Expression analysis of long non-coding RNA GAS5 and BANCR in lung cancer cell line A549 compared to IMR-90

Project report submitted to the Central University Of Punjab, Bathinda

For the award of

Master of Science

In

Life Sciences (Specialization in Animal Sciences)

By

Uttam Sharma

Supervisor

Dr. Aklank Jain

Department of Animal Sciences
School of Basic and Applied Sciences
Central University Of Punjab, Bathinda

May, 2018

DECLARATION

I, **Uttam Sharma**, declare that the project entitled **"Expression analysis of long non-coding RNA GAS5 and BANCR in lung cancer cell line A549 compared to IMR-90"** has been completed by me under the supervision of Dr. Aklank Jain, Associate Professor, Department of Animal Sciences, Central University of Punjab, Bathinda. No part of this project has formed the basis for the award of any degree or fellowship previously.

Uttam Sharma

Registration no - 16mslsas02
Department of Animal Sciences
School of Basic and Applied Sciences
Central University Of Punjab, Bathinda- 151001

Date:

CERTIFICATE

I certify that **Uttam Sharma** has completed and prepared his project entitled **"Expression analysis of long non-coding RNA GAS5 and BANCR in lung cancer cell line A549 compared to IMR-90"** under my guidance for the award of M.Sc. in Life Sciences (Specialization in Animal Sciences), Central University of Punjab, Bathinda,

Dr. Aklank Jain

Associate Professor

Department of Animal Sciences

School of Basic and Applied Sciences

Central University Of Punjab, Bathinda- 151001

Date:

ABSTRACT

Title:- Expression analysis of long non-coding RNA GAS5 and BANCR in lung cancer cell line A549 compared to IMR-90.

Name of Student : Uttam Sharma

Registration Number : 16mslsas02

Degree for which submitted : Master of Science

Name of Supervisor : Dr. Aklank Jain

Department : Animal Sciences

Name of School : School of Basic and Applied Sciences

Keywords : Long non-coding RNA, GAS5, BANCR, Quantitative Real- Time PCR, Melt curve

Lung cancer is the major cause of death worldwide. Several chemotherapeutic drugs and therapies have been established, but the early diagnosis and prognosis of lung cancer is still a question. Long non-coding RNAs are important regulator molecules in the human genome, which serves as transcriptional modulator, post transcriptional processor, chromatin remodeletor and splicing regulator during the gene modification process. Emerging studies have suggested the role of long non-coding RNA as potential biomarker for cancer diagnosis and prognosis by functioning as tumor suppressors and oncogenes. Several studies have been reported on cell lines, tissues as well as tumor and the molecular mechanism is still not clearly understood. GAS5 and BANCR are two long non-coding RNAs, which are found to be down-regulated in multiple cancers such as lung carcinoma, breast cancer, etc. In the current study, we focus on the expression analysis of GAS5 and BANCR in A549 cell line compared to IMR-90 cell line to study the role of long non-coding RNA in the pathogenesis of lung cancer. Furthermore, we investigated the expression of GAS5 and BANCR using quantitative Real-Time PCR. The result showed that GAS5 and BANCR expression was significantly down-regulated in cancerous cell line compared to non-cancerous cell line. The fold change of lncRNAs GAS5 and BANCR was 14 times (P=0.0088) and 7 times (P=0.0088) down-regulated in A549 cell line respectively. The melt curve analysis showed that there was only one sharp peak obtained for both GAS5 and BANCR

which suggests that primers bind to their specific targets and no primer dimer was observed.

Uttam Sharma **Dr. Aklank Jain**

ACKNOWLEDGEMENT

This project work was attainable only with the help of several people, and I would like to express my sincere regards to each one of them.

First and foremost, I owe my deepest gratitude to my guide, Dr. Aklank Jain, for his valuable guidance, scholarly inputs and consistent encouragement that I have received throughout my dissertation. I would like to thank him for his continuous support, scientific advice, insightful, suggestions and encouragement that he provided patiently to complete this project work. I consider it as an inordinate opportunity to accomplish my research work under his guidance and to learn from his expertise.

I offer my sincere respect to Dr. Anil Kumar Mantha, Head of the Department, Animal Sciences for providing research facilities to carry out this work.

I am fortunate to have a friendly and cheerful group of lab mates and classmates. I would like to thank them for their constant support throughout the project work. I especially thank Lovlesh Thakur, Akshay Malhotra and Akanksha Khandelwal for their endless support, encouragement and guidance. I want to give part of the credit to my project mate Arifa pp for her assistance and feedback.

The project completion was not possible without the constant words of encouragement of my family and friends. I am very thankful to them.

Uttam Sharma

TABLE OF CONTENTS

SERIAL NO.	CONTENT	PAGE NO.
1	List of Tables	vii
2	List of Figures	viii
3	List of Abbreviations	ix
4	Chapter- 1: Introduction	1-7
5	Chapter 2: Review of Literature	8-12
6	Chapter 3: Materials and methods	13-23
7	Chapter 4: Results	24-29
8	Chapter 5: Discussion	30-31
9	Conclusion and Future Aspects	32-33
10	References	34-41

LIST OF TABLES

Table no.	Table Description	Page no.
1	Participation of lncRNAs in the pathogenesis of various types of cancer	10
2	List of materials used for experimentation	15
3	Genomic DNA elimination mix for RNA isolated from A549 cell lines	19
4	Genomic DNA elimination mix for RNA isolated from IMR-90 cell lines	19
5	Reverse Transcription reaction components	20
6	Reaction mixture for Real Time PCR	21
7	Template designed for reaction	22
8	Standardized cycling conditions for Long non-coding RNA	22
9	Primer Sequence	22
10	Concentration of isolated total RNA	25

LIST OF FIGURES

Figure No.	Figure Description	Page No.
1	Location of Non-Small Cell Lung Cancer (NSCLC)	2
2	Estimated cancer incidence rates worldwide	3
3	Estimated cancer mortality rates worldwide	3
4	Diagrammatic representation of RNA types	4
5	Types of lncRNAs	5
6	Time scale of discovery of long non-coding RNAs	10
7	Experimental design for expression analysis	14
8	A549 cell line	25
9	Heat map showing change in gene expression of GAS5 and BANCR in cancerous and non-cancerous cell line	26
10	Bar diagram showing relative gene expression of GAS5 in A549 cell line compared to IMR-90 cell line	27
11	Melt curve of GAS5 showing specific binding of primer to the target.	28
12	Bar diagram showing relative gene expression of BANCR in A549 cell line compared to IMR-90 cell line	29
13	Melt curve of BANCR showing specific binding of primer to the target	29

LIST OF ABBREVIATIONS

Serial no.	Full Form	Abbreviation
1	**Ribonucleic Acid**	**RNA**
2	**Millilitre**	**ml**
3	**Microlitre**	**µl**
4	**Room Temperature**	**RT**
5	**Rotation Per Minute**	**RPM**
6	**Microgram**	**µg**
7	**Polymerase Chain Reaction**	**PCR**
8	**Quantitative Real Time PCR**	**qRT-PCR**
9	**Reverse Transcriptase Kit**	**RT-Kit**
10	**Nuclease-Free Water**	**NFW**
11	**Cycle threshold**	C_t
12	**Non- Small Cell Lung Cancer**	**NSCLC**
13	**Growth Arrest Specific Transcript 5**	**GAS5**
14	**BRAF- Activated Long Non-Coding RNA**	**BANCR**
15	**Long Non-Coding RNA**	**lncRNA**

Student approval form

Name of the author	Uttam Sharma
Department	Department of Animal Sciences
Degree	M.Sc. in Life Sciences with specialization in Animal Sciences
University	Central University of Punjab, Bathinda
Guide	Dr. Aklank Jain
Project title	Expression analysis of long non-coding RNA GAS5 and BANCR in lung cancer cell line A549 compared to IMR-90
Year of award	2018

Agreement

1. I hereby certify that, if appropriate, I have obtained and attached here to a written permission / statement from the owner (s) of each third party copyrighted matter to be included in my dissertation/thesis, allowing distribution as specified below.

2. I hereby grant to the university and its agents the non-exclusive license to archive and make accessible, under the conditions specified below, my dissertation/thesis, in whole or in part in all forms of media, now or hereafter known. I retain all other ownership rights to the copyright of the dissertation/thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis, dissertation, or project report.

Conditions:

1. Release the entire work for access worldwide	Not applicable
2. Release the entire work for Central University of Punjab only for: 1 year 2 years	After 03 years

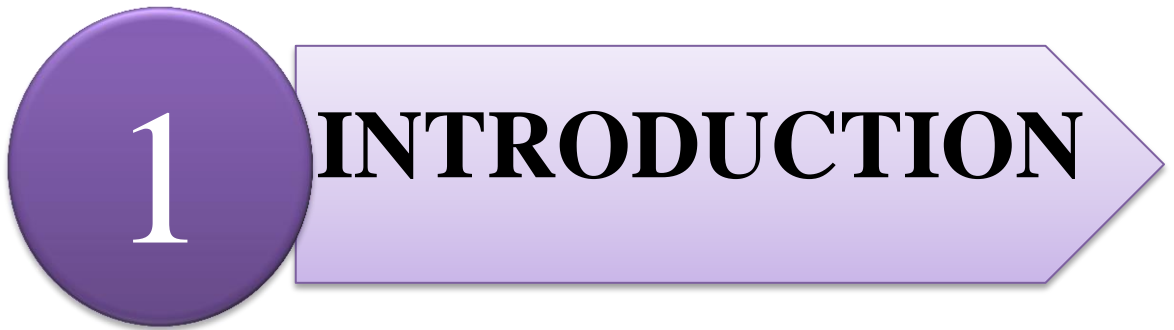
3 years and after this time release the work for access worldwide.	
3. Release the entire work for Central University of Punjab only, while at the same time releasing the following parts of the work (e.g. because other parts relate to publications) for worldwide access: a) Bibliographic details and synopsis only b) Bibliographic details, synopsis and the following chapters only c) Preview / Table of contents / page only	Not applicable
4. View only (No downloads) (world-wide)	Not applicable

Signature of the candidate

Signature and seal of the supervisor

Place: Bathinda

Date: May,2018


1
INTRODUCTION

Cancer is an abnormal growth of body cells. When the programming of a cell or a group of cells is altered, growth may become uncontrolled. Cancer can start in many different places in the body, such as lung, breast, colon, brain, blood, etc. According to American Cancer Society, the factors which contribute to altering the programmed cell is chronic irritation, tobacco, smoke and dust, radioactive substances, age, sex, race and heredity.

Amongst all types of cancer, Lung cancer is the major cause of death worldwide. Basically, there are two types of lung cancer-Small cell lung cancer (10-15%) and non-small cell lung cancer (80-85%). Non-Small cell lung cancer is further classified depending upon pathological characteristics such as lung adenocarcinoma, large cell carcinoma and lung squamous cell carcinoma. Lung adenocarcinoma is more prone than any other NSCLC (Devesa *et al.*, 2005; Topalian *et al.*, 2012; Wei and Zhou, 2016).

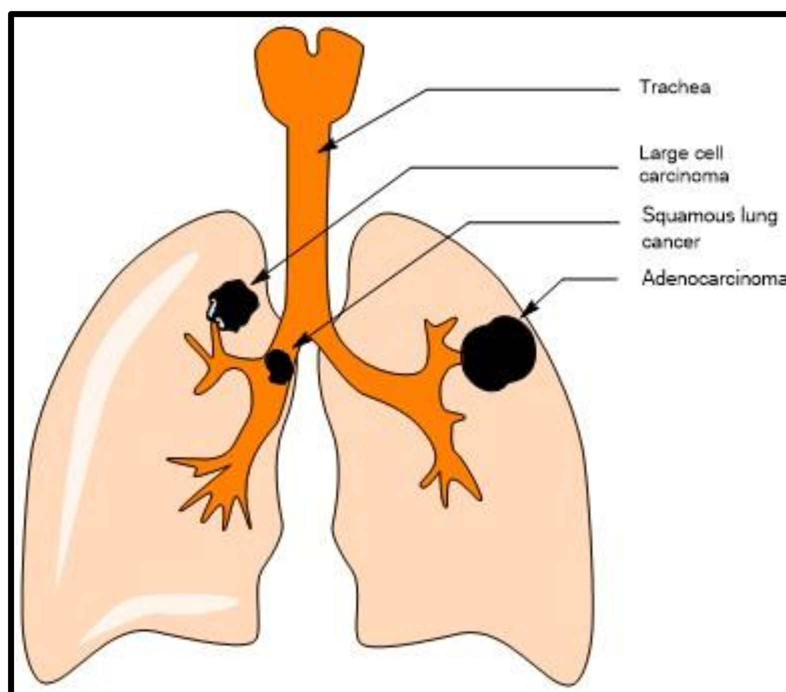


Figure 1: Location of Non-Small Cell Lung Cancer (NSCLC) (Drawn using ChemBio Draw Ultra 14)

According to the GLOBOCAN 2012 report, in India, the estimated incidence rate of lung cancer was 70,275 in all ages and both sexes and the overall estimated lung cancer mortality in 2012 was 63,759 (IARC and WHO, 2012). Out of all the

cancers incidence and mortality rate (in percentage), as shown in the figure 2 and 3, Lung cancer occupy 17% and 24% respectively of all the cancers worldwide.

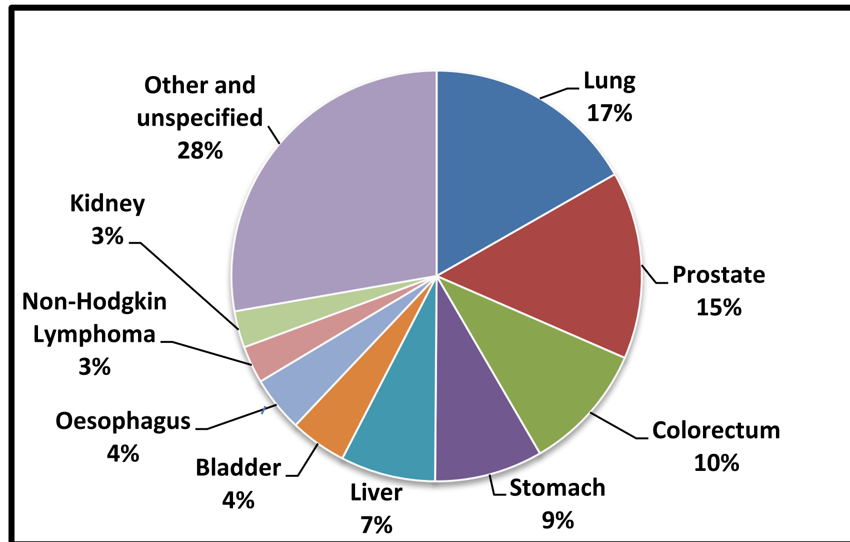


Figure 2: Estimated cancer incidence rates worldwide (IARC and WHO, 2012)

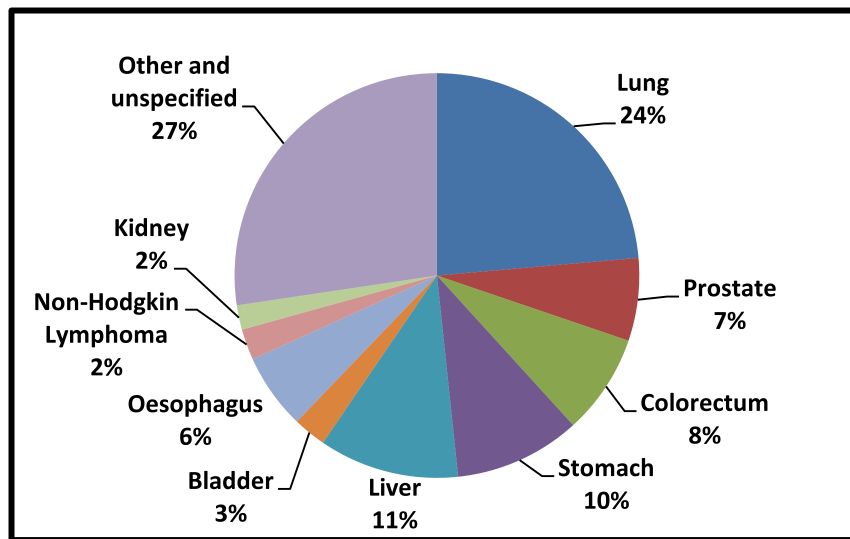


Figure 3: Estimated cancer mortality rates worldwide (IARC and WHO, 2012)

The most crucial reason for high mortality is sustained proliferation of cells and the tumor metastatic potential (X. Shi *et al.*, 2015).Therefore, it is very important to study the potential biomarkers which help us to early diagnose the lung cancer. Recent studies have revealed that more than 90% of the human genome transcribed into non-coding RNA, whereas approximately 2% transcribes into coding RNA and translated into protein (as shown in the figure 4) (Khandelwal *et*

al., 2015). Previously it was studied that mutation in a protein coding gene contributes to the pathogenesis of lung cancer, but recent evidences showed that long non-coding RNA also participate in tumorigenesis and tumor progression of lung cancer (J. X. Chen *et al.*, 2015; T. R. Mercer *et al.*, 2009; Nakagawa *et al.*, 2013; X. Shi *et al.*, 2015).

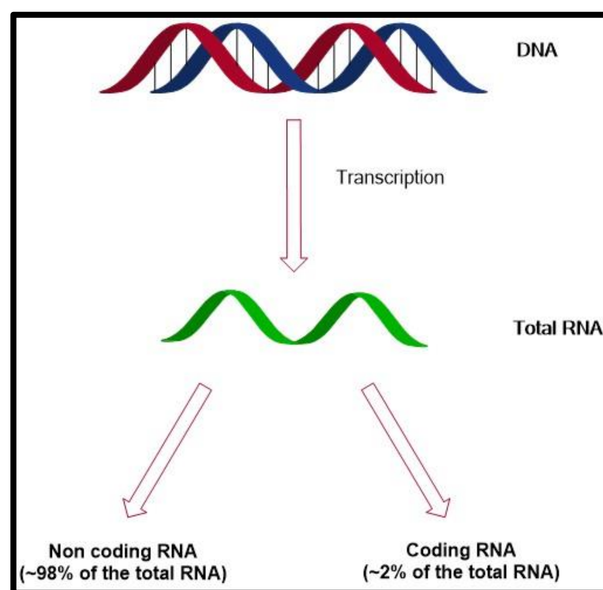


Figure 4: Diagrammatic representation of RNA types (Drawn using ChemBio Draw Ultra 14)

Long non-coding RNA is an RNA molecule which is longer than 200 nucleotides in length, which do not code for proteins (X. Shi *et al.*, 2015; Spizzo *et al.*, 2012). It was once thought that lncRNAs were the transcriptional noise and they do not play any significant role in cellular processes of life (Zou *et al.*, 2017). But with the advancement of whole genome sequencing and microarray, it has been revealed that they can be used as biomarkers for some tumors (Gao *et al.*, 2017). It performs many biological processes such as transcription, splicing, translation, protein localization, epigenetic gene expression, cell structure integrity, cell cycle, heat shock response, imprinting, stem cell pluripotency, reprogramming, embryogenesis, immune response regulation, cell differentiation, cell fate determination, proliferation and migration (Brannan *et al.*, 1990; B. Chen *et al.*, 2013; Fatica and Bozzoni, 2014; Grote and Herrmann, 2013; Gutschner and Diederichs, 2012; Khandelwal *et al.*, 2015; Kino *et al.*, 2010; Z. Li and Rana, 2014; L. Ma *et al.*, 2013; Wapinski and Chang, 2011). As the long non-coding RNA plays a significant role in multiple biological processes and gene regulation, any aberrant

expression of lncRNA leads to cancer etiology such as proliferation and metastasis of cancer cell through transcriptional and post transcriptional modifications (Geisler and Coller, 2013; Guttman and Rinn, 2012; Wei and Zhou, 2016).

1.1. Types of Long noncoding RNA

Previously, the naming of newly discovered lncRNAs was a debatable issue, however, most lncRNAs are named for the genes which are nearby or the protein-coding genes they regulate (Nakagawa *et al.*, 2013). Long noncoding RNAs are classified into six broad categories, depending upon the relationships with protein coding genes.

a. Intergenic - A lncRNA gene lies as an independent unit within the genomic interval between two genes.
b. Bidirectional- Expression of a lncRNA gene and its neighboring coding transcript on the opposite strand is initiated in close genomic proximity.
c. Intron sense-overlapping- A lncRNA gene lies in the intron of a protein-coding gene on the same strand.
d. Exon sense-overlapping- A lncRNA gene lies in the exons of protein-coding gene on the same strand.
e. Intronic-antisense - A lncRNA gene lies in the introns of protein-coding gene on the opposite strand in the same region.
f. Natural-antisense - A lncRNA gene lies in the exons of protein-coding gene on the opposite strand. lncRNA.

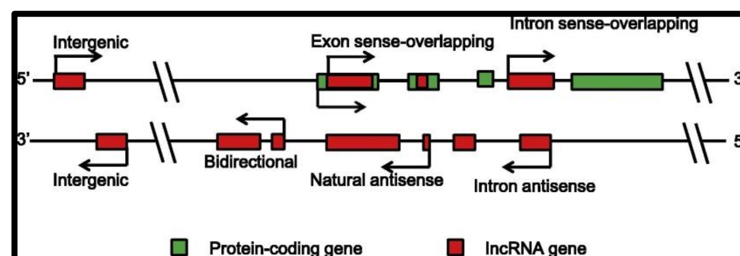


Figure 5: Types of lncRNAs (W. Li *et al.*, 2017)

1.2. Growth Arrest Specific transcript 5 (GAS5)

The lncRNA Growth Arrest Specific transcript 5 (GAS5), acts as a tumor suppressor in human cancer and has been reported to be involved in the progression of several types of cancer, such as breast cancer, prostate cancer, hepatocellular carcinoma, and colorectal cancer and lung cancer (W. Li *et al.*, 2017; C. Ma *et al.*, 2016; Pickard and Williams, 2016; Tao *et al.*, 2015), gastric cancer, cervical cancer and head and neck squamous cell carcinoma (Cao *et al.*, 2014; Gao *et al.*, 2017; Gee *et al.*, 2011; Sun *et al.*, 2014). The genomic location of GAS5 is 1q25 and nucleotide length is 630bp. Studies have shown that GAS5 has been down-regulated in multiple cancers, which leads to tumorigenesis, cell proliferation, apoptosis, metastasis and survival time (Chang *et al.*, 2016; Gao *et al.*, 2017; L. Hu *et al.*, 2016; Zhang *et al.*, 2017). Recently it has been discovered that the function of the GAS5 in mammalian cells can be regulated by the nonsense-mediated degradation pathway (C. Ma *et al.*, 2016).

1.3. BRAF-activated lncRNA (BANCR)

BRAF-activated lncRNA (BANCR) is also a tumor suppressor which is 693bp long non-coding RNA, located on chromosome 9 (J. X. Chen *et al.*, 2015). The dysregulation of BANCR is observed in many human cancers such as melanoma, colorectal cancer, retinoblastoma, lung carcinoma and hepatocellular carcinoma. The progression of lung cancer is related to the down-regulation of lncRNA BANCR by affecting tumor size, clinical stage and TNM stage of lung cancer patients (L. Li *et al.*, 2015; Liu *et al.*, 2016; Y. Shi *et al.*, 2015; Zou *et al.*, 2017). Downregulation of BANCR expression associated with reduction of E-cadherin gene expression and induction of N-cadherin, vimentin and MMPs gene expression (W. Li *et al.*, 2017).

In the current study, we tried to study the expression level of lncRNAs GAS5 and BANCR in lung cancer cell line A549 compared to IMR-90 cell line. The expression levels of GAS5 and BANCR were significantly down-regulated in cancerous (A549) cell line.

OBJECTIVES

1. To isolate the total RNA from A549 cell line (cancerous) and IMR-90 cell line (normal).
2. To study the expression levels of long non-coding RNA GAS5 and BANCR in cancerous cell line (A549) and normal lung cell line (IMR-90).

REVIEW OF LITERATURE

Earlier in the 1950s, Scientists were thought that higher organisms contain more genes than lower organisms (Comings, 1972) but it had been revealed that the developmental complexity of animals is not determined by the amount of DNA in the genome (Thomas, 1971). With the advancement of DNA-RNA hybridization techniques, researchers realized that major parts of the genome are non-coding and were considered as "Junk DNA" (Ohno, 1972).

In 1970s, Subsequent studies on the non-coding RNA had been done, which resulted the discovery of heterogenous nuclear RNAs and introns (Berget *et al.*; Holmes *et al.*, 1972; Pierpont and Yunis, 1977) and demonstrated the role of snRNAs and snoRNAs in post transcriptional RNA processing (Busch *et al.*, 1982).

In 1990s, several lncRNAs such as, H19 and XIST were known (Brannan *et al.*, 1990; Brockdorff *et al.*, 1992) but with the discovery of large number of micro RNA and sustaining keen interest on micro RNA studies resulted the suspension of lncRNAs.

Studies have shown that it was very difficult to study lncRNAs because of their low expression levels and is present in specific cell types, tissues and for very limited time frame (Bartonicek *et al.*, 2016; Cabili *et al.*, 2011; Gloss and Dinger, 2016; Tim R. Mercer *et al.*, 2008). In the early 2000s, with the advancement of whole genome sequencing and microarray techniques resulted in the identification and annotations of many lncRNAs (Bertone *et al.*, 2004; Okazaki *et al.*, 2002; Ota *et al.*, 2004) and various biological processes such as growth, differentiation and establishment of cell identity, gene expression, mRNA processing and protein translation and transport, and are commonly deregulated in cancers which serves as clinical biomarkers (Flynn and Chang; W. Hu *et al.*; Rossi and Antonangeli; Wang and Chang).

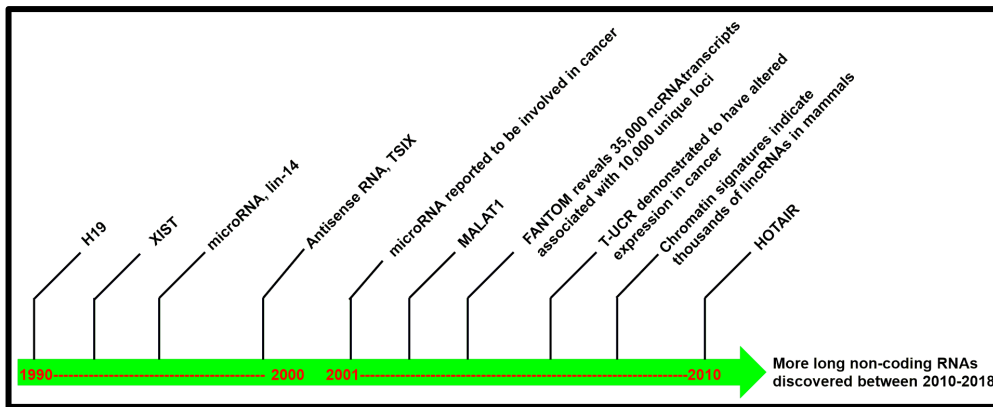


Figure 6: Time scale of discovery of long non-coding RNAs

Studies identified that numerous lncRNAs have been involved in multiple cancer types (as shown in the Table 1), either with the help of previously annotated GENCODE lncRNAs, or by de novo assembly of all available public datasets.

Table 1: Participation of lncRNAs in the pathogenesis of various types of cancer

Cancer Types	LncRNAs
Brain	MALAT1,XIST,CRNDE,POU3F3
Oral/Tongue/Nasopharyngeal	HOTAIR, UCA1, NEAT1, AFAP1-AS1
Thyroid	BANCR, PVT1
Esophageal	CCAT2, HOTAIR ,PCAT-1
Lung	CCAT2, HOTAIR, LCAL 1, ANRIL, UCA1, LUADT1, AFAP1-AS1, GAS5, BANCR, GAS6-AS1, SCAL1
Breast	ZFAS1, LSINCT5, H19, LINC00617, RP11-445H22.4
Gastric	LINC00982, PVT1, LSINCT-5,PTENP1, AA174084, CUDR
Liver	MVIH, HULC, SNHG3, ANRIL, PRAL
Pancreas	HULC, HOTAIR, H19
Colorectal	MALAT1, PCAT-1, PVT1, CCAT1-L, LOC554202
Cervical	HOTAIR, CCHE1, GAS5
Prostate	PCAT1, PCA3, PCAT5, PCAT-18, NEAT1
Melanoma	BANCR, CASC-15, SPRY4-IT1

Among the above mentioned lncRNAs, GAS5 and BANCR have been found to be involved in multiple cancer types. Few studies have been reported in lung cancer with IMR-90 as control and it needs more investigations to study the molecular mechanism, the pathways involved and their interactions with RNA binding proteins.

Pickard *et al*. (2013) provided the first report on programmed cell death promoting lncRNAs GAS5 in prostate cells. They have found that abnormal levels of GAS5 attenuates the apoptosis of cells and reduces the effect of chemotherapeutic agents.

Shi *et al*. (2013) investigated the expression pattern of GAS5 by qRT-PCR in 72 NSCLC specimen. They have shown that GAS5 expression was down-regulated in cancerous tissues compared to non-adjacent cancerous tissues and was highly related to the size of the tumor i.e. GAS5 expression is much more down-regulated in large size tumor compared to the small size tumor.

Renganathan *et al*. (2014) analyzed the role GAS5 in malignant pleural mesothelioma (MPM) and found to be downregulated in MPM cell lines. They have suggested that in in vitro MPM model GAS5 expression is upregulated by inhibition of Hedgehog and PI3K/mTOR signaling.

Tu *et al*. (2014) proved that low GAS5 expression in hepatocellular carcinoma associated with increased tumor size, lymph node metastasis and clinical stages. Their results indicated that GAS5 expression serves as an independent prognostic biomarker for hepatocellular carcinoma patients.

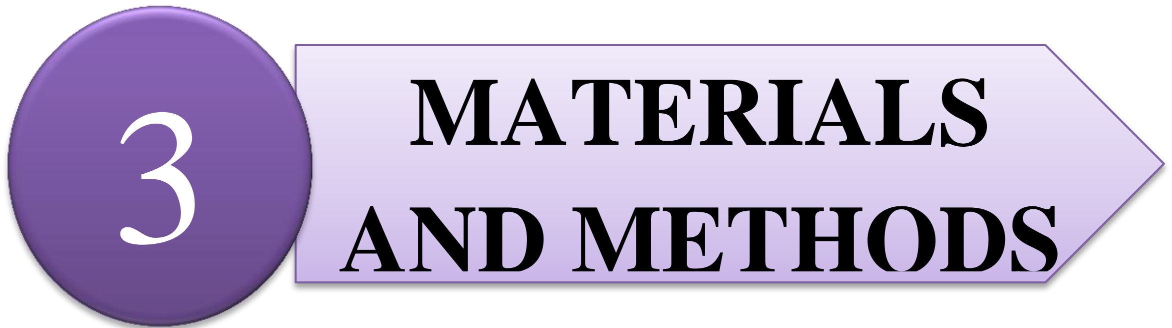
Sun *et al.* (2014) analyzed the expression of BANCR in NSCLC tissues and cell lines using quantitative polymerase chain reaction and found that BANCR expression was significantly down-regulated in NSCLC tumor tissues compared with normal tissues. Their results showed that low BANCR expression was co-related with advanced pathological stage, larger tumor size, metastasis distance, and shorter overall survival of NSCLC patients.

Chen *et al* (2015) showed that up-regulated expression of BANCR is associated with radiation therapy for lung cancer. They used C57BL/6 mice and were inoculated with Lewis lung cancer cells and exposed to radiation therapy, then

they analyzed the BANCR expression using qPCR and found that BANCR expression was significantly increased in C57BL/6 mice receiving radiation therapy compared with the control group which suggests that low expression of BANCR associated with large tumor size in C57BL/6 mice inoculated with Lewis lung cancer cells.

Liang *et al*. (2016) studied the GAS5 expression in the plasma sample of 90 patients with NSCLC and 33 healthy controls. Their results showed that GAS5 was detectable and stable in the plasma of NSCLC patients and the plasma levels of GAS5 were significantly down-regulated in NSCLC patients compared with healthy controls.

Xue *et al*. (2017) showed that GAS5 directly targets miR-135b and negatively regulate its expression. They demonstrated that GAS5 was down-regulated and miR-135b was upregulated in NSCLC tissues and cells. Their experiments revealed that GAS5 overexpression and miR-135b down-regulation significantly suppressed tumorigenesis by repressing cell proliferation and invasion, and enhanced the radiosensitivity of NSCLC cells by reducing colony formation rates.


3
MATERIALS
AND METHODS

Culture of A549 and IMR-90 Cell lines

Total RNA isolation

cDNA Synthesis

Standardization of qRT- PCR protocol

qRT- PCR

Analysis of data

Figure 7: Experimental design for expression analysis

Table 2: List of materials used for experiment

Serial No.	Required Materials	Source
1	RPMI-1640	Gibco
2	MEM	Gibco
3	FBS	Gibco
4	1X PBS	Thermo
5	Trypsin	Gibco
6	Micro centrifuge tubes 1.5 & 2 ml	Tarsons
7	TRIzol reagent	Ambion
8	Chloroform	HPLC
9	Isopropanol	HPLC
10	RNAase away	Ambion
11	Nuclease free water	Invitrogen
12	RT2 First Strand kit (50)	Qiagen
13	RT^2 lncRNA qPCR Assay for Human BANCR	Qiagen
14	RT^2 lncRNA qPCR Assay for Human GAS5	Qiagen
15	RT^2 lncRNA qPCR Assay for Human GAPDH	Qiagen
16	0.2 ml Flat PCR Tube 8-Cap Strips, optical, ultraclear	Biorad
17	iTaq Universal SYBR green supermix	Biorad

3.1. Cell culture

Cell culture is the technique of removal of cells from an organism and their subsequent growth in a favorable artificial environment. These cells may be removed from the tissue directly and separated by enzymatic or mechanical means before culture, or they may be derived from a cell line that has already been established.

Protocol of A549 cell line culture

1. Cell culture media is removed and discarded from the T-25 flask.
2. Cells are washed using 2ml PBS and the T-25 flask is rocked several times back and forth.
3. The wash solution is then removed and discarded from the T-25 flask.
4. Then Trypsin (500µl) is added to dissociate the cells from the surface of the flask. Gently rock the flask to get all the cells.
5. Cells are incubated at 37˚C for approximately 5 minutes.
6. The cells are then observed under the microscope for detachment.
7. When more than 90% of the cells have detached then add 5ml of pre-warmed complete growth medium **(RPMI + 10% FBS+ 1% Penicillin streptomycin)**.
8. The cells are then transferred to a new T-25 flask using serological pipettes for culture.

Protocol of IMR-90 cell line culture

1. Cell culture media is removed and discarded from the T-25 flask.
2. Cells are washed using 2ml PBS and the T-25 flask is rocked several times back and forth.
3. The wash solution is then removed and discarded from the T-25 flask.
4. Then Trypsin (500µl) is added to dissociate the cells from the surface of the flask. Gently rock the flask to get all the cells.
5. Cells are incubated at 37˚C for approximately 5 minutes.
6. The cells are then observed under the microscope for detachment.

7. When more than 90% of the cells have detached then add 5ml of pre-warmed complete growth medium **(MEM + 10% FBS+ 1% Penicillin streptomycin)**.
8. The cells are then transferred to a new T-25 flask using serological pipettes for culture.

3.2. RNA isolation

RNA isolation and purification technique is important for the downstream applications, such as RT-PCR assays and other molecular biology detections. TRIzol Reagent is used for the isolation of total RNA from cells and tissues. Chomczynski and Sacchi developed an improved method for the single-step RNA isolation which involves a mono-phasic solution of phenol and guanidine isothiocyanate. During sample homogenization, TRIzol Reagent maintains the integrity of the RNA as well as disrupting cells and dissolving cell components. The solution separates out into an aqueous phase and an organic phase after the addition of chloroform followed by centrifugation. RNA is recovered from the aqueous phase by precipitation with isopropyl alcohol.

Protocol

1. Collect 2ml cells in a Microcentrifuge tube.
2. Centrifuge at 500g at 4˚c for 5 minutes.
3. Wash pellet with 2ml PBS.
4. Centrifuge at 500g at 4˚c for 5 minutes.
5. Remove the supernatant.
6. Add 250µl PBS.
7. Add 750µl of TRIzol LS reagent in a sample (250µl).
8. Add 200µl chloroform (per 750µl of TRIzol reagent).
9. Shake vigorously or vortex for 15 seconds.
10. Incubate for 2-15minutes at room temperature.
11. Centrifuge the sample at 12,000g for 15 minutes at 4˚C.
12. The aqueous phase of the sample is then transferred into a new RNAse free tube by tilting the tube at 45˚.
13. Add 500µl of 100% Isopropanol to the aqueous solution (per 750µl TRIzol reagent).

14. Incubate at RT for 10 minutes.
15. Centrifuge at 12,000g for 10 minutes at 4˚C.
16. Remove all supernatant, leaving only the RNA pellet.
17. Wash RNA pellet with 1 ml of 75% ethanol (per 750µl TRIzol reagent) and vortex to mix.
18. Centrifuge at 7,500g for 5 minutes at 4˚c and remove the supernatant.
19. Air/vacuum dry the RNA pellet for 5-10 minutes.
20. Resuspend the RNA pellet in RNAase free water (20-50µl) and mix it with pipetting up and down.
21. Incubate in water bath / heat shock at 55-60˚c for 10-15 minutes.

3.3. Nanodrop reading using nanodrop 2000cc (Thermo Scientific)

To determine the amount of each sample to be used in downstream applications, such as cDNA synthesis, RT-PCR, etc, it is very important to measure the amount and purity of RNA. The spectrophotometer is based on the principle of the Beer-Lambert law. The Beer-Lambert law draws a direct correlation between absorbance and concentration. However, nucleic acids absorb at many wavelengths, they have a peak absorbance of UV light at 260nm. Thus, the amount of light absorbed in this region can be used to determine the concentration of RNA or DNA in solution by applying the Beer-Lambert law. However, the Beer-Lambert equation is only linear for absorbance between 0.1 and 1.0. RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A260/A280 of 1.8-2.0.

Protocol

1. Bring RNA samples as well as the NFW water used to elute them on ice to the spectrophotometer.
2. Wash the sample reader with nuclease free water and dry with a KimWipes.
3. Following the software's instructions, load 1 µL of nuclease free water (blank) and initialize the system.
4. Change the computer's setting to RNA and click the blank button.
5. Load 1 µL of sample and click the measure button.

6. After the read is complete, record the A260/A280 and A260/A230 ratios as well as the concentration of RNA recovered (in ng/μL).

3.4. cDNA synthesis

cDNA synthesis is useful for downstream applications such as, expression analysis of the candidate genes.

Protocol

1. Thaw the reagents of RT^2 First Strand Kit. Briefly centrifuge (10-15s) to bring the contents to the bottom of the tubes.
2. Prepare the genomic elimination mix for each RNA sample. Mix gently by pipetting up and down and then centrifuge briefly.

Table 3: Genomic DNA elimination mix for RNA isolated from A549 cell line

Components	Recommended amount	Amount taken
RNA	**25 ng-5 µg**	**41.4 ng (2 µl)**
Buffer GE	**2 µl**	**2 µl**
Nuclease Free Water (NFW)	**Variable**	**6 µl**
Total	**10 µl**	**10 µl**

Table 4: Genomic DNA elimination mix for RNA isolated from IMR-90 cell line

Components	Recommended amount	Amount taken
RNA	**25 ng-5 µg**	**42 ng (5 µl)**
Buffer GE	**2 µl**	**2 µl**
Nuclease Free Water (NFW)	**Variable**	**3 µl**
Total	**10 µl**	**10 µl**

3. Incubate the Genomic DNA elimination mix for 5 minutes at 42˚C, then place immediately on ice for atleast 1 minute.

4. Prepare the Reverse Transcriptase mix for RNA isolated from both A549 and IMR-90 cell lines.

Table 5: Reverse Transcription reaction

Components	Volume for 2 reactions
5X Buffer BC3	**8 µl**
Control P2	**2 µl**
RE3 Reverse Transcriptase mix	**4 µl**
Nuclease Free Water	**6 µl**
Total volume	**20 µl**

5. Add 10 µl RT mix to each tube containing 10 µl genomic DNA elimination mix. Mix gently by pipetting up and down.
6. Incubate at 37˚C for exactly 60 minutes, then immediately stop the reaction by incubating at 95˚C for 5 minutes.
7. Add 91 µl NFW to each reaction. Mix by pipetting up and down several times.
8. Place the reaction on ice and proceed for Real time PCR.

3.5. Standardization of qRT-PCR protocol

Standardization of Real-Time PCR protocol is one of the important steps while analyzing the gene expression of Long non-coding RNA. The temperature increment step of melt curve stage and the ramp rate percentage were not available in the literature for ABI Biosystem StepOne Plus. Melt curve data is important because the sharp peak depicts the specific binding of the primer to target and the product is amplified. Ramp rate is the maximum rate at which its heating block can change the temperature. It also determines the time to complete a specified number of Real-Time PCR cycles.

Whenever we follow up our Real-Time experiment, we got C_t value less than 37 as well as a sharp peak in all the Negative Template Control (NTC). In 40 cycles qRT-PCR protocol, if C_t value is less than 37 in NTC then there is a chance of genomic DNA contamination and if it is greater than 37 or undetermined, then

we ignore the contamination because our unknown sample already achieved C_t value between 22-35. To troubleshoot this issue we checked our primers, Nuclease free water, SYBR Green by multiple Real-Time experiment because they are the main source of cross contamination. Later, we freshly aliquoted the primers, SYBR Green and Nuclease free water. After contamination check and proper handling of the pipettes, the betterment in our results observed.

3.6. Quantitative Real Time PCR

qRT – PCR technique is used when the starting material is RNA. In this technique, total RNA is first isolated and transcribed into cDNA using reverse transcriptase. The synthesized cDNA is used as the template for the reaction. This technique can be used in a variety of applications such as gene expression analysis, pathogen detection, genetic testing, and disease research. This technique can be performed either in one-step or two-step assays. One-step assay involves combining both reverse transcription and PCR in a single tube and buffer, while two-step assay involves reverse transcription and PCR in a separate tube and buffer.

Protocol

1. Briefly centrifuge the RT^2 SYBR Green Mastermix (10-15s) to bring the contents to the bottom of the tube.
2. Prepare the PCR components in a nuclease free tube.

Table 6: Reaction mixture for Real Time PCR

Components	Recommended Volume for 1 reaction	Volume for 8 reactions
Nuclease-Fee Water	**10.5 µl**	**84 µl**
RT^2 SYBR Green Mastermix	**12.5 µl**	**100 µl**
cDNA	**1 µl**	**8 µl**
RT^2 IncRNA qPCR Assay (10 µM)	**1 µl**	**8 µl**
Total volume	**25 µl**	**200 µl**

Table 7: Template designed for reaction

NTC GAPDH	NTC GAPDH	Control cDNA	Control cDNA	Control cDNA	Sample cDNA	Sample cDNA	Sample cDNA
NTC GAS5	NTC GAS5	Control cDNA	Control cDNA	Control cDNA	Sample cDNA	Sample cDNA	Sample cDNA
NTC BANCR	NTC BANCR	Control cDNA	Control cDNA	Control cDNA	Sample cDNA	Sample cDNA	Sample cDNA

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

Table 8: Standardized Cycling conditions for Long non-coding RNA

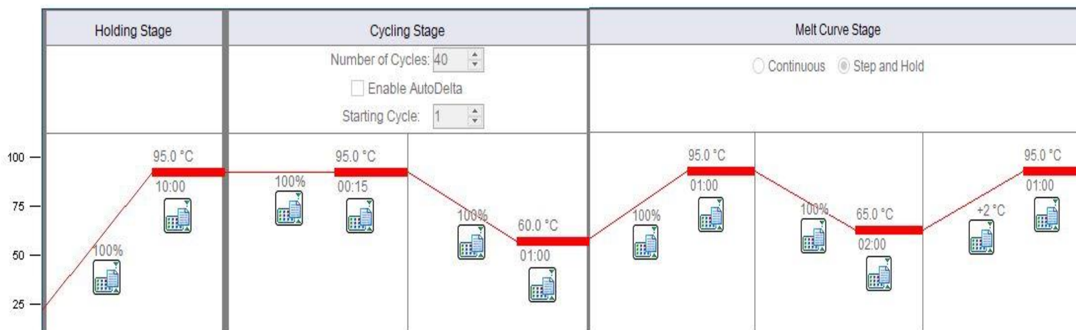


Table 9: Primer Sequence

Gene	Forward Sequence	Reverse Sequence
GAPDH	5'-GTCAACGGATTTGGTCTGTATT-3′	5′-AGTCTTCTGGGTGGCAGTGAT-3′
GAS5	5′-CTTGCCTGGACCAGCTTAAT-3′	5′-CAAGCCGACTCTCCATACCT-3′
BANCR	5’-ACAGGACTCCATGGCAAACG-3’	5’-ATGAAGAAAGCCTGGTGCAGT-3’

The reference gene GAPDH is used to normalize the C_t value of the target. The reaction was performed in duplicates in ABI Biosystem StepOne Plus Real-Time

PCR instrument. The relative change in expression of the target in the cancerous cell compared to the target in the normal cell is calculated using the $2^{-\Delta Ct}$.

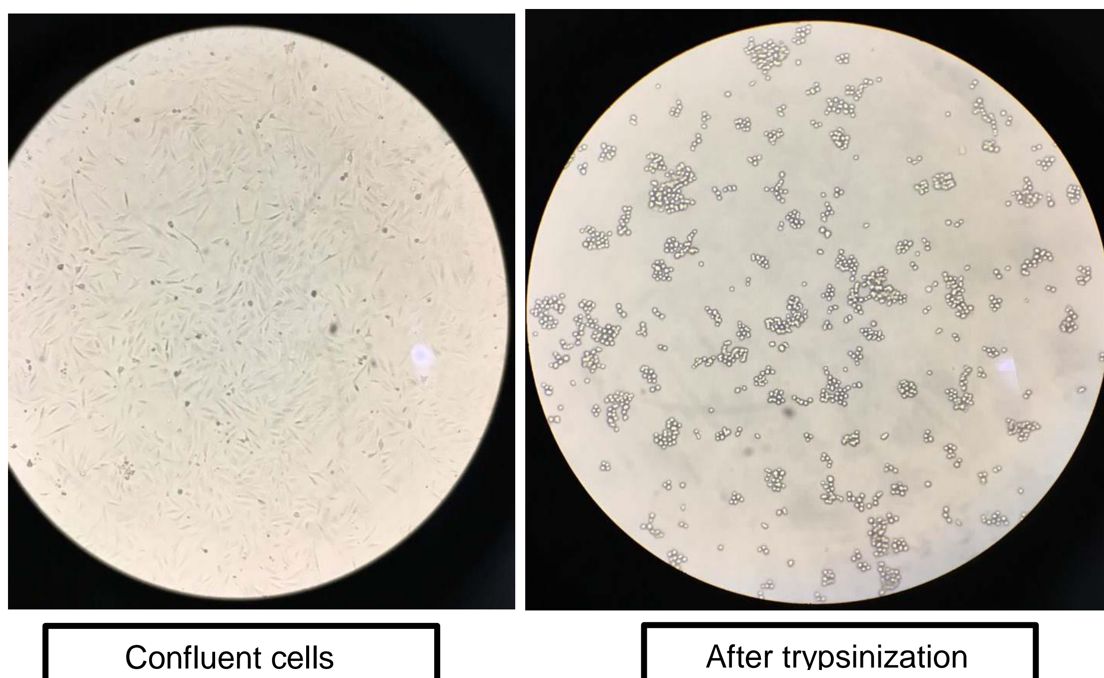
3.7. Statistical analysis

Statistical anlysis was performed using statistical program GraphPad Prism 7 software. The levels of $2^{-\Delta Ct}$ between cancerous and non-cancerous cell line were analysed by the student t-test. The level of significance was set at $P<0.05$.

4
RESULTS

4.1. Maintenance of cell lines

A549 cell lines required 3-4 days to be confluent and 2-3 days to change the media (Complete RPMI). IMR-90 cell line image was not taken as because the IMR-90 requires a longer period of time to grow and the cell number was very less during the experiment.



Confluent cells

After trypsinization

Figure 8: A549 cell lines

4.2. Nanodrop reading

Table 10: Concentration of isolated Total RNA

Cell lines	Concentration of RNA(ng/µl)	Concentration of Diluted RNA(
A549	240.5	20.7 **ng/µl**
IMR-90	8.4	No need to dilute

Calculation of Dilution of RNA (A549) :

$$\frac{\textbf{Amount of RNA taken * Concentration of RNA (ng/µl)}}{\textbf{Total volume to be prepared}}$$

$$= \frac{2\mu l \text{ RNA} * 240.5 \text{ ng}/\mu l}{20\mu l}$$

$$= 24.05 \text{ ng}/\mu l$$

After 10 times dilution, the expected concentration was 24.05 ng/µl and the actual concentration come out as 20.7 ng/µl which is near to an expected outcome.

4.3. Change in gene expression of GAS5 and BANCR in cancerous and non-cancerous cell line

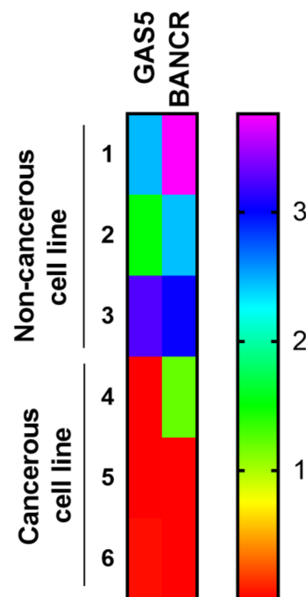


Figure : Heat map showing GAS5 and BANCR gene expression in human lung cancer cell line (A549) and the normal lung cell line (IMR-90). GAS5 and BANCR were shown to be down-regulated in cancerous cell line compared to non-cancerous cell line.

4.4. Down-regulation of GAS5 was detected in NSCLC cell line (A549)

We measured the expression level of GAS5 in NSCLC cell line using quantitative Real-Time PCR (qRT- PCR) . In qRT- PCR measurements, the C_t value of all the samples were obtained, which is defined as the fractional cycle number at which the fluorescence passed the fixed threshold. If the C_t value is higher in A549 than IMR-90 suggests that the initial template is lower in A549 than IMR-90. The ΔC_t values were normalized by GAPDH. The mean of $2^{-\Delta Ct}$ is plotted in the graph

showing the relative expression level of GAS5 in A549 compared to IMR-90 cell line (Figure 8). The expression level of lncRNA GAS5 is down-regulated in A549 (P=0.008) suggesting that GAS5 may function as a tumor suppressor in normal lung cell. lncRNA GAS5 is 14 fold down-regulated in A549 cell line compared to IMR-90 cell line. The unpaired Student t-test was applied to test the significant difference between the expression level of cancerous and the non-cancerous cell lines.

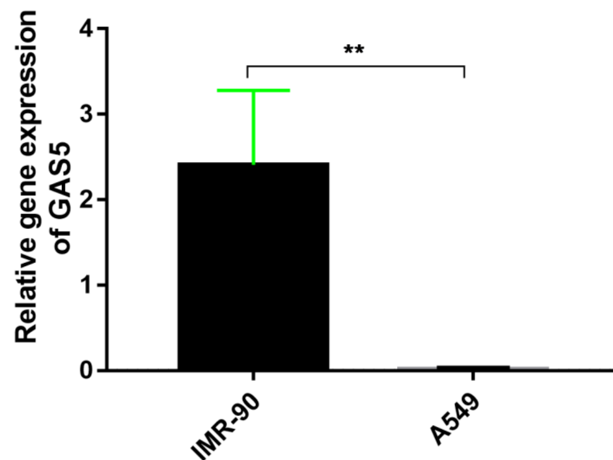


Figure 10: Bar diagram showing relative gene expression of GAS5 in A549 cell lines (n=3) compared to IMR-90 cell lines (n=3). Statistical significance is represented by ** (P<0.05). The fold change in cancerous cell line (A549) is 14 times downregulated compared to normal lung cell (IMR-90).

The dissociation curve or melt curve analysis showed that there was only one peak at the highest Tm in each dissociation curve (Figure 9) which indicates that there were no primer-dimer or non-specific amplifications.

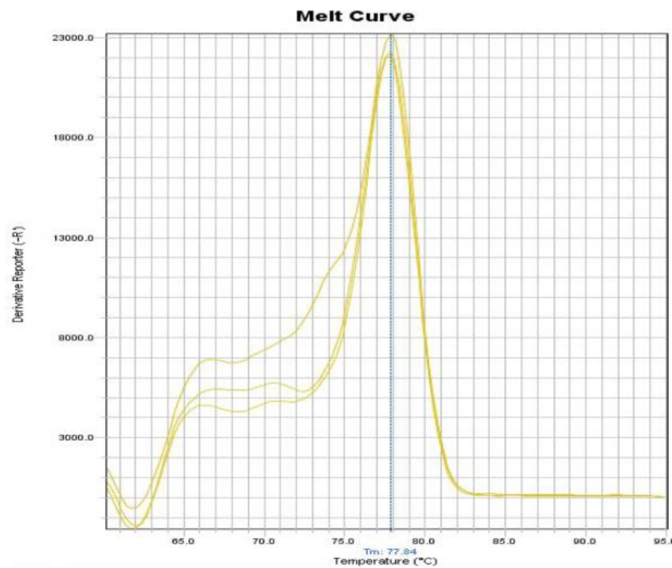


Figure 11: Melt curve of GAS5 showing specific binding of primer to the target

4.5. Down-regulation of BANCR was detected in NSCLC cell line (A549)

The ΔC_t values were normalized by GAPDH. The mean of $2^{-\Delta Ct}$ is plotted in the graph showing the relative expression level of BANCR in A549 compared to IMR-90 cell line (Figure 10). The expression level of lncRNA BANCR is down-regulated in A549 (P=0.008) suggesting that BANCR may function as a tumor suppressor in normal cell. lncRNA BANCR is 7 fold down-regulated in A549 cell line compared to IMR-90 cell line. The unpaired Student t-test was applied to test the significant difference between the expression level of cancerous and the non-cancerous cell lines.

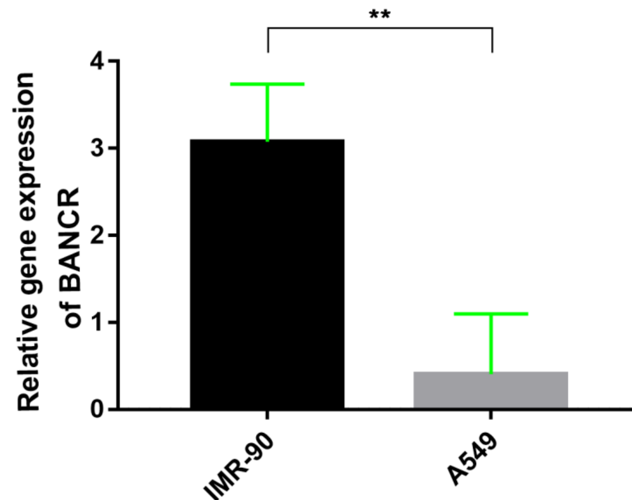


Figure 12: Bar diagram showing relative gene expression of BANCR in A549 cell lines (n=3) compared to IMR-90 cell lines (n=3). Statistical significance is represented by ** (P<0.05). The fold change in cancerous cell line (A549) is 7 times downregulated compared to normal lung cell (IMR-90)

The melt curve analysis of BANCR shows a single peak at the highest melting temperature in each melt curve (Figure 11) which indicates that there were no primer-dimer or non-specific amplifications.

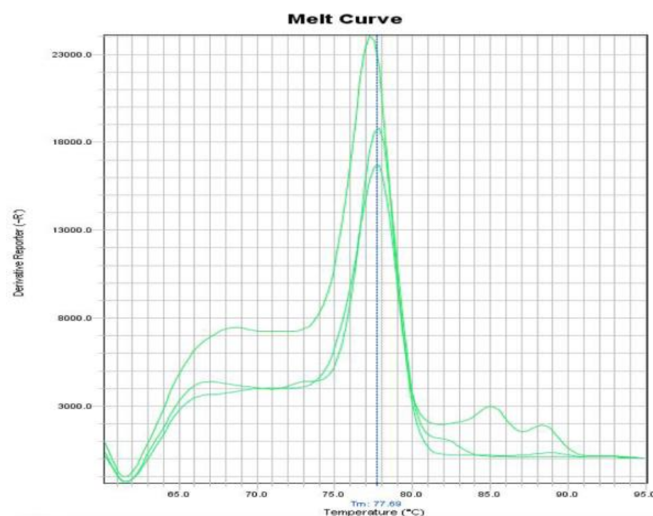


Figure 13: Melt curve of BANCR showing specific binding of primer to the target

5
DISCUSSION

Till now, multiple lncRNAs have been discovered and are found to be associated with cancer development and progression. In the present study, we focussed on GAS5 and BANCR expression in NSCLC cell lines A549 compared to normal human lung cell line IMR-90 because the published literature revealed that the GAS5 and BANCR play an important role in different types of cancers. So, we were interested to study the expression levels of GAS5 and BANCR in NSCLC because according to GLOBOCAN 2012 report, among the different types of cancers, lung cancer is the major cause of death worldwide. Moreover, to analyze the expression levels in cancerous and non-cancerous cell line, we used quantitative real time PCR (qRT- PCR). We standardized the Real-Time cycle, ramp rate for long non-coding RNA and troubleshoot the contamination. The media of A549 and IMR-90 cell lines was changed in every two days to maintain the good health of the cells. But the growth of IMR-90 was not healthy so we had to conduct experiments with a very few number of cells. The total RNA was isolated from both cancerous and non-cancerous cell lines using Trizol method and the concentration was analyzed using nanodrop. Later, cDNA was synthesized using RT^2 First Strand Kit. The qRT –PCR eperiment was done in triplicates. The heat map (Figure 9) shows the expression levels of GAS5 and BANCR in cancerous and non-cancerous cell line. The expression levels shown to be decreased in cancerous cell line. GAS5 expression was 14-fold down-regulated (P=.0.0088) (Figure 10) in A549 cell line. BANCR expression was 7-fold down-regulated (P=0.0088) (Figure 12) in A549 cell line. The decreased expression levels suggests that they may function as a tumor suppressor in normal condition. The expression levels suggests that the overall survival and prognosis rate of a patient is relatively less. It has been studied that the survival rate of the patients with ≥4 fold change of BANCR is 16 months and the patients with ≤ 4 fold change of BANCR is 31 months (W. Li *et al.*, 2017). Both GAS5 and BANCR may partially be treated as potential biomarkers of NSCLC in the early diagnosis and prognosis of lung cancer. Further studies are required to investigate the mechanism of progression of cancer through lncRNA GAS5 and BANCR and their interactions with protein coding genes.

CONCLUSION AND FUTURE ASPECTS

The field of long non-coding RNA is emerging due to the advancement of techniques and many lncRNAs have been discovered and play an important role in the pathogenesis of cancer. Presently, Scientists are more focussed on long non-coding RNA as because lncRNAs are found to play an important role in the various fundamental biological processes such as transcription, translation, splicing, epigenetic gene silencing cell cycle, etc. Any aberrations in the lncRNAs may lead to uncontrolled proliferation of cells. Therefore, we were focused to analyze the expression levels of GAS5 and BANCR in lung cancer cell line. Our result suggests that both GAS5 and BANCR may act as a tumor suppressor in NSCLC.

The future aspects of our study are-

1. To study the exact mechanism of down-regulation of GAS5 and BANCR.
2. Role of GAS5 and BANCR in the pathogenesis of lung cancer.
3. To study the signaling pathway and molecular mechanism of GAS5 and BANCR.

REFERENCES

Bartonicek, N., Maag, J. L., and Dinger, M. E. (2016). Long noncoding RNAs in cancer: mechanisms of action and technological advancements. *Mol Cancer,* **15**(1), 016-0530.

Berget, S. M., Moore, C., and Sharp, P. A. *Spliced segments at the 5' terminus of adenovirus 2 late mRNA*: Proc Natl Acad Sci U S A. 1977 Aug;74(8):3171-5.

Bertone, P., Stolc, V., Royce, T. E., Rozowsky, J. S., Urban, A. E., Zhu, X., Rinn, J. L., Tongprasit, W., Samanta, M., Weissman, S., Gerstein, M., and Snyder, M. (2004). Global identification of human transcribed sequences with genome tiling arrays. *Science,* **306**(5705), 2242-2246.

Brannan, C. I., Dees, E. C., Ingram, R. S., and Tilghman, S. M. (1990). The product of the H19 gene may function as an RNA. *Mol Cell Biol,* **10**(1), 28-36.

Brockdorff, N., Ashworth, A., Kay, G. F., McCabe, V. M., Norris, D. P., Cooper, P. J., Swift, S., and Rastan, S. (1992). The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell,* **71**(3), 515-526.

Busch, H., Reddy, R., Rothblum, L., and Choi, Y. C. (1982). SnRNAs, SnRNPs, and RNA processing. *Annu Rev Biochem,* **51**, 617-654.

Cabili, M. N., Trapnell, C., Goff, L., Koziol, M., Tazon-Vega, B., Regev, A., and Rinn, J. L. (2011). Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes & Development,* **25**(18), 1915-1927.

Cao, S., Liu, W., Li, F., Zhao, W., and Qin, C. (2014). Decreased expression of lncRNA GAS5 predicts a poor prognosis in cervical cancer. *Int J Clin Exp Pathol,* **7**(10), 6776-6783.

Chang, L., Li, C., Lan, T., Wu, L., Yuan, Y., Liu, Q., and Liu, Z. (2016). Decreased expression of long non-coding RNA GAS5 indicates a poor prognosis and promotes cell proliferation and invasion in hepatocellular carcinoma by regulating vimentin. *Mol Med Rep,* **13**(2), 1541-1550.

Chen, B., Yu, M., Chang, Q., Lu, Y., Thakur, C., Ma, D., Yi, Z., and Chen, F. (2013). Mdig de-represses H19 large intergenic non-coding RNA (lincRNA) by down-regulating H3K9me3 and heterochromatin. *Oncotarget,* **4**(9), 1427-1437.

Chen, J. X., Chen, M., Zheng, Y. D., Wang, S. Y., and Shen, Z. P. (2015). Up-regulation of BRAF activated non-coding RNA is associated with radiation therapy for lung cancer. *Biomed Pharmacother,* **71**, 79-83.

Comings, D. E. (1972). The structure and function of chromatin. *Adv Hum Genet,* **3**, 237-431.

Devesa, S. S., Bray, F., Vizcaino, A. P., and Parkin, D. M. (2005). International lung cancer trends by histologic type: male:female differences diminishing and adenocarcinoma rates rising. *Int J Cancer,* **117**(2), 294-299.

Fatica, A., and Bozzoni, I. (2014). Long non-coding RNAs: new players in cell differentiation and development. *Nat Rev Genet,* **15**(1), 7-21.

Flynn, R. A., and Chang, H. Y. *Long noncoding RNAs in cell fate programming and reprogramming*: Cell Stem Cell. 2014 Jun 5;14(6):752-61. doi:10.1016/j.stem.2014.05.014.

Gao, Q., Xie, H., Zhan, H., Li, J., Liu, Y., and Huang, W. (2017). Prognostic Values of Long Noncoding RNA GAS5 in Various Carcinomas: An Updated Systematic Review and Meta-Analysis. *Frontiers in Physiology,* **8**, 814.

Gee, H. E., Buffa, F. M., Camps, C., Ramachandran, A., Leek, R., Taylor, M., Patil, M., Sheldon, H., Betts, G., Homer, J., West, C., Ragoussis, J., and Harris, A. L. (2011). The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis. *Br J Cancer,* **104**(7), 1168-1177.

Geisler, S., and Coller, J. (2013). RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. *Nat Rev Mol Cell Biol,* **14**(11), 699-712.

Gloss, B. S., and Dinger, M. E. (2016). The specificity of long noncoding RNA expression. *Biochim Biophys Acta,* **1**, 16-22.

Grote, P., and Herrmann, B. G. (2013). The long non-coding RNA Fendrr links epigenetic control mechanisms to gene regulatory networks in mammalian embryogenesis. *RNA Biol,* **10**(10), 1579-1585.

Gutschner, T., and Diederichs, S. (2012). The hallmarks of cancer: a long non-coding RNA point of view. *RNA Biol,* **9**(6), 703-719.

Guttman, M., and Rinn, J. L. (2012). Modular regulatory principles of large non-coding RNAs. *Nature,* **482**(7385), 339-346.

Holmes, D. S., Mayfield, J. E., Sander, G., and Bonner, J. (1972). Chromosomal RNA: its properties. *Science,* **177**(4043), 72-74.

Hu, L., Ye, H., Huang, G., Luo, F., Liu, Y., Yang, X., Shen, J., Liu, Q., and Zhang, J. (2016). Long noncoding RNA GAS5 suppresses the migration and invasion of hepatocellular carcinoma cells via miR-21. *Tumour Biol,* **37**(2), 2691-2702.

Hu, W., Alvarez-Dominguez, J. R., and Lodish, H. F. *Regulation of mammalian cell differentiation by long non-coding RNAs*: EMBO Rep. 2012 Nov;13(11):971-83. Epub 2012 Oct 16 doi:10.1038/embor.2012.145.

IARC, and WHO. (2012). homepage. <http://globocan.iarc.fr/Default.aspx> Accessed 2018 Feb, 14.

Khandelwal, A., Bacolla, A., Vasquez, K. M., and Jain, A. (2015). Long non-coding RNA: A new paradigm for lung cancer. *Mol Carcinog,* **54**(11), 1235-1251.

Kino, T., Hurt, D. E., Ichijo, T., Nader, N., and Chrousos, G. P. (2010). Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. *Sci Signal,* **3**(107), ra8.

Li, L., Zhang, L., Zhang, Y., and Zhou, F. (2015). Increased expression of LncRNA BANCR is associated with clinical progression and poor prognosis in gastric cancer. *Biomed Pharmacother,* **72**, 109-112.

Li, W., Huang, K., Wen, F., Cui, G., Guo, H., and Zhao, S. (2017). Genetic variation of lncRNA GAS5 contributes to the development of lung cancer. *Oncotarget,* **8**(53), 91025-91029.

Li, Z., and Rana, T. M. (2014). Decoding the noncoding: prospective of lncRNA-mediated innate immune regulation. *RNA Biol,* **11**(8), 979-985.

Liu, Z., Yang, T., Xu, Z., and Cao, X. (2016). Upregulation of the long non-coding RNA BANCR correlates with tumor progression and poor prognosis in esophageal squamous cell carcinoma. *Biomed Pharmacother,* **82**, 406-412.

Ma, C., Shi, X., Zhu, Q., Li, Q., Liu, Y., Yao, Y., and Song, Y. (2016). The growth arrest-specific transcript 5 (GAS5): a pivotal tumor suppressor long noncoding RNA in human cancers. *Tumour Biol,* **37**(2), 1437-1444.

Ma, L., Bajic, V. B., and Zhang, Z. (2013). On the classification of long non-coding RNAs. *RNA Biol,* **10**(6), 925-933.

Mercer, T. R., Dinger, M. E., and Mattick, J. S. (2009). Long non-coding RNAs: insights into functions. *Nat Rev Genet,* **10**(3), 155-159.

Mercer, T. R., Dinger, M. E., Sunkin, S. M., Mehler, M. F., and Mattick, J. S. (2008). Specific expression of long noncoding RNAs in the mouse brain. *Proceedings of the National Academy of Sciences,* **105**(2), 716-721.

Nakagawa, T., Endo, H., Yokoyama, M., Abe, J., Tamai, K., Tanaka, N., Sato, I., Takahashi, S., Kondo, T., and Satoh, K. (2013). Large noncoding RNA HOTAIR enhances aggressive biological behavior and is associated with short disease-free survival in human non-small cell lung cancer. *Biochem Biophys Res Commun,* **436**(2), 319-324.

Ohno, S. (1972). So much "junk" DNA in our genome. *Brookhaven Symp Biol,* **23**, 366-370.

Okazaki, Y., Furuno, M., Kasukawa, T., Adachi, J., Bono, H., Kondo, S., Nikaido, I., Osato, N., Saito, R., Suzuki, H., Yamanaka, I., Kiyosawa, H., Yagi, K., Tomaru, Y., Hasegawa, Y., Nogami, A., Schonbach, C., Gojobori, T., Baldarelli, R., Hill, D. P., Bult, C., Hume, D. A., Quackenbush, J., Schriml, L. M., Kanapin, A., Matsuda, H., Batalov, S., Beisel, K. W., Blake, J. A., Bradt, D., Brusic, V., Chothia, C., Corbani, L. E., Cousins, S., Dalla, E., Dragani, T. A., Fletcher, C. F., Forrest, A., Frazer, K. S., Gaasterland, T., Gariboldi, M., Gissi, C., Godzik, A., Gough, J., Grimmond, S., Gustincich, S., Hirokawa, N., Jackson, I. J., Jarvis, E. D., Kanai, A., Kawaji, H., Kawasawa, Y., Kedzierski, R. M., King, B. L., Konagaya, A., Kurochkin, I. V., Lee, Y., Lenhard, B., Lyons, P. A., Maglott, D. R., Maltais, L., Marchionni, L., McKenzie, L., Miki, H., Nagashima, T., Numata, K., Okido, T., Pavan, W. J., Pertea, G., Pesole, G., Petrovsky, N., Pillai, R., Pontius, J. U., Qi, D., Ramachandran, S., Ravasi, T., Reed, J. C., Reed, D. J., Reid, J., Ring, B. Z., Ringwald, M., Sandelin, A., Schneider, C., Semple, C. A., Setou, M., Shimada, K., Sultana, R., Takenaka, Y., Taylor, M. S., Teasdale, R. D., Tomita, M., Verardo, R., Wagner, L., Wahlestedt, C., Wang, Y., Watanabe, Y., Wells, C., Wilming, L. G., Wynshaw-Boris, A., Yanagisawa, M., Yang, I., Yang, L., Yuan, Z., Zavolan, M., Zhu, Y., Zimmer, A., Carninci, P., Hayatsu, N., Hirozane-Kishikawa, T., Konno, H., Nakamura, M., Sakazume, N., Sato, K., Shiraki, T., Waki, K., Kawai, J., Aizawa, K., Arakawa, T., Fukuda, S., Hara, A., Hashizume, W., Imotani, K., Ishii, Y., Itoh, M., Kagawa, I., Miyazaki, A., Sakai, K., Sasaki, D., Shibata, K., Shinagawa, A., Yasunishi, A., Yoshino, M., Waterston, R., Lander, E. S., Rogers, J., Birney, E., and

Hayashizaki, Y. (2002). Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature,* **420**(6915), 563-573.

Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R., Wakamatsu, A., Hayashi, K., Sato, H., Nagai, K., Kimura, K., Makita, H., Sekine, M., Obayashi, M., Nishi, T., Shibahara, T., Tanaka, T., Ishii, S., Yamamoto, J., Saito, K., Kawai, Y., Isono, Y., Nakamura, Y., Nagahari, K., Murakami, K., Yasuda, T., Iwayanagi, T., Wagatsuma, M., Shiratori, A., Sudo, H., Hosoiri, T., Kaku, Y., Kodaira, H., Kondo, H., Sugawara, M., Takahashi, M., Kanda, K., Yokoi, T., Furuya, T., Kikkawa, E., Omura, Y., Abe, K., Kamihara, K., Katsuta, N., Sato, K., Tanikawa, M., Yamazaki, M., Ninomiya, K., Ishibashi, T., Yamashita, H., Murakawa, K., Fujimori, K., Tanai, H., Kimata, M., Watanabe, M., Hiraoka, S., Chiba, Y., Ishida, S., Ono, Y., Takiguchi, S., Watanabe, S., Yosida, M., Hotuta, T., Kusano, J., Kanehori, K., Takahashi-Fujii, A., Hara, H., Tanase, T. O., Nomura, Y., Togiya, S., Komai, F., Hara, R., Takeuchi, K., Arita, M., Imose, N., Musashino, K., Yuuki, H., Oshima, A., Sasaki, N., Aotsuka, S., Yoshikawa, Y., Matsunawa, H., Ichihara, T., Shiohata, N., Sano, S., Moriya, S., Momiyama, H., Satoh, N., Takami, S., Terashima, Y., Suzuki, O., Nakagawa, S., Senoh, A., Mizoguchi, H., Goto, Y., Shimizu, F., Wakebe, H., Hishigaki, H., Watanabe, T., Sugiyama, A., Takemoto, M., Kawakami, B., Watanabe, K., Kumagai, A., Itakura, S., Fukuzumi, Y., Fujimori, Y., Komiyama, M., Tashiro, H., Tanigami, A., Fujiwara, T., Ono, T., Yamada, K., Fujii, Y., Ozaki, K., Hirao, M., Ohmori, Y., Kawabata, A., Hikiji, T., Kobatake, N., Inagaki, H., Ikema, Y., Okamoto, S., Okitani, R., Kawakami, T., Noguchi, S., Itoh, T., Shigeta, K., Senba, T., Matsumura, K., Nakajima, Y., Mizuno, T., Morinaga, M., Sasaki, M., Togashi, T., Oyama, M., Hata, H., Komatsu, T., Mizushima-Sugano, J., Satoh, T., Shirai, Y., Takahashi, Y., Nakagawa, K., Okumura, K., Nagase, T., Nomura, N., Kikuchi, H., Masuho, Y., Yamashita, R., Nakai, K., Yada, T., Ohara, O., Isogai, T., and Sugano, S. (2004). Complete sequencing and characterization of 21,243 full-length human cDNAs. *Nat Genet,* **36**(1), 40-45.

Pickard, M. R., and Williams, G. T. (2016). The hormone response element mimic sequence of GAS5 lncRNA is sufficient to induce apoptosis in breast cancer cells. *Oncotarget,* **7**(9), 10104-10116.

Pierpont, M. E., and Yunis, J. J. (1977). Localization of chromosomal RNA in human G-banded metaphase chromosomes. *Exp Cell Res,* **106**(2), 303-308.

Rossi, M. N., and Antonangeli, F. *LncRNAs: New Players in Apoptosis Control*: Int J Cell Biol. 2014;2014:473857. Epub 2014 Jan 30 doi:10.1155/2014/473857.

Shi, X., Sun, M., Liu, H., Yao, Y., Kong, R., Chen, F., and Song, Y. (2015). A critical role for the long non-coding RNA GAS5 in proliferation and apoptosis in non-small-cell lung cancer. *Mol Carcinog,* **54 Suppl 1**, E1-e12.

Shi, Y., Liu, Y., Wang, J., Jie, D., Yun, T., Li, W., Yan, L., Wang, K., and Feng, J. (2015). Downregulated Long Noncoding RNA BANCR Promotes the Proliferation of Colorectal Cancer Cells via Downregualtion of p21 Expression. *PLoS One,* **10**(4), e0122679.

Spizzo, R., Almeida, M. I., Colombatti, A., and Calin, G. A. (2012). Long non-coding RNAs and cancer: a new frontier of translational research? *Oncogene,* **31**(43), 4577-4587.

Sun, M., Jin, F. Y., Xia, R., Kong, R., Li, J. H., Xu, T. P., Liu, Y. W., Zhang, E. B., Liu, X. H., and De, W. (2014). Decreased expression of long noncoding RNA GAS5 indicates a poor prognosis and promotes cell proliferation in gastric cancer. *BMC Cancer,* **14**(319), 1471-2407.

Tao, R., Hu, S., Wang, S., Zhou, X., Zhang, Q., Wang, C., Zhao, X., Zhou, W., Zhang, S., Li, C., Zhao, H., He, Y., Zhu, S., Xu, J., Jiang, Y., Li, L., and Gao, Y. (2015). Association between indel polymorphism in the promoter region of lncRNA GAS5 and the risk of hepatocellular carcinoma. *Carcinogenesis,* **36**(10), 1136-1143.

Thomas, C. A., Jr. (1971). The genetic organization of chromosomes. *Annu Rev Genet,* **5**, 237-256.

Topalian, S. L., Hodi, F. S., Brahmer, J. R., Gettinger, S. N., Smith, D. C., McDermott, D. F., Powderly, J. D., Carvajal, R. D., Sosman, J. A., Atkins, M. B., Leming, P. D., Spigel, D. R., Antonia, S. J., Horn, L., Drake, C. G., Pardoll, D. M., Chen, L., Sharfman, W. H., Anders, R. A., Taube, J. M.,

McMiller, T. L., Xu, H., Korman, A. J., Jure-Kunkel, M., Agrawal, S., McDonald, D., Kollia, G. D., Gupta, A., Wigginton, J. M., and Sznol, M. (2012). Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med,* **366**(26), 2443-2454.

Wang, K. C., and Chang, H. Y. *Molecular mechanisms of long noncoding RNAs*: Mol Cell. 2011 Sep 16;43(6):904-14. doi:10.1016/j.molcel.2011.08.018.

Wapinski, O., and Chang, H. Y. (2011). Long noncoding RNAs and human disease. *Trends Cell Biol,* **21**(6), 354-361.

Wei, M. M., and Zhou, G. B. (2016). Long Non-coding RNAs and Their Roles in Non-small-cell Lung Cancer. *Genomics Proteomics Bioinformatics,* **14**(5), 280-288.

Zhang, H., Guo, Y., Song, Y., and Shang, C. (2017). Long noncoding RNA GAS5 inhibits malignant proliferation and chemotherapy resistance to doxorubicin in bladder transitional cell carcinoma. *Cancer Chemother Pharmacol,* **79**(1), 49-55.

Zou, Y., Li, J., Chen, Y., Xiao, H., Zhang, F., Yu, D., and Luo, K. (2017). BANCR: a novel oncogenic long non-coding RNA in human cancers. *Oncotarget,* **8**(55), 94997-95004.

URKUND

Urkund Analysis Result

Analysed Document: CHAPTER 1 to chapter 5 - APA REF 3 updated 22052018.docx (D39117575)

Submitted: 5/24/2018 12:43:00 PM

Submitted By: aklankjain@gmail.com

Significance: 3 %

Sources included in thereport:

https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf

https://link.springer.com/article/10.1186/1476-4598-13-68

http://www.ajcr.us/files/ajcr0023134.pdf

https://www.thermofisher.com/ca/en/home/life-science/stem-cell-research/mesenchymal- stem-cells/alkaline-phosphatase-msc-cells/alk-phos-msc-passaging.html

Instances where selected sourcesappear:

5