Expression Study of Long non-coding RNA SCAL1 and GAS6-AS1 in Lung cancer Cell line A549 Compared to IMR-90

Research Project submitted to the Central University of Punjab

For the Partial Fulfilment of

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In

Life Sciences (Specialization in Animal Sciences)

By

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May 2018

DECLARATION

I declare that the dissertation entitled **"Expression Study of Long non-coding RNA SCAL1 and GAS6-AS1 in lung cancer cell line A549 compared to IMR-90"** has been done by me, under the guidance of **Dr. Aklank Jain**, Associate Professor, Department of Animal Sciences, Central University of Punjab, Bathinda. No part of this thesis/dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

This certifies that dissertation entitled **"Expression Study of Long non-coding RNA SCAL1 and GAS6-AS1 in lung cancer cell line A549 compared to IMR-90"**, submitted by **Arifa PP** in partial fulfillment of the award of the degree of **Master of Science** to the Central University of Punjab.

The work has been carried out at Department of Animal Sciences, School of Basic and Applied Sciences, the Central University of Punjab under my guidance.

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Arifa PP

ABSTRACT

Title **: Expression Study of Long non-coding RNA SCAL1 and GAS6-AS1 in Lung cancer cell line A549 compared to IMR-90**

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Degree of which submitted **:** Master of Science

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Keywords **:** Long non-coding RNA, Gene expression, Lung cancer, Therapeutic targets, Tumour Suppressors, Oncogene

Lung cancer is the fatal type of cancer owing to maximum number death worldwide. Despite the advances in clinical and experimental setup lung cancer still is the deadliest type of cancer wherein survival rate is as low as 15% five-yearly. The reason being the lack of proper candidate molecule for prognosis and diagnosis prior to invasion and metastasis. But usually, cancers are detected at later stages. The past cancer studies and investigations and investigations about tumorigenesis mechanism mostly focused on protein-coding gene considering them as principal regulators of cancer and diseases. But evidence from numerous high throughput genomic platform indicates that 98% of the eukaryotic genome is transcribed to non-coding RNA. The non-coding RNAs are significant in the regulation of many major biological processes that impact

development, differentiation, and metabolism through different pathways. Non-coding RNA also plays a major role in cancer development and progression by influencing different cellular processes like proliferation, cell cycle progression, cell growth, and apoptosis. They also influence post-transcriptional gene regulation through controlling process like transport, splicing, transcriptional gene silencing, epigenetic gene expression, cell structure integrity etc. So we can assure that the long non-coding RNA and its altered expression play an important role in cancer etiology. In this project, we studied the expression of SCAL1 and GAS6-AS1 using qRT-PCR. The expression analysis shows that the long non-coding RNA is up-regulated (approximate 5-folds, P=0.000464) and GAS6-AS1 is down-regulated (approximate 4-folds, P=0.00378) in lung cancer cell line compared to control cell line. The melt curve analysis shows only one sharp peak for both SCAL1 and GAS6-AS1 and thereby indicates that there is only one specific primer binding and the primer dimer is not formed.

Arifa PP **Dr. Aklank Jain**

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LIST OF ABBREVIATIONS

S. No.	Full Form	Abbreviation
1.	Ribonucleic Acid	RNA
2.	Long non- coding RNA	lncRNA
3.	Messenger RNA	mRNA
4.	Millilitre	mL
5.	Quantitative Real-Time PCR	qRT-PCR
6.	Reverse Transcriptase Kit	RT-kit
7.	Deoxyribonucleic acid	DNA
8.	Complimentary DNA	cDNA
9.	Ethylene-diamine-tetra-acetic acid	EDTA
10.	Growth arrest-specific transcript 5	GAS5
11.	Smoke and cancer-associated lncRNA-1	SCAL1
12.	Growth arrest-specific 6 antisense RNA1	GAS6-AS1
13.	RNA polymerase	RNAP
14.	Single polypeptide nuclear RNA polymerase	spRNAP
15.	Polymerase Chain Reaction	PCR
16.	Diethyl pyrocarbonate	DEPC
17.	World health organisation	WHO
18.	International agency for research on cancer	IARC
19.	Non-coding RNA	ncRNA
20.	Long non-coding RNA	lnc

21	Micro RNA	miRNA
22	Nucleotides	nts
23	Base pair	Bp
24	Prostate cancer associated non-coding RNA transcript1	PCAT-1
25	HOX transcript antisense RNA	HOTAIR
26	Metastasis-associated lung adenocarcinoma trancript1	MALAT1
27	Maternally expressed gene 3	MEG3
28	Room temperature	RT
29	Microliter	µL
30	Rotation per minute	Rpm
31	Nanometre	Nm
32	Microgram	µg

CHAPTER 1

INTRODUCTION

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells (American cancer society, 2017). If the spread is not controlled, it can result in death. Cancer causes include lifestyle factors (external) such as tobacco use and non-modifiable (internal) factors, such as inherited genetic mutations, hormones, and immune conditions. These risk factors may act simultaneously or in sequence to initiate and/or promote cancer growth.

1.1 Worldwide cancer statistics for the most common cancers

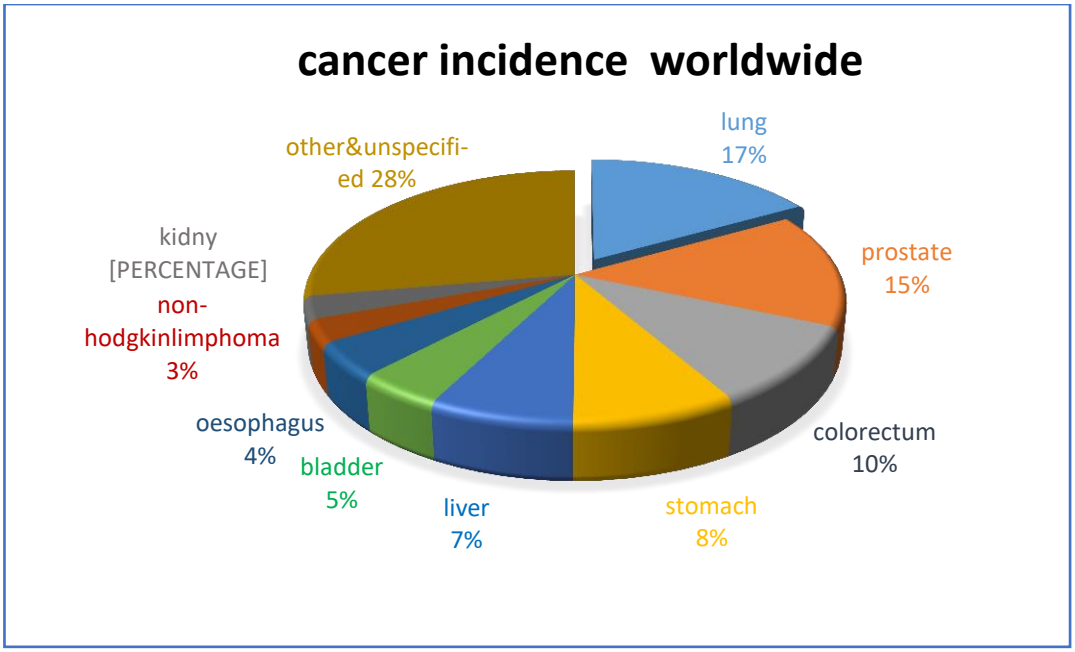


Figure 1.1: Estimated cancer incidence rates worldwide (Ref: The international Agency for Research on Cancer 2012)

The international agency for research on cancer estimated the worldwide incidence and mortality of cancer. Overall, there were 14.1 million new cases and 8.2 million deaths in 2012 (cancer statistics 2016). The most diagnosed and most causes of cancer death was lung cancer (1.82 million and 1.6 million respectively). In India, also

the incidence and mortality dramatically increased. Despite advances in clinical and experimental setup, lung cancer remains the leading cause of cancer and death; with an overall five-year survival rate is only 15%.

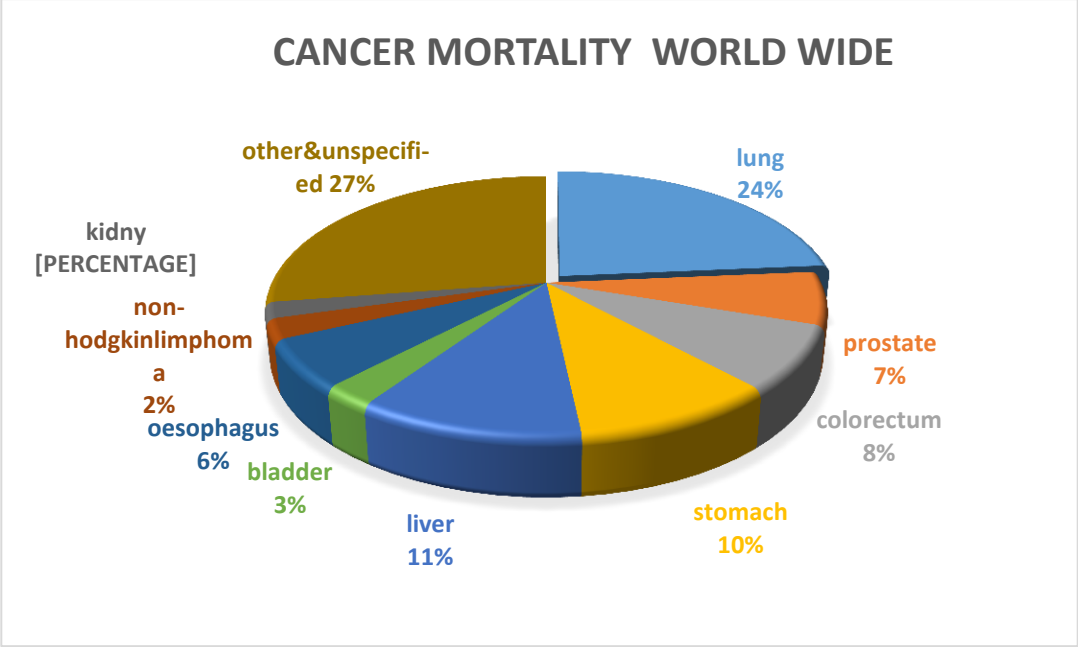
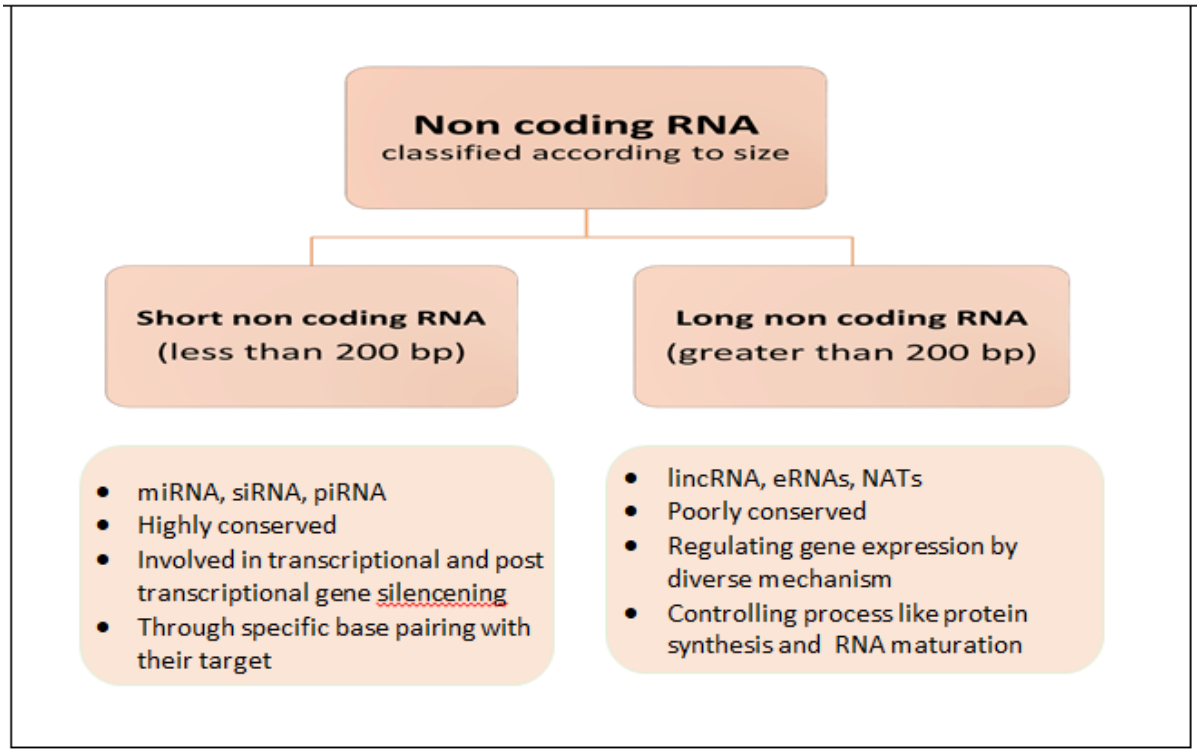


Figure 1.2: Estimated cancer mortality rates worldwide (Ref: The international Agency for Research on Cancer 2012)

Cancer is a dynamic and complex disease, which involves changes at the structural and functional level of the gene. Lung cancer occurs when gene expression goes awry leading to the mutational epithelial cell in response to exposure to different stresses. In the past, cancer studies and investigation into the mechanism of tumorigenesis mostly focused on protein-coding genes, considering them as the principal regulators of diseases and cancers. But, Mattick in 2004 enlightens that, evidence from numerous high throughput genomic platforms suggest that the evolution of developmental processes regulating the complexity of an organism is mainly due to the expansion of regulatory potential of the non-coding protein of the genome (Mattick, 2004). Recent advances in transcriptome studies also indicate that

98% of the eukaryotic genome is transcribed to non-coding RNA (Rinn jl *et al*, 2016) while only 2% is believed to be transcribed and translated into proteins. Indeed the recent explosion in knowledge demonstrating the importance of ncRNA in the regulation of multiple major biological processes impacting development, differentiation and metabolism have brought these heretofore neglected molecular players to the forefront (Mercer et al., 2009).



. Figure1.3: Classification of non-coding RNA

Based on size, Non-coding RNAs are mainly categorized into two types, small non-coding RNAs, and Long non-coding RNAs. Small non-coding RNAs include microRNAs, snoRNAs, piRNAs, etc. These are shorter than 200nts and long non-coding RNAs(lncRNAs) exceeding 200nts.

These highly transcribed long non-coding RNA might be having the major roll in cancer development and progression by involving different pathways. Former studies have demonstrated that lncRNA are capable of influencing different cellular process like proliferation, cell cycle progression, cell growth and apoptosis (Han et al, 2013)

and also involves post-transcriptional gene regulation through controlling process like transport, splicing, transcriptional gene silencing through regulating chromatin structure, epigenetic gene expression, cell structure integrity etc. Because of their roles in the regulation of multiple molecular pathways, we can assure that, the long non-coding RNA and its altered expression playing an important role in cancer etiology. For example, the lncRNA PCAT-1 (prostate cancer-associated ncRNA transcript 1) is overexpressed in prostate cancer (Han et al., 2013) and expression levels of HOTAIR (HOX transcript antisense RNA), MALAT1 (metastasis associated lung adenocarcinoma transcript 1) are found to be up-regulated in colorectal, lung, breast, gastric, and bladder cancers. And some have seen down-regulated like such as MEG3 (maternally expressed gene 3), lincRNA-p21, GAS-5 (growth arrest-specific transcript 5).

OBJECTIVES OF THE STUDY

1. Isolation of total RNA from lung cancer cell line A549 and control cell line IMR-90
2. Expression study of long non-coding RNA SCAL1 and GAS6-AS1

CHAPTER 2

REVIEW OF LITERATURE

Long non-coding RNA (lncRNA) defined as transcribed RNA molecules greater than 200nts in length – are poorly conserved and regulate gene expression by the diverse mechanism that is not yet fully understood (Bernstein *et al*., 2005).

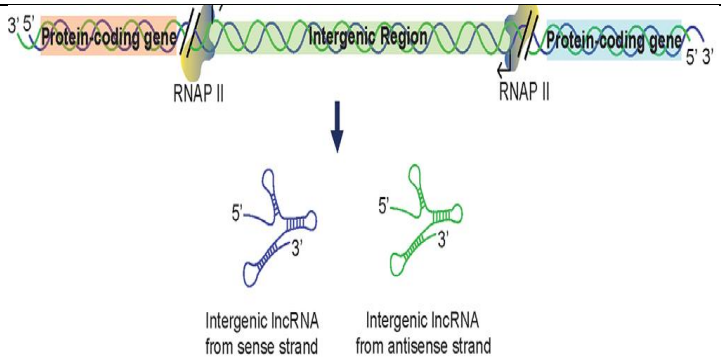
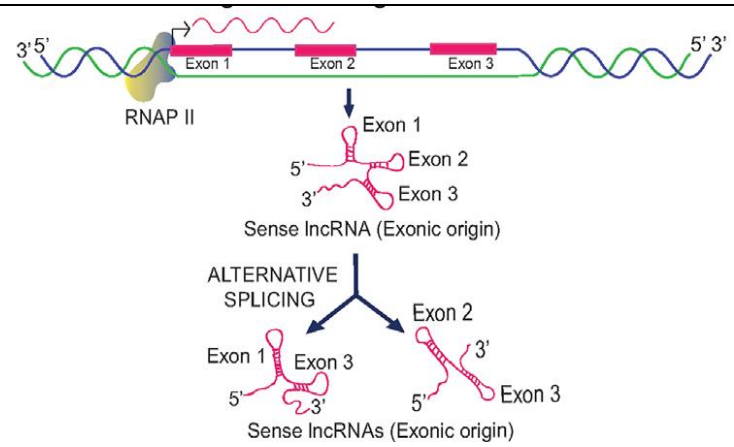
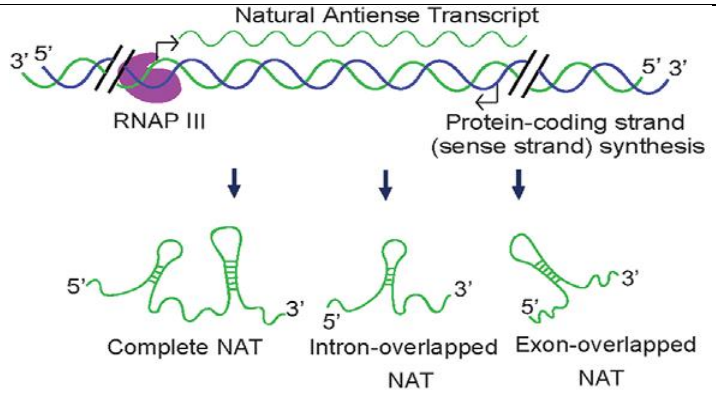
2.1 Biogenesis of long non-coding RNA

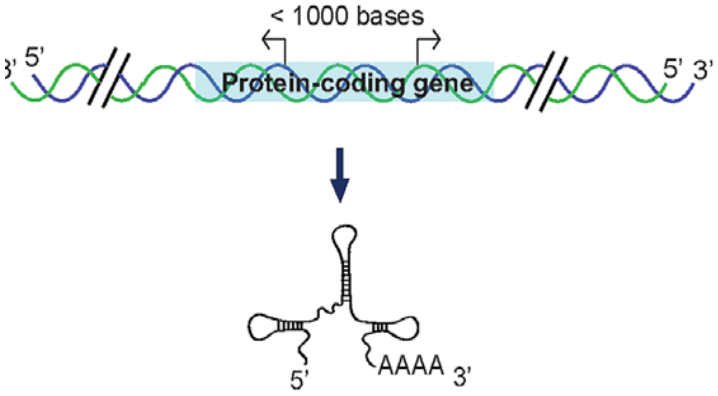
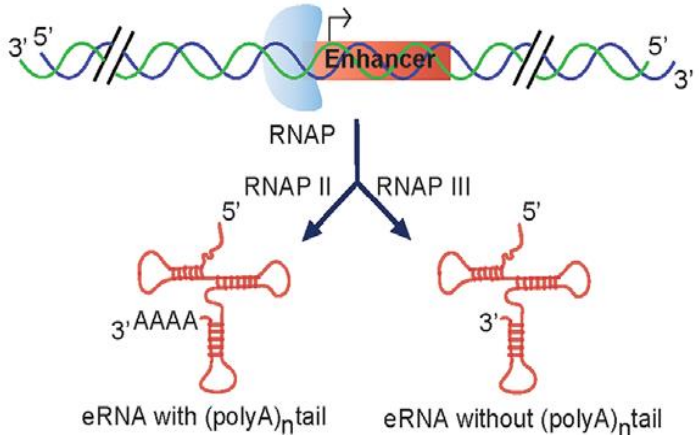
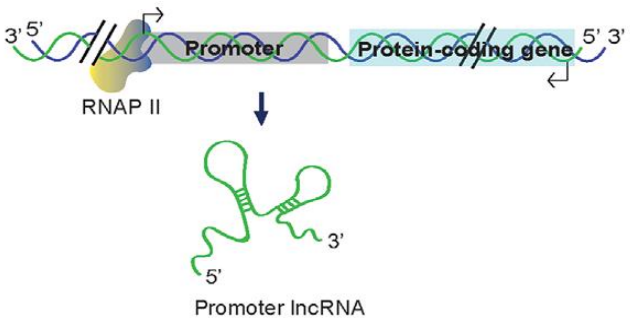
Previous studies indicate that most lncRNAs are synthesized by the RNAP II (RNA polymerase II) complex, similar to protein-coding RNAs. Also, some lncRNAs are transcribed by RNAP III and spRNAP IV (single-polypeptide nuclear RNA polymerase IV) (Khandelwal *et al*., 2016). The lncRNA transcriptional process shares a considerable number of features with mRNA transcription, including 50-capping, a multi-exonic structure, 30-polyadenylation, normal and alternative splicing mechanisms, RNA editing processes, and patterns of transcriptional activation. In some cases, transcripts may undergo post-transcriptional cleavage, whereby exposure of a cryptic 30-poly (A) tail engages in hydrogen bonds with two single-stranded poly (U) tracts to form a triple-helix that serves to protect the mature molecule against nucleolytic cleavage. Recently, it has been reported that some lncRNAs are also transcribed from the mitochondrial genome (Khandelwal, Bacolla et al. 2015)[i]

Long non-coding RNAs are mainly classified to five With respect to the genomic loci in which lncRNAs are transcribed. That are,

1. Intergenic long non-coding RNA
2. Intronic long non-coding RNA.
3. Sense long non-coding RNA
4. Antisense long non-coding RNA and
5. Bidirectional long non-coding RNA

Table 2.1 The five different groups of long non- coding RNA and their biogenesis with diagrammatic representation.

Long non-coding RNA	Biogenesis	Pictorial representation
Linc RNA **(Long intergenic RNA)**	Synthesized by RNAP2 from the intergenic region in both sense and antisense orientations between two protein-coding gene	
Exonic or sense long non-coding RNA	By RNAP 3, from the exonic sequence of protein-coding genes and processed via alternative splicing mechanism	
Natural antisense transcripts NATs)	Synthesized by RNAP III from the antisense strand of protein-coding genes, and give rise to three different forms	

Bi-directional long non-coding RNAs	Synthesized from sequences in close proximity (<1000 bases) to the transcription start sites of protein-coding genes but proceed in the opposite direction	
Enhancer long non-coding RNA (eRNAs)	Synthesized by RNAP II from the upstream enhancer region of protein-coding genes to form eRNAs with a 30-poly(A) tail, / by RNAP III without a 30-poly (A) tail modification.	
Promoter long non-coding RNAs	Synthesized from the promoter region of protein-coding genes by RNAP II and regulate the expression of the associated Protein-coding gene.	

Source of pictures: Khandelwal *et al*.,*Molecular carcinogenesis,* 2015

Involvement of Long non-coding RNAs in Cellular Activities

New studies suggest that lncRNAs show numerous regulatory functions at various stages throughout the lifespan, such as gene silencing through genetic imprinting, regulation of the cell cycle, splicing, translation, chromatin modification,

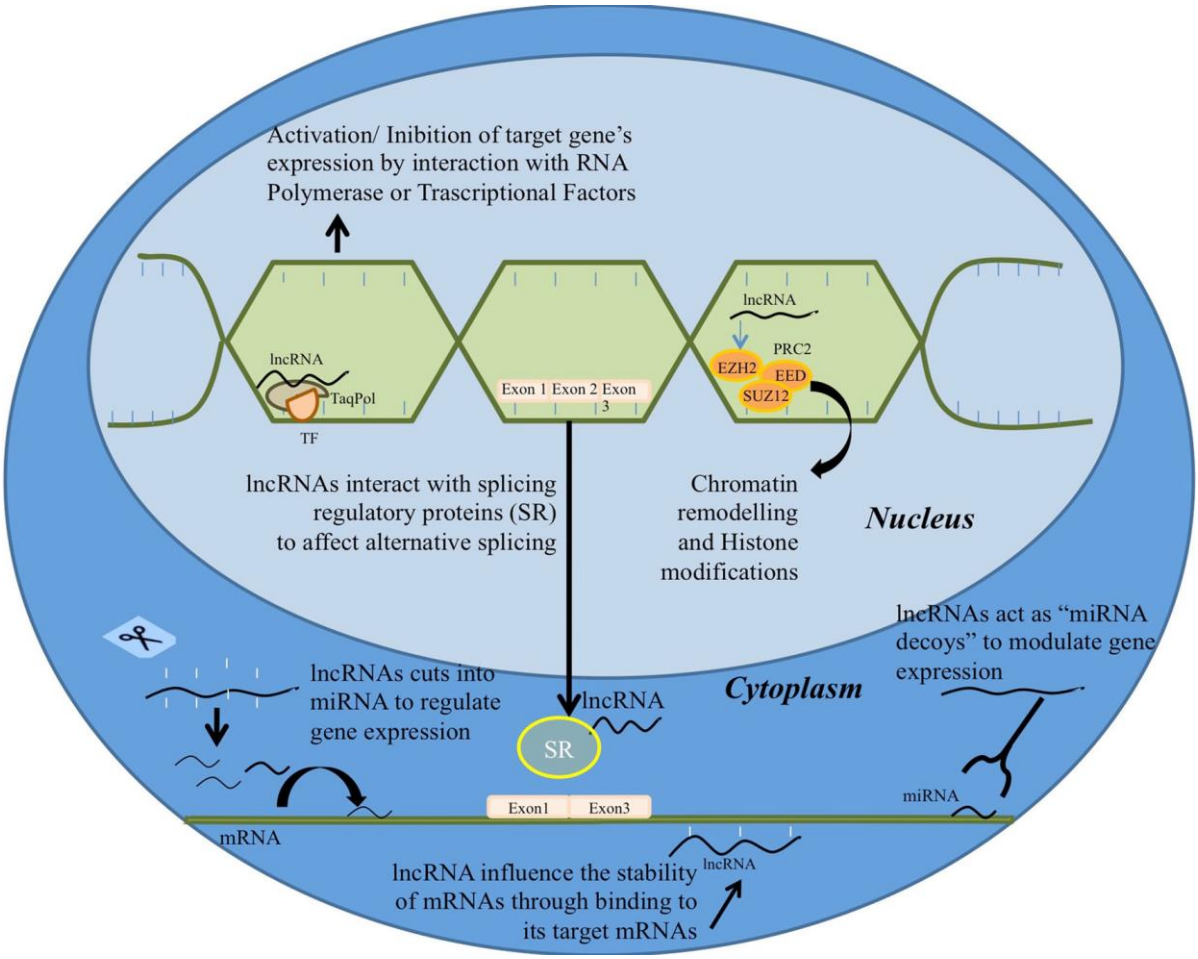


Fig. 2.1: Molecular mechanisms of lncRNAs (Ricciuti *et al.,* 2016)

Transcriptional interference, transcriptional activation, development and differentiation, proliferation, disease progression, and metastasis and invasion. Some of the most relevant cellular regulatory activities are

- LncRNAs as Signaling Molecules

- LncRNAs as Guiding Molecules
- LncRNAs as Scaffolding Molecules
- LncRNAs as Molecular Decoys

Long non-codingRNA as signaling molecule

lncRNAs control the transcription process by binding reversibly to different transcriptional components, such as RNAP II and transcription factors.

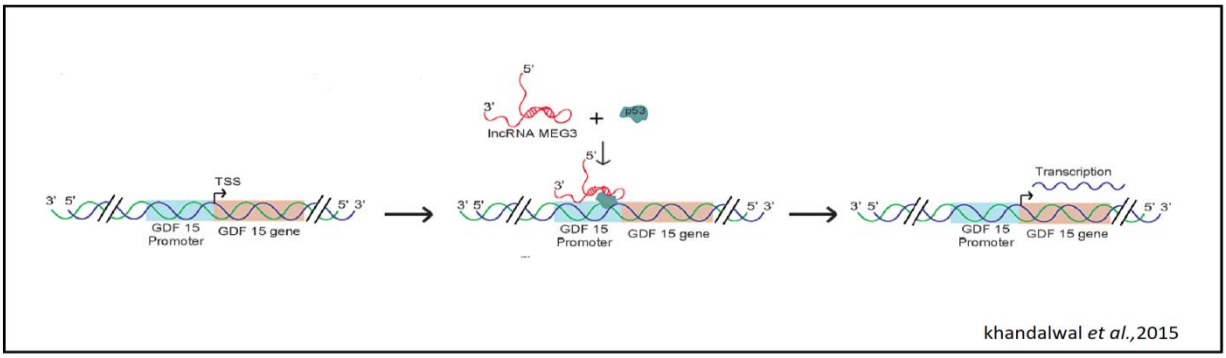


Fig: MEG3 recruits the p53 transcription factor to drive transcription of the growth differentiation factor 15 (GDF15) gene, which in turn inhibits cell proliferation in cancer cells

Long non-coding RNAs as guide molecules

They act as guiding molecules of chromatin enzymes, which are then epigenetically modified and delivered to specific genomic sites.

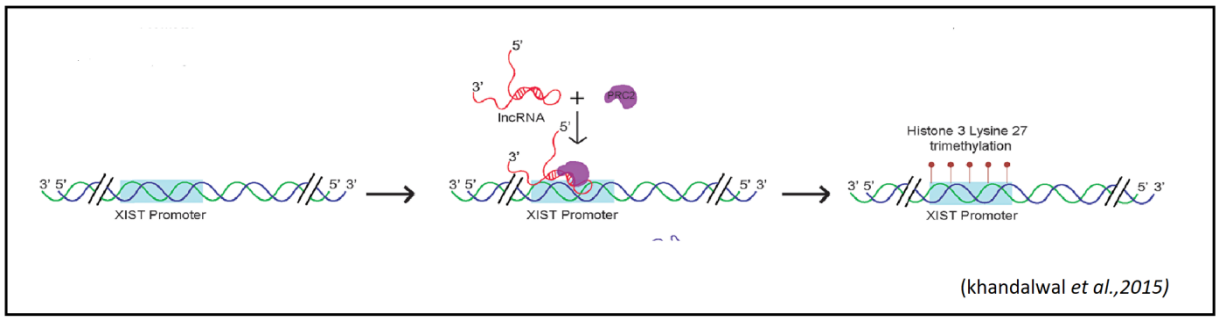


Fig: lncRNAs recruit polycomb repressive complex 2(PRC2) to the XIST promoter site and stimulate suppression by trimethylation of histone H3 at lysine 27 residues.

Long noncoding RNAs as scaffold molecules

Different domains of lncRNAs bind to distinct effector molecules and their partners, achieving either transcriptional activation or repression in time- and space-restricted manners

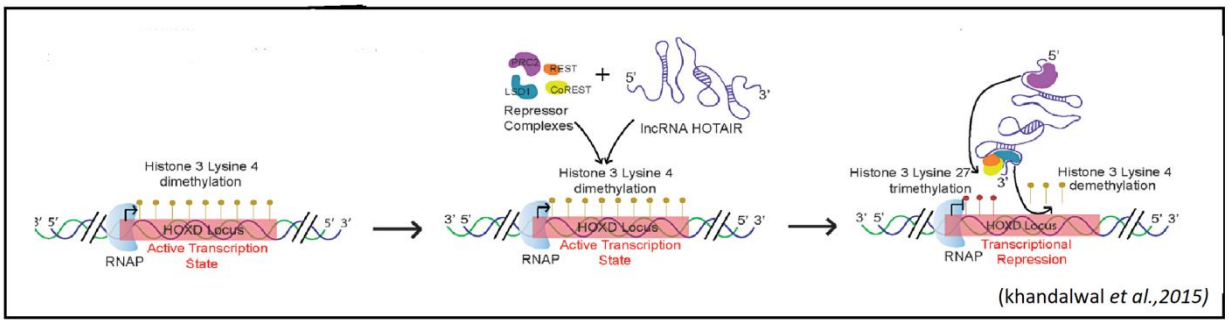


Fig: HOTAIR binds to PRC2 at its 50-end to trimethylate histone H3 at lysine 27 residues and to the transcriptional co-repressor complex LSD1/CoREST/REST at its 30-end to demethylate histone H3 at lysine 4 residues at the HOXD locus, thereby repressing transcription.

Long non-coding RNAs as molecular decoys

Elicit both positive and negative transcriptional regulation, by mimicking the target-binding site of an effector molecule on DNA or RNA.

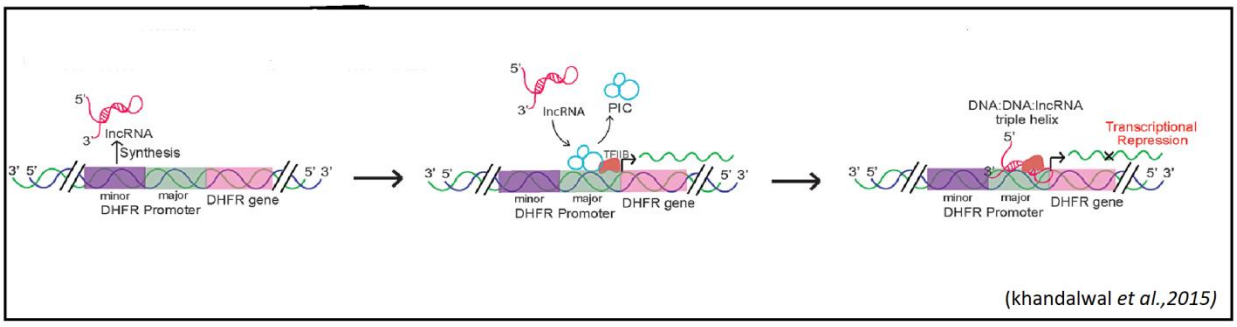


Fig: A lncRNA synthesized from the upstream minor promoter of the dihydrofolate reductase (DHFR) gene decoys the pre-initiation complex (PIC) assembly to the downstream major promoter region through DNA:DNA:lncRNA triple-helix formation.

Miscellaneous Functions

In addition to the functions discussed above, lncRNAs are also involved in the regulation of miRNA-related gene expression. Specifically, lncRNAs have shown to sequester miRNA molecules and prevent them from binding to their target mRNAs, thereby enhancing translation. In the absence of competition by lncRNAs, miRNAs bind to their complementary seed sequences available on target mRNAs, known as MREs (miRNA response elements), and inhibit translation.

Association of Long non-coding RNAs with Lung cancer

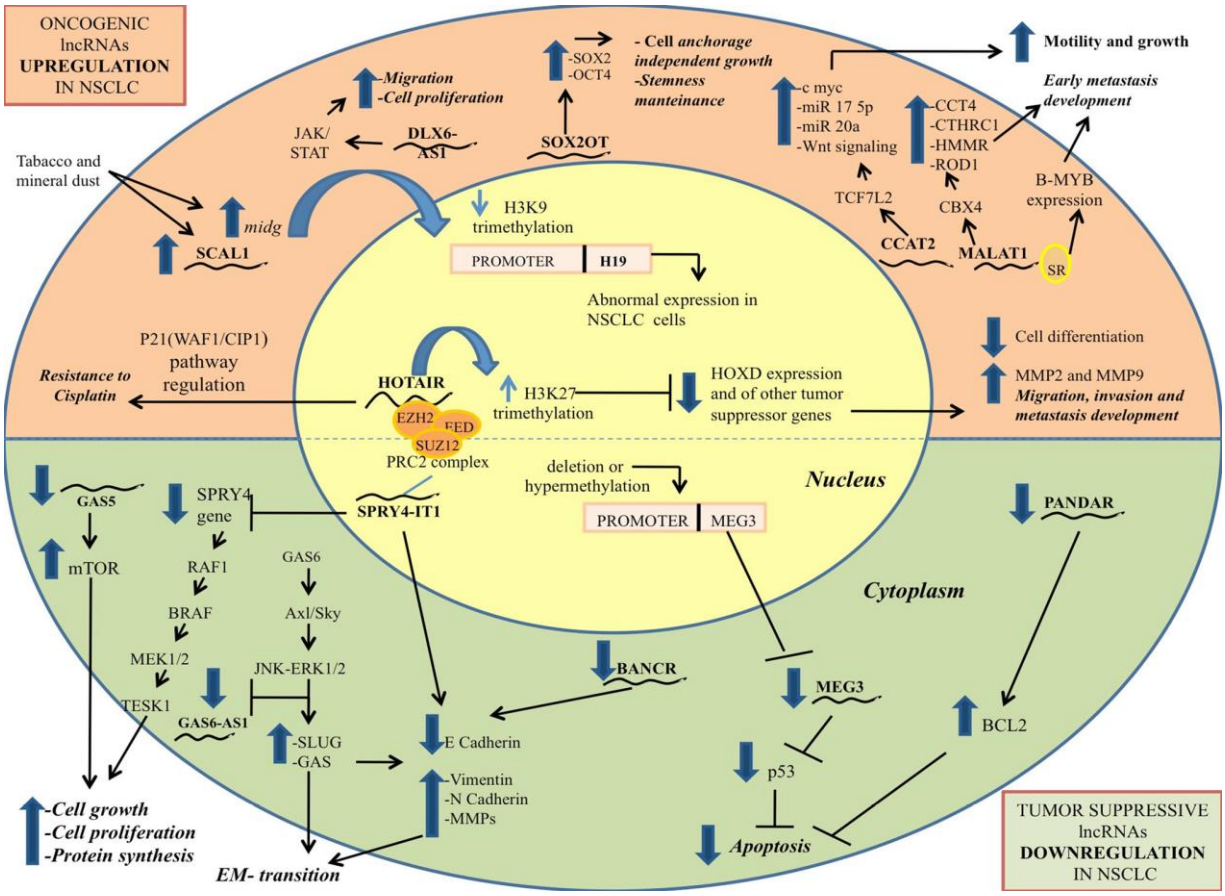


Fig.2.2: lncRNAs dys-regulation in NSCLC (Ricciuti *et al*., 2016)

As protein-coding genes, long noncoding RNAs can be classified into oncogenic lncRNAs, whose up-regulation enhances NSCLC cell growth, proliferation, migration, invasiveness and apoptosis inhibition, and tumour suppressive lncRNAs, which are conversely down-regulated in NCSLC, thus indirectly promoting the aforementioned processes and facilitating NSCLC development and progression

Table 2.2: Long-non coding RNA classified as onco lncRNA and tumour suppressor lncRNA

Onco lncRNA	Tumour suppressor lncRNA
SCAL1	GAS6-AS1
DLX6-AS1	PANDAR
SOX2OT	MEG3
CCAT2	BANCR
MALAT1	GAS5

SCAL1

LncRNA SCAL1, Smoking and Cancer-Associated lncRNA1 also known as lung cancer associated transcript 1 or LUCAT1. It is located on chromosome 5, between the intergenic region of two protein-coding genes, the GPR98, G-protein coupled receptor-98 and ARRDC3, arrestin-domain-containing-3. The SCAL1 gene contains four exons and three introns and is expressed in the metastatic-prone airway epithelial cells of lung cancer in response to exposure to cigarette smoke (Thai *et al* 2013). In vitro studies have shown higher SCAL1 expression in CL1-5, a metastasis-prone cell line, than in CL1-0, a non-invasive lung cancer-derived cell line, supporting a link between SCAL1 up-regulation and malignancy. SCAL1 is transcriptionally

regulated by the NRF2, nuclear factor erythroid 2-related factor (Thai *et al* 2013) as determined by siRNA knockdown of NRF2 and KEAP1 (Kelch-like ECH-associated protein 1). Under normal conditions, NRF2 is bonded in the cytoplasm with KEAP1. However, exposure of normal lung cells to either cigarette smoke or oxidative stress, or treatment of lung tumour cells with chemotherapeutic drugs, lead to NRF2 dissociation form KEAP1 and translocate to the nucleus, where it alters the expression of protective antioxidant genes and up regulates lncRNA SCAL1. An Increased SCAL1 expression is also observed after activation of EGFR, epidermal growth factor receptor, which is commonly found at high concentrations in lung cancer cell lines (Kubo *et al*., 2009). These findings support the activity of SCAL1 as a putative cytoprotector against cigarette smoke in airway epithelial lung cancer cells.

GAS6-AS1

The long non-coding RNA, GAS6-AS1 [growth-arrest-specific gene 6 (GAS6) antisense RNA 1], is located at 13q34 and is transcribed in the antisense direction relative to GAS6.

Table 2.3: Characteristics of GAS6 and GAS6-AS1

GAS6	GAS6-AS1
• Gene-induced in cells during growth arrest. • A Common ligand for Axl/sky tyrosin kinase family. • Increased level in many cancers • Influence JNK, ERK pathway and EMT, epithelial-mesenchymal transition	• Transcribed in the antisense direction with respect to GAS6 gene • Levels are irreversibly correlated with GAS6 • Lowered level in lung cancer • It might inhibit cancer progression by decreasing GAS6 mRNA expression

Its levels are inversely correlated with the GAS6 gene, whose product act as a ligand for the Axl/Sky family of tyrosine kinases which regulate the migration, invasion, and proliferation in many cancers by influencing EMT(Epithelial-Mesenchymal Transition).

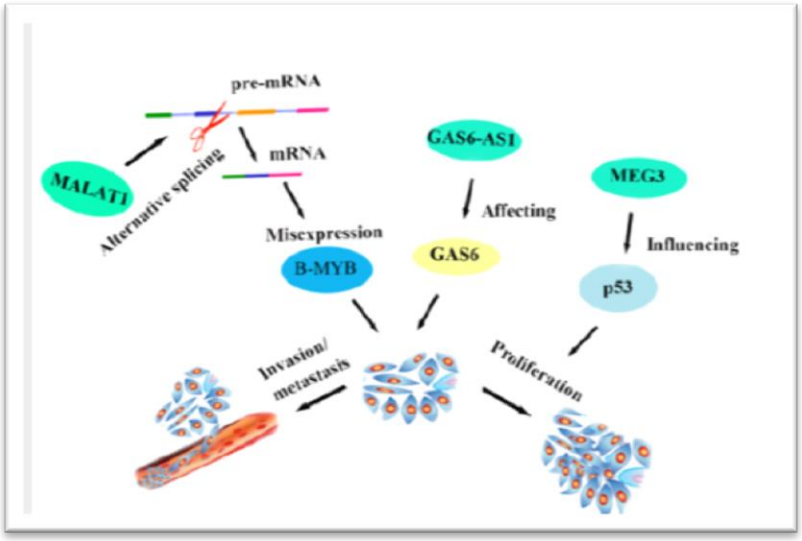


Fig 2.3: Involvement of GAS6-AS1 in invasion and metastasis (Chen *et al.,* 2014)

Han et al. reported that the expression of GAS6-AS1 was significantly down-regulated in NSCLC tissues compared with adjacent normal tissues and decreased GAS6-AS1 expression was negatively correlated with lymph node metastasis and advanced stages of tumour-node metastasis. But Levels of GAS6 were found to be higher in cancers like ovary, uterine endometrium, gliomas, and lung compared to normal tissues.GAS6-AS1 might inhibit cancer progression by decreasing GAS6 mRNA expression. GAS6-AS1 abundance also correlates inversely with clinico-pathological parameters, including the degree of lymph-node metastases, TNM staging, histological classification, and grading (low, middle or high) . In contrast, no correlations were found between levels of GAS6-AS1 and sex, age, or smoking history (Han et al.,2013). Although the study supports the role for GAS6-AS1 in the development and progression of NSCLC, further work is required to assess it is as a candidate molecule for prognosis and diagnosis as a biomarker for NSCLC.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Approach for Expression Analysis

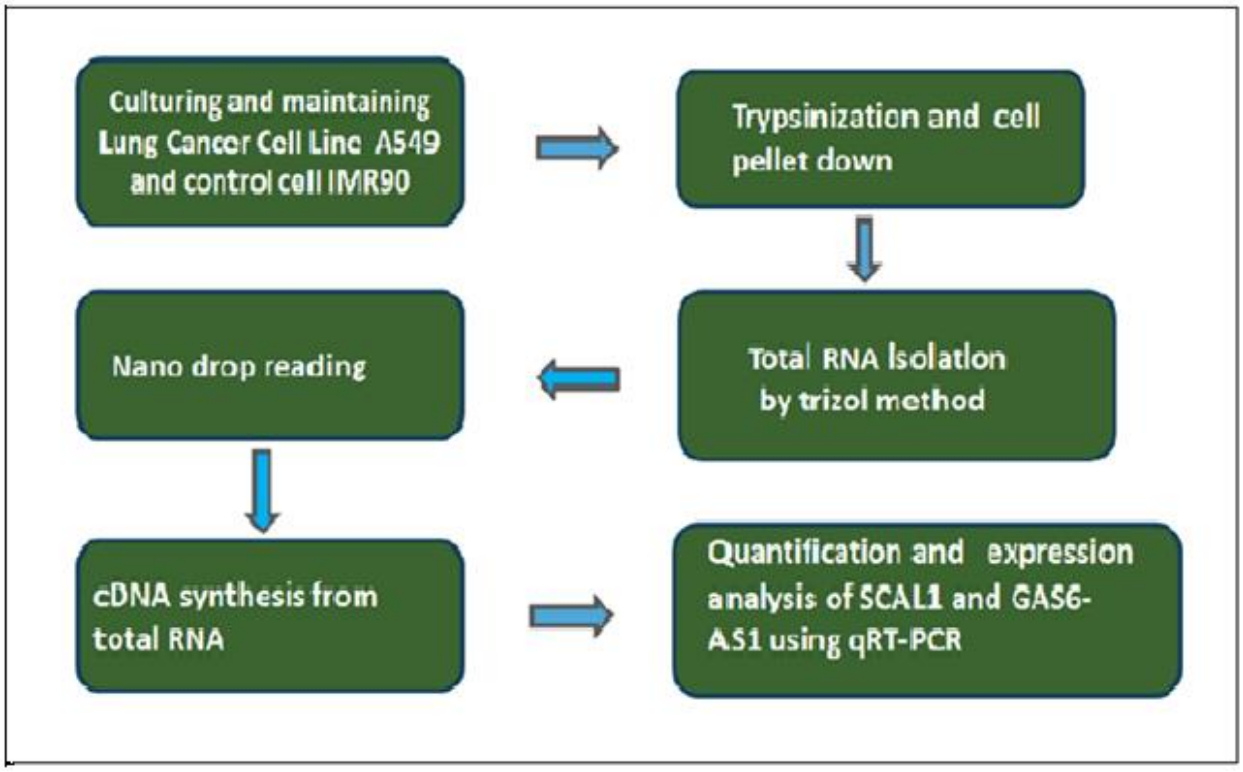


Figure 3.1: Schematic representation of experimental design for expression analysis

3.2 Materials used for the experiment

S. No	Materials used	source
1	Cell line A549	CDRI
2	RPMI media	Gibco
3	PBS buffer	Thermo scientific

4	Trypsin	Gibco
5	RT SYBR green ROX Tm qPCR master mix	Qiagen
6	Rt first strand kit	Qiagen
7	High-quality nuclease-free water	Qiagen
8	Optical flat8-cap PCR strips	Biorad

3.3 Cell culture processing

Trypsinization and cell splitting

The A549 cell line was established in 1972 by D.J. Giard, et al. (Giard et al., 1973). The cells originate from an explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. This adenocarcinomic human alveolar basal epithelial cell line has been used as a Type II pulmonary epithelial cell model. At about 70-90% confluence cells are detached from the flask by Trypsin/EDTA treatment, pelleted and reseeded cells in complete cell culture medium.

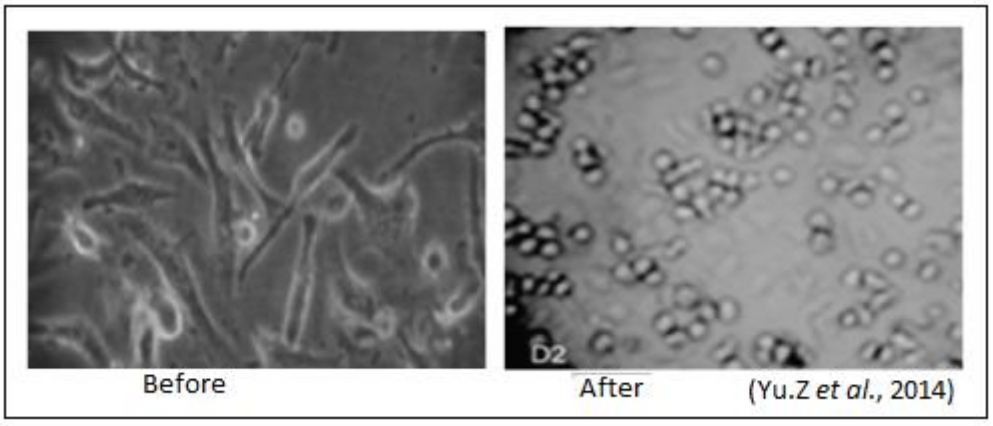


Figure 3.2: A549 cells before and after trypsinizations

Protocol

I. Sterilize fume hood working area.

II. Visualize cells under the microscope and estimate their confluency. Plates should have between 60-80% confluent in order to be split. Below 60% will not yield high numbers of cells and above 80%, the cells will have likely senesced and are no longer useful.

III. Aspirate media from each plate using the Pasteur pipette.

IV. Wash each plate with 2 mL of PBS. Wait for about 30 seconds before aspirating off PBS.

V. Add trypsin to the plate(s) to detach cells; 1 mL for single Petri dishes and 0.5 mL for individual wells in a six-well plate. Gently swirl each plate so that the trypsin completely covers the bottom surface.

VI. Incubate the trypsinized plates at 37° C for 2 minutes.

VII. Visualize cells to determine the extent of detachment. Cells should not be in large clumps and should move when the plate is gently nudged. If the cells are in clumps or still attached to the plate bottom, hold the plate in one hand and gently tap the palm of your other hand against the side of the plate. Visualize again to determine the extent of detachment. If cells are have still adhered to the plate, try incubating for another 1-2 minutes. The incubation period of cells mixed with trypsin should not exceed 5 minutes.

VIII. Add 5 micro litter media to deactivate trypsin. The amount of media added can be in 1:1, 1:2, or 1:3 ratios to the trypsin originally added.

IX. Take out 2 microliters in a polypropylene tube and centrifugation at 4 degree Celsius for 5 minutes.

3.4 Total RNA isolation from cell line A549

Trizol homogenization and extraction is a relatively recently developed general method for deproteinizing RNA. This method is particularly advantageous in situations where cells or tissues are enriched for endogenous RNases or when separation of cytoplasmic RNA from nuclear RNA is impractical. Trizol or TRI Reagent is a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously

solubilizes biological material and denatures the protein. Is an improvement to the single-step RNA isolation method developed by Chomczynskl and Sacchi. Guanidinium isothiocyanate is a powerful protein denaturant which inactivates all the proteins including RNases and simultaneously solubilizes the biological material. The acidic phenol present in the TRI reagent and chloroform addition in the subsequent step facilitates partitioning of RNA into aqueous supernatant for separation. Low pH is crucial since at neutral pH DNA but not RNA partitions into the aqueous phase. Therefore, it is advisable to check the pH of old Trizol/TRI reagents. After solubilisation, the addition of chloroform causes phase separation, much like extraction with phenol: chloroform: isoamyl alcohol, where the protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase. Therefore, RNA, DNA, and protein can be purified from a single sample hence, the name is TRIzol.

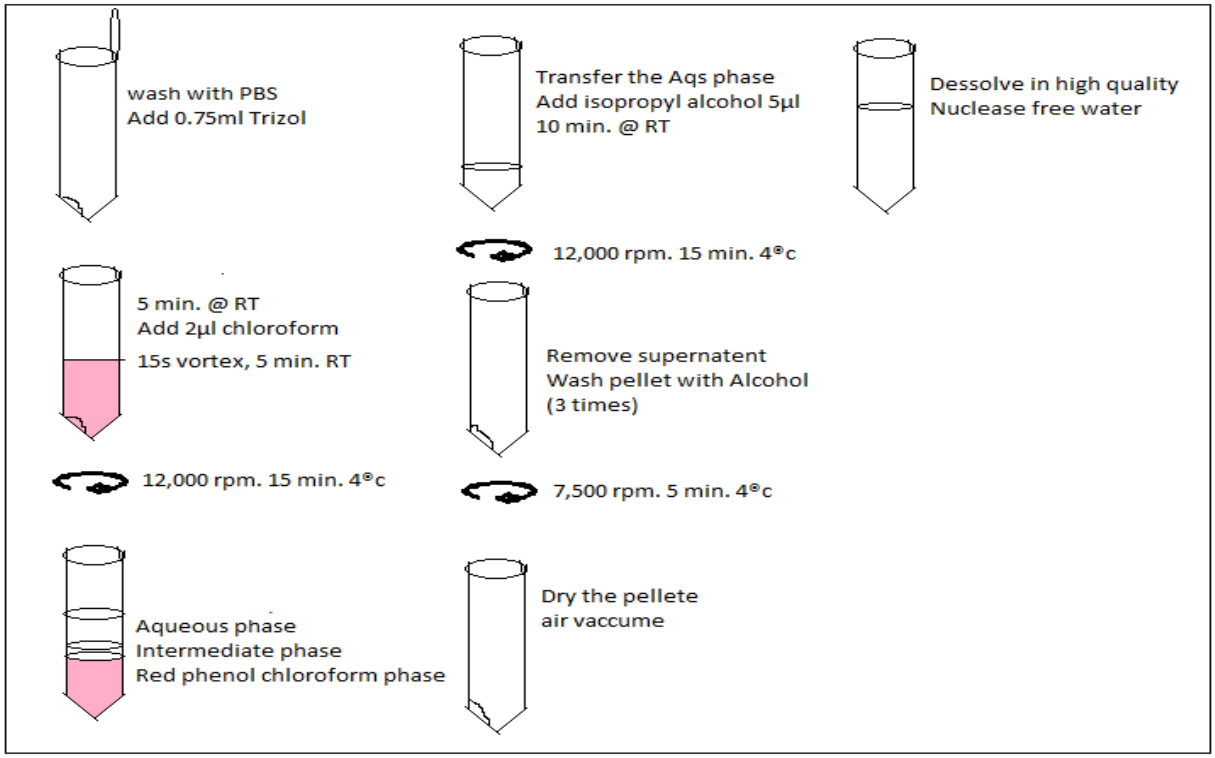


Fig.3.3: Sequential diagrammatic representation of total RNA isolation by Trizol method (made by using Paint).

Reagents required:

Trizol reagent, Chloroform, isopropyl alcohol, 75% Ethanol (in DEPC-treated water), DEPC-treated water (Add diethylpyrocarbonate (DEPC) to 0.01% (v/v) in distilled and stir for 3 h and autoclave).

Protocol:

1. Collect 20 micro litter cells in a polypropylene tube
2. Centrifuge at 4 degree Celsius for 5 minutes.
3. Pellet washed with 2 ml PBS
4. Centrifuge at 500g @ 4 degree Celsius for 5 minutes
5. Remove the supernatant
6. Add 250 micro litter PBS (now this sample volume)
7. Add 0.75 micro litter or 750 ml of Trizol LS Reagent in a sample
8. Incubate the homogenized samples for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per .75 ml of TRIZOL Reagent. Cap sample tubes securely. Shake tubes vigorously by hand or vortex for 15 sec and incubate them at room temperature for 5 min.
9. Centrifuge the samples at 12,000 rpm for 15 min at 4°C. Following centrifugation, the mixture separates into
    - A lower red, phenol-chloroform phase,
    - An interphase, and
    - A colourless upper aqueous phase.

    RNA remains exclusively in the aqueous phase which is 70%of the volume of the Trizol reagent used for homogenization.

10. Transfer the aqueous phase to a fresh tube by tilting the tue at 45 degree Celsius and pipetting out the solution.

Note: avoid draining interphase or organic layer into pipette when removing the Aqueous phase. Place the aqueous phase into a new RNA free tube and proceed to RNA precipitation

RNA precipitation

Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per .75 ml of TRIZOL Reagent used for the initial homogenization.

11. Incubate samples at room temperature for 10 min and centrifuge at 12,000 rpm for 15 min at 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. If the yield of RNA is low, the incubation step can be extended overnight at -20 °C followed by centrifugation.

RNA wash

12. Remove the supernatant. Wash the RNA pellet thrice with 75% ethanol, adding at least 1 ml of 75% ethanol per .75 ml of TRIZOL Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge 7,500 rpm for 5 minutes at 4°C. This step essentially removes all salts from the pellet.
13. At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Dissolve RNA in DEPC-treated water and store at -20 °C.

3.5 Nanodrop estimation of RNA purity and concentration

Quantification of RNA nucleic acid is done by UV absorption using a spectrophotometer. The absorbance is measured at 260 and 280 nanometres, in its simplest form. By using Beer-lambert law, the concentration of nucleic acid can be determined which predicts a leaner change in absorbance with concentration. The

concentration of RNA should be determined by measuring the absorbance at 260 nm (A260)

3.6 lncRNA Expression Analysis

The dis-regulated lncRNA expression under diseased condition can be assessed by lncRNA expression analysis of specific lncRNA in cell line versus healthy control by qRT PCR.

3.6.1 cDNA Synthesis

Quantitative reverse transcription PCR (RT-qPCR) is used when the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA or messenger RNA (mRNA). The cDNA is then used as the template for the qPCR reaction. The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA (cDNA).

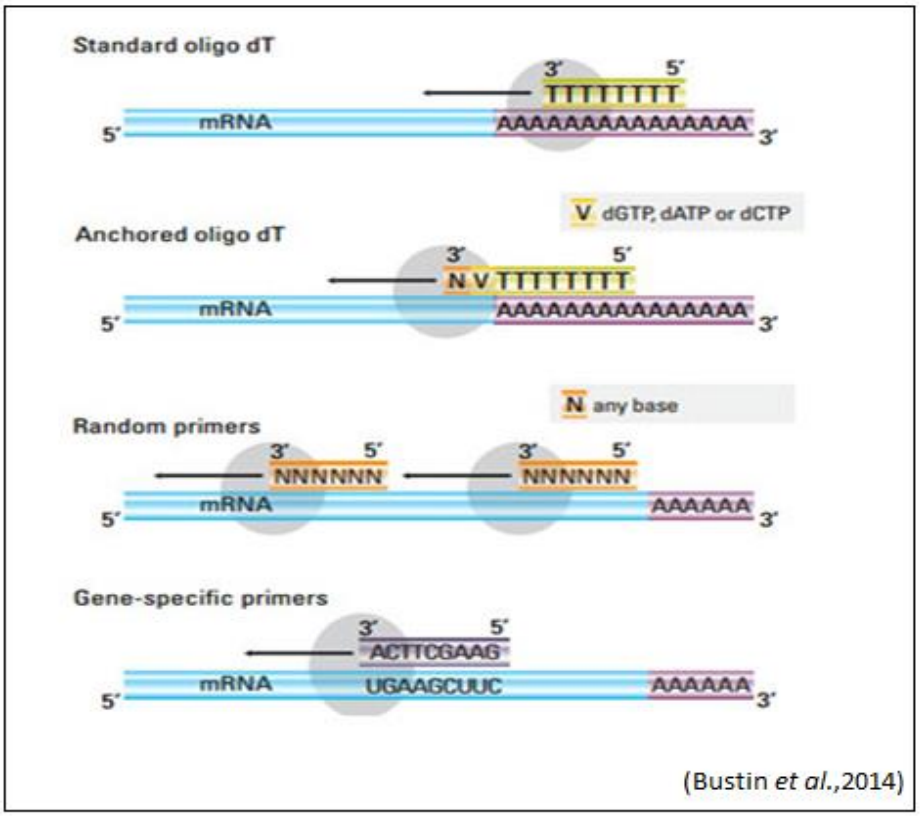


Figure 3.4: Four different priming methods of cDNA synthesis for the reverse transcription.

Reverse transcriptase (RTs) use an RNA template and a short primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, which can be used directly as a template for the Polymerase Chain Reaction (PCR). This combination of reverse transcription and PCR (RT-PCR) allows the detection of low abundance RNAs in a sample, and production of the corresponding cDNA, thereby facilitating the cloning of low copy genes. Alternatively, the first-strand cDNA can be made double-stranded using DNA polymerase I and DNA Ligase. These reaction products can be used for direct cloning without amplification. In this case, RNase H activity, from either the RT or supplied exogenously, is required

3.6.2 Choosing total RNA vs. mRNA

When designing an RT-qPCR assay it is important to decide whether to use total RNA or purified mRNA as the template for reverse transcription. mRNA may provide slightly more sensitivity, but total RNA is often used because it has important advantages over mRNA as a starting material.

DNA elimination mix

Component	Amount
RNA	5ng- 25ng
Buffer GE	2ng
Nuclease-Free water	Variable
Total Volume	10 microliter

Reverse transcription reaction components:

Component	Volume/Reaction
5x Buffer BC3	4 micro litter
Control P2	1 micro litter
RE3 Reverse transcriptase Mix	2 micro litter
Nuclease-Free Water	3 micro litter
Total volume	10 micro litter

Thermal cycler incubation conditions:

- 60 min at 37ºC
- 5 min at 95ºC

Protocol

1. Briefly centrifuge the reagents of the RT2 First Stand Kit (10-15 s) to bring the contents to the bottom of the tubes.
2. Prepare the genomic DNA elimination mix for each RNA sample in a sterile PCR tube according to Table 1. Mix gently by pipetting up and down and then centrifuge briefly. To perform a no-template control, omit the RNA and substitute with to 10 microliter Nuclease-Free Water.
3. Briefly centrifuge the reagents of the RT2 First Stand Kit (10-15 s) to bring the contents to the bottom of the tubes.
4. Prepare the genomic DNA elimination mix for each RNA sample in a sterile PCR tube according to Table 1. Mix gently by pipetting up and down and then

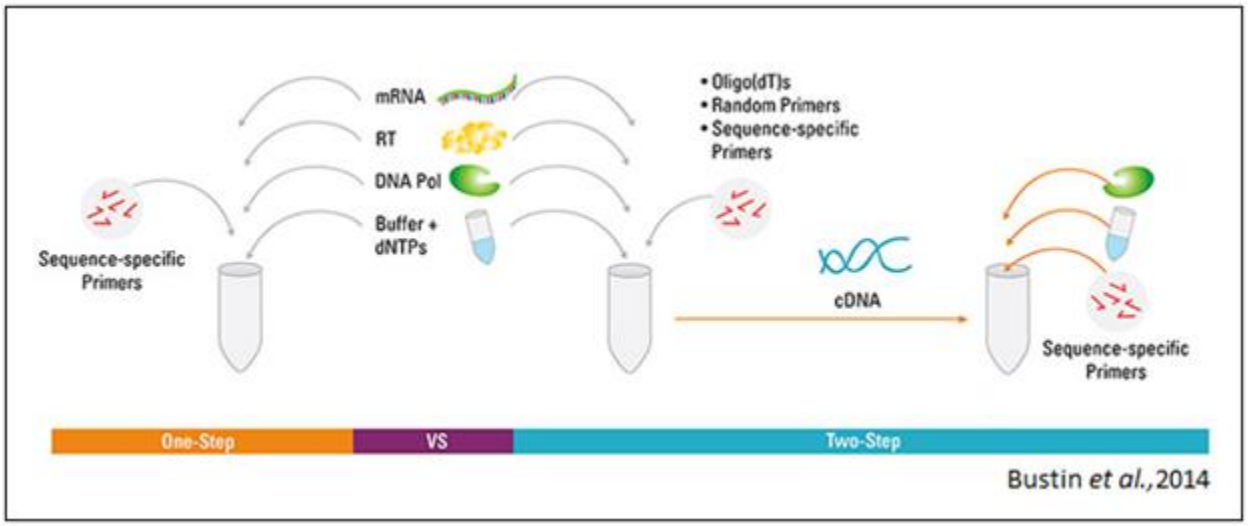
centrifuge briefly. To perform a no-template control, omit the RNA and substitute with to 10 microliter Nuclease-Free Water.

5. Incubate the genomic DNA elimination mix for 5 min at 42°C, then place immediately on ice for at least 1 min.
6. Prepare the reverse transcription mix according to Table 2.
   To prepare a no reverse transcription control, replace the RE3 Reverse transcriptase Mix with 2 microliter l nuclease-Free Water.
7. Add 10 microliter reverse transcription mix to each tube containing genomic DNA elimination mix. Mix gently by pipetting up and down.
8. Incubate at 37°C for 60 min. Then immediately stop the reaction by incubating at 95°C for 5 min.
9. Place the reactions on ice and add 91microlite Nuclease-Free Water to each reaction. Mix by pipetting up and down several times
10. Proceed with the real-time PCR protocol (store the reactions prior to real-time PCR, transfer them to a -20°C freezer).

3.6.3 Real-Time PCR

Real-time PCR is a highly sensitive and reliable method for gene expression analysis in multiple applications, such as the verifications of RNAseq and microarray data. Carefully designed primers specifically amplify genes of interest, overcoming the challenge of eliminating non-specific amplification due to the presence of thousands of genes in first strand cDNA, each potentially available as a PCR template. In addition, primers that provide efficient amplification are important to ensure accurate gene expression results from the commonly used Cq method, which requires a consistently high degree of amplification efficiency across all experiments. RT2 lncRNA qPCR assays are designed for SYBR green-based real-time PCR lncRNA detection.

One-step vs. Two-step RT-q PCR



. Figure 3.5: One-Step vs. Two-Step RT-qPCR

RT-qPCR can be performed in a one-step or a two-step assay. One-step assays combine reverse transcription and PCR in a single tube and buffer, using a reverse transcriptase along with a DNA polymerase. One-step RT-qPCR only utilizes sequence-specific primers. In two-step assays, the reverse transcription and PCR steps are performed in separate tubes, with different optimized buffers, reaction conditions, and priming strategies.

Table 3.4: Advantages and Disadvantages of two-step assays in RT-qPCR

Advantages	Disadvantages
• A stable cDNA pool is generated that can be stored for long periods of time and used for multiple reactions • The target and reference genes can be amplified from the same cDNA pool without multiplexing • Optimized reaction buffers and reaction conditions can be used for each individual reaction • Flexible priming options	The use of several tubes and pipetting steps expose the reaction to a greater risk of DNA contamination Time consuming Requires more optimization than one-step

Materials required

RT2 SYBR Green Masetmix, RT2 lncRNA qPCR Assay, Nuclease-free water, Template cDNA , RT-PCR tubes, Autoclaved pipette tips, Micropipettes, RNAase away, tissue papers

Table 3.5: Reaction setup for real-time PCR:

Component	Volume/Reaction
RT2 SYBR Green Masetmix	6.25sµl
RT2 lncRNA qPCR Assay	0.5µl
Nuclease-free water	5.25µl
Template cDNA	1.0µl

Table3.6: Cycling conditions for real-time PCR

Cycles	Time	Temperature	Additional comments
1	10 min	95ºC	HotStart DNA Taq Polymerase activated by this heating step
40	15s 1 min	95ºC 60 ºC	Perform fluorescence data collection

Thermal cycler incubation conditions for RT-PCR

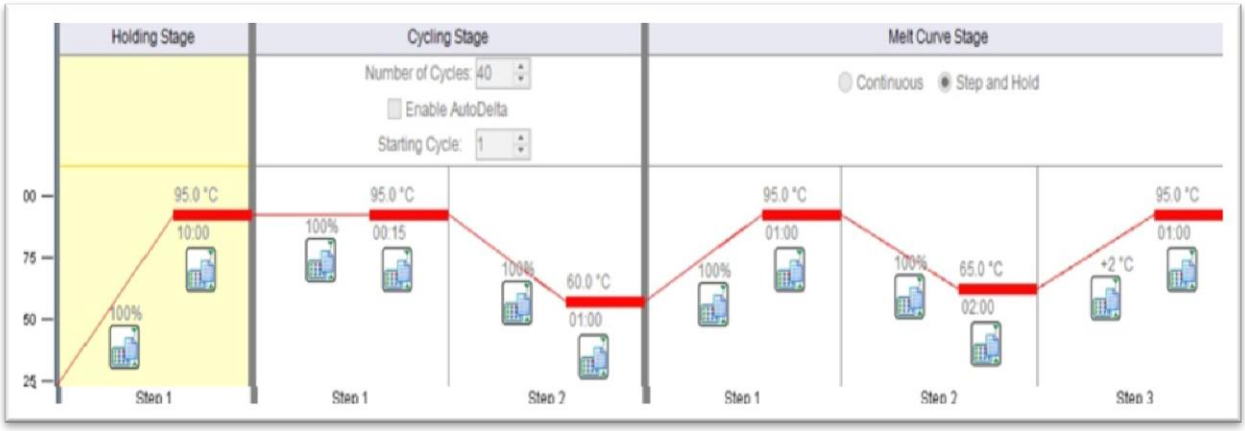


Fig 3.6: Three main stages of the RT-PCR thermal cycle

Protocol

1. Briefly centrifuge the RT2 SYBR Green Master mix, RT2 lncRNA qPCR Assay, and cDNA synthesis reaction (10-15 s) to bring the contents to the bottom of the tubes.

   As the RT2 SYBR Green Master mix contains Hot Start DNA Taq Polymerase that is active only after heat activation, reactions can be prepared at room temperature (15-25 °C).

2. Prepare the PCR components in a nuclease-free tube as described in Table 4.

3. Briefly centrifuge the PCR components mix and place the tubes into the real-time cycler.

4. Program the real-time cycler according to Table 5, 6, or 7, depending on the real-time cycler used. Run the program.

CHAPTER 4

RESULTS

4.1 RNA Concentration

Table 4.1 Nanodrop readings for isolated RNA of A549 and IMR90 samples

Samples	Concentration (ng/µl)	A260/A280	A260/A230
A549	118.4	1.70	0.26
IMR90	8.4	2.04	0.24

4.2 Results of qRT-PCR expression analysis (cT values)

	GAPDH	GAS6-AS1	SCAL1
A549	27.29 27.86 28.92 28.03 28.92 28.92 28.03 32.36	31.88 31.66 31.08 30.80 31.88 31.88 31.66 31.61	27.82 30.09 30.09 27.82 30.09 31.66
IMR 90	31.9 30.95 31.32 32.92 32.89 30.95 30.95 31.92 31.9	32.73 31.91 32.79 31.91 31.91 32.79 32.73	34.67 34.89 34.87 34.67 34.87

4.3 Melt curve of GAS6-AS1 and SCAL1

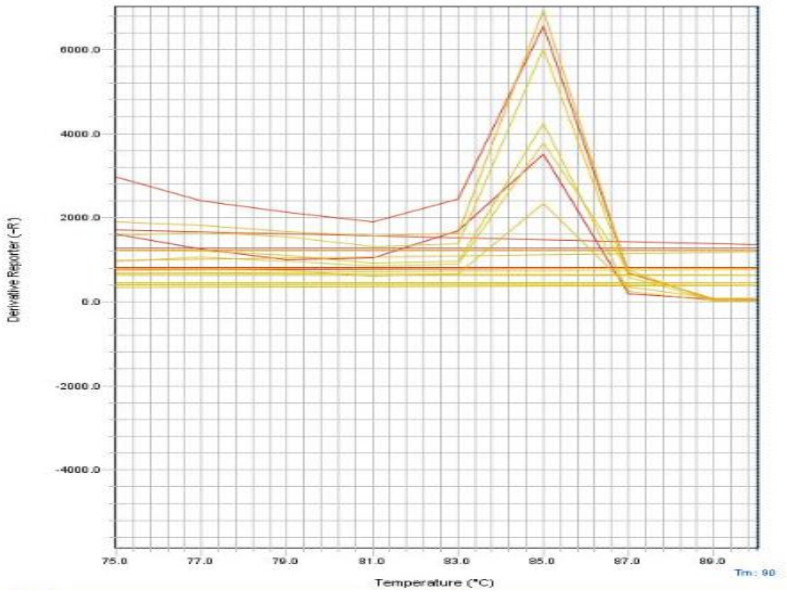


Fig 4.1 : One sharp peak of GAS6-AS1 indicates the specific primer binding

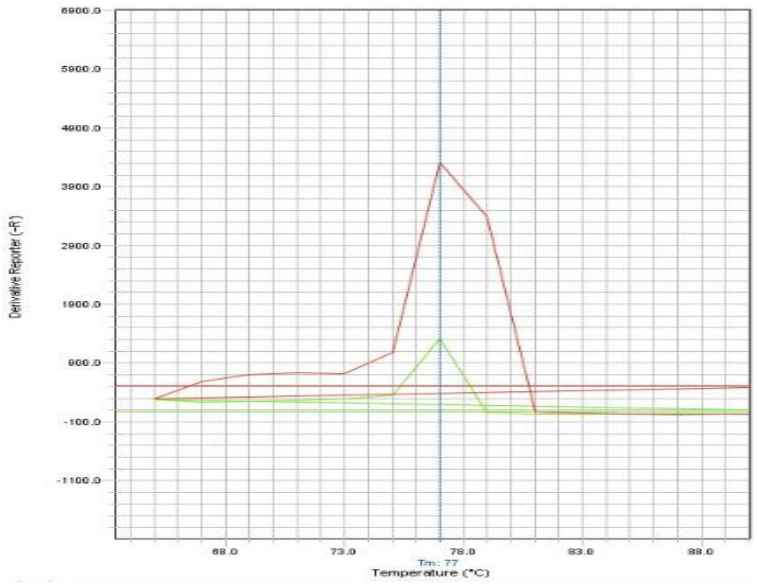
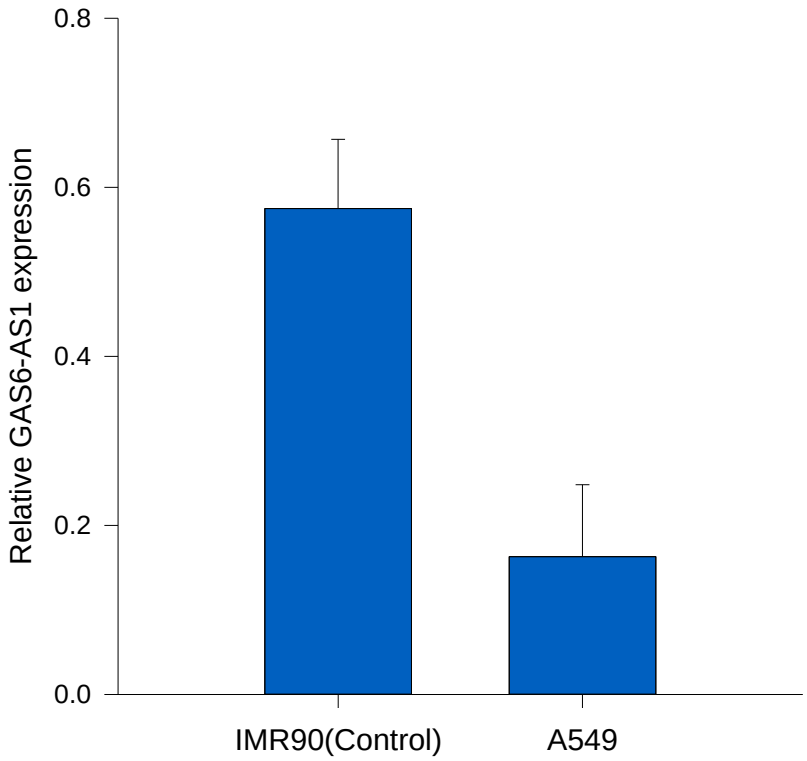


Fig 4.2: One sharp peak of SCAL1 indicates the specific primer binding

4.4 Graphical representation of Relative Expression of GAS6-AS1 in cancer cell line from control cell line



Graph1: Expression of GAS6-AS1 in A549 shows a 3.6-fold decrease compared with IMR-90.Statistically significant $P < 0.005$ (n=7). Error bar represent the SEM.

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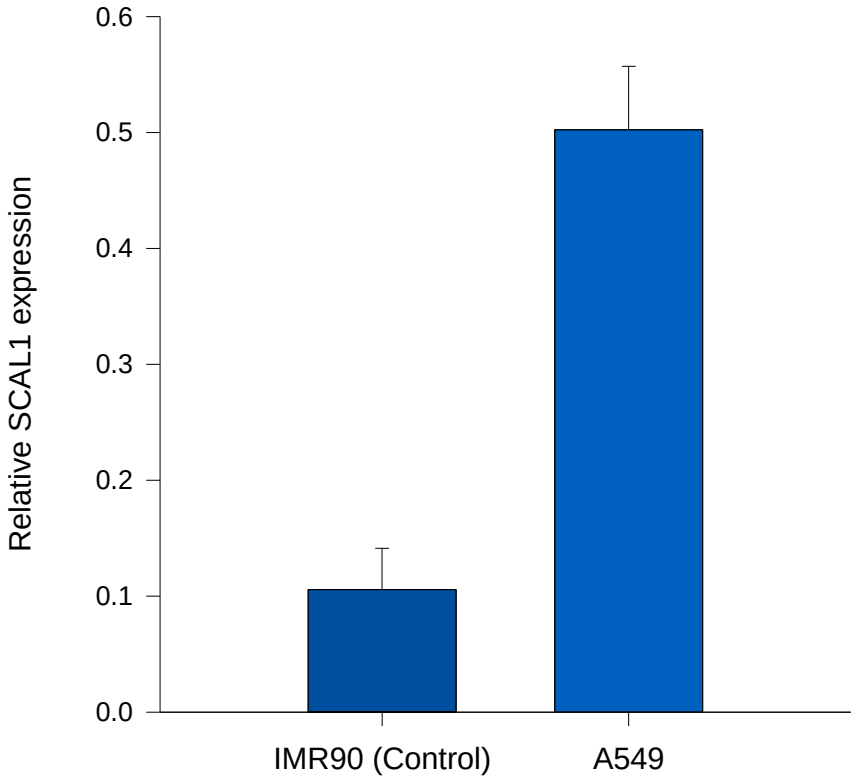
4.5 Graphical representation of Relative Expression of SCAL1 In cancer cell line from control cell line



Graph 2: Expression of SCAL1 in A549 shows a 4.8 -fold increase compared with IMR-90.Statistically significant P < 0.005 (n=7). Error bar represent the SEM.

CHAPTER 5

DISCUSSION

DISCUSSION

Expression analysis of SCAL1 by qRT-PCR shows that it is up-regulated (approximate 5-folds, P=0.000464) in lung cancer cell line compared to control cell line. And GAS6-AS1 is down-regulated (approximate 4-folds, P=0.00378) in lung cancer cell line compared to control cell line. From this data, we can say that SCAL1 might be an oncogene for lung cancer (needs further studies), and it may be used as a candidate molecule for lung cancer prognosis and diagnosis. GAS6-AS1 might be a tumour suppressor (further studies required) because it is down-regulated in lung cancer cell line than normal cell line and it may also be used as a candidate molecule for lung cancer prognosis and diagnosis.

CHAPTER 6

CONCLUSION AND FUTURE WORK

CONCLUSION AND FUTURE WORK

The project has been undertaken mainly on lack of understanding the role of SCAL1 and GAS6-AS1 as early prognostic and diagnostic biomarker in lung cancer through expression studies, with a view of contributing a little to the research society as to have an impact on the lives of people living with and suffering from lung cancer

Future prospects of the project include an extension of the current project in the following areas:-

- Study the biological role of both SCAL1 and GAS6- AS1.
- The pathway which they involve for cancer progression or cancer inhibition.
- Correlation with different stages of cancers.

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STUDENT APPROVAL FORM

Name of the author	Arifa PP
Department	Department of Animal Sciences
Degree	M.Sc. life sciences with specialization in Animal sciences
University	Central University of Punjab, Bathinda
Guide	Dr. Aklank Jain
Project title	Expression Study of Long non-coding RNA SCAL1 and GAS6-AS1 in lung cancer cell line A549 compared to IMR-90
Year of award	2018

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