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## Down-regulation of MicroRNA-137 Improves Proliferation of Oral Squamous Cell Carcinoma by Up-regulation of NF-Y

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**Aim:** The purpose of our study was to investigate the impact of miR-137 suppression on the growth of the OEEM-1 human tongue SCC cell line. In addition, by targeting the NF-Y gene, we also studied the molecular mechanism of miR-137.

**Materials and methods:** The (OEEM-1) & negative control (SCC-15) cells were transfected with microRNA137 inhibitor, MTT assay was utilized to ascertain the cytotoxic impact. SYBER green-based quantitative polymerase chain reaction was used to determine the expression level of both the miR-137 and NF-Y genes in treated and untreated cells. The collected data were subjected to One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test.

**Results:** According to our research, OEEM-1 cells transfected with an inhibitor of miR-137 significantly increased their ability to proliferate when compared to an untreated sample & negative control. Also, we reported that miR-137 is down regulated in OSCC and suppression of miR-137 significantly associated with 1.4 folds increase in NF-Y gene, compared to untreated OEEM-1 cells.

**Conclusion:** MiR-137 is down regulated in OSCC cell lines compared to normal control & its inhibition has a significant increase in cell proliferation which reflects the tumor suppressor role of miR-137 in OSCC. In addition, its inhibition is associated with marked increase in the expression of NF-Y which suggested NF-Y a new target for miR-137.

**Key words:** OSCC, MiR-137, NF-Y, Cell line.

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## Introduction

Oral cancer is a class of malignant diseases originating from the surface of the lips, gums, tongue, mouth, and palate. Worldwide, the most prevalent type of head and neck cancer is oral cancer. It is stated that there were 34,864 new cases worldwide in 2018; these cases are expected to rise to 377,713 in 2020, with a mortality rate of 178,000 cases. Greater than twofold increase from the 185,976 cases identified in 1990, indicating the cancer's rapidly increasing prevalence, that ranks the oral cancer the sixth most common malignancy all over the world.<sup>1, 2</sup>

Oral squamous cell carcinoma (OSCC) accounts for 90% of all oral malignancies as keratinocytes make up the majority of the epithelium of the oral cavity. OSCC has a poor prognosis because of its high potential for recurrence and invasiveness.<sup>3</sup> The 5-year survival rate is still below 50% without a discernible increase in its incidence, despite advancements in diagnosis and treatment, with a higher prevalence among males than females.<sup>4</sup> Oral structures conceal OSCC, which frequently advances slowly. A significant percentage of oral cancer cases are discovered in their later phases, even though the oral cavity is easily accessible for screening and clinical examination.<sup>5</sup>

As with other malignancies, oral carcinogenesis develops gradually as a consequence of a steady accumulation of genetic and epigenetic changes that permanently alter the molecular sequences of DNA regulatory molecules.<sup>6</sup> Of these regulatory molecules, noncoding RNAs—in particular, microRNA—are becoming increasingly significant in the etiology of cancer.<sup>7</sup>

MicroRNAs (miRNAs) are large family of small (22–23 nucleotides), non-coding RNAs, playing critical roles in regulating gene expression, and, consequently, The reason for this is because

miRNAs have the capacity to bind to particular messenger RNAs (mRNAs) and then induce an RNA-Induced Silencing Complex (RISC) to silence the mRNAs.<sup>8</sup>

One-third of the genes in the human genetic material are regulated by 3-4% of miRNA that is encoded by human genes. This highlights the ability of miRNA to control the expression of proteins. One miRNA has the ability to bind to up to 100 distinct mRNAs “one hit, multiple target” and regulate their corresponding protein expression. Numerous biological processes, including development, cell division, proliferation, apoptosis, signal transduction, and cell cycle, are regulated by miRNAs.<sup>9</sup>

Depending on which genes they influence, miRNAs can function as oncomirs or suppressor miRNAs—that block oncogenes. Among the numerous microRNAs with a big impact on cellular biology is miR-137. According to multiple investigations, miR-137 plays a crucial controlling role in brain function and is linked to both proliferation and differentiation during development.<sup>10</sup>

The upstream promoter region of the miR-137 gene, which is situated on chromosome 1p21, contains a sizable CpG island that significantly regulates the gene. DNA-hyper methylating substances have been demonstrated to alter these, which suppresses the transcription of miR-137.<sup>11</sup> At the G1/S phase checkpoint, miR-137 plays a key role for controlling the cell cycle. In a small clinical sample of OSCC, A recent publication found hypermethylation and concomitant downregulation of miR-137.<sup>12</sup>

One transcriptional factor that is widely distributed in creatures such as plants, animals, and other eukaryotes is nuclear transcription factor Y (NF-Y). NF-Y which is The trimeric transcription factor controls a DNA region abundant in promoters of genes called the CCAAT box, that is overemphasized in various types of cancer.<sup>13</sup>

A tight dimer made up of the B and C subunits attaches the third specific A subunit, resulting in a trimer that binds to DNA with high specificity and affinity. The A subunit of the complex participates in sequence-specific interactions, indicating that it may act as a regulatory subunit of the trimer.<sup>14</sup>

The physiological role of NF-Y complex and underlying molecular mechanism in mammals have been thoroughly studied in relation to a variety of cellular activities, which include endoplasmic reticulum stress, cell cycle regulation and DNA damage. recruits further transcription factors and proteins to promote and control more than 1,000 cancer-related genes associated with differentiation, stemness, proliferation, apoptosis, miRNA expression and metabolism.<sup>15, 16</sup>

NF-Y is essential for development, its inhibition is embryonically lethal and changes in NF-Y activity have a variety of behavioral consequences on cells<sup>17</sup>. At different phases of the cell cycle and in response to DNA damage, NF-Y at least partially controls the activity of the CCAAT box-containing promoters of the E2F1, cyclin A, cyclin B1, cyclin B2, cdk1, cdc25C, chk2, topo IIa, cdc25C, and MDR-1 genes.<sup>18</sup>

The aim of this work was to investigate the impact of miR-137 alteration (inhibition) on the proliferation rate of the human tongue SCC cell line (OECM-1) in comparison to normal control cells (SCC-15). Furthermore, we examined its impact on the NF-Y gene's expression.

### Materials and Methods

The proposal for the present study was reviewed and accepted by the Ethical Research Committee of the Faculty of Dentistry, Minia University, Egypt. The committee approval number was: 87.

### Study design

**Group (A)** human tongue squamous cell carcinoma cell line (OECM-1) (treated & untreated).

**Group (B)** normal tongue fibroblast (negative control cells (SCC-15)) (treated & untreated).

### Transduction of (OECM) cells with miRNA 137 inhibitor

This in vitro investigation was carried out on (OECM-1) that was acquired from the cell bank of the Veterinary Serum & Vaccine Research Institute (VACSERA) at Global Research Labs, Medical Center2, Nasr City, Egypt's Cairo.

In 96-well culture plates, the OECM-1 and SCC-15 cells were grown. A day prior to the experiment, 200  $\mu$ L of Dulbecco's Eagle Modified Medium (DMEM) was used. This medium contained 10% fetal bovine serum (FBS), 1% penicillin G sodium (10.000 UI), streptomycin (10 mg), and amphotericin B (25  $\mu$ g) (PSA) (Thermo scientific, Gibco, Germany). 1 x 10<sup>4</sup> OECM-1 cells on average were embedded in the culture medium. Culture plates were kept at 37 °C for 24 hours with 5% CO<sub>2</sub> in order to attain 70% confluence. The following day, a 0.5  $\mu$ L of miR-137 inhibitor was spotted in 3  $\mu$ L of RNase-free water to form the complex.

To get a final concentration of 5 nM for the miRNA mimic and 50 nM for the inhibitor, the complex was added. After adding 0.75  $\mu$ L of HI Perfect Transfection Reagent (cat no. 301704, Qiagen, Hilden, Germany) to 24.25  $\mu$ L of serum-free RPMI culture medium, the mixture is incubated for 10 minutes at 15-20°C to facilitate the creation of transfection complexes. Afterward, 25  $\mu$ L of the complex was added to each well. The transfected cells were incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub> for 48 hours, then the cell viability was tested by MTT assay.

### **Cell Proliferation Assay (MTT) to assess the cells' vitality**

For the cell cytotoxicity assay, Thermo Fisher, Germany's Vybrant® MTT Cell Proliferation Assay Kit, cat. no. M6494, was utilized. The viability of OECM-1 and SCC-15 cells transfected with miR-137 inhibitor was evaluated using the cell proliferation test. Each well acquired 20µL of MTT solution [4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1mg/mL)] (Thermo Scientific, Invitrogen, Germany).

After that, the wells were set up for 4 hours at a temperature of 37 °C with 5% CO<sub>2</sub>. finally, after eliminating the MTT solution from every well, A100 µL of sodium dodecyl sulphate with hydrochloric acid (SDS-HCL) was added. Utilizing a spectrophotometer (ELx 800; Bio-Tek Instruments Inc, Winooski, VT, USA), the optical density at 570 nm was measured to determine the cell viability. Images are captured by COLORED LC-6 USB3.0 CMOS DIGITAL CAMERAS (5MP), Labomed, USA). The magnification:100x, and the scale bar: 50µm for OECM-1 and SCC-15 cells transduced with miR-137 inhibitor, cultured in DMEM media for 48 hours.

### **Evaluation of gene expression in transfected cells**

Measuring The expression levels of the miR-137 and NF-Y genes in (OECM-1) and SCC-15 cells were determined using real-time polymerase chain reaction (PCR).

#### **Cell harvesting from culture media:**

Bead-milling was used to distribute and homogenize an average of 1x10<sup>6</sup> cells in a lysis solution containing guanidine-thiocyanate. Within 15 to 90 seconds, a rotor-stator homogenizer called the Tissue Ruptor II (Qiagen, Hilden, Germany) can completely disintegrated and mixed the tissues when a lysis solution is present. After that, the mixture was centrifuged for 20 minutes at

4000 rpm. Next, the cell supernatant was collected so that RNA may be extracted.

### **Total RNA purification using the RNeasy® Mini Kit**

The homogenate of tissues was mixed with ethanol before being placed onto RNeasy Mini spin column. The RNeasy silica membrane binds to whole RNARNA of superior quality is eluted in RNase-free water. The Qiagen, Hilden, Germany, RNeasy Mini kit, cat no. 74104, was used for the RNA extraction and purification process. The procedure followed the manufacturer's instructions.<sup>19</sup>

### **Synthesis of cDNA using reverse transcription:**

The mi Script RT Kit from Germany, Qiagen in Hilden was used to produce cDNA using reverse transcription. The following ingredients were combined to create the reverse-transcription master mixture: 4µl 5x miScript HiFlex buffer, 2µl 10x miScript dNTP mixture, 2µl miScript reverse transcriptase mixture, and 7µl RNase-free water. A precise quantity of 5 µl of RNA sample was introduced. The tubes were then inserted into the German-made Biometra Thermal Cycler. One cycle of first-strand cDNA synthesis and reverse transcription was carried out for 60 minutes at 37°C and five minutes at 95°C. Until it was amplified, the reverse transcription product, or cDNA, was stored at -20°C.

### **MiR137 and Nuclear transcription factor gamma (NF-Y) gene expression analysis:**

The 5 plex Rotor Gene PCR Analyzer (Qiagen, Germany) was used to analyze each sample. The following is how the PCR reaction mix was made: two microliters of the (10x Universal Primer and 10x Quantitect Primer Assay), plus four microliters of RNase-water free. All of them were added to ten microliters of the 2x QuantiTect SYBR Green PCR Master Mix. Following the proper pouring of the reaction mix into every

Rotor-Disc, 2  $\mu$ l of cDNA template was added, resulting in a total amount of 20  $\mu$ l.

The activation process for Hot Star Taq DNA Polymerase was run for 15 minutes at 95°C in the RT-PCR software. Three-step cycling consisting consist of 40 repetitions of 15 seconds at 94°C for denaturation, 30 seconds for annealing at 55°C, and 30 seconds for extension at 70°C. additionally, 2- $\Delta\Delta$ Ct equation test control was used to calculate the relative expression level (fold change) for the NF-Y gene compared to a calibrator (negative control sample) after normalization to an internal control ( $\beta$ -actin).

### Statistical analysis

To determine whether there was a statistically significant difference in the mean values between the groups, the obtained data were subjected to One-way analysis of variance (ANOVA). Tukey's multiple comparisons test was used to monitor the experiment-wise error rate. This test uses a standardized range distribution and pairwise post-hoc testing to see if there is a difference between the mean of all possible pairs.

### Results

#### [MTT] cytotoxicity Assay:

**Comparative analysis of the proliferation index percentage between cells transfected with miR-137 inhibitor & untreated cells:**

#### Group (A) OECM-1 cells (treated & untreated):

Our findings demonstrated that OECM-1 cells treated with miR-137 inhibitor (mean:  $148 \pm 3.95$ ) showed marked increase in cell proliferation compared to untreated cells (mean:  $102 \pm 1.68$ ), and a highly interesting difference was obtained ( $p < 0.0001$ ) which reflect the tumor suppressor function of miR-137 in OECM-1.

#### Group (B) negative control cells (SCC-15) (treated & untreated):

On the other hand, we transduced the normal human tongue fibroblast(SCC-15)(NC).<sup>20</sup> cells with miR-137, to see if suppression of miR-137 will be harmful on normal cells? The obtained results revealed no significant change occurred in normal cells (mean:  $100 \pm 2.80$ ) after suppression of miR-137 (mean  $105 \pm 2.22$ ) ( $p = 0.09$ ). Data are shown in Table 1 and Figure 1(A&B).

The cytotoxic effect was likewise considerably increased and a notable variation was seen between the two groups ( $p < 0.0001$ ) data present in (Figure2).

**Table 1. Comparative analysis for cell viability in the studied groups**

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
OECM-1 vs. miR-137 inhibitor	0.0946	-0.147 to 0.336	0.4958
OECM-1 vs. SCC-15	-0.8393	-1.08 to -0.597	0.0001**
miR-137 inhibitor vs. SCC-15	-0.9340	-1.17 to -0.691	<0.0001**

CI: confidence interval, \*\*: High statistical difference ( $p < 0.001$ ), \*: Mild statistical significance ( $p < 0.05$ )

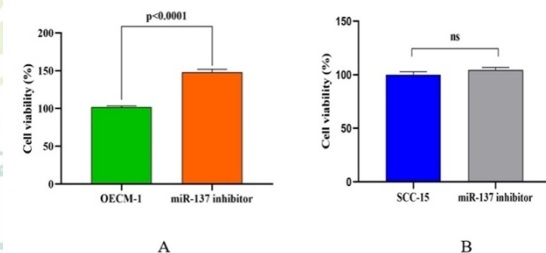


Figure 1. A) Comparative analysis between the proliferation index of OECM-1 cells transfected with miR-137 inhibitor compared to (untreated) cells showed a high significant difference ( $p < 0.05$ ).

B) Comparative analysis between the proliferation index of SCC-15 cells treated with miR-137 inhibitor compared to untreated cells showed no statistical variation between the two groups ( $p > 0.05$ ). (Mean and standard deviation are used to organize the data).

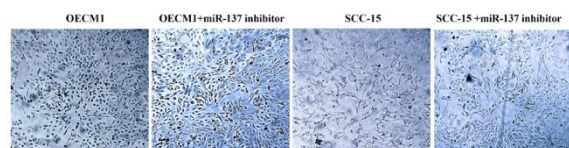


Figure 2. (OECM-1 + miR-137 inhibitor) showed marked increase in the density of cells compared to untreated (OECM-1) and no change occur in the density of (SCC-15 cells transduced with miR-137 inhibitor) compared to (untreated SCC-15).

## PCR results

### Comparative analysis for miR-137 gene expression between OECM-1 cells (treated & untreated) compared to SCC-15 cells (untreated):

Tukey's multiple comparison tests indicated a noteworthy reduction in miR-137 expression of OECM1 cells transfected with miR-137 inhibitor, compared to SCC-15 cells (mean difference: -0.93,  $p < 0.0001$ ). In addition, a marked reduction on miR-137 was significantly associated with malignancy in OECM-1 cells, compared with SCC-15 cells, underscoring the tumor suppressor role of miR-137 (mean difference: -0.83,  $p < 0.0001$ ) (table 2. figure 3A)

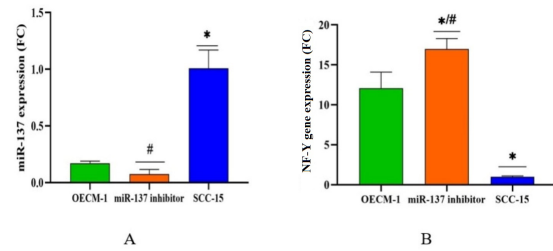
### Comparative analysis for NF-Y gene expression between OECM-1 cells (treated & untreated) compared to SCC-15 untreated cells (negative control).

The obtained results revealed that suppression of miR-137 is significantly associated with 1.4 folds increase in expression of NF-Y in treated (OECM-1), compared to untreated OECM-1 cells (mean difference: 15.98, 95%CI: 12.52 to 19.44,  $p = 0.0001$ ). Furthermore, increased expression level (12 folds) of NF-Y gene was significantly associated with (OECM-1), compared to SCC-15 "negative control" group (mean difference: 11.09,  $p = 0.0002$ ) (table 3, figure 3B).

**Table 3: Multiple comparison analysis for the NF-Y expression in the studied groups (post-Hoc test)**

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
OECM-1 vs. miR-137 inhibitor	-4.888	-8.347 to -1.428	0.011*
OECM-1 vs. SCC-15	11.09	7.632 to 14.55	0.0002**
miR-137 inhibitor vs. SCC-15	15.98	12.52 to 19.44	<0.0001**

CI: confidence interval, \*\*: High statistical significance ( $p < 0.001$ ), \*: Mild statistical significance ( $p < 0.05$ )



**Figure 3(A).** Comparative analysis between the miR-137 gene expression (FC) of OECM-1 cells transfected with miR-137 inhibitor. A significant reduction in miR-137 expression was associated with OECM-1 cells (malignant), compared to normal SCC-15 cells -15 ( $p < 0.05$ ). \*: significant difference compared to the OECM-1 cells, #: significant difference compared to the SCC-15 cells.

**(B)** Comparative analysis between the NF-Y gene expression (FC) of OECM-1 cells transfected with miR-137 inhibitor compared to untreated cells showed a significant increase in NF-Y expression (FC) ( $p < 0.05$ ) following miR-137 suppression. \*: significant difference compared to the OECM-1 cells, #: significant difference compared to the SCC-15 cells.

## Discussion

Cancer is the second most prevalent cause of illness and morbidity worldwide. The biology and incidence of many cancer types are significantly influenced by exposure to risk factors.<sup>21</sup> Oral squamous cell carcinoma (OSCC) is a common cancer of the head and neck with poor survival chance and a significant risk of relapse. Clinically, OSCC includes intra-oral cancer, which mostly affects the tongue, and lip cancer, which accounts for the majority of extra oral SCC occurrences.<sup>22</sup>

The process by which cumulative genetic and epigenetic alterations affect the surface squamous epithelium and modify cellular kinetics from mildly aberrant growth to widely distributed and invasive malignancy is known as oral carcinogenesis.<sup>23</sup>

Since numerous investigations have shown the crucial part which miRNAs play in the onset of cancer, there has been a surge of interest in miRNAs in recent years.<sup>24</sup> MiRNAs are composed of (19–22) nucleotides that bind to the 3'-untranslated

regions (3'UTRs) of the target gene(s) to silence it. This process may enhance the gene's apoptotic or carcinogenic potential.<sup>24,25</sup> MiRNAs are associated with invasion, metastasis, chemoresistance, proliferation of cells, cell cycle arrest, along with apoptosis in oral carcinomas.<sup>26</sup> Out of thousands of human miRNAs, miR137 has been verified in multiple prior research studies as a biomarker indicating dysregulated expression in OSCC.

More than 10 distinct cancer types have been shown to have hypermethylated miRNA-137 promoter regions, which has been involved in transcriptional suppression of miRNA-137 and influences the regulation of numerous biological processes in tumor cells.<sup>27</sup> It is clear that miR-137 has tumor-suppressive properties in a number of cancers, including pancreatic cancer, osteosarcoma, gastric cancer, oral cancer, ovarian cancer, and brain tumors.<sup>28</sup> There are only few studies discussing the involvement of miR-137 in oral squamous cell carcinoma.<sup>29</sup>

The target genes of miR-137 are documented and presumed to play significant roles in cell cycle signaling. An event that triggers the development of cancer, for example oncogenic Ras activation which leads to senescence of cells, is a tumor suppressor response. During Ras-induced senescence, miR-137 targets KDM4A mRNA, and is responsible for activating retinoblastoma (pRb), a tumor suppressor pathway.<sup>30</sup>

One of the original transcription factors implicated in activation of "cancer" genes is nuclear transcription factor Y (NF-Y). The trimeric transcription factor NF-Y regulates the CCAAT box, a DNA region rich in gene activators that are amplified in a range of malignant tumors. The heterotrimer NF-Y, which is made up of the sequence-specific (NF-YA) and the histone fold domain dimer (NF-YB/NF-YC), is what gives CCAAT its

specificity. The oncoming database's profiling of over 60,000 cancer specimens revealed that the two most enriched motifs in the regulators of "cancer" genes were NF-Y and E2F.<sup>31</sup>

Regarding to our MTT results, they revealed that the viability of miR-137 inhibitor-OECM-1 was increased (mean:  $148 \pm 3.95$ ) when compared to untreated OECM-1 cells (mean:  $102 \pm 1.68$ ). A rise that is statistically significant ( $p < 0.0001$ ).

Our results are in agreement with Sun and Li 2018 which undertaken to investigate and find out how human squamous cell carcinoma cell lines 2 (HSC-2 cell line) expressed miRNA-137 and how its expression affected the activity, invasion, and proliferation of OSCC. According to their findings, OSCC cells (HSC-2) treated with miRNA-137 mimics had considerably reduced cell viability, proliferation, and invasion than untreated OSCC (HSC-2) cells ( $p < 0.05$ ).<sup>32</sup>

The results are consistent with Sun et al. 2016 who demonstrated that exaggerated level of miR-137 inhibits the cell proliferation and colony formation of tongue squamous cell carcinoma cells (TSCCs) in cells treated with microRNA 137 mimic.<sup>33</sup> On the other hand, the viability of miR-137 inhibitor-SCC-15 cells was not statistically significant when compared to SCC-15 cells (mean  $105 \pm 2.22$ ) ( $p = 0.09$ ).

Our inverted microscope images support an increase in the cell proliferation of miR-137 inhibitor-OECM-1 when compared to OECM-1 cell (Figure 2). These results are in accordance with (Zhang et al 2018), who reported that miR-137 overregulation inhibited cell proliferation and migration in vitro, and transplantation in vivo in cervical cancer confirmed the suppressive effect of miR-137 on tumor.<sup>34</sup>

We measured miR-137 expression in OECM-1 and the normal (SCC-15) cells. Our obtained results revealed down regulation of

miR-137 in OECM-1 when compared to the expression of miR-137 in SCC-15. These results are agreed with He T et al.2017, They discovered that in OSCC tissues and cell lines, the amount of miR-137 was reduced. Over regulation of miR-137 suppressed the OSCC cells proliferation and colony creation, migration and invasion.<sup>35</sup>

Furthermore, Li et al.'s 2022 study demonstrated that esophageal squamous cell carcinoma (ESCC) had down-regulated miR-137. Patients with ESCC showed an inverse relationship between the expression of miR-137 & their 5-year survival rate. Overregulation of miR-137 encouraged the death of malignant diseased cells and impeded the growth of ESCC.<sup>36</sup>

This study was the first one to provide an overview of the molecular pathway of miR-137 by targeting NF-Y gene. Our results revealed that the suppression of miR-137 was considerably related to 1.4 folds an increasing in level of NF-Y gene, compared to untreated OECM-1 cells ( $p=0.0001$ ) & the difference was statistically significant.

Furthermore, elevated expression level (12 folds) of NF-Y gene was significantly associated with OECM-1 cells compared to (SCC-15) “negative control” group ( $p=0.0002$ ). This may be as a result of down regulation of miRN-137 in OECM-1 cells when compared to normal cells SCC-15(mean difference: -0.83,  $p<0.0001$ ) underscoring the tumor suppressor role of miR-137 as seen in (figure 2).

In human glioma samples, NF-Y expresses itself differently and promotes cell proliferation<sup>37</sup> & its overexpression had been reported in breast cancer<sup>38</sup>, lung adenocarcinoma (LUAD) , lung squamous cell carcinoma ( LUSC)<sup>39</sup>, of all subunits in hepatocellular carcinoma HCC & always associated with poor prognosis of these cancers.<sup>40</sup> The significant number of NF-Y sites (CCAAT) in the promoters of genes that

are highly expressed in cancer is further supported by their findings.

Although over a thousand potential miR-137 targets have been identified, only around five percent of these have been confirmed through experimentation<sup>28</sup>. From all previous data we suggest that NF-Y may be a target for miR-137 and we recommended more researches and investigations to be verified.

## Conclusion

OSCC cell lines have down-regulated MiR-137& its inhibition has a significant increase in cell proliferation which reflect the tumor suppressive role of miR-137 in OSCC. Further we find that NF-Y is up regulated in OSCC cell line than normal control cells. in addition to those findings upon inhibition of miR-137 the expression of NF-Y was markedly increased which suggested NF-Y a new target for miR-137 and may be a future target as a gene therapy for OSCC.

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