DIFFERENTIAL EXPRESSION OF miR-127 IN LUNG CANCER

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CERTIFICATE

This is to certify that the work titled "**Differential Expression of miR-127 in Lung Cancer**" submitted by "**Akriti Sharma**" in partial fulfillment for the award of degree of 4 Year Degree Program Bachelor of Technology in Biotechnology at Jaypee University of Information Technology, Waknaghat has been carried out under my supervision.

This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Name of Supervisor : Dr. Aklank Jain

Designation : Associate Professor (Biotechnology)

Signature of Supervisor :

Date :

ACKNOWLEDGEMENT

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Signature of the student :

Name of Student : Akriti Sharma

Date :

SUMMARY

The discovery of microRNA and its role in regulation of gene expression has been one of the defining developments in cancer biology over the past decade. miRNAs are known to influence numerous cancer-relevant processes such as proliferation, cell cycle control, apoptosis, differentiation, migration and metabolism. miRNAs are integral parts of feedback circuits in biological systems and as a single miRNA may target up to several hundred mRNAs, aberrant miRNA expression may affect a multitude of transcripts and profoundly influence cancer-related signalling pathways. Various microRNAs have been reported to show differential expression in different types of cancers. They behave as either oncogenic or tumor suppressors based on what genes they target and therefore their expression varies accordingly in cancer patients. Based on literature studies, I have selected four miRNAs to check for their differential expression in lung cancer. The aim of the project is to select the microRNA with maximum differential expression and study the pathway associated with that particular microRNA.

Signature of Student	**Signature of Supervisor**
Name: Akriti Sharma	**Name: Dr. Aklank Jain**
Date :	**Date :**

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Graph 5: miR-127 Expression in Untreated Lung Cancer Samples in comparison to Control Samples

CHAPTER 1

INTRODUCTION

Cancer is a complex disease in which cells in a specific tissue are no longer fully responsive to the signals within the tissue that regulate cellular differentiation, survival, proliferation and death. As a result, these cells accumulate within the tissue, causing local damage and inflammation and in later stage metastasize throughout the body. There are over 200 different types of cancer [1]. The risks factors leading to the development of cancer diverse and not yet completely understood. These may include environmental pollutants, tobacco, dietary factors, radiation exposure, certain infections, viruses, etc [2]. In 2012, 14.1 million adults in the world were diagnosed with cancer and there were 8.2 million deaths from cancer in the world in 2012 [3].

Cancer is fundamentally a disease of abnormal gene expression. The genetic changes that contribute to cancer tend to affect three main types of genes—proto-oncogenes, tumor suppressor genes, and DNA repair genes. Abnormal expression may arise due to mutations within these genes or closely linked DNA that regulates activity of these genes, fusions of two genes by recombination between DNA sequences or by disruption in the machinery responsible for production or activity of proteins being produced from these genes, such as systems that control the synthesis, modification, or degradation [4]. MicroRNAs form a part of such machinery and abundant evidences have demonstrated fundamental importance of microRNA in normal development, differentiation, growth control and cancer.

1. MicroRNA

microRNAs (miRNAs) are a class of naturally occurring, small non-coding RNA molecules of about 18–25 nucleotides in length. These are known to be evolutionarily conserved among species constituting approximately 1-5% of genes in human genome. miRNAs were first described in 1993 by Lee and colleague, and the term microRNA was coined in 2001. They are partially complementary to different messenger RNA sequences and work by binding to 3’UTR of these mRNAs thus

regulating the gene expression in a variety of manners, including translational repression, mRNA cleavage, and deadenylation [5].

Biogenesis of microRNA

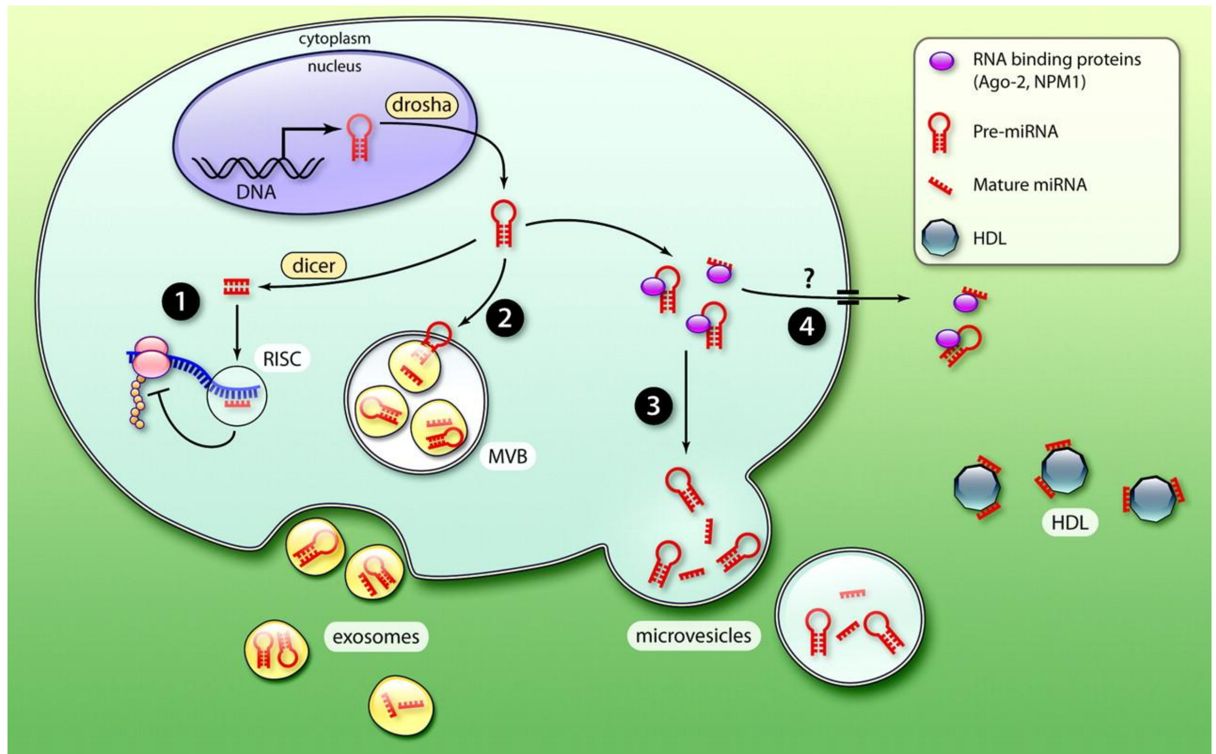


Fig. 1

The genes encoding miRNAs are much longer than the processed mature miRNA molecule. Therefore after being transcribed the pri-miRNA is acted upon by Drosha-DCGR8 microprocessor complex which cleaves it to produce pre-miRNA of about 70 nucleotides. This pre-miRNA is then transported to the cytoplasm by Exportin 5 and RAN/GTP complex where it is further processed by Dicer proteins to render mature miRNA, a double-stranded miRNA aproximately 22 nucleotides in length [5].

MicroRNA Stability

Circulating miRNAs are known for their stability against RNase treatment, freeze thaw cycles and, varying temperature and pH cycles. It is because of the extremely small size of miRNA that it is able to escape degradation. A second, more important reason for its stability is that miRNAs are protected against degradation by being

packaged in lipid vesicles or by being associated with protein or lipoprotein complexes [6].

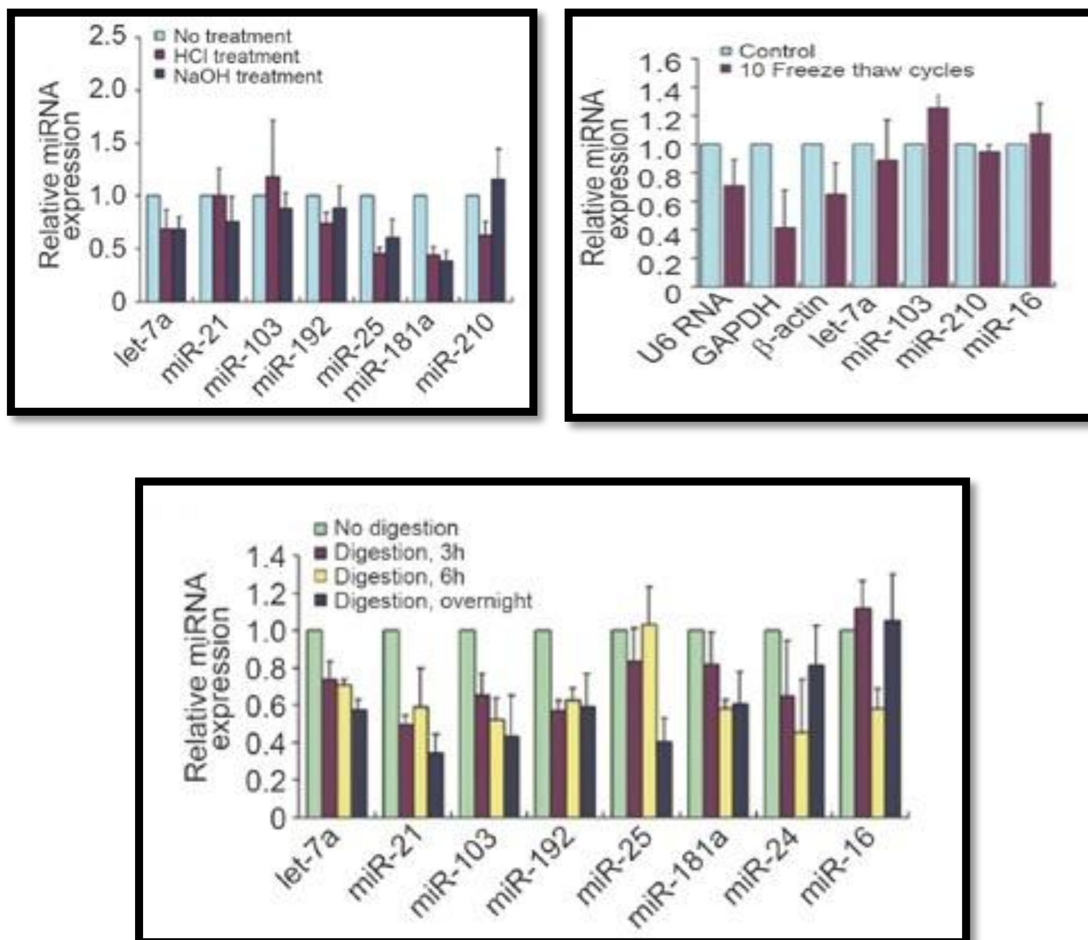


Fig. 2

MicroRNA and Cancer

The discovery of microRNAs has been one of the defining developments in cancer biology over the past decade. MicroRNA dysregulation in cancer was first reported in2002. Within few years of its discovery, microRNAs have become firmly established key molecular components that are targeted in cancer research. miRNA can act as either oncogenic or tumor suppressor or both. Oncogenic miRNA expression is upregulated in case of cancer and vice versa in case of tumor suppressor miRNA.

1.2 Rationale

Cancer, and in particular, Lung cancer is the leading cause of cancer related death worldwide. The 5-year survival rate for NSCLC patients remains low as 15%. Reasons for these huge statistics include delay in diagnosis, poor disease management, late stage detection and many more. Differential expression of various miRNAs has been reported in lung cancer. The selected miRNAs have been reported earlier in cell lines and tissue samples for lung cancer and have shown differential expression. Here we hypothesize that these miRNAs will also show differential in lung cancer plasma samples.

1.3 Main Objectives

- To evaluate the expressions of miR-218, miR-25, miR-127 and miR-372 in lung cancer patients compared to healthy controls
- To select the miRNA with maximum differential expression
- To study the pathway associated with the selected miRNA in lung cancer

CHAPTER 2

LUNG CANCER

2.1 Overview

Lung cancer is the uncontrolled growth of abnormal cells that start off in one or both lungs; usually in the cells that line the air passages. The abnormal cells do not develop into healthy lung tissue; they divide rapidly and form tumors. As tumors become larger and more numerous, they undermine the lung's ability to provide the bloodstream with oxygen. Tumors that remain in one place and do not appear to spread are known as benign tumors. Malignant tumors, the more dangerous ones, spread to other parts of the body either through the bloodstream or the lymphatic system. Metastasis refers to cancer spreading beyond its site of origin to other parts of the body. When cancer spreads it is much harder to treat successfully.

Primary lung cancer originates in the lungs; while secondary lung cancer starts somewhere else in the body, metastasizes, and reaches the lungs. They are considered different types of cancers and are not treated in the same way.

Lung cancer can be broadly classified into two main types based on the cancer's appearance under a microscope: non-small cell lung cancer and small cell lung cancer. Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancers, while small cell lung cancer accounts for the remaining 20%.Non-small cell lung cancer (NSCLC) is the most common type of lung cancer. It usually grows and spreads more slowly than small cell lung cancer [7].

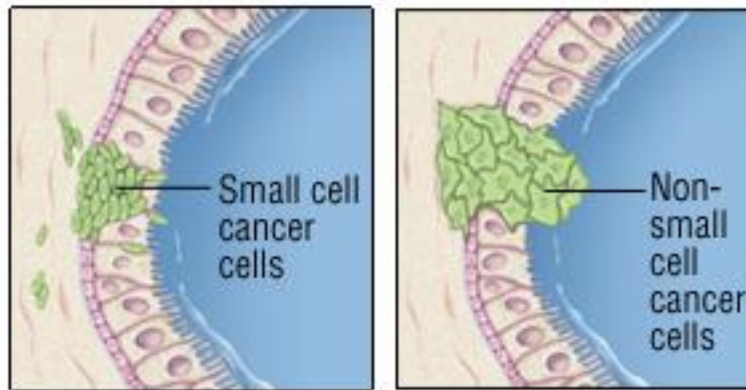


Fig. 3

NSCLC can be further divided into four different types, each with different treatment options:

• **Squamous cell carcinoma**

As the most common type of NSCLC and the most common type of lung cancer in men, squamous cell carcinoma forms in the lining of the bronchial tubes.

• **Adenocarcinoma**

As the most common type of lung cancer in women and in nonsmokers, adenocarcinoma forms in the mucus-producing glands of the lungs.

• **Bronchioalveolar carcinoma**

This type of lung cancer is a rare type of adenocarcinoma that forms near the lungs' air sacs.

• **Large-cell undifferentiated carcinoma**

A rapidly growing cancer, large-cell undifferentiated carcinomas form near the outer edges or surface of the lungs.

Small cell lung cancer (SCLC) is characterized by small cells that multiply quickly and form large tumors that travel throughout the body. Almost all cases of SCLC are due to smoking [8].

2.2.1 Causes

Smoking causes most cases of lung cancer. The risk depends upon the number of cigarettes smoked every day and for how long someone has smoked. Being around the

smoke from others (secondhand smoke) also raises your risk for lung cancer. However, people who do not smoke and have never smoked have become sick with lung cancer. High levels of air pollution and drinking water containing high levels of arsenic can increase your risk for lung cancer. Radiation therapy to the lungs can also increase the risk.

Working with or near the following cancer-causing chemicals or materials can also increase risk:

- Asbestos
- Products using chloride and formaldehyde
- Certain alloys, paints, pigments, and preservatives

2.2.2 Symptoms

Early lung cancer Symptoms includes:

- Cough that doesn't go away
- Coughing up blood
- Shortness of breath
- Wheezing
- Chest pain
- Loss of appetite
- Losing weight without trying
- Fatigue

Other symptoms that may be due to NSCLC:

- Weakness
- Swallowing difficulty
- Nail problems
- Joint pain
- Hoarseness or changing voice
- Swelling of the face
- Eyelid drooping

- Bone pain or tenderness
- Shoulder pain or weakness [9]

2.3 Lung Cancer Staging

Stage 0

This is called in situ disease, meaning the cancer is "in place" and has not grown into nearby tissues and spread outside the lung.

Stage I

A stage one (I) lung cancer is a small tumor that has not spread to any lymph nodes, making it possible for a surgeon to completely remove it. Stage I is divided into two substages: stage IA or stage IB, based on the size of the tumor. Smaller tumors, such as those less than 3 centimeters (cm) wide are stage IA, and slightly larger ones, such as those more than 3 cm but less than 5 cm wide, are stage IB.

Stage II

Stage two (II) lung cancer is divided into two substages: stage IIA or IIB. A stage IIA cancer describes a tumor larger than 5 cm but less than 7 cm wide that has not spread to the nearby lymph nodes or a small tumor less than 5 cm wide that has spread to the nearby lymph nodes.

Stage IIB lung cancer describes a tumor larger than 5 cm but less than 7 cm wide that has spread to the lymph nodes or a tumor more than 7 cm wide that may or may not have grown into nearby structures in the lung but has not spread to the lymph nodes.

Sometimes, stage II tumors can be removed with surgery, and other times, more treatments are needed.

Stage III

Stage three (III) lung cancers are classified as either stage IIIA or IIIB. For many stage IIIA cancers and nearly all stage IIIB cancers, the tumor is difficult, and sometimes impossible, to remove. For example, the lung cancer may have spread to the lymph nodes located in the center of the chest, which is outside the lung. Or, the tumor may have grown into nearby structures in the lung. In either situation, it is less

likely that the surgeon can completely remove the cancer because removal of the cancer must be performed bit by bit.

Stage IV

Stage four (IV) means the lung cancer has spread to more than one area in the other lung, the fluid surrounding the lung or the heart, or distant parts of the body through the bloodstream. Once released in the blood, cancer can spread anywhere in the body, but it is more likely to spread to the brain, bones, liver, and adrenal glands. It is called stage IVA when the cancer has spread within the chest or IVB when it has spread outside of the chest.

In general, surgery is not successful for most stage III or IV lung cancers. Lung cancer can also be impossible to remove if it has spread to the lymph nodes above the collarbone, or if the cancer has grown into vital structures within the chest, such as the heart, large blood vessels, or the main breathing tubes leading to the lungs. The doctor will recommend other treatment options [10].

2.4 Incidence Rate

Lung cancer has been the most common cancer in the world for several decades. There are estimated to be 1.8 million new cases in 2012 (12.9% of the total), 58% of which occurred in the less developed regions. The disease remains as the most common cancer in men worldwide (1.2 million, 16.7% of the total) with the highest estimated age-standardised incidence rates in Central and Eastern Europe (53.5 per 100,000) and Eastern Asia (50.4 per 100,000).

Notably low incidence rates are observed in Middle and Western Africa (2.0 and 1.7 per 100,000 respectively). In women, the incidence rates are generally lower and the geographical pattern is a little different, mainly reflecting different historical exposure to tobacco smoking. Thus the highest estimated rates are in Northern America (33.8) and Northern Europe (23.7) with a relatively high rate in Eastern Asia (19.2) and the lowest rates again in Western and Middle Africa (1.1 and 0.8 respectively).

Lung cancer is the most common cause of death from cancer worldwide, estimated to be responsible for nearly one in five (1.59 million deaths, 19.4% of the total). Because of its high fatality (the overall ratio of mortality to incidence is 0.87) and the relative

lack of variability in survival in different world regions, the geographical patterns in mortality closely follow those in incidence [11].

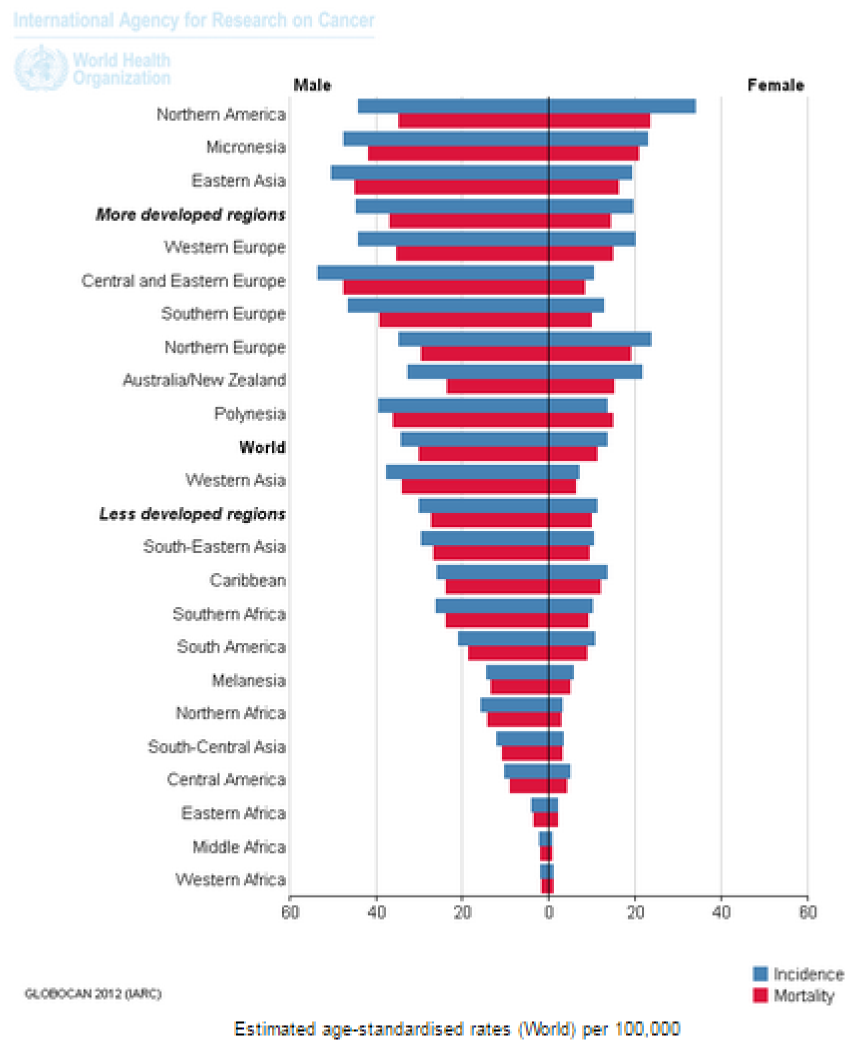


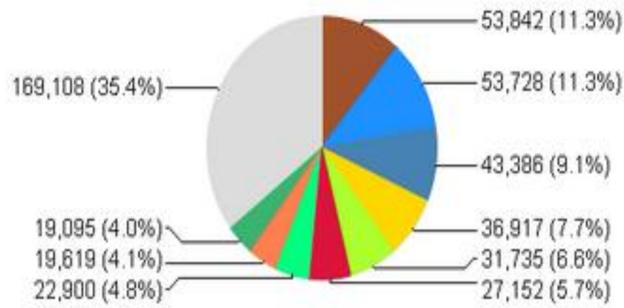
Fig. 4

Indian Statistics

One million of the current 5 million deaths in world, and 2.41 million in developing countries is contributed by India in 2010 and, in 2020, this figure is projected at 1.5 million.

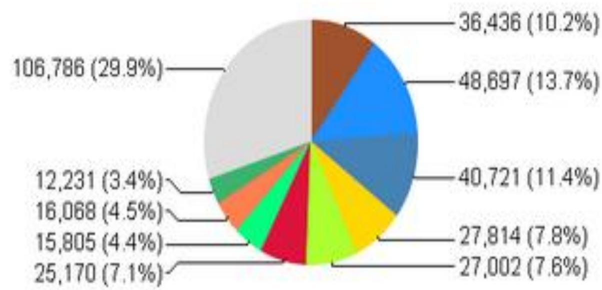
International Agency for Research on Cancer
World Health Organization

Incidence



International Agency for Research on Cancer
World Health Organization

Mortality



- Lip, oral cavity
- Lung
- Stomach
- Colorectum
- Other pharynx
- Oesophagus
- Larynx
- Leukaemia
- Prostate
- Other and unspecified

Fig. 5

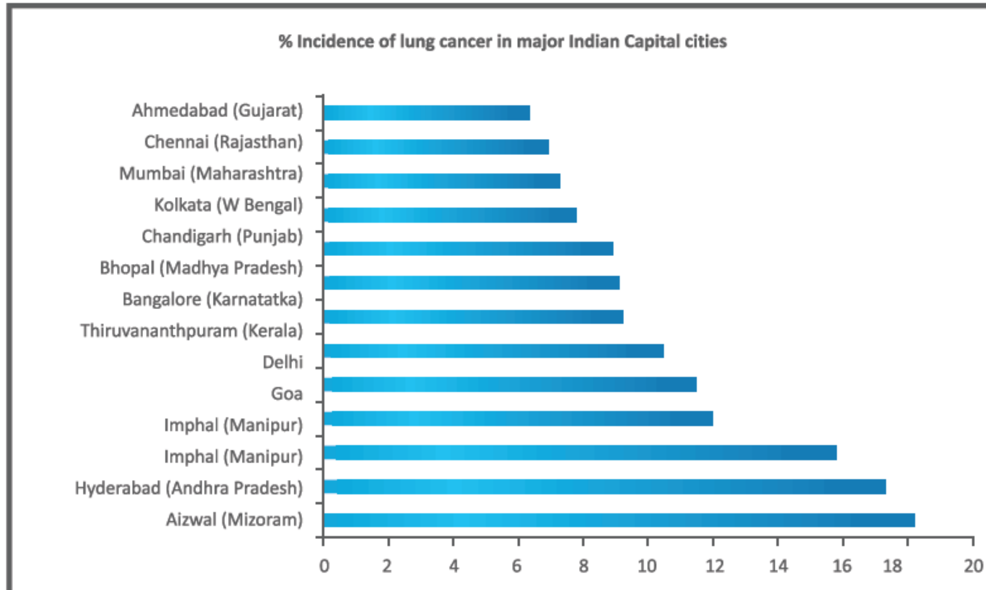


Fig. 6

The Indian council of medical research (ICMR), after studying lung cancer data of 24 years (1982-2005), has found that while new cases of lung cancer per one lakh male population has increased by around 160% in Chennai, 100% in Bangalore and 40% in Delhi during this period, such cases have fallen by 60% in Mumbai [12].

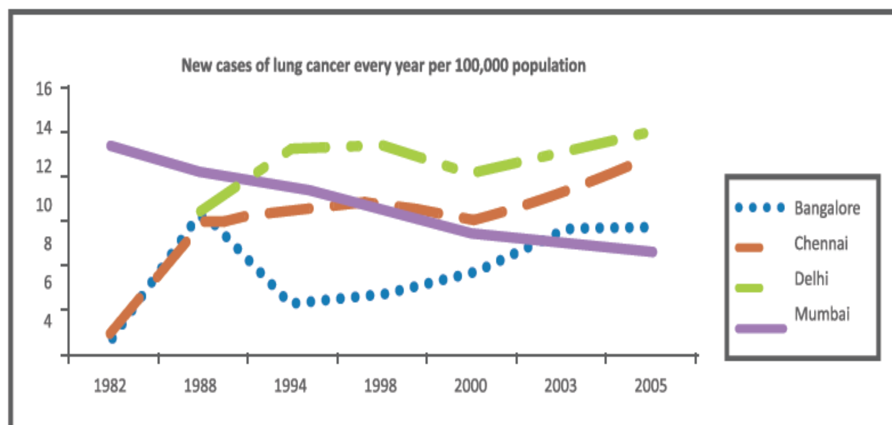


Fig. 7

2.5 Diagnosis

Chest X-ray

Patients with lung cancer often have obstructive features (37%) and pleural effusions (22%). Lung cancer patients rarely present with normal chest X-ray (only 2% in one study) .Chest radiography is a simple, cost-effective measure and it imparts very little radiation to thepatient. Naturally, it is routinely carried out at every institute.

CT / MRI scanning

Results from computed tomography (CT) scanning are subject to variation caused by different scanning techniques, but suggest that CT scanning of the chest has a high sensitivity (89 to 100%) but a relatively low specificity (56 to 63%) and a poor negative predictive value (60 to 100%).CT has now become the mainstay of staging chest malignancies and is routinely performed at all major centers in India. Superiority of magnetic resonance imaging (MRI) over CT scan for the detection of bronchial and chest wall invasion or for nodal staging is unestablished. Also as the CT is less expensive and widely available in India it is preferred and routinely advised.

PET scanning

Positron emission tomography (PET) scanning has a diagnostic sensitivity of 96% and a specificity of 78% but there is considerable variation within the studies included. The diagnostic studies indicate negative predictive values as low as 47%.

The considerable cost of the instrument imaging agents as well as the short half-life of positron emitting isotopes (which require a nearby cyclotron for generation), has prevented widespread acceptance and hence these units are available only at a few specialized centers.

Bronchoscopy

The value of bronchoscopy depends on the location of the primary tumor. Peripheral tumors in subsegmental bronchi may not be visible.Flexible bronchoscopy has good diagnostic sensitivity (83% to 88%) for central lesions.

Sputum cytology

There is a wide variation (10% to 97%) in the sensitivity of sputum cytology in the diagnosis of lung cancer. High sensitivity is only achieved by the use of specific and carefully controlled protocols for sample collection

2.6 Treatment

1. Radiotherapy

Radiotherapy has an established role in management of lung cancer, both on its own and in combination with chemotherapy. Radiotherapy has a well-documented effect in palliating thoracic symptoms and, in selected cases with NSCLC, it may be curative. Radiotherapy can also be useful in treating locally symptomatic metastases.

2. Chemotherapy

NSCLC

• Chemotherapy with a platinum-based combination double regimen should be considered in all patients who are not suitable for curative resection or radical radiotherapy and are fit enough to receive it.

• Second-line chemotherapy with docetaxel should be considered for stage IIIB/IV patients with good performance status.

SCLC

• A regimen containing a platinum agent with etoposide is recommended for first line treatment.

• Second-line in SCLC cases must be considered depending upon the duration of response to first line chemotherapy and on patient’s performance status and desire.

3. Surgery

Surgical removal of the tumor is generally performed for limited stages (stage 1 or stage 2). This is the choice of treatment for cancer that has not spread beyond the lungs .

2.7 Review

There are several databases available that provide a graphic user interface to facilitate miRNA-cancer profile database search and provide sequence analysis tools to analyze selected sequences. miRCancer is one such database that provides sequence analysis tools and microRNA-cancer relation data extracted with simple 3-occurance method. miRCancer was first launched in May 23, 2011.

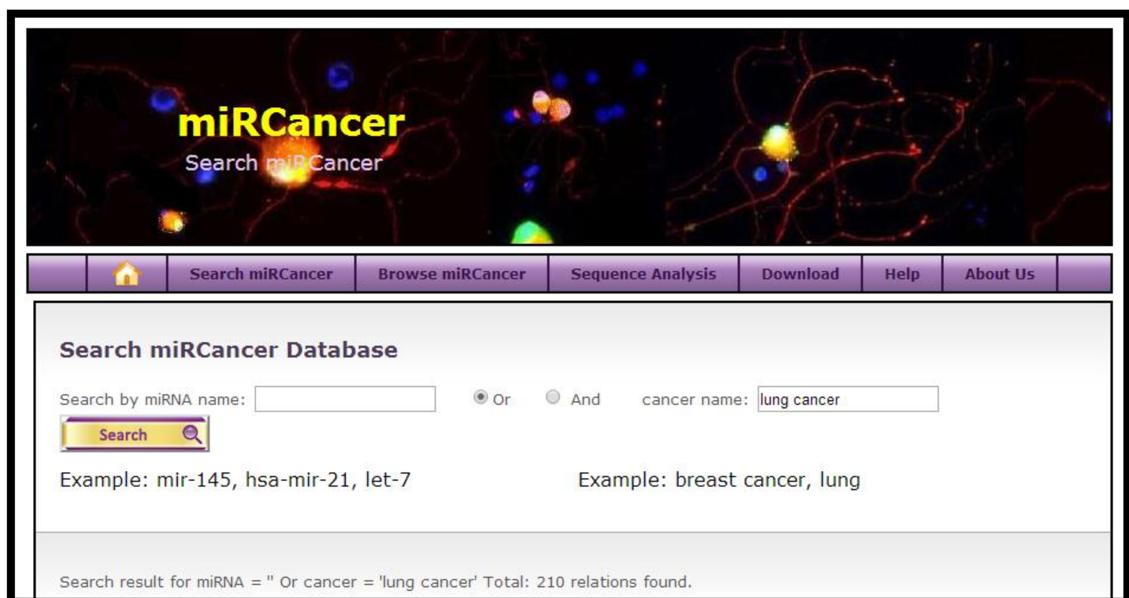


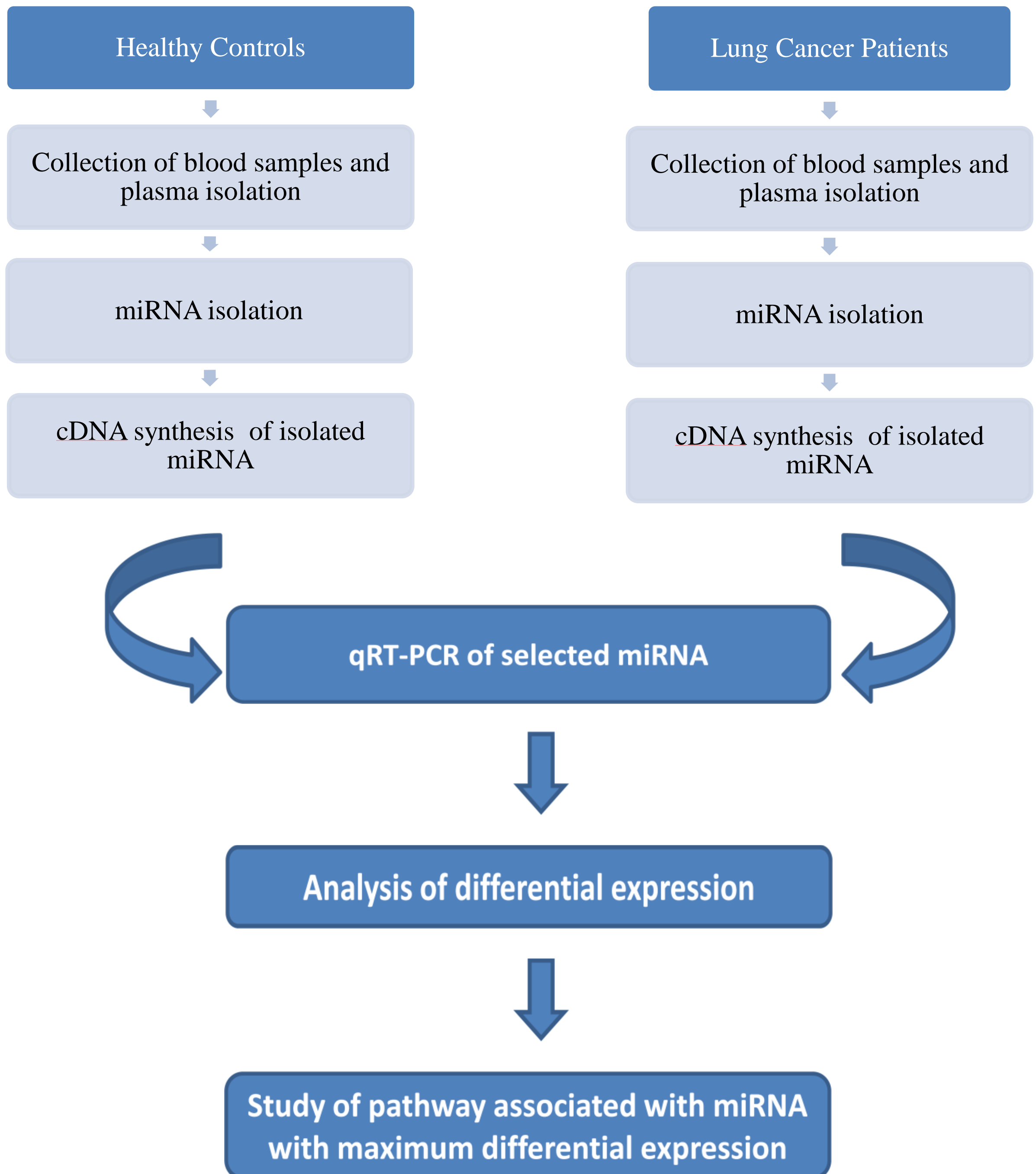
Fig. 8

After studying various cancer-miRNA combinations, four miRNAs miR-25, miR218, miR127 and miR-372 were selected were selected that were reported in lung cancer in tissue samples but no significant studies had been carried out on them. Furthermore, the literature was reviewed from NCBI to avail more information about the selected miRNAs.

Elevated expressions of miR-25 were reported in lung adenocarcinomas of female non-smokers. The exact role of miR-25 is still unknown and its effects on cancer are complicated [13]. miR-127 was reported to be up regulated in Human Lung adenocarcinoma. miR-372 over-expression showed strong correlation with the presence of NSCLC and miR-218 over-expression was also reported in a number of cancers [14].

CHAPTER 3

EXPERIMENTAL WORK OUTLINE



CHAPTER 4

METHODOLOGY

4.1 Sample Collection

The lung cancer samples were collected from IGMC hospital, Shimla in EDTA coated vials.

4.2 Plasma Isolation

Protocol

1. The cancer samples, after collection, were refrigerated at 4ºC for 1-2 days.
2. The samples were then taken and the clear liquid layer formed in the vials (plasma) was transferred to centrifuge tubes.
3. The samples were centrifuged at 10,000 rpm for 10 minutes.
4. The supernatant was taken and transferred into new centrifuge tubes.
5. The samples were then stored at -80ºC for further use.

4.3 miRNA Isolation from Blood Samples

Material Required

- Plasma samples
- Qaizol
- Chloroform
- Ethanol
- Spike-in control
- miRNA isolation kit

Protocol

1. Plasma samples were mixed well by simple inversion.
2. Qaizol was added 5 times the volume of plasma sample taken followed by gentle vortexing and were incubated for 5 minutes at room temperature.
3. 3.5 µl spike-in as internal control was added to each sample followed by gentle mixing.
4. Equal volume of chloroform as that of plasma sample was added to each tube and vortexed for 15 seconds followed by incubation for 2-3 minutes at room temperature.
5. After incubation the tubes were centrifuged for 15 minutes at 12,000 g and 4ºC.
6. The upper aqueous phase formed after centrifugation was then transferred to a fresh labeled 2 ml centrifuge tube.
7. To the aqueous phase 1.5 times the volume of initial sample absolute ethanol was added and immediately pipette up and down several times to ensure proper mixing.
8. From the above, 700 µl of sample was dispensed into the centre of RNeasy mini elute column in a 2 ml collection tube and centrifuged for 15 seconds at 8,000 g and 25 ºC.
9. The flow-through was discarded and the the above step was repeated with the remaining sample.
10. 700 µl RTW buffer was added to the centre of RNeasy mini elute column.

11. The samples were centrifuged for 1 minute at 8,000 g and 25ºC and the flow-through was discarded.
12. 500 µl of RPE pipetted into the centre of RNeasy mini elute column.
13. The samples were centrifuged for 1 minute at 8,000 g and 25ºC and the flow-through was discarded.
14. 500 µl of 80% ethanol was pipetted into the centre of RNeasy mini elute column.
15. The samples were centrifuged for 2 minute at 8,000 g and 25ºC and the flow-through was discarded alongwith the collection tube.
16. The RNeasy mini elute column was placed in a new 2 ml collection tube.
17. The samples were centrifuged for 5 minute at 15,000 g and 25ºC and the flow-through was discarded alongwith the collection tube.
18. The RNeasy mini elute column was placed in a new 1.5 ml collection tube and add 14 µl of RNase free water to it.
19. Centrifugation was carried out for 1 minute at 15,000 g and 25ºC.
20. The liquid collected in the collection tube contained the isolated miRNA which was stored at -80ºC for further use.

4.4 miRNA Quantification

Protocol

1. The upper and lower optical surfaces of the microvolume spectrophotometer sample retention system (nano plate) were cleaned using ethanol and RNase away.
2. 2 µl of sample and blank (RNase free water) was pipette onto the nano plate.
3. The plate was inserted into the spectrophotometer system.
4. The software was run and nucleic acid application was selected with sample type RNA.
5. miRNA concentrations ware obtained in ng/µl.

4.5 cDNA Synthesis

Reaction Setup for cDNA Synthesis

Component	Volume for 1 Reaction
5X miScript hispec buffer	4 µl
10X miScript nucleis mix	2 µl
RNase free water	variable
miScript reverse transcriptase	2 µl
Template miRNA	variable (100ng conc.)
Total	**20 µl**

Table 1

Protocol

1. The miRNA templates and the reaction components were thawed and kept on ice.
2. Volume of components required to prepare the master mix was calculated.
3. Volume of each miRNA template was calculated such that it contained 100 ng of miRNA.
4. The master mix was prepared by adding 5X miScript Hispec buffer, 10X miScript nucleis mix.
5. miRNA template and RNase free water was added to each PCR vial.
6. miScript reverse transcriptase was added to the master mix.
7. 8 µl of master mix was dispensed into each vial.
8. The PCR vials were placed in the thermal cycler for cDNA synthesis with following conditions.

37ºC	60 minutes
95ºC	5 minutes
4ºC	∞

Table 2

9. The cDNA was stored at -20ºC for further processing.

4.6 qRT-sPCR

Reaction Setup for qRT-PCR

Component	Volume for 1 Reaction
2X quantitech SYBER green mix	6.25 µl
10X universal primer	1.25 µl
Specific primer assay	2.50 µl
RNase free water	1.25 µl
cDNA Template	1.25 µl
Total	**12.50 µl**

Table 3

Protocol

1. cDNA templates and qRT-PCR reaction components were kept on ice for thawing.
2. Volume of components required to prepare the master mix was calculated.
3. The 96-well plate program was designed on a sheet specifying the positions of samples.
4. The program was setup on the computer system attached to the q-RT PCR machine.
5. The master mix was prepared by adding 10X universal primer, specific primer assay specific primer assay and RNase free water.
6. cDNA template was added to each PCR vial.
7. 2X Quantitech SYBR green mastermix was added to the master mix.
8. 11.25 µl of master mix was dispensed into each vial.
9. The PCR vials were placed in the q-RT PCR machine and the programme was run.
10. Data obtained was further processed to obtain fold expression.

CHAPTER 5

RESULTS AND DISCUSSION

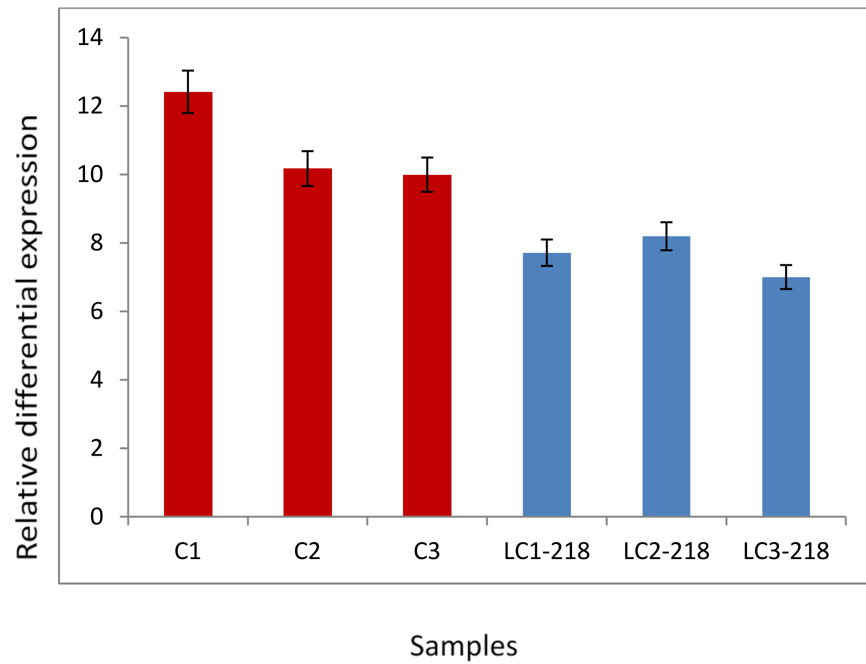
Initially the lung cancer samples were taken and miRNA was isolated followed by cDNA synthesis. Q-RT PCR was then performed using the specific primers of the selected four miRNAs to check for their relative fold expressions.

1. nucleic acid quantification of processed samples

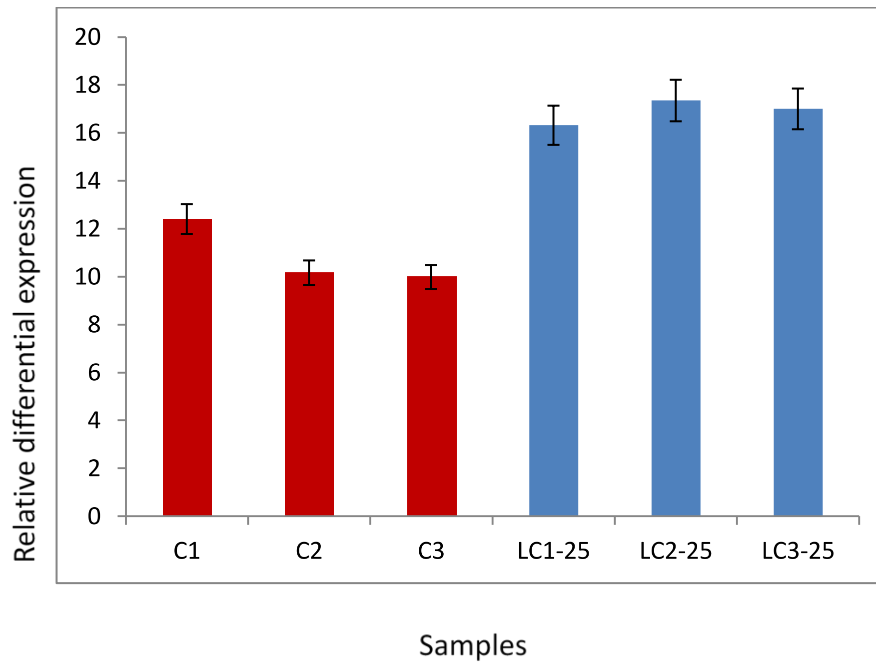
Sample	Concentration (ng/μl)	A 260/280
C1	17.6	1.89
C2	15.9	1.76
C3	17.8	1.94
LC1	21.0	1.82
LC2	12.5	1.87
LC3	18.1	1.71

Table 4

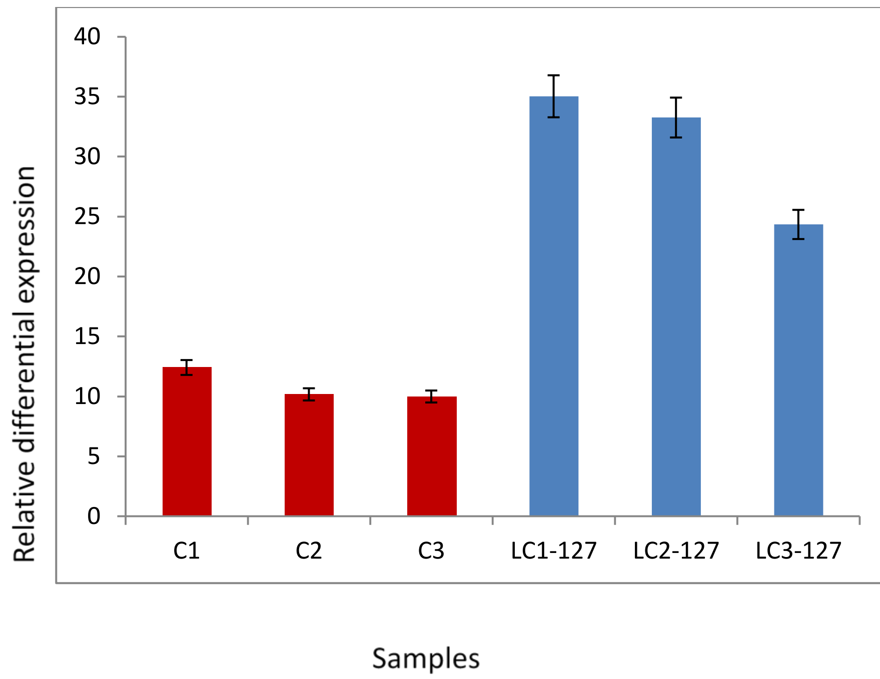
2. miR-218, miR-25, miR-127 and miR-372 Expression in Untreated Lung Cancer Samples in comparison to Control Samples



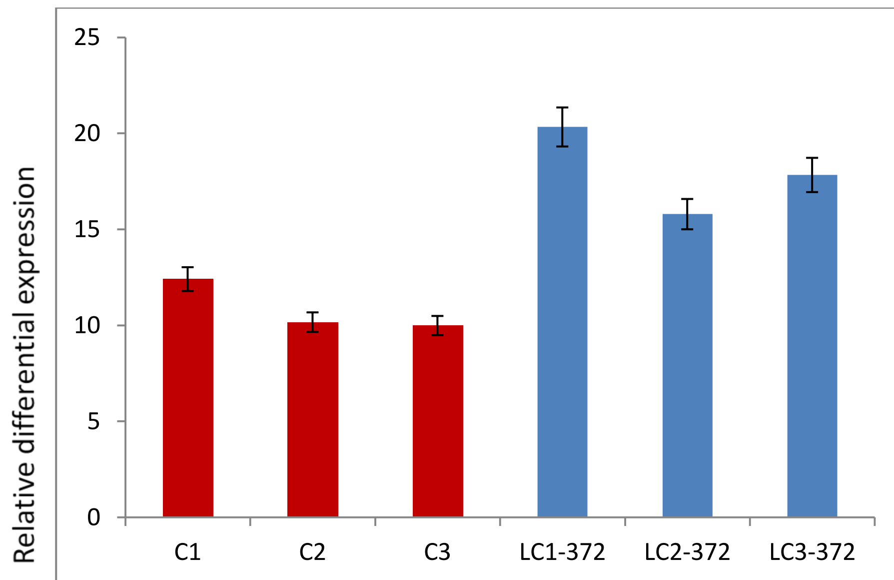
Graph 1



Graph 2



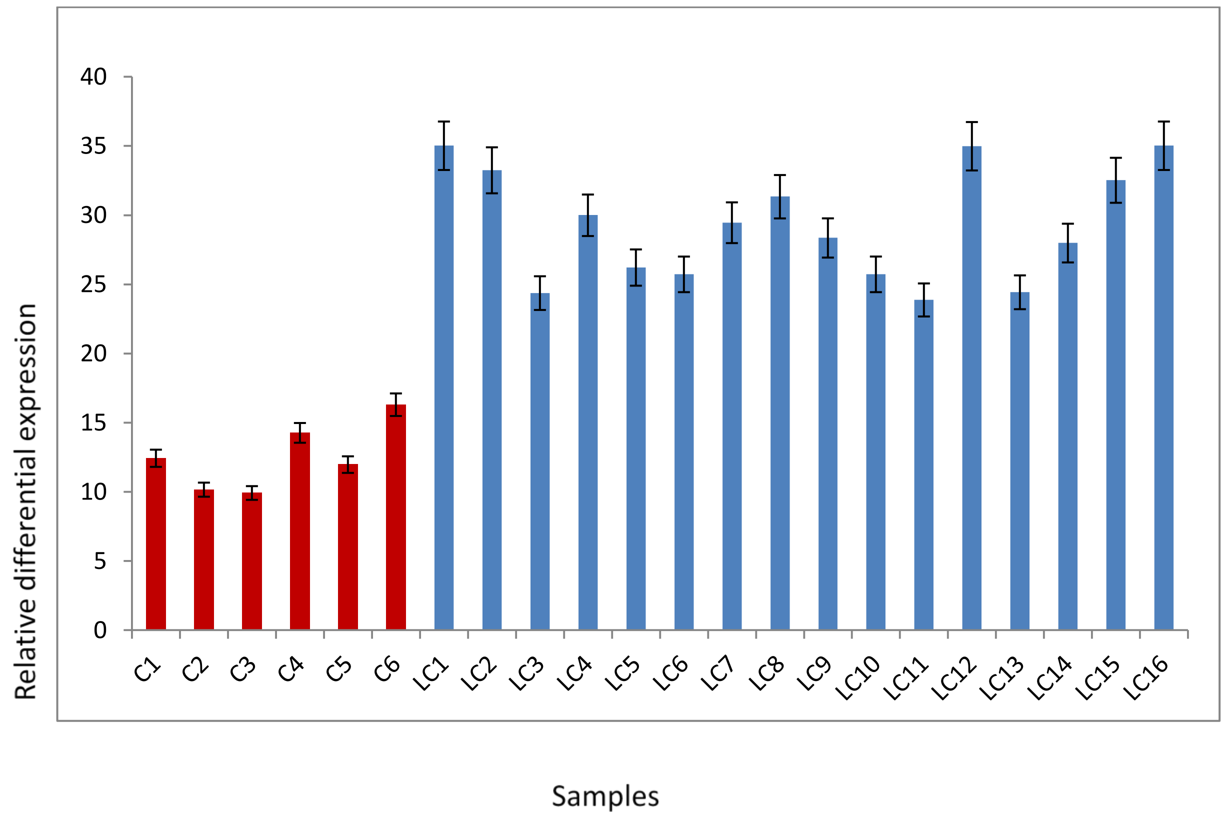
Graph 3



Graph 4

From the above data it was concluded that miRNA 127 showed maximum differential expression hence it was selected for further study.

<u>4. miR-127 Expression in Untreated Lung Cancer Samples in comparison to Control Samples</u>



Graph 5

5. Study of targets associated with miRNA 127

The human miR-127 gene is located within a cluster on chromosome 14q32.31. Huawei Jiang and colleagues have found that SEPT7 was targeted by miR-127-3p. SEPT7 is a member of the septin family gene and the members of this family can act as either tumor suppressors or promoters. The SEPT7 gene is located on human chromosome 7p14.3, is involved in several human diseases including neoplasia and neurodegenerative disorders. Previous data by Huang and colleagues suggested that SEPT7 is a tumor suppressor and has been shown to inhibit the migration and invasion of GBM cell lines. To establish the functional connection between miR-127-3p and SEPT7, Huawei Jiang and colleagues over-expressed SEPT7 and miR-127-3p in LN229 cells and found that SEPT7 protein could partly suppress the function of miR-127-3p. Therefore, SEPT7 protein might be a mediator of the regulation of cell migration and invasion by miR-127-3p [15].

CONCLUSION

From the results obtained and literature studies, we observe that miR-127 is over-expressed in lung cancer. Also, literature studies have proved via animal model, luciferase assay, western blot analysis and microarray analysis that miR-127 targets the SEPT7 gene effects cell migration and invasion in glioblastoma. Thus we can conclude that miR-127 may be explored as a novel biomarker for predicting the migratory and invasive potential of cancerous cells or may be exploited as a therapeutic target.

Future Prospects

- Micro array studies to validate miRNA expression
- Cell culture studies using mimics/inhibitors
- Use of miR-127-3p as a novel biomarker

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STUDENT RESUME

I am currently pursuing B.Tech degree programme in biotechnology and will be completing the degree in June 20015from Jaypee University of Information Technology, Waknaghat, Solan (H.P.). My current CGPA is 8.5. I believe in continuous progress on both professional and personal fronts through all round skills. I wish to seek a career to utilize my skills and abilities and offer me a challenging environment, both innovative and flexible.

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