Epigenetic regulation of key genes involved in Cervical malignancy

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE

IN

LIFE SCIENCE

SUBMITTED TO

NATIONAL INSTITUTE OF TECHNOLOGY

ROURKELA

Submitted by:

Ms. Jyoti Roy

412LS2055

Supervised By:

Dr. Bibekanand Mallick

Assistant Professor

Department of Life Science

National Institute of Technology Rourkela

राष्ट्रीय प्रौद्योगिकी संस्थान, राउरकेला
National Institute of Technology, Rourkela

Dr. Bibekanand Mallick, M.Tech., Ph.D.
Assistant Professor

RNAi & Functional Genomics Lab.
Department of Life Science
National Institute of Technology
Rourkela - 769 008, Odisha, India
Telephone: +91-661-246 2685 (O)
E-mails: vivek.iitian@gmail.com, mallickb@nitrkl.ac.in
Website: http://vvekslab.in

CERTIFICATE

*This is to certify that the thesis entitled "**Epigenetic regulation of key genes involved in Cervical malignancy**" submitted by **Ms. Jyoti Roy** (Roll No: 412LS2055) in partial fulfilment of the requirements for the award of **Master of Science in Life Science** to the National Institute of Technology, Rourkela, is an authentic and original record of research work carried out by her under my supervision and guidance.*

To the best of my knowledge, the work incorporated in this thesis has not been submitted elsewhere for the award of any degree.

11/05/2K14

Place: Rourkela

Date: 11.05.2014

(Dr. Bibekanand Mallick)

Dr. Bibekanand Mallick
Assistant Professor
Department of Life Science
NATIONAL INSTITUTE OF TECHNOLOGY
Rourkela-769008, Odisha

Fax: +91-661-2472926 | Tel: +91-661-246 2685 (O) | +91-661-246 4685 (Lab) | Website: http://vvekslab.in

ACKNOWLEDGEMENT

A project is a golden opportunity for learning and self development. I consider myself very lucky and honoured to have so many wonderful people lead me through in completion of this project. I take this opportunity to thank my Almighty God for his abundant grace which enabled me to finish my project work successfully.

I express my heartfelt gratitude & indebtness to my Guide, **Dr Bibekanand Mallick**, Assistant Professor, National Institute of Technology for his valuable guidance, encouragement, patience, & worthy suggestions for the project work, without which this project work would not have materialized.

I am grateful to the Ph.D scholars Ms. Debashree Das, Mrs. Devyani Samantrrai, Ms. Mousumi Sahu, Mrs. Garima Singh, Mr Bedanta Ballav Mohanty, for their support & guidance to work in their Laboratory.

I am indebted to my loving parents, my near & dear ones for their inspiration, support & love. Finally, thanks are due to all my friends & well-wishers for their support throughout my course & project work.

Date*:* ***Jyoti Roy***

Place:

LIST OF SYMBOLS AND ABBREVIATIONS USED

µg	Micro gram
µl	Micro litre
°	Degree
C	Centigrade
ml	Mili litre
mM	Mili molar
min	Minutes
%	Percentage
No.	Number
FC	Fold Change
Reg.	Regulation

TABLE OF CONTENTS

Sl. No.	CONTENTS	PAGE No.
1	INTRODUCTION	1-3
2	REVIEW OF LITERATURE	4-20
3	OBJECTIVES	21
4	MATERIALS AND METHODS	22-32
5	RESULTS AND DISCUSSIONS	33-37
6	CONCLUSIONS	38
7	FUTURE PROSPECTIVES	39
8	REFERENCES	40-44

LIST OF TABLES

Table-1- List of differentially expressed miRNAs in Cervical intraepithelial neoplasia (CIN) and/or Squamous Cell Carcinoma (SCC) vs. normal Squamous epithellium.

Table-2.1 Components used for mRNA-cDNA synthesis.

Table-2.2 Components used for mRNA-cDNA synthesis.

Table-3 Components used for miRNA-cDNA synthesis

Table-4 Components used for qRT-PCR of mRNA

Table-5 Components used for qRT-PCR of miRNA

Table-6 List of primers for miRNA qRT-PCR

LIST OF FIGURES

Figure-1 Progressive stage of cancer from benign to malignancy.

Figure-2 Different cancer types

Figure-3 Hallmarks of cancer

Figure-Stages of metastatic progression

Figure-5 Overview of Cervix

Figure-6 The Stage I of cervical cancer

Figure-7 The Stage II of cervical cancer

Figure-8 The Stage III of cervical cancer

Figure-9 Stage IV of cervical cancer

Figure-10 DNA modification through methylation at the CpG island

Figure-11 Mechanism of action of microRNA

Figure-12 Types of miRNA target sites

Figure-13 Cycle temperature for mRNA qRT-PCR

Figure-14 cycle temperature for miRNA qRT-PCR

Figure-15 clustering analysis for both mRNAs showing the list of altered expressed genes

Figure-16 Enrichment analysis by *NCI* pathway analysis tool

Figure-17 miRNA target Prediction using *TargetScan*

Figure-18 The melting curve of CYR61

Figure-19 The melting curve of miR-221

Figure- 20 qRT-PCR result showing upregulation of CYR61 in cervical cancer cell line.

Figure- 21 qRT-PCR result showing downregulation of miR-221 in cervical cancer cell line.

ABSTRACT

microRNAs (miRNAs) are single stranded non-coding RNAs of about 22 nucleotides that have been reported to be involved in various biological processes like embryonic development, cell proliferation, differentiation, apoptosis, developmental timing etc. The role of miRNAs as tumor suppressors/oncomiRs has been reported in many cancers. In this study, we performed genome-wide expression analysis of miRNAs as well as mRNAs in cervical cancer and obtained 257 up-regulated and 81 down-regulated mRNAs as well as 27 up-regulated & 14 down-regulated miRNAs. Enrichment analysis of differentially expressed mRNAs of cervical cancer revealed CYR61 (cysteine-rich, angiogenic inducer, 61) as a key gene involved in vascularisation of the tumors. This CYR61 was found to harbor target sites (7mer-m8) for hsa-miR-221 as inferred from TargetScan predictions. From qRT-PCR study in HeLa cell lines, we found that CYR61 is over-expressed, whereas hsa-miR-221 is down-regulated in cancer system. The altered expression of CYR61 might be due to down-regulation of hsa-miR-221 as this miRNA has target sites within 3′UTR of CYR61 which can be further confirmed by luciferase reporter assay. We hypothesize that hsa-miR-221 might be playing a role in metastatic spread and lethality in cervical cancer by altering the expression of the corresponding mRNA through RNA interference mechanism. Moreover, it can be expected that the altered expression of hsa-miR-221 may be due to promoter hypermethylation of the miRNA gene or through targeting by other non-coding RNAs, such as lncRNAs which need further studies in future.

Keywords: microRNAs, CYR61, angiogenesis, cervical cancer, metastasis, DNA methylation

INTRODUCTION

Cancer is an emerging class of disease characterized by out-of-control cell growth where some of the somatic cells '*deny to die*' and invade other tissues. Several check points or quality controls are employed in order to prevent uncontrolled proliferation of cells. The normal cells prefer to commit suicide when these cells could not pass through the quality controls, however this does not happen in case of malignancy. The abnormal and uncontrolled cell growth becomes harmful for the body when the affected cells divide rapidly to form lump of cells called tumors. These tumors in turn interfere in the normal cell metabolisms and spread to other parts of the body through circulatory and lymphatic systems. Till now, more than 100 cancer types have been reported and characterized.

All cancers begin from the basic unit of life i.e cell. For well understanding of cancer, it is better to know what actually happens when normal cells become cancerous. In case of normal cell types, they grow and divide in a controlled way to keep the body healthy. As they grow older or damaged, they prefer to die and are replaced by the newer ones. However, sometimes the orderly process goes deviated when the genetic material of the cell (DNA) become damaged, producing mutations and ultimately affecting normal cell growth and division.

Dys-regulation of the gene networks involved in maintaining healthy cellular identity, growth and differentiation result in diseases like cancer. A very tiny proportion of cancer is the result of inheritable single-gene disorder that involves non-synonymous mutation in the coding sequence of mRNA transcript such as BRCA1 in breast cancer and Rb1 in retinoblastoma (Cheetham et al., 2013). As a result of this, cells don't die when they should and new cells tend to form when they are not supposed to be created, leading to formation of mass of tissues called tumors.

Tumors that are found to be non-motile i.e do not spread to other parts of the body and are characterized by limited growth are known as benign tumors. Other type of tumors is a malignant tumor which is motile and invades nearby tissues and spread to different parts of the body. This spread of malignant tumor/cancer is known as metastasis. Cancers can be classified depending on their place of origin. These are – (i) Carcinoma- cancer of epithelial origin like skin or tissues that cover internal organs. The subtypes of this category include adenocarcinoma, basal cell carcinoma, squamous cell carcinoma and transitional cell carcinoma etc. (ii) Leukemia- cancer of blood cells or blood-forming cells such as bone marrow. (iii) Lymphoma- cancer of immune cells. (iv) Sarcoma- cancer of mesenchymal origin. (v) CNS cancer- cancer of central nervous system i.e cancer that begin in the tissue of the brain and spinal cord.

For the metastatic spread of malignant tumors, supply of vascular network is very important. The processes by which new blood vessels are formed and lymphatic system is established are called angiogenesis and lymhangiogenesis respectively. Both have essential role in the growth and maintenance of cancer cells by providing nutrients, oxygen and removing waste products. These phenomena are now getting special attention in the field of neoplastic vascularization. Without the vascular support, the tumors may become necrotic and apoptotic. Vascularization is basically a four step process- first, there will be occurrence of local injury to the basement membranes, then angiogenic factor migration causing endothelial cells activation followed by proliferation and stabilization and finally the angiogenic factors continue to influence the angiogenesis process (Nishida et al., 2006).

The potentiality of the cancer cells to multiply independently of exogenous growth inhibitory or growth promoting signals, invade nearby tissues and metastasize, exhibit angiogenic response, evade program cell death and cellular senescence are the reflection of altered cell signaling pathways. Current research strategies are mainly based on the signaling molecules that are supposed to elicit potential response in these pathways.

The cancer of prostrate, breast, lungs, colon/rectum and cervix account for more than half of the cancer cases. In developing countries, cervical cancer is the second largest class of malignant tumors which endangers the woman's health (Lei et al., 2012). It is the cancer of cervix (the connecting organ between uterus and vagina) and is generally slow growing. This cancer usually doesn't show any symptoms and can only be detected through regular pap tests (a procedure by which the cervical cells are scraped and seen under microscope).

Although cervical cancer is mainly caused by prolonged infection with Human papilloma virus (HPV), but it has been found that it may arise due to abnormal expression or functioning of oncogenes or tumor suppressor genes and/or other factors (Kaczkowski et al., 2012).

The abnormal expression involves either up or down regulation of genes, achieved by two means- Histone/DNA modification & RNA interference (RNAi) mechanism (Wilting et al., 2013a). RNAi mechanism is mainly operated by non-coding RNAs (ncRNAs) that cause translational repression or degradation of target transcript and thereby alter their normal function. DNA modification mainly occurs through promoter hyper or hypo-methylation. An association between chromosomal alteration and differential expression of miRNA has been reported in many cancer types such as breast cancer, melanoma, neuroblastoma and myeloma. It has been reported that the miRNA exhibits its regulatory mechanism through inhibition of mRNA translation or direct degradation of the coding transcript thereby promoting the development of cervical cancer. The microarray analysis performed by them indicated that there are a large number of differentially expressed miRNAs as well as mRNAs in cervical cancer, which together promote its occurrence and development (Ma et al., 2012).

The significance of DNA methylation and its impact on gene activity in many cancers have been studied. The frequency of promoter methylation has been found in case of many tumor supressor genes in cervical cancer as well. The p16 gene that play a key role in controlling cell cycle progression has been reported to be progressively methylated in 31% of the cervical cancer cases. There are also reports of methylation in other genes such as FHIT and GST enzyme families that accounts for 20-30% of cervical cancer cases (Virmani et al., 2001).

Proper understanding and unrevealing the mystery of this cancer progression and the effect of the above described control mechanisms on various key transcripts can provide a better way for the therapeutic prevention of this deadly disease. Metastasis is the main cause of invasion and spreading of cancers including cervical cancer and angiogenesis is the key mechanism involved in this process contributing to lethality of the cancer. Therefore, we have chosen some key regulatory genes involved in various signaling pathways promoting angiogenesis which will be helping in cancer progression. By exploring the control mechanism responsible for the abnormal expression of these transcripts involved in angiogenesis and signaling pathways might provide some effective preventive major of this cancer. We

executed this project by studying the differentially expressed transcripts and miRNAs and the cross-talks among them by amalgamating microarray analysis, miRNA target prediction, miRNA-mRNA interaction studies with experimental validation of selected mRNAs involved in angiogenesis and miRNA proposed to be regulating these genes in HeLa cell line (test) and HaCaT cell line (control).

REVIEW OF LITERATURE

Cancer

Cancer is a multistep process in which cells acquire their ability to proliferate uncontrollably and undergo metastasis. The crucial steps involved in transformation of normal cells in to malignant type are the ability of the cells to be self-sufficient and unresponsiveness to the growth-limiting signals (Hanahan and Weinberg, 2000). The abnormal behavior exhibited by the cancer cell that distinguishes itself from normal cell type is the result of accumulated abnormalities in diverse regulatory systems. As cancer can arise from uncontrolled proliferation of any kind of cells in the living system, so there exist more than 100 distinct types of cancer those vary in their regular behavior and response to the conventional therapies. Distinction between benign and malignant tumors is one of the most importance aspects of cancer pathology (Fig.1).

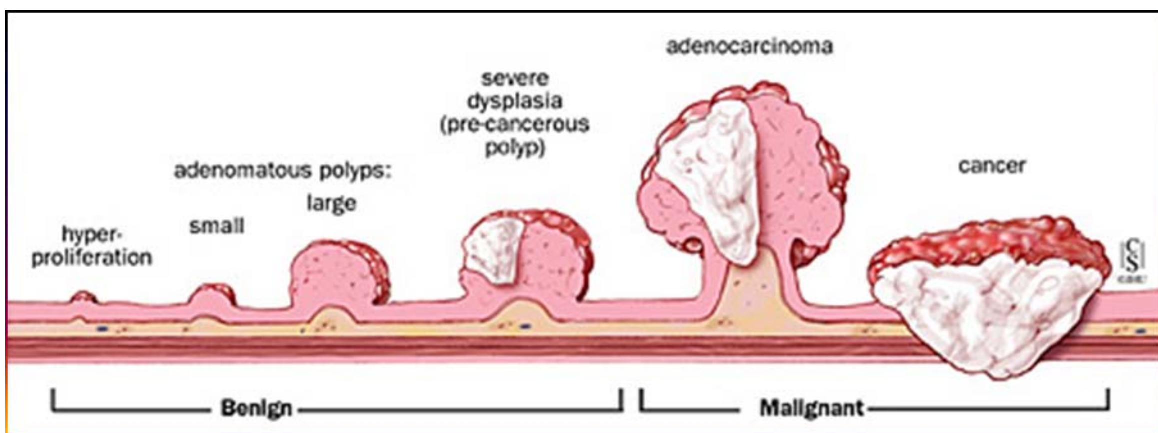


Fig.1. Progressive stage of cancer from benign to malignancy (*adopted from- www.hopkinscoloncancercenter.org*).

The marked region for benign shows hyper proliferative and non-motile tumors and that for malignant shows invasion and migration of the tumors in to the blood stream.

A tumor that remains confined to its original location, incapable of spreading or invading to the nearby tissues is known as benign. These are mostly like common skin warts. A malignant tumor on the other hand is capable of invading neighboring cells as well as distant normal tissues and thus spreads throughout the body with the help of circulatory or lymphatic systems. The spreadability of the cancer through these circulatory or lymphatic systems is known as metastasis, the only cause of most cancer lethality. The malignant tumors are mainly considered as cancers with the ability to metastasize (GM).

Types of cancer

Both the malignant and benign tumors are categorized according to their cells of origin. Most cancers can be systematized in to the following main groups (Fig.2)-

- Carcinomas
- Sarcomas
- Leukemia
- Lymphomas
- CNS cancers

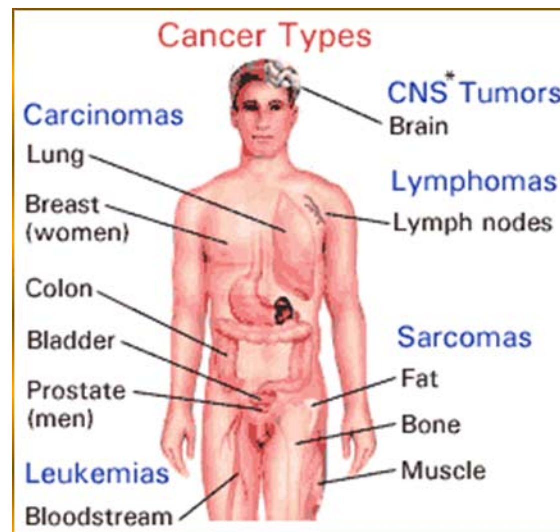


Fig.2. Different cancer types *(adopted from-HEALTH LIBRARYweb.iadmd.org)*

- **Carcinoma**- It accounts for approximately 90% of human cancers and are the malignancies of cells of epithelial origin. The subtypes of this category include adenocarcinoma, basal cell carcinoma, squamous cell carcinoma and transitional cell carcinoma etc. Depending on the tissue of origin these can be further classified accordingly such as Breast cancer, Lung cancer, colon cancer etc.

- **Sarcoma-** These are solid tumors of connective tissues or cancer of mesenchymal origin such as muscle, cartilage, fibrous tissues (fibrosarcoma) and bones.

- **Leukemia-** These are cancer of blood cells or blood-forming cells such as bone marrow which account for approximately 8% of human malignancies. Other examples of this cancer type are Erythroid leukemia arising from the red blood cells.

- **Lymphoma-** These are cancer of cells from immune system that arises from lymph-nodes

- **CNS cancer-** These are cancer of central nervous system i.e cancer that begin in the tissue of the brain and spinal cord (GM).

Hallmarks of cancer

Irrespective of the diverse types of cancer, there exists some idiosyncrasy that makes the cancer well distinguishable from normal cells. There exist various hall marks (Fig. 3) that enable metastatic growth and allocate solid foundation for understanding the biology of cancer (Hanahan and Weinberg, 2011).

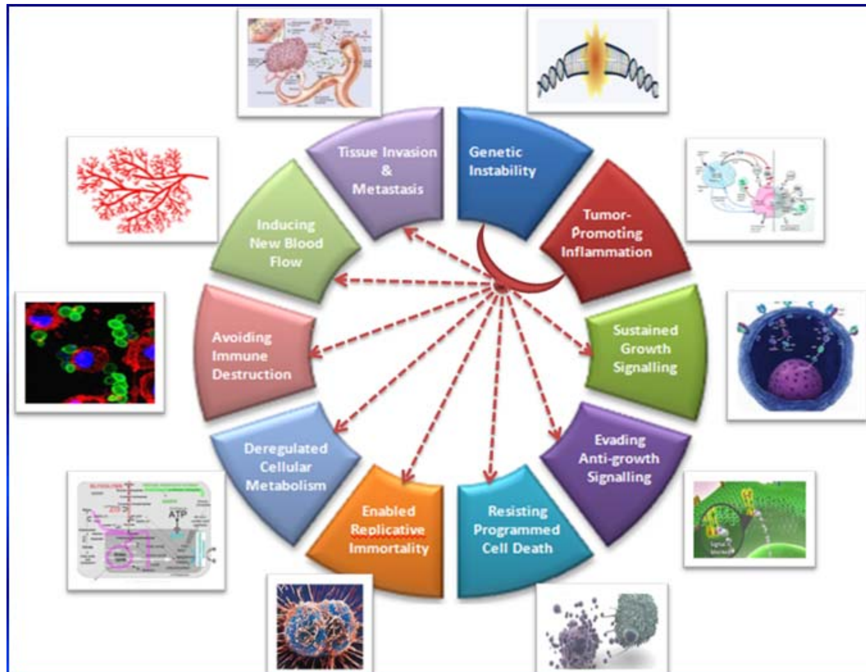


Fig. 3. Different Hallmarks of cancer

Metastasis and Cancer

Adaptation of the unstable malignant cells to a new microenvironment that is away from their origin is considered as metastasis. In due course of metastasis, some beneficial traits are selected that helps invasion as well as the survivability and stability of cancer cells. Recent understandings related to the origin and nature of cancer metastasis has been improved by new advanced technologies. Although the actual cause behind the mechanism that how the malignant tumors spread throughout the body and kill the host is not known, but the cancer biologists have predicted that it might be due to interplay between rambling tumor cells with their bialable target tissues. The mechanism about how tumor cells enter to metastasis through changing their own microenvironment, entering to the circulatory or lymphatic system, and finally colonizing at the distant tissue has been less explored till to date, irrespective of the fact that it is the crucial cause of more than 90% of deaths because of solid tumors. Recent studies indicate that, metastasis may occur due to genomic instability because of some oncogenic events like evasion of growth suppression or DNA damage check points (Gupta and Massague, 2006).

The steps involved in biological cascade of metastasis mainly include- loss of cellular attachment, transition, invasiveness, entry to the circulatory system and colonization at a distant tissue sites (Chambers et al., 2002). Recent works based on the genomic-level molecular profiling have unrevealed some key regulatory genes whose expression in primary tumor state and the metastatic state are found to be strongly co-related (Weigelt et al., 2005). These observation has provided some clue to find out how, where and when the cancer cells acquire the relevant genes to undergo metastasis in diverse cancer types (Bernards and Weinberg, 2002).

Metastasis takes place as a result of progressive implication of traits that enable the cancer cells to go for migration and invasion at the secondary site (Fig.4). All the these traits not necessarily should follow this particular order but the factors responsible for each step may facilitate the execution of multiple steps simultaneously (Gupta and Massague, 2006). It is also possible that the rate limiting steps for this metastatic progression may also vary from diverse tumor types.

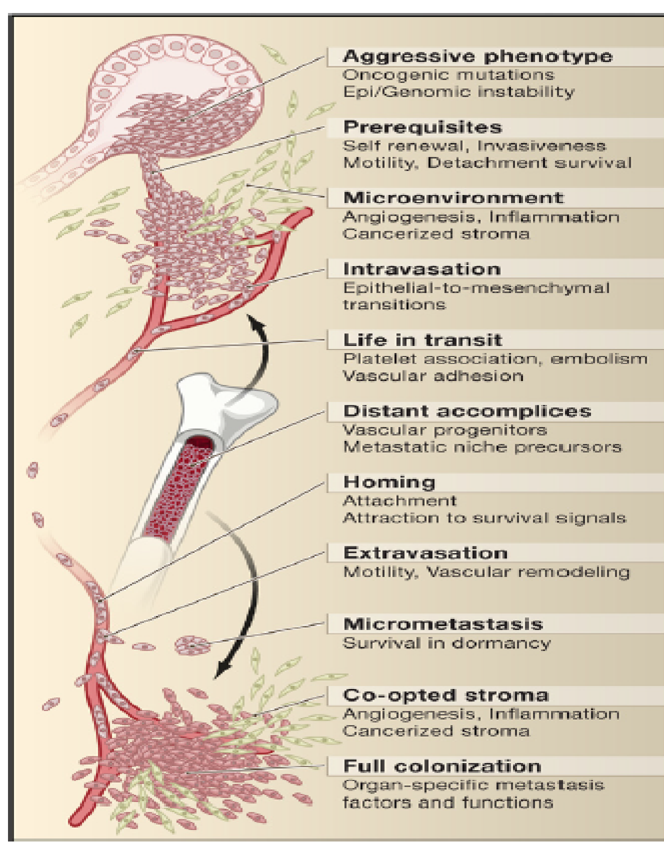


Fig.4. Stages of metastatic progression *(adopted from (Gupta and Massague, 2006)*

Cervical cancer

Cervical cancer is a carcinoma of cervix, the joining between uterus and vagina. Cervix is the lower and narrow part of vagina situated in the pelvis that constitute the major portion of the female reproductive system (Fig.5) (Elst et al., 2007). The cervix is considered as a gateway because-

- It connects the uterus with vagina. During the menstrual period, blood flow from the uterus, move to the vagina and finally to the outside.
- Mucus secreted by the cervix help in the entry of sperm in to uterus through vagina during sexual intercourse.
- In due course of pregnancy, cervix remains tightly closed to keep the baby inside the uterus and opens to allow the baby to pass through the vagina during childbirth.

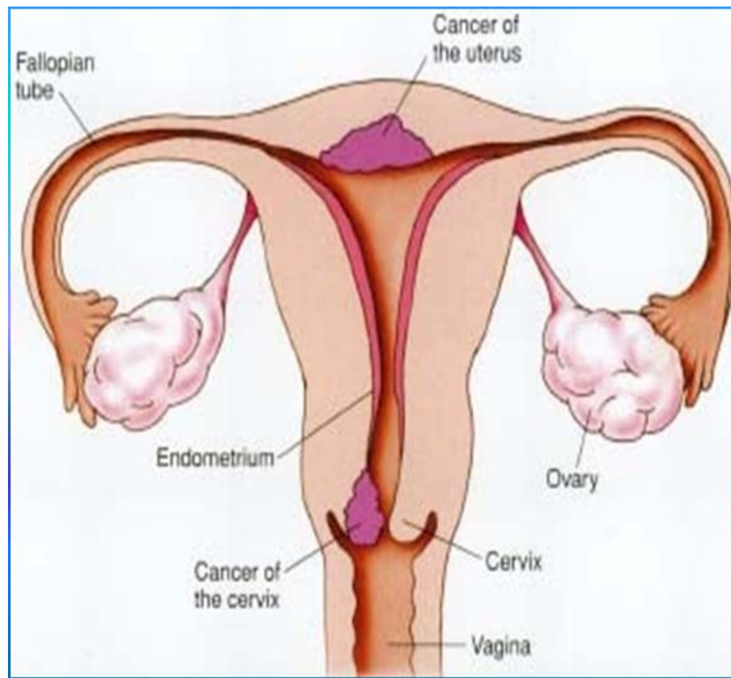


Fig.5. Overview of Cervix (adopted from www.ncis.com.sg)

There are two categories of cervical cancer-

Squamous cell cervical cancer (Ectocervix) - This type of cervical cancer arises when the flat fish scale like cells present on ectocervix region become malignant. Ectocervix also known as portio vaginialis is the portion of cervix that projects in to the vagina.

Cervical adenocarcinoma (Endocervix) - It arises in the inner lining of the cervix that are covered by glandular cells leading to adenocarcinoma of the cervix.

The region of Endocervix contains the transformation zone (most prone region to develop the cancer) situated in the endocervical canal. During the screening for cervical cancer, this region is mainly studied by the doctors (Elst et al., 2007)

Symptoms of Cervical cancer

Similar to many other cancer types, patients generally experience no symptoms at the early stages. Therefore, it is always advised to women to have regular cervical smear tests. At later stages, patients experience some symptoms like-

- Excessive vaginal bleeding after sexual intercourse, douching(cleaning out of vagina with water or some specific fluid to avoid sexually transmitted diseases especially done during pregnancy to avoid any kind of infection) or any kind of pelvic examination.
- Durable and heavy menstrual periods.
- Heavy vaginal bleeding even after going through menopause.
- Increased and smelly vaginal discharges sometimes mixed with blood.
- Pelvic pain and pain during sex (Garner, 2003)

Stages of cervical cancer

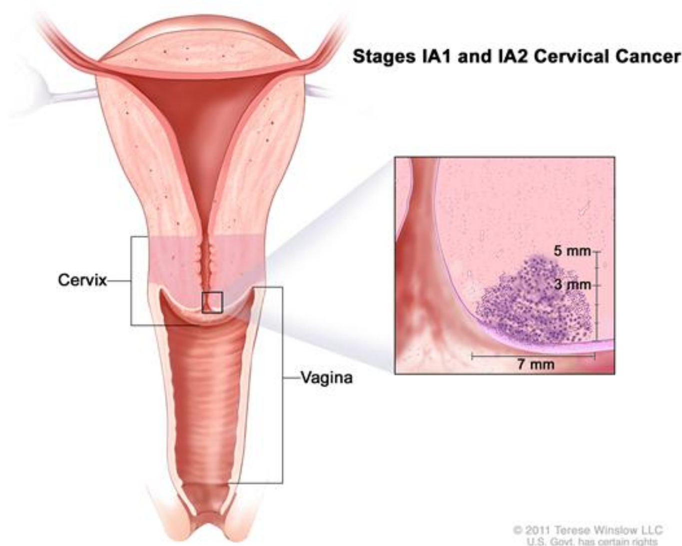
Most cancers spread from their primary location to the other mainly through lymphatic or circulatory systems or invading through the tissues. Malignant cells spread through the tissues by growing to nearby areas, while spreading through lymph systems they move through the lymph vessels and similarly travels through the blood vessels after entering to the circulatory system. Depending on the extent of cancer spread from the cervical region to other parts of the body, there are many stages found according to the survey by the National Cancer Institute. These are as follows-

Stage 0:

At this stage, the abnormal cells are localized to the innermost lining of cervix which may become cancerous and invade to the other part of the body in due course of cancer progression.

Stage I:

During this stage, cancer is found in the cervix only and depending on the amount of cancer found it can be further classified in to two sub-stages such as stage IA and IB (Fig.6). At this stage, The cancer cells can only be visualized through microscope. The cancer cells at stage IA1 and IA2 are found to be 3-5 millimeters deep and 7 millimeters wide.



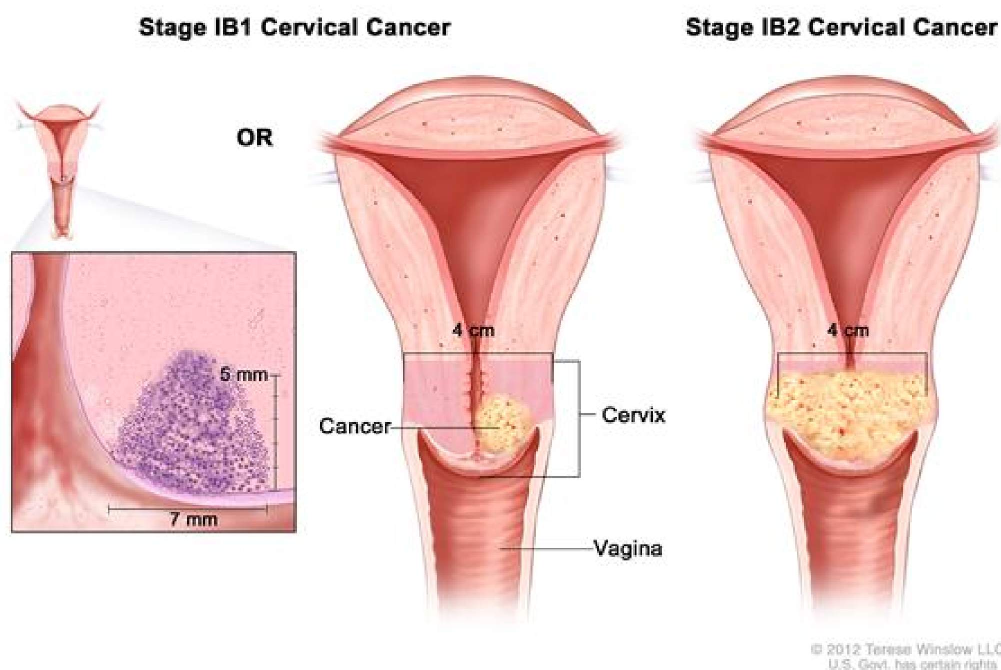


Fig. 6. The Stage I of cervical cancer (*adopted from- http://www.cancer.gov/cancertopics/pdq/treatment/cervical/Patient/page2*)

At the stage IB1 and IB2, the cells can be seen without microscope and are more than 5 millimeters deep, more than 7 millimeters wide .

Stage II:

In stage II, cancer cells tend to spread beyond the cervix, but not up to the pelvic wall or lower part of vagina. It can be further divided to IIA or IIB (Fig. 7) depending on how far the cancer has been spread. In stage IIA (IIA1 & IIA2), the cancer cells spread up to the two thirds of vagina but not till the uterine region, while in stage IIB, cancer tend to move to the tissues around the uterus.

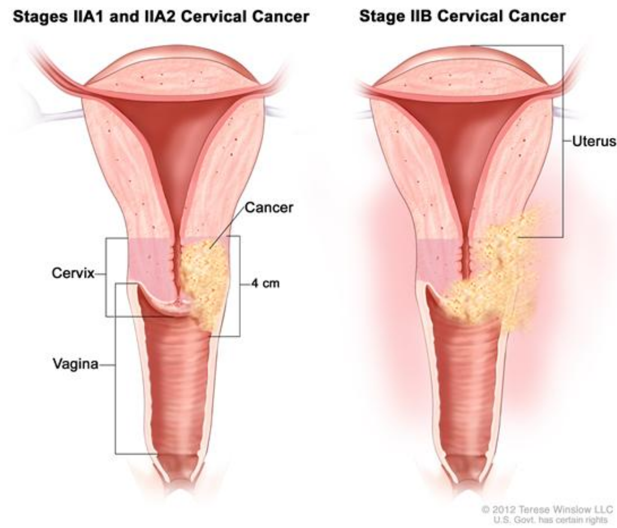


Fig.7. The Stage II of cervical cancer (*adopted from-http://www.cancer.gov/cancertopics/pdq/treatment/cervical/Patient/page2*)

Stage III:

In this stage, cancer has spread to the lower part of vagina, to the pelvic wall. Some kidney disorders are found at this stage and can be classified to IIIA or IIB (Fig. 8) depending on how far the cancer cells have migrated. At this stage the tumor cells become large enough to block the uterus as a consequence kidney starts enlarging and stop functioning.

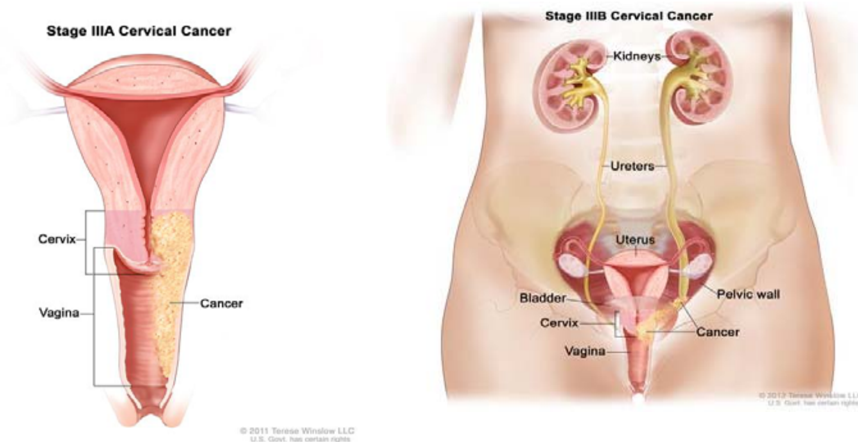


Fig. 8. The Stage III of cervical cancer (*adopted from-http://www.cancer.gov/cancertopics/pdq/treatment/cervical/Patient/page2*)

Stage IV:

At this stage, cancer starts migrating to the other parts of the body. Based on the localization of the cancer, this stage can be divided in to sub-stages like IVA (bladder, rectum) or IVB (liver, lungs, and bones) (Fig. 9).

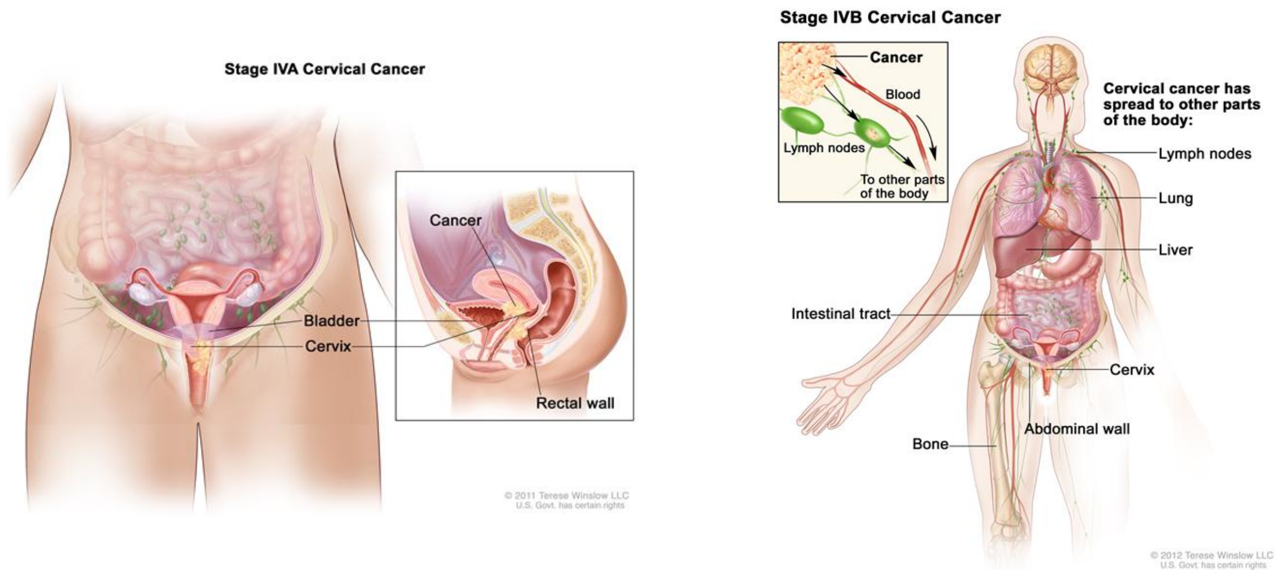


Fig. 9. Stage IV of cervical cancer *(adopted from- http://www.cancer.gov/cancertopics/pdq/treatment/cervical/Patient/page2)*

Causes of Cervical cancer

There are many factors reported that are known to increase the risk of cervical cancer as surveyed by National cancer Institute (NCI).

HPV (Human papilioma virus) infection:

The virus is a sexually transmitted virus of 100 different kinds, out of which 15 types are known to cause cervical cancer. It has been found that the HPV type 16 and 18 are known to cause approximately 70% of cervical cancer cases and type 6 & 11 cause 90% of genital warts. Other HPV types may promote the growth of abnormal cells on the surface of cervix causing cervical intra-epithelial neoplasia (CIN).

Multiple sexual partners:

The HPV causing the cervical cancer are mainly transmitted due to sexual contacts with the infected person. Therefore, it is expected that the woman having many sexual partners are generally tend to possess high risk of cancer through infection with HPV.

Smoking:

Like other cancers, smoking is known to increase the risk of cervical cancer possibly through weakening our own immune system.

Suppressed immune system:

Persons suffering from diseases like HIV/AIDS that weaken the host's immune system, are mostly in higher risk of having this cancer. This is also seen in individuals who have taken immune suppressive drugs during any transplantation therapy.

Giving birth at an early age or having several pregnancies:

Women who have given birth to child at very early age like 17 are supposed to have high risk for cervical cancer as compared to the lady who has undergone pregnancy after the age of 25. Repeated and several pregnancy also have shown to increase the risk of this cancer.

Other sexually transmitted Disease:

Women having infection with Chlamydia, gonorrhea or syphilis are highly sensitive to develop cervical cancer. It has been found by scientists at the Medical university of South Carolina that HPV infection becomes long lasting in presence of Chlamydia.

Other factors like long term mental stress, continuous use of contraceptive pills, socio-economic status also contribute to the higher probability of being infected with cervical cancer.

Other genetic and epigenetic causes:

Besides these factors associated with cervical carcinogenesis, other genetic and epigenetic factors also promote the cervical carcinogenesis. These factors are include DNA or Histone modification or the regulatory mechanism exhibited by the RNA interference which alter the normal functioning of various genes involved in diverse biological processes like cell cycle regulation, growth arrest, angiogenesis, metatstasis, tumor suppression etc. These abnormal functioning helps the tumor cells to proliferate uncontrollably by increasing their stability.

The integrated genome analysis of Squamous cell carcinoma (SCC) and Adenocarcinoma (AdCs) conducted by Wilting et al identified 83 differentially expressed genes in comparison with normal epithelium. They also identified chromosomal hot spots where the gain or loss at those chromosomes leads to alteration in expression of the corresponding genes viz. chromosomal gains at the arm of chromosome 1, 3, and 20 leads to over expression of the genes (DTX3L, PIK3RA, ATP2C1 and SLC25A36) located at 1q32.1-32.2, 3q13.32-23, 3q26.32-27.3, and 20q11.21-13.33 whereas loss in chromosome at 11q22.3-25 were related with low activity of those associated genes (Wilting et al., 2008). In another study, it was reported that alteration of mRNA expression that codes for sialytransferase was found to be associated with cervical carcinogenesis (Wang et al., 2001). Besides these genetic factors, the epigenetic control mechanisms comprising mainly of DNA/Histone modification and RNA interference are also known to be involved in carcinogenesis.

DNA/Histone modification:

The process of oncogenesis depend on the switch on/off activity of two different set of genes- Tumor supressor genes and oncogenes regulating different aspects of cell growth and survival. The alterations in these genes are essential for the malignancy and are probably modulated by either of the epigenetic changes. Epigenetic changes are heritable changes in the gene activity that are not supposed to be caused by alteration in the primary DNA sequence. These epigenetic modification may comprises of - covalent modification of some amino-acid residue of the histone protein that helps in close packing of DNA maintaining its heterochromatin state; methylation or acetylation or phosphorylation or sumoylation on the DNA itself. Methylation is carried out by enzymes like DNA methyl transferases (DNMT) that cause methylation at the specific regions of promoter known as CpG island (Fig. 10) (repeated cluster of CG dinucleotides) thereby suppressing the corresponding gene transcription. Gene silencing can also be reversible through other epigenetic mechanism like acetylation of histone which results in decondensation of chromatin network and reestablishment of the transcriptional machinery by the addition of acetyl group to the Lysine residue of histone and distorting the nucleosomal structure. This aspect of epigenetic alteration in the functionality of various transcripts are now getting more attention and have become the therapeutic target in various disease systems including cancer (Gronbaek et al., 2007).

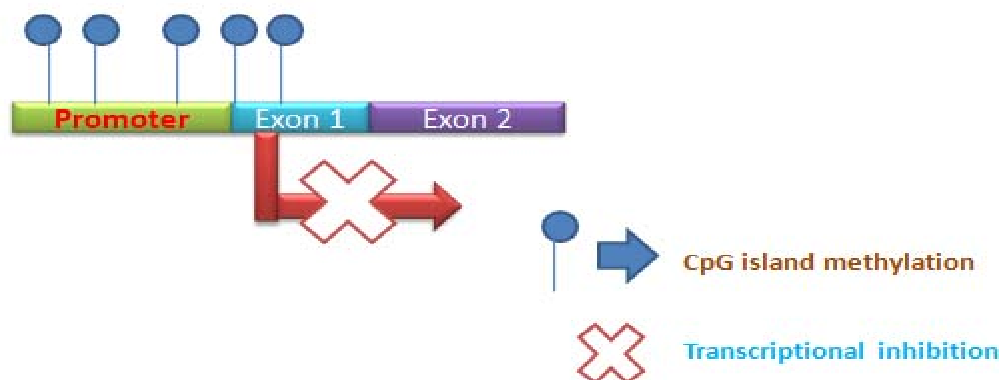


Fig. 10. DNA modification through methylation at the CpG island

DNA methylation is not only restricted for the regulation coding transcripts, there are also reports where they have been found to regulate the expression of noncoding RNAs thereby altering their expression pattern in many cancer systems including cervical. Such study was done by a group of scientists where they have found that the frequent down regulation of miR-203 was because of methylation at miR-203 promotor, thus promoting cervical tumorigenesis(Zhu et al., 2013).

Another approach in this aspect to find out the impact of methylation on the activity mRNAs in cervical cancer have shown hypermethylation miR-432, -1286, miR -1290, miR-128 and miR-95 with respect to HPV-infection (Yao et al., 2013). miR-214 that has been previously reported to inhibit cell growth and metastasis, further analysis has shown that the expression of this miRNA was correlated with expression of Bax, caspase-9 & 8 and is itself regulated by DNA methylation and histone deacetylation (Wang et al., 2013a). In an another study, deregulation of miR-149, miR-203 and miR-375 were found to be mediated by DNA methylations of the respective miRNA genes (Wilting et al., 2013b).

RNA interference:

RNA interference refers to the post transcriptional gene silencing mechanism operated by different kinds of RNA molecules. RNAs are mainly comprised of many coding as well as non-coding RNAs (ncRNAs) that interfere in the activity of other coding transcripts. The ncRNAs include small non-coding RNAs (miRNA, siRNA, piRNA etc) and long non-coding RNAs (lncRNA). The ncRNAs are involved in specific gene silencing or gene regulatory mechanisms (Gomes et al., 2013). Recent studies have shown that among the three types of small ncRNAs, miRNA have some pivotal role in normal cell metabolism and its differential expression is supposed to have diagnostic and prognostic value in many cancer cases including cervical cancer (Lui et al., 2007).

miRNAs are single stranded RNAs of 22 nucleotide in length, generated from double stranded hairpin shaped RNA precursors with the help of RNase-III enzyme (Bartel, 2004). These small ncRNAs exhibit their mode of action through post transcriptional gene silencing either by target RNA cleavage or translational repression of the target transcript (Fig.11) in association with RNA inducing silencing complex (RISC) thereby importing dramatic effects on cellular phenotypes (Filipowicz et al., 2005).

.

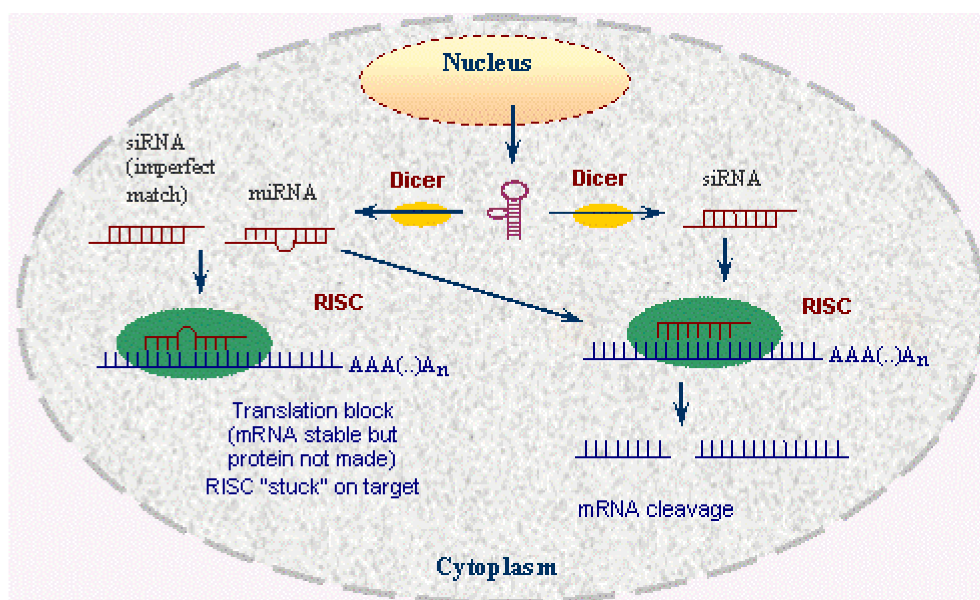


Fig.11. Mechanism of action of microRNA

The complementarity between the seed region of miRNA and with that of target mRNA has a pivotal role in miRNA-mediated gene regulation through miRNA targeting. There exist mainly three kinds of conventional target binding sites such as canonical sites, marginal sites and atypical sites (Fig. 12). In the canonical sites, the binding mainly occurs between 2-7 or 2-8 ntds position on the miRNA, while for marginal sites it lies in between 2-7 or 3-8 ntd positions. The supplementary sites are located at 12-17 position in addition to the seed region with complete Watson-crick base pairing. But compensatory binding sites are seen in between 12-19 position with respect to wobble base pairing in the traditional seed sites in order to compensate the mismatch (Bartel, 2009).

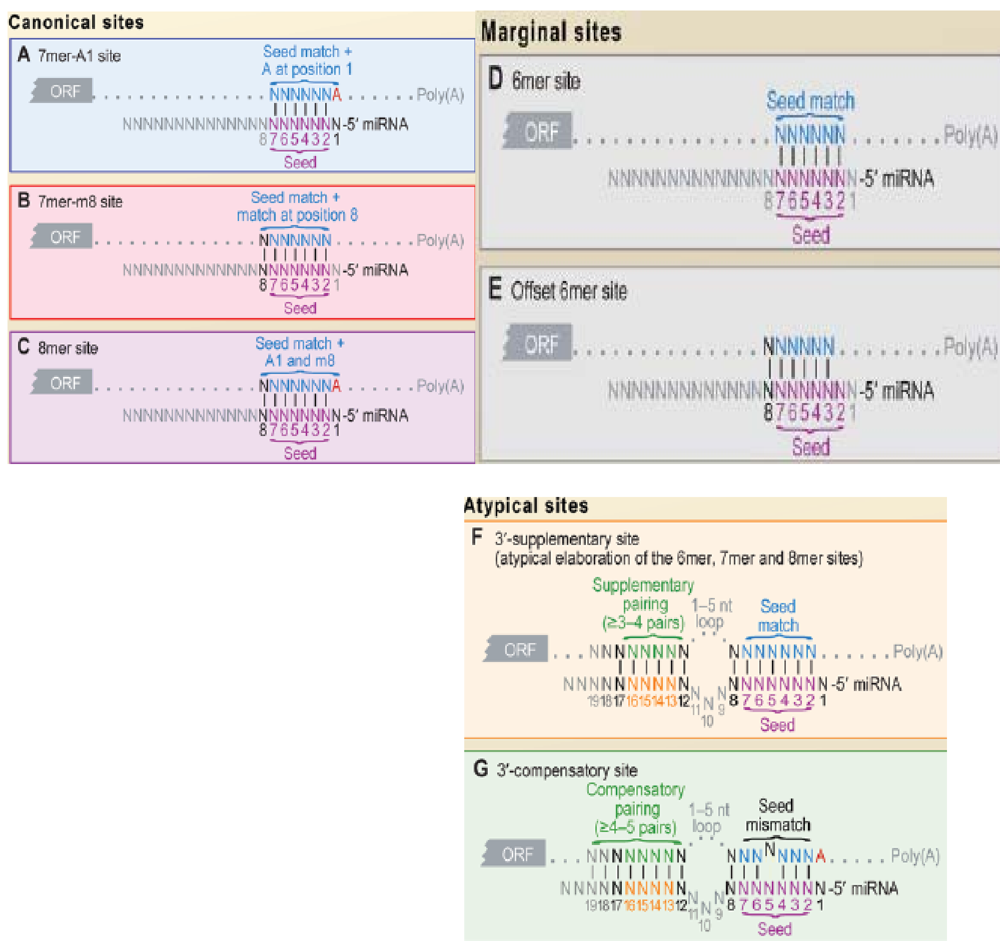


Fig.12. Types of miRNA target sites (*adapted from* (Bartel, 2009)

Role of miRNA in Cervical tumorigenesis:

The mature miRNA induce posttranscriptional gene silencing in association with RISC complex by binding to the complementary motifs on the 3'UTR (untranslated regions) of the target transcript (Fabian

and Sonenberg, 2012). As miRNA has the ability to target up to several mRNAs, therefore aberrant expression of miRNAs can have profound influence on specific genes thereby affecting cancer-associated signaling pathways (Wightman et al., 1993).

The miRNA profiling study in many tumor types suggested that they are found to have altered expression in many cases thereby by changing the expression pattern of the target transcript and further downstream signaling mechanisms. One study in such aspect have shown that, miR-29 acts as a tumor supressor in lung cancer, while in Breast cancer it was found to be involved in oncogenesis (Fabbri et al., 2007; Gebeshuber et al., 2009). Other reports like- down regulation of miR-26a was associated with hepatocellular carcinoma (Kota et al., 2009). Likewise in cervical cancer systems, many altered miRNAs have been found through genome wide miRNA expression profiling (Wilting et al., 2013a) and are listed as follows Table 1.

Table 1. List of differentially expressed miRNAs in Cervical intraepithelial neoplasia (CIN) and/or Squamous Cell Carcinoma (SCC) vs. normal Squamous epithellium (Wilting et al., 2013a).

Up-regulated miRNAs	Down-regulated miRNAs
hsa-miR-192	hsa-miR-205
hsa-miR-135	hsa-miR-27
hsa-miR-101	hsa-miR-212
hsa-miR-191	hsa-miR-770
hsa-miR-34	hsa-miR-484
hsa-miR-150	hsa-miR-636
hsa-miR-125	hsa-miR-494
hsa-miR-30	hsa-miR-125
hsa-miR-143	hsa-miR-375
hsa-miR-146	hsa-miR-99
hsa-miR-181	hsa-miR-188
hsa-let-7	hsa-miR-148
hsa-miR-26	hsa-miR-671
hsa-miR-29	hsa-miR-199
hsa-miR-10	hsa-miR-51
hsa-miR-145	hsa-miR-378
hsa-miR-425	hsa-miR-195

hsa-miR-24	hsa-miR-486
hsa-miR-331	hsa-miR-376
hsa-miR-151	hsa-miR-497
hsa-miR-151	hsa-miR-100
hsa-miR-107	hsa-miR-660
hsa-miR-17*	hsa-miR-218
hsa-miR-9	hsa-miR-203
hsa-miR-185	hsa-miR-638
hsa-miR-339	hsa-miR-370
hsa-miR-18	hsa-miR-575
hsa-miR-7	hsa-miR-193
hsa-miR-17	hsa-miR-572
hsa-miR-130	hsa-miR-149
hsa-miR-15	hsa-miR-210
hsa-miR-106	hsa-miR-622
hsa-miR-19	hsa-miR-23
hsa-miR-200c	hsa-miR-493
hsa-miR-20	hsa-miR-296
hsa-miR-363	hsa-miR-617
hsa-miR-155	hsa-miR-134
hsa-miR-141	hsa-miR-365
hsa-miR-93	
hsa-miR-15	
hsa-miR-16	
hsa-miR-28	
hsa-miR-338	
hsa-miR-206	
hsa-miR-17*	
hsa-miR-9	
hsa-miR-185	
hsa-miR-339	
hsa-miR-18	

Above mentioned altered miRNA profiling data clearly indicates that the corresponding targeted mRNA expression may also be altered in cervical cancer system. Many more studies have been carried out on this aspect in this cancer system. Recently, the CHL1 (a cell cycle regulator) gene was found to be targeted and silenced by overexpressed miR-590-5p and thus promoting cervical cancer cell growth and survival (Chu et al., 2014). In another similar kind of study, it was found that the overexpression of COX-2 gene in cervical cancer leads to cell proliferation and migration due to loss of regulation by miR-101 (Huang et al., 2013).

Moreover, it has also been found that down regulation of miR-506 was inversely correlated with KI-67 expression, a marker for cell proliferation in cervical cancer (Wen et al., 2014). In another approach, miR-181b has been seen promoting cell proliferation and inhibiting programmed cell death by targeting adenyl- cyclase 9 thereby restricting cAMP production in cervical cancer (Yang et al., 2014). Recent research has uncovered the role of miR-196a in cervical cancer proliferation and migration as a oncogenic inducer by inhibiting the expression of netrin4 (NTN4)mRNA (Zhang et al., 2013).

Another reverse interaction study between octamer-binding transcription factor 4(OCT4)mRNA and miRNA-125b has indicated that , OCT4 induces the up regulation of the miRNA by binding to its promoter and the overexpression of miR-125b reduces the activity of BAK1(another transcript) as a result reducing cell apoptosis in primary cervical cancers (Wang et al., 2013b).

Another study has demonstrated the role of miR-7 in cervical cancer. Over expression of this miRNA resulted in reduced cell viability regulating the expression of an oncogene XIAP(X-linked inhibitor of apoptosis protein) (Liu et al., 2013). Over expression of miR-125b in cervical cancer cells has been reported that results in low expression of phosphoinositide 3-kinase thereby inducing apoptosis and decreasing cell proliferation (Cui et al., 2012).

Recently it has been reported that, miR-99a and miR-99b were found to be down regulated in cervical cancer tissues and are found to target mTOR-gene thereby negatively inducing metastasis (Wang et al., 2014). Based on these observations, we have hypothesized and have found a novel miRNA and its potential target transcript which is basically an angiogenesis inducing factor, playing crucial role in metastasis of malignant

OBJECTIVES

OBJECTIVE 1

Transcriptional profiling analysis of differentially expressed mRNA in cervical cancer

OBJECTIVE 2

Identification of differentially expressed miRNA in cervical cancer

OBJECTIVE 3

Identification of miRNA-mRNA interaction network operating in cervical cancer using NCI pathway analysis database, targetscan software.

OBJECTIVE 4

Experimental validation of the selected pairs of mRNA-miRNA in the cervical cancer by qRT-PCR

MATERIALS AND METHODS

Gene Expression data:

The expression profiles of mRNAs as well as miRNAs in different stages of cervical cancer were retrieved from GEO database of NCBI. GEO provides a flexible and open design that facilitates submission, storage and retrieval of heterogeneous data sets from high-throughput gene expression and genomic hybridization experiments such as microarray. The three central data entities of GEO are platforms, samples and series, and were designed with gene expression and genomic hybridization experiments. The gene expression data are generally generated by microarray hybridization experiments that represent the expression of specific genes in a particular system. Microarray is a powerful technology for biological exploration which enables to simultaneously measure the level of activity of thousands genes.

Gene expression data are generated and stored in databases like GEO (Gene Expression Omnibus), GXD(Gene Expression Database), Gene Expression Atlas etc. For our study the microarray data were retrieved from GEO database which is the well known depository of functional genomic data. The data were retrieved for both normal (control) tissues and cancerous (test) tissues of cervical origin from different records (GSE) of GEO database. GSE records are comprised of specific experiments constituting individual samples called GSMA sample describes the set of molecules that are being probed and references a single platform used to generate its molecular abundance data. A series organizes samples into the meaningful data sets which make up an experiment Experimental data were obtained by selecting two GSE records , that were GSE-7803 for mRNA data analysis and GSE-30656 for microRNA.

GSE-7803

This Experiment was based on the expression profiling by array, taking 10 normal Squamous cervical epithelia sample and 21 invasive squamous cell carcinoma. The platform for data was Affymetrix HG-U133A with GEO platform accession number GPL96. A platform is, essentially, a list of probes that define what set of molecules may be detected. From the above mentioned GSE, individual GEO samples (GSM) were selected and are shown as follows

GSM189381, GSM189382, GSM189383, GSM189384, GSM189385, GSM189386, GSM189387, GSM189388, GSM189389, GSM189390, GSM189391, GSM189392, GSM189393 for Normal squamous cervical epithelia

GSM189401, GSM189402, GSM189403, GSM189404, GSM189405, GSM189406, GSM189407, GSM189408, GSM189409, GSM189410, GSM189411, GSM189412, GSM189413, GSM189414, GSM189415, GSM189416, GSM189417, GSM189418, GSM189419, GSM189420, GSM189421 for Invasive squamous cell carcinoma.

GSE-30656

This Experiment was based on Non-coding RNA profiling by array, taking 47 samples such as 10 Squamous cell carcinoma and 10 cervical squamous epithelial samples.

The platform for data was Affymetrix HG-U133A with GEO platform accession number GPL6955. From the above mentioned GSE, individual GEO samples (GSM) were selected and are shown as follows:

GSM760511, GSM760512, GSM760513, GSM760520, GSM760521, GSM760538, GSM760539, GSM760540, GSM760546, GSM760547 for Squamous cell carcinoma.

GSM760507, GSM760508, GSM760509, GSM760510, GSM760541, GSM760542, GSM760543, GSM760548, GSM760549, GSM760550 for cervical Squamous epithelial samples.

The process of retrieving the data from GEO is as follows:

GEO profile database of NCBI was selected and searched for studies relevant to our interests

Raw data was downloaded from the provided supplementary files with each GSM at the end of the section in .CEL format

Downloaded files were then unzipped

Files were then renamed as control and test

The files were then imported in to GeneSpring software for further statistical analysis

Analysis of gene expression data

We have used Genespring GX software (Genespring GX 11; Agilent, Santa clara, CA) for analyzing the gene expression data. This software provides powerful and accessible statistical tools for high-speed visualization and analysis of transcriptomics, genomics, proteomics and metabolomics data. It is an user friendly software that provides the user an interactive computing environment for well understanding of the microarray data in biological context. This software provides an integrative platform for any kind of data analysis. Some unique features of this software includes Gene-level expression analysis on diverse

platform such as Agilent, Affymetrix and Illumina, microRNA analysis and their potential targets, correlative analysis of miRNA and mRNA expression, real-time PCR data analysis etc.

We adapted some steps while doing gene expression analysis in genespring described as follows:

1. The raw data files from GEO database were first downloaded in .CEL format as a zip file.

2. Then the files were unzipped, extracted and renamed according to convenience.

3. GeneSpring interface was opened and a new project with new experiment was created. Workflow type was selected according to the biological significance.

4. Data was uploaded in the selected technology as Affymetrix Gene HG-U133A. Profile plot of Normalized intensity map values is obtained after Normalization of data. Normalization is done mainly for eliminating redundancy and ensuring that the data make sense with minimum number of entities.

5. The common set of genes obtained was exported along with their normalization values, gene symbol and Entrez gene IDs from the GeneSpring software as a tab delimited file for further analysis.

6. Grouping was done which further includes Addition of parameter, Assigning value etc.

8. Creation of interpretation was done by choosing average, Filter probset by error, taking co-efficient of variation as 50, Fold change >=1.54

9. Output was exported in excel that showed following headings- probset, Fold change (Test/control), Log fold change(Test/control),Regulation (up or down) Intensity value, gene symbol, entrez gene id by NCBI.

10.Using the obtained common set of genes, clustering analysis was done in GeneSpring using Hierarchical clustering algorithm. The distance metrics used was pearson uncentered and the linkage rule used was average.

Interaction study by NCI pathway analysis tool

The National Cancer Institute (NCI) in collaboration with Nature Publishing Group has established the Pathway Interaction Database (PID) in order to provide a highly structured, curated collection of information about known biomolecular interactions and key cellular processes assembled into signaling pathways. The database focuses on the bimolecular interactions that are known or believed to take place in human cells. It can be browsed as an online encyclopedia, used to run computational analyses, or employed in ways that combine these two approaches. In addition to PID's predefined pathways, search results are displayed as dynamically constructed interaction networks.

For this we selected the down regulated mRNA and run in enrichment analysis test with a web based NCI pathway analysis tool.

Prediction of miRNA targets using Target scan

Target scan is free web based software that predicts biological targets of miRNAs by searching for the presence of sites that match the seed region of each miRNA. In mammals, the user can choose whether the predictions should be ranked based on the probability of their conservation or on site number, type, and context. In mammals and nematodes, the user can also chose to extend the predictions beyond conserved sites and consider all sites.

we searched the name of the gene and selected the organism as Homo sapiens. Then it showed the all probable target binding sites of miRNAs.

Experimental validation

Cell culture

For our study HeLa cell line was procured from NCCS , those are human cervical cancer cells. HeLa cell line is the first type of human cancer cell which were successfully cultured continuously for experimental purpose. It was first derived from cervical cancer cells in 1951 from a patient named Henrietta Lacks. We took HaCaT cell line as control as it is a normal epithelial cell line, procured from NCCS. The medium used for culturing the Hela cell was MEM and that for control was DMEM (Invitrogen with NE A-A (non essential amino acids) and L-Glutamine) with 10% FBS (Fetal bovine serum from HIMEDIA) and 1% antibiotic solution (Penstrep solution from HIMEDIA). The culture flask containing the cell line is kept in the CO2 incubator with 5% level of CO2.

Cell harvesting

Before harvesting the cells were seen under microscope**.**
At first media was discarded using 1ml pipette and washed with 1ml PBS.
Then 1ml trypsin was added to the flask and kept for 5min incubation inside the CO_2 incubator.
Then 1ml media was added to neutralize the trypsin and stop the trypsinization process.
Then the cells were taken in a separate tube and centrifuged to obtain the pellet for further use.
All the procedure should be carried out inside the laminar airflow under aseptic condition to avoid contamination.

RNA Isolation

For mRNA

RNA isolation was carried out using QIAGEN RNAeasy kit. The steps involved in the isolation of RNA were as follows-

Appropriate volume of RLT buffer was to the harvested cells.
1 volume of 70% ethanol was added to the lysate followed by mixing through pipettin only.

Approximately 700µl of the sample including any precipitate was added to an RNasy mini spin column placed in a second collection tube followed by centrifugation for 15s >=8000xg.Flow through was discarded.

Then 700 µl buffer RWI was added to the RNasy spin column and centrifuged at >=8000 xg. Flow through was discarded.

500 µl buffer RPE was added to the column and centrifuged for 15s at >= 8000 xg. Flow through was discarded.

500 µl buffer RPE was added to the column and centrifuged for 2min at >= 8000 xg. Flow through was discarded.

The spin column was placed in a new 1.5ml collection tube. 30-50 µl RNase free water was directly added to the spin column membrane followed by centrifugation for 1min at.=8000 xg to elute the RNA.

<u>For miRNA</u>

miRNA isolation was carried out using mirVANA ™ miRNA isolation kit with phenol . The steps involved in the isolation of miRNA were as follows

The harvested cells were first washed with PBS and cell pellet was collected.

After removing the PBS ,600µl lysis/binding solution was added followed by vortexing to obtain homogenous lysate.

60 µl miRNA homogenate additive was added to the cell mix, mixed well by vortexing and was then kept on ice for 10 mins.

1 volume (600 µl) of Acid phenol-chloroform was added before the addition of miRNA homogenate to the lysate followed by vortexing and centeifugation at 10,000 g for 5min to separate the aqueous and organic phase at room temperature.

Then the aqueous phase was carefully removed without disturbing the lower phase and was transferred to a fresh tube.

1.25 volume of 100% ethanol (maintained at room temperature) was added to the aqueous phase.

The lysate-ethanol mix was placed in to a filter catridge placed in the collection tube followed by centrifugation at 10,000 g for 15 sec. Flow through was discarded.

700 µl of miRNA wash solution 1 was added to the filter catridge and centrifuged for 5-10 secs. Flow through was discarded.

500 µl of wash solution 2/3 was added and centrifuged for 5-10 secs. This step was repeated for one more time and flow through was discarded.

The filter catridge was again centrifuged to remove the residual volume from the filter.

The filter catridge was then transferred to a fresh collection tube, 100 µl of preheated (95° C) elution solution was added to the centre of the filter and was given short spin for 20-30secs at maximum speed to recover the RNA.

The elluent was then collected and stored at -20° C.

Precaution:

- A clean and sterilized environment was maintained while working with the RNA as there is high chance of contamination by RNase which may degrade the RNA and leading to reduced RNA yield.
- Gloves should be used while doing the reaction as there is high chance RNA degradation by the effect of nuclease secreted from the finger tip.

<u>cDNA synthesismRNA-cDNA synthesis</u>

- cDNA synthesis was carried out using SuperScript First-Strand Synthesis System for RTPCR by Invitrogen using oligo dT primers.
  The steps in cDNA synthesis are as follows:

- Each component of the kit was mixed and briefly centrifuge before use.
- For each reaction, the following components were combined in a sterile 0.2 or 0.5ml tube.

Table-2.1 Components used for mRNA-cDNA synthesis

Components	**Amount**
RNA (2µg)	n µl
10 mM dNTP mix	1 µl
Primer (0.5µg/µl oligo (dT)12-18)	1µl
DEPC treated water	To 10µl

- The RNA/primer mixture was incubated at 65·c for 5 minutes and then placed on ice for at least 1 minute.
- 4. In a separation tube, the following 2X reaction mix was prepared, by adding each component in the indicated order.

Table-2.2 Components used for mRNA-cDNA synthesis

Components	1RXn	10 RXnS
10X RT buffer	2 µl	20 µl
25mM Mgcl2	4 µl	40 µl
0.1M DTT	2 µl	20 µl
RNase out TM (400/ µl)	1 µl	10µl

- 9µl of the 2X reaction mixture was added to each RNA/primer mixture from step 3 and mixed gently and collect by briefly centrifugation.
- It was incubated at 42·c for 2 minutes.
- 1µl of super scriptTM II RT was then added to each tube and Incubated at 42·c for 50 minutes
- Then, the reaction was terminated at 70·c for 15 minutes and chilled on ice
- The reaction was collected by brief centrifugation and 1µl of RNase H was added to each tube and incubated for 20 minutes at 37·c.

Then, the reaction was stored at -20·c or used for PCR immediately.

<u>miRNA-cDNA synthesis</u>

miRNA cDNA synthesis was carried out using NCode™ miRNA First-Strand cDNA Synthesis Kit. The steps involved in miRNA- cDNA synthesis were as follows

The reaction mixture was made taking the following components-

Table-3 Components used for miRNA-cDNA synthesis

Components	**Amount (for 1RXN)**
5X Reaction Mix	4 µl
10 µl Superscript enzyme mix	2 µl
Total RNA(100pg to 1 µg)	x µl
DEPC treated water	Up to 20 µl

- After mixing all these components, the mixture was mixed properly through brief centrifugation.
- The tube was then incubated at 37° C for 60 mins.
- Then the reaction was terminated at 95° C for 5 mins and the reaction was held at 4° C until use.

<u>Quantification of mRNA and miRNA through qRT-PCR</u>

Real time PCR is a method that allows exponential amplification of DNA sequences and simultaneously quantifies it. This system is based on the detection and quantification of a fluorescent probes. Probes which are used in qRT-PCR are taqman probes, molecular beacon, SYBR® Green, displacing probes, light up probes etc. For present study we used SYBR® Green probe which is a frequently used fluorescent DNA binding probe and relies on the sequence specific detection dye. For this experiment we have taken two sets of gene, one is the reference gene.Reference genes are used to compare the expression of test genes in terms of fold change with respect to normal reference gene expression. Reference genes are basically the house keeping genes having the same copy number in all cells, having their expression in all kind cells.

For mRNA quantification experiment the test genes was CYR61 and reference gene used is Beta-actin while for miRNA quantification test was miR-221 and rehference was U6 . Beta actin and U6 are the housekeeping genes also called as constitutive genes which are required for the maintenance of fundamental cellular function, and are expressed in all cells of an organism under normal and patho-physiological conditions.

Quantification of mRNA

Total master mix was prepared for two genes i.e one housekeeping gene (Beta actin) and one test gene(CYR61) with respect to two samples HaCaT and Hela.
1:20 dilution was prepared for cDNA of both infected and control sample.
The reaction mix was prepared taking the following components:

Table-4 Components used for qRTPCR of mRNA

Components	Amounts(for 10 µl Rxn)
SyberGreen Mastermix	5 µl
Forward primer	0.5 µl
Reverse primer(10 µM)	0.5 µl
cDNA	3 µl
DEPC water	1µl

The sequence of the primer used are as follows:

Beta actin: *Forward primer*- CATGTACGTTGCTATCCAGGC

Reverse primer- CTCCTTAATGTCACGCACGAT

CYR61: *Forward primer*- CTCGCCTTAGTCGTCACCC

Reverse primer-CGCCGAAGTTGCATTCCAG

The cycle temperature for the q-RT PCR were as follows:

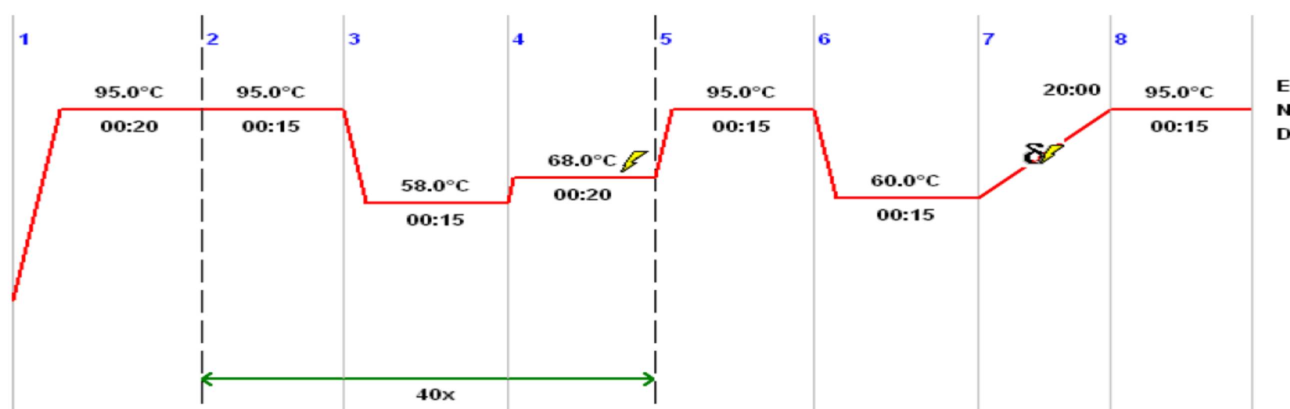


Figure-13 cycle temperature for mRNA q-RT PCR

Quantification of miRNA

Total master mix was prepared for two miRNAs i.e one reference U6 and the test has-miR-221 with respect to two samples HaCaT and Hela.

Undiluted cDNA was taken for both infected and control sample.

The reaction mix was prepared taking the following components:

Table-5 Components used for qRTPCR of miRNA

Components	**Final concentration**	**Amount (for 10 µlRxn)**
SyberGreen Mastermix(2X)	1X	5 µl
Forward primer(10 µM)	200nM	0.2 µl
Universal Reverse primer(10µM)	200nM	0.2 µl
ROX dye(25 µM)	(0.1 µM)	0.04 µl
cDNA	Up to 2 µl undiluted	1 µl
DEPC water		3.56 µl

The sequence of the primer used is as follows

Table-6 List of primers for miRNA qRTPCR

Primer type	**Sequence**
hsa-miR-221-5p Forward	ACCUGGCAUACAAUGUAGAUUU
U6 Forward	TGCTCGCTTCGGCAGCACATA
U6 Reverse	GGCGAAAGATGGAACGCTTCACGA

The cycle temperature for the q-RT PCR was as follows:

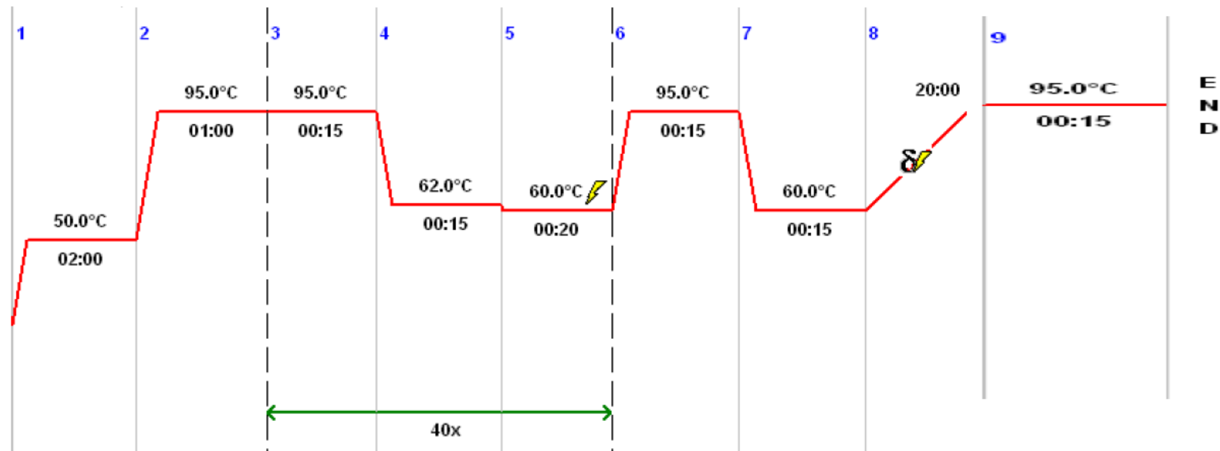


Figure-14 cycle temperature for miRNA q-RT PCR

RESULTS AND DISCUSSIONS

GENESPRING ANALYSIS

Gene spring analysis of the differentially expressed micro array for mRNA data that was comprised of 10 normal (squamous cervical epithelia) 28 Test (7 high grade squamous intraepithelial lesions & 21 inavsive squamous cell carcinoma) showed 338 differentially expressed mRNA out ofwhich no. of up-regulated down regulated mRNA were found to be 257 and 81 respectively with FC >=1.5.

Similarily for miRNA data that was comprised of 10 Normal (cervical squamous epithellium) 37 Test sample, the analysis resulted 41 differentially expressed miRNA, out of which no. of up-regulated & down regulated miRNA were found to be 27 and 14 respectively with FC>=1.5.

The clustering analysis for both mRNAs and mRNAs showed the list of altered expressed genes as well as for miRNA.

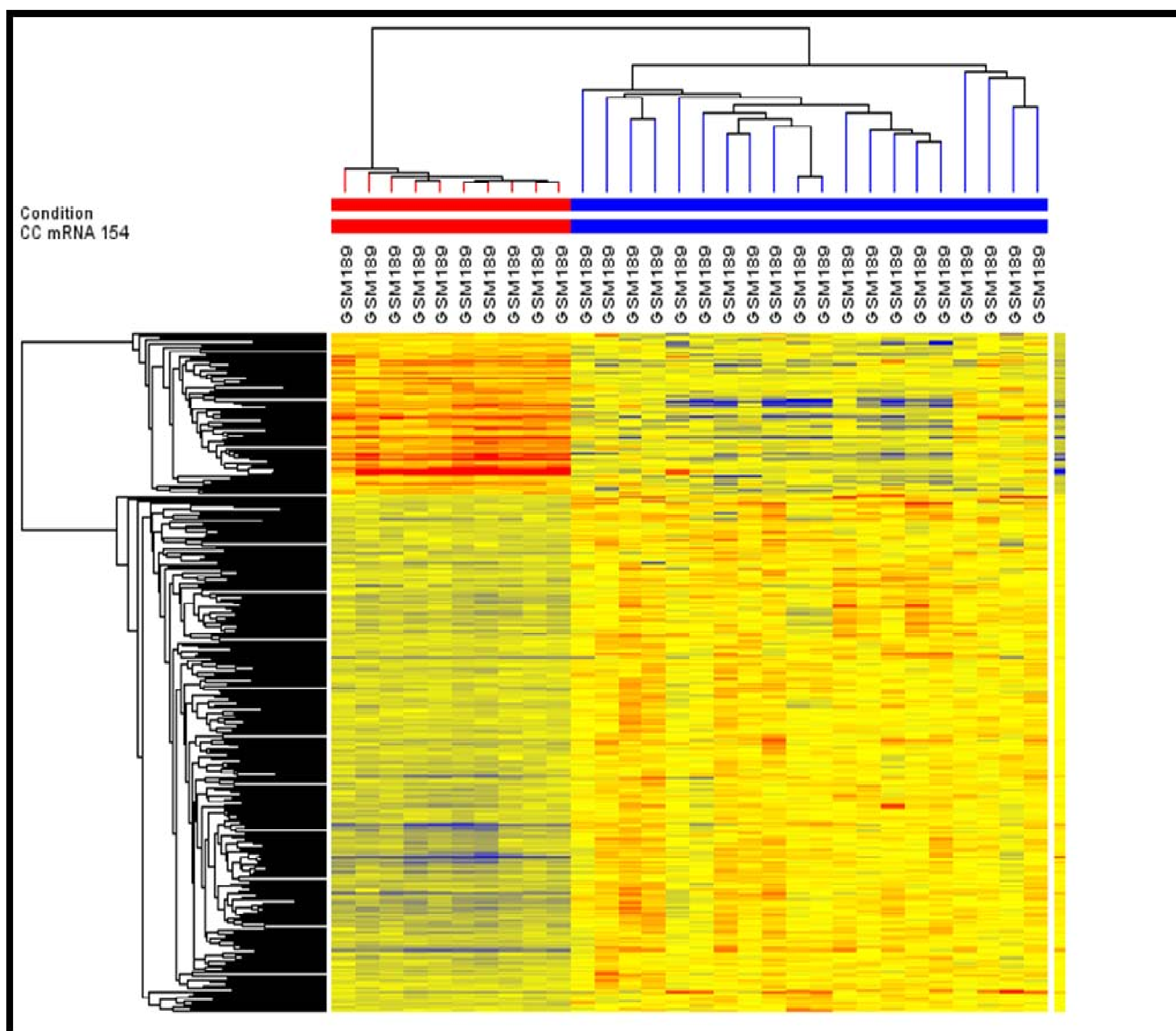


Figure-15 clustering analysis for both mRNAs showing the list of altered expressed gene

Interaction study by NCI pathway analysis tool

From the NCI pathway interaction tool,CYR61 gene was found to be interesting as it is involved in various cell signaling pathway.

It showed the following results:

NATIONAL CANCER INSTITUTE PathwayInteractionDatabase

Home > Batch query > Batch query results

Batch query results for NCI-Nature Curated data

Pathway Name	Biomolecules in Group 1	Biomolecules in Group 2	P-value ? Help
Endogenous TLR signaling	S100A8, S100A9, TIRAP		3.29e-04
AP-1 transcription factor network	CCND1, CYR61, ESR1, GJA1		5.64e-04
Regulation of nuclear beta catenin signaling and target gene transcription	AR, CCND1, CYR61, KRT1		9.20e-04
Validated transcriptional targets of AP1 family members Fra1 and Fra2	CCND1, GJA1, IVL		1.05e-03
RhoA signaling pathway	CYR61, MAL, MKL1		1.95e-03
Arf6 signaling events	ACAP1, GULP1		1.61e-02
FOXM1 transcription factor network	CCND1, ESR1		2.13e-02
FOXA1 transcription factor network	AR, ESR1		2.32e-02
ATF-2 transcription factor network	CCND1, ESR1		3.87e-02
Coregulation of Androgen receptor activity	AR, CCND1		4.33e-02
IL12-mediated signaling events	CD8A, IL18		4.80e-02
a4b7 Integrin signaling	CD44		4.85e-02
Validated nuclear estrogen receptor alpha network	CCND1, ESR1		4.92e-02
Regulation of Telomerase	CCND1, ESR1		5.04e-02
Signaling mediated by p38-gamma and p38-delta	CCND1		5.87e-02

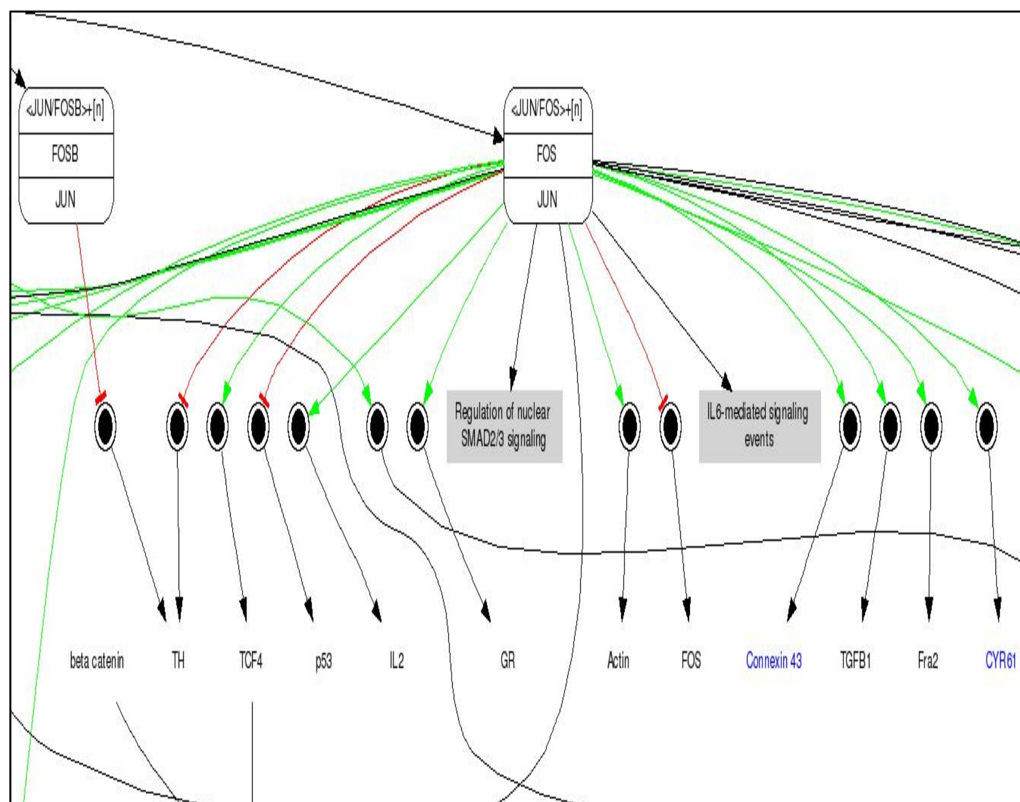
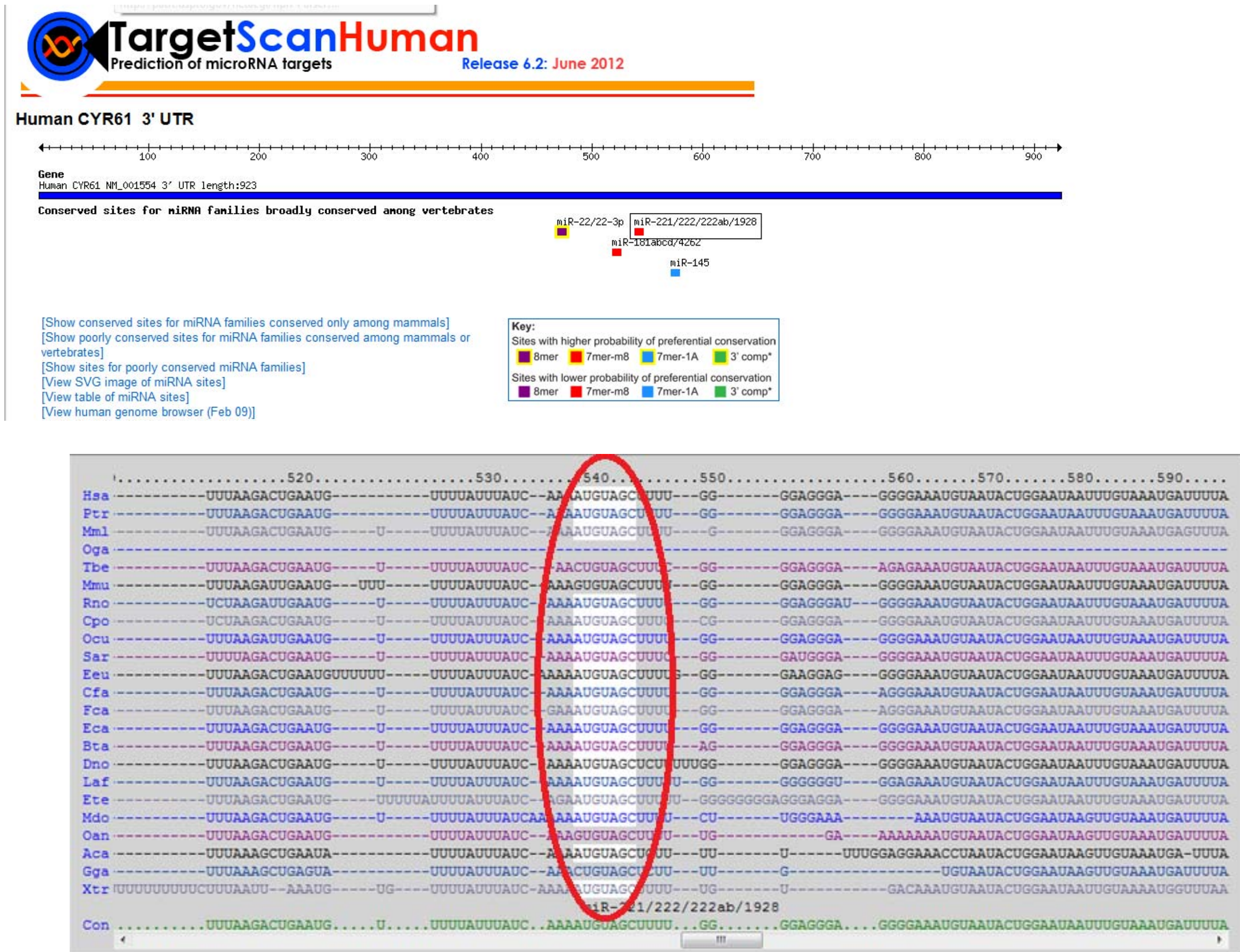


Figure-16 Enrichment analysis by *NCI* pathway analysis tool

Prediction of miRNA targets using Target scan

With the help of Target scan, we found hsa-miR-221, that targets this gene with potential binding sites (7mer-M8).

It showed the following results:



Conserved

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match
Position 539-545 of CYR61 3' UTR hsa-miR-221	5' ...GUUUUAUUUAUCAAAAUGUAGCU... ||||||| 3' CUUUGGGUCGUCUGUUACAUCGA	7mer-m8

Figure-17 miRNA target Prediction using *Target Scan*

EXPERIMENTAL VALIDATIONS

RNA isolation

mRNA Isolation:

The yield after RNA isolation in

HaCaT cell line was 828.4 µg/ml, 260/280-2.03, 260/230-2.0

The yield after RNA isolation in Hela was 870 µg/ml, 260/280-2.0, 260/230-2.08

miRNA isolation:

The yield after RNA isolation HaCaT cell line was-318.4 µg/ml, 260/280-2.05, 260/230-1.90

The yield after RNA isolation HaCaT cell line was-476 µg/ml, 260/280-2.05, 260/230-1.79

qRT PCR

The melting curve for the gene CYR61 showed the following results:

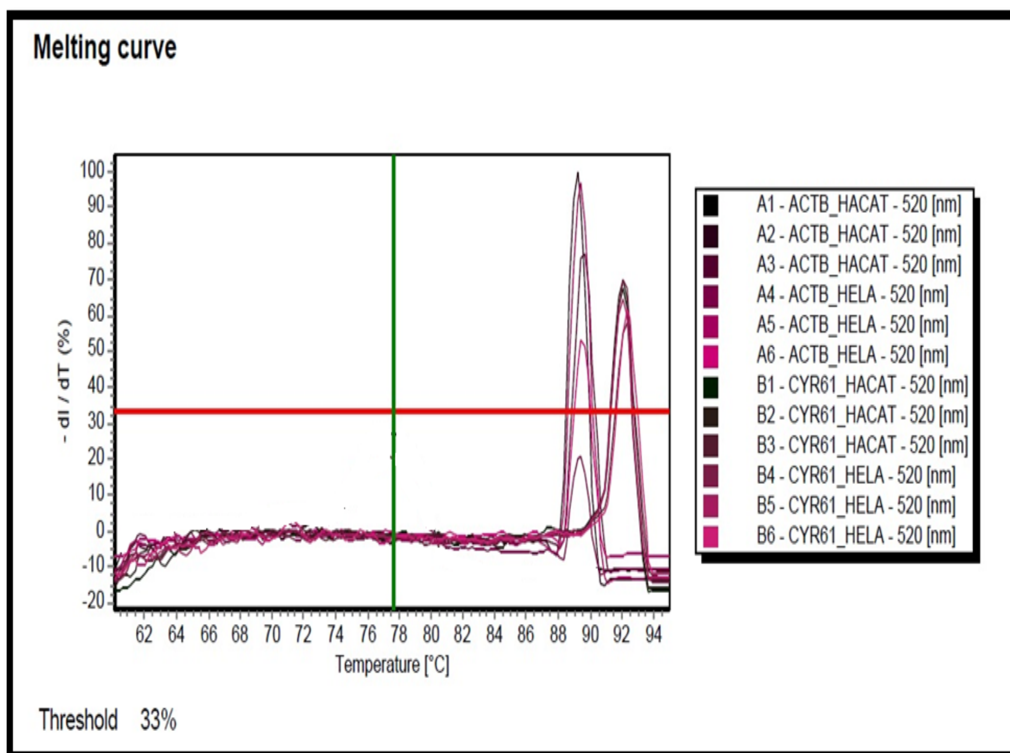


Figure-18 The melting curve of CYR61

The melting curve for miR-221 showed the following results:

Melting curve

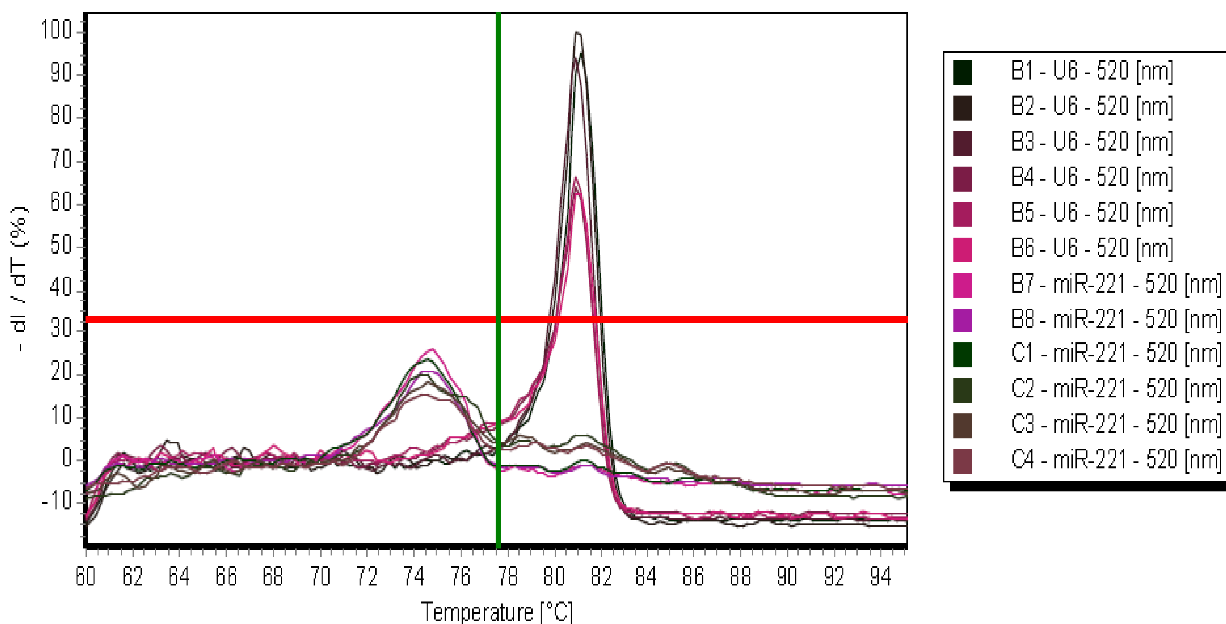
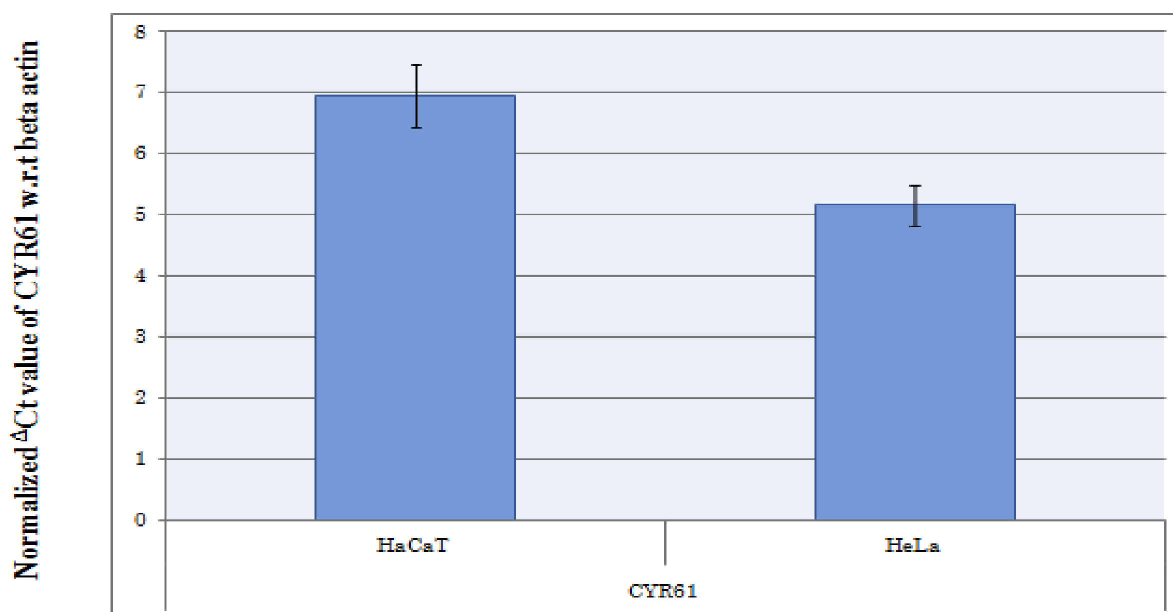


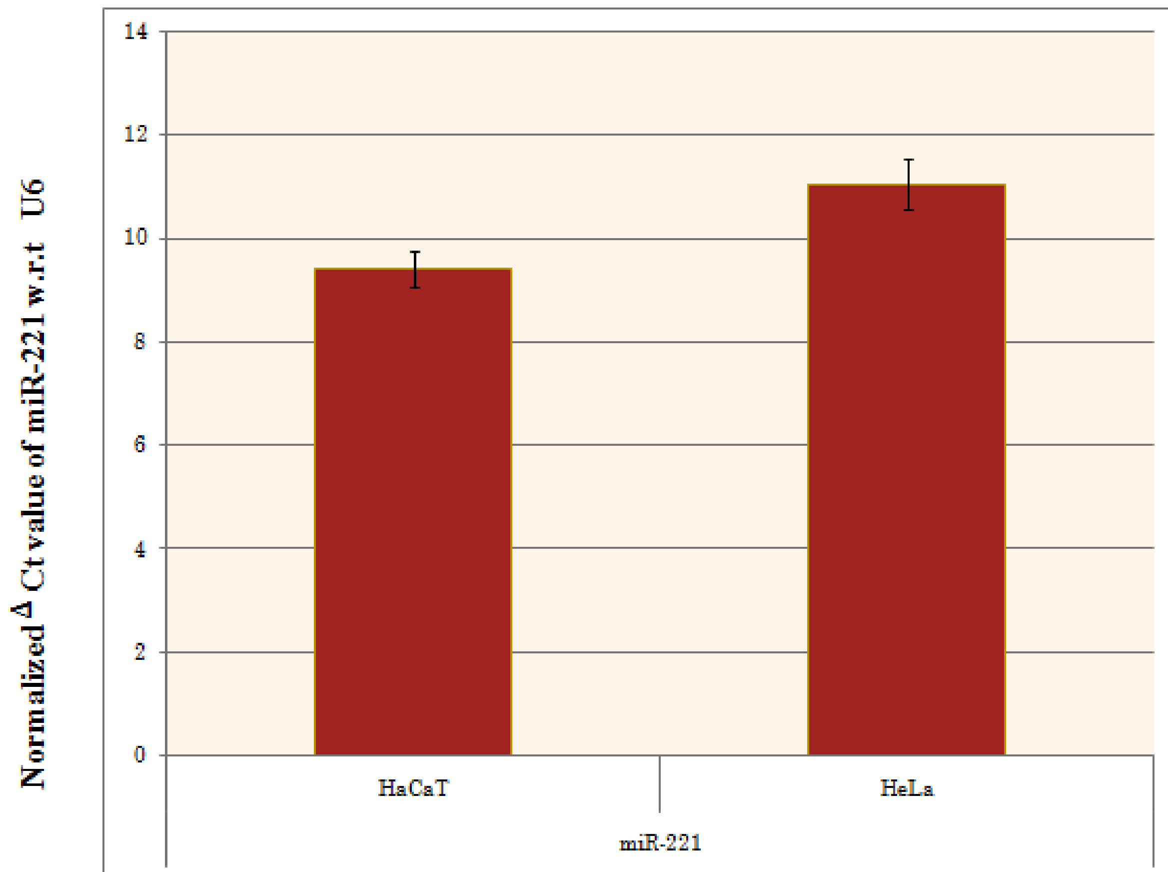
Figure-19 The melting curve of miR-221

After calculating the Fold change by taking CT values with the formula Fold change $=2^{(-\Delta\Delta Ct)}$,the following graphs were plotted for relative expression for the miRNA as well as the gene.



Fold Change- $2^{(-\Delta\Delta Ct)}$, ($\Delta\Delta$Ct =ΔCt of Test-ΔCt of control)
Obtained FC- 3.24

Figure- 20 qRT-PCR result showing upregulation of CYR61 in cervical cancer cell line



Fold Change- $2^{(-\Delta\Delta Ct)}$, ($\Delta\Delta Ct = \Delta Ct$ of Test-ΔCt of control)

Obtained FC- -3.12

Figure- 21 qRT-PCR result showing downregulation of miR-221 in cervical cancer cell line.

CONCLUSIONS

CONCLUSIONS

From genome-wide mRNA expression analysis of cervical cancer, CYR61 was found to be down-regulated, but was reported to be over-expressed in other cancer systems. Therefore, we were curious to know the expression of this gene in cervical cancer and selected it for experimental validation. Through qRT-PCR, we found that CYR61 gene is over-expressed in cervical cancer. From miRNA expression analysis followed by target prediction by TargetScan, we identified a miRNA i.e hsa-miR-221 that targets this CYR61. To know its expression, we did miRNA qRT-PCR and found this miRNA to be down-regulated. As CYR61 was reported earlier as an angiogenesis inducing factor in most cancer systems, so we hypothesize that the over-expression of this gene in cervical cancer may contribute to its metastatic spread and lethality and this over-expression can be attributed to altered expression of miRNA-221 that might have resulted in abnormal expression of CYR61. The targeting of CYR61 by miR-221 can be further confirmed by luciferase reporter assays. Moreover, the altered expression of miR-221 may be due to promoter hypermethylation of the miRNA gene and can be further confirmed through methylation specific PCR of the corresponding miRNA.

FUTURE PROSPECTIVES

FUTURE PROSPECTIVES

Based on the current findings, further studies can be performed to understand & confirm the role of miRNA in regulating CYR61 thereby confirming its role in angiogenesis and metastasis. Performing luciferase reporter assay to confirm targeting of CYR61 by miR-221 will provide evidence on the mode of regulation of this gene. Furthermore, investigating the role of promoter hyper-methylation of the miRNA gene through Methylation Specific PCR (MSP) can help us to know the root of regulation of CYR61. Understanding the basis of regulation of these mRNA and miRNA may provide a clue towards discovering RNA-based therapeutic approach for cervical cancer. Therapeutic value of this study can be further assessed by over-expressing miR-221 in HeLa cell lines thereby checking the proliferation rate along with the expression of CYR61. After successful execution of these in cell lines, it can be extended further to validate in primary cervical stages in order to check the level of angiogenesis. Final validation is supposed to be carried by taking animal models. This novel approach can thus prevent the cancer progression at the initial stages and hence can reduce the mortality among women.

REFERENCES

References

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281-297.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215-233.

Bernards, R., and Weinberg, R.A. (2002). A progression puzzle. Nature *418*, 823.

Chambers, A.F., Groom, A.C., and MacDonald, I.C. (2002). Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer *2*, 563-572.

Cheetham, S.W., Gruhl, F., Mattick, J.S., and Dinger, M.E. (2013). Long noncoding RNAs and the genetics of cancer. Br J Cancer *108*, 2419-2425.

Chu, Y., Ouyang, Y., Wang, F., Zheng, A., Bai, L., Han, L., Chen, Y., and Wang, H. (2014). MicroRNA-590 promotes cervical cancer cell growth and invasion by targeting CHL1. J Cell Biochem *115*, 847-853.

Cui, F., Li, X., Zhu, X., Huang, L., Huang, Y., Mao, C., Yan, Q., Zhu, J., Zhao, W., and Shi, H. (2012). MiR-125b inhibits tumor growth and promotes apoptosis of cervical cancer cells by targeting phosphoinositide 3-kinase catalytic subunit delta. Cell Physiol Biochem *30*, 1310-1318.

Elst, P., Ahankour, F., and Tjalma, W. (2007). Management of recurrent cervical cancer. Review of the literature and case report. Eur J Gynaecol Oncol *28*, 435-441.

Fabbri, M., Garzon, R., Cimmino, A., Liu, Z., Zanesi, N., Callegari, E., Liu, S., Alder, H., Costinean, S., Fernandez-Cymering, C*., et al.* (2007). MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci U S A *104*, 15805-15810.

Fabian, M.R., and Sonenberg, N. (2012). The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. Nat Struct Mol Biol *19*, 586-593.

Filipowicz, W., Jaskiewicz, L., Kolb, F.A., and Pillai, R.S. (2005). Post-transcriptional gene silencing by siRNAs and miRNAs. Curr Opin Struct Biol *15*, 331-341.

Garner, E.I. (2003). Cervical cancer: disparities in screening, treatment, and survival. Cancer Epidemiol Biomarkers Prev *12*, 242s-247s.

Gebeshuber, C.A., Zatloukal, K., and Martinez, J. (2009). miR-29a suppresses tristetraprolin, which is a regulator of epithelial polarity and metastasis. EMBO Rep *10*, 400-405.

GM, C., 2 edn.

Gomes, A.Q., Nolasco, S., and Soares, H. (2013). Non-coding RNAs: multi-tasking molecules in the cell. Int J Mol Sci *14*, 16010-16039.

Gronbaek, K., Hother, C., and Jones, P.A. (2007). Epigenetic changes in cancer. APMIS *115*, 1039-1059.

Gupta, G.P., and Massague, J. (2006). Cancer metastasis: building a framework. Cell *127*, 679-695.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Huang, F., Lin, C., Shi, Y.H., and Kuerban, G. (2013). MicroRNA-101 inhibits cell proliferation, invasion, and promotes apoptosis by regulating cyclooxygenase-2 in Hela cervical carcinoma cells. Asian Pac J Cancer Prev *14*, 5915-5920.

Kaczkowski, B., Morevati, M., Rossing, M., Cilius, F., and Norrild, B. (2012). A Decade of Global mRNA and miRNA Profiling of HPV-Positive Cell Lines and Clinical Specimens. Open Virol J *6*, 216-231.

Kota, J., Chivukula, R.R., O'Donnell, K.A., Wentzel, E.A., Montgomery, C.L., Hwang, H.W., Chang, T.C., Vivekanandan, P., Torbenson, M., Clark, K.R.*, et al.* (2009). Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell *137*, 1005-1017.

Lei, C., Wang, Y., Huang, Y., Yu, H., Wu, L., and Huang, L. (2012). Up-regulated miR155 reverses the epithelial-mesenchymal transition induced by EGF and increases chemo-sensitivity to cisplatin in human Caski cervical cancer cells. PLoS One *7*, e52310.

Lui, W.O., Pourmand, N., Patterson, B.K., and Fire, A. (2007). Patterns of known and novel small RNAs in human cervical cancer. Cancer Res *67*, 6031-6043.

Ma, D., Zhang, Y.Y., Guo, Y.L., Li, Z.J., and Geng, L. (2012). Profiling of microRNA-mRNA reveals roles of microRNAs in cervical cancer. Chin Med J (Engl) *125*, 4270-4276.

Nishida, N., Yano, H., Nishida, T., Kamura, T., and Kojiro, M. (2006). Angiogenesis in cancer. Vasc Health Risk Manag *2*, 213-219.

Virmani, A.K., Muller, C., Rathi, A., Zoechbauer-Mueller, S., Mathis, M., and Gazdar, A.F. (2001). Aberrant methylation during cervical carcinogenesis. Clin Cancer Res *7*, 584-589.

Wang, F., Liu, M., Li, X., and Tang, H. (2013a). MiR-214 reduces cell survival and enhances cisplatin-induced cytotoxicity via down-regulation of Bcl2l2 in cervical cancer cells. FEBS Lett *587*, 488-495.

Wang, L., Chang, L., Li, Z., Gao, Q., Cai, D., Tian, Y., Zeng, L., and Li, M. (2014). miR-99a and -99b inhibit cervical cancer cell proliferation and invasion by targeting mTOR signaling pathway. Med Oncol *31*, 934.

Wang, P.H., Li, Y.F., Juang, C.M., Lee, Y.R., Chao, H.T., Tsai, Y.C., and Yuan, C.C. (2001). Altered mRNA expression of sialyltransferase in squamous cell carcinomas of the cervix. Gynecol Oncol *83*, 121-127.

Wang, Y.D., Cai, N., Wu, X.L., Cao, H.Z., Xie, L.L., and Zheng, P.S. (2013b). OCT4 promotes tumorigenesis and inhibits apoptosis of cervical cancer cells by miR-125b/BAK1 pathway. Cell Death Dis *4*, e760.

Weigelt, B., Peterse, J.L., and van 't Veer, L.J. (2005). Breast cancer metastasis: markers and models. Nat Rev Cancer *5*, 591-602.

Wen, S.Y., Lin, Y., Yu, Y.Q., Cao, S.J., Zhang, R., Yang, X.M., Li, J., Zhang, Y.L., Wang, Y.H., Ma, M.Z.*, et al.* (2014). miR-506 acts as a tumor suppressor by directly targeting the hedgehog pathway transcription factor Gli3 in human cervical cancer. Oncogene.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell *75*, 855-862.

Wilting, S.M., de Wilde, J., Meijer, C.J., Berkhof, J., Yi, Y., van Wieringen, W.N., Braakhuis, B.J., Meijer, G.A., Ylstra, B., Snijders, P.J.*, et al.* (2008). Integrated genomic and transcriptional profiling identifies chromosomal loci with altered gene expression in cervical cancer. Genes Chromosomes Cancer *47*, 890-905.

Wilting, S.M., Snijders, P.J., Verlaat, W., Jaspers, A., van de Wiel, M.A., van Wieringen, W.N., Meijer, G.A., Kenter, G.G., Yi, Y., le Sage, C.*, et al.* (2013a). Altered microRNA expression associated with chromosomal changes contributes to cervical carcinogenesis. Oncogene *32*, 106-116.

Wilting, S.M., Verlaat, W., Jaspers, A., Makazaji, N.A., Agami, R., Meijer, C.J., Snijders, P.J., and Steenbergen, R.D. (2013b). Methylation-mediated transcriptional repression of microRNAs during cervical carcinogenesis. Epigenetics *8*, 220-228.

Yang, L., Wang, Y.L., Liu, S., Zhang, P.P., Chen, Z., Liu, M., and Tang, H. (2014). miR-181b promotes cell proliferation and reduces apoptosis by repressing the expression of adenylyl cyclase 9 (AC9) in cervical cancer cells. FEBS Lett *588*, 124-130.

Yao, T., Rao, Q., Liu, L., Zheng, C., Xie, Q., Liang, J., and Lin, Z. (2013). Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in cervical cancer. Virol J *10*, 175.

Zhang, J., Zheng, F., Yu, G., Yin, Y., and Lu, Q. (2013). miR-196a targets netrin 4 and regulates cell proliferation and migration of cervical cancer cells. Biochem Biophys Res Commun *440*, 582-588.

Zhu, X., Er, K., Mao, C., Yan, Q., Xu, H., Zhang, Y., Zhu, J., Cui, F., Zhao, W., and Shi, H. (2013). miR-203 suppresses tumor growth and angiogenesis by targeting VEGFA in cervical cancer. Cell Physiol Biochem *32*, 64-73.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281-297.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215-233.

Bernards, R., and Weinberg, R.A. (2002). A progression puzzle. Nature *418*, 823.

Chambers, A.F., Groom, A.C., and MacDonald, I.C. (2002). Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer *2*, 563-572.

Chu, Y., Ouyang, Y., Wang, F., Zheng, A., Bai, L., Han, L., Chen, Y., and Wang, H. (2014). MicroRNA-590 promotes cervical cancer cell growth and invasion by targeting CHL1. J Cell Biochem *115*, 847-853.

Cui, F., Li, X., Zhu, X., Huang, L., Huang, Y., Mao, C., Yan, Q., Zhu, J., Zhao, W., and Shi, H. (2012). MiR-125b inhibits tumor growth and promotes apoptosis of cervical cancer cells by targeting phosphoinositide 3-kinase catalytic subunit delta. Cell Physiol Biochem *30*, 1310-1318.

Elst, P., Ahankour, F., and Tjalma, W. (2007). Management of recurrent cervical cancer. Review of the literature and case report. Eur J Gynaecol Oncol *28*, 435-441.

Fabbri, M., Garzon, R., Cimmino, A., Liu, Z., Zanesi, N., Callegari, E., Liu, S., Alder, H., Costinean, S., Fernandez-Cymering, C.*, et al.* (2007). MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci U S A *104*, 15805-15810.

Fabian, M.R., and Sonenberg, N. (2012). The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. Nat Struct Mol Biol *19*, 586-593.

Filipowicz, W., Jaskiewicz, L., Kolb, F.A., and Pillai, R.S. (2005). Post-transcriptional gene silencing by siRNAs and miRNAs. Curr Opin Struct Biol *15*, 331-341.

Garner, E.I. (2003). Cervical cancer: disparities in screening, treatment, and survival. Cancer Epidemiol Biomarkers Prev *12*, 242s-247s.

Gebeshuber, C.A., Zatloukal, K., and Martinez, J. (2009). miR-29a suppresses tristetraprolin, which is a regulator of epithelial polarity and metastasis. EMBO Rep *10*, 400-405.

GM, C., 2 edn.

Gomes, A.Q., Nolasco, S., and Soares, H. (2013). Non-coding RNAs: multi-tasking molecules in the cell. Int J Mol Sci *14*, 16010-16039.

Gronbaek, K., Hother, C., and Jones, P.A. (2007). Epigenetic changes in cancer. APMIS *115*, 1039-1059.

Gupta, G.P., and Massague, J. (2006). Cancer metastasis: building a framework. Cell *127*, 679-695.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Huang, F., Lin, C., Shi, Y.H., and Kuerban, G. (2013). MicroRNA-101 inhibits cell proliferation, invasion, and promotes apoptosis by regulating cyclooxygenase-2 in Hela cervical carcinoma cells. Asian Pac J Cancer Prev *14*, 5915-5920.

Kota, J., Chivukula, R.R., O'Donnell, K.A., Wentzel, E.A., Montgomery, C.L., Hwang, H.W., Chang, T.C., Vivekanandan, P., Torbenson, M., Clark, K.R.*, et al.* (2009). Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell *137*, 1005-1017.

Lui, W.O., Pourmand, N., Patterson, B.K., and Fire, A. (2007). Patterns of known and novel small RNAs in human cervical cancer. Cancer Res *67*, 6031-6043.

Wang, F., Liu, M., Li, X., and Tang, H. (2013a). MiR-214 reduces cell survival and enhances cisplatin-induced cytotoxicity via down-regulation of Bcl2l2 in cervical cancer cells. FEBS Lett *587*, 488-495.

Wang, L., Chang, L., Li, Z., Gao, Q., Cai, D., Tian, Y., Zeng, L., and Li, M. (2014). miR-99a and -99b inhibit cervical cancer cell proliferation and invasion by targeting mTOR signaling pathway. Med Oncol *31*, 934.

Wang, P.H., Li, Y.F., Juang, C.M., Lee, Y.R., Chao, H.T., Tsai, Y.C., and Yuan, C.C. (2001). Altered mRNA expression of sialyltransferase in squamous cell carcinomas of the cervix. Gynecol Oncol *83*, 121-127.

Wang, Y.D., Cai, N., Wu, X.L., Cao, H.Z., Xie, L.L., and Zheng, P.S. (2013b). OCT4 promotes tumorigenesis and inhibits apoptosis of cervical cancer cells by miR-125b/BAK1 pathway. Cell Death Dis *4*, e760.

Weigelt, B., Peterse, J.L., and van 't Veer, L.J. (2005). Breast cancer metastasis: markers and models. Nat Rev Cancer *5*, 591-602.

Wen, S.Y., Lin, Y., Yu, Y.Q., Cao, S.J., Zhang, R., Yang, X.M., Li, J., Zhang, Y.L., Wang, Y.H., Ma, M.Z.*, et al.* (2014). miR-506 acts as a tumor suppressor by directly targeting the hedgehog pathway transcription factor Gli3 in human cervical cancer. Oncogene.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell *75*, 855-862.

Wilting, S.M., de Wilde, J., Meijer, C.J., Berkhof, J., Yi, Y., van Wieringen, W.N., Braakhuis, B.J., Meijer, G.A., Ylstra, B., Snijders, P.J.*, et al.* (2008). Integrated genomic and transcriptional profiling identifies chromosomal loci with altered gene expression in cervical cancer. Genes Chromosomes Cancer *47*, 890-905.

Wilting, S.M., Snijders, P.J., Verlaat, W., Jaspers, A., van de Wiel, M.A., van Wieringen, W.N., Meijer, G.A., Kenter, G.G., Yi, Y., le Sage, C.*, et al.* (2013a). Altered microRNA expression associated with chromosomal changes contributes to cervical carcinogenesis. Oncogene *32*, 106-116.

Wilting, S.M., Verlaat, W., Jaspers, A., Makazaji, N.A., Agami, R., Meijer, C.J., Snijders, P.J., and Steenbergen, R.D. (2013b). Methylation-mediated transcriptional repression of microRNAs during cervical carcinogenesis. Epigenetics *8*, 220-228.

Yang, L., Wang, Y.L., Liu, S., Zhang, P.P., Chen, Z., Liu, M., and Tang, H. (2014). miR-181b promotes cell proliferation and reduces apoptosis by repressing the expression of adenylyl cyclase 9 (AC9) in cervical cancer cells. FEBS Lett *588*, 124-130.

Yao, T., Rao, Q., Liu, L., Zheng, C., Xie, Q., Liang, J., and Lin, Z. (2013). Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in cervical cancer. Virol J *10*, 175.

Zhang, J., Zheng, F., Yu, G., Yin, Y., and Lu, Q. (2013). miR-196a targets netrin 4 and regulates cell proliferation and migration of cervical cancer cells. Biochem Biophys Res Commun *440*, 582-588.

Zhu, X., Er, K., Mao, C., Yan, Q., Xu, H., Zhang, Y., Zhu, J., Cui, F., Zhao, W., and Shi, H. (2013). miR-203 suppresses tumor growth and angiogenesis by targeting VEGFA in cervical cancer. Cell Physiol Biochem *32*, 64-73.