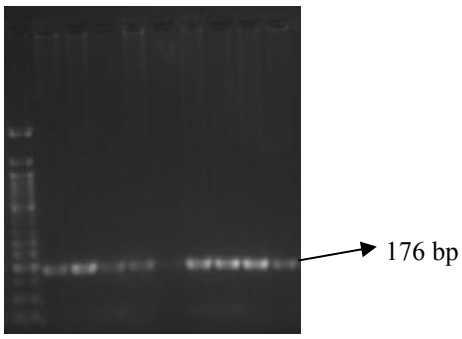


Fig 2.1: PCR product of BRCA1 185delAG (rs80357713) (176 bp) (Lane 2 to 14 (2% agarose gel) (Lane at the left contains Molecular ruler)

2.4.1. Fragmentation Pattern for BRCA1 185delAG (rs80357713)

The fragments were visualized in agarose gel (3%) after digestion of the PCR product with DdeI.

Table 2.4 Name of the restriction enzyme with its sites of digestion

Restriction Enzyme	Sites of digestion
DdeI	5′...C▼TNAG...3′ 3′...GANT▲C...5′

Table 2.5: Type of nucleotide changes, cutting sites and fragments of the allele in case of BRCA1 185delAG (rs80357713**)**

Change	Fragments	Type
When R= AG in both chromosomes (AG/AG)	150, 26	Normal Homozygote
When R= AG in one chromosome (AG/-)	174, 150, 26	Heterozygote
When X= -/- in both chromosome	174	Mutant Homozygote

When X= AG in both of the sister chromosomes: (NORMAL HOMOZYGOTE) (AG/AG)

When X=AG in both of the sister chromosomes, there will be one cutting between 150 bp and 26 in both of the chromosomes and two fragments with 150 and 26 will be obtained (Rohlfs et al., 1997; Haytural et al., 2014).

AAAATGAAGTTGTCATTTTATAAACCTTTTAAAAAGATATATATATATGTTTTTCTAATGTGT

TAAAGTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTTCGCGTTGAAGAAGTACAAAA

DdeI cutting site

TGTCATTAATGCTATGCAGAAAATCTTAGAGTGTCCCATCTGGTAAGTCAG

Yellow DdeI recognition site

5′...CTNAG...3′
3′...GANTC...5′

Restriction Enzyme DdeI

AAAATGAAGTTGTCATTTTATAAACCTTTTAAAAAGATATATATATATGTTTTTCTAATGTGT

TAAAGTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTTCGCGTTGAAGAAGTACAAAA

TGTCATTAATGCTATGCAGAAAATC **(Fragment 1: 150 bp)**

TTAGAGTGTCCCATCTGGTAAGTCAG **(Fragment 2: 26 bp)**

When X=AG in one of the sister chromosome: (HETEROZYGOTE) (AG/-)

When X=AG in one of the sister chromosome, there will be one cutting site at 150 bp. So, we shall get 3 fragments (176, 150 and 26bp) for two sister chromosomes (Rohlfs et al., 1997; Haytural et al., 2014).

AAAATGAAGTTGTCATTTTATAAACCTTTTAAAAAGATATATATATATGTTTTTCTAATGTGT

TAAAGTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTTCGCGTTGAAGAAGTACAAAA

DdeI cutting site

TGTCATTAATGCTATGCAGAAAATCTTAGAGTGTCCCATCTGGTAAGTCAG

Yellow DdeI recognition site

5'...CTNAG...3'
3'...GANTC...5'

Restriction Enzyme DdeI

AAAATGAAGTTGTCATTTTATAAACCTTTTAAAAAGATATATATATATGTTTTTCTAATGTGT

TAAAGTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTTCGCGTTGAAGAAGTACAAAA

TGTCATTAATGCTATGCAGAAAATC **(Fragment 1: 150 bp)**

TTAGAGTGTCCCATCTGGTAAGTCAG **(Fragment 2: 26 bp)**

AAAATGAAGTTGTCATTTTATAAACCTTTTAAAAAGATATATATATATGTTTTTCTAATGTGT

TAAAGTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTTCGCGTTGAAGAAGTACAAAA

TGTCATTAATGCTATGCAGAAAATCTTAGTGTCCCATCTGGTAAGTCAG **(Fragment 3: 174 bp, for uncut sister chromosome)**

When X=- (AG deleted) in both of the sister chromosomes: (Mutant Homozygote) (-/-)

When X= - (AG deleted) in both of the sister chromosomes, there will be no cutting site for DdeI. So we shall get one fragment with 174 bp (Rohlfs et al., 1997; Haytural et al., 2014).

AAAATGAAGTTGTCATTTTATAAACCTTTTAAAAAGATATATATATATGTTTTTCTAATGTGT

TAAAGTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTTCGCGTTGAAGAAGTACAAAA

No DdeI cutting site

TGTCATTAATGCTATGCAGAAAATCTTAGTGTCCCATCTGGTAAGTCAG

Yellow DdeI recognition site

5′...CTNAG...3′
3′...GANTC...5′

Restriction Enzyme DdeI

AAAATGAAGTTGTCATTTTATAAACCTTTTAAAAAGATATATATATATGTTTTTCTAATGTGT

TAAAGTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTTCGCGTTGAAGAAGTACAAAA

TGTCATTAATGCTATGCAGAAAATCTTAGTGTCCCATCTGGTAAGTCAG **(No digestion, one fragment of 174 bp)**

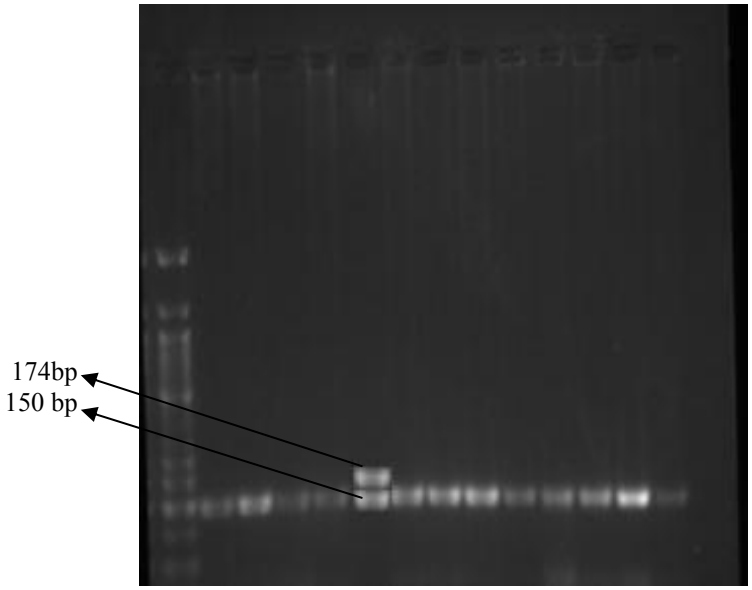


Fig. 2.2 Restriction Enzyme (DdeI) digestion fragment of BRCA1 (3% agarose gel); lane (6th from the left): Heterozygous, Rest of the lane: Normal homozygous, lane at the left: Molecular ruler

2.5 PCR-RFLP for BRCA1 5382insC (rs76171189)

CCAAAGCGAGCAAGAGAATXACCAGGACAGAAAGGTAAAGCTCCCTCCCTCAAGTTGACAAA

AATCTCACCCCACCACTCTGTATTCCACTCCCCTTTGCAGAGATGGGCCGCTTCATTTTGTAAGACTTATT

ACATACATACACAGTGCTAGATACTTTCACACAGGTTCTTTTTTCACTCTTCCATCCCAACCACATAAATAA

GTATTGTCTCTACTTTATGAATGATAAAACTAAGAGA TTTAGAGAGGCTGTGTAATTTGGATTCCCGTC

Red---------->Primer sequence Green ----------> SNP of interest

After completing PCR amplification with appropriate reagents a PCR product of BRCA1 (for BRCA1 5382insC: rs76171189) was obtained. The PCR product size was 273 bp and this was visualized in 2% (w/v) agarose gel.

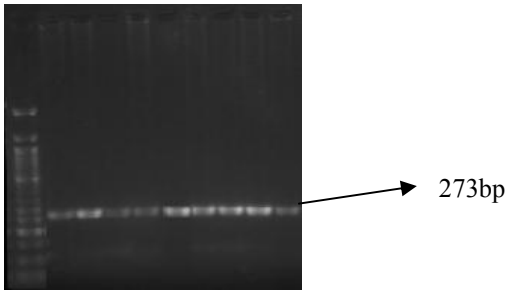


Fig 2.3 PCR product of BRCA15382insC (rs76171189) (273 bp) (Lane 1 to 13 (2% agarose gel) (Lane- 14 contains Molecular ruler)

2.5.1 Fragmentation Pattern for BRCA1 5382insC (rs76171189)

The fragments were visualized in agarose gel (2%) after digestion of the PCR product with *BstNI*.

Table 2.6: Name of the restriction enzyme with its sites of digestion

Restriction Enzyme	Sites of digestion
BstNI	5′...CCWGG...3′ 3′...GGWCC...5′

Table 2.7: Type of nucleotide changes, cutting sites and fragments of the allele in case of BRCA1 5382insC (rs76171189)

Change	Fragments	Type
When X= - in both chromosomes (-/-) (No insertion)	273	Normal Homozygote
When X= - in one chromosome (-/C) (Insertion C in one chromosome)	273, 251, 23	Heterozygote
When X=C in both chromosome (Insertion C in both chromosomes)	251, 23	Mutant Homozygote

When X= - (No insertion) in both of the sister chromosomes: (NORMAL HOMOZYGOTE) (-/-)

When X= - (No insertion) in both of the sister chromosomes, there will be no cutting site for BstNI in the 273 bp sequence of both of the chromosomes and one fragment with 273bp will be obtained (Rohlfs et al., 1997; Haytural et al., 2014).

CCAAAGCGAGCAAGAGAATCACAGGACAGAAAGGTAAAGCTCCCTCCCTCAAGTTGACAAAAATCTCAC

CCCACCACTCTGTATTCCACTCCCCTTTGCAGAGATGGGCCGCTTCATTTTGTAAGACTTATTACATACAT

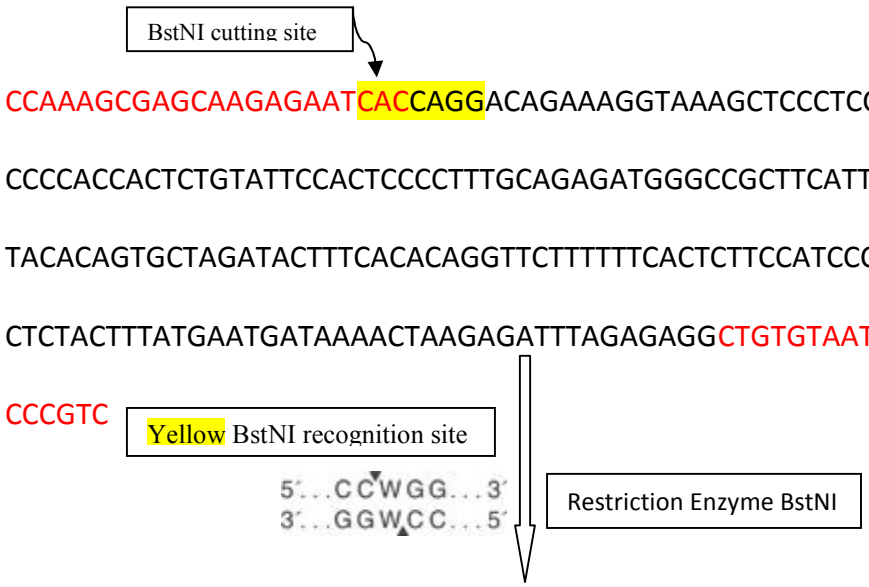
ACACAGTGCTAGATACTTTCACACAGGTTCTTTTTTCACTCTTCCATCCCAACCACATAAATAAGTATTGTC

TCTACTTTATGAATGATAAAACTAAGAGATTTAGAGAGGCTGTGTAATTTGGATT

CCCGTC **(Fragment 1: 273 bp)**

When X=C in one of the sister chromosome: (HETEROZYGOTE) (-/C)

When X= C (one insertion C) in one of the sister chromosomes, there will be one cutting site for BstNI between 23bp and 251 bp in the 273 bp sequence of one of the chromosomes and no cutting site for another chromosome and three fragments with 23 bp, 250 bp and 273 bp will be obtained (Rohlfs et al., 1997; Haytural et al., 2014).



CCAAAGCGAGCAAGAGAATCACC **(Fragment 1:23 bp)**

AGGACAGAAAGGTAAAGCTCCCTCCCTCAAGTTGACAAAAATCTCACCCCACCACTCTGTATTCCACTCC

CCTTTGCAGAGATGGGCCGCTTCATTTTGTAAGACTTATTACATACATACACAGTGCTAGATACTTTCACA

CAGGTTCTTTTTTCACTCTTCCATCCCAACCACATAAATAAGTATTGTCTCTACTTTATGAATGATAAAACT

AAGAGATTTAGAGAGGCTGTGTAATTTGGATTCCCGTC **(Fragment 2: 251 bp)**

CCAAAGCGAGCAAGAGAATCACAGGACAGAAAGGTAAAGCTCCCTCCCTCAAGTTGACAAAAATCTCAC

CCCACCACTCTGTATTCCACTCCCCTTTGCAGAGATGGGCCGCTTCATTTTGTAAGACTTATTACATACAT

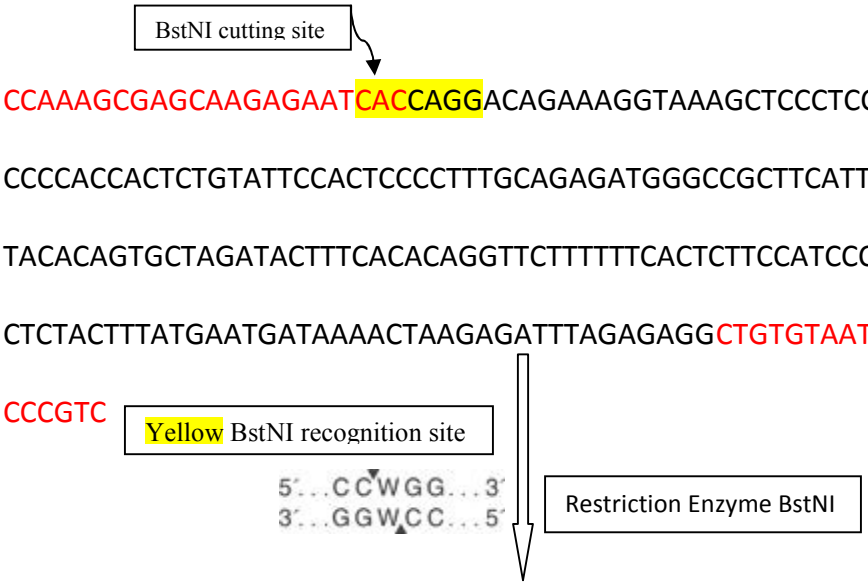
ACACAGTGCTAGATACTTTCACACAGGTTCTTTTTTCACTCTTCCATCCCAACCACATAAATAAGTATTGTC

TCTACTTTATGAATGATAAAACTAAGAGATTTAGAGAGGCTGTGTAATTTGGATT

CCCGTC **(Fragment 3: 273 bp)**

When X=C in both of the sister chromosomes: (Mutant Homozygote) (C/C)

When X= C (Insertion C in both chromosomes) in both of the sister chromosomes, there will be one cutting site for BstNI between 23bp and 251 bp in the 273 bp sequence of both of the chromosomes and two fragments with 23 bp and 250 bp will be obtained (Rohlfs et al., 1997; Haytural et al., 2014).



CCAAAGCGAGCAAGAGAATCACC **(Fragment 1:23 bp)**

AGGACAGAAAGGTAAAGCTCCCTCCCTCAAGTTGACAAAAATCTCACCCCACCACTCTGTATTCCACTCC

CCTTTGCAGAGATGGGCCGCTTCATTTTGTAAGACTTATTACATACATACACAGTGCTAGATACTTTCACA

CAGGTTCTTTTTTCACTCTTCCATCCCAACCACATAAATAAGTATTGTCTCTACTTTATGAATGATAAAACT

AAGAGATTTAGAGAGGCTGTGTAATTTGGATTCCCGTC **(Fragment 2: 251 bp)**

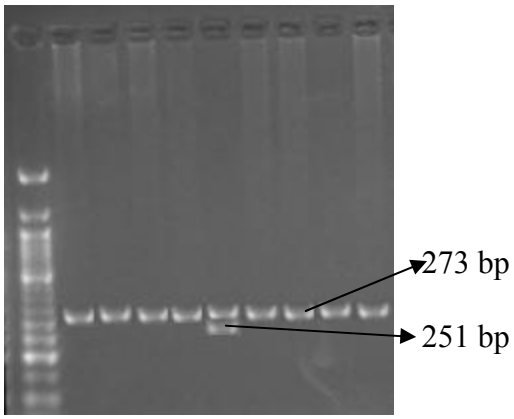


Fig. 2.4 Restriction Enzyme (BstNI) digestion fragment of BRCA2 (3% agarose gel); Lane at the left: Molecular ruler, Lane 6th from the left: Heterozygous, Rest of the lane: Normal Homozygote [Uncut PCR: (273 bp)]

2.6. PCR-RFLP for BRCA2 A/G transition (rs11571653)

TGGAATACAGTGATACTGACTTTCAATCCCAGAAAAGTCTTTTATATGATCATGAA
AATGCCAGCACTCTTATTTTAACTCCTACTTCCAAGGATGTTCTGTCAAACCTAGTCX
TGATTTCTAGAGGCAAAGAATCATACAAAATGTCAGACAAGCTCAAAGGTAACAAT
TATGAATCTGATGTTGAATTAACCAAAAATATTCCCATGGAAAAGAATCAAGATGTA
TGTGCTTTAAATGAAAATTATAAAAACGTTGAGCTGTTGCCACCTGAAAAATACATG
AGAGTAGCATCACCTTCAAGAAAGGTACAATTCAACCAAAACACAAATCTAAGAGT
AATCCAA

After completing PCR amplification with appropriate reagents a PCR product of *BRCA2* was obtained. The PCR product size was 346 bp and this was visualized in 2% (w/v) agarose gel.

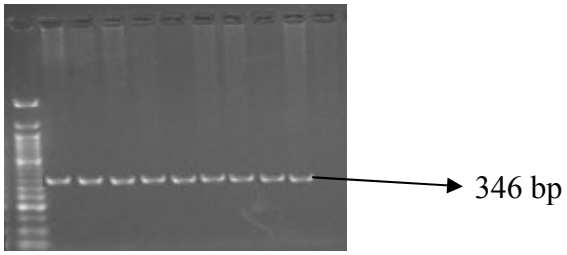


Fig: 2.5 PCR product of BRCA2 A/G transition (rs11571653) (346 bp) (Lane at the left: Molecular ruler, Rest of the Lane: PCR product (2% agarose gel)

2.6.1. Fragmentation Pattern for BRCA2 A/G transition (rs11571653)

The fragments were visualized in agarose gel (3%) after digestion of the PCR product with *BspHI*.

Table 2.8 Name of the restriction enzyme with its sites of digestion

Restriction Enzyme	Sites of digestion
BspHI	5′...T▼CATGA...3′ 3′...AGTAC▲T...5′

Table 2.9: Type of nucleotide changes, cutting sites and fragments of the allele in case of BRCA2 A/G transition (rs11571653)

Change	Fragments	Type
When X= A in both chromosomes (A/A)	235, 62, 50	Normal Homozygote
When X= A in one chromosome (A/G)	297, 235, 62, 50	Heterozygote
When X= G in both chromosome (G/G)	297, 50	Mutant Homozygote

When X= A in both of the sister chromosomes: (NORMAL HOMOZYGOTE) (A/A)

When X=A in both of the sister chromosomes, there will be two cutting sites for BspHI in the 347 bp sequence of both of the chromosomes and three fragments with 235, 61 and 50 will be obtained.

BspHI cutting site

TGGAATACAGTGATACTGACTTTCAATCCCAGAAAAGTCTTTTATATGATCATGAA

AATGCCAGCACTCTTATTTTAACTCCTACTTCCAAGGATGTTCTGTCAAACCT

BspHI cutting site

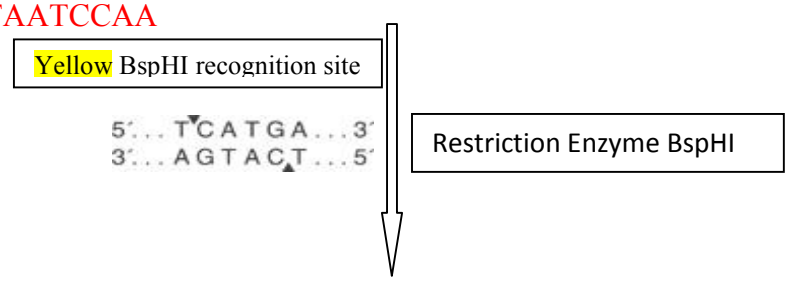
AGTCATGATTTCTAGAGGCAAAGAATCATACAAAATGTCAGACAAGCTCAAAGGTA

ACAATTATGAATCTGATGTTGAATTAACCAAAAATATTCCCATGGAAAAGAATCAA

GATGTATGTGCTTTAAATGAAAATTATAAAAACGTTGAGCTGTTGCCACCTGAAAAA

TACATGAGAGTAGCATCACCTTCAAGAAAGGTACAATTCAACCAAAACACAAATCT

AAGAGTAATCCAA



TGGAATACAGTGATACTGACTTTCAATCCCAGAAAAGTCTTTTATATGAT

(Fragment1: 50 bp)

CATGAAAATGCCAGCACTCTTATTTTAACTCCTACTTCCAAGGATGTTCTGTCAAACC
TAGT **(Fragment 2: 62 bp)**

CATGATTTCTAGAGGCAAAGAATCATACAAAATGTCAGACAAGCTCAAAGGTAACA

ATTATGAATCTGATGTTGAATTAACCAAAAATATTCCCATGGAAAAGAATCAAGATG

TATGTGCTTTAAATGAAAATTATAAAAACGTTGAGCTGTTGCCACCTGAAAAATACA

TGAGAGTAGCATCACCTTCAAGAAAGGTACAATTCAACCAAAACACAAATCTAAGA

GTAATCCAA **(Fragment 3: 235 bp)**

When X=A in one of the sister chromosome: (HETEROZYGOTE) (A/G)

When X=A in one of the sister chromosomes, there will be two cutting sites for BspHI in the 347 bp sequence in one chromosome and one cutting site in another chromosome and four fragments with 297, 235, 62 and 50 will be obtained.

BspHI cutting site

TGGAATACAGTGATACTGACTTTCAATCCCAGAAAAGTCTTTTATATGATCATGAA

AATGCCAGCACTCTTATTTTAACTCCTACTTCCAAGGATGTTCTGTCAAACCT

BspHI cutting site

AGTCATGATTTCTAGAGGCAAAGAATCATACAAAATGTCAGACAAGCTCAAAGGTA

ACAATTATGAATCTGATGTTGAATTAACCAAAAATATTCCCATGGAAAAGAATCAA

GATGTATGTGCTTTAAATGAAAATTATAAAAACGTTGAGCTGTTGCCACCTGAAAAA

TACATGAGAGTAGCATCACCTTCAAGAAAGGTACAATTCAACCAAAACACAAATCT

AAGAGTAATCCAA

Yellow BspHI recognition site

5′...TCATGA...3′
3′...AGTACT...5′

Restriction Enzyme BspHI

TGGAATACAGTGATACTGACTTTCAATCCCAGAAAAGTCTTTTATATGAT

(Fragment1: 50 bp)

CATGAAAATGCCAGCACTCTTATTTTAACTCCTACTTCCAAGGATGTTCTGTCAAACC
TAGT **(Fragment 2: 62 bp)**

CATGATTTCTAGAGGCAAAGAATCATACAAAATGTCAGACAAGCTCAAAGGTAACA

ATTATGAATCTGATGTTGAATTAACCAAAAATATTCCCATGGAAAAGAATCAAGATG

TATGTGCTTTAAATGAAAATTATAAAAACGTTGAGCTGTTGCCACCTGAAAAATACA

TGAGAGTAGCATCACCTTCAAGAAAGGTACAATTCAACCAAAACACAAATCTAAGA

GTAATCCAA **(Fragment 3: 235 bp)**

CATGAAAATGCCAGCACTCTTATTTTAACTCCTACTTCCAAGGATGTTCTGTCAAACC

TAGTCGTGATTTCTAGAGGCAAAGAATCATACAAAATGTCAGACAAGCTCAAAGGT

AACAATTATGAATCTGATGTTGAATTAACCAAAAATATTCCCATGGAAAAGAATCA

AGATGTATGTGCTTTAAATGAAAATTATAAAAACGTTGAGCTGTTGCCACCTGAAAA

ATACATGAGAGTAGCATCACCTTCAAGAAAGGTACAATTCAACCAAAACACAAATC

TAAGAGTAATCCAA **(Fragment 4: 297bp)**

When X=G in both of the sister chromosomes: (Mutant Homozygote) (G/G)

When X=G in both of the sister chromosomes, there will be one cutting sites for BspHI in the 347 bp sequence in both of chromosome between 297bp and 50bp and two fragments with 297, and 50 will be obtained.

BspHI cutting site

TGGAATACAGTGATACTGACTTTCAATCCCAGAAAAGTCTTTTATATGATCATGAA

AATGCCAGCACTCTTATTTTAACTCCTACTTCCAAGGATGTTCTGTCAAACCT

No BspHI cutting site

AGTCGTGATTTCTAGAGGCAAAGAATCATACAAAATGTCAGACAAGCTCAAAGGTA

ACAATTATGAATCTGATGTTGAATTAACCAAAAATATTCCCATGGAAAAGAATCAA

GATGTATGTGCTTTAAATGAAAATTATAAAAACGTTGAGCTGTTGCCACCTGAAAAA

TACATGAGAGTAGCATCACCTTCAAGAAAGGTACAATTCAACCAAAACACAAATCT

AAGAGTAATCCAA

Yellow BspHI recognition site

5′...TCATGA...3′
3′...AGTACT...5′

Restriction Enzyme BspHI

TGGAATACAGTGATACTGACTTTCAATCCCAGAAAAGTCTTTTATATGAT
(Fragment1: 50 bp)

CATGAAAATGCCAGCACTCTTATTTTAACTCCTACTTCCAAGGATGTTCTGTCAAACC

TAGTCGTGATTTCTAGAGGCAAAGAATCATACAAAATGTCAGACAAGCTCAAAGGT

AACAATTATGAATCTGATGTTGAATTAACCAAAAATATTCCCATGGAAAAGAATCA

AGATGTATGTGCTTTAAATGAAAATTATAAAAACGTTGAGCTGTTGCCACCTGAAAA

ATACATGAGAGTAGCATCACCTTCAAGAAAGGTACAATTCAACCAAAACACAAATC

TAAGAGTAATCCAA **(Fragment2: 297bp**

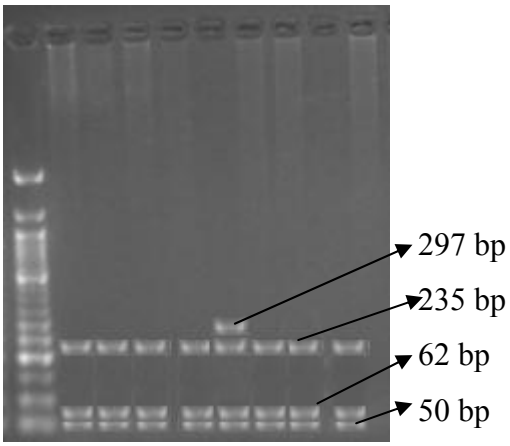


Fig 2.6: Restriction Enzyme (BspHI) digestion fragment of BRCA2 (3% agarose gel); Lane 6th from the left: Heterozygous, Lane at the left: Molecular ruler, Rest of the lane: Normal Homozygote

2.7. PCR-RFLP for HER2 Val655Ile (rs1136201)

ATCCCTGACCCTGGCTTCCGCCCCCAGCCCTCTGACGTCCATCXTCTCTGCGGTGGTT
GGCATTCTGCTGGTCGTGGTCTTGGGGGTGGTCTTTGGGATCCTCATCAAGCG

After completing PCR amplification with appropriate reagents a PCR product of *HER2* was obtained. The PCR product size was 111 bp and this was visualized in 2% (w/v) agarose gel.

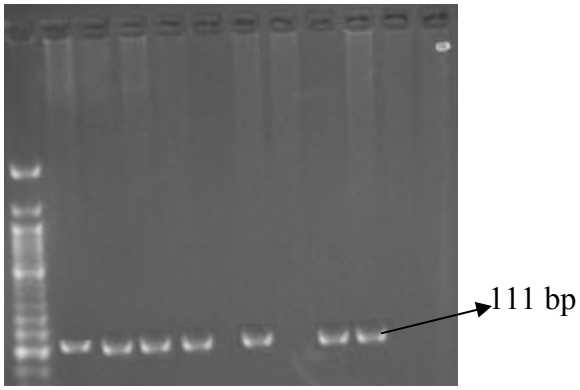


Fig: 2.7 PCR product of HER2 Val655Ile (rs1136201) (111 bp) [(Lane at the left: Molecular ruler; Rest of the lane: PCR product (2% agarose gel)]

2.7.1. Fragmentation Pattern for HER2 Val655Ile (rs1136201)

The fragments were visualized in agarose gel (3%) after digestion of the PCR product with *BsmAI*.

Table 2.10 Name of the restriction enzyme with its sites of digestion

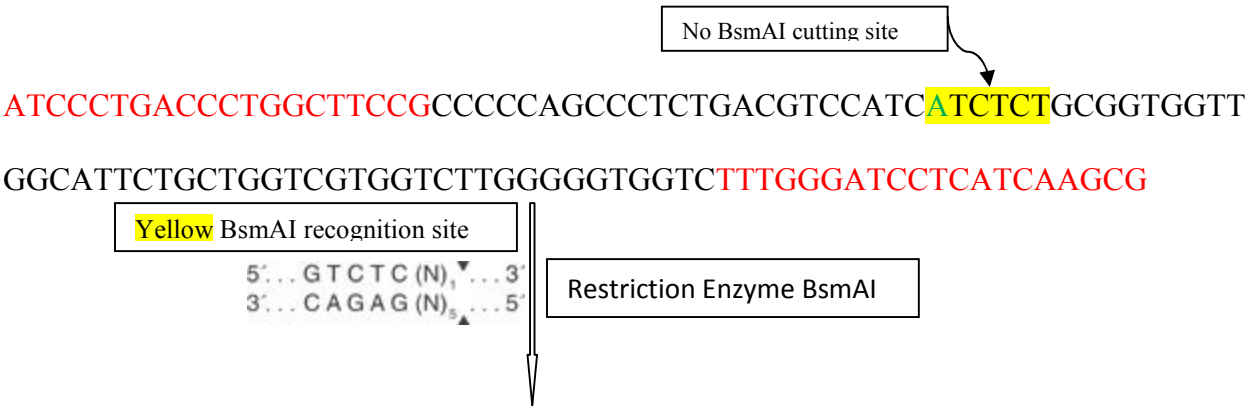
Restriction Enzyme	Sites of digestion
BsmAI	5′...GTCTC(N)$_1$▼...3′ 3′...CAGAG(N)$_5$▲...5′

Table 2.11: Type of nucleotide changes, cutting sites and fragments of the allele in case of HER2 Val655Ile (rs1136201)

Change	Fragments	Type
When X= A in both chromosomes (A/A)	111	Normal Homozygote
When X= A in one chromosome (A/G)	111, 61, 50	Heterozygote
When X= G in both chromosome (G/G)	61, 50	Mutant Homozygote

When X= A in both of the sister chromosomes: (NORMAL HOMOZYGOTE) (A/A)

When X=A in both of the sister chromosomes, there will be no cutting sites for BsmAI in the 111 bp sequence of both of the chromosomes and one fragments with 111 will be obtained.



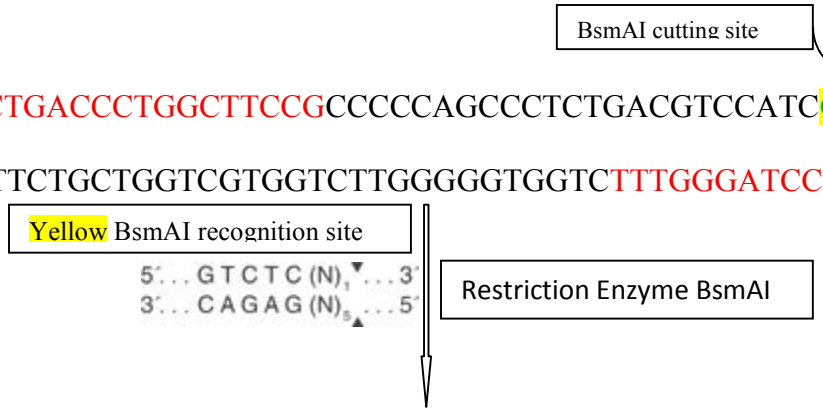
ATCCCTGACCCTGGCTTCCGCCCCCAGCCCTCTGACGTCCATCATCTCTGCGGTGGTT

GGCATTCTGCTGGTCGTGGTCTTGGGGGTGGTCTTTGGGATCCTCATCAAGCG

(Fragment1: 111 bp)

When X=A in one of the sister chromosome: (HETEROZYGOTE) (A/G)

When X=A in one of the sister chromosomes, there will be one cutting site for BsmAI in the 111 bp sequence in one chromosome and no cutting site in another chromosome and three fragments with 111 bp, 62 bp and 49 bp will be obtained.



ATCCCTGACCCTGGCTTCCGCCCCCAGCCCTCTGACGTCCATCATCTCT **(Fragment 1: 49bp)**

GCGGTGGTTGGCATTCTGCTGGTCGTGGTCTTGGGGGTGGTCTTTGGGATCCTCATC AAGCG **(Fragment 2: 62bp)**

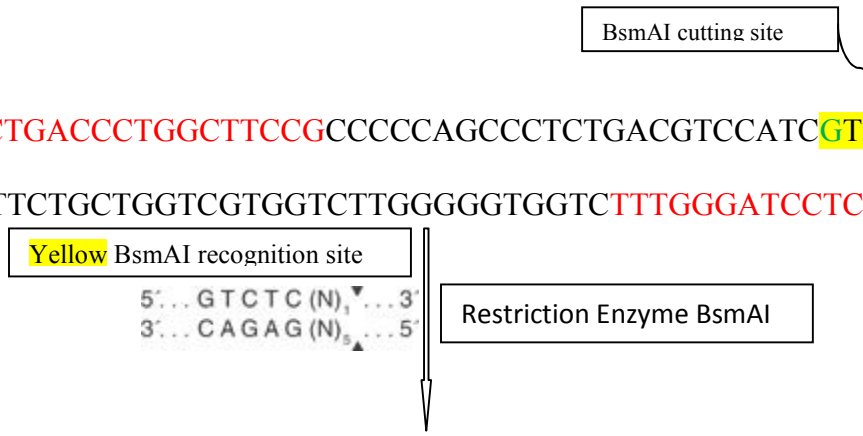
ATCCCTGACCCTGGCTTCCGCCCCCAGCCCTCTGACGTCCATCATCTCTGCGGTGGTT

GGCATTCTGCTGGTCGTGGTCTTGGGGGTGGTCTTTGGGATCCTCATCAAGCG

(Fragment 1: 111 bp)

When X=G in both of the sister chromosomes: (Mutant Homozygote) (G/G)

When X=G in both of the sister chromosomes, there will be one cutting site for BsmAI in the 111 bp sequence of both of the chromosomes and two fragments with 62bp and 49bp will be obtained.



ATCCCTGACCCTGGCTTCCGCCCCCAGCCCTCTGACGTCCATCATCTCT **(Fragment 1: 49bp)**

GCGGTGGTTGGCATTCTGCTGGTCGTGGTCTTGGGGGTGGTCTTTGGGATCCTCATC AAGCG **(Fragment 2: 62bp)**

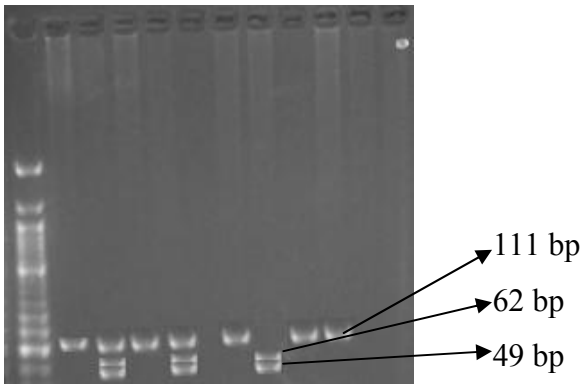


Fig 2.8: Restriction Enzyme (BsmAI) digestion fragment of HER2 (3% agarose gel); lane at the left: Molecular ruler, 2nd, 4th, 7th, 10th and 11th from the left: Normal Homozygote, lane 3rd and 5th from the left: Heterozygous, lane 8th from the left: Mutant homozygous, Lane 6th and 9th from the left contain dye only (No DNA product).

2.8. PCR-RFLP for RAD51135G/C (rs1801320)

TGGGAACTGCAACTCATCTGGGTTGTGCGCAGAAGGCTGGGGCAAGCGAG
TAGAGAAGTGGAGCGTAAGCCAXGGGCGTTGGGGGCCGTGCGGGTCGGG
CGCGTGCCACGCCCGCGGGGTGAAGTCGGAGCGCGGGGCCTGCTGGAGA
GAGGAGCGC

After completing PCR amplification with appropriate reagents a PCR product of *RAD51* was obtained. The PCR product size was 157 bp and this was visualized in 2% (w/v) agarose gel.

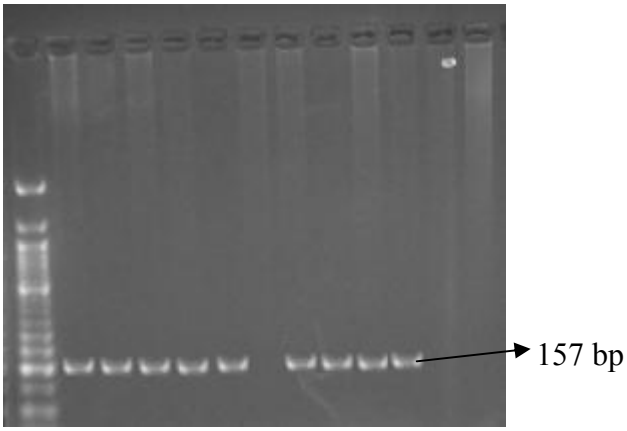


Fig 2.9: PCR product of RAD51 135G/C (rs1801320) (157 bp) [Lane at the left: Molecular ruler, rest of the lane PCR product (2% agarose gel)]

2.8.1. Fragmentation Pattern for RAD51135G/C (rs1801320)

The fragments were visualized in agarose gel (3%) after digestion of the PCR product with *MvaI*.

Table 2.12 Name of the restriction enzyme with its sites of digestion

Restriction Enzyme	Sites of digestion
MvaI	5′...CCWGG...3′ 3′...GGWCC...5′

Table 2.13 Type of nucleotide changes, cutting sites and fragments of the allele in case of RAD51 135G/C (rs1801320)

Change	Fragments	Type
When X= G in both chromosomes (G/G)	71, 86	Normal Homozygote
When X= G in one chromosome (A/C)	71, 86, 157	Heterozygote
When X= C in both chromosome (C/C)	157	Mutant Homozygote

When X= G in both of the sister chromosomes: (NORMAL HOMOZYGOTE) (G/G)

When X= G in both of the sister chromosomes, there will be one cutting sites for MvaI between at 71bp and 86bp in the 157 bp sequence of both of the chromosomes and two fragments with 71 and 86 will be obtained.

TGGGAACTGCAACTCATCTGGGTTGTGCGCAGAAGGCTGGGGCAAGCGAG

TAGAGAAGTGGAGCGTAAGCC **(Fragment1: 71 bp)**

AGGGGCGTTGGGGGCCGTGCGGGTCGGGCGCGTGCCACGCCCGCGGGGT

GAAGTCGGAGCGCGGGGCCTGCTGGAGAGAGGAGCGC **(Fragment1: 86 bp)**

When X=G in one of the sister chromosome: (HETEROZYGOTE) (G/C)

When X= G in one of the sister chromosomes, there will be one cutting sites for MvaI between at 71bp and 86bp in the 157 bp sequence in one of the chromosome and no cutting site in another chromosome and three fragments with 71 and 86 and 157 will be obtained.

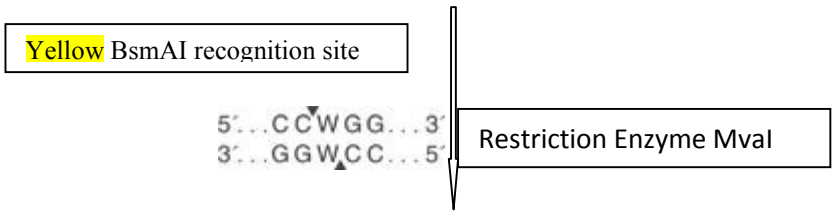
TGGGAACTGCAACTCATCTGGGTTGTGCGCAGAAGGCTGGGGCAAGCGAG

MvaI cutting

TAGAGAAGTGGAGCGTAAGCCAGGGGCGTTGGGGGCCGTGCGGGTCGGG

CGCGTGCCACGCCCGCGGGGTGAAGTCGGAGCGCGGGGCCTGCTGGAGA

GAGGAGCGC



TGGGAACTGCAACTCATCTGGGTTGTGCGCAGAAGGCTGGGGCAAGCGAG

TAGAGAAGTGGAGCGTAAGCC **(Fragment1: 71 bp)**

AGGGGCGTTGGGGGCCGTGCGGGTCGGGCGCGTGCCACGCCCGCGGGGT

GAAGTCGGAGCGCGGGGCCTGCTGGAGAGAGGAGCGC **(Fragment1: 86 bp)**

TGGGAACTGCAACTCATCTGGGTTGTGCGCAGAAGGCTGGGGCAAGCGAG

TAGAGAAGTGGAGCGTAAGCCACGGGCGTTGGGGGCCGTGCGGGTCGGG

CGCGTGCCACGCCCGCGGGGTGAAGTCGGAGCGCGGGGCCTGCTGGAGA

GAGGAGCGC **(Fragment 3: 157 bp)**

When X=C in both of the sister chromosomes: (Mutant Homozygote) (C/C)

When X= C in both of the sister chromosomes, there will be no cutting sites for MvaI in the 157 bp sequence in one of the chromosome and one fragment 157 will be obtained.

TGGGAACTGCAACTCATCTGGGTTGTGCGCAGAAGGCTGGGGCAAGCGAG

MvaI cutting site

TAGAGAAGTGGAGCGTAAGCCAGGGGCGTTGGGGGCCGTGCGGGTCGGG

CGCGTGCCACGCCCGCGGGGTGAAGTCGGAGCGCGGGGCCTGCTGGAGA

GAGGAGCGC

Yellow BsmAI recognition site

5′...CCWGG...3′
3′...GGWCC...5′

Restriction Enzyme MvaI

TGGGAACTGCAACTCATCTGGGTTGTGCGCAGAAGGCTGGGGCAAGCGAG

TAGAGAAGTGGAGCGTAAGCCACGGGCGTTGGGGGCCGTGCGGGTCGGG

CGCGTGCCACGCCCGCGGGGTGAAGTCGGAGCGCGGGGCCTGCTGGAGA

GAGGAGCGC **(Fragment 1: 157 bp)**

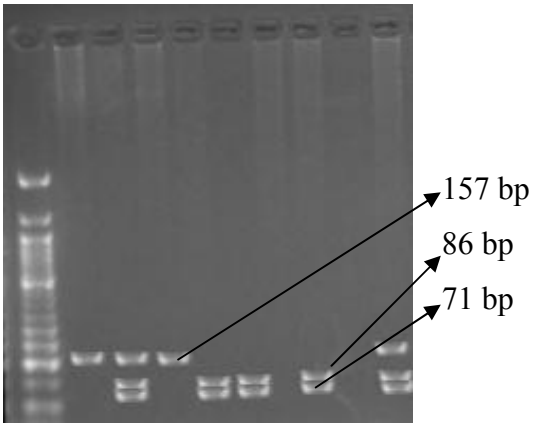


Fig 2.10: Restriction Enzyme (MvaI) digestion fragment of RAD51 (3% agarose gel); lane at the left: Molecular ruler, lane 5th, 6th and 8th from the left: Normal Homozygote, lane 3rd and 10th from the left: Heterozygous, lane 2nd and 4th from the left: Mutant homozygous, lane 9th from the left contains dye (No DNA product).

2.9 Statistical Analysis

The statistical significance of differences in genotype frequencies between patients and controls were determined by the Chi-square (χ^2) test. Binary logistic regression was applied for all analysis variables to evaluate risk as odds ratios (ORs) with 95 % confidence intervals (95 % CIs). ORs were adjusted for different types of variables like age, menstrual status, clinical TNM stage, histology, tumor grade, hormone receptor (HR) and Her-2neu status. All statistical analyses were done applying the SPSS software, version 17.0 (SPSS, Chicago, IL, USA).

CHAPTER THREE

RESULTS

3.0 Results

3.1 Demographic Distributions of the patients and Controls

We have collected and compared different categories of demographic parameters (education, family, history of breast cancer in first- and second-degree relatives, use of oral contraceptives, average months of breast feedings, and area of residence) of patient. The odds ratios ranges from 1.16 to 1.74 and all the p values were more than 0.05. The heterogeneity among the data was not significant. So in this study, the demographic parameters were not significantly associated with the risk of breast cancer (Table 3.1).

Table 3.1: Demographic Distributions of the patients and Controls

Demographic characteristics	Cases (n= 310)	Controls (n=250)	OR (95% CI)	P Value
Education				
a. Under High School	169	153	Ref.	-
b. At and Over High School	141	97	1.32 (0.94 to 1.85)	0.11
Family history of breast cancer in first- and second-degree relatives				
a. No	281	236	Ref.	-
b. Yes	29	14	1.74 (0.9- 3.37)	0.1
Use of Oral Contraceptives				
a. No	119	116	Ref.	-
b. Yes	191	134	1.39 (0.1-1.95)	0.056
Average Breast Feeding				
a. <12 months	65	39	1.55 (0.96-2.5)	0.072
b. 12-23 months	131	105	1.16 (0.8-1.68)	0.43
c. >24 months	114	106	Ref.	-
Area of Residence				
a. Urban	71	46	1.4 (0.9 -2.2)	0.13
b. Sub urban	78	58	1.22 (0.8 - 1.8)	0.34
c. Rural	161	146	Ref.	-

3.2 Correlation of different genotypes with breast cancer

3.2.1 BRCA1185del AG (rs80357713) polymorphism

We have got 7 heterozygote from 310 patient and 1from 250 controls in this study. We found no mutant homozygote in our population. In statistical calculation, the result was not significant [OR=5.75, 95% CI=0.07 to 47.07, p=0.1] as the frequency of the polymorphism is very few (Table 3.2).

Table 3.2: Statistics of BRCA1185del AG (rs80357713) polymorphism and breast cancer risk

Polymorphisms	Genotype	Cases (n=310)	Controls (n=250)	OR (95% CI)	P Value
BRCA1185del AG (rs80357713)	AG/AG	303	249	Ref.	-
	-/AG	7	1	5.75 (0.7030 to 47.07)	0.1
	-/-	0	0	-	-

3.2.2 BRCA1-5382insC (rs76171189) polymorphism

We have found 6 heterozygote from 310 patient and 1from 250 controls in the experiment. We also found no mutant homozygote in this population. In SPSS statistical calculation, as the frequency of the polymorphism is rare, no significant association was observed [OR=4.9, 95% CI=0.59 to 41.09, p=0.14] (Table 3.3).

Table 3.3: Statistics of BRCA1-5382insC (rs76171189) polymorphism and breast cancer risk

Polymorphisms	Genotype	Cases (n=310)	Controls (n=250)	OR (95% CI)	P Value
BRCA1-5382insC rs76171189	-/-	304	249	Ref.	-
	-/C	6	1	4.9 (0.59 to 41.09)	0.14
	C/C	0	0	-	-

3.2.3 BRCA1-5382insC (rs76171189) polymorphism

We have observed 11 heterozygote from 310 patient and 3 from 250 controls in the study. We also found no mutant homozygote in this population. In SPSS statistical calculation, we found moderate association of the polymorphism with breast cancer risk [OR=3.03, 95% CI=0.84 to 10.98, p=0.09] (Table 3.4).

Table 3.4: Statistics of BRCA2 -A/G transition (rs11571653) polymorphism and breast cancer risk

Polymorphisms	Genotype	Cases (n=310)	Controls (n=250)	OR (95% CI)	P Value
BRCA2 -A/G transition (rs11571653)	A/A	299	247	Ref.	-
	A/G	11	3	3.03 (0.84 to 10.98)	0.09
	G/G	0	0	-	-

3.2.4 Combined BRCA1/2 polymorphism

We have got total 22 heterozygote from 310 patients and 5 from 250 controls in BRCA1 and BRCA2 gene. In SPSS statistical calculation, we found strong association of the polymorphism with risk of breast cancer [OR=3.74, 95% CI=1.4to 10.03, p=0.0087] (Table 3.5).

Table 3.5: Statistics of BRCA1/2 polymorphism and breast cancer risk

Polymorphisms	Genotype	Cases (n=310)	Controls (n=250)	OR (95% CI)	P Value
BRCA1/2 (Combined)	NH	288	245	Ref.	-
	HE	22	5	3.74 (1.4 to 10.03)	**0.0087**
	MH	0	0	-	-

3.2.5 HER2-Val655Ile (rs1136201) polymorphism

We have found 88 heterozygote and 12 homozygote from 310 patients and 55 heterozygote and 6 homozygote from 250 controls in HER2 gene. AG plus GG genotype of HER2 rs1136201 were also found to be related with breast cancer risk in compared to AA genotype of the gene [OR=1.48, 95% CI=1.01 to 2.15, p=0.042] (Table 3.6).

Table: 3.6: Statistics of HER2-Val655Ile (rs1136201) polymorphism and breast cancer risk

Polymorphisms	Genotype	Cases (n=310)	Controls (n=250)	OR (95% CI)	P Value
HER2-Val655Ile (rs1136201)	A/A	210	189	Ref.	-
	A/G	88	55	1.44 (0.97 to 2.13)	0.065
	G/G	12	6	1.8(0.66 to 4.89)	0.249
	A/G + G/G	100	61	1.48 (1.01 to 2.15)	**0.0416**

3.2.6 RAD51 135G/C polymorphism (rs1801320) polymorphism

In this study we have found 80 heterozygote and 15 homozygote from 310 patients and 44 heterozygote and 8 homozygote from 250 controls in RAD51 gene. GC genotype and GC plus CC genotypes of RAD51rs1801320 were significantly associated with breast cancer risk in compared to GG genotype [OR=1.67, 95% CI=1.11 to 2.54, p=0.015 and OR=1.68, 95% CI=1.14 to 2.48, p=0.0088 respectively] (Table 3.7).

Table: 3.7 Statistics of RAD51 135G/C polymorphism (rs1801320) polymorphism and breast cancer risk

Polymorphisms	Genotype	Cases (n=310)	Controls (n=250)	OR (95% CI)	P Value
RAD51 135G/C polymorphism (rs1801320)	G/G	215	198	Ref.	-
	G/C	80	44	1.67 (1.11 to 2.54)	**0.015**
	C/C	15	8	1.73 (0.72 to 4.16)	0.22
	G/C + C/C	95	52	1.68 (1.14 to 2.48)	**0.0088**

3.3 Correlation of different genotypes with clinic-pathological parameters of the patients

Different clinic-pathological parameters like age, clinical stage, lymph node status, histology, tumor grade, HR status, and Her-2 neu expression were measured in patients with different genotypes and compared between wild types and mutation carriers.

3.3.1 Correlation of BRCA1/2 gene with clinico-pathological characteristics of the patients

Table 3.8: Correlation of BRCA1/2 gene with clinic-pathological characteristics of the patients

Characteristics	No. of Patients	BRCA1/2 Carrier(n=22)	BRCA1/2 Non Carrier (n=288)	OR (95% CI)	P Value
Age					
<45	141	11	130	1.22 (0.51-2.89)	0.66
45-55	123	8	115	1 (0.39-2.56)	1
>55	46	3	43	1(0.27-3.75)	1
45-55 + >55	169	11	158	Ref.	-
Menstrual status					
Premenoposal	162	11	151	-	
Perimenoposal	11	1	10	1.37(0.16-11.7)	0.77
Postmenoposal	137	10	127	1.08(0.44-2.63)	0.86
TNM stage (Clinical)					
I (Tumor ≤2 cm)	87	4	83	Ref.	-
II (Tumor 2 cm but ≤ 5 cm)	110	8	102	1.62 (0.47-5.6)	0.44
III (Tumor 5 cm)	94	8	86	1.93 (0.56-6.66)	0.29
IV (Tumor of any size with direct extension)	19	2	15	2.7 (0.46-16.48)	0.26
Lymph node status					
No (No regional lymph node metastases)	92	8	80	Ref.	-
N1 (Metastases to moveable ipsilateral axillary)	142	9	135	0.66 (0.25-1.8)	0.42
N2 (Metastases to fixed ipsilateral axillary lymph nodes)	58	4	55	0.73 (0.21-2.53)	0.61
N3 (Metastases to ipsilateral internal mammary lymph node)	18	1	17	0.59(0.07-5.02)	0.59
Histology					
Ductal	301	22			
Lobular	6	0			
Mixed	3	0			
Tumor grade					
Grade I	58	3	55	Ref.	-
Grade II	153	11	142	1.42(0.38-5.3)	0.6
Grade III	91	8	83	1.76(0.45-6.95)	0.42

Hormone receptor status					
Estrogen Receptor(ER)					
Negative	127	11	116	Ref.	-
Positive	183	11	172	0.67(0.28-1.6)	0.67
Progesterone Receptor(PR)					
Negative	157	13	144	Ref.	-
Positive	153	9	144	0.69(0.29-1.67)	0.41
Her-2 neu status					
Negative	187	15	172	Ref.	-
Positive	123	7	116	0.69(0.27-1.75)	0.44

As the frequencies of BRCA1/2 mutation carriers are few in number, it was not observed to get statistically significant variations among the different clinic-pathological characteristics (Table 3.8).

3.3.2 Correlation of HER2 gene with clinico-pathological characteristics of the patients

Patients with younger age (<45 years) were found to have significant number of AG plus GG genotype of HER2 rs1136201 in compared patients with the older age (> 45 years) [OR=1.66, 95% CI=1.03 to 2.68, p=0.038] (Table 3.9). No other parameters were found to be significantly associated with rs1136201polymorphism.

Table 3.9: Correlation of HER2 gene with clinico-pathological characteristics of the patients

Characteristics	No. of Patients	HER2 Carrier(n=100)	HER2 Non Carrier (n=210)	OR (95% CI)	P Value
Age					
<45	141	54	87	1.66(1.03-2.68)	**0.038**
45-55	123	34	89	1.02(0.60-1.72)	0.94
>55	46	12	34	0.94 (0.45-1.98)	0.88
45-55+ >55	169	46	123	Ref.	-
Menstrual status					
Premenoposal	162	57	105	Ref.	-
Perimenoposal	11	5	6	1.54(0.45-5.25)	0.49
Postmenoposal	137	38	99	0.7(0.43-1.16)	0.17
TNM stage (Clinical)					
I (Tumor ≤2 cm)	87	24	63	Ref.	-
II (Tumor 2 cm but ≤ 5 cm)	110	33	87	1.29(0.69-2.42)	0.42
III (Tumor 5 cm)	94	36	58	1.63(0.54-4.35)	0.13
IV (Tumor of any size with direct extension)	19	7	12	1.53(1.01-7.89)	

					0.42
Lymph node status					
No (No regional lymph node metastases)	92	27	65	Ref.	-
N1 (Metastases to moveable ipsilateral axillary)	142	44	98	1.08 (0.61-1.92)	1.08
N2 (Metastases to fixed ipsilateral axillary lymph nodes)	58	22	36	1.47(0.73-2.95)	0.28
N3 (Metastases to ipsilateral internal mammary lymph node)	18	7	11	1.53(0.54-4.37)	0.43
Histology					
Ductal	301	97	204	Ref.	-
Lobular	6	2	4	1.05 (0.19-5.84)	0.95
Mixed	3	1	2	1.05(0.09-11.74)	0.97
Tumor grade					
Grade I	58	15	43	Ref	-
Grade II	153	53	100	1.52 (0.77-2.99)	0.22
Grade III	91	32	59	1.55(0.75-3.22)	0.24
Hormone receptor status					
Estrogen Receptor(ER)					
Negative	127	39	88	Ref.	-
Positive	183	61	122	1.13 (0.69-1.84)	0.63
Progesterone Receptor(PR)					
Negative	157	47	110	Ref.)	-
Positive	153	53	100	1.24(0.77-2.0)	0.38
Her-2 neu status					
Negative	187	63	124	Ref.	-
Positive	123	37	86	1.18(0.72-1.93)	0.51

3.3.3 Correlation of RAD51 gene with clinico-pathological characteristics of the patients

We have got total 95 RAD51 carrier out of 310 patients in this study and different clinic-pathological parameters are compared between the carrier and non-carriers (Table 3.10).

Table 3.10: Correlation of RAD51 gene with clinico-pathological characteristics of the patients

Characteristics	No. of Patients	RAD51 Carrier(n=95)	RAD51 Non Carrier (n=215)	OR (95% CI)	P Value
Age					
<45	141	44	97	1.14 (0.7-1.84).	0.60
45-55	123	39	94	1.04(0.64-1.70)	0.87
>55	46	12	34	0.89 (0.43-1.85)	0.75
45-55 + >55	169	51	128	Ref.	-
Menstrual status					
Premenoposal	162	46	116	Ref.	
Perimenoposal	11	4	7	1.44(0.4-5.16)	0.57

Postmenoposal	137	45	92	1.23 (0.75-2.02)	0.4
TNM stage (Clinical)					
I (Tumor ≤2 cm)	87	21	72	Ref.	-
II (Tumor 2 cm but ≤ 5 cm)	110	33	87	1.3 (0.69-2.44)	0.41
III (Tumor 5 cm)	94	34	60	1.94 (1.02-3.7)	**0.043**
IV (Tumor of any size with direct extension)	19	7	12	2.0(0.7-1.72)	0.2
Lymph node status					
No (No regional lymph node metastases)	92	25	63	Ref.	-
N1 (Metastases to moveable ipsilateral axillary)	142	43	99	1.09 (0.61-1.97)	0.76
N2 (Metastases to fixed ipsilateral axillary lymph nodes)	58	20	38	1.33(0.65-2.7)	0.44
N3 (Metastases to ipsilateral internal mammary lymph node)	18	7	11	1.6(0.56-4.6)	0.38
Histology					
Ductal	301	92	209	Ref.	-
Lobular	6	2	4	1.14 (0.2-6.3)	0.88
Mixed	3	1	2	1.14(0.1-12.68)	0.92
Tumor grade					
Grade I	58	12	46	Ref.	-
Grade II	153	51	102	1.92 (0.93-3.93)	0.076
Grade III	91	34	59	2.2 (0.75-3.22)	**0.042**
Hormone receptor status					
Estrogen Receptor(ER)					
Negative	127	42	85	Ref.	-
Positive	183	53	130	0.83(0.51-1.34)	0.44
Progesterone Receptor(PR)					
Negative	157	51	106	Ref.)	-
Positive	153	44	109	0.84(0.52-1.36)	0.48
Her-2 neu status					
Negative	187	60	127	Ref.	-
Positive	123	35	87	0.85(0.52-1.4)	0.53

Patients with higher tumor size (size III) found to carry significant number of GC plus CC genotype of RAD51rs1801320 in compared to patients with lower tumor size (size I) [OR=1.94, 95% CI=1.02 to 3.70, p=0.043].

Tumor aggressiveness was found to be related to RAD51rs1801320 polymorphism. Patients with histological grade III tumor found to carry significant frequency of GC plus CC genotype of RAD51rs1801320 in compared to patients with grade III tumor [OR=2.2, 95% CI=0.75 to 3.22, p=0.042]. No significant effect of RAD51rs1801320 polymorphism on other clinic-pathological parameters of the patients was observed in this study (Table 3.10).

CHAPTER FOUR

DISCUSSION

4.0 Discussion

In the last two decades many studies different specific mutations in BRCA1 and BRCA2 occurring at a high frequency in various ethnic groups have been reported. Three specific mutations such as 185delAG, 5382insC in BRCA1, and 6174delT in BRCA2 were found to occur in 36% of breast cancer families in Israel (Levy-Lahad et al. 1997). 185delAG was reported to be at a very high frequency of 18.0% in families of Ashkenazi Jews with breast cancer (Phelan et al. 2002). This mutation also found at a frequency of 1% among the Ashkenazi general population making it one of the founder mutations which is thought to be responsible for its increased association with breast cancer (Struewing et al. 1995).

Although BRCA1 and BRCA2 mutations have been found to occur with different frequencies in the breast cancer patients of different populations, a significant frequency of 31.6% 185delAG has been reported among non-Jewish Americans of Spanish ancestry from the San Luis Valley, Colorado (Mullineaux et al. 2003). However, this mutation has been reported to occur at a comparatively lower frequencies with variations (1.13–5.9%) among white Americans, the Spanish from Spain, Polish, Iranian, Pakistani and Turkish women (Grzybowska et al. 2002; Shih et al. 2002; Guran et al. 2005; Weitzel et al. 2005; Mehdipour et al. 2006; Rashid et al. 2006). But in some previous studies the 185delAG mutation was not found among Chinese and Japanese families with breast cancer (Ikeda et al. 2001; Zhi et al. 2002).

The aim of this mutational research analysis was to study the incidences and distribution of mutations in Bangladesh and their role in the pathogenesis of breast cancer. Three important mutations resulting in alteration of the protein namely BRCA1- 185DelAG (rs80357713) BRCA1-5382insC (rs76171189) and BRCA2 -A/G transition (rs11571653) in BRCA1 and BRCA2 were selected for investigation in this study. The frequencies of the mutations were observed to be very few in number in the control group. After statistical analysis we found that combined BRCA1/2 mutation carriers were strongly associated with breast cancer risk [OR=3.74, 95% CI=1.40 to 10.03, p=0.0087].

As this is the first study with these genes in Bangladeshi population, we have no previous data to compare our results with the frequency of these mutations in this population. BRCA1-185DelAG (rs80357713) BRCA1-5382insC (rs76171189) mutations were previously reported in our neighboring Indian population (Kumar et al., 2002; Hedau et al., 2004; Valarmathi et al.,

2004; Saxena et al., 2005; Saxena et al. 2006, Kannan Vaidyanathan et al.2009; Lakhotia et al., 2010; Hansa et al., 2011; Chakraborty et al., 2013) that have the similarity with the ancestry of our population. BRCA2 -A/G transition (rs11571653) were also previously reported to be associated with breast cancer risk in different population (Haytural et al., 2014; Krupa et al., 2009; Sliwinski et al., 2005).

The present study have the similarity with the findings from most other previous studies in breast cancer patients with mutations in BRCA1/2 (Saxena et al. 2006, Kannan Vaidyanathan et al.2009; Lakhotia et al., 2010; Hansa et al., 2011; Chakraborty et al., 2013). As we have found strong association of BRCA1/2 mutation with breast cancer risk especially in the women with the younger age, the explanation may be that the mutations are very detrimental to their protein resulting in the alteration of the activity of the protein which finally may lead to the enhancement of the carcinogenesis process.

Now It would be interesting to test the carrier frequency of BRCA1- 185DelAG (rs80357713) BRCA1-5382insC (rs76171189) and BRCA2 -A/G transition (rs11571653) among different ethnic groups of the general Bangladeshi population as well as in different categories of Bangladeshi breast cancer patients to further understand its prevalence and role in the pathophysiology of breat cancer.

Genomic stability and integrity is maintained by DNA repair pathways which play a vital role in protecting against genetic mutations (Dixon & Kopras, 2004). In the prevention of genomic damage and correcting injuries done by exogenous agents and endogenous mutagens, DNA repair genes have been reported to be a considerable factors (Wood et al., 2001; Yu et al, 1999). According to some previous study it was indicated that DNA repair capacity is altered due to genetic variation in DNA repair genes leading to accumulation of DNA damage, resulting in cancer development (Krajinovic et al., 2002).

Human RAD51, one of the key proteins for homologous recombination, has been repored to plays a pivotal role in homologous recombination repair of DNA double-strand breaks (Richardson, 2005). In recent years, RAD51 gene polymorphisms have become a focusing point for the researchers and a common target is G135C (rs1801320), a G to C transversion at position

+135. This polymorphism was reported to affect mRNA stability or translational efficiency, leading to malfunctioning of encoding RAD51 protein, and alteration of DNA repair capacity (Hasselbach et al., 2005; Thacker, 2005).

Double-strand break damage is reported to be the most dangerous lesion in eukaryotic cells due to having the potentiality to permanently arrest cell cycle progression and endanger cell survival (Suwaki et al., 2011). As DNA repair mechanisms play a pivotal role to preserve genomic stability and functionality, defects in DNA repair lead to the chromosomal aberrations resulting in increased susceptibility to cancer (Dixon &Kopras, 2004; Wood et al., 2005; Berwick & Vineis, 2000).

The relationship of RAD51 variants (G135C) with the risk of breast cancer has been extensively investigated in different populations with inconsistent results. RAD51C has been reported to be associated with both Fanconi anemia–like disorders and familial breast and ovarian cancers with contradictory findings. In the recent years, several meta-analyses have been conducted to investigate the link between the RAD51 135G→C polymorphism and breast cancer risk (He et al., 2011; Lu et al., 2012; Wang et al., 2010). Nine epidemiologic studies with 13,241 cases and 13,203 controls of unknown BRCA1/2 status summarized in a meta-analysis concluded that women carrying the CC genotype had an increased risk of breast cancer compared with women with the GG or GC genotype (OR, 1.35; 95% CI, 1.04–1.74). A meta-analysis of 14 case-control studies involving 12,183 cases and 10,183 controls observed an increased risk only for women who were known BRCA2 carriers (OR, 4.92; 95% CI, 1.10–21.83) (Zhou et al., 2011). Another meta-analysis of 12 studies found null association between RAD51 135G→C and breast cancer risk (Yu et al., 2011). Among this conflicting data there is good number of evidence for association between germline mutations in RAD51C and breast cancer.

In this study we have found strong relationship of RAD51 135G→C with the breast cancer risk. Patient containing G/C genotype were more likely to be affected with breast cancer [OR=1.67, 95% CI=1.11 to 2.54, p=0.042] in compared to G/G genotype and patients those contain at least one C allele in the genetic sequence showed significant association with breast cancer (1.68 (1.14 to 2.48) [OR=1.68, 95% CI=1.14 to 2.48, p=0.0088]. This finding was consistent with the

results of some other previous studies (Blasiak et al, 2003; Dufloth et al, 2005; Brooks et al, 2008; Akisik et al, 2011; Romanowicz-Makowska et al, 2011; Romanowicz-Makowska et al, 2012; Hosseini et al, 2013) though some studies found poor association with the breast cancer risk (Jakubowska et al, 2009; Krupaet al; 2009; Brooks et al; 2008, Synowiec et al, 2008; Antoniou et al, 2007; Costa et al, 2007; Jara et al, 2007).

The effect of RAD51rs1801320 polymorphism on tumor aggressiveness was also observed in this study. Patients with higher tumor size (size III) found to carry significant number of GC plus CC genotype of RAD51rs1801320 in compared to patients with lower tumor size (size I) [OR=1.94, 95% CI=1.02 to 3.70, p=0.043]. Patients with histological grade III tumor found to carry significant frequency of GC plus CC genotype of RAD51rs1801320 in compared to patients with grade I tumor [OR=2.2, 95% CI=0.75 to 3.22, p=0.042]. This finding is consistent with the results of Costa et al., 2008.

The intron localization of the RAD51rs1801320 polymorphism is thought to play a role in the proper recognition of non-coding element of RNA splicing responsible for the correct functioning of RAD51 protein, influencing the activity of the multiprotein DNA-repair complex that includes BRCA1, BRCA2, RAD51 and other proteins resulting in malfunction of the protein and cancer development (Sassi et al, 2013).

The implication of various structural and functional genetic alterations in cancer development is indicated by molecular genetic analysis. Hence identification of molecular characteristics of breast cancer helps us to get more accurate prediction of the course of the disease and response to chemotherapy (Normanno et al., 2005). Growth factor receptors were reported to be linked with growth, differentiation, and motility of various tumor cells (Casalini et al., 2004). An important of growth factor receptors is Epidermal Growth Factor Receptor EGFR or HER2 (Uzan et al., 2009). Carcinogenesis of a variety of solid tumors was found to be related with mutations of genes and aberration of signaling pathway of HER2 (Ciardiello et al., 2001). Amplification and over expression of HER in several types of human tumors, including about 30% of all breast carcinomas have been reported in various studies (V et al., 2005; Paik et al., 1998; Marty et al., 2005).

Single nucleotide polymorphism (SNPs) at codon 655 of HER2 indicating a isoleucine-to-valine substitution (I655V) in the transmembrane domain have found to be associated increased risk of

breast cancer in some earlier studies including a meta analysis done by Tao et al., 2009 with 10,642 cases and 11,259 controls confirmed higher frequency of V allele in breast cancer cases (OR = 1.1, 95% CI 1–1.2, p = 0.04) (Tao et al., 2009). Another meta analysis performed by Lu et al., 2010 with 11504 cases and 12538 controls, found significant association among Africans (OR = 8.6, 95% CI 1.9–38.5), and Asians (OR = 1.2, 95% CI 0.01–1.38) (Lu et al., 2010). Xie et al., 2000 in the pivotal paper reported very high risk of HER2 I655V polymorphism in relation to breast cancer in Chinese population (OR 14.1, 95% CI 1.8–113.4) of the V/V versus I/I genotype (Xie et al., 2000). Millikan et al., 2003 reported a twofold increased risk of breast cancer associated with V/V or V/I genotype compared with I/I genotype among women living in North Carolina (United States) who were both less than 45 years of age and reported a positive family history of breast cancer (OR 2.3 95% CI 1–5.3) (Millikan et al., 2003).

In the current study we analyzed the frequency of HER2 655V variants in breast cancer patients, exposing significant increase of both variants in the patients in comparison to control group. Patients carrying AG plus GG genotype of HER2 rs1136201 were highly susceptible to breast cancer risk in compared to AA genotype of the gene [OR=1.66, 95% CI=1.03 to 2.68, p=0.038].

The results of the present study was important correlation with previous reports of Baxter and Campbell 2001; Mackean Cowdin et al., 2001; Montogomery et al., 2003; Lee-Hoeflich et al., 2008; and Ozturk et al., 2012, El-Mougy et al., 2012 though everal studies have shown conflicting results (Montgomery et al., 2003; Kara et al., 2010; Dahabreh and Murray 2011; and Ma et al., 2011).

Subgroup analysis in several studies demonstrated that, younger women with Val/Val genotype were associated with an increased risk of breast cancer in comparison with the Ile/Ile genotype (Xie et al., 2000); Millikan et al, 2003; Montgomery et al., 2003). Our findings of the relationship of HER2 polymorphism with breast cancer risk in younger women (> 45 years) [OR=1.75, 95% CI=1.08 to 2.83, p=0.023] was consistent with the above mentioned studies.

Since the first study of the HER2 Ile655Val polymorphism in relation to breast cancer risk with significant association (OR 14.1, 95% CI 1.8 to 113.4) of the Val/Val versus Ile/Ile genotype (Xie et al., 2000), a lots of studies have been conducted more modest risk ranging from 0.3 to

2.8, and our results is within this (wide) range. Allele frequencies in our study have the consistency to frequencies reported in other studies (Baxter & Campbell, 2001; Wang-Gohrke & Chang-Claude, 2001; Milikan et al., 2003, 2000; Keshava et al., 2001; Montgomery et al., 2003). The impact of and HER2 655V polymorphisms on clinicopathological parameters has become a controversial point with conflicting findings in the previous literatures (Naidu et al., 2008, Ozturk et al., 2012; Akisik and Dalay, 2004).

In the current study HER2 655V polymorphism there was slightly different between histopathological types as the frequency of V allele was moderately higher [OR=1.52, 95% CI=0.77 to 2.99, p=0.22 and OR=1.55, 95% CI=0.75 to 3.22, p=0.24] for grade II and grade III tumor respectively in compared to grade I tumor]. These findings may imply the some effect of HER2 655V polymorphism on tissue differentiation which is considered a poor prognostic feature of the disease.

The findings of this study indicate that implication of HER2 655V polymorphism in carcinogenesis may preferentially occur through functional modification due to alteration of tyrosine kinase activity. At last, we suggest that HER2 655V polymorphism may be considered as genetic markers for the risk of breast carcinogenesis although it needs further exploration with large samples of different background from Bangladeshi population.

Finally, we have observed positive relationship of BRCA1 (rs80357713), BRCA1 (rs76171189), BRCA2 (rs11571653), RAD51 (rs1801320), HER2 (rs113620) polymorphisms with breast cancer patients of Bangladesh. Extensive mutation screening of high-risk breast cancer primarily targeting early-onset cases should be undertaken in this country with appropriate genetic counseling, patients and presymptomatic mutation carriers would be able to make better medical and surgical decisions. Personal risk information may help in taking preventive measures and also motivate a high-risk woman to adopt breast screening that may promote early detection and improve chances of surviving breast cancer.

CHAPTER FIVE

CONCLUSION

5.0 Conclusion

Human cancer is still one of the leading causes of death worldwide, resulting in one of the most challenging global health issues confronted by mankind today. According to etiological studies, carcinogenesis of cancer is a complex, multistep and multifactor process, in which many genetic and environmental factors is involved. In recent years, it has become clear that individual variation in genetic backgrounds can lead to various consequences following the environmental exposure and may ultimately contribute to the cancer pathogenesis and progression (Bredberg, 2011; Pharoah et al., 2004; Hoeijmakers, 2001).

Development in many fields of science and technology has enabled us in some extend to understand the mechanism of carcinogenesis in breast now days. The exact aetiology of breast cancer is still under investigation, though it has the common occurrence. In the numerous epidemiological and research studies it is reported that breast cancer is a multifactorial disease resulting from interactions between genetic and environmental factors (Ponder, 2001). Over the past decades, significant development has been done, in defining risk factors that help us to identify those individuals who are highly susceptible to developing breast cancer. However, the major part of the development of genetic and bio markers of breast cancer are still unidentified, a lots of investigations are needed. As a result of several years of research, it is found that polymorphisms of some genes are associated with the risk of breast cancers with variations in findings (Pharoah et al., 2008; Stratton and Rahman, 2008). But no previous study can be cited on Bangladeshi population to evaluate their genetic susceptibility to breast cancer. In this regard we are the first team to go through this experiment on Bangladeshi breast cancer patients. On the basis of previous literature; we have selected some polymorphisms in different genes like BRCA1 (rs80357713), BRCA1 (rs76171189), BRCA2 (rs11571653), RAD51 (rs1801320), and HER2 (rs1136201) to investigate their role in the carcinogenesis of breast cancer. After the completion of the study we observed combined BRCA1/2, RAD51 (rs1801320), and HER2 (rs1136201) polymorphisms are associated with breast cancer risk for Bangladeshi population and RAD51 (rs1801320) is also related with tumor aggressiveness.

Although a large number of samples are required to validate our claim, our study may be useful in building a set of clinical and molecular markers helpful for diagnosis of breast cancer as well

as as important tool for making better decision on medical and surgical preventive measures for controlling breast cancer in Bangladesh.

CHAPTER SIX

REFERENCE

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APPENDIX

DATA COLLECTION FORM

Questionnaires

BRCA1, BRCA2, HER2 and RAD51 gene polymorphisms in breast cancer patients of Banglasesh

1. Identification

1.1 I.D. Code:

1.2 Name:

1.3 Father's/ Husband's Name:

1.4 Sex: **Male** **Female**

1.5 Marital Status: **Married** **Unmarried**

1.6 Date of Birth (dd/mm/yy): **1.7** Age (yr):

1.8 Mailing address:

1.9 Permanent address:

1.10 Telephone No.:

1.11 Religion:

1.12 Nationality:

2. Personal History

2.1 Area of residence:

	Rural	**Urban**	**S-Urban**	**Others**
ℵ Where have you spent your boyhood (1-15 y)?				
ℵ Where have you spent at least ¾th or more of your life time?				

2.2 Education level:

	Illiterate		SSC or equivalent
	Can read only		HSC or equivalent
	Can write a letter		Graduate or higher
	Other		

2.3 Occupation:

	Student		Unemployed
	Professional		Housewife
	Business		Skilled worker
	Technical		Other

2.4 Family expense/month:

2.5 Impression about social class:

	Rich		Lower middle
	Upper middle		Poor

2.6 Dietary habit:

	Rich fibrous		Moderately		Poor fibrous

2.7 Food Habit (24 hours recall method)

Morning	
Lunch	
Afternoon	
Dinner	

2.8 Number of Children	
2.9. Average lenth of breast feeding (month)	

2.10 Family History of breast or ovarian cancer	Yes	No
2.11 Habit of exercise	Yes	No

3. Biophysical Characteristics

3.1 Height (cm):		**3.4** Temperature (^{0}F):	
3.2 Weight (kg):		**3.5** BP (Sys/Dias):	
3.3 Pulses/min:		3.6 BSA	
3.4 BMI			

4.1 TNM stage:	
4.2 Tumor position:	
4.3 Initial Tumor volume (cm^3):	
4.4 Tumor volume after 4 weeks of chemotherapy (cm^3):	
4.5 Cycles	

5. Prescribed drugs

Drugs	**Dose (mg)**
Cyclophosphamide	
Epirubicin/Doxorubicin	
5-FU	
Docitaxel	
Others	

6. **Toxic effects:**

Toxicity	**Grades**			
Hematological toxicity	1	2	3	4
Anemia				
Neutropenia				
Leucopenia				
Throbocytopenia				
Gastointestinal toxicity				
Diarrhoea				
Constipation				
Allopecia				
others				

Name of the investigator:
Signature:

PATIENT CONSENT FORM

I, the undersigned, authorize the research student to consider me as a patient for his/ her research work. I understand that I can change my mind at any time to withdraw myself as patient during this research work.

Patient's consent to study treatment

Please tick as appropriate

1. Do you have complete idea about the type, ultimate goal and methodology of the research?	Yes	No
2. Are you aware that you don't have to face any physical, mental and social risk for this?	Yes	No
3. There will be no chance of injury in any of your organs; are you aware of this?	Yes	No
4. Have you got any idea about the outcome of this experiment?	Yes	No
5. Have you decided intentionally to participate in this experiment?	Yes	No
6. Do you think this experiment violate your human rights?	Yes	No
7. Are you sure that all the information regarding you will be kept Confidentially?	Yes	No
8. No remuneration will be provided for this experiment, are you aware of this?	Yes	No

After reading the above mentioned points, I am expressing my consent to participate in this experiment as a patient.

Patient's signature and Date:_____

Patients's Name:_____

Address:_____

Witness:_____

Please return the signed copy to the research student and keep an extra copy for yourself.

_____

Signature of the Researcher

Department of Clinical Pharmacy and Pharmacology

Faculty of Pharmacy

University of Dhaka