TARGETING WNT PATHWAY THROUGH miR-142-3P AS A POTENTIAL THERAPEUTIC APPROACH IN ORAL SQUAMOUS CELL CARCINOMA (IN VITRO STUDY)

Yara M. Eldahshan BDS, Sahar E. Riad PhD, Hagar R. Elkafrawy PhD, Enas M. Omar PhD, Mai M. Saleh PhD

ABSTRACT
INTRODUCTION: Oral squamous cell carcinoma (OSCC) accounts for 95% of oral cancers and is associated with a low 5-year survival rate using the conventional treatments methods. Advancements in oncological research require understanding the molecular mechanisms driving OSCC occurrence and progression to devise more effective therapeutic approaches and improving prognostic outcomes. Among these mechanisms, the WNT/β-catenin signaling pathway is recognized for its contribution in promoting tumorigenesis, cancer progression, and metastasis, making it a potential target for unconventional cancer therapies. MicroRNA-142-3p (miR-142-3p), a constituent of the miR-142 family, has demonstrated tumor-suppressive properties in certain malignancies. It was proposed that miR-142-3p positively modulates WNT signaling by directly targeting the adenomatous polyposis coli (APC) gene. This targeting destabilizes β-catenin, resulting in suppression of WNT pathway activity.

OBJECTIVES: In this current investigation, the aim was to explore the capabilities of miR-142-3p in modulating the WNT pathway and evaluating its impact on the proliferation, migration, and apoptosis of OSCC-4 cells.

METHODOLOGY: MiR-142-3p was transfected into OSCC-4 cell line. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to determine cytotoxicity, while the impact on cell migration was evaluated through scratch wound healing test. Apoptotic effects were evaluated using Annexin-V, while cell proliferation was measured through Ki-67 staining, both analyzed by flow cytometry.

RESULTS: The results indicated that miR-142-3p suppressed OSCC-4 proliferation and migration, demonstrating its anti-proliferative potential. It didn't induce early apoptosis however, it enhanced late-stage apoptosis in a dose-dependent manner.

CONCLUSIONS: miR-142-3p exhibits a promise as a potential therapeutic option for OSCCs.

KEYWORDS: Oral squamous cell carcinoma, WNT pathway, Epithelial mesenchymal transition, miR-142-3p.

INTRODUCTION
Oral squamous cell carcinoma (OSCC) represents the most prevalent type of oral and oropharyngeal cancers, amounting to about 95% of cases (1). It is ranked as the sixth most prevalent cancer globally, contributing to approximately 300,000 new cases annually (2). Despite advancements in therapeutic modalities, the overall survival rate for OSCC persists to be below 60%, with prognosis significantly influenced by the TNM staging (2).

The canonical WNT significantly impacts the development and progression of several varieties of human malignancies (3). It has been identified to have a pivotal role in triggering the epithelial-mesenchymal transition (EMT) process and the maintenance of Cancer Stem Cells (CSCs) (4-5). As a result, there has been a growing interest in targeting this pathway as a potential avenue for therapeutic intervention for neoplastic conditions characterized by elevated rates of both mortality and morbidity (3).

Epithelial-mesenchymal transition significantly contributes to the local recurrence and lymph node metastasis of OSCC, correlating with a lower patient survival outcome (6). Investigations have revealed the participation of MicroRNAs (miRNAs) in orchestrating EMT regulation at the post-transcriptional level (7). They play a critical role in EMT regulation, not solely through influencing key transcription factors, but also by interacting with the pathways that govern differentiation and transformation (8).
MicroRNAs are a subset of small, RNA molecules composed of a single strand that are noncoding in nature, with a length ranging approximately from 21 to 23 nucleotides (9). They show their influence over a range of signaling pathways including WNT, Notch, TGFβ, and Hedgehog (7). miRNAs show two-sided behavior, functioning in the capacity of tumor suppressors or oncogenes during EMT and metastasis processes (7). Studies have indicated that miRNAs have the capacity to regulate the expression of over 30% of critical genes that play pivotal role in vital physiological processes like cell differentiation, proliferation, programmed cell death, invasion, metabolic equilibrium, and survival (10-13). Circulating miRNAs present a promising potential as biomolecular indicators for both cancer diagnosis and prognosis, regardless of tumor stage or the presence of genetic mutations (14). These miRNAs, which maintain their structural integrity and can be detected in various bodily fluids, present a valuable opportunity for detecting and predicting various diseases and conditions (15).

Among the miRNAs utilized as biomarkers, miR-142-3p stands out for its distinct ability to perform both oncogenic and tumor suppressor roles in different human cancers (16-17). For instance, in hepatocellular carcinoma cell lines, it exhibits the capability to suppress the migration and invasion through the regulation of the RAC1 gene (18). Meanwhile, cervical cancer cells experience inhibition of proliferation and invasion via miR-142-3p’s interaction with FZD7 (19). However, its function shifts to that of an oncogene in non-small-cell lung carcinoma cells, where it suppresses TGFβ-induced growth inhibition via modulation of TGFβR1 (20). Some studies have indicated a connection between miR-142-3p and cellular activities such as migration, proliferation, and apoptotic responses in cases of renal cell carcinoma (21) and esophageal squamous cell carcinoma (22).

Isebo et al. revealed that miR-142 and miR-142-3p were involved in the initiation of the canonical WNT pathway in breast cancer stem cells (23). A study conducted by Hu et al, revealed that miR-142-3p has a direct inhibitory impact on β-catenin by targeting its protein translation. This interaction on a molecular level result in the suppression of cellular proliferation (24).

Although several in vivo and in vitro investigations have explored the influence of miR-142-3p in various cancer types, including its impact via the WNT pathway and other signaling routes, it's worth mentioning that none of these investigations have explored the therapeutic potential of this miRNA in OSCC. Thus, this current study aims to investigate the influence of miR-142-3p on OSCC cell proliferation, migration, and apoptosis, with a focus on its interactions with the WNT pathway.

MATERIAL AND METHODS

Cell lines and treatment

The human OSCC-4 cell line was purchased from ATCC (American Type Culture Collection). The study was conducted at the Center of Excellence for Research in Regenerative Medicine and Applications (CERRMA) at Alexandria Faculty of Medicine. Experiments were conducted in accordance with the guidelines approved by the Research Ethics Committee and the Medical Ethics Committee (IRB NO: 00010556-IORG 0008839), Faculty of Dentistry, Alexandria University, Egypt. The cells were cultivated in DMEM/high-glucose (Dulbecco's modified Eagle medium; Lonza; containing 0.2 mmol/ml L-glutamine) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin Lonza and 10% fetal bovine serum (FBS; Lonza). Cells were maintained in a humidified 37 °C, 5% CO2 incubator. Cells were monitored daily for their growth and morphology using the phase-contrast inverted microscope (CKX41SF; Olympus). Media was changed every 2 days, and cells were passaged on reaching 80%-90% confluence by suspension with 0.25% (w/v) trypsin-EDTA (Lonza), then plated in T75-cm2 flasks for maintenance or in 6- or 96-well plates according to the experiment conducted.

Cell Transfection

The OSCC-4 cells were subjected to transfection with the hsa-miR-142-3p mimic (Catalog# 4427975, Thermo Fisher Scientific, USA) using HiPerFect Transfection Reagent 0.5 ml (Catalog# 301704, QIAGEN, Germany) following the manufacturer's guidelines. Briefly, HiPerFect Transfection Reagent (1μM) was added to miRNA mimic (20 and 40 nM) diluted with serum-free medium (10% of total final volume). This complex was incubated for 15 minutes before being added to the cells. At 24 h or 48 h post-transfection, downstream experiments were performed.

MTT Cytotoxicity Assay

The MTT assay was carried out to investigate the possible cytotoxic effects of the miR-142-3p in a dose-dependent manner within a concentration range of 0 to 40 nM (25). The OSCC-4 cells were seeded in a 96-well plate at a cell density of 7000 cells per well until they reached confluence at 24 hours. Subsequently, the cells were transfected with different dose combinations of miR-142-3p (0, 5, 20 and 40 nM) and transfection reagent (0, 0.7 and 1 μM) diluted with serum-free medium (10% of total final volume). After an incubation period of 24 hours, each well received 100 μL of MTT solution (0.5 mg/mL in DMEM) and was incubated at 37°C for 4 hours. Finally, the plates were read using a 96-well plate reader at a wavelength of 550 nm. The absorbance was measured and used to calculate the percent viability of the cells.
subsequently subjected to an additional incubation period at 37 °C for 3.5 hours. After discarding the MTT solution, 100 μl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals generated by viable cells. The absorbance levels of treated and untreated control wells were measured using spectrophotometry at 550 nm using an ELISA microplate reader (Infinite F50, TECAN, Switzerland).

Grouping

All the following downstream experiments were performed on the following groups of cells:

Group I: Untreated OSCC-4 cell line served as the negative control with no treatment received.

Group II: OSCC-4 cell transfected with (20 nM) and HiPerFect transfection reagent (1 μM)

Group III: OSCC-4 cell transfected with (40 nM) and HiPerFect transfection reagent (1 μM)

Scratch Wound Healing Test (Migration Assay)

The scratch wound healing test was performed to assess the anti-proliferative potential of different doses of miR-142-3p (20 nM, 40 nM) (26).

The OSCC-4 cells were cultured in 6-well plates at a density of 25 × 10⁴ cells per well in complete media (DMEM/high-glucose, containing 0.2 mmol/ml L-glutamine; Lonza, supplemented with 100 IU/ml penicillin; Lonza, 100 μg/ml streptomycin; Lonza and 10% FBS; Lonza) until they reached 80-90% confluence after 24 hours. Subsequently, a sterile 200-μL pipette tip was used to create controlled scratches horizontally. An inverted microscope was used to capture five images of the wound region in each well at a magnification of x100. To calculate both the cell migration rate and the percentage of wound closure, Image J software (version 1.53C, NIH, USA) was used for the measurement of the width of the wound gap horizontally.

Flow cytometry-based analysis of apoptosis through Annexin-V/propidium iodide assay

Annexin assay was conducted to assess anti-apoptotic capabilities of different concentrations of miR-142-3p on early and late apoptosis. Conjugated protein annexin V-FITC binds to cell surfaces expressing early apoptosis marker phosphatidylserine. Propidium iodide (PI), a non-cell-permeable DNA dye, stains necrotic cells, while PI- and annexin V-FITC-stained cells indicate early necrosis and later stages of apoptosis (27).

The apoptotic influence of miR-142-3p was assessed through Annexin-V assay and flow cytometry using a BD FACS Calibur™ flow cytometer (San Jose, USA). OSCC-4 cells (2 × 10⁵) were cultivated in six-well plates (2 × 10⁶) and allowed to adhere for duration of 24 hours in a 5% CO2 incubator maintained at 37 °C. Following this, the cells were transfected with miR-142-3p concentrations of 20 nM and 40 nM and HiPerFect transfection reagent (1 μM) diluted with serum-free medium (10% of total final volume) for a period of 48 hours. Both control and treated cells underwent trypsinization, PBS rinsing, and subsequent centrifugation at 2000 rpm for 5 minutes.

Subsequently, cellular samples were collected and subjected to staining using the Annexin V-FITC/propidium iodide procedure, in accordance with the instructions that the manufacturer has provided. The assessment of apoptotic cells was performed by gating 20,000 cells using a flow cytometer. The experimental setup was conducted in triplicate (n = 3), with representative images captured. Cell proliferation assessment via Ki-67 staining (flow cytometer)

To evaluate miR-142-3p influence on cell proliferation, modifications in Ki-67 transcription of OSCC-4 cell lines were analyzed using flow cytometry (28).

The OSCC-4 cells (2 × 10⁵) were seeded in 6-well plates (Corning, NY), and allowed to adhere for duration of 24 hours in a 5% CO2 incubator maintained at 37 °C. Cells were then subjected to different miR-142-3p concentrations (20 nM and 40 nM) and HiPerFect transfection reagent (1 μM) diluted with serum-free medium (10% of total final volume) for 48 hours. Post trypsinization and centrifugation, the cell pellet was cautiously treated with 70% cold ethanol (3 mL) dropwise, while simultaneously vortexed for 30 seconds. The cells were then subjected to incubation at -20°C for a 2-hour duration. Subsequent steps included two washes using 4 mL of staining buffer (PBS with 1% FBS, 0.2% Tween 20), followed by a 10 minutes centrifugation at 2000 rpm and supernatant removal (repeated twice). Cells were resuspended in a solution with a density of 1 × 10⁶ cells/mL, afterward 100 μL of this cell suspension, consisting of 1 × 10⁶ cells, was loaded into individual sample tubes.

Cells were stained by Alexa Fluor 488-conjugated Ki-67 antibody (1:50, Cell Signaling Technology, Cat# 11882) and incubated in the dark at room temperature for 30 minutes. After incubation, washing procedure was performed at 2000 rpm for 5 minutes using 2 mL of staining buffer. The final step involved resuspending the samples in 0.5 mL of
staining buffer and were subjected to flow cytometric analysis. Duplicate runs were conducted for each sample, capturing around 10,000 events per tube.

Statistical Analysis
Findings were presented as mean ± standard deviation (SD), obtained from three independent trials, each executed in triplicate. Analysis of the data was carried out with GraphPad Prism version 9.1.0 (GraphPad Software, San Diego, California, USA), statistical significance was defined at a p-value < 0.05. Two-way ANOVA was applied, followed by Tukey's multiple comparisons to analyze and assess statistical significance for flow cytometric, scratch wound healing, and cell viability analyses. The same program was used for illustrations and graphs designing.

RESULTS
Cell viability Assay (MTT Assay)
The data obtained revealed that the observed differences in cell viability when comparing treated and control cells were not statistically significant (Figure 1).

Scratch wound healing assay (Migration Assay)
At the initial time point (0 h), no statistically significant differences were detected in the gap observed within the scratch lines among the groups (p > 0.5), indicating consistency.

After 24 hours, untreated OSCC-4 cells showed 66.03% closure of the cell-free area. Cells treated with 20 nM had a slight reduction in width of the cell-free area at -1.622%, while 40 nM-treated cells displayed more reduction at -6.375%.

After 48 hours of treatment, untreated OSCC-4 cells closed the cell-free zone by 86.67%. Cells treated with 20 nM reduced the width moderately to 33.68%, while 40 nM-treated cells showed a more pronounced reduction to 34.62% (Figure 2).

Flow Cytometry-Based Apoptosis Detection with Annexin-V and Propidium Iodide
The cells transfected with 20 nM of miR-142-3p showed no significant change (p > 0.05) in early apoptosis (8.090 %) compared to untreated OSSC-4 cells (9.015%). Similarly, 40 nM of miR-142-3p didn't significantly impact early apoptosis (8.163%).

Conversely, the annexin assay revealed notable variations in late apoptosis percentages among the examined groups. Untreated OSCC-4 cells showed a late apoptosis rate of (0.300 %). Treating with miR-142-3p at 20 nM significantly raised (p < 0.0001) the late apoptosis rate to (2.455 %), while at 40 nM concentration, late apoptosis further increased to (3.423 %) (Figure 3, 4).

Cell proliferation assessment via Ki-67 staining (flow cytometer)
The results displayed noticeable variations in Ki-67 expression levels across the study groups. Untreated cells exhibited a Ki-67 expression of (59.99 ± 0.9235). Treatment with 20 nM miR-142-3p decreased Ki-67 expression to (41.88 ± 1.040), and using 40 nM miR-142-3p further lowered Ki-67 expression to (30.76 ± 0.8244) (Figure 5, 6).
Figure 3: Scatter Plots for the flow cytometric assessment of apoptosis employing Annexin V-FITC/PI. The percentages of living cells, early apoptosis, late apoptosis, and necrosis were presented in the lower left, lower right, upper right, and upper left quadrant respectively. OSCC-4 cells subjected where subjected to different treatments where: (A) Untreated OSCC-4 cells, (B) miR-142-3p at a concentration of 20 nM, and (C) miR-142-3p at a concentration of 40 nM.

Figure 4: Column Bar graph for the quantification of the Annexin V assay. Results are presented as mean ± SD Results are displayed as mean ± SD from 3 independent experiments (n=3), each performed triplicates. (B.1) LR (Early apoptosis) (B.2) UR (Late apoptosis) (***) represents significance (ns) not significant (P value < 0.05)

Figure 5: Histogram graph for Flow cytometric analysis of proliferation using ki-67-FITC was performed on OSCC-4 cells subjected to different treatments where: (A) Untreated cells, (B) miR-142-3p at a concentration of 20 nM, and (C) miR-142-3p at a concentration of 40 nM.

Figure 6: Column Bar graph for the quantification of the ki-67-FITC assay. Results are presented as mean ± SD Results are displayed as mean ± SD from 3 independent experiments (n=3), each performed triplicates. (*** ) represents significance (ns) not significant (P value < 0.05)

DISCUSSION

Despite notable advancements to comprehend the intricate involved in the development of oral cancer and progression, the development of successful treatment strategies remains pending. Challenges persist in the form of unfavorable prognosis and high recurrence rate (29).

Consequently, the formulation of efficient treatment approaches is imperative to enhance OSCC clinical outcomes. In this context, impeding pivotal molecular components in clinically relevant cancer-related pathways, such as the WNT signaling cascades which holds significance for various phases of OSCC development, is essential (30).

There has been a growing focus on potential utilization of miRNAs as prognostic indicators in different tumors (31). They have a notable impact on cancer progression through the control of oncogene or tumor suppressor gene expression (32-33). Notably, certain miRNAs with tumor suppressing properties have shown promise in mitigating cancer-associated traits like cell proliferation, migration, invasion, and resistance to chemotherapy (32-33).

Among these, miR-142-3p has attracted significant interest due to its contributions in multiple cancers (33). It actively participates in regulating oncogenic factors and holds importance as a notable miR in biomarker investigations (33).

In a study conducted by Lin et al, the possibility of miR-142-3p serving as a predictive marker for esophageal squamous cell carcinoma was highlighted (22). Nonetheless, its implications in oral squamous cell carcinoma have not been fully explored. The present study sought to investigate how miR-142-
MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.

CONFLICT OF INTEREST

MicroRNA-142-3p demonstrates significant potential as a viable therapeutic option for treating OSCCs. It effectively reduces cell proliferation and migration while exhibiting no notable cytotoxicity. Although further research is required to delve into its apoptotic effects, the outcomes of our study collectively indicate that miR-142-3p holds promise as a valuable therapeutic agent against OSCCs.
FUNDING STATEMENT
Financial support for this study was not provided by any public, commercial, or nonprofit entities.

REFERENCES


25. Tolosa L, Donato MT, Gómez-Lechón MJ. General Cytotoxicity Assessment by Means of the