ASSOCIATION OF micro RNA GENE *196a2* AND *146a* POLYMORHISM TOWARDS RISK FOR BREAST CANCER

A Dissertation
Submitted in partial fulfillment of the requirement
For the award of degree of
Masters of Science in Biotechnology

Under the guidance of
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CERTIFICATE

This is to certify that the dissertation entitled "***Association of microRNA gene 196a2 and 146a polymorphism towards risk of Breast Cancer***" being submitted by **Mr Rishi Roy** in partial fulfilment for the requirement of degree of **Masters of Sciences** in **Biotechnology** in the **Department of Biotechnology, Thapar University, Patiala** is a bonafide work carried out under the esteemed supervision and conception of **Dr Siddharth Sharma**, Assistant Professor , Department of Biotechnology, Thapar University, Patiala.

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DECLARATION

I hereby declare that the work being presented in the dissertation entitled **"case control study of miRNA 146a and 196a2 genes polymorphism towards risk for Breast Cancer"** by me in the partial fulfillment of the requirements for the award of degree of Masters of Science in Biotechnology, from Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work carried under the supervision of **Dr. Siddharth Sharma,** Assistant Professor, Department of Biotechnology, 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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CHAPTER 1
INTRODUCTION

INTRODUCTION

Cancer is the first leading cause of death worldwide after cardiovascular diseases. It has been shown that micro RNAs are involved in carcinogenesis. The exact role of *miRNAs* in cancer pathogenesis was identified through expression studies of specific *miRNAs* which were over-expressed or knocked down and the initiation and development of different types of malignancies related to it. The first evidence that *miRNA*s is related to cancers came from Croce group. In their study, they found two *miRNA* genes (*miR*-15 and *miR*-16) are located at the chromosome 13q14 region, which is frequently deleted or down-regulated in the majority (~68%) of B cell chronic lymphocytic leukemia (B-CLL) cases (Calin *et al*., 2002). Subsequent investigations demonstrated that almost all cancers have alternative *miRNA* expression profile compared to their adjunct normal tissues. These cancer types include several important cancers, for example lung cancer, leukemia, brain cancer and breast cancer, which together cause the majority of cancer related death in the past decades (George and Mittal, 2010).The study related to cancer is mainly focused on protein coding genes and are considered to play a vital role in tumourigenesis. However, recent studies have also brought non-protein coding RNA as a regulator in tumourigenesis.

Altered mature Micro RNA processing, mutations, misexpression of micro RNA leads to tumor progression and carcinogenesis. Polymorphism in the micro RNA could alter the overall expression, processing and binding to the target mRNA.

The strong association of deregulated miRNA expression with breast cancer has been well documented by a vast amount of data from numerous studies. In a genome-wide miRNA expression profiling study of a large set of breast cancer and normal tissue, Iorio *et al.,* demonstrated that 29 miRNAs were differentially expressed in breast cancer versus normal tissues; miR-21 and miR-155 were upregulated, whereas miR-10b, miR125b and miR-145 were downregulated, suggesting that these miRNAs may potentially act as diagnostic markers. Subsequent studies showed that expression patterns of miR-21 and miR-145 could discriminate between cancer and normal tissues (Paranjape *et al*.,2009).

Several miRNA-associated SNPs have been shown to increase breast cancer susceptibility—
for example, an SNP, rs11614913 located in the pre-miRNA of miR-196a2, has been identified

in the miRNA hsa-mir-196a2. The variant genotypes CC/CT were associated with significantly increased breast cancer risks in a case–control study of 1009 breast cancer cases and 1093 cancer-free controls in a population of Chinese women (Hu *et al*., 2009).

Another study conducted by Shen *et al*., (2008) has identified a G to C polymorphism (rs2910164) within the sequence of the mir-146a precursor and demonstrated that a variant C allele led to increased levels of mature miR-146 in patients with breast and ovarian cancer and predisposed them to an earlier age of onset of familial breast and ovarian cancer.54 All of these findings suggest, for the first time, that common SNPs in miRNAs may contribute to breast cancer susceptibility.

So, we conducted a case control study to find out ―Association of Single Nucleotide Polymorphism in *micro RNA 196a2* and *146a* gene towards risk for BREAST CANCER in North Indian population.

CHAPTER 2

REVIEW OF LITERATURE

- Breast cancer
- Histology of Breast Cancer
- Micro RNAs
- Micro RNA *146a* gene
- Micro RNA *196a2* gene

2.1 BREAST CANCER

Breast cancer is the most common type of cancer among women in the United States (other than skin cancer). In 2012 about 227,000 American women were diagnosed with breast cancer. Breast cancer is the most common cancer among American women, except for skin cancers. About 1 in 8 (12%) women in the US suffer from invasive breast cancer during their lifetime. The American Cancer Society's estimates for breast cancer in the United States for 2014 are:

· About 232,670 new cases of invasive breast cancer will be diagnosed in women.

· About 62,570 new cases of carcinoma in situ (CIS) will be diagnosed (CIS is noninvasive and is the earliest form of breast cancer).

· About 40,000 women will die from breast cancer (American cancer society, 2014)

2.2 HISTOLOGY OF BREAST CANCER

The most common forms of breast cancers begin in the milk ducts, lobules or glands and are named accordingly. There are many types of breast cancer but a pathologist can identify the differences by observing sections of the tumor through a microscope.

Non-invasive type of breast cancers:

Ductal Carcinoma In-Situ (DCIS):

The term ―in situ‖ or "in place" refers to a very early form of cancer that has not spread. DCIS is a type of pre-cancer inside of the ductal system that has not attacked the nearby tissue. Currently, there is no way to determine if this type of breast cancer will go on to become invasive. This is a very common type of non invasive cancer with 1 in 5 diagnosed as DCIS. Nearly all patients diagnosed with this type of pre-cancer can be cured.

Lobular Carcinoma in Situ (LCIS):

This is a very rare non-invasive tumor that will not develop into invasive cancer. LCIS is more of a ―marker‖ or signal that breast cancer may develop. Described as an abnormal growth in the number of cells, LCIS has recently been renamed lobular neoplasia. Women who have these ―markers‖ are at greater risk of developing breast cancer later in life.

Types of invasive breast cancer:

The most common type is invasive ductal carcinoma (also called infiltrating ductal carcinoma and less commonly, invasive carcinoma of no special type or invasive carcinoma not otherwise specified). Invasive ductal carcinoma accounts for 50 to 75 percent of all breast cancers. Invasive lobular carcinoma is the next most common type and accounts for about 10 to 15 percent of cases. Tubular carcinoma and mucinous (colloid) carcinoma are less common types of invasive breast cancer that tend to have a good prognosis (American cancer society,2014).

2.3 Micro RNAs

Micro RNAs (miRNAs) are small, non-coding RNAs with important functions in development, cell differentiation, and regulation of cell cycle and apoptosis. miRNA expression is deregulated in cancer by a variety of mechanisms including amplification, deletion, mutation, and epigenetic silencing. Several studies have now shown that miRNAs are involved in the initiation and progression of cancer (Bartel DP, 2004).

2.3.1 BIOGENESIS: micro RNA maturation:

Alternative splicing determines whether a miRNA is intronic or exonic. MiRNAs are 5‘capped and 3‘ polyadenylated and may also be spliced similar to mRNA.

Micro RNA pathway is universal to all mammalian miRNAs. MiRNAs are generated through two step processing that converts primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting miRNA is exported from the nucleus by Exportin-5-Ran-GTP. In the cytoplasm the RNase Dicer, in complex with the double-stranded RNA-binding protein TRBP, cleaves the pre-miRNA hairpin to its mature length. TRBP binds the Argonaute protein (Ago2) and instantaneously with Dicer to form a trimeric complex. This initiates the assembly of the RNA-induced silencing complex (RISC), a ribonucleoprotein complex that leads to mRNA degradation Association of the miRNA-RISC with a target mRNA results in the repression of the target gene by promoting mRNA degradation and/or translational inhibition (Micro RNA in carcinogenesis & cancer diagnostics: A new paradigm ;Indian J Med Res 137, April 2013)

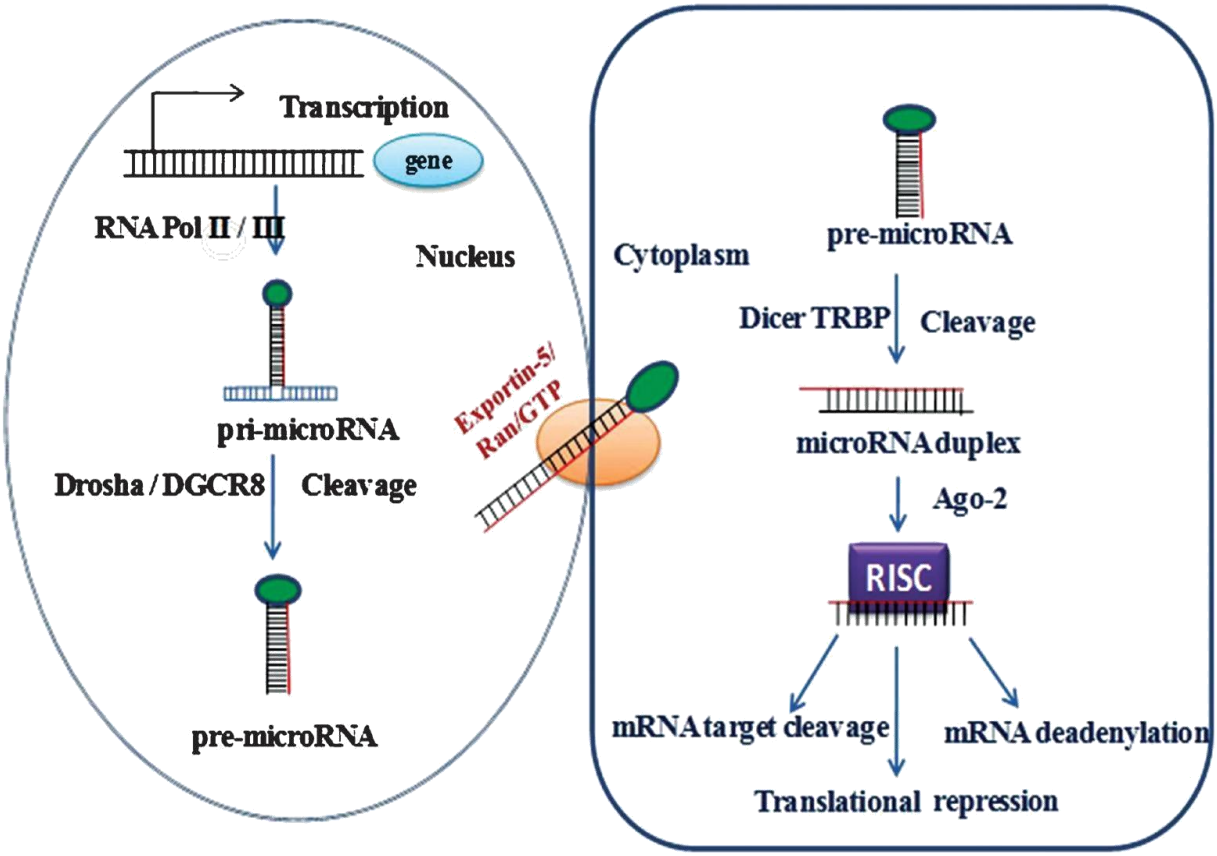

Fig 2.1: Biogenesis of miRNA (Bartel, 2004)

2.3.2 Mechanism of Action

Once incorporated into RISC, the miRNA guides the complex to target the RNA by base-pairing interactions. In case of animals, the binding is perfect or near-perfect complementarily to the miRNA, this target mRNA is cleaved and degraded, otherwise, translation is repressed. RISC contains an Ago2 protein capable of endonucleolytic cleavage. Most animal miRNAs base-pairing is imperfect with their targets, which stimulates translational repression rather than cleavage and degradation. In this type of repression, target mRNAs are not actively degraded, however, these can be destabilized by deadenylation and subsequent decapping.

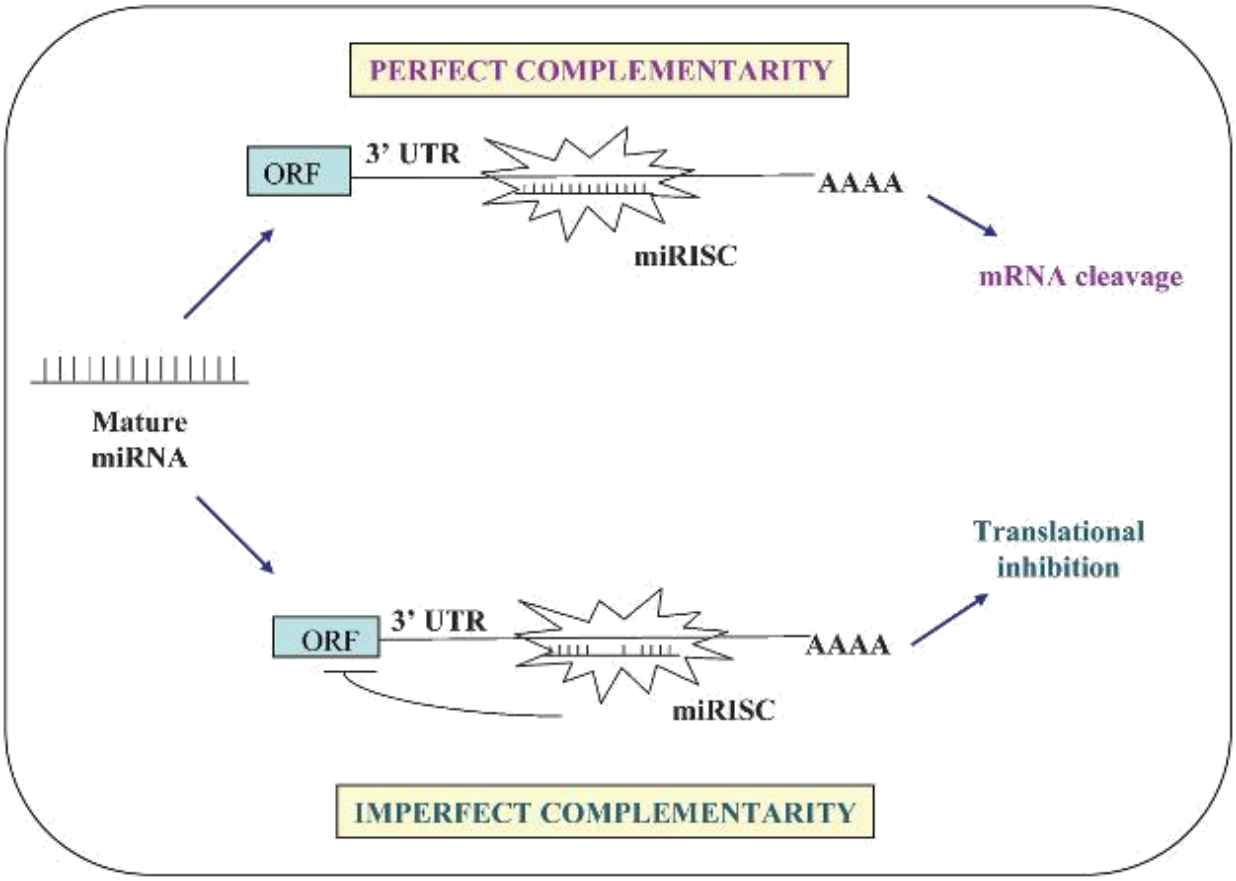

Fig 2.2: Mechanism of action (Paranjape *et al*., 2009)

2.3.3 Micro RNA as oncogene and tumor suppressor:

Micro RNAs may function as a novel class of oncogenes and tumor suppressor genes. The miRNAs with increased expression in tumors are thought to function as oncogenes and are termed as oncomirs. These oncomirs negatively inhibit tumor suppressor genes or those controlling cell differentiation or apoptosis and thereby promote tumor development. Whereas, some miRNAs exhibit decreased expression in cancerous cells and are considered as tumor suppressor genes. Tumor suppressor miRNAs usually prevent tumor development by negatively inhibiting oncogenes or genes that control cell differentiation or apoptosis.

2.3.4 Micro RNA as Therapeutic Tools

miRNAs are known to be involved in various cellular processes such as apoptosis, proliferation, receptor-driven pathways, etc. Mi RNAs or anti-micro RNA (anti-mi RNA) may be used in therapeutics either individually or in combination with other treatments that have lost efficacy.

2.3.5 Micro RNA as Diagnostic and Prognostic tools

miRNAs are promising candidates to distinguish different tumors and different subtypes of tumors as well as to predict their clinical behavior .miRNA profiling has acquired importance in resolving one of the most demanding issues in cancer diagnostics—the origin of metastasis of unknown primary tumor.

2.3.6 Micro RNA Polymorphism and Cancer:

The role of miRNA-associated SNPs in disease is just emerging. Because small variations in the quantity of miRNAs may have an effect on thousands of target mRNAs and result in diverse functional consequences, the most common genetic variation, SNPs, in miRNA sequences may also be functional and therefore may represent ideal candidate biomarkers for cancer diagnosis, prognosis and outcome. SNPs that disrupt miRNA gene sequences have been associated with cancer risk. The table given below shows the association of various SNP with different Cancers in the ethnically different populations.

Table 2.1: Association of various SNPs with different Cancers(Paranjape *et al*.,2009)

Disease	miRNA	SNP	Risk allele	Target gene	SNP in
Colorectal cancer	miR-337	rs17281995	C	CD86	Target site
	miR-582				
	miR-200a*				
	miR-184				
	miR-212				
Colorectal cancer	miR-618	rs1051690	A	INSR	Target site
	miR-612				
Lung cancer	let-7	rs61764370	G	KRAS	KRAS 3′ UTR
	miR-196a2	rs11614913	T (Chinese population)		pre-miR
Papillary thyroid carcinoma	miR-221/222	rs17084733	A	KIT	KIT 3′ UTR
	miR-146a	rs2910164	GC (heterozygous genotype)		Pre-miR-146a
Hepatocellular carcinoma	miR-146a	rs2910164	G	RAF1	MiRNA gene
Breast cancer	miR-196a2	rs11614913	C (Chinese population)		Pre-miR
	hsa-mir-499	rs3746444	G		Pre-miR
Oesophgeal cancer	miR-423	rs6505162	C		Pre-miR

miRNA, microRNA; SNP, single nucleotide polymorphism; UTR, untranslated region.

Various other SNP in the mirRNA genes have been found to be associated with the risk for Breast Cancer. A C>T (rs93410170) miR SNP in the 3'-UTR of ER-alpha, resulted in stringent miR-206 mediated regulation of ER-alpha. Since ER-alpha overexpression is associated with higher risk for breast cancer, it was suggested that the SNP may be associated with breast cancer (Brendle *et al*., 2008).

2.4 MiRNA 146a gene

Structure and Location of gene:

Resides in the LOC 285628 gene on human chromosome 5. Using the 3′- and 5′-RACE technique the two-exon structure of the *miR-146a* primary transcript pri-*miR146a* was confirmed and its full length was found to be 2,337bp (Taganov *et al*., 2006). *Pre-miR-146a* G/C polymorphism designated rs2910164 is located in the stem region exon nucleotide of *pre miR-146a* (Akkiz *et al*., 2011).

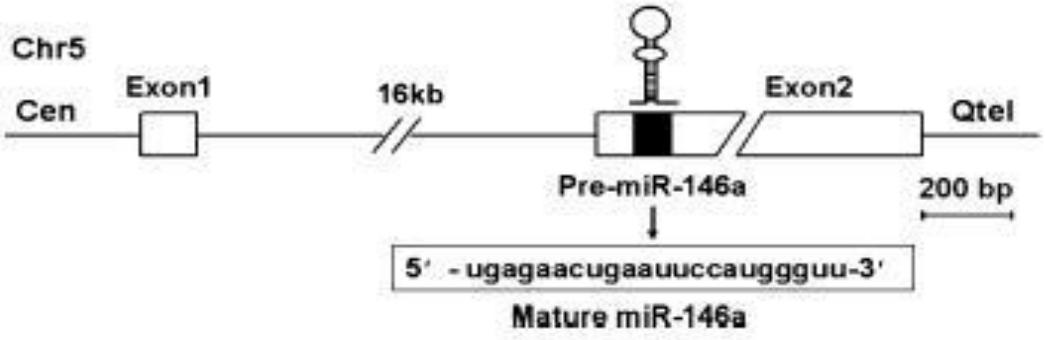

Fig 2.3: *miR-146a* loci and the detailed sequence of mature *miR-146*a on human chromosome 5 (Li *et al.*, 2010)

2.4.1 MiRNA 146a in Tumourigenesis and Tumour Metastasis

Increased Cell proliferation and colony formation in NIH/3T3 cells by *miR146* (Xu *et al*., 2008) suggest the possibility that *miR-146a* plays an important role in tumourigenesis. The initial study focused on miR-146a and tumourigenesis comes from the study by He *et al.*, who reported that

miR-146a is over expressed in unaffected part of thyroid glands. Breast cancer metastasis suppressor 1 (BRMS1) is a nuclear protein that can suppress the metastasis of numerous cancer cells. It is reported that BRMS1 increases miR-146a expression in metastatic breast cancer cells, while miR-146a transduction down-regulates expression of epidermal growth factor receptor, inhibits invasion, migration and metastatic ability of metastasis. Therefore, the expression of miR-146a modulation might provide a therapy for the suppression of cancer metastases.

2.4.2 MiRNA 146a in Senescence:

It is proposed that there is an increased secretion of the inflammatory cytokines IL-6 and IL-8 by miRNA 146a/b as a result inflammation associated with through senescence could be limited (Bhaumik *et al*., 2009).

2.4.3 *rs2910164*

rs2910164, a Single nucleotide polymorphism (SNP) located in the precursor micro RNA sequence of miR-146a, is the only micro RNA sequence SNP studied in papillary thyroid cancer (PTC).

The rs2910164 G/C SNP, which is located in the stem region of precursor miR-146a and results in a C:U miss-pair instead of a G:U pair, affects the integrity of the stem region of pre-miR-146a, as well as the processing of pre-miR-146a into mature miR-146a. This polymorphism has been reported to contribute to the susceptibility for several diseases, such as papillary thyroid carcinoma, prostate cancer, and hepatocellular carcinoma, lung cancer, prostate cancer, breast cancer etc. As a result miRNA 146a plays important role in tumourigenesis (Mediators of Inflammation Volume 2014 (2014), Article ID 916202).

Zeing *et al*., (2010), for the first time found that variant in *premiR-146a* has an increased risk of gastric cancer in Chinese population. Statistically there is no significant differences were found in the allele or genotype distributions of the miR-*146a* rs2910164 polymorphism among HCC (Hepatocellular carcinoma) and cancer-free control subjects in Turkish population thus demonstrating that the miR-*146a* rs2910164 polymorphism plays no major role in genetic susceptibility to hepatocellular carcinogenesis in the population studied (Akkiz *et al*., 2011). Another study was done to look whether rs2910164 plays any role in breast and/or ovarian

cancer; they studied associations between this polymorphism and age of diagnosis in 42 patients with familial breast cancer and 82 patients with familial ovarian cancer. Breast cancer patients who had at least one miR-146a variant allele were diagnosed at an earlier age than with no variant alleles (median age 45 versus 56, P=0.029) and ovarian cancer patients who had at least one miR-146a variant allele were diagnosed younger than women without any variant allele (median age 45 versus 50, P=0.014) (Shen *et al*., 2008).

2.5 MiR-*196a2* gene

Structure and Location of miR- *196a2* gene

mi R- *196a2* Gene resides in the region between *HOXC10* and *HOXC9* on chromosome 12 (Tian *et al*.,2009).Mature *miR-196* expression partly directs the cleavage of HOX gene cluster which are related to breast cancer development and lung cancer metastasis (Landi *et al*., 2008). SNP rs11614913 G/C polymorphism affect expression alters the miRNA–target binding site and influences cancer risks.

Three *miR-196* genes have been found. The *miR-196a-1* gene is located on chromosome 17 at a site between *HOXB9* and *HOXB10* genes, and the *miR-196a2* gene is located at a region between *HOXC10* and *HOXC9* on chromosom*e* 12 .The gene for *miR-196b* is located in a region which is highly conversed between *HOXA9* and *HOXA10* genes, on chromosome 7 in human beings and chromosome 6 in mice.

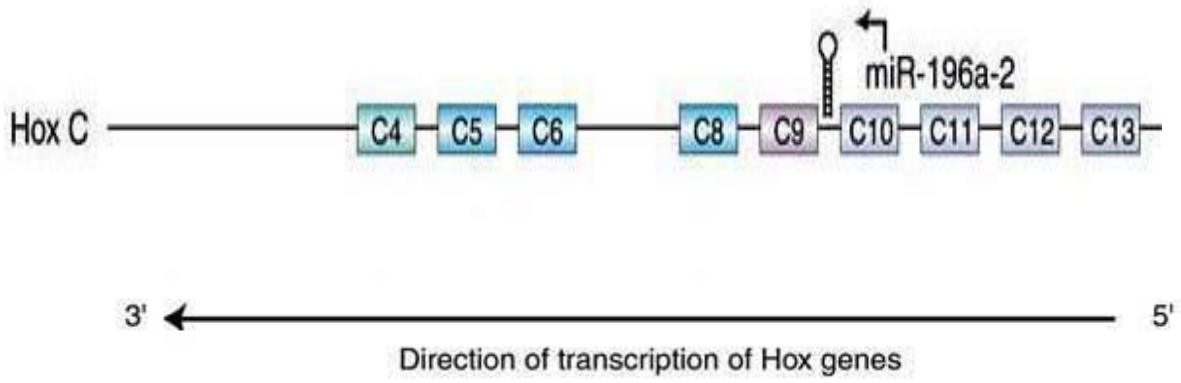

Fig 2.4: Location of *miRNA 196a2* gene (Mansfield *et al*., 2004)

2.5.1 Role of *miRNA 196a2* in Development

In the Hox gene cluster there are two miR-196a genes that generate identical mature transcripts (a third, *miR-196b*, is located upstream of *Hoxa9*). One is located upstream of *Hoxc9*, and the other is upstream of *Hoxb9*. *In vitro* experiments indicate that *miR-196a* directly binds to the *Hoxb8* 3′UTR, resulting in the cleavage of the *Hoxb8* transcript. It was determined that *miR-196a* and *Hoxb8* have complementary expression patterns in the developing embryo. (Current Opinion in Genetics & Development Volume 15, Issue 4, August 2005).

2.5.2 Target genes and functions of *Micro RNA 196a2* and *146a* gene

Table 2.2: Target genes and functions of *Micro RNA 196a2* and *146a* gene

Target genes(*miRNA 146a*)	**Function**	**Target genes (*miRNA 196a2*)**
TIMELESS,PPBP, NOTCH2	Cell Proliferation	DHFR,TYMR
EIF4G2,TIMELESS, NOTCH2	Cell Differentiation	GDF3
EIF4G2,TIMELESS, NOTCH2 CASP3	TRAF6,ARF6	Apoptosis
TRAF6,ARF6	Apoptosis	CASP3

(Zhang *et al*., 2013)

2.5.3 *rs11614913*

The *rs11614913* variant homozygote CC of *miR-196a2* was found to be associated with a significantly increased risk of lung cancer compared with its wild-type homozygote TT and heterozygote CT in Chinese population.

Recently, a few studies have examined the association between cancer risk and the miR196a2 *rs11614913* C/T polymorphism on human cancers and the CC genotype of this polymorphism. A

study evaluated the hsa-miR-196a2 *rs11614913* SNP in 388 breast cancer cases and 388 controls in Brazilian women. The results of this study indicated that the CC polymorphic genotype is associated with a decreased risk of BC and the presence of the T allele was significantly associated with an increased risk of BC (Linhares *et al*., 2012). The variant genotypes CC/CT were associated with significantly increased breast cancer risks in a case–control study of 1009 breast cancer cases and 1093 cancer-free controls in a population of Chinese women (Hu *et al*., 2009).

CHAPTER 3

AIMS AND OBJECTIVES

AIMS AND OBJECTIVE

Various parameters has to be taken in consideration that might be associated towards risk of breast cancer:

- To study the genotypic frequencies of the micro RNA genes like *miR-196a2* gene and *146a* gene in breast cancer cases and controls.

- To find out the genotypic frequencies of the *miR-196a2* and in *miR-146a* polymorphisms on the basis of clinico pathological features towards breast cancer susceptibility using statistical analysis.

CHAPTER 4

MATERIALS AND METHODOLOGY

- Isolation of DNA from peripheral blood
- Sample collect
- DNA quantification
- PCR amplification of *miRNA 196a2* and *146a* gene
- Restriction digestion of miRNA *196a2* gene and *146a* gene
- DNA polyacrylamide gel electrophoresis (PAGE)
- Statistical Analysis

4.1 Sample Collection

The cases with histologically confirmed primary breast cancer were recruited from September 2012 to May 2014 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 patient 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 *mir 146a and mir 196a2* 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 Isolation of DNA from Peripheral Blood

REQUIREMENTS:

Washing buffer

Lysis buffer

Phenol:Chloroform:Isoamylalcohol (25:24:1)

Chloroform:Isoamylalcohol (24:1)

Isopropanol

Chilled 70% Ethanol, TE buffer

Table 4.1 :Preparation 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 (8.0)	10mM Tris-HCl pH (8.0)

Table 4.2 : Preparation of lysis 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	100 µg/mlProteinase

Protocol

- Took 3ml of blood and added 3ml of washing buffer and mix it thoroughly. Centrifuged it at 3500 rpm for 5 minutes.

- Discarded the supernatant and added 3ml of washing buffer (16ml 1M Sucrose,0.5 ml Triton X-100, 2.5ml MgCl2, 0.5 ml 100mM Tris HCl and 0.26ml of water) to the pellet and resuspended the pellet in the buffer and centrifuged again (repeat this step thrice).

- Dissolved the pellet in 3ml of Lysis buffer (1 M Tris HCl 2ml,10% SDS 0.5ml ,0.5 M EDTA 0.6ml, 5M NaCl 0.15ml,10mg/ml Proteinase-K 0.05ml and water 1.7ml) and incubated at 44 °C overnight.

- Added an equal volume of Phenol: chloroform: Isoamyl alcohol (PCI) 25:24:1(2.5ml Phenol, 2.4 ml chloroform and 0.1ml isoamyl alcohol) and mixed the contents slowly.

- Centrifuged at 8000 rpm for 10 minutes at 4°C. Took the upper layer and again add PCI mix and centrifuged.

- Took the aqueous layer and added equal volume of Chloroform: Isoamyl alcohol (24:1).

- Centrifuged it at 6500 rpm for 5 minutes and took the upper layer.

- To the aqueous layer added equal volume of chilled isopropanol or 2.5 times volume of absolute ethanol and mixed it gently.

- Freeze it at -20°C for 1-2 hours.

- Centrifuged it at 12,000 rpm for 10 min at 4°C.

- Discard the supernatant and washed the pellet with 70% ethanol twice at 10,000 rpm for 5 minutes.

- Decanted ethanol and dry the pellet.

- Dissolved the pellet in 150-200µl Tris-EDTA buffer depending on the size of DNA pellet (Bartlett & White, 2003).

4.3 DNA Quantification

Spectrophometric analysis is the most common method for the quantification of DNA. The absorbance of samples were taken at 260 nm, to determine the concentration of sample.

.Nanodrop (Thermo Scientific) was used to find out the concemtration.

1 OD = 50µg/ml of DNA

Concentration (µg/ml) = O.D260nm × 50µg/ml × dilution factor.

The purity of the sample was checked by taking the ratio of its absorbance at 260 nm and 280 nm. If the ratio is less than 1.8, then it is accepted as RNA contamination, and if the ratio is more

than 1.8 it means there is proteins, phenol and other contaminants and if the ratio is equal to 1.8, DNA is pure.

Purity of DNA = A260/A280

4.4 PCR Amplification

PCR is an ingenious new tool for molecular biology that has had an effect on research. PCR (Polymerase Chain Reaction) is a very sensitive assay in which a single DNA molecule can be amplified, and single-copy genes can be extracted out of complex mixtures of genomic sequences. Mainly PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material usually known as template DNA.

REQUIREMENTS:

- Water
- BSA
- 10X PCR buffer
- Forward Primer
- Reverse Primer
- dNTP_s
- Taq DNA polymerase
- DNA sample

Table 4.3: Cycling Profile for 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	65°C for *miRNA 196a2* gene 63°C for *miRNA 146a* gene	30 sec 30 sec
Step-4	Polymerization	72°C	45 sec
Step-5	Final Extension	72°C	5 min

The reaction was carried out for 29 cycles.

Table 4.4: Different reagents used for PCR optimization		
Reagents	Stock Conc.	Working Conc.
Water		
BSA	100X	1X
Buffer	15mM	1.5mM
dNTP‘s	10mM	0.2mM
Forward Primer	10μM	0.5 μM
Reverse Primer	10 μM	0.5 μM
Taq. Polymerase	5U/μl	0.5U

4.5 Restriction Digestion of *miRNA 196a2* gene AND *146a* gene

This enzymatic technique can be used for cleaving DNA molecules at specific sites, ensuring that all DNA fragments that contain a particular sequence have the same size. Each fragment that contains the desired sequence has the sequence located at exactly the same position within the fragment.These enzymes are called restriction endonucleases or restriction enzymes, and they are able to cleave DNA molecules at the positions at which particular short sequences of bases are present.

Msp1I is an enzyme isolated from *Moraxella* species. It has following restriction site.

↓

5′….CCGG….3′

3′…. GGCC….5′

↑

Sac1 is isolated from *Streptomyces achromogenes*. It has following restriction site.

↓

5′…GAGCTC…3′

3′…CTCGAG…5′

↑

4.6 DNA Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE), describes a technique widely used to separate biological macromolecules, usually proteins or nucleic acids, on the basis of their electrophoretic mobility. Mobility is a function of the length, conformation and charge of the molecule.

REQUIREMENTS:

- Acrylamide and bis-acrylamide (30%)
- Ammonium persulfate (10mg/ml)
- TEMED
- 5X TBE
- Deionized water

Mechanism of Polymerization:

Polyacrylamide gels are formed by copolymerization of bis-acrylamide and acrylamide (N, N'-methylene-bis-acrylamide). Polymerization is initiated by ammonium persulfate and (tetramethylethylenediamine).TEMED accelerates the rate of formation of free radicals from persulfate and these in turn catalyze polymerization. The persulfate free radicals convert acrylamide monomers to free radicals which react with unactivated monomers to begin the polymerization chain reaction.

Table 4.5: Volume of reagents used in polyacrylamide gels

GEL%	WATER(ml)	Acrylamide (30%)	5X TBE Buffer (ml)	10% APS (µl)	TEMED (µl)
6%	7.2ml	2.4ml	2.4ml	200	20

4.6 Silver Staining

Silver staining is the most sensitive colorimetric method for detecting total protein. The technique involves the deposition of metallic silver onto the surface of a gel at the location of protein bands. Silver ions (from silver nitrate in the stain reagent) interact and bind with certain protein functional groups.

REQUIREMENTS:

- Fixative
- Staining Solution
- Developing Solution

Procedure

1. Preparation of Fixative (100ml): Water: Methanol: Glacial acetic acid (50:40:10)

2. Preparation of Staining Solution (0.1%):

- Dissolve 0.1gm of AgNO3 in 100ml of distilled water
- Then addition of a 150µl formaldehyde (37%). Formaldehyde act as a reductant to convert silver ion to metallic silver.

3. Preparation of Developing Solution:

- Dissolved 3gm of Na2CO3 in 100ml distilled water
- Add 150µl formaldehyde (37%)
- Add 20µl of sodium-thiosulfate (mg/ml).

Staining

- The gel was fixed in a fixative (Water: Methanol: Glacial acetic acid) for 15-20 min (or about 1-2 hrs).
- Washed it with deionized water 2-3 times.
- The gel was stained with Silver nitrate solution (0.1gm in 100 ml water) and kept it in a dark place for about 30 min.
- Again then washed it with deionized water for about 1-2 min.
- Put the gel in developing solution contains sodium carbonate(3gm in 100 ml) so to become a solution alkaline and formaldehyde (150 µl) which converts sodium ions to metallic silver and sodium thiosulphate (10mg/ml)
- Then stopping solution is added in it, containing 10% glacial acetic acid which prevents the gel from further reduction of silver ion.
- Washed the gel with deionized water and then stored it in glycerol (1%) to prevent the gel from cracking

4.7 Statistical Analysis

Crude ORs with 95% CIs was used to assess the strength of relationship between the Polymorphism and breast cancer risk. The differences in the distribution between cases and controls were tested using the $\chi 2$. The crude odd ratios (ORs) were calculated by Wolf's method (Hong *et al*., 2011) using MedCalc software.

CHAPTER 5

RESULTS AND DISCUSSION

- Genotyping
- Epidemiology
- Overall Distribution of genotypes of *miRNA 196a2* and *miRNA 146a* gene
- Frequency Distribution of genotypes of *miRNA 146a and miRNA 196a2* gene on the basis of histology.
- Frequency Distribution of genotypes of *miRNA 196a2* and *146a* gene on the basis of lymph node.
- Frequency Distribution of genotypes of *miRNA 146a* and *196a2* gene on the basis of side(left or right)

5.1 Genotyping

5.1.1 DNA Isolation:

The DNA was isolated qualitatively and the DNA samples were run on agarose gel (0.7%)**.** The DNA bands were clearly visible and distinct which indicated that the DNA isolated was of good quality and didn‘t show any shearing. Fig 5.1 shows the gel picture of genomic DNA isolated from Peripheral Blood

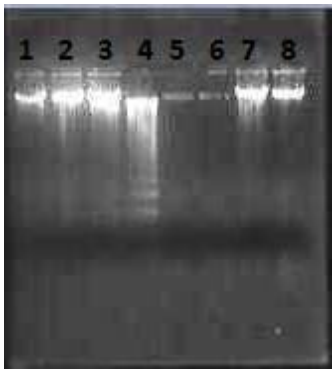

Fig 5.1: Gel pic showing Genomic DNA

5.1.2 PCR amplification:

For the amplification of *miRNA genes146a* and *196a2*, the products which are amplified by using PCR were separated on 1.7% agarose gel that contained ethidium bromide. The sharp DNA bands were clearly visible and distinct which indicated that the primer combinations worked well for both the genes. Fig 5.2 and Fig 5.3 show the PCR amplified product for both the genes respectively.

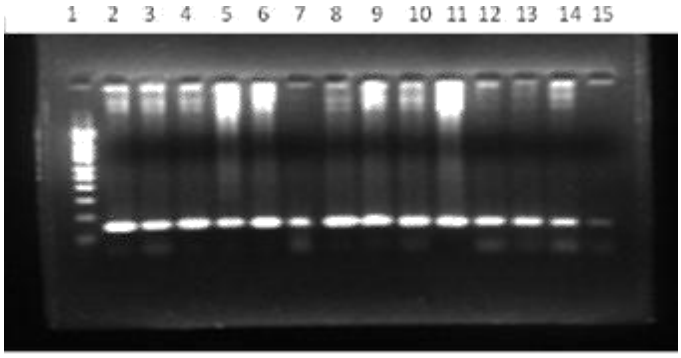

Fig 5.2: PCR amplified DNA products of *miRNA 146a* gene

Lane 1: 50bp ladder, Lane 2-15: Amplified DNA product (*147bp)*

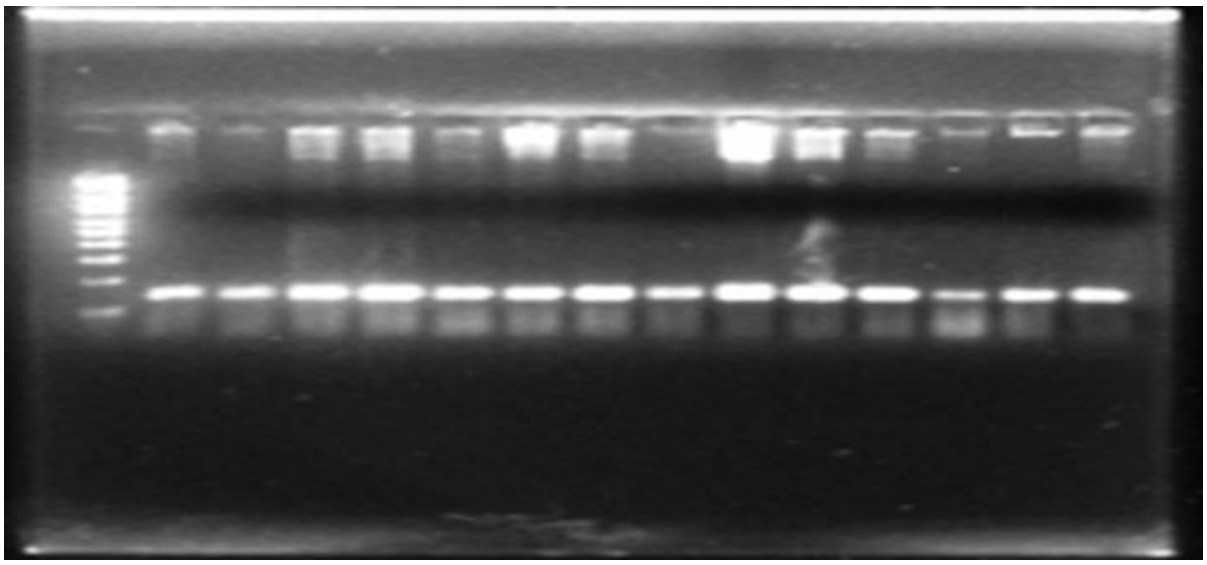

Fig 5.3: PCR amplified DNA products of *miRNA 196a2* gene

Lane 1: 50bp ladder, Lane 2-15: Amplified DNA product (*149bp)*

5.1.3 Restriction digestion:

PCR products were then digested with enzyme *Sac I* and *Msp I* for genes *146a* and *196a2*. Then the digested samples were allowed to run on Polyacrylamide gel and after that silver staining of the gels was done.The enzymatic digestion technique for cleaving DNA molecules at specific sites, ensuring that all DNA fragments that contain a particular sequence have the same size. Each fragment that contains the desired sequence has the sequence located at exactly the same position within the fragment.

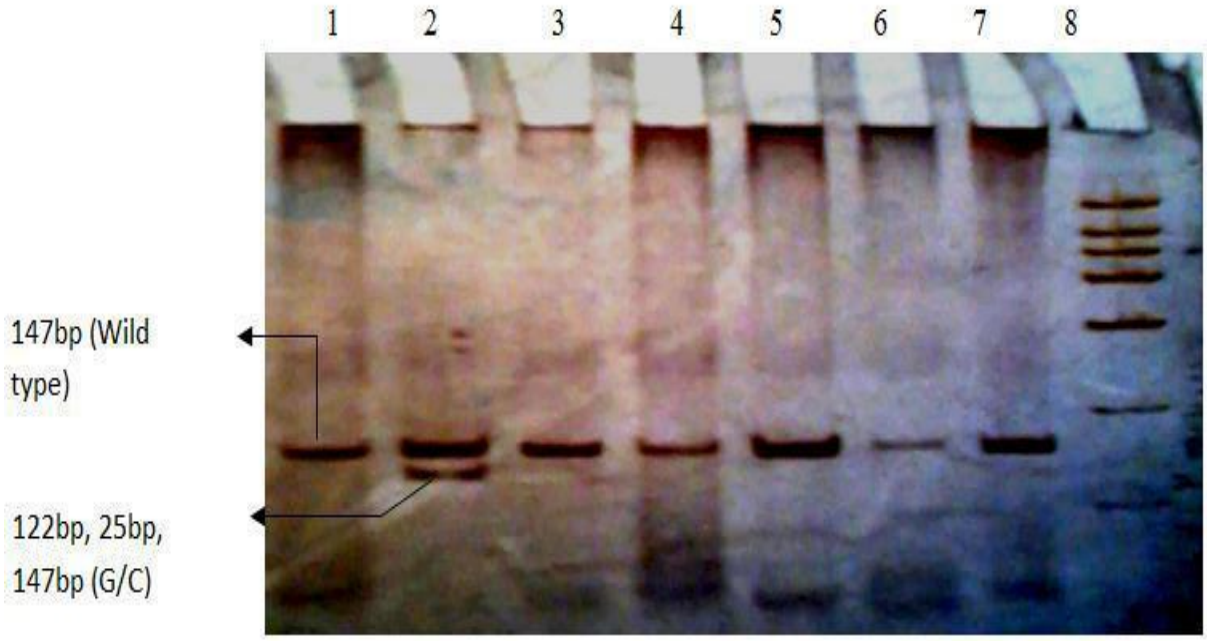

Fig 5.4: PCR RFLP genotypic analysis of *miRNA 146a* gene

Lane 1, 3-6: Homozygous uncut wild (GG) genotype (147bp), Lane 2: Heterozygous (GC) genotype (122bp, 25bp and 147bp bands), Lane 7: Control uncut

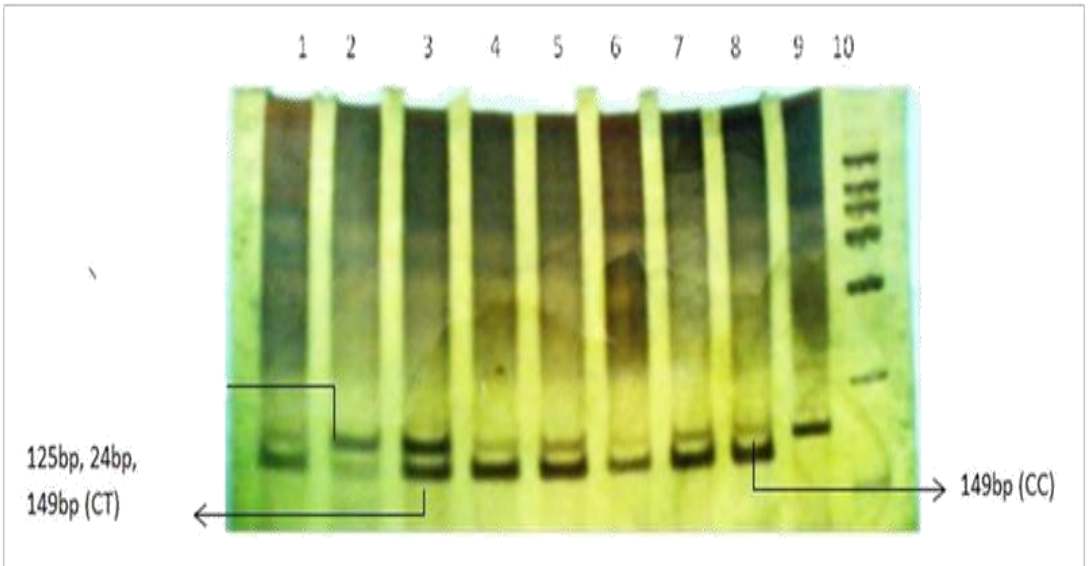

Fig 5.5: PCR RFLP genotypic analysis of *miRNA 196a2* gene

Lane10: 100bp ladder, Lane 9: Uncut Control, Lane 8, 7, 6, 5, 4, 1: Homozygous, cut, wild type (CC) genotype (149bp), Lane 3: heterozygous (CT) genotype (149,125 and 24) bp

5.2 Epidemiology

Table 5.1 Distribution of demographic variables of cases and controls

CHARACTERISTICS	CASES (n %) (98)	CONTROLS (n %) (49)
Age Mean Age	51.53±12.51	40.61±13.26
Histology IDC ILC Others	 77(78.5) 5 (5.1) 16(16.3)	
Lymph Node YES	 51(52.0)	

NO Unknown	41(41.8) 6 (6.1)	
Side Left Right Left/ right Unknown	 48(48.9) 43 (43.8) 1 (1.0) 6 (6.1)	

Focusing on the potential role of the *146a and 196a2* genes in the development of breast Malignancy .The case control study pertains to 98 breast cancer patients and 49 controls. Average age was 51.53±12.51 among the cases. We also study histology, lymph node, side (i.e. left or right) like demographic variables in cases having various risk factors concerned with the risk of breast cancer development. As per histology 78.5% (77) cases were found to have ductal carcinoma and only 5.1% (5) cases were found to have lobular carcinoma and rest 16.3%(16) cases were found to have other type or carcinomas. Case study was also done in which multivariate analysis of hsa-mir-*146a and hsa-mir-196a2*genotypes were found to have lymph node positivity 52% (51) and 41.8% (41) were having negativity in lymph node, and remaining 6.1% (6) were found to be unknown cases.

5.3 Overall Frequency distribution and their association with risk of developing Breast cancer

Table 5.2 : Overall Frequency distribution of *196a2 gene* and their association with risk of Breast Cancer:

GENOTYPE *(196a2)*	CASES	CONTROLS	OR(95%CI)	p-VALUE
TOTAL	95	49		
CC	48 (50.5)	28(57.1)	1.00(reference)	
CT	47 (49.5)	21(42.8)	1.30 (0.65-2.6)	0.45
C-allele	0.75	0.79		
T-allele	0.25	0.21		

Table 5.3 : Overall Frequency distribution of *146a gene* and their association with risk of Breast Cancer:

GENOTYPE *(146a)*	CASES	CONTROLS	OR(95%CI)	p-VALUE
TOTAL	98	48		
GG	52(53.0)	35(72.9)	1.00(reference)	
GC	46(46.9)	20(41.6)	1.54 (0.78-3.04)	0.20
G-allele	0.77	0.82		
C-allele	0.23	0.18		

In the study population of 95 cases and 45 controls for 196a gene, it was vividly seen that (50%) of them were carrying the homozygous wild genotype (CC) whereas (49.5%) of them carried the heterozygote genotype (CT) in cases. However the account of the homozygous wild in the controls was (57.1%) and the ones with heterozygote genotype were (42.8%). The allelic frequencies for the homozygous recessive genotype were found to be 0.25 and 0.21 in cases and controls respectively. In the study population of 98 cases and 48 controls for 146a gene, (53%) of them were carrying the homozygous wild genotype (GG) whereas (46.9%) of them carried the heterozygote genotype (GC)in cases. However the account of homozygous wild in the controls were(72.4%) and the ones with the heterozygote genotype were (41%). Overall Genotype distributions of the two SNPs hsa-mir-196a2 rs11614913 (p=0.45), OR 1.30(0.65-2.6) and hsa-mir-146a rs2910164 (p=0.20), OR 1.54(0.78-3.04). From the above data there was significantly increased breast cancer risks were found to be associated with variant genotypes of hsa-mir-196a2 rs11614913 (CC/CT).Similar study was done where subjects carrying variant homozygous genotypes of hsa-mir-196a2 rs11614913 had significantly increased risks of breast cancer (OR, 1.37; 95% CI, 1.08–1.74 for rs11614913 CC respectively) compared to the wild-type homozygotes in Chinese Population (Hu *et al*., 2009). But our study fails to stand in line with the former. It was also observed that there was an increased risk associated to the TT/CC genotypes with OR of 1.23(1.02-1.48) as per the study in Asian population. Another study was done to look whether rs2910164 plays any role in breast and/or ovarian cancer; they studied associations between this polymorphism and age of diagnosis in 42 patients with familial breast cancer and 82 patients with familial ovarian cancer. Breast cancer patients who had at least one miR-146a variant allele were diagnosed at an earlier age than with no variant alleles (median age 45 versus 56, P=0.029) and ovarian cancer patients who had at least one miR-146a variant allele were diagnosed younger than women without any variant allele (median age 45 versus 50, P=0.014) (Shen *et al*., 2008). One more study evaluated the hsa-miR-196a2 rs11614913 SNP in 388 breast cancer cases and 388 controls in Brazilian women where they found that the CC polymorphic genotype is associated with a decreased risk of BC and the presence of the T allele was significantly associated with an increased risk of BC. (Linhares *et al*., 2012)

5.4 Frequency distribution of genotypes among different histological types of Breast Cancer:

Table 5.4a Frequency distribution of genotypes of *m146a*among different histological types of Breast Cancer:

IDC(*m146a)*	**CASES (n= 45)**	**CONTROLS (n= 55)**	**OR(95% CI)**	**p- VALUE**
GG	24(53.3)	35(63.6)	1.00 (reference)	
GC	21(46.6)	20(36.3)	1.53 (0.68-3.4)	0.29

Table 5.4b Frequency distribution of genotypes of *m146a*among different histological types of Breast Cancer:

ILC*(m146a*)	CASES (n=4)	CONTROLS (n=55)	OR(95% CI)	p- VALUE
GG	3(7.5)	35(63.6)	1.00(reference)	
GC	1(2.5)	20(36.3)	0.58(0.05-5.98)	0.65

Table 5.5a Frequency distribution of genotypes of *m196a2*among different histological types of Breast Cancer:				
IDC(m196a2)	CASES (n=44)	CONTROLS (n=49)	OR(95% CI)	p- VALUE
CC	25(56.8)	28	1.00(reference)	
CT	19(43.1)	21	1.01(0.4-2.3)	0.97

Table 5.5b Frequency distribution of genotypes of *m196a2*among different histological types of Breast Cancer:				
ILC(m196a2)	CASES (n=3)	CONTROLS (n=49)	OR(95% CI)	p- VALUE
CC	2(66.6)	28	1.00(reference)	
CT	1(33.3)	21	0.66(0.05-7.8)	0.747

In the cases studied in has-mir146a 53.3% (24) and 7.5(3) of the cases were of those who suffered from Lobular carcinoma having homozygous variants GG, 56.8%(25) those who suffered from ductal carcinoma and 66.6(2) had lobular carcinoma having homozygous variants CC in hsa-mir-196a2 genotype. On further stratification on basis of genotypes it was found that and individuals of lobular and ductal carcinoma respectively had homozygous genotypes. On the other hand 46.6% (21) and 2.5%(1) for has-mir146a and 43.1%(19) and 33.3%(1) for has-mir196a2 respectively had heterozygote genotypes shows a significant increased risk of breast cancer when compared with the analysed data in Chinese population shows a significant increased risk of breast carcinoma.(Hu *et al*.,2009)

5.5 Frequency Distribution of genotypes of miRNA *196a2* and *146a* gene on the basis of lymph node.

Table 5.6a: Frequency Distribution of genotypes of miRNA *146a* gene on the basis of lymph node

LYMPH NODE (*m146a*)	**CASES (n=54)**	**CONTROLS (n=55)**	**OR(95% CI)**	**p- VALUE**
YES(GG) (GC)	19(35.1) 10(18.5)	35	1.00(reference) 0.92 (0.35-2.36)	0.864
NO (GG) (GC)	11(20.3) 14(25.9)	20	1.00(reference) 2.22 (0.851-5.8)	0.1028
UNKOWN	41(75.9)			

Table 5.6b: Frequency Distribution of genotypes of miRNA *196a2* gene on the basis of lymph node

LYMPH NODE (*m196a2*)	**CASES (n=53)**	**CONTROLS (n=49)**	**OR(95% CI)**	**p- VALUE**
YES(CC) (CT)	17(32.0) 13(24.5)	28	1.00(reference) 1.01 (0.40-2.55)	0.96
NO (CC) (CT)	12(22.6) 11(20.7)	21	1.00(reference) 1.22 (0.45-3.3)	0.69
UNKOWN	42(79.2)			

Case study was done in which multivariate analysis of hsa-mir-*146a and hsa-mir-196a2*genotypes with lymph node positivity having 35.1% homozygote and 18.5% heterozygote variants and 32%homozygote and 24.5% heterozygote variants in has-mir-196a2 genotypes. Patients with lymph node negativity having 20.3% homozygote and 25.9% heterozygote for has-mir-146a and 22.6% homozygote and 20.7% heterozygote variants for hsa-mir-196a2 genotypes. (p=0.864) and OR 0.92(0.35-2.36) for hsa-mir-146a and (p=0.96) and OR 1.01(0.40-2.55) for hsa-mir-196a2 those patients with lymph node negativity having (p=0.1028) and OR 2.22(0.851-5.8) for 146a and (p=0.69) and OR 1.22(0.45-3.3) for 196a2 shows a hazard ratio of expression with significant increased risk of breast cancer when compared with Chinese population .The GG genotype was considered as the reference for comparisons with GC (homozygote) variants (Hu *et al*., 2009).

5.6 Frequency Distribution of genotypes of miRNA *146a* and *196a2* gene on the basis of side (left or right) of tumor

Table 5.7a : Frequency Distribution of genotypes of miRNA *146a* gene on the basis of side (left or right) of tumor

SIDE (m146a)	CASES (n=53)	CONTROLS (n=55)	OR(95% CI)	p- VALUE
LEFT(GG) (GC)	16(30.1) 10(18.8)	35	1.00(reference) 1.09 (0.4178-2.86)	0.855
RIGHT(GG) (GC)	13(24.5) 14(26.4)	20	1.00(reference) 1.88 (0.740-4.79)	0.18
UNKOWN	42(79.2)			

Table 5.7b : Frequency Distribution of genotypes of miRNA *196a2* gene on the basis of side (left or right) of tumor				
SIDE (m196a2)	CASES (n=53)	CONTROLS (n=49)	OR(95% CI)	p- VALUE
LEFT (CC) (CT)	14(26.4) 12(22.6)	28	1.00(reference) 1.14 (0.43-2.97)	0.78
RIGHT(CC) (CT)	15(28.3) 12(22.6)	21	1.00(reference) 1.06 (0.41-2.7)	0.893
UNKOWN	42(79.2)			

Genotype distributions of the two SNPs hsa-mir-*196a*2 rs 11614913 and hsa-mir-146a *rs2910164* having left sided carcinoma with homozygous genotype(GG) having OR 1.09(0.4178-2.86) is higher as compare to the cases having right sided carcinoma with homozygous genotype(GG) having OR 1.88(0.740-4.79).whereas in case of hsa-mir-196a2, the cases having left sided carcinoma with homozygous genotype having OR 1.14(0.43-2.97) greater as compare to cases having carcinoma on their right side with homozygous genotype CC having lesser OR value are less prone to carcinoma ,when compared with genotype distribution in Chinese population (P=0.221 for hsa-mir-146a rs2910164, 0.207 for hsa-mir-196a2 *rs11614913*. from the above data there was significantly increased breast cancer risks were found to be associated with variant genotypes of hsa-mir-196a2 *rs11614913* (CC/CT).

CHAPTER 6

CONCLUSION

CONCLUSION

- There is significant increase in risk of lobular carcinoma having (p=0.65) as comparison to ductal Carcinoma having (p=0.29) in the cases found in has-mir*146*a.

- The combined presence of *miR-196a2* C/T (rs11614913) and *miR-146a* G/C (rs2910164) functional polymorphisms further increased the risk for lung cancer

- There is a significant increase in the risk of carcinoma during multivariate analysis of hsa-mir-196a2 having higher (p=0.96) value when it is compare with hsa-mir-146a lymph node positivity having value (p=0.864).

- miR-146 G/C (2910164) polymorphism was significantly associated with increased risk of breast cancer.

CHAPTER 6

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REFERENCES:

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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°C 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 (MgCl2) (100mM)**: Dissolved 0.41g of MgCl2 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.