Deregulation of miRNA-1 in Oral Squamous Cell Carcinoma and it's role in cancer development

Dissertation submitted in partial fulfillment for the degree of

Master of Science in Biotechnology

Submitted By

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CERTIFICATE

This is to certify the dissertation entitled **"Deregulation of miRNA-1 in Oral Squamous Cell Carcinoma and it's role in cancer development"** Submitted by ***AHANA DASGUPTA*** in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology/Applied Microbiology, KIIT School of Biotechnology, KIIT University, Bhubaneswar bearing Roll No. *'**1661002**'* & Registration No. *'**1664575145**'* is a *bona fide* research work carried out by her under my guidance and supervision from **4th December 2017 to 15th May 2018.**

(*Research Supervisor full Signature*)

Full name and Designation

DECLARATION

I hereby declare that the dissertation entitled **"Deregulation of miRNA-1 in Oral Squamous Cell Carcinoma and it's role in cancer development"** submitted by me, for the degree of Master of Science to KIIT University is a record of *bona fide* work carried by me under the supervision and guidance of ***Dr. Raghunath Chaterjee, Indian Statistical Institute, Kolkata, West Bengal, India.***

Date:

Place:

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ABSTRACT

- **Background:**

Head and neck squamous cell carcinoma (HNSCC) being the sixth most common neoplasm is a significant cause of cancer morbidity and mortality over the globe [1]. Oral squamous cell carcinoma (OSCC) that comprise a major part of HNSCC, accounts for about 90% of all oral malignancies. miRNAs owing to their role in post-transcriptional gene regulation have recently gained interest in the field of cancer pathogenesis.

- **Problem:**

In previous studies from our laboratory, small RNA sequencing analysis in OSCC vs. adjacent normal tissues identified 45 deregulated miRNAs. hsa-miR-1 was found to be one of the top deregulated miRNA and hence the expression of miR-1 needs to be validated in additional independent samples. The role of mir-1 is not well studied in Indian population and hence, the functional characterisation of miR-1 in Indian OSCC patients also needs to be studied.

- **Objectives:**

✓ We aim at validating the deregulated miRNA (hsa-miR-1) by quantitative real-time PCR.

✓ We aim at stably over-expressing the down regulated miRNA in OSCC cell line to find the role of this miRNA in cancer pathogenesis.

- **Methodology:**

✓ RNA isolation from OSCC vs. adjacent normal paired tissue samples and quantitative real-time PCR(qPCR) for validation of hsa-miR-1 expression.

✓ Lentiviral cloning of miR-1 in PLKO1 vector. Viral packaging and stable transduction in SCC-131 cell line to observe the effect of miR-1 over-expression in cancer.

- **Achievements:**

✓ hsa-miR-1 was validated to be significantly downregulated in OSCC tissue compared to adjacent normal.

✓ Generation of SCC-131-miR-1 stable cell-line.

✓ Migration and proliferation assay showed that miR1 might play a role in inhibiting migration and proliferation of cancer cells.

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Date:

Place:

Signature & Name

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INTRODUCTION

- **Background and Context:**

Head and neck squamous cell carcinoma (HNSCC) being the sixth most common neoplasm is a significant cause of cancer morbidity and mortality over the globe[1]. Oral squamous cell carcinoma (OSCC) that comprises a major part of HNSCC, accounts for about 90% of all oral malignancies [1]. Two thirds of all cancer deaths occur in low and middle income countries. The morbidity and mortality associated with cancer has adverse impact on all the countries especially on the lower middle income countries like India [2]. Globally, India has the highest incidence of oral cancer per year and is the leading cancer among Indian men and fifth most common in women [3-4]. Oral cancer broadly encompasses tumours arising in the lips, hard palate, upper and lower alveolar ridges, anterior two-thirds of the tongue, sublingual region, buccal mucosa, retromolar trigone and floor of the mouth [5]. The widespread practise of smoking or chewing tobacco and alcohol drinking, apart from poor oral hygiene, poor diet and Human Papilloma Virus (HPV) infections may explain this disproportionately higher incidence of OSCC in India [5]. The oral cancer pathogenesis pathway is regulated in several ways at the molecular level. miRNAs are important players in post-transcriptional gene regulation and hence, has recently gained interest in the field of cancer pathogenesis.

The role of miRNAs in gene regulation and cancer:

MicroRNAs are a class of 20-22 nucleotides long non-coding RNA, that inhibit translation of their target mRNAs binding with the 3'-untranslated region of mRNA by translational repression or mRNA cleavage [6]. Pri-miRNA are processed in the nucleus by DROSHA resulting in the formation of stem-loop Pre-miRNAs that are translocated to the cytoplasm and cleaved by Dicer into small double-stranded RNA molecules, the mature miRNA which are 20-22 nucleotides in length. The mature miRNA binds with the RNA-induced silencing complex (RISC and together they bind to the 3` UTR (untranslated regions) of target mRNAs and inhibit their translation by either sequestering the transcript, mRNA cleavage, or mRNA degradation [7]. miRNAs play a major role in gene regulation and hence, deregulation of miRNAs are associated with various diseases like cancer. In cancer, changes in protein coding tumour suppressor gene and/or oncogenes are often associated with tumour development. A single miRNA can regulate many mRNA expression regulating several biological processes, like cell proliferation, differentiation, migration and apoptosis, which are important for normal cell development. In recent studies miRNAs

have gained interest in the field of cancer owing to it's role in gene regulation. Many miRNA have been reported to show important role in OSCC tumorigenesis. miRNAs that target oncogenic mRNAs generally play a tumor-supressor role by decreasing tumor growth, proliferation, migration etc. while miRNAs targetting tumor-supressor mRNAs result in enhanced tumorigenesis [6].

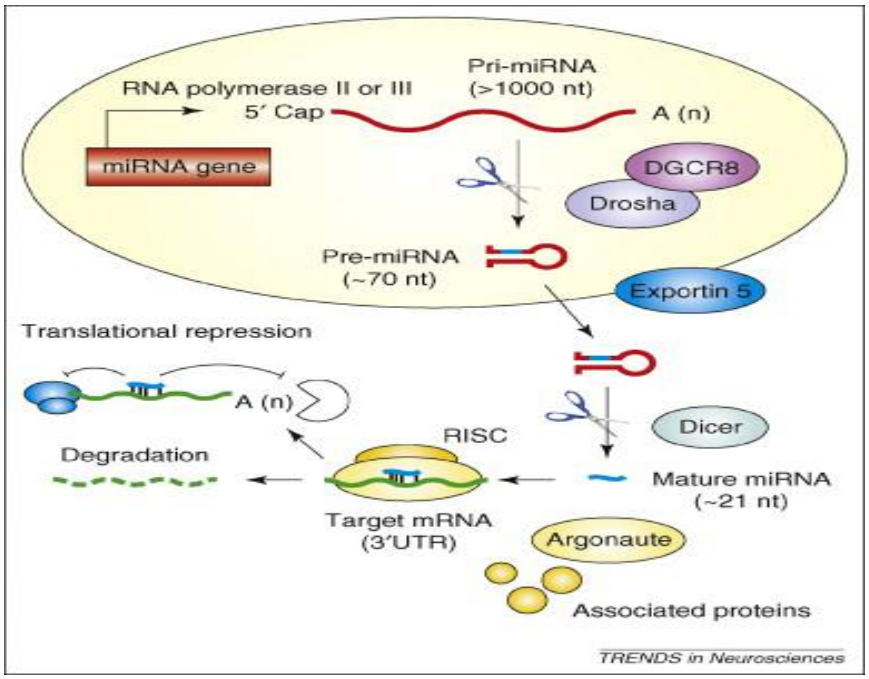


Fig1: MicroRNA biogenesis pathway

- **Objectives:**

✓ We aim at validating the deregulated miRNA (hsa-miR-1) by quantitative real-time PCR.

✓ We aim at stably over-expressing the down regulated miRNA in OSCC cell line to find the role of this miRNA in cancer pathogenesis.

- **Achievements**

✓ hsa-miR-1 was validated to be significantly downregulated in OSCC compared to adjacent normal.

✓ Generation of SCC-131-MIR-1 stable cell-line.

✓ Wound-healing migration assay and Wst1 proliferation assay showed a possible role of hsa-miR-1 in the inhibition of migration and proliferation of cancer cells

REVIEW OF LITERATURE

In many studies it has been reported that miRNAs can play an important role in tumorigenesis by acting as a tumour supressor gene or as an oncogene. miRNAs that areb dwnregulated in cancer may be potential to tumour suppressor gene. Hence, over expression of tumor-supressor miRNAs can alter tumorigenesis. In several cases it has been reported that miRNA1 is down regulated in cancer or directly effects some target mRNAs that has an impact on cell proliferation, migration and apoptosis etc. For instance, miR1 is reported to directly target MET gene and down regulates it's expression in gastric cancer [9]. MET which is upregulated in many cancer is a proto oncogene and is a potential target of miR1. MET and Pim1 which are reported to be the downstream targets of miR1 are frequently upregulated in lung cancer patients and promotes tumour growth in cancer [10]. miR1 is also reported as a direct regulator of Nanoreceptor tyrosine kinase cSrc also known as Src, a potent oncogene involved in a series of biological processes like cell growth, proliferation and apoptosis. Abnormal regulation of Src mediated by miR1 has been found in esophegal cancer [11]. In breast cancer miR1 is negatively associated with K-RAS anad MALAT-1. miR1 acts as a tumour suppressor by targeting K-RAS and MALAT-1 and it has been observed that patients having low miR1 expression has poor survival than patients with higher expression of miR1. It has been also reported that miR-1 exerts an anti-angiogenic effect on nasopharyngeal carcinoma. Recent studies have observed a functional role of miR1 in cellular transformation, tumorigenesis, apoptosis, and drug sensitivity [12]. In colon cancer, miR-1 is reported to be downregulated in 84.6% of the tumors. This decrease is significantly correlated with MET overexpression, particularly in metastatic tumors. Overexpression of metastasis-associated in colon cancer 1 (MACC1) and downregulation of miR1 are associated with MET overexpression. In rhabdomyosarcoma miR1 downregulation has been obseved. Overexpression of Mir1 may inhibit RMS cell migration and proliferation and arrest cell cycle and restrict tumour growth. Recent studies has shown transfection of miR1 in RMS cell-line causes a supression of cMet expression which leads to inhibition of cell growth and proliferation [10]. In most of the cancer it has been found that miR1 is down regulated and mainly targets protooncogenes. Hence, down-regulation of miR1 leads to protooncogene activation and cancer. In oral cancer, the functional role of miR1 is not yet studied properly although in Head and neck cancer transgelin2 (TAGLN2) is a direct target of miR1. Silencing of TAGLN2 sigificantly inhibited cell proliferation and invasion in HNSCC. Thus, miR1 may be a potential tumor supressor targetting the oncogenic mRNA TAGLN2 thus regulating tumor growth [10]. Hence, in our study we

have aimed to constitutively overexpress miR1 in oral cancer cell line (SCC-131) to see the effect of this miRNA on cancer development.

AIMS AND OBJECTIVES

- ✓ We aim at validating the deregulated miRNA-1 by quantitative real-time PCR.
- ✓ We aim at stably over-expressing the down-regulated miRNA in OSCC cell line to find the role of this miRNA in cancer pathogenesis.

MATERIALS AND METHODOLOGY

❖ SAMPLE COLLECTION

OSCC diseased tissue and adjacent normal tissue samples were collected from oral cancer patients in RNA Later and stored at -80°C.

❖ RNA ISOLATION FROM TISSUE

Materials and reagents:

- ✓ Liquid nitrogen
- ✓ DEPC treated micro-centrifuge tubes, mortar pestle and tips
- ✓ Qiagen RNeasy Mini Kit
- ✓ Qiashredder
- ✓ Chloroform
- ✓ Ethanol

Protocol:

- ➢ The tissue samples were thawed in ice.
- ➢ The tissue was minced and grounded to form a fine powder using liqui nitrogen.
- ➢ 700µl of Qiazol lysis reagent was added to each sample and mixed well and passed through a Qiashredder column at 13000g for 2min to remove cell debris.
- ➢ The supernatant was transferred to a fresh 1.5ml tube and 140µl of chloroform was added to each tube and mixed vigorously.
- ➢ The sample was incubated at room temperature for 2-3 minutes and centrifuged at 12000g for 15min at 4°C for phase separation.
- ➢ The aqueous phase was transferred to a fresh 1.5ml tube and 1.5 times of 100% ethanol was added for RNA precipitation.
- ➢ The solution was mixed well and transfered to a RNA mini spin column and centrifuged at 8000g for 1min at room temperature.
- ➢ 700µl of buffer RWT was added to the RNA column and incubated for 6-7mins and centrifuged at 8000g for 1min.

- 500µl of RPE buffer was added to the RNA column and centrifuged at 10000g for 2mins after 5mins incubation.
- 50µl of RNase free water was added to the membrane and incubated for 7-8mins and centrifuged at 9000g for 1min for elution.
- The eluted RNA was placed in ice and quantified using nanodrop spectrophotometry.

❖ CONCENTRATION AND PURITY MEASUREMENT BY NANODROP

Thermo scientific nanodrop was used to measure the concentration and purity of nucleic acid in a sample volume of 1µl.

Protocol:

- 5µl nuclease free water was added for wash.
- The water was wiped off and 1µl of RNase free water was added as blank.
- 1µl of each sample was added respectively to measure the concentration and purity of the isolated DNA.

A260/A280 : The ration of absorbance of 260nm and 280nm to measure the purity of the nucleic acid sample. Ratio of 1.8 is considered as pure DNA.

A60/A230 : The ratio of range 1.8-2.2 is considered as pure nucleic acid. Lower than that indicates that the sample contains copurified contaminants.

❖ cDNA SYNTHESIS

Principle:

cdna is complementary DNA produced from RNA with the help of Reverse transcriptase enzyme. Reverse transcriptase enzyme uses RNA strand as a template and synthesizes a single stranded DNA molecule, that can be used further to create a double stranded DNA molecule. The first strand of cDNA can be directly used as PCR template.

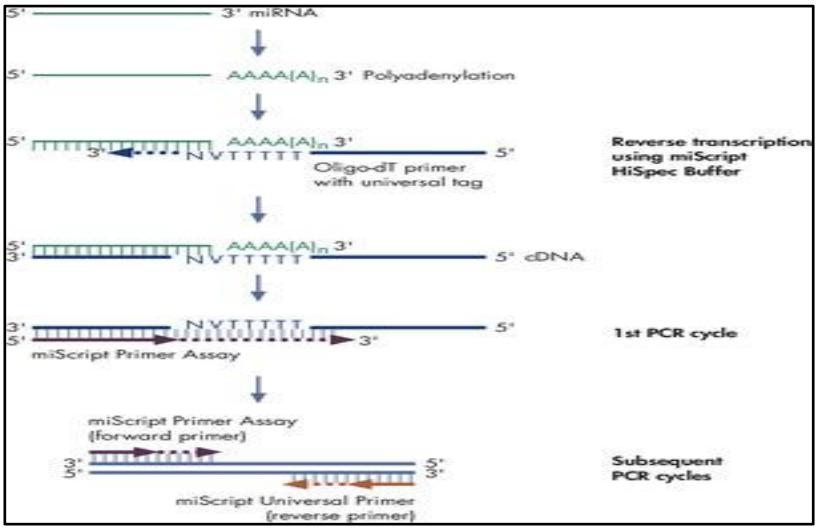


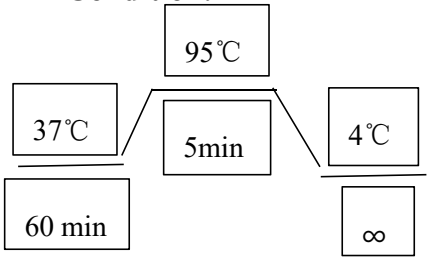
Fig2: cDNA synthesis

Protocol:

- 500ng of RNA was used for cDNA synthesis.

Reagents	**Volume (in µL)**
Water	--
5X buffer	4.0
10X nuclicmix	2.0
Reversetranscriptase mix	2.0
RNA	--
Total	20

Condition:



❖ QUANTITATIVE REAL-TIME PCR

Real time PCR is a technique based on polymarase chain reaction. It monitors amplification of a target DNA molecule in real time and quantify the amount of DNA amplified. In this process very small amount of DNA can be quantified. The quantification is based on the product fluorescent detection. The PCR product is analysed by it's Ct (cycle threshold number) value. A low Ct value indicates a higher expression of the gene while a high Ct value is indicative of low expression. The dissociation curve is analysed to detect that the specific amplicon has been produced in the process The temperature-dependent dissociation between two DNA strands can be measured using DNA intercalating fluorophore such as SYBR green. First the double strand breaks and after PCR amplification. PCR products reannelling stage SYBR green intercalate with the DNA molecule and gives florescence.

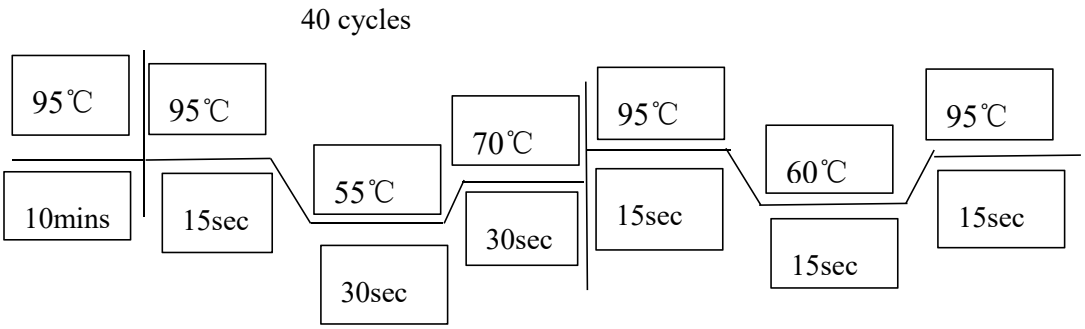
Materials:

- cDNA template for quantitative real-time PCR
- Intercalating dye: SYBR green
- dH2O, DEPC treated PCR tubes and tips
- Universal reverse primer (UF)
- Assorted primer embedded 96-well plates.

Protocol:

Reagents	**Volume/Amount**
dH_2O	--
Universal Forward primer	2.5µL
SYBR	12.5µL
c-DNA	10ng
Total	25.0 µL

Condition:



❖ **PRIMER DESIGNING**

The DNA sequence of the gene of interest was searched and extracted from UCSC genome browser. The whole sequence along with extended downstream and upstream sequence was used for primer designing using the Primer3 software.In-silico PCR was done with the primer designed by the software in UCSC genome browser to ensure that the selected primer is covering the whole gene of interest.
EcoRI restriction sequence was added to the reverse primer followed by a poly A stretch for transcriptional termination while AgeI restriction sequence was added to the forward primer for restriction digestion. A 6nucleotide sequence was added to each primer to ensure proper anchorage of the restriction enzymes.

miR1-1PLKO1F: GGTGGTACCGGTGCCTGCTTGGGAAACATACT
miR1-1PLKO1R: GGTGGTGAATTCAAAAATCCCGGCCTGAGATACATAC

Primer Standardization and determination of the annealing temperature for primer pairs:

Melting Temperature or Tm for a primer pair is usually calculated by the formula = 2(A + T) + 4(G + C); where A=Adenine, G=Guanine, T=Thymine and C=Cytosine.

Usually, Annealing Temperatures for a PCR reaction are set 2-5 °C lower than Melting Temperature.

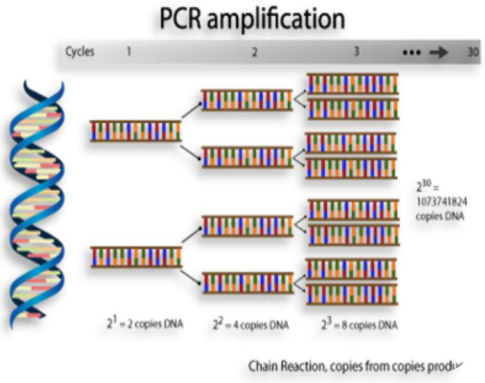
The Tm of miR1-PLKO1 primers were calculated to be 65. Hence, the annealing temperature was kept 2-5 less than the respective Tm for specific binding of the primers to the template.

❖ PCR AMPLIFICATION

Principle:

Polymerase chain reaction is used to amplify a specific region from whole genome or a very small DNA with the help of specific primers. The reaction mixture contains a template DNA fragment which will be amplified, two set of primers (forward primer and reverse primer) and thermo stable taq polymerase (isolated from *Thermus aquaticus)* is used mainly in PCR. In this polymerase chain reaction we have amplified the precursor miRNA from whole genome.

There are three major steps in a PCR, which are repeated for 30 - 35 cycles. This is done in an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.



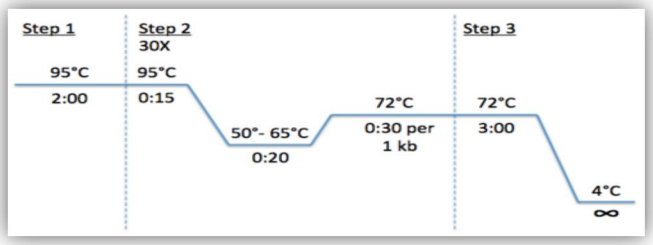
<u>Denaturation at 95°C</u>: During the denaturation, the double strand melts open to singlestranded DNA, all enzymatic reactions stop.

<u>Annealing at 55-60°C:</u>At this temperature polymerase canattach with the primer and template complex and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, which does not break anymore.

<u>Extension at 72°C:</u>This is the ideal working temperature for the Taq polymerase. At this temperature polymerase starts synthesis of a new DNA strand compliment of the template DNA strand. The polymerase adds nucleotides in the 5' to 3' direction.

After this there is an final elongation step for 5-7mins to ensure that if any single strand is left then that will be amplified. After elongation final hold step is performed at very low temperature. This step cools down the reaction chamber.

Both strands are copied during PCR,there is only one copy of the wanted gene before the cycling starts, after one cycle it will be 2, after two cycle it will be 4 and so on it will continues for 35-40 cycles. It increases in 2^n manner.



To start a PCR, it was first required to determine the annealing

temperature of primers and the optimum concentration of $MgCl_2$ at which the PCR is to be carried out. The annealing temperature is the optimum temperature at which the forward and reverse primers would bind specifically to each of the target DNA strands respectively, after the double strand DNA has been denatured. The annealing temperature of each primer pair was taken to be 5℃below the calculated melting temperature.

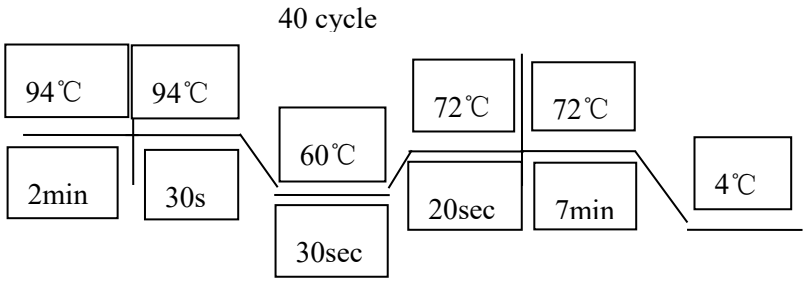
Materials:

- Template DNA
- Primers - Forward primer and Reverse primer
- Taq polymerase
- Deoxynucleotide triphosphate (dNTPs)
- 5X buffer
- $MgCl_2$

Protocol:

Reagents	**Volume (in µl)**	**Concentration**
5X buffer	10	1X
2mM $MgCl_2$	3	1.5mM
10mM dNTPs	0.5	100µM
Forward primer	1.5	500nM
Reverse primer	1.5	500nM
dH_2O	31.25	--
Taq polymerase	0.25	0.25U
DNA	2.0	
Total	50	

Condition:



❖ PCR PURIFICATION

- 5 times PB buffer was added to the digested product and the mixture was transferred into a mini-elute column
- It was centrifuged at 13000 rpm for 1min and the flow through was discarded
- 750µl of PE buffer was added and incubated for 5mins and centrifeuged at 13000rpm for 1min
- 500µl of PE buffer was added and incubated for 5mins
- Centrifuged at 13000rpm for 1min
- 12µl of EB buffer was added for elution and incubated for 5min and centrifuged at 13000rpm for 1min

❖ VECTORS USED FOR CLONING

Lentiviral packaging vectors are used to generate virus particles. Lentiviral vectors are a type of retrovirus vectors which can infect both dividing and non-dividing cells. Their preintegration complex can get through the intact membrane of the nucleus of the cell. Thus, it is very helpful for gene cloning and gene therapy. For lentivirus generation, the genes involved in virus particle generation are present on different plasmids. A lentiviral plasmid, an envelope plasmid and a packaging plasmid are transfected together for lentivirus generation.

We have used PLKO1 vector as a transfer plasmid containing the insert. It is a third generation vector. The PLKO1 vector size is 7032bp having a stuffer sequence of 1900bp. The stuffer is cleaved by restriction digestion with AgeI and EcoRI. Pre-miRNAs digested with Eco-R1 and Age-1 is cloned in place of the stuffer sequence. The PLKO1 vector has ampicillin selection marker for bacterial system and puromycin selection marker for selection in mammalian cells.

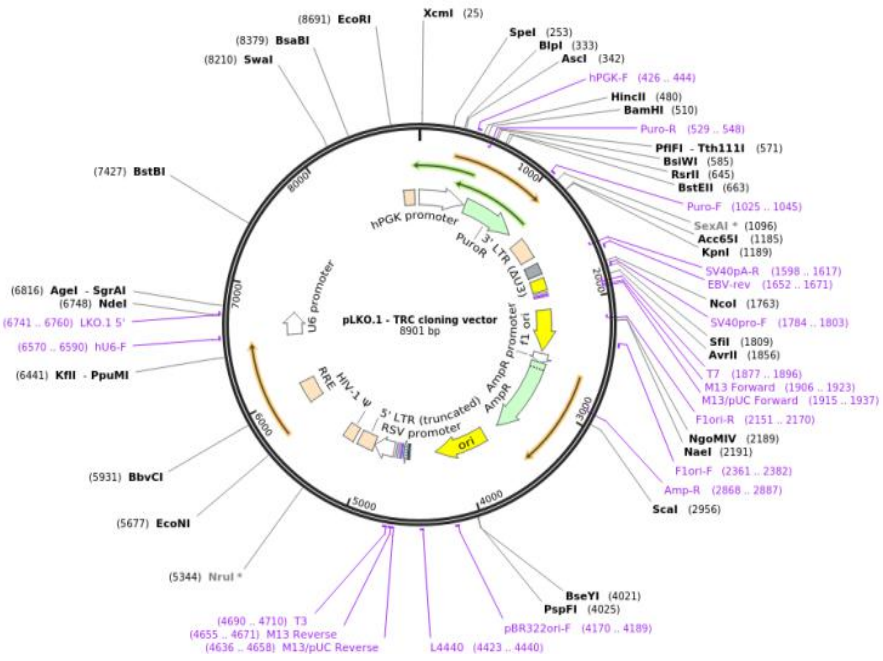


Fig3: PLKO1 vector construct

We have used psPAX2 as packageing plasmid and PMD2G as an envelope plasmid.

Vector name	**Size**	**Vector type**	**Bacterial selection marker**	**Copy no.**
psPAX2	1073bp	Mammalian expression, lentiviral packaging	Ampicillin	High
PMD2G	5824bp	Mammalian expression, lentiviral envelope	Ampicillin	High

❖ RESTRICTION DIGESTION OF THE AMPLIFIED DNA AND PLASMID

Principle:

Restriction enzymes are nucleases which can cut the sugar-phosphate backbone of DNA, found in bacteria. As they cut within the molecule, they are commonly called restriction endonucleases. They specifically cleave the nucleic acids at specific nucleotide sequence called Restriction sites to generate a set of smaller fragments with either sticky ends or blunt ends .

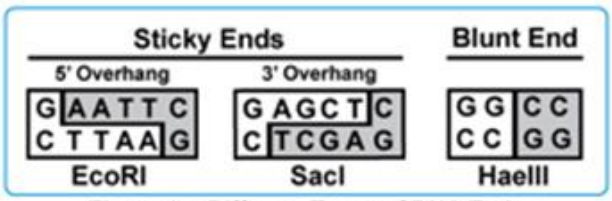


Figure 1 -- Different Types of DNA Ends Produced by Restriction Enzymes

Restriction enzymes used for pre-miRNA cloning:

Enzyme name	Cut sequence	Blunt/ sticky end
EcoRI	GAATTC	5’ STICKY END
AgeI	ACCGGT	5’ STICKY END

Double digestion with EcoRI and AgeI Reaction mix:

Solutions	Volume in µL
dH_2O	20.03(for vector) / 20 (for insert)
10X cutsmart buffer	3.0
EcoRI-HF	1.0
AgeI-HF	1.0
Vector(PLKO1)/ Insert	4.97(vector)/5.0 (insert)

The digestion mix was incubated in a water bath at 37 for 1hour and the inserts (pre-miRNA) were PCR purified.

❖ ELECTROPHORESIS

Principle:

Agarose is a polymer extracted from seaweeds. Its Purified agarose is a powder insoluble in water or buffer at room temperature but dissolves on boiling. Molten solution of agarose is poured into a mould for solidification. On cooling agarose undergoes polymerization i.e. sugar polymers cross-link with each other and cause the solution to gel, the density or pore size of which is determined by the concentration of agarose.

Electrophoresis is a technique used to separate charged molecules. DNA is negatively charged at neutral Ph and when electric field is applied across the gel. DNA migrates towards the anode. Migration of DNA through the gel depends upon:

- ✓ Molecular size of DNA
- ✓ Agarose concentration
- ✓ Conformation of DNA
- ✓ Applied current

Since DNA is not naturally coloured, it will not be visible on the gel. Hence, an intercalating dye like Ethidium bromide is added to the agarose gel and DNA bands visualized by examining the gel under UV light.

Protocol:

- ✓ A 0.8% gel was prepared to resolve the digested plasmid.
- ✓ The digested plasmid was loaded in one well and the undigested plasmid was loaded in another well.
- ✓ The DNA bands were visualized and analysed using an UV transilluminator.

❖ GEL EXTRACTION OF DIGESTED PLASMID

Principle:

Gel extraction was performed to extract and purify DNA from agarose gels . Gel extraction involves four basic steps: identifying the fragments of interest, isolating the corresponding bands, isolating the DNA from those bands, and removing the accompanying salts and stain. Gel extraction was performed by using QIAquick Gel Extraction kit.

Protocol:

- ➢ The DNA fragment was excised from the agarose gel with a clean, sharp scalpel.
- ➢ The gel slice was weighed in a colourless tube.
- ➢ 3 volumes of buffer QG was added to 1 volume of the gel (considering 100 mg = 100 µl)The gel slice with buffer QG was incubated at 56 º C for 10 mins.After the gel slice was dissolved completely, the colour of the mixture was checked to be yellow.
- ➢ 1 gel volume of isoprpapnol was added to the sample and mixed well.
- ➢ A QIAquick spin column provided with a 2 ml collection tube was taken.
- ➢ The sample was applied to the QIAquick spin column. To bind DNA it was centrifuged at 13,000rpm for 1 minute.
- ➢ The flow through was discarded and the column was placed back in the same collection tube. (The maximum volume of the column reservoir is 800µl . For sample volumes more than 800 µl the column was simply loaded again).
- ➢ 500µl of buffer QG was added to the column and was centrifuged for 1 minute. 750µl of buffer PE was added and centrifuged for 1 minute to wash the column.
- ➢ The flow through was discarded and the column was centrifuged for an additional 1minute to flow away the remaining alcohol.

➢ The column was placed into a clean 1.5 ml microcentrifuge tube.The DNA was eluted by adding buffer EB.The column was allowed to stand for 1 minute and then centrifuged at 13,000rpm for 1 minute.

❖ LIGATION

Principle:

Ligation is a method in molecular biology to join two DNA strands by enzymatic reaction. A phosphodiester bond is formed between 3' hydroxyl group of one strand and 5' phosphoryl group of the other strand. This process is mainly used in molecular cloning to join one DNA fragment to another to create a recombinant DNA fragment. T4 DNA ligase enzyme is mainly used for this process. This process is done in 37°C temperature which is the optimum temperature of T4 DNA ligase.

Ligation reaction for pre-miRNA cloning:

Reagents	**Volume/Amount**
dH_2O	--
10X buffer	1µl
Vector	100ng
Insert	25ng
Ligase	1µl

❖ TRANSFORMATION

Principle:

Transformation is one of the three forms of horizontal gene transfer that occurs in nature among bacteria in which one gene encoding a protein passes through one bacteria to another bacteria. In this process bacteria take up some other gene from nature which enters into the recipient genome by homologus recombination. In molecular biology this natural technique of bacteria is used to transfer any recombinant plasmid into the bacteria. For this competant cell is needed. Competence

is a temporary state in which the bacteria can uptake any external gene from environment.

Materials:

- LB medium
- Ice-cold 100mM CaCl2 solution
- ice cold solution of 80mM $MgCl_2$ + 20mM $CaCl_2$
- LA plates containing ampicillin (100ug/ml)
- Ligated DNA product
- 42°C water bath
- 37°C incubator with shaker, spreader, SOC media
- Stbl3 competent cells

Competent Cells Preparation and Tansformation protocol:

- Stbl3 cells were cultured overnight in a 37℃ shaker.
- 1% Stbl3 cells were cultured in fresh LB media (500ul in 50ml LB) and incubated at 37^{O}C with mild shaking for 1.5-2hrs (until log phase).
- 25ml of culture was taken in 50ml tubes and centrifuged at 4^{O}C at 4000rpm for 5mins and the media was discarded.
- The pellet was resuspended in 4ml ice cold solution of 80mM $MgCl_2$ + 20mM $CaCl_2$ and centrifugation at 4000rpm for 5mins at 4^{O}C.
- The pellet was resuspended in 1ml ice-cold 100mM $CaCl_2$ and kept on ice for 1-1.5hrs.
- 10µl of the ligated product was added to 100µl competent cell and kept in ice for half an hour and a heat shock was applied at 42 ^{O}C for 45secs.
- The tubes were immediately placed in ice and 900µl of SOC media was added and placed in a 37 ^{O}C shaker incubator for 1hr.
- It was centrifuged at 5000rpm for 8mins.
- 800µl of the supernatant was discarded and the pellet was resuspended in the remaining media and plated in LA plate (antibiotic- ampicillin)
- The plates were incubated at 37 ^{O}C for 16hours.

❖ **COLONY PCR**

Principle:

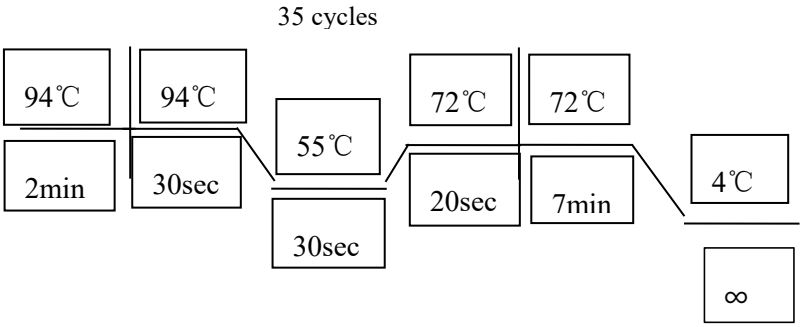
Colony PCR is used after transformation to screen colonies for the desired recombinant DNA. Individual transformants are lysed at high temperature so that they are lysed and the DNA is released. Insert specific amplification is done with insert specific primers for miR1-PLKO1 colonies. Thus, any colony which gives rise to an amplification product of the expected size are likely to contain the plasmid DNA with the desired insert. Presence or absence of a PCR amplicon and size of the product are determined by electrophoresis alongside of a DNA size marker on an agarose gel.

Protocol:

- Transformants are collected in water and an initial heat of 95°C for 10mins is applied for cell lysis.
- Colony PCR was done using colony PCR primers.

Reagents	**Volume (in µL)**	**Amount**
dH_2O	5.25	--
5X buffer	2.0	1X
25mM MgCl2	0.6	1.5mM
10mM dNTPs	0.1	100nM
Forward primer	0.5	500nM
Reverse primer	0.5	500nM
Taq polymerase	.05	0.25U
Template DNA	1.0	
Total	10.0	--

Condition:



Positive clone slection:

- PCR products were run on an agarose gel and visualized under UV light
- Positive bands were selected by measuring the band size
- Positive colonies were then cultured in LB

❖ PLASMID ISOLATION

- The overnight culture was centrifuged at 30000rpm for 5mins and discarded the supernatant.
- The cell pellet was resuspended into 250µl buffer GSPB1 with 1µl of RNase.
- 250µl of buffer GSPB2 was added.Complete cell lysis will give a pink colour
- 750µl of buffer GSPB3 was added and mixed for the solution to became white and centrifuged at 13000rpn for 15mins for phase separation.
- The aqueous phase was added to a Gmini spin column and centrifuged at 13000rpm for 1min.
- The column was washed with 600µl of wash buffer and centrifuged at 13000rpm for 1min
- 50µl elution buffer was added for elution of the plasmid and centrifuged at 1300rpm for 1min

- Concentration and purity was measured using nanodrop spectrophotometry.

❖ LENTIVIRAL PACKAGING AND TRANSDUCTION FOR STABLE CELL GENERATION

❖ TRANSFECTION

Principle:

Trnasfection is process of introduction of foreign genetic materials in a mammalian cell. It means transformation by infection. Tnasfection can be of two types - non-viral transfection and viral transfection. In non-viral transfection three methods are used- chemical based transfection, non-chemical based transfection and particle based transfection. In viral transfection, DNA is introduced into cells using virus as a carrier. This method is called tansduction.

We have used lenti-virus (genus of retrovirus) for the transfection and over-expressionof miR1 in SCC-131 cell line. We have used two plasmids, pSPAX2 and PMD2G, which contains packaging and envelope gene of the virus respectively. It is a very useful in-vitro gene delivery system in mammalian cells. Virus mediated transfection results in integration of the insert DNA in the genome of the host cell by homologous recombination and thus, constitutive expression.

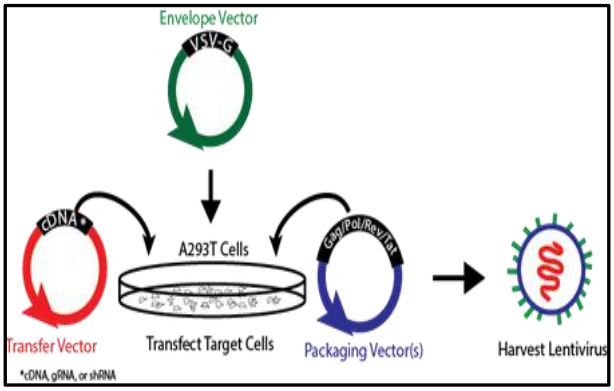


Fig4: Mechanism of Lentiviral packaging

Materials:

- Optimem media (antibiotic and serum excluded media)
- DMEM + 10%FBS(complete media)

- Lipofectamin
- Penicillin and streptomycine mixture
- HEK-293T cell line
- Plasmids used: PLKO1-miR-204, psPAX1 and PMD2G
- 60mm plate
- 1.5ml microcentrifuge tube
- 15ml tube

Protocol:

Day1:

HEK-293T cells were split into two 60mm dish.

Day2:

- OPTI-MEM media was added to HEK-293T cultured dishes and incubated for 4hours.
- 200µl of OPTI-MEM media, 2µl of lipofectamine, 1µg of miR1, 750ng of psPAX2 and 500ng PMD2G was mixed well and incubated for 25minutes and added to one 60mm dish and the other dish was kept as a transfection control.
- After 6hrs of tranfection the media was changed with complete media.

Day3:

After 24hours of transfection the media containing the virus particles was harvested and filtered using a 0.45µ filter. The virus was stored at 4℃.

Day4:

The remaining virus particles were harvested and filtered after 48hours of transfection and pooled with the 24hours harvested media.

❖ **DETERMINATION OF OPTIMAL PUROMYCIN CONCENTRATION FOR SCC-131 CELL LINE**

Materials:

- SCC-131 cell line
- 6 well plate
- Puromycin
- DMEM + 10%FBS +1%PenStrep

Protocol:

DAY1:

- SCC-131 cells were seeded in a 6-well plate in 3ml of complete media.

DAY2:

- Increasing concentrations of puromycin was added to the cells.
- Well 1: 0µg/ml; Well 2: 0.3µg/ml; Well 3: 0.5µg/ml; Well 4: 1µg/ml; Well 5: 2µg/ml; Well 6: 3µg/ml

DAY3:

- 70% of the cells in Well 4 containing 1µg/ml puromycin died after 24 hours of incubation. The cells in the wells containg lesser concentrations of puromycin did not show any significant difference with the control. Hence, 1µg/ml puromycin was determind to be minimun inhibitory concentration for SCC-131 cell-line.

❖ **TRNASDUCTION**

Materials:

- SCC131 cell line (70% confluent)
- Polybrene
- DMEM + 10% FBS + 1% PenStrep
- DMEM + 10% FBS + 1% PenStrep + 1µg/ml puromycin

Protocol:

- SCC131cells were cultured in a 6-well plate.

- 3ml of DMEM supplemented with 10%FBS and 1%Penstrep was added in each well.
- 4µg/ml polybrene was added to each well for efficient transduction.
- 50µl, 100µl and 500µl of harvested virus particle was added in 6 wells in duplicate.
- An wildtype SCC-131 cell was kept as a transduction control.
- Media was changed with puromycin(conc- 1µg/ml) containing media after 24hrs of transduction.
- Subsequent media change with puromycin containing media was performed as per the requirement.

❖ **RNA ISOLATION FROM STABLE CELLS**

➢ Media was removed from the 6-well plate and 1ml of ice-cold PBS was added for washing

➢ 1ml of trizol was added and cells were scraped with a cell scraper and transferred into a 1.5ml microcentrifuge tube

➢ 200µl of choloroform was added, mixed well and incubated for 5mins at room temperature and centrifuged at 12000rpm for 15mins at 4℃ for phase separation

➢ Aqueous phase was collected in a fresh 1.5ml microcentrifuge tube and 500µl of isopropanol was added and mixed by inverting and incubated for 10mins

➢ Centrifuged it for 10mins at 12000rpm for 10min at 4℃

➢ Supernatant was discarded and 75% of ethanol was added and mixed well

➢ It was centrifuged at 7500g for 5min at 4℃ and the pellet was air dried for 10mins to remove any traces of ethanol and dissolved in 20µl RNase free water.

➢ The concentration and purity of the isolated RNA was measured spectrophotometrically using nanodrop.

➢ cDNA was synthesized using 500ng of RNA as template.

➢ 10times diluted cDNA was used for qPCR analysis.

❖ QUANTITATIVE REAL-TIME PCR

Real time PCR is a technique based on polymarase chain reaction. It monitors amplification of a target DNA molecule in real time and quantify the amount of DNA amplified. In this process very small amount of DNA can be quantified. The quantification is based on the product fluorescent detection. The PCR product is analysed by it's Ct (cycle threshold number) value. A low Ct value indicates a higher expression of the gene while a high Ct value is indicative of low expression. The dissociation curve is analysed to detect that the specific amplicon has been produced in the process The temperature-dependent dissociation between two DNA strands can be measured using DNA intercalating fluorophore such as SYBR green. First the double strand breaks and after PCR amplification. PCR products reannelling stage SYBR green intercalate with the DNA molecule and gives florescence.

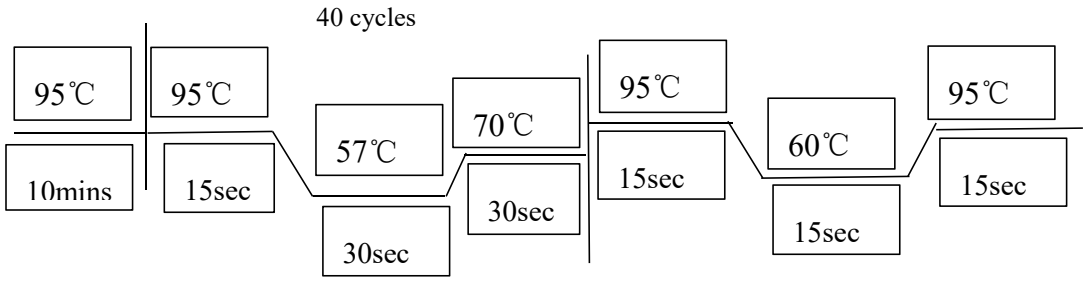
Materials:

- 10times diluted cDNA was used as template for quantitative real-time PCR
- Intercalating dye: SYBR green
- dH2O, DEPC treated PCR tubes and tips
- Universal reverse primer (UF)
- miR-1 expression primer
- 96-well plates.

Protocol:

Reagents	Volume/Amount
dH_2O	--
Universal Forward primer	0.3
emiR-1 Reverse primer	0.3
SYBR	5.0
c-DNA	12.0
Total	10.0

Condition:



- **FUNCTIONAL ASSAY**
- **WOUND HEALING ASSAY**

Principle:

Cell-cell interaction has a very important role in multicellular organism. It helps cells to communicate with each other and survive in the microenvironment. Many protein like cadherin, calnexin, integrin etc helps cells to interact and connect with each other. Any gene mutation or disfunction if affecting these proteins results in a lost of cell-cell interaction. Cadherin has a major role in contact inhibition and hence, any mutation in this protein causes a loss in contact inhibition. As a result of this, abnormal cell growth and migration is observed. In normal cells, growth is regulated by contact inhibition which prevents cells from abnormal gorwth and migration. However, cancer cells have lost the property of regulated growth and it can migrate and proliferate infinitely due to loss of cell-cell interaction.

Wound healing or migration assay is a in-vitro assay to measure cell migration. It is a cost-effective assay to measure migration of cancer cells in-vitro.

Materials:

- SCC-131 wildtype(WT) cell line
- SCC-131_miR1 stable cell line
- Media, 35mm culture dish, tips, PBS
- Inverted microscope

Protocol:

- SCC-131_WT and SCC-131_miR1 cells were seeded in 35mm culture dish containg 3ml of complete media.

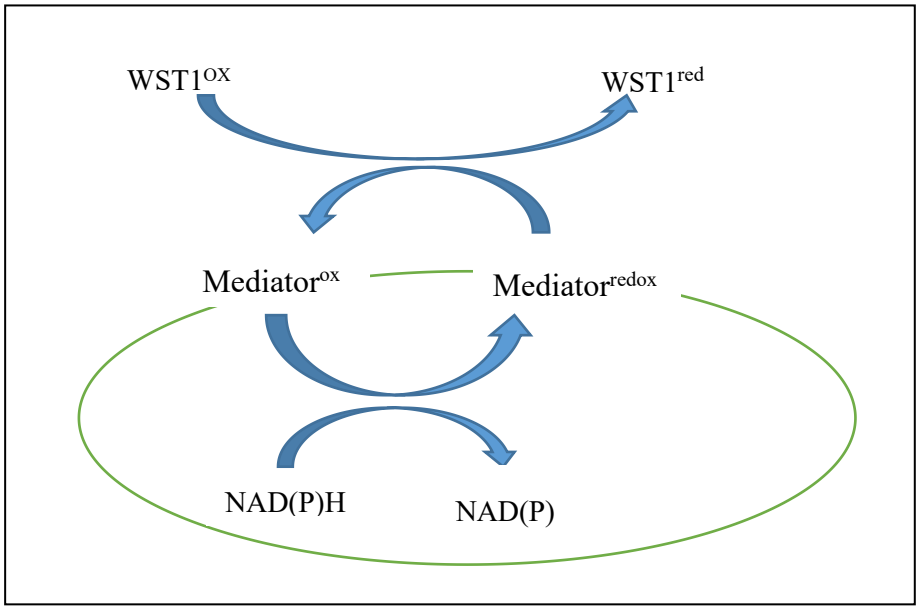
➢ After 24 hours 100% confluency was attained and hence the plates were scratched with a mini tip and washed 2-3 times with PBS to remove the scraped off cells.

➢ The cells were monitered after regular intervals under 10X magnification of an inverted microscope.

❖ CELL PROLIFERATION ASSAY

Principle:

WST1 assay or cell proliferation assay is a measure of the proliferation rate of different cell-lines in-vitro. It is a colorimetric assay to measure the relative proliferation of cells. The assay is based on the conversion of WST, a tetrazolium salt to a coloured soluble dye called formazan through a complex oxidation-reduction reaction. NAD(P)H is produced in the cell as a result of various metabolic pathways like glycolysis. The mitrocondrial dehydrogenase enzyme oxidizes the NAD(P)H to NAD(P). This reaction in turn results in a complex cellular reaction resulting in the reduction of WST1 on the cell surface to an orange coloured product called fromazan. Formazan is a water soluble dye having an absorbance maxima between 420-480nm and the amount of formazan produced is directly proportional to the number of viable cell.



Cell counting by hemocytometer

Principle: Hemocytometer is a special type of microscopic slide consisting of two chambers which is divided into nine (1.0mm*1.0mm) large squares which are separated from one another by triple lines.

Protocol:

✓ SCC131_WT and SCC131_miR1 cells were trypsinized and resuspended in 1ml of media.

✓ 10µl of 0.4% Trypan blue and 10µl of each cell were mixed and 10µl of the mix was used to count the cell number in duplicates with a hemocytometer under an inverted microscope.

✓ For WST1 assay, cells were seeded in a 96-well plate at different dilutions ranging from 2*10^4 - 5*10^4cells/well. Cells were seeded in triplicates in 100µl of media per well.

✓ After 24hours of seeding, 10µl of wst1 reagent was added to each well. 10µl wst1was added to 100µl of media in another well as blank .

✓ The abosrbance was measured at 450nm by a ELISA plate reader after regular time intervals of time (15mins).

OBSERVATION AND RESULTS

Validating the expression of miR1 in OSCC vs. adjacent normal tissues

RNA was isolated from paired tissue samples and concentration and purity was measured by nanodrop. c-DNA was produced from 500ng of RNA template for quantitative PCR. With the Ct values paired t-test was performed and P value was calculated to be 0.02. So, we can conclude that miR1 expression in OSCC tissue is significantly down-regulated.

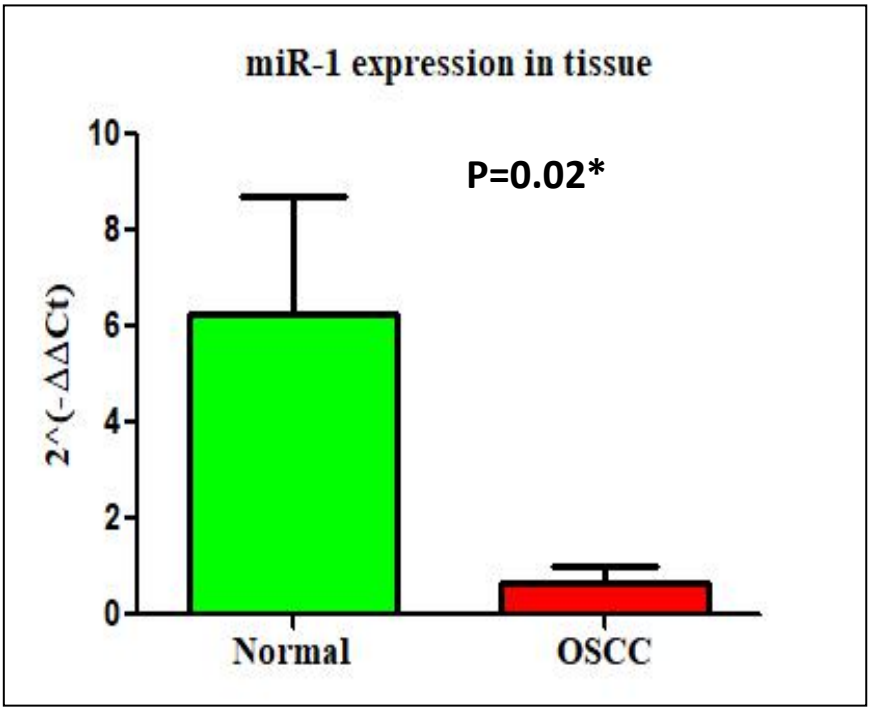


Fig5: Expression of miR1 in normal vs. OSCC tissue

PCR amplification of pre-miRNA

Genomic DNA was used to amplify the pre-miRNA gene with specific primer. The PCR product was run in a 2% agarose gel with 100 bp DNA ladder used as amplicon-size marker to check for specific product size. On comparison with the marker, the desired product band was confirmed. The product size was 98bp .

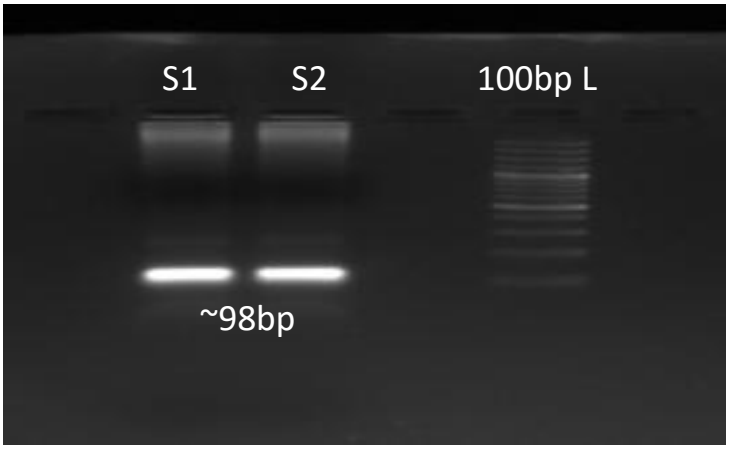


Fig6: PCR products run in a 2% agarose gel (L indicates ladder)

Since single band was observed for both of the PCR products, the remaining product was PCR purified.

Nanodrop table of PCR purified product:

Sample	Concentration(ng/µl)	Purity (260/280)
MiR-1	207	1.83

Vectors and PCR amplified products were double digested for ligation.

Double digestion:

PLKO1 double digestion

PLKO1 vector was double-digested with EcoRI and AgeI. Digested product was run in a 1% agarose gel along with 1kb DNA ladder. Presence of a 1.9kb stuffer band indicates complete digestion of vector. The linearised vector backbone was cut out from the gel and the DNA was extracted.

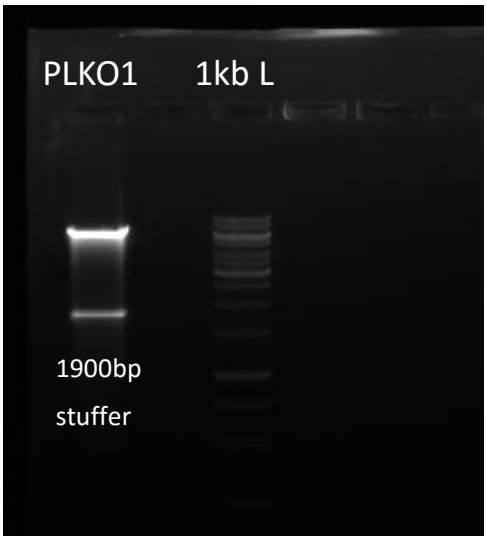


Fig7: Digested PLKO1 vector run in a 1% agarose gel

The digested vector band was excised from the gel and purified with MiniElute gel extraction kit from Qiagen . Digested inserts were purified by PCR purification method with MiniElute PCR purification kit from Qiagen.

Nanodrop table for digested product:

PCR and Gel purified product	Concentration	Purity (260/280)
Pre-miRNA-1	47 ng/µl	1.8
PLKO1 vector	20 ng/µl	1.78

100ng of digested PLKO1 vector and 25ng of digested pre-miR1 was used for ligation. PLKO1 vector was transformed in Stbl3 competent cell respectively. The ligated products were mixed with the competent cells and incubated for 30minutes. The cells were resuspended in SOC media after heat-shock. Ampicillin agar plates were used for transformation of PLKO1 vector. The plates were incubated for 16 hours at in 37℃ and 20 random colonies were selected and used for colony PCR to identify the positive clones. The colonies were also streaked in a replica plate for further experiments.

Identification of positive clones:

The transformed colonies for PLKO1-miR1 were confirmed by colony PCR with insert specific primer. The PCR product size for positive clones were confirmed to be 98bp compared to a 100bp ladder when run on a 2% agarose gel.

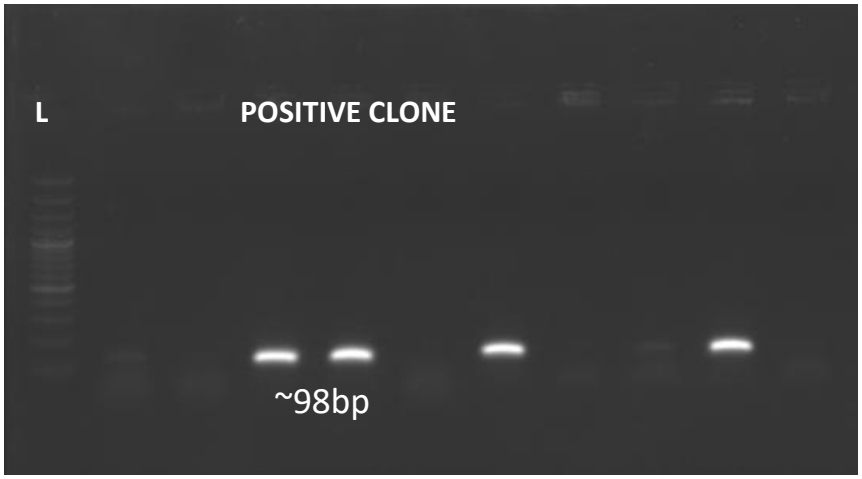


Fig8: Colony PCR products run in a 2% agarose gel for screening of positive clones

The positive colonies were cultured in 10ml LB along with 100µg/ml ampicillin for plasmid isolation. The colonies were further screened and confirmed by double digestion of the isolated plasmids with EcoR1 and Age1 restriction enzymes.

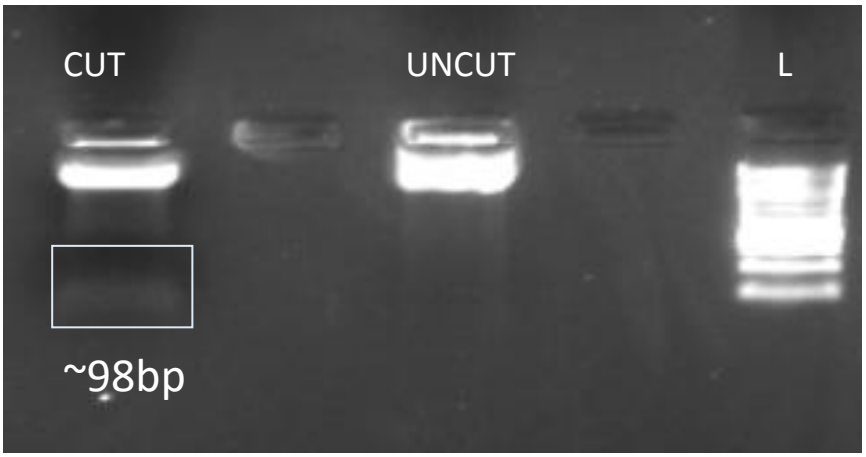


Fig9: Double digested clones run on a 2% agarose gel

PsPAX2, PMD2G and PLKO1vectors were cultured in LB along with 100µg/ml ampicillin for plasmid isolation. After 16 hours incubation in 37℃ plasmids was isolated from the culture.

Plasmid	Concentration	Purity
PLKO-1_miR-1 colony-18	431.8 ng/µl	1.81
PsPAX2	170 ng/µl	1.82
PMD2G	270 ng/µl	1.84

Lentiviral packaging and construction of stable cell lines

HEK293T cells were used for lentiviral packaging. 1µg PLKO1_miR-1-col18, 750ng PsPAX2 and 500ng of PMD2G was transfected in HEK293t cells. The virus was harvested after 24hours and 48hours respectively and three different volumes of the harvested media (50µl, 100µl and 500µl) was used for transduction in SCC-131 cell line. After 24 hours of transduction, 1µg/ml puromycin containing media was added to the cells for selection of stble cells. After 2-3 days of incubation the cell images were captured in 10X magnification under inverted microscope.

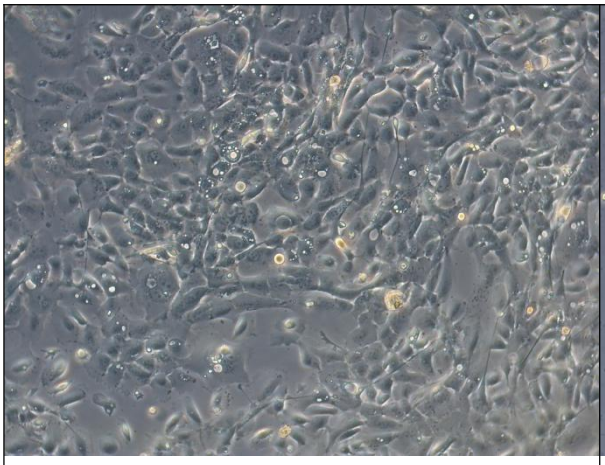


Fig: 10A

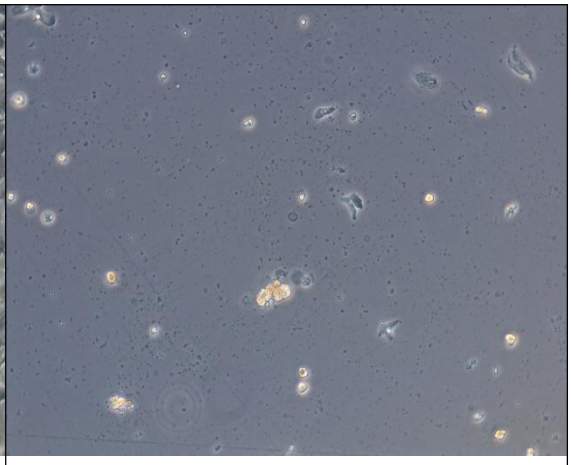


Fig: 10B

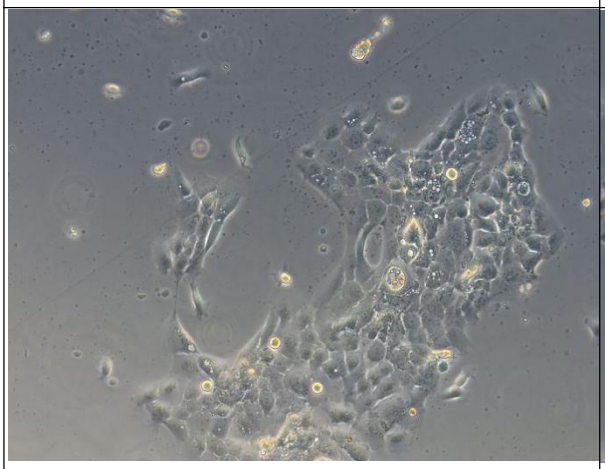


Fig: 10C

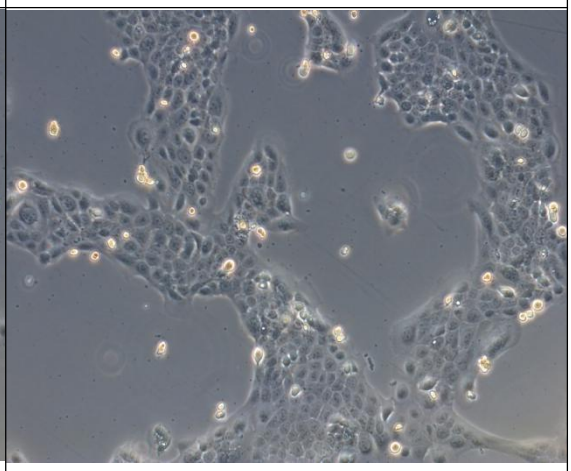


Fig: 10D

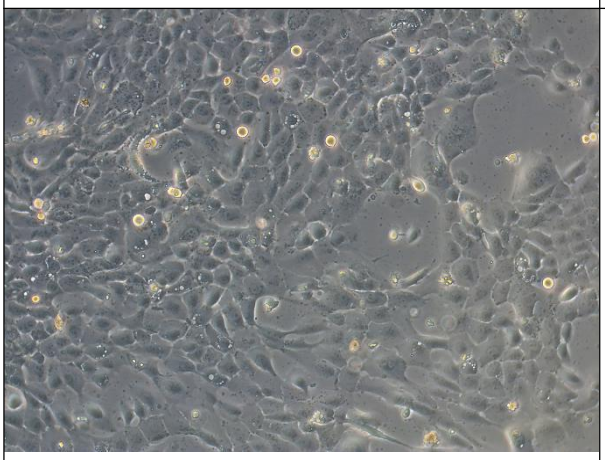


Fig: 10E

Fig 10: Puromycin selection for SCC-131_miR1 stable cells

10A- SCC-131_WT without puromycin

10B- SCC-131_WT + 1µg/ml puromycin

10C- SCC-131_miR1 (50µl virus) + 1µg/ml puromycin

10D- SCC-131_miR1 (100µl virus) + 1µg/ml puromycin

10E- SCC-131_miR1 (500µl virus) + 1µg/ml puromycin

The transducted cells were further confirmed for stable miR-1 integration and over-expression by quantitative real-time PCR. SCC-131_WT and SCC-131_miR1 cells were lysed using trizol reagent for RNA isolation. 500ng of the isolated RNA was used as template for cDNA synthesis and the product was diluted 10times. Quantitative PCR was performed using miR-1 expression primer to check for the expression of miR-1 in SCC-131_miR-1 cells compared to the wildtype. RNAU6 is used as an endogenous control.

Expression of miR-1 in stable cells and W/T cells:

Sample	Ct value for miR-1	Ct value RNAU6	ΔCt
SCC-131_miR1	28.45	24.10	4.35
SCC-131_WT	34.00	25.05	8.95

In Scc-131_mir1cells, we observed a lower Ct value for mir-1 expression, whereas a higher Ct value was observed for SCC-131_WT cells indicating a higher expression of miR-1 in SCC-131_miR1 cells and thus constitutive over-expression. Expression of miR-1 was normalised with that of RNAU6.

A single peak was observed for all the samples for RNAU6 as well as miR-1 indicating the presence of a specific product.

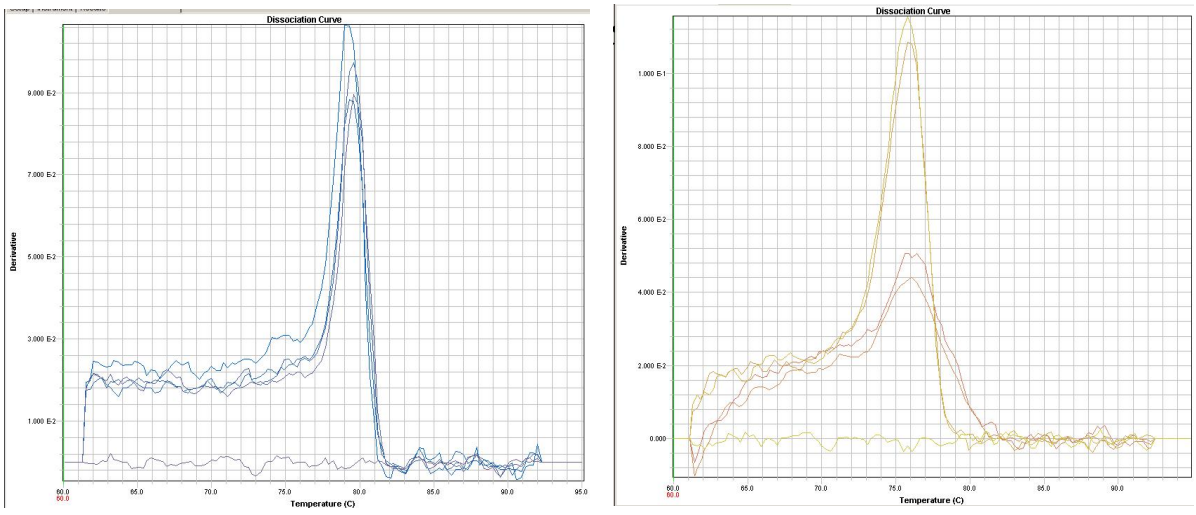


Fig11: Dissociation curves for RNAU6 (left) and miR-1 (right)

To further analyze the role of miR-1 in OSCC pathogenesis, functional studies were performed

Functional Assays:

Scratch assay (Wound-healing assay)

SCC-131_miR1 and SCC-131_WT cells were seeded in a 35mm plate for 100% confluency in 24hours. After 100% confluency the plates were scratched using a mini tip and viewed under an inverted microscope(10X) (Fig). The plates were incubated at 37C in a 5% CO_2 incubator and monitored after regular intervals.

The cells were captured using an inverted microscope(10X) after 4hours and 24hours (Fig:12). SCC-131_miR1 cells migrated very slow compaired to the SCC-131_WT cells in a 24hours time period. So we can conclude that miR1 might have inhibited the migration rat of SCC-131 cells.

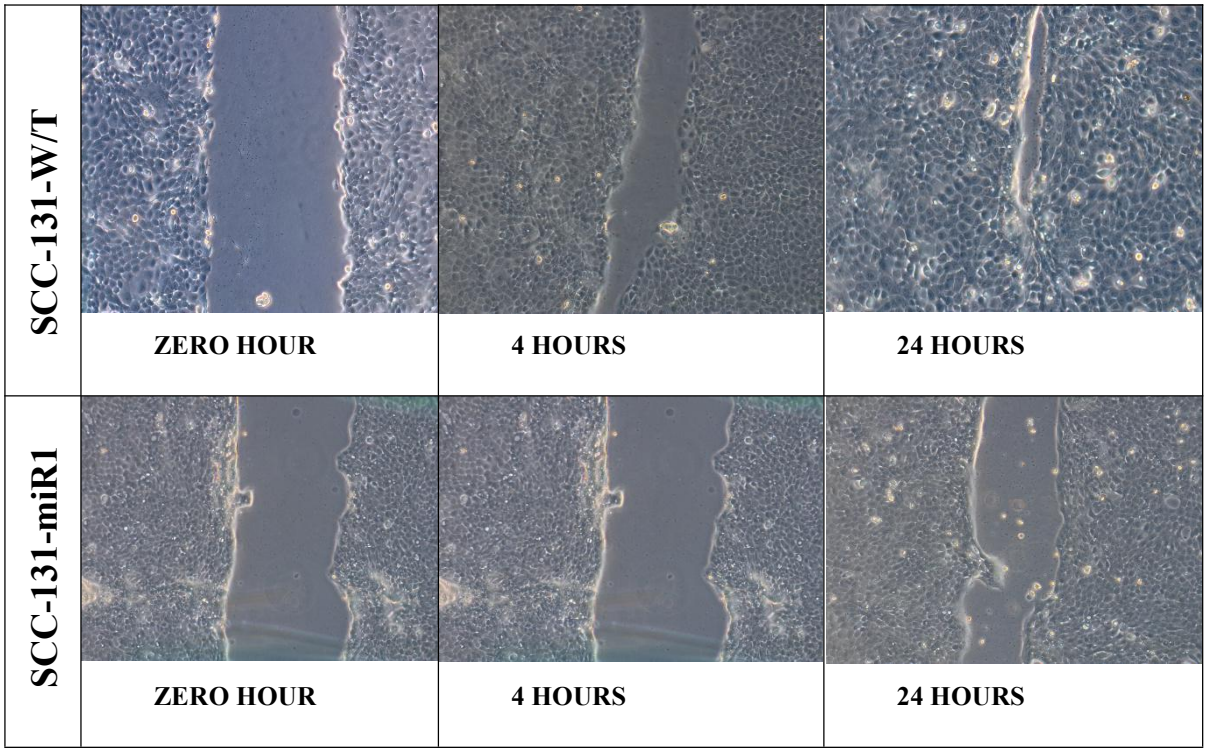


Fig12: Wound healing assay

WST1 proliferation assay:

After 24 hours of seeding, the wells with 4*10^4 cells were 60-65% confluent and hence, was used for wst1 assay. For 100µl media 10µl wst1 was added. 10µl wst1 reagent was added to 100µl of media in another well as blank. Absorbance was measured at 450nm at regular intervals of time by ELISA reader. t-test was done with the normanlised values to find the significance of our result.

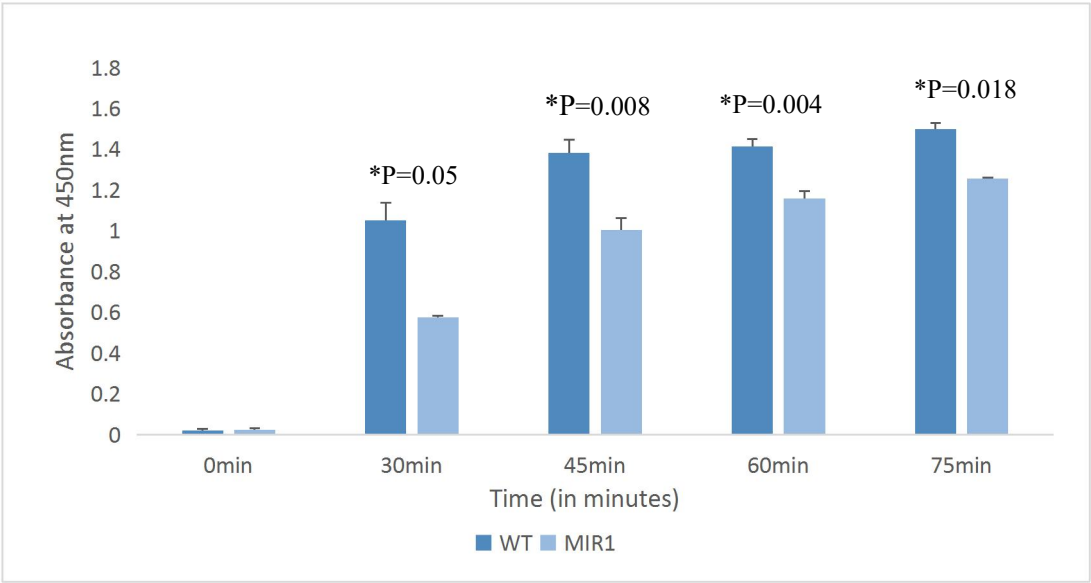


Fig13: WST1proliferation assay

From statistical analysis results it has been found that the absorbance of SCC-131_miR1 was significantly lower than SCC-131_WT cells. So we can conclude that SCC-131_miR1 proliferation rate is lower than SCC-131_WT cells. Hence, miR1 might have a role in inhibiting proliferation of cancer cells.

DISCUSSION AND CONCLUSION

SUMMARY AND EVALUATION

MicroRNAs have recently gained a lot of interest in disease pathogenesis owing to their role in post-transcriptional regulation gene expression. The role of miR1 among Indian OSCC patients has not been studied well. From the sequencing data, miR1 is found to be significantly deregulated in OSCC tissue . So, it might have an important role in cancer development. Hence, we validated the expression of miR1 in additional OSCC paired samples and observed it's role in cancer development.

In previous studies of our laboratory 45miRNAs were found to be deregulated in OSCC tissue compared to the adjacent normal tissue. So, we have validated the expression of hsa-miR-1 by quantitative real-time PCR in 17 additional independent paired tissue samples. miR1 is found to be significantly down-regulated in OSCC compared to the adjacent normal tissue. Hence, it was used for over-expression in a cancer cell-line to generate a stable cell-line and characterize its role in cancer.

Pre-miR1 was cloned into PLKO1 vector. The PLKO1 vector along with psPAX2 and pMD2G plasmids was used for lentiviral packeging in HEK-293T cells. The harvested virus particles were used for transducting SCC-131 cells to produce a stable cell line which will constitutively express miR1. transducted cells were selected using ampicillin and the constitutive over-expression of miR1 in SCC131_miR1 stable cell-line was validated by RNA isolation quantitative PCR method. After validating it's expression, the stable cell line was used for functional characterization of hsa-miR-1 in cancer development. Functional assays like Wound-healing migration assay and WST1 proliferation assay was done in SCC-131_WT vs. SCC-131_miR1 cells. From migration assay it was observed that the migration in SCC-131_miR1 cells was slower than the wild type SCC-131 cells. Hence, we can conclude that miR1 might have a role in regulating cancer cell migration. From WST1 proliferation assay it was observed that the absorbance in SCC-131_WT cells was higher compared to that of SCC-131_miR1 cells. Since, the absorbance of formazan formed in this assay is directly proportional to the number of viable cells, miR1 might have a role in inhibiting the proliferation of the cancer cells.

miR1 is also found to be down-regulated in many cancers like gastric cancer, lung cancer, esophegal cancer, nasopheryngeal cancer etc. In most of the cases it has been shown that miR1 targets proto-onco genes like MET, Pim1, cSrc etc and inhibits tumorigenesis. Hence, down-regulation of miR1 leads to protooncogene activation and cancer. In oral cancer, the functional role of miR1 is not studied elaborately although

in Head and neck cancer transgelin2 (TAGLN2) is reported to be a direct target of miR1. Silencing of TAGLN2 sigificantly inhibited cell proliferation and invasion in HNSCC [10]. Our present findings are in concordance with these reports. We have found that miR1 is down-regulated in oral cancer compared to the normal and from functional assays miR1 over-expression was observed to reduce migration and proliferation in cancer cells.

In HNSCC miR1 targets the TAGLN2 gene, hence, it may also be a probable target mRNA of miR1 in OSCC as well. So, downstream target prediction and validation of those targets would help us in determining the pathway by which miR1 regulates oral cancer pathogenesis.

FUTURE WORK

- More functional assays like colony formation assay, apoptosis assay, invasion assays will be done further to check effect of miR1.
- We have to find the exact target mRNA for the miR1. And which pathway is regulated with that mRNA and what is the exact role of that pathway in cell proliferation and apoptosis in normal cell.
- It should be also checked whether miR1 is effecting any off-target when it is being over expressed in the cancer cells.

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