A Dissertation on

CLINICAL ROLE OF MICRO RNA (miRNA) EXPRESSION PROFILES IN ORAL SQUAMOUS CELL CARCINOMAS

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BONAFIDE CERTIFICATE

This is to certify that **Dr. K.S. Rajkumar,** bonafide student of M.Ch. Surgical Oncology (August 2011 to August 2014) in the Department of Surgical Oncology, Government Royapettah Hospital, Chennai – 600 014 has done this dissertation on "**Clinical Role of Micro RNA (miRNA) Expression Profiles in Oral Squamous Cell Carcinomas"** 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 2014.

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DECLARATION

I solemnly declare that the dissertation titled "**Clinical Role of Micro RNA (miRNA) Expression Profiles in Oral Squamous Cell Carcinomas"** was done by me at Department of Surgical Oncology, Kilpauk Medical College and Government Royapettah Hospital, Chennai between August 2011 to February 2014 under the guidance and supervision of **Prof. R. Rajaraman MS MCh**. The Dissertation is submitted to **The Tamil Nadu Dr. M.G.R. Medical University** towards the partial fulfillment for the award of **MCh (Branch VII) in Surgical Oncology** to be held in August 2014.

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Date

This is my prayer to thee, my lord--strike,

strike at the root of penury in my heart.

Give me the strength lightly to bear my joys and sorrows.

Give me the strength to make my love fruitful in service.

Give me the strength never to disown the poor

or bend my knees before insolent might.

Give me the strength to raise my mind high above daily trifles.

And give me the strength to surrender my strength to thy will with love.

- ***Gitanjali - Rabindra Nath Tagore (1861 – 1941)***

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Finally nothing is possible without the blessings of the omnipotent ***Almighty.***

*"Je le pansai, Dieu le guérit" -****Ambroise Paré*** *(c. 1510 – 1590)*

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INTRODUCTION

Head and Neck Squamous Cell Carcinoma (HNSCC) include squamous epithelial cancers of the upper aerodigestive tract. HNSCCS represent the sixth most common cancer in the world, and accounts for more than 500,000 new cases annually [1]. HNSCC, especially oral cancers have a multifaceted aetiology. A plethora of lifestyle, environmental, viral and genetic factors has been identified as the risk factor for oral cancers. However, smoking, tobacco chewing, and alcohol consumption are widely considered to be major preventable risk factors. [2, 3]

Effective treatment regimens with advanced surgical procedures and new radiotherapy techniques have resulted in improvements in the survival and quality of life of oral cancer patients. However, overall 5 year survival is still around sixty percent. Most patients present with advanced disease and hence poor prognosis. [4]

The inherent genetic and phenotypic heterogeneity and scarcity of good molecular targets have hampered the development of targeted agents for HNSCC. A deeper understanding of the genetic factors and molecular mechanisms of carcinogenesis involved in HNSCC is required for the discovery of new bio markers that can be used for early detection and treatment. This is especially important in the present era where the emphasis is on early detection, prevention and organ-preserving treatment protocols. [5]

A number of high quality genomics-based researches in the recent times have unravelled different types of genetic alterations associated with these tumours. Also, new, innovative and highly accurate technologies have led to the detection of new biomarkers in HNSCC.

A new class of small non coding RNAs termed as microRNAs (miRNAs) have recently been identified as potential biomarkers in various cancers. These miRNAs are endogenous, small, non coding RNAs of 17–25 nucleotides size. They are thought to be involved in many essential biological functions of the cell like cellular differentiation, proliferation, development, apoptosis, cell cycle regulation, etc. by regulating the genes at the post transcriptional level. MiRNAs may play a crucial role in tumourigenesis by differential expression in cancer cells when compared with

noncancerous normal cells. Studies about the role of miRNAs' varied expressions in the diagnosis and prognosis of cancers have been reported. Few of these studies have shown strong clinical benefits of using miRNA expression profiles to warrant further investigation. [6]

Microarray analysis is the most prevalent technique of miRNA expression profiling. Consistently altered miRNA expressions (up/down regulation) have been demonstrated by various groups in oral squamous cell carcinomas. The aim of such expressional analysis studies is to identify potential miRNA candidates that may aid in the diagnosis, treatment and predict clinical outcomes of oral cancers. [6]

In this study, we intended to analyze the possible clinical roles that miRNA expression profiles may play in oral cancer patients.

REVIEW OF LITERATURE

Oral cancers – Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) includes epithelial cancers of the upper aerodigestive tract and ranks as the sixth most common cancer in the world. It accounts for more than 500000 new cases annually [1].The majority of oral cavity cancers (approx. 90%) are squamous cell carcinomas (SCC). The oral cavity includes the following sub sites (Figure 1):

- Anterior two thirds of the tongue
- Gingiva and alveolus
- The buccal mucosa
- Floor of the mouth under the tongue
- Hard palate
- Retro molar
- Upper and Lower Lips

Signs and Symptoms of oral cancers include:

- Pre malignant lesions:
  - White patches (leukoplakia)
  - Mixed red and white patches (erythroleukoplakia)
  - Red patches (erythroplakia)
- Ulcers
- Lumps
- Bleeding
- Loose teeth
- Dysphagia/ Odynophagia
- Trismus
- Akyloglossia
- Neck swelling
- Earache

Figure 1: Oral Cavity Sub sites

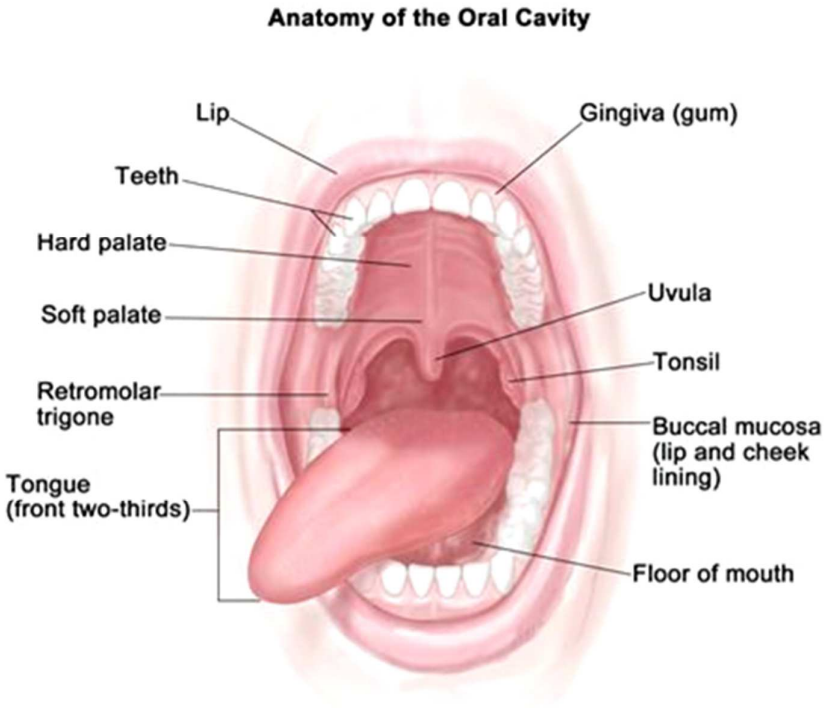
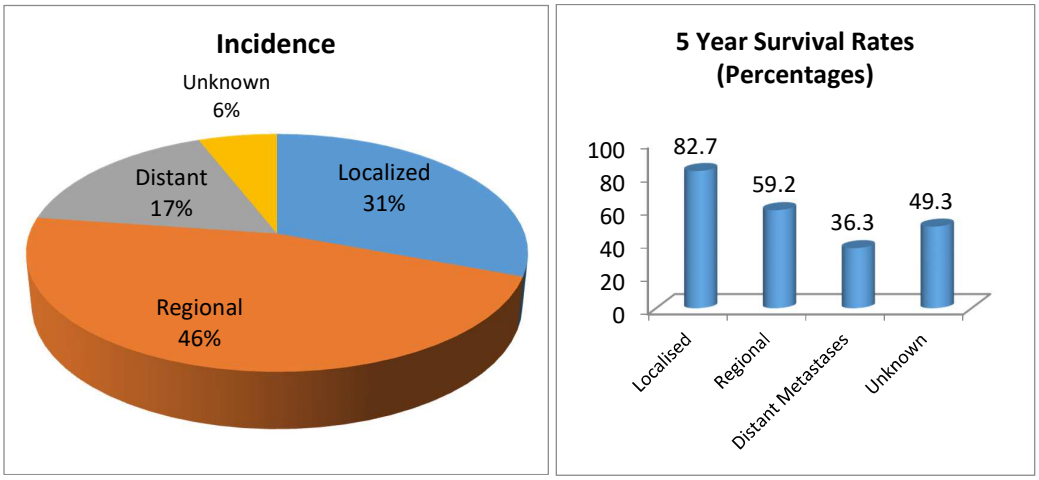


Figure 2: Incidence and 5 Year Survival of Oral Cancers (SEER Statistics)



Epidemiology of Oral Cancers:

Head and Neck cancers, especially oral cancers are a one of the most common cancers by incidence and cancer related mortality. An estimated 263,900 new oral cavity cancer (including lip cancer) cases and 128,000 deaths occurred worldwide in 2008. [1] The incidences of head and neck cancers are higher in developing countries than in developed countries. Oral cancers are the second leading cancer by incidence and cancer related mortality in South Central Asia (including India). The highest incidences of oral cavity cancer are in South-Central Asia, Central and Eastern Europe. The incidence is lowest in Africa, Central America and Eastern Asia. [2]

The increase in incidence of oral cancers among females in the west in recent years probably reflects the consequences of the upsurge in the tobacco epidemic. On the other hand, there is a decrease in oral cavity cancer trends for all ages and both sexes in places where the tobacco epidemic ended prematurely. Oral cancer is a disease of the elderly and most of the cases of occur in 50 - 70 years age group. Recently, there has been a shift towards younger age at diagnosis; about 17% of patients are below 40 years. Considering all the age groups, men are more affected than women. [2]

The commonest sub site depends on the predominant risk factors in that particular geographical region. The incidence of oral cancer sites related to HPV infections are increasing in young adults in the United States and Europe due to changes in sexual behaviour. [1] In South East Asia, Japan, India and Iran tongue is the common sub site (up to 42%) of all oral cancers of all ages. In India, tongue and buccal mucosa are the most common sub sites. Buccal mucosa cancers are also common in Pakistan and Taiwan. Lip cancers are less common in Asian countries. Tongue is also the commonest sub site in young adults (45 years or below). Older people have tendency to develop cancer of buccal mucosa. [2]

Overall 5 year survival rates vary between 37 and 62 percent in oral cancers. Oral cavity cancer death rates have shown varied trends across the globe with a decline in Asian and European males but increase in some eastern European countries and females.

Risk Factors:

Smoking, smokeless tobacco products (quid), alcohol and HPV infections are the major risk factors for oral cavity cancer. Smoking and alcohol have shown to have synergistic effects. Globally smoking (42 %) and heavy alcohol consumption (16%) account for majority of deaths due to oral and pharyngeal cancers. Smokeless tobacco products and betel nut quid are the major risk factors in the Indian subcontinent and South East Asia. [2]

Premalignant lesions for oral cancers are

- White patches (leukoplakia)
- Red patches (erythroplakia)
- Mixed red and white patches (erythroleukoplakia or "speckled leukoplakia")
- Oral lichen planus
- Oral sub mucous Fibrosis
- Actinic cheilitis.

In the Indian subcontinent, due to betel and tobacco quid chewing oral sub mucous fibrosis is very common. It is characterized by limitation of mouth opening (Trismus) and burning sensation on eating of spicy food. It is a progressive lesion sometimes leading to complete trismus. Infection with human papillomavirus (HPV), particularly HPV-16 and HPV – 18 is a known independent causative factors for oral cancer especially in young adults who are non tobacco users. HPV16 and HPV18 are also associated with cervical cancers. The common sub site in HPV related cancers is the oropharynx (tonsils, tonsillar pillars, base of the tongue). These tumours also respond better to radiation treatments than tobacco aetiology disease and have a better survival rate. [7]

Hematopoietic stem cell transplantation (HSCT) is also a risk factor in oral cancers. These tumours generally have a more aggressive behaviour with poorer prognosis, when compared to oral cancer from other causes. The probable reason for such aggressive behaviour may be the lifelong immune suppression and chronic oral graft-versus-host disease [8]

Diagnosis and Staging [9]

Diagnosis of oral cancers include

- Clinical examination of the oral cavity
- Imaging
    - Dental X-rays – Pan odantogram
    - Chest X-rays
    - CT/ MRI scan of Face and Neck
    - Ultrasound Scan of Neck
    - PET Scan

- Tissue biopsy
    - A non-invasive brush biopsy (BrushTest)
    - Punch biopsy / Incision Biopsy / Excision Biopsy
    - FNAC of Neck Nodes

Oral Cancers are staged using the TNM /AJCC Cancer Staging System (7^{th} Edition). (Figure 3) [9]

Management [9]

The management options for oral cancers include surgery, radiotherapy and chemotherapy. Surgery is the preferred treatment option for most early cancers whereas multimodal approach using a combination of radiotherapy, chemotherapy and surgery is used for most advanced cancers. Surgical options vary according to the sub site and include:

- Maxillectomy (partial, subtotal, total with or without orbital exenteration)
- Mandibulectomy (Hemi, marginal, posterior etc)
- Glossectomy (total, subtotal, hemi or partial)
- Neck dissections (Selective/comprehensive)
- Wide Excision
- Moh's procedure
- Combinational e.g. Composite Resections

- Feeding tubes for nutrition.

Reconstruction of the soft tissue and bony defects can be done by

- Primary Closure
- Skin Grafts
- Local Flaps e.g. Deltopectoral, pectoralis major, nasolabial, submental, forehead, etc
- Free Flaps e.g. Radial forearm, anterolateral thigh, latismus dorsi, etc

Radiotherapy includes external beam radiotherapy and brachytherapy (implants, coils, moulds, etc) and can be given in the neoadjuvant setting (i.e., before surgery, the primary mode of treatment) or adjuvant setting (after definitive surgery) usually in combination with chemotherapy. The primary and the neck nodes are irradiated. Treatment planning including target delineation and optimal dose distribution is done based on the disease stage, extent of involvement, differentiation, patient characteristics, etc and requires good head and neck imaging. The usual radiation dose is 66-74 Gy to gross disease, 50-60 Gy to subclinical disease and 37.5 - 50 Gy for palliation.

Common chemotherapy agents used in oral cancers are combination of cisplatin, 5 fluorouracil, paclitaxel, bleomycin and targeted agents like cetuximab. Chemotherapy is used alone or concurrently in the neoadjuvant, adjuvant and palliative settings. The NCCN guidelines for the treatment of early and advanced oral cancers are shown in figures 4 to 9. [9]

Figure 3: Oral Cancer Staging (AJCC/TNM 7th Edition)

American Joint Committee on Cancer (AJCC)
TNM Staging Classification for the Lip and Oral Cavity
(7th ed., 2010)
(Nonepithelial tumors such as those of lymphoid tissue, soft tissue, bone, and cartilage are not included)

Primary Tumor (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T1	Tumor 2 cm or less in greatest dimension
T2	Tumor more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumor more than 4 cm in greatest dimension
T4a	Moderately advanced local disease* (lip) Tumor invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face, that is, chin or nose (oral cavity) Tumor invades adjacent structures (eg, through cortical bone [mandible or maxilla] into deep [extrinsic] muscle of tongue [genioglossus, hyoglossus, palatoglossus, and styloglossus], maxillary sinus, skin of face)
T4b	Very advanced local disease Tumor invades masticator space, pterygoid plates, or skull base and/or encases internal carotid artery

*Note: Superficial erosion alone of bone/tooth socket by gingival primary is not sufficient to classify a tumor as T4.

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N2a	Metastasis in single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension

Distant Metastasis (M)

M0	No distant metastasis
M1	Distant metastasis

Histologic Grade (G)

GX	Grade cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated
G4	Undifferentiated

Anatomic Stage/Prognostic Groups

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

Figure 4: Treatment of Early oral cancers:

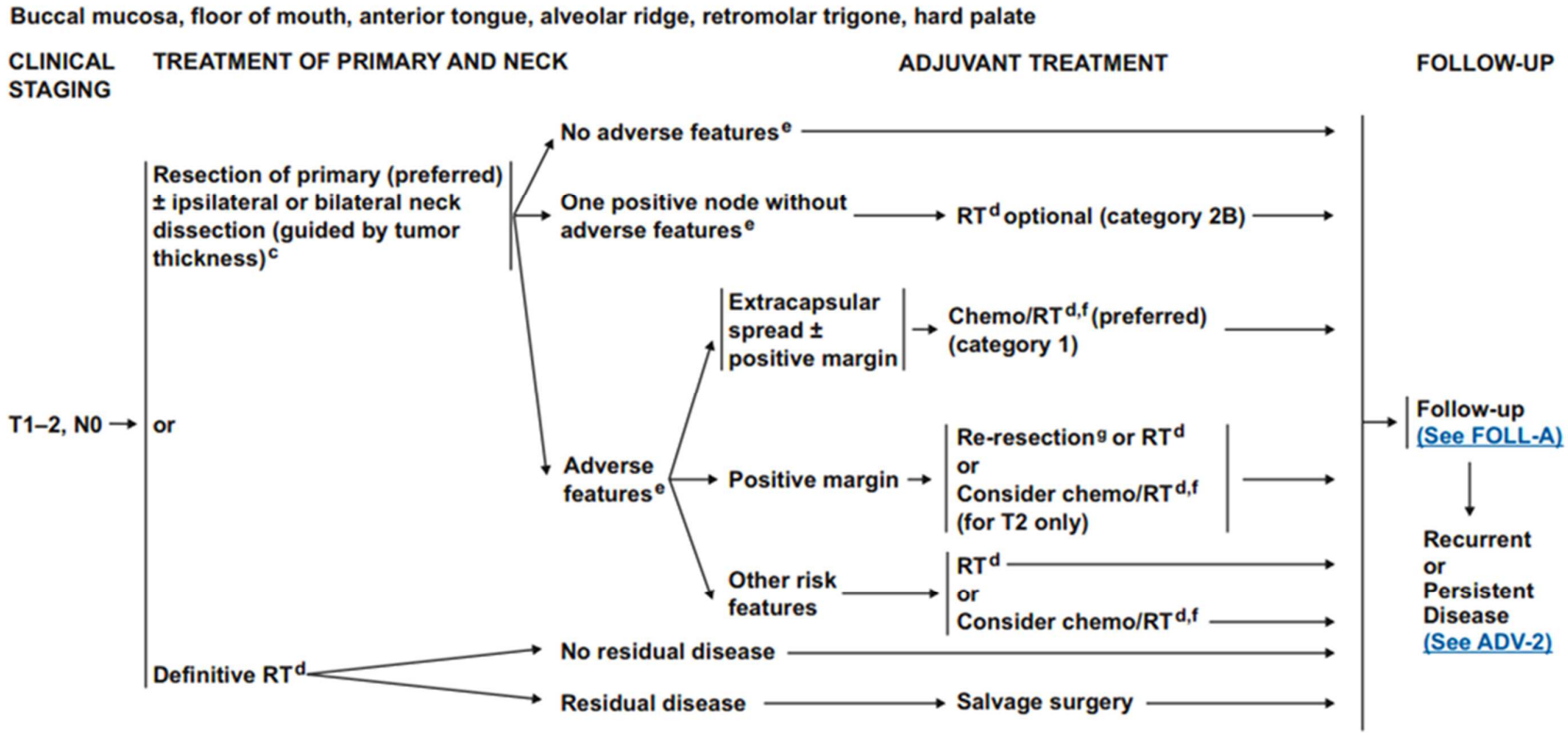


Figure 5: Treatment of Advanced Oral Cancers

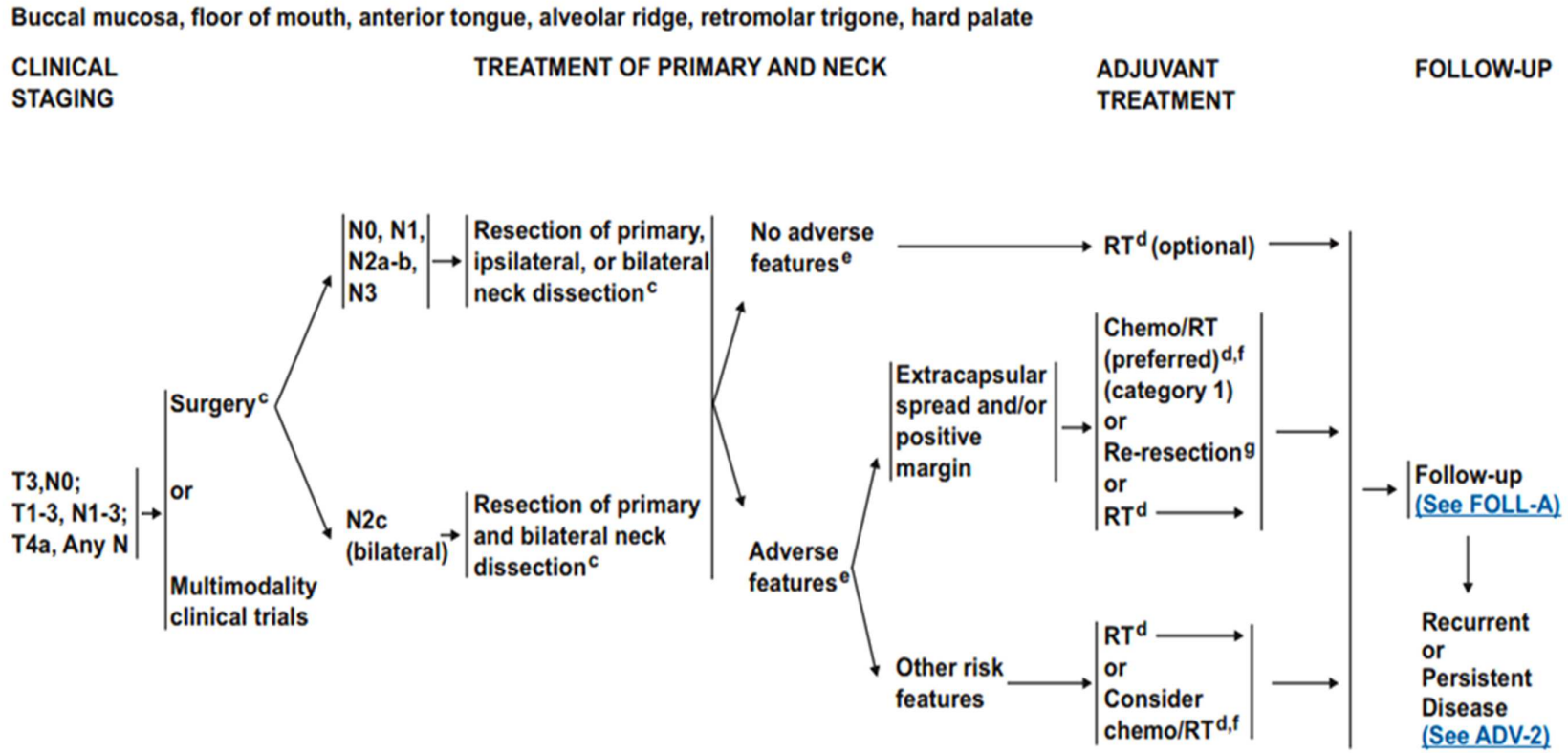


Figure 6: Treatment of Very advanced Oral Cancers

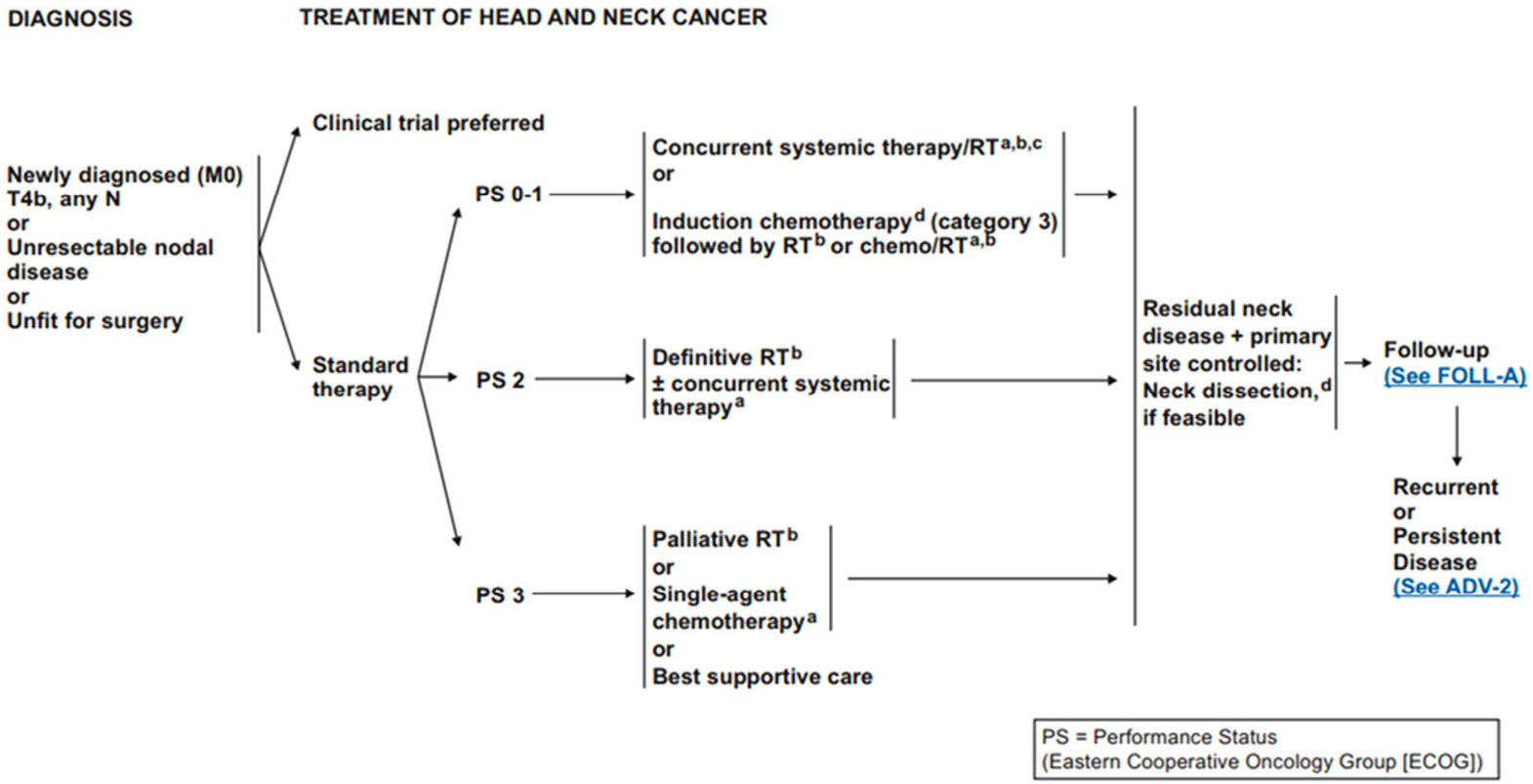


Figure 8: Treatment of Recurrent/ Persistent Oral Cancers

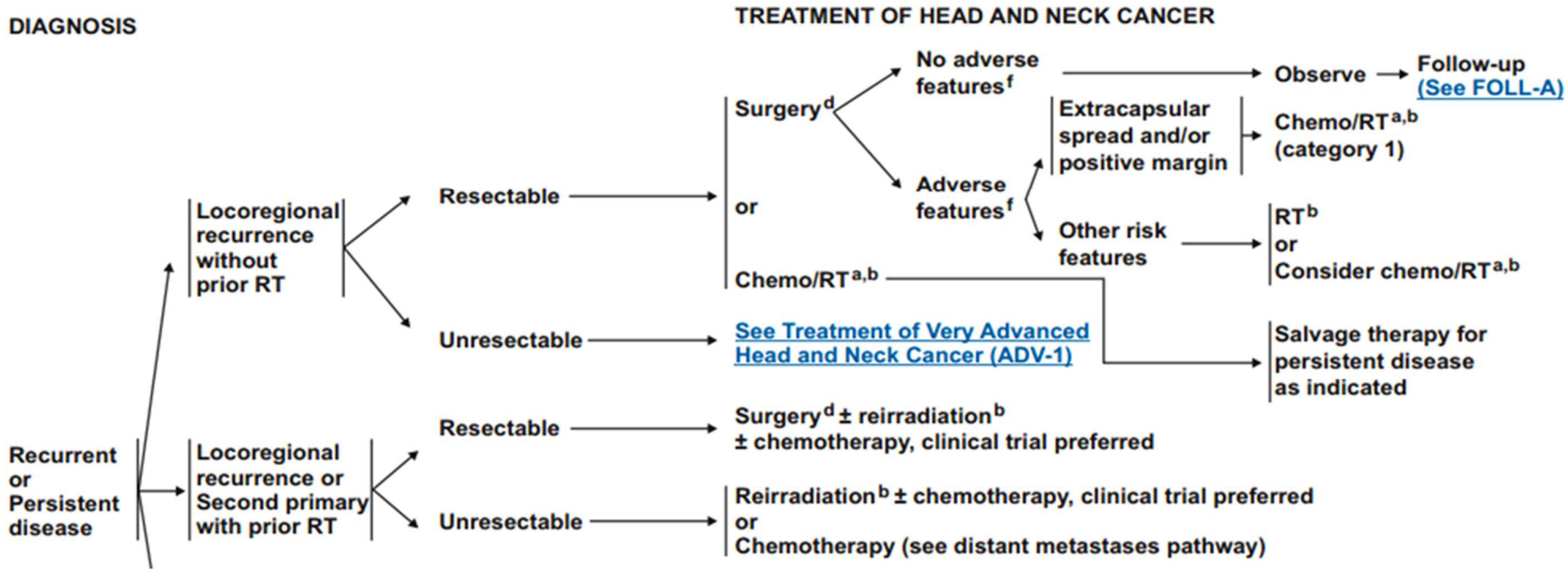
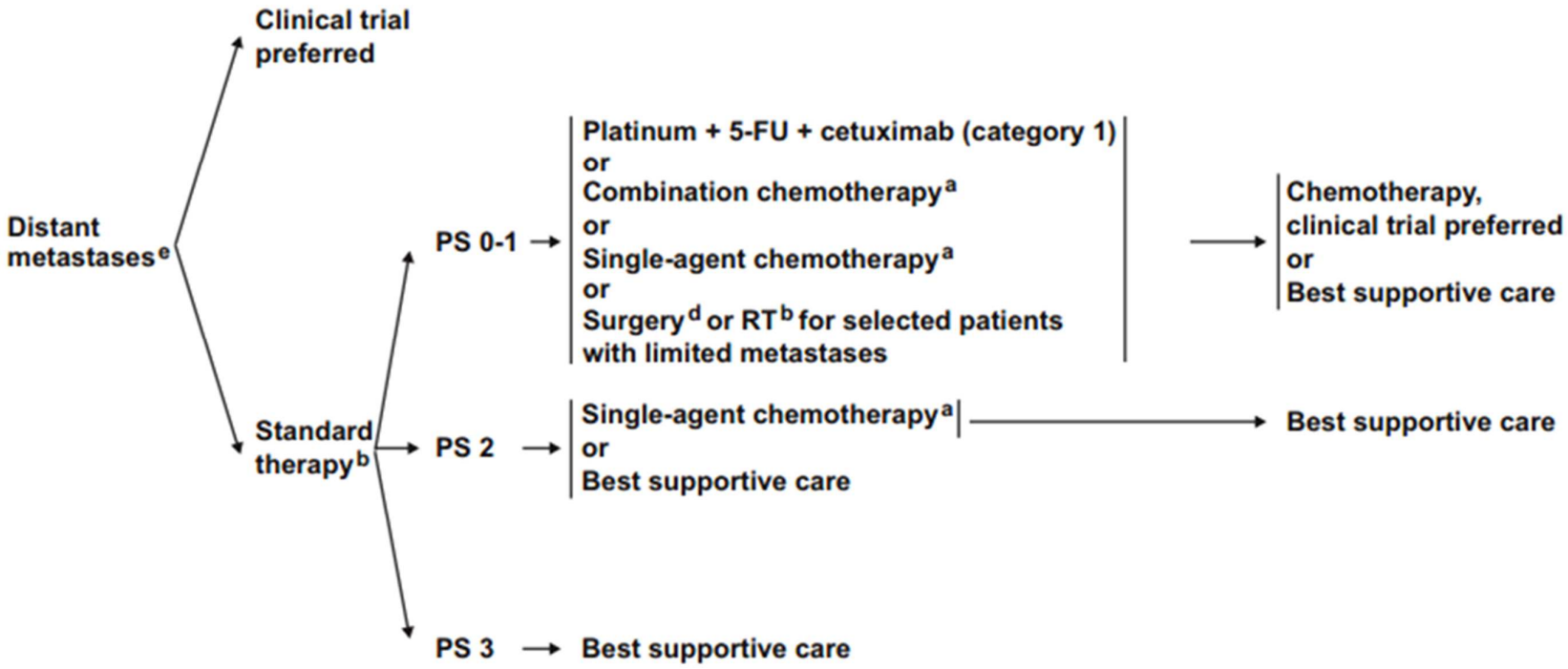


Figure 9: Treatment of Distant Metastases:



Cell Structure and Cell Cycle

The cell is the basic functional and structural unit of all organisms. Growth, development, differentiation, aging, disease, death and all other physiological processes can be described at the cellular level. The cell consists of cell membrane, cell wall, cytoplasm, various cellular organelles like cytoplasmic reticulum, Golgi bodies, mitochondria, ribosomes, vesicles, etc and nucleus (Figure 10). The normal cell cycle is shown in Figure 11.

Nucleic acids (DNA and RNA)

The two main genetic materials in all cells are Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Figure 12). DNA is used for encoding and storage of long-term biological information. RNA is used to transport and transform the information encoded in the DNA into various proteins for cellular function. The DNA and RNA have similar chemical structures with some exceptions.

DNA	**RNA**
Double Stranded	Single Stranded
Sugar Moiety– Deoxyribose	Sugar Moiety- Ribose
Adenine: Thymine Base pairing	Adenine:Uracil Base Pairing
Long Chain/ circular	Shorter Chains
Stable	Easily Prone to Hydrolysis and Degeneration

Ribonucleic Acid (RNA)

There are several types of RNA, each with its unique functions. Most biologically active RNAs contain self-complementary sequences to fold and form double helices like DNA. However, the RNA double helices are shorter helices than that found in the DNA. The enzymatic property of the RNA is due to these helical structures.

Transcription is the process by which RNA is synthesized using DNA as a template. The enzyme RNA polymerase catalyzes RNA synthesis. Transcription of RNA consists of the following steps: Initiation, Elongation and Termination.

Initiation of transcription begins with binding of RNA Polymerase enzyme to a promoter sequence in the DNA. Its helicase activity first unwinds the DNA double helix. Then synthesis and elongation of complementary RNA sequence occurs from the start codons, in the 5' to 3' direction as the RNA polymerase enzyme progresses along the opposite direction. Thus the RNA sequence is dictated by the DNA template sequence. Termination occurs at stop codons in the DNA. Primary transcript RNAs, called pre mRNA are further modified by the addition of a poly (A) tail and a 5' cap in eukaryotic cells. The introns are removed by enzymes called the spliceosomes.[10]

Types of RNA

RNAs can be classified according to their structure and function. A modified list of various types of RNA is shown in Table 1[11]

Micro RNA

Micro RNAs (miRNAs) are small non coding RNAs of approximately 18 to 22 nucleotides size. They have been found in both plants and animal cells and are a novel class of gene regulators. The first miRNA was described in 1993 in *C. elegans.* [12]

The expression of miRNA is highly heterogeneous. Different miRNAs are expressed in different cell types and tissues. MiRNAs are also found to be highly conserved in eukaryotic cell evolution. More than clinically relevant 530 miRNAs have been identified in humans. Details about all known miRNA are available in as a searchable database called miRbase which is maintained by the University of Manchester, United Kingdom. It is predicted that miRNAs regulate expression of 30-60% of human genes. Recent evidences also indicate that miRNAs may have a significant role in cancerigenesis by functioning as tumour suppressors and oncogenes. Such miRNAs with a role in cancer are called as oncogenic miRNAs (oncomiRs). [13-16]

Figure 10: Cell Structure

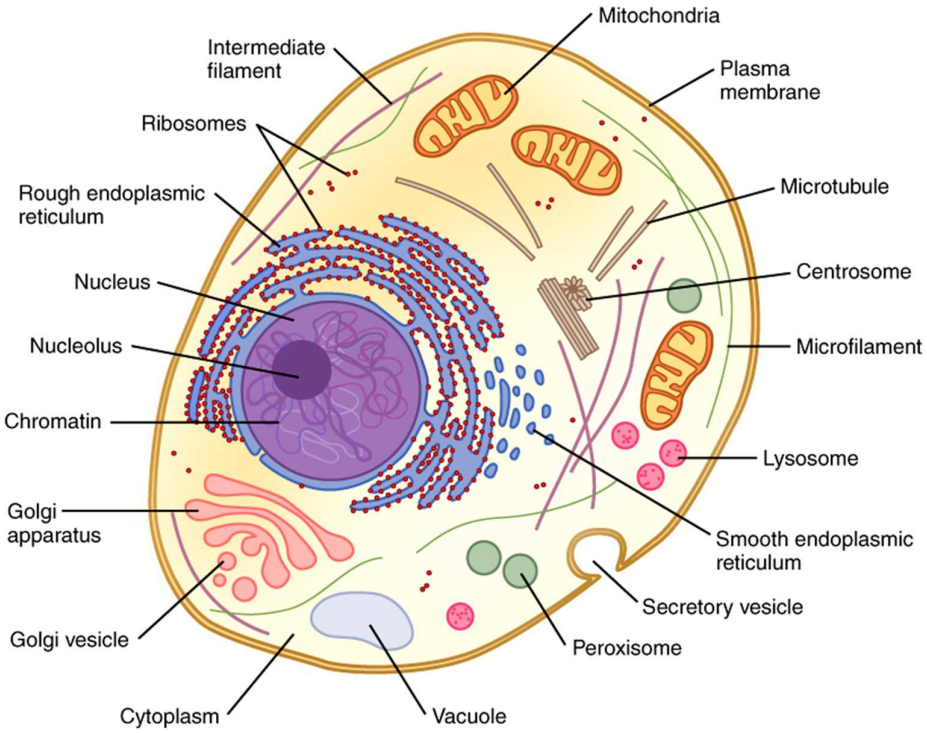


Figure 11: Human Cell Cycle

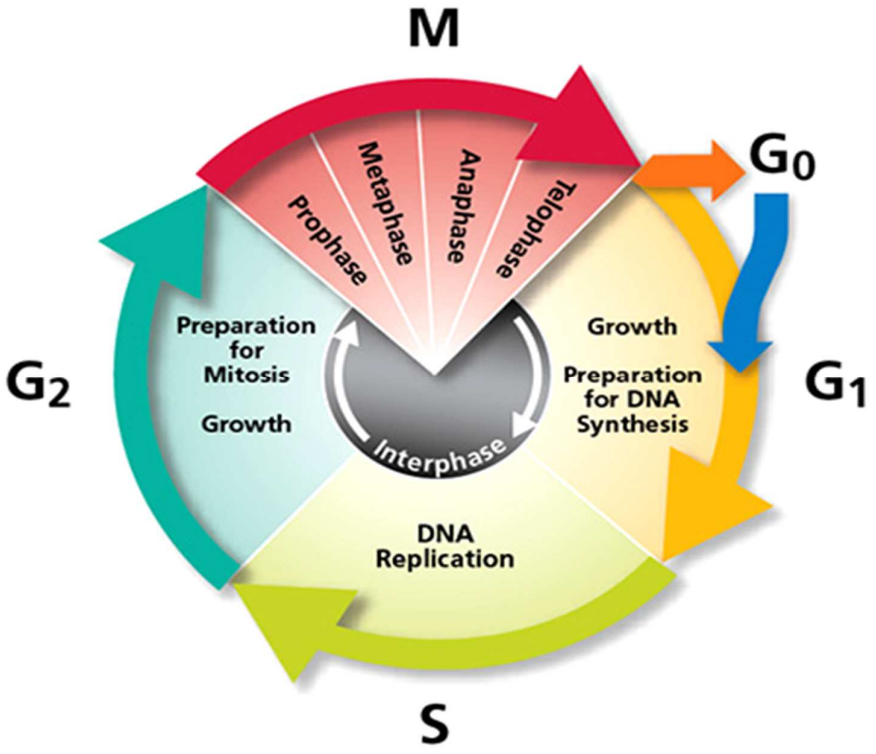


Figure 12: DNA and RNA

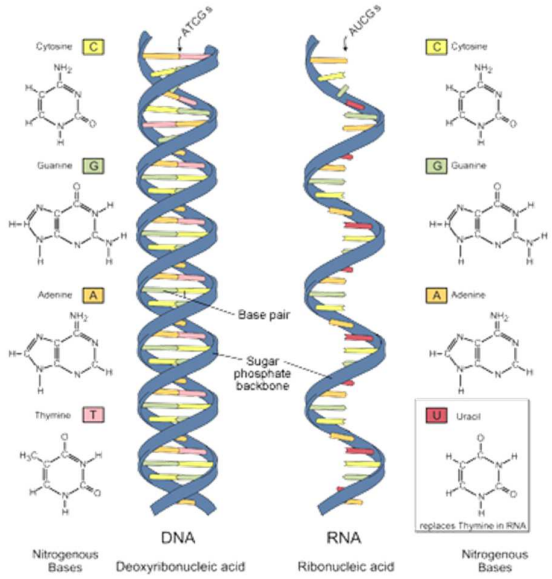


Image adapted from: National Human Genome Research Institute.

Figure 13: RNA Transcription

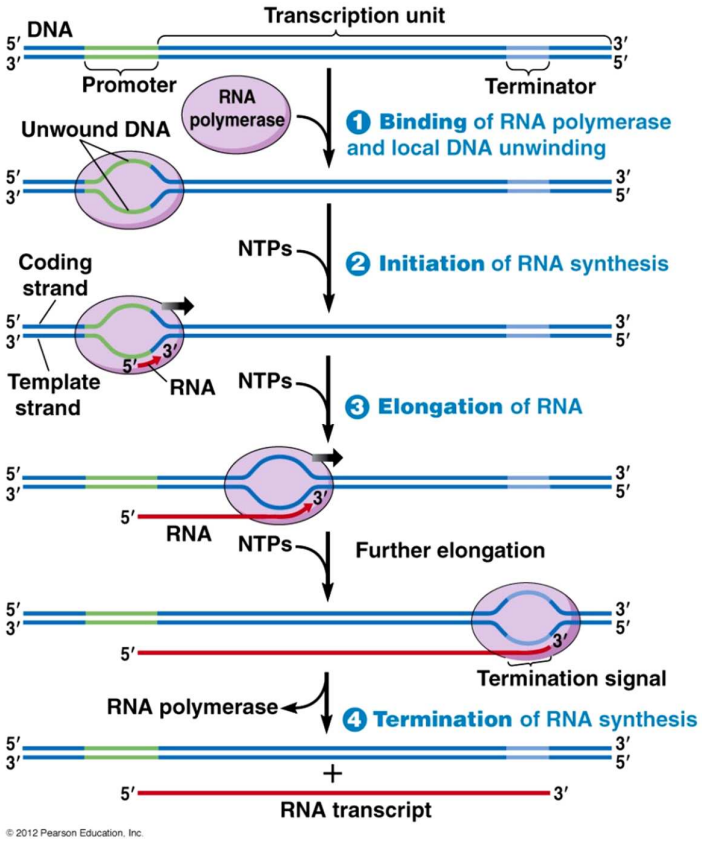


Table 1: Classification of RNA

Type	Abbreviation	Function	Distribution
RNAs involved in protein synthesis			
Messenger RNA	mRNA	Codes for protein	All organisms
Ribosomal RNA	rRNA	Translation	All organisms
Signal recognition particle RNA	7SL RNA or SRP RNA	Membrane integration	All organisms
Transfer RNA	tRNA	Translation	All organisms
Transfer-messenger RNA	tmRNA	Rescuing stalled ribosomes	Bacteria
RNAs involved in DNA replication & post-transcriptional modification			
Small nuclear RNA/ Small nucleolar RNA	snRNA/ snoRNA	Splicing and other functions, Nucleotide modification of RNAs	Eukaryotes and archaea
SmY RNA	SmY	mRNA trans-splicing	Nematodes
Guide RNA	gRNA	mRNA nucleotide modification	Kinetoplastid mitochondria
Ribonuclease P/MRP	RNase P/MRP	tRNA maturation/ rRNA maturation, DNA replication	All organisms, Eukaryotes

Type	**Abbreviation**	**Function**	**Distribution**
Y RNA		RNA processing, DNA replication	Animals
Telomerase RNA		Telomere synthesis	Most eukaryotes
Regulatory RNAs			
Antisense RNA	aRNA	Transcriptional attenuation / mRNA degradation / mRNA stabilisation / Translation block	All organisms
Long noncoding RNA	Long ncRNA	Various	Eukaryotes
MicroRNA	miRNA	Gene regulation	Most eukaryotes
Small interfering RNA	siRNA	Gene regulation	Most eukaryotes
Repeat associated siRNA	rasiRNA	Type of piRNA; transposon defense	Drosophila
Parasitic RNAs			
Retrotransposon	Self-propagating	Eukaryotes and some bacteria	Eukaryotes and some bacteria
Viral genome	Information carrier	ds RNA viruses, RT viruses, other RNA viruses	ds RNA viruses, RT viruses, other RNA viruses
Viroid	Self-propagating	Infected plants	Infected plants
Satellite RNA	Self-propagating	Infected cells	Infected cells

Synthesis of miRNA

The biogenesis of miRNA is similar to that of other RNAs. Pri-miRNA transcripts are produced in the nucleus by RNA Polymerase II (Pol II). This pri-miRNA is processed by the RNase III enzyme Drosha and its co-factor Pasha, to form the pre-miRNA precursor, which is then transported to the cytoplasm. Another RNase called Dicer processes this pre-miRNA to form a miRNA: miRNA duplex. This duplex is stored in the miRNA-associated multi protein RNA-induced silencing complex (miRISC) and mature single-stranded miRNA is released when needed. (Figure 14) [14]

Mechanisms of Action of miRNA

MiRNAs play a role in the regulation of cell development, differentiation, proliferation and apoptosis. MiRNAs regulate their targets in two ways. The method of regulation depends on complementarities between the miRNA and its target. Both methods leads to negative regulation (Figure 14). [14, 15]

1. **RNA mediation interference Pathway (RNAi):**

   MiRNAs binding to targets with perfect or near perfect complementarities cause cleavage of the target mRNA. Such target sites for miRNAare generally found in the coding sequences or open reading frame (ORF) of the target mRNA.

2. **Post transcriptional Repression of target Gene:**

   MiRNAs binding to targets with imperfect complementarities cause post transcriptional block of target gene expression at protein translation level. Such target sites are generally found in the 3′ untranslated regions (3’ UTRs) of the target mRNA genes.

Nine mechanisms of miRNA action are described:

During Transcription	Post Transcription
• 40S Cap initiation • 60S Ribosomal unit joining • Elongation • Ribosome drop-off (premature termination)	• Degradation of nascent protein during translation • Sequestration in P-bodies • mRNA Decay (destabilisation) • mRNA Cleavage • Chromatin reorganization • Gene silencing

Role of miRNAs in cancers

MicroRNAs can function both as tumour suppressors and oncogenes depending on their target gene (Figure 15). In normal cells with normal miRNA levels causes repression of target-gene expression through a block in protein translation or altered mRNA stability. The overall result is normal cell growth, proliferation, differentiation and cell death. [14]

The down regulation of a miRNA that functions as a tumour suppressor and amplification or over expression (up regulation) of a miRNA that functions as an oncogene leads to inappropriate expression of the target oncoprotein. The overall result is increased proliferation, invasiveness, angiogenesis, decreased apoptosis, de-differentiation, etc ultimately leading to tumour formation. Down regulation can occur because of defects at any stage of miRNA biogenesis. Increased miRNA levels may be due to miRNA gene amplification, constitutively active promoter region, increased efficacy of miRNA processing, increased stability of mRNA/miRNA, etc. Oncogenic miRNAs can be classified based to their function, target gene, up/down regulation; causal / non causal, etc. Table 3 lists commonly identified oncomiRs. [16]

Up or down-regulation of miRNAs observed in tumours does not necessarily indicate its causative role in tumourigenesis. It may be a secondary event due to loss of normal cell function and malignant change. To define the miRNAs as tumour suppressor or oncogene, four evidences must be provided:

1. Widespread deregulation (Up/down regulation) in diverse cancers

2. Gain or loss of function of the miRNA in the tumour cells
3. Evidence for tumour-suppressor/ oncogenic activity in animal models
4. Identification and verification of cancer related targets through which these miRNAs acts in oncogenesis.

Table 3: miRNAs Profiles in common cancers

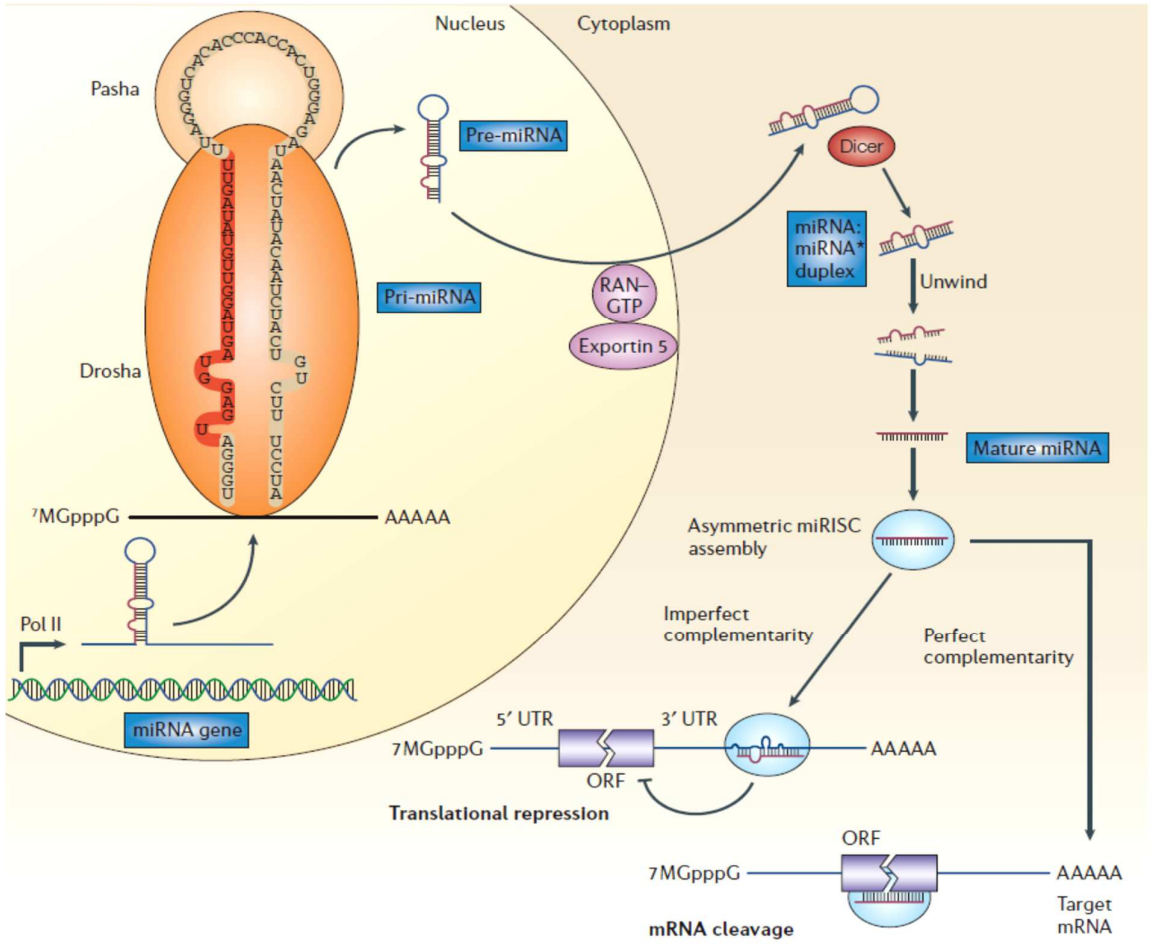
Cancer type*	MiRNA profiling data	Significance
Chronic lymphocytic leukaemia	A unique signature of 13 genes associated with prognostic factors (ZAP70 and IgVH mutation status) and progression (time from diagnosis to therapy)	MiRNAs as diagnostic markers (the identification of two categories of patients)
Lung adenocarcinoma	Molecular signatures that differ with tumour histology; miRNA profiles correlated with survival (*miR-155* and *let-7*)	MiRNAs as prognostic and diagnostic markers
Breast carcinoma	MiRNA expression correlates with specific pathological features	MiRNAs as prognostic markers
Endocrine pancreatic tumours	A signature that distinguishes endocrine from acinar tumours; the overexpression of *miR-21* is strongly associated with both a high Ki67 proliferation index and the presence of liver metastases	MiRNAs as diagnostic and prognostic markers
Hepatocellular carcinoma	MiRNA expression correlated with differentiation	MiRNAs as prognostic markers
Papillary thyroid carcinoma	MiRNA upregulation (for example, *miR-221* and *miR-222*) in tumoral cells and normal cells adjacent to tumours, but not in normal thyroids without cancers	MiRNAs probably involved in cancer initiation
Glioblastoma	A specific signature compared with normal tissues	MiRNAs as diagnostic markers
Human cancers	MiRNA-expression profiles accurately classify cancers; an miRNA classifier classes poorly differentiated samples better than a messenger RNA classifier	MiRNAs as diagnostic markers
Human solid cancers	Common signature for distinct types of solid carcinomas	Specific miRNAs are involved in common molecular pathways

MiRNAs expression patterns are highly specific to cell-type and differentiation status. Some microRNAs reported to be up-regulated in one type of cancer may be down-regulated in other cancers. For some miRNAs, both up- and down-regulation have been reported in the same cancer type by different studies.

Techniques of miRNAs Expression Profiling

Microarray analysis is the most common high-throughput technique used for assessment of cancer-specific miRNA expression levels in a large number of samples. Other techniques include bead-based flow cytometry, quantitative real-time PCR, miRAGE, RAKE assay, etc. Each of these techniques has its strengths and weaknesses (Table 4, Figures 16, 17). Usually, the first step in miRNA studies is isolation of total RNA and enrichment or direct isolation of small RNAs. The miRNAs are then labelled, cleaned-up, assessed for quality and then hybridized to arrays spotted with miRNA probes. After several washes the differential miRNAs are identified by scanning.

Figure 14: Biogenesis, Structure and Mechanism of Action of miRNA



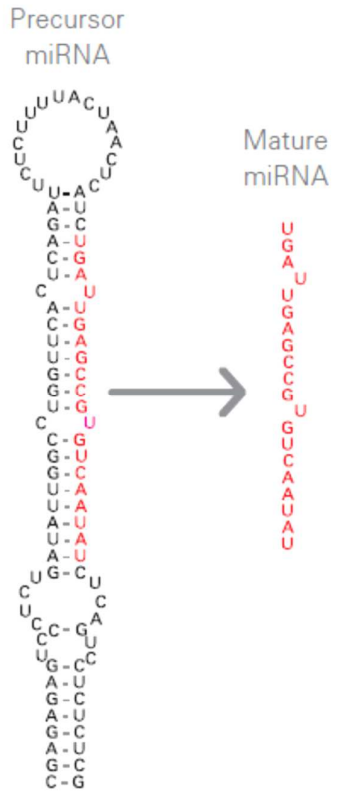
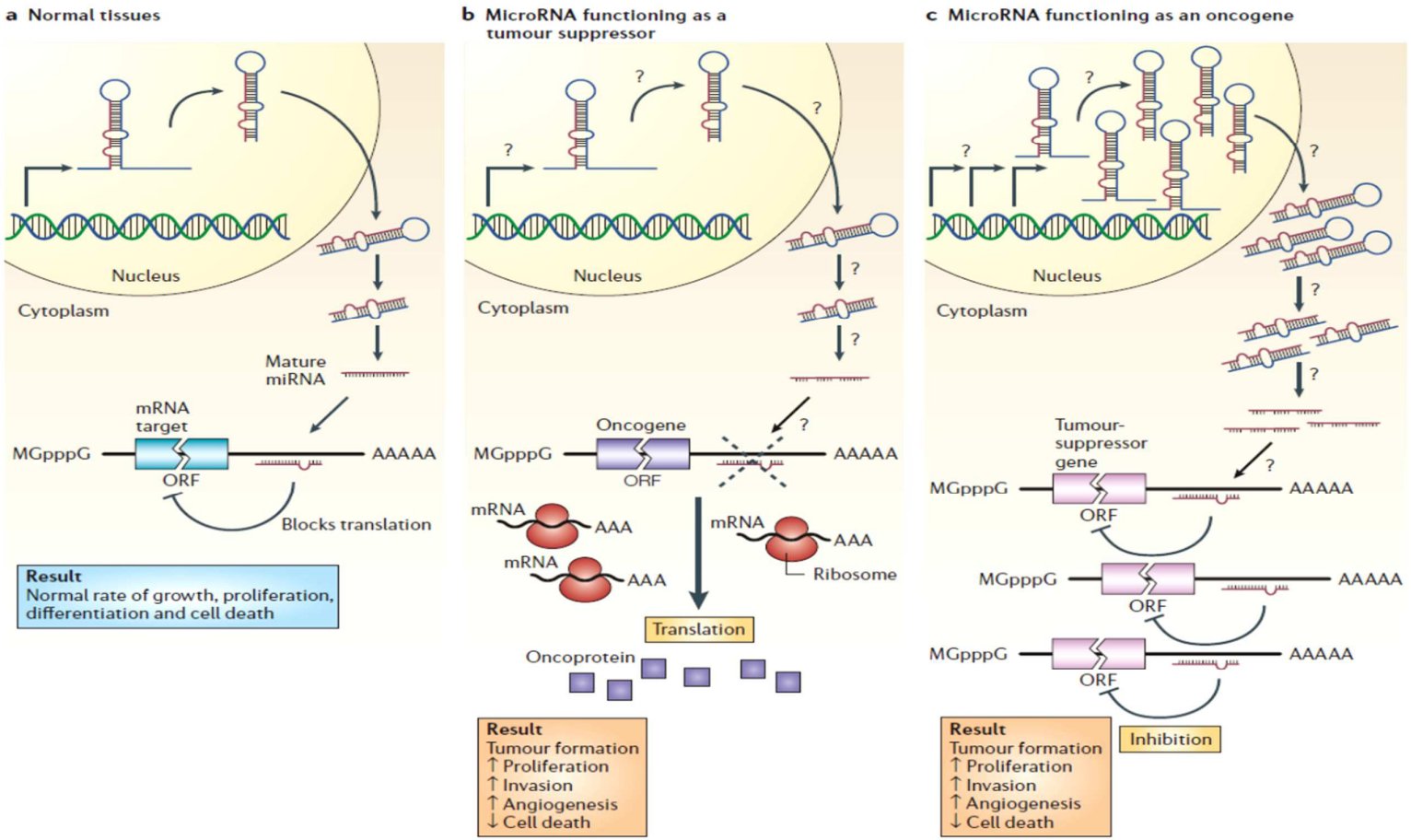


Figure 15: miRNAs Role in tumourigenesis



Validation following microarray studies is done using Northern blot, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), or other analytical methods. Recent developments in labelling methods and microarray probe designs enable study of as low as 120 ng of total RNA without fractionation or amplification, to produce precise and accurate measurements of 0.2 amol to 2 fmol of miRNA. [17]

Table 4: miRNAs Profiling methods

Method	*Advantages*	*Disadvantages*
Cloning (miRAGE)	Open ended; possibility for discovery of new miRNAs	Cost of sequencing
Northern blot	Gold standard; ability to assay miRNA precursor	Low throughput; limited sensitivity for low-abundance species
Microarrays	Low cost; high throughput	Closed-ended
Real-time PCR	Low cost; high throughput; superior detection of low-abundance species	Closed-ended
Bead-based hybridization	Cost; superior hybridization	Closed-ended
In situ detection	Ability to visualize miRNA levels in tissue context	Low throughput, closed-ended
Single molecule detection	Speed	Closed-ended; high cost

MiRNAs and Oral Cancers

MiRNAs have tremendous potential as a new class of molecular tumour biomarker. The diversity in their expression across human cancers can provide a large amount of diagnostic, prognostic, and predictive information. The list of commonly deregulated miRNAs in oral cancers and their proposed targets are given in Tables 5 & 6. [6, 18-21]

Table 5: Common miRNAs deregulated in oral cancers

MicroRNA	Chromosomal location	Mature miR sequence	Up-/down-regulation
miR-21	17q23.1	uagcuuaucagacugauguuga	Up
miR-155	21q21.3	uuaaugcuaaucgugauaggggu	Up
miR-130b	22	cagugcaaugaugaaagggcau	Up
miR-223	Xq12	ugucaguuugucaaauacccca	Up
miR-31	9p21.3	aggcaagaugcuggcauagcu	Up
miR-7	9q21.32 or 15q26.1 or 19p13.3	uggaagacuagugauuuuguugu	Up
miR-34b	11q23.1	caaucacuaacuccacugccau	Up
miR-100	11q24.1	aacccguagauccgaacuugug	Down
miR-99a	21q21.1	aacccguagauccgaucuugug	Down
miR-375	2q35	uuuguucguucggcucgcguga	Down
miR-125b	11q24.1 or 21q21.1	ucccugagacccuaacuuguga	Down

MicroRNA 125b (miR 125b)

miR 125b is known to have altered expression in oral cancers. Its target genes are KLF13 (down-regulated), CXCL11 and FOXA1 (both up-regulated). KLF13 is a transcription factor involved in proliferation and differentiation of cardiac cells, B-and T-cell. CXL11 is a chemokine and a ligand for CXCR3 involved in immunity, inflammation and angiostasis. FOXA1 a transcription factor involved in both growth stimulation and repression. [19]

MicroRNA 21 (miR 21)

MiRNA-21 is one of the most consistently deregulated miRNAs in many cancers. It is well established oncogenes. It has been demonstrated to promote cell proliferation and suppresses apoptosis. It regulates tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), PTEN (phosphatase tensin homologue) and mapsin. In one study, inhibition of miR-21 in tongue cancer cell lines reduced survival, anchorage dependant growth and induced apoptosis. It has been also found to be an independent prognosticator of poor survival for tongue cancers. [19]

MicroRNA 138 (miR 138)

Reduced miR-138 expressions have been shown to be associated with enhanced metastatic potential in oral cancers. This involves suppression of the Rho GTPase signalling cascade. The Rho GTPase cascade acts as molecular switch that regulates cell shape, polarity and locomotion by acting on intracellular actin (Figure 18). [19]

MicroRNA 184 (miR 184)

miR 184 is deregulated in tongue cancers. It is postulated that miR-184 acts as an oncogene by targeting C-MYC gene and induces proliferation and inhibiting apoptosis. [19]

Recent studies have shown that specific miRNAs expression signatures are correlated with prognosis and clinical aggressiveness. Identification of miRNAs alterations in oral cancers could facilitate our understanding and guide targeted therapy. Table 7 lists the recent studies in miRNAs deregulated in oral cancer. Pharmacological modulation of miRNAs expressions leading to ‘‘normalization’’ of malignant phenotypes is a potential approach in cancer treatment. The combination of miRNAs targeting with classical chemotherapy or radiation therapy may provide new tools against cancer. [20-30]

Against this background, we intended to study the feasibility of extracting miRNAs from oral cancer tissue samples on a real time basis and to study the clinical role of selected miRNAs expression profiles.

Figure 16: Microarray and Bead Based Techniques

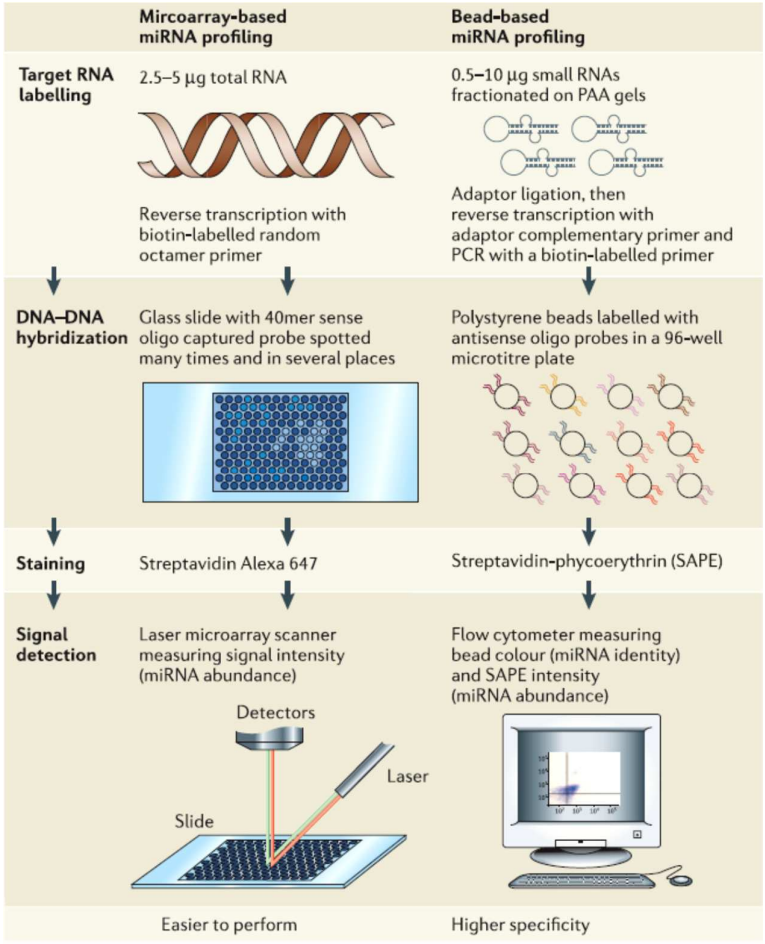


Figure 17: RT PCR Technique

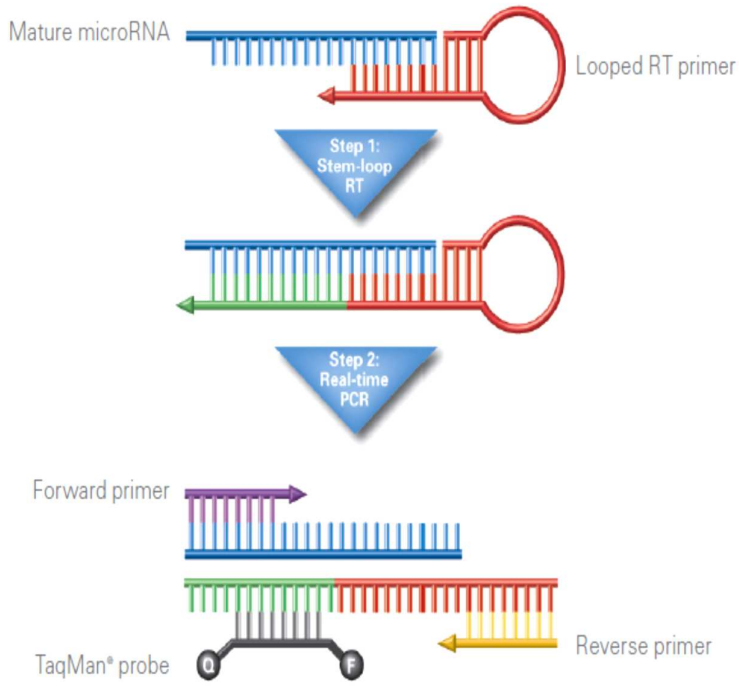


Figure 18: Proposed role of miR -138 in oral cancers

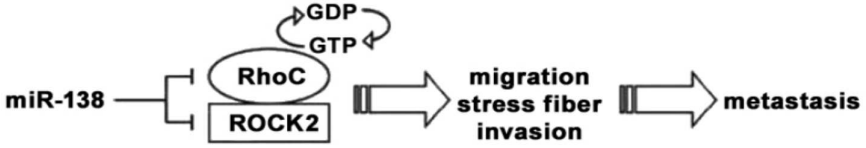


Table 6: Common Oral Cancer deregulated miRNAs and their targets [20, 21]

microRNA	**Proposed Target gene(s)**
microRNA-184	**C-MYC**
micrRNA-133a /133b	**PKM2** (pyruvate kinase type 2)
microRNa-137 and 193a	**CDK6** (cyclin dependant kinase 6)**, E2F transcription factor 6, DNA hypermethylation**
microRNA-15a	**Cyclin E, PKCa** (protien kinase c alpha) down regulates microRNA-15a that directly inhibits cyclin E
microRNA-21	**TPM1 and PTEN** (tropomyosin 1 and phosphatase tensin)
microRNA-103 and 107	**PDCD4** (programmed cell death protein 4), **TGFBR3** (tumour growth factor receptor beta 3)
microRNA-205 and let-7	**DHFR** (dehydrofolate reductase)
microRNA-125b and100	**KLF13, CXCL11 and FOXA1, EGFR3** (epidermoid growth factor receptor 3)
microRNA-222	**MMP1** (matrix metalloproteinase 1) , **SOD2** (manganese superoxide dismutase 2)
microRNA-24	DND1 (dead end 1)
microRNA-7	**IGF1 R** (insulin-like growth factor1 receptor)
microRNA-138	**GNAI 2** (G protein alpha inhibiting activity polypeptide 2)

Table 7: Recent Studies on miRNA in oral cancers [Ref 20-30].

Study	miRNAs	Tumour Samples	Comments
Tran et al 2007	23 miRNAs ↑ - miR 21,16,let 7,205 22 miRNAs ↓	9 cell lines	No controls. First study to do genome wide survey of miR in HNSCC
Chang et al 2008	8 miR ↑- miR 21,18,19,29c,155,let 7 1 miR ↓- miR 494	4 cell lines, 4 tumour samples & 4 normal controls	Used control samples
Wong et al 2008	24 miR↑ - miR 184,21 13 miR↓ - miR 100,125,133	4 cell lines, 4 tumour samples, 4 normal controls	Used paired controls
Kozaki et al 2008	11 miR↑ - 374,224,31,9,340 54 miR↓ - miR 137,139,133,138	18 cell lines 11 primary frozen tumour samples	Control - Immortalized keratinocyte line RT 17
Avissur et al 2009	11 miR ↑- miR 21 1 miR ↓- miR 375	16 fresh frozen tumour samples 5 normal epithelial tissues, 2 cell lines	
Ramdas et al 2009	16 miR↑ - miR-21,34b,155, 182,185,let7,15b,7 4 miR↓- 23b,125a,125b	5 tumour samples and adjacent normal tissue controls	Demonstrated that tumour and adjacent normal tissue can be differentiated based on miRNA differential expression
Scapoli et al 2010	13 miR ↑ - miR21 4 miR ↓- miR 155,146a	15 tumour samples	miR 146a was found to characterize disease progression and nodal metastasis
Lajer et al 2011	114 miR deregulated in oral cancers miR 375,31 ↑	51 oral and pharyngeal cancer samples and controls	Showed relation between HPV infection and miR deregulation in pharyngeal cancers

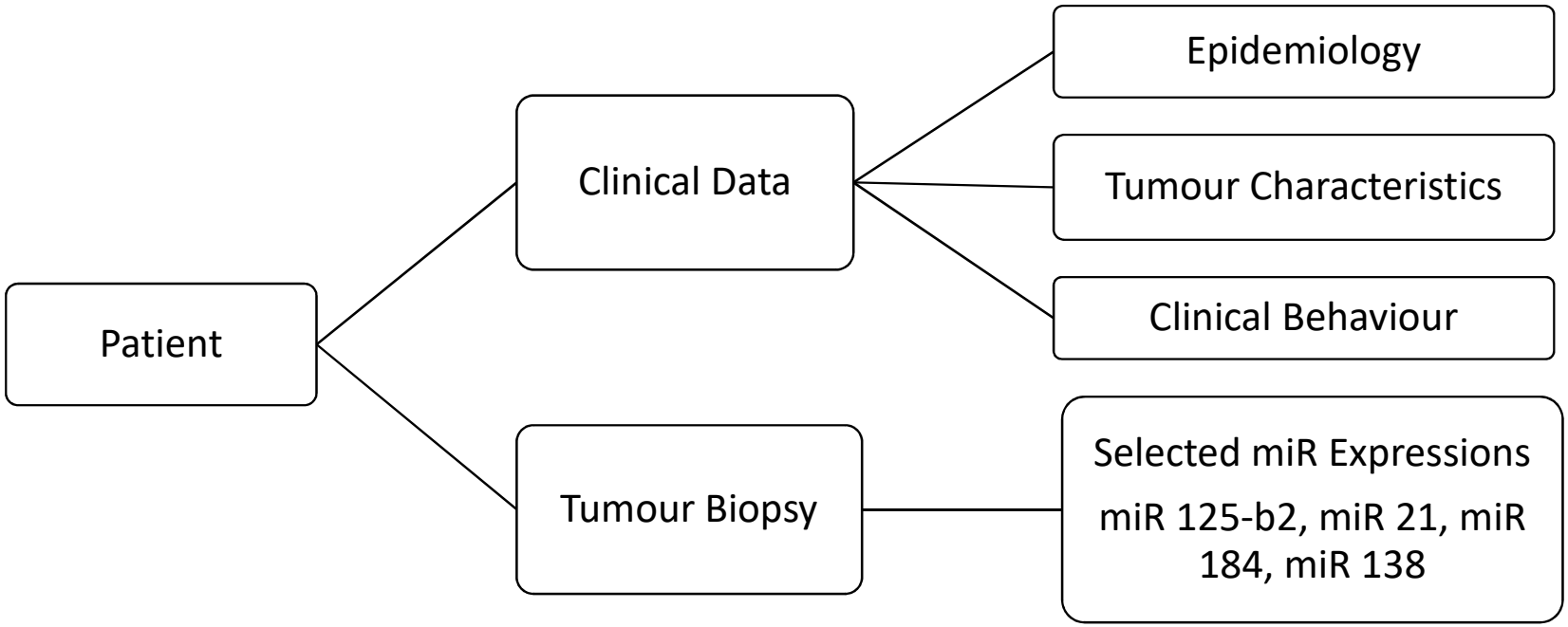
AIMS OF THE STUDY

1. To assess the feasibility of extracting miRNA in oral cancer tumour tissue samples
2. To assess selected miRNAs expression profiles and compare it with epidemiological data, tumour characteristics and clinical behaviour of oral cancer patients

STUDY DESIGN

Study design:	Prospective collaborative clinical and molecular research
Place of Study:	1. Department of Surgical Oncology, Centre for Oncology Govt. Royapettah Hospital & Kilpauk Medical College, Chennai 2. Department of Genetics Dr. ALM PG Institute of Basic Medical Sciences University of Madras, Taramani Campus, Chennai
Duration of Study:	October 2011 – December 2013
Number of Patients:	36 patients with oral squamous cell carcinomas
Inclusion criteria:	1. Patients of age 18 to 70 years 2. Oral squamous cell carcinomas, all sub sites 3. Patients on regular follow-up
Exclusion criteria:	1. Metastatic disease 2. Recurrent or second primary cancers 3. Patients who had previously undergone any treatment for the primary disease 4. Other medical illness(es) precluding full study participation
Materials:	1. Clinical data of patients **2.** Tumour tissue samples from biopsies and surgical specimens
Principal Investigator:	**Prof. R. Rajaraman MS, MCh** Professor and Head, Centre for Oncology Department of Surgical Oncology Govt. Royapettah Hospital & Kilpauk Medical College, Chennai
Co Investigator:	**Dr. A.K. Munirajan PhD** Professor, Department of Genetics Dr. ALM PG IBMS, University of Madras, Chennai

Figure 19: Study Design



MATERIALS AND METHODS

This study was part of a larger research study on the role of miRNAs expression profiles of various cancers which is currently in progress in the Department of Genetics, Dr. ALM PGIMS, University of Madras.

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

CLINICAL DATA

All patients underwent a complete clinical examination, dental evaluation, ENT evaluation and relevant radiological including Chest X-Ray and CT/MRI of Primary & neck and other investigations required for diagnosis and staging. Multidisciplinary evaluations were done as indicated. Pre-anaesthesia fitness was taken for patients undergoing surgery. Relevant history including smoking habits and other addictions, co morbidities, previous treatment and family history were obtained (As per Proforma).

All patients were staged according to UICC TNM 7th edition staging system after histopathological proof of cancer by biopsies. Patients received treatment according to their cancer site and stage as per standard practice.

PATIENT FOLLOW-UP

All patients were followed monthly in the first year, every 2 months in the second year, and every 6 months thereafter. During each follow- up, patients undergo complete clinical examination after relevant history to look for disease recurrence. Further, patients undergo yearly chest X-Ray as part of their metastatic workup and other investigations as and when indicated. Patients are referred for relevant rehabilitation programs at the end of their treatment.

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 then chopped to smaller pieces and stored in the same RNAlater® (Invitrogen Life Technologies, USA) solution at 4°C overnight. RNAlater prevents RNA from getting degraded during transportation and storage. After 24hrs, the RNAlater® (Invitrogen Life Technologies, USA) solution is drained from the tube 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 pure RNA thus obtained is dissolved in RNAs free water and stored at minus 20°C. 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 ≥ 2.0. The observation of absorbance is recorded at 280nm.

MICRORNA EXPRESSION ANALYSIS

The quality of the RNA was checked as mentioned above. MiRNAs expression profiles were studied by Real Time Quantitative Polymerase chain reaction (RT-qPCR) using TaqMan® MicroRNA Assays (Applied Biosystems, USA). The quantification of miRNA is done in two steps:

1. Reverse transcription (RT) step: cDNA is reverse transcribed using specific miRNA primers.
2. PCR step: the products are amplified from cDNA samples.

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 112 patients with oral cancers 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 76 tumour tissue samples (67.8%). Gel Photos and Ultraviolet Spectrometry were used to asses if the RNA was of sufficient quality and quantity for miRNAs Expression Studies. Of these 76 tissue samples, 52 samples (48.2 %) had sufficient quantity and quality of RNA i.e., can be used for miRNAs Expression studies. Thirty six patients with good quality RNA extracted from tumour biopsies were selected for miRNAs Expression Studies. MiRNAs Expressions of four miRNAs relevant to oral cancer based on available literature (miR-125b2, 184, 21 and 138) were analysed and compared with Clinical data of these 36 patients. Controls used were RNU 44 and RNU 48 Expression and Pooled Control Samples (normal tissues)

Figure 20: PCR System and mi RNA Assays

Applied Biosystems 7900HT Fast Real-Time PCR System

Figure 21: mi RNA Extraction Schema

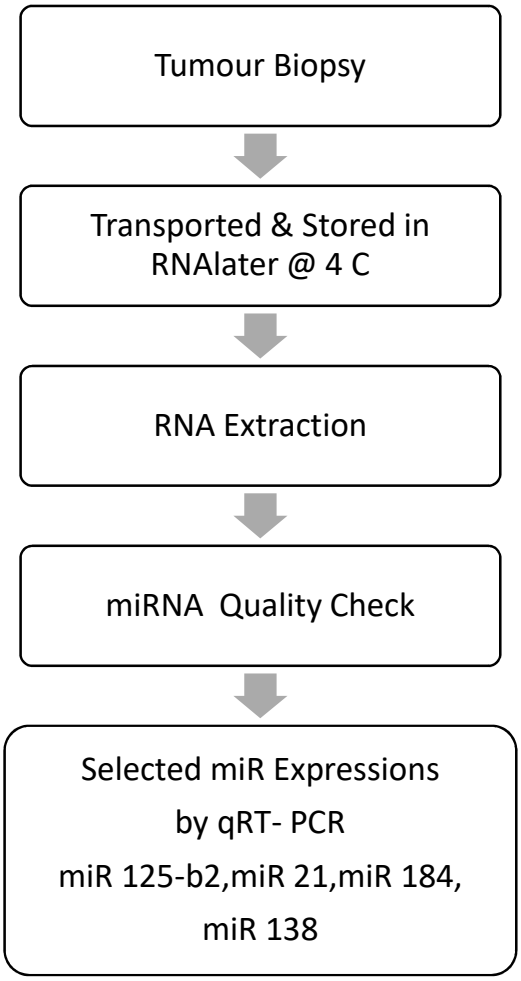
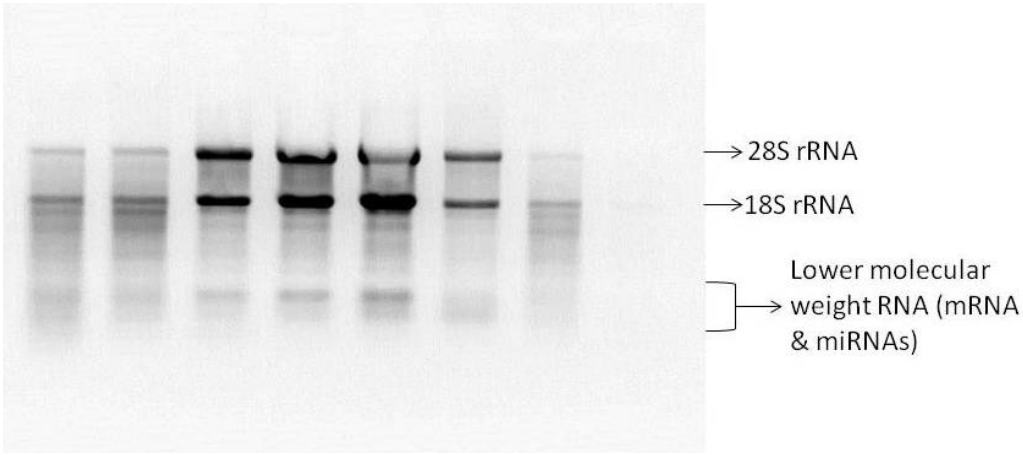


Figure 22: Gel Photo of RNA



STATISTICAL ANALYISIS

The miRNA 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. Clinical data including history of smoking, tobacco and betel chewing and other addictions; site, stage, histological grade of tumour; response to treatment and survival were analyzed. Results for continuous variables are expressed as mean +/- standard error. Comparison of group characteristics was done by chi square test and analysis of variance (ANOVA). A $p < 0.05$ is considered statistically significant. Statistical analysis was done using SPSS 16® (IBM Inc, USA) and EXCEL 2007 ® (Microsoft, USA).

RESULTS

Thirty six patients with good quality RNA in the tumour tissue were selected for miRNAs analysis. MiRNAs Expressions of four miRNAs relevant to oral cancer based on available literature (miR-125b2, 184, 21 and 138) were analysed and compared with Clinical data of these 36 patients

Epidemiology and Tumour Characteristics:

Te male: female ratio was 25:11. The mean age of the patients was 51 years (SD±11, range: 26-70 years). Tongue (n=14, 39%) and buccal mucosa (n=8, 25%) were the most common sub sites. Left sided cancers were more common. Tobacco usage (61 %) in the form of cigarettes, beedis, smokeless tobacco like pan, etc was common. The other addictions seen were betel nut (27.8%), alcohol (33.3%), hans and cocaine.

Locally advanced (T4) lesions constituted 47.2 percent of all tumours and node positivity was seen in 70 percent of patients. Using a three grade grading system, 44 percent of tumours were moderately differentiated and 19 percent of tumours were poorly differentiated. The other epidemiological details of the patients are given in table 8.

Figure 23: Age Distribution of Patients

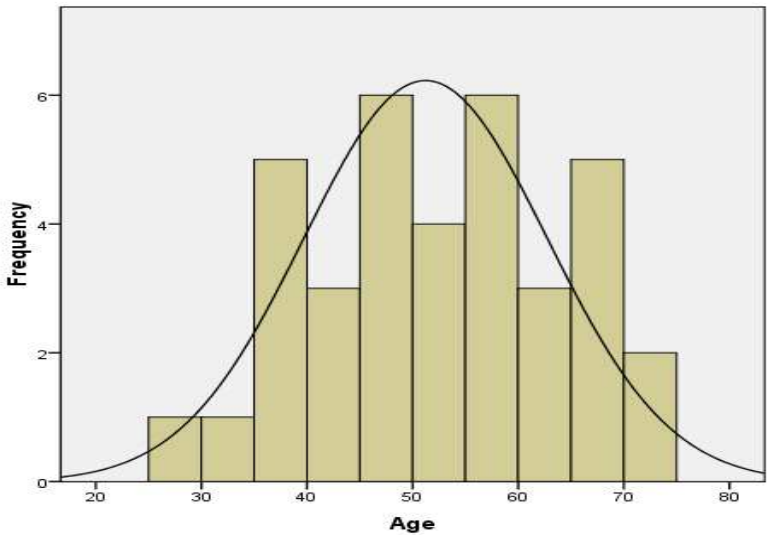
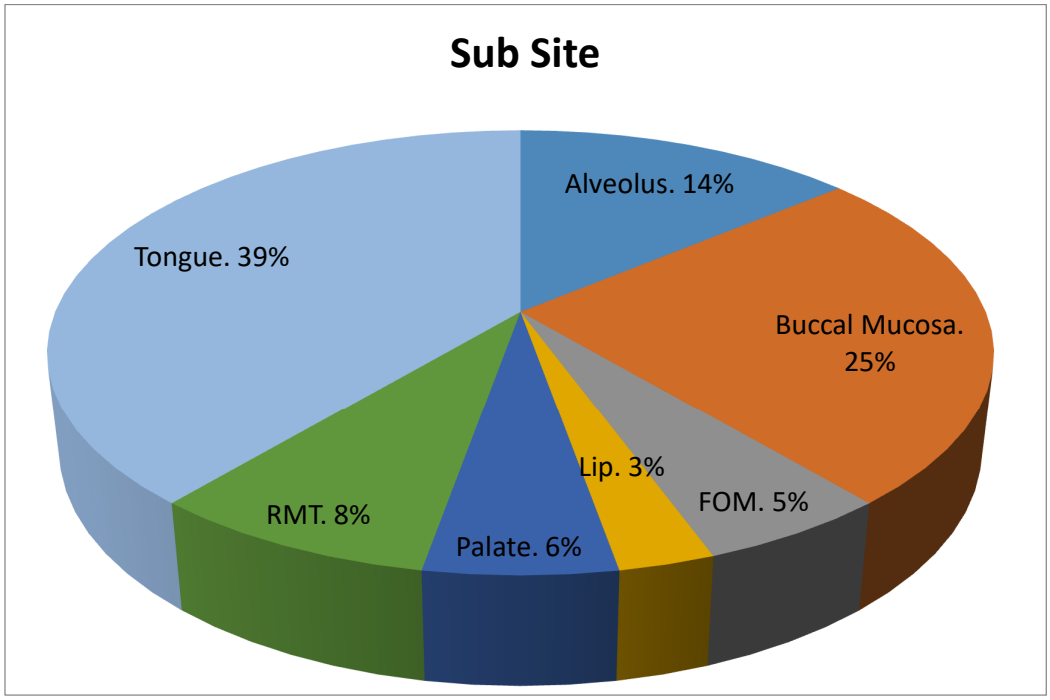
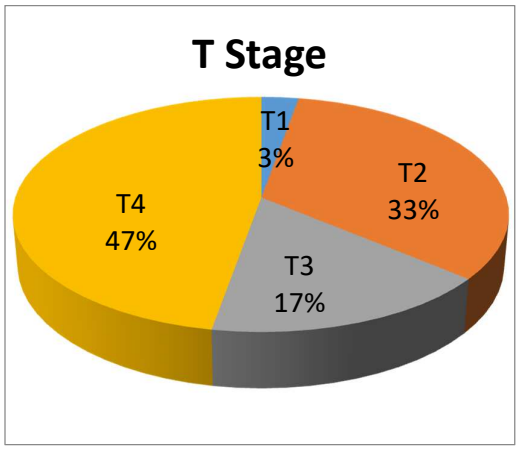


Table 8: Epidemiological Data and Tumour Characteristics

	Characteristics	**N**	**Percent (%)**
Sex	Male	25	69.4
	Female	11	30.6
Sub site	Alveolus	5	13.9
	Buccal mucosa	9	25
	FOM	2	5.6
	Lip	1	2.8
	Palate	2	5.6
	RMT	3	8.3
	Tongue	14	38.9
Side	Left	25	69.4
	Right	11	30.6
Tobacco	Users	22	61.1
	Non users	14	38.9
Betel Nut	Users	10	27.8
	Non users	26	72.2
Alcohol	Users	12	33.3
	Non users	24	66.7
Other Addictions	Users	5	14
	Non users	31	86
T Stage	T1	1	2.8
	T2	12	33.3
	T3	6	16.7
	T4	17	47.2
N Stage	0	11	30.6
	1	12	33.3
	2	12	33.3
	3	1	2.8
Grade	Well Differentiated	13	36.1
	Moderately Differentiated	16	44.4
	Poorly Differentiated	7	19.4

Figure 24 a, b, c: Tumour Sub sites, Tumour Stage and Differentiation





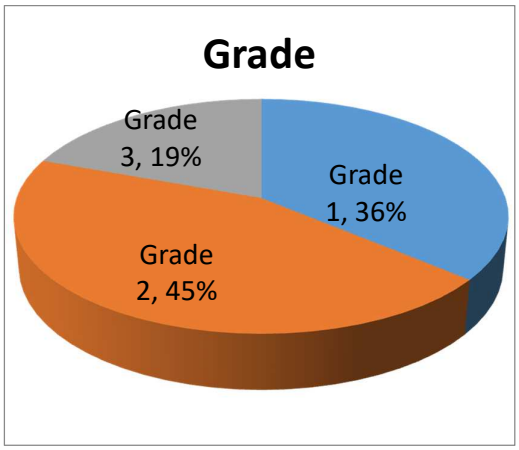


Figure 25: Addictions

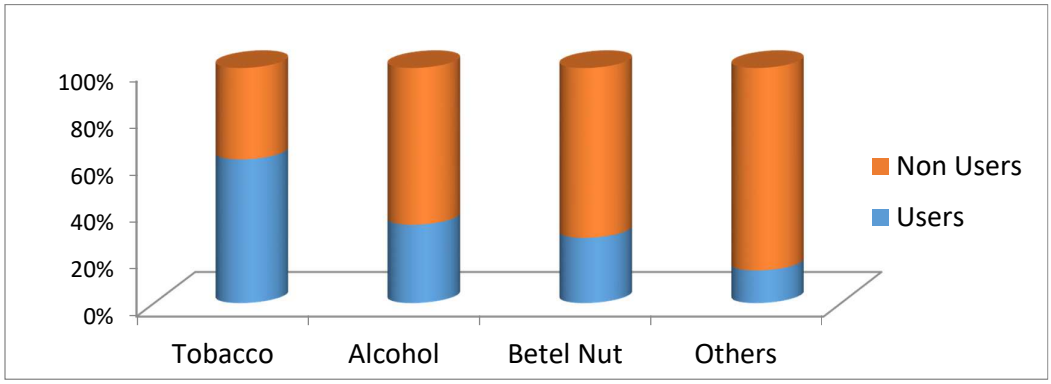


Figure 26: Surgery for the Primary

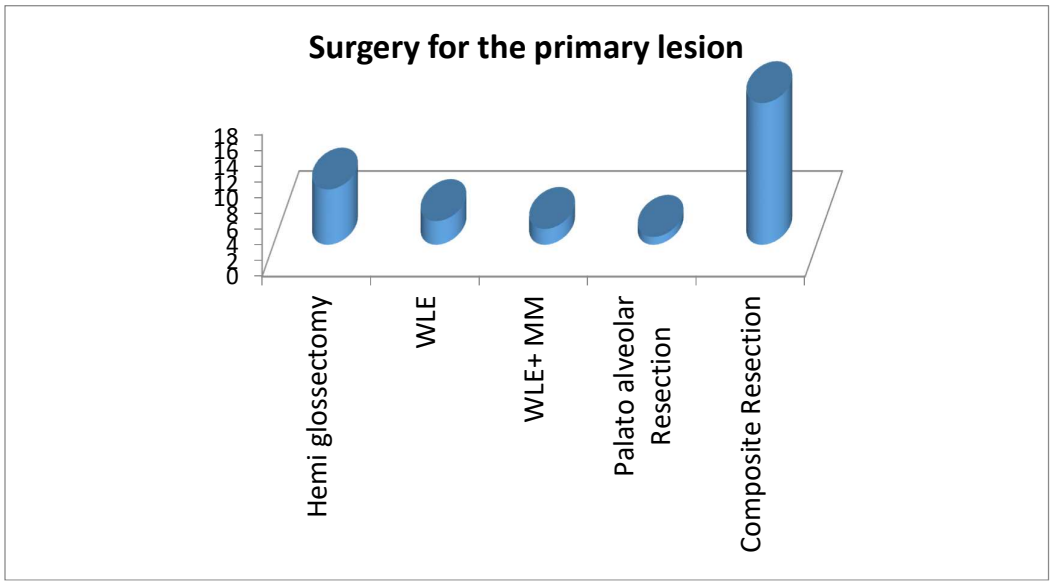
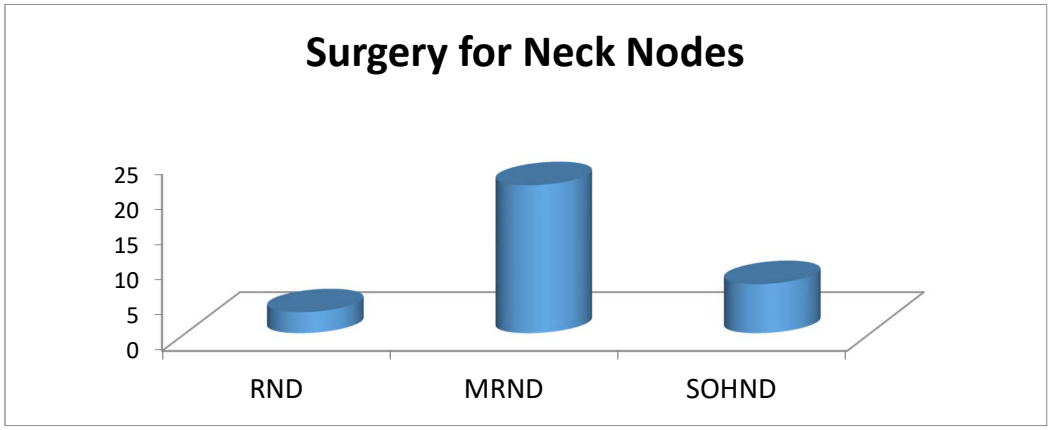


Figure 27: Surgery for the Neck



Clinical Characteristics of Patients:

Out of the 36 patients, 31 underwent surgery. Thirteen patients underwent primary surgery. Twenty two patients received neoadjuvant Chemotherapy and/or radiotherapy of which 5 patients had progressive disease (14%), 5 patients had stable disease (14%) and 12 patients had partial response. These 17 patients (PR +SD) underwent composite resections after neoadjuvant therapy. Another 3 patients received chemotherapy and/or radiotherapy adjuvantly. The details of the surgeries for the primary and neck nodes are shown in figures below.

Table 9: Clinical Data of Patients:

		N	**Percent (%)**
Surgery for Primary	Composite Resection	1	50
	Composite Resection after Neoadjuvant Therapy	17	
	Hemiglossectomy	7	19.4
	Palatoalveolar Resection	1	2.8
	Wide local Excision	3	8.3
	Wide Local Excision + Marginal Mandibulectomy	2	5.6
Surgery for Neck Nodes	RND	3	8.3
	MRND	21	58.3
	SOHND	7	19.4
Chemotherapy	CDDP+5FU	22	61.1
	CDDP+Taxol	3	8.3
Radiotherapy	Radical (60-66 Gy)	13	36.1
	Pre op (50 Gy)	11	30.6
	Palliative (48 Gy)	1	2.8
Response to CRT	Partial Response	12	54.5
	Stable Disease	5	22.7
	Progressive Disease	5	22.7

Follow up:

With a mean follow up of 12 months (range 5-21 months), 5 patients developed recurrences (distant metastases to spine =2, local recurrence =1, nodal recurrence=2) out of whom 3 patients died during follow up.

miRNA Expression

The expression of four miRNA relevant to oral squamous cell cancers were analysed using RT-PCR. The mean Relative Quantitification (mean RQ) of the miRNAs were calculated. A mean RQ ≥1.2 was taken as up regulation and mean RQ≤0.8 was taken as down regulation. (Table)

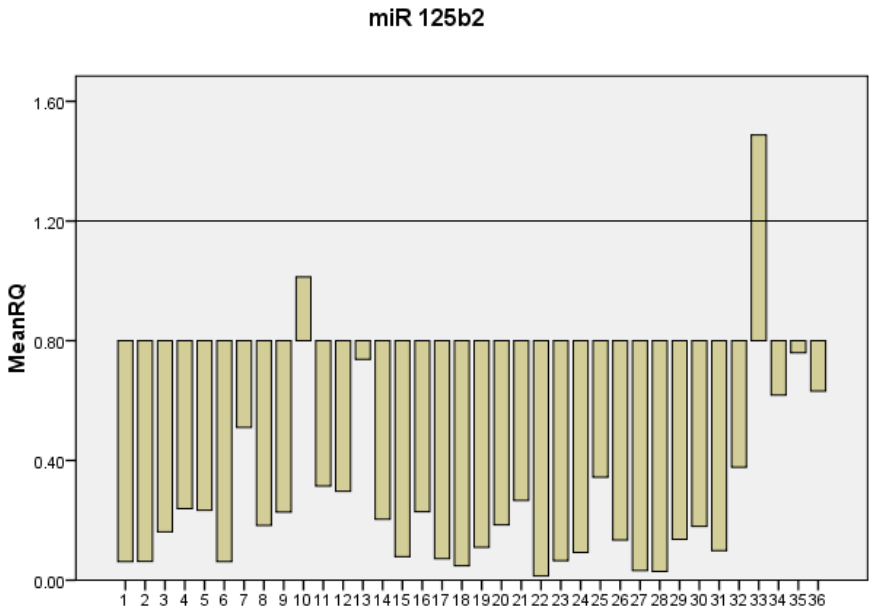
Table 10: miRNA Expression

miRNAs	Up regulation No. of Patients (N)	Normal No. of Patients (N)	Down regulation No. of Patients (N)	Deregulation (Up+ Down) No. of Patients (N)
miR 125-b2	1	1	34	35
miR 138	11	5	20	31
miR 184	6	2	28	34
miR 21	16	6	14	30

The deregulations of these miRNAs were compared with clinical and epidemiological data and their P values were derived. (Table below)

MiR 138 down regulation was seen significantly higher in younger patients (P=0.02, 46.6±11 years vs. 58.4±5.4 years). MiR 184 was significantly up regulated in non tongue cancers (P=0.035, 17 vs. 11 patients). The down regulation of miR125b2 in alcoholics was significantly higher when mean RQ was compared (P=0.02, 0.12±0.08 vs. 0.37±0.35). Similarly mean RQ values of miR 21 was significantly different in patients who responded to chemo radiotherapy versus non responders (P=0.05, 0.83±0.56 vs.1.52±0.99). Similarly miR 21 was significantly deregulated in left sided tumours and in high grade tumours.

Figure 28 a, b: miR 125b2 and 138 Deregulation



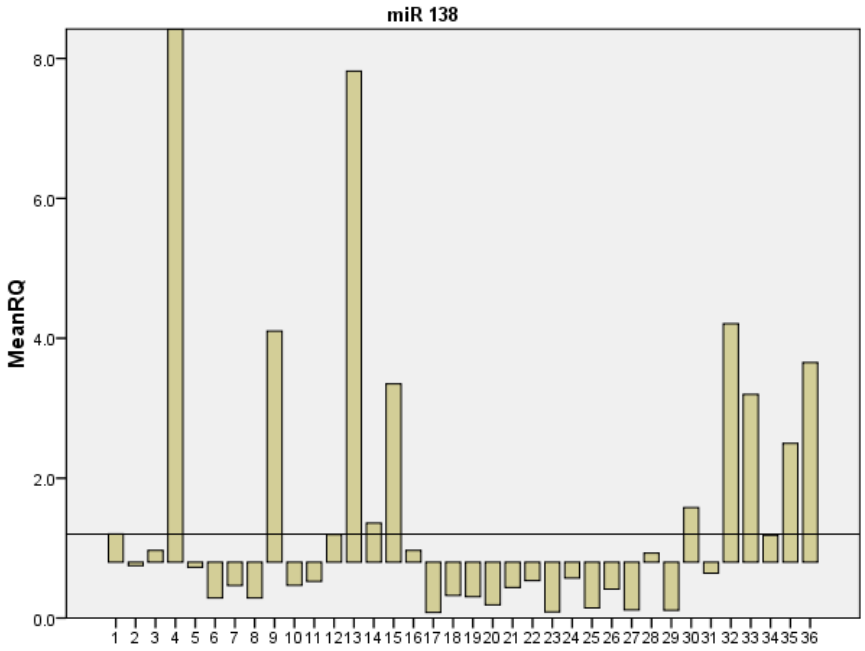
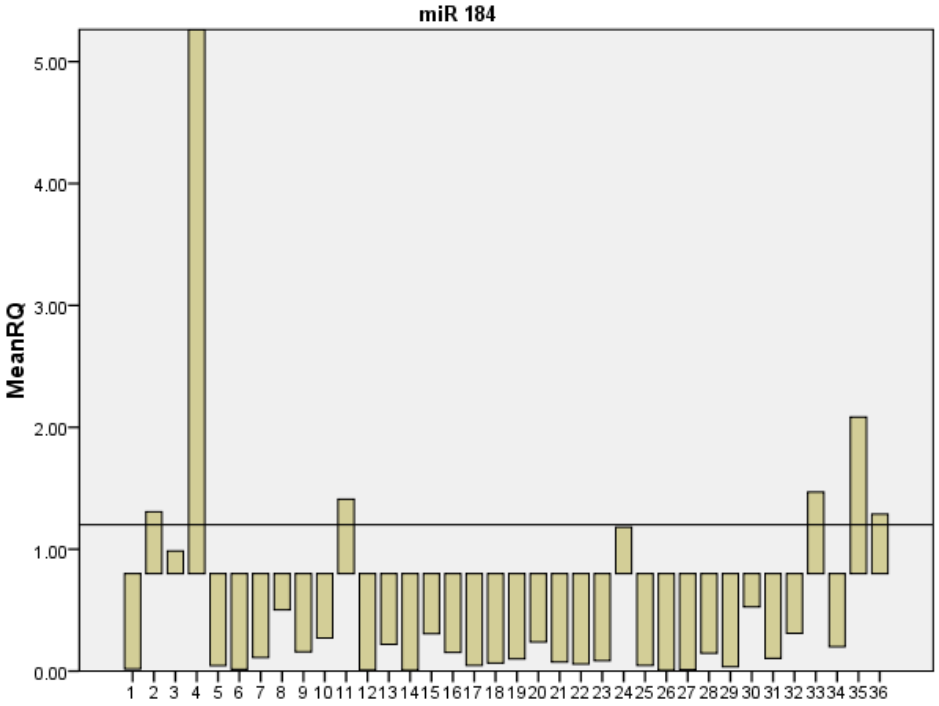


Figure 29 a, b: miR 184, miR 21 deregulation



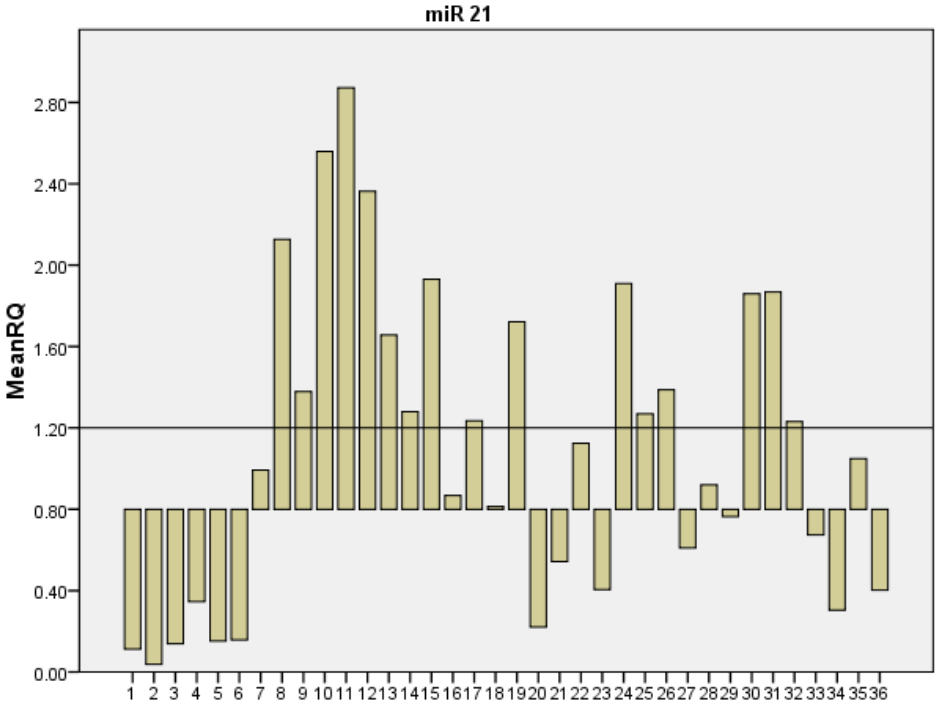


Table 11: miRNAs Expression vs. Clinical Characteristics (P Values)

Clinical Characteristics	miR 125-b2 Down	miR 138 UP	miR 138 Down	miR 184 UP	miR 184 Down	miR 21 UP	miR 21 Down
Sex	0.52	0.59	0.21	0.67	0.52	0.86	0.57
Age	0.82	0.67	**0.02**	0.08	0.12	0.60	0.81
Age Group:<40 yrs vs. > 40 yrs	0.41	0.49	0.12	0.35	0.46	0.91	0.57
Side: left VS. Right	0.13	0.54	0.31	1	0.26	**0.032**	0.11
Sub site :Tongue vs. Others	0.41	0.59	0.21	**0.035**	0.09	0.16	0.39
T stage	0.17	0.43	0.46	0.59	0.91	0.38	0.57
N Stage	0.52	0.44	0.97	1	0.52	0.19	0.09
N Pos vs. N Neg	0.49	0.35	0.84	1	0.44	0.93	0.83
Grade	0.50	0.79	0.77	051	0.8	0.59	**0.04**
Tobacco	0.43	0.59	0.69	0.67	0.76	0.22	0.11
Alcohol	0.52	0.93	0.31	0.54	0.26	0.70	0.69
Betel	0.46	0.12	0.16	0.35	0.52	0.9	0.33
Others	0.92	1	0.49	1	0.81	0.52	0.62
Response to CRT	0.28	0.57	0.9	1	1	0.57	0.32
Recurrence	0.67	0.31	0.36	1	0.51	0.25	0.33

DISCUSSION

This study was a pilot study to assess the feasibility of doing miRNAs expression analysis in oral cancer patients. It was part of a larger ongoing project to assess feasibility of miRNAs expression analysis in various cancers.

Most studies on miRNAs Expression in oral cancers 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 unregulated or down regulated.

This study is probably the first study which puts the emphasis on the patient and analyzes whether a particular miRNAs is unregulated or down regulated in a particular patient. By this patient centric analysis, we intended to study whether in a particular patient with oral cancer, whether a single miRNAs or a panel of miRNAs, whose expression when elucidated in a tumour sample from the patient, may probably correlate with the clinical behaviour of that patient. 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).

For this, we will first need to be able to extract miRNAs in that particular patient sample in sufficient quantity. The challenge for us was to safely transport and store the tumour samples collected at Govt. Royapettah hospital and transported and stored in the Dept. of Genetics, University of Madras where it was subject to further analysis. Unlike DNA, RNA gets degraded rapidly when removed from the body. Further RNAses, enzymes which degrade RNA are found ubiquitously in the environment from our hands to circulating air. The options available are immediate processing of samples, freezing the sample in liquid nitrogen to at least -20º Celsius, using solutions which degrade RNAses (e.g. RNAlater), etc. Immediate processing and freezing such large number of samples is costly and time consuming.

We used RNAlater solution to prevent RNA degradation. We were able to extract RNA with good quality bands in Gel photos in 76 out 112 patients (67.8%). During the initial phase of the study the extraction rate was only around 40 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 tumour samples. So we opted to study the expressions of select miRNAs based on our previous microarray data and available literature by quantitative RT PCR methods. Due to cost constraints, we selected 36 out of 52 samples with good quality RNA which were feasible for expression analysis for further study. We selected 4 miRNAs known to have altered expressions for our study i.e., miR 125b2, miR 138, miR 184 and miR 21.

Demographic Profile of Patients

In our study male: female ratio was approximately 2.5:1. The mean age of presentation was 51 years. Tongue was the most common site followed by buccal mucosa and alveolus or gingiva. These data are consistent with other studies from India and other parts of Asia, where oral cancers are predominantly found in males in the 50 to 70 year age group. Tongue and Buccal mucosa are the commonest sites among oral cancers in Asia unlike in the west where lip cancers predominate. This is probably due to the etiological association with smokeless tobacco and betel nut quid used in this part of the world. This is also the reason for more left sided lesions in our study and other studies from India and other parts of Asia.

Tobacco usage (both smoking and smokeless tobacco) has been implicated in etiopathogenesis of oral cancers. Alcohol has synergistic effect with tobacco. Areca nut or betel nut chewing as part of a quid is common in India and south Asia and has been implicated in oral, especially buccal mucosal cancers. In our study, around 61 percent of patients used some form of tobacco, one third was alcoholics and around 27 percent used betel nut based quid.

In our study, nearly 50 percent of the patients had T4 (locally advanced or very advanced) disease and about 70 percent had neck nodal metastases. None of the patients had distant metastases. This data is also consistent with other studies and

cancer registries from India that patients with oral cancers in India present at a later stage than in the west.

Patients with early cancers underwent surgery as the primary modality of treatment and received adjuvant chemo radiotherapy based on histopathology. Patients advanced disease at presentation need multimodal management with chemotherapy, radiotherapy and surgery. In our study, 22 patients received neoadjuvant chemo and/or radiotherapy, of which 17 patients had partial response or no response (stable disease) and underwent further surgery and adjuvant therapy as appropriate based on final histopathology. Five patients had progressive disease and received palliative chemotherapy and/or symptomatic care.

The advanced nature of presentation in turn leads to a dismal overall 5 year survival of around 37 percent in India whereas it is up to 62 percent in North America. In our study, 3 patients died during follow up, further 2 patients developed spinal metastases, two patients nodal recurrences and 1 patient had local recurrence.

MiRNAs Expression Profiles:

MiR 125b2 was down regulated in almost all patients (n=34). Similarly miR 138 and 184 were also down regulated in a majority of patients. MiR 21 was up regulated and down regulated in almost equal number of patients. MiR 138 down regulation was seen significantly associated with younger age, miR 125b2 down regulation was significantly higher in alcoholics, miR 21 altered expression affected response to chemo radiotherapy, left side predominance of oral tumours and higher grade of tumours. MiR 184 was significantly up regulated in non tongue oral cancers.

Some of these associations have already been shown in various studies. The left side predominance seen in miR 21 up regulation may probably be a spurious association warranting further study. One hypothesis may be due to use of quid in India which is usually kept on the lower gingivobuccal sulcus by patients. However, there was no association between miR 21 and tobacco or betel nut usage.

Table 12: Clinical Association of miRNAs Expressions

miRNAs	**Expression**	**Clinical Association**
miR 125B2	↓	Alcoholics
miR 184	↑	Non tongue oral cancers
miR 138	↓	Younger Age
miR 21	↓	High Grade
	↑	Left side predominance
	↑/↓	Response to CRT

There was no association between the miRNAs studied and tumour stage, nodal stage, response to chemo radiotherapy, recurrence pattern, etc. the association between miR 21 and response to chemo radiotherapy was in the degree of altered expression measured by mean RQs.

SUMMARY AND CONCLUSIONS

The present study was a pilot study of a patient centric analysis of the use of miRNAs expression profiles in the assessing clinical behaviour of oral cancer patients. The study included analysis of 4 select miRNAs (miR - 21, 125 b2, 184 and 138) Expression Profiles in 36 oral cancer patients and their impact on the epidemiological and clinical characteristics of these patients. Based on this study the following conclusions can be made:

1. The extraction and analysis of miRNAs expressions from oral cancer tumour tissue sample is feasible provided care in taken in transportation, storage and processing of the samples.
2. MiRNAs altered expressions have definite and significant associations with many clinical and epidemiological characteristics of oral cancer patients.

Development of newer, simpler, more efficient, safer and cost effective techniques for storage, transportation, extraction and analysis of RNA is the need of the hour. Further studies involving a panel of miRNAs are needed to provide conclusive evidences for use of miRNAs in assessing clinical behaviour of oral cancer patients and to influence their management.

PROFORMA

Name: **Age:** **Sex:** Male / Female **Hospital ID No:**

History: Tobacco abuse: Chewing/Smoking/None/Others
Duration: <5yrs/5-10yrs/>10yrs
Alcohol abuse: Yes/No
Duration :< 5yrs/5-10yrs/>10yrs
Other Addictions: Yes/No Name if any:
Duration :< 5yrs/5-10yrs/>10yrs

Primary Tumor Site:

Stage (TNM 7th edition): T N M Clinical/Pathological

Tumor Grade: I II III IV UNKNOWN

Samples Collected: **Date**

1. **At Diagnosis**: Tumor tissue/Normal tissue
2. **Post Chemo/RT**: Tumor tissue/Normal tissue
3. **At Surgery**: Tumor tissue/Normal tissue

Treatment Given:

1. Chemotherapy:
2. Radiotherapy:
3. Surgery:

Follow-up Record:

Date	**Follow- up Period**	**Follow up Details and Status (NED/Local Recurrence/New Cancer/Distant Disease/Death/LOFU)**

Written Informed Consent

I …….. Hereby agree to participate in the study on microRNA expression profiles in head and neck cancers 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.

Signature/Thumb Impression

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