Effect of SULT1A1, UGT2B7, CYP3A5 and CYP2D6polymorphisms on Tamoxifen response in breast cancer patients of Bangladesh

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Abstract

Background: Breast cancer is the leading cause of cancer death in females worldwide as well as in Bangladesh. Tamoxifen (TAM), selective estrogen receptor modulators (SERMs), is the most commonly used agent for the treatment of estrogen receptor positive breast cancer patients as adjuvant therapy which reduces the risk of recurrence and prolongs the survival. Though most patients are benefited from TAM, some are either fail to respond or become resistant. Tamoxifen is metabolized via the cytochrome P450-mediated pathway and metabolites are inactivated by sulfotransferases 1A1 (SULT1A1), or by the UDP-glucuronosyltransferases (UGT).This study aimed to investigate the prognostic and/or predictive value of functional polymorphisms in SULT1A1,UGT2B7,CYP3A5 and CYP2D6 in Tamoxifen-treated breast cancer patients to correlate the genotype data pharmacogeneticaly with Response, Survival Rate (SR), Hazard Ratio (HR), Breast Cancer Specific Survival (BCSS) and Recurrence Free Survival (RFS).

Methods: A total three hundred and eighty eight estrogen receptor positive female patients with invasive breast cancers and taking Tamoxifen 20mg/day orally for 8 weeks or more were recruited from different public and private hospitals of Bangladesh.The study was conducted in accordance with the International Conference of Harmonization (ICH) for Good Clinical Practice (GCP) and in compliance with the Declaration of Helsinki .Ethical permission was taken to approve the protocol. Each volunteer signed an informed consent document before entering the study.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.Performance status was done by ECOG Performance Statusdeveloped by the Eastern Cooperative Oncology Group, Robert L. Comis, MD, Group Chair.Genomic DNA was isolated by using Daly's Method (Daly et al., 1998). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method was used to analyze the genetic polymorphisms.Amplified DNA fragments was digested by restriction

enzymes followed by gel electrophoresis to identify the targeted alleles namely SULT1A1*2, UGT2B7*2, CYP3A5*3, CYP2D6*10 and CYP2D6*4.

Results: Bangladeshi breast cancer patients showed variable Tamoxifen treated response data in the study.Patients carrying mutant homo(AA) genotype (aOR=2.358 and p=0.0058)and hetero(GA) genotype(aOR=2.933 and p=3.97E-05) showed good response in comparison to Wild(GG) genotype. Patient having hetero(CT) polymorph of UGT2B7*2 also showed prominat(aOR=4.213 and p=0.0001) response in comparison to normal(CC) polymorph. Those patients who have hetero(AG) polymorph of CYP3A5*3 also showed statistically significant result(aOR=5.626 and p<0.0001) in comparison normal homozygote(AA).Both hetero(CT) and mutant homo(TT) showed significant result(aOR=29.082 & p<0.0001 and aOR=1.33E+15 & p<0.0001,respectively) for Tamoxifen associated response among Bangladeshi breast cancer patients. Other polymorphism of selected genes did no show any significant result. Patients carrying hetero(CT) polymorph of CYP2D6*10 showed significant relationship with hot flashes toxicity(aOR=8.40E+07 & p=0.0479) in comparison to wild(CC) genotype. Those breast cancer patients who had hetero(CT) polymorph(aOR=2.61 & p=0.033) of UGT2B7*2 and both hetero(CT)(aOR=1.77E+08 & p=0.0223) & mutant homo(TT)(aOR=2.32E+08 & p=0.0117) polymorphs of CYP2D6*10 showed significant depression in comparison to wild CC for UGT2B7*2 and CC for CYP2D6*10.Both hetero(GA)(aOR=1.58E+07 & p=0.0215) and mutant homo(AA)(aOR=6.31E+06 & p=0.0017) of SULT1A1*2 were found significantly related with Tamoxifen associated decreased libido in comparison to wild(GG) genotype.Hetero(CT) polymorphs of UGT2B7*2 also showed significant relation(aOR=6.34E+06 & p=0.0054) with depression in comparison to wild(CC) genotype. Both hetero(CT)(aOR=4.27E+06 & p=0.0070) and mutant homo(TT)(aOR=6.44E+06 & p=0.0027) polymorphs of CYP2D6*10 showed significant relation with depression in comparison to normal(CC) genotype.Both hetero(CT)(aOR=1.47E+08 & p=0.0364) and mutant homo(TT)(aOR=1.59E+08 & p=0.0255) polymorphs of CYP2D6*10 showed significant relation with vaginal dryness in comparison to normal(CC) homozygote. No other polymorphs of selected genes showed relationship with these toxicity responses.Patents carrying both hetero(GA)(OR=21.5680 & p=0.0364) and mutant homo(AA)(OR=24.9813 & p=0.0255) polymorphs of SULT1A1*2 showed significant relation with Hazard ratio≤1.5 in comparison to wild(GG) genotype. Hetero polymorph(CT) of UGT2B7*2 carrying patients showed significant relation(OR=4.6445 & p<0.0001) with Hazard

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ratio≤1.5 in comparison to wild CC genotype. Hetero polymorph(AG)(OR=5.5577 & p=0.0002) of CYP3A5*3 carrying patients had significant relation with Hazard ratio≤1.5 in comparison to wild(AA) genotype. Both hetero(CT)(OR=210.9091 & p<0.0001) and mutant homo(TT)(OR=28357.6667 & p<0.0001) of CYP2D6*10 showed significant result in relation to Hazard ratio≤1.5 in comparison to wild(CC) genotype. Mutant homo(TT) genotype of UGT2B7*2 showed significant relation(OR=4.0906 & p=0.0004) with survival rate>5 years in comparison to wild(CC) genotype patients.Mutant homo(GG) of CYP3A5*3 polymorph showed significant relation(OR=7.7834 & p<0.0001) with survival rate>5 years in comparison to normal(AA) genotype.Both hetero(GA)(OR=17.2757 & p<0.0001) and mutant homo(AA)(OR=6.7143 & p=0.0062) of CYP2D6*4 were found significant relationship with survival rate>5 years in comparison to wild(GG) genotype. Recurrence free survival>5 years was associated significantly among breast cancer patients with CYP2D6*10 mutant homo(TT)(OR=1.8645 & p=0.0107) in comparison to normal(CC) genotype.Mutant homo(GG) of CYP3A5*3 found strongly related(OR=192.0737 & p=0.0002) with breast cancer specific survival in comparison to normal(AA) genotype. Both hetero(GA)(OR=55.1209 & p<0.0001) and mutant homo(AA)(OR=27.5137 & p=0.229) polymorphisms of CYP2D6*4 found significant relation with breast cancer specific survival in Bangladeshi patients in comparison to normal(GG) genotype. No other polymorphs of our selected gene were found significantly associated with survival data. We did not find any correlation between performance status and SULT1A1*2, UGT2B7*2,CYP3A5*3,CYP2D6*4 and CYP2D6*10 genotype among Tamoxifen treated Bangladeshi breast cancer patients.

Conclusion:A,T,G,A and T allele of SULT1A1*2,UGT2B7*2,CYP3A5*3,CYP2D6*4 and CYP2D6*10 respectively found significant in Tamoxifen treated respone,survival,toxicity and performance status variability among Bangladeshi breast cancer patients.It is important to identifying such patients before the start of treatment in optimizing therapy with Tamoxifen. The finding of this research work has detected the actual causes of the development of side effects, response variability, survival difference and performance diversity after Tamoxifen therapy in Bangladeshi breast cancer patients and suggest the alternative treatment therapy for the disease at early stage and help to develop a safe, efficient and cost effective treatment plan for the 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.

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Dedication

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

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Achievement

The abstract of this research work has been awarded as best poster in Pharmacogenomics category in 2nd International South Asian Biotechnology Conference 2016 on Biotechnology for sustainable Development held on 5-6 February 2016 in Dhaka.

List of abbreviation

Name	**Details**
TAM	Tamoxifen
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 datacompared)
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
$MgCl_2$	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 datacompared)
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

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SRS	Substrate recognition sites
T	Thymine (where referring to a nucleotide)
TAE	Tris-acetate-EDTA
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

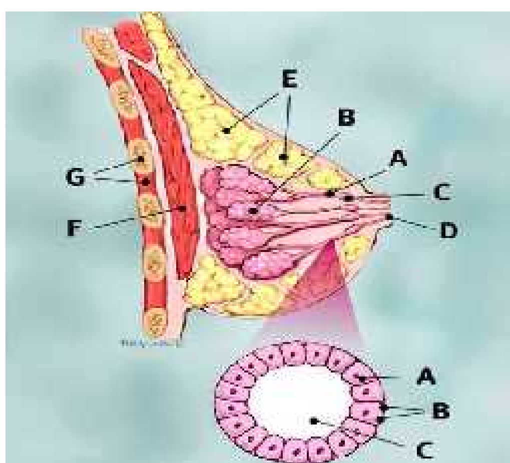
INTRODUCTION

1. INTRODUCTION

Cancer, the disease of cell, is characterized by abnormal genetic content, altered chromosomal structure and uncontrolled growth usually accompanied by loss of cellular differentiation. It occurs as a result of mutations, or abnormal changes, in the genes responsible for regulating the growth of cells and keeping them healthy.

Breast cancer is the most common cancer among women worldwide as well as in Bangladesh. Aside from family history and other environmental factors, the most well-established risk factors for breast cancer are those associated with hormonal and reproductive factors that result in greater lifetime exposure to estrogens and androgens, such as an extended reproductive life (resulting from an early age at menarche and late age at menopause), late age at first fulltime pregnancy and nulliparity. Its lifetime risk amounts to a total of 10% and approximately 15-20% of all breast cancer are associated with the occurrence of familial and/ ovarian cancer.

Breast cancer develops in either the ducts or the lobules. Development of breast cancer starts when the cells lining the ducts or lobules become abnormal in size and shape and start multiplying in an uncontrolled way (Abeloff MD *et al*., 2008).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, World Health Organization (WHO) classifies more than a dozen variants which are less common but still very well defined.



Breast cancer Profile showing different parts with cancer susceptibility

Breast profile:

A Ducts
B Lobules
C Dilated section of duct to hold milk
D Nipple
E Fat
F Pectoralis major muscle
G Chest wall/rib cage

Enlargement

A Normal duct cells
B Basement membrane
C Lumen (center of duct)

1.1 Signs and symptoms of breast cancer:

Breast cancer usually has no symptoms in its early stage. As a tumor develops, the following signs and symptoms are appeared:

- A lump in the breast or underarm is seen that persists after menstrual cycle. This is often the first apparent symptom of breast cancer. Lumps associated with breast cancer are usually painless, although some may cause a prickly sensation. Lumps are usually visible on a mammogram long before they can be seen or felt.
- Swelling is seen in the armpit.
- Pain or tenderness is felt in the breast. Although lumps are usually painless, pain or tenderness can be a sign of breast cancer.
- A noticeable flattening or indentation on the breast, which may indicate a tumor that cannot be seen or felt.
- Any change in the size, contour, texture or temperature of the breast is a sign of breast cancer. A reddish, pitted surface like the skin of an orange could be a sign of advanced breast cancer.
- A change in the nipple, such as a nipple retraction, dimpling, itching, a burning sensation or ulceration is also a sign of breast cancer. A scaly rash of the nipple is symptomatic of Paget's disease, which may be associated with an underlying breast cancer.
- Unusual discharge from the nipple that may be clear, bloody or another color is a sign of breast cancer. It's usually caused by benign conditions but could be due to cancer in some cases.
- Sometimes a marble-like area under the skin is observed.
- An area that is distinctly different from any other area on either breast could be a sign of breast cancer.

1.2 Diagnostic tests for breast cancer:

<u>Breast Self-Exam</u>

While some cancers are too tiny to feel and most lumps aren’t cancer, self-exams are a proactive way to help taken care of.

<u>Clinical Breast Exam</u>

A clinical breast exam is a breast exam performed by a health care professional. It’s a basic part of women’s check-ups, starting at age 20. The physician will

check both the patient's breasts, looking out for lumps and other possible abnormalities, such as inverted nipples, nipple discharge or change in breast shape. The patient will be asked to sit/stand with her arms in different positions, such as above her head and by her sides.

Mammogram

A mammogram is a special type of X-ray taken to look for abnormal growths or changes in breast tissue. It's a key tool in breast cancer detection, though no test is perfect. It is commonly used for breast cancer screening. If anything unusual is found, the doctor may order a diagnostic mammogram. Breast cancer screening has become a controversial subject over the last few years. Experts, professional bodies, and patient groups cannot currently agree on when mammography screening should start and how often it should occur. Some say routine screening should start when the woman is 40 years old, others insist on 50 as the best age, and a few believe that only high-risk groups should have routine screening. 3D mammograms, when used in collaboration with regular 2D mammograms were found to reduce the incidence of false positives, researchers from the University of Sydney's School of Public Health, Australia, reported in *The Lancet Oncology*.

Breast Ultrasound

Doctors sometimes use ultrasound images to check whether a breast lump is a cyst (a fluid-filled sac that is not cancer) or a solid mass.

Breast MRI

MRI stands for magnetic resonance imaging. A dye is injected into the patient. This type of scan helps the doctor determine the extent of the cancer. Researchers from the University of California in San Francisco found that MRI provides a useful indication of a breast tumor's response to pre-surgical chemotherapy much earlier than possible through clinical examination.

Breast Biopsy

When doctors perform a biopsy, they remove cells from a suspicious mass to see if it's cancer. A sample of tissue from an apparent abnormality, such as a lump, is surgically removed and sent to the lab for analysis. It the cells are found to be cancerous, the lab will also determine what type of breast cancer it is, and the grade of cancer (aggressiveness).

Minimally Invasive Breast Biopsy

This type of breast biopsy generally uses a needle, not surgery.

Sentinel Node Biopsy

In a sentinel node biopsy, doctors check a few lymph nodes under the arm to see if cancer has spread into the lymph system.

Ductal Lavage

Ductal lavage checks cells from the milk ducts for precancerous cells.

1.3 Epidemiology of Breast cancer:

Breast cancer incident varies greatly around the world: lowest in less-developed countries and greatest in the more-developed countries. In the twelve world regions, the annual age-standardized incidence rates per 100,000 women are as follows: in Eastern Asia, 18; South Central Asia, 22; sub-Saharan Africa, 22; South-Eastern Asia, 26; North Africa and Western Asia, 28; South and Central America, 42; Eastern Europe, 49; Southern Europe, 56; Northern Europe, 73; Oceania, 74; Western Europe, 78; and in North America, 90(B. W. and Kleihues P, 2003).

1.3.1 Relation of breast cancer with age:

The average age of breast cancer is at menopause. The age-incidence curve changes around the menopausal period, most likely due to hormonal changes 10 to 15 years earlier, flattens out in the 40 to 50 year old age range, and then increases as age increases. Recent data showing decreased risk of breast cancer incidence at older ages, e.g., older than 75 years of age, relative to younger ages, are likely an artifact of recent increases in breast cancer screening in the United States(Kessler LG,1992). The incidence of breast cancer increases rapidly with age during the reproductive years and then increases at a slower rate after about age 50 years. The cumulative incidence of breast cancer among women in Europe and North America is about 2.7% by age 55, about 5.0% by age 65, and about 7.7% by age 75. (Murray CJL & Lopez AD, 1997).

1.3.2 Ethnic variation and effects of migration upon breast cancer:

The US breast cancer occurrence rates (per 100,000 women) were: 139 for White women, 121 for Black women, 98 for Asian and Pacific island women, 82 for Hispanic women and 42 for American Indian and Alaska native women. Breast-cancer incidence at ages 30–49 increased in the UK in the recent period, whereas in

Japan the steep increase started after 1970, such as by 1990 the rates in younger women in Japan were similar to those in women in the UK 30 years earlier. At older ages (50–74), the difference in incidence between the two countries is greater, and incidence has increased in both countries during most of this period. (Hoel DG *et al.,* 1983). Women who are migrants to areas of higher or lower breast cancer risk adopt the level of breast cancer risk reported for women already living in the new place of residence. This suggests that, rather than genetics, there is an important role for a woman's lifestyle and environment in breast cancer risk.

1.4 Risk factors

1.4.1 Menarche and the menstrual cycle

For each 1-year delay in menarche (first time menstruation), the risk decreases by around 5% (Hunter DJ *et al,* 1997). Women with late onset of menarche had longer and more variable cycles in the 10 years after menarche than did those with early onset. Women with late onset of menopause had longer and more variable cycles in the premenopausal interval than did those with early onset. Cumulative fertility in women after marriage did not differ according to cycle length and variance. Late menopause may be a breast cancer risk factor due to relative estrogen excess and progesterone lack as reflected in longer, more varied cycle patterns (Wallace RB et al., 1978).There is also evidence that, although age at menarche is related to breast cancer risk at all ages, the effect may be stronger in younger (premenopausal) women (Kelsey JL *et al*., 1993). Cycle length and regularity have not been found in research consistently related to risk of breast cancer.

1.4.2 Breast density

Breast density may reflect lifelong hormone exposure and potentially could be used as a biomarker for breast cancer risk. Several studies have shown an inverse association between parity and mammographic density. Nulliparous women and women with a later age at first birth have higher estrogen levels than parous women and women with a younger age at first birth, respectively. Nulliparous women have denser breast tissue than parous women, and density decreases with increasing number of children. Moreover, among parous women, later age at first birth and fewer live births have

been associated with a higher proportion of dense breast tissue and greater risk for breast cancer (Mellissa Yong et al., 2010).

1.4.3 Height

Taller-than-average women have a slightly greater likelihood of developing breast cancer than shorter-than-average women. Experts are not sure why.

1.4.4 Radiation exposure

Undergoing X-rays and CT scans may raise a woman's risk of developing breast cancer slightly. Scientists at the Memorial Sloan-Kettering Cancer Center found that women who had been treated with radiation to the chest for a childhood cancer have a higher risk of developing breast cancer.

1.4.5 Hormone Replacement Therapy (HRT)

Both forms, combined and estrogen-only HRT therapies may increase a woman's risk of developing breast cancer slightly. Combined HRT causes a higher risk.

1.4.6 Certain jobs

French researchers found that women who worked at night prior to a first pregnancy had a higher risk of eventually developing breast cancer. Canadian researchers found that certain jobs, especially those that bring the human body into contact with possible carcinogens and endocrine disruptors are linked to a higher risk of developing breast cancer. Examples include bar/gambling, automotive plastics manufacturing, metal-working, food canning and agriculture.

1.4.7 Childbearing

Childbearing seems to have a dual effect on risk of breast cancer; it is increased risk immediately after a birth, but this excess risk gradually diminishes. In the longer term, the effect of a birth protect against the disease.(Beral V and Reeves G, 1993) . Pike et al. (Nature 1983;303:767-70) and Moolgavkar et al. (JNCI 1980;65:59-69) proposed quantitative theories for the effect on age-specific breast cancer risk of ages at menarche, first childbirth, and menopause. The main qualitative findings are that 1) the protective effects of late menarche and of early first full-term pregnancy are

greater in premenopausal than in postmenopausal women; 2) first full-term pregnancy initially boosts the level of risk, but incidence rates increase with age more slowly thereafter; 3) among the parous, multiparity is protective both in premenopausal and postmenopausal women, regardless of age at first full-term pregnancy; 4) both nulliparous and lean women are more protected by early menopause than are parous and overweight women; 5) increased body mass index is protective before, but detrimental after, menopause; and 6) postmenopausal incidence rates increase with age more rapidly among overweight than among lean women. In comparison with nulliparous women, women who have had at least one full-term pregnancy have around 25% reduction in breast-cancer risk.(Layde PM, *et al.,1989;* Ewertz *et al., 1990*).

1.4.8 Breastfeeding

The effect of breastfeeding on risk of breast cancer has been controversial. However, recent studies in less developed countries, where the total duration of breastfeeding can be very long, have reported substantial protective effects (Lipworth *et al*., 2000).Protection has also been seen in some, studies in more developed countries. For example, the US Cancer and Steroid Hormone Study examined the relation between breastfeeding and breast cancer in over 4500 women with the disease and found that women who had breastfed for a total of 25 months or more had a 33% lower risk of breast cancer than those who had never breastfed (Layde PM *et al.,* 1989).The protective effect of breastfeeding on breast cancer risk may be stronger in younger women than in older women (Kelsey JL *et al.,* 1993).

1.4.9 Menopause

Women experiencing menopause at a late age are at a higher risk of breast cancer than those who cease menstruating earlier, with risk increasing by about 3% for each year older at menopause (Lancet, 1997). The protective effect of menopause can be seen in the slowing in the rate of increase in breast-cancer incidence with age that occurs at around age 50. Thus, premenopausal women are at higher risk of breast cancer than postmenopausal women of the same age, and perimenopausal women are at intermediate risk.

1.4.10 Endogenous hormones

There is substantial evidence that high estrogen levels in postmenopausal women are associated with an increase in breast cancer risk, but such a relation has not yet been established in premenopausal women, despite biologic evidence that breast epithelial cell division rates are high during the luteal phase of the menstrual cycle when estradiol and progesterone levels are high. The lack of total consistency among studies that have assessed estrogen differences, whether in breast cancer patients versus controls or in subgroups of the population characterized by different risk profiles for breast cancer, is not unexpected given the extraordinarily complex methodological issues that must be addressed in these studies (Bernstastein L et al.,1993).Estrogen stimulation plays an important role in human breast cancer cell growth and development. It was reported that estrogen could affect breast cancer risk through stimulating cellular proliferation and promoting tumor progression. For postmenopausal women, studies have shown a positive association between serum oestradiol concentrations and risk; postmenopausal women with high serum oestradiol concentrations have a risk around twice that of women with lower concentrations of this hormone (Key TJ & Verkasalo PK., 1999).

1.4.11 Oral contraceptives

The risk of breast cancer is increased by around 25% in current users of combined oral contraceptives, but the excess risk falls after cessation of use, such that 10 or more years after use stops, no significant increase in risk is evident (Lancet 1996).

Because breast cancer is rare at young ages when use of oral contraceptives is common, most use of these agents does not result in a large number of breast cancer cases. However, use of oral contraceptives late in a woman's reproductive life will result in an increased relative risk of breast cancer. Thus, the later the use of oral contraceptives, the larger the number of resulting excess cases of breast cancer (Lancet 1996)

1.4.12 Hormonal therapy for the menopause

Current users of hormonal therapy for the menopause are at higher risk of breast cancer than women who have never used these preparations (Lancet 1997; Magnusson CM *et al., 1999*) Data reanalyzed by the Collaborative Group on

Hormonal Factors in Breast Cancer and another large study have shown that among current and recent users of hormonal therapy, the risk of breast cancer increases with increasing duration of use and that this excess diminishes after cessation of use (Lancet, 1997 ; Magnusson CM *et al.,*1999) Most studies so far suggest that 5 or more years after cessation of use of hormonal therapy for the menopause, there is no significant excess of breast cancer relative to never users. Collaborative Group show that, among current and recent users, use of hormonal therapy for about 10 years increases risk of breast cancer by roughly 35 % (Lancet, 1997). Estimates suggest that 10 years of use of hormonal therapy for the menopause from age 50 to 60 would result in a cumulative excess incidence of six breast cancers per 1000 women from age 50 to age 70 (51 new cancers in users compared with 45 new cancers in non-users) (Lancet ,1997).

1.4.13 Diet

The role of specific dietary factors in breast cancer causation is not completely resolved. Enthusiasm for the hypothesis that dietary fat intake was responsible for the high rates of breast cancer rates in Western countries was based largely on the weakest form of epidemiologic evidence – ecological correlation studies. Some evidence suggests that vitamin A or other compounds in vitamin A-rich foods may reduce breast cancer risk modestly, but these findings are not conclusive and deserve further consideration. Alcohol intake is the best-established specific dietary risk factor for breast cancer, and studies demonstrating that even moderate alcohol intake increases endogenous estrogen levels provide a potential mechanism, thus supporting a causal interpretation. Hypotheses relating childhood and adolescent diet to breast cancer risk decades later will be more difficult to test. Nevertheless, the available evidence that breast cancer risk can be reduced by avoiding weight gain during adult years and by limiting alcohol consumption is strong (Michelle D Holmes et al.,2004).

Recent studies are done to evaluate the effect of meat, fibre, fruit and vegetables, and phytooestrogens in breast risk and results for meat, fibre, and fruit have been inconsistent.but phyto-oestrogens, may block the effects of the potent endogenous oestrogens and thereby reduce breast-cancer risk. However, the results of studies so far are inconclusive (Gandini *et al. 2000; Key TJ et al., 1998*).

Table1.1:Age-adjusted relative risk of breast cancer according to quantile of calorie-adjusted intake of total fat (Willett,W. C. *et al* ,1987)

Quantile of intake	Age adjusted relative risk	95% confidence limit
1	1.0	---
2	0.8	0.6-1.0
3	0.9	0.7-1.1
4	0.8	0.6-1.0
5	0.8	0.6-1.1

1.4.14 Alcohol and smoking

Observational studies have repeatedly shown that alcohol consumption is associated with a moderate increase in the risk of breast cancer; risk increases by roughly 10% per 10 g alcohol (1 unit) consumed per day. (Minton, 1979)

1.4.15 Anthropometry

Adult height shows a weak positive association with breast cancer risk. Within populations, a 10 cm greater height is typically associated with an increase in risk of about 10% (Hunter DJ & Willett WC, 1993)

In postmenopausal women, obesity increases the risk of breast cancer; risk is about 50% higher in obese women (body-mass index >30 kg/m) than in lean women (bodymass index 20 kg/m). Breast-cancer risk is increased in women whose birthweight was high. This association is not observed in premenopausal women, (Lancet, 1997)

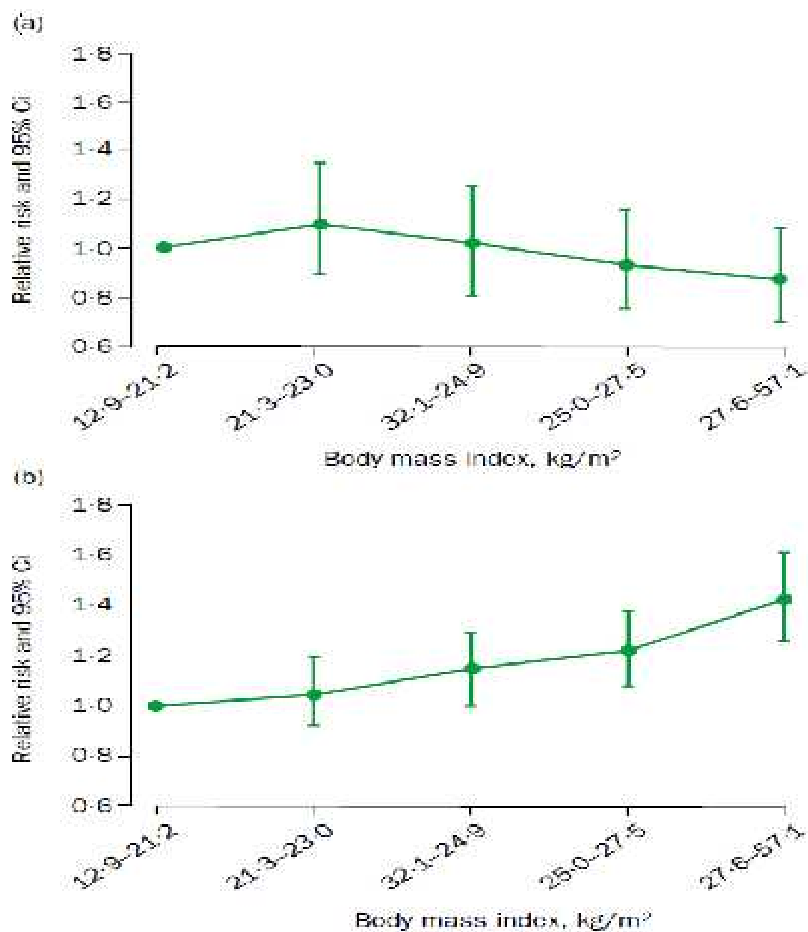


Fig1.1: Relationship between body mass index and breast cancer risk in (a) premenopausal and (b) postmenopausal women (Trentham-Dietz *et al.,* 1997; Rohan. T. E. and Bain C. J., 1997).

1.4.16 Exercise

Several studies have reported that moderate physical activity is associated with a lower risk of breast cancer. The data are not entirely consistent, although they are somewhat stronger for premenopausal women than for postmenopausal women. (Friedenreich CM, *et al, 1998*).

1.4.17 Environmental estrogens

Some chemicals have structural similarities to endogenous oestrogens and can bind to the oestrogen receptor such as DDT (dichlorodiphenyltrichloroethane) and its

metabolites, and PCBs (polychlorinated biphenyls) but so far the results have not shown an association breast cancer (Dich J et al., 1997; Wolff MS et al., 2000).

1.4.18 Family history and genetic factors

Environmental and lifestyle factors rather than inherited genetic factors account for most cases of breast cancer (Frisch et al., 1985).Most women with the disease do not have a family history of it, and most women with affected relatives never develop breast cancer. Studies of twins have allowed estimation of the overall contribution of inherited genetic characteristics. In a population-based study of 45 000 pairs of twins in three Nordic countries, hereditary factors were estimated to contribute to around a quarter and environmental and lifestyle factors to around three-quarters of the inter individual differences in susceptibility to breast cancer. However, the five-fold global variation in breast-cancer rates and the results from migrant studies indicate that, worldwide, the importance of environmental and lifestyle factors must be even greater than estimated within this Nordic population (Lichtenstein *et al.,* 2000).

1.4.19 Family history

Environmental and lifestyle factors rather than inherited genetic factors account for most cases of breast cancer (Frisch et al., 1985).Most studies on familial risk of breast cancer have found about two-fold relative risks for first-degree relatives (mothers, sisters, daughters) of affected patients (Bernstein *et al.,* 1987). With affected second-degree relatives (grandmothers, aunts, grand-daughters), there is a lesser increase in risk.

1.4.20 High-risk mutations

These mutations in the genes *BRCA1, BRCA2, P53, PTEN, and ATM.* Mutations in *BRCA1* and *BRCA2* can cause high risks of breast cancer. Germ line mutations in *P53* predispose to the Li-Fraumeni cancer syndrome (including childhood sarcomas and brain tumors, as well as early-onset breast cancer).High-risk alleles probably account for most of the families with four or more breast cancer cases, for around 20—25% of the familial breast cancer risk overall, and for around 5% of all breast cancers (Wolff MS *et al,* 2000; Ford D *et al.,* 1998; *Easton DF, 1999; Peto et al,1999*).

1.4.21 Low-risk polymorphisms

The effects of common polymorphisms of 18 genes (Dunning AM *et al.1999*). Statistically significant differences in genotype frequencies were observed for four genes (*CYP19, GSTP1, P53*, and, for postmenopausal breast cancer, *GSTM1*).

TP53: The *TP53* gene gives instructions for making a protein called p53 that helps stop the growth of abnormal cells. Inherited mutations of this gene cause *Li-Fraumeni syndrome* (named after the 2 researchers who first described it). People with this syndrome have an increased risk of developing breast cancer, as well as several other cancers such as leukemia, brain tumors, and sarcomas (cancer of bones or connective tissue). This is a rare cause of breast cancer. (American Cancer Society)

1.4.22 Benign Breast Diseases

Since benign breast disease encompasses a broad spectrum of histological changes, breast cancer risk has been further evaluated according to the histopathological characteristics of the biopsy specimens (Hutchinson *et al.,*1980; Haagensen, 1971;Kodlin, D. *et al.,*1977; Page, D. L *et al.*,1978; Humphrey and Swerdlow, 1968; Black *et al*.,1972; Dupont *et al,*1985).The small proportion of women with atypical hyperplasia who have been estimated to have approximately a 5-fold increased risk of breast cancer (Kodlin, D. *et al,*1977; Page D. L *et al.,*1978; Humphrey and Swerdlow, M. A, 1968; Black *et al,* 1972, Dupont *et al,* 1985). Calcification in the biopsy specimens (Hutchinson *et al.,* 1980; Haagensen, C. D., 1971; Kodlin, D., 1977; Kodlin, D. *et al,* 1977; Page, D. L *et al,* 1978; Humphrey and Swerdlow, 1968; Black *et al.,* 1972; Dupont *et al,* 1985).and possibly large breast size (Dupont and Page, 1987) may further increase the risk in women with proliferative disease. A family history of breast cancer and late age at first birth (or nulliparity) have also been suggested to be more important risk factors in women (Dupont and Page, 1987; Dupont *et al,* 1985).

Tab1. 2: Risk factors in breast cancer in female (Jennifer L. Kelsey and Gertrud S. Berkowitz, 1988)

Factor	High risk	Low risk	Magnitude of risk differential
Age	Old	Young	>>>
Country of birth	North America, North Europe	Asia ,Africa	>>>
Place of residence	Urban	Rural	>
Marital status	Never married	Ever married	>
Race	White	Black	>
Age at first full term pregnancy	Older than 30	Younger than 20	>>
Body build post menopausal	Obese	Thin	>>
Age at menarche	Early	Late	>
Age at menopause	Late	Early	>
Family history of premenopausal bilateral breast cancer	Yes	No	>>>
History of cancer in one breast	Yes	No	>>>
Any first degree relative with breast cancer	Yes	No	>>
Radiation to chest	Large dose	Minimum exposure	>>
Socioeconomic class	Upper	Lower	>>

1.5 Prospects for prevention:

For preventing Breast cancer measures should be considered below:

1.5.1 Lifestyle

The classic risk factors for breast cancer, such as age at menarche, age at menopause, and parity, are not possible to change. However avoidance of obesity should reduce the risk of breast cancer; limitation of alcohol intake would be beneficial; breastfeeding is also beneficial for the baby; and maintenance of at least moderate physical activity throughout life may directly reduce breast-cancer risk, indirectly reduce risk by helping to prevent the development of obesity, and have many other health benefits (Gandini *et al.,* 2000).

1.5.2 Hormonal therapies

The overall effect of oral-contraceptive use on risk of breast cancer in young premenopausal women is small, because the small increase in relative risk is acting on a very low background risk. In older premenopausal women oral contraceptives may be a less favorable choice because the absolute risk of breast cancer, and other health hazards, rises rapidly at ages that's why use of contraceptives should be avoided.(Ford *et al*., 1998; Easton DF., 1999; Peto *et al.,* 1999; Thorlacius *et al*., 1998; Struewing *et al.,*1997; Dunning *et al.,* 1999; Russo, 1999; Trentham-Dietz *et al,* 1997)

1.5.3 Reduction of risk:

For women at average risk, the emphasis is on regular screening and healthy lifestyle choices (e.g., low-fat diet, regular exercise, and breastfeeding). Women at increased risk for breast cancer are advised to consider additional risk reduction strategies in consultation with their health care providers.

1.5.4 Physical activity:

Regular physical exercise has been shown to provide some protection against breast cancer, especially in postmenopausal women. The reduction in risk for physically active women compared with women who are least active may be as much as 25%

(Lynch *et al*., 2011; Maruti *et al*., 2008; McTiernan *et al.,*2003; Patel *et al,* 2003; Bardia *et al,* 2006; Kobayashi *et al.,* 2013).

1.5.5 Diet:

A diet that is rich in vegetables, fruit, poultry, fish, and low-fat dairy products has been associated with a lower risk of breast cancer in some studies.There is also some evidence that soy-rich diets may reduce risk (Chen, W.Y., 2013).Overall, however, the influence of dietary factors on breast cancer risk remains inconclusive.

1.5.6 Breastfeeding:

The risk reducing effect of breastfeeding has been shown in multiple studies, especially if the breast-feeding lasts 1½ to 2 years. (American Cancer Society (ACS).2014, Jan. - Last revised)For every year of breastfeeding, the reduction in relative risk has been estimated at approximately 4% (Stuebe *et al.,* 2009; Zheng *et al.*, 2001; Tryggvadóttir *et al*., 2001)

1.6 Classification or subtypes of breast cancers:

Breast cancer is a genetically and clinically heterogeneous disease. In order to organize this heterogeneity and standardize the language, breast cancer classification systems have been developed. These classification schemes have been used to aid in treatment and prognosis.

Table1.3 :Tumor Location and Related Histologic Subtype (National cancer institute;2014)

Tumor Location	**Histologic Subtype**
NOS = not otherwise specified.	
Carcinoma, NOS	
Ductal	Intraductal (*in situ*)
	Invasive with predominant component

Tumor Location	**Histologic Subtype**
	Invasive, NOS
	Comedo
	Inflammatory
	Medullary with lymphocytic infiltrate
	Mucinous (colloid)
	Papillary
	Scirrhous
	Tubular
	Other
Lobular	Invasive with predominant *in situ* component
	Invasive
Nipple	Paget disease, NOS
	Paget disease with intraductal carcinoma
	Paget disease with invasive ductal carcinoma
Other	Undifferentiated carcinoma
	Metaplastic

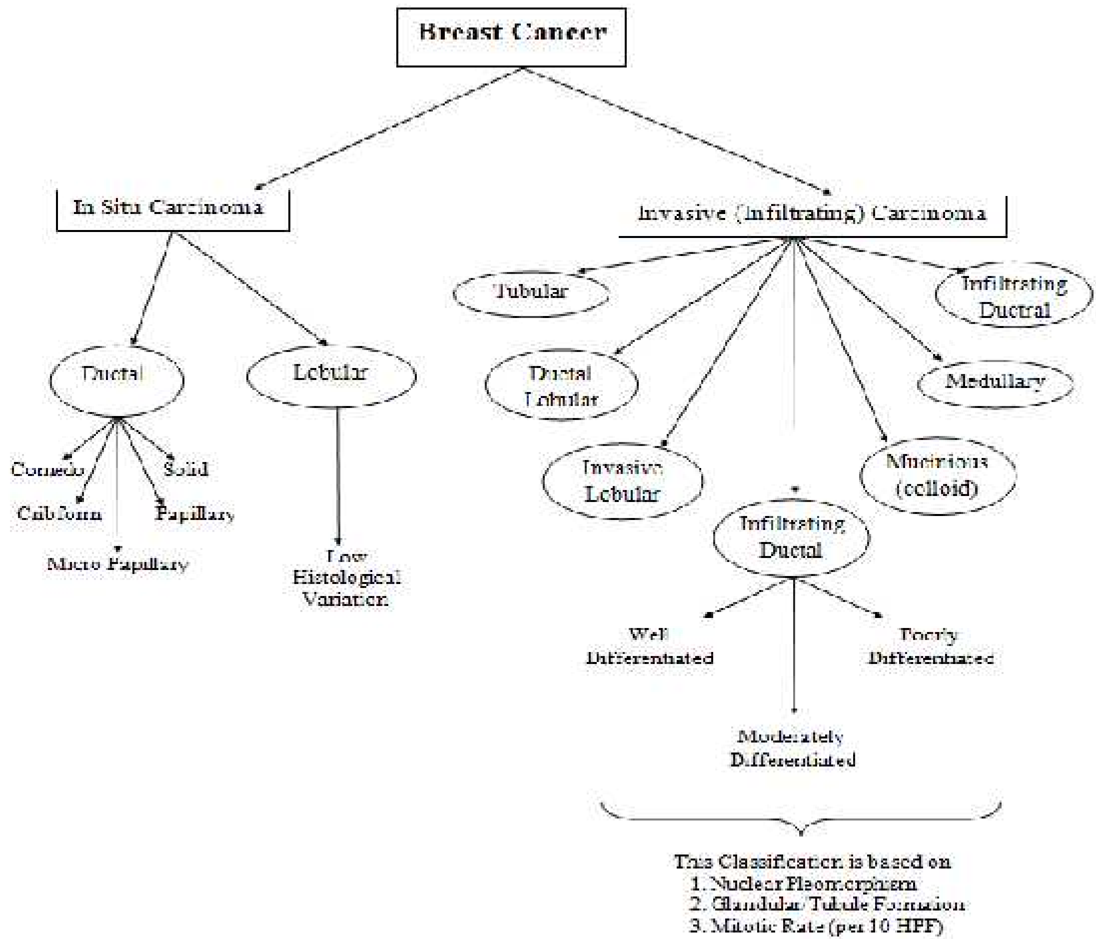


Fig1.2:Histologicalclassification of breast cancer(Gautam K Malhotra etal.,2010)

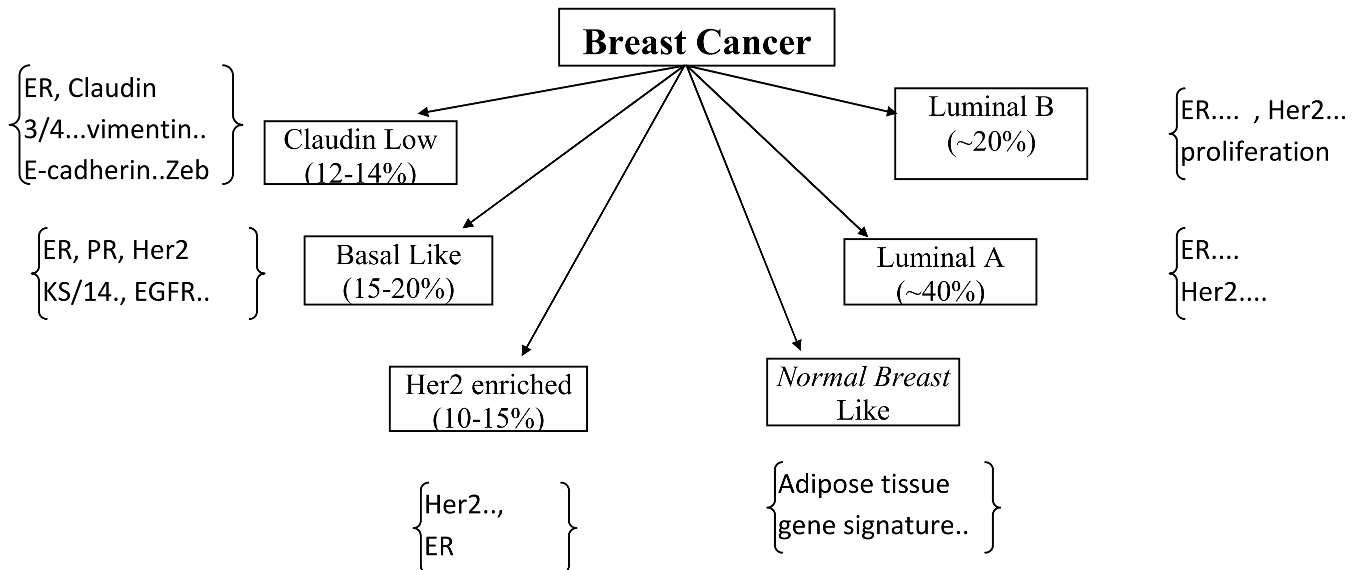


Fig1.3: Molecular classification of breast cancer (Gautam K Malhotra *et al.*, 2010)

1.7 STAGING Information for Breast Cancer (National cancer research institute, 2014)

Definitions of TNM and AJCC Stage Groupings:

The American Joint Committee on Cancer (AJCC) staging system provides a strategy for grouping patients with respect to prognosis. Definitions of TNM and AJCC Stage Groupings—

"The AJCC has designated staging by tumor, node, and metastasis (TNM) classification to define breast cancer (Edge SB *et al*., 2010). When this system was modified in 2002, some nodal categories that were previously considered stage II were reclassified as stage III (Singletary SE *et al*., 2002).As a result of the stage migration phenomenon, survival by stage for case series classified by the new system will appear superior to those using the old system (Woodward WA *et al.,* 2003)".

Table1.4: 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	node
M0	No distant metastases
M1	Distant metastases (including supraclavicular

Table1.5: 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,
IV	any T, any N, M1

1.8 Breast cancer statistics:

1.8.1 Key Statistics:

(https://qap.sdsu.edu/screening/breastcancer/facts.html)

- In 2014, an estimated 232,670 new cases of invasive breast cancer will be diagnosed in U.S. women. In addition to invasive breast cancer, an estimated 62,570 cases of carcinoma in situ (CIS) will be diagnosed. (American Cancer Society (ACS), 2014, Jan. - Last revised)

- In 2014, an estimated 40,000 U.S. women will die from breast cancer. (American Cancer Society (ACS), 2014, Jan. - Last revised)

- The risk of getting breast cancer increases with age. Approximately 77% of women with breast cancer are over the age of 50 at the time of diagnosis (U.S. Department of Health and Human Services (USDHHS), 2008, Aug. - Last revised).

- Although overall incidence is highest for Caucasian women, African Americans have the highest mortality rate from breast cancer. Caucasian women have the second highest mortality rate, followed by American Indian/Alaska Natives, Hispanic/Latinos, and Asian American/ Pacific Islanders. American Cancer Society (ACS). (2013). Breast cancer facts & figures 2013-2014. Accessed Jul. 8, 2014

- The breast cancer mortality rate has decreased since 1989, with larger decreases in women younger than 50. The decline is attributed to earlier detection, improved treatments, and possibly, decreased incidence as a result of declining use of postmenopausal hormone therapy. (American Cancer Society ACS.2013).
- When detected and treated early, 5-year relative survival for localized breast cancer is 99%. For regional disease, it is 84%. If the cancer has spread to distant organs, 5-year survival drops to 24%(American Cancer Society ACS.2013). Larger tumor size at diagnosis is also associated with decreased survival. (American Cancer Society ACS.2013).

- At this time, there are an estimated 2.8 million breast cancer survivors living in the U.S. (American Cancer Society (ACS), 2014, Jan. - Last revised).

1.8.2 World Breast Cancer ranking: (http://www.worldlifeexpectancy.com/cause-of-death/breast-cancer/by-country/;november, 2014

Table1.6: world ranking of countries with percentage of death:

Serial	**Country**	**Percentage of death**
1	**Saint Kitts**	36.1
2	**Antigua/Bar.**	**29.8**
3	**Uruguay**	**29.5**
116	**Bangladesh**	**14.7**

Number of New Cases and Deaths per 100,000: The number of new cases of breast cancer was 124.6 per 100,000 women per year. The number of deaths was 22.2 per 100,000 women per year. These rates are age-adjusted and based on 2007-2011 cases and deaths.

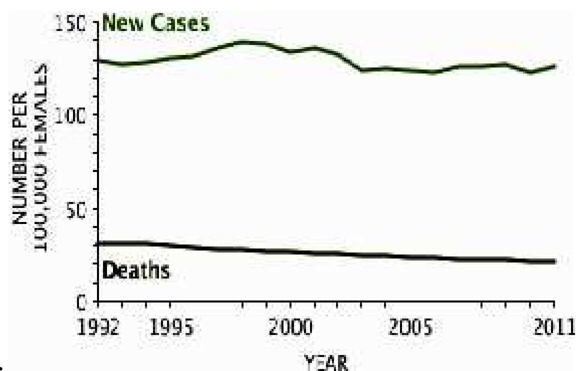


Fig1.4: New cases and death rate of patients in different years

Lifetime Risk of Developing Cancer: Approximately 12.3 percent of women will be diagnosed with breast cancer at some point during their lifetime, based on 2009-2011 data.

1.9 Worldwide prevalence:

Breast cancer is the most frequently diagnosed cancer in women worldwide.1.4 million new cases in 2014 has been occurred. About half of these cases occurred in economically developing countries, breast cancer incidence rates rose approximately 30% in westernized countries because of changes in reproductive patterns and more recently because of increased screening (Althuis *et al,* 2005).However, incidence rates in the United States decreased between 1999 and 2006, in part due to lower use of postmenopausal combined hormone therapy (Ravdin *et al.,* 2007; Edwards *et al.,* 2010). Breast cancer incidence rates have been rising in many African and Asian countries including Japan, where rates increased more than 140% and India, where rates increased 40% in the Chennai registry between 1983-1987 and 1998-2002. (Parkin *et al.,* 2005) Reasons for these rising trends are due to changes in reproductive patterns, obesity, physical inactivity and some breast cancer screening activity. In 2011, there were an estimated 2,899,726 women living with breast cancer in the United States.

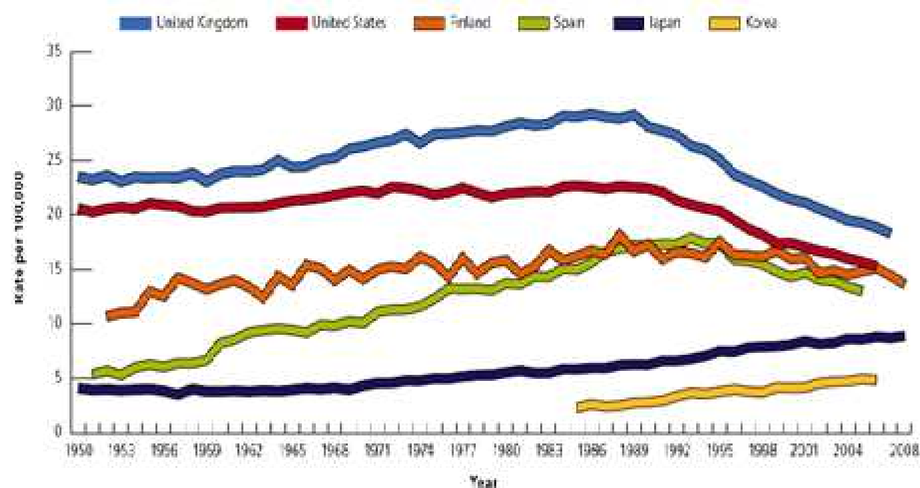


Figure1.5: Trends in Age-standardized Female Breast Cancer Death Rates in Select Countries Source: GLOBOCAN 2008

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 % .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). 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 (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).

1.9.1Breast cancer prevalence in Asian patients: (Stanley P.L. *et.al* Published online: 7 July 2010)

Breast cancer, with approximately one million new patients in each year, is the most common malignancy among women (Coley, 2008). Breast cancer is included about 10 percent of all cancers and 23 percent of women cancers in developed countries (Coley, 2008). Over 15% of healthy women have at least one first-degree relative with breast cancer and experimental data showed that the risk of breast cancer in women has been doubled in recent years (Robson *et al.,* 2007).

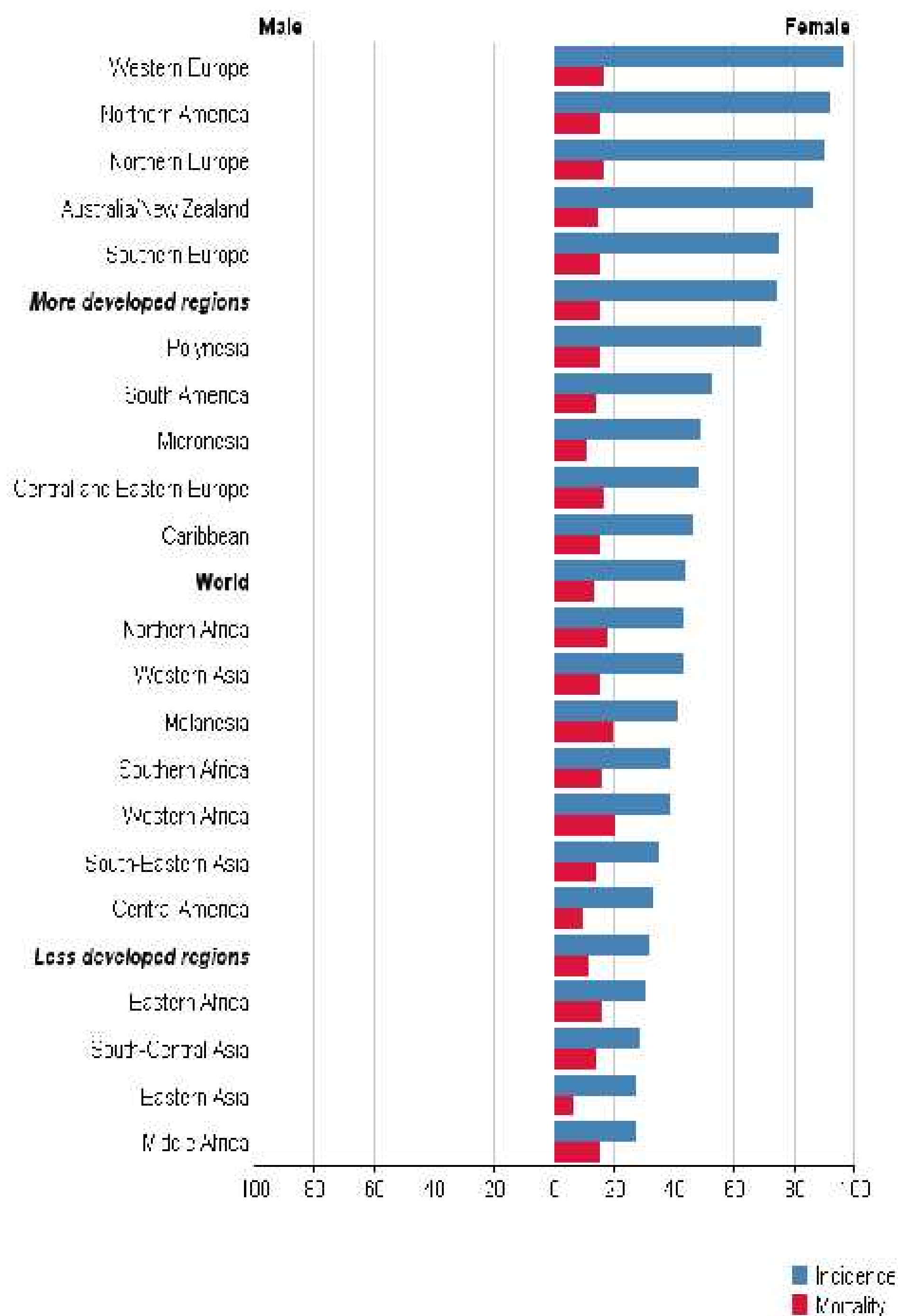


Fig: 1.6: Breast cancer Incidence and Mortality in Asian women in perspective of age (Globocan fact sheet, 2012)

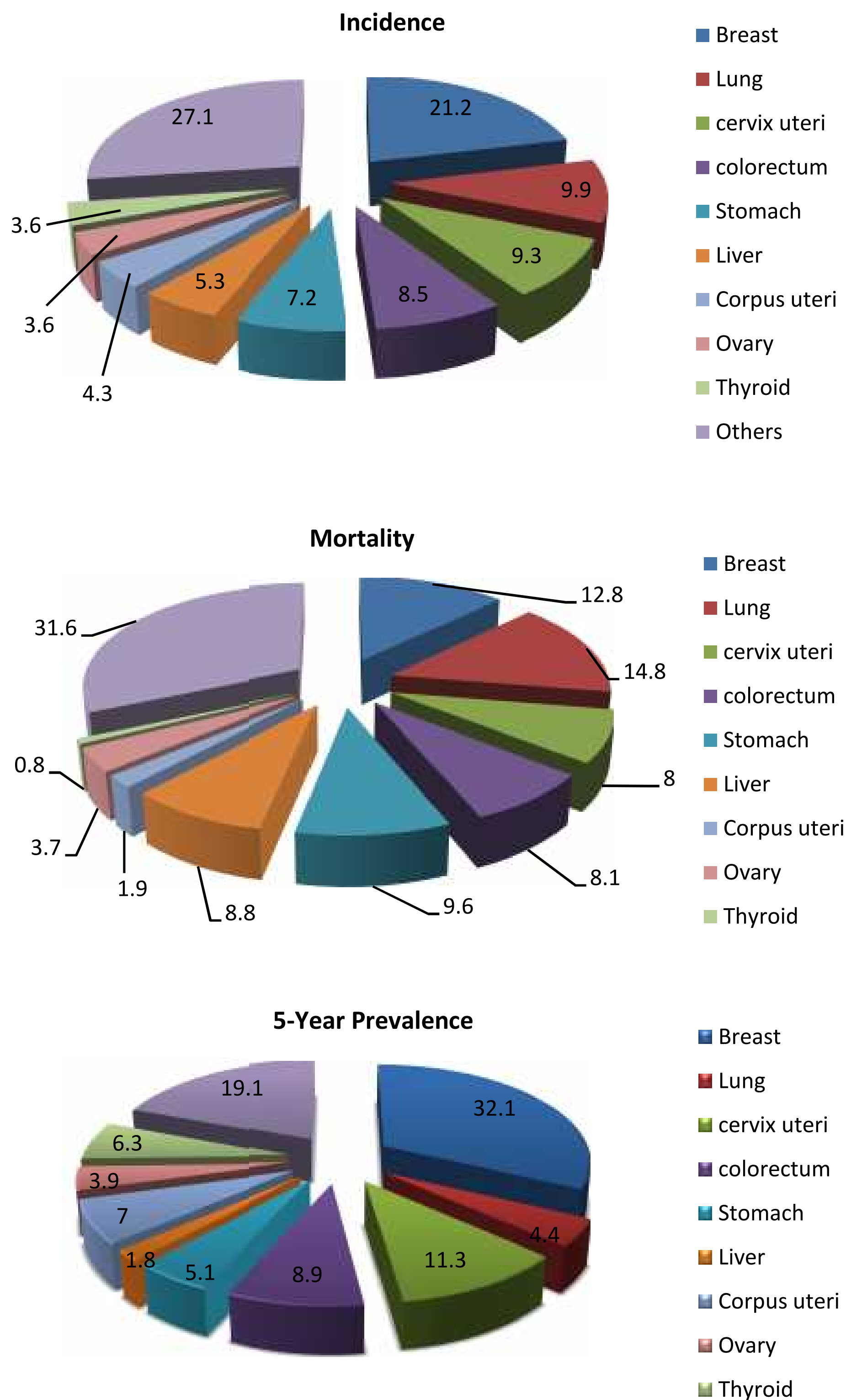


Fig1.7: Estimated incidence, mortality and 5-year prevalence of Asian Women due to different types of cancer (Globocan fact sheet, 2012).

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)

Rates of breast cancer are uniformly many times lower in Asia .The peak age of onset in Asia is 45–50 years of age. (Stanley P. L. Leong, 2010).

Table1.7: Breast cancer prevalence in Asian countries (Stanley P. L. Leong ,et al., 2010)

	China	Taiwan	India	Japan	South Korea
Incidence of breast cancer	24/100,000	43.27/100,000 females	Annual age adjusted rate 7.2 to 33.4/100,000	37/100,000 in 1993 Age-adjusted rate 43.6 in 1998	40.5/100,000 in 2004
Peak age of breast cancer	45–50 years	40–49 years, peak at age 45	45–49 years	45–49 years	Peak at 47 years
Ratio of female to male	100:0.7	100:0.3	>100:1	170–180:1	195:1(212,920 females: 1720 males)
AJCC stage of initial presentation	Mostly stage II	See descriptive analysis	St I: 1–8%; St II: 23–58%; St III: 29–52%; St IV: 6–24%	43.6 presented with Stage II disease	St I: 36% St II: 40% St III: 5%
Mastectomy rate	60–80%	69.91%	(Estimate) >90%	45.3%	64.7%
Lumpectomy rate	15–30%	15.99%	(Estimate) <10%	48–40%	35.3%
Family history	4.1%	<5%	<5%	8.3%	6.8%
Method of detection	Clinical detection and mammogram	Clinical detection, mammogram, and sonogram	Self detected palpable lump or its secondary complications	Clinical detection and mammogram	Clinical detection and mammogram
Self exam	Recommended to all women	Recommended	Advocated, data on what proportion exercised is not available	Recommended	Most common clinical manifestation was painless lump (59.5%)
Health caretaker exam	Recommended to all women	Not available	Advocated, specific data not available	Not available	Not available
Mammogram	Recommended to women <40	Yes	Available for diagnostic and screening purposes	Yes once every 2 years for women <40	Yes. Screening mammogram recommended annually after age 40, with initial mammogram at age 35

	China	Taiwan	India	Japan	South Korea
Surgical treatment					
Lumpectomy and AxLND	15–30%	31%	<5% (country-wide estimates)	48.4%	42.6%
Lumpectomy and SLNB	>5%	8%	Approx 2%		
Modified RM	75%	59%	Approx 80% (country-wide)	45.3%	50.4%
Radical	5%	1%—for local advanced breast cancer only	Rarely	1–2%	The rate of radical mastectomy was 1.2% (559casse/45964cases)
Postoperative therapy					
Radiation therapy	20–40%	Yes	Only in tertiary care centers. Palliative RT where indicated	Yes	Universally administered to most invasive patients, irrespective of nodal status
Chemo therapy	60–80%	Yes	Yes, in centers of excellence, but no in almost 25% of out-lying areas	Yes	Yes
Hormonal therapy	30–40%	Yes	Approx 53%—higher in centers of excellence	Yes	Yes
Five-year overall Survival	76.5%	78.37%	St I: 90%, St II: 78%, St III: 57%, St IV: 22%; for all patients treated: 62%	76.1–86.1% (at 5 different hospitals) 87.0% after breast cancer operation	81.2% from 1993 to 2002. 49,174 cases analyzed
10 year overall survival	79%	75%	Overall 45%	78.5%	70.3%

Estimated age standardized incidence and mortality rate per 100000 by level of economic development 2008 proves that incidence and mortality because of breast cancer in Asian countries are much lower. The incidence rate is only 27.3 per 100000; the mortality rate is 10.8 per 100000 in developing counties (Globocan, 2008).

Table 1.8: Breast Cancer Estimated Incidence, Mortality and Prevalence Worldwide in 2012 (Globocan, 2012)

Estimated numbers (thousands)	Cases	Death	5-years prevalence
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

Table 1.9: Worldwide Incidence, Mortality, 5 years prevalence of different types of Cancers in Females [Incidence and mortality data for all ages, 5-year prevalence for adult population only, ASR (W) and proportions per 100,000] (Fearly *et al*., 2010; http://globocan.iarc.fr/factsheet.asp, accessed date: 20 August, 2013).

Cancer type	Incidence			Mortality			5-year prevalence		
	Number	(%)	ASR (W)	Number	(%)	ASR (W)	Number	(%)	Pro per.
Breast	**1384155**	**22.9**	**38.9**	**458503**	**13.7**	**12.4**	**5189028**	**33.9**	**210.7**
Colorectal	571204	9.4	14.6	288654	8.6	7	1495468	9.8	60.7
Cervix uteri	530232	8.8	15.2	275008	8.2	7.8	1555341	10.2	63.2
Lung	515999	8.5	13.5	427586	12.8	10.9	555797	3.6	22.6
Stomach	348571	5.8	9.1	273489	8.2	6.9	548134	3.6	22.3
Corpus uteri	288387	4.8	8.2	73854	2.2	1.9	1097620	7.2	44.6
Liver	226312	3.7	6	217592	6.5	5.7	180006	1.2	7.3
All cancer expt non-melanoma skin cancer	6044710	100	164.4	3345176	100	87.2	15288300	100	620.8

1.10 Treatment:

A multidisciplinary team should be involved in a breast cancer patient's treatment. The team may consist of an oncologist, radiologist, specialist cancer surgeon, specialist nurse, pathologist, radiologist, radiographer, and reconstructive surgeon. Sometimes the team may also include an occupational therapist, psychologist, dietitian, and physical therapist.

The team should take into account several factors when deciding on the best treatment for the patient, including:

- The type of breast cancer
- The stage and grade of the breast cancer - how large the tumor is, whether or not it has spread, and if so how far
- Whether or not the cancer cells are sensitive to hormones
- The patient's overall health
- The age of the patient (has she been through the menopause?)
- The patient's own preferences.

The main breast cancer treatment options may include:

- Radiation therapy (radiotherapy)
- Surgery
- Biological therapy (targeted drug therapy)
- Hormone therapy
- Chemotherapy.

Surgery

- **Lumpectomy** - surgically removing the tumor and a small margin of healthy tissue around it. In breast cancer, this is often called breast-sparing surgery. This type of surgery may be recommended if the tumor is small and the surgeon believes it will be easy to separate from the tissue around it.
- **Mastectomy** - surgically removing the breast. *Simple mastectomy* involves removing the lobules, ducts, fatty tissue, nipple, areola, and some skin. *Radical mastectomy* means also removing muscle of the chest wall and the lymph nodes in the armpit.

- **Sentinel node biopsy** - one lymph node is surgically removed. If the breast cancer has reached a lymph node it can spread further through the lymphatic system into other parts of the body.
- **Axillary lymph node dissection** - if the sentinel node was found to have cancer cells, the surgeon may recommend removing several nymph nodes in the armpit.
- **Breast reconstruction surgery** - a series of surgical procedures aimed at recreating a breast so that it looks as much as possible like the other breast. This procedure may be carried out at the same time as a mastectomy. The surgeon may use a breast implant, or tissue from another part of the patient's body.

Radiation therapy (radiotherapy)

Controlled doses of radiation are targeted at the tumor to destroy the cancer cells. Usually, radiotherapy is used after surgery, as well as chemotherapy to kill off any cancer cells that may still be around. Typically, radiation therapy occurs about one month after surgery or chemotherapy. Each session lasts a few minutes; the patient may require three to five sessions per week for three to six weeks.

The type of breast cancer the woman has will decide what type of radiation therapy she may have to undergo. In some cases, radiotherapy is not needed.

Radiation therapy types include:

- **Breast radiation therapy** - after a lumpectomy, radiation is administered to the remaining breast tissue
- **Chest wall radiation therapy** - this is applied after a mastectomy
- **Breast boost** - a high-dose of radiation therapy is applied to where the tumor was surgically removed. The appearance of the breast may be altered, especially if the patient's breasts are large.
- **Lymph nodes radiation therapy** - the radiation is aimed at the axilla (armpit) and surrounding area to destroy cancer cells that have reached the lymph nodes
- **Breast brachytherapy** - scientists at UC San Diego Moores Cancer Center revealed that patients with early-stage breast cancer in the milk ducts which has not spread seem to benefit from undergoing breast brachytherapy with a strut-based applicator. This 5-day treatment is given to patients after they have undergone lumpectomy surgery. The researchers found that women who

received strut-based breast brachytherapy had lower recurrence rates, as well as fewer and less severe side effects.

Side effects of radiation therapy may include fatigue, lymphedema, darkening of the breast skin, and irritation of the breast skin.

Chemotherapy

Medications are used to kill the cancer cells - these are called *cytotoxic* drugs. The oncologist may recommend chemotherapy if there is a high risk of cancer recurrence, or the cancer spreading elsewhere in the body. This is called adjuvant chemotherapy. If the tumors are large, chemotherapy may be administered before surgery. The aim is to shrink the tumor, making its removal easier. This is called neo-adjuvant chemotherapy.

Chemotherapy may also be administered if the cancer has metastasized - spread to other parts of the body. Chemotherapy is also useful in reducing some of the symptoms caused by cancer.

Chemotherapy may help stop estrogen production. Estrogen can encourage the growth of some breast cancers.

Side effects of chemotherapy- may include nausea, vomiting, loss of appetite, fatigue, sore mouth, hair loss, and a slightly higher susceptibility to infections. Many of these side effects can be controlled with medications the doctor can prescribe. Women over 40 may enter early menopause.

You can learn more about chemotherapy in this article.

Protecting female fertility - Scientists have designed a way of aggressively attacking cancer with an arsenic-based chemo medication, which is much gentler on the ovaries. The researchers, from Northwestern University Feinberg School of Medicine in Chicago, believe their novel method will help protect the fertility of female patients undergoing cancer treatment.

Hormone therapy (hormone blocking therapy)

Hormone therapy is used for breast cancers that are sensitive to hormones. These types of cancer are often referred to as ER positive (estrogen receptor positive) and PR positive (progesterone receptor positive) cancers. The aim is to prevent cancer recurrence. Hormone blocking therapy is usually used after surgery, but may sometimes be used beforehand to shrink the tumor. If for health reasons, the patient cannot undergo surgery, chemotherapy or radiotherapy, hormone therapy may be the only treatment she receives. Hormone therapy will have no effect on cancers that are

not sensitive to hormones. Hormone therapy usually lasts up to five years after surgery.

The following hormone therapy medications may be used:

- **Tamoxifen** - prevents estrogen from binding to ER-positive cancer cells. Side effects may include changes in periods, hot flashes, weight gain, headaches, nausea, vomiting, fatigue, and aching joints.
- **Aromatase inhibitors** - this type of medication may be offered to women who have been through the menopause. It blocks aromatase. Aromatase helps estrogen production after the menopause. Before the menopause, a woman's ovaries produce estrogen. Examples of aromatase inhibitors include letrozole, exemestane, and anastrozole. Side effects may include nausea, vomiting, fatigue, skin rashes, headaches, bone pain, aching joints, loss of libido, sweats, and hot flashes.

  Ovarian ablation or suppression - pre-menopausal women produce estrogen in their ovaries. Ovarian ablation or suppression stop the ovaries from producing estrogen. Ablation is done both through surgery or radiation therapy - the woman's ovaries will never work again, and she will enter the menopause early.

  A luteinising hormone-releasing hormone agonist (LHRHa) drug called Goserelin will suppress the ovaries. The patient's periods will stop during treatment, but will start again when she stops taking Goserelin. Women of menopausal age (about 50 years) will probably never start having periods again. Side effects may include mood changes, sleeping problems, sweats, and hot flashes.

Biological treatment (targeted drugs)

- **Trastuzumab (Herceptin)** - this monoclonal antibody targets and destroys cancer cells that are HER2-positive. Some breast cancer cells produce large amounts of HER2 (growth factor receptor 2); Herceptin targets this protein. Possible side effects may include skin rashes, headaches, and/or heart damage.
- **Lapatinib (Tykerb)** - this drug targets the HER2 protein. It is also used for the treatment of advanced metastatic breast cancer. Tykerb is used on patients who did not respond well to Herceptin. Side effects include painful hands,

painful feet, skin rashes, mouth sores, extreme tiredness, diarrhea, vomiting, and nausea.

- **Bevacizumab (Avastin)** - stops the cancer cells from attracting new blood vessels, effectively causing the tumor to be starved of nutrients and oxygen. Side effects may include congestive heart failure, hypertension (high blood pressure), kidney damage, heart damage, blood clots, headaches, mouth sores. Although not approved by the FDA for this use, doctors may prescribe it "off-label". Using this drug for breast cancer is controversial. In 2011, the FDA said that Avastin is neither effective nor safe for breast cancer.
- **Low dose aspirin** - research carried out on laboratory mice and test tubes has suggested that regular low-dose aspirin may halt the growth and spread of breast cancer. Cancer campaigners cautioned that although the current results show great promise, this research is at a very early stage and has yet to be shown to be effective on humans.

1.11 Pharmacogenetics:

Pharmacogenetics is the study of inherited genetic differences in drug metabolic pathways which can affect individual responses to drugs, both in terms of therapeutic effect as well as adverse effects (Klotz, 2007). The term has been pieced together from the words pharmacology (the study of how drugs work in the body) and genetics (the study of how traits are inherited).(NH clinical genetics)

This is the study of how genes and genetic information influences the action of a drug. Most drugs do not work effectively in all people and some also have undue side effects or toxicity. The variation people show in response to a drug, whether it is for efficacy or toxicity, can in part be explained by their own genetic makeup. Understanding the interactions of genes and drugs enables us to identify the most appropriate drug or dose for each individual (UNC,Eshelman school of pharmacy ,Center for Pharmacogenomics and Individualized Therapy". Retrieved 2014-10-28). Pharmacogenetics involves the use of molecular genetic information to predict drug effectiveness and drug-induced adverse events. Pharmacogenetic data could be vital to the development of individualized treatment (Anil K. Malhotra, 2010).

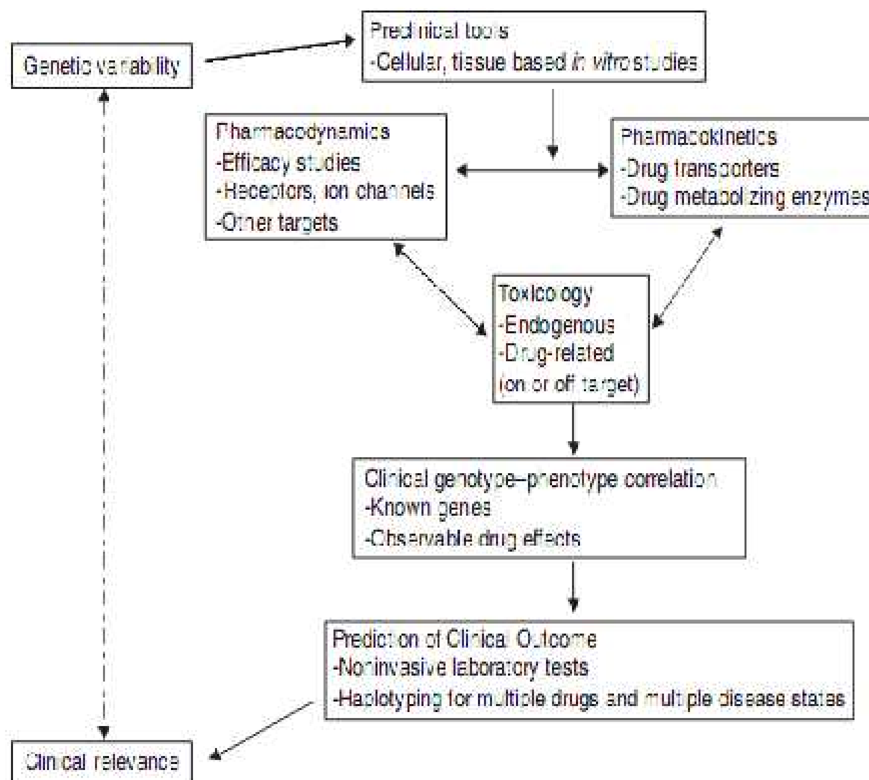


Fig 1.8 : Translation of basic pharmacogenetics research into clinical practice.

Metabolic pathways — the chains of chemical reactions that occur in each cell in the body — are affected by a wide range of DNA mutations, including single nucleotide polymorphisms (SNPs); gene deletions, duplications, and insertions; mutations in the regulatory elements of genes; and variations in gene copy number. The identification of gene variants (alleles) that influence drug metabolism is one of the goals of pharmacogenetics, a field of study focused on understanding the genetic basis for differences in drug response among individuals.

The appeal of pharmacogenetics lies in the possibility of personalized medicine. This sort of care has always been the goal of the doctor-patient relationship, with physicians considering a patient's family history and lifestyle when prescribing treatment. Access to information about an individual's genetic makeup would provide yet another source of personalized data and would therefore enable doctors to better define the nature of a disease and find the most effective treatment for a particular patient.(Sonia Y. Hunt, Ph.D;2008 Nature Education)

In fact, it is estimated that genetics is responsible for 15% to 95% (depending on the drug or class of drug) of the observed inter individual variability in drug disposition

and effects (Evans and McLeod, 2003; Eichelbaum *et al.*, 2006; McGee, 2006). With the help of pharmacogenetics studies, physicians will be able to administer treatment regimens that are personalized and adapted to each person's genetic makeup. Accordingly, two people with the same diagnosis might receive different therapies or drug dosages. This might in turn reduce health care costs, because physicians would be able to prescribe more targeted drugs and pharmaceutical companies would be able to develop and market drugs to specific groups of patients (Sonia Y. Hunt, 2008).

As the influence of pharmacogenetics on drug discovery and development and drug treatment regimens increases, there will undoubtedly be a move away from the current approach of standardized treatments towards more individualized, 'tailor-made' therapies (Roses, 2000; Liggett, 2004). Despite the fact that this concept of individualized drug treatment seems very helpful and is largely the product of recent advances in human molecular biology, the scientific foundation on which it is based has a relatively long history.

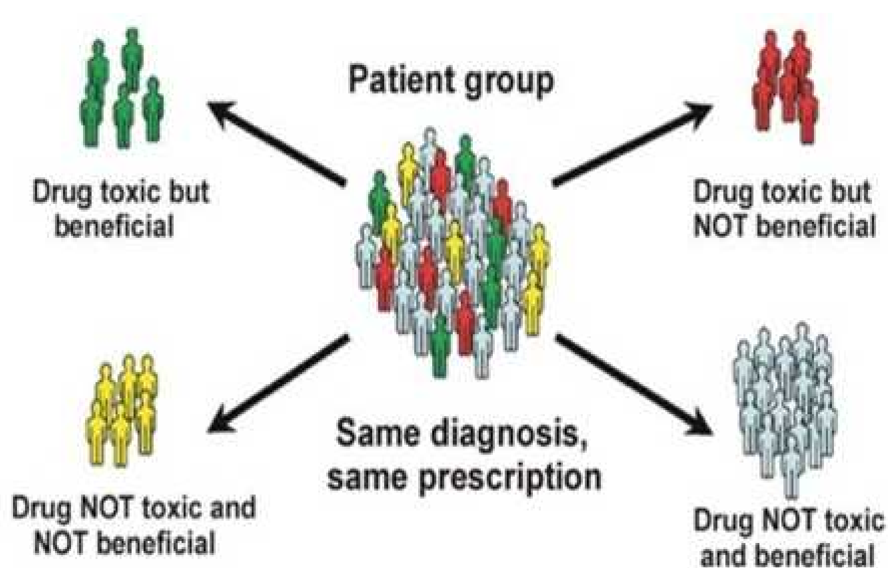


Fig: 1.9 Pharmacogenetics and personalized medicine

The rationale behind pharmacogenetics is to find genetic polymorphisms in the genes encoding proteins and enzymes involved in drug transport, metabolism and action that can predict the usefulness of a particular drug, increasing the number of responders and decreasing the number of subjects affected by adverse drug reactions. (Magnus Ingelman-Sundberg and Cristina Rodriguez-Antona, 2005)

1.11.1Benefits of Pharmacogenetics

Effective Medication:

The differential gene expressions and proteomics are indicators that detect different patterns of gene and protein expression in tissues (Blackstock WP & Weir MP 1999; Kozian DH & Kirschbaum BJ, 1999). The disease susceptible genes identification and study of the function of the gene variants can be used to identify targets that will be related to the disease in patients (Monica RP Rao & Snehal Gorey, 2007).

Enhancing efficacy

Many medicines are not effective for everyone with a particular disease. Some common treatments for diabetes, depression and asthma are only effective in around 60% of patients. Pharmacogenetics could allow doctors to prescribe medicine only for those patients most likely to respond. Alternatively, new medicines could be designed on the basis of genetic information about the cause of disease (McLeod *et al.,* 2001).

Screening for Disease Identification

Technologies such as differential gene expression, transgenic animal models, proteomics, in-situ hybridization are used to imply relationships between a gene and a disease process (Roses AD 2000). Likewise, advance knowledge of particular disease susceptibility will allow careful monitoring, and treatments can be introduced at the most appropriate stage to maximize their therapy (Licinio and Wong, 2002).

Improvements in the Drug Discovery and Approval Process

To discover a new drug sometimes many targets are chosen on the basis of scientific hypotheses and do not lead to effective medicines because the initial hypotheses are subsequently disproved (Curran ME, 1998; Marton MJ *et al.,* 1998). However, using pharmacogenetics developed optimized molecules (leads) can be obtained, which have properties of well-tolerated and effective medicines (Monica RP Rao & Snehal Gorey, 2007).

Decrease in the Overall Cost of Health Care

Decreases in the number of adverse drug reactions, the number of failed drug trials, the time it takes to get a drug approved, the length of time patients are on medication,

the number of medications patients must take to find an effective therapy, the effects of a disease on the body (through early detection), and an increase in the range of possible drug targets will promote a net decrease in the cost of health care (Duncan *et al.,* 2007).

Better Vaccines

Vaccines made of genetic material, either DNA or RNA; promise all the benefits of existing vaccines without all the risks. They will activate the immune system but will be unable to cause infections. They will be inexpensive, stable, easy to store, and capable of being engineered to carry several strains of a pathogen at once (Gage *et al.,* 2008; Eby *et al.,* 2009).

Safer Drugs

Instead of the standard trial-and-error method of matching patients with the right drugs, doctors will be able to analyze a patient's genetic profile and prescribe the best available drug therapy from the beginning. Not only will this take the guesswork out of finding the right drug, it will speed recovery time and increase safety as the likelihood of adverse reactions is eliminated. Pharmacogenetics has the potential to dramatically reduce the estimated 100,000 deaths and 2 million hospitalizations that occur each year in the United States as the result of adverse drug response (J. Lazarou *et al.,* 1998).

More Accurate Methods of Determining Appropriate Drug Dosages

The other anticipated benefits of pharmacogenetics include more accurate methods of determining appropriate drug dosages. The ability of a person's body to process a drug that is his genetic profile will form the basis for dosage calculations instead of the weight and age of the patient (Monica RP Rao & Snehal Gorey, 2007).

1.12 Genetic Polymorphism:

A combination of the Greek words *poly* (meaning multiple) and *morph* (meaning form), this term is used in genetics to describe the multiple forms of a single gene that

can exist in an individual or among a group of individuals. (polymorphism - gene sequencing - genetics - Biotech/Biomedical Theresa Phillips ,last review at Dec,2014)

Natural variations in a gene, DNA sequence, or chromosome that have no adverse effects on the individual and occur with fairly high frequency in the general population. Polymorphism involves one of two or more variants of a particular DNA sequence. The most common type of polymorphism involves variation at a single base pair. Polymorphisms can also be much larger in size and involve long stretches of DNA. Called a single nucleotide polymorphism, or SNP (pronounced "snip"), scientists are studying how SNPs in the human genome correlate with disease, drug response, and other phenotypes (Genetic home reference, 2014).

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."[Source: PHRMA Genomics Lexicon]

Genetic Mutation A change 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 incited by external agents (such as viruses or radiation). (Source: PHRMA Genomics Lexicon) Genetic polymorphisms contribute to variations in phenotypes, risk to certain diseases, and response to drugs and the environment (Ruiqiang Li, 2009).

1.13 Single Nucleotide Polymorphisms (SNPs):

SNPs are the most simple form and most common source of genetic polymorphism in the human genome (90% of all human DNA polymorphism) (Lippert *et al.,* 2002).

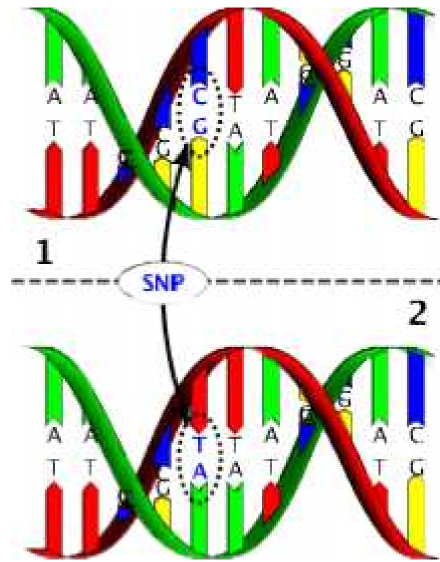


Fig1.10 : Single nucleotide Polymorphism in human genome

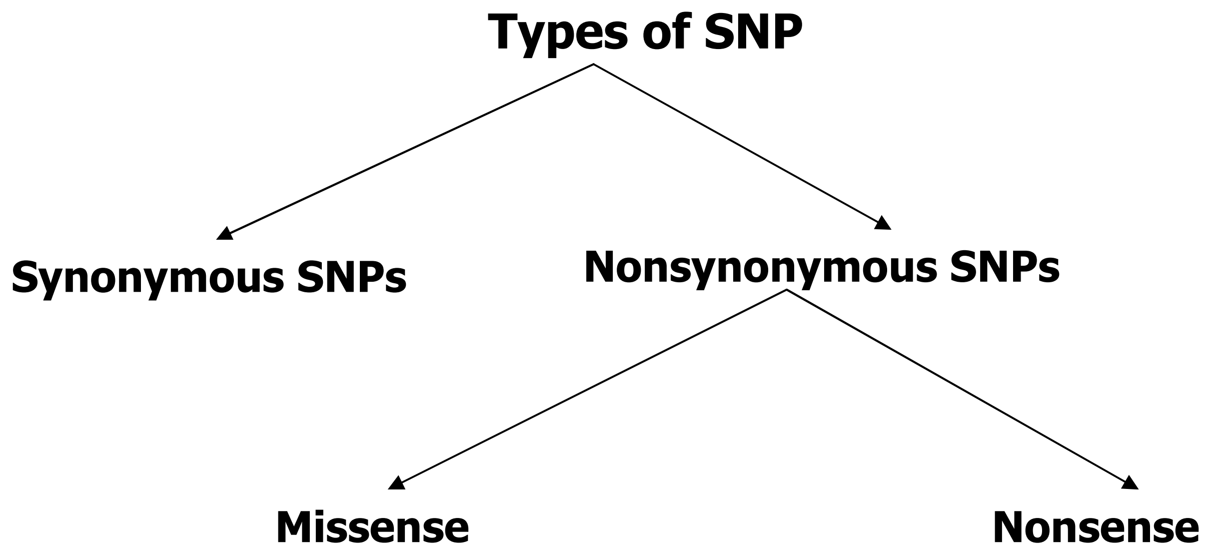
There are two types of nucleotide base substitutions resulting in SNPs:

- **A transition substitution** occurs between purines (A, G) or between pyrimidines (C, T). This type of substitution constitutes two third of all SNPs. (Schwartz *et al.,* 1996).
- **A transversion substitution** occurs between a purine and a pyrimidine.

1.13.1 Types of SNP:

Single-nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions (regions between genes). SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code.

SNPs in the coding region are of two types, synonymous and nonsynonymous SNPs. Synonymous SNPs do not affect the protein sequence while nonsynonymous SNPs change the amino acid sequence of protein. The nonsynonymous SNPs are of two types: missense and nonsense.



1.13.2 Coding Region SNPs:

A SNP in a coding region may have two different effects on the resulting protein:

Synonymous the substitution causes no amino acid change to the protein it produces. This is also called a silent mutation.

Non-Synonymous the substitution results in an alteration of the encoded amino acid. A missense mutation changes the protein by causing a change of codon. A nonsense mutation results in a misplaced termination codon. One half of all coding sequence SNPs result in non-synonymous codon changes.(Kaleigh Smith,2002)

1.13.3 SNP Applications

The major application of SNP information is towards improved and futuristic health care. Genomics and specifically SNP research can be used to improve health care

through gene therapy, to yield new targets for drug discovery, to renew the process of drug development and to discover new diagnostics.

1.13.3.1 In Pharmacogenomics

Pharmacogenomics is the science of understanding the correlation between an individual patient's genetic make-up (genotype) and their response to drug treatment. Some drugs work well in some patient populations and not as well in others. Studying the genetic basis of patient response to therapeutics allows drug developers to more effectively design therapeutic treatments. [Source: PHRMA Genomics Lexicon]

All aspects of pharmacogenomics require data from high-throughput genotyping, specifically the target population for a drug or the population of people who react poorly with the drug. Also, this type of research may lead to population specific treatments. The high cost of drug recalls have provided an initiative for advanced drug design involving drug-target validation studies as well as studies to predict adverse events and lack of efficacy.

A sample pharmacogenomic experiment may proceed as follows:

1. Define the drug response (phenotype) of interest
2. Accumulate patients/DNA/families
3. Identify candidate genes that might explain significant response variations
4. Identify polymorphisms in candidate genes
5. Relate the identified polymorphism to the phenotype

1.13.3.2 SNP Diagnostics

Genetic testing is the analysis of an individual's genetic material. Among the purposes of genetic testing could be to gather information on an individual's genetic predisposition to particular health condition, or to confirm a diagnosis of genetic disease. [Source: PHRMA Genomics Lexicon]

An individual's genotype can be determined and then analyzed according to a haplotype map to determine the patient's disease risk or reception to different treatments.

1.13.3.3 SNPs in Functional Proteomics and Gene Therapy

SNP related functional proteomics involve the identification of functional SNPs that modify proteins and protein active sites structure and function. Functional proteomics is closely tied to modern (post-genomic) drug design and function SNP information helps to discover new therapeutic targets. Most interestingly, by developing a database of the modifications generated by functional (coding) SNPs in disease related proteins, "new compounds can be designed for correcting or enhancing the effects of those mutations in the population." [Source: Genodyssee]

Aside from drugs, here are some interesting genomic therapies that may become more feasible as SNP information in the form of trees and maps become more detailed. Germ line gene therapy involves the insertion of normal genes into germ cells or fertilized eggs in an attempt to create a beneficial genetic change which can be transmitted to an organism's offspring (for example, to correct for a genetic trait associated with disease). If a change is introduced via germ line gene therapy, that change may be present in the offspring from birth in every cell in the body. See genomics, and compare with somatic cell gene therapy. [Source: PHRMA Genomics Lexicon]

Somatic cell genetic mutation is a genetic mutation in a somatic cell. Such mutations, which are not inherited from parents but occur during the lifetime of an organism, are often known as "acquired genetic mutations." Somatic cell genetic mutations are not passed on to offspring.[Source: PHRMA Genomics Lexicon]

Somatic cell gene therapy Somatic cell gene therapy involves the insertion of genes into cells for therapeutic purposes, for example to induce the treated cells to produce a protein that the body is missing. It does not affect the genetic makeup of a patient's offspring, and generally does not change all, or even most, cells in the recipient. [Source: PHRMA Genomics Lexicon]

1.13.3.4 SNP in Cancer Diagnosis:

The occurrence of different forms of an allele in a gene is called polymorphism. Researchers have shown that genetic polymorphisms are one of the causes of individual difference in cancer incidence (Karen et al., 2006). In 2001, the complete human genome was sequenced and sequence analyses showed that 99% of genome sequences of different individuals were identical (Sachidanandam et al., 2001).

Differences among individuals are due to the existence of 4.5 million single nucleotide polymorphisms distributed throughout the genome in both coding and non coding regions. These polymorphisms are individual different factors in having the unique traits (Chakravarti, 1999).SNPs are the most common type of genetic variation. SNPs can cause silent, harmless or harmful effects (http://www.cancer.gov/cancertopics/understandingcancer/geneticvariation/page39). SNPs in the genome can occur in coding and non-coding regions. More SNP occurs in non-coding regions. The researchers concluded that some of the SNPs have a useful activity. If SNP occurring with high frequency near a particular gene, it can be used as a marker for a specific gene, while SNPs occur in coding regions cause change in the produced protein of that gene and the resulting product would have effects on health(http://www.cancer.gov/cancertopics/understandingcancer/geneticvariation/AllPages). Whereas SNPs can act as genetic markers, SNPs profile can aid scientists to identify the complete set of genes that are involved in creating and developing complex diseases like cancer. For example scientists studied SNP profiles of many individuals including cancer patients, and comparison between different profiles made it possible to discover a small subset of SNPs that are present only in cancer patients. As a result, SNPs can be considered as markers for identification of cancer genes. Scientists also use SNPs to estimate the factors contributing to cancer risk in large populations.

1.14 Pharmacogenetics of Breast Cancer:

Breast cancer is the most common malignancy and cause of death in the Western world. If current breast cancer rates remain constant, a woman born today has a one in 10 chance of developing breast cancer. High-penetrance genes account for only 5% of cases, whereas polymorphic low-penetrance genes acting in concert with lifestyle/environmental risk factors are likely to account for a much higher proportion (Jana armanová *et al*., 2004).

Various genes have been implicated in the development of breast cancer and those most studied include the BRCA1 and BRCA2 genes. The lifetime risk for developing breast cancer is 65 % to 85 % for BRCA1 and 45 % to 85 % for BRCA2 mutation carriers (Chen and Parmigiani 2007). Increased frequencies of specific BRCA1 and

BRCA2 mutations have been described in the South African Caucasian (Afrikaans-speaking) and Coloured (Mixed ancestry) populations (Reeves et al. 2004; Agenbag 2005; van der Merwe et al. 2012). Three founder mutations [(BRCA1 c.1374delC (1493delC), BRCA1 c.2641G>T (2760 G>T, E881X) and BRCA2 c.7934delG (8162delG)] account for approximately 90 % of all BRCA mutation-positive families in the Afrikaner population of European descent (van der Merwe and van Rensburg 2009). A relatively frequent BRCA2 founder mutation c.5771_5774delTTCA (5999del4) has also recently been identified in 3.4 % of Coloured and 25 % of Black Xhosa breast cancer patients studied in the Western Cape region of South Africa (van der Merwe et al. 2012).

Researchers have shown that women with a family history of breast cancer have increased risk of developing breast cancer. The mutations responsible for increasing breast cancer risk within families are recognized. These include mutations in *ATM, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, MLH1, MRE11, MSH2, MSH6, MUTYH, NBN, PALB2, PMS1, PMS2, PTEN, RAD50, RAD51C, STK11* and *TP53* genes. These rare mutations are probably assigned to a small percent (2-5%) of breast cancer cases.(Kooshyar Mohammad Mahdi *et al*, 2013).Germline mutations of high penetrance breast cancer susceptibility genes such as *BRCA1, BRCA2, TP53, CHEK2, ATM, PTEN* and *PPM1D* confer a high risk of developing hereditary breast cancer (Cybulski *et al.,* 2011).Three reasonably well-defined classes of breast cancer susceptibility alleles with different levels of risk and prevalence in the population have become apparent: rare high-penetrance alleles, rare moderate penetrance alleles and common low-penetrance alleles (Michael R Stratton & Nazneen Rahman, 2008)

1.14.1Genes and SNPs associated with breast cancer:

1.14.1.1 Rare high-penetrance breast cancer susceptibility genes:

Disease-causing variants in *BRCA1* and *BRCA2* confer a high risk of breast cancer, approximately 10- to 20-fold relative risk. This translates into a 30–60% risk by age 60, compared to 3% in the general population. The relative risks are higher for early-onset breast cancers and there are also elevated risks of ovarian and other cancers (Thompson & Easton, 2002; J.Natl*,* 1999).Mutations in *BRCA1* and *BRCA2* account for ≈16% of the familial risk of breast cancer (Br. J. Cancer, 2000*;* Peto *et al., 1999).* Germ line mutations in *TP53* cause Li-Fraumeni syndrome, which includes a high

risk of breast and other cancers, but these mutations are very rare and hence account for a much smaller proportion of the familial risk. Cancer predisposition syndromes due to mutations in *PTEN* (Cowden syndrome), *STK11* (Peutz-Jeghers syndrome) and *CDH1* are also associated with elevated risks of breast cancer, although the cancer risks and prevalence of mutations in these genes are not well defined. It is unlikely that mutations in all six of these genes together account for more than 20% of the familial risk of the disease (Thompson & Easton, 2004; Antoniou & Easton, 2006).

1.14.1.2 Rare moderate-penetrance breast cancer susceptibility genes

The candidacy of the breast cancer susceptibility genes recently identified through direct interrogation for disease-causing mutations has been based primarily on involvement of the encoded proteins in biological pathways that include BRCA1 and BRCA2. To date, this strategy has identified at least four genes: *CHEK2*, *ATM*, *BRIP1* and *PALB2* (Rahman, *et al.*2007*;* Meijers-Heijboer *et al.*2002*,* Renwick *et al.*2008*)*.CHEK2 is a checkpoint kinase involved in DNA repair that directly modulates the activities of p53 and BRCA1 by phosphorylation23. *ATM* also encodes a checkpoint kinase that has key functions in DNA repair, and which also phosphorylates p53 and BRCA1 (Shiloh, 2006). BRIP1 (also known as BACH1) was discovered as a binding partner of BRCA1 and is implicated in some BRCA1 activities relating to DNA repair (Peng *et al*, 2006). PALB2 was discovered as a protein associated with BRCA2 (Xia *et al., 2006*). The patterns of susceptibility associated with these four genes have many features in common.

1.14.1.3 Common low-penetrance breast cancer susceptibility alleles:

It is comprised of common alleles that confer very small increases in risk (common low-penetrance alleles). The currently known susceptibility alleles of this type have been discovered through association studies, either targeted at individual genes on the basis of biological candidacy or, more recently, through genome-wide tag SNP searches.(Thompson, D. *et al.; 2006).*

Table1.10: Classes and key features of Breast Cancer Susceptibility alleles (Michael R Stratton & Nazneen Rahman, 2008)

Box 1 Classes and key features of known breast cancer susceptibility alleles
High-penetrance breast cancer susceptibility genes. Examples: *BRCA1*, *BRCA2*, *TP53*
• **Risk variants:** Multiple, different mutations that predominantly cause protein truncation
• **Frequency:** Rare (population carrier frequency ≤0.1%)
• **Risk of breast cancer:** 10- to 20-fold relative risk
• **Primary strategy for identification:** Genome-wide linkage and positional cloning
Moderate-penetrance breast cancer susceptibility genes Examples: *ATM*, *BRIP1*, *CHEK2*, *PALB2*
• **Risk variants:** Multiple, different mutations that predominantly cause protein truncation
• **Frequency:** Rare (population carrier frequency ≤0.6%)
• **Risk of breast cancer:** two- to fourfold relative risk
• **Primary strategy for identification:** Direct interrogation of candidate genes for coding variants in large, genetically enriched breast cancer case series and controls.
Low-penetrance breast cancer susceptibility alleles Examples: rs2981582 (*FGFR2*, 10q), rs3803662 (*TNRC9* (recently renamed *TOX3*), 16q), rs889312 (*MAP3K1*, 5q), rs3817198 (*LSP1*, 11p), rs13281615 (8q), rs13387042 (2q), rs1045485 (*CASP8*_D302H)
• **Risk variants:** Single-nucleotide polymorphisms that are causal or in linkage disequilibrium with the causal variant(s). May occur in
noncoding, nongenic regions.
• **Frequency:** Common (population frequency 5–50%)
• **Risk of breast cancer**: up to ~1.25-fold (heterozygous) or 1.65-fold (homozygous) relative risk
• **Primary strategy for identification:** Genome-wide association studies of hundreds of thousands of SNPs in large breast cancer case control series.

1.14.1.4 Hereditary Genes of Breast Cancer

About 5-10% of breast cancers are thought to be hereditary that are caused by abnormal genes (http:// www.breast cancer.org/risk/factors/genetics). This section describes genes associated with hereditary breast cancer. Some of the most important genes associated with hereditary breast cancer and their characterizations are given in the Table

1.14.1.5 Breast cancer susceptibility gene 1 and breast cancer susceptibility gene2:

Known genetic factors that are important in breast cancer include high-risk mutations in Breast Cancer susceptibility gene 1 (*BRCA1)* and Breast Cancer susceptibility gene 2 (*BRCA2)* genes. *BRCA1* gene is considered as one of the breast cancer genes. The majority mutations of *BRCA1* are nonsense mutations that they are a major part of the known mutations. The high frequency of this type of mutation showed that when the gene product is defective, it will lose its performance and this process will lead to cancer (Ahn et al., 2007). Mutated *BRCA1* gene and less likely *BRCA2* gene increases. *BRCA1* and *BRCA2* genes play an important role in DNA repair by homologous recombination, maintain chromosome stability, activation of DNA damage control points and regulation of cell cycle (Ahn et al., 2007).

Table1.11: Hereditary Genes Associated with Breast Cancer and Breast Cancer Risk: (Kooshyar Mohammad Mahdi & Mohammad Reza Nassiri *et al.,* 2013)

Gene	Chromosome	Syndrome	Cancer risk	Inheritance	Reference
BRCA1	17q21	Hereditary Breast/Ovarian cancer	Breast: 50 90%	Autosomal Dominant	(Ford et al., 1998)
BRCA2	13q12	Hereditary Breast/Ovarian cancer	Breast: 41-87%	Autosomal Dominant	(Ford et al., 1998)
CHEK2	22q12	Hereditary Breast	Breast: 2 to 3-fold	Autosomal Dominant	(Thompson et al., 2006)
PTEN	10q23	Cowden	Breast: 25-50%	Autosomal Dominant	(Black et al., 2005)
TP53	17p13	Li-Fraumeni	Breast: >50% often by age 30	Autosomal Dominant	(Birch et al., 2001)
ATM	11q22 23	Ataxia Telangiectasia	Breast: 17%	Autosomal Recessive	(Thompson et al., 2005)

A. Tumor Protein 53:

Tumor Protein 53 (TP53) gene is one of the most important tumor suppressor genes. The risk of developing breast cancer increases with changes in the TP53 gene. This gene has a key role in the maintenance of genome integrity and prevents the proliferation of cells with damaged DNA. Thus it plays a role in the inactivation of tumor-causing genes. About 40% of cases of breast carcinoma have mutated form of TP53 gene (Barnes and Cample john, 1996). Mutations in TP53 gene lead to unregulated cell growth and division (Genetic home reference, Reviewed August 2007, http://ghr.nlm.nih. gov/gene). One copy of the TP53 gene is lost in some cases of breast cancer and the remaining copy which has a mutation, thus prevents the production of any tumor suppressing protein 53 in the cell and leads to a cancerous

tumor. Several polymorphisms and their possible roles in breast cancer risk have been detected in the TP53 gene (http:// ghr.nlm.nih.gov/gene; Genetic home reference, Reviewed August 2007,).

B.Ataxia Telangiectasia Mutated:

Ataxia Telangiectasia Mutated (ATM) gene may be associated with an increased risk of breast cancer. The role of the ATM gene is controlling cell division. Reports have shown that having a mutation in one copy of the ATM gene in each cell increased the risk of developing breast cancer (Kooshyar Mohammad Mahdi, 2013)

1.15 Breast cancer-associated cancer predisposition syndromes:

Hereditary breast and ovarian cancer caused by mutations in BRCA1 or BRCA2 is the most frequent autosomal dominant disorder associated with a high breast cancer risk. (Turnbull C & Rahman N 2008;) In addition, there are several other rare cancer predisposing syndromes associated with an increased breast cancer risk (Table). In Li–Fraumeni syndrome (LFS1, MIM no. 151623),(Garber JE *et al*, 1991; Bradbury A R& Olopade OI, 2007) caused by germline mutations in TP53, breast cancer is one of the multiple neoplasias such as soft tissue sarcomas, leukaemia and brain tumours that recurrently occur at a young age (NCCN Clinical Practice Guidelines in Oncology:2008).

In addition, an increased breast cancer risk is present in individuals with Peutz–Jeghers syndrome (MIM no. 175200) carrying heterozygous germline mutations in STK11, formerly

known as LKB1, causing a polyposis syndrome characterized by melanocytic macules of the lips, buccal mucosa and digits as well as multiple gastrointestinal hamartomatous polyps and an increased risk of various neoplasms, including breast cancer.(Hearle N,2006). Cowden syndrome (MIM no. 158350), characterized by multiple hamartomas occurring in skin, breast, thyroid, gastrointestinal tract, endometrium and brain as well as an increased risk of malignant tumours, is associated with an increased breast cancer risk of up to 30–50% by the age of 70 years. In view of the increased cancer risk, intensified screening procedures are

indicated.(Lynch HT,et al,2008; NCCN Clinical Practice Guidelines in Oncology 2008) .

Table1.12 : Breast cancer-associated cancer predisposition syndromes. (Tim Ripperger *et al,* 2009)

Syndrome[a]	Gene	Name	Location	Syndrome Prevalence	Estimated breast cancer risk
Cowden syndrome	PTEN	Phosphatase and tensin Homologue	10q23.3	1 1– 9/1 000000	30 – 50% risk by the age of 70 years
Hereditary diffuse gastric cancer/familial lobular breast cancer	CDH1	Cadherin 1, E-cadherin	16q22.1	---------	52% risk by the age of 75 years
Li – Fraumeni syndrome	TP53	Transformation-related Protein53	17p13.1	1 – 9/100 000	50 – 60% risk by the age of45 years
Neurofibromatosis type I	NF1	Neurofibromin	17q11.2	1 – 5/10 000	SIR: 3.5
Nijmegen breakage syndrome	NBN[c]	Nibrin	8q21 – 24	Exceptional	OR: 2.8 for 657del5
Peutz – Jeghers syndrome	STK11[d]	Serine/threonine protein kinase 11	19p13.3	1 – 9/100 000	45% risk by the age of 70 years

OR=odd ratio; SIR= standardized incidence ratio; [a]Alphabetical order. [b]According to Orphanet (www.orpha.net, 28 June 2008). [c]Formerly known as NBS1. [d]Formerly known as LKB1

CDH1 mutations are known to cause hereditary diffuse gastric cancer and/or lobular breast cancer. Even in families without diffuse gastric cancer in their medical history, CDH1 mutations could be found in women with lobular breast cancer. (Masciari Set al 2007; Schrader KA, Masciari S, Boyd N *et al,* 2008), In view of the increased cancer risk, intensified surveillance programmes are indicated.(Schrader KA, Masciari S, Boyd N et al 2008; Lynch HT,2008) For women with neurofibromatosis type I (NF1) (MIM no. 162200), an autosomal dominant disorder caused by NF1 mutations, a moderately increased breast cancer risk was reported. Therefore, early screening has to be considered in these women by an interdisciplinary team of experts.(Sharif S, Moran A, Huson SM *et al*: 2007)

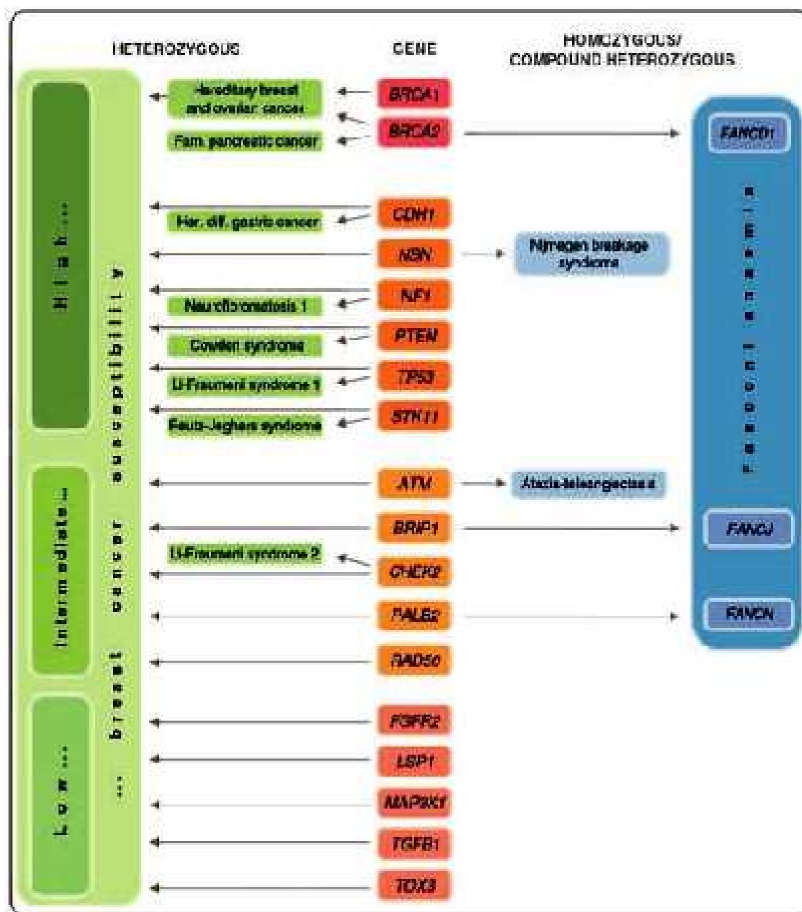


Figure1.11:Genotype –phenotype correlation of heterozygous and homozygous/compound heterozygous mutation carriers for breast cancer predisposition genes and low-penetrance breast cancer susceptibility SNPs (Tim Ripperger *et al*., 2009)

Except for the high breast cancer risk in BRCA1 and BRCA2 mutation carriers as well as the risk for breast cancer in certain rare syndromes caused by mutations in TP53, STK11, PTEN, CDH1, NF1 or NBN, familial clustering of breast cancer remains largely unexplained. Several reports have recently been published on genes involved in DNA repair and single nucleotide polymorphisms (SNPs) associated with an increased breast cancer risk. **(**Tim Ripperger *et al*., 2009)

1.16 Role of Tamoxifen in breast cancer:

1.16.1 Clinical relevance of estrogen receptor

The first major classification of breast cancer is the identification of the estrogen (ER) and progesterone (PR) receptor status. More than two thirds of breast cancers are positive on immunohistochemical staining to the estrogen receptor (ER), so hormonal therapies are the backbone of treatment for the large majority of patients with breast cancer. Both estrogen synthesis and activity through the ER have been targeted by therapies to control endocrine-dependent breast cancer; however, not all women whose tumors express ER derive benefit from hormonal therapies. Differentiating breast cancers, just on the basis of ER and PR status vastly improves treatment efficacy; the ER-positive (ER+) tumors are dependent on estrogen signaling for their growth and replication and they can be treated by antiestrogen therapy with either tamoxifen or an aromatase inhibitor (AIs). The ER-negative tumors, instead, are not estrogen-dependent and will not respond to antiestrogen therapy but are more responsive to first line chemotherapeutic combinations such as paclitaxel, 5-FU, doxorubicin and cyclophosphamide (T/FAC). Expression of the PR is highly correlated with ER expression but the effect of PR status on treatment seems to be less important than that of ER. Tamoxifen is the gold standard treatment for early and advanced disease and its activity on ER+ cancers is considered the first form of molecular targeted therapy. Recently, the introduction of aromatase inhibitors either in metastatic or in adjuvant setting has added a novel and more active tool in the breast cancer treatment. Another well characterized tumor marker is the human epidermal growth factor receptor 2 (HER2/neu). HER2 has been identified as a marker of poor prognosis and increased cancer aggressiveness and treatment of HER2+ tumors has changed significantly with the use of trastuzumab, a monoclonal antibody specific for the HER2/neuprotein.

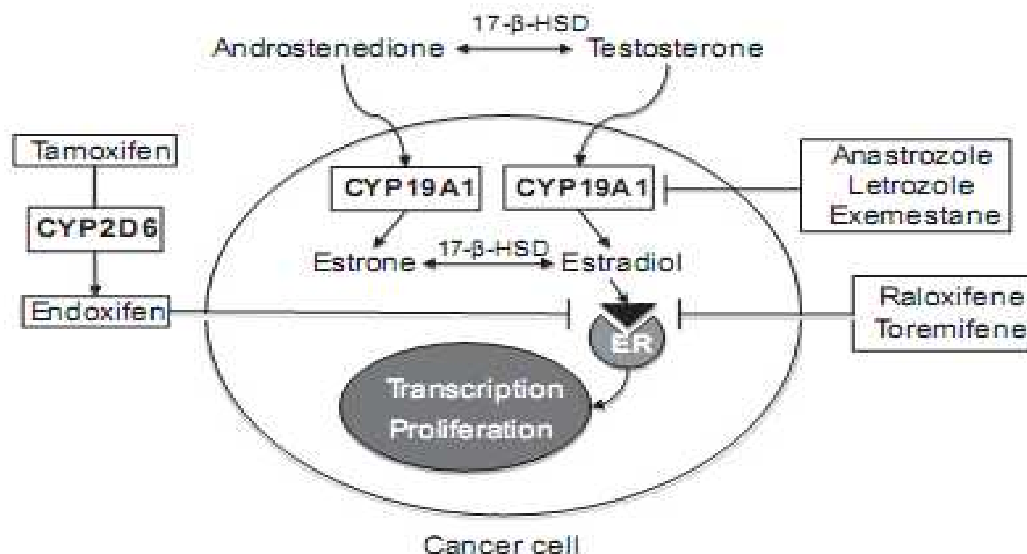


Fig. 1.12 Schematic representation of action of steroid hormones and their inhibitors on breast cancer cells. 17-b-HSD: 17b-hydroxysteroid dehydrogenase; ER: estrogen receptor.

1.17 Tamoxifen-drug of interest

Tamoxifen is the most commonly used agent for the treatment of ER+ breast cancer and belongs to the class of selective estrogen receptor modulators (SERMs). The drug was approved by the US Food and Drug Administration in 1977 for the treatment of metastatic breast cancer and subsequently approved for use in the adjuvant setting; in addition, the Early Breast Cancer Trialists' Collaborative Group demonstrated that five years of therapy with tamoxifen reduced the annual risk of breast cancer recurrence by 39%. Substantial interindividual variation exists in steady state levels of tamoxifen and its metabolites following standard dosing. A considerable portion of patients with metastatic disease and patients receiving adjuvant tamoxifen are finally relapse, suggesting that the benefit from the therapy is not uniform.

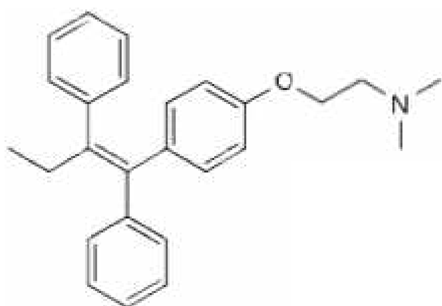
1.17.1 Chemistry of Tamoxifen

CAS Name: (Z)-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-N,N-dimethylethanamine

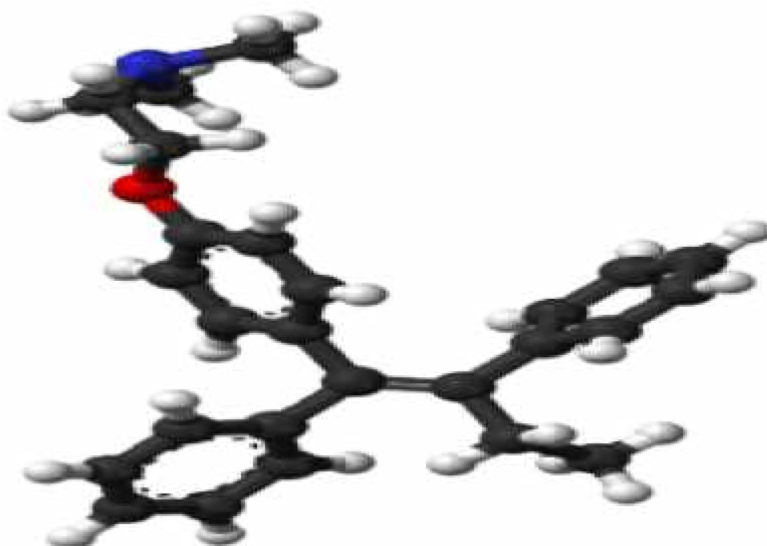
Additional Names: 1-p-b-dimethylaminoethoxyphenyl-trans-1,2-diphenylbut-1-ene

Molecular Formula: $C_{26}H_{29}NO$

Chemical structure:



Crystallographic structure:



CAS Registry Number: 10540-29-1

Molecular Weight: 371.51

Percent Composition: C 84.06%, H 7.87%, N 3.77%, O 4.31%

Literature References: Nonsteroidal estrogen antagonist.

Properties: Crystals from petr ether, mp 96-98°.

Melting point: mp 96-98°

Toxicity data: LD50 in mice, rats (mg/kg): 200, 600 i.p.; 62.5, 62.5 i.v.; 3000-6000, 1200-2500 orally (Furr, Jordan)

CAUTION: Tamoxifen is listed as a known human carcinogen: Report on Carcinogens, Eleventh Edition (PB2005-104914, 2004) p III-239.

1.17.2 History of Tamoxifen

Tamoxifen was first developed in 1962 as a morning-after birth control pill that was successful in experiments with laboratory rats.Tamoxifen (brand name Nolvadex) is the best-known hormonal treatment and the most prescribed anti-cancer drug in the world.Used for over 20 years to treat women with advanced breast cancer, tamoxifen also is commonly prescribed to prevent recurrences among women with early breast cancer.Anti-estrogens work by binding to estrogen receptors, blocking estrogen from binding to these receptors, stopping cell proliferation

Breast-cancer prevention occurred in 1998 when the National Cancer Institute (NCI) announced results of a six-year study showing that tamoxifen reduced the incidence of breast cancer by 45 percent among healthy but high-risk women.13,388 healthy women considered at high risk for breast cancer were recruited85 developed breast cancer compared to 154 of those on the placebo or dummy pill.potentially life-threatening side effects. There were 33 cases of endometrial cancer in the tamoxifen group.There were 30 cases of blood clots in major veins (deep-vein thrombosis)

lBecause these problems developed exclusively among postmenopausal women

–60-year-old, an age at which 17 out of every 1,000 women can be expected to develop breast cancer within five years

–ages of 35 and 59 were eligible to participate if their risks matched or exceeded those of a 60-year-old

Although tamoxifen has been useful both in treating breast cancer patients and in decreasing the risk of getting breast cancer.Side effects arise from the fact that while tamoxifen acts as an antiestrogen that blocks the effects of estrogen on breast cells, it mimics the actions of estrogen in other tissues such as the uterus. Its estrogen-like effects on the uterus stimulate proliferation of the uterine endometrium and increase the risk of uterine cancer.Adequate patent protection is required to develop an innovation in a timely manner. In 1962, ICI Pharmaceuticals Division filed a broad patent in the United Kingdom (UK) (Application number GB19620034989 19620913). The application stated, “The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity”.

This was published in 1965 as UK Patent GB1013907, which described the innovation that different geometric isomers of substituted triphenylethylenes had either oestrogenic or anti-oestrogenic properties. Indeed, this observation was significant, because when scientists at Merrell subsequently described the biological activity of the separated isomers of their drug clomiphene, they inadvertently reversed the naming. This was subsequently rectified.

Although tamoxifen was approved for the treatment of advanced breast cancer in post-menopausal women in 1977 in the United States (the year before ICI Pharmaceuticals Division received the Queen's Award for Technological Achievement in the UK), the patent situation was unclear. ICI Pharmaceuticals Division was repeatedly denied patent protection in the US until the 1980s because of the perceived primacy of the earlier Merrell patents and because no advance (that is, a safer, more specific drug) was recognized by the patent office in the United States. In other words, the clinical development advanced steadily for more than a decade in the United States without the assurance of exclusivity. This situation also illustrates how unlikely the usefulness of tamoxifen was considered to be by the medical advisors to the pharmaceutical industry in general. Remarkably, when tamoxifen was hailed as the adjuvant endocrine treatment of choice for breast cancer by the National Cancer Institute in 1984, the patent application, initially denied in 1984, was awarded through the court of appeals in 1985. This was granted with precedence to the patent dating back to 1965! So, at a time when world-wide patent protection was being lost, the patent protecting tamoxifen started a 17 year life in the United States. The unique and unusual legal situation did not go uncontested by generic companies, but AstraZeneca (as the ICI Pharmaceuticals Division is now called) rightly retained patent protection for their pioneering product, most notably, from the Smalkin Decision in Baltimore, 1996. (Zeneca, Ltd. vs. Novopharm, Ltd. Civil Action No S95-163 United States District Court, D. Maryland, Northern Division, March 14)

1.17.3 Pharmacology of Tamoxifen

The classic understanding of tamoxifen pharmacology has been that the parent drug and its metabolites interact with the ER in both breast and non-breast tissues to produce a complex phenotype of both agonist and antagonist effects. Although

investigators observed wide interindividual variability in the concentrations of tamoxifen and its metabolites, there was no evidence linking variability in tamoxifen concentrations (or its metabolites) with response or side effects. However, recent in vitro and in vivo human studies have characterized an important tamoxifen metabolic pathway leading to the formation of active metabolites, which exhibit significantly greater affinity for the ER and greater potency in suppressing cell proliferation (compared to tamoxifen). Knowledge of the genetic and environmental factors, which contribute to the wide variability in the formation of these active metabolites, has led to a new understanding of tamoxifen pharmacology.

1.17.4 Primary metabolism of Tamoxifen

Tamoxifen undergoes extensive hepatic oxidation by the cytochrome P450 (P450) isoforms to several primary and secondary metabolites with variable potencies toward the ER (see Figure 1). Major tamoxifen metabolites include N-desmethyl-tamoxifen (NDM), 4-hydroxy-tamoxifen (4-OH-Tam), tamoxifen-N-oxide, a-hydroxy-tamoxifen, and N-didesmethyl-tamoxifen. NDM, resulting from the CYP3A4/5-mediated catalysis of tamoxifen, is quantitatively the major primary metabolite of tamoxifen and accounts for approximately 92% of primary tamoxifen oxidation. In women receiving tamoxifen at a dose of 20mg/day, plasma steady-state concentrations of tamoxifen and NDM are 362.5 and 654.9 nM, respectively, whereas the steady-state concentrations of 4-OH-Tam are extremely low (9 nM).

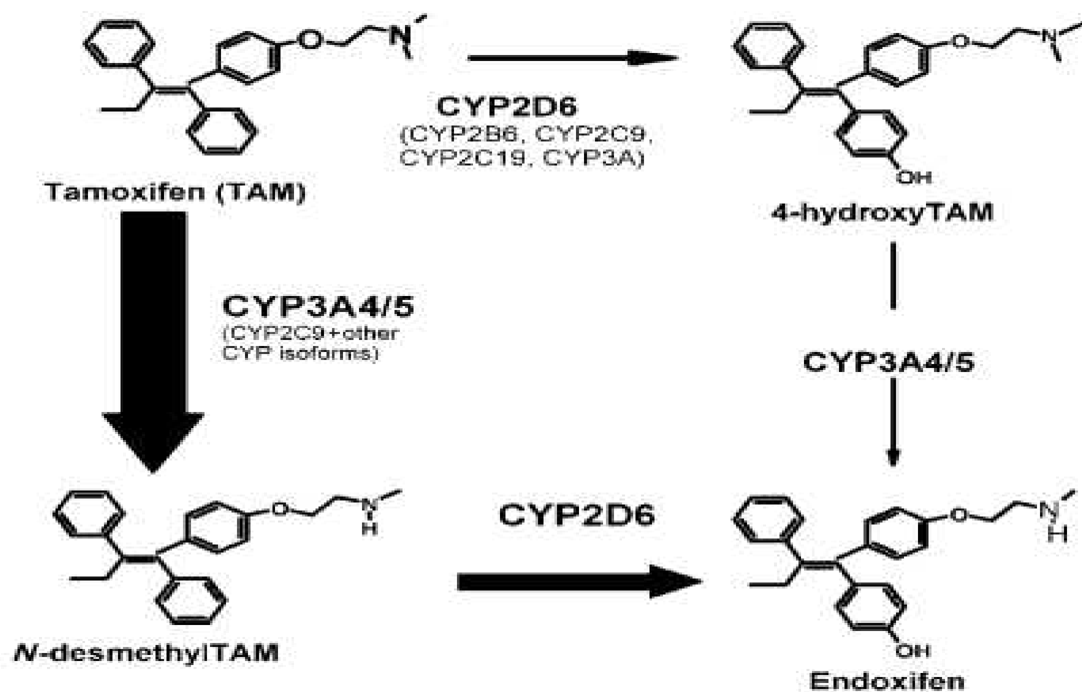


Fig 1.13: Selected transformation pathways of tamoxifen and the main CYP enzymes involved. The relative contribution of each pathway to the overall oxidation of tamoxifen is shown by the thickness of the arrow, and the principal P450 isoforms responsible are highlighted in larger fonts. Modified in part from Jin, Y. et al. J. Natl. Cancer Inst. 97, 30–39 (2005)

1.17.5 Secondary metabolism of Tamoxifen

Using NDM as an intermediary substrate, the secondary metabolism of tamoxifen has recently been more fully elucidated. NDM is predominantly biotransformed to a-hydroxy N-desmethyl-, N-didesmethyl-, and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen). Whereas the biotransformation of NDM to endoxifen is exclusively catalyzed by CYP2D6 , all other routes of N-desmethyl biotransformation are catalyzed predominantly by the CYP3A subfamily24. Recent clinical studies have demonstrated that common CYP2D6 genetic variation (leading to low or absent CYP2D6 activity), or the inhibition of CYP2D6 enzyme activity significantly lowers the plasma concentrations of endoxifen. Patients homozygous for a CYP2D6 null allele had significantly lower endoxifen concentrations (mean, 21.9 nM) than patients with one (mean, 64.2 nM) or two (mean, 88.6 nM) CYP2D6 functional alleles.

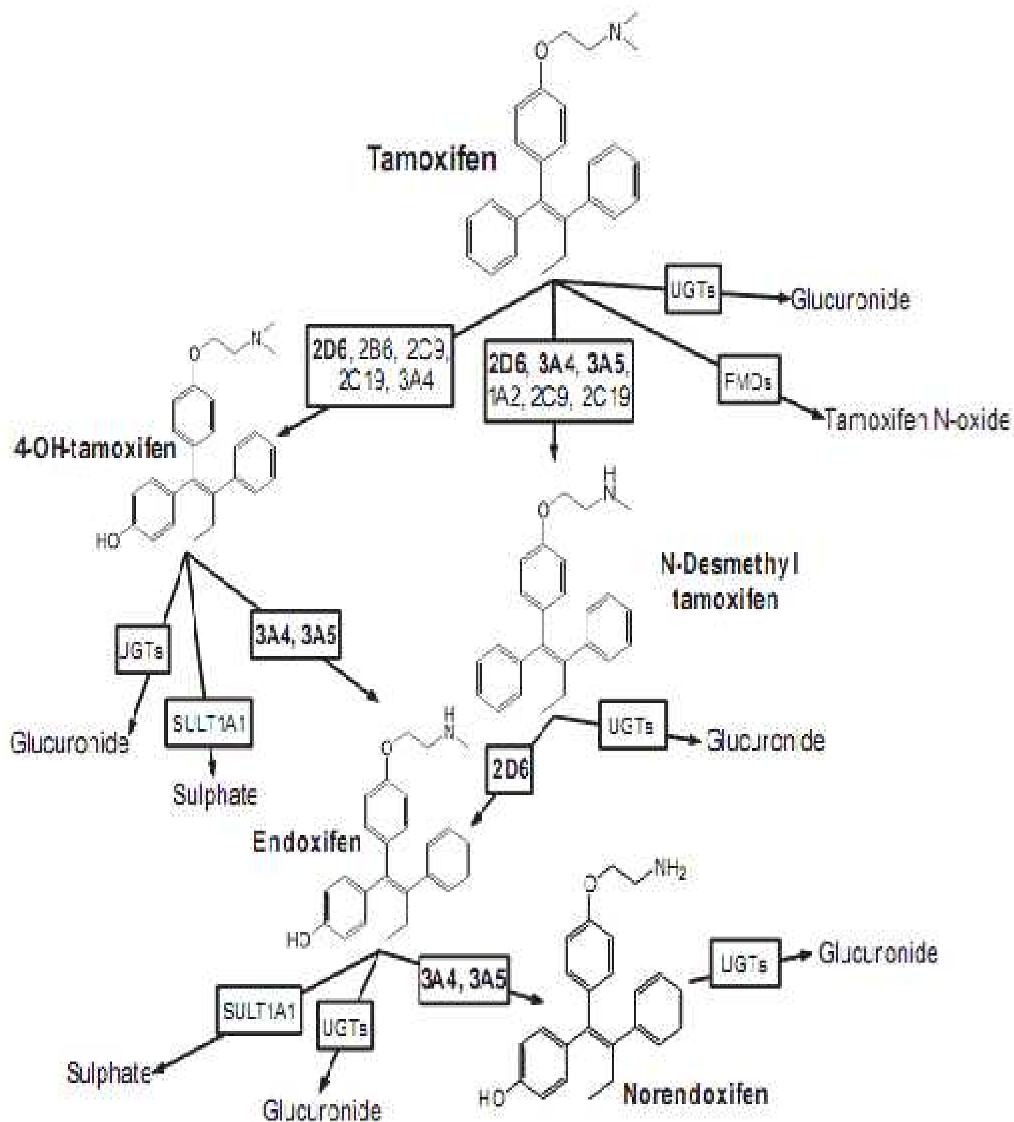


Fig1.14: Schematic representation of metabolic pathways of tamoxifen. UGTs: uridine 50-diphospho-glucuronosyltransferases; SULT1A1: sulfotransferase 1A1; FMOs: flavin-containing monooxygenases. CYP isoforms are reported in the figure by omitting the cytochrome p450 abbreviation.

1.17.6 Antiproliferative effects of Tamoxifen and its metabolites

It has been known for many years that 4-OH-Tam possesses a much higher affinity for ERs and is 30- to 100-fold more potent than tamoxifen in suppressing estrogen-dependent cell proliferation. For this reason, many considered 4-OH-Tam to be the active metabolite of tamoxifen and it is frequently used to characterize tamoxifen activity in vitro. However, new data suggest that endoxifen has identical properties

and potency as 4-OH-Tam, but is present in concentrations up to 10-fold higher than 4-OH-Tam. Lien et al. first identified ‘‘metabolite Bx’’, or endoxifen, in 1989. However, only recently did Stearns et al. publish that endoxifen exhibits potency similar to 4-OH-Tam with respect to ER-binding affinity and suppression of estrogen-dependent cell growth. Subsequent to this work, Flockhart’s group performed a series of studies to further characterize endoxifen pharmacology. These studies confirmed that endoxifen has equivalent potency in vitro similar to 4-OH-Tam in ER-a and ER-b binding, in suppression of ER-dependent breast cancer proliferation and in global ER-responsive gene expression. Recently, Buck et al. confirmed these findings through the characterization of the effects of tamoxifen and its metabolites on the antiproliferative transforming growth factor-b signal-transduction pathway in human breast cancer cells. They analyzed the growth inhibitory effect of tamoxifen and its major metabolites, and demonstrated that only 4-OH-Tam and endoxifen had significant antiproliferative activity and were able to induce transforming growth factor-b2 and transforming growth factor-b type II receptor.

1.17.7 Pharmacogenetics of Tamoxifen metabolism

Tamoxifen is metabolized via the cytochrome P450-mediated pathway to metabolites that show variable potencies toward theER (Fig. 2). N-desmethyltamoxifen, resulting from CYP3A4/5-mediated metabolism, is the major primary metabolite, accounting for 90% of tamoxifen oxidation; whereas 4-OH-tamoxifen, mediated by CYP2D6 activity, is a minor metabolite (Fig. 2). Both N-desmethyl-tamoxifen (via CYP2D6) and 4-OH-tamoxifen (via CYP3A4/5) are secondarily metabolized to 4-OH-N-desmethyl-tamoxifen (endoxifen); 4-OH-tamoxifen and endoxifen are important active metabolites, exhibiting similar potency. Norendoxifen is a potent inhibitor of CYP19A1 and is produced by CYP3A4/5. Tamoxifen metabolites are inactivated by sulfotransferases 1A1 (SULT1A1), or by the UDP-glucuronosyltransferases (UGT), including UGT1A8,1A10, 2B7, 2B15, and 2B17 isoforms (Fig. 2). In addition, CYP1B1,2B6, and 2C19 may be responsible for the isomerization of trans-4-OH-tamoxifen to its weakly estrogenic cis-isomer form, a reaction that could be associated with drug-resistant phenotypes.

CYP3A4

Because of many of the tamoxifen-metabolizing enzymes are polymorphic, genetic variations may account for interindividual or interethnic differences in tamoxifen-related outcomes. Genetic variants responsible for tamoxifen metabolism have been studied:A CYP3A4 promoter variant, CYP3A4⁄1B (392A>G) has been associated with cancer phenotype, but no studies to date have linked CYP3A4⁄1B with altered tamoxifen metabolism, although it has been reported to confer a three-fold increased risk of endometrial cancer in tamoxifen treated women.

CYP3A5

A CYP3A5 genetic polymorphism, CYP3A5⁄3 (6986A>G), results in markedly decreased CYP3A5 activity, although several studies have failed to show its association with tamoxifen metabolism orbreast cancer outcomes. However, in a study on postmenopausal patients treated with adjuvant tamoxifen, who were homo-zygous for the CYP3A5⁄3C variant, displayed significantly improved recurrence-free survival.These conflicting findings underscore the complexity of tamoxifen metabolism and resistance mechanisms and suggest that the evaluation of variants representing different interacting mechanisms may be more informative than evaluating single variants.

CYP2C19

The hypothesis that CYP2C19 681G>A and 636G>A variants, known for their lack of enzyme activity, are associated with an increased breast cancer mortality rate was studied in 80 patients given tamoxifen. CYP2C19 681AA carriers were associated with a significantly longer breast cancer survival rate than patients with the wild-type (hazard ratio 0.26), suggesting that CYP2C19 genotype may possibly be a predictive factor for survival in breast cancer patients using tamoxifen.

CYP2D6

More than 80 allelic variants have been described in CYP2D6. A selection of them is presented in Table 1. A commonly accepted classification system designates individuals homozygous for two wild-type CYP2D6 alleles as CYP2D6⁄1/1, and refers to such subjects as extensive metabolizers (EMs). Individuals with one or two variant alleles that encode enzymes with reduced or null activity are designated intermediate metabolizers (IMs) and poor metabolizers (PMs), respectively; a minority of population may carry multiple copies of CYP2D6 alleles and consequently are called ultrarapid metabolizers (UMs), such individuals have increased enzymatic activity compared to CYP2D6⁄1. The incidence of SNPs in

CYP2D6 varies by race and ethnicity.African-Americans, for example, have more than twice the median frequency of nonfunctional alleles compared with Africans (14.5% versus 6.3%). However, evidence from published studies evaluating CYP2D6 variants as predictive factors of tamoxifen efficacy and clinical outcome are conflicting. In a study on 618 breast cancer patients treated with adjuvant tamoxifen, CYP2D6 ⁄4⁄5,10 and ⁄41 alleles were genotyped and the recurrence free survival (RFS) was calculated based on inferred CYP2D6 phenotypes using Kaplan–Meier and Cox proportional hazard models, adjusted for nodal status and tumor size. Patients with at least one reduced function CYP2D6 allele (60%) or no functional alleles (6%) had a non-significant trend for worse RFS (hazard ratio (HR) 1.52). For postmenopausal women on tamoxifen monotherapy, the HR for recurrence in patients with reduced functional alleles was 1.96. However, RFS analysis limited to four common CYP2D6 allelic variants was not significant (P = 0.39). Another study investigated CYP2D6⁄1,⁄4,⁄5,⁄6b/c,⁄9,⁄10,⁄41 and ⁄UM functional variants and the correlation with breast cancer specific survival (BCSS) in 6640 DNA samples from patients with invasive breast cancer, of wich 3155 cases had received tamoxifen therapy. There were 312 deaths from breast cancer, in the tamoxifen-treated patients, with over 18000 years of cumulative follow-up. The association between genotype and BCSS was evaluated using Cox proportional hazards regression analysis. In tamoxifen treated patients, there was weak evidence that the poor-metaboliser variant, CYP2D6⁄6, was associated with decreased BCSS (P = 0.02; HR = 1.95). No other variants, including CYP2D6⁄4, previously reported to be associated with poorer clinical outcomes, were associated with differences in BCSS, in either the tamoxifen or non-tamoxifen groups. In another study the impact of CYP2D6⁄4 genotype on progression-free survival (PFS) and time to progression (TTP) was evaluated in 493 patients with ER+ tumors and tamoxifen therapy longer than 6 months. No significant difference in TTP or PFS between the CYP2D6⁄4 and ⁄1 genotype groups was found. In a subgroup of patients treated with chemotherapy the CYP2D6⁄4 poor metabolizers had a tendency towards a shorter mean TTP; in this group the mean TTP and the PFS were 1 year in the CYP2D6⁄4/4 group, 6.3 years in the 1/4 group and 4.97 years in the 1/1 group. A study on 206 patients receiving adjuvant tamoxifen and on 280 control untreated subjects (71 months median follow-up) genotyped for several polymorphisms of CYP2D6, CYP2C19, CYP2B6, CYP2C9, and CYP3A5, tamoxifen-treated patients carrying the deficient CYP2D6 alleles4/5⁄10,⁄41 had

significantly more recurrences of breast cancer,shorter relapse-free periods, and worse event-free survival rates compared with carriers of functional alleles. Patients with the high enzyme activity promoter variant CYP2C19⁄17 had a more favorable clinical outcome than carriers of 1⁄2, and 3 alleles. In a study on 190 postmenopausal breast cancer patients treated with adjuvant tamoxifen, CYP2D6⁄4 homozygotes had significantly worse RFS and DFS and lower incidence of hot flashes compared with women with either one or no CYP2D6⁄4 variant alleles. This was also confirmed in a cohort of 486 patients treated with tamoxifen who were carriers of the CYP2D6⁄4, CYP2D6⁄5, CYP2D6⁄10and CYP2D6⁄41 alleles and had significantly worse survival, whereas no association was found among the untreated. Concomitant administration of CYP2D6 inhibitors, in addition to genetic variants, may affect tamoxifen-related outcomes, converting an extensive metabolizer to a phenotypic poor metabolizer. CYP2D6 inhibitors include antidepressants such as fluoxetine and paroxetine, whereas moderate/weak inhibitors include cimetidine, amiodarone, and haloperidol. CYP2D6 extensive metabolizers who took potent CYP2D6 inhibitors had lower plasma endoxifen concentrations and poorer RFS than those who did not. Studies correlating CYP2D6 variants with tamoxifen outcome were unable to assess the predictive importance of endoxifen pharmacokinetics, probably because CYP2D6 influences the exposure to several active metabolites that collectively account for tamoxifen pharmacodynamics. The levels of tamoxifen, endoxifen, 4-hydroxytamoxifen and N-desmethyltamoxifen were measured in 1370 samples from patients with ER+ disease. Breast cancer outcomes were not associated with the concentration levels of tamoxifen, 4-OH-tamoxifen and N-desmethyltamoxifen.The usefulness of CYP2D6 testing before tamoxifen use is still a matter of debate and no specific recommendation has been issued with respect to the testing. It is not clear whether women who are intermediate or poor CYP2D6 metabolizers should avoid tamoxifen or receive an higher dose and it is not established a treatment algorithm based on CYP2D6 genotype. Several studies have evaluated pharmacogenetic influences on tamoxifen metabolism, efficacy, and safety; however, the development of third-generation aromatase inhibitors, developed in the early 1990s, including two non-steroidal triazole derivatives (anastrozole and letrozole) and one steroidal derivative (exemestane), has provided an alternative strategy for managing hormone-responsive breast cancer in postmenopausal women. However, a recent study on 119 patients treated with tamoxifen 20 mg/day for at least 4 months and not on any strong

CYP2D6 inhibiting medications demonstrated that plasma levels of endoxifen in CYP2D6 poor metabolizer were 8–10 times lower than extensive metabolizers (EM) and that increasing the tamoxifen dose to 40 mg/daily significantly increased endoxifen levels in intermediate (IM) and poor metabolizers (PM). Assuming that the concentration of the active metabolite at the target tissue is of potential crucial importance for an optimal outcome of treatment, a reasonable strategy is to adapt the drug dose based on CYP2D6 genotype, especially when a patient bearing an IM or PM phenotype is treated with strong or moderate CYP2D6 inhibitors, including buproprion, duloxetine, paroxetine, fluoxetine, quinidine, amiodarone, terbinafine, haloperidol, indinavir or ritonavir.

SULT1A1 and UGT

The enzymes responsible for elimination and inactivation of tamoxifen and its metabolites through conjugation with either a sulfate (SULT) or a glucuronide (UGT) moiety may also have functionally relevant genetic variants. The SULT1A1⁄2 variant (638G>A,Arg213His) encodes for a protein with reduced activity. However,two studies exploring its association with tamoxifen pharmacokinetics. Non-functional (homozygotes or heterozygotes had decreased plasma endoxifen concentrations on adjuvant tamoxifen) Jin et al.Non-functional (allele carriers had more recurrences and significantly worse disease-free survival) Goetz et al.Gene deletion and lack of enzyme activity (allele carriers had significantly worse event-free survival with adjuvant tamoxifen) Schroth et al. Non-functional (homozygotes or heterozygotes had decreased plasma endoxifen concentrations after adjuvant tamoxifen) Jin et al. Reduced activity (allele carriers had worse recurrence outcomes with adjuvant tamoxifen and metastatic breast cancer patients had shorter TTP with tamoxifen) Lim et al.;Xu et al. Reduced activity (allele carriers had increased recurrences, decreased relapse-free survival and significantly worse event-free survival with adjuvant tamoxifen) Schroth et al.whereas a third study on 160 patients reported conflicting results on a greater risk of death in tamoxifen-treated patients. In vitro studies suggested that the UGT1A4 variant Leu48Val shows increased glucuronidation activity against tamoxifen and its metabolites, although the clinical significance is still unexplored. A nonsynonymous polymorphism in UGT2B15(UGT2B15⁄2; 253G>T) in a putative substrate binding domain has been assessed in adjuvant tamoxifen-treated breast cancer patients and found that subjects possessing SULT1A1⁄2 and either UGT2B15⁄1/2 or UGT2B15⁄2/2 had a significantly reduced 5-year survival.

1.18 Gene of interest and justification:

Tamoxifen-treated patients carrying the CYP2D6 and CYP3A5 alleles associated with impaired formation of antiestrogenic metabolites, had significantly more recurrences of breast cancer, shorter relapse-free periods (hazard ratio [HR], 2.24; 95% CI,1.16 to 4.33; P =0.02), and worse event-free survival rates(HR, 1.89; 95% CI, 1.10 to 3.25; P =0.02) compared with carriers of functional alleles (Schroth et al., 2007).

The most active hepatic UGT is UGT2B7 which can be also found in the gastrointestinal tract and breast tissue.O-glucuronidation of both trans-4-hydroxytamoxifen and trans-endoxifen was found to be lower in theUGT2B7268Tyr allele (Lazarus et al., 2009).

The SULT1A1*2 variant allele is associated with decreased catalytic activity. 4-Hydroxytamoxifen and endoxifen are subject to conjugation leading to excretion by sulfation,via SULT1A1. (Corine Ekhart et al.,2009)

1.19 Hazard Ratio (HR):

A measure of how often a particular event happens in one group compared to how often it happens in another group, over time. In cancer research, hazard ratios are often used in clinical trials to measure survival at any point in time in a group of patients who have been given a specific treatment compared to a control group given another treatment or a placebo. Hazard ratios are commonly used when presenting results in clinical trials involving survival data, and allow hypothesis testing. They should not be considered the same as relative risk ratios. When hazard ratios are used in survival analysis, this may have nothing to do with dying or prolonging life, but reflects the analysis of time survived to an event (the event may, in some instances, include cure).A hazard is the rate at which events happen, so that the probability of an event happening in a short time interval is the length of time multiplied by the hazard. Although the hazard may vary with time, the assumption in proportional hazard models for survival analysis is that the hazard in one group is a constant proportion of the hazard in the other group. This proportion is the hazard ratio. When expressing the results of clinical trials, it is best to consider the hazard ratio alongside a measure of time, such as median time to the event under scrutiny, comparing active treatment and control groups (the points at which half the subjects have experienced the event in

each arm of the study). The hazard ratio is an expression of the hazard or chance of events occurring in the treatment arm as a ratio of the hazard of the events occurring in the control arm. The term hazard ratio is often used interchangeably with the term relative risk ratio to describe results in clinical trials. This is not strictly correct as there are subtle and important differences. It is useful to understand the meaning of the term and also be able to identify when it is used appropriately. Hazard ratios are increasingly used to express effects in studies comparing treatments when statistics which describe time-to-event or survival analyses are used. In most recent trial publications these have largely replaced direct comparisons of number of events (or 'rates') after a specific point in time or at the end of a study, seen in tests such as the t-test.

1.20 Survival Rate (SR):

The percentage of people in a study or treatment group who are still alive for a certain period of time after they were diagnosed with or started treatment for a disease, such as cancer. The survival rate is often stated as a five-year survival rate, which is the percentage of people in a study or treatment group who are alive five years after their diagnosis or the start of treatment. Also called overall survival rate.

1.21Relapse Free Survival (RFS):

In cancer, the length of time after primary treatment for a cancer ends that the patient survives without any signs or symptoms of that cancer. In a clinical trial, measuring the relapse-free survival is one way to see how well a new treatment works. It is also known as DFS, disease-free survival and RFS.

1.22Breast cancer specific survival (BCSS):

The length of time between primary detection of breast cancer and the patient survives.

1.23 Rationale and Objective of our study:

In the present study we investigated the prognostic and/or predictive value of functional polymorphisms in cytochrome P4503A5 (CYP3A5), cytochrome P4502D6 (CYP2D6) sulphotransferase 1A1, (SULT1A1) and UDP-glucuronosyltransferase 2B7 (UGT2B7) of tamoxifen-treated patients with breast cancer by Polymerase Chain reaction- restriction fragment length polymorphism (PCR-RFLP).

The aim of our study was to correlate the genotype data with response, Survival Rate (SR), Hazard Ratio (HR), Breast Cancer Specific Survival (BCSS), and Relapse Free Survival (RFS) which is —

1. Leading to preventive or screening strategies for individual or family members which allows for preventive measures, clinical management and counseling.

2. Used to find out the impact of polymorphisms on disease prognosis, the cure of the disease might be done more precisely and cost effectively. As a result prognosis of the disease will be faster and mortality rate will decrease.

3. Reducing the number of injuries or deaths resulting from incorrect or ineffective prescriptions prescribed for the cancer patients and tends to integrate into personalized medicine.

4. Detect the actual causes of the development of side effects after tamoxifen therapy in breast cancer patients and suggest the alternative treatment therapy for the disease at early stage.

5. Help us to develop a safe, efficient and cost effective treatment plan for the patients.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Selection of volunteer

2.1.1. Volunteers

The study comprises of 388 estrogen receptor positive breast cancer patients taking Tamoxifen 20mg daily dose orally with different age and smoking status. Breast cancer patients were recruited from Ahsania Mission Cancer and General Hospital, Dhaka Medical College Hospital and Bangabandhu Sheikh Mujib Medical University (PG Hospital), Dhaka, Bangladesh. Patients were histologically diagnosed with breast cancer between the periods of June 2013 to February 2016. Menarche and the menstrual cycle, breastfeeding, age, demographic characteristics, lifestyle factors data were collected through interviews by trained nurses in presence of expert physicians. The study protocol was approved by the ethical committees of the respective hospitals. Each volunteer signed an informed consent document before entering the study after briefing the purpose of the study and was free to withdraw from the study at any time without any obligation. Nodal status (UICC), Stage (UICC), Progesterone receptor status, Morphological type and adjunct therapy was recorded. The study was conducted in accordance with the International Conference of Harmonization (ICH) for Good Clinical Practice (GCP) and in compliance with the Declaration of Helsinki and its further amendments (adopted by the 18th WMA general assembly, Helsinki, Finland, June 1964 and last amendment in in Seoul, South Korea on October 2008).

2.2. Individual demographic data:

Demographic data include detailing about the height, weight, body index of patients which is attached in the appendix part of our study.

2.3 Patient consent form:

A form regarding the approval of the patients is included in the appendix portion.

2.4 Data collection form:

The form needed to encompass all the information about the patients is jostled down in a data collection form.

2.4. Materials and methods

2.4.1. Materials

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
Distillation Plant (Distinction D4000)	Bibby Sterlin Ltd., UK
Micropipette	Bio-Rad Laboratories, USA
Freeze (-80^0 C)	DAIREI,Sweden

Ultrapure Water System (Arium 611)	Sartorius, Germany
Microcentrifuge Machine (Mikro 20)	Hettich GmbH & Co., Germany
Freeze (− 20^0 C)	Siemens, USA
Vortex Mixer Machine (Rotamixer-9590)	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, German
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

2.4.3.1 Agarose

Type	DNA Size (kbp)	Gel Strength (gm/cm^2)
HS	0.5-30	>2000 (1.5%)
H	1-200	>2800 (1.5%)
X	0.01-1.0	>1000 (3.0%)

1600	0.01-1.0	>1400 (1.5%)

2.4.3.2. Other 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	BDH, UK
Sample Loading Dye, 6X	Promega Corporation, USA
Taq DNA polymerase	NEB, USA
Standard Reaction Buffer	NEB, USA
$MgCl_2$ Solution	NEB, USA
Deoxyribonucleotide Solution Mix (dNTP)	NEB, USA
Quick-Load® 50bp DNA Ladder	NEB, USA
100 bp DNA ladder	NEB, USA

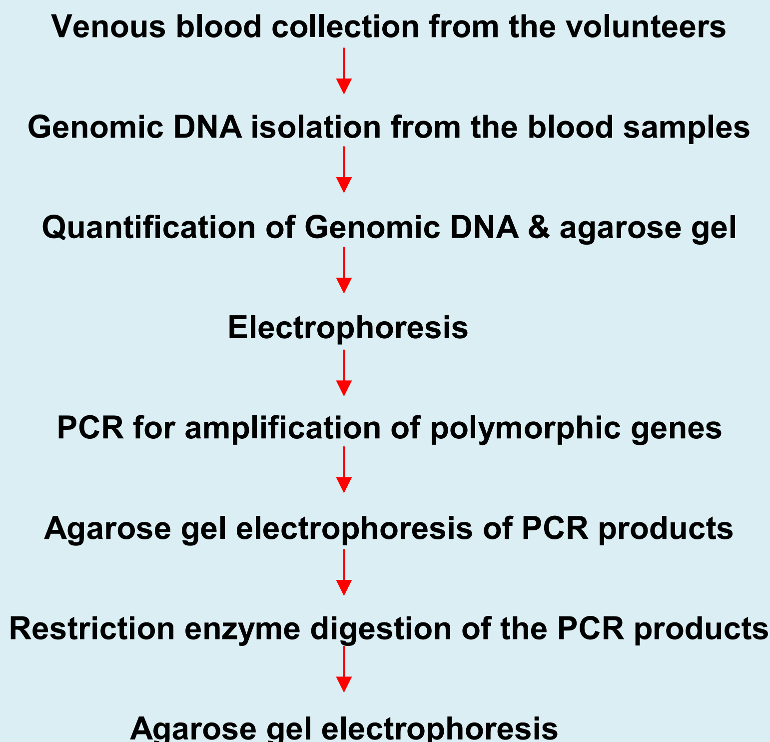
2.4.4 Restriction enzymes

Genes	RE	Recognition sites	Source
SULT1A1*2 **rs9282861**	**HaeII**	5'...RGCGCY...3' 3'...YCGCGR...5'	New England BioLabs Inc., UK.
UGT2B7*2 **rs7439366**	**FokI**	5'...GGATG(N)$_{9}$...3' 3'...CCTAC(N)$_{13}$...5'	New England BioLabs Inc., UK.
CYP3A5*3 **rs776746**	**MboI**	5'...GATC...3' 3'...CTAG...5'	New England BioLabs Inc., UK.
CYP2D6*4 **rs3892097**	**MvaI**	5'...CCWGG...3' 3'...GGWCC...5'	New England BioLabs Inc., UK.
CYP2D6*10 **rs1065852**	**HphI**	5'...GGTGA(N)$_{8}$...3' 3'...CCACT(N)$_{7}$...5'	New England BioLabs Inc., UK.

2.4.5 Solutions

Name	Composition
TAE Buffer (10x)	0.4 M Tris-Base (Tris (Hydroxymethyl)-amino methane), 11.4 % (v/v) /0.2 M Glacial acetic acid 0.01 M EDTA-Na_2, p^H to 7.6 with Glacial acetic acid or Tris.
TBE Buffer(10x)	0.89 M Tris-Base Boric acid, 20 mM EDTA-Na_2, p^H to 8.0 with Boric acid or Tris.
TE Buffer(1x)	10 mM (Tris-HCl (Tris (Hydroxymethyl) amino methane hydrochloride), 0.001M EDTA-Na_2, p^H to 8.0 with Tris.

2.5 Methods of genotyping



2.5.1 Blood collection

Venous blood (3 ml) was collected from all cases in sterile tubes containing EDTA-Na_2 and stored at -80˚C until DNA extraction.

2.6. Preparation of DNA Isolation Reagents

2.6.1. Cell Lysis Buffer

To prepare 1 L buffer, 10 mM Tris-(hydroxymethyl)-amino methane, 320 mM Sucrose, 5 mM MgCl2 were taken in a 1L buffer container and it was diluted to 850 ml with Milli-Q water. pH

was adjusted to 8.0 by adding Glacial acetic acid. After autoclaving 1% Triton X-100 was added to it and the total solution was made up to 1L by Milli-Q water and it was stored at 4˚C.

2.6.2. Nuclear Lysis Buffer

400 mM Tris-(hydroxymethyl)-amino methane, 60 mM EDTA-Na_2 , 150 mM Sodium chloride were taken in a 1L buffer container and it was added to 850 ml with Milli-Q water. pH was adjusted to 8.0 by adding Glacial acetic acid. After autoclaving, 1% Sodium lauryl sulphate was added to it and the total solution was made up to 1L by adding Milli-Q water and stored at room temperature.

2.6.3. Sodium Perchlorate (5 M)

61.22 gm of Sodium perchlorate was dissolved in 100 ml Milli-Q water and stored at 4˚C.

2.6.4. Tris-EDTA (TE) Buffer (1x)

10 ml of 1M Tris - (Hydroxymethyl) - amino methane and 2 ml of 500 mM EDTA-Na_2 were mixed in buffer container and then diluted to 1L by adjusting pH to 8.0 and stored at 4˚C. The final concentration of Tris - (Hydroxymethyl) - amino methane and EDTA-Na_2 were 10Mm and 1mM respectively.

2.6.5. TAE buffer (10x)

0.4 M Tris -(Hydroxymethyl)-amino methane, 11.4 %(v/v)/0.2 M Glacial acetic acid and 0.01 M EDTA-Na were taken in a buffer container and diluted to 1L after adjusted pH to 7.6 and stored at 4˚C.

2.7 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 (-20°C).

2.8. Quantification of Genomic DNA

The quantity and purity of DNA isolated from blood samples were assessed by using a UV Spectrophotometer (UV Prove v2.1) at 260 nm. In order to ensure complete sample homogeneity, which is critical when measuring genomic DNA concentration and purity with this instrument, 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 mg of DNA/ml.DNA concentration in µg/µl was calculated as follows:

$$\text{DNA Concentration } (\mu g/\mu ml) = \frac{\text{OD 260X50 (dilution factor)X 50 } \mu g/ml}{1000}$$

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

A value out of this range is not acceptable. It may indicate that the DNA sample is not in solution or that there are contaminants (i.e., protein) in the sample that may inhibit subsequent reactions. All working solutions of genomic DNA were stored at -20ºC until genotype analysis. 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 2% (w/v) agarose gel.

2.9. Genotyping of Single Nucleotide Polymorphism (SNPs) of genes

In order to facilitate the accurate genotyping of the volunteer's DNA samples for the selected SNPs, PCR-RFLP was employed due to its affordability, efficiency, ease of use and reliability. This method of genotyping produces the restriction enzyme (REase) digestion of polymerase chain reaction (PCR) amplification product. The subsequent digestion or lack of digestion, of PCR amplification 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 available from the National Centre for Biotechnological Information (NCBI) Entrez Nucleotides Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

2.9.1 DNA Amplification by PCR (Polymerase Chain Reaction)

The relevant genomic target regions, containing the SNPs of interest, were amplified by means of primer-directed PCR using thermo stable DNA polymerase, as 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.

2.9.2 Primer Design

There are some guidelines for primer design:

i. PCR primers should be generally 15-30 nucleotides long.
ii. Optimal GC content of the primer is 40-60%. Ideally, C and G nucleotides should be distributed uniformly along the primer.

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

iv. Should avoid primer self-complementarities or complementarities between the primers to prevent hairpin formation and primer dimerization.

v. Should check for possible sites of non-desirable complementarities between primers and the template DNA.

vi. Differences in melting temperatures (Tm) of the two primers should not 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 2.4

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	**SULT1A1*2 (rs9282861)** FP	**5'-GGTTGAGGAGTTGGCTCTGC-3'**	60.5	20
	SULT1A1*2 (rs9282861) RP	**5'-ATGAACTCCTGGGGGACGGT-3'**	60	20
2	**UGT2B7*2 (rs7439366) FP**	**5'-GACAATGGGGAAAGCTGACG-3'**	81.4	20
	UGT2B7*2 (rs7439366) RP	**5'-GTTTGGCAGGTTTGCAGTG-3'**	80	19

3	**CYP3A5*3 (rs776746)** FP	**5’-TGTTGCATGCATAGAGGAAGGATCG-3′**	57.5	25
	CYP3A5*3 (rs776746) RP	**5′-TACTGTCCCAAACACTGTCCC-3′**	55.5	21
4	**CYP2D6*4 (rs3892097)** FP	**5’-GCCTTCGCCAACCACTCCG-3**	62.5	19
	CYP2D6*4 (rs3892097) RP	**5’-AAATCCTGCTCTTCCGAGGC-3’**	65	20
5	**CYP2D6*10 (rs1065852)** FP	**5’-GTGCTGAGAGTGTCCTGCC-3’**	58	19
	CYP2D6*10 (rs1065852) RP	**5’-CACCCACCATCCATGTTTGC-3’**	60.5	20

FP=Forward Primer; RP=Reverse Primer; M.T=Melting Temperature
Primers are obtained from Jena Bioscience.

2.10 Required Conditions for PCR

GoTaq® Flexi DNA Polymerase, reaction buffer, dNTPs and $MgCl_2$ were used for the PCR amplification of the relevant genomic target regions, containing the SNPs of interest. A 25 µl reaction volume was used containing 1.0 µl of genomic DNA (50-70 ng/µl), 20.0 µl of 10X Colorless GoTaq® reaction buffer,4.0 µl of dNTPs (5 mM), 2.0 µl of each primer (20 µM), 1 µl of GoTaq® DNA polymerase (50 U/µl), and 180 µl of Nuclease free water.

PCR conditions to synthesize various alleles with their respective lengths are given in Table-2.5.

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

POLYMORPHS	PCR Conditions (35 Cycles)	Size of PCR Products (bp)
SULT1A1*2 (rs9282861)	94^0C For 30s 63^0C For 45s 72^0C For 2min	281
UGT2B7*2 (rs7439366)	95^0C For 30s 65^0C For 30s 72^0C For 30s	116
CYP3A5*3 (rs776746)	95^0C For 30s 56^0C For 30s 72^0C For 30s	468
CYP2D6*4 (rs3892097)	95^0C For 30s 63^0C For 30s 72^0C For 30s	414
CYP2D6*10 (rs1065852)	95^0C For 30s 59^0C For 30s 72^0C For 30s	344

2.11 Restriction Enzyme Digestion

After PCR amplification, 25 µl of the PCR products were digested with approximately 1 unit of respective restriction enzyme obtained from New England BioLabs Inc., UK. Incubation conditions are listed in Table 2.6. Electrophoreses was done for the digested products using 2% agarose gel.

Table 2.3: The restriction enzymes, digestion condition and length of the expected fragments on digestion to diagnose genes:

SNP	RESTRICTION ENZYME	DIGESTION CONDITIONS	EXPECTED FRAGMENTS (bp)
SULT1A1*2-rs9282861	HaeII	Incubation at 37°C overnight	NH: 281 HE: 104 ,177,281 MH: 177,104
UGT2B7*2-rs7439366	FokI	Incubation at 37°C overnight	NH: 116 HE: 45,71,116 MH: 45,71
CYP3A5*3-rs776746	MboI	Incubation at 37°C overnight	NH: 468 HE: 218,250,468 MH: 218,250
CYP2D6*4-rs3892097	MvaI	Incubation at 37°C overnight	NH: 414 HE:163,251,414 MH: 163,251
CYP2D6*10-rs1065852	HphI	Incubation at 37°C overnight	NH: 344 HE: 159,185,344 MH: 159,185

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

2.12 Visualization of PCR Products and REase Digestion Fragments

PCR amplification products were visualized by means of agarose gel electrophoresis in order to allow for size estimation and thus confirmation of amplification of the desired genomic target region. REase digestion fragments that were 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, which served 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 used for size estimation of all REase digestion fragments, allowing for accurate and reliable genotyping of samples. EZ Load™ 100 bp DNA ladder is thus evident in lane 1 of all agarose gel photos. All agarose gels were visualised under ultraviolet (UV) light and photographed with a Gel Documentation and Analysis System.

2.13 Gel Electrophoresis

Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose gel electrophoresis of DNA is used to determine the presence and distinguish the type of nucleic acids obtained after extraction and to analyze digestion products. Desired DNA fragments can be physically isolated for various purposes such as sequencing, probe preparation, or for cloning fragments into other vectors. Both agarose and polyacrylamide gels are used for DNA analysis. Agarose gels are usually run to size larger fragments (greater than 200 bp) and polyacrylamide gels are run to size fragments less than 200 bp. Typically agarose gels are used for most purposes and polyacrylamide gels are used when small fragments, such as digests of 16S rRNA genes, are being distinguished. Regular agarose gels may range in concentration from 0.6 to 3.0%.

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

Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, 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 through the agarose faster than larger molecules. DNA in the gel will be visualized by the use of Ethidium Bromide, added to the gel. Ethidium bromide binds to DNA and illuminates when exposed to ultraviolet light, causing the DNA to 'glow'.

All PCR products were resolved by electrophoresis in 2% (w/v) agarose gel at 80 volts (V). The REase digestion fragments were also observed in 2% (w/v) agarose gel. All REase digestion fragments were resolved at 80 V, so as to ensure sufficient resolution to allow for accurate genotyping.

2.14 Agarose Gel Electrophoresis Procedure:

All agarose gels were made with and resolved in 1X tris acetate ethylenediaminetetraacetic acid (TAE) buffer, which was made and stored as a 10X stock solution and diluted to the required working concentration as was needed. In order 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)

Procedure:

A. 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 mixed in a conical flask. The flask was swirled to evenly distribute the agarose.

2. The solution was then heated in the microwave oven for 1 minute. Protective gloves were worn and 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. When it started to boil, boiling was stopped and swirled again repeating the process until all of the agarose went into solution.

3. The flask was allowed to cool. The gel was poured when the temperature of the solution was 55-65° C.

4. The gel apparatus was prepared for casting the gel while the agarose was cooling.

5. Prior to pouring the gel, Ethidium bromide was added to the dissolved agarose and swirled to mix.

6. The gel was poured into the casting tray and the comb was adjusted to keep the wells perpendicular. The gel was allowed to cool and was hardened (20-30 minutes) prior to use.

7. No bubble or black particles were allowed to settle in the tray.

B. Preparing the gel for electrophoresis

8. A few ml of 1X TAE buffer was added to the well area of the gel and the comb was carefully removed by pulling straight up.

9. The electrophoresis tank was filled with buffer solution (1X TAE) and the gel was placed (In the casting tray) on the tank platform.

C. Preparing samples for loading/running the gel

10. An appropriate volume of loading dye (6X) was added to the sample (1 µl of 6X sample dye for every 5 µl of sample).

11. The sample was loaded using a 1-10 µl micropipette. The marker was also loaded.

12. After the gel had been loaded, the cover was gently placed on the apparatus and the Power leads were hooked up. The power was adjusted to 80 volts (constant voltage). The gel was run until the first dye front (bromophenol blue) had migrated about two-thirds the length of the gel and the second dye front (xylene cyanol) had migrated approximately one-third of the length of the gel.

13. The power was turned off before removing the gel for photographing.

14. The gel was placed on the UV transilluminator to visualize the DNA.

2.15 Study End Point

Prospective study was done to evaluate the role of the SULT1A1*2, UGT2B7*2, CYP3A5*3, CYP2D6*4 and CYP2D6*10 polymorphism in the response of 388 patients with intact tumor taking Tamoxifen therapy. Response Evaluation Criteria in Solid Tumors (RECIST) (Therasse et al., 2000) was applied to assess 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 selected polymorphisms on Tamoxifen therapy were evaluated on the patients. Patient

showing different drug induced toxicities were assessed according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.0. The highest grade toxicity occurred during the course of treatment of an individual patient was taken as a tool for the analysis.

2.16 Adverse event evaluation method:

We followed Common Terminology Criteria for Adverse Events (CTCAE) v4.0 for adverse event analysis. Different diagnostic test report and expert doctors' advice were taken for grading of adverse events and staging of cancer. Several criteria and signs and symptoms as stated in the aforesaid guide line were considered as tools for adverse event identification and grading.

Common Terminology Criteria for Adverse Events v4.0 (CTCAE)

Publish Date: May 28, 2009

CTCAE Terms

An Adverse Event (AE) is any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medical treatment or procedure that may or may not be considered related to the medical treatment or procedure. An AE is a term that is a unique representation of a specific event used for medical documentation and scientific analyses. Each CTCAE v4.0term is a MedDRA LLT (Lowest Level Term).

Definitions

A brief definition is provided to clarify the meaning of each AE term.

Grades

Grade refers to the severity of the AE. The CTCAE displays Grades 1 through 5 with unique clinical descriptions of severity for each AE based on this general guideline:

Grade 1 Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.

Grade 2 Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL*.

Grade 3 Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL**.

Grade 4 Life-threatening consequences; urgent intervention indicated.

Grade 5 Death: related to AE.

A Semi-colon indicates 'or' within the description of the grade. A single dash (-) indicates a grade is not available. Not all Grades are appropriate for all AEs. Therefore, some AEs are listed with fewer than five options for Grade selection. Grade 5 (Death) is not appropriate for some AEs and therefore is not an option.

Activities of Daily Living (ADL)
*Instrumental ADL refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.
**Self care ADL refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.
Critical and common adverse event were graded as below criteria:

<table>
<tr><td colspan="7">Common Terminology Criteria for Adverse Events (CTCAE) v.4.0</td></tr>
<tr><td rowspan="2">Sl no.</td><td rowspan="2">Adverse Event</td><td colspan="5">Grade</td></tr>
<tr><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td></tr>
<tr><td rowspan="2">1.</td><td>Hot flashes</td><td>Mild symptoms; intervention not indicated</td><td>Moderate symptoms; limiting instrumental ADL</td><td>Severe symptoms; limiting self care ADL</td><td>-</td><td>-</td></tr>
<tr><td colspan="6">Definition: A disorder characterized by an uncomfortable and temporary sensation of intense body warmth, flushing, sometimes accompanied by sweating upon cooling.</td></tr>
<tr><td>2.</td><td>Depression</td><td>Mild</td><td>Moderate</td><td>Severe</td><td>Life-</td><td>Death</td></tr>
</table>

		depressive symptoms	depressive symptoms; limiting instrumental ADL	depressive symptoms; limiting self care ADL; hospitalization not indicated	threatening consequences, threats of harm to self or others; hospitalization indicated	
	Definition: A disorder characterized by melancholic feelings of grief or unhappiness.					
3.	Libido decreased	Decrease in sexual interest not adversely affecting relationship	Decrease in sexual interest adversely affecting relationship	-	-	-
	Definition: A disorder characterized by a decrease in sexual desire.					
4.	Vaginal dryness	Mild vaginal dryness not interfering with sexual function	Moderate vaginal dryness interfering with sexual function or causing frequent discomfort	Severe vaginal dryness resulting in dyspareunia or severe discomfort	-	-
	Definition: A disorder characterized by an uncomfortable feeling of itching and burning in the vagina.					

2.17 Performance status evaluation method:

To conduct clinical trials for the treatment of cancer in a consistent manner across many participating hospitals, cancer centers, and clinics requires the use of standard criteria for measuring how the disease impacts a patient's daily living abilities (known to physicians and researchers as a patient's performance status). The ECOG Scale of Performance Status is one such measurement. It describes a patient's level of functioning in terms of their ability to care for themself, daily activity, and physical ability (walking, working, etc.).

Researchers worldwide take the ECOG Performance Status into consideration when planning trials to study a new treatment method. This numbering scale is one way to define the population of patients to be studied in the trial, so that it can be uniformly reproduced among physicians who enroll patients. It is also a way for physicians to track changes in a patient's level of functioning as a result of treatment during the trial.

The scale was developed by the Eastern Cooperative Oncology Group (ECOG), now part of the ECOG-ACRIN Cancer Research Group, and published in 1982. It circulates in the public domain and is therefore available for public use. It is displayed below both for future reference and to spur further standardization among researchers who design and evaluate cancer clinical research.

ECOG Performance Status

Developed by the Eastern Cooperative Oncology Group, Robert L. Comis, MD, Group Chair.*

GRADE	ECOG PERFORMANCE STATUS
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all self care but unable to carry out any work activities; up and about more than 50% of waking hours
3	Capable of only limited self care; confined to bed or chair more than 50% of waking

	hours
4	Completely disabled; cannot carry on any self care; totally confined to bed or chair
5	Dead

2.18 Response evaluation method:

We evaluated response of drug in accordance with Response Evaluation Criteria in Solid Tumor (RECIST).This method is internationally recognized and followed method. We categorized our patients in different level of this method considering patient's different histopathological data and diagnostic test report. We took the help of expert physicians, oncologist, pathologist and laboratory expert of respective hospital to categorize patients. The method is described in brief below:

Response Evaluation Criteria in Solid Tumors (RECIST)

(Source: https://www.cibmtr.org/datamanagement/trainingreference/manuals/datam)

The Response Evaluation Criteria in Solid Tumors (RECIST) were published in February 2000 by the European Organization for Research and Treatment of Cancer (EORTC), the National Cancer Institute of the United States, and the National Cancer Institute of Canada Clinical Trials Group. RECIST criteria are used to evaluate a patient's response to the therapy used to treat their disease. The content of this appendix has been modified to fit the needs of the CIBMTR data collection forms. For the complete text and more detailed information regarding confirmation of response, methods of measurement, and use of RECIST in clinical trials, see
http://ctep.cancer.gov/protocolDevelopment/docs/quickrcst.doc
and
http://ctep.cancer.gov/protocolDevelopment/docs/therasserecistjnci.pdf.

Baseline documentation of Target and Non-Target lesions:

•All measurable lesions up to a maximum of five lesions per organ and 10 lesions in total that are representative of all involved organs should be identified as target lesions recorded, and measured at baseline. Target lesions should be selected on the basis of their size (lesions with the longest diameter) and their suitability for accurate repeated measurements (either by imaging techniques or clinically). A sum of the longest diameter (LD) for all target lesions will be calculated and reported as the baseline sum LD. The baseline sum LD will be used as the reference by which to characterize the objective tumor.

•All other lesions (or sites of disease) should be identified as non-target lesions and should also be recorded at baseline. Measurements of these lesions are not required, but the presence or absence of each should be noted throughout follow-up.

Response Criteria for Target and Non-Target lesions:

Evaluation of target lesions

Complete Response (CR)	Disappearance of all target lesions for a period of at least one month
Complete Response Unknown (CRU)	Complete response with persistent imaging abnormalities of unknown significance
Partial Response (PR)	At least a 30% decrease in the sum of the longest diameter of measures lesions (target lesions), taking as reference the baseline sum of the longest diameter
Stable Disease (NR/SD)	Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum of the longest diameter since the treatment started
Progressive Disease (PD)	A 20% or greater increase in the sum of the longest diameter of measured lesions (target lesions), taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions

Evaluation of non-target lesions

Complete Response (CR)	Disappearance of all non-target lesions and normalization of tumor marker level
Incomplete Response/ Stable Disease (SD)	Persistence of one or more non-target lesion(s) or/and maintenance of tumor marker level above the normal limits
Progressive Disease (PD)	Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions(1)

(1) Although a clear progression of “non target” lesions only is exceptional, in such circumstances, the opinion of the treating physician should prevail and the progression status should be confirmed later on by the review panel (or study chair).

Evaluation of best overall response:

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for PD the smallest measurements recorded since the treatment started). In general, the recipient’s best response assignment will depend on the achievement of both measurement and confirmation criteria.

Target lesions	Non-Target Lesions	New Lesions	Overall response
CR	CR	No	CR
CR	Incomplete response/SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

Source: **http://ctep.cancer.gov/protocolDevelopment/docs/quickrcst.doc**

2.19 Statistical Analysis

The statistical significance analysis of differences in genotype frequencies between patients with different survival outcomes and toxicities were determined by the Chi-square test. Multivariate logistic regression was applied for all analysis variables to evaluate response, toxicity, and survival as adjusted odds ratios (ORs) with 95 % confidence intervals (95 % CIs). All statistical analyses were done applying the SPSS software, version 23.0 (SPSS, Chicago, IL, USA).Odd Ratio was adjusted considering age, body mass index(BMI) data. P value<0.05 was considered as significance for all data.Uncorrected Odd ratio was calculated following Altman, 1991 method.

The odds ratio is given by

$$OR = \frac{a/b}{c/d} = \frac{a \times d}{b \times c}$$

Where, a=no. in exposed group of bad outcome

b=no. in exposed group of good outcome

c=no. in control group of bad outcome

d=no. in control group of good outcome

and 95% confidence interval

$$95\%\ \text{CI} = \exp\left(\ \ln(OR) - 1.96 \times \text{SE}\{\ln(OR)\}\ \right) \quad \text{to} \quad \exp\left(\ \ln(OR) + 1.96 \times \text{SE}\{\ln(OR)\}\ \right)$$

Where, OR=Odd Ratio and SE=Standard Error

Where zeros cause problems with computation of the odds ratio or its standard error, 0.5 is added to all cells (a, b, c, d) (Pagano & Gauvreau, 2000; Deeks & Higgins, 2010).

CHAPTER THREE

RESULTS and DISCUSSIONS

3.1. Genomic DNA Extraction

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., 1995).

The purity of the DNA and their concentrations were measured by SHIMADZU UV-Spectrophotometer at 260 nm. The purity (OD 260/OD 280) of the entire DNA was found to be in the range between 1.7to 1.9 and the average concentration was found to be 50 to 70 µgm/ml of whole blood. Agarose gel electrophoresis was done for all the DNA samples.

D1 D2 D3 D4 D5 D6 D7 D8

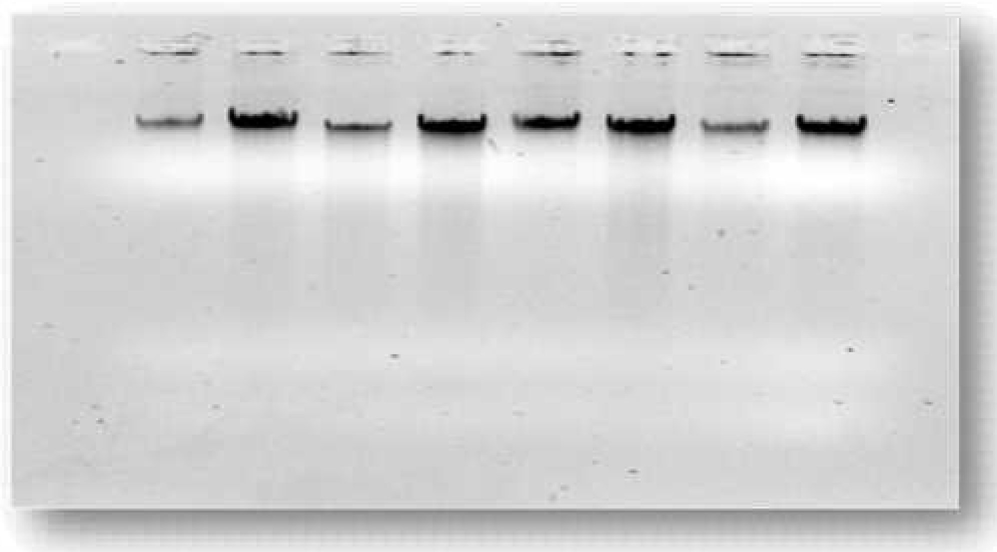


Figure 3.1: Agarose gel electrophoresis (1% w/v agarose) of genomic DNA (DNA No. 1 to 8)

3.2. Genotyping of genes:

The allelic distribution of selected genes in Bangladeshi population was done by using a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method.The genomic target regions, which contain the SNPs of interest, were amplified by means of primer-directed PCR using DNA polymerase originally described by 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.

3.2.1 PCR-RFLP OF SULT1A1*2 (rs9282861)

The size of PCR product is 281 bp. The PCR product was visualized in 2% (w/v) agarose. Later on, after cutting the PCR product, two sequences will be obtained containing two fragments of 104 and 177 bp.

GGTTGAGGAGTTGGCTCTGCAGGGTTTCTAGGAGAAGTGGCCAGATC GCCTCTGAGGTTAGAGAAGGGGACCCCTTTTACTTTTCCTGAATCAGT AATCCGAGCCTCCACTGAGGGGCCCTCTGCTGCTCAGAACCCGAAAA GGGAGATTCAAAAGATCCTGGAGTTTGTGGGGCRGCGCCTGCCAGAG GAGACCGTGGACTTCGTGGTTCAGCACACGTCGTTCAAGGAGATGAA GAAGAACCCTATGACCAACTACACCACCGTCCCCCAGGAGTTCAT(Fragment=281)

RED---------------> **PRIMER SEQUENCE** **YELLOW** --------------> **SNP OF INTERES**

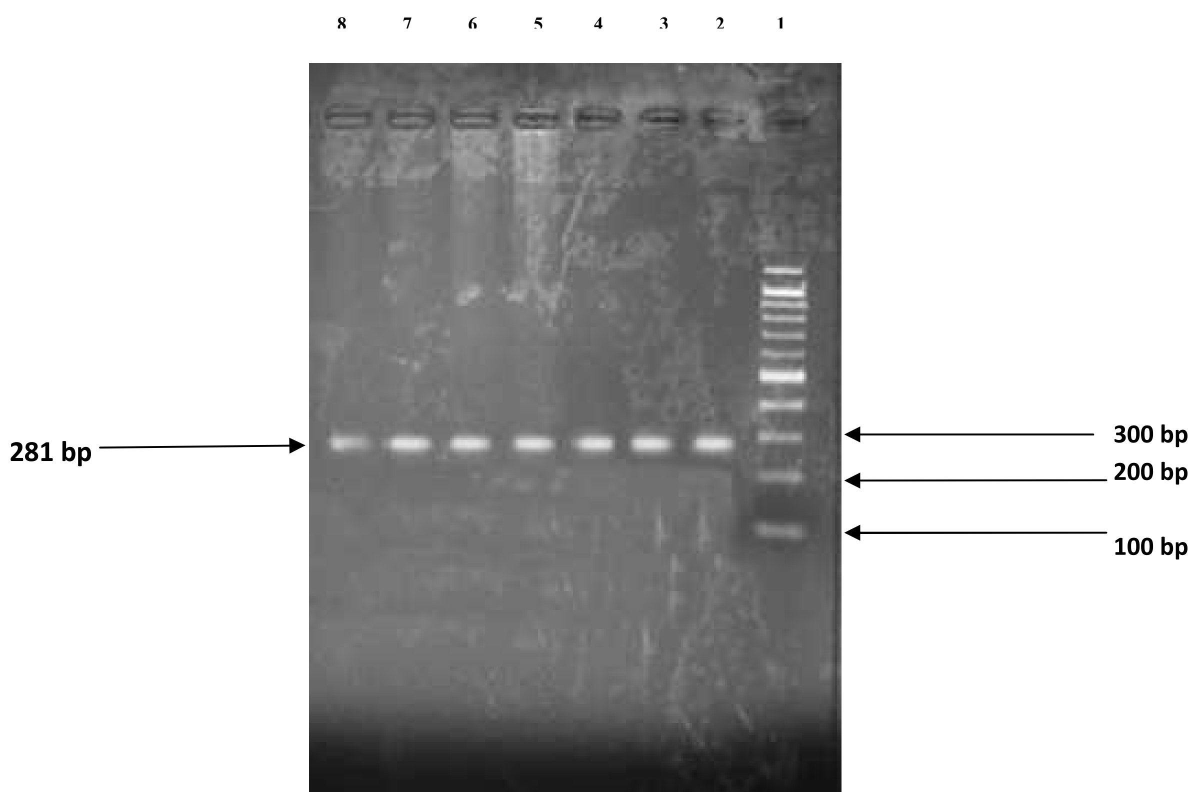


Figure 3.2: PCR product of SULT1A1*2 (281bp) (Lane 2 to 8) (2% agarose gel) (Lane-1 contains Molecular ruler).

Fragmentation Pattern:

The PCR product was digested with *HaeII*. The fragments were visualized in agarose gel (2%) as well as on polyacrylamide gel (10%).After cutting the PCR product of 281 bp, two sequences were obtained containing two fragments of 104 and 177 bp.

Table 3.1: Name of the restriction enzyme with its sites of digestion

Restriction enzyme	Sites of digestion
HaeII	5'...RGCGC▼Y...3' 3'...Y▲CGCGR...5'

GGTTGAGGAGTTGGCTCTGCAGGGTTTCTAGGAGAAGTGGCCAGATCGCCTCT
GAGGTTAGAGAAGGGGACCCCTTTTACTTTTCCTGAATCAGTAATCCGAG
CCTCCACTGAGGGGCCCTCTGCTGCTCAGAACCCGAAAAGGGAGATTCA
AAAGATCCTGGAGTTTGTGGGGCRGCGCCTGCCAGAGGAGACCGTGGAC
TTCGTGGTTCAGCACACGTCGTTCAAGGAGATGAAGAAGAACCCCTATGA
CCAACTACACCACCGTCCCCCAGGAGTTCAT(Fragment=281bp)

Blue HaeII recognition site

HaeII Cutting site

5'...RGCGCY...3'
3'...YCGCGR...5'

GGTTGAGGAGTTGGCTCTGCAGGGTTTCTAGGAGAAGTGGCCAGATCGCCTCT
GAGGTTAGAGAAGGGGACCCCTTTTACTTTTCCTGAATCAGTAATCCGAG
CCTCCACTGAGGGGCCCTCTGCTGCTCAGAACCCGAAAAGGGAGATTCA
AAAGATCCTGGAGTTTGTGGGGCRGCGC
(Fragment=177bp)

and

CTGCCAGAGGAGACCGTGGACTTCGTGGTTCAGCACACGTCGTTCAAGGAGA
TGAAGAAGAACCCTATGACCAACTACACCACCGTCCCCCAGGAGTTCAT(
Fragment=104bp)

Table 3.2: Type of nucleotide changes, cutting sites and fragments of the allele in case of arginine/arginine, arginine/proline, proline/proline.

Changes	Fragments	Type	Digestion conditions
When R=G in both chromosome (G/G)	281	Arginine/Arginine	
When R=A in one chromosome (G/A)	281,177,104	Arginine/Histidine	Incubation at 37° C overnight with HaeII restriction enzyme
When R=A in both chromosome (A/A)	177,104	Histidine/Histidine	

- **When G is present in both of the sister chromosomes:** There is no cutting site for both the chromosomes. So there will be one fragment for each chromosome.
- **When A in one of the sister chromosome:** There is ONE cutting site for the polymorphic chromosome (R=A), but for the other chromosome (R=G) there will be NO cutting site. So there will be three fragments for the two sister chromosomes.
- **When A in both of the sister chromosomes:** There will be cutting site for both the chromosomes. So there will be TWO fragments for each chromosome.

Observed Results:

Restriction enzyme digestion products were visualized in agarose gel (2%).

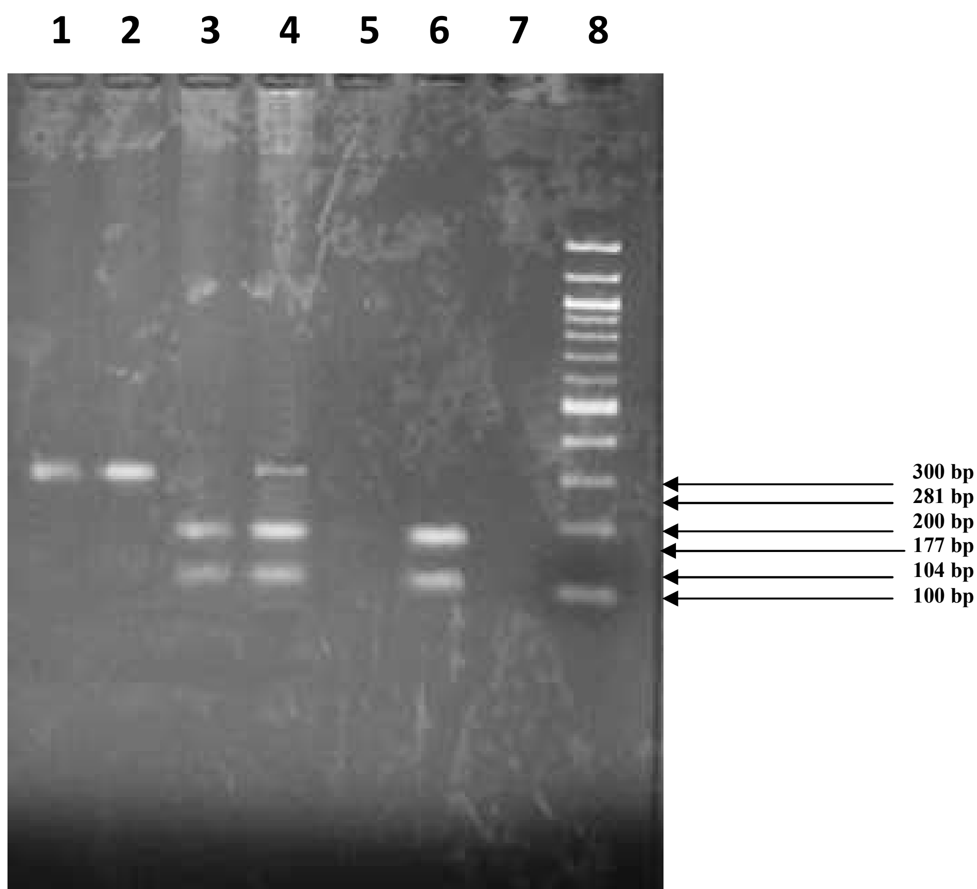


Figure 3.3: Restriction Endonuclease (*HaeII*) digestion fragment of SULT1A1*2 (Lane 1 to 7) (2% agarose gel)

(Lane-8 contains Molecular ruler)

3.2.2 PCR-RFLP OF UGT2B7*2 (rs7439366)

GACAATGGGGAAAGCTGACGTATGGCTTATTCGAAACTCCTGGAATTTTC
AGTTTCCATATCCACTCTTACCAAATGTTGATTTTGTTGGAGGACTCC
ACTGCAAACCTGCCAAAC(Fragment=116)

DARK RED---------------> **PRIMER SEQUENCE**

BLUE------------------> **POLYMORPHISM RECOGNITION SITE**

By using the appropriate pair of primers and other PCR reaction program parameters the PCR product of UGT2B7*2 was obtained. The PCR product size was 116 bp.

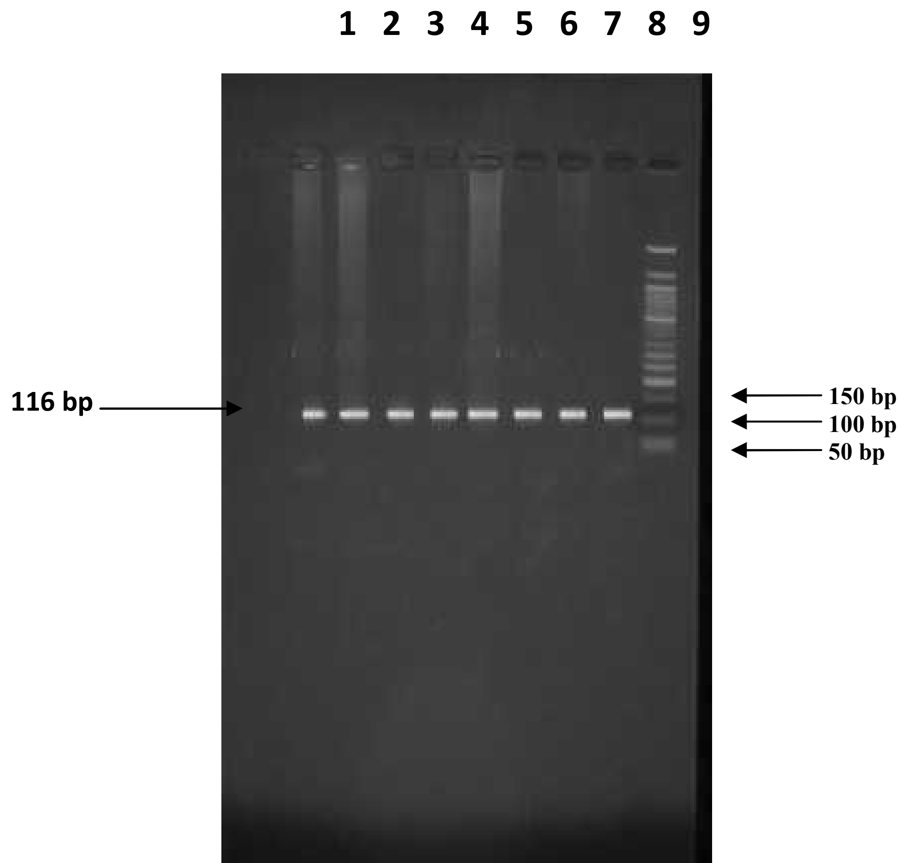


Figure 3.4: PCR product of UGT2B7*2 (116 bp) (Lane 1 to 8) (2% w/v agarose gel) (Lane-9 contains Molecular ruler).

Fragmentation Pattern:

The PCR product was digested with *Fok*I. The fragments were observed in agarose gel (2%).

Table 3.3: Name of the restriction enzyme with its sites of digestion for UGT2B7*2

Restriction enzyme	Sites of digestion
***Fok*I**	5'...GGATG(N)$_9$▼...3' 3'...CCTAC(N)$_{13}$▲...5'

GACAATGGGGAAAGCTGACGTATGGCTTATTCGAAACTCCTGGAATTTTCAGT
TTCCATATCCACTCTTACCAAATGTTGATTTTGTTGGAGGACTCCACTGC
AAACCTGCCAAAC

(Fragment=116)

T → Site of polymorphism

Blue → FoKI recognition site

FokI Cutting site

5'...GGATG(N)$_9$▼...3'
3'...CCTAC(N)$_{13}$▲...5'

GACAATGGGGAAAGCTGACGTATGGCTTATTCGAAACTCCTGGAA

(Fragment=45bp)

and

TTTTCAGTTTCCATATCCACTCTTACCAAATGTTGATTTTGTTGGAGGACTCCA
CTGCAAACCTGCCAAAC

(Fragment=71bp)

Table 3.4: Type of nucleotide changes, cutting sites and fragments of the allele in case of normal homozygote, heterozygote, and mutant homozygote.

Changes	Fragments	Type	Digestion conditions
When T=T in both chromosome (T/T)	116	Valine/Valine	Incubation at 37° C overnight with FokI restriction enzyme
When T=C in one chromosome (T/C)	45,71,116	Valine/Proline	
When T=C in both chromosome (C/C)	45,71	Proline/Proline	

- **When T is present in both of the sister chromosomes:** There is no cutting site for both the chromosomes. So there will be one fragment for each chromosome.
- **When C in one of the sister chromosome:** There is ONE cutting site for the polymorphic chromosome (T=T), but for the other chromosome (T=C) there will be NO cutting site. So there will be three fragments for the two sister chromosomes.
- **When C in both of the sister chromosomes:** There will be cutting site for both the chromosomes. So there will be TWO fragments for each chromosome.

Observed Results:

Restriction enzyme digestion products were visualized in agarose gel (2%).

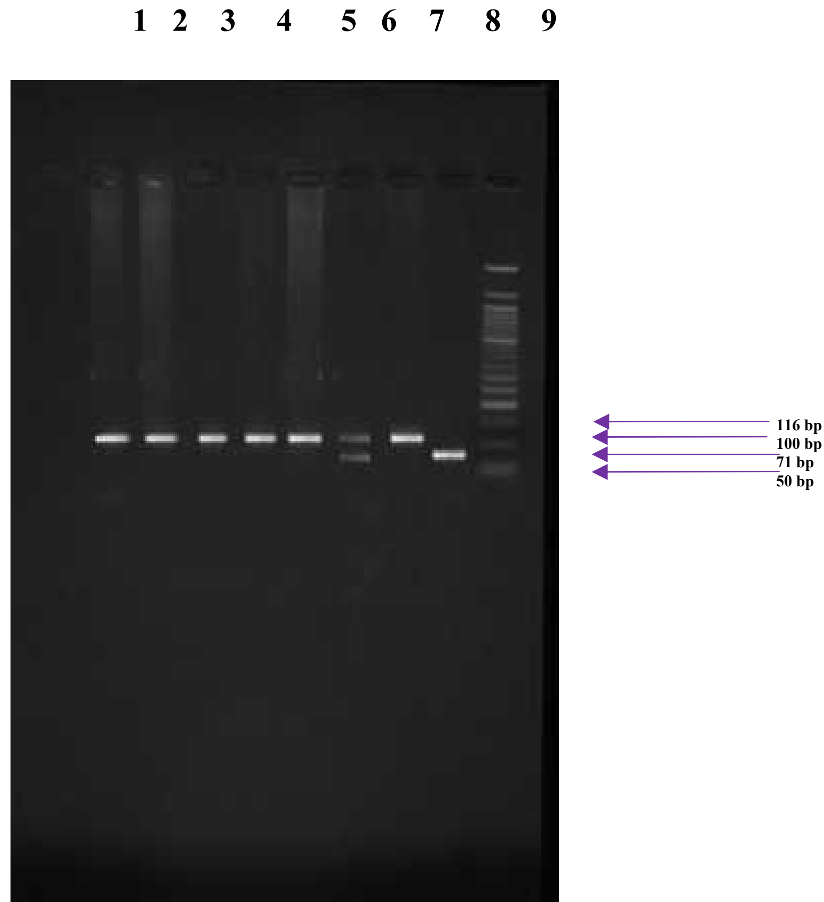


Figure 3.5: Restriction Endonuclease (*Fok*I) digestion fragments of UGT2B7*2 (Lane 1 to 8) (2% Agarose gel) (Lane-9 contains Molecular ruler)

3.2.3 PCR-RFLP OF CYP3A5*3 (rs776746)

TGTTGCATGCATAGAGGAAGGATCGAGCTTTGCCYCTTTGTACYTCTTCAT
CTTCTCCCCTCAAGTCYTCAGAATCCACAGCRCTGACTGTGGAGYGCTGTG
GAGCTGGCATGGCCCATACAGGCAACATGACTTAGYAGACAGATGACAC
AGCTCTAGATGTCCATGGGCCCCACRCCAACTGCCCTTGCAGCATTTAGTC
CTTGTGAGCAYYTGATGATCTACCTGCCTTCAATTTTTCACTGACCTAATA
TTCTTTTTGATAATGAAGTATTTTAAACATAKAAAACATTATGGAGAGTGG
CATAGGAGAKACCCAYGTATGTACCACCCAGCTTAACGAATGCTCTACTG
TCATTTCTAACCATAATCTCTTTAAAGAGMTCTTTBGTCTTTCARYATCTCT

TCCCTGTTTGGACCACATTACCCTTCATCRTATGAAGCCTTTACTGTCCCA AACACTGTCCC(Fragment=468)

DARK RED--------------> **PRIMER SEQUENCE**

BLUE------------------> **POLYMORPHISM RECOGNITION SITE**

By using the appropriate pair of primers and other PCR reaction program parameters the PCR product of CYP3A5*3 was obtained. The PCR product size was 468 bp.

1 2 3 4 5 6 7 8 9

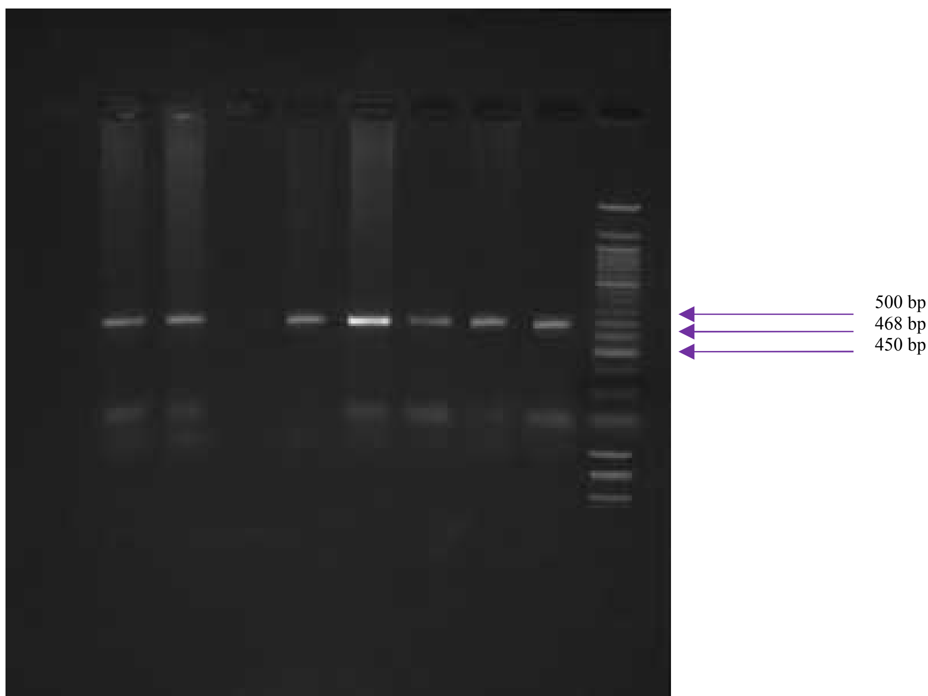


Figure 3.6: PCR product of CYP3A5*3 (468 bp) (Lane 1 to 8) (2% w/v agarose gel) (Lane-9 contains Molecular ruler).

Fragmentation Pattern:

The PCR product was digested with *MboI*. The fragments were observed in agarose gel (2%).

Table 3.5: Name of the restriction enzyme with its sites of digestion for CYP3A5*3

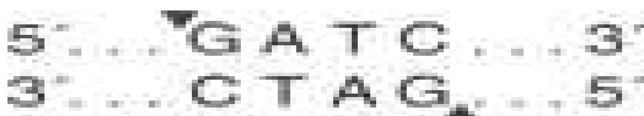
Restriction enzyme	Sites of digestion
*Mbo*I	5'...GATC...3' 3'...CTAG...5'

TGTTGCATGCATAGAGGAAGGATCGAGCTTTGCCYCTTTGTACYTCTT
CATCTTCTCCCCTCAAGTCYTCAGAATCCACAGCRCTGACTGTGGAGY
GCTGTGGAGCTGGCATGGCCCATACAGGCAACATGACTTAGYAGACA
GATGACACAGCTCTAGATGTCCATGGGCCCCACRCCAACTGCCCTTG
CAGCATTTAGTCCTTGTGAGCAYYTGATGATCTACCTGCCTTCAATTT
TTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTTAAACATAKAA
AACATTATGGAGAGTGGCATAGGAGAKACCCAYGTATGTACCACCCA
GCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAAAGA
GMTCTTTBGTCTTTCARYATCTCTTCCCTGTTTGGACCACATTACCCT
TCATCRTATGAAGCCTTTACTGTCCCAAACACTGTCCC(Fragment=468)

R ⟶ **Site of polymorphism**

Green ⟶ **MboI recognition site**

MboI Cutting site



TGTTGCATGCATAGAGGAAGGATCGAGCTTTGCCYCTTTGTACYTCTT
CATCTTCTCCCCTCAAGTCYTCAGAATCCACAGCRCTGACTGTGGAGY
GCTGTGGAGCTGGCATGGCCCATACAGGCAACATGACTTAGYAGACA
GATGACACAGCTCTAGATGTCCATGGGCCCCACRCCAACTGCCCTTG
CAGCATTTAGTCCTTGTGAGCAYYTGAT(Fragment=218bp)

and

GATCTACCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAAT GAAGTATTTTAAACATAKAAAACATTATGGAGAGTGGCATAGGAGAK ACCCAYGTATGTACCACCCAGCTTAACGAATGCTCTACTGTCATTTCT AACCATAATCTCTTTAAAGAGMTCTTTBGTCTTTCARYATCTCTTCCC TGTTTGGACCACATTACCCTTCATCRTATGAAGCCTTTACTGTCCCAA ACACTGTCCC(Fragment=250bp)

Table 3.6: Type of nucleotide changes, cutting sites and fragments of the allele in case of normal homozygote, heterozygote, and mutant homozygote.

Changes	Fragments	Type	Digestion conditions
When R=A in both chromosome (A/A)	468	Proline/Proline	
When R=G in one chromosome (A/G)	218,250,468	Proline/Threonine	Incubation at 37° C overnight with MboI restriction enzyme
When R=G in both chromosome (G/G)	218,250	Threonine/Threonine	

- **When A is present in both of the sister chromosomes:** There is no cutting site for both the chromosomes. So there will be one fragment for each chromosome.
- **When G in one of the sister chromosome:** There is ONE cutting site for the polymorphic chromosome (R=A), but for the other chromosome (R=G) there will be NO cutting site. So there will be three fragments for the two sister chromosomes.

- **When G in both of the sister chromosomes:** There will be cutting site for both the chromosomes. So there will be TWO fragments for each chromosome.

Observed Results:

Restriction enzyme digestion products were visualized in agarose (2%).

1 2 3 4 5 6 7 8 9 10 11

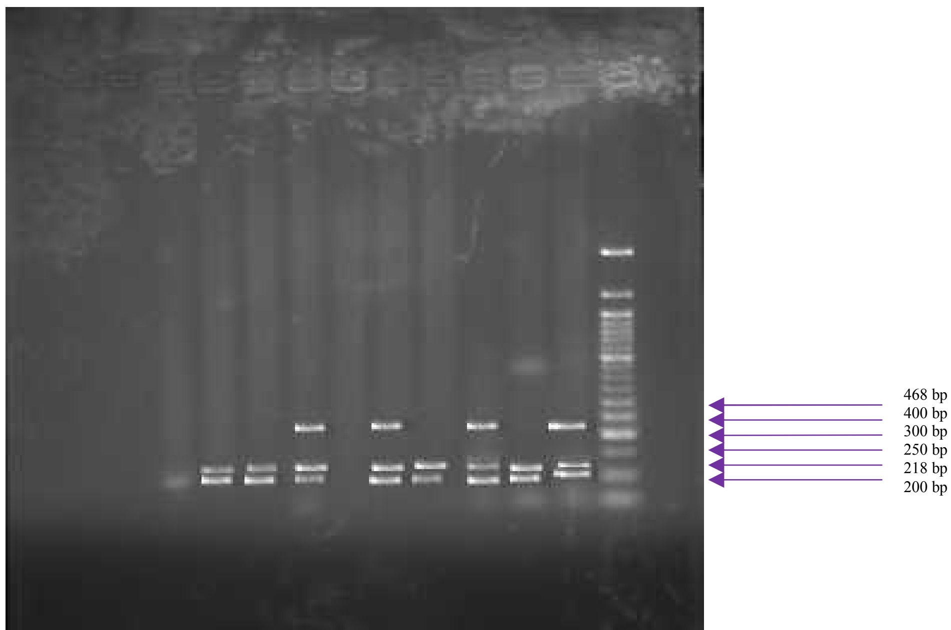


Figure 3.7: Restriction Endonuclease (*MboI*) digestion fragment of CYP3A5*3 (Lane 1 to 10) (2% agarose gel)
(Lane-11 contains Molecular ruler)

3.2.4 PCR-RFLP OF CYP2D6*4 (rs3892097)

GCCTTCGCCAACCACTCCGYTKGAGSRGTGRGTRACBRMGGMSGCYRCCT
GCCTBTKYGCYRCYTTYRYYRACCAHKMYDGTGSSTRATGGRYAGA
AGRDCACAMARCRGGRACKGGRAARGYRRGGGAYRGRGAMGKYRA
CCBYTTACCYBCATCTYYCACCCCYGGGAYGCCCYTTTYRCCCCAAY
VGYYTCTKRRAYAAAGCCRTGAGCRAYSTGATYGCCYCCYTCRMCTR
YVGRYGMCRBTTSRARTAYRAYKMYSCTVVCTTCYTYAGRCTRCTGR
ACYYAGCTCAGGAGRRAYYGRARRARRMSYHGSGCTYYCTGYGYRA
GRTRYRGAGCRAGASAMHVARGRRTCTYTGYMGGRYRRGCTCYHRV
GARGKKCCGGGGCTGGAYTGGGGCCTCGAAGAGCAGGATTT(Fragment=414)

Red ⟶ Primer sequence   Yellow ⟶ SNP of interest

By using the appropriate pair of primers and other PCR reaction program parameters the PCR product of CYP2D6*4 was obtained. The PCR product size was 414 bp.

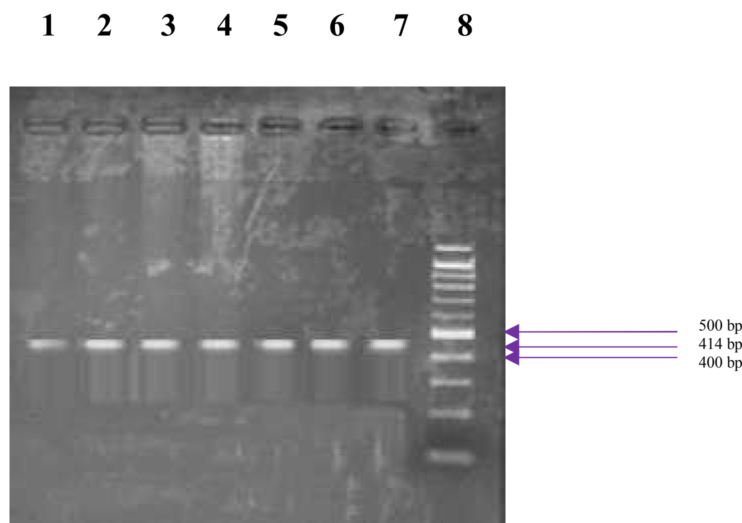


Figure 3.8: PCR product of CYP3A5*3 (468 bp) (Lane 1 to 7) (2% w/v agarose gel) (Lane-8 contains Molecular ruler).

Fragmentation Pattern:

The PCR product was digested with *MboI*. The fragments were observed in agarose gel (2%).

Table 3.7: Name of the restriction enzyme with its sites of digestion for CYP2D6*4

Restriction enzyme	Sites of digestion
*Mva*I	5'...CC^WGG...3' 3'...GGW^CC...5'

GCCTTCGCCAACCACTCCGYTGAGSRGTGRGTRACBRMGGMSGC
YRCCTGCCTBTKYGCRCYTTYRYYRACCAHKMYDGTGSSTRATGG
RYAGAAGRDCACAMARCRGGRACKGGRAARGYRRGGGAYRGRG
AMGKYRACCBYTTACCYBCATCTYYCACCCCWGGGAYGCCCYTT
TYRCCCCAAYVGYYTCTKRRAYAAAGCCRTGAGCRAYSTGATYGC
CYCCYTCRMCTRYVGRYGMCRBTTSRARTAYRAYKMYSCTVVCTT
CYTYAGRCTRCTGRACYYAGCTCAGGAGRRAYYGRARRARRMSY
HGSGCTYYCTGYGYRAGRTRYRGAGCRAGASAMHVARGRRTCTY
TGYMGGRYRRGCTCYHRVGARGKKCCGGGGCTGGAYTGGGGCC
TCGAAGAGCAGGATTT(Fragment=414)

R ⟶ Site of polymorphism

Green ⟶ MvaI recognition site

MvaI Cutting site

5'...CC^WGG...3'
3'...GGW^CC...5'

GCCTTCGCCAACCACTCCGYTGAGSRGTGRGTRACBRMGGMSGCYRC
CTGCCTBTKYGCRCYTTYRYYRACCAHKMYDGTGSSTRATGGRYA
GAAGRDCACAMARCRGGRACKGGRAARGYRRGGGAYRGRGAMG
KYRACCBYTTACCYBCATCTYYCACCCC(Fragment=163bp)

And

WGGGAYGCCCYTTTYRCCCCAAYVGYYTCTKRRAYAAAGCCRTG
AGCRAYSTGATYGCCYCCYTCRMCTRYVGRYGMCRBTTSRARTAY
RAYKMYSCTVVCTTCYTYAGRCTRCTGRACYYAGCTCAGGAGRR
AYYGRARRARRMSYHGSGCTYYCTGYGYRAGRTRYRGAGCRAGA
SAMHVARGRRTCTYTGYMGGRYRRGCTCYHRVGARGKKCCGGG
GGCTGGAYTGGGGCCTCGAAGAGCAGGATTT(Fragment=251bp)

Table 3.8: Type of nucleotide changes, cutting sites and fragments of the allele in case of normal homozygote, heterozygote, and mutant homozygote.

Changes	Fragments	Type	Digestion conditions
When R=G in both chromosome (G/G)	414	Arginine/Arginine	
When R=A in one chromosome (G/A)	163,251,414	Arginine/Histidine	Incubation at 37° C overnight with MvaI restriction enzyme
When R=A in both chromosome (A/A)	163,251	Histidine/Histidine	

- **When G is present in both of the sister chromosomes:** There is no cutting site for both the chromosomes. So there will be one fragment for each chromosome.
- **When A in one of the sister chromosome:** There is ONE cutting site for the polymorphic chromosome (R=A), but for the other chromosome (R=G) there will be NO cutting site. So there will be three fragments for the two sister chromosomes.
- **When A in both of the sister chromosomes:** There will be cutting site for both the chromosomes. So there will be TWO fragments for each chromosome.

Observed Results:

Restriction enzyme digestion products were visualized in agarose (2%).

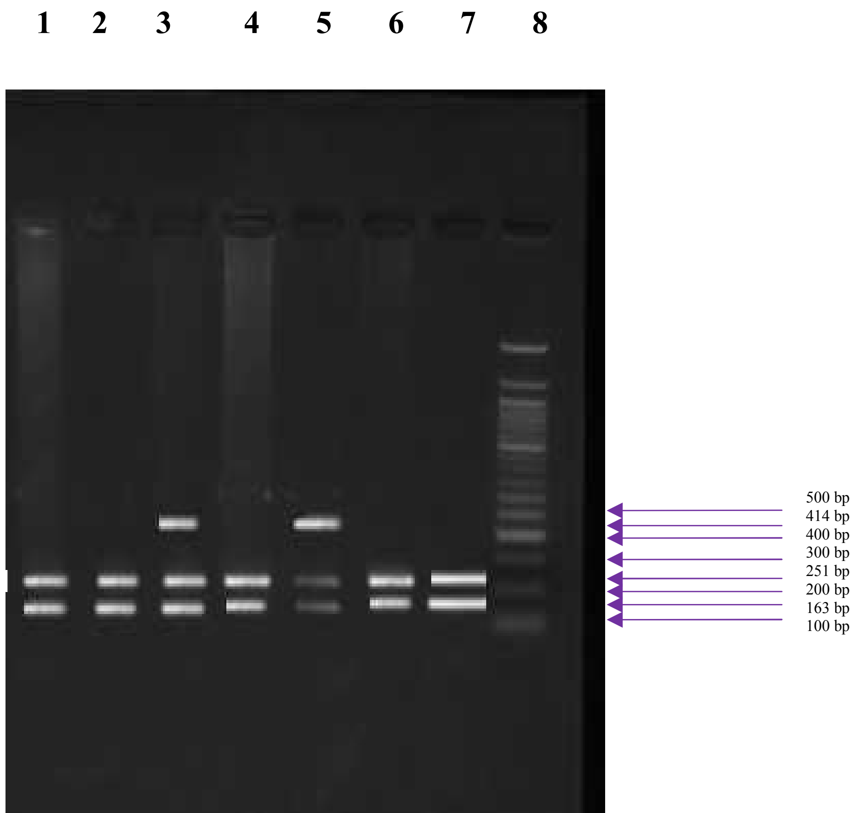


Figure 3.9: Restriction Endonuclease (*MvaI*) digestion fragment of CYP2D6*4 (Lane 1 to 7) (2% agarose gel)

(Lane-8 contains Molecular ruler)

3.2.5 PCR-RFLP OF CYP2D6*10 (rs1065852)

GTGCTGAGAGTGTCCTGCCTGGTCCTCTGTGCCTGGTGGGTGGRGGTGCC
AGGTGTGTCYAGAGGASMCCAKTTKGTAGTGAGGCAKSYATGGGSCTAGA
AGYABTRRTKCCCCTGGCMRTGATAGTRGYYATCTTCCTGCTCCTGGTGG
ACCTGATGCACYRGYRCCAAYDVTGGGCTGMAYGCTAYYMRYCAGGYCC
MCTGCCACWGYYYRRGYKGRGCAAYCTGCTGYATGTGGRYTTYYAGAAC
ACRCCMTACTGCTTCGACYAGGTGRRGGAGGARGTCCTRGAGGGYDGYA
GAGGWBCYGAGGMTNCCCTACCASMAGCAAACATGGATGGTGGGTG(Fragment=344)

Red ⟶ Primer sequence Yellow ⟶ SNP of interest

By using the appropriate pair of primers and other PCR reaction program parameters the PCR product of CYP2D6*10 was obtained. The PCR product size was 344 bp.

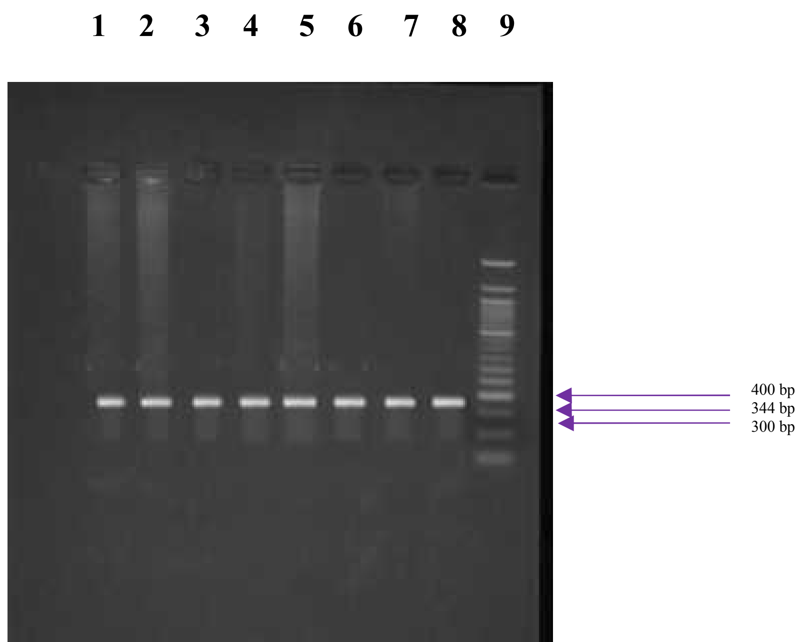


Figure 3.10: PCR product of CYP2D6*10 (344 bp) (Lane 1 to 8) (2% w/v agarose gel) (Lane-9 contains Molecular ruler).

Fragmentation Pattern:

The PCR product was digested with *HphI*. The fragments were observed in agarose gel (2%).

Table 3.9: Name of the restriction enzyme with its sites of digestion for CYP2D6*10

Restriction enzyme	Sites of digestion
*Hph*I	5′... G G T G A (N)$_8$▼... 3′ 3′... C C A C T (N)$_7$▲... 5′

GTGCTGAGAGTGTCCTGCCTGGTCCTCTGTGCCTGGTGGGGTGGRG
GTGCCAGGTGTGTCYAGAGGASMCCAKTTKGTAGTGAGGCAKSYAT
GGGSCTAGAAGYABTRRTKCCCCTGGCMRTGATAGTRGYYATCTTC
CTGCTCCTGGTGAACCTGATGCACYRGYRCCAAYDVTGGGCTGMAY
GCTAYYMRYCAGGYCCMCTGCCACWGYYYRRGYKGRGCAAYCTGC
TGYATGTGGRYTTYYAGAACACRCCMTACTGCTTCGACYAGGTGRR
GGAGGARGTCCTRGAGGGYDGYAGAGGWBCYGAGGMTNCCCTACC
ASMAGCAAACATGGATGGTGGGTG

(Fragment=344)

Y ⟶ Site of polymorphism

Green ⟶ HphI recognition site

HphI Cutting site

5'... G G T G A (N)8 ... 3'
3'... C C A C T (N)7 ... 5'

GTGCTGAGAGTGTCCTGCCTGGTCCTCTGTGCCTGGTGGGGTGGRG
GTGCCAGGTGTGTCYAGAGGASMCCAKTTKGTAGTGAGGCAKSYAT
GGGSCTAGAAGYABTRRTKCCCCTGGCMRTGATAGTRGYYATCTTC
CTGCTCCTGGTGAACCTGATG(Fragment=159bp)

and

CACYRGYRCCAAYDVTGGGCTGMAYGCTAYYMRYCAGGYCCMCTG
CCACWGYYYRRGYKGRGCAAYCTGCTGYATGTGGRYTTYYAGAACA
CRCCMTACTGCTTCGACYAGGTGRRGGAGGARGTCCTRGAGGGYD
GYAGAGGWBCYGAGGMTNCCCTACCASMAGCAAACATGGATGGTG
GGTG(Fragment=185bp)

Table 3.10: Type of nucleotide changes, cutting sites and fragments of the allele in case of normal homozygote, heterozygote, and mutant homozygote.

Changes	Fragments	Type	Digestion conditions
When Y=C in both chromosome (C/C)	344	Valine/Valine	
When Y=T in one chromosome (C/T)	159,185,344	Valine/Proline	Incubation at 37° C overnight with HphI restriction enzyme
When Y=T in both chromosome (T/T)	159,185	Proline/Proline	

- **When C is present in both of the sister chromosomes:** There is no cutting site for both the chromosomes. So there will be one fragment for each chromosome.
- **When T in one of the sister chromosome:** There is ONE cutting site for the polymorphic chromosome (Y=C), but for the other chromosome (Y=T) there will be NO cutting site. So there will be three fragments for the two sister chromosomes.
- **When T in both of the sister chromosomes:** There will be cutting site for both the chromosomes. So there will be TWO fragments for each chromosome.

Observed Results:

Restriction enzyme digestion products were visualized in agarose (2%).

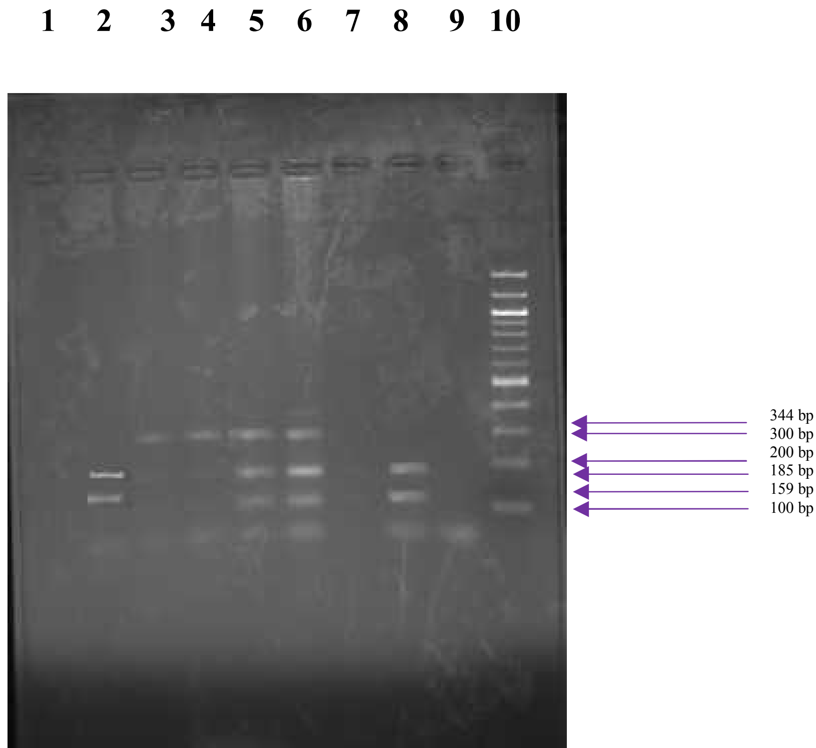


Figure 3.11: Restriction Endonuclease (*HphI*) digestion fragment of CYP2D6*10 (Lane 1 to 9) (2% agarose gel)

(Lane-10 contains Molecular ruler)

3.3 Individual Results:

3.3.1 Allelic frequency

Allelic frequency of different genotypes were distributed as below….

Table 3.11: Allelic frequency of SULT1A1*2

SULT1A1*2					
Types of polymorphism	Polymorphs	No. of patients	% of polymorphs	G allele frequency (%)	A allele frequency (%)
Normal Homozygote(NH)	GG	196	50.52	65.99	34.02
Heterozygote(HE)	GA	120	30.93		
Mutant Homozygote(MH)	AA	72	18.56		

Graph3.1: allelic distribution of SULT1A1*2

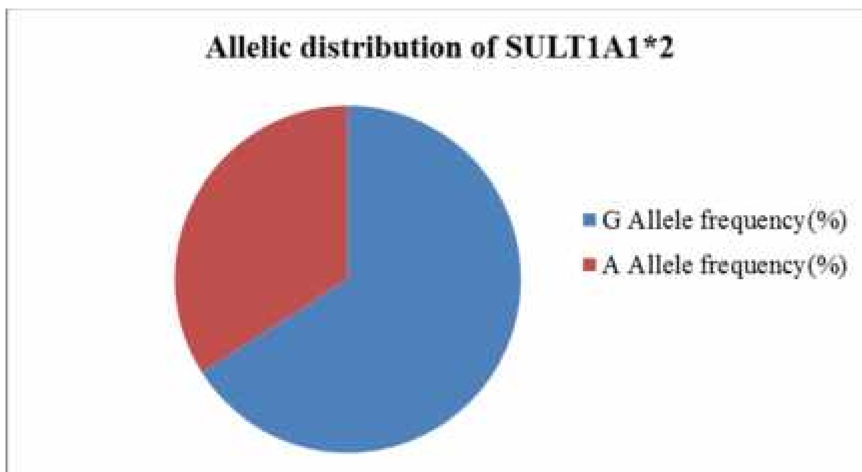


Table3.12: Allelic frequency of UGT2B7*2

UGT2B7*2					
Types of polymorphism	Polymorphs	No. of patients	% of polymorphs	C allele frequency (%)	T allele frequency (%)
Normal Homozygote(NH)	CC	140	36.08	62.24	37.76
Heterozygote(HE)	CT	203	52.32		
Mutant Homozygote(MH)	TT	45	11.60		

Graph3.2: Allelic distribution of UGT2B7*2

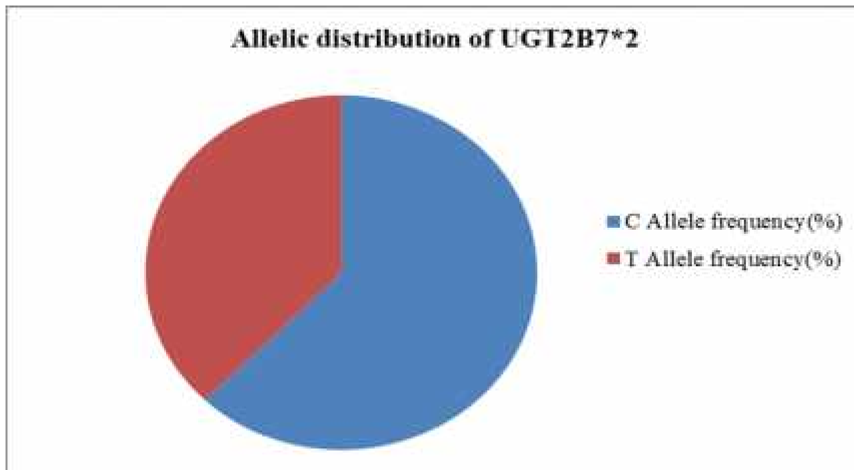


Table3.13: Allelic frequency of CYP3A5*3

CYP3A5*3					
Types of polymorphism	Polymorphs	No. of patients	% of polymorphs	A allele frequency (%)	G allele frequency (%)
Normal Homozygote(NH)	AA	82	21.13	44.07	55.93
Heterozygote(HE)	AG	178	45.88		
Mutant Homozygote(MH)	GG	128	32.99		

Graph3.3: Allelic distribution of CYP3A5*3

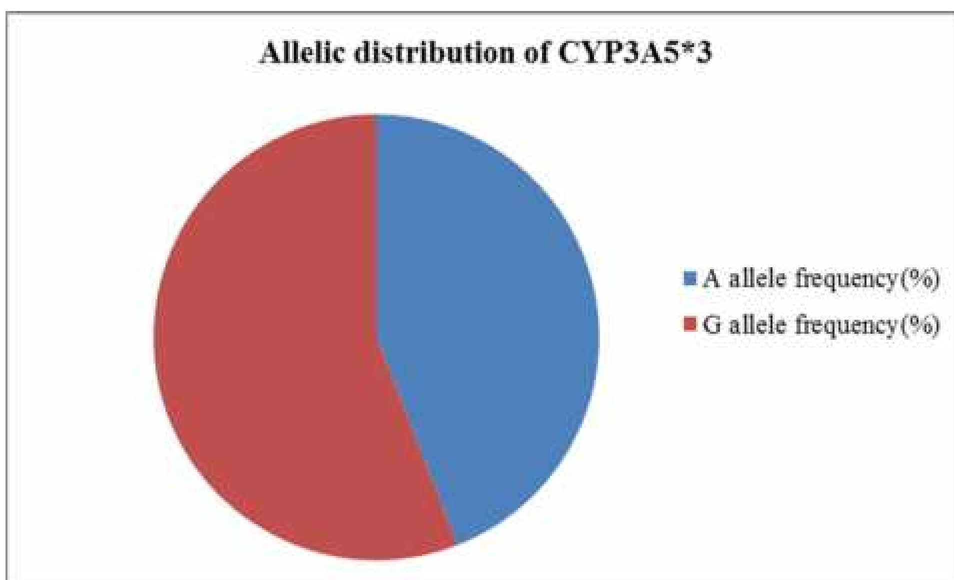


Table3.14: Allelic frequency of CYP2D6*4

CYP2D6*4					
Types of polymorphism	Polymorphs	No. of patients	% of polymorphs	G allele frequency (%)	A allele frequency (%)
Normal Homozygote(NH)	GG	223	57.47	77.58	22.43
Heterozygote(HE)	GA	156	40.21		
Mutant Homozygote(MH)	AA	9	2.32		

Graph3.4: Allelic distribution of CYP2D6*4

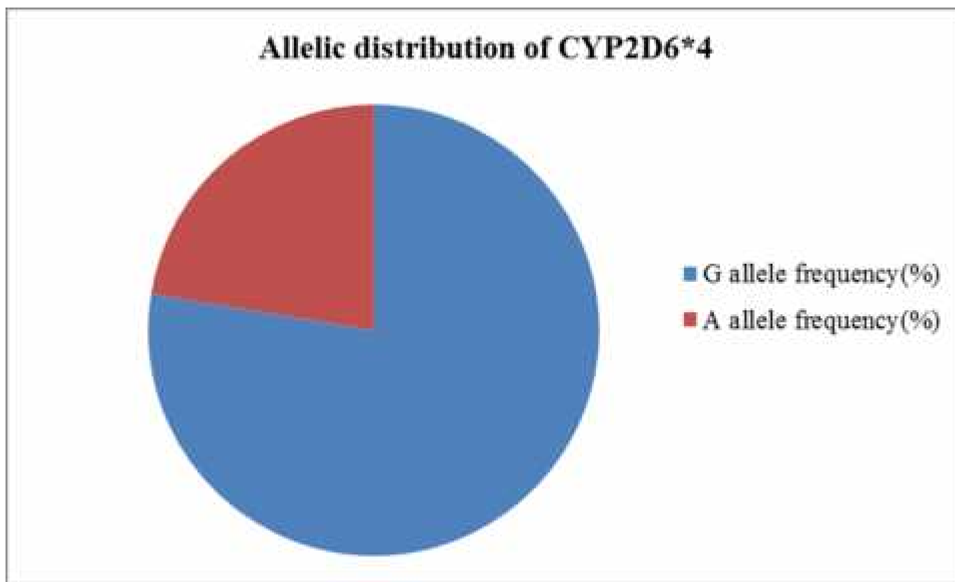
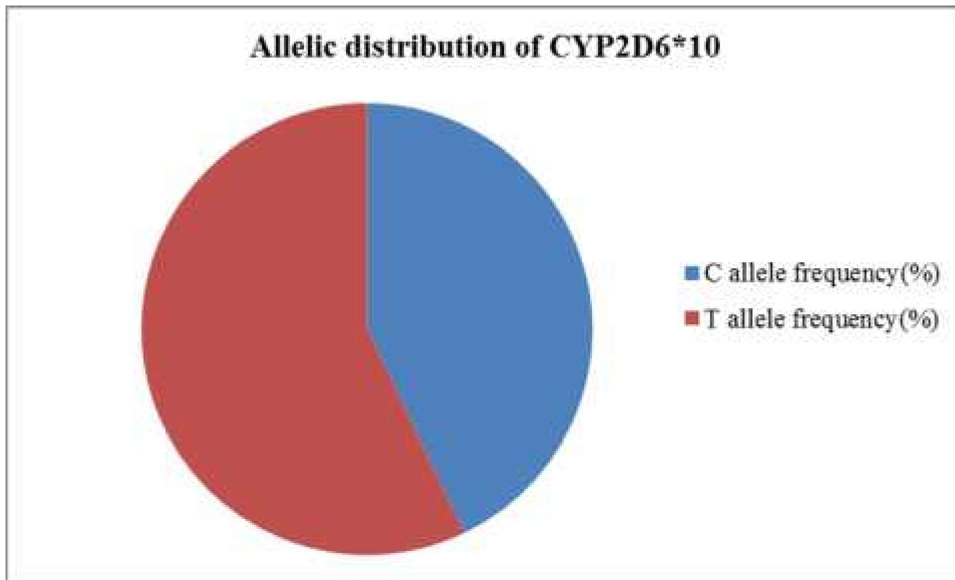


Table3.15: Allelic frequency of CYP2D6*10

CYP2D6*10					
Types of polymorphism	Polymorphs	No. of patients	% of polymorphs	C allele frequency (%)	T allele frequency (%)
Normal Homozygote(NH)	CC	121	31.19	42.92	57.09
Heterozygote(HE)	CT	91	23.45		
Mutant Homozygote(MH)	TT	176	45.36		

Graph3.5: Allelic distribution of CYP2D6*10



From the allelic frequency distribution, it is clear that G Allele for SULT1A1*2,C Allele for UGT2B7*2,G Allele for CYP3A5*3,G Allele for CYP2D6*4 and T allele for CYP2D6*10 is very prominent among Bangladeshi breast cancer population. Among polymorphism, Normal Homozygoate(GG) for SULT1A1*2(50.52%), Heterozygote(CC) for UGT2B7*2(52.32%),Heterozygote(AG) for CYP3A5*3(45.88%), Normal Homozygoate(GG) for CYP2D6*4(57.47%), Mutant Homozygoate(TT) for CYP2D6*10(45.36%) are prevalent type polymorphism.

3.3.2 Clinicopathological characteristics of patients

Clinicopathological characteristics of patients were much diversified. They were different among their clinical outcomes, survival parameters, toxicity grades.

Table 3.16: Clinicopathological characteristics of patients receiving Tamoxifen and comparison of responder vs non responder and their performance status

Characteristics	Non responders	Responders	P value	Performance status≤2	Performance status>2	P value
Age						
<45	24	33	0.367	48	9	0.446
45-55	137	193		304	16	
>55	1	0		1	10	
Menstrual status						
Premenopausal	21	47	0.568	53	4	0.675
Perimenopausal	7	57		26	5	
Postmenopausal	108	148		289	11	
Body Mass Index(BMI),Kg/m^2						
< 20	55	74	0.346	125	4	0.568
20-25	104	152		249	7	
> 25	3	0		3	0	
TNM stage (Clinical)						
I	42	133	0.567	135	2	0.789
II	13	27		152	2	
III	57	105		61	0	
IV	3	8		27	7	
Lymph node status						
No	52	87	0.234	134	4	0.345
N1	57	80		132	5	
N2	52	58		108	3	
N3	1	1		2	0	
Histology						
Ductal	107	167	0.567	201	13	0.787
Lobular	27	73		110	2	
Mixed	1	13		54	8	
Tumor grade						
Grade I	45	72	0.345	136	3	0.567
Grade II	54	78		157	5	
Grade III	27	54		81	6	
Hormone receptor status						
1. Estrogen Receptor(ER)						
Negative	7	0	0.561	20	2	0.178
Positive	123	258		327	39	
2. Progesterone Receptor(PR)						
Negative	43	79	0.719	156	37	0.543
Positive	119	147		167	27	
3. Her-2/ neu status						
Negative	47	94	0.578	155	25	0.563
Positive	65	162		181	27	

Features : The above data shows diversified distribution of response and performance status considering clinicopathological cheracteristics. The total no. of responders was very very high in comparison to non responders. Performance status≤2 was much greater than performance status >2 which means more patients are physically active after or during taking Tamoxifen.Patients with age group 45-55 and post menoposal were prominent for responders and better physical fitness (Performance status≤2).Body Mass index(BMI) between 20-25kg/m^2 showed better response and performance status(≤2) in comparison to 20 and >25 group. Most of the patients were affected either ductal or lobular type breast cancer. Very few of them were found mixed type. Nodular grade N0 and Tumor grade II showed better response and performance status. We were trying to select estrogen receptor positive patients though some of them were negative. Most of the estrogen receptor positive patients showed positive response and performance status though some other showed different. Progesterone and Her-2 receptor positive patients showed better response and performance status in comparison to negative patients.

Table3.17 : Clinicopathological characteristics of patients receiving Tamoxifen and comparison of different survival data of patients

Characteristics	HR ≤1.5	HR >1.5	P value	SR ≤5 yrs	SR >5 yrs	P value	RFS ≤5 yrs	RFS >5 yrs	P value	BCSS		P value
										Yes	No	
Age												
<45	19	38	0.243	20	31	0.547	20	31	0.643	35	16	0.436
45-55	134	196		139	170		139	170		242	67	
>55	0	1		0	1		0	1		1	0	
Menstrual status												
Premenopoasal	48	20	0.425	17	51	0.646	19	49	0.536	33	35	0.562
Perimenopoasal	58	6		4	60		8	56		6	58	
Postmenopoasal	141	115		109	147		112	144		147	109	
Body Mass Index(BMI),Kg/m^2												
< 20	51	78	0.634	55	63	0.356	60	58	0.636	103	15	0.647
20-25	99	157		103	137		107	133		174	66	
> 25	3	0		1	2		0	3		1	2	
TNM stage (Clinical)												
I	41	66	0.474	41	78	0.242	60	52	0.858	97	21	0.456
II	50	77		61	68		53	74		90	45	
III	30	51		34	50		39	45		56	23	
IV	25	48		23	33		26	39		25	31	
Lymph node status												
No	56	83	0.687	60	78	0.367	66	72	0.47	102	36	0.463
N1	53	84		58	70		62	66		101	27	
N2	44	66		41	53		38	56		74	20	
N3	0	2		0	1		1	0		1	0	

Histology												
Ductal	199	15	0.467	101	161	0.356	117	157	0.477	105	169	0.467
Lobular	108	4		30	70		25	75		24	76	
Mixed	51	11		5	9		2	12		6	8	
Tumor grade												
Grade I	49	68	0.637	49	68	0.467	61	56	0.467	98	19	0.376
Grade II	56	76		69	63		55	77		97	35	
Grade III	29	52		32	48		37	43		58	22	
Hormone receptor status												
1.Estrogen Receptor(ER)												
Negative	28	4	0.367	6	1	0.467	5	2	0.467	4	3	0.467
Positive	325	41		121	260		129	252		133	248	
2. Progesterone Receptor(PR)												
Negative	18	4	0.467	40	82	0.467	46	76	0.367	41	81	0.365
Positive	321	45		115	151		129	137		118	148	
3. Her-2/ neu status												
Negative	145	35	0.123	43	98	0.678	49	92	0.469	44	97	0.467
Positive	180	28		64	163		66	161		62	165	
HR:Hazard Ratio;SR:Survival Rate;RFS:Recurrence Free Survival;BCSS:Breast Cancer Specific Survival												

Features : Age group 45-55years and postmenoposal woman showed better survival(less HR,SR>5yrs,RFS>5yrs) in comparison to other groups.Body Mass Index 20-25Kg/m^2 showed better survival result.Nodular grade N1 and Tumor grade II showed slightly better result than other groups. Ductal carcinoma patients showed better survival after taking Tamoxifen in comparison to other carcinoma.Estrogen,progesterone and Her-2 positive patients showed better survival after taking Tamoxifen comparing others.

Table3.18: Clinicopathological characteristics of patients receiving Tamoxifen and comparison of different toxicity data of patients

Characteristics	**Hot flashes**			**Depression**			**Decreased libido**			**Vaginal dryness**		
	Toxicity grade (≤II)	**Toxicity grade (III)**	**P value**	**Toxicity grade (≤II)**	**Toxicity grade (III+IV)**	**P value**	**Toxicity grade (I)**	**Toxicity grade (II)**	**P value**	**Toxicity grade (≤II)**	**Toxicity grade (III)**	**P value**
Age												
<45	56	1	0.789	52	5	0.789	48	9	0.868	55	2	0.965
45-55	315	15		303	27		284	45		309	21	
>55	1	0		0	0		0	0		0	0	
Menstrual status												
Premenoposal	46	22	0.245	45	23	0.345	41	27	0.234	48	20	0.349
Perimeno	54	10		57	7		56	8		53	11	

posal												
Postmenoposal	140	116		139	117		140	116		144	112	
Body Mass Index(BMI),Kg/m^2												
< 20	122	7	0.677	122	7	0.123	111	18	0.278	120	9	0.271
20-25	247	9		231	25		219	37		242	14	
> 25	3	0		3	0		3	0		3	0	
TNM stage (Clinical)												
I	118	3	0.567	117	11	0.922	99	28	0.299	111	12	0.488
II	121	8		123	17		101	7		121	8	
III	86	3		79	5		63	11		76	4	
IV	39	10		26	10		59	20		40	16	
Lymph node status												
No	132	7	0.437	127	12	0.579	118	21	0.389	128	11	0.381
N1	132	5		126	11		117	20		131	6	
N2	107	3		101	9		96	14		104	6	
N3	1	1		2	0		2	0		2	0	
Histology												
Ductal	198	16	0.278	191	23	0.468	189	25	0.356	193	21	0.476
Lobular	104	8		106	6		101	11		107	5	
Mixed	50	12		53	9		45	16		52	10	
Tumor grade												
Grade I	113	4	0.382	107	10	0.378	99	18	0.482	110	7	0.372
Grade II	126	6		121	11		131	1		127	5	
Grade III	76	5		76	5		69	12		73	8	
Hormone receptor status												
1.Estrogen Receptor(ER)												
Negative	21	11	0.267	27	5	0.356	28	4	0.467	20	12	0.847
Positive	320	46		322	44		319	47		321	45	
2.Progesterone Receptor(PR)												
Negative	17	5	0.256	17	5	0.478	11	11	0.367	18	4	0.267
Positive	320	46		326	40		311	55		319	47	
3.Her-2/ neu status												
Negative	143	37	0.357	141	39	0.376	148	32	0.425	140	40	0.463
Positive	181	27		178	30		181	27		179	29	

Features : Age group 45-55years showed less toxicity grade in comparison to other age group considering only Tamoxifen related toxicity. Though postmenoposal showed slightly better result for toxicity in comparison to others but there were not too much different. Body Mass Index group <20 and 20-25Kg/m^2 showed less toxicity in comparison to >25 group. No significant differences were found among different nodular or tumor grade and toxicities. Different types of carcinoma showed similar data for toxicity. Estrogen, progesterone and Her-2 receptor positive patients showed less toxicity in comparison to negative patients.

3.3.3 Correlation of different genotype with response

3.3.3.1 Impact of SULT1A1*2 polymorphism on response

Below tables show the response data of different polymorphism of SULT1A1*2

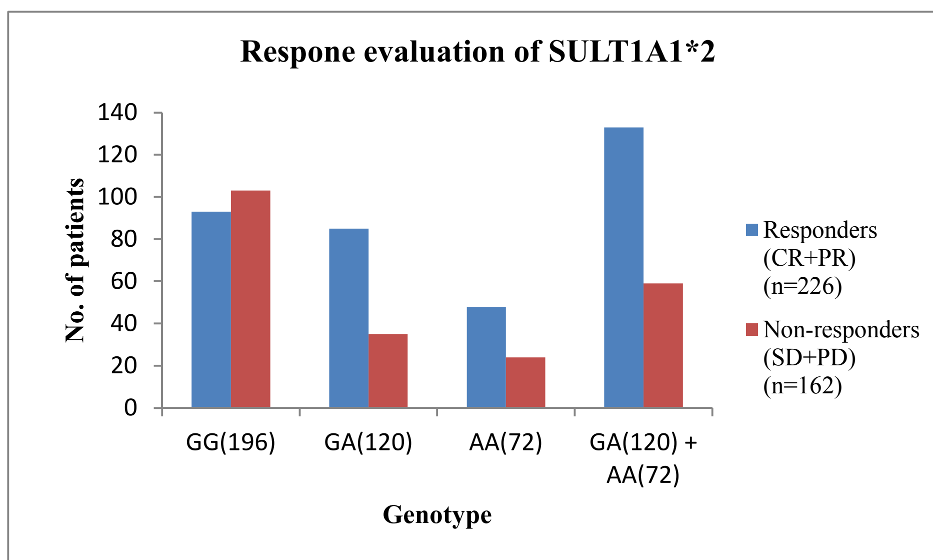
Table 3.19: Distribution of responders vs non responders of different genotype of SULT1A1*2

SULT11A1*2						
	Total Responders			**Total Non responders**		
Genotype	**Complete Response**	**Partial Response**	**Total Responders**	**Stable Disease**	**Progressive Disease**	**Total Non-responders**
GG(196)	80	13	93	17	86	103
GA(120)	75	10	85	10	25	35
AA(72)	42	6	48	2	22	24
GA(120) + AA(72)	117	16	133	12	47	59

Table 3.20 : Response evaluation of different polymorphism for SULT1A1*2

SULT1A1*2					
Genotype	**Responders (CR+PR) (n=226)**	**Non-responders (SD+PD) (n=162)**	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(196)	93	103	Ref	Ref	Ref
GA(120)	85	35	0.3718(0.2293-0.6028)	2.933	3.97E-05
AA(72)	48	24	0.4515().2567-0.7939)	2.358	0.0058
GA(120) + AA(72)	133	59	0.4005(0.2644-0.6068)	2.692	<0.0001

Graph3.6: Comparison of responders and non responders of different genotype of SULT1A1*2



Features : Mutant homo showed significant response OR= 2.358 and p value=0.0058<0.05.Hetero polymorph also showed significant relationship. Here odd ratio and p value were 2.933 and 3.97E-05.The results indicate presence of A allele contributes response data.

3.3.3.2 Impact of UGT2B7*2 polymorphism on response

Below tables show the response data of different polymorphism of UGT2B7*2

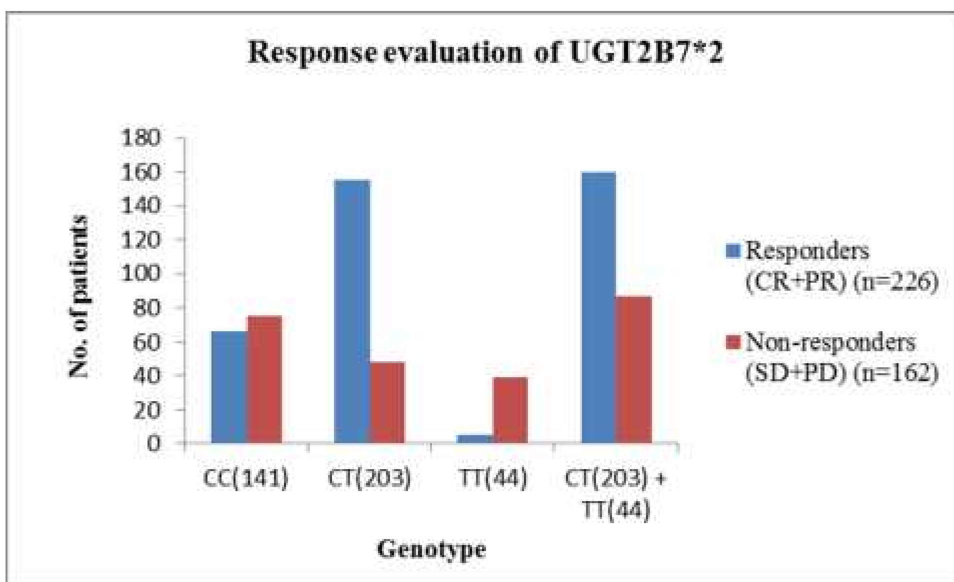
Table3.21: Distribution of responders vs non responders of different genotype of UGT2B7*2

UGT2B7*2						
	Total Responders			**Total Non responders**		
Genotype	**Complete Response**	**Partial Response**	**Total Responders**	**Stable Disease**	**Progressive Disease**	**Total Non-responders**
CC(141)	55	11	66	14	61	75
CT(203)	139	16	155	10	38	48
TT(44)	3	2	5	6	33	39
CT(203) + TT(44)	142	18	160	16	71	87

Table3.22: Response evaluation of different polymorphism for UGT2B7*2

UGT2B7*2					
Genotype	**Responders (CR+PR) (n=226)**	**Non-responders (SD+PD) (n=162)**	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(141)	66	75	Ref	Ref	Ref
CT(203)	155	48	0.2725(0.1715-0.4329)	4.213	<0.0001
TT(44)	5	39	6.8640(2.5555-18.4367)	0.126	0.0001
CT(203) + TT(44)	160	87	0.4786(0.3140-0.7293)	2.177	0.001

Graph3.7: Comparison of responders and non responders of different genotype of UGT2B7*2



Features : Hetero polymorph showed significant response OR= 4.213 and p value=0.0001<0.05.

3.3.3.3 Impact of CYP3A5*3 polymorphism on response

Below tables show the response data of different polymorphism of CYP3A5*3

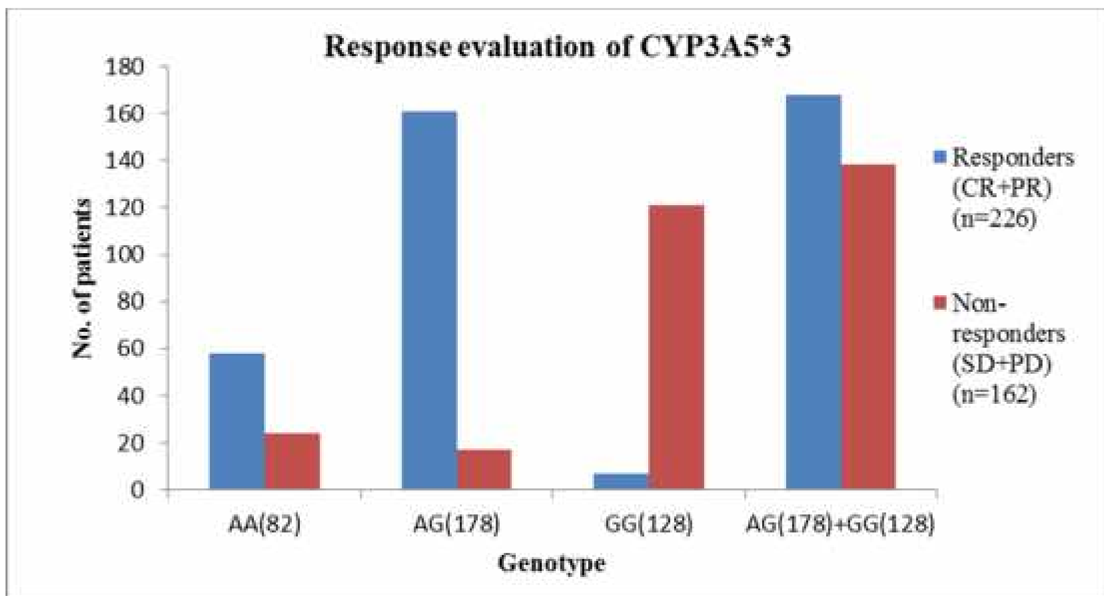
Table3.23: Distribution of responders vs non responders of different genotype of CYP3A5*3

CYP3A5*3						
	Total Responders			**Total Non responders**		
Genotype	**Complete Response**	**Partial Response**	**Total Responders**	**Stable Disease**	**Progressive Disease**	**Total Non-responders**
AA(82)	52	6	58	2	22	24
AG(178)	145	16	161	0	17	17
GG(128)	0	7	7	27	94	121
AG(178)+GG(128)	145	23	168	27	111	138

Table3.24: Response evaluation of different polymorphism for CYP3A5*3

CYP3A5*3					
Genotype	**Responders (CR+PR) (n=226)**	**Non-responders (SD+PD) (n=162)**	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
AA(82)	58	24	Ref	Ref	Ref
AG(178)	161	17	0.2552(0.1280-0.5088)	5.626	0.0001
GG(128)	7	121	41.7738(17.0137-102.5672)	0.016	<0.0001
AG(178)+GG(128)	168	138	1.9881(1.1728-3.3602)	0.437	0.0107

Graph3.8: Comparison of responders and non responders of different genotype of CYP3A5*3



Features : Hetero showed significant response OR=5.626 and p value<0.0001.

3.3.3.4 Impact of CYP2D6*4 polymorphism on response

Below tables show the response data of different polymorphism of CYP2D6*4.

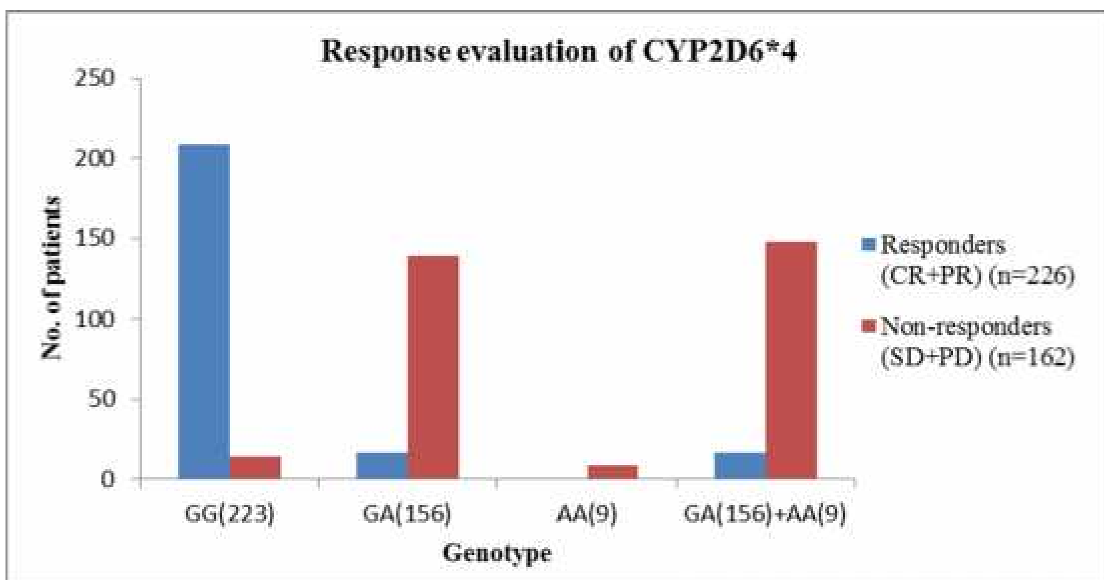
Table 3.25: Distribution of responders vs non responders of different genotype of CYP2D6*4

CYP2D6*4						
	Total Responders			**Total Non responders**		
Genotype	**Complete Response**	**Partial Response**	**Total Responders**	**Stable Disease**	**Progressive Disease**	**Total Non-responders**
GG(223)	190	19	209	0	14	14
GA(156)	7	10	17	27	112	139
AA(9)	0	0	0	2	7	9
GA(156)+AA(9)	7	10	17	29	119	148

Table 3.26: Response evaluation of different polymorphism for CYP2D6*4

CYP2D6*4					
Genotype	**Responders (CR+PR) (n=226)**	**Non-responders (SD+PD) (n=162)**	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(223)	209	14	Ref	Ref	Ref
GA(156)	17	139	122.0630(58.2854-255.6279)	<0	0.993
AA(9)	0	9	274.5172(15.2072-4955.5174)	<0	0.998
GA(156)+AA(9)	17	148	129.9664(62.1300-271.8698)	<0	0.993

Graph3.9: Comparison of responders and non responders of different genotype of CYP2D6*4



Features : No polymorphs showed significant response for this gene.

3.3.3.5 Impact of CYP2D6*10 polymorphism on response

Below tables show the response data of different polymorphism of CYP2D6*10.

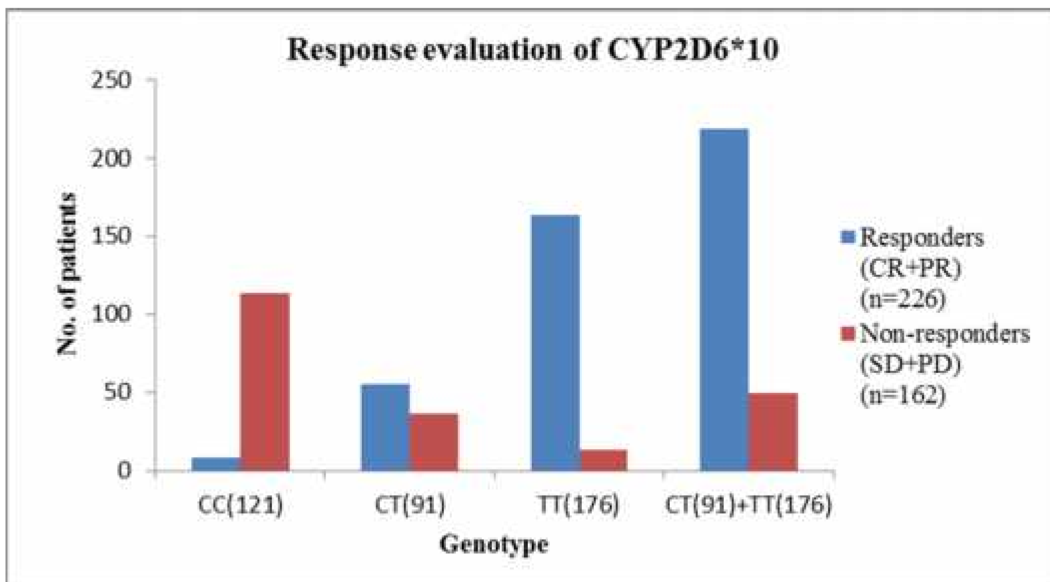
Table 3.27 : Distribution of responders vs non responders of different genotype of CYP2D6*10

CYP2D6*10						
	Total Responders			**Total Non responders**		
Genotype	**Complete Response**	**Partial Response**	**Total Responders**	**Stable Disease**	**Progressive Disease**	**Total Non-responders**
CC(121)	1	7	8	22	91	113
CT(91)	46	9	55	7	29	36
TT(176)	150	13	163	0	13	13
CT(91)+TT(176)	196	22	218	7	42	49

Table 3.28: Response evaluation of different polymorphism for CYP2D6*10

CYP2D6*10					
Genotype	**Responders (CR+PR) (n=226)**	**Non-responders (SD+PD) (n=162)**	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(121)	8	113	Ref	Ref	Ref
CT(91)	55	36	0.0463(0.0202-0.1064)	29.082	<0.0001
TT(176)	163	13	0.0056(0.0023-0.0141)	1.33E+15	<0.0001
CT(91)+TT(176)	218	49	0.0159(0.0073-0.0348)	104.23	<0.0001

Graph3.10: Comparison of responders and non responders of different genotype of CYP2D6*10



Features : Both hetero(CT) and Mutant homo(TT) showed significant response data. Adjusted odd ratio for hetero was 29.082 and p value <0.0001.For mutant homo adjusted odd ratio was 1.33E+15 and p value was <0.0001.

3.3.4 Correlation of different genotype with toxicities

3.3.4.1 Impact of SULT1A1*2 polymorphism on Hot flashes

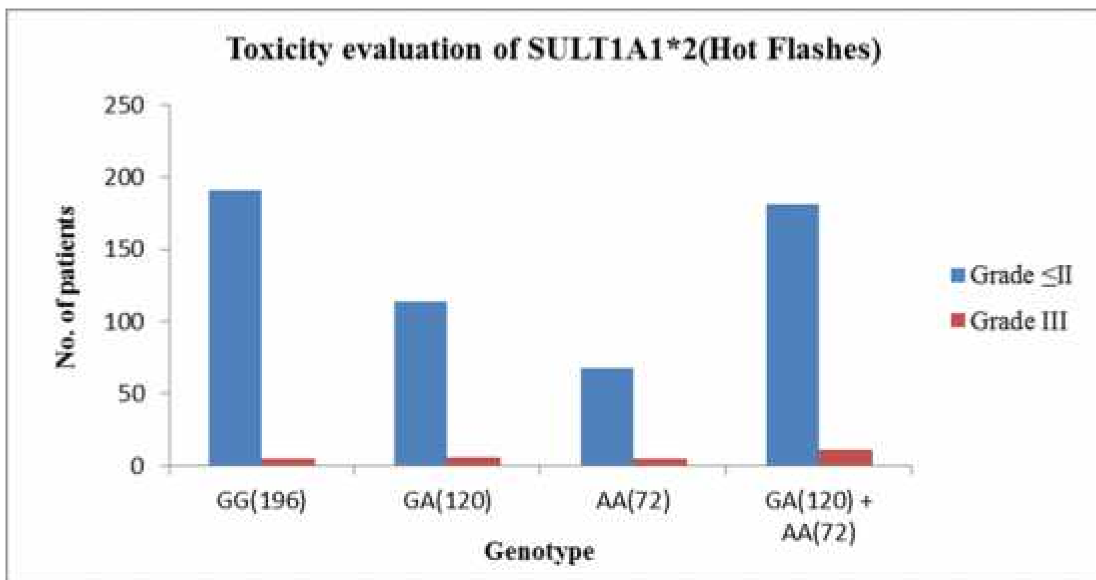
Table 3.29: Distribution of different grades of toxicity among different genotype of SULT1A1*2

Hot Flashes					
SULT11A1*2					
	Total	GG (196)	GA (120)	AA (72)	GA(120) + AA(72)
Grade ≤II	372	191	114	67	181
Grade III	16	5	6	5	11

Table3.30: Toxicity evaluation of different polymorphism for SULT1A1*2

Hot Flashes					
SULT1A1*2					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(196)	191	5	Ref	Ref	Ref
GA(120)	114	6	2.0105(0.6000-6.7375)	1.974	0.274
AA(72)	67	5	2.8507(0.8002-10.1558)	2.575	0.151
GA(120) + AA(72)	181	11	2.3215(0.7911-6.8125)	2.174	0.16

Graph3.11: Comparison of different grades of toxicity among different genotype of SULT1A1*2



Features : No significant association was found among different polymorphism of SULT1A1*2 and Hot flashes.

3.3.4.2 Impact of UGT2B7*2 polymorphism on Hot flashes

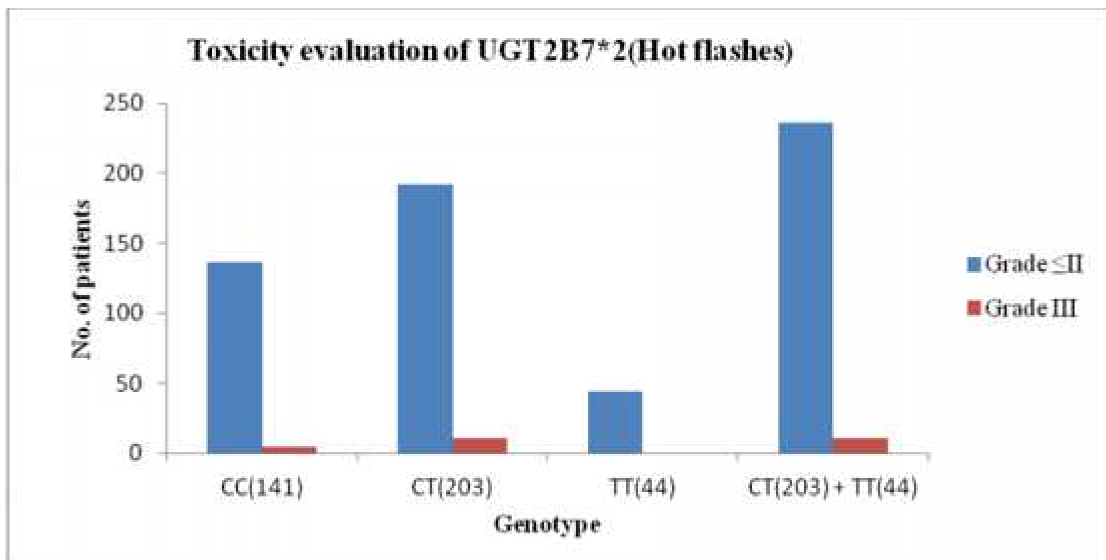
Table3.31: Distribution of different grades of toxicity among different genotype of UGT2B7*2

Hot Flashes					
UGT2B7*2					
	Total	CC (141)	CT (203)	TT (44)	CT(203) + TT(44)
Grade ≤II	372	136	192	44	236
Grade III	16	5	11	0	11

Table3.32: Toxicity evaluation of different polymorphism for UGT2B7*2

Hot Flashes					
UGT2B7*2					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(141)	136	5	Ref	Ref	Ref
CT(203)	192	11	1.5582(0.5293-4.5875)	1.503	0.462
TT(44)	44	0	0.2789(0.0151-5.1436)	<0	0.997
CT(203) + TT(44)	236	11	1.2678(0.4314-3.7258)	1.216	0.724

Graph3.12: Comparison of different grades of toxicity among different genotype of UGT2B7*2



Features : No significant association was found among different polymorphism of UGT2B7*2 and Hot flashes.

3.3.4.3 Impact of CYP3A5*3 polymorphism on Hot flashes

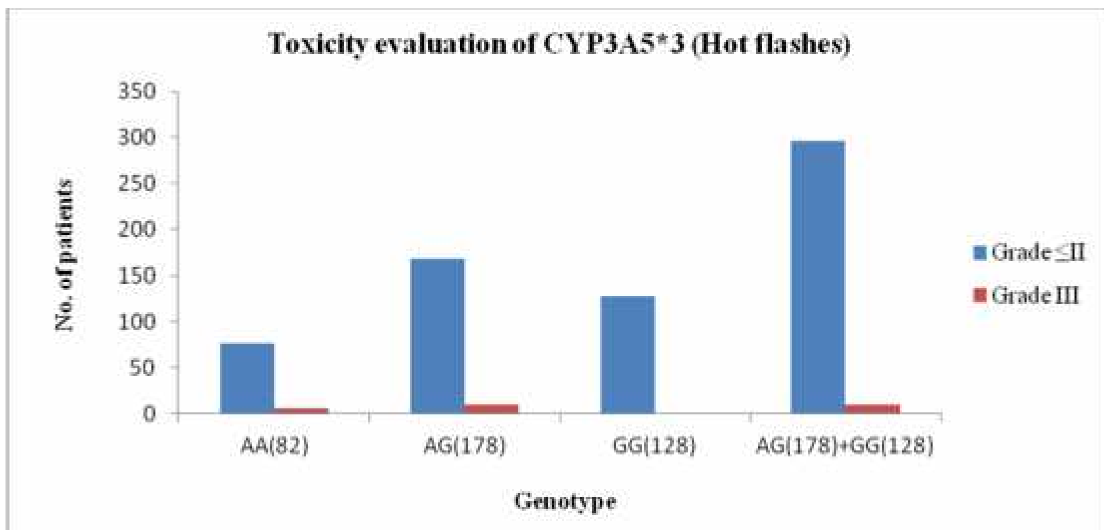
Table3.33: Distribution of different grades of toxicity among different genotype of CYP3A5*3

Hot Flashes					
CYP3A5*3					
	Total	AA (82)	AG (178)	GG (128)	AG(178) + GG(128)
Grade ≤II	372	76	168	128	296
Grade III	16	6	10	0	10

Table3.34: Toxicity evaluation of different polymorphism for CYP3A5*3

Hot Flashes					
CYP3A5*3					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
AA(82)	76	6	Ref	Ref	Ref
AG(178)	168	10	0.7540(0.2644-2.1498)	0.655	0.44
GG(128)	128	0	0.0458(0.0025-0.8243)	0	0.996
AG(178)+GG(128)	296	10	0.4279(0.1508-1.2144)	0.37	0.066

Graph3.13: Comparison of different grades of toxicity among different genotype of CYP3A5*3



Features : No significant association was found among different polymorphism of CYP3A5*3 and Hot flashes.

3.3.4.4 Impact of CYP2D6*4 polymorphism on Hot flashes

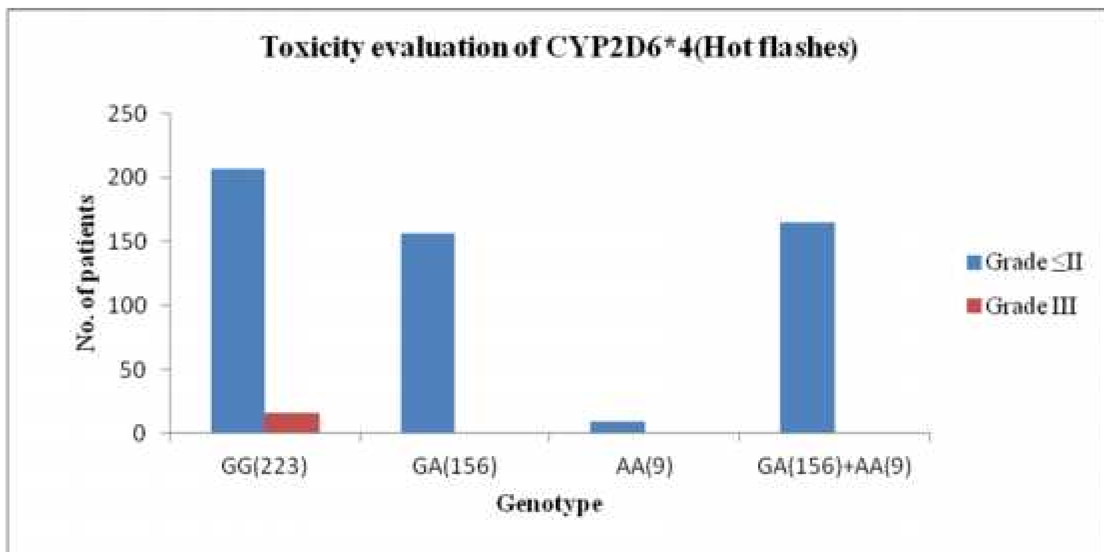
Table3.35: Distribution of different grades of toxicity among different genotype of CYP2D6*4

Hot Flashes					
CYP2D6*4					
	Total	GG (223)	GA (156)	AA (9)	GA(156) + AA(9)
Grade ≤II	372	207	156	9	165
Grade III	16	16	0	0	0

Table3.36: Toxicity evaluation of different polymorphism for CYP2D6*4

Hot Flashes					
CYP2D6*4					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(223)	207	16	Ref	Ref	Ref
GA(156)	156	0	0.0402(0.0024-0.6749)	0	0.995
AA(9)	9	0	0.6619(0.0369-11.8823)	<0	0.994
GA(156)+AA(9)	165	0	0.0380(0.0023-0.6380)	<0	0.995

Graph3.14: Comparison of different grades of toxicity among different genotype of CYP2D6*4



Features : No significant association was found among different polymorphism of CYP2D6*4 and Hot flashes.

3.3.4.5 Impact of CYP2D6*10 polymorphism on Hot flashes

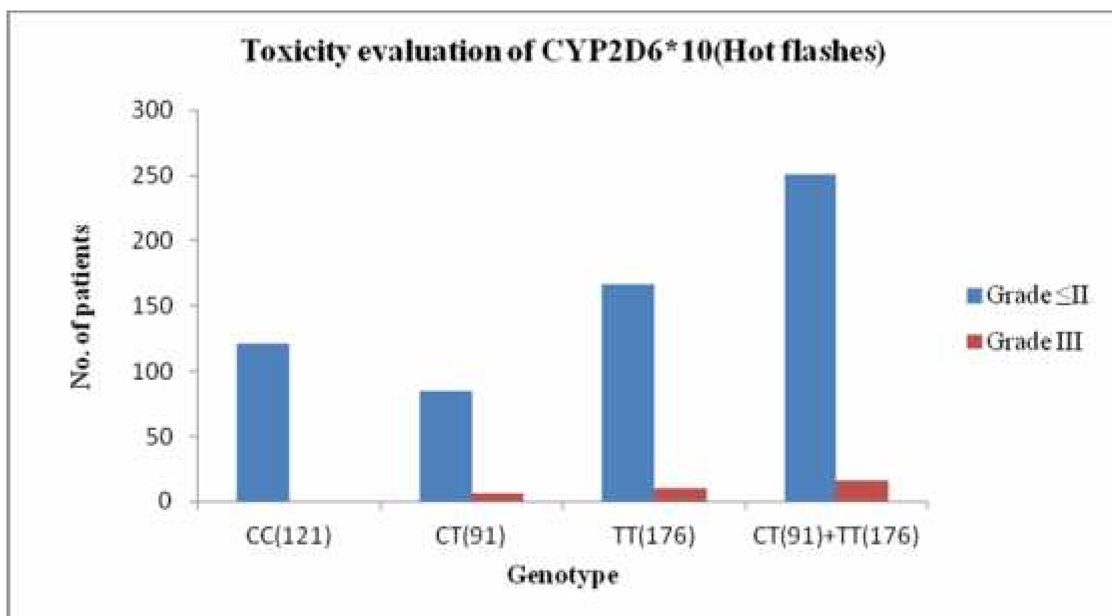
Table3.37: Distribution of different grades of toxicity among different genotype of CYP2D6*10

Hot Flashes					
CYP2D6*10					
	Total	CC (121)	CT (91)	TT (176)	CT(91) + TT(176)
Grade ≤II	372	121	85	166	251
Grade III	16	0	6	10	16

Table3.38: Toxicity evaluation of different polymorphism for CYP2D6*10

Hot Flashes					
CYP2D6*10					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(121)	121	0	Ref	Ref	Ref
CT(91)	85	6	18.4737(1.0269-332.3259)	8.40E+07	0.0479
TT(176)	166	10	15.3243(0.8893-264.0534)	1.04E+08	0.0602
CT(91)+TT(176)	251	16	15.9423(0.9485-267.9614)	1.03E+08	0.0544

Graph3.15: Comparison of different grades of toxicity among different genotype of CYP2D6*10



Features : Significant association was found among hetero polymorphism of CYP2D6*10 and Hot flashes.p value for hetero was 0.0479<0.05 and odd ratio was 8.40E+07.

3.3.4.6 Impact of SULT1A1*2 polymorphism on Depression

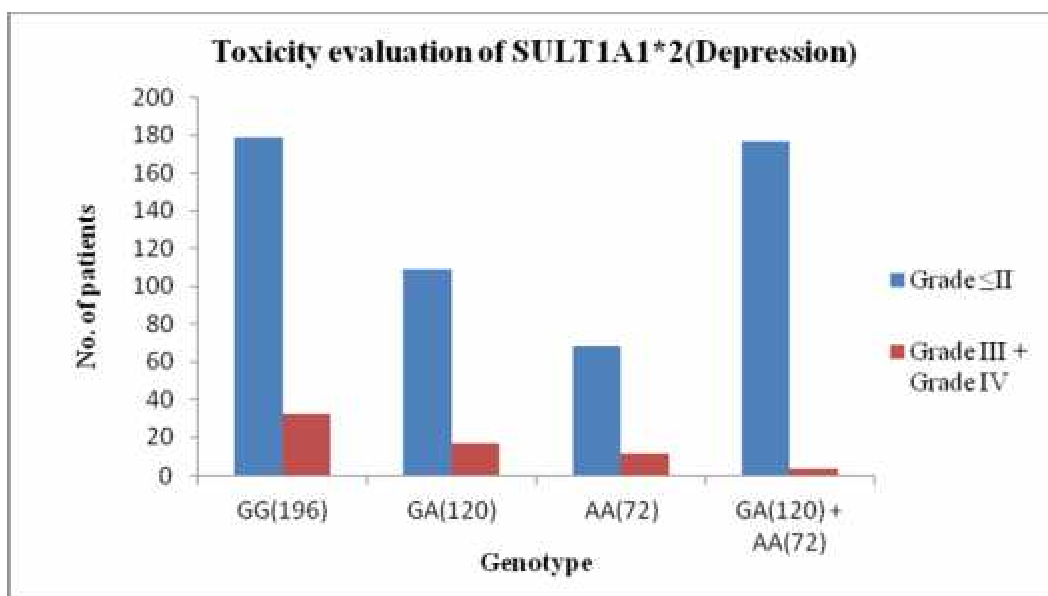
Table3.39: Distribution of different grades of toxicity among different genotype of SULT1A1*2

Depression					
SULT11A1*2					
	Total	GG (196)	GA (120)	AA (72)	GA(120) + AA(72)
Grade ≤II	356	179	109	68	177
Grade III	32	17	11	4	15
Grade IV	0	0	0	0	0

Table3.40: Toxicity evaluation of different polymorphism for SULT1A1*2

Depression							
SULT1A1*2							
Genotype	Grade ≤II	Grade III	Grade IV	Grade III + Grade IV	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(196)	179	32	0	32	Ref	Ref	Ref
GA(120)	109	17	0	17	0.8724(0.4625-1.6456)	1.126	0.773
AA(72)	68	11	0	11	0.9049(0.4318-1.8961)	0.676	0.51
GA(120) + AA(72)	177	4	0	4	0.1264(0.0438-0.3649)	0.93	0.848

Graph3.16: Comparison of different grades of toxicity among different genotype of SULT1A1*2



Features : No significant association was found among different polymorphism of SULT1A1*2 and Depression.

3.3.4.7 Impact of UGT2B7*2 polymorphism on Depression

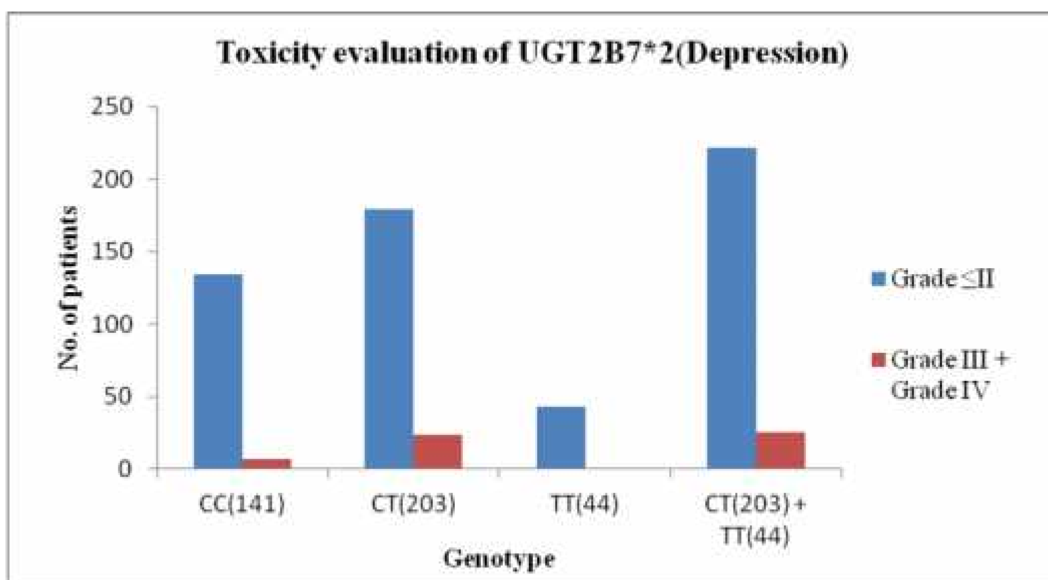
Table3.41: Distribution of different grades of toxicity among different genotype of UGT2B7*2

Depression					
UGT2B7*2					
	Total	CC (141)	CT (203)	TT (44)	CT(203) + TT(44)
Grade ≤II	356	134	179	43	222
Grade III	32	7	24	1	25
Grade IV	0	0	0	0	0

Table3.42: Toxicity evaluation of different polymorphism for UGT2B7*2

Depression							
UGT2B7*2							
Genotype	Grade ≤II	Grade III	Grade IV	Grade III + Grade IV	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(141)	134	7	0	7	Ref	Ref	Ref
CT(203)	179	24	0	24	2.5666(1.0740-6.1337)	2.61	0.033
TT(44)	43	1	0	1	0.452(0.0533-3.7211)	0.537	0.574
CT(203) + TT(44)	222	25	0	25	2.1557(0.9076-5.1205)	2.224	0.075

Graph3.17: Comparison of different grades of toxicity among different genotype of UGT2B7*2



Features : Significant association was found among hetero polymorphism of UGT2B7*2 and Depression(P value=0.033<0.05 and OR=2.61).

3.3.4.8 Impact of CYP3A5*3 polymorphism on Depression

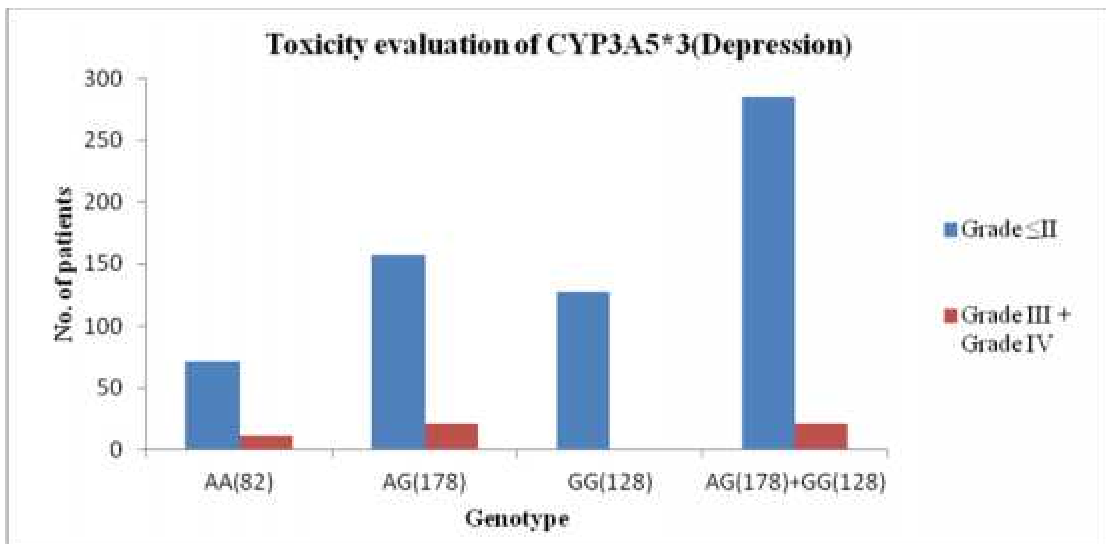
Table3.43: Distribution of different grades of toxicity among different genotype of CYP3A5*3

Depression					
CYP3A5*3					
	Total	AA (82)	AG (178)	GG (128)	AG(178) + GG(128)
Grade ≤II	356	71	157	128	285
Grade III	33	11	21	0	21
Grade IV	0	0	0	0	0

Table3.44: Toxicity evaluation of different polymorphism for CYP3A5*3

Depression							
CYP3A5*3							
Genotype	Grade ≤II	Grade III	Grade IV	Grade III + Grade IV	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
AA(82)	71	11	0	11	Ref	Ref	Ref
AG(178)	157	21	0	21	0.8633(0.3952-1.8862)	0.927	0.854
GG(128)	128	0	0	0	0.0242(0.0014-0.4166)	<0	0.995
AG(178)+GG(128)	285	21	0	21	0.4756(0.2192-1.0318)	0.511	0.098

Graph3.18: Comparison of different grades of toxicity among different genotype of CYP3A5*3



Features : No significant association was found among different polymorphism of CYP3A5*3 and Depression.

3.3.4.9 Impact of CYP2D6*4 polymorphism on Depression

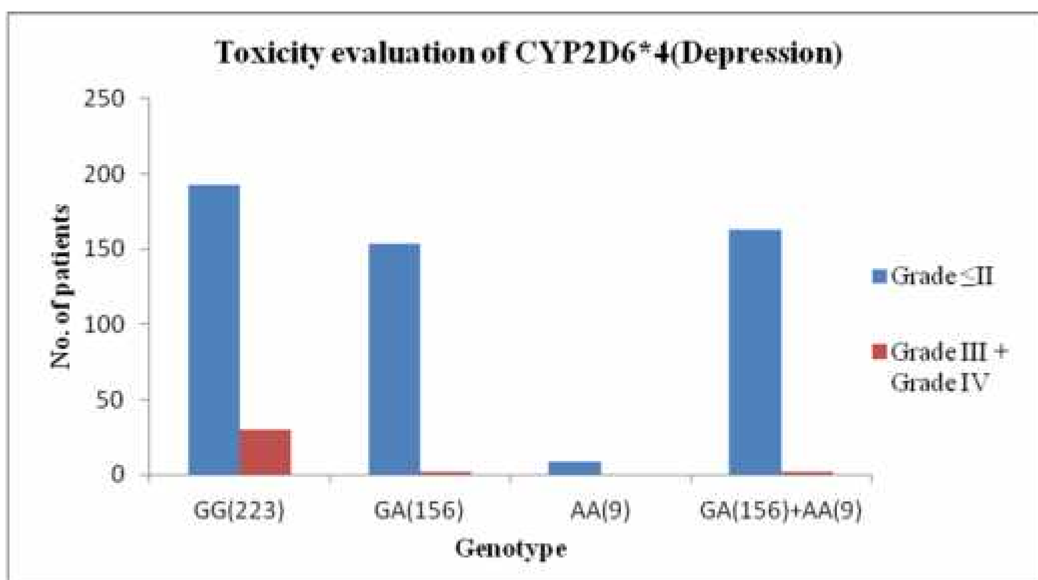
Table3.45: Distribution of different grades of toxicity among different genotype of CYP2D6*4

Depression					
CYP2D6*4					
	Total	GG (223)	GA (156)	AA (9)	GA(156) + AA(9)
Grade ≤II	356	193	154	9	163
Grade III	33	30	2	0	2
Grade IV	0	0	0	0	0

Table 3.46: Toxicity evaluation of different polymorphism for CYP2D6*4

Depression							
CYP2D6*4							
Genotype	Grade ≤II	Grade III	Grade IV	Grade III + Grade IV	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(223)	193	30	0	30	Ref	Ref	Ref
GA(156)	154	2	0	2	0.0835(0.0197-0.3551)	0.078	0.001
AA(9)	9	0	0	0	0.3339(0.0189-5.8855)	<0	0.999
GA(156)+AA(9)	163	2	0	2	0.0789(0.0186-0.3353)	0.074	0.0006

Graph3.19: Comparison of different grades of toxicity among different genotype of CYP2D6*4



Features : No significant association was found among different polymorphism of CYP2D6*4 and Depression. Though hetero showed significant p value but odd ratio was very negligible.

3.3.4.10 Impact of CYP2D6*10 polymorphism on Depression

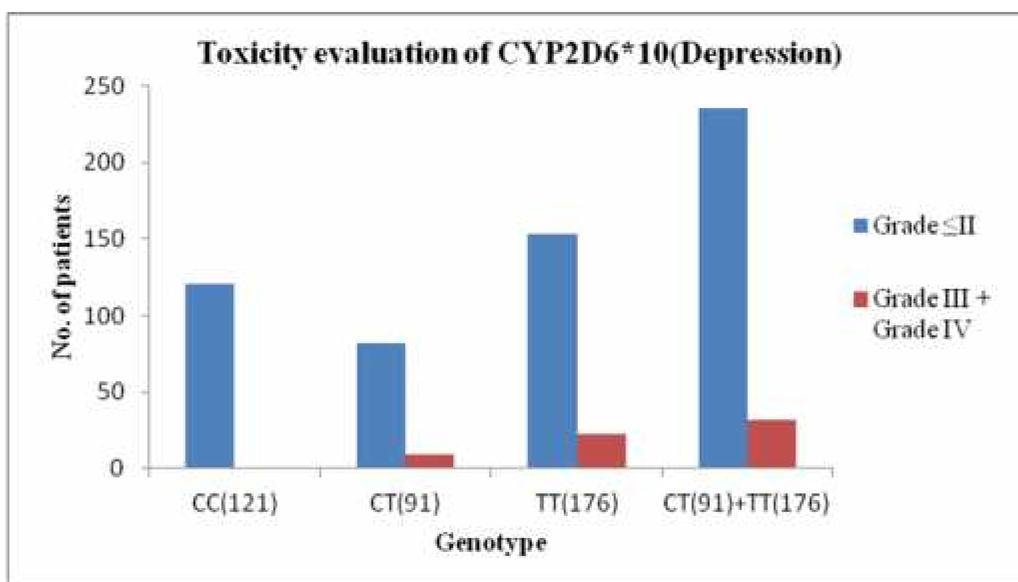
Table3.47: Distribution of different grades of toxicity among different genotype of CYP2D6*10

Depression					
CYP2D6*10					
	Total	CC (121)	CT (91)	TT (176)	CT(91) + TT(176)
Grade ≤II	356	121	82	153	235
Grade III	33	0	9	23	32
Grade IV	0	0	0	0	0

Table3.48: Toxicity evaluation of different polymorphism for CYP2D6*10

Depression							
CYP2D6*10							
Genotype	Grade ≤II	Grade III	Grade IV	Grade III + Grade IV	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(121)	121	0	0	0	Ref	Ref	Ref
CT(91)	82	9	0	9	27.9818(1.6064-487.4241)	1.77E+08	0.0223
TT(176)	153	23	0	23	37.2020(2.2369-618.7153)	2.32E+08	0.0117
CT(91)+TT(176)	235	32	0	32	33.5350(2.0359-552.3914)	2.15E+08	0.0140

Graph3.20: Comparison of different grades of toxicity among different genotype of CYP2D6*10



Features : Significant association was found among both hetero and mutant homo polymorphism of CYP2D6*10 and Depression. Odd ratios were 1.77E+08 and 2.32E+08 respectively whereas p values were 0.0223 and 0.0117.

3.3.4.11 Impact of SULT1A1*2 polymorphism on Decreased libido

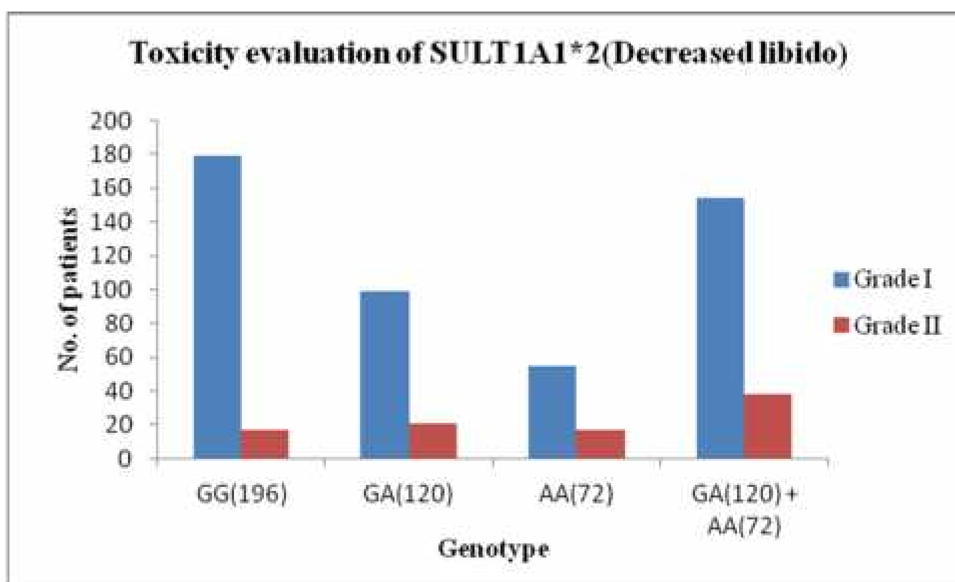
Table3.49: Distribution of different grades of toxicity among different genotype of SULT1A1*2

Decreased libido					
SULT11A1*2					
	Total	GG (196)	GA (120)	AA (72)	GA(120) + AA(72)
Grade I	333	179	99	55	154
Grade II	55	17	21	17	38

Table3.50: Toxicity evaluation of different polymorphism for SULT1A1*2

Decreased libido					
SULT1A1*2					
Genotype	Grade I	Grade II	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(196)	179	17	Ref	Ref	Ref
GA(120)	99	21	2.2335(1.1259-4.4306)	1.58E+07	0.0215
AA(72)	55	17	3.2545(1.5574-6.8013)	6.31E+06	0.0017
GA(120) + AA(72)	154	38	2.5982(1.4101-4.7871)	3.14E+07	0.0022

Graph3.21: Comparison of different grades of toxicity among different genotype of SULT1A1*2



Features : Significant association was found among both hetero and mutant homo polymorphism of SULT1A1*2 and Decreased libido. Odd ratios were 1.58E+07 and 6.31E+06 respectively whereas p values were 0.0215 and 0.0017.

3.3.4.12 Impact of UGT2B7*2 polymorphism on Decreased libido

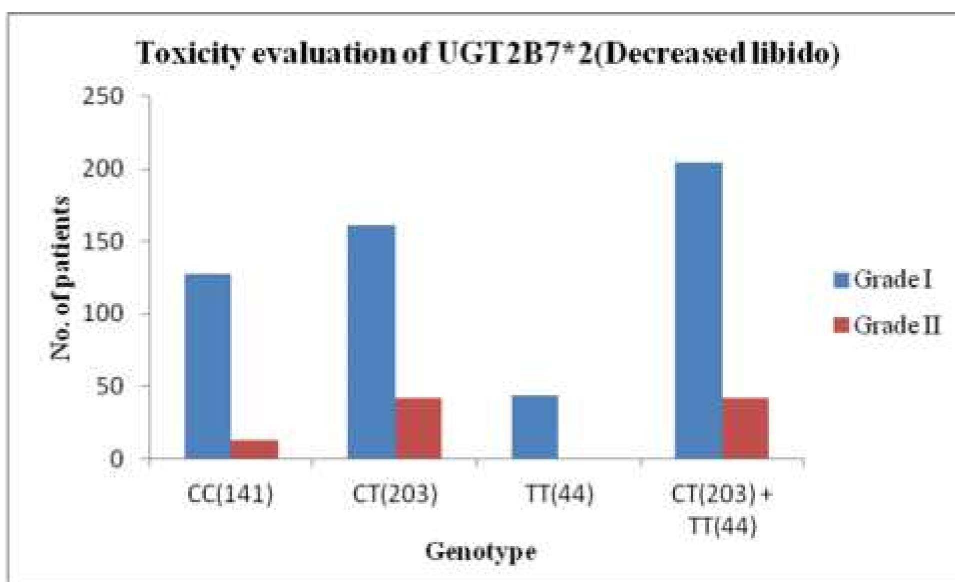
Table3.51: Distribution of different grades of toxicity among different genotype of UGT2B7*2

Decreased libido					
UGT2B7*2					
	Total	CC (141)	CT (203)	TT (44)	CT(203) + TT(44)
Grade I	333	128	161	44	205
Grade II	55	13	42	0	42

Table3.52: Toxicity evaluation of different polymorphism for UGT2B7*2

Decreased libido					
UGT2B7*2					
Genotype	Grade I	Grade II	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(141)	128	13	Ref	Ref	Ref
CT(203)	161	42	2.5686(1.3223-4.9894)	6.34E+06	0.0054
TT(44)	44	0	0.1069(0.0062-1.8365)	5.10E+06	0.1233
CT(203) + TT(44)	205	42	2.0173(1.0425-3.9034)	4.98E+06	0.0372

Graph3.22: Comparison of different grades of toxicity among different genotype of UGT2B7*2



Features : Significant association was found among hetero polymorphism of UGT2B7*2 and Decreased libido. Odd ratio was 6.34E+06 and p values was 0.0054.

3.3.4.13 Impact of CYP3A5*3 polymorphism on Decreased libido

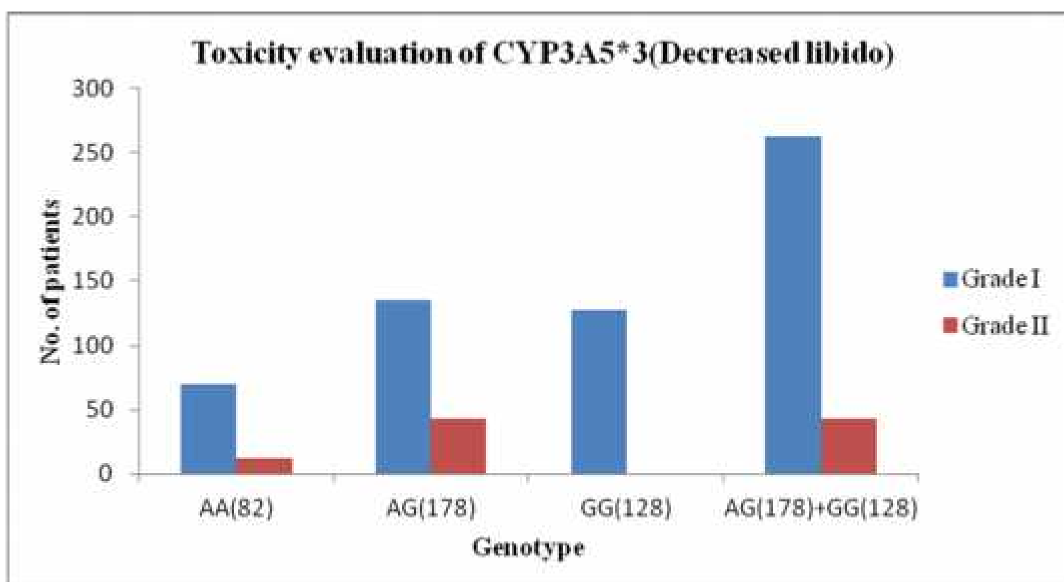
Table3.53: Distribution of different grades of toxicity among different genotype of CYP3A5*3

Decreased libido					
CYP3A5*3					
	Total	AA (82)	AG (178)	GG (128)	AG(178) + GG(128)
Grade I	356	70	135	128	263
Grade II	33	12	43	0	43

Table3.54: Toxicity evaluation of different polymorphism for CYP3A5*3

Decreased libido					
CYP3A5*3					
Genotype	Grade I	Grade II	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
AA(82)	70	12	Ref	Ref	Ref
AG(178)	135	43	1.8580(0.9208-3.7491)	5.92E+06	0.997
GG(128)	128	0	0.0219(0.0013-0.3762)	4.68E+06	0.997
AG(178)+GG(128)	263	43	0.9537(0.4774-1.9054)	3.69E+06	0.996

Graph3.23: Comparison of different grades of toxicity among different genotype of CYP3A5*3



Features : No significant association was found among different polymorphism of CYP3A5*3 and Decreased libido.

3.3.4.14 Impact of CYP2D6*4 polymorphism on Decreased libido

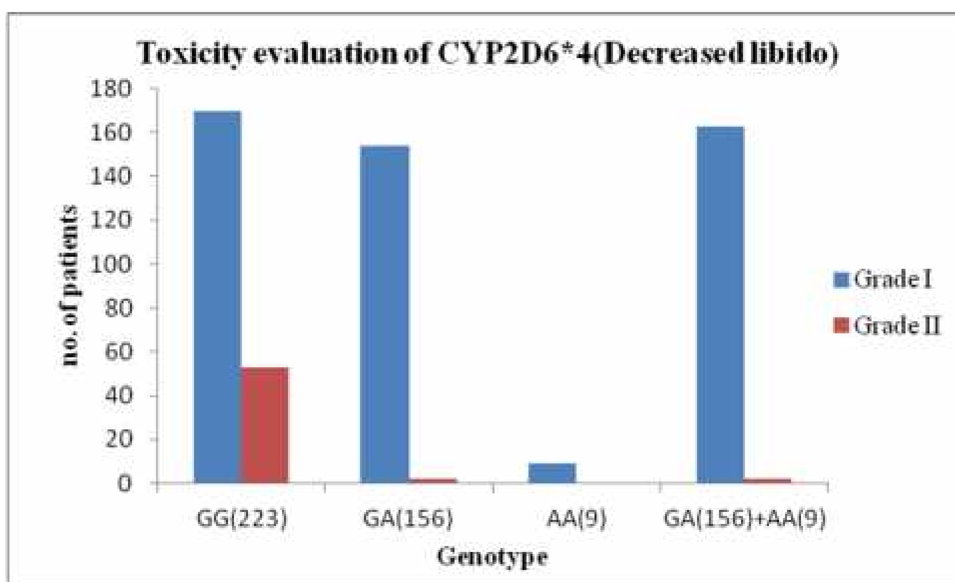
Table3.55: Distribution of different grades of toxicity among different genotype of CYP2D6*4

Decreased libido					
CYP2D6*4					
	Total	GG (223)	GA (156)	AA (9)	GA(156) + AA(9)
Grade I	356	170	154	9	163
Grade II	33	53	2	0	2

Table3.56: Toxicity evaluation of different polymorphism for CYP2D6*4

Decreased libido					
CYP2D6*4					
Genotype	Grade I	Grade II	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(223)	170	53	Ref	Ref	Ref
GA(156)	154	2	0.0417(0.0100-0.1738)	0	0.996
AA(9)	9	0	0.1677(0.0096-2.9301)	0	0.999
GA(156)+AA(9)	163	2	0.0394(0.0044-0.1641)	0	0.996

Graph3.24: Comparison of different grades of toxicity among different genotype of CYP2D6*4



Features : No significant association was found among different polymorphism of CYP2D6*4 and Decreased libido.

3.3.4.15 Impact of CYP2D6*10 polymorphism on Decreased libido

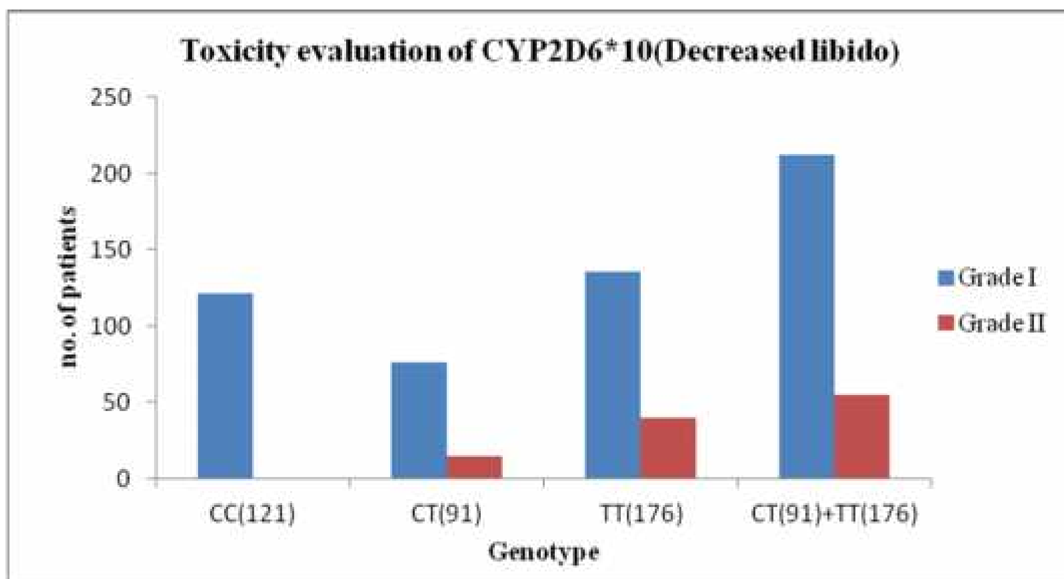
Table3.57: Distribution of different grades of toxicity among different genotype of CYP2D6*10

Decreased libido					
CYP2D6*10					
	Total	CC (121)	CT (91)	TT (176)	CT(91) + TT(176)
Grade I	356	121	76	136	212
Grade II	33	0	15	40	55

Table3.58: Toxicity evaluation of different polymorphism for CYP2D6*10

Decreased libido					
CYP2D6*10					
Genotype	Grade I	Grade II	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(121)	121	0	Ref	Ref	Ref
CT(91)	76	15	49.2353(2.9035-834.9016)	4.27E+06	0.0070
TT(176)	136	40	72.0989(4.3860-1185.1786)	6.44E+06	0.0027
CT(91)+TT(176)	212	55	63.4659(3.8856-1036.6286)	4.17E+06	0.0036

Graph3.25: Comparison of different grades of toxicity among different genotype of CYP2D6*10



Features : Significant association was found among both hetero and mutant homo polymorphism of CYP2D6*10 and Decreased libido. Odd ratios were 4.27E+06 and 6.44E+06 respectively whereas p values were 0.0070 and 0.0027.

3.3.4.16 Impact of SULT1A1*2 polymorphism on Vaginal dryness

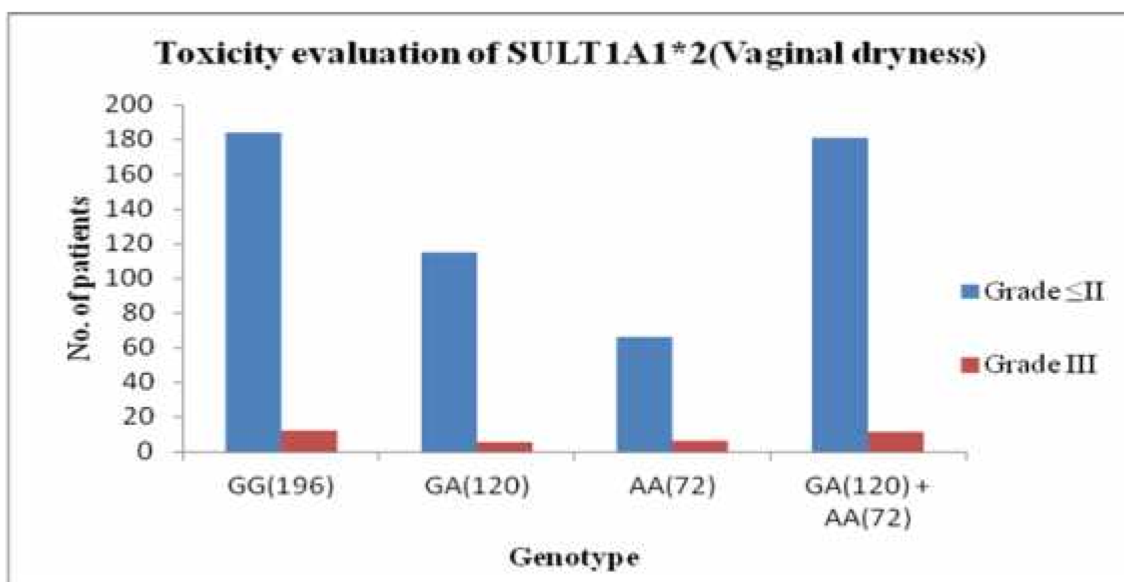
Table3.59: Distribution of different grades of toxicity among different genotype of SULT1A1*2

Vaginal dryness					
SULT11A1*2					
	Total	GG (196)	GA (120)	AA (72)	GA(120) + AA(72)
Grade ≤II	365	184	115	66	181
Grade III	23	12	5	6	11

Table3.60: Toxicity evaluation of different polymorphism for SULT1A1*2

Vaginal dryness					
SULT1A1*2					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(196)	184	12	Ref	Ref	Ref
GA(120)	115	5	0.6667(0.2289-1.9416)	0.698	0.514
AA(72)	66	6	1.3939(0.5029-3.8639)	1.707	0.321
GA(120) + AA(72)	181	11	0.9319(0.4009-2.1661)	1.004	0.993

Graph3.26: Comparison of different grades of toxicity among different genotype of SULT1A1*2



Features : No significant association was found among different polymorphism of SULT1A1*2 and Vaginal dryness.

3.3.4.17 Impact of UGT2B7*2 polymorphism on Vaginal dryness

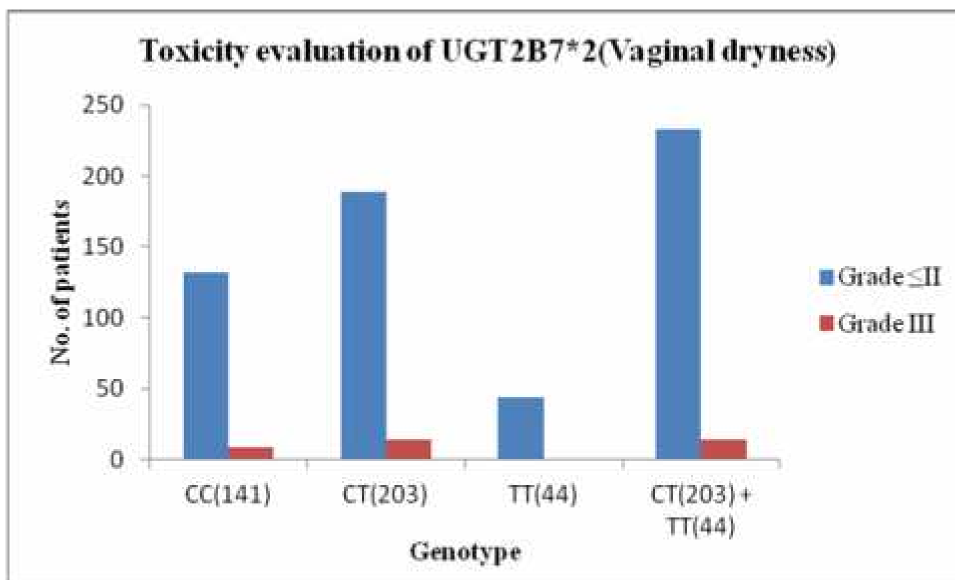
Table3.61: Distribution of different grades of toxicity among different genotype of UGT2B7*2

Vaginal dryness					
UGT2B7*2					
	Total	CC (141)	CT (203)	TT (44)	CT(203) + TT(44)
Grade ≤II	365	132	189	44	233
Grade III	23	9	14	0	14

Table3.62: Toxicity evaluation of different polymorphism for UGT2B7*2

Vaginal dryness					
UGT2B7*2					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(141)	132	9	Ref	Ref	Ref
CT(203)	189	14	1.0864(0.4568-2.5839)	1.064	0.89
TT(44)	44	0	0.1567(0.0089-2.7476)	0	0.998
CT(203) + TT(44)	233	14	0.8813(0.3714-2.0913)	0.867	0.748

Graph3.27: Comparison of different grades of toxicity among different genotype of UGT2B7*2



Features : No significant association was found among different polymorphism of UGT2B7*2 and Vaginal dryness.

3.3.4.18 Impact of CYP3A5*3 polymorphism on Vaginal dryness

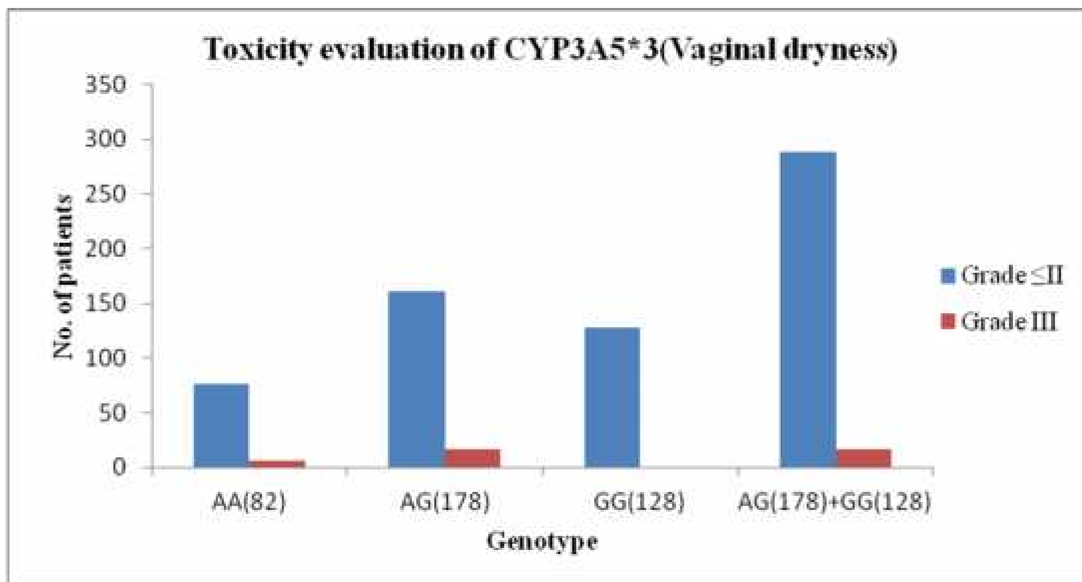
Table3.63: Distribution of different grades of toxicity among different genotype of CYP3A5*3

Vaginal dryness					
CYP3A5*3					
	Total	AA (82)	AG (178)	GG (128)	AG(178) + GG(128)
Grade ≤II	365	76	161	128	289
Grade III	23	6	17	0	17

Table3.64: Toxicity evaluation of different polymorphism for CYP3A5*3

Vaginal dryness					
CYP3A5*3					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
AA(82)	76	6	Ref	Ref	Ref
AG(178)	161	17	1.3375(0.5071-3.5277)	1.489	0.428
GG(128)	128	0	0.0458(0.0025-0.8243)	9.58E-09	0.0365
AG(178)+GG(128)	289	17	0.7451(0.2840-1.9546)	0.823	0.697

Graph3.28: Comparison of different grades of toxicity among different genotype of CYP3A5*3



Features : No significant association was found among different polymorphism of CYP3A5*3 and Vaginal dryness.

3.3.4.19 Impact of CYP2D6*4 polymorphism on Vaginal dryness

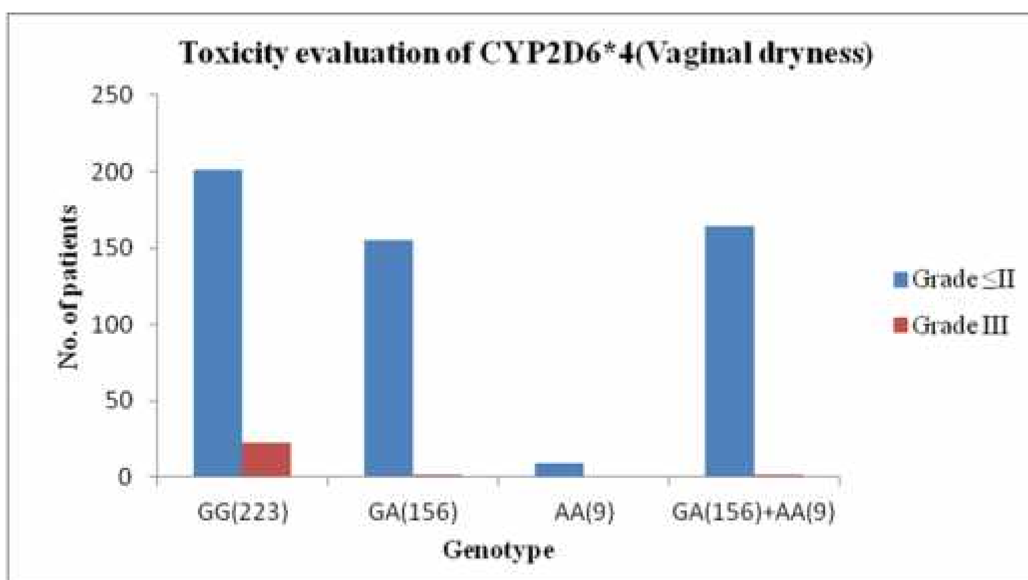
Table3.65: Distribution of different grades of toxicity among different genotype of CYP2D6*4

Vaginal dryness					
CYP2D6*4					
	Total	GG (223)	GA (156)	AA (9)	GA(156) + AA(9)
Grade ≤II	365	201	155	9	164
Grade III	23	22	1	0	1

Table3.66: Toxicity evaluation of different polymorphism for CYP2D6*4

Vaginal dryness					
CYP2D6*4					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
GG(223)	201	22	Ref	Ref	Ref
GA(156)	155	1	0.0589(0.0079-0.4421)	0.057	0.005
AA(9)	9	0	0.4713(0.0265-8.3719)	0	0.999
GA(156)+AA(9)	164	1	0.0557(0.0074-0.4177)	0.054	0.0050

Graph3.29: Comparison of different grades of toxicity among different genotype of CYP2D6*4



Features : No significant association was found among different polymorphism of CYP2D6*4 and Vaginal dryness.

3.3.4.20 Impact of CYP2D6*10 polymorphism on Vaginal dryness

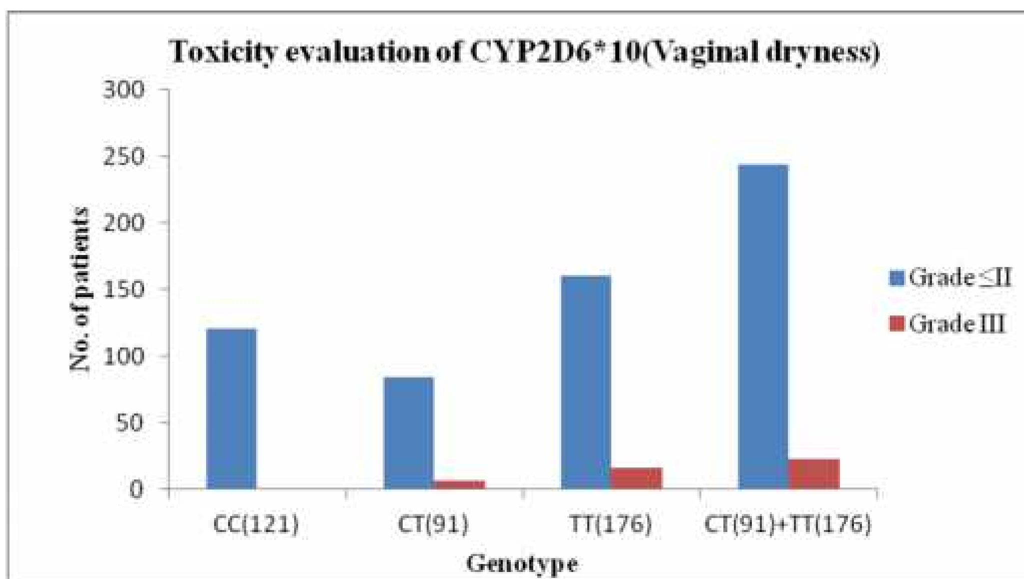
Table3.67: Distribution of different grades of toxicity among different genotype of CYP2D6*10

Vaginal dryness					
CYP2D6*10					
	Total	CC (121)	CT (91)	TT (176)	CT(91) + TT(176)
Grade ≤II	365	121	84	160	244
Grade III	23	0	7	16	23

Table3.68: Toxicity evaluation of different polymorphism for CYP2D6*10

Vaginal dryness					
CYP2D6*10					
Genotype	Grade ≤II	Grade III	**Odds Ratio (95 % CI)**	**Adjusted odd ratio**	**P Value**
CC(121)	121	0	Ref	Ref	Ref
CT(91)	84	7	21.5680(1.2153-382.7597)	1.47E+08	0.0364
TT(176)	160	16	24.9813(1.4840-420.5343)	1.59E+08	0.0255
CT(91)+TT(176)	244	23	23.3558(1.4067-387.7936)	1.59E+08	0.0279

Graph3.30: Comparison of different grades of toxicity among different genotype of CYP2D6*10



Features : Significant association was found among both hetero and mutant homo polymorphism of CYP2D6*10 and Vaginal dryness. Odd ratios were 1.47E+08 and 1.59E+08 respectively whereas p values were 0.0364 and 0.0255.

3.3.5 Correlation of different genotype with survival

3.3.5.1 Impact of SULT1A1*2 polymorphism on Hazard ratio

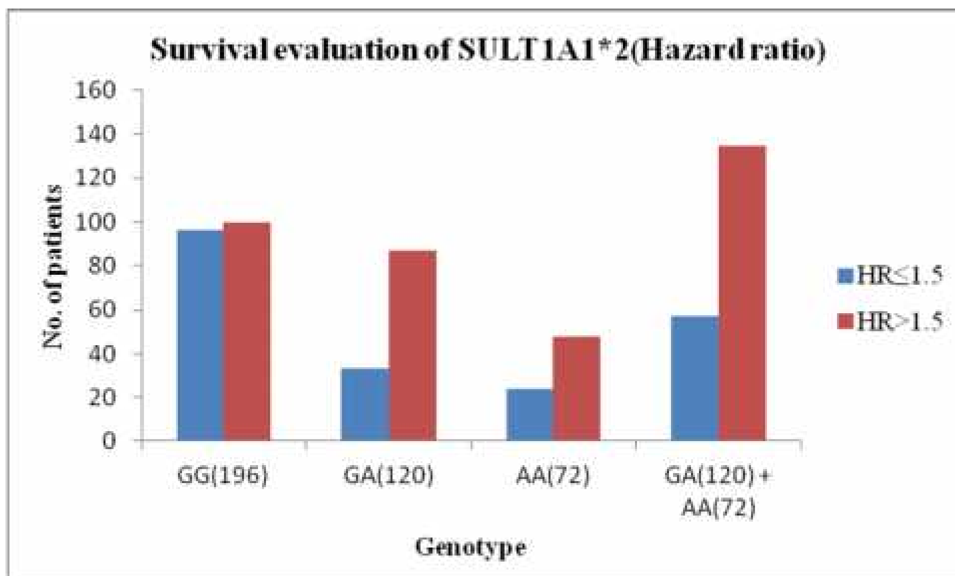
Table3.69: Distribution of different survival data among different genotype of SULT1A1*2

Hazard Ratio(HR)					
SULT11A1*2					
	Total	GG (196)	GA (120)	AA (72)	GA(120) + AA(72)
HR≤1.5	153	96	33	24	57
HR>1.5	235	100	87	48	135

Table3.70: Survival evaluation of different polymorphism for SULT1A1*2

Hazard Ratio(HR)				
SULT1A1*2				
Genotype	HR≤1.5	HR>1.5	**Odds Ratio (95 % CI)**	**P Value**
GG(196)	96	100	Ref	Ref
GA(120)	33	87	2.5309(1.5522-4.1266)	0.0002
AA(72)	24	48	1.9200(1.0919-3.3761)	0.0235
GA(120) + AA(72)	57	135	2.2737(1.4977-3.4517)	0.0001

Graph3.31: Comparison of survival data among different genotype of SULT1A1*2



Features : Significant association was found among both hetero and mutant homo polymorphism of SULT1A1*2 and Hazard ratio. Odd ratios were 21.5680 and 24.9813 respectively whereas p values were 0.0364 and 0.0255.

3.3.5.2 Impact of UGT2B7*2 polymorphism on Hazard ratio

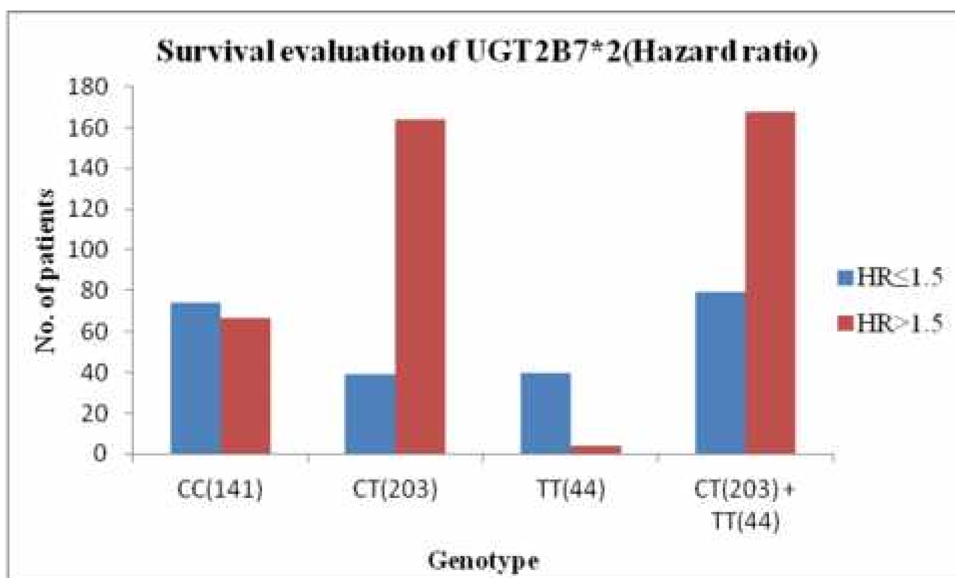
Table3.71: Distribution of different survival data among different genotype of UGT2B7*2

Hazard Ratio(HR)					
UGT2B7*2					
	Total	CC (141)	CT (203)	TT (44)	CT(203) + TT(44)
HR≤1.5	153	74	39	40	79
HR>1.5	235	67	164	4	168

Table3.72: Survival evaluation of different polymorphism for UGT2B7*2

Hazard Ratio(HR)				
UGT2B7*2				
Genotype	HR≤1.5	HR>1.5	**Odds Ratio (95 % CI)**	**P Value**
CC(141)	74	67	Ref	Ref
CT(203)	39	164	4.6445(2.8716-7.5119)	<0.0001
TT(44)	40	4	0.1104(0.0375-0.3251)	0.0001
CT(203) + TT(44)	79	168	2.3488(1.5353-3.5932)	0.0001

Graph3.32: Comparison of survival data among different genotype of UGT2B7*2



Features : Significant association was found among hetero polymorphism of UGT2B7*2 and Hazard ratio. Odd ratios was 4.6445 whereas p values was <0.0001.Other polymorphs were not significant.

3.3.5.3 Impact of CYP3A5*3 polymorphism on Hazard ratio

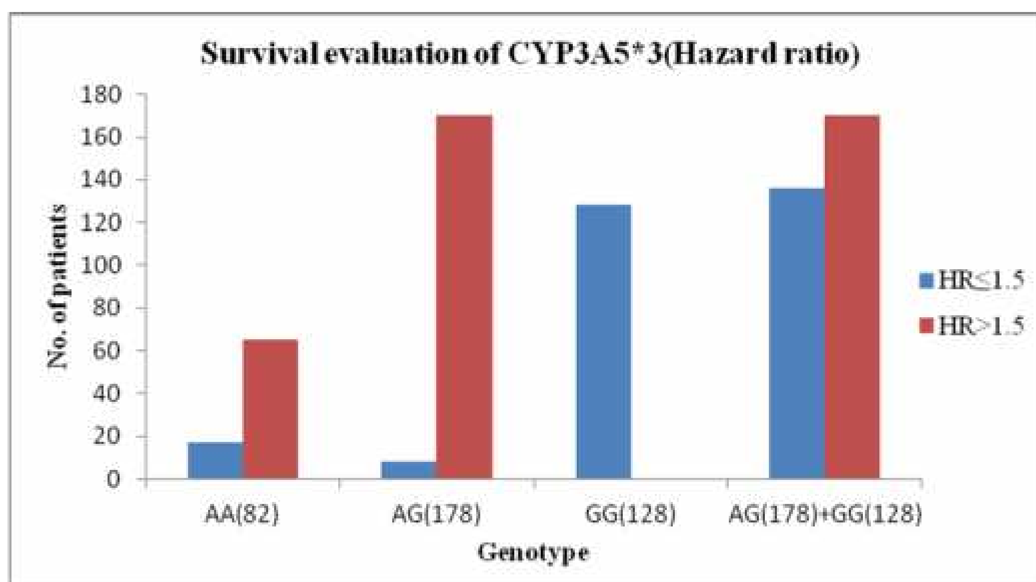
Table3.73: Distribution of different survival data among different genotype of CYP3A5*3

Hazard Ratio(HR)					
CYP3A5*3					
	Total	AA (82)	AG (178)	GG (128)	AG(178) + GG(128)
HR≤1.5	153	17	8	128	136
HR>1.5	235	65	170	0	170

Table3.74: Survival evaluation of different polymorphism for CYP3A5*3

Hazard Ratio(HR)				
CYP3A5*3				
Genotype	HR≤1.5	HR>1.5	**Odds Ratio (95 % CI)**	**P Value**
AA(82)	17	65	Ref	Ref
AG(178)	8	170	5.5577(2.2877-13.5016)	0.0002
GG(128)	128	0	0.0010(0.0001-0.0176)	<0.0001
AG(178)+GG(128)	136	170	0.3269(0.1831-0.5837)	0.0002

Graph3.33: Comparison of survival data among different genotype of CYP3A5*3



Features : Significant association was found among hetero polymorphism of CYP3A5*3 and Hazard ratio. Odd ratios was 5.5577 whereas p values was 0.0002.Other polymorphs were not significant.

3.3.5.4 Impact of CYP2D6*4 polymorphism on Hazard ratio

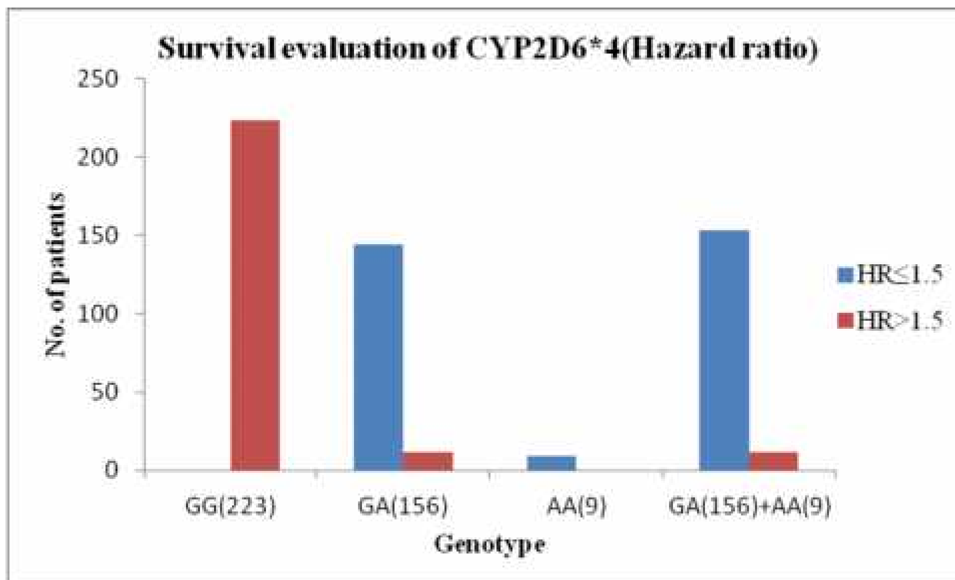
Table3.75: Distribution of different survival data among different genotype of CYP2D6*4

Hazard Ratio(HR)					
CYP2D6*4					
	Total	GG (223)	GA (156)	AA (9)	GA(156) + AA(9)
HR≤1.5	153	0	144	9	153
HR>1.5	235	223	12	0	12

Table3.76: Survival evaluation of different polymorphism for CYP2D6*4

Hazard Ratio(HR)				
CYP2D6*4				
Genotype	HR≤1.5	HR>1.5	**Odds Ratio (95 % CI)**	**P Value**
GG(223)	0	223	Ref	Ref
GA(156)	144	12	0.0002(0.0000-0.0033)	<0.0001
AA(9)	9	0	0.0001(0.0000-0.0063)	<0.0001
GA(156)+AA(9)	153	12	0.0002(0.0000-0.0031)	<0.0001

Graph3.34: Comparison of survival data among different genotype of CYP2D6*4



Features : No significant association was found among polymorphisms of CYP2D6*4 and Hazard ratio.

3.3.5.5 Impact of CYP2D6*10 polymorphism on Hazard ratio

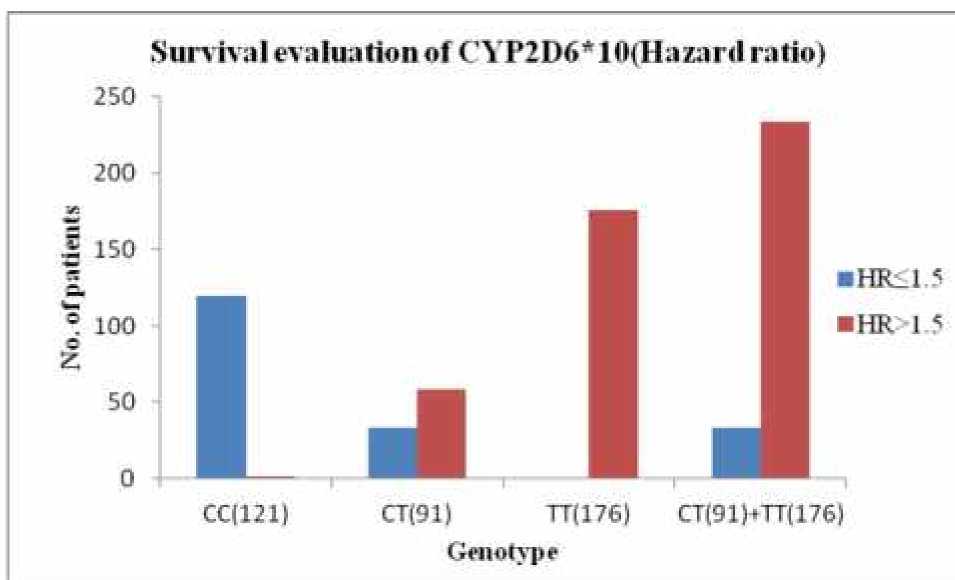
Table3.77: Distribution of different survival data among different genotype of CYP2D6*10

Hazard Ratio(HR)					
CYP2D6*10					
	Total	CC (121)	CT (91)	TT (176)	CT(91) + TT(176)
HR≤1.5	153	120	33	0	33
HR>1.5	235	1	58	176	234

Table3.78: Survival evaluation of different polymorphism for CYP2D6*10

Hazard Ratio(HR)				
CYP2D6*10				
Genotype	HR≤1.5	HR>1.5	**Odds Ratio (95 % CI)**	**P Value**
CC(121)	120	1	Ref	Ref
CT(91)	33	58	210.9091(28.1462-1580.4155)	<0.0001
TT(176)	0	176	28357.6667(1145.5230-702000.0738)	<0.0001
CT(91)+TT(176)	33	234	850.9091(114.9728-6297.5458)	<0.0001

Graph3.35: Comparison of survival data among different genotype of CYP2D6*10



Features : Significant association was found among both hetero and mutant homo polymorphisms of CYP2D6*10 and Hazard ratio. Hetero had odd ratio 210.9091and p value <0.0001 whereas mutant homo had odd ratio 28357.6667 and p value <0.0001.

3.3.5.6 Impact of SULT1A1*2 polymorphism on Survival Rate

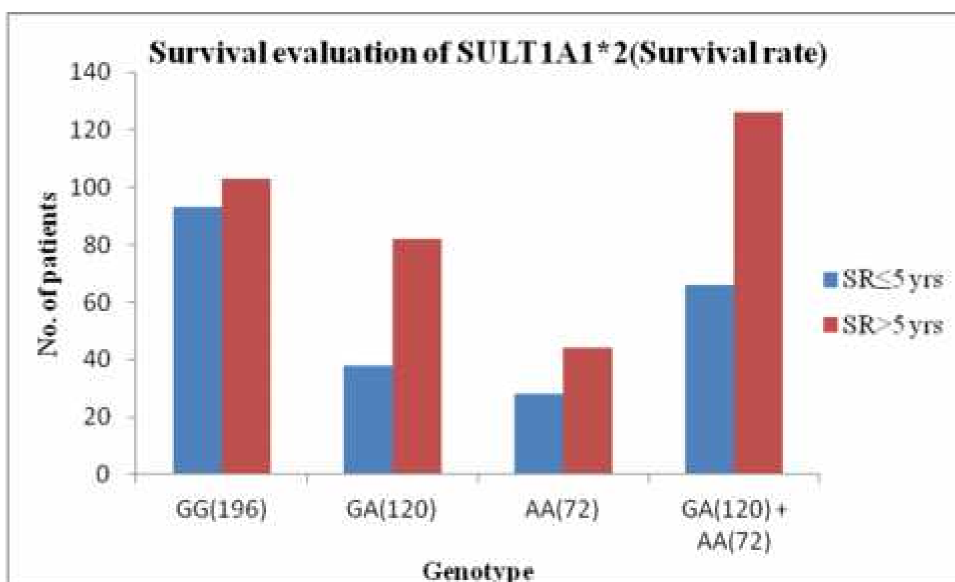
Table3.79: Distribution of different survival data among different genotype of SULT1A1*2

Survival Rate (SR)					
SULT11A1*2					
	Total	GG (196)	GA (120)	AA (72)	GA(120) + AA(72)
SR≤5 yrs	159	93	38	28	66
SR>5 yrs	229	103	82	44	126

Table3.80: Survival evaluation of different polymorphism for SULT1A1*2

Survival Rate (SR)				
SULT1A1*2				
Genotype	SR≤5 yrs	SR>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
GG(196)	93	103	Ref	Ref
GA(120)	38	82	0.5132(0.3189-0.8261)	0.0060
AA(72)	28	44	0.7048(0.4064-1.2223)	0.2129
GA(120) + AA(72)	66	126	0.5801(0.3854-0.8733)	0.0091

Graph3.36: Comparison of survival data among different genotype of SULT1A1*2



Features : No significant association was found among polymorphisms of SULT1A1*2 and Survival Rate. Though p value was significant for hetero but odd ratio was too small.

3.3.5.7 Impact of UGT2B7*2 polymorphism on Survival Rate

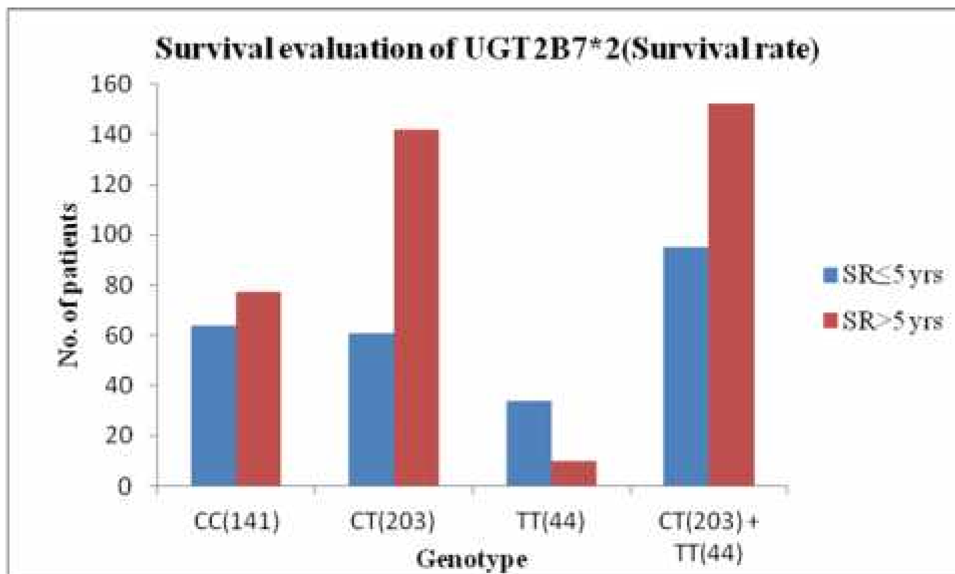
Table3.81: Distribution of different survival data among different genotype of UGT2B7*2

Survival Rate (SR)					
UGT2B7*2					
	Total	CC (141)	CT (203)	TT (44)	CT(203) + TT(44)
SR≤5 yrs	159	64	61	34	95
SR>5 yrs	229	77	142	10	152

Table3.82: Survival evaluation of different polymorphism for UGT2B7*2

Survival Rate (SR)				
UGT2B7*2				
Genotype	SR≤5 yrs	SR>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
CC(141)	64	77	Ref	Ref
CT(203)	61	142	0.5168(0.3305-0.8083)	0.0038
TT(44)	34	10	4.0906(1.8768-8.9159)	0.0004
CT(203) + TT(44)	95	152	0.7520(0.4945-1.1434)	0.1824

Graph3.37: Comparison of survival data among different genotype of UGT2B7*2



Features : Significant association was found among mutant homo polymorphisms of UGT2B7*2 and Survival Rate. p value was for 0.0004 mutant homo and odd ratio was 4.0906.

3.3.5.8 Impact of CYP3A5*3 polymorphism on Survival Rate

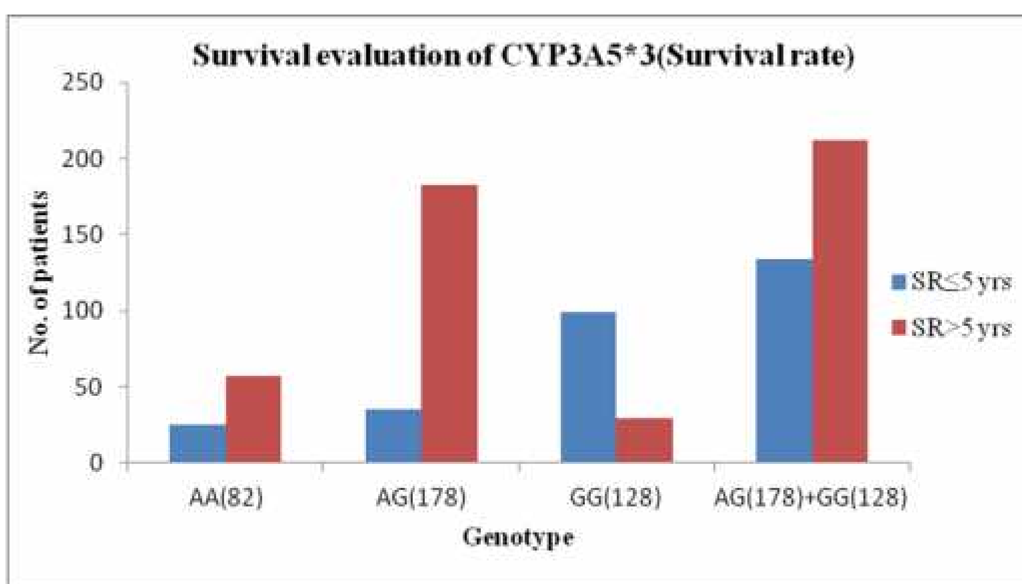
Table3.83: Distribution of different survival data among different genotype of CYP3A5*3

Survival Rate (SR)					
CYP3A5*3					
	Total	AA (82)	AG (178)	GG (128)	AG(178) + GG(128)
SR≤5 yrs	159	25	35	99	134
SR>5 yrs	269	57	183	29	212

Table3.84: Survival evaluation of different polymorphism for CYP3A5*3

Survival Rate (SR)				
CYP3A5*3				
Genotype	SR≤5 yrs	SR>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
AA(82)	25	57	Ref	Ref
AG(178)	35	183	0.4361(0.2410-0.7891)	0.0061
GG(128)	99	29	7.7834(4.1605-14.5613)	<0.0001
AG(178)+GG(128)	134	212	1.4411(0.8589-2.4181)	0.1664

Graph3.38: Comparison of survival data among different genotype of CYP3A5*3



Features : Significant association was found among mutant homo polymorphisms of CYP3A5*3 and Survival Rate. p value was for <0.0001 mutant homo and odd ratio was 7.7834.

3.3.5.9 Impact of CYP2D6*4 polymorphism on Survival Rate

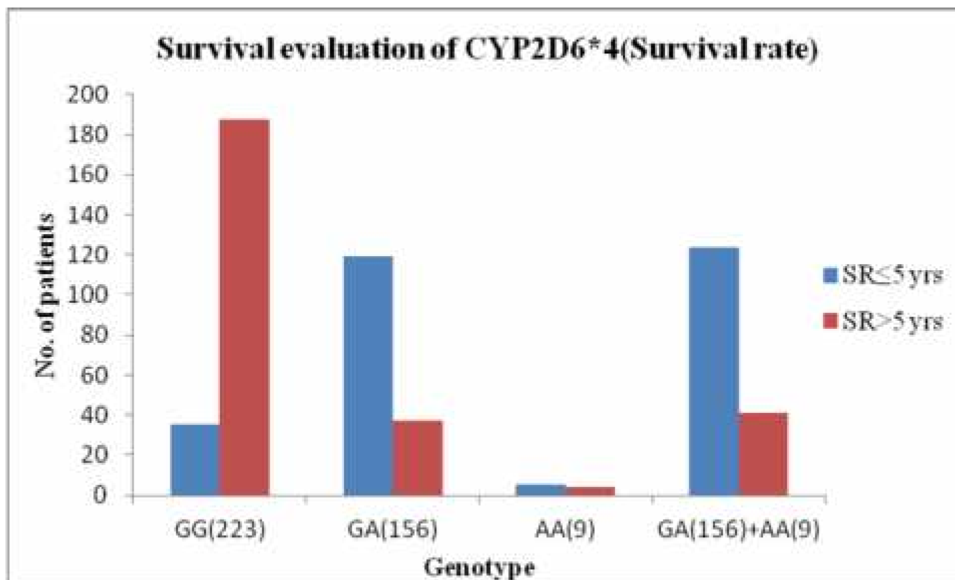
Table3.85: Distribution of different survival data among different genotype of CYP2D6*4

Survival Rate (SR)					
CYP2D6*4					
	Total	GG (223)	GA (156)	AA (9)	GA(156) + AA(9)
SR≤5 yrs	159	35	119	5	124
SR>5 yrs	269	188	37	4	41

Table3.86: Survival evaluation of different polymorphism for CYP2D6*4

Survival Rate (SR)				
CYP2D6*4				
Genotype	SR≤5 yrs	SR>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
GG(223)	35	188	Ref	Ref
GA(156)	119	37	17.2757(10.3114-28.9435)	<0.0001
AA(9)	5	4	6.7143(1.7174-26.2498)	0.0062
GA(156)+AA(9)	124	41	16.2453(9.8056-26.9141)	<0.0001

Graph3.39: Comparison of survival data among different genotype of CYP2D6*4



Features : Significant association was found among both hetero and mutant homo polymorphisms of CYP2D6*4 and Survival Rate. p value were <0.0001 and 0.0062 for hetero and mutant homo respectively whereas odd ratio were 17.2757 and 6.7143.

3.3.5.10 Impact of CYP2D6*10 polymorphism on Survival Rate

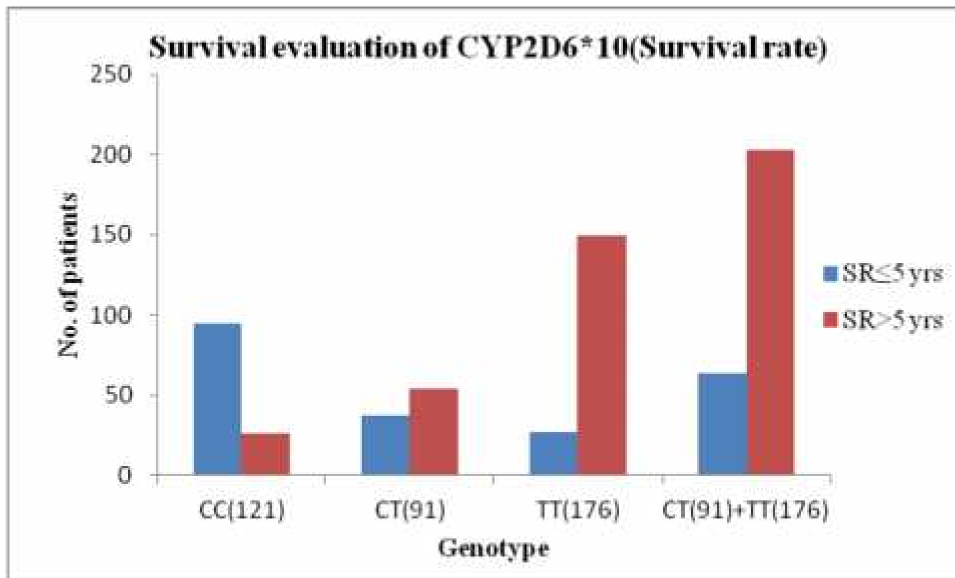
Table3.87: Distribution of different survival data among different genotype of CYP2D6*10

Survival Rate (SR)					
CYP2D6*10					
	Total	CC (121)	CT (91)	TT (176)	CT(91) + TT(176)
SR≤5 yrs	159	95	37	27	64
SR>5 yrs	269	26	54	149	203

Table3.88: Survival evaluation of different polymorphism for CYP2D6*10

Survival Rate (SR)				
CYP2D6*10				
Genotype	SR≤5 yrs	SR>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
CC(121)	95	26	Ref	Ref
CT(91)	37	54	0.1875(0.1026-0.3426)	<0.0001
TT(176)	27	149	0.0496(0.0273-0.0901)	<0.0001
CT(91)+TT(176)	64	203	0.0863(0.0515-0.1447)	<0.0001

Graph3.40: Comparison of survival data among different genotype of CYP2D6*10



Features : No significant association was found among polymorphisms of CYP2D6*10 and Survival Rate. Though p value were found significant for both hetero and mutant homo but odd ratio found insignificant.

3.3.5.11 Impact of SULT1A1*2 polymorphism on Recurrence Free Survival

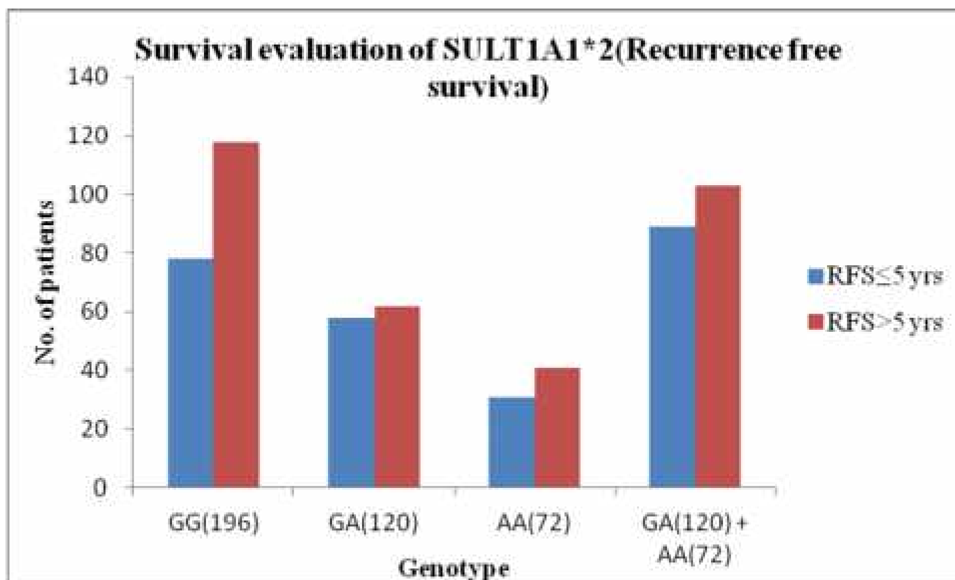
Table3.89: Distribution of different survival data among different genotype of SULT1A1*2

Recurrence Free Survival (RFS)					
SULT11A1*2					
	Total	GG (196)	GA (120)	AA (72)	GA(120) + AA(72)
RFS≤5 yrs	167	78	58	31	89
RFS>5 yrs	221	118	62	41	103

Table3.90: Survival evaluation of different polymorphism for SULT1A1*2

Recurrence Free Survival (RFS)				
SULT1A1*2				
Genotype	RFS≤5 yrs	RFS>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
GG(196)	78	118	Ref	Ref
GA(120)	58	62	1.4152(0.8950-2.2379)	0.1375
AA(72)	31	41	1.1438(0.6618-1.9770)	0.6303
GA(120) + AA(72)	89	103	1.3072(0.8738-1.9556)	0.1924

Graph3.41: Comparison of survival data among different genotype of SULT1A1*2



Features : No significant association was found among polymorphisms of SULT1A1*2 and Recurrence Free Survival.

3.3.5.12 Impact of UGT2B7*2 polymorphism on Recurrence Free Survival

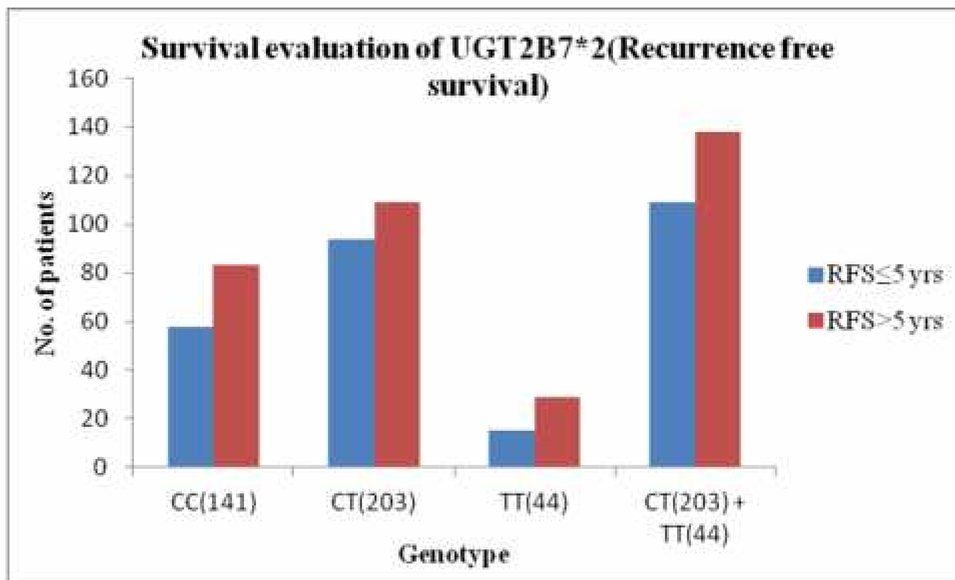
Table3.91: Distribution of different survival data among different genotype of UGT2B7*2

Recurrence Free Survival (RFS)					
UGT2B7*2					
	Total	CC (141)	CT (203)	TT (44)	CT(203) + TT(44)
RFS≤5 yrs	167	58	94	15	109
RFS>5 yrs	221	83	109	29	138

Table3.92: Survival evaluation of different polymorphism for UGT2B7*2

Recurrence Free Survival (RFS)				
UGT2B7*2				
Genotype	RFS≤5 yrs	RFS>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
CC(141)	58	83	Ref	Ref
CT(203)	94	109	1.2341(0.7993-1.9053)	0.3425
TT(44)	15	29	0.7402(0.3647-1.5024)	0.4048
CT(203) + TT(44)	109	138	1.1303(0.7434-1.7186)	0.5667

Graph3.42: Comparison of survival data among different genotype of UGT2B7*2



Features : No significant association was found among polymorphisms of UGT2B7*2 and Recurrence Free Survival.

3.3.5.13 Impact of CYP3A5*3 polymorphism on Recurrence Free Survival

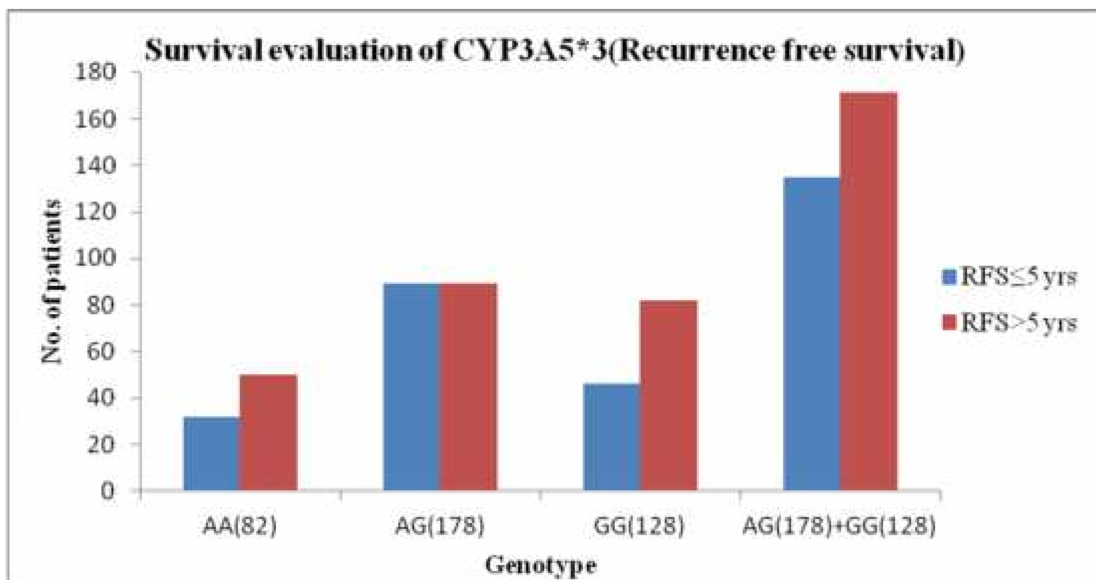
Table3.93: Distribution of different survival data among different genotype of CYP3A5*3

Recurrence Free Survival (RFS)					
CYP3A5*3					
	Total	AA (82)	AG (178)	GG (128)	AG(178) + GG(128)
RFS≤5 yrs	167	32	89	46	135
RFS>5 yrs	221	50	89	82	171

Table3.94: Survival evaluation of different polymorphism for CYP3A5*3

Recurrence Free Survival (RFS)				
CYP3A5*3				
Genotype	RFS≤5 yrs	RFS>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
AA(82)	32	50	Ref	Ref
AG(178)	89	89	1.5625(0.9177-2.6604)	0.1002
GG(128)	46	82	0.8765(0.4947-1.5531)	0.6516
AG(178)+GG(128)	135	171	1.2336(0.7498-2.0293)	0.4086

Graph3.43: Comparison of survival data among different genotype of CYP3A5*3



Features : No significant association was found among polymorphisms of CYP3A5*3 and Recurrence Free Survival.

3.3.5.14 Impact of CYP2D6*4 polymorphism on Recurrence Free Survival

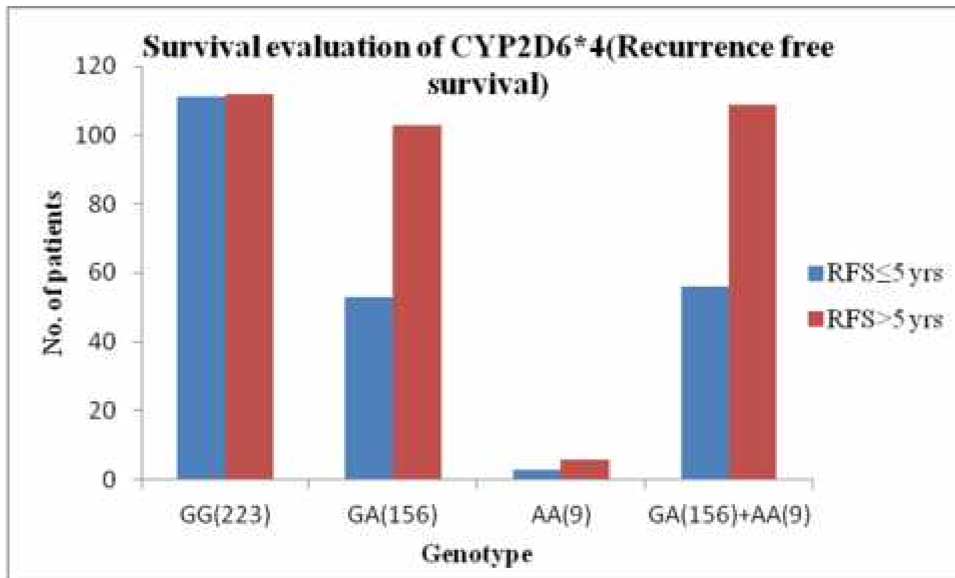
Table3.95: Distribution of different survival data among different genotype of CYP2D6*4

Recurrence Free Survival (RFS)					
CYP2D6*4					
	Total	GG (223)	GA (156)	AA (9)	GA(156) + AA(9)
RFS≤5 yrs	167	111	53	3	56
RFS>5 yrs	221	112	103	6	109

Table3.96: Survival evaluation of different polymorphism for CYP2D6*4

Recurrence Free Survival (RFS)				
CYP2D6*4				
Genotype	RFS≤5 yrs	RFS>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
GG(223)	111	112	Ref	Ref
GA(156)	53	103	0.5192(0.3402-0.7924)	0.0024
AA(9)	3	6	0.5045(0.1231-2.0676)	0.3418
GA(156)+AA(9)	56	109	0.5184(0.3421-0.7855)	0.0019

Graph3.44: Comparison of survival data among different genotype of CYP2D6*4



Features : No significant association was found among polymorphisms of CYP2D6*4 and Recurrence Free Survival. Though p value was found below 0.05 but odd ratio was insignificant.

3.3.5.15 Impact of CYP2D6*10 polymorphism on Recurrence Free Survival

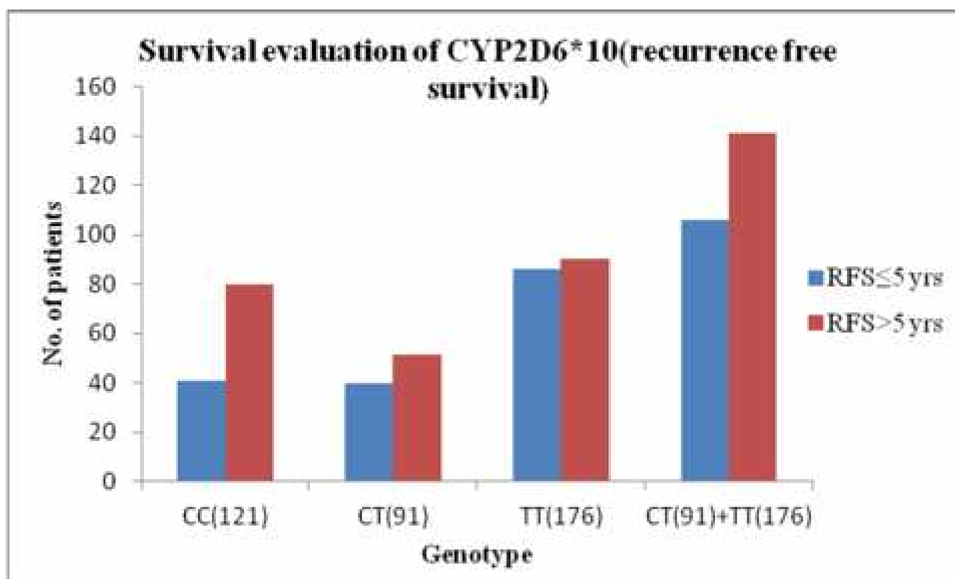
Table3.97: Distribution of different survival data among different genotype of CYP2D6*10

Recurrence Free Survival (RFS)					
CYP2D6*10					
	Total	CC (121)	CT (91)	TT (176)	CT(91) + TT(176)
RFS≤5 yrs	167	41	40	86	106
RFS>5 yrs	221	80	51	90	141

Table3.98: Survival evaluation of different polymorphism for CYP2D6*10

Recurrence Free Survival (RFS)				
CYP2D6*10				
Genotype	RFS≤5 yrs	RFS>5 yrs	**Odds Ratio (95 % CI)**	**P Value**
CC(121)	41	80	Ref	Ref
CT(91)	40	51	1.5304(0.8746-2.6779)	0.1361
TT(176)	86	90	1.8645(1.1553-3.0090)	0.0107
CT(91)+TT(176)	106	141	1.4669(0.9325-2.3074)	0.0974)

Graph3.45: Comparison of survival data among different genotype of CYP2D6*10



Features : Significant association was found among mutant homo polymorphisms of CYP2D6*10 and Recurrence Free Survival. p value was found 0.0107 and odd ratio was 1.8645.

3.3.5.16 Impact of SULT1A1*2 polymorphism on Breast Cancer Specific Survival

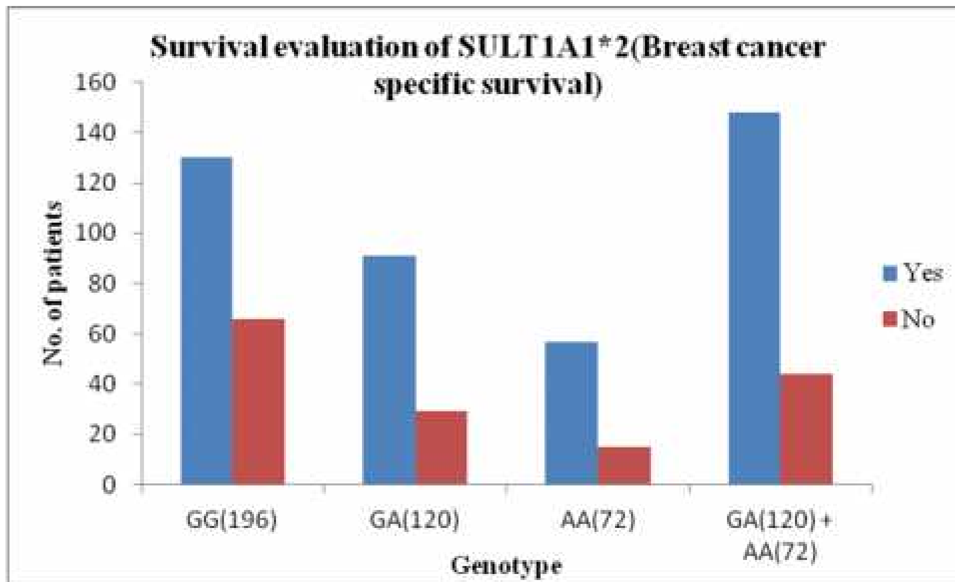
Table3.99: Distribution of different survival data among different genotype of SULT1A1*2

Breast Cancer Specific Survival(BCSS)					
SULT11A1*2					
	Total	GG (196)	GA (120)	AA (72)	GA(120) + AA(72)
Yes	278	130	91	57	148
No	110	66	29	15	44

Table3.100: Survival evaluation of different polymorphism for SULT1A1*2

Breast Cancer Specific Survival(BCSS)				
SULT1A1*2				
Genotype	Yes	No	**Odds Ratio (95 % CI)**	**P Value**
GG(196)	130	66	Ref	Ref
GA(120)	91	29	0.6277(0.3761-1.0477)	0.0748
AA(72)	57	15	0.5183(0.2730-0.9843)	0.0446
GA(120) + AA(72)	148	44	0.5856(0.3740-0.9169)	0.0193

Graph3.46: Comparison of survival data among different genotype of SULT1A1*2



Features : No significant association was found among polymorphisms of SULT1A1*2 and Breast cancer specific survival.

3.3.5.17 Impact of UGT2B7*2 polymorphism on Breast Cancer Specific Survival

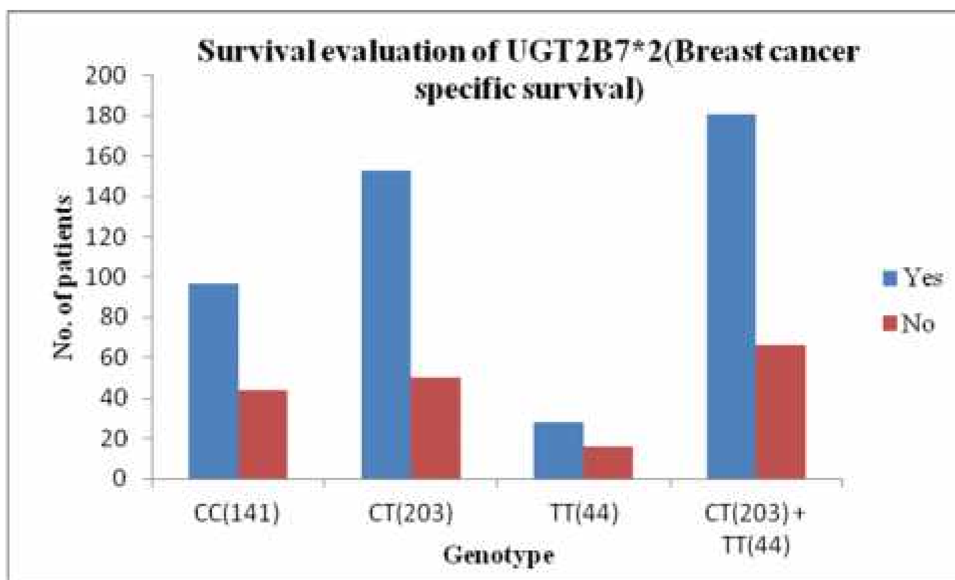
Table3.101: Distribution of different survival data among different genotype of UGT2B7*2

Breast Cancer Specific Survival(BCSS)					
UGT2B7*2					
	Total	CC (141)	CT (203)	TT (44)	CT(203) + TT(44)
Yes	278	97	153	28	181
No	110	44	50	16	66

Table3.102: Survival evaluation of different polymorphism for UGT2B7*2

Breast Cancer Specific Survival(BCSS)				
UGT2B7*2				
Genotype	Yes	No	**Odds Ratio (95 % CI)**	**P Value**
CC(141)	97	44	Ref	Ref
CT(203)	153	50	0.7204(0.4465-1.1624)	0.1791
TT(44)	28	16	1.2597(0.6193-2.5625)	0.5239
CT(203) + TT(44)	181	66	0.8039(0.5104-1.2661)	0.3462

Graph3.47: Comparison of survival data among different genotype of UGT2B7*2



Features : No significant association was found among polymorphisms of UGT2B7*2 and Breast cancer specific survival.

3.3.5.18 Impact of CYP3A5*3 polymorphism on Breast Cancer Specific Survival

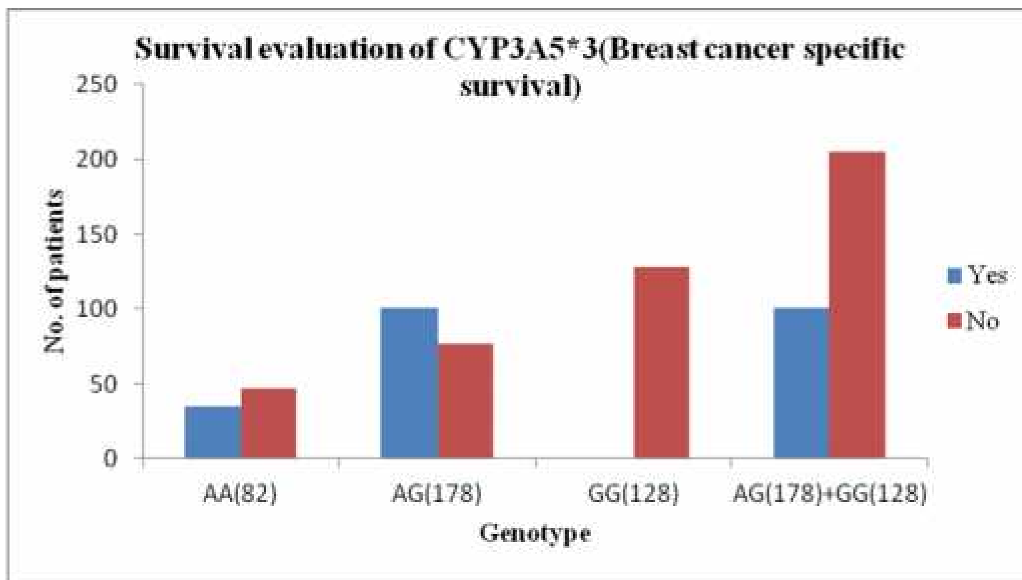
Table3.103: Distribution of different survival data among different genotype of CYP3A5*3

Breast Cancer Specific Survival(BCSS)					
CYP3A5*3					
	Total	AA (82)	AG (178)	GG (128)	AG(178) + GG(128)
Yes	136	35	101	0	101
No	252	47	77	128	205

Table3.104: Survival evaluation of different polymorphism for CYP3A5*3

Breast Cancer Specific Survival(BCSS)				
CYP3A5*3				
Genotype	Yes	No	**Odds Ratio (95 % CI)**	**P Value**
AA(82)	35	47	Ref	Ref
AG(178)	101	77	0.5677(0.3346-0.9632)	0.0358
GG(128)	0	128	192.0737(11.5513-3193.7840)	0.0002
AG(178)+GG(128)	101	205	1.5115(0.9183-2.4877)	0.1042

Graph3.48: Comparison of survival data among different genotype of CYP3A5*3



Features : Significant association was found among mutant homo polymorphisms of CYP3A5*3 and Breast cancer specific survival. Odd ratio was 192.0737 whereas p value was found 0.0002.

3.3.5.19 Impact of CYP2D6*4 polymorphism on Breast Cancer Specific Survival

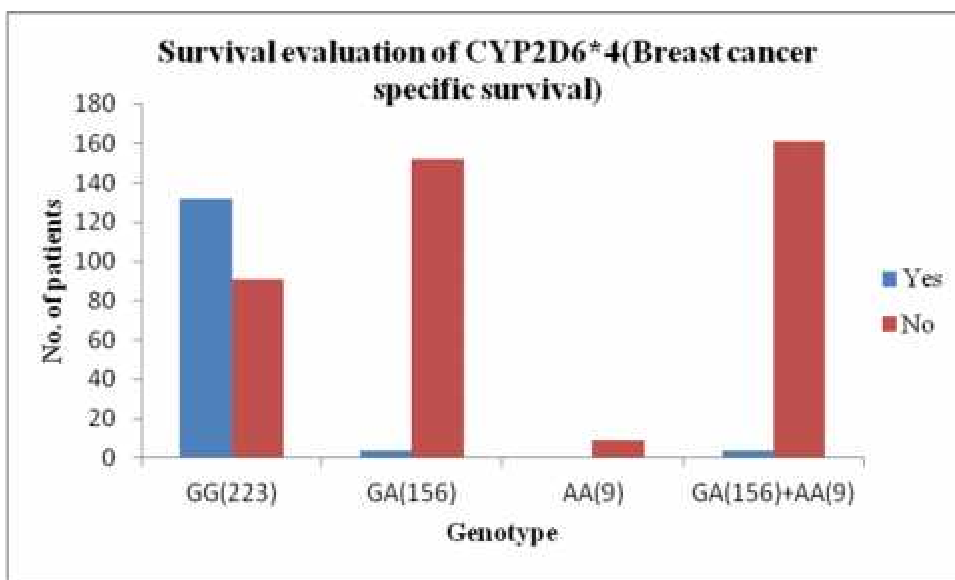
Table3.105: Distribution of different survival data among different genotype of CYP2D6*4

Breast Cancer Specific Survival(BCSS)					
CYP2D6*4					
	Total	GG (223)	GA (156)	AA (9)	GA(156) + AA(9)
Yes	136	132	4	0	4
No	252	91	152	9	161

Table3.106: Survival evaluation of different polymorphism for CYP2D6*4

Breast Cancer Specific Survival(BCSS)				
CYP2D6*4				
Genotype	Yes	No	**Odds Ratio (95 % CI)**	**P Value**
GG(223)	132	91	Ref	Ref
GA(156)	4	152	55.1209(19.7159-154.1043)	<0.0001
AA(9)	0	9	27.5137(1.5815-478.6652)	0.0229
GA(156)+AA(9)	4	161	58.3846(20.8977-163.1168)	<0.0001

Graph3.49: Comparison of survival data among different genotype of CYP2D6*4



Features : Significant association was found among both hetero and mutant homo polymorphisms of CYP2D6*4 and Breast cancer specific survival. Odd ratio were 55.1209 and 27.5137 respectively whereas p value were found <0.0001 and 0.0229.

3.3.5.20 Impact of CYP2D6*10 polymorphism on Breast Cancer Specific Survival

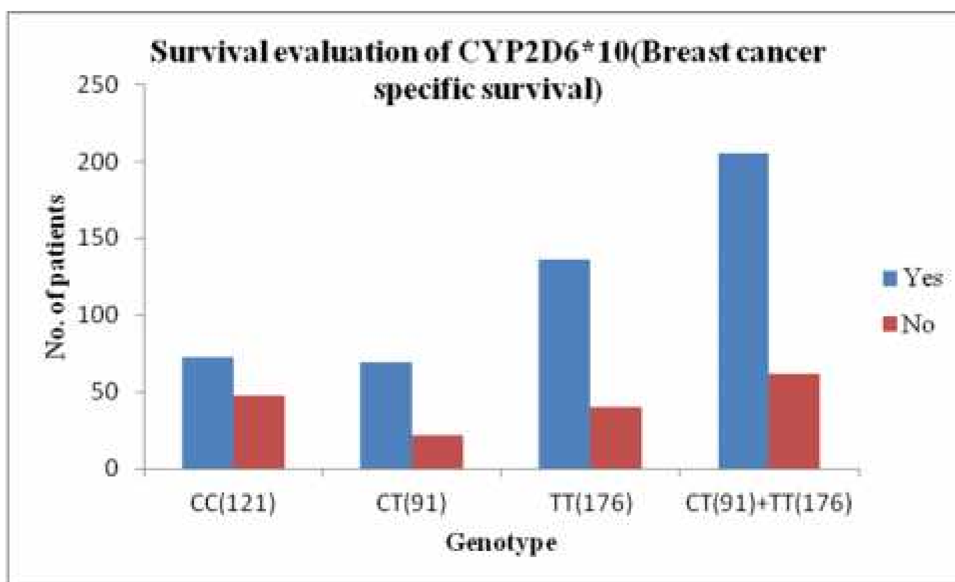
Table3.107: Distribution of different survival data among different genotype of CYP2D6*10

Breast Cancer Specific Survival(BCSS)					
CYP2D6*10					
	Total	CC (121)	CT (91)	TT (176)	CT(91) + TT(176)
Yes	136	73	69	136	205
No	252	48	22	40	62

Table3.108: Survival evaluation of different polymorphism for CYP2D6*10

Breast Cancer Specific Survival(BCSS)				
CYP2D6*10				
Genotype	Yes	No	**Odds Ratio (95 % CI)**	**P Value**
CC(121)	73	48	Ref	Ref
CT(91)	69	22	0.4849(0.2655-0.8857)	0.0185
TT(176)	136	40	0.4473(0.2694-0.7426)	0.0019
CT(91)+TT(176)	205	62	0.4600(0.2898-0.7300)	0.0010

Graph3.50: Comparison of survival data among different genotype of CYP2D6*10



Features : No significant association was found among polymorphisms of CYP2D6*10 and Breast cancer specific survival. Though p value were found significant for both hetero and mutant homo but odd ratio were insignificant.

3.3.6 Correlation of different genotype with performance status

3.3.6.1 Impact of SULT1A1*2 polymorphism on performance status

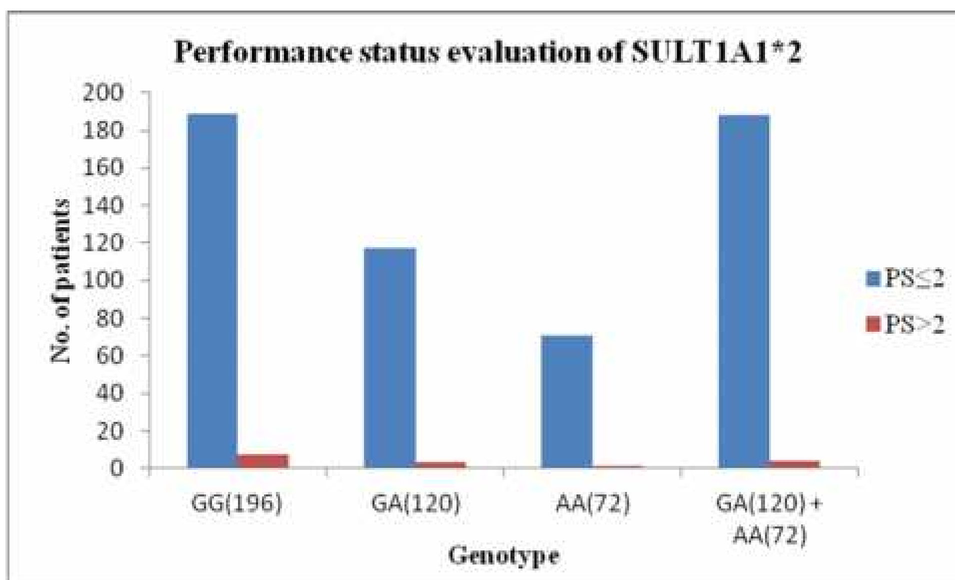
Table3.109: Distribution of different performance status data among different genotype of SULT1A1*2

Performance Status(PS)					
SULT11A1*2					
	Total	GG (196)	GA (120)	AA (72)	GA(120) + AA(72)
PS≤2	**377**	189	117	71	188
PS>2	**11**	7	3	1	4

Table3.110: Performance status evaluation of different polymorphism for SULT1A1*2

Performance Status(PS)				
SULT1A1*2				
Genotype	**PS≤2**	**PS>2**	**Odds Ratio (95 % CI)**	**P Value**
GG(196)	189	7	Ref	Ref
GA(120)	117	3	0.6923(0.1756-2.7300)	0.5994
AA(72)	71	1	0.3803(0.0460-3.1461)	0.3698
GA(120) + AA(72)	188	4	0.5745(0.1654-1.9950)	0.3828

Graph3.51: Comparison of performance status data among different genotype of SULT1A1*2



Features : No significant association was found among polymorphisms of SULT1A1*2 and performance status.

3.3.6.2 Impact of UGT2B7*2 polymorphism on performance status

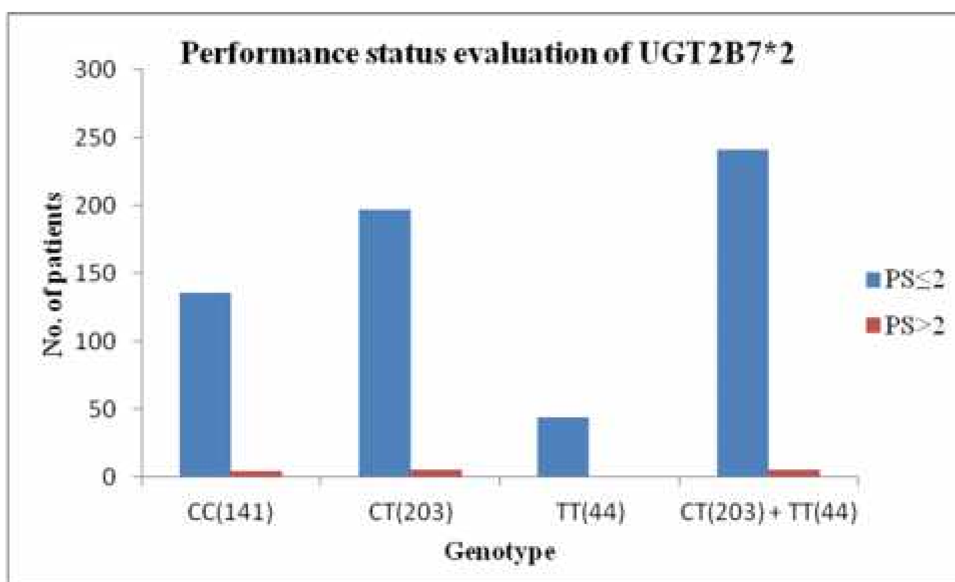
Table3.111: Distribution of different performance status data among different genotype of UGT2B7*2

Performance Status(PS)					
UGT2B7*2					
	Total	CC (141)	CT (203)	TT (44)	CT(203) + TT(44)
PS≤2	**377**	136	197	44	241
PS>2	**11**	5	6	0	6

Table3.112: Performance status evaluation of different polymorphism for UGT2B7*2

Performance Status(PS)				
UGT2B7*2				
Genotype	**PS≤2**	**PS>2**	**Odds Ratio (95 % CI)**	**P Value**
CC(141)	136	5	Ref	Ref
CT(203)	197	6	0.8284(0.2478-2.7692)	0.7598
TT(44)	44	0	0.2789(0.0151-5.1436)	0.3905
CT(203) + TT(44)	241	6	0.6772(0.2029-2.2603)	0.5261

Graph3.52: Comparison of performance status data among different genotype of UGT2B7*2



Features : No significant association was found among polymorphisms of UGT2B7*2 and performance status.

3.3.6.3 Impact of CYP3A5*3 polymorphism on performance status

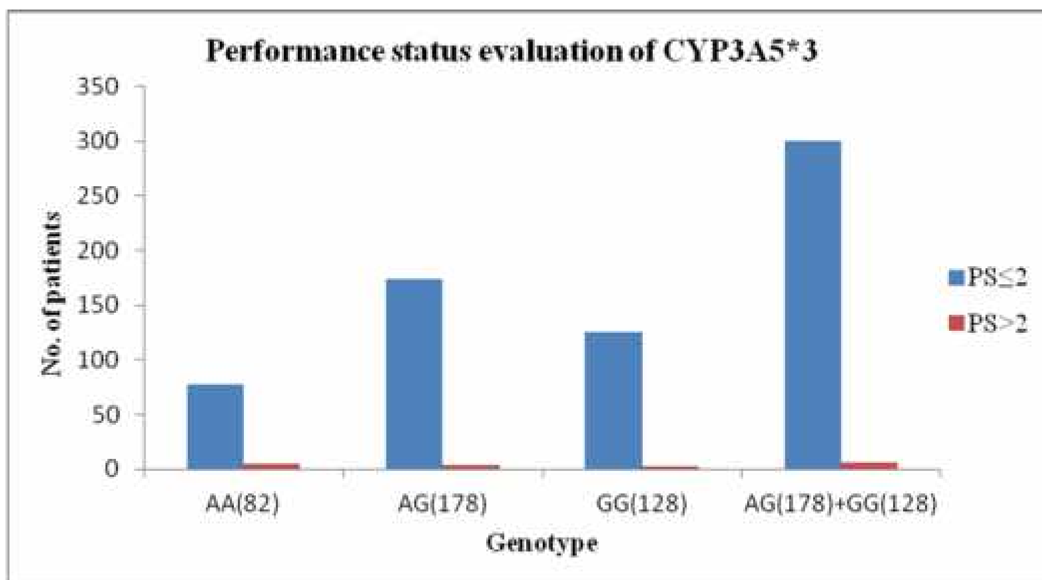
Table3.113: Distribution of different performance status data among different genotype of CYP3A5*3

Performance Status(PS)					
CYP3A5*3					
	Total	AA (82)	AG (178)	GG (128)	AG(178) + GG(128)
PS≤2	377	77	174	126	300
PS>2	11	5	4	2	6

Table3.114: Performance status evaluation of different polymorphism for CYP3A5*3

Performance Status(PS)				
CYP3A5*3				
Genotype	PS≤2	PS>2	**Odds Ratio (95 % CI)**	**P Value**
AA(82)	77	5	Ref	Ref
AG(178)	174	4	0.3540(0.0925-1.3546)	0.1293
GG(128)	126	2	0.2444(0.0463-1.2910)	0.0971
AG(178)+GG(128)	300	6	0.3080(0.0916-1.0359)	0.0570

Graph3.53: Comparison of performance status data among different genotype of CYP3A5*3



Features : No significant association was found among polymorphisms of CYP3A5*3 and performance status.

3.3.6.4 Impact of CYP2D6*4 polymorphism on performance status

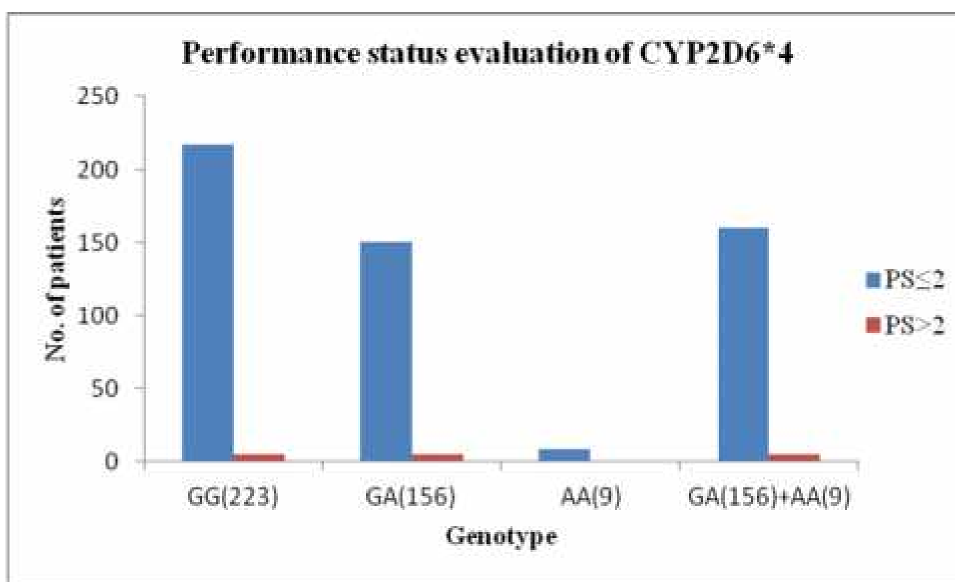
Table3.115: Distribution of different performance status data among different genotype of CYP2D6*4

Performance Status(PS)					
CYP2D6*4					
	Total	GG (223)	GA (156)	AA (9)	GA(156) + AA(9)
PS≤2	377	217	151	9	160
PS>2	11	5	5	0	5

Table3.116: Performance status evaluation of different polymorphism for CYP2D6*4

Performance Status(PS)				
CYP2D6*4				
Genotype	PS≤2	PS>2	**Odds Ratio (95 % CI)**	**P Value**
GG(223)	217	5	Ref	Ref
GA(156)	151	5	1.4371(0.4089-5.0506)	0.5718
AA(9)	9	0	2.0813(0.1071-40.4520)	0.6282
GA(156)+AA(9)	160	5	1.3563(0.3861-4.7638)	0.6345

Graph3.54: Comparison of performance status data among different genotype of CYP2D6*4



Features : No significant association was found among polymorphisms of CYP2D6*4 and performance status.

3.3.6.5 Impact of CYP2D6*10 polymorphism on performance status

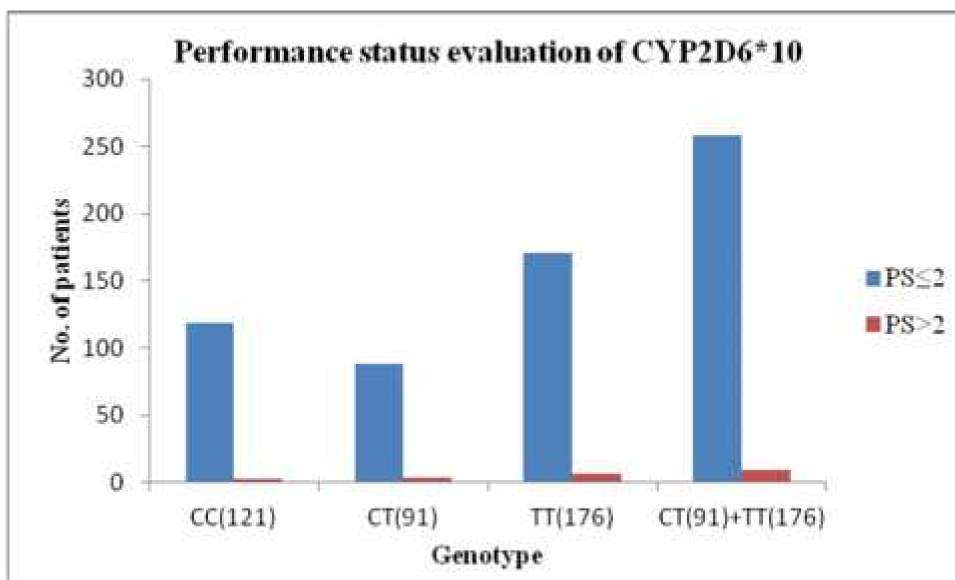
Table3.117: Distribution of different performance status data among different genotype of CYP2D6*10

Performance Status(PS)					
CYP2D6*10					
	Total	CC (121)	CT (91)	TT (176)	CT(91) + TT(176)
PS≤2	377	119	88	170	258
PS>2	11	2	3	6	9

Table3.118: Performance status evaluation of different polymorphism for CYP2D6*10

Performance Status(PS)				
CYP2D6*10				
Genotype	PS≤2	PS>2	**Odds Ratio (95 % CI)**	**P Value**
CC(121)	119	2	Ref	Ref
CT(91)	88	3	2.0284(0.3319-12.3985)	0.4438
TT(176)	170	6	2.1000(0.4167-10.5838)	0.3686
CT(91)+TT(176)	258	9	2.0756(0.4416-9.7548)	0.3050

Graph3.55: Comparison of performance status data among different genotype of CYP2D6*10



Features : No significant association was found among polymorphisms of CYP2D6*10 and performance status.

3.4 DISCUSSION:

3.4.1 Effect of different polymorphism on response evaluation

No significant association was found between responders (both complete response and partial response) and different polymorphism of CYP2D6*4 among Bangladeshi population.

We found statistically significant association of both hetero (Adjusted OR=2.933 and p value=3.97E-05) and mutant homo (Adjusted OR=2.358 and p value=0.0058) polymorphisms of SULT1A1*2 and response among our study population. It means A allele and at least one variant of A allele of this genotype contribute to the response. As A allele for SULT1A1*2 is low in our population (only 34.02%).So, fewer people of our population will be responsive to this drug with this polymorphism.

Significant association was observed among hetero polymorph of UGT2B7*2(Adjusted OR=4.213 and P value=0.0001). It signifies presence of both allele C and T contributes to the response. As UGT2B7 expression has been detected in a variety of tissues including liver, the gastrointestinal tract and breast, variations in UGT2B7 function or expression could potentially significantly impact individual response to drugs or chemotherapeutic agents (Philip Lazarus et al, 2009).

Significant association between hetero polymorph of CYP3A5*3 and drug response was also found prominent (Adjusted OR=5.626 and $p<0.0001$).In this case G and A allele both are responsible for drug response.

We also found both hetero (Adjusted OR=29.082 and p $value<0.0001$) and mutant homo (Adjusted OR=1.33E+15 and p $value<0.0001$) polymorphisms of CYP2D6*10 associated significantly with response. It means T allele and at least one T allele contribute significantly in response in Bangladeshi population. As T allele for CYP2D6*10 is high in our population (57.09%).So, larger population of our study people group will be responsive to this drug with this polymorphism.

In subgroup analysis we found all the responders carrying aforesaid polymorphism are postmenoposal, BMI 20-25Kg/m^2 and estrogen receptor positive patients. All

belongs to the age group 45-55 yrs. Our result is in compliance with previous study on different population. Among the postmenopausal women with ER-positive breast cancer who were randomized to 5 years of tamoxifen, there was a significantly higher response of the disease event for those with different allele of CYP2D6, but this was not observed in patients treated with anastrozole following tamoxifen (Goetz et al,2012). The polymorphisms of *CYP2D6**4 was significantly associated with lower endoxifen and higher N-desmethyltamoxifen (NDM) concentrations. Patients who were **1/*1* carriers exhibited 2.4- to 2.6-fold higher endoxifen concentrations and 1.9- to 2.1-fold lower NDM concentrations than either **10/*10* or **5/*10* carriers ($P < 0.001$). Similar relationships were observed between the *CYP2D6* polymorphisms and metabolic ratios of tamoxifen and its metabolites (Joanne S L Lim et al,2011). Genetic variants in UGTs that are highly active against Tamoxifen metabolites significantly alter Tamoxifen metabolism in vitro and, potentially, its elimination in Tamoxifen-treated individuals. Similar to that described above for CYP2D6, this could potentially affect overall patient response to Tamoxifen (Blevins-Primeau et al., 2010). Other polymorphisms of different genes were not found significant. This may be due to poor contribution on drug metabolism. Specially we did not find any correlation among CYP2D6*4 polymorphism and response data among Bangladeshi population. Our result is in contrast with the findings of Balram Chowbay et al,2011 for CYP2D6*4.

3.4.2 Effect of different polymorphism on toxicities evaluation

Significant association was found among hetero polymorphs of CYP2D6*10 and Hot flashes. We got Adjusted odd ratio of 8.40E+07 and p value of 0.0479 for this polymorph. It denotes presence of C and T alleles contribute equally to this toxicity. Other polymorphs of other selected genes were not found significant.

Significant association was found for hetero polymorph of UGT2B7*2 and depression. Here we got Adjusted odd ratio of 2.61 and p value of 0.033. We also found significant association of both hetero and mutant homo polymorph of CYP2D6*10 and depression. For hetero type polymorph, we got Adjusted odd ratio of 1.77E+08 and p value 0.0223 and for mutant homo type polymorph, we got Adjusted odd ratio of 2.32E+08 whereas p value was 0.0117. Here the contributory allele for this toxicity is T allele. As T allele for CYP2D6*10 is high in our population

(57.09%).So, larger population of our study people group will show toxicity to this drug with this polymorphism.

We found significant association of SULT1A1*2 hetero and mutant polymorph and degreased libido.Here Adjusted odd ratio were 1.58E+07 and 6.31E+06 respectively whereas p value were 0.0215 and 0.0017 respectively.Here A allele and at least one A allele are responsible for this toxicity. As A allele for SULT1A1*2 is low in our population (only 34.02%).So, fewer people of our population will show toxicity to this drug with this polymorphism. SULT1A1 allele would increase the rate of elimination of CPA, thus decreasing the individual's exposure to its cytotoxic effects (Vesa Kataja et al,2012). The most frequent polymorphism in the SULT1A1 gene is a G→A transition at nucleotide 638, defining the SULT1A1*2 allele, which is correlated with a diminished capacity to sulphate SULT1A1 substrates which contributes to toxicity(Sten Wingren et al,2006).UGT2B7*2 hetero polymorph was also showed Significant association with decreased libido. Adjusted odd ratio was 6.34E+06 and p value was 0.0054.Both the allele of C and T contribute significantly in this toxicity. Again both hetero and mutant homo of CYP2D6*10 showed significant association in this toxicity. For hetero, adsjusted odd ratio was 4.27E+06 and p value was 0.0070 whereas Adjusted odd ratio and p value for mutant homo were 6.44E+06 and 0.0027.It denoted presence of T allele and at one T allele contribute significantly in this toxicity. As T allele for CYP2D6*10 is high in our population (57.09%).So, larger population of our study people group will show toxicity to this drug with this polymorphism. Rest of the polymorphs of different genotype did not show any significant relationship.

Significant association was also found among hetero and mutant homo polymorphs of CYP2D6*10 and Vaginal dryness. Adjusted odd ratios were 1.47E+08 and 1.59E+08 respectively and p values were 0.0364 and 0.0255 respectively. Here also presence of T allele contribute in this toxicity. As T allele for CYP2D6*10 is high in our population (57.09%).So, larger population of our study people group will show toxicity to this drug with this polymorphism.No other polymorphs of different genes showed significant relationship with this toxicity.

In subgroup analysis, we found postmenoposal, age between 45-55 and more, BMI between 20-25Kg/m^2 showed severe toxicity in comparison to other groups. Both

ductal and lobular carcinoma patients showed toxicity with higher grade with different TNM stage especially more than II.

CYP2D6 is a first line metabolic gene of Tamoxifen. It leads to the conversion of Tamoxifen to it's active metabolites endoxifen and N-Desmethyl tamoxifen. If polymorphism leads to stop this conversion, toxic or less active metabolites will be generated which leading to severe toxicity outcome. Another two gene SULT1A1 and UGT2B7 are second line metabolic gene and they contribute for safe excretion of tamoxifen. These two genes polymorphisms are leading to toxic metabolic end product resulting severe toxicity.

3.4.3 Effect of different polymorphism on survival evaluation

Significant association was found among both hetero and mutant homo polymorphisms of SULT1A1*2 and Hazard ratio. Hetero showed odd ratio and p value 21.5680 and 0.0364 whereas mutant homo showed odd ratio and p value 24.9813 and 0.0255.Here presence of A allele rules the Hazard ratio data. As A allele for SULT1A1*2 is low in our population (only 34.02%).So, fewer people of our population will affected in Hazard ratio≤1.5 to this drug with this polymorphism. We also found significant association between UGT2B7*2 hetero polymorphism and Hazard ratio. Here the odd ratio and p value were 4.6445 and <0.0001.Here the presence of C and/or T allele contributes this survival data.CYP3A5*3 hetero type polymorph also carried significant association with Hazard ratio. We got p value and odd ratio were 0.0002 and 5.5577.Presence of G allele and/or A allele contribute this survival data. Not only those,CYP2D6*10 had also showed association with Hazard ratio significantly. Both mutant homo and hetero showed association in this respect.Here odd ratio and p value were 28357.6667 and <0.0001 for mutant homo whereas 210.9091 and <0.0001 for hetero. This means presence of T allele and at least one T allele contribute this survival data. As T allele for CYP2D6*10 is high in our population (57.09%).So, larger population of our study people group will affected in Hazard ratio≤1.5 to this drug with this polymorphism. No other polymorphism of any selected gene was showed significant association.

Association between Survival rate more than 5 years and UGT2B7*2 mutant homo polymorphism was found significant. Here odd ratio and p value were 4.0906 and

0.0004.This means presence of T allele contribute this data. CYP3A5*3 mutant homo polymorph and Survival rate (>5 years) was significantly associated. Here odd ratio was 7.7834 and p value was <0.0001.Presence of G allele contributes this survival data. Association was also found between both hetero and mutant homo polymorph of CYP2D6*4 and Survival rate more than 5 years. Odd ratio and p value for hetero were 17.2757 and <0.0001 whereas odd ratio and p value for mutant homo were 6.7143 and 0.0062. A allele and at least one A allele roles this survival data for this type of polymorphism. As A allele for CYP2D6*4 is low in our population (only 22.43%).So, fewer population of our study people group will affected survival rate>5 years to this drug with this polymorphism. No other polymorphs of our selected genes showed significant association with survival rate more than 5 years.

Prominent association was found among Recurrence free survival for more than 5 years and CYP2D6*10 mutant homo polymorph. Odd ratio and p value were 1.8645 and 0.0107.It means presence of T allele contributes this polymorphism. Rest of the selected genes were showed no significant association with Recurrence free survival for more than 5 years.

We also found significant association between Breast cancer specific survival and mutant homo polymorphs of CYP3A5*3. Here odd ratio and p value were 192.0737 and 0.0002.G allele for this polymorphism carries significant role in this survival data.We also got significant association between both hetero and mutant homo polymorphs of CYP2D6*4 and Breast cancer specific survival. Here odd ratio and p value for hetero were 55.1209 and <0.0001 respectively. Odd ratio and p value were 27.5137 and 0.0229. A allele and at least one A allele signifies their contribution in this survival data. As A allele for CYP2D6*4 is low in our population (only 22.43%).So, fewer population of our study people group will affected in Breast cancer specific survival to this drug with this polymorphism. No other selected genes were showed prominent association statistically with this survival parameter.

Our result is supported by the findings of previous study for SULT1A1*2.In the multivariate analysis for SULT1A1*2 adjusting for age, stage and adjuvant radiation therapy, the odd ratio did not differ significantly between the patients homozygous for the variant A allele and the patients carrying the wild-type G allele (HR = 0.53, 95% CI = 0.27-1.08, P = 0.079) . In terms of BCSS or RFS, there were no statistically

significant differences according to the rs9282861 genotype (Maria Tengström et al,2012). After adjusting for age, stage, adjuvant radiation therapy, and hormone receptor status, the multivariate analysis showed that patients with the homozygous variant rs9282861 AA genotype had statistically significantly improved odd ratio (HR = 0.50,95% CI = 0.29-0.88, P = 0.015). A parallel although statistically insignificant pattern was seen in BCSS (HR = 0.53, 95% CI = 0.26-1.05, P = 0.069). No statistically significant difference was seen in the RFS (P = 0.091) (Maria Tengström et al,2012).

We found significant relationship between different alleles of CYP2D6 and different survival data like Breast cancer specific survival, Survival rate more than 5 years,Hazard ratio less than 1.5,Recurrence free survival for more than 5 years among Bangladeshi breast cancer women taking Tamoxifen. This correlation also in line with previous findings on different population.Polymorphisms associated with the CYP genes, especially CYP2D6, may also have a substantial effect on the outcome of Tamoxifen therapy; CYP2D6 contributes to the formation of 4-OH-TAM in human liver . Moreover,Tamoxifen is metabolized to 4-desmethyl-TAM via CYP-dependent pathway by CYP3A4 and secondarily to endoxifen by CYP2D6, and decreased CYP2D6 enzyme activity has been associated with worse event-free survival and disease-free survival in patients treated with adjuvant Tamoxifen (Veli-Matti Kosma et al,2012). Our results were validated by Schroth et al.,2011. They found the significance of CYP2D6 and CYP2C19 genetic variation in a retrospective cohort of 206 tamoxifen-treated and 280 tamoxifen-untreated patients of breast cancer. Tamoxifen-treated women with CYP2D6 alleles associated with absent or reduced enzyme function (*4, *5, *10, *41) had significantly more recurrences, shorter relapse-free time (HR¼2.24; 95% CI (confidence interval)¼1.16–4.33;P¼0.02), and worse event-free survival (HR¼1.89; 95%CI¼1.10–3.25; P¼0.02) compared to carriers of functional alleles. As expected, there was no association between treatment outcome and CYP2D6 genotype in the cohort of patients that did not receive Tamoxifen(MP Goetz et al,2008).

Our findings are in contrast with two findings. One is Nowell et al. findings which found no association between CYP2D6*4 genotype and overall survival in a retrospective cohort of 162 patients treated with tamoxifen for ER-positive and

negative disease. Another one is Wegman et al. who demonstrated no association between the CYP2D6*4 allele with distant disease-free survival or overall survival. The reasons for the conflicting outcomes of these studies are likely due to one or more of the following: (1) the lack of central testing of ER, resulting in the probable inclusion of ER-negative patients; (2) use of retrospectively assembled(MM Ames et al,2008). CYP2D6*4 polymorphism generates a truncated non-functional gene product by a G→A transition at the first nucleotide of exon 4 in the CYP2D6 gene.

After a median follow-up of 10 years, no statistically significant associations were observed between CYP2D6 genotype and recurrence in tamoxifen-treated patients (PM vs EM: HR for distant recurrence = 1.25, 95% CI =0.55 to 3.15, P = .64; HR for any recurrence = 0.99, 95% CI = 0.48 to 2.08, P = .99). A near-null association was observed between UGT2B7 genotype and recurrence in tamoxifen-treated patients(James M. Rae et al,2012). Though we found correlation of recurrence free survival for more than 5 years and CYP2D6*10 in Bangladeshi breast cancer population but we did not found any relationship between recurrence and UGT2B7 polymorphism in our population.

Another findings for CYP3A5 by Pia Wegman et al,2006 demonstrated that homozygous carriers of the *3 allele tended to have an increased risk of recurrence when treated for 2 years with tamoxifen, although this was not statistically significant (hazard ratio (HR) = 2.84,95% confidence interval (CI) = 0.68 to 11.99, P = 0.15). In the group randomised to 5 years' tamoxifen the survival pattern shifted towards a significantly improved recurrence-free survival (RFS) among CYP3A5*3-homozygous patients (HR = 0.20,95% CI = 0.07 to 0.55, P = 0.002). No reliable differences could be seen between treatment duration and the genotypes of CYP2D6, SULT1A1 or UGT2B15. The significantly improved RFS with prolonged tamoxifen treatment in CYP3A5*3 homozygotes was also seen in a multivariate Cox model (HR =0.13, CI = 0.02 to 0.86, P = 0.03), whereas no differences could be seen for CYP2D6, SULT1A1 and UGT2B15.But we did not find any correlation between recurrence free survival and CYP3A5*3 polymorphs. Rather we found correlation between Survival rate and breast cancer specific survival with CYP3A5*3 polymorphism in Bangladeshi breast cancer population. As another study by Sauli Elingarami et al,2006 supported our findings which quoted that CYP3A5

homozygous carriers of the *3 allele tended to have an increased risk of recurrence when treated with 2 years of tamoxifen, although this was not statistically significant (HR= 2.84, 95% CI = 0.68 to 11.99, P = 0.15).

When comparing Hazard Ratios the benefit of 5 years of tamoxifen associated with homozygous carriers of the CYP3A5*3 allele persisted(John Carstensen et al,2006).This result also support our findings on Hazard ratio.We also found correlation of Hazard ratio less than 1.5 and CYP3A5*3 polymorphism among Bangladeshi breast cancer population.

3.4.4 Effect of different polymorphism on performance status evaluation

We did not find any correlation between performance status in our population and different polymorphs of selected gene SULT1A1*2, UGT2B7*2, CYP3A5*3, CYP2D6*4 and CYP2D6*10.

CHAPTER FOUR

CONCLUSION

Conclusion

Breast cancer is the most common malignancy among women in Bangladesh. Its lifetime risk amounts to a total of 10% and approximately 15–20% of all breast cancers are associated with the occurrence of familial breast and/or ovarian cancer. Aside from family history, the most well-established risk factors for breast cancer are those associated with hormonal and reproductive factors that result in greater lifetime exposure to estrogens and androgens (Easton et al.1993). These observations, along with the finding that higher plasma concentrations of total and free estradiol (E2) in the early follicular phase and total and free testosterone (T) in both menstrual cycle phases are associated with an increased risk of breast cancer in premenopausal women (Lancet, 2005), suggest that cumulative estrogen exposure is sufficient to alter breast cancer risk later in life. Scientists had detected various high- and low-risk cancer susceptibility genes including high-risk susceptibility genes such as breast cancer gene1 (BRCA1) and breast cancer gene 2 (BRCA2). Despite the well established role of inheritance in breast cancer carcinogenesis, an emerging area of research is pharmacogenetics, a field that studies the role of genetic inheritance in individual variation in drug response and toxicity. For last two or three decades, Tamoxifen has been widely used as a standard endocrine therapy for the treatment of ER-positive breast cancer. When administered to women with ER-positive breast cancer for 5 years after surgery, Tamoxifen almost halves the annual recurrence rate and reduces the breast cancer mortality rate by one-third in both pre- and post-menopausal women. Tamoxifen is extensively used for the prevention of breast cancer, the treatment of ductal carcinoma in situ, and the treatment of pre and post-menopausal breast cancer. Even though the benefit of adjuvant Tamoxifen persists for years, some patients will eventually relapse and die of breast cancer. Moreover, In addition to causing hot flushes, depression etc., Tamoxifen increases the risk of endometrial cancer and thromboembolic complications. Recently, genetic and drug-induced variation in the phase I

drug-metabolizing enzyme cytochrome P450 2D6 (CYP2D6) has emerged as an important contributor to the inter individual variability in response after the administration of Tamoxifen. The most important metabolites of Tamoxifen in terms of therapeutic efficacy are 4-hydroxy-TAM (4-OH-TAM) and 4-OH-N-desmethyl-TAM (endoxifen). The detoxification of 4-OH-TAM is catalyzed by the phase II enzymes human sulfotransferase 1A1 (SULT1A1) and uridine diphosphate glucuronosyltransferase isoform 2B7 (UGT2B7). Endoxifen is formed by the CYP2D6-mediated oxidation of n-desmethyl tamoxifen . SULT1A1 is a member of the sulfotransferase family, which has the capability to sulphate phenolic and steroid compounds. A G683A base substitution (rs9282861) in exon 7 of SULT1A1 results in an Arg213His amino acid change with functional consequences; the variant A allele encodes an enzyme with lower catalytic activity and thermostability compared with the wild-type G allele. Common genetic variations in CYP2D6 and/or drug-induced inhibition of CYP2D6 enzyme activity are associated with significant reductions in endoxifen concentrations in tamoxifen-treated humans. These data led to the hypothesis that CYP2D6 variation may affect the clinical outcomes of women treated with tamoxifen.

In this study significant association was found between response, survival data (Hazard ratio≤1.5, Recurrence free survival>5 years), common toxicities (Hot flashes,depression,decresed libido,vaginal dryness) of Bangladeshi breast cancer patients and homo & hetero polymorphs of CYP2D6*10. T allele and at least one T allele of this polymorphism is responsible for this result. Again we found significant relationship between SULT1A1*2 polymorphism and response, survival data(Hazard ratio≤1.5),toxicities(decreased libido) among Bangladeshi population.We got association between response, survival data(Hazard ratio≤1.5,Survival rate>5 years,Breast cancer specific survival) and CYP3A5*3 polymorphism among Tamoxifen treated Bangladeshi breast cancer patients.Our study also revealed significant association between response,survival

data(Hazard ratio≤1.5,Survival rate>5 years),common toxicities(depression, decreased libido) and different polymorphisms of UGT2B7*2 among study population. Unfortunately, we did not get any relationship between response and CYP2D6*4.This is due to lack of sufficient sample size. But we found relationship between different survival data (Survival rate>5 years, Breast cancer specific survival) and CYP2D6*4 polymorphism among Tamoxifen treated Bangladeshi breast cancer patients. No association was found among performance status and any genotype of selected gene of our study population.

However it is the first study among Bangladeshi population in Tamoxifen response, survival rate and toxicities with a limited number of cases. Studies with larger samples may reveal more statistically significant facts and reveal to us vital genetic events that actually lead to the development of breast cancer and discover newer anticancer drugs that can heal breast cancer and save the life of thousands of people in the world. Though this study have some limitation as the number of patients are small and only metabolic gene's SNP is considered, but it opens scope for further research in personalised medicine. Now our suggestion goes to GWAS investigation to find out root cause of toxicities, survival and response evaluation. Expression level testing should be done to ensure the purpose. This study can help the physician to serve safe and effective treatment plan for patients to save many precious life. Patients medical cost will be minimized which will contribute economically developing country like Bangladesh. National health budget can cover more people by saving money through pharmacogenetic application like this.

CHAPTER FIVE

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Appendix

DATA COLLECTION FORM

Questionnaires

Effect of SULT1A1, UGT2B7, CYP3A5 and CYP2D6polymorphisms on Tamoxifen response in breast cancer patients of Bangladesh

1. Identification

1.1 I.D. Code:

1.2 Name:

1.3 Father's/ Husband's Name:

1.4 Sex:

Male		**Female**	

1.5 Marital Status:

Married		**Unmarried**	

1.6 Date of Birth (dd/mm/yy):

1.7 Age (yr):

1.8 Mailing address:

1.9 Permanent address:

1.10 Telephone No.:

1.11 Religion:

1.12 Nationality:

2. Personal History

2.1 Area of residence:

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

2.2 Education level:

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

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	

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2.8 Number of Children:

2.9 Brest Feeding

	Yes		No

2.10. Early sign of breast cancer:

2.11a Family History of breast cancer (Disease):

2.11b Relative with breast cancer (Disease) affected on ------------------------ age.

2.12 Habit of Exercise:

3.Biophysical Characteristics

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

4.Other diseases: A) B) C)

5. Menarche and Menstrual cycle :

6. Other Prescribed drugs

Drugs	**Dose (mg)**
Cyclophosphamide	
5-FU	

Paclitaxel	
Methotrexate	
Others1)…………………	
2)…………………	

7.Hypersensitivity reaction diseases:

	Grades			
	1	2	3	4
Toxic Epidermal Nacrosis				
Severe Skin rash				
Others(………………….)				

8.Toxic effects:

Toxicity	**Grades**			
	1	2	3	4
Hot flashes				
Depression				
Decreased libido				
Vaginal dryness				
Vomiting				
Blurred vision				

Others(……………….)				

9.Response Evaluation:

Response

	Put Tick(√) mark
Complete response	
Partial response	
Stable disease	
Progressive disease	

10. Performance status:

	Grades					
	0	1	2	3	4	5
Performance						

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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