Case-control study of HER2/ neu Ile^{655}Val single nucleotide polymorphism in relation towards risk for breast cancer

A dissertation submitted
in partial fulfillment of the requirements
for the award of degree of

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IN
MICROBIOLOGY

Submitted by

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July, 2013

CERTIFICATE

This is to certify that the dissertation entitled **"Case- control study of HER2/ neu Ile^{655}Val single nucleotide polymorphism in relation towards risk for breast cancer"** being submitted by **Ms. Rashmi Pal** in partial fulfillment for the requirement of degree of **Masters of Science** in **Microbiology** in the **Department of Biotechnology and Environmental Sciences, Thapar University, Patiala** is a record of candidate's own work carried out by her under my supervision. To the best of our knowledge, the content of this dissertation does not form a basis for the award of any other degree.

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DECLARATION

I hereby declare that the work being presented in the dissertation entitled **"Case- control study of HER2/ neu Ile^{655}Val single nucleotide polymorphism in relation towards risk for breast cancer"** by me in the partial fulfillment of the requirements for the award of degree of Masters of Science in Microbiology, from Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work carried under the supervision of **Dr. Siddharth Sharma**, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala. The matter presented in this report has not been submitted in any other University/Institute for the award of Masters of Science or any other degree.

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ABSTRACT

Proto-oncogene HER2 (also known as erbB-2or neu) plays an important role in the causing cancer and in the prognosis of breast cancer. Genetic alterations in this proto-oncogene (HER-2/neu) have been shown to induce abnormal cell proliferation and prevent apoptosis. Genotyping studies have shown the association of single nucleotide polymorphism at codon ^{655} isoleucine to Valine polymorphism located in the transmembrane coding region and the risk of breast cancer, but the results remains controversial and varies in different population. In this study, we investigated the association of HER-2/neu Ile^{655}Val polymorphism and the risk of breast cancer in a North Indian population.

Single nucleotide polymorphism (SNP) in HER-2/neu Ile^{655}Val [dbSNP rs1136200] was done on in 41 breast cancer patients and in 42 healthy controls, data of demographic features and clinical features were also collected. Genotyping was performed by using DNA, extracted from blood and than polymerase chain reaction methodology, followed by the restriction fragment-length polymorphism (PCR-RFLP) analysis. Association between genotype and breast cancer risk was determined by statistical analysis.

There was a 6.67-fold (OR=6.67,95% CI=1.35–32.70 *p*=**0.00955**) increase in the risk of patients with breast cancer who are (Ile/Val and Val/Val genotypes). Furthermore, for the early onset (less than 40 years old) breast cancers with Ile/Val and Val/Val genotypes, there was an 11.2-fold (OR=11.2, 95% CI=1.23–101.8; *p* =**0.01**) increase in the risk of breast cancer.

Our results show that the HER-2/neu Ile^{655}Val polymorphism may contribute to breast cancer risk. A somewhat increased overall breast cancer risk was seen among women with the HER-2/neu Ile/Val heterozygosity, and younger patient(less than 40 years) have more risk towards risk for breast cancer. On the basis of clinicopathological data, there is significant increase risk of occurrence of ductal carcinoma in comparison to lobular carcinoma.

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ABBREVATIONS

BSA	Bovine Serum Albumin
CI	Confidence Interval
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate (N = A, C, T, G)
ECD	Extracellular domain
EDTA	Ethylene Diamine Tetraacetic Acid
EtBr	Ethidium_bromide
Ile	Isoleucine
kDA	Kilo Daltons
µl	Microlitre
ng	Nanogram
OR	Odds Ratio
R.R.	Relative Risk
SNP	Single Nucleotide Polymorphism
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TEMED	Tetramethylethylenediamine
TM	Transmembrane
T_m	Melting temperature
UV	Ultraviolet
Val	Valine

Chapter1

Introduction

Breast cancer incidence has been increasing in the general population all over the world. In India health care facilities are heterogeneous so in spite of having advanced diagnostic techniques, there are regular enhancements in breast cancer incidence every year. Many risk factors are associated with this which are modifiable or non modifiable. Somewhere modifiable risk factor can be reduced. In India according to different researches, westernization of lifestyle has led to increase in number of cases of breast cancer.

There are many possible causes of occurrence breast cancer but as study reveals around 20% of population having increased amount of HER2/neu protein. (Nelson *et al.,* 2005). The name for the HER-2 protein is derived from "*Human Epidermal growth factor Receptor*" as it features substantial homology with the epidermal growth factor receptor (EGFR) (Han *et al.,* 2005). HER2 protein is a member of HER family, which include 4 homologous receptor namely HER1, HER2, HER3and HER4. HER2 is a proto-oncogene and located on chromosome 17q11.2-12 and encodes a 185 kDa (p185HER2) transmembrane glycoprotein with tyrosine kinase activity (Siddig *et al.,* 2008).

Normally HER2 protein involve in cell growth, proliferation and angiogenesis (Siddig *et al.,*2008) but excessive production of this protein leads to cancer or tumor formation .Clinically HER2 gene amplification and/or over expression are associated with a more aggressive tumor biological behavior and poorer response to anticancer treatments in breast cancer(Lu *et al.,*2010) . HER2/neu is a member of HER family and plays a central role in the family and it's the most preferred hetrodimerization partner for other family member and by this activity it has potency to activate certain signalling pathway. Major signalling pathway include RAS/mitogen activated protein kinase pathway, PI3K/Akt pathway ultimately affects cell proliferation, adhesion, survival and motility (Ross *et al.,* 2003). Further degree of over expression of HER2 protein varies according to the type of breast cancer. So HER2/neu is an important factor in breast cancer

diagnosis and gene encoding it, is a target of detecting any genetic factor, which may help in diagnosis of disease and therapeutics.

Single nucleotide polymorphism (SNP) is genetic factor that might detect breast cancer development. So, SNP might indicate resistant or susceptibility towards disease progression (Nelson *et al.,* 2005). Genomic distribution of SNP is not homogenous and it varies from population to population. An isoleucine to valine polymorphism at codon ^{655} ($I^{655}V$) of HER2/neu gene was reported to be associated with an increased risk of breast cancer in different populations (Han *et al.,* 2005). This is an important issue in the context of significant ethnic differences in the incidence of breast cancer and other solid tumors (Ameyaw *et al.,* 2002). But according to different studies on different population the result remains controversial .Single nucleotide polymorphism can be inherited from generation to generation so other factor such as age and prior history of breast cancer in family also plays an important role. In this study we are going to compare the association of HER2/neu and $Ile^{655}Val$ polymorphism in North Indian population and by this evaluate the association of HER2 $Ile^{655}Val$ polymorphism and breast cancer risk in this population.

Chapter 2

Literature Review

2.1 Breast Cancer Current Statistics:

Breast cancer has been described as an alarmingly health problem in India (Ross *et al.*, 2003).According to the reports, breast cancers has badly attacked women population in India. A survey carried out by Indian Council of Medical Research (ICMR) in the metropolitan cities *viz.* Delhi, Mumbai, Bangalore and Chennai; from 1982 to 2005; has shown that the incidences of breast cancer have doubled (Yeole *et al.*, 2003).

According to ICMR statistics, 100,000 breast cancers are being diagnosed every year in India & more than 70% of them are diagnosed in advanced stage. In India, breast cancer is the second most common cancer (after cervical cancer) with an estimated 115,251 new diagnoses and the second most common cause of cancer-related deaths with 53,592 breast cancer deaths in 2008 (Dhillon *et al.*,2008). By 2020, the incidence of breast cancer in developing nations like India is expected to double. Further ICMR revealed that the incidence of breast cancer nearly doubled in metropolitan cities. The statistics now show that one in eleven women will develop breast cancer at some stage in their lives. Many studies have reported that young women are far less likely to develop breast cancer than their older counterparts and that the risk of breast cancer increases significantly with age. Breast cancer in urban areas of India is three times higher than in rural parts of the country (Agarwal *et al.*, 2011)

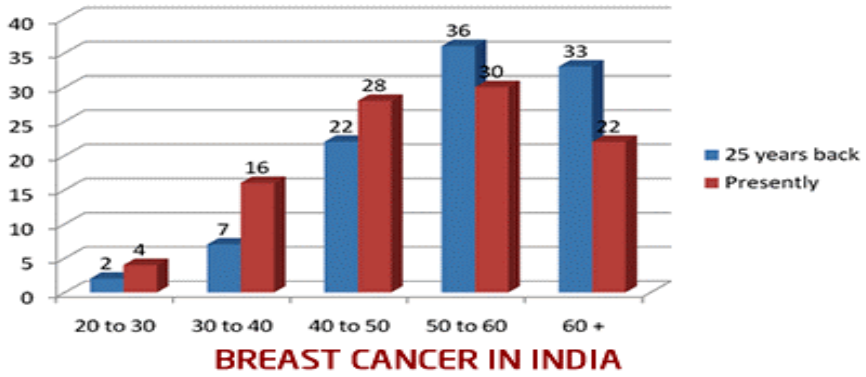

Figure 2.1: Bar graph showing comparison in breast cancer in 2008 and 25 years back.

The horizontal line lower down represents the age groups: 20 to 30 years, 30 to 40 yrs and so on. The vertical line represents the percentage of cases. The blue color represents the incidences 25 years back, and maroon color represents the situation in 2008. Owing to the lack of awareness of this disease and in absence of a breast cancer screening program, the majority of breast cancers are diagnosed at relatively in advanced stage (Ali *et al.*, 2011).

(http://www.breastcancerindia.net/bc/statistics/trends.html)

2.2 Risk factors for breast cancer:

The increasing burden of disease may be associated with lifestyle factors such as later age at marriage, age at first birth, reduced breastfeeding and westernization of diet and physical activity patterns. There is some evidence to suggest that environmental factors with estrogenic properties (e.g., pesticides) may play a role in the etiology of this disease, however there is no consistent epidemiological evidence or long-term data in humans to support this. Rather than this there is certain modifiable and non modifiable risk factor associated with breast cancer risk.

According to some research there are many risk factors responsible for occurrence for breast cancer, which are as follows:

2.2.1 Non-modifiable risk factors:

1. Age
2. Height
3. Personal history of benign breast or other breast disease.
4. Family history.
5. BRCA1/BRCA2
6. Menstrual history: ages at menarche and menopause
7. Breast density on mammogram.
8. Medical history of Hodgkin's lymphoma.

2.2.2 Modifiable risk factors:

1. Age at first child.
2. Hormone replacement therapy.

3. Breastfeeding

4. Smoking

The risk of breast cancer may be lowered to the extent that one can make lifestyle changes consistent with modifiable risk factors and may be modified by menopausal and/or hormone replacement therapy status.(Dhillon *et al.*,2008)

Table 2.1: Established and probable risk factors for breast cancer

Factor	**Relative risk**	**High risk group**
Age	> 10	Elderly
Geographical location	5	Developed country
Age at menarche	3	Menarche before age 11
Age at menopause	2	Menopause after age 54
Age at first full pregnancy	3	First child in early 40s
Family history when young	<2	Breast cancer in first degree relative
Previous benign disease	4-5	Atypical hyperplasia
Cancer in other breast	> 4	
Socioeconomic group	2	Groups I and II
Diet	1.5	High intake of saturated fat
Body weight:		
Premenopausal	0.7	Body mass index > 35
Postmenopausal	2	Body mass index > 35
Alcohol consumption	1.3	Excessive intake
Exposure to ionizing radiation	3	young females after age 10
Taking exogenous hormones:		
Oral contraceptives	1.24	Current use
Hormone replacement therapy	1.35	Use for >10 years
Diethylstilbestrol	l 2	Use during pregnancy

(McPherson *et al.*, 2000)

2.3 Definition and types of breast cancer:

Breast cancer is a type of malignant tumor that has developed from cells in the breast. Cells are made up of glands called lobules, and the ducts. The remainder of the breast is made up of fatty, connective, and lymphatic tissue. Breast cancer can be benign or malignant. Usually benign breast cancer is not life threatening but malignant tumors is invasive and can be spread to other organ such as bones and other tissues so they are life threatening (Abeloff *et al.*, 2008).

2.3.1 Types of breast cancer:

There are several types of breast cancer, but some of them are quite rare. In some cases a single breast tumor can be a combination of these types or be a mixture of invasive and in situ cancer.

- **Ductal carcinoma *in situ*:** DCIS means that the cancer cells are inside the ducts but have not spread through the walls of the ducts into the surrounding breast tissue. About 1 in 5 new breast cancer cases will be DCIS.

- **Lobular carcinoma *in situ*:** This is not a true cancer or pre-cancer. But, because it is a marker for the development of all types of invasive and non-invasive breast cancers, LCIS is often thought of as a form of breast cancer (http://www.breastcancer.org).

- **Invasive (or infiltrating) Ductal carcinoma:** This is the most common type of breast cancer. Invasive (or infiltrating) ductal carcinoma (IDC) starts in a milk duct of the breast, breaks through the wall of the duct, and grows into the fatty tissue of the breast. At this point, it may be able to spread (metastasize) to other parts of the body through the lymphatic system and bloodstream. About 8 of 10 invasive breast cancers are infiltrating ductal carcinomas.

- **Invasive (or infiltrating) lobular carcinoma:** Invasive lobular carcinoma (ILC) starts in the milk-producing glands (lobules). Like IDC, it can spread (metastasize) to other parts of the body. About 1 invasive breast cancer in 10 is an ILC.

Other less common types of breast cancer include inflammatory breast cancer, triple negative breast cancer, phyllodes cancer and angiosarcoma (http://www.cancer.org).

2.4 HER2/neu:

Breast cancer is considered as a highly heterogeneous group of cancer arising from different cell types and each having its own clinical implications. Currently, all breast cancers are tested for expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2/neu proteins. Clinical studies have shown that HER-2/neu over expression is observed in 60% of ductal carcinoma *in situ* and in 20–30% of infiltrating breast carcinoma, which is correlated with other parameters which are indicative of tumor progression, such as tumor size, nodal involvement (Maggie *et al.,* 2008). The activation and over expression of cellular oncogenes has been considered to play an important role in the development of human cancer. HER2/neu gene having, sequence length: 40522 bp ; CDS: 3678. 30 exons, 26 coding exons; total exon length: 4816 bp, max exon length: 969 bp, min exon length: 48 bp. Number of SNPs: 17 (Braccioli *et al.,* 2011).

The HER-2(rs1136200) is a proto-oncogene,and this gene was discovered in 1982(Nahta *et al.,*2012). It is a human epidermal growth factor receptor belongs to a family of receptors involved in the tyrosine kinase-mediated regulation of normal breast tissue growth and development (Siddig *et al.,* 2008). HER2 (Human Epidermal Growth Factor Receptor 2) also known as Neu, ErbB-2, CD340 (cluster of differentiation 340) or p185, is a protein that in humans is encoded by the *ERBB2* gene. Neu is named because it was derived from a rodentglioblastoma cell line, a type of neural tumor. ErbB-2 was named for its similarity to *ERBB* (avian erythroblastosis oncogene B). It is located on long arm of human chromosome 17q11.2-12 and encodes a 185 kDa transmembrane glycoprotein (Braccioli *et al.,* 2011). The HER2/neu protein is a component of a four-member family of closely related growth factor receptors, including EGFR or HER-1 *(erb*-B1); HER-2 *(erb*-B2); HER-3 *(erb*-B3) and HER-4 *(erb*-B4). In addition to its association with disease outcome in gastrointestinal, pulmonary, genitourinary and other neoplasm (Sassen *et al.,*2008).

HER2/ neu is a member of family of receptor which belongs to tyrosine kinase mediated regulation. Normally members of this family of cell-surface receptors have emerged as key regulators of critical cellular processes, such as proliferation and differentiation, cell survival and metabolism, cell migration, and cell-cycle control. Amplification or over-expression of the ERBB2 gene occurs in approximately 30% of breast cancers. It is strongly associated with increased disease recurrence and poor prognosis (Schlessinger *et al.,* 2000). HER2 proteins have

been shown to form clusters in cell membranes that may play a role in tumorgenesis (Tan *et al.*, 2007). Alterations in the proto-oncogene HER-2/neu have been extensively studied in Western and East Asian females with breast cancer and proposed as prognostic markers of potential clinical utility (Kaufmann *et al.*, 2011).

2.5 Structure of HER2/neu protein:

HER2 belongs to the tyrosine kinase family receptor. HER2 encode an 185kda, 1255 amino acid protein. The receptor consists of an extracellular domain, with four subdomains including two cysteine rich domains, a transmembrane domain, and an intracellular domain, consisting of a juxtamembrane region, a tyrosine kinase domain, and a carboxyl tail harboring autophosphorylation sites. These domains have different structural and functional features (Sarel *et al.*, 2002).

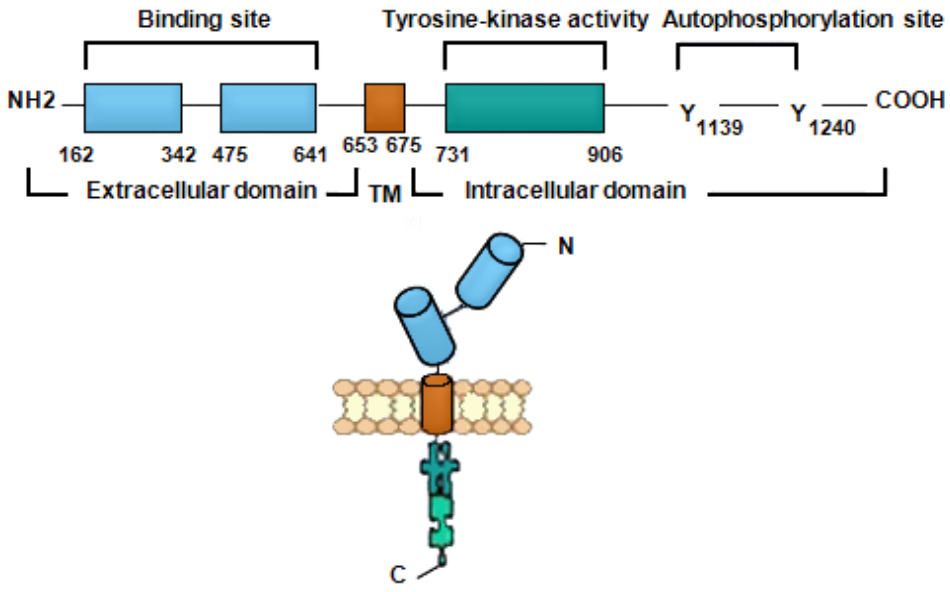

Figure 2.2: HER2 protein: schematic representation. An extracellular ligand-binding domain (blue); a single transmembrane I domain has an extensive homology to the epidermal grow factor receptor (brown); a cytoplasmic domain with catalytic activity (green) (www.ncbi.nlm.nih.gov).

2.5.1 Function of different domains:

Receptor tyrosin-kinases (RTKs) are cell surface allosteric enzymes consisting of different domains. Each domain play important role in dimerization and activation of signalling pathways.

- **Extracellular sub-domain I and sub-domain III**

  Unlike other receptors in the HER family, sub-domain I on HER2 is constantly in contact with sub-domain III. Permanent interaction between these 2 sub-domains keeps the receptor in an open conformation, exposing sub-domain II, and ensuring the receptor is always ready to dimerize.

- **Extracellular sub-domain II**

  Sub-domain II is the dimerization domain. This domain enables HER2 to bind with other receptors in the HER family to initiate downstream signalling.

- **Extracellular sub-domain IV**

  While the exact role of sub-domain IV in HER functioning is still unknown, it is believed to stabilize and lock the receptor in an open conformation. Other approaches to targeting HER2 bind to sub-domain IV to interfere with HER2 signalling, but sub-domain IV is not directly involved with dimerization.

- **Transmembrane domain**

  The transmembrane domain of HER2 has been implicated in receptor activation control, but further research is required to understand its function in signalling initiation (David *et al.,* 1996).

- **Intracellular domain**

  The intracellular domain has a cytoplasmic linker, a tyrosine kinase component, and a tail that phosphorylates and recruits adapter proteins following dimerization. These adapter proteins can then initiate cell proliferation signalling, leading to cell growth, differentiation, and angiogenesis. Evidence suggests that the intracellular domain plays more than a passive role in dimerization and it suggests that the intracellular domain may stabilize dimer formation (Patrick *et al.,* 2000) & (http://www.biooncology.com).

2.6 Other member of HER family:

HER2 is thought to be an orphan receptor, with none of the EGF family of ligands able to activate it. While no known ligand for HER2/neu receptor identified so its activation required the other

family receptor .The preferred partner for HER2 activation is other member of HER family. The details about the members of HER family are given below:

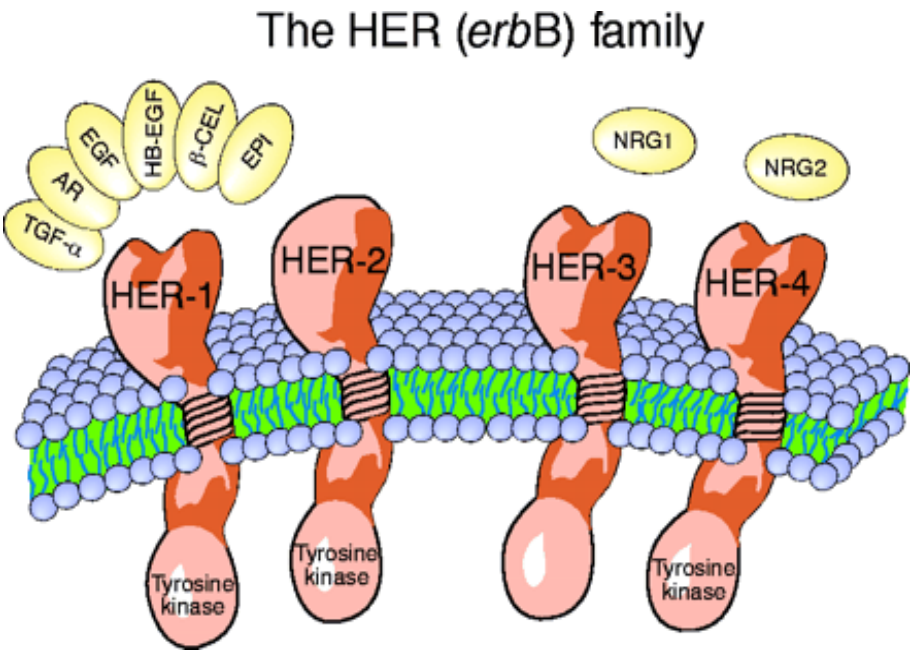

Figure 2.3: Structure of HER family member and ligand related to the members

Abbreviations (Ross *et al*., 2003)**:**

TGF- Transforming growth factor alpha

AR - Amphiregulin

EGF - Epidermal growth factor

HB-EGF - Heparin binding EGF,

β-CEL – β- Cellulin

EPI - Epinephrine

NRG1 - Nergulin-1

NRG2 - Nergulin-2

As figure shows that HER2 has no ligand and HER-3 has no intrinsic kinase activity, so it is not functional. Eleven ligands have been reported to bind with HER family receptor. These ligands can directly bind with HER1, HER3 and HER4 (Landau *et al.*, 2007). On the basis of their common evolutionary origin these receptors share a high degree of structural and functional homology, which is the molecular basis for receptor interaction and cross-activation. Thus, HER-

receptor's activity and functionality depend on one another and thus the impact on tumour cell proliferation and growth is likely to be dependent on HER receptor co expression and communication (Sassen *et al.*, 2008).

Table2.2. Types of cancer and Dysregulation caused by member of HER family:

Name	**Other Names**	**Type(s) of Dysregulation**	**Cancer Types**
HER1	EGFR	Overexpression Mutation leading to constitutive (non-stop) activity	Head and neck, bladder, prostate, renal, non-small-cell lung cancer, ovarian, pancreatic and glioblastoma.
HER2	c-erbB-2 ErbB2	Overexpression Co-expression with HER-1 improves ability to predict aggressiveness of breast cancer	Breast, ovarian
HER3	ErbB3	Co-expression with HER-2 improves ability to predict aggressiveness of breast cancer	Breast, colon, gastric, prostate, other carcinomas
HER4	ErbB4	Reduced expression produces differentiated phenotype Co-expression with HER-2 has prognostic Value	Breast, prostate, childhood medulloblastoma

2.7 HER2 activation:

The mechanism of activation of the HER-2/*neu* pathway starts by dimerization, as studies have shown that the ECDs of the HER family of receptor tyrosine kinases form homodimers and heterodimers and that receptor dimerization activates a cascade of events in the HER-2/*neu* signalling pathway (Patrick *et al.*,2000).

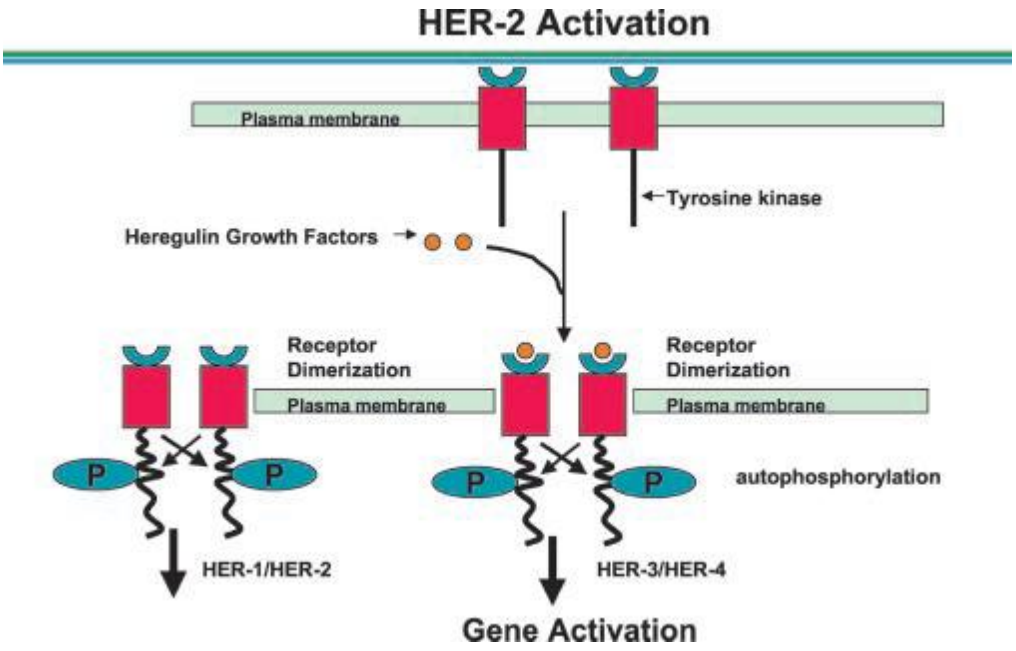

Figure2.4. HER2 gene activation by ligand and dimer formation. (Carney *et al.,* 2003)

A physical feature of the HER pathway is that the signalling system always involves two receptors in combination, in a formation called a "dimer." Homodimers are combinations of two similar receptor types, such as HER1/HER1 and HER3/HER3. Heterodimers contain two different receptors, such as HER1/HER2; HER1/HER3; and HER4/HER3 (Ross *et al.,* 2003). The various hetero- and homo-dimer pairs affect the signal strength within the cell. For example, the co-expression of certain pairs (such as HER3/HER2) is more powerful than others such as the HER3/HER3 Homodimers, which is inactive. (Yarden *et al.,* 2000) dimerization/oligomerization has been recognized as an integral component of signalling by erbB family receptors.erbB-2 is an orphan receptor; it plays an active role in ligand-mediated signalling through heterodimerization with other erbB family members. The family of receptor involved in cell to cell and cell to stroma communication through signalling transduction process.

HER activation is usually dependent on the presence of small molecules called ligands (Yarden *et al.,* 2000), these ligands and many other growth factors affect the transcription of various genes by phosphorylation or dephosphorylation activity. Ligands activate the dimer formation. The formation of these dimers leads to activation of the intrinsic tyrosine kinase

domain and subsequent phosphorylation on specific tyrosine residues, which serve as docking sites for a variety of molecules. Recruitment of these molecules leads to the activation of different downstream signalling cascades, including the MAPK proliferation pathway and/or the PI3K/Akt prosurvival pathway (Olayioye *et al.,* 2001).

Abnormal MAPK signalling may lead to:

- Increased/uncontrolled cell proliferation
- Resistance to apoptosis (programmed cell death)
- Resistance to chemotherapy, radiotherapy, and targeted therapies.

Abnormal PI3K/Akt/Mtor signalling pathway leads to:

- Abnormal Cell growth
- Abnormal Cell proliferation
- Abnormal Cell survival

(http://www.biooncology.com)

2.8 Single nucleotide polymorphism (Ile^{655}Val) at codon ^{655} :

Single nucleotide polymorphisms, frequently called SNPs, are the most common type of genetic variation among people. Single nucleotide polymorphism is the variation in DNA sequences that occurs when a single nucleotide in a DNA sequences differ from the normal and present in a population. SNP is help in detection of either resistance or susceptibility towards risk of breast cancer. A single nucleotide polymorphism at codon ^{655} leads to guanine to adenine substitution in transmembrane domain coding region of HER/2neu gene (Nelson *et al.,* 2005).

However several studies have shown that relation of HER2/neu and Ile^{655}Val polymorphism remains controversial. In humans, the Ile^{655}Val amino acid substitution might alter the formation of active HER2 dimers, which would then alter the activity of the protein (Nelson *et al.,* 2005). Substitution of Val for a bulkier Ile residue in this position of the transmembrane domain will destabilize the formation of active erbB2 dimers that are mediated by the N-terminal dimerization motif. Consequently, receptor activation caused by over expression of erbB2 will be reduced even at high levels of erbB2 over expression **(**Fleishman *et al.*, 2002). Therefore, the usefulness of the HER2 SNP in the determination of risk, prognosis, and treatment response of breast cancer will be dramatically different between ethnic populations (Ameyaw *et al.*, 2000).

The research studies done on total number of Ile/Ile, Ile/Val and Val/Val genotypes in cases and controls of HER2/neu Ile^{655} Val polymorphism in different population are tabularized in Table 2.3.

Table 2.3: Case-Control studies of the HER2/neu Ile^{655}Val in different populations

Authors	**Population**	**Cases**			**Controls**		
		Ile/Ile	**Ile/Val**	**Val/Val**	**Ile/Ile**	**Ile/Val**	**Val/Val**
Nelson *et al.*,2005	European	637	396	61	551	356	69
Montgomery *et al.*,2003	Australian	240	138	31	196	94	9
Ozturk *et al.*,2012	Turkish	61	57	-	87	41	-
Lee *et al.,* 2007	Taiwan	341	83		273	45	
Sidding *et al.*,2008	Sudan	56	11	1	75	5	1
Zubor *et al.*,2006	Slovakia	22	22	3	42	17	1
Benusiglio *et al.*,2006	White British	1134	752	113	1229	791	134
Papadopoulos *et al.,* 2007	Northern Greece	15	22	19	19	16	10
Rajkumar *et al.,* 2007	India	181	62	73	63	119	18

Chapter3

Aim of study

- To study the prevalence and genotypic frequencies of the Ile^{655}Val polymorphism in breast cancer and control samples

- To check the association between HER2/neu and Ile^{655}Val polymorphism in relation towards risk of breast cancer and also in relation to demographic and clinicopathological features

Chapter4

Methodology

4.1 Study population:

In this case–control study, we evaluated the HER2 codon Ile^{655}Val polymorphism in 41 breast cancer patients with a mean age of 55 (35-75) years and 42 control subjects with a mean age of 47.5 (35-60) years. . The cases with histologically confirmed primary breast cancer were recruited from September 2012 to June 2013 from Government Medical College, Rajindra Hospital, Patiala, and Punjab. The study proposal and ethical procedures were approved by the Ethics Committee of Government Medical College and Rajindra Hospital Patiala. Written informed consent was obtained from all participants or from patients' representatives if direct consent could not be obtained. Demographic and clinical characteristics of the breast cancer patients were gathered from dept. of pathology GMC Patiala .All the participants lived in northern India. To evaluate the relationship of HER2 Ile^{655}Val with the risk of breast cancer and ages, we divided the patients into two groups (cases and control). Blood samples were collected in 3 ml EDTA containing tubes and their DNA was extracted from the blood.

4.2 DNA extraction from blood:

DNA was isolated from blood by Phenol-Chloroform Isoamyl Alcohol (PCI) method. This is most commonly used method of purifying and concentrating DNA from samples. Phenol/chloroform extraction is an easy way to remove proteins from nucleic acid samples and can be carried out in a manner that is very close to quantitative. Nucleic acids remain in the aqueous phase and proteins separate into the organic phase or lie at the phase interface (Brawerman *et al.,* 1972).

Firstly washing was done by washing buffer. Stock and working concentration of washing buffer are as follows:

Table 4.1: Composition of washing buffer

Stock Concentration	Working Concentration
1M sucrose	320 mM sucrose
100% Triton X-100	1% Triton X-100
100mM Magnesium Chloride	5mM Magnesium Chloride
100mM Tris-HCl pH=8	10mM Tris-HCl pH=8

After this lysis was done by lysis buffer. Stock and working concentration of lysis buffer are as follows:

Table 4.1: Composition of washing buffer

Stock Concentration	Working Concentration
1M Tris HCl pH=8	400mM Tris HCl pH=8
10% SDS	1% SDS
0.5M EDTA	60mM EDTA
5M NaCl	150mM NaCl
10mg/ml Proteinase-K	100 µg/ml Proteinase –K

Procedure:

3ml of blood was taken and added equal amount of washing buffer and mixed it thoroughly. Centrifuged it at 3500 rpm for 5 minutes. Discarded the supernatant and added equal amount of washing buffer to the pellet and re-suspend the pellet in the buffer and centrifuged it again (repeated this step thrice). Dissolved the pellet in equal amount of Lysis buffer and incubated at 44 °C for overnight. For purification of DNA, added an equal volume of Phenol: chloroform: Isoamyl alcohol (25:24:1) and mixed the contents slowly. Centrifuged it at 8000 rpm for 10

minutes at 4°C. Upper aqueous layer was taken and again added PCI. Mixed it thoroughly and centrifuged. Aqueous layer was again separated and equal volume of Chloroform: Isoamyl alcohol (24:1) was added .Centrifuged it at 6500 rpm for 5 minutes and separated the upper layer. To the aqueous layer, equal volume of chilled isopropanol was added and gently mixed. Sample was kept at -20°C for 1-2 hours for freezing and again centrifuged at 12,000 rpm for 10 min at 4°C. Discarded the supernatant and washed the pellet of DNA with chilled 2 ml of 70% ethanol and centrifuged twice at 10,000 rpm for 5 minutes. Decanted ethanol and air dried the pellet. Dissolved the pellet in 150µl Tris-EDTA buffer.

4.3 Agarose gel electrophoresis:

Agarose gel electrophoresis is method of visualization of DNA bands. After DNA isolation we performed Agarose gel electrophoresis to check the quality of the DNA as isolated from the blood samples. 0.7% Agarose gel containing 0.5µg/ml of Ethidium Bromide dye was made in Tris-EDTA buffer then gel was casted in casting tray along with comb and allowed it to solidify. Agarose gel for DNA electrophoresis was submerged in 0.5X TBE buffer. Buffer is essential to maintain a constant state of ionization of the molecule being separated and to maintain the rate of migration in applied field. After then carefully remove the comb and 2µl sample was prepared in 4µl water and 2µl 6X loading dye. Sample was loaded in the well, and run at 55 volts. DNA band was visualized under UV transilluminator and gel imaging was performed under UV light in Bio Rad gel documentation system using Quantity-1-D analysis software.

4.4 Quantification of DNA:

Quantification of DNA was done to estimate and also to check the purity of DNA. Quantitative estimation was done by spectrophotometric analysis. The ratio of the absorbance at 260nm and 280nm ($A_{260/280}$) is used to assess the purity of nucleic acids. For pure DNA, $A_{260/280}$ is ~1.8 and for pure RNA $A_{260/280}$ is ~2. According to literature, 1 O.D. at 260 nm for double-stranded DNA is equal to 50 ng/µl of ds DNA. So, the DNA concentration was calculated by using given formula:

$$\text{Concentration µg/ml} = \text{OD} \times 50\ \text{ng/µl} \times \text{dilution factor}$$

4.5 Analysis of HER2 I ^{655} Val polymorphism

The HER2 $I^{655}V$ polymorphism was analyzed with a polymerase chain reaction–restriction fragment-length polymorphism- based (PCR-RFLP) assay in the 42 patients with breast cancer and 41 normal controls. After the extraction, the DNA was amplified in a PCR system. So, as to produce large amount of desired fragment.

4.5.1 Polymerase Chain Reaction:

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA and generating thousands to millions of copies of a particular DNA sequence (Joshi *et al.,* 2005). The basic PCR principle is as the name implies, it is a chain reaction, and one DNA molecule is used to produce two copies, then four, then eight and so on. (http://www.biosmart.ch). There are three major steps involved in the PCR technique: denaturation, annealing, and extension. In step one; the DNA is denatured at high temperatures (From 90 - 97°C). In step two, primers anneal to the DNA template strands to prime extension. In step three, extension occurs at the end of the annealed primers to create a complimentary copy strand of DNA (Innis *et al.,* 1990). PCR was done in Bio-Rad T100^{Tm} thermocycler.

For PCR, we required working concentration of following reagent;
1x BSA, 1.5Mm PCR buffer, 0.2 Mm dNTP's, 0.5µM of forward and reverse primer and 0.8 U Taq polymerase.

Firstly PCR optimization was done, for optimization we selected four temperature i.e. 65^{o}C, 67^{o}C, 65^{o}C with DMSO and 67^{o}C with DMSO. Temperature range was decided according to T_m Value of primers. The annealing temperature is 5^{O}C below the true T_m of primer (Innis *et al.,* 1990). Then, a master mix for 20 reactions was prepared. The reaction mixture contained 205.33 µl autoclaved double distilled water, 44µl BSA, 22µl each HER2 primers; F-5′AGA GAG CCA GCC CTC TGA CGT CCA T3′ and R-5′ TCC GTT TCC TGC AGC AGT CTC CGC A3′ (Lee *et al*., 2007), 44µl PCR buffer , 8.80µl dNTP, and 5.87µl Taq polymerase in respective order. So for single reaction 16µl of this master mix was taken with 4µl template. The HER2 primer was cited from the previous study. The PCR reaction was set on following temperature ;initial melting for 5 min at 94 °C, 30 cycles at 94 °C for 30 seconds, 67 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 7 min. The PCR product was verified by 1.7% agarose gel

electrophoresis in 0.5X TBE and run at 50 volts. Gel imaging was performed under ultraviolet light in Bio-Rad gel documentation system using Quantity 1-D analysis software.

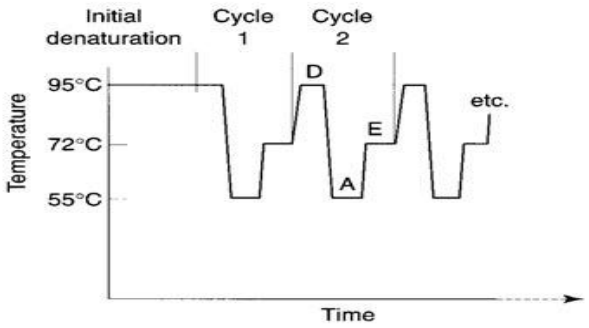

Figure 4.1 .Ray diagram of PCR

4.5.2 Restriction fragment length polymorphism:

Restriction fragment length polymorphism or RFLP analysis is used to identify a change in the genetic sequence that occurs at a site where a restriction enzyme cuts. RFLPs can be used to trace inheritance patterns, identify specific mutations, and for other molecular genetic techniques. Restriction enzymes are proteins isolated from bacteria that recognize specific short sequences of DNA and cut the DNA at those sites. (http://www.ncbi.nlm.nih.gov)

The PCR products (148 base pairs) were digested with *Bsm*AI enzyme (NEB). For single reaction, 10 µl of PCR product, 7.5µl autoclaved water, 2.2 µl 10X NEB4 buffer and 3 units of *Bsm*AI (10U/µl) enzyme was taken. After then incubated it for overnight at 37^{o}C and digested product was separated by 6% polyacrylamide gel electrophoresis. Digestion of each PCR product with *Bsm*AI gives 116- and 32-bp fragments for the Val allele and a single 148-bp fragment for the Ile allele. *Geobacillus stearothermophilus* A664 is the genomic source for *Bsm*AI.

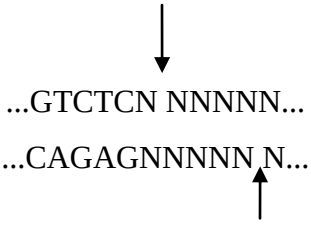

Figure 4.2.Restriction Site Sequence

4.6 Polyacrylamaide gel electrophoresis:

Several methods have been used to estimate molecular weight of small DNA molecules. For molecules of 10-1000 base pairs or nucleotides, the method of polyacrylamide gel electrophoresis provides the highest degree of resolution and is in principle, the simplest. (Maniatis *et al.*,1975).

Reagent required:

30% Acrylamide solution (29:1, acrylamide, bisacrylamide solution w/v)

TEMED

10% Ammonium persuphate

1.5 M Tris buffer

5X TBE

Procedure:

6% gel was prepared by using 2.4 ml acrylamide, 7.2 ml water, 2.4 ml 5X TBE, 200µlAPS, 10µl TEMED. Gel was casted in vertical gel casting tray and insert 10 well comb in it. Allowed it to solidify and carefully remove the comb. Gel was submerged in 1X TBE buffer, buffer is essential to maintain a constant state of ionization of the molecule being separated, and to maintain the rate of migration in applied field. After then 10µl sample were loaded in presence of 2 µl 6X loading dye. And run the sample at 60 volts. DNA band was visualized by performing silver staining.

4.7 Silver staining:

Silver staining is the most sensitive method for permanent staining of proteins or nucleic acids in polyacrylamide gels. It creates a record of the electrophoresis result that can be viewed without any special equipment (Bassam *et al.,* 1991). In silver staining, polyacrylamide gels are impregnated with fixative and soluble silver ion (Ag+) and developed by treatment with a reductant. Macromolecules in the gel promote the reduction of silver ion to metallic silver (Ag), which is insoluble and visible, allowing nucleic acid-containing bands to be seen. Fixative renders the macromolecules in the gel insoluble and prevents them from diffusing out of the gel during subsequent staining steps. (https://www.gelifesciences.com)

Reagents required:

Fixative solution:

Water: Methanol: glacial acetic acid in 50:40:10 ratio

Staining solution:

0.1% $AgNO_3$ and150 µl of 37% formaldehyde

Developing solution

3% sodium carbonate, 150µl of 37% formaldehyde and 10mg/ml of 20µl sodium thiosuphate

Procedure:

- Gently separate the two glass plates with the aid of a thin plastic spatula to remove gel
- Put gel in a plate contains 100 ml of fixative for 10 minutes.
- Rinsed by distilled water for 2 min.
- Stained the gel by 100 ml staining solutions for 30 min and kept in dark.
- Again rinsed for 10 sec gently.
- The gel was developed in 100 ml developing solution for 10 min.
- Shake for developing the bands.
-

4.8 Statistical analysis::

The Hardy−Weinberg equilibrium was tested among patients and controls separately with the $\chi 2$-test. Crude ORs with 95% CIs was used to assess the strength of relationship between the HER2 $Ile^{655}Val$ Polymorphism and breast cancer risk. All statistical tests and *p* value were calculated, and a result was considered significant when the *p* value was less than 0.05. Relative Risk was also calculated along with *p* value. The same statistical analysis was applied to subgroups according to menopausal status, and age (≤40 and >40 years) and on clinicopathological features. Data were analyzed by using the computer software MedCalc (Lee *et al.*, 2008).

Chapter 5

Result and Discussion

5.1 Result of DNA isolation :

Gel image showing genomic DNA picture. Gel imaging was performed under UV light in Bio-Rad gel documentation system using Quantity-1-D analysis software.

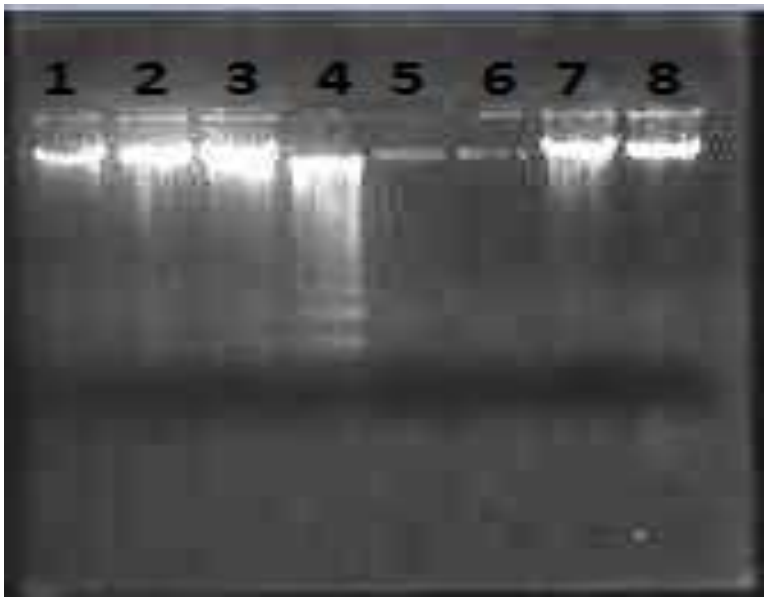

Figure 5.1: Lane 1-8 showing genomic DNA

The bands observed were intact showing that genomic DNA was free from shearing.

5.2 Result of PCR:

PCR product of 148 base pair was obtained after amplification.Gel imaging was performed under UV light in Bio Rad gel documentation system using Quantity-1-d analysis software.

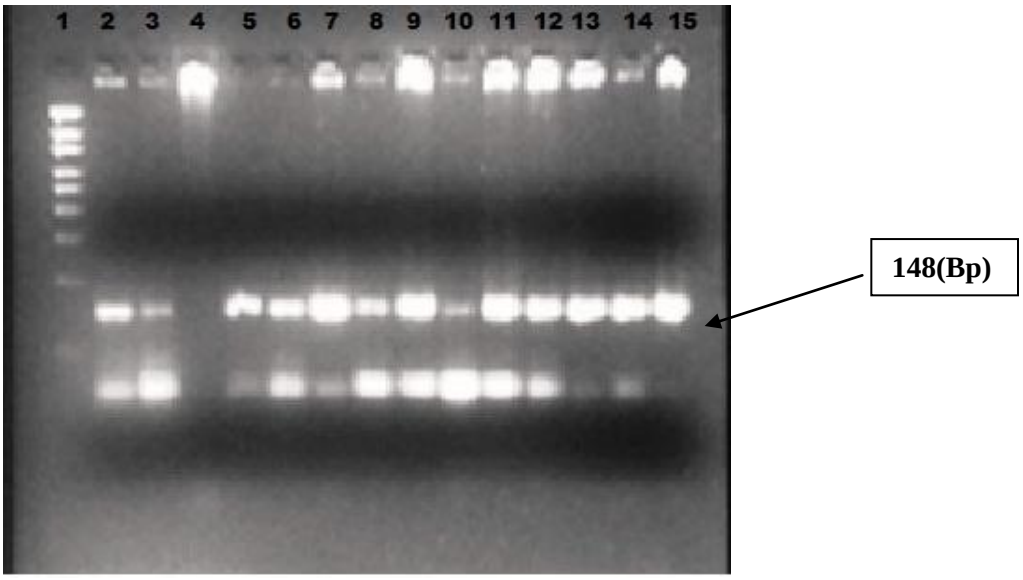

Figure 5.2: Lane 1 show marker of 100bp and lane 2-9 –PCR product of patients sample.

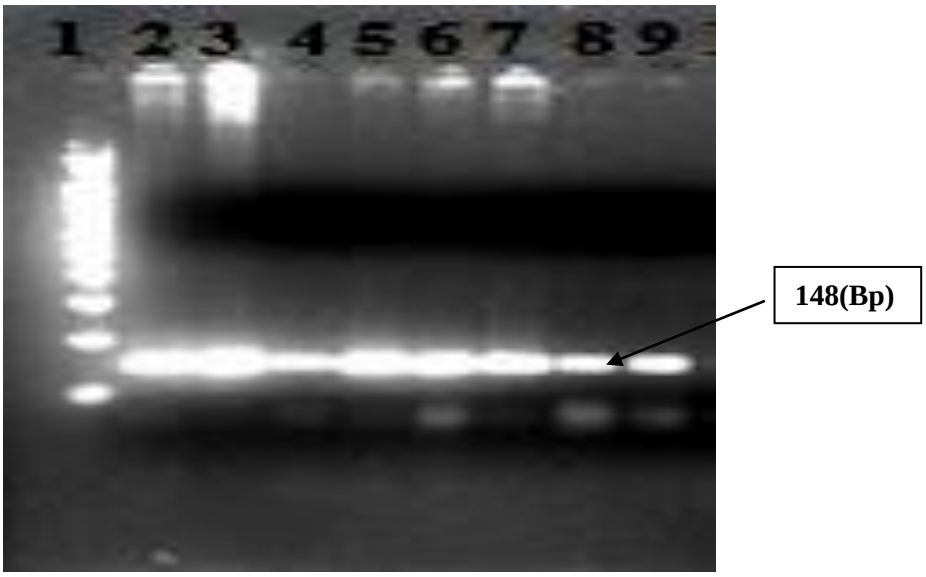

Figure 5.3: Lane 1 show marker of 100bp and lane 2-9 –PCR product of control sample.

5.3 Genotyping:

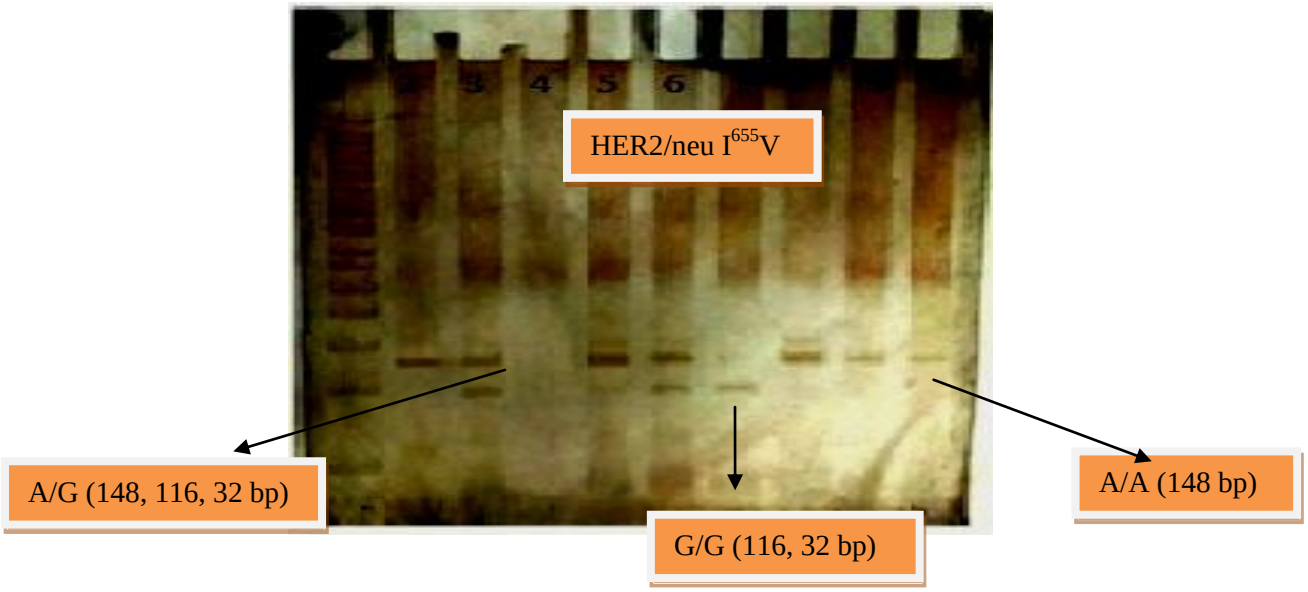

Figure 5.4: Restriction fragment-length polymorphism analysis of the 148-base-pair (bp) HER2 polymerase chain reaction product of patient sample. Representative genotypes are shown.

Lane 1 molecular size standards and **lane 2** for uncut PCR product Lanes **5, 8,9and 10** homozygous for the Ile allele; **lanes 3** and **6** heterozygous for the Val and Ile alleles; **lanes 7** homozygous for the Val allele

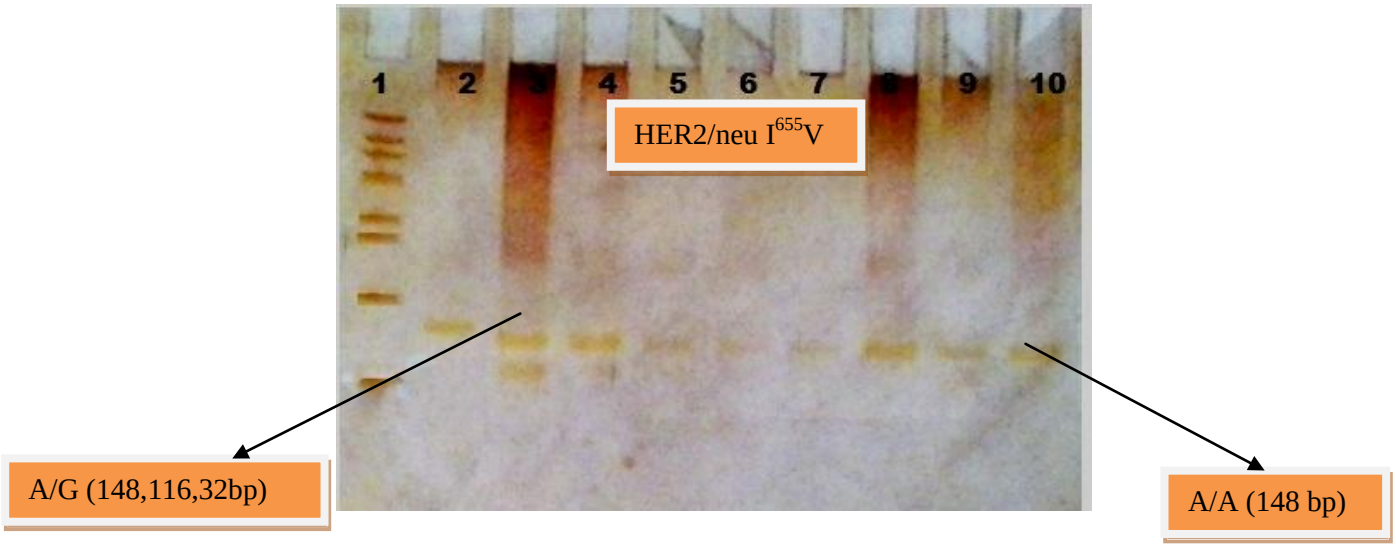

Figure 5.5: Restriction fragment-length polymorphism analysis of the 148-base-pair (bp) HER2 polymerase chain reaction product of control sample. Representative genotypes are shown.

5.4: Epidemiology:

In this case–control study, we evaluated the HER2 codon Ile^{655}Val polymorphism in 41 breast cancer patients with a mean age of 49.15 years and 42 control subjects with a mean age of 45.59 years. Among 41 cases, 1 male and 40 female subject is evaluated. Further we categorized on the basis of age menopausal status (early menopause or late menopause) and pathological features. Demographic and clinical characteristic of breast cancer patients and controls are listed below.

Table 5.1: Demographic and Clinical Characteristics of 41 Breast Cancer Patients and 42 controls

	Patient (N =41)		**Controls (N=42)**	
Characteristics	N	(%)	N	(%)
Age				
<40 years	21	51.21	30	71.42
≥40 years	20	48.78	12	28.57
Gender				
Female	40	97.56	42	100
Male	1	2.4	-	-
Age at time of Menopause				
≥50	6	14.63	9	21.42
<50 or no menopause	35	85.36	33	78.57
Pathological diagnosis*				
Ductal carcinoma	16	41.0	-	
Lobular carcinoma	23	58.97		

N=Total no of subjects
*- Total no. of subject is 39 for pathological diagnosis

We found that patients with breast cancer and control groups were comparable in the major known risk factors that were studied together with demographic features. Some of the, data was missed. According to data patient having no menopause or menopause in less than 50 years of age have high risk in comparison to control group i.e. 85.36% and 78.57% respectively. Pathological diagnosis show two major type of breast cancer in patient *i.e.* ductal and lobular carcinoma in 41.025% and 58.97 % respectively.

Table 5.2: The frequency of the HER2 allele and genotypes, and OR data in the patients with breast cancer and the controls

	Patients (N=41)	**Controls(N=42)**	**OR(95%CI)**	***p***
Allele frequency	**N (%)**	**N (%)**		
Ile	68(82.92)	82(97.61)		
Val	14(17.07)	2(2.38)		
Genotype frequency				
Ile/Ile	30(73.17)	40(95.23)		
(Ile/Val +Val/Val)	10(24.39)	2(4.76)	6.6 (1.3-32.7)	**0.009**
Allele frequency				
Age<40 years				
Ile	34(41.46)	58(69.04)		
Val	8(9.75)	0		
Age≥40 years				
Ile	34(41.46)	22(26.19)		
Val	6(7.31)	2(2.38)		
Genotype frequency				
Age<40 years				
Ile/Ile	15 (36.58)	28(66.66)		
(Ile/Val +Val/Val)	5 (12.19)	1(2.38)	11.2(1.2-101.8)	0.01
Age≥40 years				
Ile/Ile	15(36.58)	11(26.19)		
(Ile/Val +Val/Val)	4(9.75)	1(2.38)	2.9(0.28-30.0)	0.34

As shown in table 5.2 the Val allele is more prevalent among case patient (17.07%) than among control subjects (2.38%).We found that 17.07% of the patients and 4.76% of the control subjects were heterozygous for Ile/Val genotype, whereas 7.31% of the breast cancer cases and none of the controls subject were homozygous for Val/Val allele. The prevalence of HER2 Ile/Val genotype and Val/Val genotype in controls of Chinese population is 21.7% and 0.3% respectively (Xie *et al.*,2000). This data is substantially higher than our result. Other then Chinese population, Caucasian women also have high representation of the Ile/Val and Val/Val genotype in control subjects (40% and 12%) (Papewalis *et al*., 1991). This data correspond well with traditionally lower risk of breast cancer compared with Chinese and Caucasian population. Meanwhile, in the present study, the frequencies of Val/Val genotype in cases and controls are 7.31 and 0

respectively. Keshava *et al.,* 2001 suggested the possibility that any influence of the Valine allele on cancer susceptibility may vary considerably between different ethnic populations. The prevalence of the Val/Val genotype ranges from 0% to 7.1% in white British (Benusiglio,*et al.*,2005),Iranian (Kamali-Sarvestani *et al.*,2004),Slovakian (Zubor *et al.*,2006) populations. This genotype may be less common or unobserved in people who are Asian or African descent (Xie et *al.,* 2000, Keshava *et al.,* 2001).

When compared with patients with the Ile/Ile genotype, individuals with Val/Val genotype showed a significant risk towards breast cancer with this genotype (OR=4.00; 95%CI=0.463–186.73.*p*=**0.05**). In case of Ile/Val a similar trend was also seen which was statistically more significantly for the increased risk towards breast cancer with the above genotype (heterozygous Ile/Val) (OR=4.44; CI=1.125-17.56, *p*=**0.023**). Since a few Val/Val genotypes were detected (only 3 cases), we categorized Ile/Val and Val/Val genotypes as a single genotype. Thus, risks between the cases and the controls were estimated for Ile/ Ile and (Ile/Val / Val/Val) genotypes. The frequency of the Ile/Val +Val/Val carrier genotype is also significantly higher in the patients than that of the controls (24.39% vs.4.76%, OR=6.66 95% CI=1.359–32.701, *p*=**0.009**), which shows a 6.66-fold increased risk in breast cancer women with the Ile/Val and Val/Val carrier genotype.

According to the age stratified analysis, as shown in Table 5.2, there was an 11-fold increased risk for developing breast cancer in case whose age was less than 40 years and who were carrying the Ile/Val+Val/Val genotype (OR=11.2; 95% CI=1.23–101.88; *p*= **0.01**). However, at the elder group (more than or equal to 45 years of age and carrying the Ile/Val+Val/Val genotype), the ORs were 3-fold less as compared to the patients whose age was less than 40 (OR=2.91; 95% CI=0.287-30.008; *p*= 0.34). In a related study in Melbourne and Sydney women diagnosed before the age of 40 years, has demonstrated that homozygosity for the Valine allele was associated with an increased risk of early onset breast cancer (Montgomery *et al.,* 2003).

Table 5.3: The genotype distribution at codon ^{655} of HER2 among the controls and patients

Genotype/allele	**Patients (n = 41)**	**Controls (n =42)**	**p Value**	**OR(CI)**	**Overall χ2**
Ile/Val	7(17.07)	2(4.76)	0.02	4.6(0.9-24.0)	5.1
Ile/Val+Val/Val	10(24.39)	2(4.76)	0.009	6.6(1.3-32.7)	
Val/Val	3(7.31)	-	0.05	9.2(0.46-186.7)	

In our study, the OR between the controls and the patients with the Ile/Val and Ile/Val+Val/Val carrier genotype is significantly higher than the OR between the controls and the patients with the Val/Val genotype, which is consistent with the study of Xie *et al.*, 2000. However, some other studies disagreed about the relationship between the HER2 polymorphism and breast cancer. To explore this discrepancy, Xie *et al.*, 2000 found that the HER2 Val allele was associated with an increased risk of breast cancer, particularly in the younger women. In this study, the association of Val carrier genotype and breast cancer risk was more apparent in early onset of breast cancer that approved the finding of previous studies (Lee *et al.*, 2007,Lu *et al.*, 2010, Ozturk *et al.*,2012) .Furthermore, the Val allele frequency in case patients (17.07%) was higher than that in the controls (2.38%).In India related study was done by Rajkumar *et al.*,2008 in South Indian population .They found all genotype statistically significant with reference to Ile/Ile genotype (OR =2.44 ;*p* = 0.03). OR and *p* Value for Ile/Val

And Val/Val genotype was OR=2.32 with a *p*= 0.03 and 0.04, respectively. The result is approximately similar with our result.

Table 5.4: The genotype distribution of pathological diagnosis among patients

	Ile/Ile(N=39)%	**Ile/Val(N=39)%**	**Val/Val(N=39)%**	**OR95%(CI)**	***p***
Ductal	11(28.20)	2(5.12)	3(7.69)	10.9(1-115)	0.04
Lobular	18(46.15)	5(12.8)	0	5.5(.9-31.3)	0.05

According to study done in a Chinese population 79% of tumors were ductal carcinomas and 11% of tumors were categorized as tubular or lobular of special or variant types, with good prognoses. (Breyer *et al.*,2009).Same result was found in Taiwanese populations ,they found 75 % patient with ductal carcinoma and 9% patient with lobular carcinoma.(Lee *et al*.,2007). Val/Val genotype show significant association with ductal carcinoma (OR=10.9, 95%CI=1-115, *p*=0.04) and Ile/Val genotype show significant association with lobular carcinoma (OR=5.5.9, 95%CI=.9-31.3, *p*=0.05). As shown in table 5.4 there was significant 2- fold increased risk in ductal carcinoma in comparison to lobular carcinoma, these result favors the result of previous studies.

5.5 Relative risk:

Relative risk is an absolute risk factor of occurrence of genotypes at 95 % CI. We compared the relative risk of Ile/Val, Val/Val ,Ile/Val+Val/Val. If the relative risk is more than 1 it indicates significant increased risk of genotype in case group when compared to control group.

Table5.5: The relative risk of the HER2 genotypes, and OR

Genotype	Relative Risk	95%CI	*p*
Ile/Val	1.8	1.1-2.8	0.0082
Val/Val	1.8	1.1-3.12	0.01
Ile/Val+Val/Val	1.94	1.3-2.8	0.0004

As shown in table 5.4 individuals who were carrying the Val/Val genotype had a Relative risk factor 1.8 which was statistically significant (R.R=1.8, 95% CI=1.1-3.12,*p* =0.01). Similarly individuals having the Ile/Val genotypes also had a R.R of 1.8 as compared to those who had the Wild Ile/Ile genotype. (95%CI=1.1-2.8, *p* =**0.008**). But when both the heterozygous and mutant genotype was combined a single genotype *i.e.* Ile/Val+Val/Val, the R.R. (1.94) showed a modest increase as compared to the other two genotypes as mentioned above (95%CI=1.3-2.8, *p* =**0.0004**). Since a few Val/Val genotypes were detected (only 3 cases), relative risk for homozygous Val allele is 1.8, which is slight lower than combined (Ile/Val+Val/Val) genotype.

Chapter 6

Conclusion

- In conclusion, our results show that the HER-2/neu $Ile^{655}Val$ polymorphism may contribute to breast cancer risk. A somewhat increased overall breast cancer risk was seen among women with the HER-2/neu Ile/Val heterozygosity.

- According to result based on demographic features patient less than 40 years of age was more susceptible towards risk for breast cancer.

- There is significant increase risk of ductal carcinoma in comparison to lobular carcinoma.

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APPENDIX- I

1. Sucrose (1M): Dissolved 3.41 g of sucrose in 10 ml of deionized water and sterilized by autoclaved.

2. Magnesium chloride ($MgCl_2$) (100mM): Dissolved 0.41gms of $MgCl_2$ in 20ml of deionised water and sterilized by autoclaved.

3. Triton X- 100 (10%): Taken 100 µl of TritonX-100 and mixed with 900 µl of deionised water and mixed properly.

4. 100mM Tris-Cl (pH 8.0): Dissolved 0.32g of Tris-Cl in 10 ml of deionsed water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilize the solution by autoclaving.

5. 10% SDS: Dissolved 1g of SDS in 10ml of deionsed water.

6. 10mg/ml Proteinase K: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilize the solution by autoclaving.

7. 0.5M EDTA: Dissolved 9.306g of disodium salt of EDTA in 20ml of deionsed water, and then adjusted the pH to 8.0 by 1 M sodium hydroxide. Sterilize the solution by autoclaving.

8. 5M Sodium chloride (NaCl): Dissolved 5.85g of sodium chloride in 20ml of deionsed water. Sterilize the solution by autoclaving.

9. TE buffer (pH 8.0): added 1ml of 100mM Tris-Cl (pH 8.0) and 200 µl of 0.5M EDTA solution to 8.8 ml of deionsed water. Sterilize the solution by autoclaving.

10. 1mg/ml BSA: Dissolved 100mg of BSA in 100ml of deionsed sterile water and kept at 4℃ overnight.

11. Ethidium Bromide (10mg/ml): Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.

12. DMSO: Mixed 50ml of 100% DMSO in 50ml of deionsed sterile water. Sterilize the solution by autoclaving and stored at -20˚C.

13. 5X TBE buffer: Dissolved 54g of Tris base and 27.5g of boric acid in 980ml of double distilled water and then added 20ml of 0.5 EDTA. Sterilize the solution by autoclaving.

14. Acrylamide solution (30%, 29:1, acrylamide: bisacrylamidew/v):30g acrylamide and 0.8 g N'N'-bis-methylene-acrylamide dissolved in 100 ml of deionized water.

15. Ammonium per sulphate (10%): 1 g APS dissolved in 10 ml of deionized water.

16. Silver nitrate (0.1%): 0.1 g silver nitrate in 100 ml of deionized water.

17. Sodium carbonate(3%): 3g sodium carbonate dissolved in 100 ml of deionized water.

18. Sodium thiosulphate(10mg/ml):10 mg sodium thiosulphate dissolved in in 1 ml of distilled water