ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN microRNA146a AND 196a2 GENE TOWARDS RISK FOR LUNG CANCER IN NORTHINDIAN POPULATION

Dissertation

Submitted in partial fulfillment of the requirement for the award of degree of

Master of Technology in Biotechnology

Submitted

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DECLARATION

I, the undersigned, hereby declare that the research work presented in the M.Tech dissertation entitled ***"Association of Single Nucleotide Polymorphism in microRNA 146a and 196a2 Gene towards Risk for Lung Cancer in North Indian Population "*** has been carried out by me under the supervision and guidance of Dr. Siddharth Sharma, Department of Biotechnology, Thapar University, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

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CERTIFICATE

This is to certify that dissertation entitled, "***Association of Single Nucleotide Polymorphism in microRNA 146a and 196a2 Gene towards Risk for Lung Cancer in North Indian Population***" submitted by Ms. Kushaldeep Kaur Sodhi in partial fulfillment of the requirements for the award of M. Tech in Biotechnology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance. To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/ institute for award of any Degree or Diploma.

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ABBREVIATIONS

ADCC	Adenocarcinoma
SQCC	Squamous cell carcinoma
FADD	Fas-Associated protein with Death Domain
miRNA	microRNA
B-CLL	B cell chronic lymphocytic leukemia
SCLC	Small cell lung carcinoma
SCCHN	Squamous cell carcinoma of head and neck
LC	Lung Cancer
TRAF	Tumor necrosis factor receptor associated factors
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
SNP	Single Nucleotide Polymorphism
IRAK1	Interleukin-1 receptor associated kinase 1
ESCC	Esophageal squamous cell carcinoma
HCC	Hepatocellular Carcinoma
NSCLC	Non-small cell lung cancer
UTR	Untranslated region
PLZF	promyelocytic leukemia zinc finger
EGFR	Epidermal growth factor receptor
RACE	Rapid Amplification of cDNA Ends
BRMS 1	Breast Cancer Metastasis Suppressor 1

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ABSTRACT

MicroRNAs (miRNAs) are small non-protein-coding RNAs that regulate the expression of corresponding messenger RNAs (mRNAs). Variations in the level of expression of distinct miRNAs have been observed in the genesis, progression and prognosis of multiple human malignancies. The present study was aimed to investigate the association between two highly studied miRNA polymorphisms (*mir-146a* rs2910164, *mir-196a2* rs11614913) and cancer risk. A case-control study was performed on North Indian population. Odds ratio (OR) and 95% confidence interval (95% CI) were used to investigate the strength of the association. Participants who possessed heterozygous GC genotype and combined GC/CC genotypes for *miRNA 146a* gene showed high risk for LC especially for ADCC (OR=1.82, 95%C.I; 1.04-3.20, *p*=0.03) when compared to wild type GG genotype. Significant association of GC genotype was more pronounced among light smokers (OR=1.86, 95%C.I; 0.90-3.82, *p*=0.002) indicating elevated risk of LC. On the other hand mutant (TT) genotype for *miRNA 196a2* gene also showed high risk for LC (OR=3.51, 95%C.I; 1.36-9.04, *p*=0.009) especially SQCC (OR=4.98, 95%C.I; 1.55-15.9, *p*=0.006) and ADCC (OR=3.28, 95%C.I; 1.01-10.6, *p*=0.04). An interesting finding of this study was that non-smokers with mutant (TT) and combined CT/TT genotype were more susceptible for LC risk as compared to smokers. When analyzed in combinations of *miR-146a* and *196a2* polymorphism, combined heterozygous and mutant genotypes did show an association with overall LC and further these combinations had an elevated risk for ADCC and SCLC. The present study implicate the important role of *mir-146a G>C and miR-196a2* C>T polymorphism with overall LC risk especially in North Indian population. Further studies with large sample size are needed to evaluate and confirm this association.

CHAPTER 1

INTRODUCTION

Cancer is the outcome of abnormal proliferation of cells without differentiation and apoptosis. The transition of a normal cell into cancerous cell is a "multistep process" involving mutation and selection of cells with progressively increasing capacity for proliferation, invasion, and metastasis. It is the process of acquiring additional genetic changes in key genes that drives tumorigenesis. Each successive genetic change is thought to provide the developing tumor cell with important growth advantage that allow cell clones to outgrow their normal neighbouring cells.

Amongst all the cancers LC is the leading cause of substantial morbidity and mortality worldwide. There were estimated 1.8 million new cases in 2012 of which 58% occurred in the less developed regions of the world. Worldwide LC is the most common cancer in men. It has been estimated that more than 1 in 10 of all cancers diagnosed in men are LCs. Survival rate directly correlates with the stage of diagnosis. The 5 –year overall survival rate is approximately 50% if patient's presents early in clinical stage I, whereas it decreases precipitously in cases with lymph node involvement and metastasis (Hu *et al*., 2008). The reason for poor survival rate attributes to non-symptomatic features during early phase resulting in late diagnosis of disease. Given the bleak prognosis and scanty treatment options for patients diagnosed at advanced stage, need of the hour is the identification of new, reliable biomarker that allow non-invasive early detection of LC.

Epidemiological studies have viewed LC as a multifactorial disease. Tobacco smoking is one of the greatest risk factors contributing to the staggering rise in LC incidence. It has been estimated that about 90% of LCs occur among cigarette smokers. However it is also true that most smokers live LC free until a late age and that conversely, some non-smokers develop the disease, implying that genetic factors might contribute to carcinogenic mechanism. Hence there is a clear need to identify genetic variation in genes which are associated with disease susceptibility that would help to stratify those individuals into high risk population in addition, improve risk assessment and early diagnosis.

Recent advances, supports the role of miRNA in LC development and/or progression. MicroRNAs (miRNAs) are small (~22 nucleotide), non- protein coding, single stranded RNA that function as posttranscriptional negative gene regulators. Their function is to induce m-RNA cleavage or translation repression by interacting with 3`UTR of the target m-RNA (Kshitij *et al*., 2012). Computational analysis has revealed that a single miRNA can bind to hundred of mRNA targets and these targets are involved in regulation of various biological processes such as cell differentiation, proliferation, apoptosis etc. The loss or gain of specific miRNA expression may promote carcinogenesis by targeting various oncogenes or tumor suppressor genes associated with the development and progression of diverse cancers. For instance, miRNA *let-7* targeting *Ras* oncogene is downregulated in LC, whereas miR17-92 cluster was found to be overexpressed in LC (Croce *et al*., 2009).

Infact, more than 50% of the miRNA are located at fragile sites and cancer associated genomic regions or common breakpoint regions that are genetically altered in human cancer

suggesting that miRNA plays a vital role in the pathogenesis of various cancers. For example- correlation between the location of *miRNA-196a2* and *Homeobox (HOX)* genes. HOX proteins belong to the family of transcription factors that are involved in development and in oncogenesis (Zhang *et al.,* 2007).

SNP is the most common type of genetic variation found in human genome. However, many studies have been done showing the association of various SNP in different genes towards the risk of LC but very few studies have been carried out showing the biological function of SNP in pre-miRNA genes. Accumulating evidences have suggested that SNPs in miRNA could alter miRNA processing, expression, and/or binding to target miRNA. In addition, several reports identified genetic variants in the precursor or mature miRNA sequence of *miR-146a* (rs2910164, guanine to cytosine, G→C), *miR-196a2* (rs11614913, cytosine to thymine, C→T) and, as possible biomarkers, which were associated with multiple kind of malignant tumors in various populations (Xu *et al*., 2011). So, we conducted a case control study to find out "*Association of Single Nucleotide Polymorphism in microRNA 146a and 196a2 Gene towards Risk for LC in North Indian Population*".

CHAPTER 2

REVIEW OF LITERATURE

- Histology of LC
- MicroRNAs
- *microRNA146a* Gene
- *microRNA-196a2* Gene

2.1 Histology of LC

Since treatment depends on the histological subtype, accurate classification of tumors into histological subtypes of LC becomes a necessity. This is quite challenging in cases in which biopsy/aspirate specimens are small or tumors are poorly differentiated. (Lebanony *et al*., 2009) identified *miR-205* as a suitable marker for differentiating SCLC from NSCLC cells. Landi *et al.,* (2010) also reported a five-miRNA signature (*miR-25, miR-34c-5p, miR-191, let-7e, and miR-34a*) that accurately differentiated SQCC from adenocarcinoma, and the lower expression level of this signature correlated with poor overall survival among SQCC patients.

Human LCs are classified into two major types,

(1) Small cell LC (SCLC)

(2) Non–small cell LC (NSCLC)

2.1.1 Small Cell Carcinoma:

It accounts for 10% to 15% of all LCs incidence, named so due to small size of the cancer cells when viewed under the microscope. Some other names for SCLC are oat cell cancer, small cell undifferentiated carcinoma and oat cell carcinoma. It is very rare for the person who has never smoked in his lifetime to have small cell LC. SCLC often starts in the bronchi near the centre of the chest (mediastinum region), and tends to spread widely throughout the body that too early in the course of the disease (American cancer Society, 2014).

2.1.2 Non Small Cell Carcinoma:

It consists of mainly adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Previously, squamous cell carcinoma was the predominant form of NSCLC, but in the last few changing trend has witnessed adenocarcinoma taking over SQCC as the most common histological cell type.

Squamous-cell carcinoma:

About 25% to 30% of all LCs are squamous cell carcinomas. These cancers start in squamous cells, which are flat cells lining the inside of the airways in the lungs. They are often linked to the history of smoking and tend to be found in the mediastinum region, near a bronchus (American cancer Society, 2014).

Adenocarcinoma:

About 40% of LCs cases are adenocarcinoma. These cancers start in early versions of the cells that would normally secrete substances such as mucus. This type of LC has more occurrences in current or former smokers. At the same time it is also the most common type of LC seen in non-smokers. It is more common in women than in men, and more likely to occur in younger people. Adenocarcinoma is usually found in outer parts or periphery of the lung. Peripheral location of the lesions is due to the use of the filters in cigarettes which prevent the large particles from entering the lungs. It tends to grow slower than other types of LC, and is more likely to be found before it has spread outside of the lung. People with adenocarcinoma *in situ* (previously called bronchioloalveolar carcinoma), another type of adenocarcinoma tend to have a better outlook (prognosis) in contrast to those with other types of LC (American cancer Society, 2014).

Large cell (undifferentiated) carcinoma:

This type of cancer accounts for about 10% to 15% of LCs. It can appear in any part of the lung such as bronchi or alveoli. It has he capacity to grow and spread quickly, which makes it harder to treat. Another subtype of large cell carcinoma, known as large cell neuroendocrine carcinoma, is a fast-growing cancer is very similar to small cell LC.

2.1.3 TNM Staging

Staging of cancer at the time of diagnosis is the most important predictor of survival, and treatments options should also be based on the stage. Some clinicopathological features such as tumor stage and lymph node metastasis have been correlated with miRNA expression levels. For example, reduced *let-7* expression is associated with increase in lymph node metastasis. According to American Joint Committee on Cancer (AJCC) **TNM** staging system is based on 3 key pieces of information:

T indicates the size of the main (primary) **tumor** and whether it has grown into nearby areas.

N describes the spread of cancer to nearby (regional) lymph **nodes**. Lymph nodes are small bean-shaped collections of immune system cells to which are connected by lymphatic vessels carrying lymph (white clear fluid).

M indicates whether the cancer has spread (**metastasized**) to other organs of the body. (The most common sites are the brain, bones, adrenal glands, liver, kidneys, and the other lung.)

2.2 MicroRNAs

MicroRNAs are also known as “micromanagers of gene expression”. The critical region for miRNA binding is 2-8 nucleotides from the 5' end of the miRNA, called the ‘seed region’. The binding of miRNA to mRNA is critical for regulating the mRNA level and protein expression. SNP in the *pre-miR* genes can affect this binding, which can either abolish

existing binding sites or create illegitimate binding sites. SNP may either abolish or weaken a miRNA target or create a perfect sequence match to the seed of a miRNA which was otherwise not associated with the given mRNA. The increase or decrease in miRNA binding caused by the SNP variation would probably result in decrease or increase in protein translation (George *et al*., 2010).

2.21 Biogenesis: miRNA maturation

MicroRNA genes are transcribed by RNA polymerase II into primary miRNA transcripts (pri-miRNAs). Further these pri-miRNAs are cleaved into ~70 nucleotide-long precursor miRNAs (premiRNAs) with a stem–loop structure by the nuclear microprocessor complex formed by Drosha (a RNase III endonuclease) and an essential cofactor DGCR8/Pasha (protein containing two double-stranded RNA binding domains).

Pre-miRNAs are exported from the nucleus to the cytoplasm with the help of Exportin-5 (Exp5) in a Ran-guanosine triphosphate (GTP)-dependent manner, where they are processed by another RNase III enzyme, Dicer. This causes the release of a double-stranded RNA duplex of ~22 nucleotides that is incorporated into the RNA-induced silencing complex (RISC) containing Argonaut proteins. In this complex, one strand is retained as the mature miRNA, whereas the other strand is generally degraded. The mature miRNA binds the 3`-UTR of target mRNAs through imperfect base pairing. Perfectly matched sequence complementarity occurs only between the 'seed' region of the miRNA (nucleotides 2–7 of mature sequence) and the target mRNA. Such binding leads to degradation, destabilisation or translational inhibition of the mRNA and consequently silencing gene expression. Approximately one-third of the protein-coding genes are controlled by miRNAs; thus, almost all cellular pathways are directly or indirectly influenced by miRNAs such as cell proliferation, differentiation, apoptosis and metabolism (Visone and Croce *et al*., 2009).

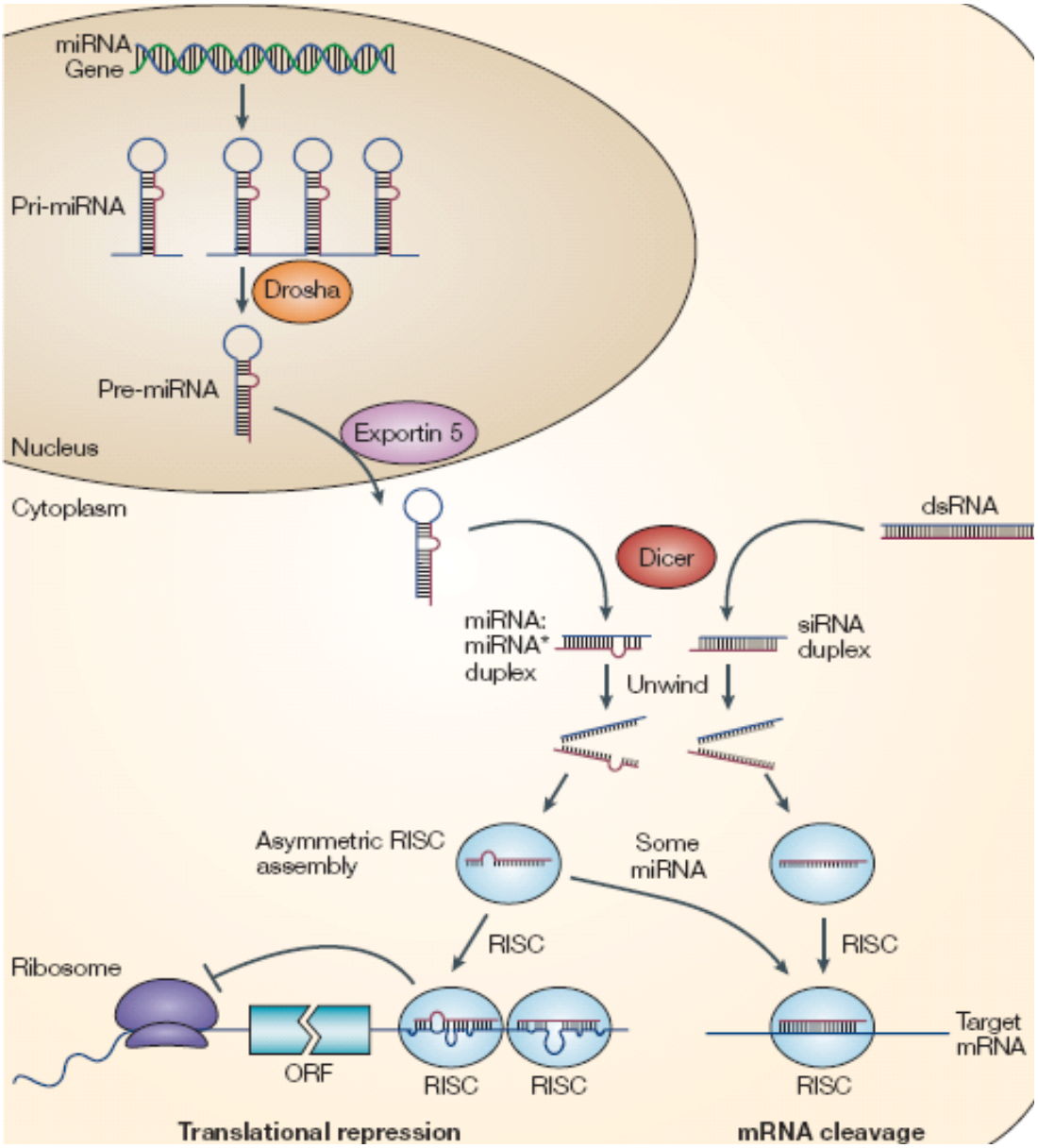

Figure 2.1: Biogenesis of miRNA (Bartel et al., 2004)

2.22 MicroRNAs function as tumor supressors or oncogenes in LC

Genes that help cells grow, divide, or stay alive are called *oncogenes*. Genes that slow down cell division or allow the cells to enter apoptosis at the right time are called *tumor suppressor genes*. Cancers can be caused by DNA changes that turn on oncogenes or turn off tumor suppressor genes. Those miRNAs whose expression is upregulated or increased in tumors are considered as oncogenes. These oncogenic miRNA (oncomirs) usually promote tumor development by negatively regulating tumor suppressor genes. On the other hand some miRNA expression is decreased or downregulated in cancerous cell. This type of miRNA is called as tumor suppressor miRNA (Ahmad *et al*., 2013).

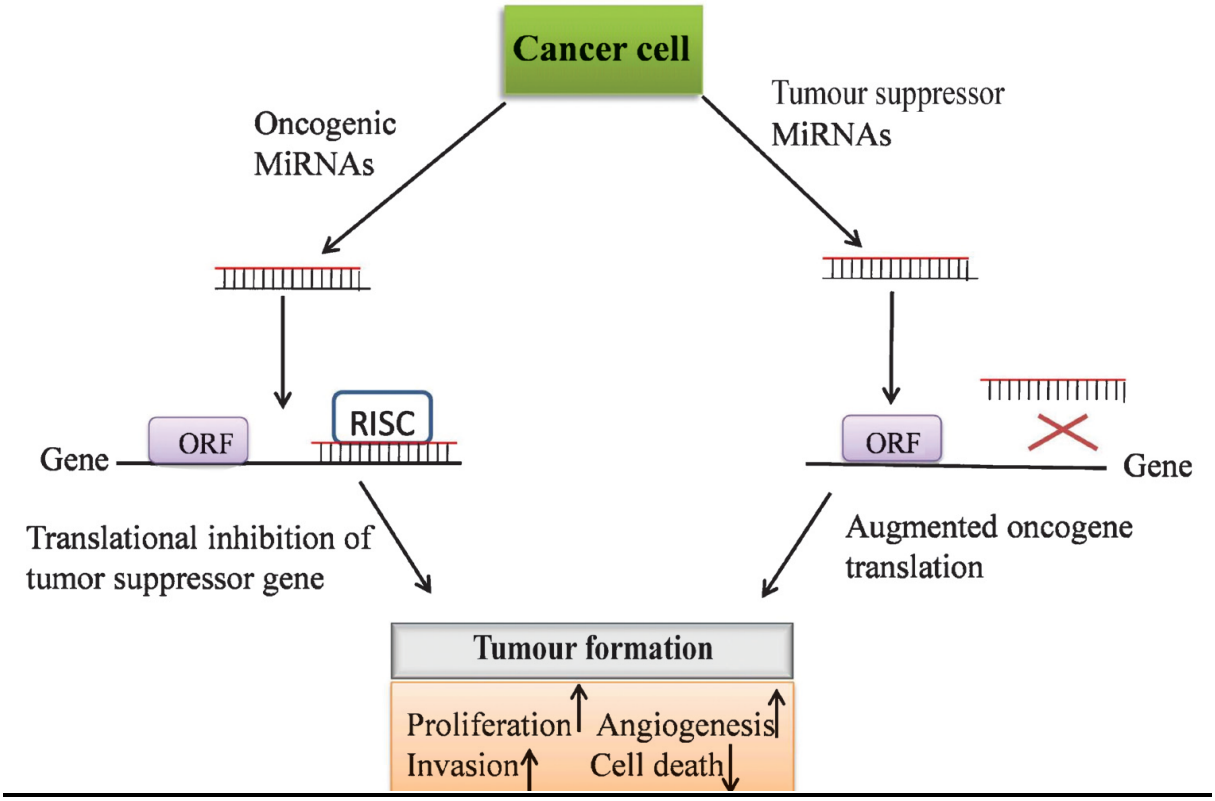

Figure 2.2: microRNAs as oncogenes and tumor supressors (Ahmad et al., 2013)

2.2.3 Causes of abnormal microRNA expression

miRNA expression can be altered by several mechanisms in human cancer including chromosomal abnormalities, epigenetic changes, mutations and Single nucleotide polymorphisms (SNPs), and defects in the miRNA biogenesis machinery.

Table 2.1 Evidence for deregulated microRNAs in cancer

microRNA (Tumor suppressor)	***Target Gene***	***Function of Target gene***	***Cancer***
miR-15 and miR-16	*BCL 2* (B cell lymphoma 2)	Antiapoptotic gene	CLL (Chronic lymphocytic leukemia)
let-7	*RAS, MYC, HMGA2*	Transcription factors	LC
miR-34	*c-myc, Bcl-2*	Antiapoptotic factors	Lung and pancreatic cancer

microRNA (Oncogenic)	***Target Gene***	***Function of Target gene***	***Cancer***
miR-17-92 cluster	*p 21*	G1-S phase checkpoint	Upregulated in lung, gastric cancers, lymphomas.
	BIM	Pro-apoptotic factor	
	PTEN	PTEN promote apoptosis through P13K-Akt-PKB pathway	
miR-155	*AGTR1, TP53INP1*	Inhibits TP53INP1-mediated apoptosis	Burkitt's Lymphoma
miR-372 and 373	*LATS2*	Inhibits p53 directed CDK signaling	Promote tumorigenesis in testicular germ cells

2.2.4 microRNAs as diagnostic and prognostic tools

Diagnostic: The exact role of miRNAs in cancer pathogenesis was identified through expression profiles of specific miRNAs which were over-expressed or knocked down (George *et al*., 2010). For instance, miRNA genes (*miR-15* and *miR-16*) located at the chromosome 13q14 region, were found to be frequently deleted or down-regulated in the majority of B cell chronic lymphocytic leukemia (B-CLL) cases. Recognition of miRNAs that are differentially expressed between tumour and normal tissues may help to identify those miRNAs that are involved in human cancers and further establish the role of miRNAs as biomarkers in cancer diagnostics (Zhang *et al.,* 2007).

Prognostic: The accumulated data on miRNA expression levels in tumors demonstrate that miRNAs are promising candidates to distinguish different tumors and different subtypes of tumors as well as to predict their clinical behavior. For example, reduced expression levels of *let-7,* was observed both in vitro and in vivo studies; and its significant association with shortened post-operative survival. (Yanaihara *et al*, 2006) reported that high *miR-155* and low *miR-let7a2* expression correlated with poor overall survival in lung adenocarcinoma patients.

Classify Poorly Differentiated Tissue: miRNA profiling has gained importance in resolving one of the most demanding issues in cancer diagnostics—to identify the tissue origin of poorly differentiated tumors (Lu *et al*., 2005).

2.2.5 microRNAs as therapeutic tools

Because of the significance of miRNAs in cancer, the management of miRNAs with altered expression in cancer can be considered as a therapeutic strategy. Restoring miRNA expression in diseases in which expression is consistently reduced/ knocked down or using antisense oligonucleotide that competes with cellular m-RNA to bind mi-RNA. This antisense mi-RNA pairs with mi-RNA and inhibits its function (Zhang *et al.,* 2007).

2.3 MicroRNA146a GENE

2.3.1 Structure and location of gene

Human *miR-146a* resides in the *LOC285628* gene on human chromosome 5. Analysis of the two ESTs encompassing *LOC285628* (GenBank accession nos. BQ430527 and BQ425371) suggests that the gene consists of two exons separated by ≈16 kb of genomic sequence, with the mature *miR-146a* sequence situated in the second exon. Notably, the LOC285628 transcript contains no significant ORF, implying that it probably belongs to a class of noncoding RNAs. Using the 3′- and 5′- RACE (Rapid Amplification of cDNA Ends) 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). Figure 2.3 shows the diagrammatic representation of *miRNA 146a* gene.

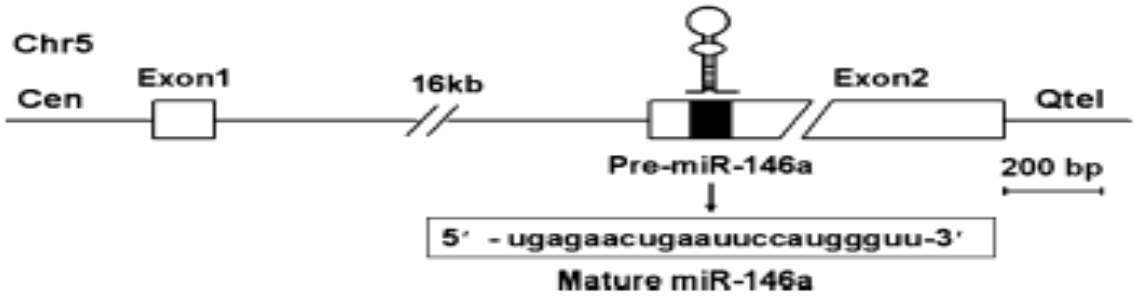

Figure 2.3: Schematic diagram of miR-146a loci and the detailed sequence of mature miR-146a on human chromosome 5 (Li et al., 2010)

Table 2.2 Molecular targets of microRNA-146a

Target Molecules	***Biological consequences***
TRAF6, IRAK1 (adaptor molecules downstream of Toll-like and cytokine receptors)	Innate immunity Response
EGFR	Cell proliferation and survival
CXCR4	Cell migration, proliferation and differentiation
FADD	Anti-apoptotic effect Adaptive immune response

2.3.2 microRNA-146a regulates NF-Kβ activation pathway

The analysis of the *miR-146a* promoter provided evidence that miR-146a is a NF-kappaβ-dependent gene. *miR-146a* was predicted to base-pair with sequences in the 3' UTR of the TNF receptor-associated factor 6 (*TRAF6*) and IL-1 receptor-associated kinase 1 (*IRAK1*) genes. Both these two genes encode key adaptor molecules downstream of Toll-like and cytokine receptors involved in cell growth and immune recognition. These two adaptor molecules positively regulate NF-kappaβ activity (Labbaye *et al*., 2012).

2.3.3 microRNA-146a and tumour metastasis

miR-146a also targets other mRNAs encoding for proteins which are involved in cellular activities, such as cell proliferation, differentiation and migration. In a study carried out on breast cancer cells have identified *EGFR* (Epidermal growth factor receptor) as a possible target of *miR-146a* (Bhamik *et al*., 2008). These studies arose from the observations showing that overexpression of *miR-146a* in breast cancer cells determines an inhibition of NF-kβ activity with reduction of metastatic potential. After this initial observation, in a second study it was shown that the capacity of the Breast Cancer Metastasis Suppressor 1 (BRMS1) protein was dependent on *miR-146a* upregulation that in turn reduces the metastatic potential of breast cancer cells and was associated with a downregulation of the Epidermal Growth Factor Receptor. Similar observations also reported in gastric cancer cells, where it was shown that enforced expression of *miR-146a* inhibited migration and invasion of tumor cells and downregulated EGFR and IRAK1 expression (Kogo *et al*., 2011). Importantly, this study showed also that the *miR-146a* downregulates EGFR expression through a direct mechanism involving targeting of the 3' UTR of the EGFR mRNA. In cardiomyocytes *miR-146a* targets ERB4, another member of the EGFR family; in these cells *miR-146a* up-regulation was related to cardiotoxic events (Labbaye *et al*., 2012).

2.3.4 microRNA-146a and CXCR4, a chemokine receptor

Studies carried out in leukemic cell lines and in normal megakaryocytes have led to the identification of another target of *miR-146a*, the mesenchymal marker CXCR4, a CXCL12/SDF-1 chemokine recptor- involved in homing / mobilization of stem cells. Particularly, the identification of this target was related to studies on PLZF (promyelocytic leukemia zinc finger). During the differentiation of hematopoietic progenitor cells to megakaryocytes, *miR-146a* is transcriptionally repressed by PLZF transcription factor. Downregulation of *miR-146a* permits the expression of chemokine receptor 4 (CXCR4). Through *in vitro* assays it was shown that PLZF interact with the *miR-146a* promoter, while *miR-146a* targets the 3' UTR of the CXCR4 mRNA, impeding its translation. During normal megakaryocytic differentiation a functionally regulatory loop was observed, involving PLZF suppressing *miR-146a* transcription and thereby activating CXCR4 translation. Abnormal

miR-146a expression (downregulation) in tumors may have important consequences for malignancy through enhanced CXCR4 expression in these cells (Labbaye *et al*., 2008).

2.3.5 microRNA-146a role in adaptive immunity

Mounting evidence indicates that *miR-146a*, in addition to acting as a modulator of the innate immune response, also play a vital role as a modulator of the adaptive immune response. It was shown that *miR-146a* is scarcely expressed in naïve T-cells, while it is abundantly expressed in human memory T-cells. *miR-146a* expression is clearly induced following T-cell activation via T-cell receptor stimulation. During T-cell activation upregulation of *miR-146a* plays an anti-apoptotic role, mediated through the targeting of FADD (Fas-Associated protein with Death Domain) mRNA. FADD is an adaptor protein that forms death-inducing silencing complex during apoptosis. *miR-146a* modulates also IL-2 production in activated T lymphocytes. Taking into effect all these observations it was suggested that *miR-146a* also regulate adaptive immunity (Curtale *et al*., 2010).

2.3.6 Polymorphism in microRNA-146a gene (rs2910164)

G>C polymorphism rs2910164 is located at position +60 relative to the first nucleotide of pre-miR-146a with a G to C change in the passenger strand, which results in change from G:U pair to C:U mismatch in stem structure of *pre-miR-146a* and reduced production of mature miR-146a. The rarer C allele is associated with decreased mature *miR-146a* expression and results in less efficient inhibition of the target genes including *TRAF6, IRAK1* and papillary thyroid carcinoma 1 gene (*PTC1*). This polymorphism is also associated with risk for breast cancer, ovarian cancer, and prostate cancer. Findings from these association studies further suggest that *miR-146a* is important in tumourigenesis.

The G>C polymorphism affects susceptibility to various human cancers, although results are inconsistent in different types of cancer in different ethnicity. The variant C allele has been associated with increased risk of adult glioma in Caucasians (Wey *et al*., 2012). Association was found to be stronger among the subjects having at least one C allele and mortality was stronger among minor allele carriers with association largely restricted to females. (Hung *et al*., 2012) reported that rs2910164 mutant (CC) genotype increases *miR-146a* expression in oral SCCs in a Chinese population. On the other hand it was reported that the wild genotype (GG) reduces *miR-146a* expression and increases cervical cancer risk in Chinese population, implying that miR-146a acts as tumor suppressor (Yue *et al*., 2011). A Korean study demonstrated that the heterozygous (CG) genotype was found to be associated towards risk for colorectal cancer (Hu *et al*., 2014). In contrast, some case control studies witnessed a protective effect of the mutant (CC) genotype in development of prostate cancer in Chinese populace (Xu *et al*., 2012). A meta-analysis study has shown no significant association of G/C polymorphism with overall cancer risk (Weng *et al*., 2012). These discrepancies in relation to the SNPs in the miRNA genes towards cancer risk can be attributed to varying genetic backgrounds and different molecular pathogenic mechanisms in different types of tumors.

2.4 microRNA-196a2 GENE

2.4.1 Structure and location of microRNA- 196a2 gene

The gene familiy for *miR-196* resides in the regions of homeobox (HOX) clusters (Chen *et al*., 2011). Three *miR-196* genes have been found. The *miR-196a-1* gene is located on chromosome 17 (17q21.32) 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 (12q13.13). The gene for *miR-196b* is located in a highly evolutionarily conserved region between *HOXA9* and *HOXA10* genes, on chromosome 7 (7p15.2) in human beings and chromosome 6 in mice. *miR-196a2* genes transcribe the functional mature miRNA sequence (3′-GGGUUGUUGUACUUUGAUGGAU-5′), whereas *miR-196b* gene produces a small RNA (3′-GGGUUGUUGUCCUUUGAUGGAU-5′), which differs from the sequence of *miR-196a* by one nucleotide (Chen *et al*., 2011). Figure 2.4 gives the schematic representation of location of *miRNA 196a2* gene.

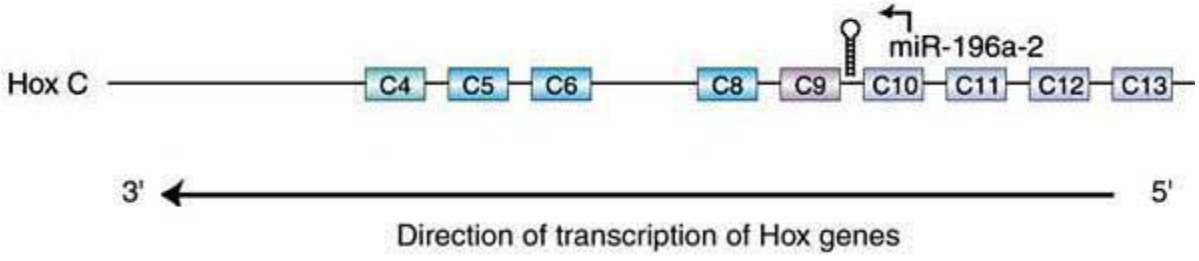

Fig 2.4: Location of miRNA 196a2 gene (Mansfield et al., 2004)

Table 2.3 Molecular targets of microRNA 196a2 gene

Target Gene	***Biological Function***
HOXB8 Homeobox protein Hox-B8	Transcription factor
HMGA2	Nuclear architectural factor
Annexin A1 (ANXA1)	Apoptosis
Keratin 5 (KRTS)	Structural cytokeratin protein

2.4.2 microRNA 196a2 gene as a regulator of apoptosis

Annexin A1 (ANXA1), also known as lipocortin or *p35*, is a well-characterized member of the caLCium and phospholipid binding protein family of annexins and is involved in modulating arachidonic acid metabolism and the epidermal growth factor receptor tyrosine kinase pathway. Significant inverse correlation between *ANXA1* mRNA levels and *miR-196a* in 12 different esophageal, breast and endometrial cancer cell lines and in esophageal tumors from patients supports the putative role of *miR-196a* in regulating *ANXA1* expression (Luthra *et al*., 2008). *ANXA1* is known to be a mediator of apoptosis and suppressor of cell proliferation

(Solito *et al.*, 2001). By targeting and suppressing ANXA1 levels, *miR-196a* may promote deregulated growth characteristics in cells.

2.4.3 Role of microRNA 196a2 in development

miR-196a2 appears to play an important role in development. Its relationship to the *HOX* gene family is crucial for embryonic development and is well known (Chen *et al*., 2011). A site in the *Hoxb8* 3' UTR containing 21 nucleotides of complementarity to *miR-196a.* The perfect complementarity between *miR-196a* and the *Hoxb8* 3' UTR suggests that *miR-196a2* could mediate degradation rather than translational repression of Hoxb8 mRNA. This indicate that micro-RNAs help define the regions where Hox genes are expressed, thereby contributing to the precise spatial and temporal patterns by which Hox genes regulate developmental processes (Pasquinelli *et al*, 2005).

2.4.4 Polymorphism in microRNA-196a2 gene (rs 11614913)

Since it has been experimentally validated that polymorphism located in *pre-miR*-196a2 alter its processing and/or binding to target mRNA, it seems plausible that genetic variation of pre-*miR*-196a2 could modulate cancer susceptibility (Chen *et al*., 2011). In compliance with this finding, the C > T substitution polymorphism in *miR*-196a2 has been reported in wide variety of malignancies, such as non-small cell LC (NSCLC) (Hong *et al*., 2011), gastric cancer (Peng *et al*., 2010), breast and digestive cancers (Guo *et al*., 2012). However many studies in different types of cancers and populations have given contradictory results. A study done in East China has reported that the variant genotype of *miR*-196a2 (CT/TT) is associated with lesser risk towards hepatocellular carcinoma (HCC) as compared to wild type (CC) genotype (Li *et al*., 2010). This is in contradiction to the study done in HCC patients of Turkish population where a pronounced effect of the mutant (TT) genotype towards susceptibility for HCC was observed (Akkiz *et al*., 2011). A study carried out in Chinese population, has shown that individuals with CC genotype are at decreased risk of Esophageal squamous cell carcinoma (ESCC) (Wei *et al*., 2013). Recently, miR-196a2 C>T SNP was found to be associated with survival in individuals. Decreased survival rate was reported for individuals homozygous (CC) for SNP suggesting that miR-196a2 could be a prognostic marker for NSCLC (Hu *et al*., 2008).

AIMS AND OBJECTIVES

The present work was aimed at to study the following aspects that might be associated towards the risk for LC.

- To study the genotypic frequencies of the microRNA genes, *miR-146a* and *196a2* in LC cases and controls

- To investigate the association between genetic polymorphisms of *miR-146a and 196a2* genes and LC risk and also the clinico-pathological features associated with it

- To find out the combined association of the *miR-146a and 196a2* polymorphisms as a risk factor for LC development and also the clinico-pathological features associated with it

- To evaluate the role of smoking on the gene polymorphism of *miR-146a and 196a2* polymorphisms in association with Lung cancer in North Indian Population.

CHAPTER 3

METHODOLOGY

- Study subjects and specimen collection
- Isolation of DNA from peripheral blood
- DNA quantification
- Resolution of DNA fragments on agarose gels
- Polymerase chain reaction (PCR) amplification of *miRNA 146a* and *196a2* gene
- Restriction digestion of miRNA *146a* gene and *196a2* gene
- DNA polyacrylamide gel electrophoresis
- Statistical Analysis

3.1 Study subjects and specimen collection:

A total of 250 LC patients were recruited for this study from the Department of Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, India. This study had been reviewed and approved by the Institute ethics committee of PGIMER. Informed written consent was obtained from all participants or their representatives. In brief, eligible cases included all patients who were newly diagnosed with primary LC. All the recruited patients were histopathologically diagnosed as having NSCLC and SCLC. There were no age, gender, smoking, histological, or TNM stage restrictions, but patients with a prior history of cancer were excluded from this study. During the same time, 250 unrelated individuals with no evidence of lung or other cancer who entered the hospital for health check-ups were enrolled as control group. Each control was pair-matched by sex, age (±10 years) and smoking parameters to a patient with LC. These characteristics allowed us to obtain control population without any possible risk bias for LC. A detailed questionnaire was completed for each case and control by a trained interviewer. The questionnaire included information on demographic and smoking characteristics. Smokers reported tobacco habits such as smoking of cigarette and/or beedi (a native cigarette like stick of coarse tobacco hand-rolled in a dry tembuhurni leaf). As an indication of cumulative smoking exposure, pack-years were calculated by the following formula: [(cigarettes or beedis per day / 20) X years smoked]. While medical information of cases, including Histology, TMN classification, clinical staging, primary tumor size, involvement of lymph node and metastasis were obtained from medical records of the hospital. Approximately 3-5ml of venous blood was collected from each participant.

3.2 Isolation of DNA from peripheral blood

Blood samples from patients with LC and from control subjects were collected into a test tube containing EDTA as anticoagulant. From each blood sample, a leukocyte cell pellet obtained was used for DNA extraction by using Phenol / Choloroform isoamyl method.

REAGENTS:

- Washing buffer
- Lysis buffer
- Phenol:Chloroform:IsoamylaLCohol (25:24:1)
- Chloroform:IsoamylaLCohol (24:1)
- Isopropanol
- 70 % Ethanol
- TE buffer

PROCEDURE:

Buffer Preparation

Washing buffer and Lysis buffer were prepared as shown in tables below.

TABLE 3.1: Preparation of Washing and Lysis Buffer

WASHING BUFFER		***LYSIS BUFFER***	
Stock Concentration	***Working Concentration***	***Stock Concentration***	***Working Concentration***
1M sucrose	320 mM sucrose	1M Tris HCl pH (8)	400mM Tris HCl pH (8)
100% Triton X-100	1% Triton X-100	10 % SDS	1 % SDS
100mM Magnesium Chloride	5mM Magnesium Chloride	0.5 mM EDTA	60 mM EDTA
100mM Tris-HCl pH (8.0)	10mM Tris-HCl pH (8.0)	10mg/ml Proteinase-K	100 µg/ml Proteinase –K
		5M NaCl	150mM NaCl

DNA Isolation:

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

- Discarded the supernatant and added 5ml of washing buffer (1.6ml 1M Sucrose,0.5 ml Triton X-100, 0.25ml $MgCl_2$, 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 5ml 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 Phenol: chloroform: Isoamyl aLCohol (PCI) 25:24:1(2.5ml Phenol, 2.4 ml chloroform and 0.1ml isoamyl aLCohol) in equal volume and mixed the contents slowly.

- Centrifuged at 8000 rpm for 10 minutes at 4°C. Took the upper aqueous 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. Discarded the supernatant and washed DNA pellet with chilled 70% ethanol twice at 10,000 rpm for 5 minutes.

- Decanted ethanol and air dry the pellet. Dissolved the pellet in 50µl-150µl Tris-EDTA buffer depending on the size of DNA pellet (Bartlett & White *et al*., 2003).

3.3 DNA Quantification

The quality and quantity of DNA were determined by agarose gel electrophoresis and spectrophotometric analysis using Nanodrop (NanoDrop ND-1000 spectrophotometer). DNA was diluted to 100ng/µl and was stored at -20°C for further use as template. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA.

3.4 Resolution of DNA fragments on agarose gels

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. DNA being negatively charged at neutral pH migrates towards anode when electric field is applied. Migration of DNA through agarose gel depends on –molecular size of DNA, agarose concentration, conformation of DNA, applied current.

Matrix of agarose gel behaves like a molecular sieve allowing movement of DNA fragments on application of electric current. Higher concentration of agarose gives firmer gels, with less spaces between cross linked molecules thus permit smaller DNA fragments to easily pass through these spaces. As the length of the DNA increases, it becomes harder for the DNA to pass through the spaces, while lower concentration of agarose helps in movements of larger DNA fragments as the spaces between the cross-linked molecules is more. The progress of gel electrophoresis is monitored by observing the migration of a visible dye (tracking dye) which contains glycerol to sediment DNA in well. Two commonly used dyes are xylene cyanol and bromophenol blue that migrate at the same speed as double stranded DNA of size 400 bps & 4000 bps respectively. These tracking dyes are negatively charged, low molecular weight compounds that are loaded along with each sample at the start of run, when the tracking dye reaches towards the anode, run is terminated.

Preparation of reagents:

5X TBE (1000 ml)		***6X LOADING DYE (20 ml)***	
Tris base	54 g	0.25% Bromophenol blue	0.05 g
Boric Acid	27.5 g	0.25% Xylene Cyanol	0.05 g
EDTA (0.5M)	20 ml	40% Sucrose	8 g
Make up final volume with water		Make up final volume with TE buffer	

Requirements:

- Electrophoresis buffer (TAE or TBE)
- Ethidium bromide solution
- Electrophoresis-grade agarose
- 6X loading dye
- DNA molecular weight markers
- Horizontal gel electrophoresis apparatus
- Gel casting platform
- Gel combs (slot formers)
- DC power supply

Agarose gel electrophoresis protocol

Making a gel

- Prepared an adequate volume of electrophoresis buffer.
- Added the desired amount of electrophoresis-grade agarose to a volume of electrophoresis buffer sufficient for constructing the gel. For exmple for genomic DNA 0.7% gel (0.7g agarose in 100ml 0.5X TBE) was prepared while for the PCR products 1.7% gel (1.7g agarose in 100ml 0.5X TBE buffer) was prepared.
- Melted the agarose in a microwave or hot water bath until the solution becomes clear. Allowed the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.
- Before pouring ethidium bromide solution was added to the electrophoresis buffer to a final concentration of 0.3µg/ml to facilitate visualization of DNA when seen under UV transilluminator.
- Poured the melted agarose onto gel casting apparatus between 0.5 and 1 cm thick and inserted the gel comb, making sure that no bubbles are trapped underneath the combs and all bubbles on the surface of the agarose were removed before the setting of the gel.

Loading and running the gel

- After the gel got hardened, gel comb was withdrawn taking care not to tear the sample wells.
- Placed the gel casting platform containing the set gel in the electrophoresis tank.

Added sufficient electrophoresis buffer so that there is about 2-3 mm of buffer over the gel. Made sure no air pockets were trapped within the wells.

- DNA samples were prepared by mixing 5µl DNA with 2µl of 6X loading dye and 2ul water in case of genomic DNA or by mixing 5µl DNA with 2µl of 6X loading dye in case of PCR product.

- Samples were typically loaded into the wells with micropipette. Care was taken to prevent mixing of the samples between wells. Appropriate DNA molecular weight marker was also loaded in case of PCR products.

- Connected the electrodes to a power pack, turned on the power, and allowed the electrophoresis run at 60 V until the marker dyes migrated the desired distance. Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – it should not exceed 5 volts/ cm between electrodes.

- Turned off the electric power, disconnected the leads, and discarded the electrophoresis buffer from the reservoirs. DNA was visualized under UV transilluminator and then photographed.

3.5 Polymerase chain reaction (PCR) amplification of microRNA 146a and 196a2 gene

Polymerase chain reaction (PCR) is an *in vitro* DNA amplification protocol. It selectively amplifies a specific DNA sequence from any source (i.e. virus, bacteria, plant, human) hundreds of millions of times in a matter of hours. PCR was performed to amplify the fragments that contain polymorphisms of *mir-196a2* (C>T, rs11614913), *mir-146a* (G>C, rs2910164). PCR was performed for 25µl reaction volume.

Requirements

- 10X PCR buffer
- BSA
- Forward Primer
- Reverse Primer
- dNTP‘s
- Taq DNA polymerase
- Water
- DNA sample

Note: Primer sequences chosen to should be unique for a particular region of DNA, avoiding the possibility of mishybridization. Primers should not easily anneal with other primers in the mixture (either other copies of same or the reverse direction primer); this phenomenon can lead to the production of 'primer dimer'. Primers should also not anneal strongly to themselves, as internal hairpins and loops as that could hinder the annealing with the template DNA. Given below are the primers used.

Table 3.2: Primers used for miRNA 146a AND 196a2 Gene				
Gene	***Base Change***	***Genotyping Assay***	***Primers***	***PCR Product***
rs2910164	G→C	PCR-RFLP mismatch, sense primer -3C→G$_A$	CATGGGTTGTGTCAGTGTC AGAGCT (sense); TGCCTTCTGTCTCCAGTCTT CCAA (antisense)	147 bp
rs11614913	C→T	PCR-RFLP mismatch, antisense primer +2T→G$_B$	CCCCTTCCCTTCTCCTCCAG ATA (sense); CGAAAACCGACTGATGTAA CTCCG (antisense)	149 bp
Underlining indicates mismatched site.A-3,3bp upstream to polymorphic site.B+2, 2bp downstream from polymorphic site (Source: Hu *et al*., 2008)				

Table 3.3 Reaction mixture for PCR			
Reagent	***Stock Concentration***	***Working Concentration***	***Quantity Used***
Additive 1 BSA	100 µg/ml	10 µg/ml	2.5 µl
PCR Buffer (Mg Conc.)	10 X 15 mM	1 X 1.5 mM	1.5 µl
Primer (Forward)	10 µM	0.5 µM	1.25 µl
Primer (Reverse)	10 µM	0.5 µM	1.25 µl
Taq Polymerase	5.0 U/µl	0.8 U	0.3 µl
dNTP	10 mM each	0.2 mM each	0.5 µl
PCR Grade Water			16.7 µl

The thermal cycling parameters were set as follows:

Table 3.4 Cycling profile of PCR		
Steps	***Tempertaure (°C)***	***Time***
Initial Denaturation	95°C	5 min
Denaturation	94°C	30 sec
Annealing	65°C for *miRNA 196a2* gene 63°C for *miRNA 146a* gene	30sec 30sec
Polymerization	72°C	45 sec
Final extension	72°C	5 min

The reaction was carried out for 30 cycles.

3.6 Restriction digestion of miRNA 146a and196a2 gene

Msp1 isolated from *Moraxella* species. It has following restriction site.

5'...C↓C G G...3'
3'...G G C↑C...5'

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

5'...G A G C T↓C...3'
3'...C↑T C G A G...5'

PROCEDURE

196a2 Gene Digestion

The total reaction mixture of 20µl consisted of 2.4 µl 10X NEB 4 buffer, 0.2µl (2U) of 10U/µl *MspI* enzyme (NEB), 10µl of PCR amplified product and 7.4µl water. The buffer used for the process was provided with the enzyme by NEB for increased activity of enzyme. All the samples were incubated at 37°C overnight. The enzyme reaction was stopped by keeping the samples at -20°C and the samples were loaded in 6% Polyacrylamide gel and developed and observed by silver staining. PCR product of 149bp was digested into fragments of 125bp and 24bp which was indicative of wild type genotype (CC), whereas the presence of three bands of 149bp, 125bp and 24bp represented the heterozygous (CT) genotype and an undigested 149-bp band represented the mutant genotype (TT). (Hu et al., 2008).

146a Gene Digestion

The total reaction mixture of 20µl consisted of 2.2 µl 10X NEB 4 buffer, 0.2µl (2U) of 10U/µl *SacI* enzyme (NEB), 10µl of PCR amplified product, 0.2 µl BSA and 7.4µl water. The buffer used for the process was provided with the enzyme by NEB for increased activity of enzyme. All the samples were incubated at 37°C overnight. The enzyme reaction was stopped by keeping the samples at -20°C and the samples were loaded in 6% Polyacrylamide gel and developed and observed by silver staining. PCR product of 147bp was digested into fragments of 122bp and 25bp for CC and was indicative of the mutant type genotype whereas three fragments of 147bp, 122bp and 25bp represented the heterozygous (GC) genotype and an undigested 147bp band was indicative of the wild type genotype (GG) (Hu *et al*., 2008).

3.7 DNA polyacrylamide gel electrophoresis

In a polyacrylamide gel electrophoresis nucleic acids are separated according to their size and charge, using a gel matrix in an electric field.

Requirements

- Acrylamide bisacrylamide solution (29:1)
- Ammonium persulfate (10mg/ml)
- TEMED
- 5X TBE
- Deionized water

Mechanism of polymerization:

Polyacrylamide gels are formed by copolymerization of acrylamide and bis-acrylamide (N,N'-methylene-bis-acrylamide). The reaction is a vinyl addition polymerization initiated by a free radical-generating system. Polymerization is initiated by ammonium persulfate and TEMED (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. The elongating polymer chains are randomly cross linked by bisacrylamide, resulting in a gel with a characteristic porosity which depends on the polymerization conditions and monomer concentrations.

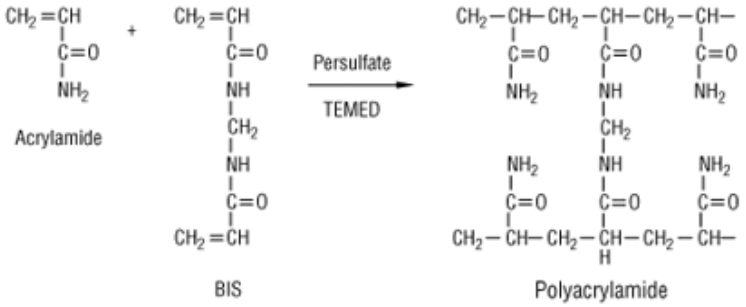

Fig 3.1: Polymerization of Polyacrylamide gels.

PROCEDURE

- Cleaned the glass plates thoroughly. Held the plates by the edges or after wearing gloves, so that oils from the hands do not become deposited on the working surfaces of the plates. Rinsed the plates with deionized water and ethanol and set them aside to dry. The glass plates must be free of grease spots to prevent air bubbles from forming in the gel.

- Assembled the glass plates (with spacers) in gel caster. Prepared the gel solution with 6% polyacrylamide percentage as shown below:

Table 3.5 Volume of reagents used to cast polyacrylamide gel

Gel %	***30% Acrylamide bisacrylamide solution (ml)***	***Water (ml)***	***5X TBE buffer (ml)***	***10% APS (µl)***	***Temed (µl)***
6%	2.4	7.2	2.4	200	10

- Poured the gel solution prepared quickly after addition of TEMED between the gel casting plates in order to prevent its polymerization in test tube.

- Immediately inserted the appropriate comb into the gel, carefully not to allow air bubbles to become trapped under the teeth. Allowed the acrylamide to polymerize for 5-10 minutes at room temperature.

- When ready to proceed with electrophoresis, removed gels from gel caster, carefully cleaned the spilled gel from back of white plates and inserted gels into BIORAD gelbox filled with 1X TBE buffer. Add running buffer and carefully pulled the combs from the polymerized gel.

- It is important to use the same batch of electrophoresis buffer in both of the reservoirs and in the gel. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the migration of DNA.

- Mixed the DNA samples with the appropriate amount of 6X loading dye. Loaded the mixture into the wells using a micropipette. Connected the electrodes to a power pack, turned on the power, and allowed the electrophoresis run.

- Allowed the gel to run at 60 V, until the marker dyes migrated the desired distance. Turned off the electric power, disconnected the leads, and discarded the electrophoresis buffer from the reservoirs.

- Detached the glass plates. Laid the glass plates on the bench and used the separators to lift a corner of the upper glass plate. Pulled the upper plate smoothly away. Similarly removed the lower plate and did silver staining (Sambrook *et al*., 1989).

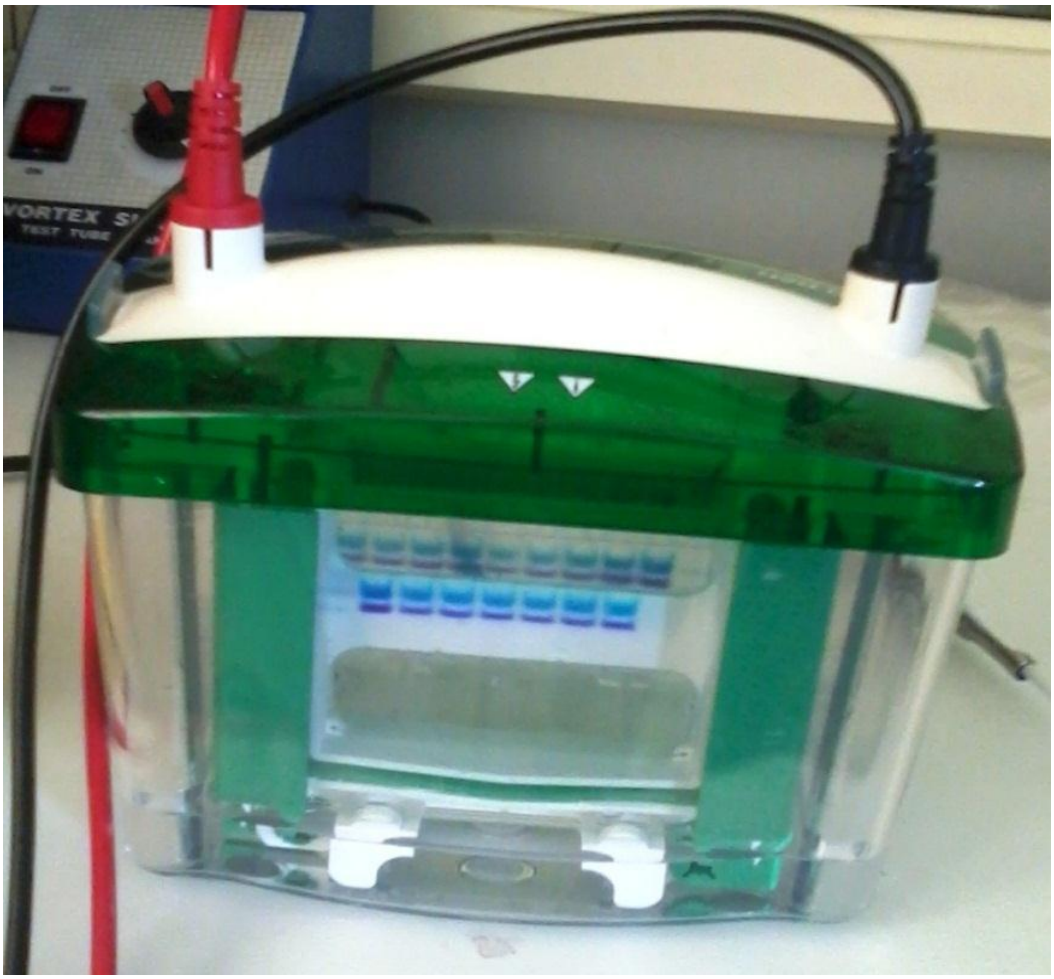

Figure 3.2: PAGE apparatus

3.8 Silver staining

Silver staining is the most sensitive method for permanent staining of proteins or nucleic acids in polyacrylamide gels. It creates a record of the electrophoresis result that can be viewed without any special equipment. It is, however, a complex multi-step process, and many variables can influence the result. High purity reagents and precise timing are necessary for reproducible, high-quality results.

REQUIREMENTS

- Fixative
- Staining Solution
- Developing Solution
- Stop Solution

PROCEDURE

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

Preparation of Staining Solution (0.1%)
Dissolved 0.1gm of $AgNO_3$ in 100ml of distilled water followed by addition of a 150µl 37% formaldehyde.
***NOTE*:** Formaldehyde serves as a reductant to convert silver ion (Ag^+) to metallic silver (Ag^0)

Preparation of Developing Solution
Dissolved 3g of Na_2CO_3 in 100ml distilled water and added 150µl 37% formaldehyde and 20µl of 10mg/ml sodium thiosulfate.

***NOTE*:** Sodium thiosulfate compound serves a dual purpose. It serves as a source of sulfide ion (S^{2-}), which reacts directly with silver, accelerating and enhancing development. Thiosulfate ion also forms a complex with free silver ion and prevents its reduction to metallic silver. This reduces back-ground staining.

NOTE: Sodium carbonate shifts the pH to approximately 12, which allows development (Ag + to Ag^0) to proceed.

Staining

- The gel was fixed in a fixative for about half an hour to render the macromolecules in the gel insoluble and prevents them from diffusing out of the gel during subsequent staining steps.
- Washed it thrice with deionized water for 2 min each.

- The gel was stained with 100ml silver nitrate solution and kept in dark for 30min.
- It was washed for 1min each in deionized water and then developing solution was added. The development solution contains formaldehyde, which reduces silver ion to metallic silver. This reaction only proceeds at high pH, so sodium carbonate was included to render the development solution alkaline. Stopping and preservation.
- The gel was then kept on gel rocker or shaker for band development
- As soon as the bands developed, stop solution containing 10% glacial acetic acid was added and kept for 5 min on shaker. The stopping solution prevents further reduction of silver ion.
- The gel was washed twice with deionized water for 5 min each.
- The gel was stored in 1% glycerol to prevent the gel from cracking during drying (Goldman and Merril, 1982).

3.9 Statistical analysis:

Differences in the distributions of demographic characteristics between the cases and controls were evaluated using the Chi-square tests (χ^2 test) for the categorical data and student *t* test for continuous variables. The Hardy–Weinberg equilibrium theory ($p^2+2pq+q^2=1$; where p is the frequency of the wild-type allele and q is the frequency of the variant allele) was used both in cases and controls to caLCulate the genotype frequencies of both *miR-146a* G>C and *miR-196a2* C>T polymorphisms using χ^2 test. Pearson's χ^2 test was used to determine whether there was any significant difference in allele and genotype frequencies between cases and controls. To assess the risk for LC and miRNA polymorphisms adjusted Odds Ratio (ORs) along with 95% Confidence Intervals (CI) were caLCulated using logistic regression analysis with adjustment for possible confounders (age and pack-years of smoking as continuous variables; and gender as a nominal variable). The homozygous wild genotype for the GG and CC allele of both *miR-146a* (rs2910164) and *miR-196a2* (rs11614913) was used as the reference in caLCulating the ORs and 95%CI. All *p* values were two sided, and a *p* value of <0.05 was considered statistically significant. In addition to overall association analysis, stratified analysis was performed to estimate risk for subgroups by smoking status, tumor histology and clinicopathological characteristics. All the statistical analyses were performed with MedcaLC version 9.3.6.0 (MedcaLC Software, Ostend, Belgium) and SPSS Version 20.0. (Chicago, IL, USA).

CHAPTER 4

RESULTS AND DISCUSSIONS

- Genotyping
- Epidemiology
- Genotypic and allelic frequency distribution of *miRNA 146a* as well as their association with LC risk
- Genotypic Distribution and association of *miRNA 146a* gene among patients with different histological types of LCs
- Distribution of genotypes of *miRNA 146a* gene among smokers and non smokers and assessment of risk towards LC
- Genotypic and allelic frequency distribution of *miRNA-196a2* as well as their association with LC risk
- Distribution of genotypes of *miRNA 196a2* gene and its association among patients with different types of LCs
- Distribution of genotypes of *miRNA 196a2* gene according to smoking habit and pack years
- Effects of combined polymorphisms in *miRNA- 196a2* and *miRNA-146a* towards LC susceptibility
- Cumulative effect of joint polymorphism (*miRNA-146a* G/C rs2910164 and *miRNA-196a2* C/T rs11614913) on LC risk, stratified according to histology
- Relationship of clinical TNM stages and *miRNA 146a* and *196a2* genotypes in LC patients

4.1 Genotyping

DNA was isolated from blood and samples were run on 0.7% gel. This total DNA from blood samples were used as template in PCR.

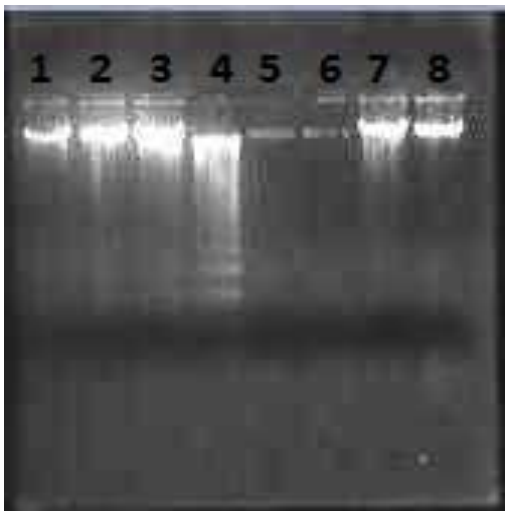

Fig 4.1: Genomic DNA isolated from peripheral blood

In order to amplify *miRNA 146a* and *196a2* gene suitable sets of primers were used as shown in Table 3.3. The PCR amplified products were separated on 1.7% agarose gel containing ethidium bromide. The DNA bands were clearly visible and distinct. Fig 4.2 shows the PCR amplified DNA products obtained using set of primer pair specific for *146a* gene. DNA bands of 147 bp were obtained.

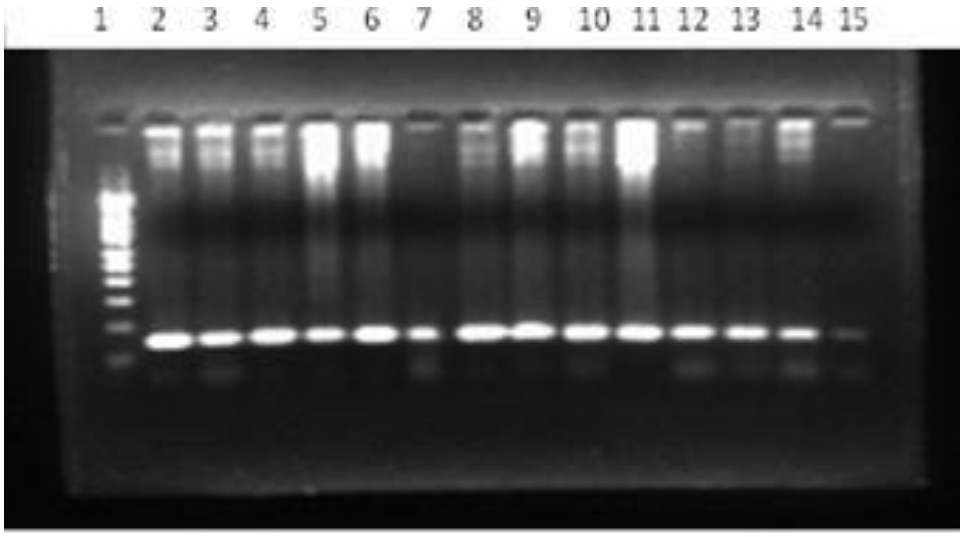

Figure 4.2: PCR amplified DNA products of miRNA 146a gene
Lane 1: 100bp ladder, Lane 2-15: Amplified PCR product (147bp)

The PCR amplified DNA products for *196a2* gene is shown in figure 4.3.

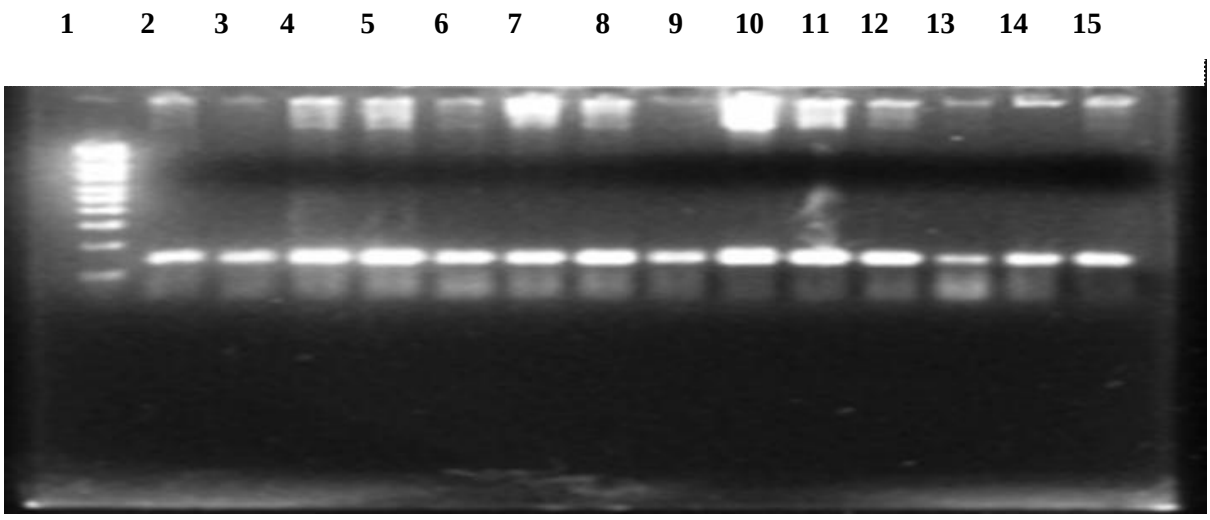

Figure 4.3: PCR amplified DNA products of miRNA 196a gene
Lane 1: 100bp ladder, Lane 2-15: Amplified DNA product (149bp)

PCR products were then digested with *SacI* and *MspI* enzyme for *146a* and *196a2* gene respectively. Then the digested samples were allowed to run on Polyacrylamide gel followed by silver staining. After the staining of the gels genotypic analysis was done as shown in Figure 4.4

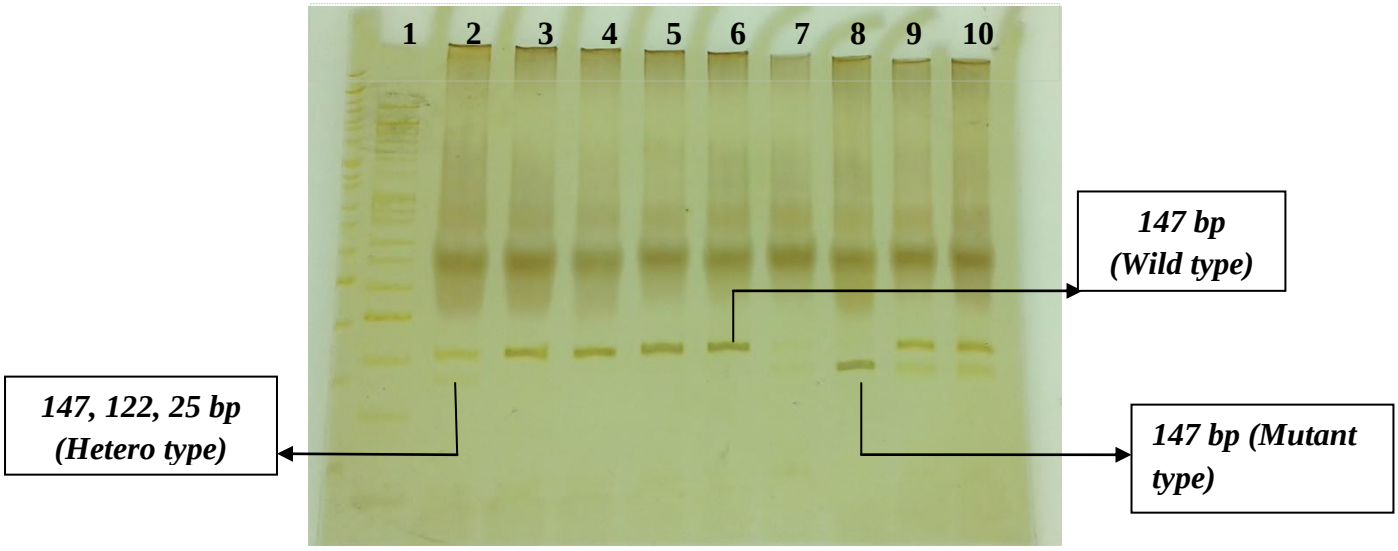

Figure 4.4: PCR RFLP genotype analysis of miRNA 146a gene.

Lane1: 50 bp ladder, Lane 8: Mutant type (CC) genotype,Lane 2, 3, 4, 5, 6: Wild type (GG)genotype,Lane 1, 7, 9, 10: Heterozygous type (GC) genotype.

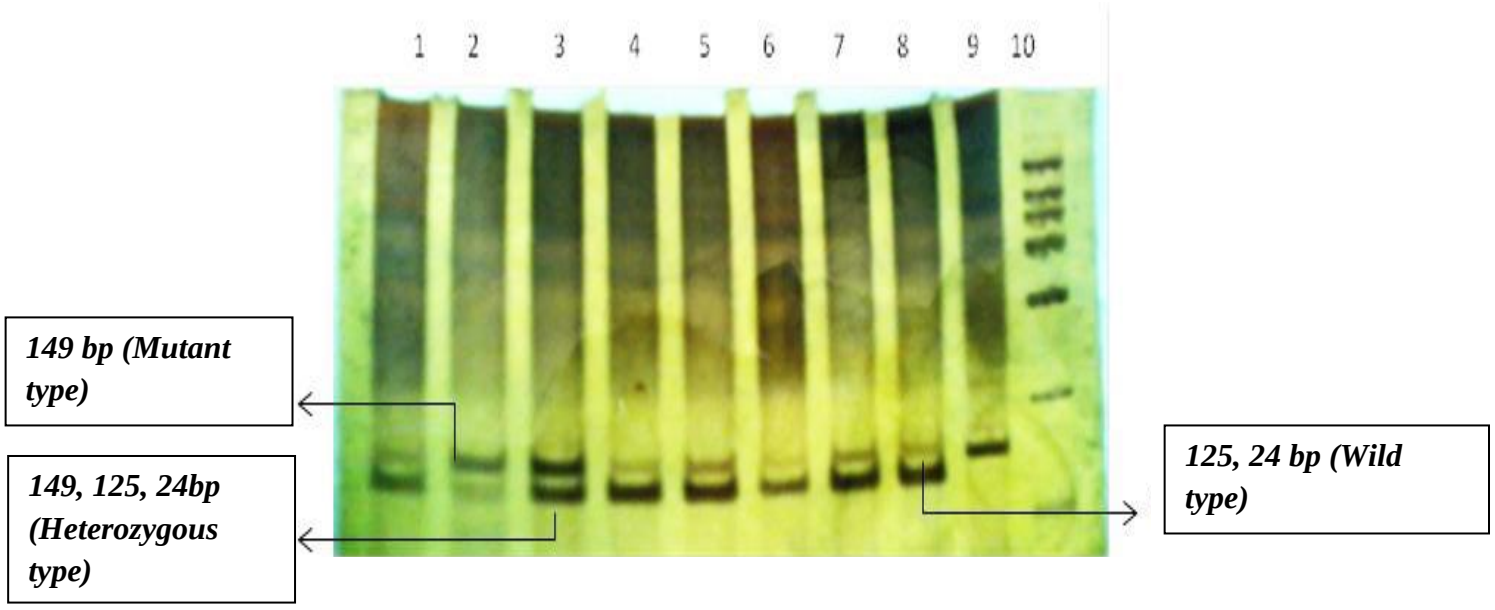

Figure 4.5: PCR RFLP genotypic analysis of miRNA 196a2.

Lane10: 100bp ladder, Lane 9: Uncut Control, Lane 8, 7, 6, 5, 4, 1: wild type (CC) genotype, Lane 3: heterozygous (CT) genotype, Lane 2: mutant (TT) genotype

4.2 Epidemiology

Demographic characteristics of the study groups including age, gender, smoking status, pack years, histological subtypes, TNM staging and other clinical parameters are shown in Table 1. The case-control study pertains to 250 LC cases and 255 controls. The mean age of cases was 57.43±10.51 (range 29-86), whereas the mean age of all control subjects were 53.00± 10.79 (range 22-83). The study comprised of 218 (87.2%) males and 32 (12.8%) of females in the case group, and 223 (87.4%) males and 32 (12.6%) females in the control group. There was no significant difference in distribution of males and females between controls and patients suggesting adequate matching (p=0.960). In the present study, 79.6% of the cases were smokers and 20.4% were non-smokers, where as in the control group 70.5% were smokers and 29.5% were non-smokers. Smoking came out to be a significant risk factor for LC suggesting an association between smoking and LC risk ($p < 0.02$). Furthermore, among the smoker's numbers of pack years were significantly higher in cases as compared to controls (34.86 ± 32.57 vs 26.10 ± 20.83: $p < 0.002$). Variables such as age, gender, smoking exposure were further adjusted for any residual confounding effect using multivariate logistic regression analyses. Of the 250 LC cases 109 (43.6 %) were squamous cell carcinoma, 76 (30.4 %) were adenocarcinoma, 64 (25.6%) small cell carcinoma, and 1 (0.4 %) undifferentiated carcinoma. TNM stage data was available in 236 (94.4 %) of 250 patients (Stage I: 2, II: 12, III: 115, IV: 107).

Table 4.1: Distribution of demographic variables for patients and controls			
Variable	**Cases, n (%) N = 250**	**Controls, n (%) N = 255**	***p* – value [a]**
Age (years)			
Mean ± SD	57.43 ± 10.51	53.00 ± 10.79	**< 0.0001**
Range	(29 – 86)	(22 – 83)	
Gender			
Male	218 (87.2)	223 (87.4)	0.960
Female	32 (12.8)	32 (12.5)	
Smoking Status			
Smokers	199 (79.6)	180 (70.5)	**0.02**
Non – Smokers	51 (20.4)	75 (29.4)	
Pack Years			
Mean ± SD	34.86 ± 32.57	26.10 ± 20.83	**0.002**
Histological Types			
SQCC	109 (43.6)		
ADCC	76 (30.4)		
SCLC	64 (25.6)		
Other carcinoma	1 (0.4)		
TNM Staging			
I	2 (0.8)		
II	12 (4.8)		
III	115 (46.0)		
IV	107 (42.8)		
Unclassified	14 (5.6)		
Tumor Size			
T1	12 (4.8)		
T2	31 (12.4)		
T3	63 (25.2)		
T4	112 (44.8)		
Unknown	32 (12.8)		
Lymph Node Involvement	45 (18)		
N0	179 (71.6)		
N1	88 (35.2)		
N2	57 (22.8)		
N3	4 (1.6)		
N4	26 (10.4)		
Unknown			
Metastasis			
M0	125 (50)		
M1	100 (40)		
Unknown	25 (10)		

Abbreviations: SD = Standard Deviation, n = total number of case patients or control subjects

[a] p-values were derived from Pearson Chi – square test except age; Student t-test was used for age. All p-values are two – sided.

$P < 0.05$ was considered statistically significant

4.3 Genotypic and allelic frequency distribution of miRNA-146a as well as their association with LC risk

The genotypes of patients and controls for *146a2* gene were obtained by PCR RFLP. Out of total patients (250) studied 64.4 % of the individuals were found to have homozygous wild type (GG) genotypes, 33.6 % had heterozygous genotypes (GC) and 2 % individuals had mutant genotype (CC). On the other hand in case of (255) controls 74.9 % of the individuals had homozygous wild type genotypes (GG), 24.3 % of the individuals had heterozygous genotypes (GC). In the present study only 2 mutant genotype were observed. The genotype and allele frequencies of the *mir-146a* (G>C, rs2910164*)* SNP and their associations with risk of LC are summarized in Table 4.2

Table4.2: Frequency distribution of miRNA 146a genotypes and their association with risk towards LC

Gene miR-146a rs2910164 G/C	***Cases, n (%) N = 250***	***Controls, n (%) N = 255***	***Adjusted OR (95% CI)*** [b]	***p-value***[a]
GG	161 (64.4)	191 (74.9)	1.00 (Reference)	
GC	84 (33.6)	62 (24.3)	1.61 (1.08-2.41)	**0.01**
CC	5 (2)	2 (0.7)	3.19 (0.58-17.5)	0.18
GC + CC vs GG	89 (35.6)	64 (25.0)	1.62 (1.09-2.41)	**0.01**
G	406 (81.2)	444 (87.05)	1.00 (Reference)	
C	94 (18.8)	66 (12.94)	1.55 (1.1-2.1)	**0.01**
Minor allele frequency	0.18	0.12		

[a] Two-sided χ^2 test for either genotype distribution or allelic frequencies between the cases and controls.

[b] Odds ratios (95% confidence intervals and their corresponding P-values were calculated by unconditional logistic analysis, adjusted for age, gender, smoking status

The allelic and genotypic frequencies of the *miR*-146a (G>C rs2910164) polymorphisms is summarized in Table II. The genotypic frequencies of *miR*-146a G>C polymorphism in the control group (n=255; $\chi 2$=1.03 df=1, *p*=0.30) were in Hardy–Weinberg equilibrium, suggesting that there was no population stratification and no sampling bias. The patients frequencies were also in Hardy–Weinberg equilibrium (n=250; $\chi 2$=2.5 df=1, *p*=0.11). Distribution of the *miR-146a* G>C genotypes was significantly different between the cases and controls (GG, GC, and CC genotypes; 66.4%, 33.6% and 2%, respectively vs. 74.9%, 24.3% and 0.7%, respectively; *p*=0.02). As for the minor allele frequency (MAF) of rs2910164, C allele was 0.18 in patients and 0.12 in controls respectively.

Using the GG genotype as the reference, it was observed that the GC genotype increased the risk of LC (OR=1.61; 95% CI=1.08–2.41, *p*=0.01), however when only the mutant genotype (CC) was evaluated it had a 3-fold increased risk towards LC, which however was not statistically significant (OR=3.19; 95% CI=0.58–17.5, *p*=0.18). Because of low prevalence of homozygous mutant-type genotype, we combined both the heterozygous (GC) and mutant (CC) genotypes as a single genotype and compared it with the reference genotype (GG). Significant association was observed towards risk for LC in case of individuals with combined GC+CC genotype

Several case-control studies have investigated the association between rs2910164 G>C polymorphism and risk factor of various cancers. Nonetheless it should be noted that some molecular epidemiological studies have reported that G allele or GG genotype is associated with the increased risk of cervical cancer (Yue *et al*, 2011), hepatocelluar carcinoma (Zhou *et al* 2013), gastric cancer (Zeng *et al* 2010), esophageal cell carcinoma (Guo *et al*, 2010). In contrast with these studies, some authors have reported opposite findings. For instance, C allele or CC genotype is associated with increased risk of earlier age onset of familial breast and ovarian cancer (Shen *et al*, 2008), gastric cancer (Okubo *et al*., 2010). Our results are in line with these findings showing that GC and combined GC/CC genotype confers the increased risk for LC. Interestingly, Jazdzewski *et al*, (2008) reported a rare feature of rs2910164, the GC heterozygous genotype being associated with an increased risk of papillary thyroid carcinoma but both homozygous genotypes protective. Discrepancy in the results may be due to differences in studied population as well as several environmental factors that influence the population. Geographic or ethnic differences have been reported regarding the genotypic frequency of several polymorphisms.

4.4 Genotypic Distribution and association of miRNA 146a gene among patients with different histological types of LCs

Among the cases studied 43.6% (109) of the cases were of those who were diagnosed with SQCC, 25.6% (64) had SCLC and 30.4% (76) were diagnosed with ADCC. There is less evidence for SCLC as compared to other two types of LCs. On further stratification on basis of genotypes it was found that 65.1%, 60.5% and 67.1% individuals of SQCC, ADCC and SCLC respectively had wild type genotype. On the other hand 31.1%, 38.1% and 32.8% respectively had heterozygote genotypes. No mutants were observed in case of SCLC while 3.6% of SQCC patients and 1.3% of the adenocarcinoma patients were mutants as shown in Table 4.3

Table 4.3 shows the associated risk for the SNP's *miR*-146a G>C when stratified according to tumor histology after adjusting for age, gender and smoking habit. In case of *miR*-146a we evaluated the risk for G/C polymorphism towards different types of LC histology. As evident from table 4.3, it was observed that ADCC cases had a significantly higher risk associated for individuals who carried the heterozygous GC genotype (OR=1.82; 95% CI= 1.04-3.20, p=0.03). No statistically significant association was observed between *miR*-146a G/C

polymorphism and risk for LC in SQCC and SCLC groups. An increasing risk towards adenocarcinoma is observed in overall analysis. However our findings are in complete contradiction to those reported in Korean population by (Jeon *et al*.2014) where no significant association was observed between the genotypes and the risk of adenocarcinoma or SCLC.

Table 4.3: Frequency distribution miRNA 146a genotypes among different histological types of LCs

Histology miR 146a rs2910164	***Cases, n (%) N = 250***	***Controls, n (%) N = 255***	***Adjusted OR (95% CI)*** [a]	***p - value***
SQCC	***109 (43.6)***			
GG	71 (65.1)	191 (74.9)	1.00 (Reference)	
GC	34 (31.1)	62 (24.3)	1.42 (0.84-2.41)	0.18
CC	4 (3.6)	2 (0.7)	4.91 (0.84-28.5)	0.07
GC + CC	38 (34.8)	64 (25.0)	1.54 (0.93-2.56)	0.09
ADCC	***76 (30.4)***			
GG	46 (60.5)	191 (74.9)	1.00 (Reference)	
GC	29 (38.1)	62 (24.3)	1.82 (1.04-3.20)	**0.03**
CC	1 (1.3)	2 (0.7)	1.85 (0.15-21.6)	0.62
GC + CC	30 (39.4)	64 (25.0)	1.82 (1.05-3.18)	**0.03**
SCLC	***64 (25.6)***			
GG	43 (67.1)	191 (74.9)	1.00 (Reference)	
GC	21 (32.8)	62 (24.3)	1.54 (0.83-2.87)	0.16
UNKNOWN	***1(0.4)***			

[a] Odds ratios (95% confidence intervals and their corresponding P-values were calculated by unconditional logistic analysis, adjusted for age, gender, smoking status

4.5 Distribution of genotypes of miRNA 146a gene among smokers and non smokers and assessment of risk towards LC

Individuals under study were classified as smokers and non-smokers to find out association between smoking and risk of developing LC. 79.6% of the cases studied were smokers while 20.4% were non-smokers. On the other hand 70.5% of controls were smokers and 29.4% were non-smokers. Smokers were further stratified as heavy smokers (pack years >= 25) and light smokers (pack years < 25) according to pack years. There were about 55.2% of heavy smokers and 44.7% of light smokers in cases whereas in controls 43.8% were heavy and 56.1% were light smokers. Table 4.5 shows genotypic distribution of *miRNA 146a* gene on basis of smoking status and cumulative smoking dose (pack years).

Table 4.4: Frequency distribution of miRNA 146a genotypes among

- ***Smokers and non-smokers***
- ***Heavy smokers and light smokers***

Variable miR-146a rs2910164	***Cases, n (%) N = 250***	***Controls, n (%) N = 255***	***Adjusted[a] OR (95% CI)***	***P-value***
Non-smokers	***51(20.4)***	***75 (29.4)***		
GG	30 (58.8)	54 (72)	1.00 (Reference)	
GC	19 (37.2)	21 (28)	1.53 (0.69-3.42)	**0.02**
GC+CC	21 (41.1)	21 (28)	1.76 (0.80-3.84)	0.15
Smokers	***199 (79.6)***	***180 (70.5)***		
GG	131 (65.8)	137 (76.1)	1.00 (Reference)	
GC	65 (32.6)	41 (22.7)	1.62 (1.01-2.60)	**0.04**
GC+CC	68 (34.1)	43 (23.8)	1.62 (1.02-2.57)	**0.03**
Heavy smokers smokers (PY >= 25)	***110 (55.2)***	***79 (43.8)***		
GG	68 (61.8)	54 (68.3)	1.00 (Reference)	
GC	41 (37.2)	24 (30.3)	1.26 (0.66-2.40)	**0.001**
GC+CC	42 (38.1)	25 (31.6)	1.25	0.48
Light smokers smokers (PY < 25)	***89 (44.7)***	***101 (56.1)***		
GG	63 (70.7)	83 (82.1)	1.00 (Reference)	
GC	24 (26.9)	17 (16.8)	1.86 (0.90-3.82)	**0.002**
GC+CC	26 (29.2)	18 (17.8)	1.91 (0.94-3.84)	0.06

[a]Odds ratios (95% confidence intervals and their corresponding P-values were calculated by unconditional logistic analysis, adjusted for age, gender, smoking status

Plausible contribution of microRNA gene variants to LC risk modified by environmental factors such as smoking was also examined. Subjects were stratified according to smoking status and further smokers were stratified as heavy smokers (pack years >=25) and light smokers (pack year>25). In our study, for *miR*-146a G>C polymorphism, smokers with GC (OR=1.62, 95%C.I: 1.01-2.60, *p*=0.04) and combined GC/CC genotype (OR=1.62, 95%C.I: 1.02-2.57, *p*=0.03) were found to be at marginal risk for LC. Further sub grouping the smokers into heavy and light smokers, the data concerning GC genotypes were found to be associated with risk for LC which was more evident in light smokers.

4.6 Genotypic and allelic frequency distribution of miRNA-196a2 as well as their association with LC risk

The genotypes of patients and controls for *196a2* gene were obtained by PCR RFLP. Out of total patients (250) studied 28 % of the individuals were found to have wild type genotypes, 64.4% had heterozygous CT genotype and 7.6 % individuals had mutant TT genotype. On the other hand in case of (255) controls 39.6 % of the individuals had wild type CC genotypes, 57.2 % of the individuals had heterozygous CT genotypes and 3.1 % had mutant TT genotype.

Table4.5: Frequency distribution of miRNA 196a2 genotypes and their association with risk towards LC

Gene miR-196a2 rs11614913C/T	***Cases, n (%) N = 250***	***Controls, n (%) N = 255***	***Adjusted OR (95% CI)*** [b]	***P value*** [a]
CC	70 (28)	101 (39.6)	1.00 (Reference)	
CT	161 (64.4)	146 (57.2)	1.49 (1.0-2.21)	**0.04**
TT	19 (7.6)	8 (3.1)	3.51 (1.36-9.04)	**0.009**
CT + TT vs CC	180 (72)	154 (60.3)	1.58 (1.07-2.33)	**0.01**
C	300 (60.0)	348 (68.23)	1.00 (Reference)	
T	200 (40.0)	162 (31.76)	1.43 (1.1-1.85)	**0.006**
Minor allele frequency	0.39	0.31		

[a] Two-sided χ^2 test for either genotype distribution or allelic frequencies between the cases and controls.

[b] Odds ratios (95% confidence intervals and their corresponding P-values were caLCulated by unconditional logistic analysis, adjusted for age, gender, smoking status

In case of the *miR*-196a2 (rs11614913) polymorphism it was observed that the genotypic distribution was highly significant between cases and controls (CC, CT, TT genotypes: 28%, 64.4% vs 7.6% vs. 39.6%, 57.2% and 3.1% respectively; (χ2 value: 10.78, df=2, p=0.004). Furthermore, as for our data on the minor allele frequency of rs11614913, T allele was 0.39 in LC group in contrast to 0.31 in the control group. It was observed that subjects carrying the mutant genotype (TT) had 3.5 fold increased risk for LC (OR=3.51, 95%CI=1.36-9.05, p=0.009) when compared to the wild type genotype (CC). The heterozygous carriers (CT) also showed a risk towards LC risk (OR=1.49, 95%CI=1.04-2.21, p=0.04) and this risk increased furthermore when both the variant genotypes (CT+TT) were combined and compared with the wild type (CC) genotype (OR=1.58, 95%CI=1.07-2.23, p=0.01).

Our results are opposite to other studies done on various populations. For example, In Korean population, carriers with combined TC/CC genotype had higher risk for NSCLC compared with TT carriers. In another study on Chinese population, it was observed that CC genotype

have significantly increased LC risk as compared to CT+TT as reference (Tian *et al*., 2009). Similar results were also found in glioma (Dou *et al*., 2010), prostate cancer (George *et al*., 2011) and other kinds of cancer.

4.7 Distribution of genotypes of miRNA 196a2 gene and its association among patients with different types of LCs

Among the cases studied 43.6 % (109) of the cases were of those who were diagnosed with SQCC, 25.6 % (64) had SCLC and 30.4 % (76) had ADCC. There is less evidence for SCLC as compared to other two types of LCs. On further stratification on basis of genotypes it was found that frequency of C/C genotype was highly associated with SQCC (31.1 %) as compared to SCLC and ADCC (14 % and 22 % respectively). On the other hand frequency of heterozygote genotypes was high in ADCC and SCLC (63.1 %, 73.4 % respectively) as compared to SQCC (59.6 %). 9.1 %, 7.8% and 4.6 % mutants were observed in case of SQCC, adenocarcinoma, SCLC.

Table 4.6: Frequency distribution miRNA196a2 genotypes among different histological types of LCs

Histology miR 146a	***Cases, n (%) N = 250***	***Controls, n (%) N = 255***	***Adjusted OR (95% CI)*** [a]	***P - value***
SQCC	***109 (43.6)***			
CC	34 (31.1)	101 (39.6)	1.00 (Reference)	
CT	65 (59.6)	146 (57.2)	1.00 (0.64-1.8)	0.73
TT	10 (9.1)	8 (3.1)	4.98 (1.55-15.9)	**0.006**
CT + TT	75 (68.8)	154 (60.3)	1.23 (0.74-2.0)	0.4
ADCC	***76 (30.4)***			
CC	22 (28.9)	101 (39.6)	1.00 (Reference)	
CT	48 (63.1)	146 (57.2)	1.81 (0.98-3.3)	0.5
TT	6 (7.8)	8 (3.1)	3.28 (1.01-10.6)	**0.04**
CT + TT	54 (71.0)	154 (60.3)	1.87 (1.03-2.37)	**0.03**
SCLC	***64 (25.6)***			
CC	14 (21.8)	101 (39.6)	1.00 (Reference)	
CT	47 (73.4)	146 (57.2)	1.73 (0.87-3.42)	0.11
TT	3 (4.6)	8 (3.1)	2.8 (0.59-14.7)	0.2
CT +TT	51 (79.6)	154 (60.3)	1.76 (0.89-3.46)	0.09
UNKNOWN	***1(0.4)***			

[a] Odds ratios (95% confidence intervals and their corresponding P-values were calculated by unconditional logistic analysis, adjusted for age, gender, smoking status

For *miR*-196a2C>T polymorphism we observed that both variant alleles (CT+TT) had a 1.87 fold risk for ADCC (OR=1.87; 95%CI=1.03-2.37, *p*=0.03), however when only mutant alleles (TT) were considered this risk became more pronounced (OR=3.28; 95%CI=1.01-10.6, *p*=0.04). Unlike ADCC, no risk was observed for SQCC with both the variant

genotypes (CT+TT) whereas a 5-fold risk was observed when only the mutant genotype was taken into consideration (OR=4.98; 95%CI=1.55-15.9, *p*=0.006). On the other hand no significant association was observed between the variant genotypes and risk for SCLC.

The pattern of LC has been changing in Indian population. LC is being increasingly diagnosed in women and adenocarcinoma has over taken SQCC as the most common histological cell type. However, in our study the pattern observed was different. SQCC was still the commonest, followed by ADCC and SCLC. Our data reported a remarkable risk of 4.9 fold for SQCC and 3.2 fold for ADCC in relation to *mir*-196a2 mutant (TT) genotype.

4.8 Distribution of genotypes of miRNA 196a2 gene according to smoking habit and pack years

Table 4.8 shows the effect of smoking and LC risk in relation with the genotypes of *196a2*. As observed, 79.6 % of the cases studied were smokers while 20.4 % were non-smokers. On the other hand 70.5 % of controls were smokers and 29.4 % were non-smokers. Smokers further stratified as heavy and light smokers accounts for 55.2 % (heavy) and 44.7 % (light) in cases, 43.8% (heavy) and 56.1% (light) in controls.

Table 4.7: Frequency distribution of miRNA 196a2 genotypes among

- ***Smokers and non-smokers***
- ***Heavy smokers and light smokers***

Variable miR-196a2 rs11614913	***Cases, n (%) N = 250***	***Controls, n (%) N = 255***	***Adjusted OR (95% CI)***	***P-value***
Non-smokers	***51(20.4)***	***75 (29.4)***		
CC	13 (25.4)	49 (65.3)	1.00 (Reference)	
CT	35 (68.6)	22 (29.3)	6.05 (2.59-14.0)	**P < 0.0001**
TT	3 (5.8)	4 (5.3)	2.80 (0.54-14.3)	0.41
CT+TT	38 (74.5)	26 (34.6)	5.4 (2.41-12.35)	**0.0001**
Smokers	***199 (79.6)***	***180 (70.5)***		
CC	57 (28.6)	52 (28.8)	1.00 (Reference)	
CT	126 (63.3)	124 (68.8)	0.93 (0.58-1.48)	0.77
TT	16 (8.0)	4 (2.2)	4.05 (1.22-13.38)	**0.02**
CT+TT	142 (71.3)	128 (71.1)	1.02 (0.65-1.62)	0.90
Heavy smokers (PY >= 25)	***110 (55.2)***	***79 (43.8)***		
CC	37 (33.6)	29 (36.7)	1.00 (Reference)	
CT	63 (57.2)	50 (63.2)	0.99 (0.52-1.86)	**0.002**
CT+TT	73 (66.3)	50 (63.2)	1.14 (0.61-2.15)	0.66
Light smokers (PY < 25)	***89 (44.7)***	***101 (56.1)***		
CC	20 (22.4)	23 (22.7)	1.00 (Reference)	
CT	63 (70.7)	74 (73.2)	0.95 (0.47-1.92)	**0.008**
TT	6 (6.7)	4 (3.9)	1.94 (0.46-8.11)	0.36
CT+TT	69 (77.5)	78 (77.2)	0.99 (0.49-2.00)	0.99

In our study as shown in Table 4.8, non-smokers with C/T genotype (heterozygous) had 6 fold higher risk of LC (OR= 6.05; 95% CI=2.59–14.0, $p<0.0001$) when compared with *miR*-196a2 CC genotype, whereas no such association was observed in case of smokers. Both heterozygous (CT) and mutant (TT) genotype were combined as a single genotype and were compared with reference genotype (CC). Among non-smokers, the combined genotype (CT+TT) was also significantly associated with an elevated risk for LC (OR=5.4; 95% CI=2.41–12.35, $p=0.0001$) but this pattern was not evident for smokers (OR=1.02; 95% CI=0.65-1.62, $p=0.90$). Significant protective effect of *mir*196a2 heterozygous genotype (CT) was observed for both heavy smokers (OR=0.99; 95% CI=0.52–1.86, $p=0.002$) and light smokers (OR=0.95; 95% CI=0.47–1.92, $p=0.008$).

Our results are in line with the results of study conducted on LC in Korean population where risk genotype has more pronounced association in non smokers (Jeon *et al*., 2014). A possible explanation for this can be differences in the lung carcinogenesis pathways between smokers and non-smoker, exposure to second-hand smoke or passive smoke, nitrogen oxide from traffic fumes, biogas fuel, and exposure to occupational, environmental and other potential carcinogens. Furthermore, no association between the *miR-196a2* polymorphism and increased risk of LC was observed in heavy and light smokers suggesting that smoking habit did not modify the association between *miR-196a2* variants and LC risk. When the *mutant TT* genotype was considered for smokers we had 4 fold increased risk to LC. Similarly in one study conducted on LC in Korean population (Hong *et al.*, 2011) significant association was found between smokers and mutant genotype of *miRNA 196a2* gene.

4.9 Effects of combined polymorphisms in miRNA- 196a2 and miRNA-146a towards LC susceptibility

rs11614913 in *miR-196a2* and rs2910164 in *miR-146a* are known to be associated with increased/decreased cancer risk. Here in our study we tried to find out association of combined *miR-196a2* C/T (rs11614913) and *miR-146a* G/C (rs2910164) functional polymorphisms and LC risk.

Table4.8: Frequency distribution of combined miRNA 146a and 196a2 and their association with risk towards LC

Gene (miR-146a &196a2)	***Cases, n (%) N = 250***	***Controls, n (%) N = 255***	***Adjusted OR (95% CI)*** [b]	***P value*** [a]
CC+ GG	43	70	1.00 (Reference)	
CT+GC	51	31	2.29 (1.24-4.24)	**0.007**
CT+TT+GC+CC	62	33	2.81 (1.55-5.1)	**0.0006**

[a] Two-sided χ^2 test for either genotype distribution or allelic frequencies between the cases and controls.

[b] Odds ratios (95% confidence intervals and their corresponding P-values were caLCulated by unconditional logistic analysis, adjusted for age, gender, smoking status

Combined analysis of these two SNPs to evaluate potentially modifying effect of combined genotypes towards risk for LC (Table 4.8). Because of insufficient number of subjects in the mutant genotype of both the SNPs, we combined the heterozygous and mutant genotypes of both *miR*-196a2 and *miR*-146a as one single genotype (GC+CC+CT+TT). A positive association was found between individuals who had heterozygote genotype (CT+GC) for both the genes and LC risk (OR=2.29, 95% CI=1.24-4.24, *p*=0.007)**.** This risk appeared to be more evident and statistically significant for combined mutant and heterozygote (CT+TT+GC+CC) genotype (OR=2.81; 95%CI= 1.55-5.1, *p*=0.0006). In a similar study conducted on non-Hispanic white population, it was found that individuals with 4 risk genotypes (rs2910164, rs2292832, rs3746444 and rs11614913) had a 40% significantly increased risk of SCHNN compared with individuals with 0-1 genotypes (Liu *et al*., 2010).

4.10 Cumulative effect of joint polymorphism (miR-146a G/C rs2910164 and miR-196a2 C/T rs11614913) on LC risk, stratified according to histology

Table 4.9 Stratification analysis of the miRNA 196a2 C/T (rs11614913) and miRNA 146a G/C (rs2910164) functional polymorphisms on LC risk according to histology

Histology rs11614913 C/T +rs2910164 G/C	***Cases, n (%) N = 250***	***Controls,n (%) N = 255***	***Adjusted OR (95% CI)*** [a]	***P - value***
SQCC	***44***			
CC+ GG	22	70	1.00 (Reference)	
CT+GC	20	31	1.45 (0.65-3.25)	0.3
CT+TT+GC+CC	26	33	1.85 (0.85-4.0)	0.1
ADCC	***32***			
CC+ GG	14	70	1.00 (Reference)	
CT+GC	18	31	2.71 (1.15-6.30)	**0.02**
CT+TT+GC+CC	22	33	3.32 (1.47-7.48)	**0.003**
SCLC	***20***			
CC+ GG	7	70	1.00 (Reference)	
CT+GC	13	31	2.91 (1.01-8.4)	**0.04**
CT+TT+GC+CC	14	33	3.00 (1.05-8.5)	**0.03**

[a]Odds ratios (95% confidence intervals and their corresponding P-values were calculated by unconditional logistic analysis, adjusted for age, gender, smoking status

The combined presence of *miR-146a* G/C and *miR-196a2* polymorphisms showed increased risk for SCLC as well as adenocarcinoma. Three-fold elevated risk was observed for adenocarcinoma when combined heterozygous and mutant genotypes were considered in *miR-146a* and *miR-196a2* combination. None of the combinations of genotype revealed any association with SQCC. Our findings suggest an increasing trend towards the risk of adenocarcinoma as compared to SQCC and SCLC.

4.11 Relationship of clinicopathological stage and miRNA 146a and 196a2 genotypes in LC patients.

Table 4.10 Relationship of clinical TNM stages and miRNA 146a and 196a2 genotypes in LC patients

Variables	***Clinical stage***		***Adjusted Or (95% CI)***	***P value***	***TNM stage***		***Adjusted OR (95% CI)***	***P value***	***Metastasis***		***Adjusted OR (95% CI)***	***P value***
	IV	***III***			***T3+ T4***	***T2+ T1***			***Positive***	***Negative***		
miR-146a												
GG	65	79	Reference		115	29	Reference		64	82	Reference	
GC	40	34	1.42(0.79-2.5)	0.23	58	12	1.24(0.58-2.62)	0.56	34	40	1.06(0.82-1.89)	0.82
GC+CC	42	36	1.38(0.78-2.44)	0.25	60	14	1.08(0.4-2.5)	0.50	36	43	1.02(0.58-2.18)	0.93
miR-196a2												
CC	28	35	Reference		48	15	Reference		26	39	Reference	
CT	71	70	1.14(0.62-2.11)	0.65	114	27	1.34(0.65-2.77)	0.52	68	77	1.26(0.69-2.3)	0.44
TT	8	10	0.95(0.31-2.89)	0.93	13	1	3.92(0.46-32.8)	0.48	6	9	1.0(0.29-3.3)	0.99
CT+TT	79	80	1.12(0.61-2.04)	0.70	127	28	1.43(0.7-2.94)	0.48	74	86	1.22(0.67-2.2)	0.50

Clinicopathologic stage is the most accurate predictor of clinical outcomes of LC patients. In order to determine whether there is any association between *miR*-146a G>C, *miR*-196a2 C>T polymorphism and certain clinicopathologic characteristics, we further divided LC patients into subgroups according to clinical TNM stage, metastatic status and primary tumor extension. The clinicopatholoical parameters were dichotomized as follows: the clinical stage IV vs. III, primary tumor extension, T3+T4 vs. T1+T2, the metastatic status, positive vs. negative (Table 4.11). We did not find any significant association between *miR-146a* and *196a2* SNP and clinicopathological parameters examined such as clinical stage, tumor extension, and metastatic status.

CONCLUSION

The present case/control study pertains to patients visiting the Post Graduate Institute of Medical Education and Research, which is a referral centre for patients from states like Haryana, Himachal Pradesh, Punjab, Uttar Pradesh, Jammu & Kashmir and Chandigarh. The following points are evident from the present study

- *miR-146a* G>C and *miR*-196a2 C>T was significantly associated with risk for LC
- Individuals with *miR-196a2* rs11614913 CT or CT/TT genotypes have high susceptibility to LC. The effect of this SNP on LC risk was more significant in never smokers.
- Significant association was found between smokers and GC genotype for *mir-146a* gene indicating elevated risk of LC especially among light smokers.
- Combined presence of *mir-196a2* C/T (rs11614913) and *mir-146a* G/C (rs2910164) functional polymorphisms further increased the risk for LC. Further on histology stratification of data elevated risk of developing ADCC was observed for both SNP separately as well as in combination.
- Both the variants can be considered as biomarkers for LC thus our present work despite the limitation of small sample size will provide guidelines for future work.

In conclusion, this is the first study to address the potential role of rs11614913 and rs2910164 in genetic predisposition to LC including 250 individuals from North Indian population. However, limited sample size especially in stratification analysis allowed us to draw preliminary conclusions. Large sample size is required in order to find out the small effects especially in stratified analysis. Further larger studies in different populations are warranted to confirm current findings.

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APPENDIX- I

- 0.5M EDTA: Dissolved 9.306g of disodium salt of EDTA in 20ml of deionised water, and then adjusted the pH to 8.0 by 1 M sodium hydroxide. Sterilized the solution by autoclaving.
- 10% SDS: Dissolved 1g of SDS in 10ml of deionised water.
- 100mM Tris-Cl (pH 8.0): Dissolved 0.32g of Tris-Cl in 10 ml of deionised water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilized the solution by autoclaving.
- 10mg/ml Proteinase K: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilized the solution by autoclaving.
- 1mg/ml BSA: Dissolved 100mg of BSA in 100ml of deionised sterile water and kept at 4°C overnight.
- 5M Sodium chloride (NaCl): Dissolved 5.85g of sodium chloride in 20ml of deionised water. Sterilized the solution by autoclaving.
- 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.
- Ethidium Bromide (10mg/ml): Dissolved 1g of Ethidium bromide in 100ml of water. Mixed the solution properly.
- Magnesium chloride ($MgCl_2$) (100mM): Dissolved 0.41g of $MgCl_2$ in 20ml of deionised water and sterilized by autoclaving.
- Sucrose (1M): Dissolved 3.41g of sucrose in 10 ml of deionised water and sterilized by autoclaving.
- TE buffer (pH 8.0): Added 1ml of 100mM Tris-Cl (pH 8.0) and 200 µl of 0.5M EDTA solution to 8.8 ml of deioinsed water. Sterilized the solution by autoclaving.
- Triton X- 100 (10%): Took 100 µl of TritonX-100 and mixed with 900 µl of deionised water and mixed properly.