Effect of GSTP1 and ABCC4 gene polymorphisms on response and toxicity of cyclophosphamide-epirubicin-5-fluorouracil based chemotherapy in Bangladeshi breast cancer patients

A thesis submitted by
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Abstract:

Background: Chemotherapy is one of the most widely used treatments of breast cancer with the limitation of significant inter-individual heterogenecity in response as well as in toxicity. The most important cytotoxic drug namely cyclophosphamide (CPA) used in breast cancer along with epirubicin and 5-fluorouracil, is transported by ABCC transporters and detoxified by glutathione S-transferases (GSTs). It has been established that the activities of these enzymes and transporters vary in different population due to the presence of polymorphisms in their genetic sequence but no such type of study has been conducted on Bangladeshi breast cancer patients. The goal of this study was to evaluate the effects of genetic polymorphism of GSTP1 (rs1695) and ABCC4 (rs9561778) genes on the response and toxicities produced by chemotherapy used in the treatment of breast cancer.

Methods: Two hundred and nineteen patients with invasive breast cancer were recruited from different public and private hospitals of Bangladesh of which 117 patients received neoadjuvant chemotherapy to examine the response as well as toxicity and another 102 patients received adjuvant chemotherapy to evaluate only the toxicity produced by the therapy.

The American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging system (sixth edition) and Response Evaluation Criteria In Solid Tumors (RECIST) were used to evaluate the pathological response of primary tumor and axillary lymph nodes and the assessment of chemotherapy induced toxicity was done with the help of common terminology criteria for adverse events (CTCAE) v4. Genetic polymorphisms of the mentioned genes were detected by using Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR RFLP) and the PCR RFLP method for ABCC4 (rs9561778) polymorphism detection has been applied for the 1st time that was developed in our laboratory.

Results: Patients carrying AG and AG plus GG genotypes of GSTP1 (rs1695) were more likely to have good response (OR = 2.5, 95% Cl = 1.13 to 5.69, p = 0.025; and OR = 2.69, 95% Cl = 1.26 to 5.76, p = 0.011, respectively) in compared to AA genotype that is statistically significant whereas GG carriers also showed good response (OR = 3.4, 95% Cl = 0.84 to 13.93, p = 0.085) that is not statistically significant. No significant association of ABCC4 gene was found with the response of chemotherapy.

Patients carrying ABCC4 (rs9561778) polymorphism was associated with chemotherapy induced toxicities including anemia, neutropenia, leukopenia and gastrointestinal toxicities. In neoadjuvant chemotherapy, patients carrying GT and GT plus TT genotypes were found to be associated with anemia (OR = 2.87, 95% Cl = 1.04 to 7.89, p = 0.042 and OR = 2.78, 95% Cl = 1.15 to 6.71, p = 0.023 respectively) in compared to GG genotype. Neutropenia and gastrointestinal toxicities were also found to have significant association with patients having at least one variant T allele (GT+ TT) of rs9561778 (OR = 2.64, 95% Cl = 1.09 to 6.40, p = 0.032 and OR = 2.38, 95% Cl = 1.10 to 10.37, p = 0.034, respectively) with the comparison of wild genotype (GG).

In case of adjuvant chemotherapy, neutropenia and leukopenia were found to have the association with the patients carrying any variant T allele (GT+ TT) of rs9561778 (OR = 2.80, 95% Cl = 1.11 to 7.05, p = 0.029 and OR = 2.75, 95% Cl = 1.06 to 7.14, p = 0.038 respectively) and patients having TT genotype of ABCC4 (rs9561778) were associated with thrombocytopenia (p = 0.034) with the comparison of GG genotype.

Combining the adjuvant and neoadjuvant chemotherapy, we found that patients carrying GT and at least one variant T allele of ABCC4 were found to be associated with anemia (OR = 2.75, 95% Cl = 1.33 to 5.67, p = 0.006 and OR = 2.62, 95% Cl = 1.39 to 4.93, p = 0.003 respectively), leukopenia (OR = 2.41, 95% Cl = 1.14 to 5.08, p = 0.021 and OR = 2.45, 95% Cl = 1.26 to 4.77, p = 0.008 respectively) and gastrointestinal toxicities (OR = 3.45, 95% Cl = 1.41 to 8.43, p = 0.007; OR = 3.17, 95% Cl = 1.40 to 7.19, p = 0.006 respectively) in comparison with GG genotype. Neutropenia was associated with patients carrying GT, TT and at least one variant T allele of ABCC4 (OR = 2.51, 95% Cl = 1.22 to 5.16, p = 0.012; OR = 3.35, 95% Cl = 1.14 to 9.79, p = 0.027 and OR = 2.72, 95% Cl = 1.44 to 5.15, p = 0.002 respectively) in compared to GG genotype.

GSTP1 gene was not found to be significantly associated with the chemotherapy induced toxicities. The response to the treatment as well as toxicity was not associated with different clinicophathological characteristics like estrogen receptor, progesterone receptor and Her2/neu status of tumors. No correlation of response and toxicity with patient's age, tumor staging and menopause status was found in this study.

Conclusion: Our result indicates that GSTP1 (rs1695) polymorphism was strongly associated with the response of chemotherapy whereas ABCC4 (rs9561778) polymorphism was significantly related with chemotherapy induced adverse effects in studied breast cancer patients.

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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
3HC	Trans-3'-Hydroxy cotinine
A	Adenine (where referring to a nucleotide)
ADRs	Adverse drug reactions
bp	Base pair
BPDE	Benzo(a)pyrene-7,8 diol-9,10-epoxide
C	Cytosine (where referring to a nucleotide)
cDNA	Complementary DNA
CI	confidence interval (where genotype, haplotype and allelic data compared)
Cl^-	Chloride ion
CMV	Cytomegalovirus
CPA	Cyclophosphamide
CR	Complete Response
CTCAE	Common Terminology Criteria for Adverse Events
CYP	Cytochrome P450
Cys	Cysteine
D'	normalized disequilibrium constant
DF	Dilution factor
DMEs	Drug metabolizing enzymes
DNA	Deoxyribonucleic acid
dNTP	De-oxy nucleotide triphosphate
EGFR	Epidermal growth factor receptor
EM	Extensive metabolizer

EPA	Environmental Protection Agency
EtBr	Ethidium Bromide
FMO3	flavin-containing monooxygenase 3
FEC	Cyclophosphamide-epirubicin-5-fluorouracil
FP	Forward Primer
G	Guanine (where referring to a nucleotide)
GSTs	Glutathione S-transferases
GWAS	Genome-wide association studies
HapMap	Haplotype map
HCAs	Heterocyclic amine
HE	heterozygote
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
Ile	Isoleucine
IM	Intermediate metabolizer
IREB2	iron-responsive element-binding protein
JPT	Japanese in Tokyo
JNK	Jun N-terminal Kinase
K^+	Potassium ion
L	Liter
LD	Linkage disequilibrium
LWK	Luhya in webuye, Kenya
Met	Methionine
mg	Miligram

MgCl2	Magnesium Chloride
MH	mutant homozygote
ml	Milliliter
mM	Millimolar
MPI	Mannose phosphate isomerase
mRNA	Messenger Ribonucleic acid
Na^+	Sodium ion
NAS	National Academy of Sciences
NC	Not Calculated
NCBI	National Centre for Biotechnological Information
NCI	National Cancer Institute
NDEA	N-nitrosodiethylamine
NE buffer	New England Biolab buffer
NEB	New England Biolab
ng	Nanogram
NH	normal homozygote
NNK	4- (methylnitrosamine)-1-(3-pyridyl)-butanone
NNN	N-nitrosonornicotine
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical Density
OR	Odds Ratio (where genotype, haplotype and allelic data compared)
PAHs	Polycyclic aromatic hydrocarbons
PCR	Polymerase Chain Reaction
PD	Progressive Disease

PM	Poor metabolizer
PR	Partial Response
Pre-mRNA	preliminary-mRNA
Pro	Proline
REase	Restriction endonuclease
RECIST	Response Evaluation Criteria In Solid Tumor
RefSeq	NCBI Reference Sequences
REs	Restriction Enzymes
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RP	Reverse Primer
rs	DbSNP record ID number
RUL	Right Upper Lobe
SCLC	Small Cell Lung Cancer
Ser	Serine
SHS	second-hand smoke
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Product and Service Solutions
SD	Stable Disease
SRS	Substrate recognition sites
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
TSI	Tuscan in Italy
U	unit
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UM	Ultrarapid metabolizer
USA	United States of America
USDHHS	U.S. Department of Health and Human Services
USEPA	United States Environmental Protection Agency
UTR	3´-untranslated region
UV	Ultraviolet
Val	Valine
WCRF	World Cancer Research Fund
WHO	World Health Organization
WMADH	World Medical Association Declaration of Helsinki
α	Alpha
β	Beta
γ	Gamma
δ	Delta
µg	Microgram
µl	Microlitre

CHAPTER ONE

INTRODUCTION

1. Introduction

1.1 Breast cancer

Breast cancer is a malignant tumor that starts in the ductal or lobular cells of the breast. A malignant tumor is a group of cancer cells that can invade surrounding tissues or spread (metastasize) to distant areas of the body. The disease occurs almost entirely in women, but men can get it, too. The most common beast cancer namely invasive breast cancer is a heterogeneous disease in its presentation, pathological classification and clinical course. Most tumors are derived from mammary ductal epithelium, principally the terminal duct-lobular unit, and up to 75% of the diagnosed infiltrating ductal carcinoma are defined as invasive ductal carcinoma, not otherwise specified (IDC-NOS). The second most common epithelial type is invasive lobular carcinoma which comprises of 5%–15% of the group. However, there are more than a dozen variants which are less common but still very well defined by the World Health Organization (WHO) classification.

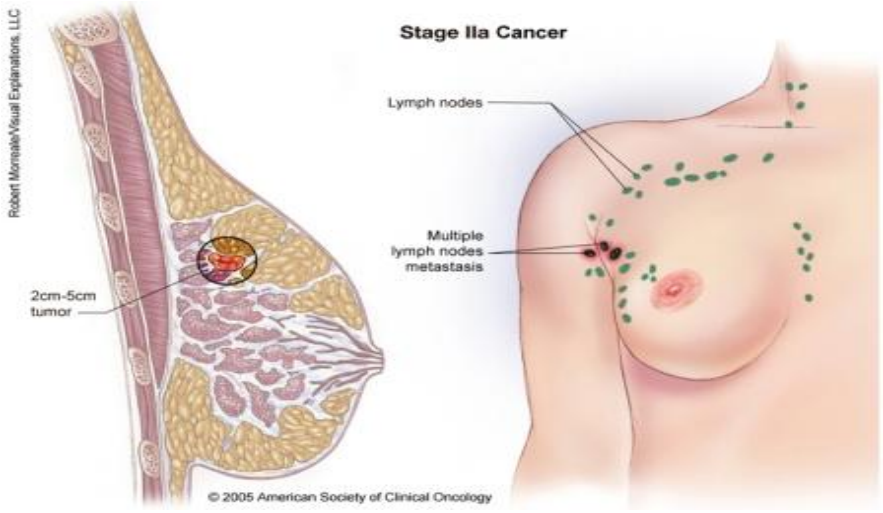

Fig 1.1: Breast cancer

1.1.1 Epidemiology

Breast cancer, the commonly occurring cancer in women comprises almost one third of all malignancies in females. As a cause of cancer mortality it is second only to lung cancer and is the leading cause of death for American women between the ages of 40 and 55 (Harris et al., 1992). The lifetime risk of a woman developing invasive breast

cancer is 12.6 % 2 one out of 8 females in the United States is under risk of developing breast cancer at some point in her life (Greenlee et al., 2001). The death rate for breast cancer has been slowly declining over the past decade, and the incidence has remained level since 1988 after increasing steadily for nearly 50 years. Twenty-five percent to 30% of women with invasive breast cancer are predicted to die of their disease (Harris et al., 1992). Mortality rates are highest in the very young (less than age 35) and in the very old (greater than age 75) (Smith et al., 1996). It appears that the very young have more aggressive disease, and that the very old may not be treated aggressively or may have comorbid disease that increases breast cancer fatality (Costanza et al., 2001). Although 60% to 80% of recurrences occur in the first 3 years, the chance of recurrence exists for up to 20 years (Shapira et al., 2001; McKay et al., 1992).

1.1.2 Pathology of breast cancer

Ninety-five percent of breast cancers are carcinomas, which arise from breast epithelial elements and are divided into 2 major types, in situ carcinomas and invasive (or infiltrating) carcinomas. The in situ carcinomas normally arise in either ductal or lobular epithelium, but remain confined there, with no invasion of the underlying basement membrane that constitutes extension beyond epithelial boundaries and is found that with such localized and confined malignancy, there is negligible potential for metastases. The extension of the ductal or lobular malignancy beyond the basement membrane constituting the epithelial border is considered invasive (or infiltrating) ductal or lobular carcinoma which leads to metastases and ultimately death (Richie et al., 2003).

1.1.3 Risk factors for developing breast cancer

Breast cancer incidence is highest in North America and Northern Europe and lowest in Asia and Africa. Studies of migration patterns to the United States suggest that genetic factors alone do not account for the incidence variation among countries, as the incidence rates of second-, third- and fourth-generation Asian immigrants increase

steadily in this country which implies that environmental and/or lifestyle factors appear to be important determinants of breast cancer risk (Costanza et al., 1991).

Gender is by far the greatest risk factor and it occurs 100 times more frequently in women than men. In women, incidence rates of breast cancer rise sharply with age until ages 45 to 50, when the rise becomes less steep (Smith et al., 1996) which probably reflects the impact of hormonal change (menopause) that occurs about this time and by ages 75 to 80, the curve actually flattens and then decreases. Although the incidence curve at younger ages is steep, the more important issue is the increasing prevalence of breast cancer with advancing age, and any breast mass in a postmenopausal woman should be considered cancer until proven otherwise (Cady et al., 1998).

Genetics plays a limited but vital role as a risk factor for breast cancer and 5% to 6% of breast cancers are considered hereditary (Malone et al., 1998). BRCA-1 and BRCA-2 account for an estimated 80% of hereditary breast cancer, but only represents 5% to 6% of all breast cancers. BRCA-1 and/or BRCA-2 positive women have a 50% to 85% lifetime risk to develop breast cancer, and a 15% to 65% risk to develop ovarian cancer which begins at age 25 (Haber et al., 2002). Familial breast cancer is also considered a risk if a first-degree relative develops breast cancer before menopause with affecting both breasts, or occurring in conjunction with ovarian cancer (Hoskins et al., 1995) and is a 2-fold relative risk in a woman with a single first degree relative (mother, sister or daughter) and the risk increased to 5-fold in a woman with 2 first-degree relatives having breast cancer (Greene, 1997).

A woman's hormonal history appears to be a risk factor due to the relative risk of breast cancer seems to be related to the breast's cumulative exposure to estrogen and progesterone. Early menarche (onset of menstruation , age 13), having no children or having them after age 30, and menopause after age 50 and especially age 55 lead to more menstrual cycles which results in greater hormone exposure (Grady, 2002). The Women's Health Initiative (WHI), a randomized controlled trial of 16,608 postmenopausal women comparing effects of estrogen plus progestin with placebo on chronic disease risk, provided the evidence that combined estrogen plus progestin use increases the risk of invasive breast cancer (2003 et al., 2003).

Hormone replacement therapy (HRT) users have a breast cancer risk of 53% higher for combination therapy and 34% higher for estrogen alone, especially in the use of more than 5 years. Although earlier studies suggested that this increased risk of cancer was offset by the fact that the cancers induced by HRT were of more benign pathology and had a more favorable prognosis (Smith et al., 1996), further studies on the WHI data reveals this impression to be incorrect. Invasive breast cancers associated with estrogen plus progestin use were larger (l.7 cm Vs 1.5 cm), had more possibility to be node positive (26% Vs 16%, p = 0.03), and were getting diagnosed at a significantly more advanced stage (regional/metastatic 25.4% Vs 16%). The percentages and distribution of invasive ductal, invasive lobular, mixed ductal, and lobular as well as tubular carcinomas had the similarity in the estrogen plus progestin group vs the placebo group (Chlebowski et al., 2003). Over observation within than a year, there was a statistically significant increase in breast density in the estrogen plus progestin group which resulted in increased incidence of abnormal mammograms (9.4% Vs 5.4%, p = 0.001) (Chlebowski et al., 2003). As noted by Gann and Morrow in a JAMA editorial, ‘‘the ability of combined hormone therapy to decrease mammographic sensitivity creates an almost unique situation in which an agent increases the risk of developing a disease while simultaneously delaying its detection’’ (Gann et al., 2003). Li et al reported that women using unopposed estrogen replacement therapy (ERT) had no notable increase in the risk of breast cancer. However, use of combined estrogen and progestin hormone replacement therapy provided an overall 1.7-fold (95% CI 1.3– 2.2) increased risk of breast cancer, including a 2.7-fold (95% CI 1.7–4.3) increased risk of invasive lobular carcinoma, a 1.5-fold (95% CI, 1.1–2.0) increased risk of invasive ductal carcinoma, and a 2-fold (95% CI 1.5–2.7) increased risk of ER1/PR1 breast cancers (Li et al., 2003).

Other risk factors for breast cancer include alcohol, which has been linked to increased blood levels of estrogen that interfere with folate metabolism providing protection against tumor growth. Women who drink 0.2 ounces of alcohol per day have 40% more possibility to develop breast cancer than women who drink no alcohol (Singletary et al., 2001).

The Nurses’ Health Study revealed that in postmenopausal women a weight gain of more than 45 pounds after age 18 had linked to an independent risk factor for breast

cancer (fat tissue produces hormones that are converted to estrogen) (Huang et al., 1997) which was stronger in postmenopausal women who had never taken estrogen replacement therapy. The relative risk to develop breast cancer was 1.6 with a 10–20 kg weight gain, and 2.0 with a weight gain of more than 20 kg, compared to women with minimal weight gain. In contrast, among women taking estrogen with weight gained did not have an increased risk of breast cancer. The differing effects of obesity and weight gain in premenopausal and postmenopausal women is due to obesity which decreases estradiol and progesterone concentrations in premenopausal women because of an increased frequency of anovulation (Potischman et al., 1996) leading to less circulating estrogen available to target tissues such as the breast.

The Nurses' Health Study also found that postmenopausal women who got at least 1 hour of physical exercise per week were 15% to 20% less likely to develop breast cancer compared to those with complete sedentary. In regularly exercising women, participating in a health-screening program in Norway, the reduction in risk was observed to be greater in premenopausal women than in postmenopausal women (relative risk 0.38; 95% CI 0.19–0.79) (Thune et al., 1997). The reason for the reduction of risk in exercising women may be due to delayed menarche in young girls involved in strenuous physical activity. Also, moderate levels of physical activity in premenopausal women are found to be associated with anovulatory cycles, which may also result in decreased risk (Briton et al., 1997).

Women treated for breast cancer have about a 1% greater chance per year to develop a new second cancer in either the treated breast or the other breast. Therefore, previous breast cancer is reported to be an accepted risk factor for development of breast cancer (Fisher et al., 1999). Ten percent of women with breast cancer have the tendency to develop a second breast cancer, and women with breast cancer have a 3 to 7 fold increased relative risk to develop cancer in the opposite breast. Women who received high doses of radiation to the chest before age 45 usually for Hodgkin's disease have significantly increased risk of breast cancer than adults. The most vulnerable ages is reported to be the pre-pubertal years of 10 to 14. These women should get yearly mammograms and clinical breast exams beginning either 10 years after the radiation treatments or by age 35 (John et al., 1993).

1.1.4 Relationship of benign breast disease with breast cancer

This is an issue of great concern for patients and physicians as there are conditions that confer no risk of malignancy and others that definitely lead to increased risk. Breast biopsies conferring no countable increased risk for malignancy include any lesion with non-proliferative change (Dupont and Page, 1995; Pike et al., 1993), which includes duct ectasia, and simple fibroadenomas, benign solid tumors containing glandular and fibrous tissue. Solitary papillomas are also benign lesions conferring no increased risk of future malignancy, although they are often (in 21 of 24 women in a single study) (Woods et al., 1995) with sanguineous or serosanguineous nipple discharge. Fibrocystic change (cysts and/or fibrous tissue without symptoms) or fibrocystic disease (fibrocystic changes occurring in conjunction with pain, nipple discharge, or a degree of lumpiness sufficient to cause suspicion of cancer) does not carry increased risk for cancer (other than the potential for missing a malignant mass) (Dupon et al, 1994). Some clinicians differentiate fibrocystic change or disease into those of hyperplasia, adenosis, and cystic change due to their differentiation into age distributions. Hyperplasia characteristically occurs in women in their 20s, often with upper outer quadrant breast pain and an indurated axillary tail, because of stromal proliferation. Women in their 30s exhibits solitary or multiple breast nodules 2–10 mm in size, due to the proliferation of glandular cells. Women in their 30s and 40s are found with solitary or multiple cysts. Acute enlargement of cysts may lead to pain, and because breast ducts are usually patent, nipple discharge is common with the discharge having the variation in color from pale green to brown (Fiorica, 1994). Conditions with getting increased risk of malignancy include ductal hyperplasia without atypia. This is the most commonly encountered breast biopsy result that definitely has the association with increased risk of future development of breast cancer and confers a 2-fold increased risk. The number, size and shape of epithelial cells lining the basement membrane of ducts are identified to be increased, but the histology does not fulfill criteria for malignancy.

The loss of expression of transforming growth factor-b receptor II in the affected epithelial cells is reported to be associated with an increased risk of invasive breast cancer (Gobbi et al., 1999). A number of other benign lesions are also found to confer a roughly 2-fold increased risk for development of breast cancer, which include

sclerosing adenosis, where lobular tissue undergoes hyperplastic change with increased fibrous tissue and interspaced glandular cells diffuse papillomatosis which is the formation of multiple papillomas, and fibroadenomas with proliferative disease, which are tumors that contain cysts greater than 3 mm in diameter, with sclerosing adenosis, epithelial calcification, or papillary apocrine alteration. Radial scars are found as benign breast lesions of uncertain pathogenesis, which are usually, discovered incidentally when a breast mass is removed for other reasons. Radial scars are determined by a fibroelastic core from which ducts and lobules radiate (Jacobs et al., 1999).

Atypical hyperplasia of either ductal or lobular cells, where the cells are normally uniform but have lost their apical-basal cellular orientation, confers a 4-fold increased risk unless there is also a family history of 1 or more first-degree relatives with breast cancer, where the risk is reported to increases to 6-fold. HER-2/neu is known as a proto-oncogene with intrinsic tyrosine kinase activity. Women with atypical hyperplasia with over-expression of HER-2/neu possess a greater than 7-fold increased risk of developing invasive breast carcinoma, as compared with women with non-proliferative benign breast lesions and no evidence of HER-2/neu amplification (Stark et al., 2000). Nipple discharge is often of concern to women as a sign of malignancy, but the reality is that non-bloody nipple discharge and bilateral nipple discharge are usually found to be benign causation. Women with papillomas often get bloody discharge. Nipple discharge is uncommon in invasive breast cancer and if present is invariably unilateral and is usually found to be associated with a palpable mass (Donegan, 1995). Similarly, breast pain is an uncommon manifestation of breast cancer. In a study of 987 women referred for breast imaging because of breast pain alone, only 4 women (0.4%) were found to be affected with invasive breast cancer, a number that was not different from a control asymptomatic group (Duijan et al., 1998).

1.1.5 Detection of breast cancer

As breast cancer rarely found to causes pain but a painless mass is much more worrisome for malignancy than is one causing symptoms. Mammography done yearly beginning at age 40 is the current recommendation for women with having no risk

factors (Smith et al., 2001). Despite mammograms detecting malignancy as small as 0.5 cm, 10% to 20% of malignancies elude detection by mammography, even when they occur at a much larger size (Donegan, 1992). In a patient with a solid and dominant mass (suspicious mass) the primary purpose of the mammogram is to screen the normal surrounding breast tissue and the opposite breast for non palpable cancers, not to make a diagnosis of the palpable mass (Cady et al,. 1998). That's why a negative mammogram is no guarantee of absence of malignancy, and a mass that does not disappear or collapse with aspiration lead to an assuming for being a malignancy and biopsied.

1.1.6 Diagnosing breast cancer: the biopsy

There are 3 established methods to obtain material from a suspicious breast lump. Fine-needle aspiration is not a reliable means of diagnosis, due to its inability to distinguish ductal carcinoma in situ from invasive cancer leading to a false-negative result. Fine needle aspiration (FNA) is generally reserved for palpable cyst-like lumps which are visible on a mammogram or ultrasound. False positives are negligible but false-negative results occur in 15% to 20%, which lead to the recommendation that if the cyst or lump doesn't disappear with FNA, further biopsy is mandatory (Cady et al,. 1998).

Core needle biopsy has found to be generally replaced fine needle aspiration in all cases except the obvious cysts. Core needle biopsies fail to identify areas of invasion in approximately 20% of cases that are originally diagnosed as ductal carcinoma in situ. Atypical ductal hyperplasia in a core needle biopsy is found to get a relatively high incidence of coexistent carcinoma (approximately 50%). This diagnosis, therefore, requires excisional biopsy (Bassett et al., 1997). 75% to 80% of excisional biopsies are predicted to be benign. Of the remaining 20% to 25% that reveal cancer, a second surgery is often demanded to ensure removal of all cancerous tissues. Axillary lymph node involvement is found to be the most important routinely-available predictor of relapse and of survival (Albertini et al., 1996). Axillary recurrence or tumor involvements in internal mammary or supraclavicular lymph nodes always demonstrate a poor prognosis (Donegan, 1997).

Sentinel lymph node biopsy is the biopsy of level I axillary lymph nodes. It contains a positive predictive value approaching 100%, with a sensitivity of 89% and a specificity of 100% (Krag et al., 1998). Three percent of positive sentinel nodes, however, are identified in non-axillary regions. There appears to be a 15% occurrences of ‘‘skip’’ metastases, defined as metastases to level II and III axillary nodes without involvement of level I nodes (Albertini et al., 1996). Thus, the cost of performing sentinel node biopsy alone is reflected in a study in which the 10- year survival rate of 85% for stage I breast cancer patients who have full axillary dissection decreases to 66% when axillary dissection was not performed (Bland et al., 1999). High nuclear grade (high nucleus-to-cytoplasmic ratio), high mitotic index and poorly differentiated all lead to poor prognosis. Infiltrating ductal carcinoma is by far the most common type of invasive breast cancer, with getting relatively poorer survival. Tubular, medullary, mucinous, and papillary cancers have a more favorable prognosis, but responsible for only 6% of invasive cancers (Donegan, 1997). Peritumoral lymphatic and blood vessel invasion results in a much poorer prognosis. Estrogen and/or progesterone receptor positive tumors are found to have a better prognosis and a better response to hormone treatment than receptor-negative tumors.

Flow cytometry measures DNA Index (or DNA content), with diploid cancer cells (normal DNA content, DNA index of 1) which have a better prognosis than those with aneuploidy (Hutter et al., 1991). S-phase fraction is referred to the number of cells actively synthesizing DNA. Tumors with high S-phase cells are found to have a poorer differentiation and poorer prognosis (Sigurdsson et al., 1990). Tumor marker CA 15–3 is reported to get increased in many women with metastatic breast cancer. HER-2/neu oncoprotein (also called c-erbB- 2) is found to be associated with shorter survival, shorter time-to-relapse, and an overall worse prognosis (Harris et al., 1992). This tumor marker becomes especially important with the introduction of trastuzumab for treatment. CA (Woods et al., 1992; Fiorica, 1994) is the first introduced FDA-approved (in June 1996) blood test for breast cancer recurrence (Keyomarsi et al., 1990).

1.1.7 Intraductal (ductal) carcinoma in situ (DCIS)

Intraductal (or ductal) carcinoma in situ (DCIS) is a special type of breast cancer with the proliferation of malignant epithelial cells which is confined to ducts, with no evidence of invasion through the basement membrane. Prior to mammography, DCIS was found to have an uncommon diagnosis. With the introduction of routine mammography, the age adjusted incidence of DCIS was reported to rise from 2.3 to 15.8 per 100,000 females, a 587% increase. A new case of invasive breast cancer was found to be increased 34% over the same time period (Eunster et al., 1996). About 85% of all intraductal cancers, often less than 1 cm is identified by the appearance of clustered micro calcifications on mammography. Other condition, including sclerosing adenosis and atypical ductal hyperplasia, is also found on mammography with micro calcifications. Morphology of the micro calcifications is reported to be the most important factor in differentiating benign from malignant calcification suggesting malignancy include heterogeneous clustered calcifications, fine linear branching calcifications, or calcifications in a segmental distribution. Magnification views of benign findings often exhibit multiple clusters of finely granular micro calcification, whereas those associated with DCIS usually appear to be coarse micro calcifications (Holland et al., 1994). For women getting poorly differentiated DCIS, the microscopic extent of disease correlates well with the radiographic extent. In contrast, the mammographic appearance of well-differentiated DCIS may sometimes substantially underestimate the microscopic extent. Residual micro calcifications on the post-surgery mammogram demonstrates residual tumor with a positive-predictive value of 65% to 70% (Aref et al., 2000). The likelihood of residual cancer increases to 90% if greater than 5 micro calcifications are found on post-operative mammography (Gluck et al., 1993). Occult invasion is more common if the lesion is clinically palpable in comparison with one found only by mammography.

Axillary node involvement in DCIS is found to be very uncommon. In a National Center Data Base review of 10,946 patients with DCIS who underwent axillary node dissection between 1985 and 1991, only 3.6% had axillary metastases (Winchester et al., 1995). In another series of 189 women with DCIS all of whom underwent axillary node dissection, none had positive nodes (Silverstein et al., 1994). Some experts have argued that presence of axillary lymph node metastases in DCIS means that the

pathologist missed the stromal invasion on initial reading of the pathologic material. Comedo-type DCIS is more malignant than other types of DCIS and is probably mid-way between DCIS and invasive cancer. Invasive breast cancer was ultimately found in 12 of 19 cases (63%) of DCIS with comedo necrosis, vs 4 of 36 (11%) without comedo necrosis (Patchefsky et al., 1989). An on-going controversy among breast surgeons and pathologists is the so-called micro-invasive DCIS lesion. The American Joint Committee on Cancer (AJCC) defines micro invasion as the extension of cancer cells beyond the basement membrane into adjacent tissues, with no focus more than 0.1 cm in greatest dimension. Lesions that fulfill such criteria are staged as T1mic, a subset of T1 breast cancer (Lippincott-Raven, 1997). Ideally, the term micro invasion in the breast should be applied in the same way as it is in the cervix, ie, to identify those invasive lesions of such limited extent that have virtually no risk of metastases. Unfortunately, the available data are inadequate to permit the reproducible identification of such a subset. In considering treatment of DCIS, mastectomy is nearly curative (98%) (Silverstein, 1993; Cataliotti et al.; 1993; Ward et al., 1992). Breast conserving therapy (‘‘lumpectomy’’) is almost as curative if certain criteria are met: the lesion is 3 cm, the histologic margins are negative, and the nuclear grade is low or intermediate, or at least certainly not high grade (Schwartz et al., 1999). Most commonly, breast-conserving surgery is followed by radiation. The rate of local failure in the treated breast is 16% at 15 years, with the median time to local failure being 5.0 years (mean 5.7 years, range 1.0– 15.2 years) (Solin et al., 2001). The importance of age and margin status in treating DCIS was revealed in a study of 418 women who underwent breast-conserving surgery (‘‘lumpectomy’’) and breast radiation. Recurrence occurred in 48 (11%) within 10 years. Recurrence developed in 24% of women who retrospectively had positive margins, 12% in women with unknown margin status, and 9% of women with negative margins. The likelihood of local recurrence is statistically related to age of the woman at initial diagnosis and surgery, with recurrences of 31% for those less than 39 years of age, 13% for ages 40–49, 8% for ages 50–59, and 6% for those older than age 60 (p5 0.0001) (Solin et al., 2001). When local recurrence does occur following lumpectomy and radiation for DCIS, roughly half of the women again have DCIS and half have invasive ductal carcinoma. Salvage therapy for recurrence usually consists of mastectomy (88%)

without adjuvant chemotherapy or tamoxifen (69%), and at 8 years post salvage treatment in 1 series, 92% of patients were alive and 88% were free of any evidence of recurrent disease. Favorable prognostic factors after salvage treatment were DCIS as the histology of the local recurrence and mammography only as the method of detection of the local recurrence (Solin et al., 2001). Interestingly, a diagnosis of DCIS vs the more ominous invasive ductal breast cancer does not automatically imply a simpler surgical solution. In 1 series, contraindications to breast preservation surgery were present in 33% of women with DCIS, compared to only 10% of women with stage I invasive ductal carcinoma (Morrow et al., 1998).

Two randomized clinical trials have compared where lumpectomy alone for DCIS with lumpectomy with radiation were applied (Fisher et al., 1998; Fisher et al., 1998). Both trials favored lumpectomy with radiation in regard to recurrence of malignancy (whether the recurrence was DCIS or invasive ductal disease), but overall survival of the 2 groups was similar (95% vs 94%), a reflection of the efficacy of salvage mastectomy. There appears to be a select group of patients with DCIS who have low histologic grade, absence of comedo- type necrosis and small tumor size, who can be managed with lumpectomy alone (Boyages et al., 1999). The time course to local failure is usually prolonged, and when local failure occurs, invasive cancer is present in the same one-half of cases as occurs with lumpectomy with radiation therapy (Solin et al., 2001; Fisher, 1999; Lagios et al., 1989). Tamoxifen is indicated for women with DCIS who have undergone either lumpectomy or lumpectomy with radiation. In a trial to specifically address this issue, 1804 women with DCIS undergoing breast conservation therapy were randomly assigned to receive either tamoxifen (20 mg daily for 5 years) or placebo. After a mean follow-up of 62 months, tamoxifen reduced the rate of invasive recurrence from 9 to 5 per 1000 patients (relative risk reduction 0.56, p 5 0.03) and reduced the rate of recurrent DCIS from 11% to 9% per 100 patients (relative risk reduction 0.82, p 5 0.043). Overall, the ipsilateral recurrence of either local or invasive cancer was reduced from 13% to 8% at 5 years in the tamoxifen group (Fisher et al., 1993).

1.1.8 Lobular carcinoma in situ (LCIS)

As it is difficult to diagnose clinically (it is never a palpable mass and it has no distinguishing mammographic features), the true incidence of LCIS is unknown (Pope et al., 1988). LCIS incidence in breast masses removed has varied from 0.05% to as high as 10% (Frykberg et al., 1988; Page et al., 1991; Asashi-Tanaka et al., 2000), and the incidence of LCIS is 10-fold higher in white compared to African-American women in the United States (Rosner et al., 1980). This diagnosis is always made incidental to a needle biopsy or resected mass done for fibrocystic change, fibroadenoma, or a mass suspected as being cancer (Morrow and Schnitt, 1995). LCIS is more often detected in premenopausal than postmenopausal women, suggesting a hormonal influence in the development or maintenance of these lesions (Schnitt, 2001; Walt et al., 1992). LCIS requires no specific therapy per se. Although the cells of LCIS are in fact small, well-differentiated neoplastic cells, they do not behave as a true malignant neoplasm in that these cells may distend and distort the terminal-lobular units, but invasion of and through the basement membrane does not occur, so the lesion never results in invasive breast malignancy. Rather, the clinical significance of LCIS is that it serves as an important marker for subsequent invasive breast cancer, in a magnitude of risk of approximately 1% per year for the remainder of the woman's life (7- to 10- fold higher risk of invasive breast cancer than the average US woman27), with the invasive cancer occurring with equal frequency in either breast. Subsequent invasive cancers are also more often of the infiltrating ductal type (Schnitt, 2001). The recommended management of LCIS is careful follow-up and semiannual physical breast exam and yearly mammography. The NSABP tamoxifen prevention trial (NSABP protocol P1) included 826 women with LCIS. At 4 years of follow-up, invasive breast cancer was less common in the tamoxifen arm (2% vs 4% with placebo, 5.7 vs 13 per 1000 women, a 56% risk reduction) (Fisher et al., 1998). However, many experts do not recommend tamoxifen in this group, citing the adverse effects of tamoxifen (hot flashes, an estrogen antagonist effect, and in postmenopausal women the increased occurrence of endometrial cancer and venous thromboembolism) and costs (tamoxifen is given in 20 mg tablets daily for 5 years).

1.1.9 Staging and prognosis of breast cancer

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

1.1.10 Surgical treatment of breast cancer

According to the Consensus Development Conference on the Treatment of Early-Stage Breast Cancer (June 1990, NCI) breast conservation treatment is an appropriate method of primary therapy for the majority of women with Stage I and Stage II breast cancers. This treatment is preferable in many cases because it provides survival equivalent to total mastectomy and axillary dissection while preserving the breast (JAMA, 1991). Subsequent studies have confirmed that there is no difference in long-term survival between surgical removal of the breast (mastectomy) and excision of the tumor mass and radiation therapy to residual breast tissue (breast conservation therapy) (Winchester et al., 1997; Lee-Feldstein et al., 1994). Breast-conserving surgery includes lumpectomy, re-excision, partial mastectomy, quadrantectomy, segmental excision, and wide excision. Axillary lymph nodes are removed for evaluation through a separate incision. The most common breast-removal procedure is a modified-radical mastectomy, which involves making an elliptical incision around an area including the nipple and biopsy scar, removing that section, and tunneling under the remaining skin to remove the breast tissue and some lymph nodes. Radical mastectomy, which removes the entire breast, chest wall muscles, and all axillary lymph nodes, is rarely done today because it offers no survival advantage over a modified radical mastectomy. A simple, or total mastectomy, removes the entire breast but none of the axillary lymph nodes. This is usually done for women with DCIS, or prophylactically for women at especially high risk for developing breast cancer. A newer procedure is the skin-sparing mastectomy, which involves removing the breast tissue through a circular incision around the nipple and replacing the breast with fat taken from the abdomen or back.

1.1.11 Adjuvant therapies for breast cancer

Radiation adjuvant therapy is given routinely after breast-conserving surgery (eg, lumpectomy) to prevent recurrence of cancer in the breast, and it may be used after mastectomy to prevent recurrence on the chest wall and axilla. Radiation therapy is generally given 5 days a week over a 5- or 6-week time span, with care taken to try to avoid damage to the heart or lungs. The only usual changes with breast radiation are skin erythema and possibly some transient lymphedema.

Systemic adjuvant chemotherapy is avoided for non-invasive, in situ cancer (DCIS). Hormone adjuvant therapy helps to prevent recurrence by blocking the effects of estrogen, which is known to stimulate cancer cell growth. Hormones are most effective in women whose primary tumor has hormone receptors (ie, estrogen-receptor or progesterone- receptor positive). Tamoxifen is the standard first choice of most experts (Lancet. 1998). Other hormonal therapeutic agents include aromatase inhibitors, which interfere with the enzyme aromatase, which plays a critical role in the production of estrogen in postmenopausal women. Examples of this class include anastrozole, letrozole and exemestane (Nabholtz et al., 2000; Mouridsen et al., 2001). A recent study of women who had received a 5 years of tamoxifen therapy and were assigned to either no therapy or continuing therapy with letrozole was prematurely ended when preliminary results revealed a greater than 40% reduction in recurrent breast cancers in the letrozole arm. Unanswered questions are whether women should take letrozole for 5 years (the original study design) or indefinitely, and whether women should take letrozole (or one of the other aromatase inhibitors) instead of tamoxifen initially. An earlier head-to-head comparison of anastrozole and tamoxifen found that it was somewhat more effective in reducing the risk of a recurrence than tamoxifen (Goss et al., 2003). Biological adjuvant therapy includes trastuzumab, which blocks the action of a growth promoting protein called Her-2/neu that is found in larger-than-normal amounts in about 30% of breast cancers (Pietras et al., 1994). Trastuzumab more specifically targets cancer cells and thus has fewer side effects than standard chemotherapy, although it may have some effects on normal heart tissue when used with chemotherapy (Cobleigh et al, 1999). The drug has been approved for metastatic breast cancer and is currently under study as a first-line agent in combination with other chemotherapy (Vogel et al., 2002).

Table1.3: Standard Adjuvant Chemotherapy Regimens

Standard Regimens	Components
AC (w or w/o T)	Adriamycin(doxorubicin), cyclophosphamide,Taxo
CMF	Cyclophosphamide, methotrexate, fluorouracil (5-FU)
CEF	Cyclophosphamide, epirubicin, fluorouracil (5-FU)
CAF	Cyclophosphamide, adriamycin (doxorubicin), fluorouracil (5-FU)

1.1.12 Prevalence of breast cancer

Prevalence of breast cancer is found to be higher in the more developed regions than the less developed regions but the death rate is high in the less developed regions and it is higher in the IARC membership (24 countries) (Fearly et al., 2012).

Table-1.4: 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

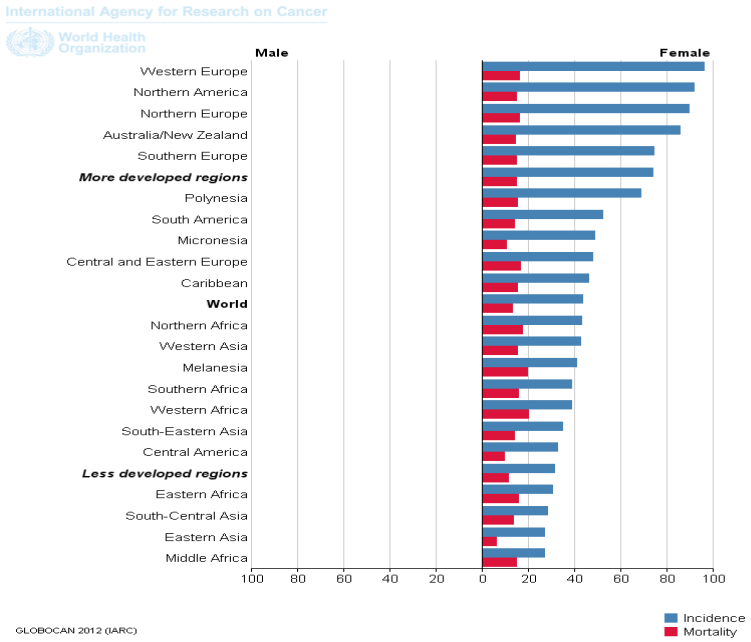

Fig 1.2: Breast Cancer Estimated Incidence Worldwide in 2012

1.1.13 Breast cancer in Bangladesh

Prevalence of breast cancer is very high in Bangladesh and it is in second in number and next to the lung cancer.

Table 1.5: 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 system	461	0.7	0.7	376	0.9	0.6	621	0.4	1.2
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.

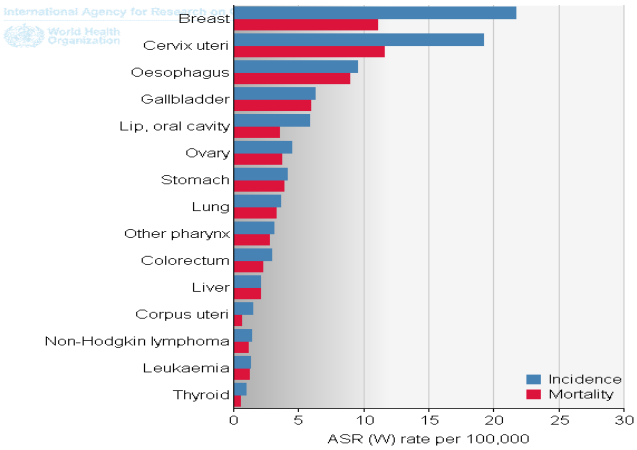

Fig1.3: Estimated age-standardised incidence and mortality rates: women

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

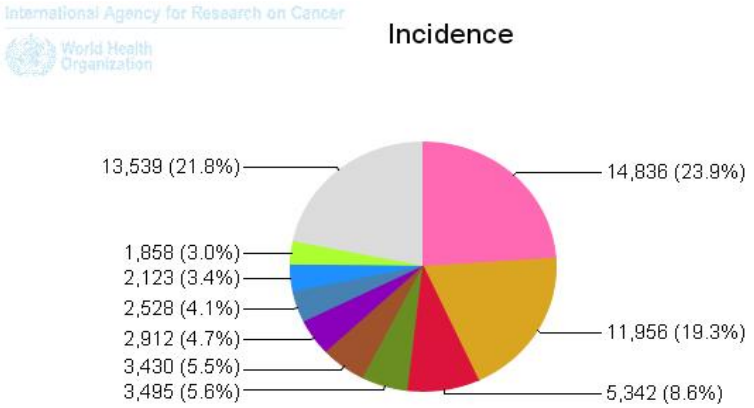

Fig 1.4: Incidence of breast cancer in Bangladesh (2012)

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

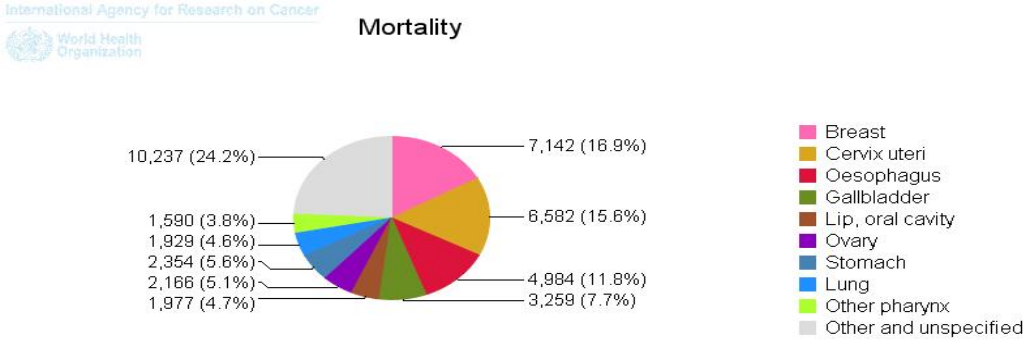

Fig 1.5: Mortality rate of breast cancer in Bangladesh (2012)

5-year prevalence rate of breast cancer in Bangladeshi women is found to be 35.6% in 2012 which is higher than any other cancers (Fearly et al., 2012).

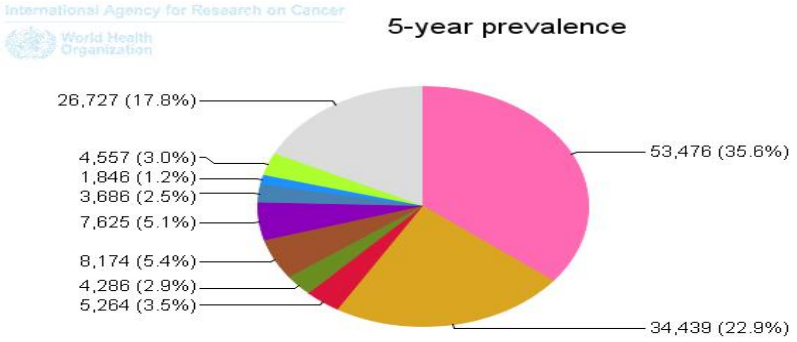

Fig1.6: 5-year prevalence of breast cancer in Bangladesh (2012)

1.2 Pharmacogenetics

Although modern pharmacotherapy has improved profoundly, it still faces many challenges such as adverse drug reactions, sometimes serious or even lethal, and non response to standard therapy. The observed prominent variability in individual response to pharmacotherapy, in part, depends on well-known factors easily assessable, like age, sex, weight, liver and renal function, co-medication, heterogeneity in the disease, nutritional state or smoking. Furthermore, inherited variants in drug-metabolizing enzymes (DMEs), transporters, receptors and molecules of signal transduction cascades may have a major impact on drug response. The last thirty years have seen unprecedented international research programs endeavoring to identify polymorphisms that are either causative of, or affect the susceptibility to, human disease. The programs are beginning to have a visible impact on medical care in a growing number of ways. Firstly, in the late 80s and 90s, identification of causative mutations of monogenic diseases and the subsequent correlation studies linking phenotype and genotype have resulted in much greater definition of the sub classification of these diseases. The resulting genetic sub classification of many of these diseases has allowed clarification of their phenotypic heterogeneity and pathogenesis (Weatherall, 2001). This knowledge has greatly facilitated implementation of effective prevention programs around the world, significantly reducing the impact and burden of some of these diseases.

Pharmacogenetics aims at understanding how genetic variation contributes to variations in response to medicines. "Pharmacogenetics" is the study of the heritable basis of individual differences in response to pharmaceutical agents (Weinshilboum et. al., 1999; Nebert, 1999a; Nebert and Dieter, 2000). "Pharmacogenomics" is a concept that has recently created 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). Pharmacogenetics is the study of how genetic variations influence a person's response to drugs. These variations underlie the response to therapy, including possible adverse effects. It also deals with the assessment of clinical efficacy and the pharmacological phenotype. These are the central tenets of pharmacogenetics. Some health care leaders view pharmacogenetics as providing 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 genetics. 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 contribute significantly to an individual's drug response. The variation that exists 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. This can lead to different responses to these drugs. Measuring the DNA differences can thus predict the variation in response to the medicine (Roses, 2000).

Genetic diversity provides a good contribution to both disease susceptibility and variability in response to drug therapy. Pharmacogenomics is a discipline focused on examining the genetic basis for individual variations in response to therapeutics (Zimmet, 1992; Dinneen et. al., 1992). Although the task of developing individualized medicines tailored to patient's genotypes poses a major scientific challenge, pharmacogenomics is already starting to influence how physicians / scientists design clinical trials and its impact on the practice of medicine is forthcoming (Kaprio et. al., 1992; Weyer et. al., 1999). Recent evidence suggests 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 that regulate 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 (Committee on diabetic twins, 1988; Medici et. al., 1999).

Pharmacogenetics and Pharmacogenomics give us an especial young field of research in the domain of pharmacology. Both work on genetic variations which occur in individuals resulting reduced drug efficacy and more adverse drug reactions. Pharmacogenetics, emphasizes the diversity of patients and their genetic background, set their response to a given drug therapy, making understood the biological variability whereas pharmacogenomic considers the effects they cause in an individual (patient) different medications. The differences are studied on gene expression induction and repression of genes.

The specific type of gene that is expressed by an individual will dictate the molecular subtype of protein that gene expresses. Depending upon subtle molecular variations in that protein (e.g., for an enzyme, receptor, or growth factor), this hypothetically alters the efficiency of information processing in brain circuits and, thus, dictates differences in behaviors mediated by those circuits (Grossman, 2007). Changing neurotransmission at these circuits with drugs acting by specific mechanisms may have different functional interactions within these circuits; this can theoretically determine whether the drug alters information processing there and, subsequently, whether it works to reduce symptoms (Stahl, 2008).Three of the most clinically important CYP450 drug metabolizing enzymes are CYP2C9, CYP2C19, and CYP2D6. Together, these three enzymes metabolize up to 40% of all currently prescribed drugs. Testing for a panel of genetic variants in each of these CYP450 genes allows the prediction if a person will have impaired or increased metabolism of drugs processed by these enzymes (Daly et. al., 1995). This knowledge can help to individualize drug selection and dosing based on individual's genetic makeup. The Pharmacological phenotype defined the response of the individual or group of individuals with common genetic characteristics to a particular drug substance. Genotypes with dedicated single microarray assay or gene chip variations of genes opened new avenues to study the metabolism of secretion and transport of drugs. In

addition, the method of microarrays is most appropriate for the analysis of many polymorphisms simultaneously, which is necessary in pharmacology. In the past, systematic research into the basis of adverse drug reactions has been hampered by the fact that these events are rare and individuals are difficult to trace and study while sufferings are action. The ability to conduct genetic research retrospectively, at the end of a clinical trial or after a medicine has been launched, using stored samples of DNA, gives researchers a powerful new tool to explore how medicines work.

1.2.1 Genetic Polymorphism

Polymorphism is a term which literally provides the meaning of variability of form, shape, size, structure and composition and it has a currency in a wide variety of disciplines in science and art. Genetic polymorphism is a much more specific term describing frequent variation at a specific locus in a genome. A useful practical definition implies 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 responsible for producing a metabolising enzyme has a variant allele with the arbitrary frequency of 1% (Meyer, 2000). For many such genes single nucleotide polymorphisms (SNP) exist and an allelic site may have more than one SNP. Genotype is the detailed gene structure of an individual whereas the more commonly measured phenotype is the outcome of metabolism of a drug in an individual. Genetic Polymorphism is a difference in DNA sequence among individuals, groups, or populations. Sources include SNPs, sequence repeats, insertions, deletions and recombination. (e. g., a genetic polymorphism might give rise to blue eyes versus brown eyes, or straight hair versus curly hair). Genetic polymorphisms may 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 called a genetic mutation. Changes in DNA sequence which have been confirmed to be caused by external agents are also generally called "mutations" rather than "polymorphisms". The mutations in the CYP genes can cause enzyme products with abolished, reduced, altered or increased enzyme activity. Abolished enzyme activity is commonly seen

where the whole gene has been deleted, but has also its origin in mutations causing altered splicing, stop codons, abolished transcriptional start sites and deleterious amino acid changes. Mutations in substrate recognition sites (SRS) can cause the synthesis of enzymes with an altered substrate specificity as seen with CYP2D6*17 found entirely in black African populations and with CYP2C9*3. Furthermore, mutations in the folding region might lead to an altered protein folding and different substrate specificity as seen with CYP2D6*10 (Fukuda et. al., 2000). Mutation is one of the factors causing DNA polymorphisms, and which therefore contributes to disease onset. DNA polymorphisms may be due to the deletion, insertion, or substitution of a nucleotide, may occur at coding or non-coding regions of the DNA, and may or may not alter gene function. The occurrence of DNA polymorphism makes it possible to associate a person's response to drugs with particular DNA regions, for example, by correlating the occurrence of the polymorphism with the response. This is the basis of current phamacogenetics, which is the study of the impact of individual genetic variants on drug response.

Genetic Mutation is a alteration in the nucleotide sequence of a DNA molecule. Genetic mutations are 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 indeed suggested 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 may affect the intracellular stability of the mRNA gene transcript (Quirk et al., 2004).

1.2.2 Single Nucleotide Polymorphism

SNPs within the coding regions of a gene which cause 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 a single base mutation in DNA and a source variance in a genome. SNPs are 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 thus multiple, as are their effects. Depending on their location within the genome and their patterns of co-occurrence (i.e. haplotypes), 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. These effects of SNPs can, as is the case with many other phenotypic characteristics, greatly affect the manner in which a patient responds to drug therapy.

1.2.3 Personalized medicine

Personalized medicine is the conventional approach to pharmacotherapy to prescribe drugs for an individual based on population studies and clinical trials. If a particular drug was not effective or had unwanted side effects, the dosage may be adjusted or alternative drug may be used. Finding an effective drug and dosage may, therefore, take several months. Physicians must rely heavily on their clinical experience and population-based studies. Therapeutic drug monitoring in blood has been used for years to guide clinicians in cases where maintaining safe and effective blood levels for the patient were critical and achievable. On the other hand, if clinicians could predict which drug would be effective and at what dose, then pharmacotherapy could be individualized. This is the goal of pharmacogenetics. Pharmacogenetics has the promise of removing much of the uncertainty of the reliability of the drugs. Physicians will be able to use a medicine response profile to predict an individual's likely response before a medicine is prescribed (Rawlins and Thompson, 1991). Although not immediately obvious, the pharmaceutical industry and the public should also benefit by faster and more efficient clinical trials, more treatments for more patients, reduced costs of drug development, expansion of research to cover more

diseases, and improved drug surveillance (Meyer, 2000). Over the past decade, advanced research into genomics (the study of an organism's genes) and proteomics (the study of the proteins that genes create or "express") has accelerated our understanding of individual differences in genetic makeup, opening the door to a more personalized approach to healthcare. The science of genomics and proteomics has the potential to personalize healthcare, enabling providers to match drugs to patients based on their genetic profiles, identify who is susceptible to which health conditions, and determine how a given patient will respond to a particular therapy (a field known as pharmacogenomics). That could eliminate unnecessary treatments, minimize the potential for adverse events, and ultimately, improve patient outcomes. The pharmacogenomics, developed in these 10 years, already permitted the identification of the patients with side drug effects risk by detection of the presence of SNPs from enzymatic systems implied in drugs metabolism such as CYP450 (Guţiu et al., 2010). Current drug development and patient treatment strategies target large patient populations as homogeneous groups on the basis of population means, irrespective of the potential for variation among patients. This "one drug fits all" method of drug development and use is often neither effective nor safe, with the consequence of high costs to the health-care system. Evidence suggests that, in a significant proportion of patients (ranging from 30% to 60%), many important classes of therapeutic drugs show no clinically significant efficacy, resulting in unnecessary costs to the health-care systemand failure to effectively treat disease in individual patients. Morbidity, mortality, and economic costs associated with the occurrence of adverse drug reactions also represent a large burden on the health-care system, representing the fourth leading cause of hospitalization and being responsible for roughly 100,000 deaths per year in the United States, with an estimated annual cost to the health-care system ranging from $30 to $150 billion. Pharmacogenomics testing attempts to predict how an individual will respond to a drug based on their genetic makeup. The two main areas to which pharmacogenomics can be applied are drug metabolism and responsiveness. The primary way in which drugs are metabolized is through the cytochrome P450 (CYP) isoenzyme system found mostly in the liver. Most drugs are metabolized by at least one CYP; many are metabolized by multiple

CYPs. Specific dosing guidelines based on pharmacogenetic data are being developed and are beginning to appear in the literature to predict adverse drug reactions (ADR). Medicine personalizing overcame the experimental-theoretical frame. Recently, Food and Drug Administration imposed the labeling of warfarin vials with the mention that it would be indicated for the patient, prior to drug administration, to perform a pharmacogenomic test in order to reduce drug therapy risks. In fact, currently, in the U.S.A. there are around 120 drugs for human use labeled with mentions on pharmacogenomic data, as concluded by the study of Frueh et al. (2008). New technologies permitting parallel genetic testing (testing for many genetic variations) developed near the end of the 20thcentury (Fodor, 1997), and mapping of the human genome was completed in 2000. Both brought hope for a new era in medicine (Mc Kusick, 2001). One of the major technological breakthroughs that allowed the genetic revolution was the introduction of computerized genotyping systems such as the Affymetrix Gene Chip (Fodor, 1997). Currently, more advanced forms of these types of DNA microarray technologies (Koch, 2004), including the Illumina Bead Array platform (Steemers and Gunderson, 2007), allow testing more than a half million SNPs at a cost of less than $1000 per sample, and further price reductions are in sight.

The therapeutic as well as side effects of a drug may vary according to genetic make-up of an individual. This is particularly important for drugs with narrow therapeutic index, or a wide dose response curve. Because of genetic variation in the drug metabolizing capacity, a predisposed individual may show one of the following variant responses:

i. Lack of efficacy at normal drug dose, requiring higher dose to achieve the expected therapeutic response.
ii. Much higher effect at the normal dose leading to development of significant side effects which are otherwise expected only at the higher dose.

For these individuals, a lower dose of the drug may be effective and acceptable. If the number of such individuals in the population is large (which may vary from population to population) then even an otherwise good drug may be discarded as ineffective or too toxic.

The historical precedents for modern personalized medicine, which stretch back several decades, but clearly momentum is building now for a more rapid

transformation. Segmenting populations into groups of patients who have a greater likelihood of responding to a particular treatment or avoiding side effects is changing the dynamic of drug development and the practice of medicine, and creating opportunities to introduce new business and health care economic models. These changes are beginning to take place as the field builds a solid track record, which demonstrates that it can:

a. Shift emphasis in medicine from reaction to prevention;
b. Select optimal therapy and reduce trial-and-error prescribing;
c. Make drugs safer by avoiding adverse drug reactions;
d. Increase patient adherence to treatment;
e. Improve quality of life;
f. Revive drugs that failed in clinical trials or were withdrawn from the market;
g. Help control the overall cost of health care.

1.3 Pharmacogenomics of drug metabolizing enzyme and transporter

The new era of personalized medicine, which integrates the uniqueness of an individual with respect to the pharmacokinetics and pharmacodynamics of a drug, holds promise as a means to provide greater safety and efficacy in drug design and development. Personalized medicine is particularly important in oncology whereby most clinically used anticancer drugs have a narrow therapeutic window and exhibit a large interindividual pharmacokinetic and pharmacodynamic variability. This variability can lead to therapeutic failure or severe toxicity. Understanding of how genetic variations influence drug disposition and action could help in tailoring cancer therapy based on individual's genetic makeup. Pharmacogenomics is the study of how variations in the human genome affect the response to medications. Each drug, after it enters the body, interacts with numerous proteins, such as carrier proteins, transporters, metabolizing enzymes, and multiple types of receptors. These protein interactions determine drug pharmacokinetics (i.e., drug absorption, distribution, metabolism, and excretion) and pharmacodynamics (i.e., target site of action, pharmacological and toxicological effects).

Moreover, drugs trigger downstream secondary events which may impact additional gene or protein expression responses and can also vary among patients. As a result,

the overall response to a drug is determined by the interplay of multiple genes that are involved in the pharmacokinetic and pharmacodynamic pathways of a drug. In general, important genetic variation in drug effect can be envisioned at the level of drug metabolizing enzymes, drug transporters, and drug targets.

1.3.1 Drug metabolizing-enzymes

Drug metabolizing enzymes are proteins that catalyze the biochemical modifications of xenobiotics (eg, drugs) and endogenous chemicals (e.g., hormones, neurotransmitters).

Broadly, drug metabolizing enzymes are divided into two categories: Phase I (functionalizing) enzymes that introduce or remove functional groups in a substrate through oxidation, reduction, or hydrolysis; and Phase II (conjugating) enzymes that transfer moieties from a cofactor to a substrate. Essentially all of the major human metabolizing enzymes exhibit genetic polymorphisms at the genomic level, and many of these enzymes have clinically relevant genetic polymorphisms (Evans and Relling, 1999). A gene is considered to be polymorphic when the frequency of a variant allele in a specific population is at least 1%.

1.3.2 Phase I enzymes

Phase I metabolizing enzymes include those involved in:

• Oxidation – cytochrome P450, alcohol dehydrogenase, aldehyde dehydrogenase, dihydropyrimidine dehydrogenase, monoamine oxidase, and flavin-containing monooxygenase;

• Reduction – nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase and reduced cytochrome P450;

• Hydrolysis – epoxide hydrolase, esterases, and amidases.

1.3.3 Phase II enzymes

The most important Phase II enzymes that exhibit functional and clinical relevant genetic polymorphisms are uridine diphosphate glucuronosyltransferase (UGT), sulfotransferase (SULT), glutathione S-transferases (GST), N-acetyltransferase (NAT), and thiopurine methyltransferase (TPMT).

1.3.4 Glutathione S-transferases (GST)

The super family of human GST catalyzes the conjugation of glutathione (GSH) to a wide range of endogenous metabolites and xenobiotics including alkylating and free radical generating anticancer drugs (Lo and Ali-Osman, 2007). Human GSTs are categorized into three main families: cytosolic/nuclear, mitochondrial, and microsomal. The cytosolic GSTs are further divided into seven classes: alpha, mu, omega, pi, sigma, theta, and zeta. Besides their enzymatic function, GSTs also possess nonenzymatic functions, in which they act as regulators of cell signaling and post-translational modification pathway in response to stress, growth factors, and DNA damage, and in cell proliferation, cell death, and other processes that ultimately lead to tumor growth and drug resistance. These multiple functionalities establish the importance of GSTs as determinants of cancer susceptibility, therapeutic response, and prognosis ((Lo and Ali-Osman, 2007). Most human GSTs have SNPs and, less frequently, deletions.

1.3.5 Drug-transporters

In addition to drug metabolizing enzymes, uptake and efflux transporters that facilitate the movement of drugs in or out of the cell are important determinants of drug disposition and response. Broadly, drug transporters are classified into two families, namely efflux transporters of the adenosine triphosphate (ATP)-binding cassette (ABC) family and uptake transporters of the solute carrier (SLC) family. In the ABC transporter family, 49 genes have been identified and classified into seven subfamilies from ABCA through ABCG based on the sequence homology (http://nutrigene.4t.com/ humanabc.htm). The ABC transporters are responsible for transport of diverse substrates out of the cell using ATP as an energy source. Among these, ABCB1, ABCC1/2, and ABCG2 have been well characterized for their roles in drug disposition and response. In the SLC family, 360 genes have been identified and classified into 46 subfamilies (http://www.bioparadigms.org/slc/menu.asp). Of particular relevance to drug disposition are members of the organic anion transporting polypeptides (OATP), organic cation transporter (OCT), and organic anion transporter (OAT) subfamilies. Table 3 summarizes the pharmacologically most important efflux ABC transporters (including ABCB1, ABCC1/2, and ABCG2) and uptake SLC

transporters (including OATP, OCT, and OAT families), their tissue distributions, and representative drug substrates. These transporters play crucial roles in the intestinal absorption, biliary excretion, renal excretion, and tissue/cellular penetration of a wide variety of therapeutic drugs, and therefore they are important determinants of drug exposure in the system and at the site of action. Genetic polymorphisms may influence the expression, subcellular localization, substrate specificity, and/or intrinsic transport activity of the transporter proteins and therefore, influence the disposition and response of drug substrates. The following sections serve to highlight the functional and clinical significance of the most commonly naturally occurring genetic polymorphisms within the pharmacologically most important ABC and SLC transporters with respect to drug disposition and response. A comprehensive list of genetic variants in the ABC and SLC transporters and related information are available in Pharmacogenetics Research Network databases at http://www.pharmGKB.org.

1.4 The drugs: Cyclophosphamide, epirubicin, fluorouracil

1.4.1 Cyclophosphamide

Cyclophosphamide (CP), an oxazophosphorine, bifunctional DNA alkylating agent, that is incorporated into the treatment of most pediatric and adult malignancies and was one of the first nonhormonal agents to show anti-tumor activity in humans (Colvin, 1999). At lower doses, CP shows a potent immunomodulatory effect and is used as second-line therapy in many autoimmune disorders (Colvin, 1999). Like all chemotherapies, variation in the efficacy and toxicity associated with CP exist. A better understanding of the pharmacogenetic factors influencing the variation in response and toxicity to CP offers the ability to individualize treatment. To date, most, if not all, studies have taken a candidate gene approach, focusing on known targets involved in CP bioactivation and/or detoxification; however, a whole-genome approach may allow for a more comprehensive, unbiased approach to identify factors important in CP clinical activity.

Cyclophosphamide is a prodrug that goes into the liver and is metabolized by the hepatic P450 system to both active and inactive compounds. N-dechloroethylation of CP, mediated primarily by CYP3A4/3A5, gives 2-dechloroethylcyclophosphamide,

which is generally believed to have no cytotoxic effects, and the neurotoxic chloroacetaldehyde (Ludeman, 1999). Oxidation at the C-4 position of CP generates 4-hydroxycyclophosphamide; this reaction is mediated by various isoforms including CYP2A6, 2B6, 2C8, 2C9, 2C19, 3A4 and 3A5 (Ludeman, 1999). 4-hydroxycyclophosphamide interconverts with aldophosphamide, which undergoes further chemical decomposition by fragmentation to phosphoramide mustard, the active anti-tumor metabolite, and acrolein, a metabolite responsible for urotoxicity (Cox, 1979). Detoxification of the metabolites of CP occurs mainly through NADPH-mediated oxidation by various aldehyde dehydrogenases (ALDH1A1 and ALDH3A1) (Parekh and Sladek, 1993). Another detoxification pathway includes the conjugation of CP with glutathione by various glutathione S-transferases (GSTs; GSTA1, GSTM1, GSTP1 and GSTT1) (Hayes and Pulford, 1995). GST-mediated conjugations of various CP metabolites with glutathione have been reported, but the significance of enzyme catalysis in these reactions is unclear as they readily occur spontaneously (in the absence of enzymatic intervention) (Shulman-Roskes, 1998). Pharmacogenetic studies in both the ALDH and GST genes have discovered polymorphisms important to response and/or toxicity associated with CP-based therapies. DNA repair proteins including MGMT and ERCC (Cai, 1999; Andersson et al., 1996), and efflux of CP and its metabolites out of the cell (MRP) (Qiu et al., 2004) have been shown to be important; however, the role of genetic variants within these genes has not been extensively evaluated. Cyclophosphamide has a relatively narrow therapeutic index, and adverse effects include cardiotoxicity, nephrotoxicity, neurotoxicity, infertility, bladder toxicity, myelosuppression and leukemogenesis. Both toxicity and response to CP is quite variable. Pharmacogenetics offers clinicians the ability to individualize therapy based on a patient's risk of untoward effects as well as their likelihood of response. This brief review aims to highlight the clinically significant pharmacogenetic discoveries pertinent to CP-based treatment regimens.

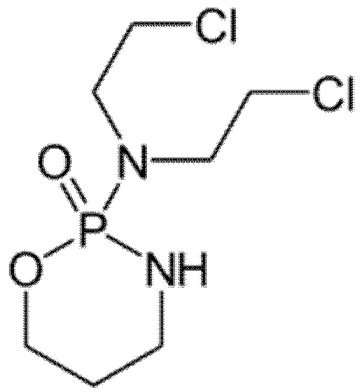

Fig1.7: Cyclophosphamide structure

Cyclophosphamide pathway

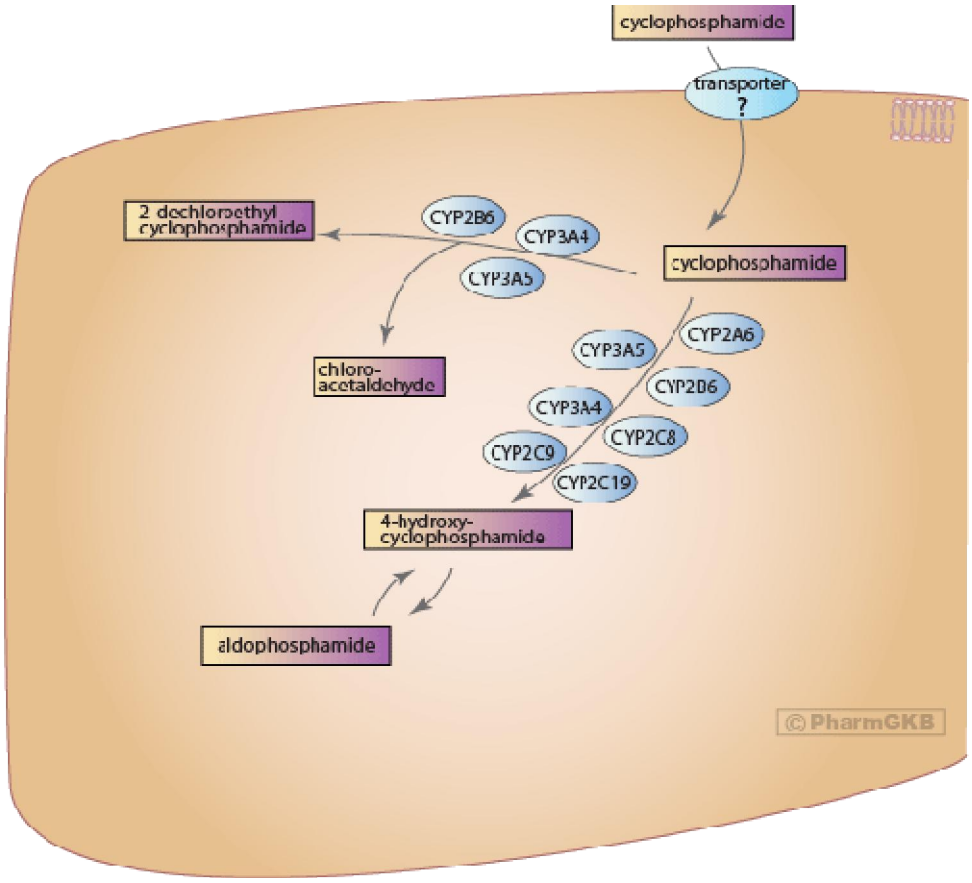

Fig 1.8 a: Cyclophosphamide pathway

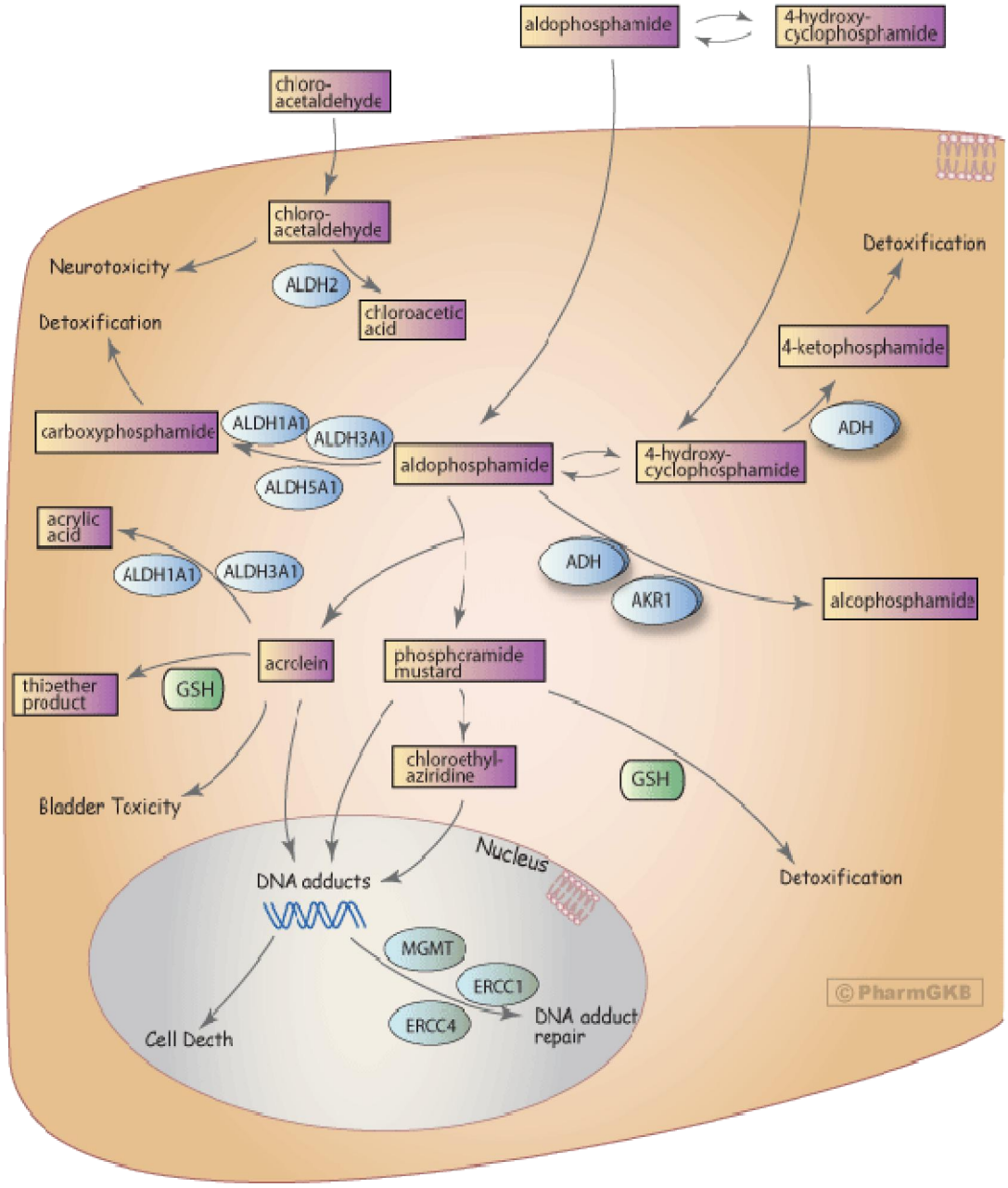

Fig 1.8 b: Cyclophosphamide pathway

1.4.2 Epirubicin

Epirubicin an important anthracycline drug used in combination with other medications to treat breast cancer in patients who have had surgery to remove the tumor. Similarly to other anthracyclines, epirubicin acts by intercalating DNA strands. Intercalation results in complex formation which inhibits DNA and RNA synthesis and also triggers DNA cleavage by topoisomerase II, which is resulting in mechanisms that lead to cell death. Binding to cell membranes and plasma proteins may be involved in the compound's cytotoxic effects. Epirubicin also generates free radicals resulting in cell and DNA damage.

Epirubicin is found to be favored over doxorubicin, the most popular anthracycline, in some chemotherapy regimens as it appears to cause fewer side-effects. Epirubicin possess a different spatial orientation of the hydroxyl group at the 4' carbon of the sugar - it has the opposite chirality - which may account for its faster elimination and reduced toxicity. Epirubicin is primarily used against breast and ovarian cancer, gastric cancer, lung cancer and lymphomas.

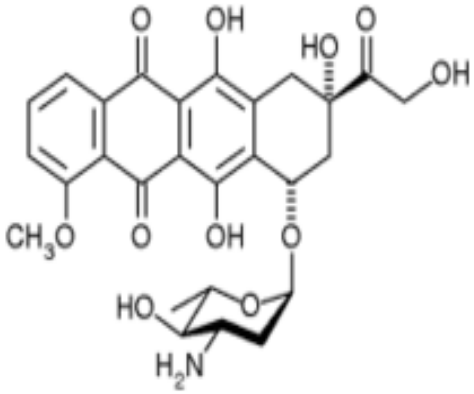

Fig 1.9: Structure of Epirubicin

The mechanism of action of epirubicin found to be related to its ability to bind to nucleic acids. It forms a complex with DNA by intercalation between base pairs, resulting in inhibition of DNA and RNA synthesis (McEvoy, 2005). Intercalation also triggers DNA cleavage by topoisomerase II, which is resulted in cytocidal activity (McEvoy, 2005) where binding to cell membranes and plasma proteins may also be involved. Epirubicin also reported to generate cytotoxic free radicals (McEvoy, 2005). Epirubicin is the 4'-epimer of doxorubicin; i.e., there is a different spatial orientation of the hydroxyl group at the 4' carbon of the sugar moiety (McEvoy, 2005). This difference may account for faster elimination and reduced toxicity.

1.4.3 5-Fluorouracil (5-FU)

5-Fluorouracil (5-FU) is still a widely used anticancer drug which plays an important role in the treatment of colon cancer and is used for patients with breast and other cancers, like those of the head and neck (Grem, 2000). 5-FU is a heterocyclic aromatic organic compound with a structure similar to that of the pyrimidine molecules of DNA and RNA; an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen (Rutman et al., 1954). Only one crystal structure is reported in the literature for pure 5-FU, in which the compound crystallizes with four molecules in the asymmetric unit and the molecule adopts a hydrogen-bonded sheet structure (Hulme et al., 2005). Due to its structure, 5-FU interferes with nucleoside metabolism and can be incorporated into RNA and DNA, leading to cytotoxicity and cell death (Thomas and Zalcberg, 1998). Over the past 50 years, despite its many advantages, clinical applications have been greatly limited due to drug resistance. The overall response rate for advanced colorectal cancer of 5-FU alone is still only 10–15% (Giacchetti et al., 2000), and the combination of 5-FU with other anti-tumor drugs has merely improved the response rates to 40–50% (Douillard et al., 2000). Therefore, new strategies for therapy and resistance reversal are urgently needed. Meanwhile, understanding the mechanisms by which tumors become resistant to 5-FU is an essential step towards predicting or overcoming that resistance. Fortunately, the development of microarray techniques offers us a chance to identify new genes

which have key roles in drug resistance. Now, we can move forward to investigate the mechanism of these molecules, which might contribute to clinical chemotherapy in the future.

In mammalian cells, 5-FU is converted to fluorodeoxyuridine monophosphate (FdUMP), which forms a stable complex with thymidylate synthase (TS) esulting in inhibition of deoxythymidine monophosphate (dTMP) production. dTMP is essential for DNA replication and repair and its depletion which therefore, causes cytotoxicity (Longley et al., 2003). Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumor cells. Up to 80% of administered 5-FU is broken down by DPD in the liver (He et al., 2008).

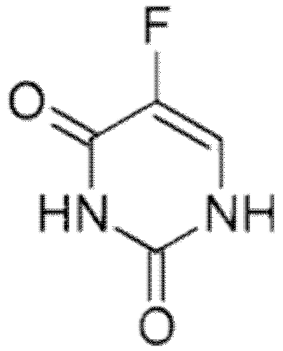

Fig 1.10 Structure of 5-FU

1.5 The gene: GSTP1 and ABCC4

Phase II metabolising enzymes have a advantage of carrying electrophilic groups intrinsically in a structure, or obtained from phase I metabolism, for making conjugation of xenobiotics with donor molecules like glutathione (GSH), UDP glucuronic acid, or 3_-phosphoadenosine-5_- phosphosulfate (PAPS). The glutathione S-transferase (GST) family of phase II metabolizing enzymes possessing catalytic properties for detoxifying endogenous reactions with GSH to protect cellular macromolecules from damage resulted from the toxic effect of a wide range of

endogenous and exogenous molecules such as cytotoxic, mutagens, carcinogens, and chemotherapeutic agents. The resulting glutathione adducts get increased property of the solubility and polarity leading to either excretion or further metabolism.

The 17 human cytosolic GST subunits are divided into seven gene families in accordance with their biochemical characteristics as well as amino acid sequence similarities: (GSTA), (GSTM), (GSTT), (GSTP), (GSTO), (GSTZ), and (GSTS) (Mannervik et al., 2005). Human GSTs have nearly a widely expression, and GSTP1, the most abundant subunit is found ubiquitously in different human epithelial tissue (Terrier et al., 1990). GSTP1 gets conjugation to provide protection particularly against the cytotoxic effects of different chemotherapeutic agents, such as anthracyclines, alkylating agents, and their metabolites (D'Al`o et al., 2004). The GST gene families, specially the glutathione S-transferase P1 (GSTP1), contain several polymorphic loci and have been found to have functional polymorphisms which are frequently present among general populations (Rebbeck, 1997). The role of GSTs in the detoxification of antitumor agents indicates the possible implication of GST polymorphisms to the response of the chemotherapeutic agents. Patients with a variant GSTP1 genotype are believed to show impaired activity in the detoxification of environmental genotoxic agents and chemotherapeutic drugs, which push forth the hypothesis that allelic variation in the gene associated with less effective detoxification of potential anticancer agents may result in an enhanced sensitivity to chemotherapy.

In addition to the enzyme, drug transporters are reported to play a vital role in determining drug absorption, drug distribution to tissues, and drug excretion in the urine and bile. Some studies have exhibited that the conjugates that are formed with the help of GSTs are transported by ABCC protein, the part of the phase III biotransformation system, in an ATP-dependent manner (Haimeur et al., 2004). Furthermore, GSTP1 is suspected to have the synergistical participation with ABCC transport as a tri-GSH conjugates (Leslie et al., 2004).

The multidrug resistance protein 4 (MRP4), which is encoded by ABCC4, a member of the superfamily of ATP-binding cassette (ABC) transporters has been implicated in the transport of antiviral agents, such as the nucleoside/nucleotide analogs azidothymidine (AZT), adefovir [9-(2-phosphonylmethoxyethyl) adenine or PMEA],

tenofovir, lamivudine, and ganciclovir (Imaoka et al., 2007), anticancer drugs [methotrexate, 6-mercaptopurine, 6-thioguanine, camptothecins (Tian et al., 2005), as well as endogenous molecules, such as prostaglandins, steroids, bile acids, cyclic nucleotides, and folate (Denk et al., 2004). MRP4 is widely expressed, with a high expression in the prostate and in hematopoietic stem cells and blood cells (Su et al., 2004) (http://symatlas.gnf.org/SymAtlas/). It is also found in the kidney proximal tubules (van Aubel et al., 2002), in the liver (Rius et al., 2003), and in the brain (Leggas et al., 2004). It is interesting that its localization in most tissues is apical, but basolateral localization has been demonstrated in brain choroid plexus epithelial cells, in prostate, and in hepatocytes (Leggas et al., 2004). Similar to most efflux transporters, no disease has been directly linked to altered MRP4 activity. Recently, a study with ABCC4_/_ mice showed that the absence of this transporter did not induce obvious abnormalities. However, it resulted in acute PMEA toxicity, suggesting a protective role of MRP4 in the bone marrow, spleen, thymus, and gastrointestinal tract. Moreover, these data suggested that MRP4 may reduce the passage of PMEA and probably other nucleotide analogs into the brain (Belinsky et al., 2007). This is in agreement with a previous report stating that ABCC4_/_ mice accumulated more topotecan in the brain and cerebrospinal fluid than wild-type mice (Leggas et al., 2004). Therefore, its physiological role could include detoxification of drugs, as well as that of endogenous molecules. With respect to endogenous substrates, up-regulation of MRP4 in the liver of rats and humans with obstructive cholestasis provides a mechanism to eliminate excess bile salts (Denk et al., 2004; Gradhand et al., 2008). Although ABCC4 is a highly polymorphic gene (Saito et al., 2002), few data are available concerning the function of its variants. Recent studies have investigated the functional effects of several ABCC4 single-nucleotide polymorphisms (SNPs) on drug disposition. Anderson et al. (2006) showed a 20% increase in lamivudine-triphosphate intracellular concentrations in patients carrying the 4131T_G variant, whereas the 3724G_A variant was associated with a trend for elevated AZT-triphosphate, suggesting a reduced MRP4 efflux function (Anderson et al., 2006). It is interesting that the 4131T_G variant is in the 3_-untranslated region (UTR) of the gene, whereas the 3724G_A variant is synonymous and there is no clear mechanism explaining these effects. In another study, no association was observed

between two nonsynonymous and seven synonymous ABCC4 variants and tenofovir disoproxil fumarateinduced renal proximal tubulopathy (Izzedine et al., 2006). Most recently, 74 genetic variants in ABCC4 were shown to have no effect on MRP4 mRNA and protein expression in Caucasian cholestatic and noncholestatic patients (Gradhand et al., 2008).

1.6 Justification of the study

Most of the patients with breast cancer were treated with chemotherapy where Anthracycline and cyclophosphamide (CPA) based chemotherapy regimen is commonly used as by the recommendation of National Comprehensive Cancer Network (NCCN) clinical practice guidelines of breast cancer (Zhang et al., 2011).

Cyclophosphamide (CPA) is frequently used anticancer drug acombined with other chemotherapeutic agents; like anthracyclin (adriamycin, epirubicin) termed the CA regimen, with methotrexate and 5-fluorouracil called CMF, with adriamycin and 5-flurouracil known as CAF, or only with 5-fluorouracil named as CF (Pritchard et. al, 2006). The CPA-based combination treatment has been known to be vary much effective for breast cancer, but most of the cases it cause adverse drug reactions (ADRs), such as anemia, leukopenia/neutropenia, and gastrointestinal symptoms such as vomiting, anorexia and nausea (http://www.cancercare.on.ca/pdfdrugs/cyclopho.pdf). CPA is administered orally as a prodrug that requires metabolic activation to 4-hydroxycyclophosphamide (4-OH-CPA) by CYP2B6 and CYP2C9 as well as to a lesser extent by CYP3A4 and CYP3A5 in the liver to exert its effect (Zhang et. al, 2005 Chang et. al, 1993; De Jonge et. al, 2005).

The 4-OH-CPA goes rapid interconversion with its tautomer, aldophosphamide and then spontaneous degradation to form phosphoramide mustard that provides therapeutic activity. Both 4-OHCPA and aldophosphamide are cytotoxic that are detoxified by glutathione (GSH) conjugation with the help of multiple glutathione S-transferases (GSTA1, GSTM1, GSTP1 and GSTT1) and aldehyde dehydrogenase (ALDH1A1 and ALDH3A1) to carboxycyclophosphamide (Dirven et al., 1994; Moreb et al., 2005) and these metabolites passively diffuse out of hepatic cells, circulate, and then passively go into other cells (Scripture et. al, 2005). Furthermore, it has been reported that transporters such as ABCC2 (also known as MRP2) (Qiu et. al,

2004) and ABCC4 (also known as MRP4) (Tian et al, 2005) are also involved in transportation of CPA and its metabolites.

Most of the drug-metabolizing enzymes and transporters which are genetically polymorphic might cause a large interindividual difference in the plasma concentration of drugs. In addition, anticancer therapies are notoriously reported due to having a narrow therapeutic range; a higher concentration in the patient's body causes toxicity and a lower concentration reduces the efficacy of the drugs. Hence, the role of pharmacogenomics, which is expected to provide a predictive way for severe drug toxicity, comes to the front line.

The toxicity profile of the regimen containing cyclophosphamide-epirubicin-5-fluorouracil is characterized by myelosuppression (anemia, nutropenia leucopenia) cardiotoxicity, gastrointestinal toxicity and urotoxicity (Siew-Kee et al., 2009; Sonam et al., 2013). Through intercalation with DNA, eventually inducing DNA cleavage by topoisomerase II epirubicin (EPI) may produce a cytotoxic effect.

Phase II metabolising enzymes have a advantage of carrying electrophilic groups intrinsically in a structure, or obtained from phase I metabolism, for making conjugation of xenobiotics with donor molecules like glutathione (GSH), UDP glucuronic acid, or 3_-phosphoadenosine-5_- phosphosulfate (PAPS). The glutathione S-transferase (GST) family of phase II metabolizing enzymes possessing catalytic properties for detoxifying endogenous reactions with GSH to protect cellular macromolecules from damage resulted from the toxic effect of a wide range of endogenous and exogenous molecules such as cytotoxic, mutagens, carcinogens, and chemotherapeutic agents (Strange et al., 2001). The resulting glutathione adducts get increased property of the solubility and polarity leading to either excretion or further metabolism.

The 17 human cytosolic GST subunits are divided into seven gene families in accordance with their biochemical characteristics as well as amino acid sequence similarities: _ (GSTA), _ (GSTM), (GSTT), (GSTP), (GSTO), (GSTZ), and (GSTS) (Mannervik et al., 2005). Human GSTs have nearly a widely expression, and GSTP1, the most abundant subunit is found ubiquitously in different human epithelial tissue (Terrier et al., 1990). GSTP1 gets conjugation to provide protection particularly against the cytotoxic effects of different chemotherapeutic agents, such as

anthracyclines, alkylating agents, and their metabolites (D'Al`o et al., 2004). The GST gene families, specially the glutathione S-transferase P1 (GSTP1), contain several polymorphic loci and have been found to have functional polymorphisms which are frequently present among general populations (Rebbeck, 1997). The role of GSTs in the detoxification of antitumor agents indicates the possible implication of GST polymorphisms to the response of the chemotherapeutic agents. Patients with a variant GSTP1 genotype are believed to show impaired activity in the detoxification of environmental genotoxic agents and chemotherapeutic drugs, which push forth the hypothesis that allelic variation in the gene associated with less effective detoxification of potential anticancer agents may result in an enhanced sensitivity to chemotherapy.

In addition to the enzyme, drug transporters are reported to play a vital role in determining drug absorption, drug distribution to tissues, and drug excretion in the urine and bile. Some studies have exhibited that the conjugates that are formed with the help of GSTs are transported by ABCC protein, the part of the phase III biotransformation system, in an ATP-dependent manner (Haimeur et al., 2004; Rebbeor et al., 2002). Furthermore, GSTP1 is suspected to have the synergistical participation with ABCC transport as a tri-GSH conjugates (Leslie et al., 2004).

Neoadjuvant (preoperative) chemotherapy provides an opportunity for the direct assessment of tumor response and both the adjuvant and neoadjuvant chemotherapy help us to estimate the toxicity to therapy without interference of other treatments.

On the basis of these different types of preclinical and clinical data, we made the hypothesis that the genetic polymorphisms in the major drug-metabolizing enzyme and transporter involved in cyclophosphamide-epirubicin-5-flurouracil predict interindividual variability in the treatment response as well as toxicity. To test this hypothesis, we examined the effect of genetic polymorphisms in GSTP1 and ABCC4 genes on the response and toxicity of the therapy with 256 patients recruited from different public and private hospitals of Bangladesh, received neoadjuvant (n=117) and adjuvant chemotherapy (n=102) of cyclophosphamide-epiubicin-5-flurouracil (CEF) regimen.

Besides no pharmacogenetic study have yet done in Bangladeshi population and the studies on the effect of GSTP1 gene polymorphism on the response and toxicities

produced by CEF regimen are conflicting. Further more, No study on the relationship of ABCC4 gene with the chemotherapy response can be cited which gave us the motivational force to go through the study.

CHAPTER TWO

MATERIALS AND METHODS

2. Materials and Methods

2.1 Subject Selection

Two hundred and nineteen Bangladeshi cases with histologically proven invasive breast carcinoma were recruited from different private and public hospitals of Bangladesh (Ahsania Mission Cancer and General Hospital, Dhaka Medical College Hospital, Bangabandhu Sheikh Mujib Medical University and Delta Medical College and Hospital) from the mid of 2009 to the end of 2013. The patients were treated with cyclophosphamide-epirubicin-5-fluorouracil (FEC) based chemotherapy (102 adjuvant and 117 neoadjuvant) and genetic study was conducted in the laboratory of pharmacogenetics and pharmacokinetics of the Departments of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh. A written informed consent has been obtained from all participants of the study and ethical approval is got from the respective hospital. Patients were staged according to the classification of TNM staging and the treatment was provided as per standard institutional multi-modal protocols involving appropriate surgical treatment, radiation therapy, chemotherapy, hormone treatment, and biological therapy as appropriate for individual patients.

2.2 Response assessment and toxicity evaluation criteria

Clinical response of tumor was estimated according to the Response Evaluation Criteria in Solid Tumors (RECST) criteria (Therasse et. al, 2000) where complete response is defined as disappearance of tumor for at least four weeks; at least a 30% decrease of the longest diameter of tumor for more than 4 weeks is determined as partial response; progressive disease is termed as at least a 20% increase of the longest diameter of tumor; neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease is evaluated as stable disease. The sixth edition of American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging system (Greene et. al, 2002) was applied for evaluation of both clinical stages before chemotherapy and pathological response of primary tumor and axillary lymph nodes after the treatment. Chemotherapy induced toxic reaction was assessed according to the Common Terminology Criteria for Adverse Events (CTCAE v4.0)(Cancer Therapy Evaluation Program, Common Terminology Criteria

for Adverse Events, Version 4.0, DCTD, NCI, NIH, DHHS 2003. *http://ctep.cancer.gov)*

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2.3 Study End Point

Prospective study was done to evaluate the role of the GSTP1 and ABCC4 polymorphism in the response of intact tumor to NACT with 117 patients. Response Evaluation Criteria in Solid Tumors (RECIST) (Therasse et al., 2000) was applied to asses the Tumor response after 3 weeks from the completion of three cycles chemotherapy. Patients were divided into two groups as responders (complete + partial response) and non-responders (static + progressive disease).

In the second part of the study, the role of the GSTP1 polymorphism on Cyclophosphamide-epirubicin-5-fluorouracil (FEC) based chemotherapy was evaluated on 219 (102 adjuvant and 117 NACT) patients. Patient showing drug induced hematological toxicities and gastrointestinal toxicities were assessed according to the Common Terminology Criteria for Adverse Events. The highest grade toxicity occurred during the course of treatment of an individual patient was taken as a tool for the analysis.

2.4. Materials

2.4 .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

2.4.2 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

2.4.3 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	Wako Pure Chemicals Ltd., Japan
Sample Loading Dye,6x	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'-Methylenebisacrylamide)	Sigma Chemical Company, USA
Ammonium Persulfate (APS)	Sigma Chemical Company, USA
TEMED (N, N, N', N'-tetramethylethylenediamine)	Sigma Chemical Company, USA

2.4.4 Restriction Enzymes (REs)

Genes	RE	Recognition sites	Source
GSTP1	*Bsm*AI	**5'…….GTCTC(N)$_{1}$……3'** **3'…….CAGAG(N)$_{5}$……5'**	New England BioLabs® Inc., USA
ABCC4	*HpAII*	**5'……..……C↓CGG……..3'** **3'……GGC↑C……5'**	New England BioLabs® Inc., USA.

→ Cutting site

2.4.5 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

2.4.6 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.

2.5 Genomic DNA isolation

2.5.1 Venous blood collection

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

2.5.2 Genomic DNA Isolation

There are many differing protocols and a large number of commercially available kits used for the extraction of genomic DNA from whole blood. In this study we isolated DNA by using Daly's Method (Daly *et al.*, 1998). This procedure is routinely used in both research and clinical service provision in our laboratory and is cheap and robust.

2.5.2.1 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	Room Temperature

	to it and the total solution was made up to 1L by adding distilled water.	
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

2.5.2.2 Genomic DNA isolation procedure

1. 3 ml blood was taken in a 50 ml Falcon centrifuge tube containing 2 mg of EDTA.
2. 20 ml Lysis Buffer was added to it and it was mixed gently for 2 minutes by inversion. It 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 supernatant was discarded into a bottle containing enough savlon. The pellet was collected.
4. 2 ml Nuclear Lysis Buffer and 0.5 ml of 5 M Sodium Perchlorate were added to it.
5. Then the tube was mixed in a rotary mixture at room temperature for about 15 min so that pellet was dissolved completely.
6. Then the sample tube was incubated at 65°C for 30 min. (Heidolph Unimax-2010 Incubator, Wolf Laboratories Limited, UK).
7. Then 2.5 ml of chilled Chloroform was added to it.
8. Then it was mixed in a rotary mixture for 10 min at room temperature.
9. Then the tube was centrifuged at 1500 rpm for 5 min. (37°C).

10. The DNA containing phase (uppermost phase) was transferred to a fresh autoclaved 15 ml polypropylene tube using a disposable Pasteur pipette.
11. Two volumes of Ethanol (double that of DNA phase) was added to it.
12. It was then mixed immediately by slow gentle inversion until all cloudiness was disappeared.
13. DNA was seen to come out of the solution as a white 'cotton-wool' pellet.
14. The white 'cotton-wool pellet' was collected with a disposable microbiology loop.
15. The loop was air dried.
16. The DNA was dissolved in 5 mM Tris-HCl Buffer contained in a 1.5 ml screw cap tube.
17. Then the tube was kept at 65°C overnight.
18. Then it was taken back and was stored in Freezer.(-40°C)

2.5.2.3 Quantification of genomic DNA

The quantity and purity of DNA isolated from blood samples were evaluated by using a UV Spectrophotometer (UV Prove v2.1) at 260 nm. To ensure complete sample homogeneity, samples were very gently shaken on a vortex shaker for approximately 30 minutes before measurements were taken. A sample volume of 1.5 to 2 µl was pipetted onto the fibre optic measurement surface. Working solutions of genomic DNA were made up to a standard concentration of 50 ng/µl with Nuclease free water, except in cases where the sample had an initial concentration of less than 50 ng/µl, in which case an undiluted aliquot was taken as a working solution.

For calculation of DNA concentration of samples free of 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 out of this range is not acceptable due to the contamination (i.e., protein) in the DNA sample that may inhibit subsequent reactions. The purity and integrity of isolated genomic DNA were also assessed by means of Agarose Gel Electrophoresis. A sample volume of 5 µl (50-70 ng/µl) was resolved on a 1% (w/v) agarose gel.

2.5.3 Genotyping of single nucleotide polymorphisms (SNPs) of GSTP1 and ABCC4

To facilitate the accurate genotyping of the DNA samples for the selected SNPs, PCR-RFLP was employed due to its affordability, ease of use and reliability. This method of genotyping entails the restriction enzyme (REase) digestion of polymerase chain reaction (PCR) amplification product. The subsequent digestion or lack of digestion, of PCR amplified product due to the presence or absence of an SNP within the REase recognition site allows for accurate and reliable genotyping and the consequent determination of SNP frequencies within a sample cohort.

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

2.5.4 DNA amplification using PCR

The relevant genomic target regions, which contain the SNPs of interest, were amplified by means of primer-directed PCR using thermostable DNA polymerase originally described by (Saiki *et al.,* 1985; Saiki *et al.*, 1988). This primer-directed PCR method facilitates the in vitro amplification of single-copy genomic DNA sequences by a factor of more than ten million with extremely high sequence specificity.

2.5.5 Primer design

There are some guidelines for primer design:

• PCR primers should be generally 15-30 nucleotides long.

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

• Should avoid placing more than three G or C nucleotides at the 3'-end to reduce the risk of non-specific priming.

• Should avoid primer self-complementarity or complementarity between the primers to avoid hairpin formation and primer dimerization.

• Should examine possible sites of non-desirable complementarity between primers and the template DNA.

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

By considering all the factors, 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 and melting point

No.	Allele	Primer sequence	M.T (°C)	Size (bp)
1	GSTP1 **FP**	5-ACCCCAGGGCTCTATGGGAA-3	69.5	20
2	GSTP1 **RP**	5-TGAGGGCACAAGAAGCCCCT-3	66.6	20
3	ABCC4 **FP**	5-GTGCACAGGGTTCCAATTTC-3'	61	20
4	ABCC4 **RP**	5'- AGAGCAAAACCCAGGCAGTA-3'	63	20

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

Primers were procured from Bio Basic Inc, USA.

2.5.6 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 GSTP1 and ABCC4 alleles with their respective lengths are showed in Table-2.2.

Table 2.2: PCR conditions to synthesize GSTP1 and ABCC4 alleles and their respective lengths.

Allele	PCR conditions (35 cycles)	Size of PCR products (bp)
GSTP1	94^0C 30 sec 57^0C 30 sec 72^0C 1 min	176
ABCC4	94^0C 30 sec 57^0C 30 sec 72^0C 30 sec	226

2.5.7 Restriction enzyme digestion

After PCR amplification, 10 µl of the PCR products of GSTP1 and ABCC4 were digested with approximately 2 units of *BsmA*I *and HpAII* respectively 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 GSTP1 and ABCC4 alleles

Allele	REs	Digestion conditions	Expected fragments (bp)
GSTP1	*BsmA*I (5000 U/ml)	Incubation at 37° C overnight	**NH** 176 **HE** 83, 93, 176 **MH** 83, 93
ABCC4	*HpAII* (5000 U/ml)	Incubation at 55° C overnight	**NH** 96, 130 **HE** 96, 130,226 **MH** 96, 130

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

2.5.8 Visualization of PCR products and REase digestion fragments

PCR amplified products were visualized by means of agarose gel electrophoresis for size estimation. REase digestion fragments of sufficient size (>100 bp) and size differential between fragments (>30 bp) were also visualized on agarose gel. EZ Load™ Molecular ruler (100 bp) was used for size estimation of PCR amplification

products serving as confirmation that amplification of the desired genomic target region had occurred, as well as for quantification of PCR product prior to REase digestion reactions. EZ Load™ 100 bp DNA ladder was also applied for size estimation of all REase digestion fragments to allowing for accurate and reliable genotyping of samples. All agarose gels were visualized on the UV transilluminator (Alpha Innotech Corporation, San Leandro, California).

2.5.9 Gel electrophoresis

Electrophoresis is a method of separating substances on the basis of the rate of movement under the influence of an electric field. Agarose gel electrophoresis of DNA is used to estimate the presence and distinguish the type of nucleic acids obtained after extraction and to evaluate digestion products.

Agarose is a polysaccharide that is purified from seaweed. An agarose gel is produced by suspending dry agarose in a buffer solution, boiling until the solution to be clear, and then pouring it into a casting tray allowing it to cool resulting in a flexible gelatin-like slab. During electrophoresis, the gel is allowed to submerse in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is passed through the pores of the gel by the electrical current.

Under an electrical field, DNA is moved to the positive electrode (red) and away from the negative electrode (black). Several factors influence the rate of the DNA movement, including the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move faster through the agarose than larger molecules. DNA in the gel is visualized by the use of Ethidium Bromide that is added to the gel. Ethidium bromide binds to DNA and illuminates when exposed to ultraviolet light that cause the DNA to 'glow'. All PCR products and REase digestion fragments were resolved by electrophoresis in 2% and 3% (w/v) agarose gel respectively at 80 volts (V).

2.5.10 Agarose gel electrophoresis procedure

All agarose gels were made with and resolved in 1X tris acetate ethylenediaminetetraacetic acid (TAE) buffer made and stored as a 10X stock solution and diluted to the required working concentration as required. To facilitate the visualization of DNA within the agarose gel under UV light, 1 µg of ethidium

bromide (EtBr) per ml agarose solution was added -i.e. 0.01% (v/v) EtBr stock solution (10 mg/ml).

2.5.10.1 Casting a gel

1. An appropriate volume of 1X Tris-acetate-EDTA (TAE) buffer with an appropriate amount of agarose (these values are determined based on the gel dimensions and the desired percentage of agarose) was allowed to mix in a conical flask. The flask was swirled to evenly distribute the agarose.
2. Then the solution was heated in the microwave oven for 1 minute. The flask was removed from the microwave oven (before it boiled over), swirled, and reheated while keeping constant watch to be sure it did not boil over.
3. The flask was allowed to cool and poured when the temperature of the solution was 55-65^0 C.
4. The gel apparatus was prepared for casting the gel while cooling.
5. Prior to pouring the gel, Ethidium bromide was added to the agarose and swirled to mix.
7. The gel was poured into the casting tray adjusting the comb to keep the wells perpendicular. The gel was allowed to cool and harden (20-30 minutes) prior to use.

2.5.10.2 Preparing the gel for electrophoresis

8. A few ml of 1X TAE buffer was added to the well area of the gel and then the comb was carefully removed by pulling straight up.
9. The electrophoresis tank was filled with buffer solution (1X TAE) placing the gel (In the casting tray) on the tank platform.

2.5.10.3 Preparing samples for loading/running the gel

10. An appropriate volume of loading dye (6X) was used to the sample (1 µl of 6X sample dye for every 5 µl of sample).
11. The sample was loaded with the help of a 1-10 µl micropipette. The marker was also loaded at Lane-1.

12. After the loading the gel, the cover was gently placed on the apparatus and the power leads were hooked up. The power was then adjusted to 80 volts (constant voltage). The gel was run until the migration of the first dye front (bromophenol blue) about two-thirds the length of the gel and the migration of the second dye front (xylene cyanol) about one-third of the length of the gel.
13. The gel was then placed on the UV transilluminator to visualize the DNA.

2.6. PCR-RFLP OF GSTP1

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACAYGGTGAAT
GACGGCRTGGAGGACCTCCGCTGCAAATACRTCTCCCTCWTCTAYACCAACTATGW
RAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGAACAAAGAAAGGGGC
TTCTTGTGCCCTCA

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACA
YGGTGAATGACGGCRTGGAGGACCTCCGCTGCAAATACXTCTCCCTCWTC
TAYACCAACTATGWRAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGA
ACAAAGAAAGGGGCTTCTTGTGCCCTCA

Red---------->Primer sequence Green ---------->Other possible SNP

Red---------->Exone sequence Yellow----------> SNP of interest

After completing PCR amplification with appropriate reagents a PCR product of *GSTP1 was* obtained. The PCR product size was 176 bp and this was visualized in 2% (w/v) agarose gel.

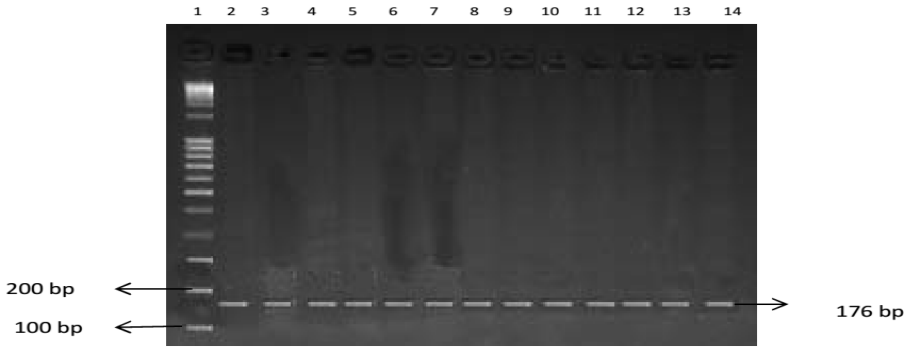


Fig: 2.1 PCR product of *GSTP1* (176 bp) (Lane 2 to 14 (2% agarose gel) (Lane-1 contains Molecular ruler)

2.6.1. Fragmentation Pattern

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

Table 2.4 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 2.5: Type of nucleotide changes, cutting sites and fragments of the allele in case of *GSTP1*

Change	Fragments	Type
When X= A in both chromosomes (A/A)	83, 93	Normal Homozygote
When X= G in one chromosome (A/G)	83, 93, 176	Heterozygote
When X=G in both chromosome	176	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 in the both chromosome and only one fragment with 176bp will be obtained and this is considered as normal homozygote.

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGAC

No BsmA1 recognition site

AYGGTGAATGACGGCRTGGAGGACCTCCGCTGCAAATACATCTCCCTC

WTCTAYACCAACTATGWRAGCATCTGCACCAGGGTTGGGCACKGGGR

GCTGAACAAAGAAAGGGGCTTCTTGTGCCCTCA

Restriction enzyme BsmA1

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGAC

AYGGTGAATGACGGCRTGGAGGACCTCCGCTGCAAATACATCTCCCTC

WTCTAYACCAACTATGWRAGCATCTGCACCAGGGTTGGGCACKGGGR

GCTGAACAAAGAAAGGGGCTTCTTGTGCCCTCA (No digestion, One fragment of 176 bp)

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

When X=G in one of the sister chromosome, there will be one cutting site at 93 bp. So, there will be 3 fragments (83, 93 and 176bp) for two sister chromosomes.

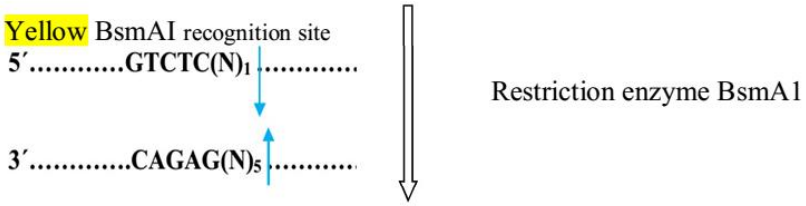
ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGAC

BsmA1 recognition site

AYGGTGAATGACGGCRTGGAGGACCTCCGCTGCAAATACGTCTCCCTC

WTCTAYACCAACTATGWRAGCATCTGCACCAGGGTTGGGCACKGGGR

GCTGAACAAAGAAAGGGGCTTCTTGTGCCCTCA



ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGAC

AYGGTGAATGACGGCRTGGAGGACCTCCGCTGCAAATACG (Fragment 1

93 bp)

TCTCCCTCWTCTAYACCAACTATGWRAGCATCTGCACCAGGGTTGGGC

ACKGGGRGCTGAACAAAGAAAGGGGCTTCTTGTGCCCTCA (Fragment 2

83 bp)

Fragment 3: 176 bp (For uncut sister chromosome)

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 between at 93 bp in both of the chromosomes and two fragments with 83 and 93 will be obtained

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGAC

BsmA1 recognition site

AYGGTGAATGACGGCRTGGAGGACCTCCGCTGCAAATACGTCTCCCTC

WTCTAYACCAACTATGWRAGCATCTGCACCAGGGTTGGGCACKGGGR

GCTGAACAAAGAAAGGGGCTTCTTGTGCCCTCA

Yellow BsmAI recognition site

5′.....GTCTC(N)$_1$.....

3′.....CAGAG(N)$_5$.....

Restriction enzyme BsmA1

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGAC

AYGGTGAATGACGGCRTGGAGGACCTCCGCTGCAAATACG (Fragment 1: 93 bp)

TCTCCCTCWTCTAYACCAACTATGWRAGCATCTGCACCAGGGTTGGGC

ACKGGGRGCTGAACAAAGAAAGGGGCTTCTTGTGCCCTCA (Fragment 2: 83 bp)

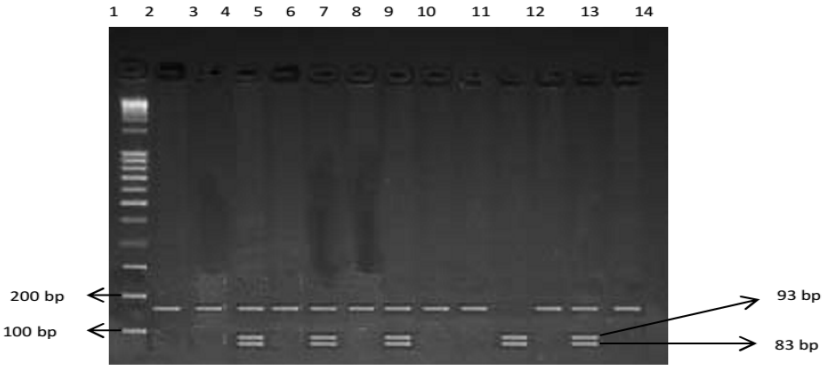


Fig. 2.2

Restriction Enzyme (BsmAI) digestion fragment of GSTP1 (3% agarose gel); lane (2, 3, 9, 10, 12, 14): Normal Homozygote, lane- (4, 6, 8, 13): Heterozygous, lane-11: Mutant homozygous, lane-1: Molecular ruler

2.7 PCR-RFLP of ABCC4

After completing PCR amplification with appropriate reagents a PCR product of *ABCC4 was* obtained. The PCR product size was 226 bp and this was visualized in 2% (w/v) agarose gel.

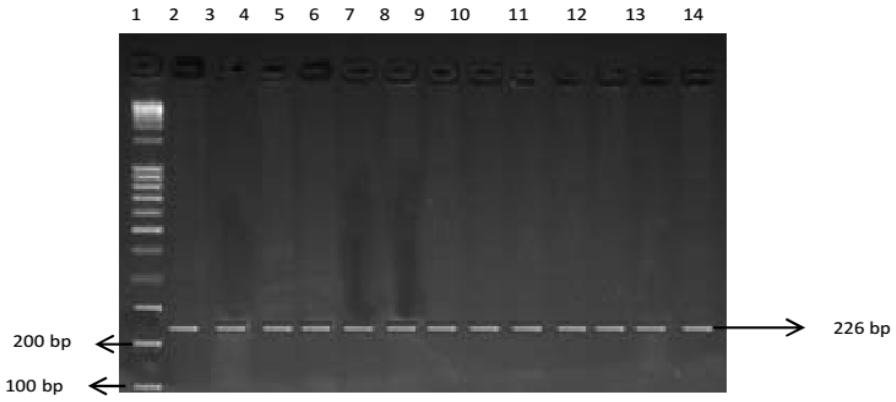


Fig 2.3 PCR product of ABCC4 (226 bp) (Lane 1 to 13 (2% agarose gel) (Lane-14 contains Molecular ruler)

2.7.1 Fragmentation Pattern

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

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

Restriction Enzyme	Sites of digestion
HpAII	**5´……C↓CGG……3´** **3´……GGC↑C……5´**

Table 2.7: Type of nucleotide changes, cutting sites and fragments of the allele in case of ABCC4

Change	Fragments	Type
When X= G in both chromosomes (A/A)	96, 130	Normal Homozygote
When X= T in one chromosome (A/G)	96, 130, 226	Heterozygote
When X=G in both chromosome	226	Mutant Homozygote

GTGCACAGGGTTCCAATTTCTTCACATCCTCCAAATTACTTAAG
CGTGATTCCAATTGTTTTAGAGGTCAGTGCTAGGATAGCCATA
ACTGTACTTGGTCTAAGATAAAAATCACATTCTCCTTCCCTTCC
XGTGGCACGTTCTCTATGCTTCCTACTAAACAGGCATTGAAGA
GTTGTTTATATCCACATGCCCAGAAAGCTCATTACTGCCTGGGT
TTTGCTCT

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

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 in the both sister chromosomes and two fragment with 96 and 130 bp will be obtained.

GTGCACAGGGTTCCAATTTCTTCACATCCTCCAAATTACTTAAGCGTG

ATTCCAATTGTTTTAGAGGTCAGTGCTAGGATAGCCATAACTGTACTT

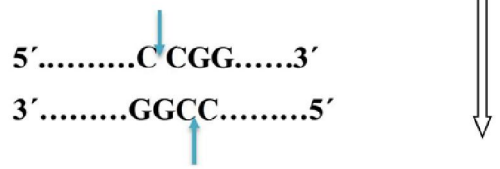
HpAII recognition site

GGTCTAAGATAAAAATCACATTCTCCTTCCCTTCCGGTGGCACGTTCTC

TATGCTTCCTACTAAACAGGCATTGAAGAGTTGTTTATATCCACATGC

CCAGAAAGCTCATTACTGCCTGGGTTTTGCTCT

Yellow HpAII recognition site



GTGCACAGGGTTCCAATTTCTTCACATCCTCCAAATTACTTAAGCGTGA

TTCCAATTGTTTTAGAGGTCAGTGCTAGGATAGCCATAACTGTACTTGG

TCTAAGATAAAAATCACATTCTCCTTCCCTTC (Fragment 1: 130 bp)

CGGTGGCACGTTCTCTATGCTTCCTACTAAACAGGCATTGAAGAGTTG

TTTATATCCACATGCCCAGAAAGCTCATTACTGCCTGGGTTTTGCTCT

(Fragment 2: 96 bp)

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

When X= G in one of the sister chromosome, there will be one cutting at 96 bp in one sister chromosome and other sister chromosome remained uncut. Three fragments with 96, 130 and 226bp will be obtained in this case.

GTGCACAGGGTTCCAATTTCTTCACATCCTCCAAATTACTTAAGCGTG

ATTCCAATTGTTTTAGAGGTCAGTGCTAGGATAGCCATAACTGTACTT

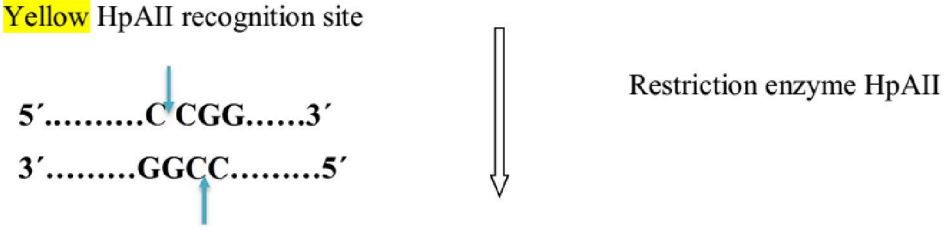
HpAII recognition site

GGTCTAAGATAAAAATCACATTCTCCTTCCCTTCCGGTGGCACGTTCTC

TATGCTTCCTACTAAACAGGCATTGAAGAGTTGTTTATATCCACATGC

CCAGAAAGCTCATTACTGCCTGGGTTTTGCTCT

Yellow HpAII recognition site



GTGCACAGGGTTCCAATTTCTTCACATCCTCCAAATTACTTAAGCGTGA

TTCCAATTGTTTTAGAGGTCAGTGCTAGGATAGCCATAACTGTACTTGG

TCTAAGATAAAAATCACATTCTCCTTCCCTTC (Fragment 1: 130 bp)

CGGTGGCACGTTCTCTATGCTTCCTACTAAACAGGCATTGAAGAGTTG

TTTATATCCACATGCCCAGAAAGCTCATTACTGCCTGGGTTTTGCTCT

(Fragment 2: 96 bp)

Fragment 3: 226 bp (For uncut sister chromosome)

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

When X= T in both of the chromosome, there will be no cutting site and only a fragment with 226 will be found and this is considered as Mutant homozygote.

GTGCACAGGGTTCCAATTTCTTCACATCCTCCAAATTACTTAAGCGTG

ATTCCAATTGTTTTAGAGGTCAGTGCTAGGATAGCCATAACTGTACTT

No HpAII recognition site

GGTCTAAGATAAAAATCACATTCTCCTTCCCTTCCTGTGGCACGTTCTC

TATGCTTCCTACTAAACAGGCATTGAAGAGTTGTTTATATCCACATGC

CCAGAAAGCTCATTACTGCCTGGGTTTTGCTCT

Yellow HpAII recognition site

5′.....C CGG.....3′

3′.....GGCC.....5′

Restriction enzyme HpAII

GTGCACAGGGTTCCAATTTCTTCACATCCTCCAAATTACTTAAGCGTGA

TTCCAATTGTTTTAGAGGTCAGTGCTAGGATAGCCATAACTGTACTTGG

TCTAAGATAAAAATCACATTCTCCTTCCCTTCCTGTGGCACGTTCTCTAT

GCTTCCTACTAAACAGGCATTGAAGAGTTGTTTATATCCACATGCCCAG

AAAGCTCATTACTGCCTGGGTTTTGCTCT (1 fragment of 226 bp)

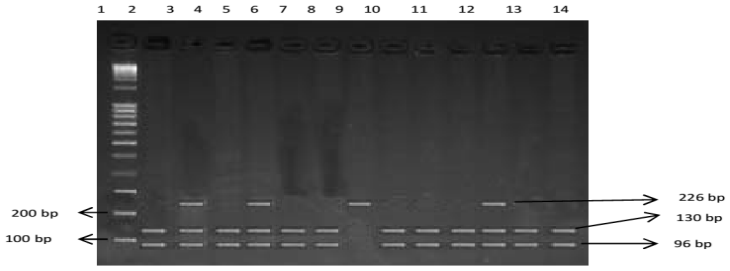


Fig. 2.4 Restriction Enzyme (HpAI) digestion fragment of ABCC4(3% agarose gel); lane (2,4,6,7,9,10,11,13,14): Normal Homozygote, lane- (3,5,12): Heterozygous, lane-8: Mutant homozygous; Uncut PCR : (226 bp); lane-1: Molecular ruler

2.8 Statistical Analysis

The statistical significance of differences in genotype frequencies between patients with different treatment outcomes and toxicities were determined by the Chi-square test. Binary logistic regression was applied for all analysis variables to evaluate risk as odds ratios (ORs) with 95 % confidence intervals (95 % CIs). All statistical analyses were done applying the SPSS software, version 17.0 (SPSS, Chicago, IL, USA).

CHAPTER THREE

RESULTS

3. Results

3.1 Clinicopathological characteristics of patients

3.1.1 Clinicopathological characteristics of patients receiving neoadjuvant chemotherapy

The clinicopathological characteristics of all the recruited patients receiving neoadjuvant chemotherapy (n= 117) including patient's age, menstrual status, TNM staging, lymph node status, histology, tumor grade, hormone receptor status like estrogen receptor, progesterone receptor, her2/neu status were recorded and a chi square test was done to analyze these recorded values in terms of response and toxicity.

No significant variation of response and toxicities (P>0.05) was observed among the patient with the above mentioned characteristics (table-3.1).

Table 3.1: Clinicopathological parameters of non-responders versus responders to NACT and patients who suffered from grade II–IV chemotoxicity versus those with grade ≤ II chemotoxicity (n= 117)

Characteristics	Non responders (49)	Responders (68)	P value	Toxicity Grade (III+IV) (53)	Toxicity Grade ≤II (64)	P value
Age						
<45	8	13	0.6692	7	14	0.2556
45-55	19	26		18	25	
>55	22	29		28	25	
Menstrual status						
Premenoposal	15	26	0.4636	17	24	0.8708
Perimenoposal	1	4		2	3	
Postmenoposal	33	38		34	37	
TNM stage (Clinical)						
I	0	0	0.9174	0	0	0.8658
II	1	1		1	1	
III	42	60		47	55	
IV	6	7		5	8	
Lymph node status						
No	4	7	0.9679	6	5	0.8474
N1	23	33		26	30	
N2	17	22		17	22	
N3	5	6		4	7	

Table 3.1 (cont.)

Characteristics	Non responders (49)	Responders (68)	P value	Toxicity Grade (III+IV) (53)	Toxicity Grade ≤II (64)	P value
Histology						
Ductal	48	67	0.3483	53	62	0.4306
Lobular	1	0		0	1	
Mixed	0	1		0	1	
Tumor grade						
Grade I	2	1	0.6780	2	1	0.6696
Grade II	28	40		27	39	
Grade III	19	27		20	26	
Hormone receptor status						
Estrogen Receptor(ER)						
Negative	31	35	0.2800	29	35	0.8545
Positive	18	33		24	29	
Progesterone Receptor(PR)						
Negative	34	40	0.3296	31	43	0.8485
Positive	15	28		18	25	
Her-2/ neu status						
Negative	29	40	0.8797	31	38	0.9267
Positive	20	28		22	26	

Significance level: P <0.05

3.1.2 Clinicopathological characteristics of the patients receiving adjuvant chemotherapy

Patients showing chemotherapy (adjuvant, n=102) induced toxicities were graded according to Common Terminology Criteria for Adverse Events (CTCAE) v4 and divided into two groups as severe toxicity (grade III and grade IV) groups and average toxicity (grade ≤II) group. Recording of the distribution of these toxicities among the patients with different clinicophathological parameters such as age, menstrual status, TNM staging, Lymph node status, histology, tumor grade, hormone receptor status like estrogen receptor, progesterone receptor, her2/neu status was done and a subsequent chi square test was performed. No significant difference was found among the patients with different clinic-pathological characteristic parameters (P >0.05) (table-3.2)

Table 3.2: Clinico-pathological parameters of patients who received adjuvant chemotherapy and suffered from grade II–IV chemotoxicity versus those with grade ≤II chemotoxicity (n= 102)

Characteristics	**Toxicity Grade (III+IV) (46)**	**Toxicity Grade ≤II (56)**	**P value**
Age			
<45	6	10	0.5441
45-55	15	21	
>55	25	25	
Menstrual status			
Premenoposal	15	20	0.8689
Perimenoposal	2	2	
Postmenoposal	29	34	
TNM stage (Clinical)			
I	0	1	0.8375
II	1	1	
III	41	49	
IV	4	5	
Lymph node status			
No	4	5	0.9838
N1	22	27	
N2	16	18	
N3	4	6	
Histology			
Ductal	45	55	0.3607
Lobular	0	1	
Mixed	1	0	
Tumor Grade			
Grade I	2	1	0.7449
Grade II	26	33	
Grade III	18	22	
Hormone receptor status			
Estrogen Receptor(ER)			
Negative	26	32	0.8903
Positive	20	24	
Progesterone Receptor(PR)			
Negative	27	38	0.8774
Positive	16	23	
Her-2/ neu status			
Negative	27	34	0.8670
Positive	18	23	

Significance level <0.05

3.1.3 Clinicopathological characteristics of the patients receiving adjuvant and neoadjuvant chemotherapy

Toxicities of different graded caused by adjuvant and neoadjuvant chemotherapy were combined. Incidence of these toxicities among patients with different clinicophathological characteristics such as age, menstrual status, TNM staging, Lymph node status, histology, tumor grade, hormone receptor status like estrogen receptor, progesterone receptor, her2/neu status were recorded. No significant variation of toxicities was observed among the patients with different clinicophathological parameters after performing a chi-square test (P>0.05) (table-3.3).

Table-3.3: Clinico-pathological parameters of total patients who received adjuvant and neoadjuvant chemotherapy and suffered from grade II–IV chemotoxicity versus those with grade ≤II chemotoxicity (n= 102 +117=219)

Characteristics	**Toxicity Grade (III+IV) (46+53)**	**Toxicity Grade ≤II (56+64)**	**P value**
Age			
<45	13	24	0.1505
45-55	33	46	
>55	53	50	
Menstrual status			
Premenoposal	32	44	0.7949
Perimenoposal	4	5	
Postmenoposal	63	71	
TNM stage (Clinical)			
I	0	1	0.7876
II	2	2	
III	88	104	
IV	9	13	
Lymph node status			
No	10	10	0.8906
N1	48	57	
N2	33	40	
N3	8	13	

Table-3.3 (Cont.)

Characteristics	Toxicity Grade (III+IV) (46+53)	Toxicity Grade ≤II (56+64)	P value
Histology			
Ductal	98	117	0.4315
Lobular	0	2	
Mixed	1	1	
Tumor grade			
Grade I	4	2	0.5017
Grade II	53	72	
Grade III	38	48	
Hormone receptor status			
Estrogen Receptor(ER)			
Negative	55	67	0.9239
Positive	44	53	
Progesterone Receptor(PR)			
Negative	58	81	0.9180
Positive	34	48	
Her-2 /neu status			
Negative	58	72	0.9280
Positive	40	49	

Significance level <0.05

3.2 Single-Locus Analysis: Correlation with Treatment Outcomes

Among the 117 patients who treated with NACT, 17 patients showed complete response and 51 patients got partial response, 47 patients exhibited stable conditions and 2 patients had disease progression according to RECIST criteria (table-3.4). Estrogen receptor, progesterone receptor status and expression of the her2 protein were measured by immunohistochemical analysis.

3.2.1 Effect of GSTP1 (rs1695) polymorphism on chemotherapy response

From the response variation among the patients with GSTP1 (rs1695) polymorphism, we observed that patients with AA genotype were less likely to give response (28 responders and 32 non responders) in compared to patients with AG genotype (31 responders and 14

non-responders) or GG genotype (9 responders and 3 non-responders) and a significant relationship of response was found in the patients with AG genotype and patient carrying any variant G allele(AG + GG) (OR = 2.53, 95% Cl = 1.13-5.69, p = 0.025 and OR = 2.69, 95% Cl = 1.26-5.76, p = 0.011 in compared to AA genotype) (table-3.5).

Table-3.4: Response variation of patients with GSTP1 (rs1695) polymorphism

GSTP1 (Neoadjuvant: n=117)						
	Total Responders (68)			**Total Non-responders (49)**		
Genotype	Complete Response (17)	Partial Response(51)	**Total Responders**	Stable Disease (47)	Progressive Disease (2)	**Total Non-responders**
AA(60)	5	23	**28**	31	1	**32**
AG(45)	7	24	**31**	13	1	**14**
GG(12)	5	4	**9**	3	0	**3**
AG(45)+GG(12)	12	28	**40**	16	1	**17**

Table-3.5: Comparison of responders and non responders with GSTP1 (rs1695) polymorphism

GSTP1 (Neoadjuvant: n=117)				
Genotype	Responders (CR+PR) (n=68)	Non-responders (SD+PD) (n=49)	Odds Ratio (95 % CI)	P Value
AA(60)	28	32	Reference	
AG(45)	31	14	2.5306 (1.1261 to 5.6867)	**0.0246**
GG(12)	9	3	3.4286 (0.8441 to 13.9265)	0.0849
AG(45)+GG(12)	40	17	2.6891 (1.2562 to 5.7563)	**0.0109**

CR: Complete Response; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease

3.2.2 Effect of ABCC4 (rs9561778) polymorphism on chemotherapy response

From the variation of response among the different ABCC4 genotype carriers, it is found that patients with GG genotype have the slightly poorer tendenciy to give response (50 responders and 39 non responders) than the patients with GT genotype (13 responders and 7 non-responders) or TT genotype (5 responders and 3 non-responders (table-3.6). No significant relationship was observed in the patients carrying ABCC4 (rs9561778) polymorphism and response variation of the chemotherapy (table-3.7)

Table-3.6: Response variation of patients with ABCC4 (rs9561778) polymorphism

ABCC4 (Neoadjuvant: n=117)						
	Total Responders(68)			**Total Non-responders (49)**		
Genotype	Complete response (17)	Partial Response(51)	**Total Responders**	Stable Condition(47)	Progressive Disease (2)	**Total Non-responders**
GG (89)	12	38	**50**	38	1	**39**
GT (20)	3	10	**13**	6	1	**7**
TT(8)	2	3	**5**	3	0	**3**
GT(20) +TT(8)	5	12	**17**	10	1	**11**

Table-3.7: Comparison of responders and non responders with ABCC4 (rs9561778) polymorphism

ABCC4 (Neoadjuvant: n=117)				
Genotype	**Responders (CR+PR) (n=68)**	**Non-responders (SD+PD) (n=49)**	**Odds Ratio 95 % CI**	**P Value**
GG (89)	50	39	Reference	-
GT (20)	13	7	1.4486 (0.5277 to 3.9763)	0.4720
TT(8)	5	3	1.3000 (0.2926 to 5.7761)	0.7302
GT(20) +TT(8)	18	10	1.4040 (0.5828 to 3.3821)	0.4494

CR: Complete Response; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease

3.3 Toxicity evaluation

3.3.1 Toxicities caused by neoadjuvant chemotherapy

Different types of hematological toxicities including anemia, nutropenia, leukopenia, thrombocytopenia and gastrointestinal toxicities caused by chemotherapy (neoadjuvant, n=117) among the patients containing genetic polymorphism of GSTP1 (rs1695) and ABCC4 (rs9561778) genes were observed and graded (grade ≤II, grade III and grade IV). Variation of toxicities in the patients with different genotypes of both GSTP1 (rs1695) and ABCC4 (rs9561778) genes were recorded. In this study we have found that frequency of anemia and nutropenia is higher than the other types of toxicities and tendency of showing toxicities is found to be higher in patients carrying variant alleles of GSTP1 (rs1695) and ABCC4 (rs9561778) genes (table-3.8)

Table 3.8: Variation of toxicities caused by chemotherapy (neoadjuvant, n=102) in the patients with different genotype of GSTP1 (rs1695) and ABCC4 (rs9561778) genes

Neoadjuvant: n=117		GSTP1				ABCC4			
		AA(60)	AG(45)	GG(12)	AG(45) +GG(12)	GG(89)	GT(20)	TT(8)	GT(20) +TT(8)
Hematological Toxicity	Total								
Anemia									
Grade ≤II	64	34	25	5	30	54	7	3	10
Grade III	34	17	13	4	17	24	8	2	10
Grade IV	19	9	7	3	10	11	5	3	8
Neutropenia									
Grade ≤II	82	43	31	8	39	67	11	4	14
Grade III	18	9	7	2	9	12	4	2	7
Grade IV	17	8	7	2	9	10	5	2	7
Leukopenia									
Grade ≤II	89	47	33	9	42	71	13	5	19
Grade III	19	9	8	2	10	12	5	2	7
Grade IV	9	4	4	1	5	6	2	1	3

Table 3.8 (cont.)

Neoadjuvant: n=117		GSTP1				ABCC4			
		AA(60)	AG(45)	GG(12)	AG(45) +GG(12)	GG(89)	GT(20)	TT(8)	GT(20) +TT(8)
Thrombocytopenia									
Grade ≤II	115	59	45	11	56	88	19	8	27
Grade III	1	1	0	0	0	1	0	0	0
Grade IV	1	0	0	1	1	0	1	0	1
Gastrointestinal Toxicity									
Grade ≤II	102	53	40	9	24	81	15	6	21
Grade III	14	7	5	2	7	8	5	1	6
Grade IV	1	0	0	1	1	0	0	1	1

3.3.1.1 Anemia induced by neoadjuvant chemotherapy

Anemia is the most common types of hematological adverse effect caused by the chemotherapeutic agents. In our study we observed that the tendency of higher grades of anemia in neoadjuvant treatment is more frequent in the patients having genetic change in both of the GSTP1(rs1695) and ABCC4(rs9561778) genes and the frequency further increased in patients with mutant homozygote in both of the genes (table-3.9) but only the ABCC4(rs9561778) polymorphism showed the significant relationship with this tendency of the toxicity with odds ratio and P value for GT and GT plus TT genotype (OR = 2.87, 95% Cl = 1.04-7.89, p = 0.042 and OR = 2.78, 95% Cl = 1.15-6.71, p = 0.023 respectively) in respect to GG genotype (Table-3.10). No association was found between GSTP1 (rs1695) gene polymorphism and variation of anemia.

Table-3.9: Different grades of anemia caused by neoadjuvant chemotherapy in patients carrying GSTP1 and ABCC4 gene polymorphisms

Neoadjuvant: n=117		**GSTP1**				**ABCC4**			
		AA(60)	AG(45)	GG(12)	AG(45) +GG(12)	GG(89)	GT (20)	TT(8)	GT(20) +TT(8)
Anemia									
Grade ≤II	64	34	25	5	30	54	7	3	10
Grade III	34	17	13	4	17	24	8	2	10
Grade IV	19	9	7	3	10	11	5	3	8

Table-3.10: Anemia caused by neoadjuvant chemotherapy in patients carrying GSTP1 and ABCC4 gene polymorphisms

Anemia					
Gene	**Genotype**	**Grade III(34)+ Grade IV(19)**	**Grade ≤II (64)**	**Odds Ratio (95% CI)**	**P value**
GSTP1	AA(60)	26	34	Reference	-
	AG(45)	20	25	1.0462 (0.4802 to 2.2794)	0.9096
	GG(12)	7	5	1.8308(0.5213 to 6.4293)	0.3454
	AG(45)+GG(12)	27	30	1.1769(0.5679 to 2.4392)	0.6613
ABCC4	GG(89)	35	54	Reference	-
	GT (20)	13	7	2.8653(1.041 to 7.8869)	**0.0416**
	TT(8)	5	3	2.5714(0.5777 to 11.4467)	0.2151

3.3.1.2 Neutropenia induced by neoadjuvant chemotherapy

Neutropenia is the most severe type of hematological toxicity found in breast cancer chemotherapy. In this study we observed that the tendency of showing different grades of neutropenia in patients containing polymorphism of GSTP1 (rs1695) and ABCC4 (rs9561778) gene was similar to anemia (table-3.11). In the study we found that GSTP1 gene polymorphism had no relationship with variation of neutropenia but a significant relationship was observed with GT plus TT genotype of ABCC4 (rs9561778) gene (OR = 2.64, 95% Cl = 1.09-6.40, p = 0.032) with the comparison of GG genotype (table-3.12)

Table-3.11: Different grades of neutropenia caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neoadjuvant: n=117		**GSTP1**				**ABCC4**			
		AA(60)	AG(45)	GG(12)	AG(45) +GG(12)	GG(89)	GT (20)	TT(8)	GT(20) +TT(8)
Neutropenia									
Grade ≤II	82	43	31	8	39	67	11	4	15
Grade III	18	9	7	2	9	12	4	2	6
Grade IV	17	8	7	2	9	10	5	2	7

Table-3.12: Neutropenia caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neutropenia					
Gene	**Genotype**	**Grade III(18)+ Grade IV(17)**	**Grade ≤II (82)**	**Odds Ratio (95% CI)**	**P value**
GSTP1	AA(60)	17	43	Reference	-
	AG(45)	14	31	1.1423(0.4908 to 2.6587)	0.7575
	GG(12)	4	8	1.2647(0.3361 to 4.7586)	0.7283
	AG(45)+GG(12)	18	39	1.1674(0.5288 to 2.5774)	0.7017
ABCC4	GG(89)	22	67	Reference	-
	GT (20)	9	11	2.4917(0.913 to 6.8005)	0.0747
	TT(8)	4	4	3.0455(0.7022 to 13.2087)	0.1368
	GT(20)+TT(8)	13	15	2.6394(1.0891 to 6.3965)	**0.0316**

3.3.1.3 Leukopenia induced by neoadjuvant chemotherapy

We found 19 patients with grade III and 9 patients with grade IV leukopenia caused by neoadjuvant chemotherapy and the tendency of producing leukopenia is higher in patients with carrying variant allele in both of the genes of GSTP1 and ABCC4 (table-3.13) and ABCC4 showed more possibility of having relationship with different grades of leukopenia in neoadjuvant breast cancer chemotherapy than GSTP1 gene but no significant relationship with both of GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms and leukopenia could be drawn from this study (table-3.14).

Table-3.13: Different grades of leukcopenia caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neoadjuvant: n=117		**GSTP1**				**ABCC4**			
		AA(60)	AG(45)	GG(12)	AG(45) +GG(12)	GG(89)	GT (20)	TT(8)	GT(20) +TT(8)
Leukopenia									
Grade ≤II	89	47	33	9	42	71	13	5	18
Grade III	19	9	8	2	10	12	5	2	7
Grade IV	9	4	4	1	5	6	2	1	3

Table-3.14: Leukopenia caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Leukopenia					
Gene	**Genotype**	**Grade III(19)+ Grade IV(9)**	**Grade >II (89)**	**Odds Ratio (95% CI)**	**P value**
GSTP1	AA(60)	13	47	Reference	-
	AG(45)	12	33	1.3147(0.5334 to 3.2404)	0.5522
	GG(12)	3	9	1.2051(0.2844 to 5.1060)	0.8000
	AG(45)+GG(12)	15	42	1.2912(0.5511 to 3.0251)	0.5563
ABCC4	GG(89)	18	71	Reference	-
	GT (20)	7	13	2.1239 (0.74 to 6.0964)	0.1615
	TT(8)	3	5	2.3667 (0.5166 to 10.8422)	0.2672
	GT(20)+TT(8)	10	18	2.1914 (0.8645 to 5.5546)	0.0983

3.3.1.4 Thrombocytopenia induced by neoadjuvant chemotherapy

The frequency of getting higher grades of thrombocytopenia is very small in breast cancer chemotherapy. In this study we got only one patient with grade III and one patient with grade IV thrombocytopenia and rest of the patients were with grade II or less than grade II thrombocytopenia (table-3.15). We did not find any association of GSTP1 (rs1695) gene polymorphism with thrombocytopenia but a significant association was observed in patients carrying TT genotype of ABCC4 (rs9561778) gene with thrombocytopenia ((OR = 35.34, 95% Cl = 1.18-827.73, p = 0.040) in compared to GG genotype (table-3.16)

Neoadjuvant: n=117		**GSTP1**				**ABCC4**			
		AA(60)	AG(45)	GG(12)	AG(45) +GG(12)	GG(89)	GT (20)	TT(8)	GT(20) +TT(8)
Thrombocytopenia									
Grade ≤II	115	59	45	11	56	88	19	8	27
Grade III	1	1	0	0	0	1	0	0	0
Grade IV	1	0	0	1	1	0	1	0	1

Table-3.15: Different grades of thrombocytopenia caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Table-3.16: Thrombocytopenia caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neoadjuvant: n=117	**Thrombocytopenia**				
Gene	**Genotype**	**Grade III(1)+ Grade IV(1)**	**Grade ≤II (115)**	**Odds Ratio(95% CI)**	**P value**
GSTP1	AA(60)	1	59	Reference	-
	AG(45)	0	45	0.4359 (0.0173 to 10.951)	0.6137
	GG(12)	1	11	5.3636(0.3116 to 92.3200)	0.2473
	AG(45)+GG(12)	1	56	0.2473(0.0643 to 17.2543)	0.9708
ABCC4	GG(89)	1	88	Reference	-
	GT (20)	1	19	4.6316(0.2772 to 77.382)	0.2860
	TT(8)	0	8	31.2353(1.1787 to 827.729)	**0.0396**
	GT(20)+TT(8)	1	27	3.2593(0.1972 to 53.8767)	0.4091

3.3.1.5 Gastrointestinal toxicity induced by neoadjuvant chemotherapy

GI toxicity is one of the major types of chemotherapy induced toxicities in the treatment of breast cancer. In our study we found 14 patients with grade III and 1 patient with grade IV gastrointestinal toxicity caused by neoadjuvant chemotherapy (table-3.17). No association of GSTP1 (rs1695) gene polymorphism with gastrointestinal toxicity was found but at least one variant T allele carrier patients of ABCC4 (rs9561778) showed a significant relationship with gastrointestinal toxicity (OR = 3.38, 95% Cl = 1.10-10.37, p = 0.034) in compared to GG genotype (table-3.18)

Table-3.17: Different grades of gastrointestinal toxicity caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neoadjuvant: n=117		**GSTP1**				**ABCC4**			
		AA(60)	AG(45)	GG(12)	AG(45) +GG(12)	GG(89)	GT (20)	TT(8)	GT(20) +TT(8)
Gastrointestinal Toxicity									
Grade ≤II	102	53	40	9	24	81	15	6	21
Grade III	14	7	5	2	7	8	5	1	6
Grade IV	1	0	0	1	1	0	0	1	1

Table-3.18: Gastrointestinal toxicity caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Gastrointestinal Toxicity					
Gene	**Genotype**	**Grade III(14)+ Grade IV(1)**	**Grade ≤II (102)**	**Odds Ratio**	**P value**
GSTP1	AA(60)	7	53	Reference	-
	AG(45)	5	40	0.9464(0.2797 to 3.2021)	0.9294
	GG(12)	3	9	2.5238(0.5487 to 11.6088)	0.2344
	AG(45)+GG(12)	8	49	1.2362(0.4172 to 3.6628)	0.7021
ABCC4	GG(89)	8	81	Reference	-
	GT (20)	5	15	3.3750 (0.971 to 11.7307)	0.0557
	TT(8)	2	6	3.3750(0.5821 to 19.5674)	0.1749
	GT(20)+TT(8)	7	21	3.3750(1.0988 to 10.3668)	**0.0336**

3.3.2 Toxicities caused by adjuvant chemotherapy

Similar trends of hematological toxicities like anemia, nutropenia, leukopenia, thrombocytopenia and gastrointestinal toxicities induced by chemotherapy (adjuvant) among the patients containing genetic polymorphism of GSTP1 (rs1695) and ABCC4 (rs9561778) genes were observed as it was in case of neoadjuvant chemotherapy and subsequent grading (grade ≤II, grade III and grade IV) was done. It was found that frequency of anemia and nutropenia is greater than the other types of toxicities and tendency of toxicities were found in patients carrying variant alleles of GSTP1 (rs1695) and ABCC4 (rs9561778) genes (table-3.19)

Table 3.19: Variation of toxicities caused by chemotherapy (adjuvant) in the patients with different genotype of GSTP1 (rs1695) and ABCC4 (rs9561778) genes

Adjuvant: n=102		**GSTP1**				**ABCC4**			
		AA(52)	AG(39)	GG(11)	AG(39) +GG(11)	GG(76)	GT (19)	TT(7)	GT(19) +TT(7)
Hematological Toxicity	Total								
Anemia									
Grade ≤II	56	30	21	5	26	46	7	3	10
Grade III	29	14	11	4	15	20	7	2	11
Grade IV	17	8	7	2	9	10	5	2	7
Neutropenia									
Grade ≤II	69	36	26	7	34	56	10	3	12
Grade III	17	8	7	2	9	11	4	2	6
Grade IV	16	8	6	2	8	9	5	2	7
Leukopenia									
Grade ≤II	75	39	29	7	36	60	11	4	15
Grade III	16	8	6	2	8	10	4	2	6
Grade IV	11	5	4	2	6	6	4	1	5

Table 3.9(cont.)

Adjuvant: n=102		**GSTP1**				**ABCC4**			
		AA(52)	AG(39)	GG(11)	AG(39) +GG(11)	GG(76)	GT (19)	TT(7)	GT(19) +TT(7)
Thrombocytopenia									
Grade ≤II	101	52	38	11	49	76	19	6	25
Grade III	1	0	1	0	1	0	0	1	1
Grade IV	0	0	0	0	0	0	0	0	0
Gastrointestinal Toxicity									
Grade ≤II	89	46	34	9	43	69	14	6	20
Grade III	12	6	4	2	6	7	4	1	5
Grade IV	1	0	1	0	1	0	1	0	1

3.3.2.1 Anemia induced by adjuvant chemotherapy

Anemia, the most common types of hematological adverse effect caused by the chemotherapeutic agents, was found in this study in a good number of patients (29 patients with grade III and 17 patients with grade IV anemia) induced by adjuvant treatment (table-3.20). We also noticed that the tendency of showing higher grades of anemia in adjuvant treatment is higher in the patients having genetic change in both of the GSTP1 (rs1695) and ABCC4 (rs9561778) genes. Though the occurrence of anemia is higher in patients carrying ABCC4 (rs9561778) polymorphism than GSTP1 (rs1695) polymorphism carriers, statistically we did not find any significant effect of either of the genes on anemia produced by adjuvant chemotherapy (3.21).

Table-3.20: Different grades of anemia caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Adjuvant: N=102		**GSTP1**				**ABCC4**			
		AA(52)	AG(39)	GG(11)	AG(39) +GG(11)	GG(76)	GT (19)	TT(7)	GT(19) +TT(7)
Anemia									
Grade ≤II	56	30	21	5	26	46	7	3	10
Grade III	29	14	11	4	15	20	7	2	9
Grade IV	17	8	7	2	9	10	5	2	7

Table-3.21: Anemia caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Anemia					
Gene	**Genotype**	**Grade III(29) + Grade IV(17)**	**Grade ≤II (56)**	**Odds Ratio (95% CI)**	**P value**
GSTP1	AA(52)	22	30	Reference	-
	AG(39)	18	21	1.1688(0.5066 to 2.6968)	0.7146
	GG(11)	6	5	1.6364(0.4423 to 6.0534)	0.4606
	AG(39)+GG(11)	24	26	1.2587(0.5762 to 2.7496)	0.5638
ABCC4	GG(76)	30	46	Reference	-
	GT (19)	12	7	2.6286(0.9296 to 7.4329)	0.0684
	TT(7)	4	3	2.0444(0.427 to 9.7881)	0.3708
	GT(19)+TT(7)	16	10	2.4533(0.9834 to 6.1207)	0.0544

3.3.2.2 Neutropenia induced by neoadjuvant chemotherapy

Neutropenia, the most severe type of hematological toxicity caused breast cancer chemotherapy, was found in a variety of patients (17 patients with grade III and 16 patients with grade IV neutropenia) (table-3.22).We also observed that the tendency of showing higher grades of neutropenia was related with the patients containing variant allele of GSTP1 (rs1695) and ABCC4 (rs9561778) gene. Patient carrying at least one variant T allele of ABCC4 (rs9561778) gene was found to have significant relationship with nutropenia (OR = 2.80, 95% Cl = 1.11-7.05, p = 0.029) but no association of GSTP1 (rs1695) gene polymorphism was found with this toxicity (table-3.23).

Table-3.22: Different grades of neutropenia caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Adjuvant: n=102		**GSTP1**				**ABCC4**			
		AA(52)	AG(39)	GG(11)	AG(39) +GG(11)	GG(76)	GT (19)	TT(7)	GT(19) +TT(7)
Neutropenia									
Grade ≤II	69	36	26	7	34	56	10	3	13
Grade III	17	8	7	2	9	11	4	2	6
Grade IV	16	8	6	2	8	9	5	2	7

Table-3.23: Neutropenia caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neutropenia					
Gene	**Genotype**	**Grade III(17)+ Grade IV(16)**	**Grade ≤II (69)**	**Odds Ratio (95% CI)**	**P value**
GSTP1	AA(52)	16	36	Reference	-
	AG(39)	13	26	1.1250(0.4625 to 2.7364)	0.7951
	GG(11)	4	7	1.2857(0.3292 to 5.0212)	0.7177
	AG(39)+GG(11)	17	33	1.1591(0.5053 to 2.6586)	0.7274
ABCC4	GG(76)	20	56	Reference	-
	GT (19)	9	10	2.5200(0.895 to 7.0956)	0.0801
	TT(7)	4	3	3.7333(0.7677 to 18.1551)	0.1026
	GT(19)+TT(7)	13	13	2.8000 (1.1127 to 7.0462)	**0.0288**

3.3.2.3 Leukopenia induced by adjuvant chemotherapy

We found 16 patients with grade III and 11 patients with grade IV leukopenia induced by adjuvant chemotherapy and the tendency of producing leukopenia was found to be higher in patients with carrying variant allele in both of the genes of GSTP1 (rs1695) and ABCC4 (rs9561778) (table-3.24). Patient carrying at least one variant T allele of ABCC4 (rs9561778) gene showed significant association with adjuvant chemotherapy induced leukopenia (OR = 2.75, 95% Cl = 1.06-7.14, p = 0.038). No association of GSTP1 (rs1695) gene polymorphism was found with leukopenia (table-3.25)

Table-3.24: Different grades of leukopenia caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Adjuvant: n=102		**GSTP1**				**ABCC4**			
		AA(52)	AG(39)	GG(11)	AG(39) +GG(11)	GG(76)	GT (19)	TT(7)	GT(19) +TT(7)
Leukopenia									
Grade ≤II	75	39	29	7	36	60	11	4	15
Grade III	16	8	6	2	8	10	4	2	6
Grade IV	11	5	4	2	6	6	4	1	5

Table-3.25: Leukopenia caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Leukopenia					
Gene	**Genotype**	**Grade III(16)+ Grade IV(11)**	**Grade ≤II (75)**	**Odds Ratio**	**P value**
GSTP1	AA(52)	13	39	Reference	-
	AG(39)	10	29	1.0345(0.3984 to 2.6863)	0.9445
	GG(11)	4	7	1.7143(0.4315 to 6.8112)	0.4438
	AG(39)+GG(11)	14	36	1.1667(0.4837 to 2.8139)	0.7315
ABCC4	GG(76)	16	60	Reference	-
	GT (19)	8	11	2.7273(0.9405 to 7.9089)	0.0647
	TT(7)	3	4	2.8125(0.5705 to 13.8654)	0.2039
	GT(19)+TT(7)	11	15	2.7500 (1.0597 to 7.1368)	**0.0376**

3.3.2.4 Thrombocytopenia induced by adjuvant chemotherapy

The frequency of producing higher grades of thrombocytopenia is rare in breast cancer chemotherapy. In this study we got only one patient with grade III thrombocytopenia and rest of the patient were with grade II or less than grade II thrombocytopenia induced by adjuvant chemotherapy (table-3.26). We found no association of GSTP1 (rs1695) gene polymorphism with thrombocytopenia but a significant association was observed in patients carrying TT genotype of ABCC4 (rs9561778) gene with thrombocytopenia ((OR = 35.31, 95% Cl = 1.30-956.63, p = 0.034) in compared to GG genotype (table-3.27)

Table-3.26: Different grades of thrombocytopenia caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Adjuvant: N=102		**GSTP1**				**ABCC4**			
		AA(52)	AG(39)	GG(11)	AG(39) +GG(11)	GG(76)	GT (19)	TT(7)	GT(19) +TT(7)
Thrombocytopenia									
Grade ≤II	101	52	38	11	49	76	19	6	25
Grade III	1	0	1	0	1	0	0	1	1
Grade IV	0	0	0	0	0	0	0	0	0

Table-3.27: Thrombocytopenia caused by neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Thrombocytopenia					
Gene	**Genotype**	**Grade III(1)+ Grade IV(0)**	**Grade ≤II (101)**	**Odds Ratio**	**P value**
GSTP1	AA(52)	0	52	Reference	-
	AG(39)	1	38	4.0909(0.1622 to 103.166)	0.3923
	GG(11)	0	11	4.5652(0.08602 to 242.2738)	0.4536
	AG(39)+GG(11)	1	49	3.1818(0.1266 to 79.9649)	0.4817
ABCC4	GG(76)	0	76	Reference	-
	GT (19)	0	19	3.9231(0.07543 to 204.0334)	0.4978
	TT(7)	1	6	35.3077(1.3031 to 956.632)	**0.0342**
	GT(19)+TT(7)	1	25	9.0000(0.3553 to 227.9481)	0.1827

3.3.2.5 Gastrointestinal toxicity induced by adjuvant Chemotherapy

GI toxicity is one of the major types of chemotherapy induced toxicities observed in the treatment of breast cancer. In this study we found 12 patients with grade III and 1 patient with grade IV gastrointestinal toxicity caused by adjuvant chemotherapy (table-3.28). Tendency of showing higher grades of gastrointestinal toxicity was found to be more in patients carrying variant allele of ABCC4 (rs9561778) gene than those of GSTP1 (rs1695) gene but statistically both of the genes had no significant association with gastrointestinal toxicity caused by adjuvant chemotherapy.

Table-3.28: Different grades of gastrointestinal toxicity caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Adjuvant: n=102		**GSTP1**				**ABCC4**			
		AA(52)	AG(39)	GG(11)	AG(39) +GG(11)	GG(76)	GT (19)	TT(7)	GT(19) +TT(7)
Gastrointestinal Toxicity									
Grade ≤II	89	46	34	9	43	69	14	6	20
Grade III	12	6	4	2	6	7	4	1	5
Grade IV	1	0	1	0	1	0	1	0	1

Table-3.29: Gastrointestinal toxicity caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Gastrointestinal Toxicity					
Gene	**Genotype**	**Grade III(12)+ Grade IV(1)**	**Grade ≤II (89)**	**Odds Ratio**	**P value**
GSTP1	AA(52)	6	46	Reference	-
	AG(39)	5	34	1.1275(0.3176 to 4.0023)	0.8528
	GG(11)	2	9	1.7037(0.2953 to 9.8291)	0.5513
	AG(39)+GG(11)	7	43	1.2481(0.3885 to 4.0093)	0.7098
ABCC4	GG(76)	7	69	Reference	-
	GT (19)	5	14	3.5204 (0.9755 to 12.7052)	0.0546
	TT(7)	1	6	1.6429 (0.1722 to 15.6697)	0.6662
	GT(19)+TT(7)	6	20	2.9571(0.8919 to 9.8051)	0.0763

3.3.3 Toxicities caused by adjuvant and neoadjuvant chemotherapy

The numbers of the recorded patients carrying polymorphism of GSTP1 (rs1695) and ABCC4 (rs9561778) genes and suffered from various types of toxicities of different grades induced by adjuvant (n=102) and neoadjuvant (n=117) chemotherapy were combined. It was observed that anemia and neutropenia were the most predominant types of toxicities and the tendency of showing these toxicities was more frequent in patients with at least one variant allele in compared to wild genotype.

Table 3.30: Variation of toxicities caused by chemotherapy (adjuvant and neoadjuvant) in the patients with different genotype of GSTP1 (rs1695) and ABCC4 (rs9561778) genes

Neoadjuvant, n=117+Adjuvant, n=102		GSTP1				ABCC4			
		AA(112)	AG(84)	GG(23)	AG(84)+ GG(23)	GG(165)	GT (39)	TT(15)	GT(39) +TT(15)
Hematological Toxicity	Total								
Anemia									
Grade ≤II	120	64	46	10	56	100	14	6	20
Grade III	63	31	24	8	32	44	15	4	19
Grade IV	36	17	14	5	19	21	10	5	15
Neutropenia									
Grade ≤II	151	79	57	15	72	123	21	7	28
Grade III	35	17	14	4	18	23	8	4	12
Grade IV	33	16	13	4	17	19	10	4	14
Leukopenia									
Grade ≤II	164	86	62	16	78	131	24	9	33
Grade III	35	17	14	4	18	22	9	4	13
Grade IV	20	9	8	3	11	12	6	2	8
Thrombocytopenia									
Grade ≤II	216	111	83	22	105	164	38	14	52
Grade III	2	1	1	0	1	1	0	1	1
Grade IV	1	0	0	1	1	0	1	0	1
Gastrointestinal Toxicity									
Grade ≤II	191	99	74	18	92	150	29	12	41
Grade III	26	13	9	4	13	15	9	2	11
Grade IV	2	0	1	1	2	0	1	1	2

3.3.3.1 Anemia induced by adjuvant and neoadjuvant chemotherapy

Anemia, the most frequent type of hematological adverse effect caused by the chemotherapeutic agents, was obseved in this study in a variety of patients (63 patients with grade III and 36 patients with grade IV anemia) induced by adjuvant and neoadjuvant treatment (table-3.31). We also found that the tendency of showing higher grades of anemia in both adjuvant and neoadjuvant treatment is higher in the patients with having genetic polymorphism in both of the GSTP1 (rs1695) and ABCC4 (rs9561778) genes. Association of GSTP1 (rs1695) gene polymorphism with chemotherapy induced anemia was not established in this study but we found a significant relationship of patient carrying GT and GT plus TT genotype of ABCC4 (rs9561778) gene with this toxicity (OR = 2.75, 95% Cl = 1.33-5.67, p = 0.006 and OR = 2.62, 95% Cl = 1.39-4.93, p = 0.003 respectively) in compared to GG genotype (table-3.32)

Table-3.31: Different grades of anemia caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neoadjuvant:n= 117+ Adjuvant:n=102		**GSTP1**				**ABCC4**			
		AA(112)	AG(84)	GG(23)	AG(84) +GG(23)	GG(165)	GT (39)	TT(15)	GT(39) +TT(15)
Anemia									
Grade ≤II	120	64	46	10	56	100	14	6	20
Grade III	63	31	24	8	32	44	15	4	19
Grade IV	36	17	14	5	19	21	10	5	15

Table-3.32: Anemia caused by adjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

NACT+ACT(219)	**Anemia**				
Gene	**Genotype**	**Grade III(63)+ Grade IV(36)**	**Grade ≤II (120)**	**Odds Ratio**	**P value**
GSTP1	AA(112)	48	64	Reference	-
	AG(84)	38	46	1.1014(0.623 to 1.9473)	0.7396
	GG(23)	13	10	1.7333(0.7009 to 4.2863)	0.2338
	AG(84)+GG(23)	51	56	1.2143(0.7127 to 2.069)	0.4752
ABCC4	GG(165)	65	100	Reference	-
	GT (39)	25	14	2.7473(1.3306 to 5.6722)	**0.0063**
	TT(15)	9	6	2.3077(0.7843 to 6.7898)	0.1288
	GT(39)+TT(15)	34	20	2.6154(1.3867 to 4.9328)	**0.0030**

3.3.3.2 Neutropenia induced by adjuvant and neoadjuvant chemotherapy

Neutropenia, the most predominant type of hematological toxicity induced by breast cancer chemotherapy, was found in a good number of patients (35 patients with grade III and 33 patients with grade IV neutropenia) (table-3.33).We also observed that the tendency of showing higher grades of neutropenia was related with the patients carrying variant allele of GSTP1 (rs1695) and ABCC4 (rs9561778) gene. Patients carrying GT, TT and at least one variant T (GT + TT) allele of ABCC4 (rs9561778) gene were significantly associated with nutropenia (OR = 2.51, 95% Cl = 1.22-5.16, p = 0.012; OR = 3.35, 95% Cl = 1.14-9.79, p = 0.027 and OR = 2.72, 95% Cl = 1.44-9.79, p = 0.012 respectively) in compared to GG genotype. No significant relationship of GSTP1 (rs1695) gene with this toxicity was observed (table-3.34).

Table-3.33: Different grades of neutropenia caused by adjuvant and neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neoadjuvant: n=117+ Adjuvant: n=102		GSTP1				ABCC4			
		AA(112)	AG(84)	GG(23)	AG(84)+ GG(23)	GG(165)	GT (39)	TT(15)	GT(39)+ TT(15)
Neutropenia									
Grade ≤II	151	79	57	15	72	123	21	7	28
Grade III	35	17	14	4	18	23	8	4	12
Grade IV	33	16	13	4	17	19	10	4	14

Table-3.34: Neutropenia caused by adjuvant and neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neutropenia					
Gene	**Genotype**	**Grade III(35)+ Grade IV(33)**	**Grade ≤II (151)**	**Odds Ratio**	**P value**
GSTP1	AA(112)	33	79	Reference	-
	AG(84)	27	57	1.1340(0.6148 to 2.0915)	0.6873
	GG(23)	8	15	1.2768(0.4941 to 3.2993)	0.504
	AG(84)+GG(23)	35	72	1.1637(0.6562 to 2.0636)	0.6039
ABCC4	GG(165)	42	123	Reference	-
	GT (39)	18	21	2.5102(1.2213 to 5.1594)	**0.0123**
	TT(15)	8	7	3.3469(1.1444 to 9.7886)	**0.0274**
	GT(39)+TT(15)	26	28	2.7194(1.4361 to 5.1494)	**0.0021**

3.3.3.3 Leukopenia induced by adjuvant and neoadjuvant chemotherapy

We found 35 patients with grade III and 20 patients with grade IV leukopenia induced by adjuvant and neoadjuvant chemotherapy and the tendency of producing leukopenia was found to be higher in patients with carrying variant allele in both of GSTP1 (rs1695) and ABCC4 (rs9561778) gene (table-3.35). Patient carrying GT and at least one variant T allele of ABCC4 (rs9561778) gene showed significant association with adjuvant and neoadjuvant chemotherapy induced leukopenia (OR = 2.41, 95% Cl = 1.14-5.08, p = 0.021and OR = 2.45, 95% Cl = 1.26-4.77, p = 0.008 espectively) in compared to GG genotype. No association of GSTP1 (rs1695) gene polymorphism was found with leukopenia (table-3.36)

Table-3.35: Different grades of leukopenia caused by adjuvant and neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neoadjuvant: n=117 + Adjuvant: n=102		**GSTP1**				**ABCC4**			
		AA(112)	AG(84)	GG(23)	AG(84) +GG(23)	GG(165)	GT (39)	TT(15)	GT(39) +TT(15)
Leukopenia									
Grade ≤II	164	86	62	16	78	131	24	9	33
Grade III	35	17	14	4	18	22	9	4	13
Grade IV	20	9	8	3	11	12	6	2	8

Table-3.36: Leukopenia caused by adjuvant and neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Leukopenia					
Gene	**Genotype**	**Grade III(35)+ Grade IV(20)**	**Grade ≤II (164)**	**Odds Ratio**	**P value**
GSTP1	AA(112)	26	86	Reference	-
	AG(84)	22	62	1.1737(0.6097 to 2.2595)	0.6317
	GG(23)	7	16	1.4471(0.5374 to 3.8969)	0.4646
	AG(84)+GG(23)	29	78	1.2298(0.6671 to 2.2671)	0.5075
ABCC4	GG(165)	34	131	Reference	-
	GT (39)	15	24	2.4081(1.1406 to 5.0843)	**0.0212**
	TT(15)	6	9	2.5686(0.8552 to 7.7146)	0.0927
	GT(39)+TT(15)	21	33	2.4519(1.2615 to 4.7656)	**0.0082**

3.3.3.4 Throbocytopenia induced by adjuvant and neoadjuvant chemotherapy

The frequency of producing higher grades of thrombocytopenia was found to be rare in breast cancer chemotherapy. In this study we got only two patients with grade III and one patient with grade IV thrombocytopenia and rest of the patient were with grade II or less than grade II thrombocytopenia induced by adjuvant and neoadjuvant chemotherapy (table-3.37). We found no association of GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms with thrombocytopenia caused by adjuvant and neoadjuvant chemotherapy.

Table-3.37: Different grades of thrombocytopenia caused by neoadjuvant and neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neoadjuvant:n=117 + Adjuvant: n=102		**GSTP1**				**ABCC4**			
		AA(112)	AG(84)	GG(23)	AG(84) +GG(23)	GG(165)	GT (39)	TT(15)	GT(39) +TT(15)
Thrombocytopenia									
Grade ≤II	216	111	83	22	105	164	38	14	52
Grade III	2	1	1	0	1	1	0	1	1
Grade IV	1	0	0	1	1	0	1	0	1

Table-3.37: Thrombocytopenia caused by neoadjuvant and neoadjuvant chemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Thrombocytopenia					
Gene	**Genotype**	**Grade III(2)+ Grade IV(1)**	**Grade ≤II (216)**	**Odds Ratio**	**P value**
GSTP1	AA(112)	1	111	Reference	-
	AG(84)	1	83	1.3373(0.08244 to 21.6954)	0.8380
	GG(23)	1	22	5.0455(0.304 to 83.7498)	0.2588
	AG(84)+GG(23)	2	105	2.1143(0.1889 to 23.6651)	0.5435
ABCC4	GG(165)	1	164	Reference	-
	GT (39)	1	38	4.3158(0.264 to 70.5633)	0.3050
	TT(15)	1	14	11.7143(0.6947 to 197.5279)	0.0878
	GT(39)+TT(15)	2	52	6.3077(0.5605 to 70.9838)	0.1359

3.3.3.5 Gastrointestinal toxicity induced by adjuvant and neoadjuvant chemotherapy

GI toxicity is one of the most predominant types of chemotherapy induced toxicities observed in the treatment of breast cancer. In this study we found 26 patients with grade III and 2 patients with grade IV gastrointestinal toxicity caused by adjuvant and neoadjuvant chemotherapy (table-3.39). Tendency of showing higher grades of gastrointestinal toxicity was found to be more in patients carrying variant allele of ABCC4 (rs9561778) gene than those of GSTP1 (rs1695) gene. Statistically we found a significant relationship among the patients showing gastrointestinal toxicity and GT and GT plus TT genotype of ABCC4 (rs9561778) gene (OR = 2.45, 95% Cl = 1.41-8.43, p = 0.007 and OR = 3.17, 95% Cl = 1.40-7.19, p = 0.0076 respectively) in compared to GG genotype. No significant effect of GSTP1 (rs1695) gene polymorphism on the chemotherapy induced gastrointestinal toxicity was found in this study (table-3.40).

Table-3.39: Different grades of gastrointestinal toxicity caused by adjuvant and neoadjuvantchemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Neoadjuvant: N=117+ Adjuvant: N=102		**GSTP1**				**ABCC4**			
		AA(112)	AG(84)	GG(23)	AG(84) +GG(23)	GG(165)	GT (39)	TT(15)	GT(39) +TT(15)
Gastrointestinal Toxicity									
Grade ≤II	191	99	74	18	92	150	29	12	41
Grade III	26	13	9	4	13	15	9	2	11
Grade IV	2	0	1	1	2	0	1	1	2

Table-3.40: Gastrointestinal toxicity caused by adjuvant and neoadjuvantchemotherapy in patients carrying GSTP1 (rs1695) and ABCC4 (rs9561778) gene polymorphisms

Gastrointestinal Toxicity					
Gene	**Genotype**	**Grade III(26)+ Grade IV(2)**	**Grade ≤II (191)**	**Odds Ratio**	**P value**
GSTP1	AA(112)	13	99	Reference	-
	AG(84)	10	74	1.0291 (0.4278 to 2.4754)	0.9489
	GG(23)	5	18	2.1154(0.6717 to 6.6621)	0.2005
	AG(84)+GG(23)	15	92	1.2416(0.5606 to 2.7498)	0.5937
ABCC4	GG(165)	15	150	Reference	-
	GT (39)	10	29	3.4483(1.4111 to 8.4265)	**0.0066**
	TT(15)	3	12	2.5000(0.634 to 9.8581)	0.1905
	GT(39)+TT(15)	13	41	3.1707(1.3977 to 7.1927)	**0.0058**

3.4 Summary of the results

When the response of the therapy was evaluated in terms of different polymorphism of GSTP1 (rs1695) and ABCC4 (rs9561778) gene, it was found that the patient containing variant alleles in both of the genes had better response in compared to wild genotype and the frequency of the responders increased more with the patients with mutant homozygous. Patients carrying AG, GG and AG plus GG genotype of GSTP1 (rs1695) showed good response (OR = 2.5, 95% Cl = 1.13 to 5.69, p = 0.025; OR = 3.4, 95% Cl = 0.84 to 13.93, p = 0.085 and OR = 2.69, 95% Cl = 1.26 to 5.76, p = 0.011, respectively) in compared to AA genotype. No significant association of ABCC4 gene was found with the response of chemotherapy.

In the second phase of the study where the role of genetic change GSTP1 (rs1695) and ABCC4 (rs9561778) gene on the chemotherapy induced adverse drug reaction were evaluated and we show higher frequencies of toxicity in case of variant allele carriers in both of the genes and more toxicity was observed in the patients carrying mutant homozygous genotypes. However, ABCC4 gene polymorphism (rs9561778) had the statistical significance in different types of toxicities like anemia, nutroepenia, leukopenia and gastrointestinal toxicity.

In neoadjuvant chemotherapy, patients carrying GT and at least one variant T allele of ABCC4 (rs9561778) were found to be associated with anemia (OR = 2.87, 95% Cl = 1.04 to 7.89, p = 0.042 and OR = 2.78, 95% Cl = 1.15 to 6.71, p = 0.023 respectively) in compared to GG genotype. Neutropenia and gastrointestinal toxicity were also found to have significant association with patients having any variant T allele of ABCC4 (rs9561778) (OR = 2.64, 95% Cl = 1.09 to 6.40, p = 0.032 and OR = 2.38, 95% Cl = 1.10 to 10.37, p = 0.034 respectively) with the comparison of wild genotype (GG).

In adjuvant chemotherapy, neutropenia and leukopenia were found to have the association with the patients carrying any variant T allele of ABCC4 (rs9561778) (OR = 2.80, 95% Cl = 1.11 to 7.05, p = 0.029 and OR = 2.75, 95% Cl = 1.06 to 7.14, p = 0.038 respectively) and patients having TT genotype of ABCC4 (rs9561778) were associated with thrombocytopenia (OR = 35.31, 95% Cl = 1.30 to 956.63, p = 0.034) with the comparison of GG genotype.

In both adjuvant and neoadjuvant chemotherapy, patients carrying GT and at least one variant T allele of ABCC4 (rs9561778) were found to be associated with anemia (OR = 2.75, 95% Cl = 1.33 to 5.67, p = 0.006 and OR = 2.62, 95% Cl = 1.39 to 4.93, p = 0.003 respectively), leukopenia (OR = 2.41, 95% Cl = 1.14 to 5.08, p = 0.021 and OR = 2.45, 95% Cl = 1.26 to 4.77, p = 0.008 respectively) and gastrointestinal toxicities (OR = 3.45, 95% Cl = 1.41 to 8.43, p = 0.007; OR = 3.17, 95% Cl = 1.40 to 7.19, p = 0.006 respectively) in compared to GG genotype. Neutropenia was associated with patients carrying GT, TT and at least one variant T allele of ABCC4 (rs9561778) (OR = 2.51, 95% Cl = 1.22 to 5.16, p = 0.012; OR = 3.35, 95% Cl = 1.14 to 9.79, p = 0.027 and OR = 2.72, 95% Cl = 1.44 to 5.15, p = 0.002 respectively) in compared to GG genotype.

Our results indicate that GSTP1 gene is significantly associated with the response of the treatment but not with chemotherapy induced toxicities where as ABCC4 gene polymorphism is strongly correlated with chemotherapy induced toxicities but not with the response of the therapy. The response to the treatment as well as toxicity was not associated with different clinicopathological characteristics like estrogen receptor, progesterone receptor and her2/neu status of tumors. No correlation of response and toxicity effects with patient's age, tumor staging and menopause status was established in this study.

CHAPTER FOUR

DISCUSSION

4. Discussion

Most of the genes that encode the enzymes which are involved in the activation and detoxification pathways are reported to contain a wide variety of polymorphisms. There are several reports which indicate that the polymorphic sites in such genes were associated with the response to the therapy and risk of the toxicity caused by CPA combination therapy, but there are also a lots of inconsistencies in the results due to the low sample size (Zhong et. al, 2006; Takada et. al, 2004; Singh et. al, 2007; Ekhart et. al; 2009; Goekkurt et. al, 2007) which suggests that it is urgently needed to confirm those reports with further research. In this study, we examined 219 patients receiving cyclophosphamide based combined chemotherapy (CEF) of which 117 patients received neoadjuvant and 102 received adjuvant chemotherapy to establish the relationship between GSTP1 and ABCC4 gene polymorphisms and response as well as the toxicity produced by this chemotherapy. Although the role of chemotherapy in improving disease-free and overall survival from breast cancer patients is praiseworthy (Levine and Whelan, 2006) , a great challenges comes forward to identify patients who are benefited from chemotherapy and limited use of chemotherapy in case of those who actually do not get proper improvement from the therapy. In locally advanced breast cancer, the use of preoperative systemic chemotherapy has been shown to provide improvement to reduce the tumor size and hence facilitate to control locally with the help of subsequent surgery and proper radiation therapy. As the standard of care for patients who are invaded with locally advanced breast cancer preopearative chemotherapy is well established (Ragaz et. al, 2006, Chia et. al, 2006). Breast cancers comprise a spectrum of different cancer subtypes but are closely related which contains different causal genetic changes, may require different clinical courses, and follow different treatments tailored to the phenotype (Sims et. al, 2007; Kapp et. al, 2006).

Our initial hypothesis was that the functional polymorphisms in GSTP1 and ABCC4 gene would lead to distinct phenotypes of drug metabolism and transport that would provide the prediction about the response and toxicity of the chemotherapy in breast cancer patients. In this study, a significant relationship between genetic variability in GSTP1 and treatment response as well as genetic change in ABCC4 and chemotherapy related

toxicity was observed. Patients who have GG and AG genotype of GSTP1 (rs1695) gene were more likely to get good response from the therapy and patients those contains variant T allele of ABCC4 (rs9561778) gene like GT and TT were susceptible to produce severe toxicity compared to those with GG genotype. The contribution of genotypes on the response and the toxicity of the treatment have the statistical significance. These findings make the suggestion that genetic change in drug metabolism and transporter may play a significant role in the efficacy and toxicity of chemotherapy in breast cancer.

As we have the limitation about the knowledge of mechanism of anthracycline (epirubicin) metabolism, the metabolic pathway of cyclophosphamide serves as a paradigm to determine the role of drug-metabolizing enzymes to make the prediction on treatment outcome (Roy et. al, 1999). GSTs, the major type of detoxifying engymes play a vital role in activation and detoxification of cyclophosphamide and its metabolytes. So GSTP1, the most abundant GST found in many normal and malignant tissues have the significant role in the metabolic pathway of this drug (Townsend and Tew, 2003; Sau et. al, 2010; Arun et. al, 2010). Polymorphism of single-nucleotide substitutions in the coding sequence of GSTP1 (rs1695 A>G) give rise to Ile105Val amino acid substitutions present within the substrate-binding site of GSTP1 (Townsend and Tew, 2003; Johansson et. al, 1998) are reported to have the differences in its catalytic activity (Zhang et al., 2011). The GSTP1 ^{105}Val variant is known to have the relationship with a lower thermal stability and altered catalytic activity to a wide range of substrates in comparison with GSTP1 ^{105}Ile (Yang et. al, 2005). Patients with homozygous isoleucine (Ile/Ile) are known to have the highest level of GSTP1 activity and it reduced in heterozygotes (Ile/Val) to some extent and further reduction in activity is observed in those patients who have mutant homozygotes and contain two copies of valine (Val/Val**)** (Watson et al., 1998; Yang et. al, 2005; Sun et. al, 2010; Kadouri et. al, 2008)**.**.

In recent years, it has become more evident that GSTs not only take part in the process of drug detoxification, but also play an important role in the control of apoptosis through the inhibition of Jun N-terminal kinase (JNK) signaling pathway (Sau et. al, 2010). As thus in the research of chemotherapy resistance they have become the focusing point. Several studies can be reported which make the assumption that GSTP1 expression may be an

important factor for making prediction of early recurrence, drug resistance and bad prognosis in different cancers including breast cancer (Bewick et. al, 2008; Sau et. al, 2010; Arun et. al, 2010).

GSTP1 also found to have the involvement in response to chemotherapy in different types of cancers, like gastric cancer, myeloid leukemia, colorectal cancer and ovarian cancer (Mossallam et al., 2006; Nagle et al., 2007; Ott et al., 2008; Funke et al., 2010).

As far our knowledge goes, no pharmacogenetic study has yet done on Bangladeshi breast cancer patients which promote us to go through this study. Only several studies can be cited that were conducted in different western, Latin and Asian countries which tried to investigate the association of GSTs with chemotherapy response of breast cancer, but the results are conflicting (Satta et al., 1992; Whelan et al., 1992; Ott et al., 2008; Lourenço et al., 2010; Oliveira et al., 2010; Mishra et al., 2011; Zheng et al., 2011; Ji et al., 2013; Yun-Lu Bai et al., 2013). A study conducted in Brasil found that the combination of null GSTT1 and GSTP1 105Val have the tendency to show poor response than combination of non-null GSTT1 and GSTP1 105Ile (Oliveira et al., 2010). However, two different studies in Indian did not find any significant relationship between glutathione S-transferases and responses to chemotherapy (Mishra et al., 2011; Sonam et al., 2013), and similarly a study conducted in Germany found no significant variation in the responses to chemotherapy among individuals with GSTT1, GSTM1 and GSTP1 (Ott et al., 2008). However, the GSTs polymorphisms were reported to be associated with resistance to chemotherapy in other vivo and in vitro studies (Wang et al., 2003; Zheng et al., 2011). These differences in the findings may be due to the various influential factors like ethnicities, sources of patients, disease state, hormone receptor status, sample size and by chance.

In our study, increasing numbers of GSTP1 G allele were observed to have the relationship with increased treatment benefit (table -3.4), which had the similarity to the finding for colorectal and lung cancer patients (Stoehlmacher et al., 2002; Ning et al., 2009) and maintained the consistency with the results of some recent studies on breast cancer patients (Romeo et al., 2011; Yun-Lu Bai et. al, 2013; Ji et. al, 2013, Ge et. al, 2013) The explanation might be due to the reduced metabolism and slower elimination of

chemotherapeutic agents leading to prolonged cytotoxic effect which could be resulted in better treatment response.

The variant G allele of GSTP1 polymorphism has been reported to be associated with increased risk of toxicity in colorectal cancer patients (Braun et al, 2009) and increased risk of neutropenia in patients with lupus erythematosus who were treated with CPA (Zhong et al, 2006). In a small group of 94 women receiving 6 cycles of anthracycline-based cyclophosphamide, epirubicin, and 5-fluorouracil (CEF) regimen for breast cancer, where GG genotypes of GSTP1 were found to have the association with increased risk of grade 3 and 4 hematologic toxicity (Zarate et al, 2007) that was similar to findings of Jhung et al., 2011 but conflicted with Yao et al. 2010). Our study indicated to have the increased number of frequency of toxic events with the variant G allele in GSTP1 but did not meet the requirement of statistical significance which was supported by the findings of some other previous studies (Low et. al, 2009; Ekhart et. al, 2008, and Jl et. al, 2013.)

ABCC4, a member of the superfamily of ATP-binding cassette (ABC) transporters is expressed relatively ubiquitously in most of the organs including the kidney (Van et. al, 2002), lung (Torky et. al, 2005), liver (Rius et. al, 2003), prostate (Lee et. al, 2000), brain (Nies et. al, 2004), pancreas (Ko¨nig et. al, 2005), lymphocytes (Schuetz et. al, 1999) and platelets (Jedlitschky et. al, 2004). Some of the substrates of ABCC4 in GSH-dependent manner is transported by it and depletion of intracellular GSH by GSH synthesis inhibitor, DLbuthionine-(S, R)-sulfoximine, ABCC4-mediated export of the substrates are blocked, such as bile acid and cAMP (Lai et. al, 2002).A previous study suggested that CPA and/or its active metabolites are the substrates to ABCC4 as the in vitro CPA cytotoxicity was increased significantly by the addition of DL-buthionine-(S, R)-sulfoximine (Tian et al, 2005). The expression of ABCC4 in the kidney may play a significant role in the removal of CPA, and its metabolites from the body and genetic changes within this gene may affect the amount or nature of this transporter, can cause impairment of elimination and subsequent manifestation of overdose (Low et al., 2009). This idea was forwarded by several other previous studies that reported specific localization of ABCC4 in the kidney at the apical membrane of proximal tubules and suggested its possible role as one of the efflux pumps for urinary excretion. The substrates for ABCC4 so far observed are purine metabolites urate, cAMP, cGMP and

methotrexate (Van et. al, 2002; Van et. al, 2005; El-Sheikh et. al, 2007). Another previous report indicated that not only CPA, but also its active metabolites are substrates to ABCC4, (Tian et al, 2005) and a large proportion of them are likely to be excreted through the urine (Bagley et. al, 1973).

Hence, ABCC4 might act as one of the most important efflux pumps for urinary excretion for CPA as well as its metabolites. However, for the confirmation of the hypothesis containing the functional activity of ABCC4 in the renal excretion of CPA and its metabolites, further studies are needed. In addition, the expression of ABCC4 in the sinusoidal membrane of hepatic cells might enhance the secretion of active metabolites of CPA which is produced from the liver goes to the systemic circulation. Polymorphisms on this gene might cause a more amount of efflux of CPA and its metabolites, leading to consequent increasing of systemic drug concentration in the body (Low et al., 2009). In our study, the SNP (rs9561778) located in intron 26 of the ABCC4 gene showed a significant association with CPA-induced ADRs. Hence, our assumsion is that the SNP in rs9561778, possess the possibility to influence the expression levels of the gene product. The SNP function prediction software (FastSNP, http://fastsnp.ibms.sinica.edu.tw /pages/input_ SNPListAnalysis.jsp) provided the indication that the SNP, rs9561778, might be located within a transcription factor binding site possibly within an intronic enhancer sequence serving as a causative variant and affect the expression level of the gene (Low et al., 2009). However, further functional analyses are needed to make clarification on how this SNP have the influence on the drug activity. We got a significant association of rs9561778 with CPA-induced ADRs, possessing similar trends of odds ratio in the gastrointestinal toxicity and anemia, leucopenia, neutropenia which maintained the consistency with the findings of one recent study (Low et. al, 2009) might indicate that the two toxicities might be caused by an overdose manifestation of CPA. So, we assumed that the impairment of ABCC4 might cause an insufficient CPA clearance resulting in subsequent increase of the CPA concentration in the body. We also observed that with the increasing of T allele frequency in ABCC4, frequency of treatment response increased but did not reach to the significance level. The explanation of these inconsistencies in the findings of both of genes in response and toxicities might be due to differential capacities of normal and

malignant cells in dealing with drug cytotoxicity, which could be further attributed to somatic changes incurred during tumorigenesis in cancer cells (Yao et al., 2010).Hence our suggestion is that it is needed to clarify the issue in further investigation with a large amount of samples in different ethnicities.

In summary, our study provided information about the role of GSTP1 polymorphisms in outcome after neoadjuvant therapy of breast cancer as well as the associations between ABCC4 genotypes and CPA-induced ADRs. Although the association and the mechanism to induce ADRs should be further validated by using a larger number of samples with different back grounds or by molecular analysis, this study has contributed another piece of the puzzle into the mist of the prediction system, which may help us to identify those patients who will get more benefit from the neoadjuvant therapy and those who are at risk of CPA-induced ADRs leading to a better prognosis and quality of life for patients with breast cancer.

CHAPTER FIVE

CONCLUSION

5. Conclusion

Breast cancer, the most frequent cancer of women which estimated about 1.15 million new cases all over the world in 2002 (Parkin et. al, 2002) and of all new cancer cases among women in the United States in 2009 was 26% (about 0.19 million) (Jemal et. al, 2009). Although the prognosis of breast cancer is good due to the advance of systematic therapy (Bonadonna et. al, 2005, Lancet et. al, 2005) significant variations in the response and toxicity of different chemotherapeutic agents are also observed (Evans et. al, 1999). While resistance to chemotherapy and toxicity of specific agents are greatly determined by multifaceted enzymatic systems that are cytotoxic targets or different molecules of the metabolic pathway of the administered drug, different polymorphic drug transporters also play an important role (Siew-Kee et al., 2009). Though many clinical characters like age, organ function, tumour biology, and concurrent medications contribute to the difference of treatment outcomes, genetic differences in drug transport, metabolism and drug targets also play a vital role (Evans and McLeod, 2003).

The finding of human genome project provided the indication that 99% of DNA within various types of individuals possessed the similarity, and only 1% contained variation, of which the major one was single nucleotide polymorphism (SNP). SNP is a point mutation located on the genome of some individuals of a population. The study of pharmacogenetics suggested that such small amount of diversity in sequence of genome have the significant influence on response, toxicity, and survival of individual treatment in cancer patients. As the knowledge of inter-individual difference is necessary for the optimization of medication, the information regarding the genetic polymorphisms comes as a factor of potential significance in drug disposition and pharmacokinetics. In addition, SNP has become a part of greater clinical significance in terms of its ease of clinical application, rather than it's mRNA, providing some clinical difficulties to obtain tissue samples from cancer patients (Ning et al., 2009).

Most of the patients with beast cancer are treated with cyclophosphamide-epirubicin-5fluorouracil (FEC) or cyclophosphamide-doxorubicin (adiramicin)-5fluorouracil (FAC)

regimen. These drugs are metabolized by different types of enzymes which contain a wide range of polymorphic sites in their genetic sequence. Not only the enzymes but also the drug transporters that play a vital role in drug distribution and clearance and have an important influence on efficacy and toxicity of the drug therapy contain a vast majority of genetic polymorphisms. These genetic polymorphisms became our interest of research to investigate its impact on the out comes of the chemotherapy used in breast cancer treatment. We examined two polymorphisms each of GSTP1 (rs1695) and ABCC4 (rs9561778) gene, the two important genes having a key role the in the drug metabolic and transpor pathway and evaluated their effects on the response of cyclophosphamide-epirubicin-5-FU (CEF) based regimen used in neoadjuvant chemotherapy as well as drug induced toxicities in both adjuvant and neoadjuvant chemotherapy. We observed that polymorphism of GSTP1(rs1695) gene had a good association with the treatment response and genetic variation of ABCC4 (rs9561778) gene had strong relationship with the chemotherapy induced toxicities which further focus to the possibility of individualizing therapy based on the polymorphisms in genes involved in drug detoxification and transportation. Although we have some limitations in this study and major one is that we selected only GSTP1 and ABCC4 gene, which account for the phase II metabolism and part of transportation pathway, our findings open some windows in further wide range of researches in the field of personalized medicine. Now our suggestion in this point is to go though further investigation in a large sample with multilocus strategies involving genetic variations in the whole drug metabolism and transportation pathway to get conclusive evidence of the role of genetic variants in predicting response to, and toxicity of, chemotherapy in breast cancer patients.

CHAPTER SIX

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6. Reference

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APPENDIX

DATA COLLECTION FORM

Questionnaires

Effect of GSTP1 and ABCC4 gene polymorphisms on response and toxicity of cyclophosphamide–epirubicin-5-fluorouracil based chemotherapy in Bangladeshi breast cancer patients

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**
✗ Where have you spent your boyhood (1-15 y)?				
✗ 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	
5-FU	
Adiramycin	
Docitaxel	

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