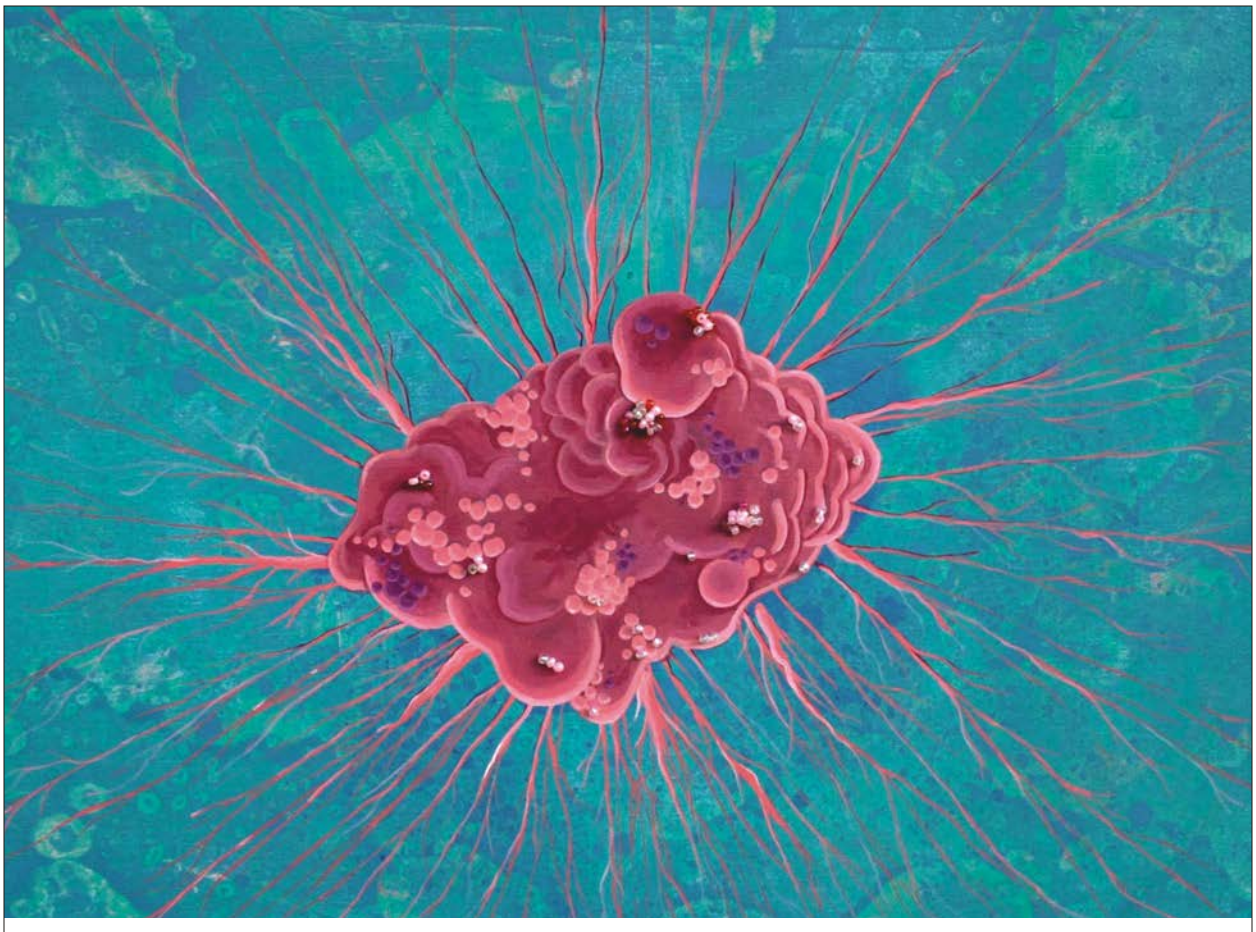

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Research Article
Expression of miR-34a/p53 and Their Apoptotic Target Bax in Oral Squamous Cell Carcinoma

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Abstract

Background and Objective: Identification of molecular targets for oral cancer is essential for developing therapeutic approaches that target those molecules. The miR-34a is a tumor suppressor gene and a transcriptional target of p53 protein. Bax is a pro-apoptotic protein which is activated by p53 and a target of miR-34a. The present study was designed to evaluate the expression levels of p53 and its transcriptional target miR-34a in oral squamous cell carcinoma cell line and to correlate this expression with their apoptotic target Bax. **Materials and Methods:** A total of 26 paraffin blocks were selected including 6 cases of normal oral mucosa, 10 cases of well differentiated oral squamous cell carcinoma and 10 cases of poorly differentiated oral squamous cell carcinoma. Sections from each block were subjected to quantitative real-time polymerase chain reaction analysis for quantification of miR-34a and p53 relative gene expression and immunohistochemical analysis was done to determine Bax protein expression. **Results:** The three studied proteins showed higher expression in normal oral mucosa than well differentiated oral squamous cell carcinoma and a lower expression in poorly differentiated oral squamous cell carcinoma. A statistically significant direct correlation was observed between the gene expression level of miR-34a and the expression level of Bax protein among studied cases and between the level of p53 gene expression and Bax protein expression. **Conclusion:** p53 dysfunction in oral carcinogenesis might lead to dysregulation of miR-34a and loss of apoptotic mechanism which gives the chance for the cancerous cells to proliferate and survive.

Key words: Oral squamous cell carcinoma, miR-34a, p53, Bax, apoptosis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oral cancer is one of the most common types of cancer throughout the world where squamous cell carcinoma is the main histological type occurring in the oral cavity and hence given the name "oral cancer"[1,2]. Identification of molecular targets for oral cancer is essential for developing therapeutic approaches which target those molecules[3].

MicroRNAs (miRNAs) are short, endogenous noncoding mRNA sequences of 18-24 nucleotides length which act as important regulator of gene expression through degradation of target mRNAs[4]. Approximately, half of all human miRNAs can function as tumor-suppressor or oncogenic miRNAs, depending on their targets[5-7]. One of these is miR-34a which was originally discovered as a potential tumor suppressor that is down regulated and induced apoptosis in neuroblastoma cells[8]. Several studies reported the role of miR-34a as a tumor suppressor in a number of tumor types including prostate cancer, hepatocellular carcinoma, neuroblastoma and colon cancer[9-13].

The p53 is a tumor suppressor gene which induces growth arrest and initiates apoptosis after exposure to DNA damage[14]. It was reported that miR-34a is a transcriptional target of p53 protein[15]. Thus, any inactivating mutations of p53 or genomic mutations at the p53 binding site in binding site of the miR-34a gene might cause loss of the expression of miR-34a[16]. Moreover, Chang *et al.*[17] observed that miR-34a induced apoptosis dependent on the presence of wild-type p53. Furthermore, p53 induces apoptosis through transcriptional activation of Bax and down regulation of Bcl-2[18].

Bax is a pro-apoptotic protein which activates both intrinsic and extrinsic apoptotic pathways[19,20]. Kontos *et al.*[21] noticed that cancer cells which highly express Bax are sensitized to apoptosis , while Bax-negative cells are resistant to apoptosis. In addition, it was found that miR-34a target Bax gene and helps in its upregulation as a mean of the cell to protect itself from virus infection by promoting apoptosis in cells induced by influenza A virus[22].

The present study aimed to evaluate the gene expression level of both p53 and miR-34a in Oral Squamous Cell Carcinoma (OSCC) cell line and to correlate their expression with their apoptotic target Bax in order to add information about their benefits and mutual function as masters and mediators of apoptosis in oral cancer cells.

MATERIALS AND METHODS

This study was carried out in the period between November, 2016 and June, 2017. The immunohistochemistry was done in Theodor Belharz Institute, Cairo, Egypt and RT-PCR for p53 and miR-34a was done at the Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt.

Case selection: A total of 26 paraffin blocks were used in this study including 6 cases of normal oral mucosa, 10 cases of well differentiated OSCC and 10 cases of poorly differentiated OSCC. All cases were retrieved from the archives of Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Cairo University. A four micron section was cut from each block, stained with haematoxylin and eosin (H and E) and re-examined for the confirmation of diagnosis. The cases of OSCC were rediagnosed and graded according to Pindborg *et al.*[23], however the cases of normal oral mucosa were selected from normal oral epithelium adjacent to benign oral mesenchymal neoplasms and were used as the control group.

Paraffin sections from each case were mounted on positively charged glass slides (Optiplus, Biogenex, Milmont Drive, CA, USA) for immunostaining with anti-Bax antibody. In addition, ten sections of 5 µm thickness were cut from each case and placed in plastic Eppendorf tubes to be subjected equally to a qRT-PCR analysis for miR-34a and p53 gene expression.

Immunohistochemistry: The 26 paraffin embedded tissue sections on positively charged slides were immunostained with anti-Bax antibody by super sensitive biotin-streptavidin staining technique. Tissue sections were deparaffinized, rehydrated and treated with endogenous peroxidase in 0.3% H_2O_2 for 30 min to block the endogenous peroxidase activity. For antigen retrieval, the slides were boiled in 10 mM citrate buffer, pH 6.0 for 10-20 min followed by cooling at room temperature for 20 min. The positive test slides were incubated with the primary antibody mouse monoclonal anti-Bax antibody (Cat #MS-711-R7) Thermo Scientific, Labvision, Kalamazoo, MI, USA) for 30 min at room temperature in a humified chamber. On the other hand, the negative control slides were not exposed to the primary antibody. After washing with phosphate buffer solution (PBS), the slides were treated with the biotin-labeled link antibody for 30 min and then the streptavidin conjugated horseradish peroxidase was used. The diaminobenzidine chromogen was applied to visualize the antigen antibody reaction. All these reagents belong to the Universal Labeled Streptavidin-Biotin 2 System, Horseradish Peroxidase (code No. K0673 Dako Cytomation, Glostrup, Denmark).Then all the slides were immersed in Mayer's hematoxylin for counter staining. Finally, the sections were covered by cover slips using aqueous mounting medium.

Immunohistochemical evaluation: The ordinary light microscope was used to detect and localize the immunostaining of anti-Bax antibody. Cells with cytoplasmic staining were considered positive. Then, all the sections were examined by an image analyzer computer system using the software Leica Qwin 500 (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Five random fields in each specimen were captured using a magnification (400X) to determine the area percentage and immunostaining intensity of the positive tumor cells. The area percentage and immunostaining intensity were scored from 1-3 according to the difference between the largest and smallest mean value of each parameter in the studied cases. The scores of both area percentage and immunostaining intensity were then summed to obtain a single total score. The overall reaction was considered mild (score 1 and 2), moderate (score 3 and 4), or strong (score 5 and 6) according to the single total score[24].

MicroRNA extraction: The miRNAs were extracted from sections of normal oral mucosa and oral squamous cell carcinoma by miRNeasy extraction kit (Qiagen, Valencia, CA, USA) using 750 µL QIAzollysis reagent. The tissue was incubated for 5 min at room temperature, then 200 µL chloroform were added, vortexed for 15 sec and incubated for 2-3 min at room temperature. This was followed by centrifugation at 12000 rpm at 4°C for 15 min. The upper watery phase was removed and 1.5 times of its volume (100%) ethanol was added. About 700 µL of this mixture were placed in RNeasy Mini spin column in 2 mL collection tube and centrifuged at 8000 rpm at room temperature for 15 sec. After the mixture had completely passed the column, 700 µL of buffer RWT were added to each column and again centrifuged at 8000 rpm at room temperature for 15 sec. About 500 µL buffer RPE were added to the column and centrifuged at 8000 rpm at room temperature for 15 sec. The previous process was repeated for 2 min at full speed. The column was transferred to new 1.5 mL collection tube and 50 µL RNAse-free water was pipetted directly onto the column and centrifuged for 1 min at 8000 rpm to elute RNA. The extracted microRNA was then stored at -80°C until use.

Quantitative real-time PCR (qRT-PCR) analysis for miR-34a expression: Reverse transcription was carried out on miRNA in a nal volume of 20 µL RT reactions (incubated for 60 min at 37°C, followed by 5min at 95°C) using the miScript II RT kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using a MiScript SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and miScript primer assay miR-34a (Qiagen, Valencia, CA, USA). The 20 ng of cDNA were used as a template in a total volume of 20 µL reaction with the following conditions: Denaturation at 95°C for 15 min followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec, in which fluorescence was acquired and detected by Rotor-gene Q Real-time PCR system (Qiagen, USA). After the PCR cycles, melting curve analyses were performed to validate the specific generation of the expected PCR product. The SNORD 68 was used as an endogenous control. The expression level of miR-34a was evaluated using the ΔCt method. The cycle threshold (Ct) value is the number of quantitative PCR cycles required for the fluorescent signal to cross a specified threshold. The ΔCt was calculated by subtracting the Ct values of SNORD 68 from those of target microRNA. The ΔΔCt was calculated by subtracting the ΔCt of the control samples from the ΔCt of the disease samples. The fold change in miR-34a expression was calculated by the equation 2-ΔΔCt.

Quantitative real-time PCR (qRT-PCR) analysis for p53 expression: Total RNA from sections of normal oral mucosa and oral squamous cell carcinoma were extracted with Trizol reagent. The RNA integrity and concentration was determined by nanodrop measurement at 260 nm. One microgram of extracted RNA was reverse transcribed to cDNA with High cDNA Reverse Transcriptase Kit. The cDNA was amplified for the expression of p53 and β-actin with SYBR green fluorophore following the manufacturer's recommended amplification procedure. The sequence of primers used for real-time PCR analysis were: p53 forward 5′-GCCCAACAACACCAGCTCCT (sense) and 5′-CCTGGGCAT CCTTGAGTTCC-3′ and human GAPDH, 5′-GAGTCAACGGATTT GGTCGT-3′ (sense) and 5′-GACAAGCTTCCCGTTCTCAG-3. The relative quantification of p53 gene was determined using the comparative CT method. The ΔCt was calculated as the difference between the average Ct values of the β-actin from the average Ct value of p53 gene. The ΔΔCt was determined by subtracting the ΔCt of the control from the ΔCt of the oral squamous cell carcinoma. The thermal cycling profile consisted of 95°C for 4 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 18 sec. Samples were run in triplicate. Relative expression of the target gene was calculated by the equation 2-ΔΔCt which was the amount of p53 product, normalized to the endogenous control (GAPDH) and relative to the control sample.

Statistical analysis: The total scores of immuno-expression of Bax in the studied cases, values of p53 and miR-34a gene expression were expressed as median (min-max). Kruskal-Wallis test was used to investigate the significant difference between the studied cases for each parameter. For correlation analysis, Spearman's rank correlation coefficient was performed to determine any correlation between the mean values of Bax protein expression, p53 and miR-34a expression. At 95% confidence interval, $p \leq 0.05$ was considered significant.

RESULTS

Immunohistochemical detection of Bax: All normal oral mucosa cases (100%) showed weak cytoplasmic immunopositivity for Bax. The positivity was diffuse within layers of normal stratified squamous epithelium. However, the positivity tends to be concentrated within basal and parabasal cell layers (Fig. 1). Regarding The OSCC, all cases (100%) of well differentiated OSCC revealed Bax immunopositivity. The positivity was diffuse through layers of keratin pearls and cell nests (Fig. 2). However, 70% of poorly differentiated OSCC cases showed less Bax immunostaining among the tumour cells (Fig. 3). There was significant decrease in the total score of Bax immuno-expression from well to poorly differentiated carcinoma ($p \leq 0.0035$) (Fig. 4, Table 1).

Expression of miR-34a by qRT-PCR: The miR-34a was detected in normal oral mucosa cases, well differentiated OSCC as well as poorly differentiated OSCC cases. The expression level of miR-34a was less in poorly differentiated carcinoma than well differentiated OSCC cases. There was a statistically significant decrease regarding the levels of miR-34a gene expression in poorly differentiated cases in comparison to well differentiated cases, $p \leq 0.0255$ (Fig. 4, Table 1).

Expression of P53 gene by qRT-PCR: The p53 gene was expressed in all studied cases. The p53 expression was decreased in cases of poorly differentiated OSCC in comparison to well differentiated OSCC cases. A statistically significant decrease was detected, $p \leq 0.009$ (Fig. 4, Table 1).

Spearman's rank correlation coefficient analyses: A positive correlation was detected between the gene expression levels of p53 and miR-34a ($p \leq 0.0001$). A statistically significant direct correlation was shown between the gene expression level of miR-34a and the expression level of Bax protein among studied cases, ($p \leq 0.0001$). A statistically significant positive

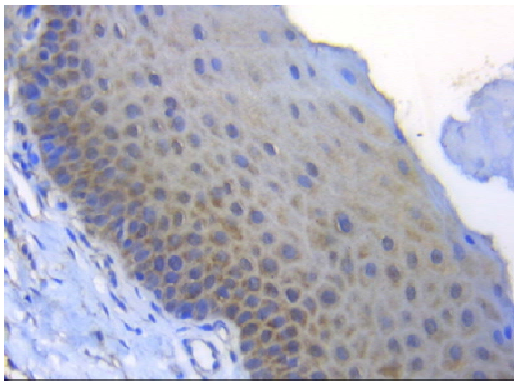

Fig. 1: A photomicrograph of normal oral mucosa showing cytoplasmic Bax immuno-expression among the different layers of the epithelium (anti-Bax antibody 400X)

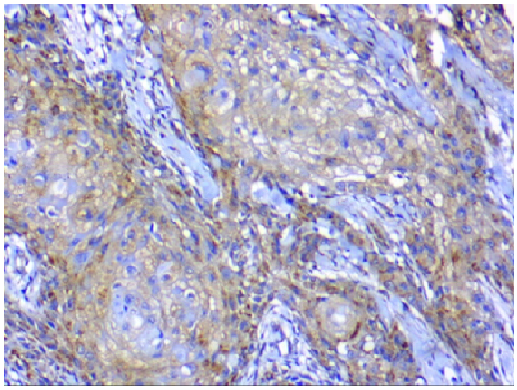

Fig. 2: A photomicrograph of well differentiated squamous cell carcinoma showing cytoplasmic Bax immuno-expression in cell nests (anti-Bax antibody 200X)

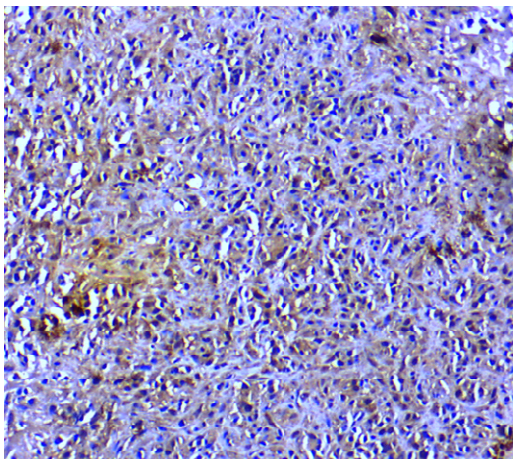

Fig. 3: A photomicrograph of poorly differentiated squamous cell carcinoma showing Bax immuno staining in some of the tumor cells (anti-Bax antibody 200X)

Table 1: Statistical difference in protein expression of Bax, gene expression of miR-34a and p53 among studied cases

	Studied cases			
Expression	Normal oral mucosa median	Well differentiated OSCC median	Poorly differentiated OSCC median	p-value
Bax protein expression	1.80	4.800	2.80	0.0035*
miR-34a gene expression	0.12	0.512	0.302861	0.0250*
p53 gene expression	0.08	0.550	0.28	0.0090*

*Significant value = $p \leq 0.05$

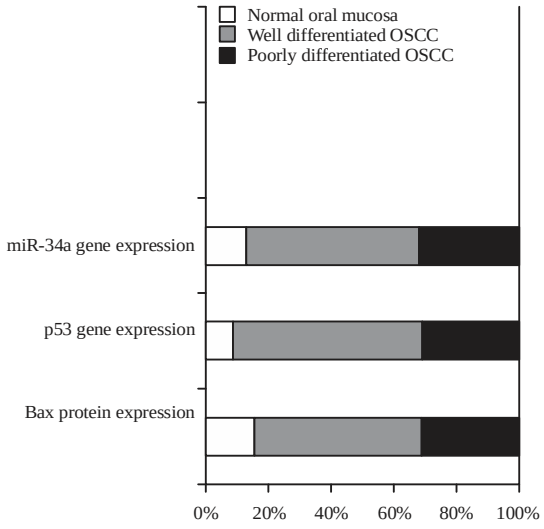

Fig. 4: Median range of expression levels of Bax protein, p53 gene and miR-34a gene in studied cases

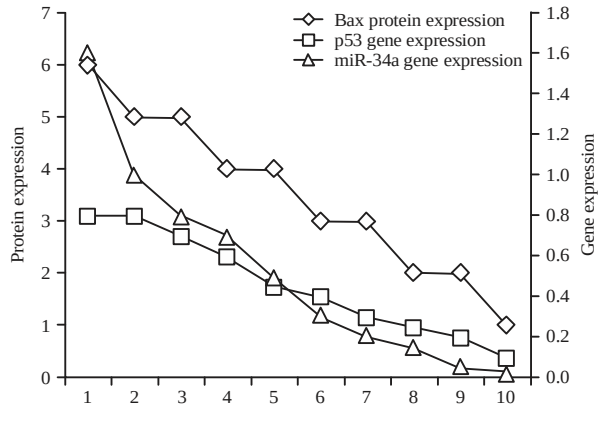

Fig. 5: Positive correlation between the expression of p53, miR-34a and Bax among studied groups

correlation was revealed between the level of p53 gene expression and Bax protein expression ($p \leq 0.0001$) (Fig. 5).

DISCUSSION

Abnormalities of miRNAs play a role in cancer formation as they control several biological processes as differentiation, proliferation and apoptosis[25]. This study was performed to investigate the expression of miR-34a in OSCC and its role in apoptosis. In the present study, the qRT-PCR analysis of miR-34a showed a high expression in normal oral mucosa than well differentiated OSCC and a lower expression in poorly differentiated OSCC. This might indicate that the decreased expression of miR-34a plays a role in the proliferation and progression of OSCC. Present study finding was in accordance with Kumar *et al.*[26] and Li *et al.*[3], who found a significant decrease of miR-34a expression in oral cancer cell lines compared to adjacent normal tissue. In addition, author concluded that miR-34a could be used as a prognostic biomarker for OSCC where its deregulated expression was associated with poor prognosis and aggressive phenotype of oral cancer.

Welch *et al.*[8] observed that miR-34a plays a role in tumor suppression by inducing apoptosis in neuroblastoma cell lines when re-introduced after showing decreased expression of miR-34a. In addition, the tumor suppressor function of miR-34a has been reported in several tumor types including prostate cancer, hepatocellular carcinoma and colon cancer[9,10,12,13]. These results are inconsistent with the statistically significant difference of the levels of miR-34a gene expression in present study which demonstrated that the dysregulation of miR-34a might affect its tumor suppressor action and its role in apoptosis.

Moreover, p53 gene mutation has been reported in OSCC[27,28]. In present study, p53 gene expression was noticed in normal oral mucosa then decreased in well differentiated OSCC, meanwhile poorly differentiated OSCC showed a more decreased expression of p53. This inactivation of p53 suggested a loss of its apoptotic potential and high proliferation of cancer cells. Iamaroon *et al.*[29] studied the correlation between p53 and Ki-67 in oral cancer. They concluded that alterations in the p53 protein might lead to increased cell proliferation. Furthermore, the loss of p53 tumor suppressor function and its association with poor prognosis in oral cancer has been reported[30].

The miR-34a is a transcriptional target of p53 protein[15]. Chang *et al.*[17] stated that miR-34a induced apoptosis is dependent on the presence of wild-type p53. In addition, it

has been concluded that miR-34-mediated apoptosis might be suppressed by inactivation of p53 and/or miR-34 genes[31]. In the present study, there was a positive correlation between the gene expression of miR-34a and p53 as both are decreased from well differentiated to poorly differentiated carcinoma. This result could be explained that p53 inactivation led to dysregulation of miR-34a transcription, thus decreasing miR-34a gene expression which provided an opportunity for cancer cells to proliferate and to escape apoptosis.

Bax is the most characteristic death-promoting member of the Bcl-2 family[32]. In the current study, Bax immuno-expression was confined to basal and parabasal layers of normal mucosa and it was stronger in well differentiated squamous cell carcinoma than poorly differentiated carcinoma. This was in agreement with Thomas and Sethupathy[33] and Zhang *et al.*[34], who found higher expression of Bax in well differentiated tumors than in poorly differentiated tumors .This decrease of Bax expression indicated the cease of apoptotic cell death and the advance of proliferation and growth of the cancerous cells. In addition, this down regulation of Bax could reflect its role in the development of a more aggressive oral cancer stage[35,36].

A statistically significant positive correlation was shown in this study between the gene expression levels of miR-34a and the expression levels of Bax protein. In addition, a statistically significant positive correlation was revealed between levels of p53 gene expression and Bax protein expression. It has been reported that p53 may induce apoptotic cell death by down-regulating Bcl-2 and up-regulating Bax expression[18,37]. Moreover, miR-34a is one of the potent mediators of tumor suppression by p53 through down regulation of several of its transcripts including Bcl-2[31]. This might explain that when there was a loss in gene expression of both p53 and miR-34a in present study, this affected Bax expression and hence led to loss of apoptosis and promotes proliferation of cancerous cells.

The significant positive correlation between the gene expression level of p53 and miR-34a and their apoptotic target Bax in this study confirmed their crucial role in inducing apoptosis. Therefore, the loss or mutation in any of these proteins will lead to proliferation of the oral cancer cells and hence it is recommended to perform further studies by using gene therapy for restoring these proteins in OSCC as it might help in its treatment.

CONCLUSION

The p53 dysfunction in oral carcinogenesis might lead to dysregulation of miR-34a and loss of apoptotic mechanism which gives the chance for the cancerous cells to proliferate and survive. In addition, restoring both p53 and miR-34a could aid in anticancer gene therapy of oral cancer.

SIGNIFICANCE STATEMENT

This study discovers a positive correlation between the gene expression levels of both miR-34a and p53 in oral squamous cell carcinoma cell line. In addition, a significant direct correlation was observed of both p53 and miR-34a with the expression of their apoptotic target Bax protein that can be beneficial for adding information about their benefits and mutual function as masters and mediators of apoptosis in oral cancer cells. This study will help the researchers to cover the area of apoptosis at the gene expression level of its regulating proteins that many researchers were not able to explore. Thus, a new theory for new therapeutic treatments for oral cancer through gene targeted therapy for such apoptosis inducing proteins may be arrived at.

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