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Transduction of miRNA-155 and miRNA-34a in Oral Squamous Cell Carcinoma Cell Line

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ABSTRACT

Objective: to assess the impact of miRNA-155 and miRNA-34a suppression and replacement on the possibility of human Tongue squamous cell carcinoma (SCC) cell line (HNO97).

Design: In vitro study.

Setting: Global Research Labs, Medical Center2, Nasr City, Cairo, Egypt

Interventions: The HNO97 cells were transfected with miR-155 and miR-34a imitates and inhibitors, then the cell capability was assessed using the MTT assay, and the c-Myc and CDK6 genes expression was calculated in treated and untreated cells using SYBER green based quantitative polymerase chain reaction.

Main outcome measure: To detect how transduction of oral SCC (OSCC) cell line with miRNA-155 and miRNA-34a mimic and inhibitor affects the expression of c-Myc and CDK6 genes.

Results: The cell viability was precisely as well as directly linked with the expression of miR-155 and the case was the opposite when miRNA-34a was used, in addition, higher expression level of c-Myc gene was combined with miR-155 mimic and miR-34a suppression and, the CDK6 gene expression showed an increase in cells treated with miR-155 mimic as well as miR-34a mimic and inhibitor.

Conclusions: miR-155 has a significant oncogenic effect on OSCC cell lines by increasing the c-Myc and CDK6 gene expression, but miR-34a lacks that well defined effect.

Keywords: *Tongue, Research, Cells, Line*

1.INTRODUCTION

Oral cancer is one of the most significant public health issues worldwide. Based on International Agency for Research on Cancer (IARC), it is one among the top 10 malignancies. Oral squamous cell carcinoma (OSCC), which is responsible for 90% of all occurrences of oral cancer, is diagnosed in over 300,000 new cases per year. In particular, individuals with advanced TNM stages still have a dismal survival rate 1.

Similar to other oral malignancies, oral carcinogenesis occurs gradually due to a persistent accumulation of genetic and epigenetic modifications that result in permanent alterations in the DNA sequence of regulatory molecules 2. Among these regulatory molecules, the relevance of noncoding RNAs, especially microRNA, in the etiology of carcinogenesis is growing 3.

Numerous pathways are active in OSCC, including MAPK, WNT, and PI3K/AKT/mTOR. Furthermore, overexpression of the Ras and Myc gene families has been accompanied with bad prognosis, also, overexpression of cyclins is a main characteristic feature of both pre-malignant lesions and OSCC 4-6.

 A common mutual group of serine/threonine protein kinases, which is made up of a cyclin-dependent protein kinase (CDK) catalytic subunit and the regulatory subunit cyclin, is the principal regulator of the cell cycle 7. CDKs are classified in to1, 2, 4, and 6 subtypes and cyclins are A, B, E, and D (D1, D2, and D3). D-type cyclins positively bind and activate CDK4 and CDK6 at the G1 phase of the cell cycle, and this phosphorylates retinoblastoma protein (RB), which initiates and promotes the activation of the E2F transcription factors that transcribe the genes necessary for G1-S phase cell cycle development 8.

Moreover, CDK inhibitory proteins (CKIs), which are divided into two groups and include the INK4 family (p16, p15, p18, and p19) and the CIP/KIP family, regulate the cell cycle (p21, p27, and p57). By binding to CDK4 and CDK6; INK4 family stops the link between CDK4 /CDK6 and D-type cyclins lead to halt the cell cycle in G1 phase, in the same way the CIP/KIP family binds to a number of Cyclin-CDK complexes that have already been developed, preventing advancement at every cell-cycle phase 9.

The C-Myc protein is a nuclear protein that functions as a transcription factor to regulate a vast array of biological activities like cell division, differentiation, metabolism, angiogenesis, cell adhesion and motility 10. Throughout the modulation of several cell-cycle control-related genes, c-Myc promotes cell-cycle progression. Although favorably and constructively modifying cyclins (D, E, A, and B1), CDKs (1, 2, 4, and 6), and E2F transcription factors, it negatively affects p15, p21, and p27 (E2F1, 2, and 3) 11. In spite of being involved in cell cycle progression, c-Myc is crucial for regulating apoptosis as it creates more susceptible cells to a variety of factors that cause cell death, such as DNA damage, hypoxia, and signifying via the tumor necrosis factor, CD95, and TRAIL receptors 12.

MicroRNAs (miRNAs) are noncoding single-stranded (19 to 24 nucleotide length) RNAs that are concerned in multiple cellular processes like cell differentiation, growth, and apoptosis 13. The human miRNA base has information on around 8,000 miRNAs, which are supposed to be the primary regulators of about 30% of all genes 3, 14. At the molecular level, miRNAs join to the 3'-untranslated region (3'UTR) of the target mRNAs, so they hinder the expression of those mRNAs by translational repression or mRNA cleavage 15.

Numerous miRNAs, including miR-499a, miR-491-5p, miR-155, miR-27a, miR-99a, miR-21, miR-9, miR-483-3p, and miR34a, have been concerned in the development of OSCC as reported in many previous studies 16.

The B-cell integration cluster (BIC) gene, which is located on chromosome 21, is responsible for producing miRNA-155. A 13 kb area is covered by the three exons that make up this gene. and include a 1500 bp noncoding precursor to the mature miR-155, known as the primary miR-155 (pri-miR-155) transcript, in exon 3 17.

MiR-155 was initially connected to the oncogenesis of hematological malignancies based on the discovery of BIC/miR-155 over manifestation in chronic lymphocytic leukemia

and B-cell lymphomas 17, 18. Therefore, miR-155 was shown to be overexpressed and linked with a bad prognosis in several solid tumors, including breast, lung, liver, thyroid, pancreatic, and cervical malignancies 18, 19. Several previous investigations have demonstrated that miRNA-155 is upregulated in head-and-neck squamous cell carcinoma (HNSCC) 18, 20.

MiR-34a, miR-34b, and miR-34c are the three members that make up the miRNA-34 family. Contrary to miR-34b and miR-34c, which are located on chromosome 11q23.1, miR-34a is located on chromosome 1p36.22 and contains an identifiable transcript 21. While miR-34b is distinct from miR-34a and miR-34c, they develop the same seed region between the second and ninth nucleotides at the 5′ end of mature miRNAs, indicating that their mRNA targets are comparable 22.

MiR-34a was identified as a tumor suppressor for the first time in neuroblastoma cells, where it downregulates and triggers apoptosis. Furthermore, several studies on prostate cancer, hepatocellular carcinoma, colon cancer, and HNSCC have confirmed and established the tumor suppressor activity of miR-34a 23.

In this study, we planned to examine and research the effect of alternation (stimulation and inhibition) of miR-155 and miR-34a on the proliferation of the human tongue SCC cell line (HNO97). In addition, we investigated their effects on the expression of c-Myc and CDK6 genes.

2. MATERIALS AND METHODS

This in vitro study was conducted in Global Research Labs, Medical Center2, Nasr City, Cairo, Egypt; on HN097 that was obtained from NAWAH Centre, Cairo, Egypt.

2.1. Transduction of HNO97 cells with miRNAs mimic and inhibitors

The HNO97 cells were sown earlier, prior to the experimentation, in a 96-well culture plate. About 1x105 HNO97 cells were planted in 200 L of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermosientific, Germany). This

medium contains 10% foetal bovine serum (FBS) and 1% of these antibiotics; streptomycin (10 mg), amphotericin B (25 g) (PSA), and penicillin G sodium (10.000 UI) (Gibco, Thermosientific, Germany). To achieve 70% confluence, 24 hours were spent incubating culture plates at 37 °C in a 5% CO_2 environment. The next day, a complex was created by spotting 0.05 L of miR-155, 0.5 L of miR-155 and miR-34a inhibitors, and 0.05 L of miR-34a mimic in 3 L of RNase-free water. Adding 25 L of the complex to the corresponding well. A 25 µL of the complex is added into the matching well of the 96 well culture plates. This will afford a final concentration of 5nM and 50nM for miRNA mimic and inhibitor; respectively, after being added to the cells. To create the transfection complexes, 0.75 L of HI Perfect Transfection Reagent, cat no. 301704, Qiagen, Hilden, Germany, is added to 24.25 L of RPMI culture media without serum. After 10 minutes at 15-20°C of incubation, the complex is then divided into 25 L portions and added to every well, followed by the adding 175 L of DMEM media. The viability of the transfected HNO97 cells was assessed using the MTT test after they had been cultured for 48 hours at 37 °C in a 5% CO_2 environment.

To assess the validity of the transfection experiment, the AllStars siRNA negative control, cat no: 1027280, Qiagen, Hilden, Germany, was used as negative controls for the miRNA mimic and miRNA inhibitor experiments. Moreover, the has-miR-1 mimic, cat no: MSY0000416, Qiagen, Hilden, Germany, was utilized as a positive control. The un-transfected cells were used for normalization.

2.2 Cell proliferation assay (MTT) for assessing cell viability

For the cell cytotoxicity experiment, the Vybrant® MTT Cell Proliferation Assay Kit, cat. no. M6494, was utilized (Thermo Fisher, Germany). A cell proliferation assay was used to gauge the health of the HNO97 cells that had been transfected with miR-155, a miR-34a mimic, and inhibitors. A 100 L of media were withdrawn and new media was added at the conclusion of incubation. From Invitrogen, ThermoScientific in Germany, 20 L of a 4, 5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/mL) were placed in every well. The plates were incubated for four hours at a temperature of 37 °C and 5% CO2. The MTT solution was then discarded, and 100 L of sodium dodecyl sulphate with hydrochloric acid (SDS-HCL) were placed in the wells as a last step. The vitality of the cells was assessed using a spectrophotometer (ELx 800; Bio-Tek Instruments Inc., Winooski, VT, USA) to measure the optical density at 570 nm.

2.3. Gene expression analysis in transfected cells

Utilizing quantitative Real-time polymerase chain reaction (PCR), the expression of the genes c-Myc and CDK6 in HNO97 cells was determined as follows:

2.3.a. Cell lysis

An average of 1x106 cells were dispersed and homogenized by bead-milling in a lysis solution that included guanidine-thiocyanate. Depending on the sample size and hardness, the Tissue Ruptor II (Qiagen, Hilden, Germany), a rotor-stator homogenizer, was used to thoroughly disrupt and homogenize samples in the presence of lysis solution in 15 to 90 seconds. Following that, the mixture is centrifuged for 20 minutes at 4000 rpm. After that, the cell supernatant is collected for RNA extraction.

2.3.b. Purification of total RNA using the RNeasy® Mini Kit

After combining the ethanol and tissue homogenate, the sample was loaded onto the RNeasy Mini spin column. High-quality RNA is eluted in RNase-free water when complete RNA binds to the RNeasy silica membrane and impurities are successfully removed. The RNA was extracted and purified with the RNeasy Mini kit, part number 74104, offered by Qiagen in Hilden, Germany. The instructions of the manufacturer for the procedure were strictly followed.

2.3.c. Reverse Transcription (cDNA synthesis)

Using the QuantiTect Reverse Transcription Kit, cat. no. 205310, the reverse transcription step was conducted (Qiagen, Hilden, Germany). The reverse-transcription master mix, which contains 14 L of genomic DNA, 1 L of Quantitect reverse transcriptase enzyme, 4 L of RT buffer, and 1 L of RT primer mix, was made on ice in a 20 L total volume. Except for template RNA, every component required for first-strand cDNA synthesis is included in the reverse-transcription master mix. The Quantiscript Reverse Transcriptase was inactivated by incubating the reaction mixture for three minutes at 95°C after 15 minutes at 42°C. After placing the reverse-transcription reactions on ice, real-time PCR started right away.

2.3.d. c-Myc and CDK6 genes expression analysis

With a 5 plex Rotor Gene PCR Analyzer, the QuantiTect SYBR Green PCR Kit (cat no. 204141: (Qiagen, Germany) and Hs ACTB 1 SG QuantiTect Primer Assay (-actin) cat no. 249900 were used to analyze and examine each sample. The Hs c-Myc, QuantiTect Primer Assay, and Hs CDK6 were also used (Qiagen, Germany). The PCR reaction mix was made up of 10 l of 10x QuantiTect Primer Assay, 2 l of 10x Universal Primer, 2 l of 10x QuantiTect Primer Assay, and 4 l of RNase-free water for a total volume of 18 l per well. The reaction mixture was carefully but gently blended and distributed in the appropriate amounts into the Rotor-Disc wells before 2 l of template cDNA was put, resulting in a final volume of 20 l. Rotor-Disc Heat-Sealing Film was used to carefully and firmly seal the disc.

Subsequently, a 15-minute activation phase for HotStarTaq DNA Polymerase was programmed into the real-time cycler Initial. A total of three procedures—denaturation at 94 °C for 15 seconds, annealing at 55 °C for 30 seconds, and extension at 70 °C for 30 seconds— were carried out 40 times. The expression levels were also standardized using the reference gene -actin. With the use of the 2-Ct test control equation, the relative level of expression (fold change) for the TP53 gene was normalized using an internal

control (-actin) and a calibrator (negative control sample).

2.4. Statistical Analysis

The acquired data were analyzed with GraphPad Prism Software 8.4.2. (San Diego, US). The experiment-wise error rate was monitored while using the Tukey's multiple comparisons test to identify discrepancies between the means of several groups. One-way analysis of variance (ANOVA) was performed to determine the statistical significant difference in mean values between the groups.

3. RESULTS

3. a Comparative analysis for the percentage of cell viability between HNO97 cells transfected with miR-155, and miR-34a mimics and inhibitors compared to untreated cells

Our results showed that transduction of HNO97 cells with miR-155 inhibitor showed a marked reduction in cell proliferation compared to untreated cells and a highly significant difference was reached ($p < 0.0001$). However, transduction with miR-155 mimic promotes cell proliferation index (140.2%) to exceed the proliferation index of untreated cells, and a significant difference was reached ($p < 0.0001$). On the contrary, the HNO97 cells transduced with miR-34a inhibitor showed the least cytotoxic effect, with a % of viability 122.2% compared to 58.21% in cells transduced with miR-34a mimic ($p < 0.0001$). With HNO97 cells transduced with either a miR-155 inhibitor or a miR-34a mimic, the cytotoxic effect was likewise considerably increased; however, the effect was more intense in cells treated with the miR-155 inhibitor, and a significant difference was seen between the two groups ($p = 0.0001$). Data are shown in Figures 1 and 2.

3.b c-Myc and CDK6 genes expression level in HNO97 cells transfected with miR-155, miR-34a mimics and inhibitors

Regarding miR-155, while the expression of the c-Myc and CDK6 genes significantly increased in the cells treated with miR-155 mimic ($p = 0.0001$) compared to the untreated cells, there

was no significant difference seen for either gene when the cells were transduced with a miR-155 inhibitor (c-Myc ($p = 0.97$), and CDK6 ($p = 0.93$)).

Regarding miR-34a, cells treated with miR-34a inhibitor had a substantial rise in the expression of the c-Myc and CDK6 genes ($p = 0.0001$), and the expression of both genes similarly increased when cells were transduced with miR-34a mimics (c-Myc, $p = 0.01$, and CDK6, $p = 0.001$). Figures 3 and 4 show the data analysis for the c-Myc and CDK6 genes.

4. DISCUSSION

Oral carcinogenesis is a successive procedure that occurred when the surface squamous epithelium is affected by accumulated genetic and epigenetic changes that alters cellular kinetics from minor deregulated growth to highly invasive and metastatic malignancy 13.

MiRNAs have received increased attention in recent years as a result of multiple studies highlighting their crucial involvement in cancer development 24. MiRNAs are formed of 19-22 nucleotides that silence targeted genes by binding to the 3'-untranslated regions (3'UTRs) of its mRNA, so it could improve either the apoptotic or oncogenic function of that genes 24, 25.

In oral carcinomas, miRNAs are correlated with cell cycle arrest, apoptosis, cell proliferation, chemoresistance, invasion and metastasis 26. Amongst thousands of human miRNAs, miR155 and miR34a are reported in previous numerous studies as a biomarker that their expression is dysregulated in OSCC 19, 23.

So, we try to find whether miR-155 and miR-34a affect the proliferation of OSCC by targeting c-Myc and CDK6. Both genes were calculated in an OSCC cell line (HNO97) transfected with miR-155, miR-34 mimic, and inhibitor using quantitative Real-time PCR. Results were standardized to the level of gene expression in untreated cells.

As regards to miRNA155, our results exhibited that there was a direct correlation between the increased expression of miR-155 and cell viability, c-Myc, and CDK6 higher expression.

The Increased rate of cell proliferation and invasion when cancerous cells were treated with miR-155 mimic is also reported by Zeng et al. 19, Baba et al. 27, KIM et al. 28, Wu et al. 29, Rather et al. 30.

Although Zeng et al. 19 highlighted that overexpression of miR-155 upregulates cyclin D2 expression and downregulates BCL6 expression, Baba et al. 27 found that miR-155 also upregulates the expression of signal transducer and activator of transcription 3 (STAT3). A negative connection between miR-155 and E-cadherin expression was also reported by KIM et al. 28 and Wu et al. 29. Moreover, Rather et al. 30 found that the tumor suppressor gene CDC73 was targeted by miR-155.

While miR-155 directly binds to the cyclin-dependent kinase inhibitor 1B (CDKN1B) 3'-UTR as reported by Fu et al. 31, there is no specific c-Myc 3'-UTR site for miR-155 to bind. Based on KIM et al. 28, and Wu et al. 29 studies, miR-155 regulates c-Myc through PIK3R1-FOXO3a- c-Myc pathway. It acts as an upstream regulator of pi3k to activate PI3K/AKT signaling.

When cells were treated with a miR-34a inhibitor, the maximum levels of CDK6 and c-Myc expression increased dramatically, and cell viability was enhanced. Manikandan et al. 32, Kumar et al. 23, Li et al. 16, Scapoli et al. 33, and Zhang et al. 34 all observed downregulated expression of miR-34a and its negative connection with cell proliferation.

However, Kalfert et al. 35, reported that miR-34a was increased in oropharyngeal carcinomas when normal epithelial cells are compared to neoplastic cells. The underlying antiproliferative mechanism of miR34a in Kumar et al. 23 study is due to the miR-34a ability to target SIRT1, E2F3a, and CDK4. Also, Manikandan et al. 32 showed that minimal miR-34a levels may relieve SIRT1 and MDM4, which deacetylate p53.

The indirect miR-34a impacts on c-Myc were also elucidated by Yamamura et al's 36 studies on prostate cancer cells. They discovered that miR-34a diminished the production of the positive transcription elongation factor (P-TEF) b (c-Myc imm) as a result of inhibiting the Myc-Skp2-Miz1 transcriptional complex, which mainly regulates cell migration and invasion RhoA. In addition to these outcomes, Christoffersen et al. 37 research found direct miR-34a binding to the Myc 3'UTR, proving that miR-34a can limit the activity of a wild-type Myc 3'UTR but not a mutant variant.

According to Zahi et al. 38, c-Myc knockdown boosted the expression of miR-34a, suppressed forkhead box P1 (Foxp1), and accelerated diffuse large B-cell lymphoma cell death. These results indicate that a negative feedback loop involving miR-34a and c-Myc is present.

On contrary, c-Myc and CDK6 genes are overexpressed in cells treated with a miR-34a mimic, but cell viability is diminished. This raises the question of the involvement of c-Myc in apoptosis and other kinase-independent biological activities of CDK6.

Functions of CDK6 in cancer are both kinase-independent and kinase-dependent. Regarding kinase-independent, it acts as a chromatin-bound cofactor stimulating genes' transcription that control angiogenesis, cell cycle inhibition, activation of stem cell, and immunological response 39.

The work by Kollmann et al. 40 emphasises CDK6's antiproliferative function. They evaluated the cell viability in mice models of T cell lymphoma and transformed B cell leukemia/lymphoma cells when p16INK4a is silenced and overexpressed, respectively. They came to the conclusion that overexpression of CDK6 and p16INK4 in transformed B cell leukemia/lymphoma cells is related with a reduction in cell proliferation, whereas an overexpression of CDK6 and an increase in cell growth were seen in an inactivated p16INK4 T cell lymphoma mouth model. This indicates that overexpressed CDK6 requires the silencing of p16INK4a by cancer cells in order to generate its oncogenic effect. By creating a negative feedback loop, CDK6-induced p16INK4a acts as a failsafe mechanism to control CDK6 activity 41.

5. CONCLUSIONS

miR-155 has a significant oncogenic effect on OSCC cell lines by increasing the c-Myc and CDK6 gene expression, but miR-34a lacks that well defined effect.

Research ethics and patient consent

Not applicable as in our study the cells were obtained from the institution not from actual patients.

Conflict of Interest

The Authors declare that there is no conflict of interest

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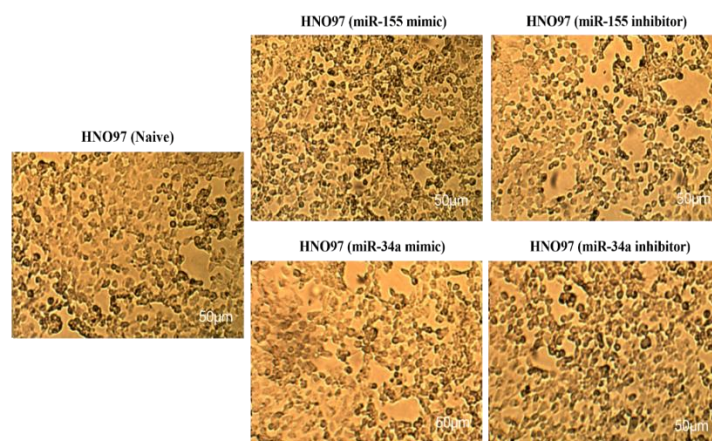
FIGURE 1: Microscopic images of HNO97 cells after transfected with miR-155, miR-34a mimic, and inhibitors for 48 hours. The images were captured by Labomed Vega Digital Camera (Labomed, USA), and the magnification power is 10x.
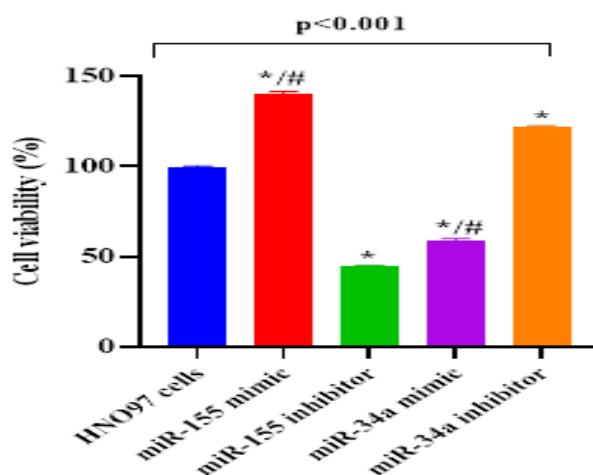

FIGURE 2: Comparative analysis for the percentage of viability in HNO97 cells transfected with miR-155 and miR-34a mimics and inhibitors compared to untreated cells, ANOVA: Analysis of variances. The data are presented in mean and SEM. *: statistical significance compared to untreated cells. #: statistical significance between miRNA mimic compared to the inhibitor of the same miRNA, ns: non-significant difference ($p > 0.05$).
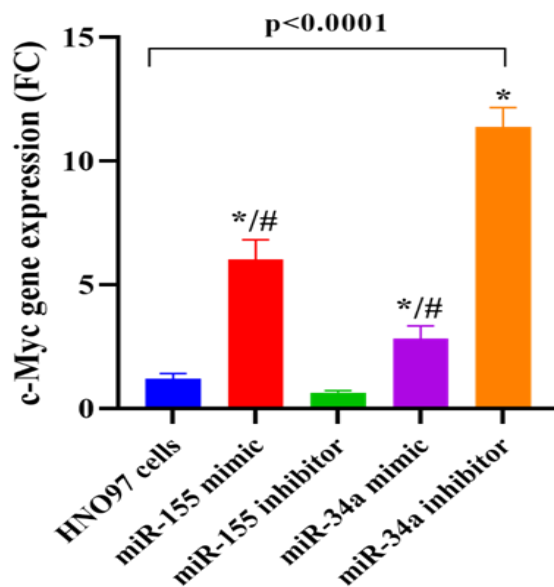
FIGURE 3: Comparative analysis between the c-Myc gene expression (FC) in HNO97 cells treated with miR-155 and miR-34a mimics and inhibitors compared to untreated cells.
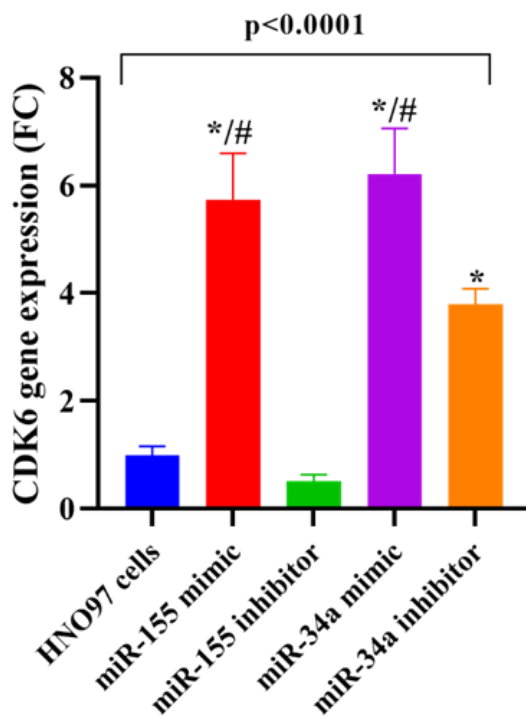

FIGURE 4: Comparative analysis between the CDK6 gene expression (FC) in HNO97 cells treated with miR-155 and miR-34a mimics and inhibitors compared to untreated cells.