ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORHISM IN microRNA 196a2 AND 146a GENE TOWARDS RISK FOR LUNG CANCER IN NORTH INDIAN POPULATION

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CERTIFICATE

This is to certify that the thesis entitled **"Association of single nucleotide polymorphism in *microRNA196a2* and *146a* gene towards risk for lung cancer in North Indian population "** submitted by Inderjot Kaur Arora (Roll no-301101015) in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, to Thapar University (Deemed University), Patiala, is a record of student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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ABBREVIATIONS

SCLC	Small cell carcinoma
ADCC	Adenocarcinoma
SQCC	Squamous cell carcinoma
LCC	Large cell carcinoma
miRNA	microRNA
LCNEC	Large cell neuro-endocrine carcinoma
C.I.	Confidence interval
SCCHN	Squamous cell carcinoma of head and neck
PTC	Papillary thyroid carcinoma
TRAF	Tumor necrosis factor receptor associated factors
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
SASP	Senescence associated secretory phenotype
IRAK	Interleukin-1 receptor-associated kinase
ESCC	Esophageal squamous cell carcinoma
HCC	Hepatocellular Carcinoma
NSCLC	Non-small cell lung cancer
UTR	Untranslated region
CARD 10	Caspase recruitment domain containing protein 10

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ABSTRACT

MicroRNAs (miRNAs) are small RNA molecules 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 CC (mutant type) genotypes for *miRNA 146a* gene showed high risk for lung cancer (OR=5.648, 95%C.I; 0.64-49.8, *p*=0.08) especially for SQCC and SCLC compared to those who possessed GC or GG genotypes. The association also persisted among non-smokers.

On the other hand TT (mutant genotype for *miRNA 196 a2* gene also showed high risk for lung cancer (OR=3.78, 95%C.I; 0.94-15.2, *p*=0.04) especially SCLC and ADCC. Smokers with mutant genotype were more susceptible for lung cancer risk as compared to nonsmokers.

The present study suggests an important role of *mir-196a2* rs11614913 and rs291014 polymorphism with overall lung cancer 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 a complex dynamic disease which develops because of a multistep process resulting in the accumulation of several genomic alterations. It is a generic term for large group of disease that can affect any part of the body and is characterized by unrestricted proliferation, invasion, and metastasis. In cancer, many molecular pathways are affected, involving canonical protein-coding genes as well as recently discovered noncoding genes.

According to WHO Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008. The main types of cancers are:

• Lung (1.37 million deaths)

• Stomach (736 000 deaths)

• Liver (695 000 deaths)

• Colorectal (608 000 deaths)

• Breast (458 000 deaths)

• Cervical cancer (275 000 deaths)

As it is clear from above data, Lung cancer is currently the leading cause of cancer related deaths. The two major known risk factors for bronchogenic carcinoma in humans are smoking and asbestos exposure. Investigators have reported a 10-fold increase in the risk of lung cancer with smoking and a 3 to 4 fold increase associated with asbestos exposure Epidemiologic data favor a synergistic model for the two risk factors so that individuals who are exposed to both tobacco smoke and asbestos have roughly a 30- to 50-fold increase in risk of developing lung cancer (Lee *et al.,* 1998*).*

If lung cancer is diagnosed at the localized stage, the 5-year survival rate is about 50%, whereas it decreases precipitously in cases with lymph node involvement or metastasis. Discovery of

biomarkers and their application in conjunction with traditional cancer diagnosis, staging, and prognosis could to a large extent help improve early diagnosis. However, despite the efforts that have been made, reliable markers are still lacking, and the prognosis remains poor (Hu *et al*., 2008).

Traditionally, the study of cancer has focused on protein-coding genes, considering these as the principal effectors and regulators of tumorigenesis. Recent advances, however, have brought non-protein-coding RNA into the spotlight. MicroRNAs (miRNAs), one such class of non-coding RNAs, have been implicated in the regulation of cell growth, differentiation, and apoptosis.

MicroRNAs (miRNAs) are non-coding, single-stranded RNAs of ~22 nucleotides and constitute a novel class of key regulators of gene expression that regulate important oncogenes and tumor suppressors. Many miRNAs act as oncogenes or tumor suppressors, and the altered misexpression of miRNAs is a hallmark of many cancer types. Dysregulated miRNAs are a potentially powerful new tool that could be used to enable the characterization of tumor environments and identify novel and important oncogenic pathways. More recently, there has been growing interest in the field of miRNAs as biomarkers for cancer risk, diagnosis and response to therapy. Understanding the associations between miRNA expression and cancer phenotypes and the potential of miRNA profiling in clinical applications, promises to be highly rewarding in the field of cancer research (Chan *et al*., 2011).

Mutation, misexpression and altered mature miRNA processing are implicated in carcinogenesis and tumor progression. Because SNPs in miRNAs could alter miRNA processing, expression, and/or binding to target mRNA and moreover recently, several reports identified genetic variants in the precursor or mature miRNA sequence of *miR-196a2* (rs11614913 [*Homo sapiens*], cytosine to thymine, C→T) and *miR-146a* (rs2910164 [*Homo sapiens*], guanine to cytosine, G→C), 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 "ASSOSCIATION OF SINGLE NUCLEOTIDE POLYMORHISM IN *microRNA 196a2* AND *146a* GENE TOWARDS RISK FOR LUNG CANCER IN NORTH INDIAN POPULATION".

CHAPTER 2

REVIEW OF LITERATURE

- Histology of lung cancer
- MicroRNAs
- miRNA*146a* Gene
- miR-*196a2* Gene

2.1 HISTOLOGY OF LUNG CANCER

Histological classification of malignant epithelial tumors of the lung presents many difficulties and numerous classifications have been put forward. Majority of tumors arise in the epithelium lining the bronchi and bronchioles. It appears that they arise in the basal layer, and, as in many nonmalignant conditions these cells are capable of differentiation into cells of different function and structure, it is not surprising that this adaptability is mirrored in tumor formation (Waltere and Pryce, 1955).

Human lung cancers are classified into two major types,

(1) Small cell lung cancer (SCLC)

(2) Non–small cell lung cancer (NSCLC)

2.1.1 SMALL CELL CARCINOMA:

SCLC accounts for 20-25% of all new cases of primary lung cancer cases and unlike major forms of lung cancer, it is highly sensitive to both chemotherapy and radiation therapy (Broers *et al.,* 1985). Ultra structurally, SCLC displays a number of neuroendocrine features in common with pulmonary neuroendocrine cells, including dense core vesicles or neurosecretory granules. These dense core vesicles are associated with a variety of secretory products, cell surface antigens, and enzymes (Cook *et al*., 1993). SCLC expresses neuroendocrine (NE) cell markers and on the other hand most Non-SCLC tumors (NSCLC) are chemo resistant and do not express NE cell markers. Among "potentially useful markers" the four that have been most extensively tested are NSE (neuron specific enolase), CPKBB (creatinephosphokinase), GRP (gastrin releasing peptide) and chromogranin A (Weynants *et al*., 1990)

2.1.2 NON SMALL CELL CARCINOMA:

It consists of several types mainly adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Previously, squamous cell carcinoma was the predominant form of NSCLC, but in the last few decades it has been replaced by adenocarcinoma.

- **Squamous-cell carcinoma**

  Squamous cell lung carcinomas, for which diagnostic criteria include evidence of squamous differentiation such as keratin formation, form a discrete cluster with high-level expression of transcripts for multiple keratin types and the keratinocyte-specific proteins. The squamous tumors also show overexpression of *p63*, a *p53*-related gene essential for the formation of squamous epithelia (Bhattacharjee *et al*., 2001)

- **Adenocarcinoma**

  These tumors show evidence of glandular function or structure. The so-called alveolar cell carcinoma is regarded as a type of well differentiated adenocarcinoma having a multifocal origin from bronchioles and showing extensive implantation metastasis. In less differentiated tumors, tubule formation is less marked and in a few cases, absent. The cells, however, present a characteristic appearance; they are polygonal in shape and show large, spherical, or slightly oval vesicular nuclei and abundant ground-glass or vacuolated cytoplasm containing mucus (Waltere and Pryce, 1955).

- **Large cell carcinoma**

  Large cell carcinoma accounts for 9% of all lung cancers. There are no specific cytological features associated with large cell carcinoma, although LCNEC (Large cell neuro-endocrine carcinoma) can be distinguished on a cytological basis from small cell carcinoma by the presence of prominent nucleoli and nuclei larger than three times the diameter of a small resting lymphocyte. Large cell carcinoma occurs preferentially in lung periphery. Neuro-endocrine differentiation is confirmed by the demonstration of immune histochemically positive neuro-endocrine markers chromogranin, synaptophysin and NCAM CD56. (Brambile, 2005).

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. Since the introduction of tumor, node, metastasis (TNM) staging by Pierre Denoix between the years 1943 and 1952, there have been significant changes including the TNM staging for lung cancer. The International Union Against Cancer (UICC) TNM Prognostic Factors Project continued to develop the TNM classification as more data became available.

- T – Stands for the size of the tumor. Tumor size is usually given in centimeters (cm). To understand this in inches, 5 cm is same as 2 inches.
- N – N stands for lymph nodes and tells whether the tumor has spread to lymph nodes, and if so, how far away from the tumor they are.
- M – M stands for metastasis, that is, the spread of the tumor to other parts of the body.

Traditionally the TNM classification has been used for non-small-cell lung cancer (NSCLC). Even though the TNM classification was applicable to the small-cell lung cancer (SCLC), this was not practiced. SCLC was classified as "local" and "extensive" disease. The new classification is also applicable to both types of lung cancers (Mirsadraee et *al*., 2011)

2.2 MicroRNAs

MicroRNAs (miRNAs) are endogenous ∼22 nt RNAs that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression. Although they escaped notice until relatively recently, miRNAs comprise one of the most abundant classes of gene regulatory molecules in multicellular organisms and likely influence the output of many protein-coding genes (Kerscher & Slack, 2005).

2.21 BIOGENESIS: miRNA MATURATION

The biogenesis of miRNAs starts with the transcription of genomic regions located within or between protein-coding genes, resulting in the synthesis of miRNA precursor molecules (pri-miRNAs). Pri-miRNAs are currently thought to be transcribed primarily by RNA polymerase II and, less frequently by RNA polymerase III. Drosha, a specific ribonuclease of the RNase III endonuclease family, then enzymatically cuts the transcribed pri-miRNA in a smaller fragment (∼70 nucleotides). This hairpin pre-miRNA is then exported to the cytoplasm by Exportin-5 in a Ran-GTP-dependent manner and cleaved into an imperfect double-strand RNA (dsRNA), duplex-designated miRNA, which is termed miRNA. This process is performed by Dicer, an RNase III endonuclease composed of a helicase domain and a dsRNA-binding domain. One strand of the miRNA/miRNA duplex is then selected to function as a mature miRNA and

preferentially loaded into a miRNA ribonucleoprotein (miRNP) complex, whereas the other strand is likely degraded (Visone and Croce, 2009). Fig 2.1 describes the process of miRNA biogenesis.

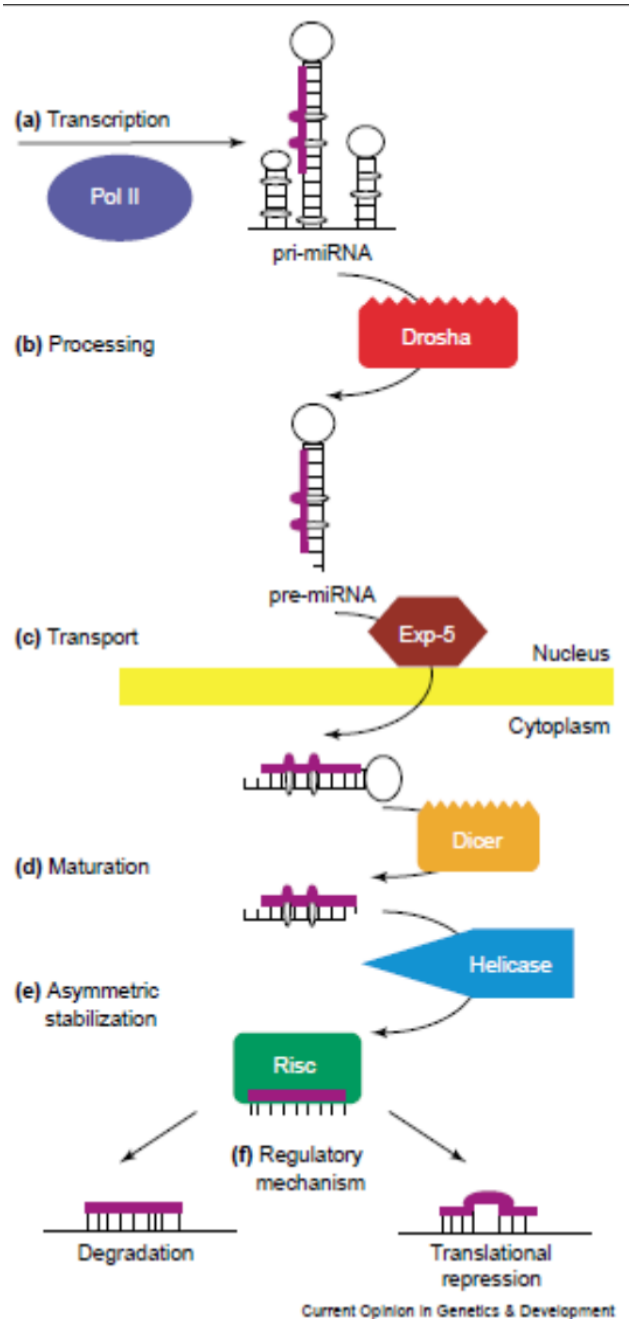


Figure 2.1: Biogenesis of miRNA (Pasquinelli *et al*., 2005)

2.22 MECHANISM OF ACTION

MicroRNAs can direct the RISC to downregulate gene expression by either of two post transcriptional mechanisms: mRNA cleavage or translational repression (Figure 2.2a and 2.2b). According to the prevailing model, once incorporated into a cytoplasmic RISC, the miRNA will specify cleavage if the mRNA has sufficient complementarity to the miRNA, or it will repress productive translation if the mRNA does not have sufficient complementarity to be cleaved but does have a suitable constellation of miRNA complementary sites (Bartel, 2004).

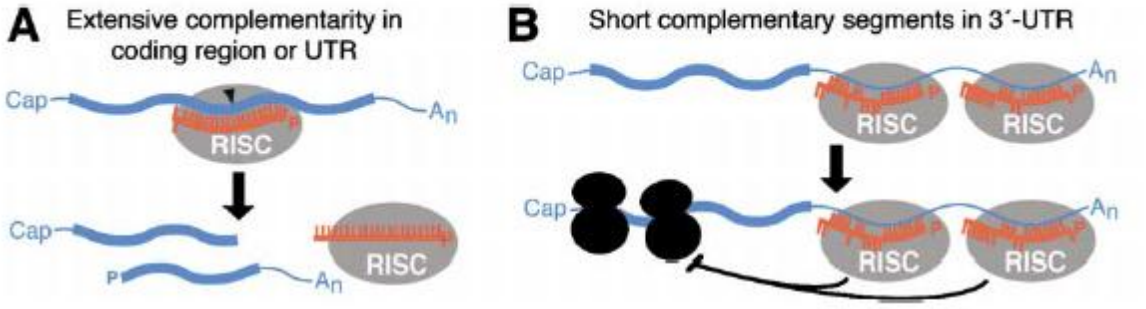


Figure 2.2: The Actions of Small Silencing RNAs (A) Messenger RNA cleavage specified by miRNA. Black arrowhead indicates site of cleavage. (B) Translational repression specified by miRNAs (Bartel, 2004)

An alternative possibility is that translation continues at the same rate but is nonproductive because the newly synthesized polypeptide is specifically degraded. In this review, both of these mechanistic possibilities are lumped together as translational repression, as is common practice, even though in the second possibility polypeptide synthesis per se is not repressed. The Argonaute proteins present in the RNA-induced silencing complex (RISC) appear to dictate the mode of regulation elicited by the miRNA–target duplex. Recruitment of specific Argonaute proteins can catalyze cleavage of mRNA sequences perfectly base-paired to the miRNA, or inhibit translation of mRNAs that form an imperfect duplex with the miRNA. (Pasquinelli *et al*., 2005)

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 polymorphisms (SNPs), and defects in the miRNA biogenesis machinery**.**

2.2.4 MicroRNAs AS ONCOMIRS

Recent evidence indicates that miRNAs can function as tumour suppressors and oncogenes, and they are therefore referred to as 'oncomirs'. Factors that are required for the biogenesis of miRNAs have also been associated with various cancers and might themselves function as tumour suppressors and oncogenes. For example: Expression of Dicer has been shown to be down regulated in lung cancer and the Argonaute proteins, which are crucial components of the RISC complex that direct both short interfering siRNA and miRNA-mediated gene regulation, have also been associated with various cancers (Kerscher & Frank, 2006).

2.2.5 MicroRNAs AS THERAPEUTIC TOOLS

Because of the significance of miRNAs in cancer, the management of miRNAs with altered expression in cancer should be considered as a therapeutic strategy. Restoring miRNA expression in diseases in which expression is consistently reduced or antisense oligonucleotides that bind directly to miRNAs and block their activity (named anti-miRNAs) could be used as novel therapeutic approaches (Visone and Carlo, 2009).

2.2.6 MicroRNAs AS DIAGNOSTIC AND PROGNOSTIC TOOLS

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. miRNA profiling has acquired importance in resolving one of the most demanding issues in cancer diagnostics—the origin of metastasis of unknown primary tumor. In a recent study, a miRNA-based tissue classifier was constructed to identify the tissue of origin of metastatic tumors (Visone and Carlo, 2009).

2.3 *miRNA146a* 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 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.

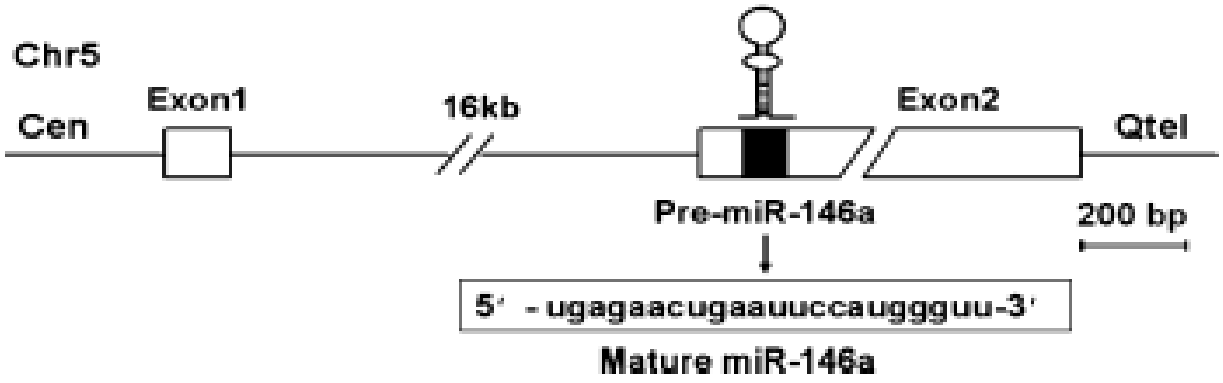


Figure 2.3: Schematic diagram of *miR-146a* loci and the detailed sequence of mature *miR-146a* on human chromosome 5 (Li *et al.*, 2010)

2.3.2 *miR-146a* IN TUMOURIGENESIS

The study by He *et al*., (2005) reported that *miR-146a* is overexpressed in unaffected part of thyroid glands of patients with papillary thyroid carcinoma (PTC). Increase in *miR-146a* expression in PTC is also proved by Jazdzewski *et al*., (2008). Recent study (Zhang *et al.,* 2009) demonstrated that *miR-146a* is the only miRNA that is highly expressed in both paedi-atric acute lymphoblastic leukaemia and paediatric acute myeloid leukaemia thus indicating the role of *miR146a* gene in tumorigenesis.

Promotion of 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.

2.3.3 *miR-146a* AND TUMOUR METASTASIS

Activation of NF-jB in promoting the survival and metastatic potential of cancer cells is widely accepted (Inoue *et al*., 2007). *miR-146a* can suppress the metastatic ability of breast cancer cells partially through decreasing constitutive NF-jB activity. It is reported that BRMS1 (Breast cancer metastasis suppressor) 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 ability of metastasizing metastasis MDA-MB-231 cells (Hurst *et al.,*2009). Therefore, modulation of *miR-146a* expression might provide a potential therapy to suppress cancer metastasis.

2.3.4 *miR-146a* IN SENESCENCE

It is reported that the levels of two related miRNAs, miR-*146a* and *146b* (*miR-146a/*b*),* increase in senescent human fibroblasts in an interleukin IL1α dependent manner, but only when high levels of IL-6 and IL-8 secretion accompany senescence. In the context of the SASP (senescence associated secretory phenotype), it is proposed that increased expression of *miR-146a/b* serves to restrain excessive secretion of the inflammatory cytokines IL-6 and IL-8, thereby limiting senescence-associated inflammation. (Bhaumik *et al*., 2009)

2.3.5 *miR-146a* TARGETS MULTIPLE NF-KB ACTIVATION PATHWAYS

miRNA-146a (*miR-146a*) is regulated by NF-κB and inhibits interleukin-1 receptor (IL-1R) and toll-like receptor (TLR)- induced activation of NF-κB by targeting interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6). *miR-146a* has been reported to be aberrantly expressed in several inflammatory diseases and cancers. CARD10 and COPS8 are new direct targets of *miR-146a*. Both are part of the G protein-coupled receptor (GPCR) mediated signal transduction that mediates activation of NF-κB. This suggests that *miR-146a* acts tumor suppressing by inhibiting GPCR mediated activation of NF-κB and the resulting expression of tumor-promoting cytokines and growth factors (Crone *et al*., 2012)

2.3.6 rs2910164

C⁄G polymorphism (designated rs2910164) is observed on the passenger strand of the precursor of *miR-146a*. The rarer C allele decreases *pri-miR-146a* nuclear processing efficiency, reduces the expression of mature *miR-146a* and results in less efficient inhibition of the target genes including TRAF6, IRAK1 and papillary thyroid carcinoma 1 gene (PTC1). The polymorphism is associated with increased risk of acquiring PTC in an over dominant manner. Interestingly, the polymorphism also undergoes somatic mutation in PTC tumor tissue. The 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.

Individuals with 4 risk genotypes (rs2910164, rs2292832 and rs3746444 andrs11614913) had a 40% significantly increased risk of SCCHN (Squamous cell carcinoma of head and neck) compared with individuals with 0–1 genotypes, and the risk was more pronounced in subgroups of younger age, men, and never smokers and patients with oropharyngeal cancer.

Zeng *et al*., (2010), for the first time found that variant in *premiR-146a* conferred an increased risk of gastric cancer in Chinese population. It was also found that the elevated gastric cancer risk was especially evident in the individuals aged ≤ 58 years, nonsmokers and males.

In a microarray-based miRNA expression analysis, *miR-146a* was found upregulated in ESCC (esophageal squamous cell carcinoma) tissues. Variant genotype of *miR-146a* rs2910164 was

found to be associated with significantly increased risk of ESCC in the case–control study of esophageal cancer in Chinese (Guo *et al*., 2010).

In a meta-analysis (Xu *et al*., 2011), the data on the association between the *miR-196a2* or *miR-146a* functional polymorphism and cancer risks was summarized. A significantly protective effect of rs11614913 T variant for cancer especially, in the subgroups of Asians and breast cancers was found. Besides, it was concluded that the C allele of the rs2910164 might be associated with protection from digestive cancer.

No statistically 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 had no major role in genetic susceptibility to hepatocellular carcinogenesis in the population studied (Akkiz *et al*., 2011).

In a case control study conducted on Chinese population it was found that the rs11614913 SNP in hsa-*mir-196a2* was associated with survival in individuals with NSCLC. Specifically, survival was significantly decreased in individuals who were homozygous CC at SNP rs11614913. Furthermore, binding assays revealed that the rs11614913 SNP can affect binding of mature hsa-mir-196a2-3p to its target mRNA. Therefore, the rs11614913 SNP in hsa-mir-*196a2* may be a prognostic biomarker for NSCLC. Further characterization of *miRNA* SNPs may open new avenues for the study of cancer and therapeutic intervention.

2.4 ***miR-196a2*** GENE

2.4.1 STRUCTURE AND LOCATION OF miR- *196a2* GENE

The gene familiy for *miR-196* is in the regions of homeobox (HOX) clusters within the genome of vertebrates (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 (6qB3) in mice. *miR-196a1* and *miR-196a2* genes transcribe the same 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.

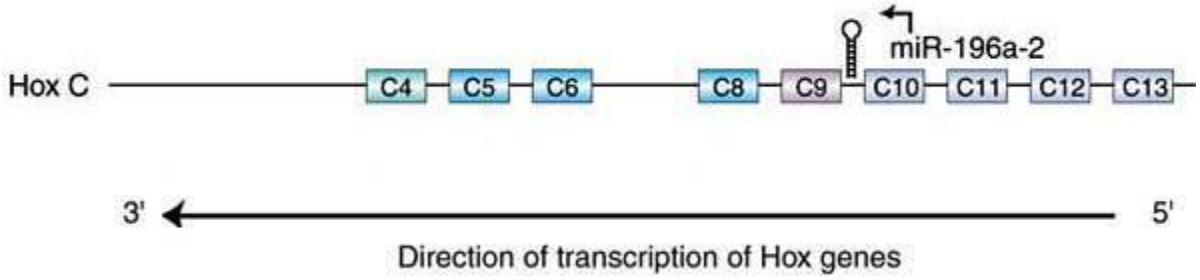


Fig 2.4: Location of *miRNA 196a2* gene (Mansfield *et al*., 2004)

2.4.2 ROLE OF *miRNA 196a2* in DEVELOPMENT

miR-196 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* is shown below in Figure 2.5.

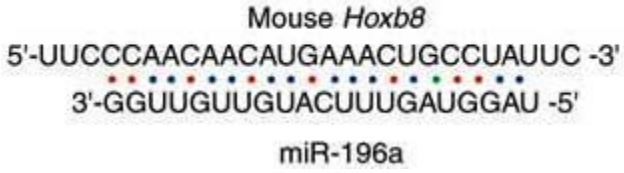


Figure 2.5: A site complrmentary to *miR-196a* in *Hoxb8* 3’ UTR (Mansfield *et al*., 2004)

This site is highly conserved across vertebrate species. The perfect complementarity between *miR-196a* and the *Hoxb8* 3' UTR suggests that *miR-196a* 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).

2.4.5 *miRNA 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. *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. Stimulation of cell proliferation, anchorage-independent growth and suppression of apoptosis by *miR-196a* observed (Luthra *et al*., 2008).

2.4.6 ANTIMETASTATIC ROLE OF miRNA *196a2* GENE

Although *miR-196a* may target multiple genes potentially pertinent to cell migration, the antimetastatic role of *miR-196* is apparently mainly associated with *HOXC8* because *miR-196a* significantly inhibits *HOXC8* expression and ectopic expression of *HOXC8* transgene reverses *miR-196*–caused inhibition in cell migration, *in vitro* invasion, and metastasis. Members of the *miR-196* family could suppress breast cancer cell migration and metastasis by inhibiting *HOXC8* expression. The ratio of *miR-196s* to *HOXC8* mRNA, rather than *miR-196* levels, correlates with breast cancer metastasis (Li *et al*., 2010).

2.4.7 TARGETS OF *miRNA 196a2* GENE

The details of various experimentally verified target genes of *miR-196* are shown in table 2.1 given below.

Table 2.1 Experimentally verified target genes of *miR-196*

Gene	Gene name	Function
HOXB8	Homeobox protein Hox –B8	Transcription factor
HOXC8	Homeobox protein Hox-C8	Transcription factor
HOXD8	Homeobox protein Hox-D8	Transcription factor

HOXA7	Homeobox protein Hox-A7	Transcription factor
HOXB7	Homeobox protein Hox-B7	Transcription factor
HMGA2	HMGA	Nuclear architectural factor
ANXA1	Annexin A1	Apoptosis
S100A	S100 calcium binding protein A9	Cross linker of epidermal differentiation complexes
KRTS	Keratin 5	Structural cytokeratin protein

2.4.8 rs 11614913

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 However, no main effects were found between the other three SNPs and lung cancer risk.

Zhibin *et al*., (2008) evaluated in detail the association of 4 SNPs (rs2910164, rs2292832 and rs3746444 andrs11614913) with the survival of individuals with non–small cell lung cancer (NSCLC). rs11614913 SNP in hsa-*mir-196a2* was found to be associated with survival in individuals with NSCLC. Specifically, survival was significantly decreased in individuals who were homozygous CC at SNP rs11614913.

There appeared to be an association between a significantly increased risk of NSCLC and variant genotypes of the *miR-196a2* rs11614913 CT/CC genotypes in a study conducted on Korean population. Therefore, *miR-196a2* rs11614913 may influence role regulation processes in Korean NSCLC patients. However, no association was observed between the homozygous *miR-196a2* rs11614913 CC polymorphism and the risk of NSCLC lung cancer patients (Hong *et al*., 2011).

The variant homozygous genotype of *miR-196a-2* is significantly associated with an increased gastric cancer risk, and lymph node metastasis of gastric cancer in the Chinese population. The results suggest that the genetic variant of *miR-196a-2* could play an important role in the evolution of gastric cancer (Peng *et al*., 2009).

A sequence variant in hsa-*miR-196a-2* (rs11614913, C→T) was significantly associated with decreased breast cancer risk (for homozygous variant). Hypermethylation of a CpG island upstream (700 bp) of the *miR-196a2* precursor was also associated with reduced breast cancer risk (Hoffman *et al*., 2009).

AIMS AND OBJECTIVES

The present piece of work is an attempt to study the following aspects that might be associated towards the associative risk for lung cancer.

1. The epidemiological factors associated with lung cancer in North Indian population
2. To study the genotypic frequencies of the microRNA genes like *miR-196a2* gene and *146a* gene in lung cancer cases and controls
3. To find a correlation between genetic polymorphisms of *miR-196a2* and *146a* gene towards the risk for lung cancer and also the clinico-pathological features associated with it
4. To find out the combined association of the *miR-196a2* and in *miR-146a* polymorphisms towards lung cancer susceptibility using statistical analysis

CHAPTER 3

METHODOLOGY

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

3.1 SUBJECTS

A case control study of 85 histopathologically confirmed lung cancer patients were recruited from the Post Graduate Institute of Medical Research, Chandigarh (PGIMER) and 90 cancer free controls from North Indian population were obtained. The current study was ethically approved by the Institute ethics committee of PGIMER, Chandigarh. Written informed consent was obtained from all participants or from patients' representatives if direct consent could not be obtained. Each patient donated 5 ml venous blood upon admission to the hospital and was interviewed to collect demographic data and clinical information.

3.2 ISOLATION OF DNA FROM PERIPHERAL BLOOD

REQUIREMENTS –

- Washing buffer
- Lysis buffer
- Phenol:Chloroform:Isoamylalcohol (25:24:1)
- Chloroform:Isoamylalcohol (24:1)
- Isopropanol
- TE buffer

PROCEDURE

Preparation of Buffers

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

Table 3.1: 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 3.2: 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-K	100 µg/ml Proteinase –K

Isolation of DNA

- 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 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 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 the pellet of DNA 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, 2003).

3.3 DNA QUANTIFICATION

The most comprehensive way to evaluate DNA concentration and purity is to use UV spectrophotometeric measurements. DNA absorbs light most strongly at 260nm so the absorbance value at this wavelength (called A_{260}) can be used to estimate the DNA concentration. Since tyrosine and tryptophan residues absorb strongly at 280nm, the absorbance at this wavelength is used as an indicator of protein contamination. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA.

PROCEDURE

- The spectrophotometer was set at double wavelength 260 nm and 280nm.
- DNA samples were diluted 300 times with TE buffer.
- Quartz Cuvettes were washed with 70% ethanol.
- Spectophotometer was set blank by using TE buffer and then O.D for various samples was noted.
- Concentration and purity of DNA samples were calculated as follows:

DNA concentration (µg/ml) = O.D at 260nm X 50 X Dilution factor

Where 50µg/ml of DNA is equal to 1 O.D

Purity of DNA = O.D at 260nm/O.D at 280nm

NOTE: A ratio of ~1.8 indicates "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants.

3.4 RESOLUTION OF DNA FRAGMENTS ON AGAROSE GELS

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

PROCEDURE

Preparing 5X TBE (1000ml)

Tris base - 54 g
Boric Acid - 27.5g
EDTA (0.5M) - 20ml
Make up final volume with water.

Preparing 6X Loading Dye (20ml)

0.25% Bromophenol blue - 0.05gm
0.25% Xylene Cyanol - 0.05gm
40% Sucrose - 8gm
Make up final volume with TE buffer

Preparing the 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 agarose was cooled to 55°C in a water bath before pouring onto the gel platform. to prevent warping of the gel apparatus.
- 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 to cover the gel until the tops of the wells are submerged. 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.

- Turned off the electric power, disconnected the leads, and discarded the electrophoresis buffer from the reservoirs
- DNA was visualized by placing the gel on a UV transilluminator and then photographed.

3.5 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION OF *MIRNA*

196a2 and 146a gene

Polymerase Chain Reaction (PCR) 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. PCR can also be utilized for rapid screening and/or sequencing of inserts directly from aliquots of individual phage plaques or bacterial colonies.

REQUIREMENTS

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

Note: Primer sequences need to be chosen to uniquely select for a region of DNA, avoiding the possibility of mishybridization to a similar sequence nearby. 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' products contaminating the mixture. Primers should also not anneal strongly to themselves, as internal hairpins and loops could hinder the annealing with the template DNA. Given below are the primers used.

Table 3.3: Primers used for *miRNA 196a2* and *146a gene*				
GENE	**BASE CHANGE**	**GENOTYPING ASSAY**	**PRIMERS**	**PCR PRODUCT**
rs2910164	G→C	PCR-RFLP mismatch, sense primer -3C→G[A]	CATGGGTTGTGTCAGTGTCAGA GCT (sense); TGCCTTCTGTCTCCAGTCTTCCA A (antisense	147bp
rs11614913	C→T	PCR-RFLP mismatch, antisense primer +2T→G[B]	CCCCTTCCCTTCTCCTCCAGATA (sense); CGAAAACCGACTGATGTAACTC CG (antisense)	149bp

Underlining indicates mismatched site.[A]-3,3bp upstream to polymorphic site.[B]+2, 2bp downstream from polymorphic site (Source: Hu *et al*., 2008)

PCR was performed in 25µl of reaction mixture containing the following:

Table 3.4: Reaction mixture for PCR			
REAGENT	**STOCK CONCENTRATION**	**FINAL REACTION CONCENTRATION**	**QUANTITY USED**
Additive 1 BSA	1000 µg/ml	100 µg/ml	2.5 µl
PCR Buffer (Mg Conc.)	10 X 15mM	1 X 1.5mM total	1.5µl
Primer (Forward)	10µM	0.5µM	1.25µl
Primer (Reverse)	10µM	0.5µM	1.25µl
Taq Polymerase	5.0U/µl	0.8U	0.3µl
dNTP	10mM each	0.2mM each	0.5µl
PCR Grade Water			16.7µl
DNA Template	100ng/µl	400ng	1µl

The thermal cycling parameters were set as follows:

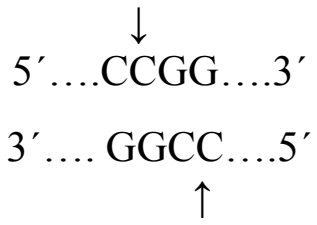
Table 3.5: Cycling profile of PCR		
STEPS	**TEMPERATURE(°C)**	**TIME**
Initial Denaturation	95°C	5min
Denaturation	94°C	30sec
Annealing	65°C for *miRNA 196a2* gene 63°C for *miRNA 146a* gene	30sec 30sec
Polymerization	72°C	45sec
Final Extension	72°C	5min

The reaction was carried out for 30 cycles

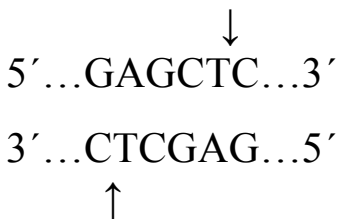
3.6 RESTRICTION DIGESTION OF *miRNA 196a2* GENE AND *146a* GENE

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; furthermore, each fragment that contains the desired sequence has the sequence located at exactly the same position within the fragment. The enzymes used by us were:

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



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



PROCEDURE

196a2 gene digestion

The total reaction mixture of 20µl consisted of 2.4 µl 10X NEB 4 buffer, 0.2µl (2U) of 10U/ml *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 (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/ml *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 (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.

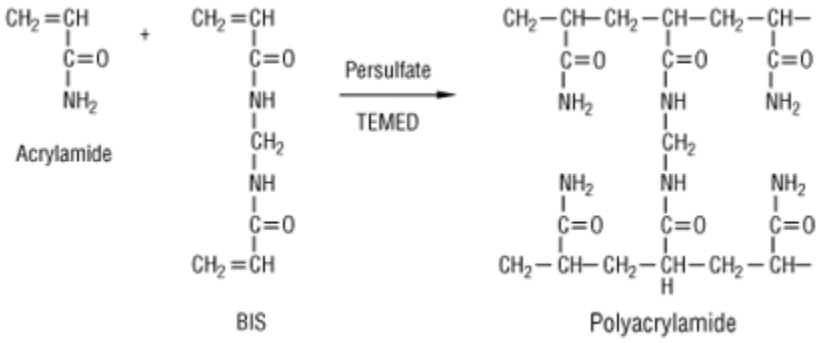


Fig 3.7: Polymerization of Polyacrylamide gels

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 3.7: Volume of reagents used to cast polyacrylamide gels					
Gel %	**30% Acrylamide solution (29:1)**	**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).

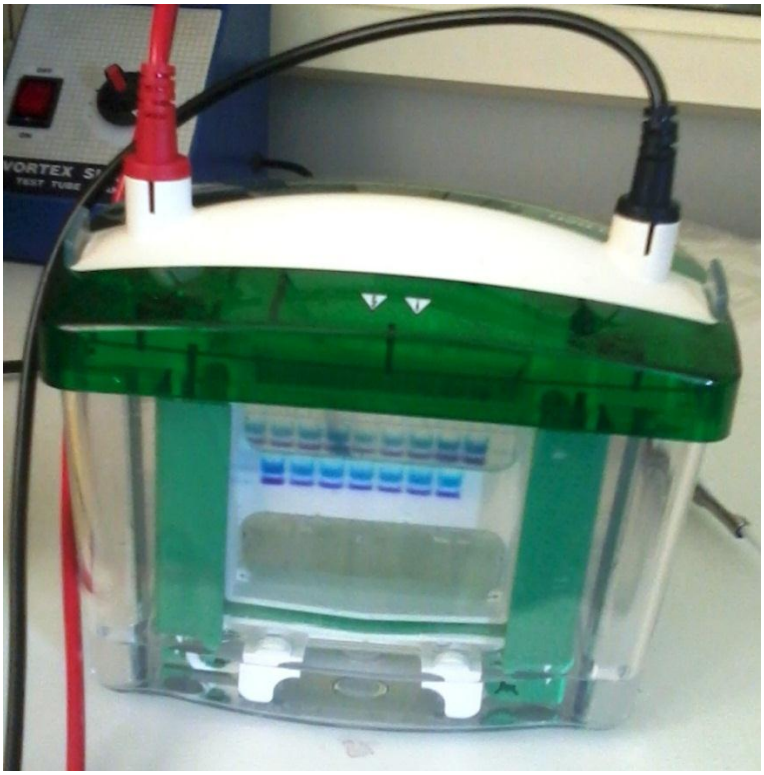


Figure 3.1: PAGE apparatus

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 3gm 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:

Crude Odds Ratio (OR) at 95% confidence interval was also calculated. All *p* values which were less than 0.05 were taken to be significant. The differences in the distribution between cases and controls were tested using the χ^2 and Hardy Weinberg wherever possible. The crude odd ratios (ORs) were calculated by Wolf's method, The Odds ratios (ORs) with 95% confidence interval (CI) calculated were computed to estimate the association between certain genotypes or tobacco smoking and disease. Smokers were considered current smokers if they smoked up to one year before the date of diagnosis for cancer or up to the date of interview for controls. Information was collected on the number of cigarettes smoked per-day, the age at which the subject started smoking and the age at which the subject stopped smoking if the person was an ex-smoker (Hong *et al*., 2011 and Tian *et al*., 2009). Various parameters were calculated using software "Med Calc version 2.0".

CHAPTER 4

RESULTS AND DISCUSSIONS

- Genotyping
- Epidemiology
- Relationship of lung cancer risk with *miRNA 146a* genotypes
- Genotypic distribution of *miRNA 146a* gene among patients with different histological types of lung cancers:
- Distribution of genotypes of *miRNA 146a* gene among smokers and non-smokers
- Distribution of genotypes of *miRNA 146a* gene among smokers and non-smokers
- SNP of *miRNA 196a2* gene and lung cancer risk
- Lung cancer risk with genotypes of *miRNA 196a2* gene.
- Distribution of genotypes of *miRNA 196a2* gene among patients with different types of lung cancers
- Distribution of genotypes of *miRNA 196a2* gene among smokers and non- smokers
- Distribution of genotypes of *miRNA 196a2* gene on basis of sex
- Distribution of genotypes of *mirna 196a2* gene among patients at different stages of lung cancer
- Effects of Common Polymorphisms rs11614913 in *miR- 196a2* and rs2910164 in miR-*146*a on Lung Cancer Susceptibility

4.1 GENOTYPING

DNA was isolated from blood (as discussed in section 3.2) using a simple and efficient procedure and the samples were run on 0.7% gel. This total DNA from blood samples were used as template in PCR.

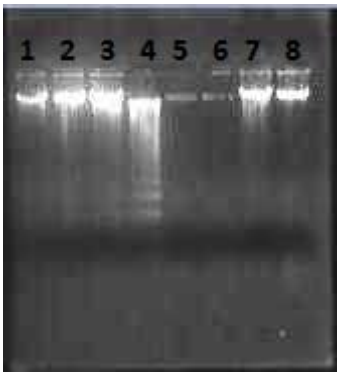


Fig 4.1: Genomic DNA isolated from peripheral blood

In order to amplify *miRNA 146a* and *196a2* gene suitable sets of primers were used as shown in table. The temperature cycling parameters as employed during PCR are given in section 4.6. The PCR amplified products were separated on 1.7% agarose gel containing ethidium bromide. The DNA bands were clearly visible and distinct which indicated that the primer combinations worked well for both the genes. Fig 4.2 shows the PCR amplified DNA products obtained using set of primer pair specific for *146 a* gene. DNA bands of 149bp were obtained.

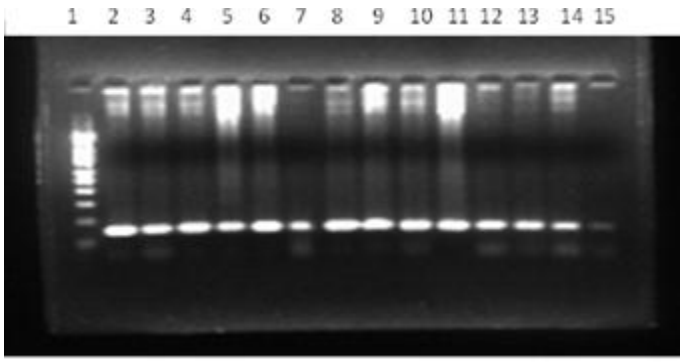


Figure 4.2: PCR amplified DNA products of *miRNA 146a* gene

Lane 1: 100bp ladder, Lane 2-15: Amplified PCR product (*147*bp)

The PCR amplified DNA products obtained using set of primer pair specific for *196a2* gene are shown in figure 4.3.

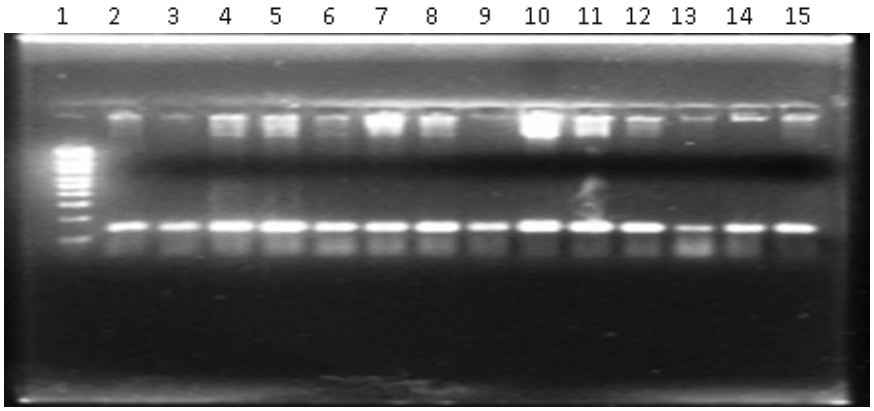


Figure 4.3: PCR amplified DNA products of *miRNA 196a* gene

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 and after silver staining of the gels, genotypic analysis was done as shown in figure 4.4

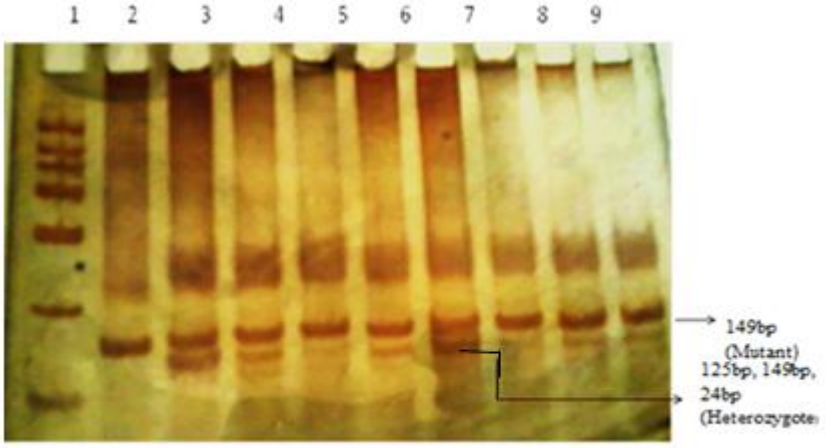


Figure 4.4: PCR RFLP genotype analysis of *miRNA 196a2* gene among cancer patients.

Lane1: 100bp ladder, Lane 2: Uncut Control, Lane 3, 4, 6, 7: Heterozygous (CT) genotype (149, 125 and 24bp bands) Lane 5, 8, 9: Homozygous, uncut mutant (TT) genotype (149bp band)

The genotypic analysis of *microRNA196a2* gen among controls was done as shown and explained in figure 4.5.

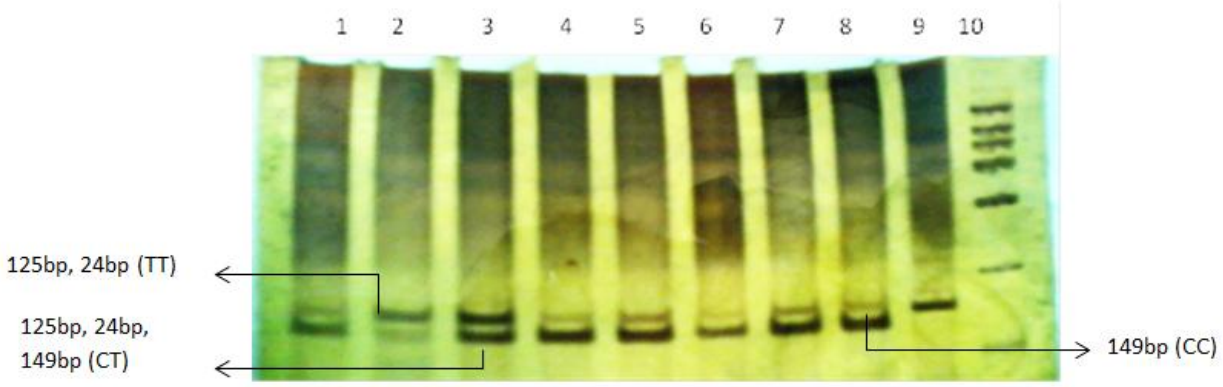


Figure 4.5: PCR RFLP genotypic analysis of *miRNA 196a2* gene among controls.

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 lane 2: uncut mutant TT genotype (125 and 24bp)

The genotypic analysis of *microRNA196a2* gene among controls was done as shown and explained in figure 4.5

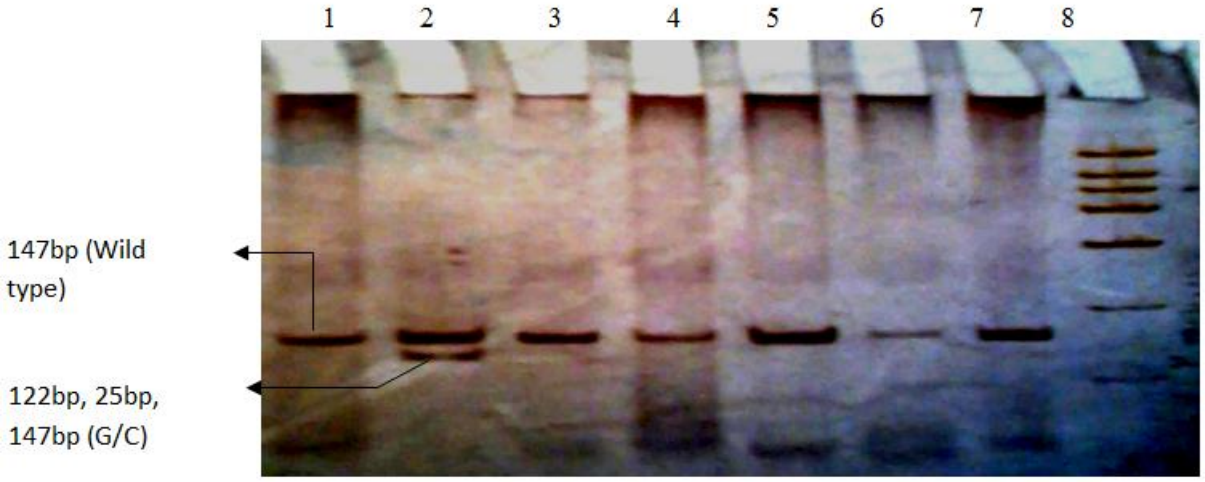


Figure 4.6: PCR RFLP genotypic analysis of *miRNA 146a* gene among controls

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

The genotypic analysis of microRNA196a2 gen among controls was done as shown and explained in figure 4.5

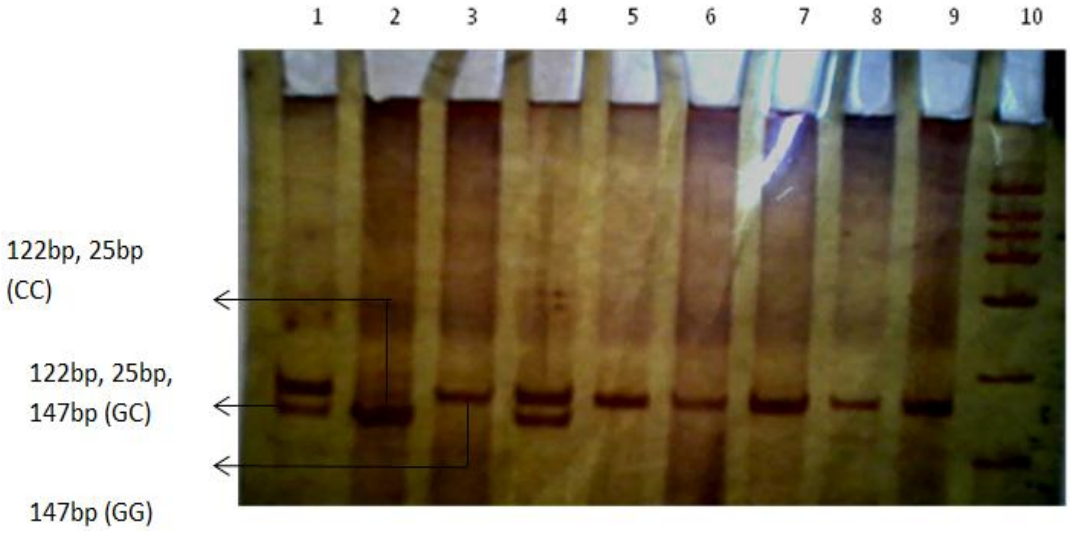


Figure 4.6: PCR RFLP genotype analysis of *miRNA 146a* gene among cancer patients

Lane1, 4: Heterozygous (GC) genotype (122bp, 25bp and 147bp bands), Lane 2: Cut, mutant (CC) genotype (122bp, 25bp). Lane 5, 6, 7, 8, 3: Homozygous (GG) wild genotype (147bp), Lane 9: Uncut control, Lane 10 100bp ladder

4.2 EPIDEMIOLOGY

The case control study pertains to 82 lung cancer patients and 81 controls. Average age was (57.18±11.22) among the cases and (43.16 ± 8.44) among the controls. 73% of the cases were more than 50 years of age and just 20% of the cases were of 50 or below, while in controls 30% of the population was above 50 years of age and 51% were below.

Of the various risk factors concerned with the risk of lung cancer development, smoking is the primary cause. In present study, about 82% of the cases were smokers which were divided into two groups i.e. heavy and light smokers on basis of their smoking index. Those who had smoking Index greater than or equal to 400 were considered to be heavy smokers and those having smoking index less than 400 were considered to be light smokers. The relevant characteristics of subjects studied are shown in Table 4.2.

Table 4.2 Distribution of demographic variables for patients and controls		
CHARACTERSTIC	**CASES (n %)**	**CONTROLS (n%)**
GENDER		
MALE	72 (87.8)	75 (92.5)
FEMALE	10 (12.1)	1 (1.23)
UNKNOWN		5 (6)
AGE		
<50	60 (73.1)	25 (30.8)
≥50	20 (24.3)	51 (62.9)
Unknown	2	5 (6.1)
RANGE	35-80	30-54
SMOKING		
NO	14 (17.03)	41 (50.6)
YES	67 (81.7)	36 (44.4)
Unknown	1	4 (4.9)
SMOKING INDEX		
≤400	47 (57.3)	
> 400	26 (31.7)	
UNKNOWN	9	
HISTOLOGY		
SQCC	36 (43.9)	
ADCC	14 (17.07)	
SCLC	11 (10.9)	
UNKNOWN*	21	
TNM STAGING		
T_1NM	1 (1.21)	
T_2NM	17 (20.7)	
T_3NM	19 (23.1)	
T_4NM	22 (26.8)	
Unknown*	23	

*Data not known

The frequencies of males in cases and controls were 87.8% and 92.5% respectively while those of females were 12.1% and 1.23% respectively thus indicating more or less similarity in case of sex among patients and cases. As per histology 43.9% of cases were found to have SQCC,

17.07% cases were those of ADCC while 11% had SCLC. On further stratification of patients according to TNM staging, 26.8% of the patients were diagnosed to be at stage 4 while 23% and 20% were found to be at stage 3 and 2 respectively.

4.3 RELATIONSHIP OF LUNG CANCER RISK WITH miRNA *146a* GENOTYPES

The genotypes of patients and controls for *146a2* gene were obtained by PCR RFLP. Out of total patients (82) studied 65.9% of the individuals were found to have homozygous wild type (GG) genotypes, 28% had heterozygous genotypes (GC) and 6% individuals had mutant genotype (CC). On the other hand in case of controls 75.3% of the individuals had homozygous wild type genotypes (GG), 24.7% of the individuals had heterozygous genotypes (GC). In the present study not a single mutant genotype was observed. The genotype and allele frequencies of the *mir-146a* (G>C, rs2910164*)* SNP and their associations with risk of lung cancer are summarized in Table 4.3

Table4.3: Frequency distribution of miRNA genotypes and their association with risk towards Lung Cancer

GENOTYPES	**NUMBER (%)**		**O.R (95%C.I)**	***p* value**
miRNA 146a	**CASES**	**CONTROLS**		
Total	82 (100)	81 (100)		
GG	54 (65.9)	61 (75.3)		
GC	23 (28)	20 (24.7)	1.13	0.12
CC	5 (6.1)	0	5.6	0.08
CC/GC	28 (34.1)	20	1.5	0.23
C allele	0.21	0.12		
G allele	0.79	0.88		

The genotypic distribution of SNP in *146a* gene was in agreement with that of the Hardy-Weinberg equilibrium ($\chi 2 = 5.62$, df= 2, p=0.05 for *mir-146a*). However, compared with the *mir-146* GC+CC genotype (crude OR=1.5 95%CI; 0.76-2.95, p=0.232) the mutant CC were associated with a statistically significant, 5.6 fold increased risk (OR 5.6, 95% C.I; 0.640–49.8, p=0.08 respectively).

Several case-control studies have investigated the association between *miR-146a* rs2910164 polymorphism and risk of various cancers including non-small cell lung cancer (*Hu et al., 2008*), SQCC of head and neck (Liu *et al*., 2010) and bladder cancer (Mittal *et al*., 2011). Nonetheless, it should be noted that some molecular epidemiological studies have suggested that G allele or GG genotype of *miR-146a* polymorphism is associated with increased risk of hepatocellular carcinoma (Xu *et al.*, 2008), prostate cancer (Xu *et al.*, 2010), esophageal cell carcinoma (Guo *et al.*, 2010), gastric cancer (Zeng *et al.*, 2010), and cervical squamous cell carcinoma (Zhou *et al.*, 2011). In contrast with these studies, some authors have reported opposite findings. For instance, C allele or CC genotype of *miR-146a* polymorphism is associated with increased risk of earlier age of onset of familial breast and ovarian cancers (Shen *et al.*, 2008) and gastric cancer (Okubo *et al.*, 2010). Our results are in line with these findings showing that there is significant association between CC genotype (*miR-146a* rs2910164 polymorphism) and risk for Lung cancer. Interestingly, Jazdzewski *et al.*, (2008) reported marked differences in genotype distribution of *miR-146a* rs2910164 polymorphism, the GC heterozygous genotype being associated with an increased risk of papillary thyroid carcinoma but both homozygous genotypes protective. It is possible that the significant difference in the results of studies may be due to differences in the studied population, as well as on several environmental and other factors that influence that population. Geographic or ethnic differences have been reported regarding the genotype frequency of several polymorphisms.

4.4 GENOTYPIC DISTRIBUTION OF *miRNA 146a* GENE AMONG PATIENTS WITH DIFFERENT HISTOLOGICAL TYPES OF LUNG CANCERS:

Among the cases studied 43.9% (36) of the cases were of those who were diagnosed with SQCC, 13.4% (11) had SCLC and 17.0% (14) were diagnosed with ADCC. There is less evidence for SCLC as compared to other two types of lung cancers. On further stratification on basis of genotypes it was found that 61%, 78.5% and 54.5% individuals of SQCC, ADCC and SCLC respectively had homozygous genotypes. On the other hand 28%, 21% and 36% respectively had heterozygote genotypes. No mutants were observed in case of adenocarcinoma while 5% of SQCC patients and 9% of the SCLC patients were mutants as shown in Table 4.4

The mutant genotype was found to be significantly associated with SQCC (OR=11.1, 95%C.I; 1.17-104.6, ***p*=0.014**). Thus individuals with mutant TT genotype are at 11 fold elevated risk of

Table 4.4: Frequency distribution *miRNA146a* genotypes among different histological types of Lung Cancers

	G/G	**G/C**	**C/C**	**O.R(95% C.I)**	***p* value**
CONTROL (81)	61	20	0		
CASES (82)	54	23	5		
SQCC (36)	22 (61%)	10 (28%)	4 (5%)	11.09	**0.014**
ADCC (14)	11 (78.5%)	3 (21%)	0	1.78	0.67
SCLC (11)	6 (54.5%)	4 (36%)	1 (9%)	10.1	0.058
UNKNOWN*	15	6	0		

*Data not known

developing SQCC (*p*=0.01), which was statistically significant Similarly significant association was also found between mutant genotype and SCLC and individuals who were carrying both mutant alleles were at 10 fold elevated risk of developing SCLC (OR=10.1, 95%C.I; 0.56-14.0, *p*=0.058) while no significant association was found for combined mutant (CC) and heterozygous (GC) genotype and risk of developing SCLC. On the other hand no significant association was found for combined (CC/GC) mutant and heterozygote genotype (OR=1.8, 95%CI; 0.08-4.25, ***p*=0.14**). In case of ADCC no association was observed in case of mutants (CC) as well combined mutant and heterozygote genotypes.

4.5 DISTRIBUTION OF GENOTYPES OF *miRNA 146a* GENE AMONG SMOKERS AND NON SMOKERS

Individuals under study were classified as smokers and non-smokers to find out association between smoking and risk of developing lung cancer. 82% of the cases studied were smokers while 18% were non-smokers. On the other hand 36% of controls were smokers and 41% were non-smokers. Smokers and Non Smokers with lung cancer were further stratified according to histology and it was found that 26% and 33% of non-smokers had ADCC and SQCC

respectively. Table 4.5 shows genotypic distribution of *miRNA 146a* gene on basis of smoking status.

Table 4.5: Frequency distribution of *miRNA 146a* genotypes among smokers and non-smokers

		N	**G/G**	**G/C**	**C/C**	**OR (95%C.I)**	***p* value**
Cases Controls		82 81	54 61	23 20	5 0		
Cases Controls	NON-SMOKERS ADCC SQCC Unknown*	15 (18%) 4 (27%) 5 (33%) 6 41 (36%)	9 (60%) 3 (33%) 2 (22%) 4 30 (73%)	4 (27%) 1 (25%) 1 (25%) 2 11(27%)	2 (13%) 2 (100%)	16.05	0.016
Cases Controls	SMOKERS ADCC SQCC SCLC Unknown*	68 (82%) 10 (15%) 31 (45%) 11 (16%) 16 36 (41%)	45 (55%) 8 (18%) 19 (42%) 6 (13%) 12 27 (75%)	19 (23%) 2 (11%) 10 (53%) 4 (21%) 3 9 (25%)	4 2 1 1	5.4 7.05 12.6	0.127 0.104 0.04
Cases Controls	Unknown*	**0** **4**	**0** **4**	**0** **0**			

*Data not known

Among 15 nonsmokers 9 (60%) had homozygous wild type GG genotype (33.3% ADCC and 22% SQCC) while 4 (26.6%) had heterozygous GC genotype (25% ADCC and 25% SQCC) and 13% (all SQCC) had mutant TT genotypes. In case of controls 30 (73%) were homozygous wild type individuals and 11 (27%) had heterozygous genotype and mutants were not observed.

Out of 68 smokers 45 (54.8%) had homozygous wild type genotype (18% ADCC and 42% SQCC and16% SCLC) while 19 (23%) had heterozygous genotype (10.5% ADCC and 53% SQCC and 21.0% mutants) and 23.17% had mutant genotypes (10.5% ADCC, 52.6% 52.6% SQCC and 21.05% SCLC). In case of controls 30 (73.17%) were homozygous wild type individuals and 11(26.8%) had heterozygous genotype and mutants were not observed

Table 4.5 shows that the Smokers were not found to be significantly associated either with mutant genotype (OR=1.26, 95% C.I; 0.5-3.1, *p*=0.61) or with combined mutant and heterozygote genotype (OR=1.53, 95%C.I; 0.6-3.7, *p*=0.35) but strong association was found

between mutant genotype and SCLC smokers (OR=12.7, 95%C.I; 0.46-348.55**, *p*=0.04**) as compared to smokers who were diagnosed with ADCC or SQCC. Thus we can conclude that smokers who were carrying the CC genotype were at 12 fold elevated risk of developing SCLC cancer which was statistically significant. Similarly significant association was found between non-smokers (OR=16.05, 95%C.I; 0.70-364.4, ***p*=0.016**) and mutant genotype indicating elevated risk of lung cancer among non-smokers, this might be as a result of changing environmental conditions. Our results are in line with the results of study conducted on head and neck cancer in Chinese population where risk genotypes were more evident in never smokers than forever smokers. However on the contrary a study conducted on esophagal cancer in Chinese population showed that smoking was significant risk factor towards esophageal cancer risk for those individuals who had the mutant genotype (Guo *et al.,* 2010*)*

According to one study strong evidence from multiple sources supports the causal association of SHS (second hand smoking) exposure in lung cancer in never smokers. Similarly, exposure to radon, common in indoor environments, is a well-established cause of lung cancer in never smokers. In the United States, these two factors may account for the majority of cases of lung cancer in never smokers. Indoor air pollution, including combustion of coal or solid fuels for cooking or heating in poorly ventilated spaces, has been clearly associated with increased risk of lung cancer in never smokers, and may be a particularly important factor contributing to the high incidence of lung cancer in never smokers in the East Asia. In addition to radon, other exogenous ionizing radiation exposures have been clearly linked to lung cancer risk. Additional exposures associated with lung cancer in never smokers in multiple studies include asbestos, which has known carcinogenic synergy with tobacco smoke, arsenic, and silica (Samet *et al.,* 2009*).*

4.6 DISTRIBUTION OF GENOTYPES OF *miRNA 146a* GENE AMONG PATIENTS AT DIFFERENT STAGES OF LUNG CANCER

Genotypes of *miRNA 146a* gene were distributed among patients at different stages of lung cancer. Table 4.6 shows frequency distribution of *miRNA 146a* genotypes.

Table 4.6: Frequency distribution of *miRNA 146a* genotypes among patients at different stages of lung cancer						
	N (%)	**G/G**	**G/C**	**C/C**	**OR(95%CI)**	***p* value**
CONTROL (86)	81	61	20	0		
CASES (79)	82	54	23	5		
STAGES						
1	1(1.2)		1			
2	4 (4.8)	2 (50%)	2 (50%)			
3	18 (21.9)	10 (55.5%)	7(38.8%)	1(5.5%)		
4	17 (20.7)	10 (58.8%)	7 (41.1%)		6.1	0.161
UNKNOWN*	41					

*Data not known

As shown in table 4.6 20.7% of the patients were at the fourth stage (OR=6.1, 95%C.I; 0.3-105.5, *p*= 0.161), 22% were at third stage, 5% and 1% were at second and first stage of lung cancer. Patients diagnosed in second stage were found to have equal frequency of wild type GG and heterozygote genotypes (GC). Among third stage patients 55% had wild type homozygote genotypes, 39% were heterozygotes and in case of patients at the fourth stage, frequency of heterozygote individuals (59%) predominated while wild type genotype was found in 41% of the patients.

4.7 SNP OF *miRNA 196a2* GENE AND LUNG CANCER RISK

The baseline characteristics of the 76 Lung cancer patients and 81 control subjects included in the analysis are summarized in Table 4.6.

Table 4.7: Distribution of demographic variables for patients and controls		
CHARACTERSTIC (*196)*	**CASES (n%)**	**CONTROLS (n%)**
GENDER		
MALE	68 (86)	72 (83.7)
FEMALE	11 (13.9)	1 (1.16)
UNKNOWN		13 (15.11)
AGE		
<50	60 (73.1)	53 (61.6)
≥50	20 (24.3)	20 (23.2)
UNKNOWN*	2	13 (15.11)

RANGE	35-80	30-54
SMOKING		
NO	15 (19)	37 (43.02)
YES	64 (81)	36 (41.8)
UNKNOWN*		13 (15.11)
SMOKING INDEX≤400	48 (30.3)	
SMOKING INDEX > 400	24 (30.3))	
SMOKING INDEX UNKNOWN*	7 (8.86)	
HISTOLOGICAL TYPE		
SQCC	33 (41.77)	
AC	16 (20.2)	
SCLC	8 (10.12)	
OTHERS	3 (3.79)	
UNKNOWN*	19 (24.05)	
TNM staging		
T_1NM	1 (1.2)	
T_2NM	5 (6.3)	
T_3NM	18 (22.7)	
T_4NM	25 (31.6)	
UNKNOWN*	30	

*Data not known

Average age was (57.04±9.72) among the cases and (43.16 ± 8.44) among the controls 73% of the cases were more than 50 years of age and just 24% of the cases were of 50 or below, while in controls 53% of the population was above 50 years of age and 20% were below. 50.81% of the patients were smokers and only 18% were non-smokers. 48% of the smokers were classified as heavy smokers as their smoking index was more than 400 while 24 % were light smokers. On the other hand among controls frequency of smokers and non-smokers was more or less same. When stratified according to histology 41.8% (33) of the cases were found to be of those who were diagnosed with SQCC, 12.6% (10) had SCLC and 20.2% (16) had ADCC. On further stratification of patients according to TNM staging, 31% of the patients were diagnosed to be at stage 4 while 22% and 6% were found to be at stage 3 and 2 respectively.

4.8 LUNG CANCER RISK WITH GENOTYPES OF *miRNA 196a2* GENE

The genotypes of patients and controls for *196a2* gene were obtained by PCR RFLP. Out of total patients (79) studied 65.9% of the individuals were found to have homozygous wild type genotypes, 28% had heterozygous genotypes and 6% individuals had mutant genotype. On the other hand in case of controls 75.3% of the individuals had homozygous wild type CC genotypes, 24.7% of the individuals had heterozygous CT genotypes and 3% had mutant TT genotype. Table 4.8 shows the genotype and allele frequencies of the *mir-196a2* (C>T, rs11614913 SNPs and their associations with risk of lung cancer are summarized in Table 4.8

Table 4.8: Frequency distribution of *miRNA 196a2* genotypes and associated risk

GENOTYPES	**NUMBER (%)**		**OR (95%C.I)**	***p* value**
miRNA 196a2	**CASES**	**CONTROLS**		
Total	79 (100)	86 (100)		
CC	38 (48)	54 (63)		
CT	33 (42)	29 (34)		
TT	8 (10)	3 (3.5)	3.78	**0.047**
CT/TT	41 (52)	32 (37)	1.82	0.057
C allele	0.689	0.796		
T allele	0.310	0.203		

The genotypic distribution SNP of *196a2* gene among controls was in agreement with that of the Hardy- Weinberg equilibrium (p=0.08 for *mir-196a2*, χ^2=5.62, df=2) and there was no overall difference in the genotype distributions between cases and controls. However, both *mir-196* CT+CC genotype and the variant TT were associated with a statistically significant, increased risk for developing cancer(OR=1.821 95% CI; 0.978-3.38 p=0.05) and (OR=3.7, 95%CI; 0.94–15.2, p=0.04), respectively There is 4-fold risk for lung cancer among mutants with TT genotype.

Our results are opposite to other studies done on different populations For e.g. In Korean population, carriers with combined TC/CC genotype of *miR-196a2* had higher risks for non-

small cell lung cancer (NSCLC) comparing with TT carriers (*Hong et al*.,2011). In another study on Chinese women, it was observed that CC or CC/CT genotypes have significantly increased breast cancer risks as compared to TT variants (Hu *et al*., 2009). Similar results were also found in glioma, (Dou *et al*., 2010) prostatic cancer (George *et al*., 2011) and other kinds of cancers.

4.9 DISTRIBUTION OF GENOTYPES OF *miRNA 196a2* GENE AMONG PATIENTS WITH DIFFERENT TYPES OF LUNG CANCERS

Among the cases studied 42% (33) of the cases were of those who were diagnosed with SQCC, 12.6% (10) had SCLC and 20% (16) had ADCC. There is less evidence for SCLC as compared to other two types of lung cancers. On further stratification on basis of genotypes it was found that frequency of C/C genotype was highly associated with SQCC (47%) as compared to SCLC and ADCC (18% and 8% respectively). On the other hand frequency of heterozygote genotypes was high in SQCC and SCLC (33%, 25% respectively) as compared to ADCC (18%). No mutants were observed in case of small cell carcinoma while 50% of SQCC patients 25% of the ADCC patients were mutants.

The mutant genotype was found to be significantly associated with squamous cell carcinoma (O.R=4, 95%C.I; 0.81-19.6, p=0.07). Thus we can conclude that there is 4 fold elevated risk of SCLC among individuals with TT genotype of *miRNA 196a2* gene. On the other hand no significant association was found for combined (CC/GC) mutant and heterozygote genotype (OR=1.4.06, 95%C.I; 0.62-3.17, p=0.41). Similarly significant association was found between mutant genotype and ADCC (O.R:5.14, 95%C.I; 0.72-36.3, p=0.074) thus indicating 5 fold increased risk of ADCC among individuals with TT genotype, while no significant association was found for combined mutant and heterozygous genotype and risk of developing SCLC. In case of SCLC no association was observed in case of mutants as well combined mutant and heterozygote genotypes. Table 4.9 shows frequency distribution of *miRNA196a2* genotypes among different histological types of lung cancers and risk associated.

Table 4.9: Frequency distribution *miRNA196a2* genotypes among different histological types of lung cancers						
	N	**C/C**	**C/T**	**T/T**	**OR(95% CI)**	***p* value**
CONTROL	86	54	29	3		
CASES	79	38	33	8		
SQCC	33	18 (47%)	11 (33%)	4 (50%)	4.0	0.070
ADCC	16	7 (18%)	6 (18%)	2 (25%)	5.14	0.073
SCLC	10	3 (7.8%)	7 (21%)	0	2.24	0.68
UNKNOWN*	20	7	11	2		

*Data not known

4.10 DISTRIBUTION OF GENOTYPES OF *miRNA 196a2* GENE AMONG SMOKERS AND NON SMOKERS

Table 4.10 shows the effect of smoking and lung cancer risk in relation with the genotypes of *196a2*. As observed, 81% of the cases studied were smokers while 18% were non-smokers. On the other hand 43% of controls were smokers and 41% were non-smokers. Smokers and Non Smokers with lung cancer were further stratified according to histology. 28.5% of non-smokers had adenocarcinoma and SQCC respectively.

Among 14 nonsmokers 7 (50%) had homozygous wild type genotype (both ADCC and SQCC 28.5%) while 7 (50%) had heterozygous genotype (both ADCC and SQCC 28.5%) and 8 (12.5%) had mutant genotype(25% ADCC and 50% SQCC). In case of controls 37 (43.02%) were homozygous wild type individuals and 13 (35.1%) had heterozygous genotype and 1 (2.7%) mutants were observed.

Table 4.10: Frequency distribution of *miRNA 196a2* genotypes among smokers and non-smokers							
		N	**G/G**	**G/C**	**C/C**	**O.R(95%C.I)**	**P value**
Cases Controls		79 86	34 54	35 29	8 3		
Cases Controls	NON-SMOKERS Adenocarcinoma SQCC SCLC	14(17.7%) 4(28.5%) 4(28.5%) 37(43%)	7(50%) 2(28.5%) 2(28.5%) 23(62.2%)	7(50%) 2(28.5%) 2(28.5%) 13(35.1%)	 1(2.7%)		
Cases Controls	SMOKERS Adenocarcimoma SQCC SCLC	64(81.0%) 10(15.6%) 29(45.3%) 10(15.6%) 36(42%)	28(43.7%) 5(17.8%) 16(57.1%) 3(10.7%) 23(63.8%)	28(43.7%) 3(10.7%) 9(32%) 7(25%) 11(30.55%)	8(12.5%) 2(25%) 4(50%) 2(5.5%)	4.23 7.20 4.5	0.031 0.03 0.048
Cases Controls	UNKNOWN*	1 13	0 8	1 5			

*Data not known

Out of 64 smokers 28 (43.75%) had homozygous wild type genotype (17.8% ADCC and 57% SQCC and 10.7% SCLC) while 28(43.7%) had heterozygous genotype (10.7% ADCC and 32.1% SQCC and 25% SCLC) and 12.5% had mutant genotypes (25% ADCC and 50% SQCC. In case of controls 23 (63.8%) were homozygous wild type individuals and 11 (30.5%) had heterozygous genotype and 2 (5.5%) mutants were observed.

Significant association was found between mutant genotype *196a2* gene and smokers (O.R=4.235, 95%CI; 1.050-17.082, ***p*=0.03**) thus proving that the smokers are at 4-folds elevated risk of developing lung cancer. On the other hand there was a 2-fold risk for lung cancer risk for smokers when both the variant and mutant genotypes were combined (OR=2.13, 95%CI; 1.14-3.99, *p*=0.017) as one single genotype.

Similarly in one study conducted on lung cancer in Korean population (Hong *et al.,* 2011) significant association was found between smokers and mutant genotype of *miRNA 196a2* gene while no such significant association was found in Chinese population.(Hu *et al.*,2008). In

another study conducted on gastric cancer there was no significant association between SNP rs11614913 and gastric cancer risk for subjects with different smoking status (Peng *et al*., 2009).

In our study as shown in table 4.10, smokers were also found to be at 7.2 and 4.5 fold elevated risk for ADCC and SQCC as strong association was observed between mutant genotype and smokers suffering from adenocarcinoma (OR=7.2, 95%C.I: 0.96-53.3, *p*=0.03) and SQCC (OR=4.5, 95%C.I; 0.91-22.2, *p*=0.048)

4.11 DISTRIBUTION OF GENOTYPES OF *miRNA 196a2* GENE ON BASIS OF SEX

Both in patients and controls, frequency of males was higher as compared to females. Patients were 86% males and just 13.9% females while 83% of the controls were males and females were only 1% (as shown in table 4.11). No significant association was found between *miR-196a2* rs11614913 genotypes and the Lung cancer risk in the subgroups of sex (OR=2.14, 95%C.I; 0.47-9.58). Similarly in studies conducted on Chinese (Tian *et al*., 2009) and Korean (Hong *et al.*, 2011) populations no heterogeneity of the risk was found to be associated with sex.

Table 4.11: Frequency distribution *miRNA196a2* genotypes on basis of sex

		N	C/C	C/T	T/T	OR	*P*
CASES CONTROLS	TOTAL	79 86	38 54	33 29	8 3		
CASES	FEMALES MALES	11(13.9%) 68(86.07%)	3(27.2%) 35(51.4%)	5 (45.4%) 28 (41.7%)	3 (27.2%) 5 (7.3%)		
CONTROLS	FEMALES MALES	1 (1.1%) 72 (83.7)	1 45 (62.5)	0 24 (33.3)	0 3(4.16)	2.14	0.30
CASES CONTROLS	UNKNOWN*	0 13	0 8	0 5	- 0		

*Data not known

4.12 DISTRIBUTION OF GENOTYPES OF *miRNA 196a2* GENE AMONG PATIENTS AT DIFFERENT STAGES OF LUNG CANCER

Genotypes of *miRNA 196a2* gene were distributed among patients at different stages of lung cancer. Table 4.12 shows frequency distribution of *miRNA 196a2* genotypes.

Table 4.12: Frequency distribution of *miRNA 146a* genotypes among patients at different stages of lung cancer

	N (%)	**C/C**	**C/T**	**T/T**	**OR (95%CI)**	***p* value**
CONTROL (86)	86	54	29	3		
CASES (79)	79	38	33 (C/T)	8 (T/T)		
STAGES						
1	1(1.2)		1			
2	4 (5)	3 (75%)	1 (25%)			
3	14 (17.7)	7 (50%)	5 (35.7%)	2 (14.2%)	5.14	0.07
4	19 (24.05)	8 (42%)	10 (52.6%)	1 (5.2%)	2.25	0.49

As shown in table (4.12) 24% of the patients were at the fourth stage (OR=2.25, 95%C.I; 0.2-6.54 *p*=0.07) 17% were at third stage (OR=5.14, 95%CI; 0.72-36.3, *p*=0.07), 5% and 1% were at second and first stage of lung cancer respectively. Patients diagnosed in second stage were majorly found to have wild type genotype GG (75%) and rest 25% were heterozygotes (GC). Similarly among third stage patients 50% had wild type homozygote genotypes, 35% were heterozygotes and 14% had mutant genotypes TT. Whereas in case of patients at the fourth stage, frequency of heterozygote individuals (52%) predominated, wild type genotype was found in 42% of the patients and mutant genotype was in 5.2% of the patients.

4.13 EFFECTS OF COMBINED POLYMORPHISMS IN *miR- 196a2* AND *miR-146a* TOWARDS LUNG CANCER 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

lung cancer risk. Table shows pooled ORs and stratification analysis of the *miR-196a2* C/T (rs11614913) and *miR-146a* G/C (rs2910164) functional polymorphisms on lung cancer risk.

Table 4.13 Main results of pooled ORs and stratification analysis of the *miR-196a2* C/T (rs11614913) and *miR-146a* G/C (rs2910164) functional polymorphisms on lung cancer risk

VARIABLES	**N (%)**	**G/G+C/C (%)**	**G/C+C/T (%)**	**C/C+T/T (%)**	**OR**	***p* VALUE**
CASES CONTROLS	38 38	24 (63) 33 (86.8)	12 (34.2) 5 (13.1)	2 (5.2) 0	3.8	0.02
SQCC COMMON	14 (36.8)	10 (30.3)	2 (16.6)	2 (14.2)	2.6	0.2
ADCC COMMON	8 (21.05)	6 (75)	2 (25)	0	2.2	0.4
SCLC COMMON	5 (13.15)	2 (40)	3 (60)	0	9.9	0.02
SMOKERS CASES CONTROLS	31 (81.5) 16 (42)	19 (61.2) 15 (93)	10 (32.2) 1 (6.25)	2 (6.45) 0	9.4	0.04
NON SMOKERS CASES CONTOLS	7 (18.4) 19 (50)	5 (71.4) 16 (84.2)	2 (28.5) 3 (15.7)	0 0	10	0.02

Out of total common patients studied 63% of the individuals were found to have homozygous wild type genotypes both for *miRNA 146* (GG) and *miRNA 196a2* (TT), 12% had heterozygous genotypes for both genes (GC for *miRNA 146a* and CT for *196a2*) and 5% individuals had common mutant genotype (CC for *146a* and TT for *196a2* gene). On the other hand in case of controls 87% of the individuals had homozygous wild type genotypes, 13% of the individuals had heterozygous genotypes and mutants were not observed. Significant association was found between individuals who had mutant or variant genotype for both the gene and lung cancer risk (OR=3.8, 95%C.I; 1.2-12.1 and ***p*=0.02**). Thus from this data we know that due to joint effect of both the polymorphisms, risk for lung cancer is elevated 3.8 folds. In a similar study conducted on non-Hispanic white population, it was found that individuals with 4 risk genotypes (rs2910164, rs2292832 and rs3746444 and rs11614913) had a 40% significantly increased risk of SCCHN compared with individuals with 0–1 genotypes (Liu *et al*., 2010).

These total cases were again stratified according to histology. 36.8% (30.3% GG for *miRNA 146* and CC for *196a2* gene, 16.6% GC for *146a* and CT for *196a2* gene, 14.2% CC for 146a and TT for *196a2* gene) of the common cases were diagnosed with SQCC, 21% of the cases diagnosed with ADCC consisted of 75% wild type homozygous (GG for *146a* and CC for *196 a2* gene) and 25% heterozygous genotypes. Out of the SCLC diagnosed patients, 60% had wild type genotype and 40% had heterozygote genotypes for both the genes while no combined mutants were found. Significant association was found between patients having variant genotype for both the genes and SCLC especially for SCLC (O.R=9.9, 95%C.I; 1.3-74, *p*=0.02), thus individuals with variant genotype are at about 10 fold elevated risk for SCLC which is much higher than risk associated with either of the two genes individually.

31(81%) of the common cases were found to be smokers and 18.4% were non-smokers. Among smokers 61.2% had wild type genotypes for both the genes, 22% and 6% were heterozygous and mutants respectively. In non-smokers most of the common cases were heterozygous (71.4%) followed by 28% mutant genotypes and 18% homozygous genotypes. In controls 42 % were smokers (32% had common wild type homozygous genotypes while 7% had heterozygous genotypes) and 50% were non-smokers (85% wild type homozygous and 15% heterozygous).

Significant association was found among smokers having common mutant or variant genotype for both the genes and risk for lung cancer, (OR=9.4, 95%CI; 1.1-81.2, ***p*=0.04).** Thus smokers having mutant or variant genotype for both *mir146a* and *196a2* genes had 9.4 folds elevated risk for lung cancer. On the other hand non-smokers with mutant or variant genotypes (OR=10, 95%C.I; 1.3-71.8 and ***p*=0.02)** for both the genes had much higher elevated risk (10 folds) for lung cancer as compared to smokers.

Thus we conclude that smokers with joint polymorphisms rs291064 and rs1161493 had more pronounced risk of lung cancer as compared to individuals with single risk genotype

CONCLUSION

The present case/control study pertains to patients visiting the Post Graduate Institute of Medical Education and Research, which is a referral center 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 (rs2910164) polymorphism was significantly associated with risk for lung cancer.
- Significant association was found between non-smokers and mutant genotype (CC) for *miRNA 146a* gene indicating elevated risk of lung cancer among non-smokers,
- However, both *mir-196* CT+CC genotype and the variant TT were associated with a statistically significant, increased risk for developing cancer
- Smokers with mutant genotype *196 a2* were at elevated risk of developing lung cancer and the presence of combined mutant genotypes for both *146a* and *196a2* gene further enhanced the risk for disease.
- Individuals with *miRNA 196a2* or *146a* mutants genotypes were at an elevated risk of developing SCLC.
- The combined presence of *miR-196a2* C/T (rs11614913) and *miR-146a* G/C (rs2910164) functional polymorphisms further increased the risk for lung cancer.

In conclusion, this case-control study provides evidence that *miR-196a2* rs11614913 C/T polymorphisms and *miR-146a* G/C (rs2910164) are associated with a significantly increased risk of lung cancer and moreover the disease is more prevalent in populations having habit of smoking. Both the variants can be considered as biomarkers for lung cancer thus our present work despite the limitation of small sample size will provide guidelines for future work.

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APPENDIX- I

1. 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.
2. 10% SDS: Dissolved 1g of SDS in 10ml of deionised water.
3. 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.
4. 10mg/ml Proteinase K: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilized the solution by autoclaving.
5. 1mg/ml BSA: Dissolved 100mg of BSA in 100ml of deionised sterile water and kept at 4℃ overnight.
6. 5M Sodium chloride (NaCl): Dissolved 5.85g of sodium chloride in 20ml of deionised water. Sterilized the solution by autoclaving.
7. 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.
8. Ethidium Bromide (10mg/ml): Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.
9. Magnesium chloride ($MgCl_2$) (100mM): Dissolved 0.41gms of $MgCl_2$ in 20ml of deionised water and sterilized by autoclaving.
10. Sucrose (1M): Dissolved 3.41 g of sucrose in 10 ml of deionised water and sterilized by autoclaving.
11. 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.
12. Triton X- 100 (10%): Took 100 µl of TritonX-100 and mixed with 900 µl of deionised water and mixed properly.

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