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MicroRNA-16, Bcl-2, and MAPK: Potential Biomarkers for Oral Squamous Cell Carcinoma

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Aim: The objectives of the current study were to assess the level of miRNA-16 expression in oral squamous cell carcinoma and its effect on cell apoptosis and proliferation following its suppression. Additionally, the study aimed to investigate the mechanisms that underlie the involvement of miRNA-16 and its target genes, Bcl-2 and, MAPK in the progression of OSCC.
Materials and methods: The Oral Cavity Squamous Cell Carcinoma cell line (OECM-1) and Normal Human Tongue Fibroblasts (HOrF) were used in this in vitro study. The MTT assay was used to evaluate the cytotoxic effect following the cells' transfection with a miRNA-16 inhibitor. In both treated and untreated cells, the expression levels of the miRNA-16, Bcl-2, and MAPK genes were measured using SYBER green-based quantitative PCR. Tukey's multiple comparisons test and One-way analysis of variance (ANOVA) were used to analyze the data.
Results: OECM-1 cells transfected with miRNA-16 inhibitor exhibited a significant increase in proliferation in comparison with the untreated and normal cells. Additionally, a significant increase in the expression of Bcl-2 and MAPK was associated with miRNA-16 inhibition compared to the untreated OECM-1 cells.
Conclusion: The findings suggest that miRNA-16 is a tumor suppressor miRNA that acts by cell proliferation inhibition and apoptosis induction in OSCC by directly targeting Bcl-2 and MAPK. This implies that miRNA-16 could be a potential therapeutic target for OSCC.

Keywords: OSCC, MiRNA-16, Bcl-2, MAPK.

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Introduction

Oral cancer is an oral cavity or lips-originated malignant neoplasia. It is among the top ten most prevalent malignancies in the global.[1]

According to the National Cancer Institute survey, the incidence rates of oral cancer are significantly higher in males than in females. Its incidence increases with age, with a more rapid increase after age 50, especially in adults aged 65 and older.[2]

Oral squamous cell carcinoma (OSCC) is the most prevalent among oral malignancies, accounting for 90% of all malignant neoplasms in the head and neck. The patients' 5-year survival rate is about 50%. however, when tumors are identified at advanced clinical stages, this rate declines to fewer than 30%.[3]

Similar to other cancers, OSCC develops as a result of a multistep process marked by distinct genetic and epigenetic alterations that result in permanent alterations in the DNA sequence of the regulatory molecules. The importance of noncoding RNAs, particularly microRNA, in the etiology of cancer, is becoming increasingly apparent among these regulatory molecules.[4]

Non-coding RNAs (ncRNAs) are gene expression and cellular processes regulating molecules, which have a crucial role in various physiological processes and disease pathogenesis. There are two types of ncRNAs: Small ncRNAs (miRNAs) and long ncRNAs.[5]

Small ncRNAs (i.e., microRNAs and small interfering RNAs) are associated with the pathogenesis of cancer as they influence multiple processes of the cell such as proliferation and differentiation by regulating gene expression post-transcriptionally.[6]

MicroRNAs (miRNAs) are 22–23 nucleotide, single-stranded, noncoding post-transcriptional regulator RNAs. According to miRNA-base, there are around 8,000 human miRNAs that are involved in the regulation of up to 60% of the genome and about 30% of all gene expression.[4]

MiRNAs can bind to particular messenger RNAs (mRNAs) and induce an RNA-Induced Silencing Complex (RISC) to silence the mRNAs. Up to 100 different mRNAs can bind to one miRNA "one hit, multiple targets", which allows it to regulate the expression of the corresponding protein.[7]

There have been reports of some miRNAs having tumor suppressor or oncogenic properties, which suggests their involvement in invasion, metastasis, and carcinogenesis.[8]

MicroRNA-16 (MiRNA-16) is found on chromosome 13q14 and its absence contributes to malignancy pathogenesis as in B-cell chronic lymphocytic leukemia.[9] It may have a direct effect on tumor apoptosis, invasion, metastasis, cell proliferation, and tumorigenicity.[10]

As a tumor suppressor, miRNA-16 decreases expression of PI3K/Akt signaling pathway encoding genes such as MTOR and Bcl-2. PI3K/Akt signaling pathway is affiliated with the progression of cell cycle, proliferation, and survival.[11]

The B-cell lymphoma 2 (Bcl-2) protein family members possess pro- and anti-apoptotic activities and their highly conserved BH domain is vital to exert their function. Bcl-2 family molecules are maintained at a state of equilibrium in healthy cells and based on the homology and function of individual proteins are categorized into three subfamilies.[12]

These subfamilies include the pro-apoptotic BAX and BAK proteins, the anti-apoptotic Bcl-2 and Bcl-XL proteins, and BH3-domain-only BAD and BID proteins.[13]

Bcl-2, as an anti-apoptotic Bcl-2 family member protein, inhibits apoptotic pathways as a result promotes cell survival. Bcl-2 is frequently overexpressed in cancers, such as OSCC.[14]

Additionally, Bcl-2 has been proposed as a useful early-stage predictor of normal oral

mucosa malignant transition to dysplastic lesions in oral carcinogenesis.[15] Thus, Bcl-2 can be regarded as a potential target for tumor drugs or a biomarker for tumor diseases.[13]

Members of the serine/threonine protein family, Mitogen-activated protein kinases (MAPKs) are established regulators of cellular processes. The MAPK pathway is a crucial signaling hub that integrates extracellular signals to regulate drug resistance, as well as cell survival, proliferation, differentiation and senescence.[16] MAPK pathway-activating mutations are prevalent in approximately 20% of head and neck squamous cell carcinoma (HNSCC) cases.[17]

The MAPKs family (i.e., ERK1/2, ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase (JNK) 1/2/3, and p38 α/β/γ (ERK6)/δ) phosphorylate target protein substrate serine and threonine residues and hence modulate programmed death cell and gene expression.[18]

It has been shown that various miRNAs target ERK-MAPK pathway members. Deregulation of these miRNAs in cancer cells plays a role in carcinogenesis by causing abnormal activation of the ERK-MAPK pathway.[19]

Material and Methods

Ethical Approval

The Ethical Research Committee of the Faculty of Dentistry, Minia University, Egypt approved this study with a committee approval number (87) in June 2022.

Study design

Group (A): Human Oral cavity squamous cell carcinoma (OECM-1) (treated and untreated).

Group (B): Normal Human Tongue Fibroblasts (HOrF) (treated and untreated).

Material

Human Oral Cavity Squamous Cell Carcinoma cell line (OECM-1) and the Normal Human Tongue Fibroblasts (HOrF) were purchased from the Veterinary Serum and Vaccine Research Institute (VACSERA) cell bank and miRNA-16 inhibitor was obtained from Qiagen, Hilden, Germany.

Methods

1-Transduction of OECM-1 cells with miRNA-16 inhibitor

The OECM-1 cells, an average of $1x10^4$ cells, were plated in a 96-well culture plate with 200 µL of Dulbecco's Modified Eagle Medium (DMEM) a day before carrying out the transduction.

The medium included antibiotics such as streptomycin (10 mg), 1% penicillin G sodium (10.000 UI), amphotericin B (25 µg) (PSA), and 10% fetal bovine serums purchased from Gibco Thermoscientific, Germany.

To achieve a confluence of 70%, the plate was incubated at 37 °C for 24 hours in a 5% CO2 atmosphere. After incubation completion, 25 µL of the complex formed by the addition of 0.5 µL of miRNA-16 inhibitor to 3 µL of RNase-free water was pipetted into each well of the culture plate, resulting in a miRNA inhibitor final concentration of 50nM upon addition to cells.

To form the transfection complexes, 0.75 µL of HI Perfect Transfection Reagent supplied by Qiagen, Hilden, Germany (Cat no.301704) was introduced to 24.25 µL of serum-free RPMI culture medium and incubated for 10 minutes at 15-20°C. Afterward, 25 µL of the complex and 175 µL of DMEM media were pipetted to each plate well.

The transfected OECM-1 cells were incubated at 37 °C for 48 hours in an atmosphere of 5% CO2. The transfection experiment was validated by using the miRNA inhibitor negative control AllStars

siRNA and the positive control miR-1 mimic purchased from Qiagen, Hilden, Germany (cat no: 1027280 and cat no: MSY0000416, respectively).

Whereas The un-transfected cells were utilized for the normalization of the experiment. The same experiment was performed on normal human fibroblast cells to ensure that miRNA-16 inhibition does not affect healthy cell viability. MTT assay was used to estimate the viability of the cells.

2- Methyl Thiazole Tetrazolium Assay (MTT Assay) for vitality and proliferation assessment

The viability of the OECM-1 cells transfected with miRNA-16 inhibitor was assessed by the Vybrant® MTT Cell Proliferation Assay Kit from Thermo Fisher, Germany (cat no: M6494).

A 100µL of the medium was substituted by a new medium after the incubation. Afterward, 20 µL of MTT solution 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1mg/mL) purchased from Invitrogen, ThermoScientific, Germany was pipetted into every well of the plate. Subsequently, the plate was incubated in a 5% CO2 atmosphere for 4 hours at 37 °C. To each well, 100 µL of sodium dodecyl sulfate with hydrochloric acid (SDS-HCL) was pipetted after the MTT solution removal.

Optical density was used to evaluate cell viability by measurement at 570 nm using the ELx 800 spectrophotometer (Bio-Tek Instruments Inc, Winooski, VT, USA).

3- Real-time Polymerase Chain Reaction (Real-time PCR) for gene expression analysis

a- Cell harvesting from culture media

Cells were lysed using trypsin, after cell growth in a monolayer 6 cell-culture well and collected as a cell pellet, followed by washing two times in phosphate buffer saline (pH 7.4). The Tissue Ruptor II (Qiagen, Hilden, Germany) was used to disrupt and homogenize a 1x10^6 cell sample in 700 µL of Qiazol lysis buffer.

This rotor-stator homogenizer can disrupt and homogenize single tissue samples in 15-90 seconds. For 20 minutes at 4000 rpm, the mixture was centrifuged. Subsequently, the cell supernatant was collected for RNA extraction.

b- Total RNA and miRNAs extraction and purification

Ethanol was added to the tissue homogenate before loading it onto an RNeasy Mini spin column. Total RNA adhered to the RNeasy silica membrane, contaminants were quickly expunged away, and high-quality RNA was eluted in RNase-free water. RNA extraction and purification were carried out utilizing the RNeasy Mini kit (Qiagen, Hilden, Germany, cat no: 74104) adhering to the manufacturer's procedure.[20]

c- Synthesis of cDNA using Reverse Transcription

The miScript RT Kit (Qiagen, Hilden, Germany) was used to produce cDNA by reverse transcription. To prepare the reverse-transcription master mixture for first strand cDNA synthesis, 4 µl of 5x miScript HiFlex buffer were combined with 2 µl 10x miScript dNTP mixture, 2 µl miScript reverse transcriptase mixture, and 7 µl RNase-free water on ice.

Each tube contained 5 µl of RNA sample, was gently mixed, and centrifuged before loading into the Thermal Cycler (Biometra, Germany). The first strand of cDNA was reverse transcribed and synthesized in one cycle for 60 minutes at 37 ºC and 5 minutes at 95 ºC. At -20 ºC, the cDNA created by reverse transcription was kept until amplified.

d- MiRNA-16 expression analysis

The miScript Hs_miRNA-16 primer (cat no: 218300, ID: MS00031493) and miScript Syber green Master mix purchased from Qiagen, Hilden, Germany were utilized to

evaluate the gene expression levels of miRNA-16. The RUN6 primer assay [ID: MS00033740] was used as a housekeeping gene.

To prepare The PCR mix for the reaction, at room temperature thawed 5µL of 2x miScript Syber green master mix, 1µL of 10x miScript Universal,1µL of 10x miScript Hs_miRNA-16 primer assay, and 1µL of template cDNA were added. Afterward, 2 µL of RNase-free water was added, resulting in a final volume of 10 µl in each well. The reaction mixture was carefully mixed and sealed in rotor-disc wells with heat-sealing film The 5-plex Rotor Gene PCR Analyzer (Qiagen, Germany) was used for sample analysis.

e- Bcl-2 and MAPK genes expression analysis

The Qiagen, Germany obtained QuantiTect Hs_BCL2 primer assay (Cat no: 249900, ID: QT0025011), QuantiTect Hs_MAPK primer Assay (ID: QT02589314), and the QuantiTect SYBR Green PCR kit (Cat no: 204141) were used to evaluate the Bcl-2 and MAPK gene expression levels. Whereas the QuantiTect β-actin Primer Assay Hs_ACTB_1_SG (Cat no: 249900, ID: QT00095431) was used as a housekeeper gene.

Afterward, 10 µl of 2x QuantiTect SYBR Green PCR Master Mix, 2 µl of 10x t Universal Primer, 2 µl of 10x Quantitect Primer Assay, and 4 µl of RNase-free water were added to the PCR reaction mix, resulting in a final volume of 18 µl in each well. In rotor-disc wells with heat-sealing film, the reaction mix was carefully mixed and sealed.

The 5-plex Rotor Gene PCR Analyzer (Qiagen, Germany) was used to analyze the sample and was programmed for 40 cycles as follows: activation of HotStarTaq DNA Polymerase for 15 minutes at 95ºC, denaturation for 15 seconds at 94ºC, annealing for 30 seconds at 55ºC, and extension for 30 seconds at 70ºC.

The relative level of expression (fold change) for Bcl-2 and MAPK genes was normalized using the internal control (β-actin). The 2-ΔΔCt test control equation was used to calculate relative to the calibrator (negative control sample).

4- Statistical analysis

The statistical analysis was performed using GraphPad Prism Software version 8.4.2 (GraphPad Software Inc, San Diego, US). The data was presented as standard deviation (SD), mean, and range. The statistical significance of the differences among the research groups was evaluated using the independent t-test, one-way analysis of variance (ANOVA) test, and Tukey's multiple comparison tests. A *P*-value ≤0.05 was considered significant.

Results

I. MTT Assay

Comparative analysis of the proliferation index between OECM-1 cells transfected with miRNA-16 inhibitor and the untreated cells

Group (A) OECM-1 cells (treated and untreated)

The results of this study revealed that transfection of OECM-1 cells with miRNA-16 inhibitor significantly increased cell proliferation (mean: 128±3.01, range: 125-131) in comparison to the untreated cells (mean: 99.9±4.79, range: 94.4 – 103), (Table 1 and Figures 1, 2).

Group (B) HOrF cells (treated and untreated)

In contrast, the HOrF cells were transduced with a miRNA-16 inhibitor to investigate the potential harm that could result from suppressing miRNA-16 in normal cells. The obtained results showed that no significant change occurred in normal cells

following miRNA-16 suppression (*P*=0.102), (Table 1 and Figures 1, 2).

Table 1: Comparative analysis for cell viability in the studied groups

Group	Mean±SD	Range	*P*-value
Untreated OECM-1	99.9±4.79	94.4 - 103	t:8.61, *P*=0.001**
MiRNA-16 inhibitor-OECM-1	128 ± 3.01	125 - 131	
HOrF cells	99.9±2.80	97.0 - 103	t:2.1, *P*=0.102
MiRNA-16 inhibitor- HOrF	95.8±1.87	94.4 – 97.9	

t: independent t-test value, * (*P*<0.05), ** (*P*<0.01)

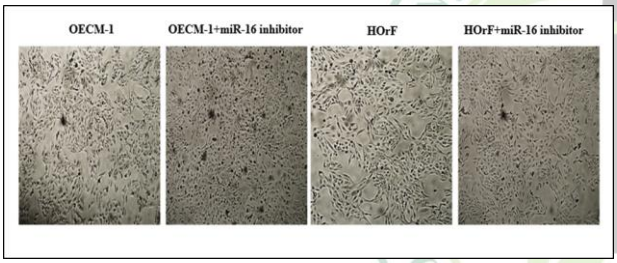

Figure 1: Photomicrographs of OECM-1 and HOrF cells provided with transduced with miRNA-16 inhibitor, taken with a digital camera from Labomed, USA, called the Vega (LC-6 USB 3.0 COLORFUL CMOS DIGITAL CAMERAS, 5MP).

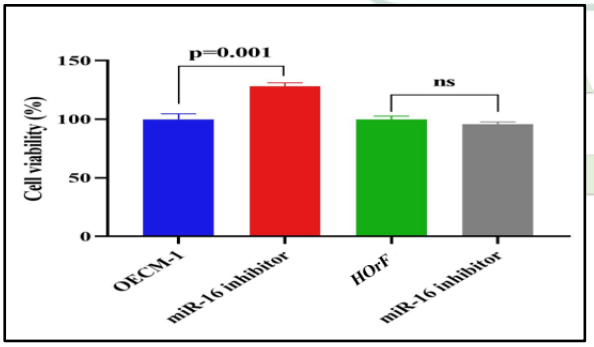

Figure 2: Comparative analysis between the proliferation index of both treated and untreated OECM-1 and HOrF cells.

II. Real-time PCR

1-Comparative analysis for miRNA-16 gene expression

Tukey's multiple comparison tests revealed a significant decrease in miRNA-16 expression in untreated OECM-1 cells compared to HOrF cells (mean difference: -0.79, *P*=0.0007).

A significant decrease in the expression of miRNA-16 in OECM-1 cells following miRNA-16 suppression, compared to untreated HOrF cells (mean difference: -0.90, *P*=0.0003)

OECM-1 cells transduced with the miRNA-16 inhibitor demonstrated no significant change in miRNA-16 expression compared to untreated OECM-1 cells (mean difference: 0.1130, *P*=0.558). These results are illustrated in (Table 2, Figure 3-A).

Table 2: Multiple comparison analysis for the miRNA-16 expression in the studied groups (post-Hoc test)

Tukey's multiple comparisons test	Mean	95.00% CI	*P*-value
OECM-1 vs. miRNA-16 inhibitor	0.1130	-0.2075 to 0.4335	0.558
OECM-1 vs. HOrF	-0.7930	-1.113 to -0.4725	0.0007**
MiRNA-16 inhibitor vs. HOrF	-0.9060	-1.226 to -0.5855	0.0003**

CI: confidence interval, **: (*P*<0.001), *: (*P*<0.05)

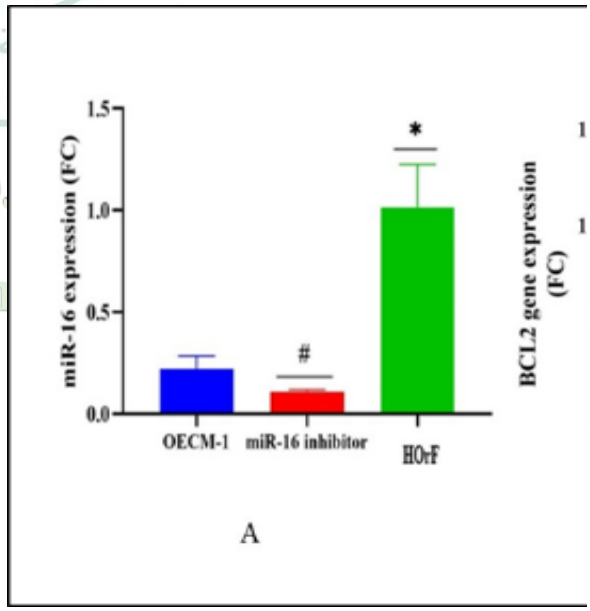

Figure 3. A. Comparative analysis between the miRNA-16 gene expression (FC) of OECM-1 cells transfected with miRNA-16 inhibitor.

2-Comparative analysis for Bcl-2 gene expression

Compared to untreated OECM-1 cells, the data showed that inhibition of miRNA-16 was significantly associated with a 2.0-fold increase in Bcl-2 expression (mean difference: -5.227, 95%CI: -8.31 to 2.14, *P*=0.005).

Also, there was a 4.5-fold increase in Bcl-2 expression in untreated OECM-1 cells, compared to HOrF cells (mean difference: 3.50, *P*=0.03). These results are illustrated in (Table 3, Figure 3-B)

Table 3: Multiple comparison analysis for Bcl-2 and MAPK expression in the studied groups (post-Hoc test)

	Bcl-2			MAPK		
Tukey's multiple comparisons test	**Mean**	**95.00% CI**	***P*-value**	**Mean**	**95.00 % CI**	***P*-value**
OECM-1 vs. miRNA-16 inhibitor	-5.227	-8.313 to -2.141	0.005*	-7.569	-10.21 to -4.925	0.0003**
OECM-1 vs. HOrF	3.502	0.4153 to 6.588	0.03*	10.78	8.139 to 13.43	<0.0001**
MiRNA-16 inhibitor vs. HOrF	8.729	5.642 to 11.82	0.0003*	18.35	15.71 to 21.00	<0.0001**

CI: confidence interval, **: (*P*<0.001), *: (*P* <0.05)

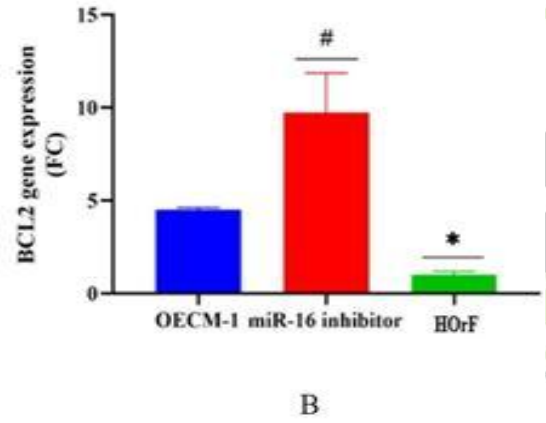

Figure 3. B. Comparative analysis between the Bcl-2 gene expression (FC) in miRNA-16 inhibitor transfected OECM-1 cells and untreated cells.

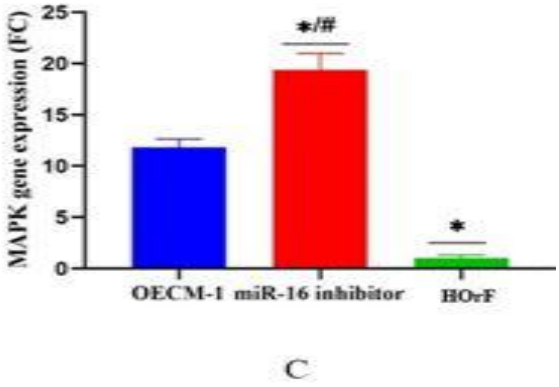

Figure 3. C. Comparative analysis between the MAPK gene expression (FC) in miRNA-16 inhibitor transfected OECM-1 cells and untreated cells.

3- Comparative analysis for MAPK gene expression

A significant 1.6-fold increase in MAPK expression in OECM-1 cells was observed after the inhibition of miRNA-16 compared to untreated OECM-1 cells, as detected by the ANOVA test (mean difference: -7.56, 95%CI: -10.21 to -4.92, *P*=0.0003).

In addition, there was a highly significant difference between the untreated OECM-1 and HOrF cells (*P*<0.001), higher expression of MAPK was associated with untreated OECM-1cells. These results are presented in (Table 3, Figure 3-C).

Discussion

Head and neck squamous cell carcinoma (HNSCCs) are ranked as the sixth most prevalent type of cancer world, with approximately 900,000 new cases in 2020, according to Globocan 2020. Its prevalence is predicted to increase by an additional 30% by 2030.[21]

Similar to other cancers, the accumulation of genomic alterations, that characterize oral squamous cell carcinoma (OSCC), jeopardizes numerous cellular processes and establishes cancer hallmarks. The unavailability of OSCC clinical outcomes and recurrence prediction biomarkers remains a concern.[22]

Non-coding RNAs (ncRNAs) comprise almost 90% of the genome whereas protein-coding genes make no more than 2% of all sequences. Because ncRNAs are so stable in biological fluids and tissues, they have become more and more recognized as potential novel causes of disorders, including cancer.[23]

These molecules regulate both non-coding and protein-coding genes through transcriptional and post-transcriptional silencing. Moreover, multiple reports indicate the proficiency of ncRNAs as tumor suppressor genes and oncogenes regulators; as such, we could potentially be able to utilize them as sensitive biomarkers for diagnosis, prognosis, and the development of effective treatment plans.[24]

MicroRNA (MiRNAs) play important roles in the pathophysiology of HNSCC as numerous miRNAs' dysregulated expression correlates with cancer behavior and clinical determinants. In HNSCC, tumor suppressor miRNAs primarily control the expression of genes that promote proliferation or inhibit apoptosis.[25]

MiRNA-16 is located on chromosome 13 between two exons of the DLEU2 gene in the 13q14.3 region. Because miRNA-16 and some messenger RNAs (mRNAs) have complementary antiparallel sequences, miRNA-16 can be used to prevent protein synthesis and regulate gene expression post-transcriptionally.[26]

In this research we evaluate the expression of microRNA-16, B-cell lymphoma 2 (Bcl-2), and mitogen-activated protein kinase (MAPK) using Methyl Thiazole Tetrazolium Assay (MTT), Real-time Polymerase Chain Reaction (Real-time PCR), and statistical analysis.

The results of the MTT assay in the present study showed that OECM-1 cells transfected with miRNA-16 inhibitor showed a much higher rate of cell proliferation compared to the un-transfected cells.

Zhao et al.[27] revealed that the expression of miRNA-16 was aberrantly down-regulated in cancer of oral tissues, which agrees with the current findings. Compared to human normal oral keratinocyte cells, human oral cancer cell lines had a lower abundance of miRNA-16, which its overexpression inhibits oral cancer cell viability, migration, and invasion.

Moreover, Wang and Li.[28] aimed to investigate if miRNA-16 could affect OSCC proliferation. The miRNA-16 mimic reduced the cell viability of the cells compared to the normal cell group. Furthermore, the results indicated that the miRNA-16 inhibitor markedly increased cell viability.

The MTT and apoptotic assays were carried out by Liu et al.[29] to assess the roles of miRNA-16 in oral cancer revealed that the cells transfected with miRNA-16 exhibited a notable nuclear fragmentation and low viability.

The current study revealed a significant reduction in miRNA-16 expression in untreated OECM-1 cells compared to normal human tongue fibroblasts (HOrF) cells. In addition, a more significant reduction in miRNA-16 expression was noticed in OECM-1 cells following miRNA-16 suppression which highlights the tumor-suppressing role of miRNA-16.The same findings were revealed by Liu et al.[29]

The results are consistent with those of Hu et al.[10] They discovered that five OSCC cell lines (Tca83, HN4, Cal27, SCC9, and SCC25) had lower levels of miRNA-16 expression than in normal human oral keratinocytes (HOK) cells and this was associated with a poor prognosis. They explained this by stating that miRNA-16 inhibited TLK1 expression in SCC9 cells.

The effect of miRNA-16 on Bcl-2 and MAPK expression in OECM-1 and HOrF cells after miRNA-16 inhibitor transfection was examined in this study to figure out the

gene regulatory frameworks that miRNA-16 modulates.

Numerous studies have established the involvement of miRNA-16 as a tumor suppressor in ovarian, colorectal, pancreatic, and prostate cancers. MiRNA-16 targets oncogenes to suppress angiogenesis, epithelial-to-mesenchymal transition, cell survival, and proliferation.[30]

According to miRNA/target interaction analysis, miRNA-16 is bioinformatically predicted to target nine of a custom panel of 15 important oncogenes linked to OSCC such as Bcl-2 and PIK3CA genes.[11]

Wang et al.[9] investigated the underlying mechanism of miRNA-16 in the process of OSCC tumor growth inhibition. MiRNA-16 has been shown to target various oncogenes, including Bcl-2.

The current study found that suppressing miRNA-16 was strongly associated with an increase in Bcl-2 expression in treated OECM-1 cells compared to the untreated cells. Furthermore, when compared to HOrF cells, OECM-1 cells showed significantly higher Bcl-2 gene expression.

The current study's findings agree with those of Munjal et al.[31] They found that Bcl-2 positivity was highest in poorly differentiated SCC patients. They concluded that this oncoprotein may play a critical role in the oral neoplasia progression and genesis in its early stages. Bcl-2 expression appears negatively related to the degree of epithelial cell differentiation.

Xiong et al.[32] found that Bcl-2 levels are significantly elevated in numerous OSCC cell lines. This indicated that Bcl-2 overexpression plays a crucial role in OSCC cell proliferation and survival. Furthermore, there was a complementary homology of miRNA-16 and Bcl-2 at the mRNA level suggesting the role of Bcl-2 oncoprotein as a target of posttranscriptional repression by miRNA-16. MiRNA-16 inhibited Bcl-2 expression leading to activation of the intrinsic apoptosis pathway.

Wang and Li.[28] found that miRNA-16 inhibited Bcl-2 mRNA and protein expression. OSCC tissues showed an inverse correlation between miRNA-16 and Bcl-2 levels, supporting this result. These findings revealed that Bcl-2 is a true downstream target of miRNA-16 in OSCC cells, suggesting that miRNA-16 performs a tumor-suppressing effect in OSCC. Downregulation of miRNA-16 causes cancer, but upregulation plays an anticancer role in OSCC.

MAPK is a complex interconnected signaling cascade that frequently contributes to oncogenesis, tumor development, and treatment resistance the mutations in the signaling pathway genes RAS/RAF/MEK/ERK are present in the majority of solid tumors.[33]

In this study, OECM-1 cells were transduced with a miRNA-16 inhibitor, and the expression of the MAPK gene was assessed. An ANOVA test revealed a significant correlation between the miRNA-16 inhibition and increased MAPK expression in OECM-1 cells. Furthermore, MAPK expression was higher in OECM-1 cells compared to HOrF cells.

Li et al.[34] stated that the activated ERK1/2 expression was significantly increased in OSCC of the tongue compared to normal oral mucosa, which is consistent with the findings of the current investigation. Downregulating ERK1/2 signal pathways was found to reduce cellular proliferation, MMP-9 expression, and OSCC metastasis. Activating ERK appears to increase cell development, migration, proliferation, and metastases.

Various investigations found similar results; approximately 20% of cases of HNSCC have MAPK pathway mutations. Ngan et al.[35] found that the frequency of MAPK-activating mutation alleles (i.e.,

HRAS and MAPK1) was high among HNSCC patients' tissues and blood samples.

The present findings agree with those of the study of Gurbi et al.[36] who proved that overexpression of epidermal growth factor receptor activates Ras/Raf/MEK/ERK signaling pathway and stimulates the progression of HNSCCs.

Many studies have emphasized miRNAs' function in regulating the activity and specificity of the ERK/MAPK cascade. Dysregulation of miRNA activity has been linked to abnormal activation of the ERK/MAPK cascade and tumor development.[37]

The results of overexpression of MAPK signaling pathway were also, observed by other studies but on different tumor tissues. He et al.[38] discovered that miRNA-16, a tumor suppressor, was downregulated in nasopharyngeal carcinoma (NPC) cell lines. MiRNA-16 reduced nasopharyngeal cancer cell proliferation, invasion, and metastasis by modulating the MAPK signaling cascade by down-regulating fibroblast growth factor 2 (FGF2) expression.

Similarly, a study performed on lung cancer cell line showed that decreased miRNA-16 expression may contribute to upregulating MEK1 expression and increasing lung cancer cell proliferation and invasion. Overexpression of miRNA-16 can lower the ERK/MAPK pathway activity by suppressing MEK1 expression.[39]

Interestingly, other miRNAs showed a tumor-suppressing role by targeting the MAPK signaling pathway in OSCC. MiR-148a expression was down-regulated in the OSCC cell line, and through targeting the Insulin-like growth factor-I receptor (IGF-IR), it may be able to limit the progression of OSCC by inactivating the ERK/MAPK signaling cascade. It was shown that the transmembrane tyrosine kinase receptor IGF-IR, which binds to IGF-I to control cell growth, development, and metabolism, was significantly upregulated in OSCC cells and that miR-148a was negatively regulating its expression.[40]

These findings support those of the current study and highlight for the first time the importance of the interplay between miRNA-16 and MAPK signaling pathways in OSCC. The overexpression of MAPK could suggest that MAPK is a novel target for miRNA-16. Therefore, further studies are required to understand the underlying patho-mechanism in this cancer type.

Conclusion

Aberrant expression of microRNA-16 is strongly associated with various tumor progression and prognosis, including OSCC. This study found that miRNA-16 was specifically downregulated in OSCC cell line. Its inhibition reflects the tumor-suppressive function of miRNA-16 in OSCC by significantly increasing cell proliferation and inhibiting apoptosis.

Furthermore, the observed upregulation of Bcl-2 and MAPK genes in OSCC cell lines became markedly manifested upon inhibition of miRNA-16 suggesting that miRNA-16 acts by directly targeting Bcl-2 and MAPK genes**.** These findings will help us to understand more about the mechanisms underlying cancer progression and identify potential targets for molecular therapy of OSCC.

Funding

There is none to be declared.

Data availability

Data are available whenever requested.

Declarations

There is none to be declared.

Ethics approval and consent to participate

The Ethical Research Committee of the Faculty of Dentistry, Minia University, Egypt

approved this study with a committee approval number (87) in June 2022.

Competing interests

The authors declare that there are no competing interests.

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