FOXP3 rs2294021 POLYMORPHISM TOWARDS THE SUSCEPTIBILITY TO BREAST CARCINOMA IN NORTH INDIAN POPULATION

Submitted in partial fulfilment for the requirements of the Degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

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CERTIFICATE

This is to certify that the thesis entitled **"FOXP3 rs2294021 polymorphism towards the susceptibility to breast carcinoma in north Indian population"** submitted by Ajit Mourya in partial fulfilment of the requirement for the award of Degree of Masters in Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled **"FOXP3 rs2294021 polymorphism towards the susceptibility to breast carcinoma in north Indian population"** in the partial fulfilment of the requirement for the award of the degree of Master in Biotechnology, Department of Biotechnology and Environment Sciences, Thapar University, Patiala is an authentic record of my own work during the period of six months from January 2013 to June 2013, under the supervision of **Dr.Manoj Baranwal**, and co-supervision of **Dr.siddharth sharma** Assistant Professor, Department of Biotechnology and Environment Sciences, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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Date: July 15, 2013 Ajit Mourya
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DEDICATED TO
THE ALMIGHTY AND MY FAMILY

CONTENTS

ABBREVATIONS

ECM	Extracellular matrix
ER positive cancer	Estrogen receptor positive cancer
EDTA	Ethylene diaminetetra acetic acid
FOXP3	forkhead box p3
Treg cells	Regulatory T cells
SNP	Single nucleotide polymorphism
MHC	Major histocompatibility complex
TCR	T cell receptor
NFAT	Nuclear factor of activated T- Cell
Ap1	Activator protein
Sp1	Specific protein 1
CREB	cAMP response element binding
ATF	Activating Transcription Factor
Ld	Linkage disequilibrium
CDK	Cyclin dependent kinases
NSCLC	Non-small cell lung cancer
YAP	Yes-associated protein
EtBr	Ethidium bromide
TBE	Tris Borate EDTA

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ABSTRACT

FOXP3, the main regulator of T_{reg} (regulatory T) cells, is down-regulated in breast carcinoma and other cancers. The rs2294021 *FOXP3* polymorphism contributes to *FOXP3* down-regulation thereby weakens its tumor suppressing activity. The aim of our study was to evaluate the potential influence of *FOXP3* polymorphism on breast cancer, we conducted a case-control study in north Indian women. *FOXP3* genotyping was conducted in 33 breast carcinoma patients and 28 age-frequency matched cancer-free controls. Genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Experiment data was analyzed using Hardy−Weinberg equilibrium. The C/C genotype was found to be significantly associated with increased risk of breast carcinoma occurrence (OR= 3.0, 95% C.I; 0.372–24.171, *p-value*=0.29184) compared with the T/T or T/C (OR=1.800, 95%CI; 0.278-11.635, *p-value*=0.53250) genotype .The increased risk for breast carcinoma related to heterozygous genotype was more pronounced in subjects over 40 years .Our findings suggest that rs2294021 *FOXP3* polymorphism may be a potential contributor for development of breast carcinoma in north Indian women.

CHAPTER 1
INTRODUCTION

INTRODUCTION

Homeostasis maintained in the organisms, a great balance between cell proliferation and cell death. When the homeostasis is disturbed, either by an increased proliferation rate or a decrease in cell death, a tumor might occur, which can further lead into a cancer.

Breast cancer is the most common malignancy in women. Breast cancer can be defined as a growth of malignant cells within the breast tissue. Breast cancer is considered to be one of the leading malignancies among women in most developed and developing regions of the world with nearly a million new cases each year. Breast cancer is considered to be one of the most common malignancies in women causing over 3,000 deaths every year. Breast cancer is the most common cancer in urban areas in India and accounts for about 25% to 33% of all cancer in women. (Dhillon *et al*., 2005) However, known risk factors of breast cancer explain approximately 50% of breast cancer risk; thus other risk factors that are environmental, estrogenic and/or related to development might be responsible for the remnant risk and different genes are responsible for causing cancer. (http://www.breastcancerindia.net).

Most patients die of distant metastases that are frequently unresponsive to cancer therapy. In order to metastasize, cells need to be able to migrate and invade into the surrounding tissue, intravasate in to a blood vessel or lymphatic system, survive in circulation, extravasate and finally proliferate at a distant site. During mammary gland development, several biological processes occur in the mammary gland that also takes place during breast cancer development and progression. Many of the stromal factors involved in mammary gland development which promote or protect against breast cancer. Epithelial and stromal cells communicate via the extracellular matrix (ECM) and disruption of this interaction and respective communication can induce breast cancer (Hinck *et al.,* 2005). It is also important to gain understanding of the normal function of the mammary gland and genes which are involved in mammary gland development to understand the process of breast cancer and metastasis formation.

Breast cancer can be controlled by a healthy immune system of the body. It has been reported that immune dysregulation is associated with cancer progression. Immune suppression is a key function of regulatory T (T_{reg}) cells which acts by limit antigen-specific immune responses and thus gives rise to suppressed immunity to cancer. T_{reg} cells are defined by the expression of forkhead family transcription factor *FOXP3* (forkhead box p3), a gene that maps to the p arm of the X chromosome (specifically, Xp11.23). Expression of *FOXP3* is required for T_{reg} cell development. *FOXP3* is a

member of the forkhead-box/winged-helix transcription factor family. It was identified during positional cloning of *Scurfin*, a gene responsible for X-linked autoimmune diseases in humans (Owenv *et al*., 2006; Nesselhut *et al*., 2009; Whiteside *et al*., 2006; Merlo *et al*., 2009). *FOXP3* protein is commonly present in the nucleus, and it functions as a sequence-specific transcription factor. *FOXP3* is highly expressed in regulatory T cells and functions as the master regulator during the development of immune response and in the biological functions of regulatory T cells. In addition, *FOXP3* is also expressed in epithelial cells of breast, prostate, and lung (Douglass *et al.,* 2012).

Although, a direct link between *FOXP3* and the occurrence of cancer has yet to be established, the *FOXP3* gene has been shown to be down-regulated in cancer tissues (Han *et al.,* 2010). In the case of breast cancer, *FOXP3* appears to be a transcriptional suppressor for a breast cancer oncogene. In addition, the expression of the *FOXP3* gene has been shown to be influenced by estrogen, a known risk factor for breast cancer.

A single nucleotide polymorphism (SNP), a variation at a single site in DNA, is the most frequent type of variation in the genome. There are around 10 million SNPs that have been identified in the human genome. These SNPs can occur due to deletion, insertion or single base pair shift. It has been reported that SNPs are associated with several diseases. Hence, it is important to detect the variation in gene which causes different diseases. Different methods such as AFLP, RFLP can be used for the detection of polymorphism.

FOXP3 inhibits breast tumor growth through directly by repressing the transcription activity of two oncogenes, *HER2/ErbB2*, *c-Myc,* SKP2, while inducing the transcription activity of tumor suppressor gene p21 (p21 is a protein encoded by *CDKN1A* gene).

FOXP3 is an X-linked breast cancer suppressor gene in both male and female. If some polymorphism is seen in *FOXP3* gene, the regulation of *FOXP3* will be interrupted or structural variation in protein is occurs due to which the expressions of *FOXP3* will down regulated or up regulated. Role of *FOXP3* in breast cancer progression is not much clear; hence it is interesting to look for *FOXP3* polymorphism in breast cancer patients. In the present, study we are focussing on *FOXP3* rs2294021 polymorphism in North Indian populations.

CHAPTER 2
REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Breast cancer

Breast cancer is one of the most common malignancies in women. Breast cancer is one of the first diseases on record and still has a poor prognosis, with metastatic spread being the major cause of mortality. There are two main type of breast cancer; ductal and lobular. Cancer can be invasive or non invasive.

Ductal and Lobular carcinoma are the two main types of breast cancer.

- Ductal carcinoma starts in the tubes (ducts) that move milk from the breast to the nipple.
- Lobular carcinoma starts in the parts of the breast, called lobules, which produce milk.

Invasive breast cancer - Cancer cells break out from inside the lobules or ducts and invade nearby tissue. In this type of cancer, the abnormal cells can reach the lymph nodes, and eventually make their way to other organs (metastasis), such as the bones, liver or lungs. The abnormal (cancer) cells can travel through the bloodstream or the lymphatic system to other parts of the body, either early on in the disease, or later.

Non-invasive breast cancer - The cancer cells are still inside its place of origin and have not broken out. When the cancer cells are still inside the lobules, they are Lobular carcinoma *in situ*; On the other hand when the cancer cells are still inside the milk ducts, they are ductal carcinoma *in situ*. Sometimes, this type of breast cancer is called "pre-cancerous"; this means that although the abnormal cells have not spread outside their place of origin, they can eventually develop into invasive breast cancer.

2.1.1 Epidemiology

The established risk factors are linked to oestrogens. Risk is increased by early menarche, late menopause, and obesity in postmenopausal women. Childbearing reduces risk, with greater protection for early first birth and a larger number of births; breastfeeding probably has a protective effect. Both oral contraceptives and hormonal therapy for menopause cause a small increase in breast-cancer risk, which appears to diminish once use stops. Alcohol increases risk, whereas physical activity is probably protective. Mutations in certain genes greatly increase breast-cancer risk, but these account for a minority of cases (Key *et al*., 2001).

Gender

Breast cancer is relatively uncommon in men; the female-to-male ratio is approximately

100:1. The incidence of breast cancer in men has remained relatively stable over the past decades, except in Africa, where, for unclear reasons, the incidence is rising. *BRCA2* mutations are associated with an increased risk of breast cancer in men. (Bermejo Perez *et al.,* 2008)

Age

The risk of developing breast cancer increases with age. The disease is less common in women younger than 40 years of age; only about 0.8% of breast cancers occur in women < 50 years old, and approximately 6.5% develop in women between 30 and 50 years old (Han *et al*., 2010).

Geography

There is at least a fivefold variation in the incidence of breast cancer reported in different countries, although this difference appears to be narrowing. The incidence of breast cancer is significantly lower in Japan, Thailand, Nigeria, and India than in Denmark, the Netherlands, New Zealand, Switzerland, the United Kingdom, and the United States. Women living in North America have the highest rate of breast cancer in the world. It has been suggested that these trends in breast cancer incidence somehow may be related to dietary influences, particularly dietary fat consumption.

Socioeconomic Status

The incidence of breast cancer is higher in women of higher socioeconomic background. This relationship is most likely related to lifestyle differences, such as age at first birth and dietary fat intake (Key *et al*., 2001).

Survival

Survival rates for patients with nonmetastatic breast cancer have improved in recent years. These improvements may be secondary to advances in screening, systemic therapy, and loco regional radiation therapy. The contribution of screening mammography to breast cancer specific survival is variable, favouring a reduction in breast cancer mortality of up to 25% in some series. Its impact on overall survival is less certain. (Key *et al*., 2001)

Etiology and risk factors

The development of breast cancer has been associated with numerous risk factors, including genetic, environmental, hormonal, and nutritional influences. Despite all of the available data on breast cancer risk factors, 75% of women with this cancer have no

readily identifiable risk factors.

Genetic factors

There are different genetic factors responsible for causing breast cancer. Mutations in *BRCA*1 confer a high risk of developing breast or ovarian cancer. (Bermejo-Perez *et al.,* 2008) A woman carrying a mutation in this gene has a risk of developing breast cancer between 40% and 90% and of developing ovarian cancer between 20% and 70%.

2.2 *FOXP3* structure and function

Introduction

Gene name *FOXP3* and protein name Forkhead box protein p3 alternative name surfin. CD4+*FOXP3*+ regulatory T cells have been called "naturally-occurring" regulatory T cells to distinguish them from "suppressor" T cell populations that are generated *in vitro*. Additional suppressor T cell populations include Tr1, Th3, CD8+CD28- T cells. The contribution of these populations to self-tolerance and immune homeostasis is less well defined. *FOXP3* can be used as a good marker for CD4+CD25+ T cells and recent studies also shows evidence for *FOXP3* in CD4+CD25- T cells. Additional regulatory T cell subsets, induced regulatory T cells, are also needed for tolerance and suppression. (Ronacador *et al*., 2005).

Regulatory T cells develop in the thymus. Expression of *FOXP3* is required for regulatory T cell development and appears to control a genetic program specifying this cell fate. The large majority of *FOXP3*-expressing regulatory T cells are found within the major histocompatibility complex (MHC) class II restricted CD4-expressing helper T cell population and express high levels of the interleukin-2 receptor alpha chain (CD25). In addition to the *FOXP3*-expressing CD4+CD25+, there also appears to be a minor population of MHC class I restricted CD8+ *FOXP3*-expressing regulatory T cells. Unlike conventional T cells, regulatory T cells do not produce IL-2 and are therefore anergic at baseline (Wang *et al*., 2006).

***FOXP3* structure and gene regulation**

FOXP3 contains 11 coding exons and three non-coding exons. The two 5′ non-coding exons (−2a and −2b) are located significantly up-stream of the coding exons and are spliced into a common non-coding exon (−1) (Lal *et al*., 2009). Exons −2a and −1 encompass regulatory cis-elements. There remains controversy in the literature over the number of *FOXP3* exons; those who describe 11 exons do not count the initial −1 non-coding exon which lies outside the protein coding region (Ebert *et al*., 2008). *FOXP3* is a forkhead (FKH) box transcription factor, which is a member of the FOX protein family. All members of this family contain a characteristic DNA-binding FKH box domain which

acts as both transcriptional activator and repressor of specific genes. This domain is highly conserved and consists of approximately 100 amino acids of helix–turn–helix class proteins which produce three alpha helices, beta strands and two loops. The arrangement of these helices, loops and strands resembles the wings on a butterfly. As a consequence, this region is often referred to as a winged helix. The FKH domain is typically invariant across the species it is expressed in. However, the location of this domain often varies. The FKH domain is located toward the C terminus of *FOXP3*. However, in FOXP1, FOXP2, FOXP4 and other FOX proteins, the FKH domain is centrally located.

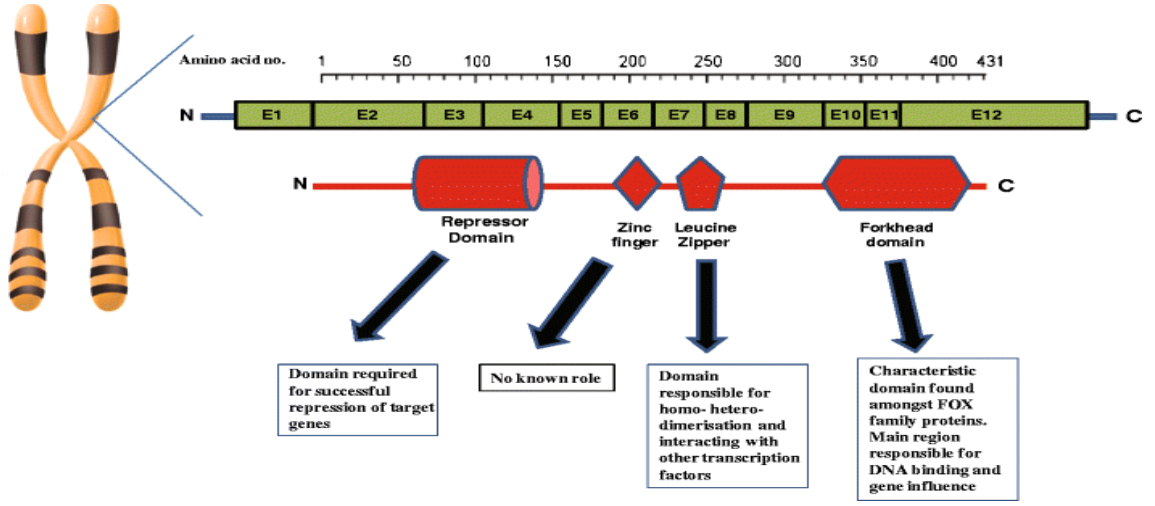


Fig. 2.1 Schematic representation of the structure and function of human *FOXP3* gene and protein. (Douglass *et al*., 2012)

FOXP3 gene expression is regulated by signalling through T cell receptor (TCR) and TGF-β receptor in periphery. We demonstrated that *FOXP3* gene expression is regulated by a promoter activity is regulated by NFAT, AP1, Sp1 and STAT5 and two enhancers, and the Enhancer 1 activity is regulated by transcription factors NFAT and Smad3, which are activated by TCR and TGF-β signalling, respectively and enhancer 2 activity is regulated by transcription factors STAT5 and CREB-ATF, which is regulated by transcription factors IL-2. Expression of IL-2 and activation of the T-cell antigen receptor (TCR) both modulate the differentiation and function of T_{reg} cells by activating the Akt, Erk and STAT5 signalling pathways (Wu *et al*., 2006).

Smad3 and NFAT (nuclear factor of activated T-cell) is required for activation of *FOXP3* enhancer1 region and induction of *FOXP3* (Bettelli *et al*., 2005). **ATF** is an activating transcription factor **CREB** (cAMP response element binding). **STAT5** refers to two highly related proteins, STAT5A and STAT5B, which are part of the seven membered STAT family of proteins. Though STAT5A and STAT5B are encoded by separate genes, the proteins are 90% identical at the amino acid level. STAT5 proteins are involved in cytosolic signalling and in

mediating the expression of specific genes. Aberrant STAT5 activity has been shown to be closely connected to a wide range of human cancers. (Heinze *et al*., 2011).

Ap1 (activator protein) is transcription factor which regulates gene expression in response of variety of stimuli include cytokines, GF (growth factors), stress and bacterial and viral infection**.** Ap1 controls number of cellular process including differentiation, proliferation and apoptosis. Ap1 upregulates the transcription of gene containing TPA DNA response element (TRE 5'-TGAGCTCA-3') and leucine zipper.

Sp1 (specific protein 1), human transcription factor contain zinc finger protein motif by which it binds directly to DNA and enhances gene transcription.

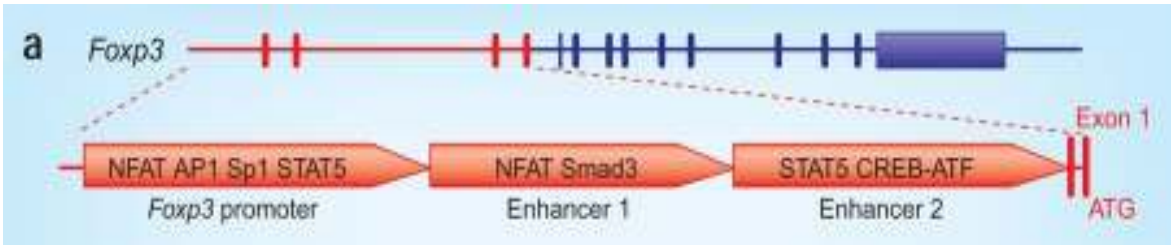


Fig. 2.2 *FOXP3* promoter and enhancer, with consensus binding sites for transcription factors NFAT, AP1, Sp1, STAT5, Smad3 and CREB-ATF (Boehmer *et al*., 2008).

2.3 *FOXP3* in breast cancer

FOXP3 is also expressed in epithelial cells of the normal human breast. Aggressive cancer of this epithelial tissue often correlates with abnormal expression of *FOXP3*, which can be either absent or underexpressed at transcript or protein levels. It is clear that this failure of normal *FOXP3* expression can result in dysregulation of the expression of a range of oncogenes which have been implicated in the development and metastasis of cancer. *FOXP3* might also regulate chemokine receptor expression, providing a possible explanation for the chemokine-driven, tissue-specific spread that is characteristic of many cancers. (Douglass *et al*., 2012) The expression of the *FOXP3* gene has been shown to be influenced by estrogen, a known risk factor for breast cancer. There are indications, therefore, that *FOXP3* has an important role to play in the development of breast cancer. (Polanczyk *et al.,* 2005).

2.4 *FOXP3* regulates numerous genes involved in breast carcinogenesis

It is now known that *FOXP3* can play an important role in controlling oncogenic factors in epithelial cells by controlling the expression of a number of genes implicated in cancer. These genes are discussed below.

2.4.1 *ErbB2/HER2*

Wild-type *FOXP3* in normal breast epithelium is able to bind and repress the expression of *ErbB2*. *ErbB2* encodes the *HER2* protein which is a transmembrane receptor tyrosine kinase and member of the epidermal growth factor receptor family. Over-expression of *HER2* has been linked to more aggressive forms of breast cancer. Indeed, the status of *HER2* expression is often used for diagnosing and grading breast cancers as cases where overexpression of *HER2* is associated with worse prognosis than $HER2^{-}$ cancers. When specific tyrosine residues on the intracellular region of *HER2* are phosphorylated, they can act as binding sites for molecules linking *HER2* to downstream pathways which include mitogen-activated protein kinase and phosphoinositide 3-kinase leading to cell growth and survival (Zuo *et al*., 2007).

Mice with mammary cancers express significantly raised levels of *ErbB2* compared with normal mammary epithelium. However, when a *FOXP3*-expressing vector was transfected into TSA cells (*HER2*-overexpressing mouse breast cancer cell line), the level of *ErbB2* was significantly reduced, suggesting that *FOXP3* is a repressor of the *ErbB2* oncogene. Further evidence of this repression was provided when the 5′ sequence of the ErbB2 gene was analysed and revealed multiple binding motifs for the FKH domain of *FOXP3*. In addition, a deletion in *FOXP3* binding sites resulted in increased *ErbB2* expression.

Analysis of ten malignant breast cancer cell lines with a significantly reduced level of *FOXP3* revealed the presence of seven which over-expressed *ErbB2* transcripts in comparison to normal epithelial cells. However, the fact that the other three cell lines did not over express *HER2* but had reduced *FOXP3* transcripts suggests that additional changes are needed, or alternative genes are involved in *HER2* up-regulation. Finally, when wild-type *FOXP3* was silenced using siRNA in normal mammary epithelial cells, a sevenfold increase was observed in ErbB2 mRNA and cell-surface expression of *HER2*, suggesting that *FOXP3* can influence mammary carcinogenesis (Zuo *et al.*, 2007).

2.4.2 SKP2

S-phase kinase-associated protein 2 (SKP2) has been reported in a wide variety of cancers and is overexpressed in nearly 50 % of breast cancers. Such cancers have a poorer prognosis than those not overexpressing SKP2 (Sonoda *et al*., 2006). The progression of the cell cycle is controlled by a number of CDK and CDK inhibitors. These enzymes are short-lived and frequently degraded by the proteolytic ubiquitin–proteosome pathway. This pathway consists of three enzymes, ubquitin-activating enzymes (E1), ubquitin-conjugating enzymes (E2) and ubquitin ligase enzymes (E3). SKP2 is an essential component of E3 ubiquitin ligase. SKP2 is expressed at its highest levels during the S and G2 phases of the cell cycle where it degrades the CKD inhibitor p27. SKP2 is therefore important for progression into mitosis during the cell cycle (Zuo et. al., 2007).

The expression of SKP2 and *FOXP3* has been studied malignant human breast tissues. It was seen that 30 % of *FOXP3*$^{+}$ samples also expressed SKP2. However, 56 % of *FOXP3*$^{-}$ samples over-expressed SKP2. After the removal of *FOXP3* expression SKP2 transcription increase eight fold in 48 hr. This shows that *FOXP3* is able to rapidly reduce SKP2 expression. *FOXP3* is able to directly repress the expression of SKP2 by binding to specific regions within the SKP2 gene, and a deletion in either binding site results in an increased level of SKP2 expression. SKP2 expression was also increased when *FOXP3* was silenced in an early passage of HMEpC, whereas when wild-type *FOXP3* was transfected into TSA cell lines, SKP2 expression was reduced between 10- and 20-fold (Zuo et. al., 2007).

2.4.3 *c-Myc*

c-Myc is one of the most studied genes in cancer. It is overexpressed in more than 30 % of all human cancer cases and in 80 % of prostate cancer samples. The *c-Myc* gene is located on chromosome 8 and encodes a transcription factor which acts as a CDK inhibitor with high affinity for p21 and p15 (Iavarone et. al., 1997). When *FOXP3* was investigated in 18 benign and malignant prostate epithelia from matched patients, 14 of the malignant samples showed significantly down-regulated *FOXP3* mRNA transcripts in comparison to the normal tissue (Jung *et al*., 2008).

FOXP3 is able to repress the expression of *c-Myc* (Wang *et al*., 2009). A clear correlation between *FOXP3* and *c-Myc* was demonstrated by knocking down the expression of *FOXP3* in human prostate cells which led to an increase in both *c-Myc* transcripts and protein resulting in an increased rate of proliferation. Corresponding to this, when *FOXP3* was transfected into two human prostate cancer cell lines, the expression of *c-Myc* was almost completely abrogated in both. With prostate epithelial cells having minimal levels of c-Myc, this demonstrates a clear correlation between *FOXP3* down-regulation and *c-Myc* up-regulation in prostate cancer.

2.4.4 p21

Expression of p21 has been implicated in many forms of cancer, particularly breast cancers. p21 is a protein encoded by the *CDKN1A* gene (Deiry *et al*., 1993). Interestingly, in these cancers, p21 itself is often not mutated, suggesting that the association between p21 expression and cancer is due to its role as a downstream target of other genes (Liu *et al*., 2009).

CDK inhibited by p21 with specificity for CDK1 and CDK2. P21 is able to cause cell cycle arrest in the G1 phase and, therefore, is important in cell cycle progression and senescence. As well as causing cell cycle arrest, it can interact with proliferating cell nuclear antigen, which is an accessory factor for DNA polymerase and is important in DNA synthesis and repair.

The expression of several oncogenes, *FOXP3* can also up-regulate factors involved in reducing tumor growth. *FOXP3* binds to intron 1 in CDKN1A and increases histone H3 acetylation by reducing the binding of HDAC2 and HDAC4, providing a potential mechanism for up-regulation of p21 expression (Liu *et al.,* 2009). When *FOXP3* was knocked down in MCF-7 and human mammary epithelial cells, there was a decrease in the expression of p21 transcripts and protein. When the same cell lines had p21 knocked down using siRNA, the size and number of the colonies was significantly increased compared with wild-type cells. The importance of the link between *FOXP3* and p21 in breast cancer was further validated when 62 human breast cancer cases were assessed for *FOXP3* and p21; only 30 % of *FOXP3* negative cases expressed p21. Despite these important findings, it should also be noted that tumor suppressor genes such as p53 and *BRCA1* are also able to control p21 levels (Deiry *et al*., 1993 and Somasundaram, *et al*., 1997).

2.4.5 LATS2

The significance of LATS2 within the human body is supported by evidence demonstrating significant down-regulation in cancers of the breast, prostate, brain and various blood-borne cancers (Takahashi et. al., 2005). LATS2 is an enzyme which is an important part of the Hippo-pathway. This pathway largely contributes to regulating cell cycle proliferation and apoptosis of cells by repressing expression of the oncogene YAP (Yes-associated protein). YAP over-expression has been reported within several cancers including the prostate, liver, breast, colon, lung and ovaries (Steinhardt *et al*., 2008).

FOXP3 is a direct transcriptional activator of LATS2 in epithelial cells of the prostate and breast where mutations in *FOXP3* often result in decreased levels of LATS2 and an increase in YAP expression (Li w *et al*., 2011).

Mice deficient in *FOXP3* have significantly reduced levels of LATS2 mRNA and protein and develop spontaneous tumor rapidly. When wild-type *FOXP3* is transfected back into MCF-7 breast cancer cells, there was a significant increase in LATS2 expression (Li *et al*., 2011).

2.5 *FOXP3* IN CANCER

2.5.1 *FOXP3* as an X-linked Tumor Suppressor Gene in Prostate Cancer

Xp11.22 and Xq27-28 two loci, each at respectively, are associated with the susceptibility to prostate cancer, but the genes in these regions have not been identified. Because *FOXP3* resides near the Xp11.22 region and reveals significant linkage disequilibrium (LD) between them, these results raised an interesting possibility that the *FOXP3* locus may contribute to X-linked prostate cancer susceptibility (Flammiger *et al.,* 2012).

FOXP3 expression is observed in a large panel of human prostate cancer samples. Nuclear *FOXP3* is expressed in normal human prostate epithelial cells but is lost in approximately 70% of human prostate cancers. *FOXP3* is frequently inactivated in prostate cancer samples by deletion or somatic mutation. The significance of such inactivation is confirmed by strong growth inhibition of prostate cancer cell lines upon addition of *FOXP3*. Importantly, prostate-specific ablation of the *FOXP3* in the mouse caused early onset of prostatic hyperplasia and prostatic intraepithelial neoplasia. Functional analysis has shown that *FOXP3*-mediated transcriptional repression of *c-Myc* is necessary to control *c-Myc* levels in normal prostate epithelial cells, which accounts for much of the widespread overexpression of *c-Myc* in prostate cancer. Therefore, *FOXP3* is also an X-linked tumor suppressor gene for prostate cancer (Wang *et al*., 2009).

2.5.2 *FOXP3* and Ovarian Cancer

FOXP3 was shown to be weakly or not expressed in ovarian cancer cells. Transfection of ovarian cancer cells with *FOXP3* inhibited cell proliferation, decreased cell migration, and reduced cell invasion. Cells with up-regulated *FOXP3* showed decreased expression of Ki-67 and cyclin-dependent kinases. *FOXP3* can inhibit cell migration and invasion by reducing the expression of matrix metalloproteinase-2 and urokinase-type plasminogen activator in ovarian cancer cells. These data suggest that upregulation of *FOXP3* could be a novel approach for inhibiting ovarian cancer (Wolf *et al*., 2005).

2.5.3 *FOXP3* in lung cancer

Expression of the transcription factor *FOXP3* characterizes regulatory T cells (T_{regs}) that engage in the maintenance of immunological self-tolerance and immune homeostasis. Intra-tumoral accumulation

of T_{regs} is associated with unfavorable prognosis in several kinds of cancers. Recently, expression of *FOXP3* and its association with prognosis have also been shown in some cancer cells in clinical studies. For non-small cell lung cancer (NSCLC), however, prognostic significance of tumor *FOXP3* expression and its relationship with T_{regs} remain unknown. *FOXP3* expression in cancer cells and tumor-infiltrating lymphocytes was examined by immunohistochemical staining of surgical specimens from 87 patients with NSCLC. Prognostic values of the tumor-infiltrating T_{regs} count and tumor *FOXP3* expression status were evaluated retrospectively. *FOXP3*-positive cancer cells were observed in 27 of 87 (31.0%) patients. There was no significant relationship between T_{regs} count and tumor *FOXP3* status. Increased T_{regs} counts were associated with worse overall and relapse-free survival whereas the influence of tumor *FOXP3* status on survival was not significant. However, when *FOXP3*-positive cancer cells were present, the relationship between T_{regs} accumulation and worse prognosis was attenuated. In contrast, patients without tumor *FOXP3* expression and high T_{regs} count had significantly worse overall and relapse-free survival than other groups. These results suggest that tumor *FOXP3* expression has a better prognostic potential in NSCLC and that in combination with tumor-infiltrating T_{regs} count the absence of tumor *FOXP3* allows the selection of high-risk patients (Tao *et al* ., 2012).

2.6 POLYMORPHISM

SNPs are highly conserved throughout evolution and within a population, the map of SNPs serves as an excellent genotypic marker for research.

SNPs are often found to be a cause in many human diseases and are also of particular interest in pharmacogenetics. A variation is to be considered a SNP, it must occur in at least 1% of the population. While many SNPs have no effect on trait or condition, others could predispose people to disease or influence their response to a drug.

A DNA polymorphism is any difference in the nucleotide sequence between individuals. These differences can be single base pair changes, deletions, insertions, or even changes in the number of copies of a given DNA sequence. SNPs (single nucleotide polymorphisms) are the most common type of DNA polymorphism in humans. An example of an SNP would be if a cytosine (C) nucleotide is present at a particular locus in one person's DNA but a thymine (T) nucleotide occurs at the same locus in another person's DNA. It might be surprised to know that around 90% of all human genetic variation is due to SNPs (Boehmer *et al*., 2008).

Mutation in *FOXP3* rs2294021is occurs in the intronic region which results in transcription defects (Shen *et al*., 2008). *FOXP3* intron mutation has been shown to inactivate its tumor repressor activity

and cancer tissues with intron mutations have an inactive *FOXP3* locus (Zuo *et al*., 2007). *FOXP3* polymorphism has been associated with increased risk for immune-mediated diseases (Bjørnvold *et al*., 2006). These evidences strongly implicate that *FOXP3* polymorphism as a contributor to the increased risk of breast carcinoma.

CHAPTER 3
OBJECTIVE

OBJECTIVES

- To study the prevalence and genotype frequencies of the *FOXP3*rs2294021 (T/C) polymorphism in breast cancer of North Indian populations
- To check the association between *FOXP3* rs 2294021 (T/C) polymorphism in relation towards risk of breast cancer and also in relation to demographic and clinico-pathological features

Chapter 4
Methodology

4.1 Sample collection

In this case–control study, we evaluated the *FOXP3* polymorphism in 33 breast cancer patients with a mean age of 55 (35-75) years and 28 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 *FOXP3* 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 isolation

After the collection of blood from breast cancer samples and breast cancer controls then next step was the isolation of DNA from blood of both breast cancer samples and breast cancer controls. For the isolation of DNA from blood Phenol:Chloroform:Isoamyl Alcohol method was used. 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 Reagents used in DNA isolation are given below. (Bartlett and White, 2003).

4.2.1 Reagents:-

1. Washing buffer
2. Lysis buffer
3. Phenol:choloroform:isoamyl alcohol solution
4. Choloroform:isoamyl alcohol solution
5. Isopropanol
6. 70% Ethanol
7. TE buffer

4.2.2 Washing buffer:-

Table 4.2.1. Different reagents used for washing buffer preparation

Working concentration	Stock concentration
320mM Sucrose	1M Sucrose
1% TritonX-100	100% TritonX-100
5mM $MgCl_2$	100mM $MgCl_2$
10mM Tris Cl (pH=8)	100mM Tris Cl (pH=8)

4.2.3 Lysis buffer:-

Table 4.2.2. Different reagents used for Lysis buffer preparation

Working concentration	Stock concentration
400mM Tris Cl (pH=8)	1M Tris Cl (pH=8)
1% SDS	10% SDS
60mM EDTA	0.5M EDTA
150mM NaCl	5M NaCl
100µg/ml Proteinase K	10mg/ml Proteinase K

4.2.4 DNA isolation procedure

First blood was taken in tube and then equal volume of washing buffer was added and then mixed this solution properly for 5 minutes and after mixing then centrifuged the solution at 3500 rpm for 5 minutes. After centrifugation supernatant was discarded and again washing buffer was added to the pellet and centrifuge, first step was repeated three times. After proper washing of cells then equal volume of lysis buffer was added and incubated this at 42°C overnight. Next day after incubation phenol:chloroform:isoamyl alcohol (25:24:1) solution was added in equal amount and proper mixing was done and then it was left for 5 minutes in ice and after that the solution was centrifuged at 8000 rpm for 10 minutes and repeat the step twice. After centrifugation two layers were formed and aqueous layer was extracted in the new tube and then solution of choloroform : isoamyl (24:1) alcohol was added in equal amount of layer which was separated and mixed properly. Then solution was centrifuged at 6500 rpm for 5 minutes. Again two layers were formed upper layer was extracted in the new tube and isopropanol was added to it in equal volume and mixed properly and then again centrifuged the solution at 12000 rpm for 10 minutes. Then pellet was formed and supernatant was discarded and washing of pellet was done with 70% ethanol with 2ml of it and centrifuged at 12000

rpm for 5 minutes, then again supernatant was discarded, repeat this step two times and when pellet was proper dried then dissolved it in TE buffer.

4.3. Agarose gel electrophoresis

After the DNA isolation then gel electrophoresis was done for the qualitative estimation of DNA which was isolated. 0.7% agarose gel having ethidium bromide (EtBr) at the concentration of 0.3µg/ml was made in 5X Tris Borate EDTA and casted in electrophoretic apparatus. The gel was allowed to solidify and after solidification comb was carefully removed. Electrophoretic running buffer (0.5X TBE) was put into the tank so that gel is fully immersed into buffer. The DNA samples were mixed with the 6X loading dye (xylene cyanol & bromophenol blue) and with water. The samples were loaded into wells and allowed to run at 60 volts. The gel was observed under UV transilluminator for the presence of DNA. Gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software.

4.4. DNA quantification

Quantitative estimation of DNA was done by Spectrometric analysis of the sample. The absorbance of samples were taken at 260 nm, to determine the concentration of sample. 1 OD is equivalent to 50µg/ml DNA sample so, accordingly the concentration of sample is calculated.

$$\text{Concentration } (\mu g/ml) = O.D_{260nm} \times 50\mu g/ml \times \text{dilution factor}$$

The purity of the sample was checked by taking the ratio of its absorbance at 260 nm and 280 nm. The ratio if is less than 1.8, then it means there is RNA contamination, if the ratio is more than 1.8 it means there is protein contamination and if the ratio is equal to 1.8 that mean DNA is pure.

$$\text{Ratio} = A_{260/}A_{280}$$

And for amount = concentration/1000 × volume of TE in which pellet was dissolved. And dilution factor taken was 300 times. For dilution of DNA 1.5µl of DNA sample was taken and 498.5µl of TE buffer was taken.

4.5. PCR amplification

Polymerase chain reaction is one of the mainstays of molecular biology. The main reason for the wide adoption of PCR is the simplicity of reaction and relative ease of practical manipulation steps.

The PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material usually known as template DNA. 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 ºC). 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)

Primer used were, forward primer 5'-CACACACAATCCATCCCAGTCACCC-3'and reverse primer used was 5'-ATCTCCATGCCCTAAGAAGGCCACC-3' (Han *et al*., 2010).

Table 4.5.3. Different reagents used for Lysis buffer preparation.

	Stock concentration	Working concentration
Forward primer	10 µM	0.5 µM
Reverse primer	10 µM	0.5µM

PCR buffer ($MgCl_2$) used was 1.5mM. Reaction volume used was 20µl. concentrations and solutions were used for optimization of PCR are given below:-

Table 4.5.4. Different reagents used for PCR optimization with their different concentrations.

Reagents	Stock concentration	Working concentration
BSA	100X	1X
Buffer	15mM	1.5mM
dNTP's	10mM	0.2mM
Forward primer	10µM	0.5µM
Reverse primer	10µM	0.5µM
Taq. Polymerase	5U/µl	0.8U
Water		

Master mix was made from all these solutions first water was added then BSA was added after that buffer was added and then primers were added and then dNTP's and then Taq. polymerase was added after any addition of solution mixing was done properly by vortex and then PCR tubes were taken and marked them one as p temperature 67°C. After marking then 21µl of master mix was taken and added into each one of three PCR tubes and then breast cancer control was used as template DNA and 4µl of it was added into each one of three PCR tubes and then after addition proper mixing was done with vortex. Then a PCR tubes were put in Thermocycler (Bio Rad) and was left for PCR

cycles. The PCR cycling conditions consisted of initial denaturation at 95°C for 5 min followed by 29 cycles of 94°C for 30 sec, 67°C for 30 sec, 72°C for 45 sec followed by final extention at 72°C for 5 minutes. PCR temperature profile is given below:

Table 4.5.5. Showing temperature profile of PCR reaction.

Steps	Step name	Temperature	Time
Step 1	Initial Denaturation	95°C	5 min
Step 2	Denaturation	94°C	30 sec
Step 3	Annealing	67°C	30 sec
Step 4	Extention	72°C	45 sec
Step 5	Step 2 to step 4 repeated 29 times		
Step 6	Final Extention	72°C	5 min
Step 7	Store	12°C	∞

The PCR products were examined using gel electrophoresis in 1.7% agarose gel dissolved in 0.5X TBE at 60 volts for 1 hour. Gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software. 429bp fragment was obtained from above mentioned amplification protocol.

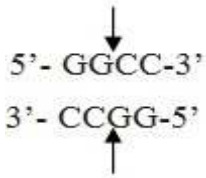
PCR amplification of both breast cancer samples and breast cancer controls was done of samples and controls. But for samples and controls reaction volume used was 16µl was master mix and 4µl was template DNA.

4.6. Restriction fragment length polymorphism (RFLP)

RFLP is a difference in homologous DNA sequences that can be detected by the presence of fragments of different length on the gel electrophoresis after digestion of DNA samples with a specific restriction endonuclease. Enzyme will have its specific restriction site.

First restriction digestion was done for both breast cancer samples and also for breast cancer controls. The reaction mixture consists of PCR product (10µl), 1X NEB 4 buffer having a stock concentration 10X, 3U *Hae III* having a stock concentration of 10U/µl, the total reaction volume was made up to 20 µl using sterile water. The reaction mixture was incubated at 37°C overnight.

Enzyme used was *HaeIII* isolated from the *Haemophilus aegyptius* bacteria and its restriction site is



The digested product was resolved 2 % agarose gel at 60 volts. Imaging was done under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software. Then genotyping was done by scoring the gel for each sample using the information given below (Han *et al.,* 2010)

Table 4.6.6. Banding pattern of *FOXP3* gene (SNP)

Wild type	**322, 87, 20 bp**
Heterozygous (TC)	322, 216, 106, 87,20 bp
Homozygous (TT)	322, 87, 20 bp
Mutant (CC)	216, 106, 87,20 bp

4.7. Statistical analysis

The Hardy−Weinberg equilibrium was tested among patients and controls separately with the χ^2-test. Crude ORs with 95% CIs was used to assess the strength of relationship between the *FOXP3* (T/C) Polymorphism and breast cancer risk. All statistical tests and *p value*s 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*s. The same statistical analysis was applied to subgroups according to menopausal status, and age (≤40 and >40 years) and on clinico- pathological features. Data were analyzed by using the computer software MedCalc. (Han *et al.,* 2010).

Chapter 5
Results and discussion

RESULTS & DISSCUSION

5.1. Genomic DNA isolation

The isolated DNA was qualitatively estimated using agarose gel electrophoresis. Fig 5.1.3 shows the gel image of the genomic DNA which is having an approximate size of 1kbp when compared to 100bp DNA ladder (G-Biosciences).The sharp bands show that isolated DNA was intact and was free from shearing.

Fig.5.1.3 Genomic DNAIsolation in agarose gel (1-8)

5.2. PCR Amplification

The amplified PCR products were resolved in1.7% agrose gel. In the fig. 5.2.4 amplified *FOXP3* gene was shown where lane 1 is ladder (100bp) and lane 4, 13 is un- amplified DNA and rest of lanes is amplified gene of 429bp (2-3,5-12,14-15)

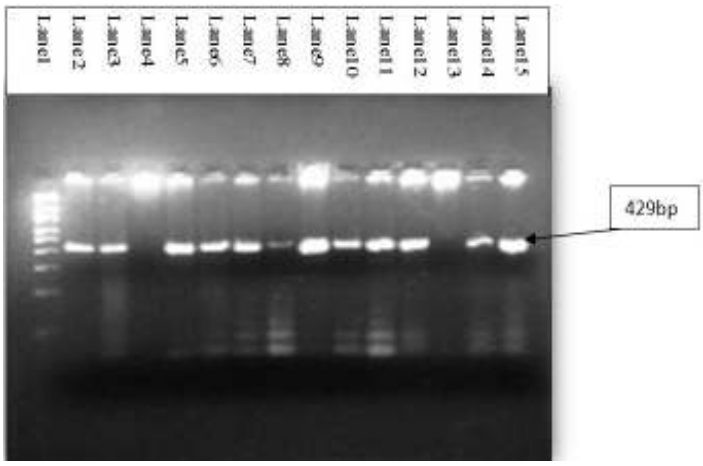


Fig.5.2.4 PCR amplified product.

5.3. Restriction digestion product

PCR sample is digested with HaeIII and separated on 2% agarose. Fig. 5.3.5 shows the different bands of PCR digested product (Lane1is ladder, lane2 is uncut PCR product and rest of lanes (1-18) are PCR digested product) in some bands there is 429bp band is also seen due to high concentration of PCR product . The T allele had two restriction sites that resulted in 3 bands (322 bp, 87 bp and 20 bp), while the C allele had three restriction sites that produced 4 bands (216 bp, 106 bp, 87 bp and 20 bp).

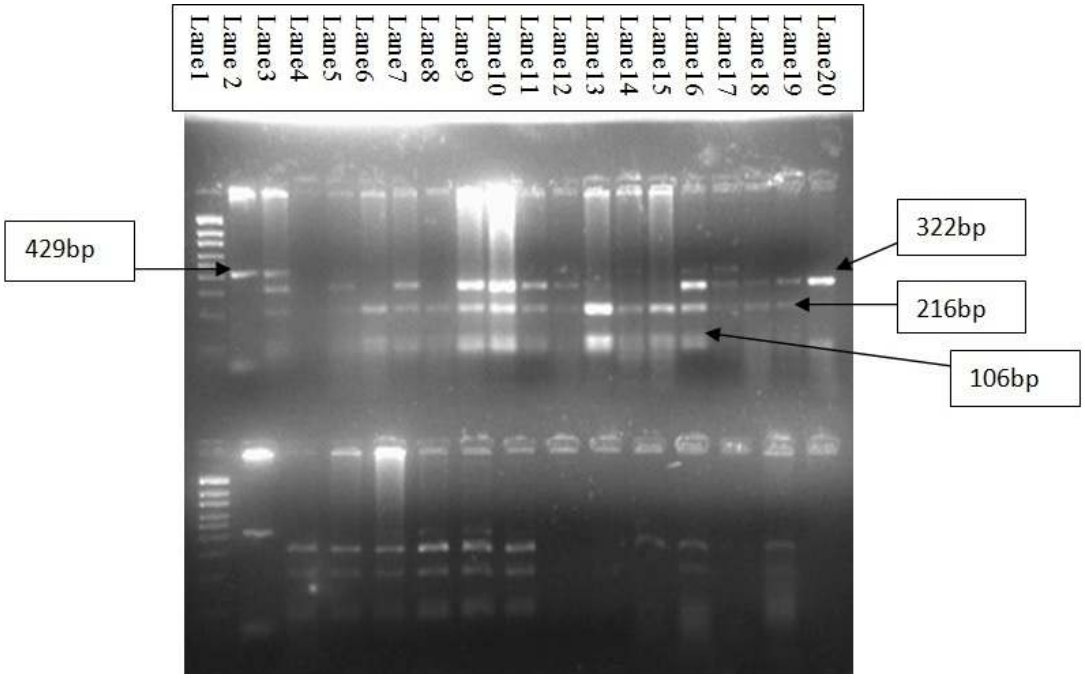


Fig 5.3.5 PCR digested product with ladder and uncut PCR product.

5.4.Epidemiology

Focusing on the potential role of the *FOXP3* gene in the development of breast malignancy, we studied haplotype tag-SNP rs2294021 (C/T) polymorphism located in the intron region in this gene. The case control study pertains to 33 breast cancer patients and 28 controls. Average age was (57.18±15.26) among the cases and (43.16 ± 12.38) among the controls. 51.5% of the cases were more than 40 years of age and just 48.5% of the cases were of below 40, while in controls 71.4% of the population was above 40 years of age and 28.5% were below 40. We also study menopause in cases (48.8%) and control (10.7%).The relevant characteristics of subjects studied are shown in Table

5.4.7. Of the various risk factors concerned with the risk of breast cancer development. The frequency of females in cases and controls is high only 1 case is seen in male so that there is less similarity among sex. In a study it was found that increased risk of breast carcinoma associated with the T/C genotype, compared with the T/T genotype, was much higher for the group aged > 50 years at diagnosis (OR 1.631; 95% CI 1.116–2.383, *p-value* = 0.011) than for the group aged ≤ 50 years at diagnosis (OR 1.374; 95% CI 1.036–1.824, *p-value*= 0.027) and but there is no significant difference was evident between breast carcinoma patients and controls in terms of age distributions. They analysed 677 breast carcinoma patents in comparison to 828 controls (Han et. al., 2010).

Table.5.4.7. Distribution of demographic variables for patients and controls.

CHARACTERSTIC	**CASES (n%)**	**CONTROLS (n%)**
GENDER		
MALE	1(3.04)	
FEMALE	32 (96.96)	28(100)
AGE		
≤40	**16 (48.4)**	**8 (28.5)**
C/C	6(18)	1(3.6)
T/C	10(30.3)	7(25)
>40	**17 (51.5)**	**20(71.4)**
C/C	5(15.15)	4(12.1)
T/C	10(30.3)	13(39.4)
T/T	2(6.06)	3(9.09)
MENOPAUSE	16 (48.8)	3 (10.7)

RELATIONSHIP OF BREAST CANCER RISK WITH GENOTYPES OF *FOXP3*

The genotypes of patients and controls for *FOXP3* gene were obtained by PCR RFLP. Out of total patients (28) studied 65.9% of the individuals were found to have homozygous wild type genotypes,28% had heterozygous genotypes and 6% individuals had mutant genotype. On the other hand in case of controls 75.3% of the individuals had homozygous wild type genotype, 24.7% of the

individuals had heterozygous genotypes and mutants were observed. The genotype and allele frequencies of the *FOXP3* (T/C, rs2294021*)* SNPs and their associations with risk of breast cancer are summarized in Table 5.4.8.

Table 5.4.8. Frequency distribution of *FOXP3* genotypes and their association with risk**.**

GENOTYPES	**NUMBER (%)**		**O.R(95%CI*)***	***p-value***
FOXP3	**CASES**	**CONTROLS**		
Total	33	28		
TT	2(6.06)	3(10.7)		
CC	11(33.3)	5(17.8)	3.000	0.29
CC+TC	20(60.6)	20(71.4)	1.800	0.53
T allele	0.37	0.32		
C allele	0.63	0.48		

The genotype distribution of both SNPs in the controls (*p-value*=0.0538, chi sq=1.29) and cases (*p-value*=0.127, chi sq= 1.13) was all in agreement with that of the Hardy- Weinberg equilibrium. However, compared with the CC+TC genotype (OR=1.800, 95%CI; 0.278-11.635, *p-value*=0.53250) the variant CC were associated with a statistically non-significant, 3 fold increased risk (OR 3.0, 95% C.I; 0.372–24.171, *p-value*=0.29184, respectively). Extremely high O.R can be neglected because of small sample size but *p-value* assures the correlation between mutant CC genotype and risk of breast cancer. It is seen that subjects carrying the T/C genotype had an increased risk of developing breast carcinoma. In contrast, neither of the homozygous C/C or T/T genotypes seems to be associated with increased breast cancer risk (Han *et al*., 2010). *FOXP3* mutation have a comparably higher incidence of mammary cancer, the *FOXP3* mutation is likely responsible for the increased rate of breast cancer. Unlike essentially all cancer suppressor genes identified till date. (Tao *et al.,* 2007). *FOXP3* genotypes did not deviate from Hardy Weinberg equilibrium in controls *(p-value*=50.637, 0.066, 0.594 for the three htSNPs, rs2294020, rs3761548, and rs5906761, respectively). *FOXP3* is a biologically relevant gene in the pathogenesis of breast cancer; germline variation was not meaningfully associated with risk of the disease (Raskin *et al*., 2009).

DISTRIBUTION OF GENOTYPES *FOXP3* GENE AMONG PATIENTS WITH DIFFERENT HISTOLOGICAL TYPES OF BREAST CANCERS:

In the cases studied 54.5% (18) of the cases were of those whose who suffered from Lobular carcinoma, 36.36%(12) had ductal carcinoma. On further stratification on basis of genotypes it was found that 3.03% and 3.03% individuals of lobular and ductal carcinoma respectively had homozygous genotypes. On the other hand 36.4% and 18.2% respectively had heterozygote genotypes. While 15.2% of Lobular carcinoma patients 15.2% of the ductal carcinoma patients were mutants as shown in Table 5.4.9

Table 5.4.9. Frequency distribution *FOXP3* genotypes among different histological types of Breast Cancer.

	Total (%)	**T/T (%)**	**C/C (%)**	**T/C (%)**
lobular	18(54.5)	1(3.03)	5(15.2)	12(36.4)
Ductal	12(36.4)	1(3.03)	5(15.2)	6(18.2)
unknown	3		1(3.03)	2(6.06)

Case study was done in which multivariate analysis *FOXP3* resulted an independent prognostic factor and the hazard ratio of *FOXP3* expression and of lymph node positivity were similar. In the Milan 3 trial, the probability of 10-year survival in node-negative subgroup was 100% for *FOXP3* -negative and 82% for *FOXP3*-positive patients; in node-positive subgroup 82% for *FOXP3* -negative and 41% for *FOXP3*-positive patients. Even in the Milan 1 trial the lack of *FOXP3* expression in node-positive subgroup was related to a significantly better prognosis than in *FOXP3*-positive patients. (Merlo *et al.,* 2009).

Chapter 6
Conclusion

Conclusion

- In conclusion, our results show that the *FOXP3* T/C polymorphism may contribute to breast cancer risk. A significant increased breast cancer risk was seen among women with the *FOXP3* T/C in mutant and less in heterozygous.

- In our study patients less than 40 years of age will be more susceptible towards risk for breast cancer

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

- Our sample size is too small to get a significant result. If a large sample size is studied a significant result showing an association between the *FOXP3* (rs2294021) polymorphism and susceptibility towards breast cancer in North Indian population can be obtained.

CHAPTER 7
APPENDIX

APPENDIX

1. **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 1M sodium hydroxide. Sterilized the solution by autoclaving.

2. **10% SDS**: Dissolved 1g of SDS in 10ml of deionsed water.

3. **100mM Tris-Cl (pH 8.0)**: Dissolved 0.32g of Tris-Cl in 10ml of deionsed water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilized the solution by autoclaving.

4. **10mg/ml Proteinase K**: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilized the solution by autoclaving.

5. **1mg/ml BSA**: Dissolved 100mg of BSA in 100ml of deionised sterile water and kept at 4℃ overnight.

6. **5% DMSO**: Mixed 50ml of 100% DMSO in 50ml of deionised sterile water. Sterilize the solution by autoclaving and stored at -20˚C.

7. **5M Sodium chloride (NaCl)**: Dissolved 5.85g of sodium chloride in 20ml of deionised water. Sterilize the solution by autoclaving.

8. **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. Sterilized the solution by autoclaving.

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

10. **Magnesium chloride ($MgCl_2$) (100mM)**: Dissolved 0.41g of $MgCl_2$ in 20ml of deionised water and sterilized by autoclaving.

11. **Sucrose (1M)**: Dissolved 3.41g of sucrose in 10ml of deionised water and sterilized by autoclaving.

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

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

CHAPTER 8

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