A Dissertation on

CLINICAL ROLE OF MICRO RNA (miRNA) 196a-5p EXPRESSION IN SQUAMOUS CELL CARCINOMA OF UTERINE CERVIX

Submitted to

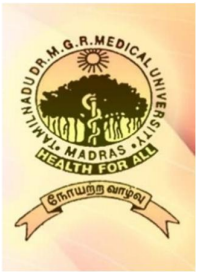
The TamilNadu Dr.M.G.R Medical University

in partial fulfillment of the requirement

for the award of degree of

M.Ch (SURGICAL ONCOLOGY)

BRANCH VII



KILPAUK MEDICAL COLLEGE

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY

CHENNAI, TAMILNADU

AUGUST 2015

BONAFIDE CERTIFICATE

This is to certify that **Dr.V.S.Ajay Chandrasekar**, bonafide student of **M.Ch Surgical Oncology (August 2012 to August 2015)** in the Department of Surgical Oncology, Government Royapettah Hospital, Chennai - 600014 has done this dissertation on **"Clinical Role of MicroRNA (miRNA) 196a-5p expression profiles in Squamous Cell Carcinomas of Uterine Cervix"** under my guidance and supervision in partial fulfillment of the regulations laid down by The TamilNadu Dr.M.G.R. Medical University, Chennai for **M.Ch Surgical Oncology Examination** to be held in August 2015.

PROF.R.RAJARAMAN M.S, M.Ch.
Head, Centre for Oncology,
Head of Department of Surgical Oncology,
Govt. Royapettah Hospital & Kilpauk Medical College,
Chennai

DEAN
Kilpauk Medical College
Chennai

ACKNOWLEDGEMENTS

I take this opportunity to express my deep sense of gratitude and thanks to all those who have been instrumental in the successful completion of this work.

I would like to express my profound gratitude and regards to my esteemed teacher and Guide, Prof. R. Rajaraman, Head of Centre for Oncology and HOD, Department of Surgical Oncology, Government Royapettah Hospital, Kilpauk Medical College, Chennai, for his supervision and invaluable suggestions throughout this study. Without his guidance and support, this work would not have seen the light of the day.

I would like to express my deep gratitude and regards to my Co Guide Prof. A.K. Munirajan, Professor, Department of Genetics at Dr. ALM PG IBMS, University of Madras, Chennai for his guidance, invaluable suggestions and constant encouragement.

I am extremely grateful to Prof . Subbiah Shanmugam, Associate Professor, Department of Surgical Oncology, Government Royapettah Hospital, Chennai for his constant support, valuable comments and suggestions.

Special gratitude is due to the Assistant Professors Dr.Sujay Susikar, Dr.Gopu and Dr.Syed Afrose Hussain , fellow postgraduates, technical, nursing and paramedical staff of our department. Special mention is needed for Mr.G.Arun kumar , Mr.Vinoth Kumar & Mr.A.K.Devamagendra Rao , research scholars at the Dept. of Genetics, University of Madras for their help and guidance.

I would like to express my gratitude to my parents and my family who have always been an epitome of courage and fortitude for me. Their blessing has given me the strength to carry on through moments of uncertainty.

My acknowledgment will be incomplete if I do not thank all my patients without whose co-operation, I would not have been able to conduct this study.

DECLARATION

I solemnly declare that the dissertation titled **CLINICAL ROLE OF MICRO RNA (miRNA) 196a-5p EXPRESSION IN SQUAMOUS CELL CARCINOMA OF UTERINE CERVIX** was done by me at Department of Surgical Oncology, Kilpauk Medical College, Chennai between August 2011 to February 2014 under the guidance and supervision of **Prof. R. Rajaraman MS MCh**. The Dissertation is submitted to **The TamilNadu Dr.M.G.R. Medical University** towards the partial fulfillment for the award of **MCh (Branch VII) in Surgical Oncology (August 2014).**

Chennai

Date :

Dr .V.S.Ajay Chandrasekar
Post Graduate in Surgical Oncology
Dept of Surgical Oncology, Centre for Oncology
Kilpauk Medical College & Govt Royapettah Hospital

CONTENTS

INTRODUCTION

AIMS AND OBJECTIVES

REVIEW OF LITERATURE

MATERIALS AND METHODS

STATISTICAL ANALYSIS

RESULTS

DISCUSSION

CONCLUSION

BIBLIOGRAPHY

ANNEXURES

- **PATIENT PROFORMA**
- **ETHICAL JUSTIFICATION**
- **MASTERCHART**
- **PLAGIARISM**

Introduction

INTRODUCTION

Carcinoma cervix is the most common cancer in the female population in the non-metropolitan cities in India. Breast cancer ranks second and carcinoma cervix ranks seventh according to incidence of cancers around the world.[1] According to IARC GLOBOCAN worldwide cancer incidence and mortality database, the incidence of carcinoma cervix in the world is estimated to around 5.2 million new cases diagnosed annually with a mortality of 2.6 million cases per year. The annual incidence of cervical carcinoma is on the rise with the emergence of oncogenic HPV subtypes which is one of the etiological factors for this cancer.

Persistent HPV infection of the metaplastic cells in the cervical transformation zone leading to formation of an insitu lesion and progression to an invasive carcinoma with a continued infection are the steps involved in the multistep carcinogenesis. With the incidence of infection common in young women , the risk of invasive carcinoma also peaks or plateaus within 35-55 years with a latency of 5-10 years.[2]

The advent of newer screening modalities has improved the chances of early diagnosis and treatment of this deadly disease, thus enhancing the quality of their survival in these patients. Although great progresses have been seen in the screening techniques, surgical modalities and newer chemotherapy regimens for cancer cervix, the exact molecular mechanism of carcinogenesis is not fully elucidated. Hence the need for an

understanding of a potential molecular mechanism becomes essential in this modern era of oncology.

Biomarkers provide an approach to understanding the molecular mechanisms in oncology. Their applications in screening, diagnosis, prognostication, prediction of recurrences, and monitoring of therapy of cancers is emerging. Advances in genomics, proteomics, metabolomics, and bioinformatics have revolutionized the search for numerous putative markers that may be informative with regard to carcinoma cervix.

One among them is the discovery of a class of "small non-coding RNAs" termed as microRNAs (miRNAs) which are thought to greatly influence the molecular pathogenetic mechanisms of various cancers. These are a small group of non-coding RNAs which are essential for gene expression and are thought to regulate cell proliferation and metabolism, cellular differentiation, cell death, DNA methylation and chromatin modification, hematopoiesis, neuronal development and tumorigenesis. With increasing evidence of altered microRNA profiles attributed to various cancers, the microRNA expression signatures have been closely associated with disease patterns and clinical outcomes.[3]

Using microarray analysis, altered expression of microRNAs is being evaluated in carcinoma cervix patients. In this study we evaluate and analyze the possible role of a subset of microRNAs which may be consistently associated with carcinoma cervix possibly playing a major role in tumorigenesis and may aid in the diagnosis and prediction of the clinical outcome.

Aims & Objectives

AIMS & OBJECTIVES

The principal aims of our study are:

1. To assess the feasibility of extracting microRNA in carcinoma cervix tumor samples.
2. To study the of miR-196a-5p expression profiles in various tumor samples of carcinoma cervix.
3. To compare miR-196a-5p expression profiles in relation with the tumor characteristics and clinical behavior of carcinoma cervix.
4. To analyse of miR-196a-5p expression profiles in relation to HPV status of the tumor samples studied.

Review of Literature

REVIEW OF LITERATURE

Carcinoma cervix represents the fifth most common tumor among all cancers and the second most common tumor in the female genital tract accounting for an annual incidence of more than 2 million new cases worldwide. Carcinoma of cervix was the leading cause of mortality in females next to breast cancers two decades back. With the invent of screening procedures such as PAP smears, colposcopic and visual examination of the cervix using acetic acid or Lugol's iodine, the frequency at which carcinoma cervix is detected at a precancerous level has tremendously risen.

While there are an estimated 11,000 new cases of invasive carcinoma in the United States annually, there are nearly 1 million precancerous lesions of varying grade that are discovered yearly by cytologic examinations. Thus, it is evident that Pap smear screening not only has increased the detection of potentially curable, low-stage cancers but has also allowed the detection and eradication of preinvasive lesions, some of which would have progressed to invasive cancer if not detected and treated.

Human papilloma virus (HPV) is the main etiological factor in the causation of carcinoma cervix, with the low risk subtypes 6, 11, 42, 43 and 44 causing condyloma or genital warts and the high risk subtypes 16 and 18 accounting for 80% of carcinoma cervix and subtypes 31, 33, 35, 45, 52 and 58 accounting for another 20% of the cases.[4] (Fig.1)

The risk factors for carcinoma cervix are related to both host and viral characteristics such as HPV exposure, viral oncogenicity, efficiency of immune response, and presence of co-carcinogens.[2] These include:

- Multiple sexual partners
- A male partner with multiple previous or current sexual partners
- Young age at first intercourse
- Persistent infection with a high oncogenic risk HPV 16 or HPV18.
- Immunosuppression
- Certain HLA subtypes
- Use of nicotine and oral contraceptives

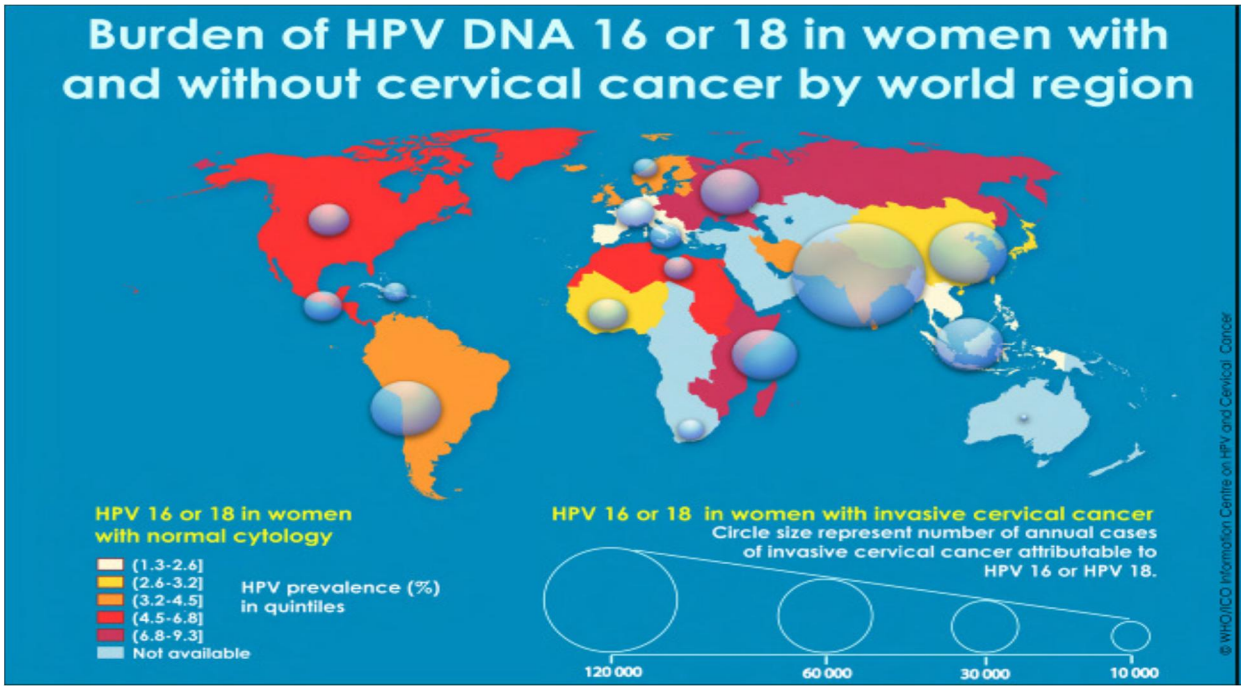


Fig1: Epidemiological data showing the burden of HPV in carcinoma cervix

The vast majority of cancer of the cervix is squamous cell carcinoma accounting for 70–80% of invasive carcinomas, adenocarcinoma and adenosquamous carcinoma comprising 10–15% of all cases, and the rest another 10–15%.

The World Health Organization (WHO) recognizes three general categories of invasive carcinoma of the cervix: squamous cell carcinoma, adenocarcinoma, and ‘‘other epithelial tumors’’.[5]

The histological classification is as follows :

- “Squamous cell carcinoma
    - Microinvasive (early invasive) squamous cell carcinoma
    - Invasive squamous cell carcinoma
        - Keratinizing
        - Nonkeratinizing
        - Basaloid
        - Verrucous
        - Warty
        - Papillary
        - Squamotransitional
        - Lymphoepithelioma-like carcinoma”

- “Adenocarcinoma
    - Usual type adenocarcinoma
    - Mucinous adenocarcinoma

        - Endocervical type
        - Intestinal type
        - Signet-ring type”
    - Minimal deviation
    - Villoglandular
    - Endometrioid adenocarcinoma
    - Clear cell adenocarcinoma
    - Serous adenocarcinoma
    - Mesonephric adenocarcinoma

- Other epithelial tumors
    - Adenosquamous carcinoma
        - Glassy cell variant
    - Adenoid cystic carcinoma
    - Adenoid basal carcinoma
    - Neuroendocrine tumors
        - Carcinoid
        - Atypical carcinoid
        - Small cell carcinoma
        - Large cell neuroendocrine carcinoma
    - Undifferentiated carcinoma

Other tumors which are included are mesenchymal tumors and tumor like conditions, mixed epithelial and mesenchymal tumors, melanocytic tumors, miscellaneous tumors which include tumors of germ cell type, lymphoid and hematopoietic tumors and secondary tumors.

Molecular mechanism in HPV carcinogenesis:

Integration of the HPV genome with the host genome plays a pivotal role in the oncogenesis of HPV 16 and 18 infection in the cervix. Cells with the viral genome integration showed more genomic instability as this process interrupts the viral DNA within the E1/E2 open reading frame, leading to loss of E2 viral repressor protein and overexpression of oncoproteins E6 and E7.

The oncogenic potential is related to the products of two viral genes E6 and E7 interacting with various growth regulating proteins encoded by protooncogenes and tumor-suppressor genes. The E7 protein has a higher affinity for Rb gene, binding to it and displacing the E2F transcription factors that are normally sequestered by Rb gene, promoting progression through the cell cycle. Moreover, E7 also inactivates p21 which allows activation cell cycle promoters such as cyclin D, cyclin dependent kinases (CDK4) causing unchecked growth of cells.

Like E7, E6 also has an increased affinity for p53. The E6-p53 interaction happens at the polymorphic site at aminoacid 72 in p53. This p53 Arg72 variant is more susceptible to degradation by E6. Meanwhile E6 also promotes the degradation of a bax,

a proapoptotic member of the bcl-2 family activating telomerase causing immortalization of the tumor cells. To summarize, high risk HPV express oncogenic proteins which eventually lead to a conglomerate of events inactivating tumor suppressors, activating cyclins, inhibiting apoptosis and combating cellular senescence.[6] (Fig.2)

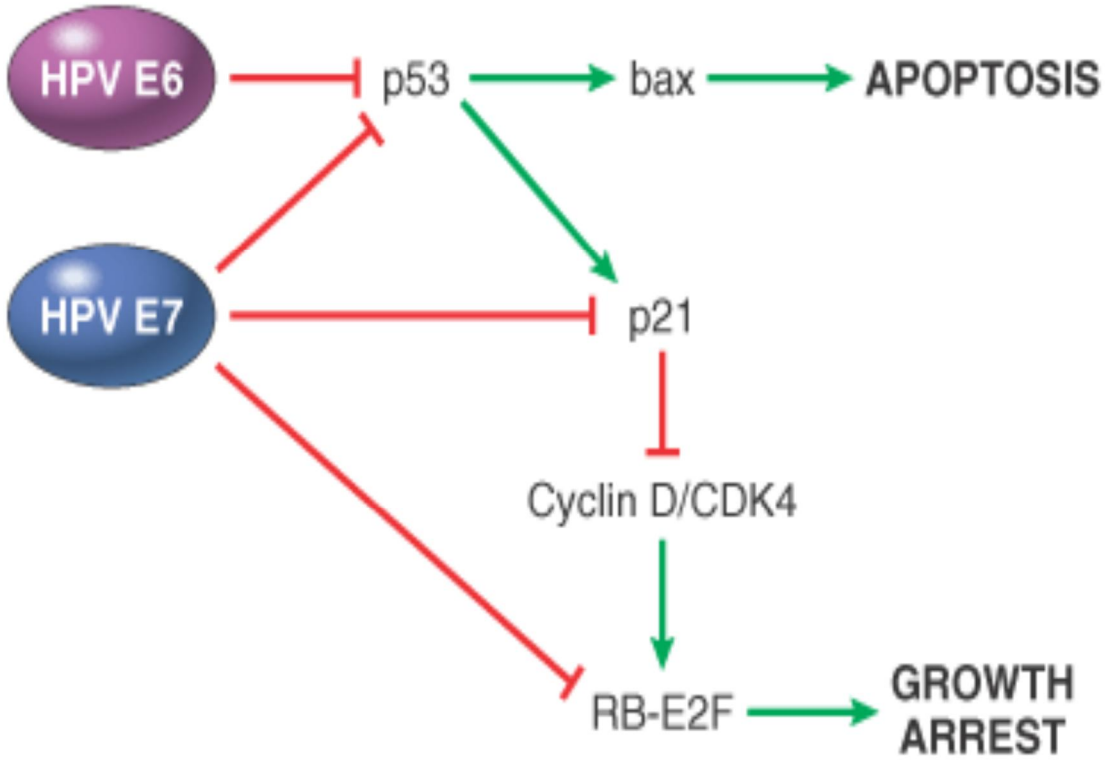


Fig.2: Molecular mechanism of HPV oncogenesis

Grading of carcinoma cervix:

Grade 1, well-differentiated squamous cell carcinoma - This tumor is composed of cells that resemble "normal squamous epithelium, with frequent formation of concentrically laminated keratin pearls and evident intercellular bridges and abundant eosinophilic cytoplasm, and their nuclei show mild atypia, with inconspicuous nucleoli and occasional mitosis".

Grade 2, moderately differentiated squamous cell carcinoma - This tumor has cells with "moderate amounts of cytoplasm and pleomorphic nuclei with Squamous differentiation of the tumor cells still recognizable by occasional keratin pearl formation and individual cell keratinization". Mitoses are easily identified.

Grade 3, poorly differentiated squamous cell carcinoma - The tumor cells have a "high nucleocytoplasmic ratio, marked nuclear pleomorphism, and abundant mitosis, including some atypical forms but no keratin pearl formation identified".

Staging of carcinoma cervix

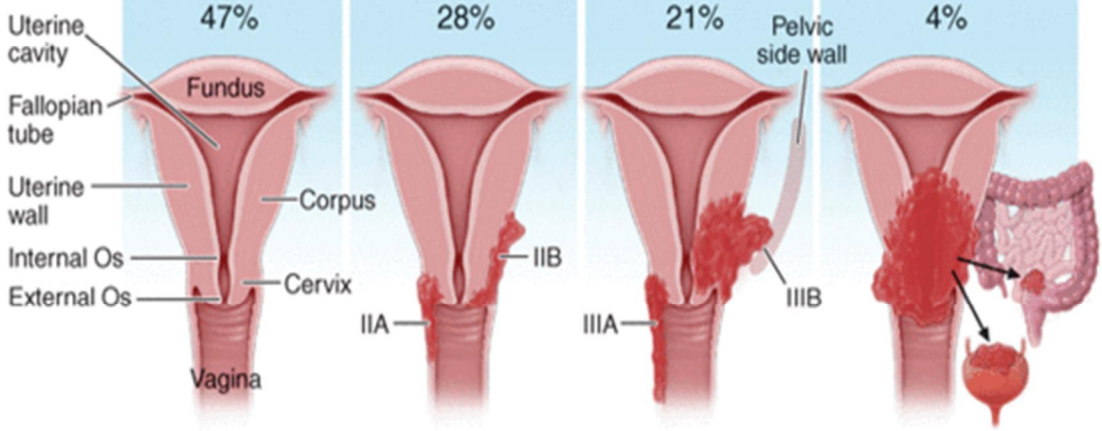
The most widely accepted staging system for tumors of the cervix is the four-stage system of the International Federation of Gynecologists and Obstetricians (FIGO)[7]. The classification is as follows:

FIGO Staging for carcinoma cervix

Stage I	The carcinoma is strictly confined to the cervix (extension to the corpus would be disregarded)
IA	Invasive carcinoma which can be diagnosed only by microscopy, with deepest invasion ≤5 mm and largest extension ≥7 mm
IA1	Measured stromal invasion of ≤3.0 mm in depth and extension of ≤7.0 mm
IA2	Measured stromal invasion of >3.0 mm and not >5.0 mm with an extension of not >7.0 mm
IB	Clinically visible lesions limited to the cervix uteri or pre-clinical cancers greater than stage IA*
IB1	Clinically visible lesion ≤4.0 cm in greatest dimension
IB2	Clinically visible lesion >4.0 cm in greatest dimension
Stage II	Cervical carcinoma invades beyond the uterus, but not to the pelvic wall or to the lower third of the vagina
IIA	Without parametrial invasion
IIA1	Clinically visible lesion ≤4.0 cm in greatest dimension
IIA2	Clinically visible lesion >4 cm in greatest dimension
IIB	With obvious parametrial invasion
Stage III	The tumor extends to the pelvic wall and/or involves lower third of the vagina and/or causes hydronephrosis or non-functioning kidney†
IIIA	Tumor involves lower third of the vagina, with no extension to the pelvic wall
IIIB	Extension to the pelvic wall and/or hydronephrosis or non-functioning kidney
Stage IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous edema, as such, does not permit a case to be allotted to Stage IV
IVA	Spread of the growth to adjacent organs
IVB	Spread to distant organs

Staging of cervix cancer

Stage	0	I	II	III	IV
Extent of tumor	Carcinoma in-situ	Confined to cervix	Disease beyond cervix but not to pelvic wall or lower 1/3 of vagina	Disease to pelvic wall or lower 1/3 vagina	Invades bladder, rectum or metastasis
5-year survival	100%	85%	65%	35%	7%
Stage at presentation		47%	28%	21%	4%



Source: Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine, 18th Edition*: www.accessmedicine.com

American Joint Committee on Cancer

Cervix Uteri Cancer Staging

7th EDITION

Definitions

Primary Tumor (T)

TNM CATEGORIES	FIGO STAGES	
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Tis*		Carcinoma in situ (preinvasive carcinoma)
T1	I	Cervical carcinoma confined to uterus (extension to corpus should be disregarded)
T1a**	IA	Invasive carcinoma diagnosed only by microscopy. Stromal invasion with a maximum depth of 5.0 mm measured from the base of the epithelium and a horizontal spread of 7.0 mm or less. Vascular space involvement, venous or lymphatic, does not affect classification
T1a1	IA1	Measured stromal invasion 3.0 mm or less in depth and 7.0 mm or less in horizontal spread
T1a2	IA2	Measured stromal invasion more than 3.0 mm and not more than 5.0 mm with a horizontal spread 7.0 mm or less
T1b	IB	Clinically visible lesion confined to the cervix or microscopic lesion greater than T1a/IA2
T1b1	IB1	Clinically visible lesion 4.0 cm or less in greatest dimension
T1b2	IB2	Clinically visible lesion more than 4.0 cm in greatest dimension
T2	II	Cervical carcinoma invades beyond uterus but not to pelvic wall or to lower third of vagina
T2a	IIA	Tumor without parametrial invasion
T2a1	IIA1	Clinically visible lesion 4.0 cm or less in greatest dimension
T2a2	IIA2	Clinically visible lesion more than 4.0 cm in greatest dimension
T2b	IIB	Tumor with parametrial invasion
T3	III	Tumor extends to pelvic wall and/or involves lower third of vagina, and/or causes hydronephrosis or nonfunctioning kidney
T3a	IIIA	Tumor involves lower third of vagina, no extension to pelvic wall
T3b	IIIB	Tumor extends to pelvic wall and/or causes hydronephrosis or nonfunctioning kidney
T4	IVA	Tumor invades mucosa of bladder or rectum, and/or extends beyond true pelvis (bullous edema is not sufficient to classify a tumor as T4)

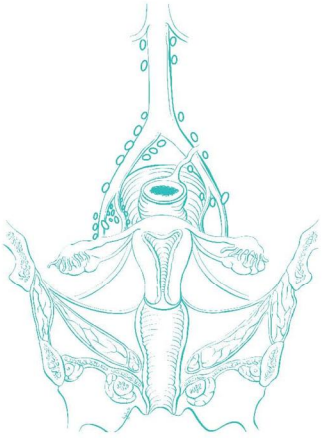
Regional Lymph Nodes (N)

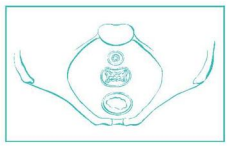
TNM CATEGORIES	FIGO STAGES	
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1	IIIB	Regional lymph node metastasis

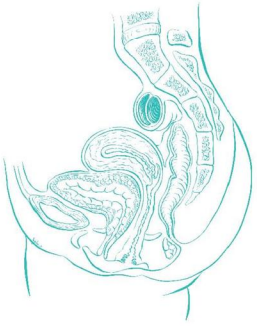
Distant Metastasis (M)

TNM CATEGORIES	FIGO STAGES	
M0		No distant metastasis
M1	IVB	Distant metastasis (including peritoneal spread, involvement of supraclavicular, mediastinal, or paraaortic lymph nodes, lung, liver, or bone)

ANATOMIC STAGE/PROGNOSTIC GROUPS (FIGO 2008)			
Stage 0*	Tis	N0	M0
Stage I	T1	N0	M0
Stage IA	T1a	N0	M0
Stage IA1	T1a1	N0	M0
Stage IA2	T1a2	N0	M0
Stage IB	T1b	N0	M0
Stage IB1	T1b1	N0	M0
Stage IB2	T1b2	N0	M0
Stage II	T2	N0	M0
Stage IIA	T2a	N0	M0
Stage IIA1	T2a1	N0	M0
Stage IIA2	T2a2	N0	M0
Stage IIB	T2b	N0	M0
Stage III	T3	N0	M0
Stage IIIA	T3a	N0	M0
Stage IIIB	T3b	Any N	M0
	T1–3	N1	M0
Stage IVA	T4	Any N	M0
Stage IVB	Any T	Any N	M1







Notes

* FIGO no longer includes Stage 0 (Tis).

** All macroscopically visible lesions—even with superficial invasion—are T1b/IB.

American Cancer Society®

Financial support for AJCC 7th Edition Staging Posters provided by the American Cancer Society

ajcc®

Management of carcinoma cervix

Locoregional treatment in cervical cancers are influenced by number of factors like tumor size, histological features, evidence of lymph node metastasis, risk factors for complications of surgery or radiotherapy and patient's preference.

As a rule, high grade squamous intraepithelial lesions (HSILs) are managed with a loop electroexcision procedure. Microinvasive cancers invading less than 3 mm (stage IA1) are managed with conservative surgery like excisional conization or extrafascial hysterectomy. Early invasive cancers (stage IA2, IB1 and stage IIA) tumors are managed with radical or modified radical hysterectomy, radical trachelectomy (if fertility is desired) or radiotherapy.

Locally advanced cervical cancers (stages IB2, stages IIB -IVA) are managed by combined chemoradiation therapy. Selected patients with centrally recurrent disease after maximum radiotherapy may be treated with pelvic exenteration. Isolated pelvic recurrences after hysterectomy are treated by irradiation. Metastatic carcinoma cervix (stage IVB) are treated by chemotherapy.

THE RNA FAMILY

The nucleus is the most important part of the cell which is largely made by chromosomes. DNA is the component of the chromosomes that carry the "genetic message," the blueprint for all the heritable characteristics of the cell and its descendants. Each chromosome contains a segment of the DNA double helix.

The genetic message is encoded by the sequence of purine and pyrimidine bases in the nucleotide chains. The text of the message is the order in which the amino acids are lined up in the proteins manufactured by the cell. The message is transferred to ribosomes, the sites of protein synthesis in the cytoplasm, by RNA. RNA differs from DNA in that it is single-stranded and has uracil in place of thymine. (Fig.5)

Different mRNAs can be formed from a single gene, with each mRNA dictating formation of a different protein. The proteins formed from the DNA blueprint include all the enzymes, and these in turn control the metabolism of the cell. In short, DNA makes RNA which makes proteins, and proteins are the cellular workhorses that carry out all the crucial tasks. However, recently a group of RNAs called non coding small RNAs that control the transcription and translation of protein-coding RNAs are found. MicroRNA is one such member of the family of small RNAs which has been implicated in various disease conditions. Various researches are been recently done to unravel the mystery of these microRNAs, which may provide a further clue to the mysterious 98% of the human genome that doesn’t direct the production of protein.

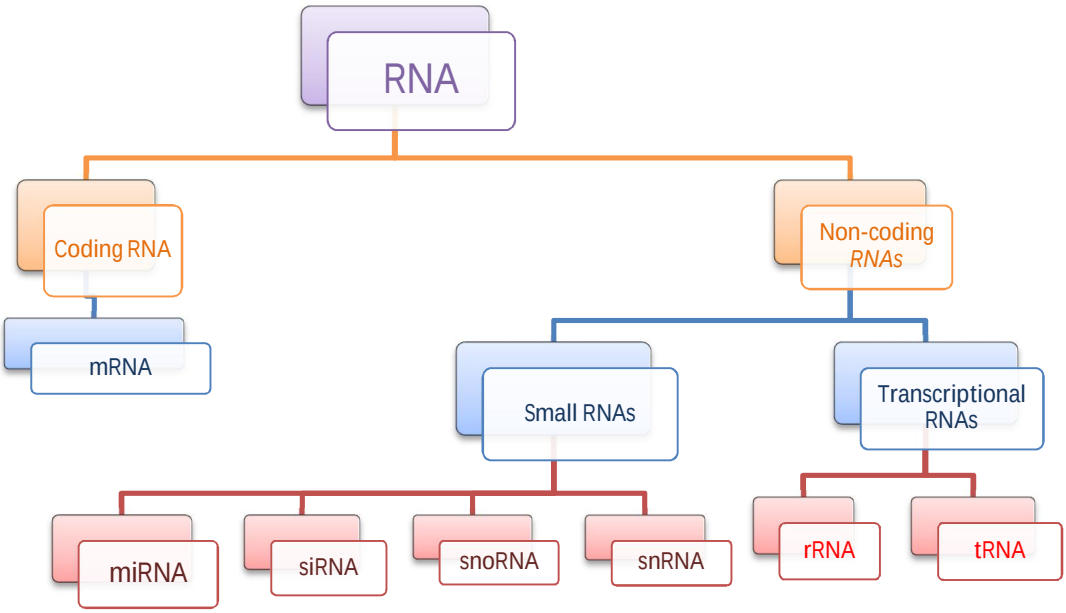


Fig.5: Different members of the RNA family

Various other RNAs of the RNA family are transfer-messenger RNA, SmY RNA, small Cajal body-specific RNA, Guide RNA, Y RNA, Telomerase RNA, Spliced leader RNA, Ribonuclease P, Ribonuclease MRP which help in post transcriptional modification or DNA replication and a few regulatory RNAs such as Piwi-interacting RNA (piRNA), cis-natural antisense transcript, repeat associated RNA, 7SK RNA, trans-acting siRNA are present in most eukaryotes, bacteria and animals.

Micro RNA:

MicroRNAs (miRNAs) are a class of non-coding RNA gene whose final product is a ~22 nucleotide length functional RNA molecule. They play important roles in the

regulation of target genes by binding to complementary regions of messenger transcripts to repress their translation or regulate degradation.[18,19,20] miRNAs have been implicated in cellular roles as diverse as developmental timing in worms, cell death and fat metabolism in flies, haematopoiesis in mammals, and leaf development and floral patterning in plants.[21,22] They were first discovered in a nematode worm, *Caenorhabditis elegans* and were shown to regulate expression of partially complementary mRNAs.[23]

Recent research have shown that microRNAs are implicated in the pathogenesis of various cancers in humans which had lead to the discovery of a whole array of microRNAs and their relation to cellular function such as cellular differentiation, proliferation and development, apoptosis, cell cycle regulation etc. These data imply that the complexity of genomic control is greater than previously imagined, and that microRNAs could represent a new order of genomic control.

Biogenesis of miRNA:

Biogenesis of miRs typically involves the transcription of pri-miRNA by RNA polymerase II from the introns (referred to as mirtrons).Pri-miRNA forms a distinct hairpin structure that is processed in the nucleus by Drosha to form a shorter Pre-miRNA and then, exported to cytosol, whereby Dicer cleaves the hairpin to generate the mature microRNA.

The mature microRNA loads onto the Argonaute protein in the RNA induced silencing complex (RISC), which directs the mature microRNA toward its targets.

Perhaps, the most important element in repression of gene expression by microRNAs is the conserved Watson-Crick base pairing between nucleotide 2 to 7 at the 5' end of the microRNA, which is referred to as the Seed Sequence, and the target mRNA.

With the exception of the seed sequence, base pairing between microRNA and its target mRNA need not be perfect which allows targeting of multiple different mRNAs by a single microRNA. (Fig.6) Targeting of multiple different mRNAs leads to suppression of expression of multiple proteins and hence, a number of secondary effects resulting from the changes in the expression of the primary targets.[24]

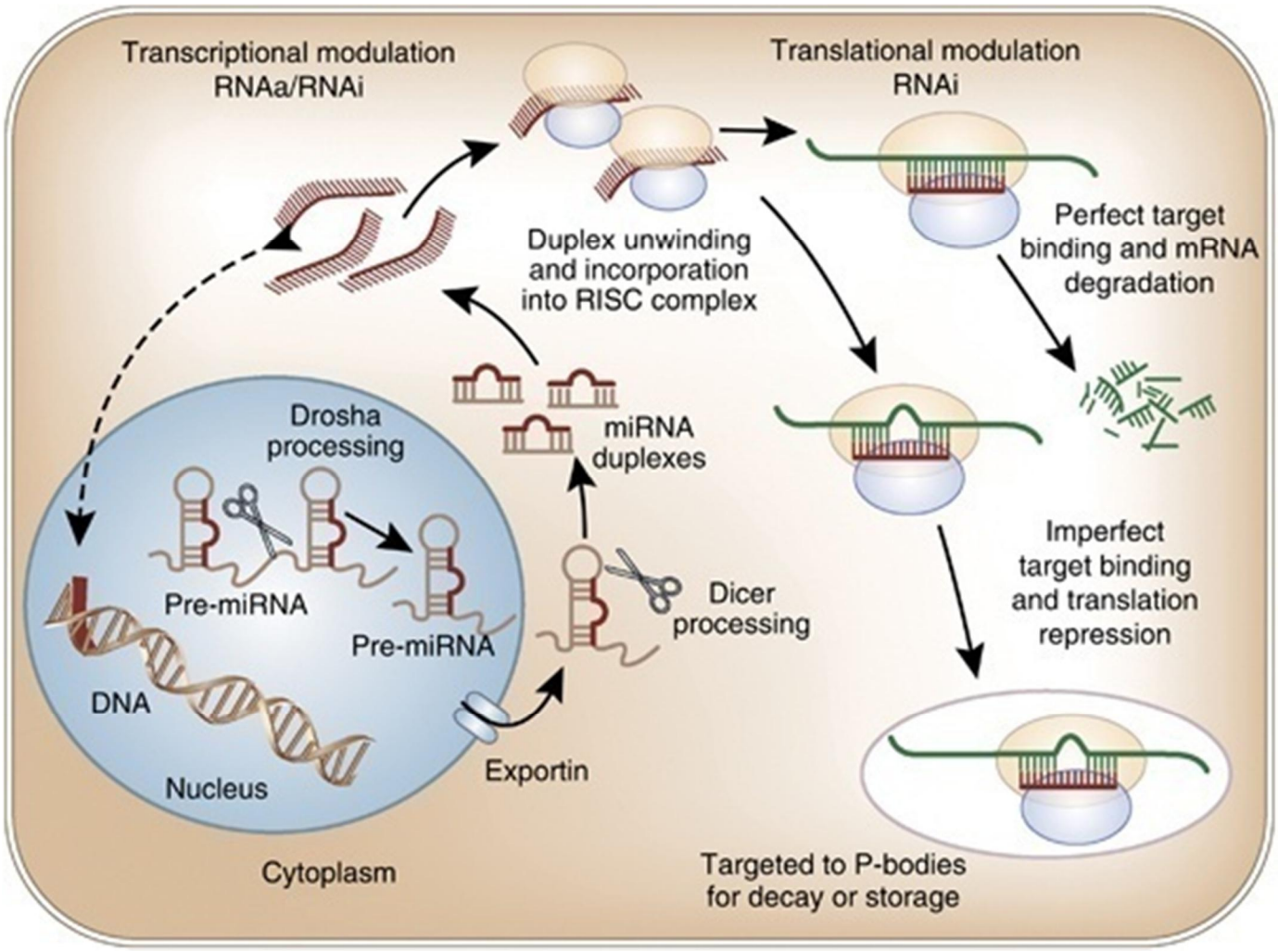


Fig 6: Biogenesis of microRNA with its functions

Functions of microRNA:

MicroRNAs are considered as key regulators of the transcriptional processes such as cap40S initiation, joining of 60S ribosomal unit, elongation and premature termination of ribosomal activity during transcription and two important post-transcriptional mechanisms that sequence to downregulate gene expression.

- mRNA cleavage and degradation through destabilization mediated by RNA induced silencing complex (RISC) at the open reading frame of the target mRNA.
- Translational repression by a complementarity imperfect RISC binding at the 3' untranslated regions of the target mRNA genes.

MicroRNAs are thought to play diverse roles in the regulation of many biological processes such as development, proliferation, differentiation and apoptosis. The known function of expressed miRNAs in an organism can be divided into three groups.[25]

- "The dysfunctional expression of specific microRNAs, known as oncomiRs which has a major role in the biogenesis of human malignancies"
- "The expression of particular microRNAs which has no functional consequence unless some specific type of stress occurs"
- "The expressions of certain microRNAs are required to preserve metabolic pathways, such as cholesterol and insulin biosynthesis"

With clinical parameters correlating with microRNA expression profiles, a wide list of varied diseases like cardiovascular diseases, diabetes and cancers etc., have shown a consistent association. Thus microRNAs serve as clinically relevant biomarkers and accurate determination of their level of expression might describe the biological, pathological and clinical roles of microRNA in health and disease.

MICRORNAs & CANCER:

microRNAs and their relation to carcinogenesis has started with the discovery of the first oncogenic microRNA described in 2005. (Fig.7) microRNAs which are related to oncogenesis are hence referred to as “Oncomirs”. Recently, microRNA profiling and data have elucidated the role of various microRNAs in different carcinogenic processes such as cell proliferation, cytotoxicity, mutagenecity, metastatic potential etc., which has made them a potential tool for diagnosis and prognostication of various cancers.

Fig 7: Historical evolution of microRNAs

ONCOMIRS:

With the discovery of the gene silencing by microRNAs by Andrew fire and Craig Mello for which they received the Nobel prize, the role of microRNAs in various disease processes have been continuously explored. By current estimates, there are more than 3000 microRNAs which encode approximately about 1000 genes in humans accounting only about 5% of the genome. Since microRNAs control various cellular processes of growth differentiation and cell survival, it is not surprising that they play a role in carcinogenesis.[26]

After the identification of two clustered miRNAs as the targets in human B-cell chronic lymphocytic leukemias (B-CLL; the search for a microRNA fingerprint investigation in normal and cancer cells by miRNA microarrays was developed. Since then cancer-specific microRNA finger prints have been identified in every type of analyzed cancer, including B-CLL, breast carcinoma, glioblastoma, hepatocellular carcinoma, papillary carcinoma of thyroid, bronchogenic carcinoma, gastric carcinoma, colonic carcinoma and endocrine pancreatic tumors, it represents that microRNAs are involved in the initiation of the malignant process.[27]

miRNAs can participate in oncogenesis either by upregulating the expression of oncogenes or by downregulating the expression of tumor suppressor genes. If a miRNA inhibits the translation of an oncogene, a reduction in the quantity or function of that miRNA will lead to overproduction of the oncogene product; thus, the miRNA acts as a tumor suppressor. Conversely, if the target of a miRNA is a tumor suppressor gene, then overactivity of the miRNA can reduce the tumor suppressor protein; thus, the miRNA acts as an oncogene. The role of these oncomirs either as an oncogene or as a tumor suppressor gene is predicted due to a mutational deregulation of the normal microRNA activity leading onto their aberrant expression. (Fig.8)

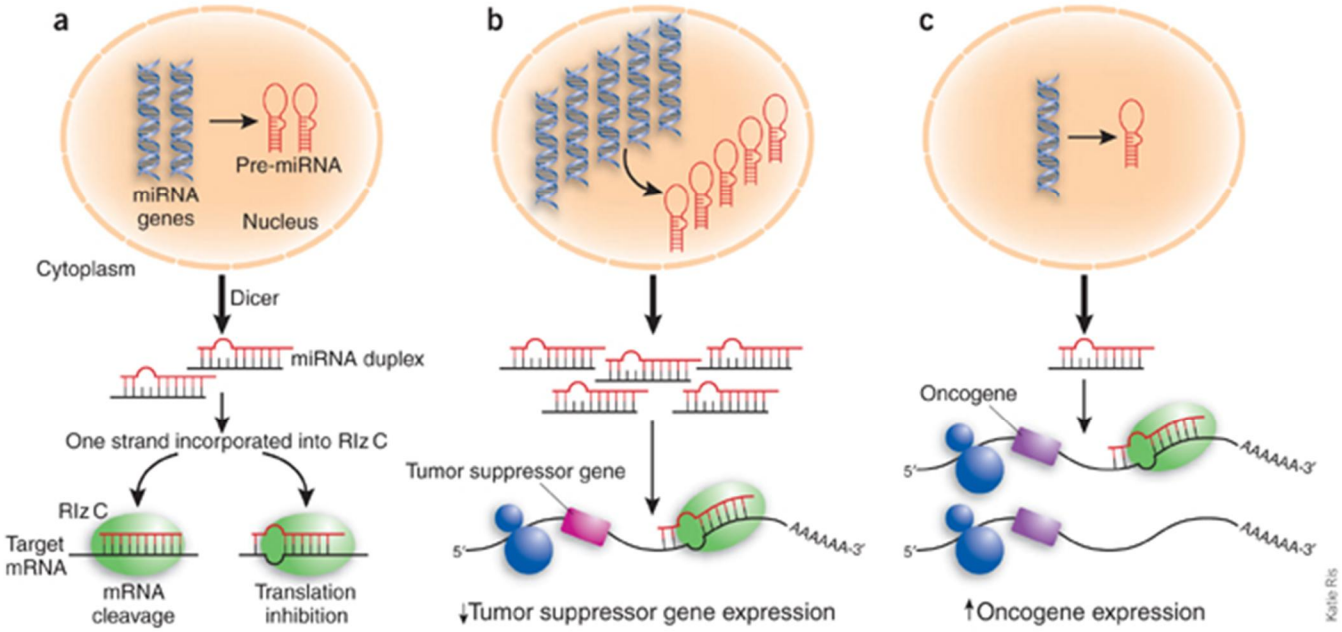
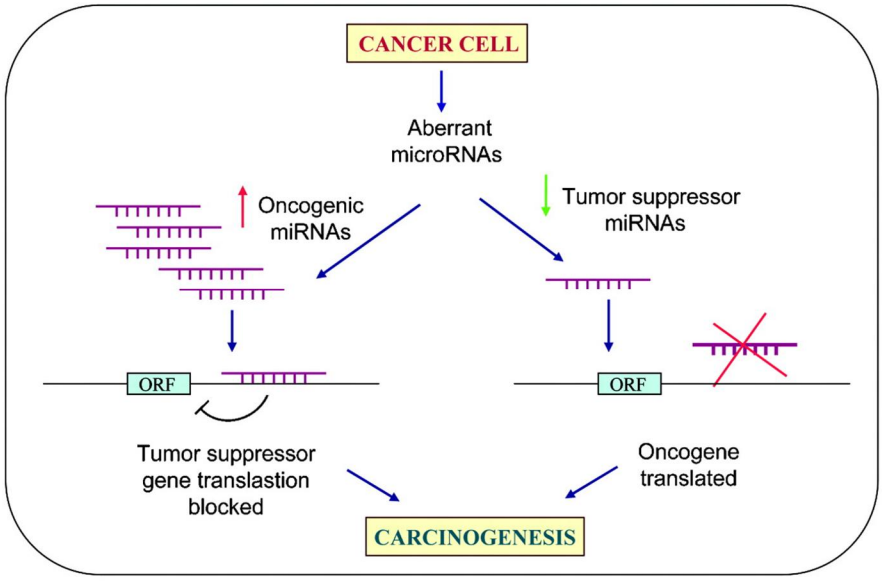


Fig.8: Molecular mechanisms in microRNA pathways

Many miRNAs reside in genomic regions involved in cancer, including minimal regions of loss of heterozygosity (LOH), minimal amplicons, or breakpoint cluster regions.[28] The aberrant expression in cancers is attributed to their location in these genomic regions i.e, overexpressed oncogenic miRNAs are located in amplified regions and the downregulated suppressor miRNAs are present in deleted regions. (Fig.9)

Fig.9: Mechanisms of microRNA induced carcinogenesis



These aberrantly expressed microRNAs, either overexpressed oncogenic miRs or underexpressed tumor suppressor miRs along with various other protein coding genes (PCGs) will dysregulate the cascade of cell cycle leading to inability of a cell to repair cytogenetic abnormalities which will accumulate independently in different cells generating clones of tumor cells with varying properties of tumor progression and heterogenecity such as tumor growth, differentiation, invasion, metastasis and resistance to chemotherapy. (Fig.10)

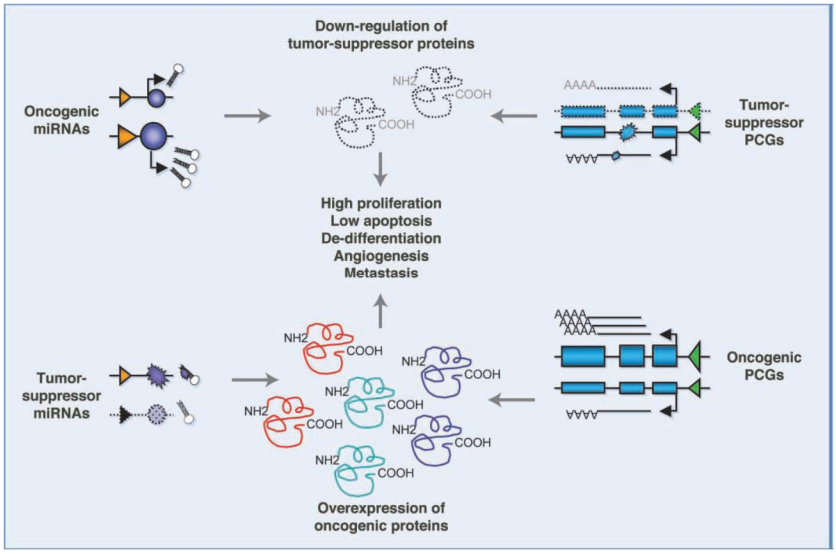


Fig 10: Oncogenesis by aberrantly expressed microRNAs

MicroRNAs expression levels assessed through oligonucleotide microarray analysis, validated by RT-PCR has shown their association with various cell cycle controls and checkpoints. A list of microRNAs and the genes they regulate has been shown below.[29] (Fig.11)

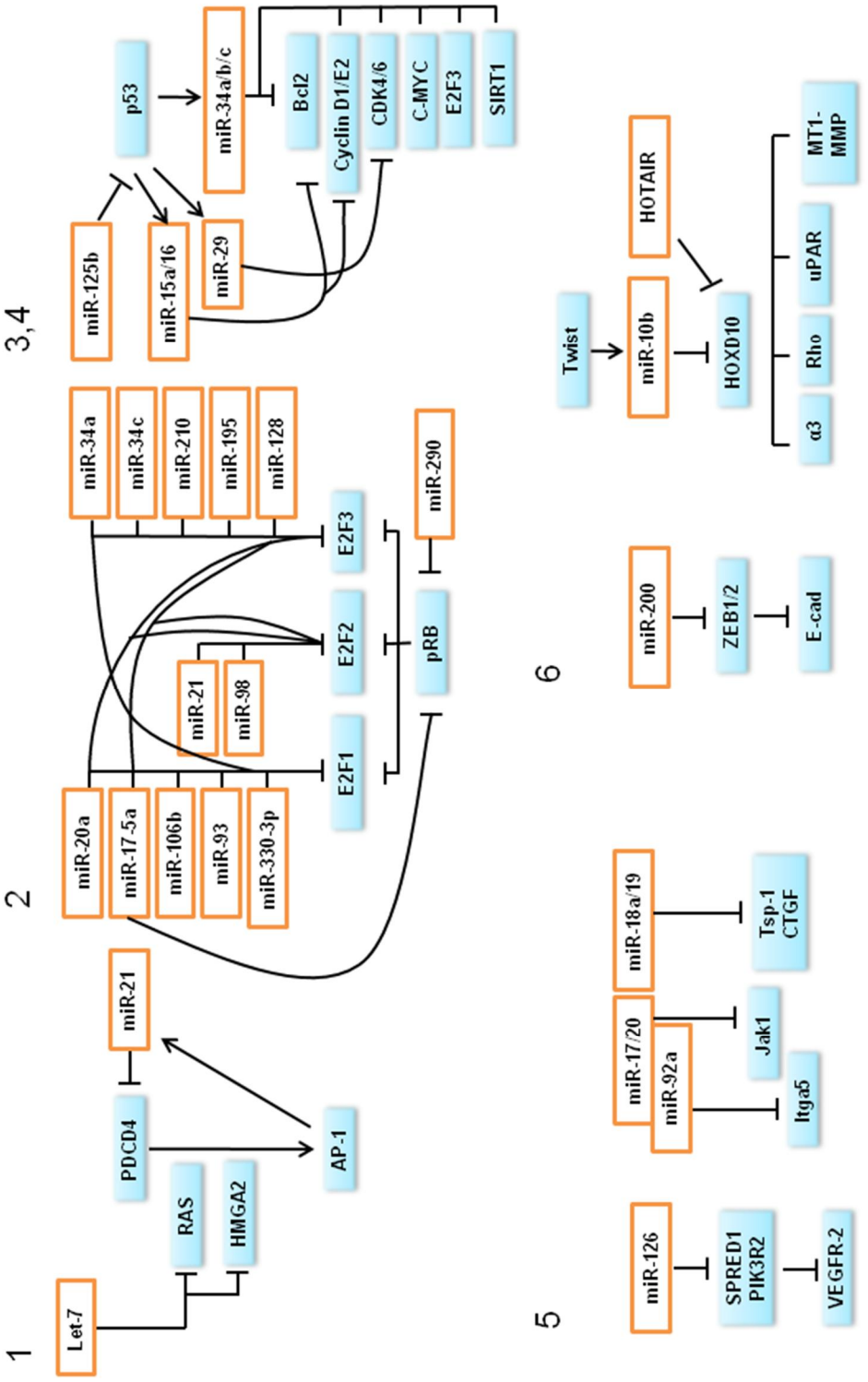


Fig.11: Different microRNAs and their downstream regulatory pathways

MICRORNAs IN CARCINOMA CERVIX:

Various microRNAs have been related in the sequential molecular mechanisms of either HPV infection or tumor progression. A list of the varied microRNAs related either to HPV or the various molecular mechanisms in oncogenesis of carcinoma cervix is shown below.[30] (Fig.12)

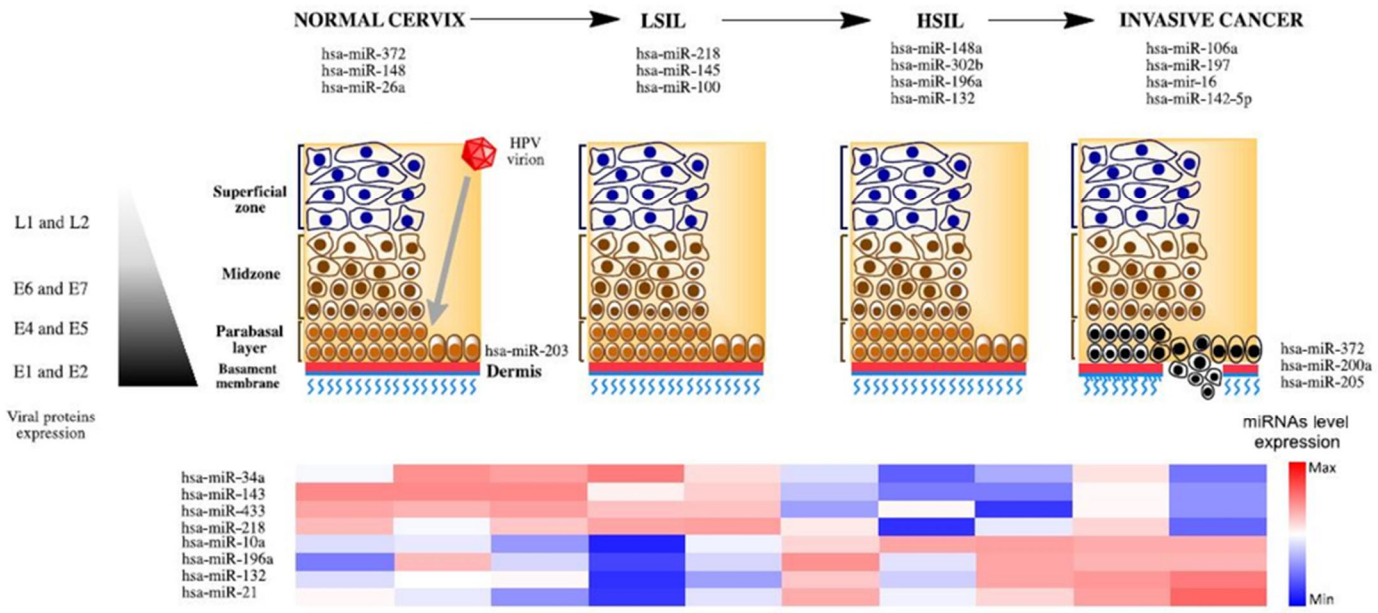


Fig.12: Frequently expressed microRNAs in cervical cancer

These microRNAs function either as oncomirs or as tumor suppressor microRNAs by altering various downstream intracellular signaling pathways mediating critical tumoral mechanisms such as growth and differentiation of cells, susceptibility to apoptosis, lymphatic or vascular invasion and metastasis, susceptibility to chemotherapeutic agents like cisplatin etc. and radiotherapy.

Understanding the mechanism of microRNA and its role in carcinoma cervix thus play a very important step in prediction and prognostication of the disease such as use of serum microRNA as markers or aberrant hypermethylation of miRs in tissues. The huge array of microRNAs have unraveled a possibility of therapeutics at a gene level, either by inhibition of miRNAs by antisense miRNA oligonucleotides called antogomirs or supplementing complementary miRNA nucleic acid sequences.[31] The commonly overexpressed and underexpressed miRs in carcinoma cervix and their location in chromosomes is listed below[32,33,34] in Table 1.

Table 1. Expression of various microRNAs in carcinoma cervix

Overexpressed miRNAs	Chromosome	Underexpressed miRNAs	Chromosome
miR-15a	13	miR-1	1
miR-15b	3	miR-7	10
miR-16	13	miR-10b	2
miR-17-5p	13	miR-29a	7
miR-19a	4	miR-30b	8
miR-20a	13	miR-100	11
miR-20b	X	miR-124	6
miR-21	17	miR-126	9
miR-27b	9	miR-138	13

miR-93	7	miR-143	5
miR-106a	X	miR-145	5
miR-133b	18	miR-149	2
miR-146	5	miR-194	4
miR-183	8	miR-195	19
miR-185	22	miR-214	1
miR-193b	16	miR-218	1
miR-196a	**17**	miR-376a	14
miR-199a	19	miR-422a	15
miR-203	14	miR-424	X
miR-210	11	miR-450	X
miR-224	X	miR-451	17
miR-324-5p	17	miR-455	9
miR-372	11	miR-487b	14
miR-373	3	miR-495	14
miR-375	2	miR-574	4
miR-432	14		
miR-641	19		
miR-1286	22		
miR-2392	26		

Lui et al. in 2007 found out an increased expression of mir-21 and reduced expression of mir-143 were reproducibly displayed in cancer samples among 6 substantially expressed miRNAs compared to normal cervix sample controls. Interestingly, there were no viral-encoded microRNAs identified from the HPV infected cells by molecular cloning approach. In addition, there were a few novel miRNAs and an additional set of small RNAs, which do not meet the microRNA criteria were also found.[35]

Wang X et al. in 2008 has found an aberrant expression of a few microRNAs such as downregulation of growth suppressing mirs such as mir-143 and mir-145 and upregulation of cell proliferating miR such as mir-146a plays an important role in carcinoma cervix growth.[36] Martinez et al. in 2008 found out that HPV 16 E6 downregulates mir-128 which is a major regulator of LAMB3 mRNA, leading to consequent overexpression of the LAMB 3 protein contributing to tumorigenesis of cancer cervix, suggesting that mir-128 regulates LAMB3 expression at the transcriptional level.[37]

Lee et al. in 2008 analysed 70 different miRs with significantly different expression in early stage invasive squamous cell carcinomas and found that an increased mir-127 expression is significantly associated with lymph node metastasis. In addition, they also reported that a novel class of chemically engineered oligonucleotides called “antagomirs” such as anti-miR-199a that effectively silenced endogenous miRNAs in

vivo, leading to reduced carcinoma cervix cell growth and increased chemotherapeutic response to cisplatin.[38]

Yang et al in 2009 has found that mir-214 level is downregulated in carcinoma cervix which is validated by their reduced expression in human cancer cervix cell lines, Hela. It was also found that mir-214 negatively regulates MEK3 and JNK1 mRNAs by binding and targeting to their 3'UTR non-coding regions inhibiting Hela cell growth.[39]

Wilting et al. in 2010 observed the tumor suppressive effects of mir-124 and that methylation mediated silencing of mir-124 is associated with a high proliferation rate in cervical cancer cell lines and hence was concluded that mir-124 may provide as a valuable marker for risk stratification in the triage of high risk HPV positive women.[40]

Apoptosis is a major barrier that must be circumvented during malignant transformation. Cancer cells evolve to evade apoptosis so that they can escape from being cleared away by the surveillance system and can survive in crucial tumor microenvironment. This mechanism is tightly regulated by a balance between pro-apoptotic and anti-apoptotic proteins and microRNAs dysregulate this balance through various methods leading to attenuation of tumoral response. Wang X et al. in 2009 found out a compelling evidence for the first time that the HPV viral oncoprotein E6 is involved in the regulation of cellular tumor suppressive miRNAs such as mir-34a. HPV E6 suppresses mir-34a by destabilizing and degrading the tumor suppressor p53 causing induction of cancer cell growth and reduced apoptosis, providing new insights to the molecular mechanisms of high risk HPV-induced carcinogenesis.[41]

Liu L et al. in 2012 found a microRNA, mir143 that targets the anti-apoptotic protein bcl-2, leading to tumor regression by promoting apoptosis.[42] Overexpression of bcl-2 is closely related to multiple epithelial tumors including cervical carcinomas resulting from DNA damage and continued survival and subsequently aggregated mutations and hence mir143 downregulation of bcl-2 causes tumoral regression.

Cui et al. in 2012 found out that the overexpression of mir-125b promotes downregulation of PIK3CD, inhibiting tumor growth and promoting apoptosis of carcinoma cervix cells both in vitro and in vivo. PIK3CD (Phosphoinositide 3 kinase catalytic subunit delta) is a special target in the mir-125b mediated apoptosis, regulating the PI3K/Akt/mTor pathway.[43]

Wang F et al. in 2013 found out mir-214 causes increased tumor cell sensitivity and cytotoxicity to cisplatin by downregulating the antiapoptotic protein bcl-2, inducing apoptosis in cervical cancer cell line Hela and another cervical cancer cell line C-33A. Furthermore, it was found that mir-214 enhanced the expression of proapototic proteins Bax and caspases 9,3 and 8 inducing the intrinsic pathway of apoptosis by altering the bax/bcl2 ratio.[44]

Liu S et al. in 2013 found out an oncogene XIAP (X-linked inhibitor of apoptosis protein) as a candidate target gene of the miR-7, which plays an important role in regulating cellular proliferation and apoptosis. Hence overexpression of mir-7 in carcinoma cervix lead to downregulation of XIAP leading on to suppressed cell viability and promoted apoptosis in cervical cancer cell lines, Hela and C-33A.[45] Huang et al. in

2013 explored the upregulation of mir-101 causing inhibition of cell proliferation, migration and invasion by increasing apoptosis of cervical cancer cells functionally targeting cycloxygenase-2 (COX-2) protein.[46]

mir 218 is commonly downregulated in carcinoma cervix and hence overexpression of mir-218 has been associated with reduced proliferation in carcinoma cervix. Li et al. in 2012 found out mir-218 directly targets Rictor (Rapamycin-insensitive companion of mTor), thus blocking the Akt-mTor signaling pathway, increasing the chemosensitivity to cisplatin and decreased tumor cell growth and survival.[47] Wei et al. in 2012 identified mir-17-5p as a tumor suppressor microRNA that downregulates a key regulator of p53 posttranscriptional activity, TP53INP1(Tumor protein p53 induced nuclear protein 1) by posttranscriptional repression. It was also found that there was an inverse correlation between the levels of mir-17-5p and TP53INP1 in carcinoma cervix tissues.[48]

Tankyrase 2 (TNKS2), a new member of the telomere-associated poly (ADP-ribose) polymerase (PARP) family, binds the telomere binding protein TRF1 and protects the end of linear chromosomes. TNKS2 upregulation in tumor cells can increase telomere length when overexpressed, which suggests that TNKS2 can promote tumor progress and function as an oncogene. Kang et al. in 2012 found out a positive upregulation of TNKS2 by mir-20a which induces cell migration and invasion of cervical cancer cells.[49]

Ma et al. in 2014 identified a serum microRNA, mir-205 which is consistently elevated in carcinoma cervix patients and was correlated with poor tumor differentiation,

metastasis and increased tumor stage.[50] The concentration of serum mir-205 could distinguish carcinoma cervix patients from normal individuals, making it a potential and novel non-invasive biomarker for carcinoma cervix screening and disease progression.

Wang WT et al. in 2014 demonstrated the differential expression of three specific serum microRNAs such as miR-646, miR-141 and miR-542-3p before and after surgery for cervical carcinoma. The levels of these serum markers were deregulated in carcinoma cervix patients suggesting the possibility of serum microRNAs as effective markers in the post-therapeutic monitoring of disease progression.[51]

Role of microRNA-196a in cervical cancer:

microRNA 196a is a class of microRNAs which was first cloned from osteoblast sarcoma cells.[52] The microRNA 196 family has 3 genes, miR196a-1, miR196a-2 and miR196b, located at three paralogous loci in the mammalian HOX clusters which encodes homeodomain-containing transcription factors regulating different genetic programs during embryonic development.[53] The class I homeodomain containing HOX transcription factors encoded by those genes are expressed throughout embryogenesis in a highly arranged temporal and spatial manner
which is responsible for embryonic patterning and involved in the control of cell identity, cell growth and differentiation, as well as cell–cell and cell–matrix interactions.[54]

Upregulation of miR196a has been implicated in the pathogenesis of various tumors by suppressing the expression of specific homeobox genes activating oncogenic

pathways inside cells leading to tumor cell proliferation and migration, thereby inducing tumor cell dissemination.[55]

Ruiz et al in 2014 determined the extreme complexity of family of microRNA 196a expression and downstream target genes in cervical carcinoma cells developing a Gene Ontology Chart, (Fig.13) describing the heterogeneity of microRNAs in cervical carcinogenesis.[56]

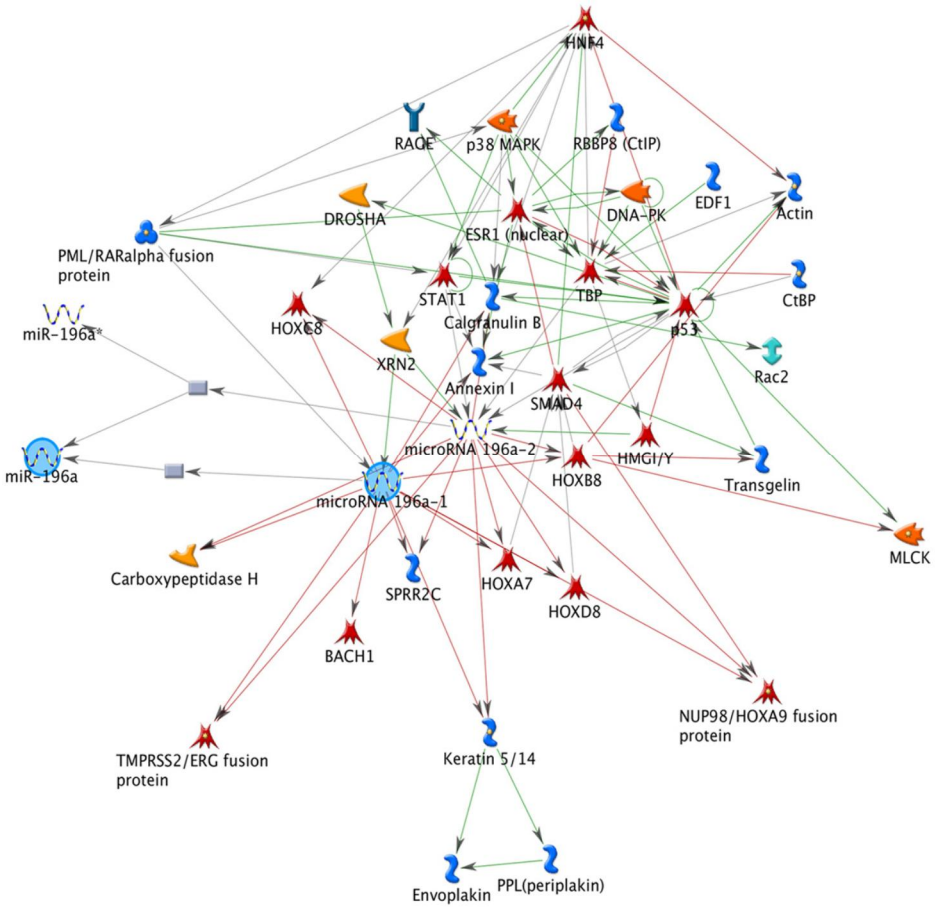


Fig 13: Gene Ontology Chart depicting the complexity of upstream regulators and downstream target genes related to miR196a

Zhang J et al. in 2013 found out an inverse relationship between a target gene netrin-4 (NTN4) and the levels of miR196a suggesting an overexpression of miR-196a plays an important role in the regulation of cell proliferation and migration of cervical cancer cells by targeting netrin-4.[57]

Hou et al. in 2014 found an upregulation in the expression of miR-196a directly targets FOXO1 and p27Kip1 that are two key effectors of PI3K/Akt signaling pathways, thus promoting cervical cancer proliferation. Upregulation of miR-196a was correlated with advanced tumour stage and poor overall and recurrence-free survival in patients with carcinoma cervix.[58]

Materials & Methods

MATERIALS AND METHODS

This study was an analysis about the role of miRNAs expression profiles of carcinoma cervix conducted on 58 patients and correlating the resultant data with various parameters such as HPV status, grade, stage of the tumor and its relationship with prognosis.

Patients diagnosed with carcinoma cervix fitting the inclusion and exclusion criteria were enrolled in the study after written informed consent.

Inclusion criteria

1. Patients with age of 18-75 years
2. All stages of carcinoma cervix
3. Cervical cancers of squamous cell histology only

Exclusion criteria

1. Cervical cancers with histology other than squamous cell carcinoma
2. Patients unfit for any treatment
3. Recurrent or residual diseases
4. Second primary cancers

CLINICAL DATA

All patients underwent a complete clinical examination, biopsy and histopathological evaluation and relevant radiological investigations including ultrasound and CT scan of abdomen and pelvis required for diagnosis and staging. Cystoscopy was done in all cases with suspicion of urinary bladder involvement. Examination under anaesthesia was done all cases to confirm the clinical staging.

All patients were staged according to FIGO staging system . Patients received treatment according to their cancer site and stage as per standard practice. Microinvasive carcinomas (Stage 1A) were treated by type I hysterectomy and FIGO stages I B1 and II A1 were treated by radical hysterectomy with bilateral pelvic lymphadenectomy . Patients with FIGO Stage I B2,
II A2 , II B , III & IV A were treated by radical chemoradiation therapy. FIGO Stage IV B was treated by systemic chemotherapy. 4 patients with FIGO stage I B2 and 6 patients with FIGO II B were treated by 3 cycles of protocol preoperative chemotherapy followed by surgery.

PATIENT FOLLOW-UP

All patients were followed monthly in the first year, every 2 months in the second year, every 3 months in the 3rd year and twice yearly for the next 2 years and annually thereafter. During each follow- up, patients undergo complete clinical examination after relevant history to look for disease recurrence. Chest x-ray and ultrasound abdomen and

pelvis is done in all patients annually. Imaging like CT scan of abdomen and pelvis and cytology were not routinely recommended and was done in symptomatic patients as required .

TUMOUR TISSUE SAMPLE

Tumour tissue samples were collected as described below at the time of diagnosis from tumour biopsies and/or from the surgical specimens in patients who undergo surgery. (Figure 1) Biopsy or surgical tissue sample (tumour tissue and tumour free normal tissue from margins) was collected in a storage tube with 3 ml of RNAlater® (Invitrogen Life Technologies, USA) solution and transferred to the Department of Genetics, University of Madras, Chennai in icepacks. The amount of tumour tissue needed for DNA/RNA studies is about 500-1000 mg.

The tissue is minced to smaller pieces and stored in the same RNAlater® (Invitrogen Life Technologies, USA) solution at 4°C overnight for percolation. RNAlater prevents RNA from getting degraded during transportation and storage. After 24hrs, the RNAlater® (Invitrogen Life Technologies, USA) solution is decanted and the tissue is stored at minus 20°C until isolation of RNA is done.

RNA ISOLATION PROTOCOL

The frozen tissues are allowed to thaw on ice and excess of RNAlater® (Invitrogen Life Technologies, USA) solution (or crystals if any) removed using a clean tissue paper and transferred to sterile 2 ml Eppendorf tubes. One ml of TRIzol® (Invitrogen Life Technologies, USA) and zirconium beads are added to the tissue sample.

The tubes are then subjected to repeated homogenization in MicroSmash® MS100 (Tomy Digital Biology Co.Ltd, Japan) homogenizer at 3500 rpm for 30 seconds with equal intervals of 1 minute incubation on ice until a clear homogenate is obtained. The homogenate is then transferred to a new sterile 2 ml Eppendorf tube and 0.2 ml of chloroform is added. The mixture is vigorously shaken for 15-30 seconds until it turns milky. All the steps of handling the tubes after step 4 should be carried out on ice.

The above mixture is centrifuged at 10,000 rpm for 20 minutes at 4°C. After centrifugation, three layers are formed: a clear aqueous layer, a turbid organic layer and an intermediate insoluble layer. The RNA containing upper aqueous layer is transferred to a new 1.5 ml Eppendorf tubes and 0.5 ml of cold ethanol is added and shaken gently to precipitate the RNA.

The tubes are then centrifuged again at 10,000 rpm for 20 minutes at 4°C to obtain a white pellet of RNA. The supernatant is discarded. To these RNA pellets, 70% ethanol is added, mixed gently and centrifuged again at 10,000 rpm for 20 minutes at 4°C.

The isolated RNA is dissolved in DEPC treated Nuclease free distilled water and stored at -20°C. RNA samples were quantified by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Germany). All the steps of handling the tubes after homogenization are carried out on ice and RNase free environment. Utmost care is taken to use RNAase free reagents and RNase free environment.

QUALITY VERIFICATION OF RNA

The quality verification of RNA is carried out to ensure RNA is free from DNA and residual phenol/alcohol. The electrophoresis on a 0.7% agarose gel is used to verify the quality of RNA.

Procedure for gel electrophoresis:

0.7 gram of agarsose is weighed and transferred into a 250 ml conical flask. 100 ml of 0.5x TAE buffer is added to it, stirred well and melted on a magnetic stirrer cum hot plate until the agars' dissolves completely. The appropriate sized gel tray and comb is washed and wiped with 70% Ethanol. The gel tray is placed inside the casting unit. The comb is placed on the gel tray and left on an even surface. After the agarose cools down to hand bearing temperature, 5 µl of ethidium bromide is added and mixed well. It is poured on the gel tray and allowed to polymerize. After polymerization the comb is removed.

The gel tray is then removed from the casting unit and placed in the electrophoresis tank. 0.5x TAE buffer is poured into the tank until the gel gets immersed. 1 µl of each RNA sample is mixed with 2µl of 6x RNA loading dye (formaldehyde added for denaturation of RNA) and 8ml of sterile double distilled water in a PCR tube. The mixture is subjected to denaturation by warming at 65˚C for 10 min. The RNA samples are allowed to cool and loaded into the wells. The electrodes are connected; power set at 100 V and run for 20 minutes.

When bromophenol blue dye is in the middle of the gel, power is switched OFF. The gel is taken to the transilluminator and observed under UV light and documented. The good quality RNA is identified by two distinct bands (28s and 18s rRNA).

SPECTROPHOTOMETRY

The nucleic acid samples are analysed at 260nm and 280nm by using Nanodrop Spectrophotometer (Thermo scientific, Germany). The concentration and purity of the sample is analysed using the following formula:

CONCENTRATION OF RNA: Concentration of double stranded RNA sample (µg/µl) = A260 x 40

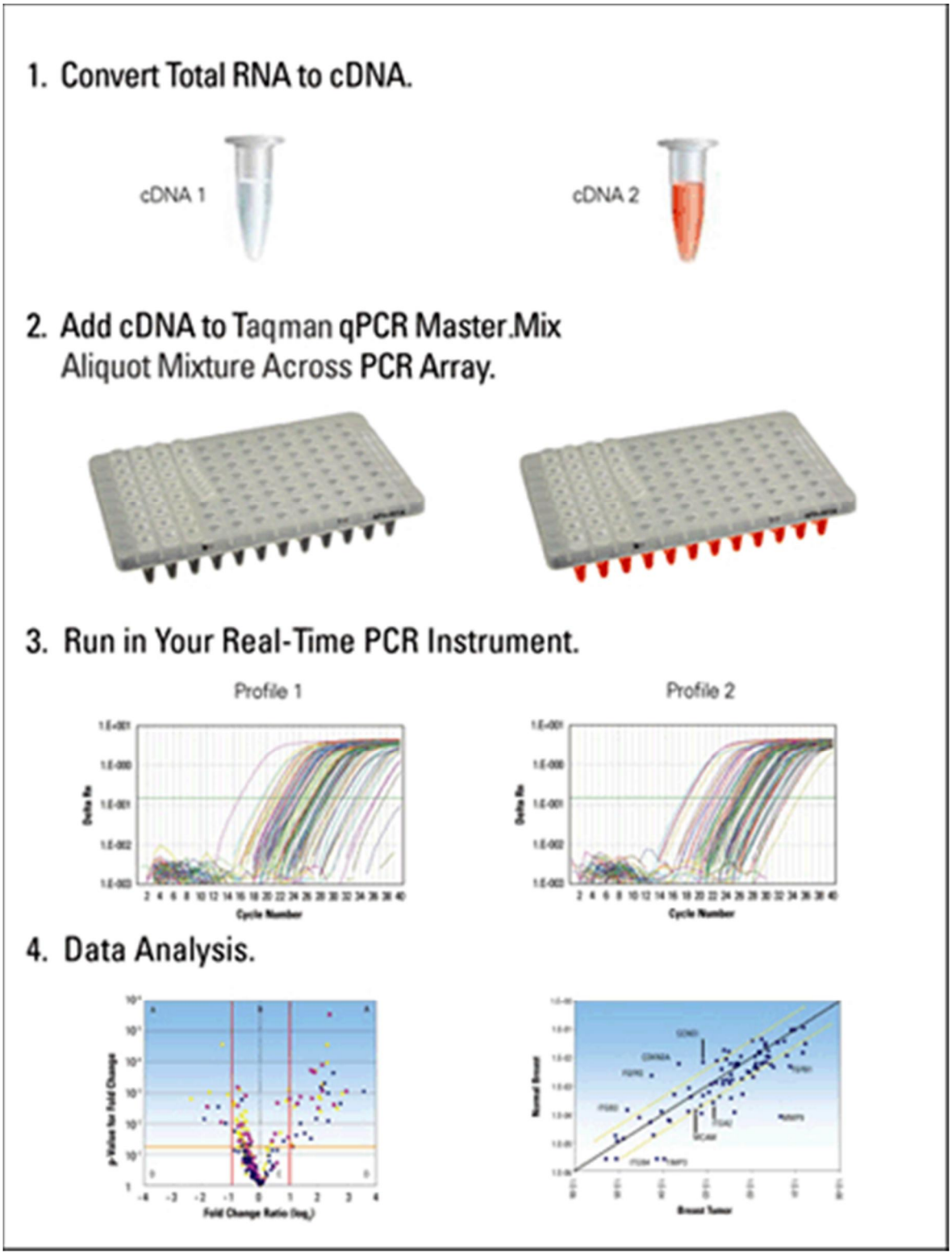
PURITY OF RNA: Pure RNA = A260 /A280 $\geq$ 2.0. The observation of absorbance is recorded at 280nm. The quality of the RNA was checked by the above mentioned methods.

MICRORNA EXPRESSION ANALYSIS

Complementary DNA (cDNA) was synthesized from the total RNA by using SuperScript® III Reverse Transcriptase (Life Technologies, CA, USA) with 10ng RNA. This cDNA was diluted 1:25 times for semi-quantitative PCR analysis and quantitative PCR.

Primer	Sequence
miR196a-5p RTstemloop primer	GTCGTATCCAGTGCGTCGAGTGACACGCAGAGCCACCTG GGCAATTTGCACTGGATACGACCCCAAC
miR196a-5p Forward primer	GCAGTAGGTAGTTTCATGTTGT

Fig.14: Method of analysis using TaqMan Gene Expression Master Mix



The relative expression was subsequently normalized to the median expression values of control genes using the ΔCt calculation in ABI 7900HT Real time PCR using TaqMan® Gene Expression Master Mix and miR196a-5p specific forward with Universal reverse. (Fig.14) Data acquired from the PCR reactions was analyzed using RQ Manager and the resulting text file was exported to DataAssist™ software (Applied Biosystems, CA, USA).

TaqMan® Protocol:

The TaqMan® Protocol as described the supplier was used to analyze the miRNAs. cDNA is first synthesized from the total RNA in 15 µl reaction volumes. The cDNA are then first incubated at 16°C for 5 minutes to anneal the stem loop primer and then at 42 °C for 30 minutes. They are further incubated at 85 °C fir 10 minutes to inactivate the enzymes. Each cDNA are then be amplified by qPCR in 20 µl PCR reactions containing 10 µl of 2x TaqMan® Universal PCR Master Mix, 2 µl of 10x TaqMan® MicroRNA Assay mix and 1.5 µl of reverse transcription product. The reaction are carried out under the following conditions: 95 °C for 10 minutes and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute using sequence specific primers.

Tumour tissue samples were collected from 119 patients with carcinoma cervix from tissue biopsies and surgical specimens. Tissue sample from normal non cancerous tissues were also collected from some patients and pooled to form a control sample.

We were able to extract RNA from 72 tumour tissue samples. Gel Photos and Ultraviolet Spectrometry were used to asses if the RNA was of sufficient quality and quantity for miRNAs Expression Studies. Of these 72 tissue samples, 63 samples (52.9 %) had sufficient quantity and quality of RNA i.e., can be used for miRNAs Expression studies. 58 patients with good quality RNA extracted from tumour biopsies were selected for miR196a-5p expression study. Expression of miR196a-5p relevant to carcinoma cervix based on available literature was analysed and compared with Clinical data of these 58 patients. Controls used were RNU 44 and RNU 48 Expression and Pooled Control Samples (normal tissues)

STUDY DESIGN

Patients with carcinoma cervix

⇩

FIGO Staging

⇩

Tumor Biopsy

⇩

Squamous cell carcinoma

⇩

Differentiation (well differentiated, moderately differentiated ,poorly differentiated)

⇔

HPV status (HPV-16 &18) **microRNA 196a-5p**

CORRELATION BETWEEN

1. **Grade of the tumor and the HPV status**
2. **Grade of the tumor and the miRNA 196a-5p expression analysis**
3. **HPV status and the miRNA 196a-5p expression analysis**
4. **miRNA196a-5p expression analysis and prognosis**

Statistical Analysis

STATISTICAL ANALYSIS

The microRNA 196a-5p expression data from tumour tissues were expressed as mean values and were compared with normal pooled control tissue samples and also normalized with external control miRNAs. The HPV status with respect to each tumor tissue was also studied and analyzed. Clinical data with the FIGO stage, histological grade of tumour, type of treatment given and the response to treatment and survival on followup were analyzed. Chi square test was employed for the comparison of group characteristics and $p < 0.05$ was considered to be statistically significant. Statistical analysis was done using SPSS 16® (IBM Inc, USA) and EXCEL 2007 ® (Microsoft, USA).

Results

RESULTS

Purified microRNA extract from 58 patients with carcinoma cervix were analyzed. These patients were categorized based on the stage of carcinoma cervix at the time of presentation by FIGO staging and the histological grade of the tumor. Out of these 58 patients with squamous cell carcinoma of cervix, 9 patients (15.5%) presented at FIGO Stage I, 21 patients (36.2%) at Stage II disease, 25 patients (43.1%) at Stage III and 3 patients (5.2%) with FIGO Stage IV at the time of presentation (Fig.15).

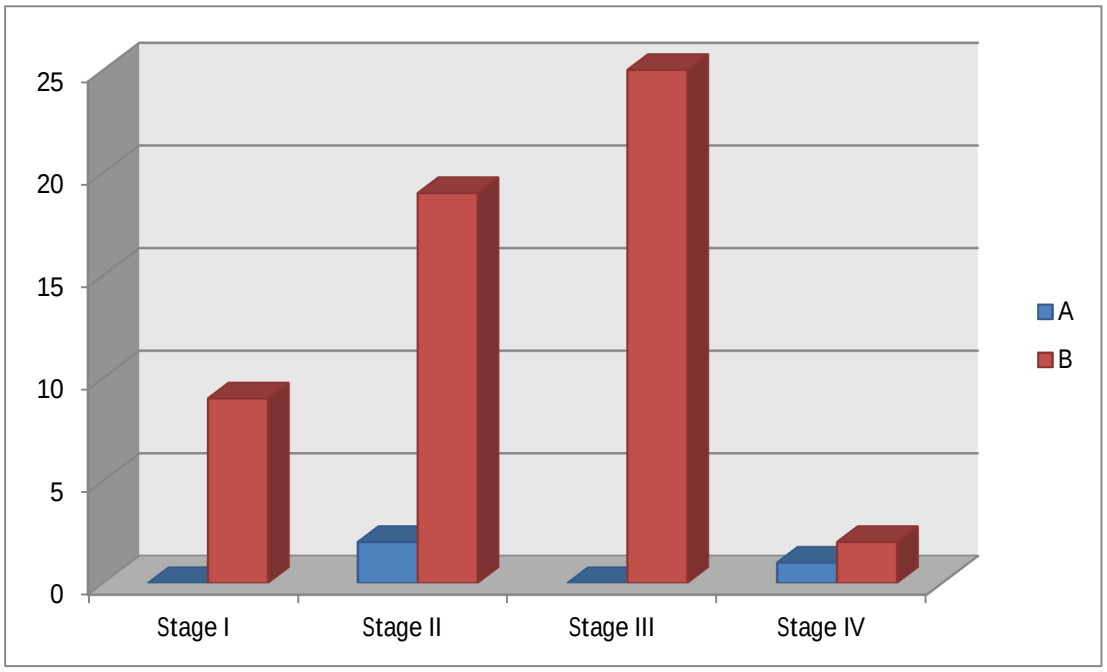
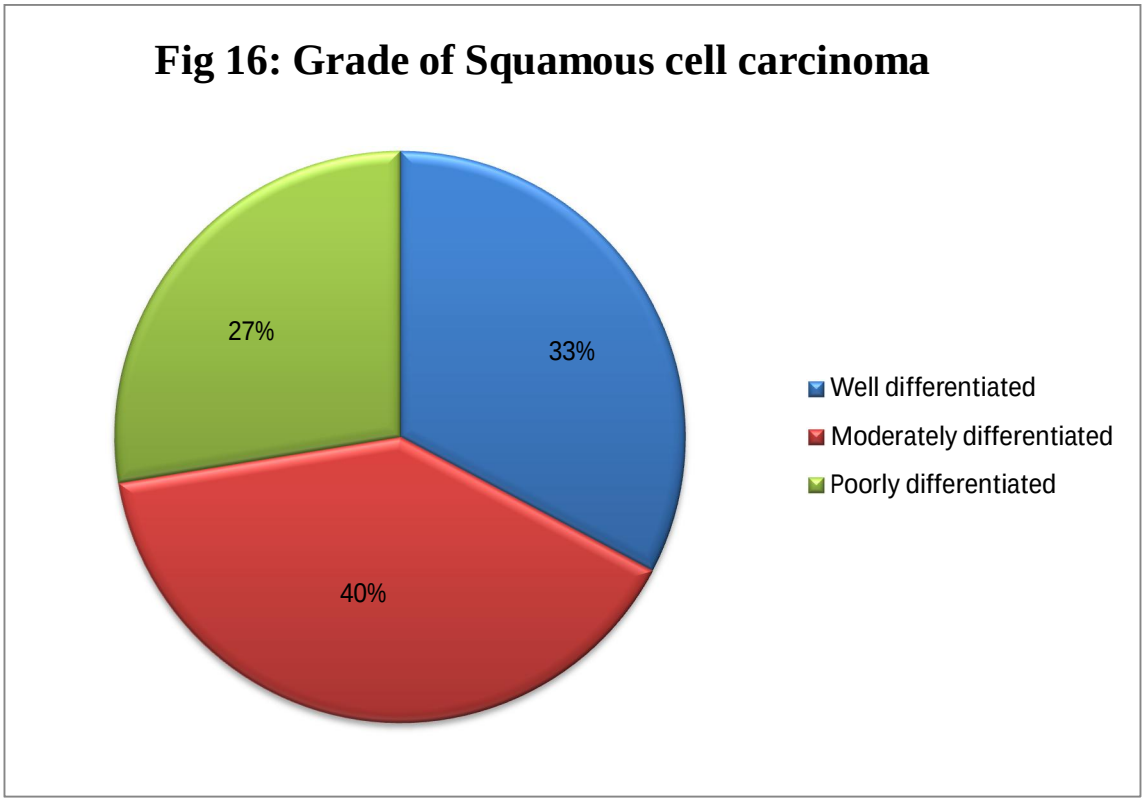
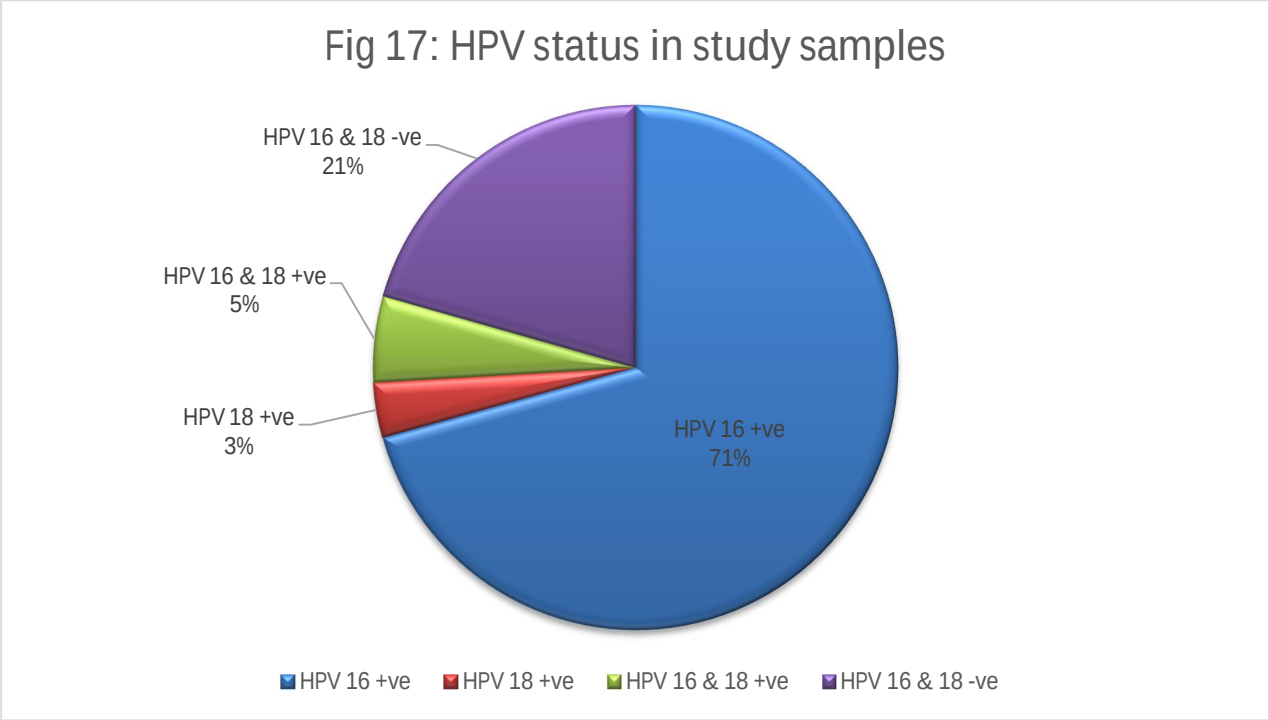


Fig.15: FIGO Stage of carcinoma cervix at the time of presentation

Among these patients presenting with different stages of carcinoma cervix, 19 patients had a well differentiated squamous cell carcinoma (33%), 23 carcinoma cases (40%) had a moderately differentiated tumor and 16 patients (27%) with a poorly differentiated carcinoma (Fig. 16).



Among these 58 patients, 41 patients (71%) of them are positive for HPV 16, and 2 patients (3%) are positive for HPV 18. 3 patients (5%) of them were positive for both and 12 patients (21%) were negative for both HPV 16 and HPV 18 (Fig.17).



To identify the dysregualtion of microRNA in cervical cancers, we attempted to correlate the relationship between the 3 parameters - HPV status, the grade of the lesion and the miR196-5p expression. The group of 58 patients who were categorized based on the grade of the tumors is further correlated with the HPV status and miR expression status. The expression of miR in different HPV categories and the statistical significance between these parameters is also studied.

Table 2: Grade of the tumor vs HPV Status

Grade of the tumor	HPV status			
	HPV 16 +	**HPV 18 +**	**HPV 16 & 18 +**	**HPV 16 & 18 -**
Well differentiated	13 (31%)	0	0	6 (50%)
Moderately differentiated	17 (42%)	0	2 (67%)	4 (33%)
Poorly differentiated	11 (27%)	2 (100%)	1 (33%)	2 (17%)
Total	41	2	3	12

From Table 2, it is evident that out of 41 patients with only HPV 16 positivity, 31% had a well differentiated tumor, 42% had a moderately differentiated tumor and 27% had a poorly differentiated tumor. All the patients in the HPV 18 positivity group presented with a poorly differentiated tumor. Among 3 patients who were positive for HPV 16 & 18, 67% presented with a moderately differentiated carcinoma and 33% with a poorly differentiated tumor. Out of 12 patients negative for both HPV 16 and 18, 50% presented with a well differentiated tumor, 33% with a moderately differentiated tumor and 17% with a poorly differentiated carcinoma. (Fig.18).

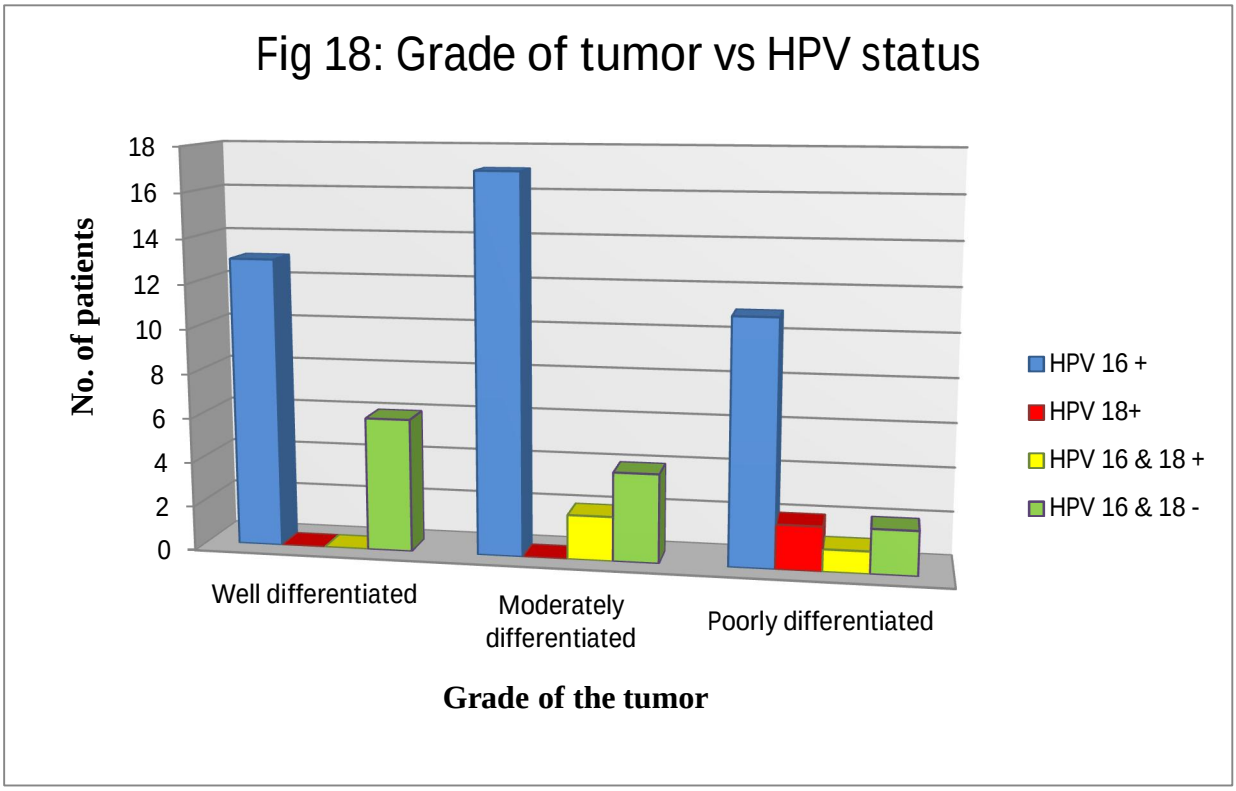
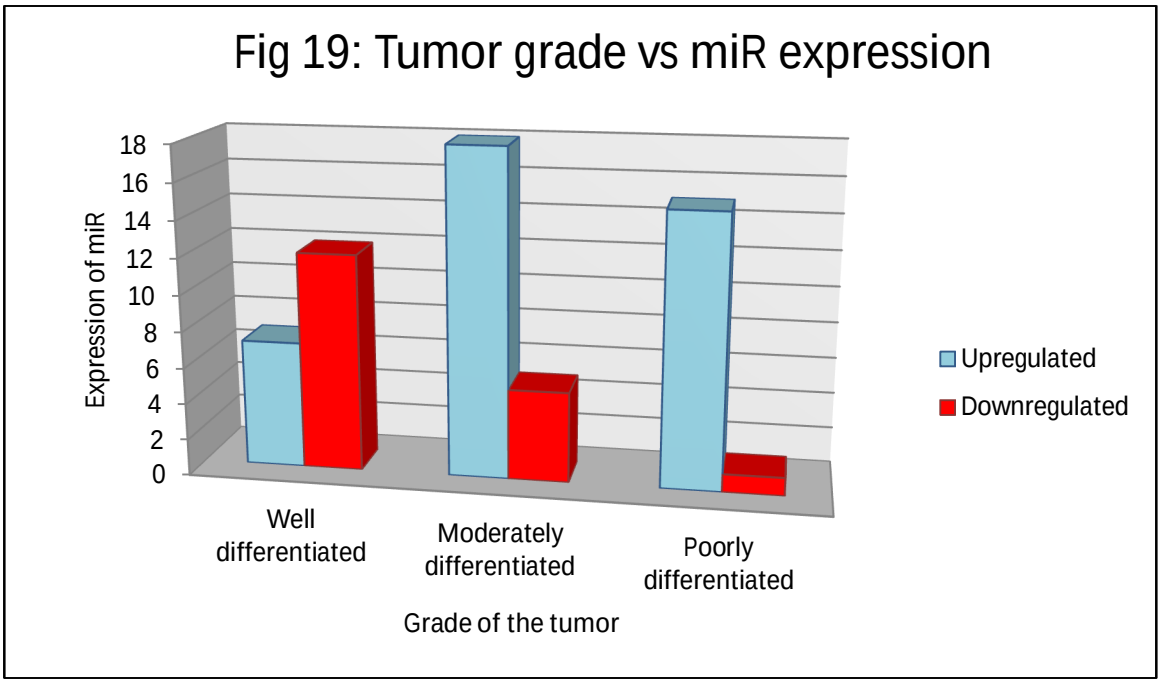


Table 3: Grade of the tumor vs miR196a-5p expression

Grade of the tumor	miR196a-5p expression	
	Upregulated	Downregulated
Well differentiated	7 (18%)	12 (67%)
Moderately differentiated	18 (45%)	5 (28%)
Poorly differentiated	15 (37%)	1 (5%)
Total	40	18

The relative expression of miR196a-5p with respect to different grades of tumor is analysed and tabulated in Table 3. It is evident that out of 40 tumors which showed an upregulation of miR, 18% of them were well differentiated tumors, 45% of them were moderately differentiated and 37% were poorly differentiated. Among the tumors which showed a downregulation of miR196a-5p, well differentiated tumors formed 67% with moderately differentiated and poorly differentiated forming 28% and 5% respectively. (Fig.19) There is a significant positive association observed between the grade of the tumor and the expression of miR196a-5p with p value < 0.001 by Chi-square test.

The above data suggests that as the grade of the lesion progresses from a well differentiated tumor towards a poorly differentiated carcinoma, a significant upregulation of mir196a-5p is observed in the tumor samples.



HPV status vs miR196a-5p expression:

The mean expression of miR196a-5p in relation to different HPV status is analyzed in Table 4. The expression of miR196a-5p in patients was expressed as either upregulated or downregulated. Out of 40 cases which showed an upregulation of miR196a-5p, 75% of the cases were positive for HPV 16, 2.5% were positive for HPV 18, 7.5% were positive for both HPV 16 and 18 and 15% were negative for both HPV 16 and 18. Out of 18 cases which showed a downregulation of miR in the tumor samples, 61% of them were positive for HPV 16, 6% of them were positive for HPV 18 and 33% of them were negative for both HPV 16 and 18. (Fig.20) However, in our studies there was no statistically significant correlation found between the HPV status and the miR expression of the individual tumors.

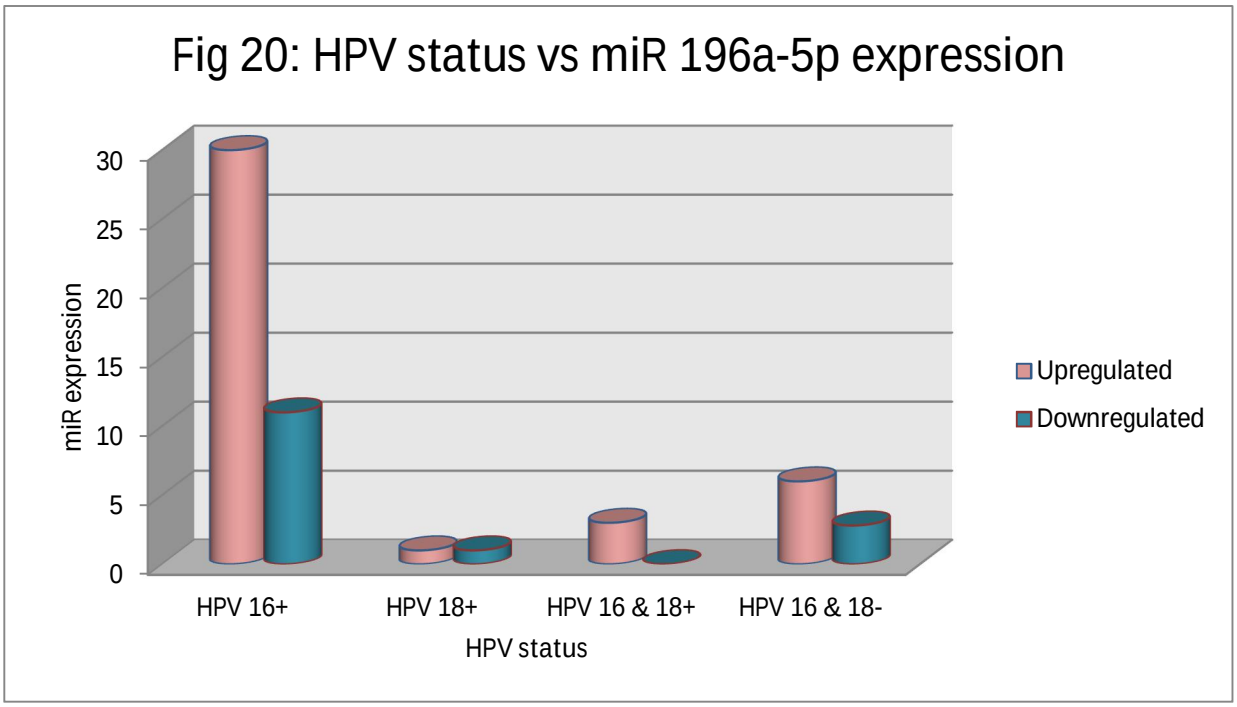


Table 4: HPV status vs miR196a-5p expression

HPV status	Expression of miR196a-5p in tumor samples		Total No. of HPV cases
	Upregulated	Downregulated	
HPV 16 +ve	30 (75%)	11 (61%)	41
HPV 18 +ve	1 (2.5%)	1 (6%)	2
HPV 16 & 18 +ve	3 (7.5%	0	3
HPV 16 & 18 -ve	6(15%)	6 (33%)	12
Total miR expression in HPV	40	18	58

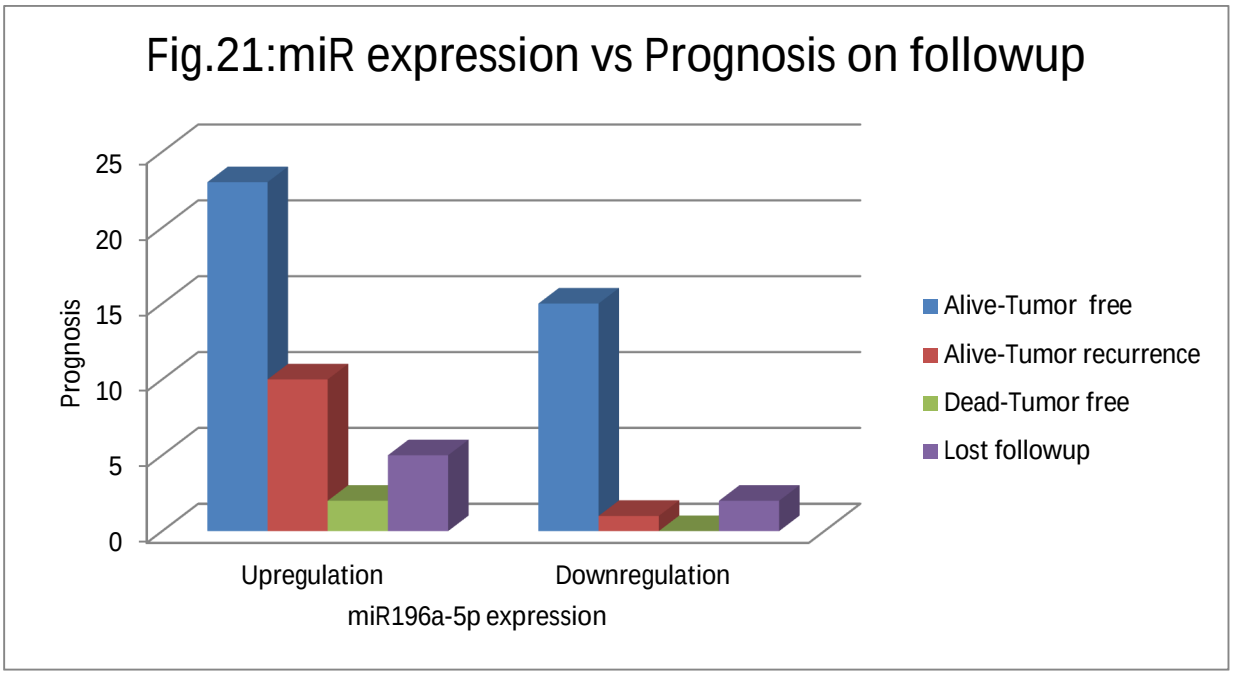
miR196a-5p expression vs Prognosis on followup:

The mean expression of miR196a-5p and its relationship with the prognosis of the disease on followup is analyzed in Table.5 and survival analysis was done by Kaplan Meier method. The median follow up period was 18 months (maximum – 53 months & minimum- 3 months). It is evident that out of 40 cases showing an upregulated expression of miR, 23 cases (57.5%) of the cases were alive & tumor free after treatment, 10 cases (25%) were alive with tumor recurrence, 2 cases (5%) were dead due to non tumor causes and 5 cases (12.5%) were lost for followup. Out of the 18 cases that showed a downregulation of expression of miR196a-5p, 15 cases (83.3%) were alive & tumor

free after treatment, 1 case (5.6%) had a tumor recurrence and 2 cases (11.1%) were lost for followup. (Fig.21)

Table 5: miR expression vs prognosis on followup

miR 196a-5p expression	Prognosis of patients on followup				Total patients
	Alive - Tumor free	Alive-Tumor recurrence	Dead- Tumor free	Lost for followup	
Upregulated	23 (57.5%)	10 (25%)	2 (5%)	5 (12.5%)	40
Downregulated	15 (83.3%)	1 (5.6%)	0	2 (11.1%)	18



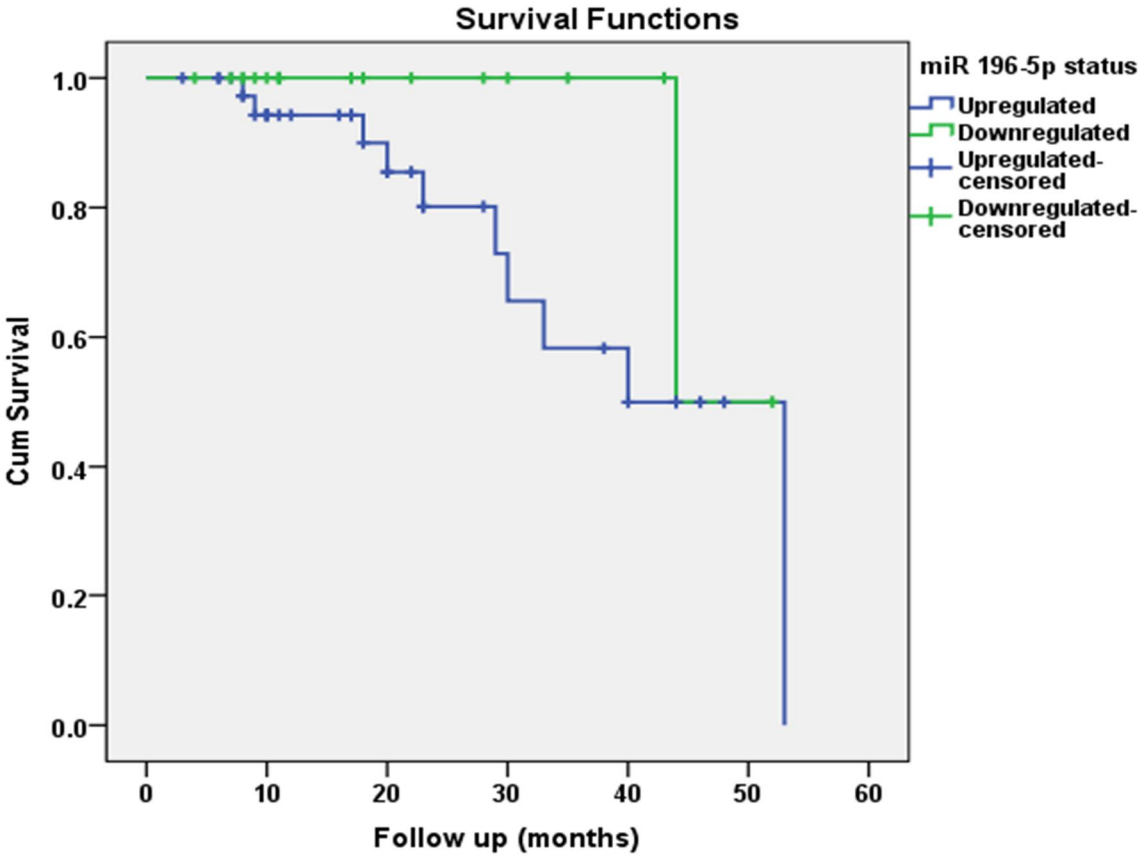
Kaplan-Meier survival analysis

Table 6: Case Processing Summary				
Micro RNA 196a - 5p Expression	Total N	N of Events	Censored	
			N	Percent
Upregulated	40	10	30	75.0%
Downregulated	18	1	17	94.4%
Overall	58	11	47	81.0%

Table 7: Means for Survival Time

MicroRNA Expression	Mean			
	Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Upregulated	39.902	3.527	32.989	46.814
Downregulated	48.000	2.828	42.456	53.544
Overall	42.398	2.834	36.843	47.952

Fig 22: Kaplan-Meier survival analysis



From the observed data, though there was an increase in the recurrence rate with cancers showing an upregulated expression of miR196a-5p compared to that of those with a downregulated expression, there was no statistically significant correlation observed between the miR expression and the prognosis on followup as determined by Log Rank test ($p = 0.177$).

Discussion

DISCUSSION

Carcinoma of cervix has been the most common tumor and is the one of the major cause of mortality and morbidity in the female population. Human papilloma virus has been implicated in the causation of carcinoma cervix and its relationship to the tumor has been a field of extensive research in unraveling the possible mechanisms of oncogenesis. Though various mechanisms have been postulated in HPV carcinogenesis, the exact mechanisms which govern the molecular sequence remains still unclear and less understood.

microRNAs are a group of endogenous non protein-coding RNAs that regulate various intracellular signaling mechanisms such as stem cell differentiation, organ development, cell death etc. are recently implicated with the causation of various cancers [59]. Due to an aberrant expression, these microRNAs function either as oncogenes or as tumor suppressor genes, suppressing or facilitating specific gene targets which could be either a proto-oncogene or a tumor suppressor gene. Hence upregulation or downregulation of various microRNAs are found to play a major role in the causation of various cancers. miR196a-5p is a member of the mir196 family, a redundant sequence in a genome, which is related to proliferation and migration of tumor cells by targeting downstream regulatory pathways.

This study was a pilot study to assess the feasibility of doing microRNA 196a-5p expression analysis in 58 patients with carcinoma cervix. Most studies on miRNAs

expression in carcinoma cervix are of small sample size based on cancer cell line cultures or few tissue samples. Also, the emphasis in these studies was to find out which miRNAs where upregulated or downregulated.

By this analysis, we aimed at correlating the expression of this novel microRNA 196a-5p and its relationship to the possible parameters like grade of the tumor, HPV status of the individual etc. that might help us in predicting the clinical behavior of the patient, response to chemotherapy or radiotherapy and probability of a disease-free survival period. This in future may help us prepare a commercially viable miRNAs Expression analysis (either single or multiple) similar to gene based testing in cancers (e.g. Oncotype DX and Mammaprint in breast cancer).

The methods of isolation of microRNA from tissue samples pose a great challenge from the time of collection to the extraction of sufficient quantity of microRNA 196a-5p from the particular tumor sample. Unlike DNA, RNA gets degraded rapidly when removed from the body. Furthermore, enzymes which degrade RNA called RNAases are found ubiquitously in the environment, that necessitates the immediate processing of samples and freezing the samples in liquid nitrogen to at least -20º Celsius, using solutions which degrade RNAses (e.g. RNAlater). The immediate processing and maintenance of cold chain until the extraction of microRNA of such large number of samples involves a larger cost and is time consuming.

To prevent RNA degradation, we used an RNAlater solution and extracted purified RNA with good quality bands in Gel photos in 58 patients. During the initial phase of the study, the extraction rate was only around 50 percent which improved during the second half of the study with careful avoidance of sample mishandling and early processing of samples.

Most studies use microarray technique to study their expression. However microarray technique, though high throughput, is not a quantitative method and has to be validated using other techniques like northern blot or PCR. We wanted to study miRNAs expressions in a larger number of tumor samples. So we opted to study the expressions of miRNA196a-5p based on our previous microarray data and available literature by quantitative RT PCR based amplification of micro RNA expression.

In our analysis, out of 41 patients with only HPV 16 positivity, 31% had a well differentiated tumor, 42% had a moderately differentiated tumor and 27% had a poorly differentiated tumor. All the patients in the HPV 18 positivity group presented with a poorly differentiated tumor. Among 3 patients who were positive for HPV 16 & 18, 67% presented with a moderately differentiated carcinoma and 33% with a poorly differentiated tumor. Out of 12 patients negative for both HPV 16 and 18, 50% presented with a well differentiated tumor, 33% with a moderately differentiated tumor and 17% with a poorly differentiated carcinoma.

Among 58 patients presenting with different stages of carcinoma cervix, 19 patients had a well differentiated squamous cell carcinoma (33%), 23 carcinoma cases (40%) had a moderately differentiated tumor and 16 patients (27%) with a poorly differentiated carcinoma. In our study, there was a significant positive correlation observed ($p<0.001$) between the grade of the tumor and the mir196a-5p expression which indicates that poorly differentiated carcinoma of cervix will show an upregulation in the expression of miR196-5p as compared to that of well differentiated tumors. Molecular researches by Zhang et al. (2013. loc ćit) and Hou et al. (2014. loc ćit) also reveal the role of upregulation of microRNA196a targets various downstream pathways which causes progression of tumor cells, correlating with the findings of our study. Similarly, Lu J et al. assayed microRNAs on poorly differentiated tumor samples with non-diagnostic histologic appearance and found out that microRNA expression by microarrays could establish an accurate diagnosis of poorly differentiated tumors which support the findings in our study [60]. This relationship of miR with the grade of the tumor explains the various intracellular regulatory mechanisms on which microRNAs play an important role that could cause the progression of the disease. Upregulation of mir196a-5p expression associated with poorly differentiated carcinomas suggest that mir-196a-5p could be used as a prognostic indicator.

The mean expression of miR196a-5p in relation to different HPV status were analyzed. The expression of miR196a-5p in patients was expressed as either upregulated or downregulated. Out of 40 cases which showed an upregulation of

miR196a-5p, 75% of the cases were positive for HPV 16, 2.5% were positive for HPV 18, 7.5% were positive for both HPV 16 and 18 and 15% were negative for both HPV 16 and 18. Out of 18 cases which showed a downregulation of miR in the tumor samples, 61% of them were positive for HPV 16, 6% of them were positive for HPV 18 and 33% of them were negative for both HPV 16 and 18. With respect to the miR expression in HPV positive tumors, though most of the tumors that are positive for HPV 16, 18 or both showed an upregulation of miR196a-5p as compared to that of tumors with negativity for both HPV 16 & 18, there was no statistical significance observed between these two parameters. This observation has been substantiated by a study done by Ruiz et al. (2014. loc ćit) on cancer cell lines suggesting the presence of specific HPV does not influence specific microRNA in cell lines, explaining the complexity and heterogeneity of microRNAs.

The mean expression of miR196a-5p and its relationship with the prognosis of the disease on followup was analyzed. It is evident that out of 40 cases showing an upregulated expression of miR, 27 cases (67.5%) of the cases became disease free after treatment, 6 cases (15%) had a recurrence of the disease, 2 cases (5%) were dead and 5 cases (12.5%) of the cases were lost for followup. Out of the 18 cases that showed a downregulation of expression of miR196a-5p, 12 cases (67%) became disease free after treatment, 1 case (6%) had a recurrence and 5 cases (27%) were lost for followup. Though about two third of the tumors which showed either an upregulation or downregulation of miR196a-5p expression were disease free on followup, there was no

statistical significance observed between the miR expression against the prognosis of the disease on followup. Since these patients were on a varied period on followup during the study, this statistical insignificance could be analyzed over a longer followup period.

MicroRNA 196a expression has been correlated with various tumors such as melanoma, oesophageal, laryngeal, pancreatic, lung and colorectal carcinomas. Most of these tumors show an upregulated or an overexpression of this novel microRNA. However in a few tumors there is a significant downregulation of miR196a which causes progression of the tumor. These controversial expressions were explained by various mechanisms, such as the heterogeneity of microRNA in these tumor cells with different target genes which are involved in embryonic development and cellular remodeling such as Homeobox genes (HOXC8, HOXA7, HOXD8, HOX A9 and HOXB8) and Actin and Keratin. The extreme complexity of miR196a regulating the various downstream gene targets as indicated previously in the Gene Ontology Chart (Fig.18) suggest that carcinoma cervix and its relation with microRNA is a highly complex event and the inability to ascertain a relationship between the HPV status and microRNA suggests that presence of fine molecular events which are yet to be unraveled.

Out of 58 patients representing a subset of female population in Chennai city with the diagnosis of carcinoma cervix, HPV positivity was observed in 46 patients (79%) and HPV negativity was observed in 12 patients (21%) as against the prevalence rate of 82.7% of HPV positivity in invasive cervical cancers in Indian women as reported

by Indian Human Papilloma virus & related cancers, Fact sheet – 2014 and more than 99% prevalence rate of HPV in western studies.

Hence further studies with a greater subset of population with carcinoma cervix would give us an idea about the correlating factors between HPV status, level of microRNA expression and the prognostic factors of the disease which would reveal the intricate molecular mechanisms involved in carcinogenesis.

Conclusion

CONCLUSIONS

Based on this study the following conclusions are made.

(1) The extraction and analysis of microRNAs from carcinoma cervix patients is feasible, provided proper care is ensured in transportation, storage and processing of the samples. Development of newer, simpler, more efficient, safer and cost effective techniques are needed for storage, transportation, extraction and analysis of microRNA.

(2) Altered expression of miR196a-5p has shown definite and significant positive correlation with the grade of the tumor. Upregulation of mir196a-5p expression associated with poorly differentiated carcinomas suggest that mir-196a-5p could be used as a prognostic indicator.

(3) There was no significant correlation observed between the grade of the tumor and the HPV status of the individual.

(4) There was no significant correlation between the HPV status and miR-196a-5p expression.

(5) There was no significant correlation observed between the prognosis of the disease with respect to the expression of miR196a-5p.

miR-196a-5p expression, if used in combination with other microRNAs with a high diagnostic relevance can be used as a potential marker in the diagnosis, prediction and prognostication of the disease.

Bibliography

BIBLIOGRAPHY

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: *IARC Cancer Base No. 10. Lyon, France: International Agency for Research on Cancer; 2010.*
2. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human Papilloma virus and cervical cancer. *Lancet. 2007. 8; 370(9590):890-907.*
3. Jeong-Won Lee, Chel Hun Choi, Jung-Joo Choi, et al. Altered microRNA expression in cervical carcinomas. *Clin Cancer Res 2008;14:2535-2542*
4. Bosch FX and de Sanjosé S, "Chapter 1: Human Papillomavirus and Cervical Cancer – Burden and Assessment of Causality". *Journal of the National Cancer Institute Monographs No. 31* (2003)
5. Wells M, Ostor AG et al. Tumours of the uterine cervix. In: Tavassoli FA, Devilee P (eds) Tumors of the breast and female genital organs. *IARC, Lyon, 2002. pp 260–286.*
6. Kumar V, Abbas A, Fausto N, Aster JC. Microbial carcinogenesis. *In: Robbins and Cortran Pathologic basis of disease, 2010; 8th edition; 516-524.*
7. Pecorelli S, Zigliani L et al. Revised FIGO staging for carcinoma of the cervix. *Int J Gynaecol Obstet. 2009;105(2):107–108*
8. Roman LD, Felix JC, Muderspach LI, Agahjanian A, Qian D, Morrow CP. Risk of residual invasive disease in women with microinvasive squamous cancer in a conisation specimen. *Obstet Gynecol 1997;90:759-764*

9. Elliott P, Coppleson M, Russell P, Liouros P, Carter J. Macleod C et al. Early invasive (FIGO Stage IA) carcinoma of the cervix: a clinicopathologic study of 476 cases. *Int J Gynecol Cancer 2000;10:42-52*
10. Landoni F, Maneo A, Colombo A, et al: Randomized study of radical surgery versus radiotherapy for stage IB-IIA cervical cancer. *Lancet 1997; 350:535-40.*
11. Peters III WA, Liu PY, Barrett II RJ et al: Concurrent chemotherapy and pelvic radiation therapy compared with pelvic radiation therapy alone as adjuvant therapy after radical surgery in high-risk early-stage cancer of the cervix. *J Clin Oncol 2000; 18(8):1606-13.*
12. Sedlis A, Bundy BN, Rotman M, Lentz S, Muderspach LI, Zaino R. A randomized trial of pelvic radiation versus no further therapy in selected patients with Stage IB carcinoma of the cervix after radical hysterectomy and pelvic lymphadenectomy: a Gynecologic Oncology Group Study. *Gynecol Oncol 1999; 73:177-183*
13. Rose PG, Bundy BN, Watkins ET, Thigpen T, Deppe G, Maiman MA et al: Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. *N Eng J Med 1999; 340:1144-53*.
14. Boronow RC. The bulky 6-cm barrel-shaped lesion of the cervix: primary surgery and postoperative chemotherapy. *Gynecol Oncol 2000; 78:313-317*
15. Stewart LA, Tierney JF. Neoadjuvant chemotherapy and surgery versus standard radiotherapy for locally advanced cervix cancer. A metaanalysis using individual patient data from randomized controlled trials. *Int J Gynecol Cancer 2002; 12:579 (abst) 22.*

16. Whitney CW, Sause W, Bundy BN et al. Randomized comparison of fluorouracil plus cisplatin vs. hydroxyurea as an adjunct to radiation therapy in stage IIB-IVA carcinoma of the cervix with negative paraaortic lymph nodes: A Gynecologic Oncology Group and Southwest Oncology Group study. *J Clin Oncol 1999; 17:1339-48*.
17. Morris M, Eifel PJ, Lu J, Grigsby PW, Levenback C, Stevens RE et al. Pelvic radiation with concurrent chemotherapy compared to pelvic and para aortic radiation for high-risk cervical cancer. *N Engl J Med 1999; 340:1137-43*
18. Bartel,D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell 2004; 116, 281–297*.
19. Filipowicz,W., Jaskiewicz,L., Kolb,F.A. and Pillai,R.S. Post-transcriptional gene silencing by siRNAs and miRNAs. *Curr. Opin. Struct. Biol.2005; 15, 331–341*.
20. Sontheimer,E.J. and Carthew,R.W. Silence from within: endogenous siRNAs and miRNAs. *Cell 2005;122, 9–12*.
21. Ambros,V. The functions of animal microRNAs. *Nature 2004; 431, 350–355*.
22. Kidner,C.A. and Martienssen,R.A. The developmental role of microRNA in plants. *Curr. Opin. Plant Biol.2005; 8, 38–44*.
23. Lee,R.C. and Ambros,V. An extensive class of small RNAs in Caenorhabditis elegans. *Science 2001; 294, 862–864*.
24. The Editors. Thematic Synopsis: MicroRNAs. *Circ Res. 2013; 113: 30-41*.

25. Benes V, Castoldi M. Expression profiling of microRNA using real time quantitative PCR, how to use it and what is available. *Methods 2010; 50:244-249.*
26. Dutta A, Lee YS. MicroRNAs in cancer. *Ann Rev Path Mech Dis 2009; 4:175.*
27. Calin GA, Croce CM. MicroRNA-Cancer connection: The beginning of a new tale. *Cancer Res 2006; 66:7390-7394.*
28. Calin GA, Sevignani C, Dumitru CD et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA 2004; 10:2999-3004.*
29. Lee KS. Non coding RNAs and cancer: New paradigms in oncology. *Discov Med 2011; 11(58):245-254.*
30. Torres AP, Urrutia EL, Castillo VG et al. MicroRNAs in cervical cancer: Evidences for a miRNA profile degreulated by HPV and its impact on radio-resistance. *Molecules 2014; 19:6263-6281*.
31. Banno K, Iida M, Yanokura M et al. MicroRNA in cervical cancer. OncomiRs and tumor suppressor miRs in diagnosis and treatment. *The Scientific World Journal 2014; 178075:1-8.*
32. Reshmi G, Pillai MR. Beyond HPV: Oncomirs as new players in cervical cancer. *Federation of Eur Biochem Societies Letters 2008; 582 (30): 4113-4116.*
33. Torres A, Torres K, Maciejewski R, Harvey WH. Micro-RNAs and their role in gynecological tumors. *Med Res Rev 2011; 31(6):895-923.*

34. Gocze K, Gombos K, Juhasz K et al. Unique microRNA expression profiles in cervical cancer. *Anticanc Res 2013; 33 (6):2561-2567.*
35. Lui WO, Pourmand N, Patterson BK et al. Patterns of known and novel small RNAs in human cervical cancer. *Cancer Res 2007; 67: 6031-6043.*
36. Wang X, Tang S, Le SY et al. Aberrant expression of oncogenic and tumor suppressive microRNAs in cervical cancer is required for cancer cell growth. *PLoS ONE 2008. e2557; 3 (7): 1-11*.
37. Martinez I, Gardiner AS, Board KF, Monzon FA, Edwards RP, Khan SA. Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. *Oncogene 2008; 27, 2575–2582.*
38. Lee JW, Choi CH, Choi JJ et al. Altered microRNA expression in cervical carcinomas. *Clin Cancer Res 2008; 14: 2535-2542*.
39. Yang Z, Chen S, Luan X, Li Y et al. MicroRNA-214 is aberrantly expressed in cervical cancers and inhibits the growth of HeLa cells. *IUBMB Life 2009; 61(11): 1075–1082.*
40. Wilting SM, van Boerdonk RAA, Henken FE et al. Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. *Mol Cancer 2010; 9:167.*
41. Wang X, Wang HK, Mccoy JP, Banerjee NS et al. Oncogenic HPV infection interrupts the expression of tumor-suppressive miR-34a through viral oncoprotein E6. *RNA 2009; 15:637–647*

42. Liu L,Yu X, Guo X, Tian Z, Su M et al. miR-143 is downregulated in cervical cancer and promotes apoptosis and inhibits tumor formation by targeting bcl-2. *Mol Med Reports 2012; 5: 753-760*
43. Cui F, Lib X, Zhu X, Huang L et al. miR-125b inhibits tumor growth and promotes apoptosis of cervical cancer cells by targeting phosphoinositide 3-kinase catalytic subunit delta. *Cell Physiol Biochem 2012; 30:1310-1318.*
44. Wang F, Liu M, Li X, Tang H. miR-214 reduces cell survival and enhances cisplatin-induced cytotoxicity via down-regulation of Bcl2 in cervical cancer cells. *Federation of Eur Biochem Societies Letters 2013; 587: 488-495*.
45. Liu S, Zhang P, Chen Z, Liu M, Li X,Tang H. MicroRNA-7 downregulates XIAP expression to suppress cell growth and promote apoptosis in cervical cancer cells. *Federation of Eur Biochem Societies Letters 2013, 587: 2247-2253*.
46. Huang F, Lin C, Shi YH, Kuerban G. MicroRNA-101 inhibits cell proliferation, invasion, and promotes apoptosis by regulating cyclooxygenase-2 in Hela cervical carcinoma cells. *Asian Pac J Cancer Prev 2013; 14 (10), 5915-5920.*
47. Li J, Ping Z, Ning H. miR-218 impairs tumor growth and increases chemo-sensitivity to cisplatin in cervical cancer. *Int. J. Mol. Sci. 2012; 13, 16053-16064*.
48. Wei Q, Li YX, Liu M, Li X, Tang H. miR-17-5p targets TP53INP1 and regulates cell proliferation and apoptosis of cervical cancer cells. *IUBMB Life 2012; 64(8): 697–704.*

49. Kang HW, Wang F, Wei Q, Zhao YF, Liu M, Li X, Tang H. miR-20a promotes migration and invasion by regulating TNKS2 in human cervical cancer cells. *Federation of Eur Biochem Societies Letters 2012; 586: 897-904.*
50. Ma Q, Wan G, Wang S, Yang W, Zhang J, Yao X. Serum microRNA-205 as a novel biomarker for cervical cancer patients. *Cancer Cell International 2014; 14(81). 1-7.*
51. Wang WT, Zhao YN, Yan JX, Weng MY, Wang Y, Chen YQ, Hong SJ. Differentially expressed microRNAs in the serum of cervical squamous cell carcinoma patients before and after surgery. *J of Hemat & Onco 2014; 7(6): 1-10*.
52. Lagos-Quintana M, Rauhat R, Meyer J, Borkhardt A, Tuschl T. New microRNAs from mouse and human. RNA 2003; 9:175-179.
53. Yu H et al. Evolution of coding and non-coding genes in HOX clusters of a marsupial. BMC Genomics 2012; 13:251.
54. Mueller DW, Bosserhoff AK. MicroRNA miR196a controls melanoma-associated genes by regulating HOX-C8 expression. *Int J Cancer 2010; 129:1064-1074.*
55. Schimanski et al. High miR-196a promote the oncogenic phenotype of colorectal cancer cells. *World J of Gastroenterology 2009; 15(17): 2089*.
56. Ruiz VV et al. Heterogeneity of microRNA expression in cervical cancer cells: overexpression of miR196a. *Int J Clin Exp Pathol 2014; 7(4): 1389-1401*.

57. Zhang J, Zheng F, Yu G, Yin Y, Lu Q. miR196a targets netrin-4 and regulates cell proliferation and migration of cervical cancer cells. *Biochem Biophys Res Commun 2013; 440(4): 582-588*.

58. Hou t, Ou J, Zhao X, Huang X, Huang Y, Zhang Y. MicroRNA 196a promotes cervical cancer proliferation through regulation of FOXO1 and p27Kip1. *Br J Cancer 2014; 110(5): 1260-1268*.

59. Yin JQ, Zhao RC, Morris KV. Profiling microRNA expression with microarrays. *Trends in Biotech 2007: 26 (2): 70-76.*

60. Lu J et al. MicroRNA expression profiles classify human cancers. *Nature 2005; 435: 834-838.*

Annexure

PATIENT PROFORMA

Name: Age: Sex: CD/COP No:

Performance status: Height: Weight: BSA:

Clinical features:

Imaging Report: HPE:

Stage (FIGO): IA1 / IA2 / IB1 / IB2 / IIA1 / IIA2 / IIB / IIIA / III B / IV

Tumor grade: Well / Moderately / Poorly / Undifferentiated / Unknown

Samples collected:

1. At diagnosis: Blood / Tumor tissue / Normal tissue Date:
2. Post Chemo/RT: Blood / Tumor tissue / Normal tissue Date:
3. At surgery: Blood / Tumor tissue / Normal tissue Date:

Treatment given:

1. Surgery
2. Radiotherapy
3. Chemoradiation
4. Chemotherapy

Follow up Record:

Date	Follow-up period	Status at followup (Alive no disease/ Alive with disease/ Distant metastases/ Death/ LOFU)

Written Informed Consent

I …… hereby agree to participate in the study on microRNA expression profiles in carcinoma cervix and agree to provide my Tumor/Blood samples. I have been clearly explained about the procedure and have been explained well about the study in my own language. I have also been told that I could withdraw from the study at any time and I hereby declare that all my questions have been clarified.

Date: Signature / Thumb impression

INSTITUTIONAL ETHICAL COMMITTEE
GOVT.KILPAUK MEDICAL COLLEGE,
CHENNAI-10
Ref.No.12117/ME-1/Ethics/2012 Dt:03.01.2013.
CERTIFICATE OF APPROVAL

The Institutional Ethical Committee of Govt. Kilpauk Medical College, Chennai reviewed and discussed the application for approval "A study on Role of microRNA Expression Profiles in the Diagnosis and Prognosis of cervical cancers" for dissertation purpose submitted by Dr.V.S.Ajay Chandrasekar, MCh (Surgical Oncology), PG Student, Govt. Kilpauk Medical College, Chennai.

The Proposal is APPROVED.

The Institutional Ethical Committee expects to be informed about the progress of the study any Adverse Drug Reaction Occurring in the Course of the study any change in the protocol and patient information /informed consent and asks to be provided a copy of the final report.

21/1/13.

CHAIRMAN
Ethical Committee
Govt.Kilpauk Medical College,Chennai

Master chart

S.No	Name	Age	Sex	Patient ID	FIGO stage	Grade	Treatment given	HPV 16	HPV 18	miR 196-5p status	Status	Followup (Months)
1	VARATHAMMAL	58	F	156/11	IIB	MD	Chemoradiation	-	-	Upregulated	Alive -Tumor free	44
2	VADIVAMBAL	66	F	295/11	IIIB	PD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	44
3	ADHILAKSHMI	75	F	383/10	IIIB	WD	Chemoradiation	+	-	Upregulated	Alive-Recurrence	53
4	JOTHI	48	F	348/11	IIB	PD	Chemoradiation	-	+	Downregulated	Alive -Tumor free	43
5	PARAMESHWARI	38	F	311/11	IIB	WD	Chemoradiation	+	-	Downregulated	Alive-Recurrence	44
6	MARAGHADAM	65	F	018/11	IIIB	MD	Chemoradiation	-	-	Upregulated	Alive -Tumor free	46
7	LAKSHMI	65	F	525/12	IIIB	MD	Chemoradiation	-	-	Downregulated	Lost follow up	11
8	VELLAKARCHI	65	F	117/12	IIB	MD	Chemoradiation	+	-	Upregulated	Alive-Recurrence	33
9	SHAKUNTHALA	48	F	1195/11	IIB	MD	Chemoradiation	+	-	Upregulated	Alive-Recurrence	40
10	LAKSHMI	50	F	1330/10	IIB	MD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	48
11	CHINNAKULANTHAI	72	F	1773/12	IIIB	MD	Chemoradiation	+	-	Upregulated	Alive-Recurrence	30
12	PAPPA	57	F	681/10	IB1	WD	Surgery	+	-	Downregulated	Alive -Tumor free	52
13	VASANTHA	48	F	854/12	IIB	WD	Chemoradiation	-	-	Downregulated	Alive -Tumor free	35
14	AMARAVATHY	75	F	1125/12	IB1	WD	Surgery	+	-	Downregulated	Alive -Tumor free	30
15	BELA	45	F	1178/11	IB2	WD	Chemotherapy + Surgery	+	-	Upregulated	Alive -Tumor free	40
16	SENTHAMARAI	56	F	1162/11	IIIB	MD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	38
17	JAGDEESHWARI	30	F	1129/12	IIB	MD	Chemotherapy + Surgery	+	+	Upregulated	Dead – tumor free	16
18	RAJALAKSHMI	74	F	1178/12	IIIB	MD	Chemoradiation	+	-	Upregulated	Lost follow up	12
19	INBAVALLI	40	F	186/13	IIIB	PD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	23
20	SARASWATHY	48	F	243/13	IIIB	PD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	23
21	UNNAMALAI	45	F	159/13	IIIB	PD	Chemoradiation	+	-	Upregulated	Lost follow up	10
22	RAJAMMA	50	F	1162/12	IIB	WD	Chemo + Surgery	+	-	Downregulated	Alive -Tumor free	28
23	SELVI	45	F	580/13	IIIB	WD	Chemoradiation	+	-	Upregulated	Alive-Recurrence	18
24	CHINNA PONNU	50	F	630/13	IIB	MD	Chemotherapy + Surgery	+	-	Downregulated	Alive -Tumor free	22
25	DEVI	33	F	1349/12	IIB	MD	Chemotherapy + Surgery	+	-	Upregulated	Alive-Recurrence	29
26	ESHWARI	30	F	1127/12	IIB	MD	Chemotherapy + Surgery	+	+	Upregulated	Alive -Tumor free	23
27	RENUKA	37	F	1135/12	IIIB	WD	Chemoradiation	+	-	Up regulated	Alive -Tumor free	28
28	SUGHANTHINI	53	F	823/13	IIB	WD	Chemoradiation	+	-	Up regulated	Alive-Recurrence	20

WD – well differentiated, MD – moderately differentiated , PD – poorly differentiated

Master chart

29	MARI	67	F	836/13	IV A	PD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	20
30	PRABAVATHY	50	F	813/13	IIIB	MD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	22
31	MARY	40	F	1352/13	IIIB	PD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	17
32	KUPPU	40	F	1024/13	IIB	MD	Chemoradiation	+	-	Downregulated	Alive -Tumor free	18
33	AMARAVATHY	65	F	742/13	IIIB	MD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	20
34	SARASU	45	F	155/13	IIB	PD	Chemoradiation	+	+	Upregulated	Alive-Recurrence	23
35	RANI	65	F	493/13	IIIB	PD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	18
36	BAKKIYAM	60	F	1526/13	IB2	WD	Chemoradiation	+	-	Downregulated	Alive -Tumor free	17
37	PAPAMMAL	60	F	149/14	IIIB	MD	Chemoradiation	+	-	Upregulated	Lost follow up	11
38	SIVAMMAL	45	F	60/14	IB2	WD	Chemotherapy + surgery	+	-	Downregulated	Alive -Tumor free	11
39	SUMATHY	62	F	25/13	IB2	WD	Chemotherapy + surgery	-	-	Downregulated	Alive -Tumor free	10
40	LALITHA	60	F	615/14	IIIB	PD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	10
41	BANU	60	F	1068/13	IIIB	MD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	17
42	MUNIAMMAL	56	F	378/14	IIB	WD	Chemotherapy + surgery	+	-	Downregulated	Lost follow up	11
43	PERIAMMAL	40	F	983/14	IB2	WD	Chemotherapy + surgery	+	-	Downregulated	Alive -Tumor free	9
44	POONGAVANAM	45	F	1098/14	IIIB	MD	Chemoradiation	+	-	Upregulated	Lost follow up	3
45	SRIMATHY	35	F	1078/14	IB1	WD	Surgery	-	-	Downregulated	Alive -Tumor free	8
46	KOTTI	38	F	739/14	IIB	PD	Chemotherapy + Surgery	-	+	Upregulated	Alive -Tumor free	10
47	ANJALAI	50	F	1211/14	IIIB	PD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	8
48	SAROJA	65	F	1394/14	IIB	MD	Chemoradiation	+	-	Downregulated	Alive -Tumor free	7
49	RUKSHANA	72	F	780/14	IIA	MD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	9
50	ROSE	60	F	1273/14	IIIB	WD	Chemoradiation	-	-	Upregulated	Alive-Recurrence	8
51	SAROJINI	45	F	1262/14	IB1	MD	Surgery	+	-	Upregulated	Alive -Tumor free	8
52	POORANI	60	F	2117/14	IIIB	MD	Chemoradiation	-	-	Downregulated	Alive -Tumor free	4
53	RAJAKUMARI	50	F	603/14	IVB	PD	Chemotherapy	-	-	Upregulated	Alive-Recurrence	9
54	KAMATCHI	37	F	850/14	IIIB	PD	Chemoradiation	+	-	Upregulated	Alive -Tumor free	10
55	ARUNTHATHI	67	F	1649/14	IV B	PD	Chemotherapy	-	-	Upregulated	Dead-Tumor free	6
56	MALLIGA	50	F	1589/14	IIB	WD	Chemoradiation	-	-	Upregulated	Lost follow up	7
57	SARASWATHY	41	F	1393/14	IIA2	WD	chemoradiation	-	-	Downregulated	Alive -Tumor free	8
58	VALLIAMMAL	60	F	1637/14	IIIB	PD	chemoradiation	+	-	Upregulated	Alive -Tumor free	6

WD – well differentiated, MD – moderately differentiated , PD – poorly differentiated

Plagiarism

A Dissertation on

CLINICAL ROLE OF MICRO RNA (miRNA) 196a-5p EXPRESSION IN SQUAMOUS CELL CARCINOMA OF UTERINE CERVIX

Submitted to

The TamilNadu Dr.M.G.R Medical University

in partial fulfillment of the requirement

for the award of degree of

M.Ch (SURGICAL ONCOLOGY)

BRANCH VII

KILPAUK MEDICAL COLLEGE

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY

CHENNAI, TAMILNADU

AUGUST 2015

https://turnitin.com/s_class_portfolio.asp?r=81.1224317877748&svr=04&lang=en_us&aid=80345&cid=8539677

181217001. Mch (Surgical Oncology) AJAY CHANDRASEKAR V S | User Info | Messages | Student | English | Help | Logout

turnitin

Class Portfolio | Peer Review | My Grades | Discussion | Calendar

NOW VIEWING: HOME > THE TAMIL NADU DR.M.G.R.MEDICAL UTY 2014-15 EXAMINATIONS

Welcome to your new class homepage! From the class homepage you can see all your assignments for your class, view additional assignment information, submit your work, and access feedback for your papers.

Hover on any item in the class homepage for more information.

Class Homepage

This is your class homepage. To submit to an assignment click on the "Submit" button to the right of the assignment name. If the Submit button is grayed out, no submissions can be made to the assignment. If resubmissions are allowed the submit button will read "Resubmit" after you make your first submission to the assignment. To view the paper you have submitted, click the "View" button. Once the assignment's post date has passed, you will also be able to view the feedback left on your paper by clicking the "View" button.

Assignment Inbox: The Tamil Nadu Dr.M.G.R.Medical Uty 2014-15 Examinations

	Info	Dates	Similarity	
TNMGRMU EXAMINATIONS	ⓘ	Start 01-Sep-2014 11:27AM Due 15-Aug-2015 11:59PM Post 15-Aug-2015 12:00AM	17%	Resubmit View