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Original Article

Assessment of the Methylene Blue Mediated Photodynamic Therapy on *BCL2* and *BAX* Genes Expression at mRNA Level and Apoptosis of Head and Neck Squamous Cell Carcinoma Cell Line

Shadi Kahalian[1], Maryam Koopaie[1], Neda Hakimiha[2], Sajad Kolahdooz[3]

[1] *Department of Oral Medicine, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran*

[2] *Laser Research Center of Dentistry, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran*

[3] *Universal Scientific Education and Research Network (USERN), Tehran, Iran*

Corresponding author: Maryam Koopaie, Department of Oral Medicine, School of Dentistry, Tehran University of Medical Sciences, Tehran, North Kargar St, Po. Code: 14399-55991 P.O.BOX:14395 -433, Iran; Email: mariakoopaie@gmail.com; Tel.: 0098-9120330580

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Abstract

Aim: This study aimed to assess the effect of photodynamic therapy (PDT) on apoptosis of head and neck squamous cell carcinoma (HNSCC) cells by flow cytometry and evaluating *BAX* and *BCL2* genes expression.

Materials and methods: In this in vitro study, human HNSCC cell line (HN5; NCBI. C196) was used and after cell culture, they were divided into four groups: controls (group C), cells irradiated by a diode laser with a wavelength of 660 nm, 150 mW power, and 45 J/cm^2 energy density (group L), cells treated by methylene blue (group MB), and cells treated using PDT (group MB plus L). The RNA was then extracted and subjected to quantitative reverse transcription polymerase chain reaction (qRT-PCR) to assess *BCL2* and *BAX* genes expression. Flow cytometry analysis was performed to assess apoptosis. Data were analysed using ANOVA.

Results: PDT caused significant down-regulation of *BCL2* (p<0.001) and significant overexpression of *BAX* (p<0.05) and PDT induced apoptosis HNSCC cell line. Changes in expression of these genes were not significant in other groups (p>0.05).

Conclusions: Considering the down-regulation of *BCL2* and overexpression of *BAX* after PDT using a 660-nm diode laser and MB with 3.2 μg/mL concentration and flow cytometry results, it is suggested that this modality can be introduced for induction of apoptosis in the HNSCC cell line.

Keywords

apoptosis, *BAX, BCL2,* head and neck squamous cell carcinoma (HNSCC), photodynamic therapy, qRT-PCR

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the most common malignancy of the head and neck region with relatively low survival rate and response and high resistance to treatment.[1] It can affect any part of the oral cavity but the tongue and floor of the mouth are the most commonly involved sites. The treatment of HNSCC mainly aims at improving the quality of life of patients. The main treatment modalities for HNSCC include surgery, radiotherapy, and chemotherapy which have adverse short-term and long-term effects on oral health, general health, and patient's quality of life.[2] The treatment of choice is surgery in the initial stages. However, squamous cell carcinoma (SCC) is often detected in advanced stages and requires a multidisciplinary approach comprising of surgery, radiotherapy and chemotherapy.[3] Nonetheless, these modalities have adverse consequences such as oral mucositis and aesthetic disturbance after surgery.[4] Thus, attempts are ongoing to find alternative treatment modalities for management of SCC.

Evidence shows that laser therapy has several advantages for resection of oral mucosal lesions such as excellent hemostasis, precise incision, and minimal damage to the adjacent tissues.[5] Photodynamic therapy (PDT) is a successful clinical modality recently suggested for management of cancerous lesions.[6] Successful management of the HNSCC with PDT in its initial stages has also been reported.[7] In PDT, photosensitizers are used locally or systemically, and selectively accumulate in the target tissue. Next, the target tissue is irradiated leading to cell damage. Since only the target tissue is irradiated, deformity and injury of the adjacent structures is uncommon.[8] PDT uses non-toxic photosensitizers and harmless visible light in combination with oxygen to create cytotoxic reactive oxygen species which can eliminate malignant cells by apoptosis, necrosis or both.[9] In superficial skin lesions, the injured tissue is directly irradiated, while in involvement of internal organs, the light source is connected to a fiber optic catheter to allow the exposure of the target organ.[10] PDT also significantly changes the level of oxygen and blood supply of the tumour and leads to its hypoxia.[11] Diode lasers are commonly used as the light source for PDT due to their easy handling and cost-effectiveness.[12]

Apoptosis refers to programmed cell death, and defects in apoptosis can lead to carcinogenesis.[13] In primary phases of apoptosis, the permeability of the mitochondrial outer membrane allows for pro-apoptotic factors such as cytochrome C to enter the cytosol from the intermembrane space and induce the apoptotic cascade. This phenomenon is precisely regulated by the *BCL2* family of proteins. [14] In this family, *BAX* is an important protein that allows penetration into mitochondrial outer membrane. On the other hand, anti-apoptotic members such as *BCL2* inhibit this process by direct binding to pro-apoptotic proteins. The pro-apoptotic *BAX* and anti-apoptotic *BCL2* genes are the best regulators of apoptosis, and the ratio of the protein products of these two genes determines the rate of apoptosis and plays an important role in cell homeostasis and tumorigenesis.[15] Abnormal expression of *BCL2* has been reported in 50% of human cancers.[16] Overexpression of *BCL2* oncogene protein has been reported as a strong anti-apoptotic factor in HNSCC, causing its resistance to radiotherapy and chemotherapy.[17] On the other hand, activation of *BAX* would enhance the permeability of mitochondrial membrane, which is an essential step in response to cell death and subsequent tumour suppression.[18]

AIM

Considering all the above, this study aimed to assess the effect of PDT with diode laser on apoptosis and expression of pro-apoptotic *BAX* and anti-apoptotic *BCL2* gene in HNSCC cell line.

MATERIALS AND METHODS

Cell cultures and treatments

In this in vitro experimental study, HN-5 head and neck carcinoma cell line (NCBI. C196) was obtained from the Pasteur Institute of Iran (Iran, Tehran). The cells were cultured in the Dulbecco's modified Eagle's medium along with 10% fetal bovine serum and incubated at 37°C with 5% CO_2 and 1-2% humidity for 24 hours.[19] After culture, the cells were divided into four groups of controls (C), laser (L), methylene blue (MB), and PDT (MB plus L). MB (Merck, Germany) was used as photosensitizer in this study, which was activated at the wavelength of 660 nm. In order to find the effective concentration of MB for use in this study, HN-5 cells were exposed to MB at 0.2, 0.4, 0.8, 1.6, 2, 3.2, 4, and 8 μg/mL concentrations for 1 hour and their cytotoxicity was evaluated in dark conditions. The methyl thiazolyl tetrazolium (MTT) assay was used to assess the cytotoxicity of the tested concentrations. Phosphate buffered saline (PBS) was used to eliminate dead cells and optical density of the solution was measured by spectrophotometer (Ava Spec 2048, Avantes, Apeldoorn, Netherlands). The effective concentration of MB was found to be 3.2 μg/mL by the aforementioned assay, which did not cause cytotoxicity effects on cells.

Diode laser

Diode laser (Klas DX-61, Konftec, Taiwan) with 660 nm wavelength, 150 mW power and 45 J/cm^2 energy density was used in this study.[20] Each well was irradiated with the sweeping motion of the laser hand-piece for 2.5 minutes in a continuous-wave mode and the distance between the laser hand-piece and the well was 1 mm. Optic Cap B hand-piece tip was used in this study measuring 0.5 cm^2 in cross-sectional area. Cells in the L and PDT groups underwent laser irradiation and were analysed after 24 hours of incubation.

Flow cytometry

In order to assess apoptosis using flow cytometry, 1 mL of the single cell suspension of each group (containing 1×10^6 cells) was prepared as described. 1 mL of cell suspension was collected and washed with cold PBS once and suspended in 100 ml of binding buffer. Afterwards, 10 ml of Annexin V-FITC was added, and the mixture was placed on ice for 15 min in the dark. Next, 380 ml of 1X binding buffer and 10 ml of PI were added, incubated on ice for 15 min in the dark, washed with cold PBS once, and suspended with 1 mL of PBS. The apoptosis of the cells was measured using flow cytometry (Beckman Coulter, Inc.). The EXPO32 ADC software (Beckman Coulter, Inc.) was used to analyse the immunofluorescence data and evaluate the apoptosis rate.

RNA extraction using RNX-Plus

For RNA extraction, after reaching 90% cell confluence in a plate, the overlaying medium was removed and the cells were rinsed with PBS. Then, they were detached from the bottom of the plate using trypsin. After neutralizing the enzyme with a medium containing 10% fetal bovine serum, all contents of the plate were transferred into a microtube and centrifuged at 12000 rpm for 2-5 minutes. Next, the supernatant was discarded and 1 mL of ice cold RNX-Plus (Sinaclone, Iran) was added to the tube containing 2 mL of homogenized sample and centrifuged for 5 to 10 seconds. It was then incubated at room temperature for 5 minutes. 200 µL of chloroform (per 1000 mL of RNX-Plus) was added to the contents of the microtube and manually mixed for 15 seconds. It was then placed on ice for 5 minutes and then the microtube was centrifuged at 12000 rpm at 4°C for 15 minutes.

A colourless layer formed on the top while an off-white layer formed at the bottom of the microtube. The interface of these two layers contained DNA and protein and appeared white. The supernatant (aqueous phase) was carefully transferred to a 1.5 mL RNase-free microtube and the same volume of isopropanol was also added to it. The mixture was gently mixed and incubated on ice for 15 minutes. The microtube was then centrifuged at 12000 rpm at 4°C for 15 minutes. The supernatant was discarded and 1 mL of 75% ethanol was added to it and shortly mixed. Next, it was centrifuged at 7500 rpm at 4°C for 8 minutes. The supernatant was discarded. The microtube was placed at room temperature to cool down. After drying, the pellet was dissolved in 50 µL of diethylprocarbonated (DEPC)-treated water. For better dissolution, the microtube was incubated at 55°-60°C for 10 minutes.

Complementary DNA (cDNA) using peqLAB kit

Complementary DNA (cDNA) was synthesized using a cDNA synthesis kit (PeqLab Biotechnologie GmbH, Switzerland) according to the manufacturer's instructions. The final volume of cDNA synthesis reaction was 20 µL. For 20 µL of cDNA, 1 µg of the total RNA was used. For this purpose, 1 µL of random hexamer (Sinaclone, Iran) and 1 µL of oligo (dT) primer (Sinaclone, Iran) were used. For reverse transcription reaction, a mixture of both primers along with 8 µL of DEPC-treated H_2O + RNA (1 µg) was used. The thermal protocol for cDNA synthesis reaction included incubation at 65°C for 5 minutes. After complete fixing of primer on RNA at 65°C, Enzyme Mix was added to initiate the enzymatic reaction. The Enzyme Mix included 1 µL of dNTP mix (Sinaclone, Iran), 2 µL of 10X cDNA synthesis MMLUV buffer (Sinaclone, Iran) and 0.5 µL of MMLUV enzyme (Sinaclone, Iran). Around 10 µL of this enzyme was well mixed with the initial primer/RNA and incubated at 42°C for 1 hour. The obtained cDNA was stored at −20°C. The concentration of extracted RNA and cDNA was measured by NanoDrop spectrophotometer (NanoDrop 2000™; Thermo Fisher Scientific, Waltham, MA, USA). The optical density of RNA and cDNA at 260 and 280 nm wavelengths was measured and the first value was divided by the second value. Regarding RNA, this value had to be between 1.8 and 2 in order to be acceptable. For cDNA, this value had to be between 1.6 to 1.8 in order to be acceptable and ensure optimal purity of the sample. To observe the quality of extracted RNA and cDNA, RNA and cDNA were run on agarose gel. For this purpose, 1.5% agarose gel was used. It was mixed with 10X TBE buffer. Agarose gel was added to the electrophoresis tank. The voltage of the device was adjusted at 150 V. The gel was evaluated under UV light. Good-quality RNA had two distinct bands on agarose gel; one for 28SrRNA, which is longer and is located on the top and the other one is 18SrRNA, which is shorter and forms at the bottom of the gel.

For designing specific primer, primer-free software was used and for this purpose the number of respective genes was extracted from the NCBI website. By entering the number of each gene in the site, its general characteristics including name and respective sequence were automatically extracted from the database of website. Data were then transferred to primer-free software and the primer was designed taking into account the physical and chemical parameters such as primer length, PCR product, and melting temperature. After obtaining the primer in the form of lyophilized powder from the manufacturer, the primer solution was prepared in 100 pmol/µL concentration by adding sterile distilled water to the powder. This solution was kept at −20°C until use. For final use, the primer was diluted and entered into the final reaction in 10 pmol/µL concentration. The primer sequences for *BAX* and *BCL2* are shown in **Table 1**.

qRT-PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using Corbett Rotor-Gene™ 3000 (Corbett, Australia) and the QuantiFast™ SYBR®

Table 1. Sequence of primers used for *BAX* and *BCL2* genes expression

Primer	Sequence
GAPDH	F: TCCTGCACCACCTGCTTAG R: TCCACAGTCTTCTGGGTGGCAG
BAX	F: GGACGAACTGGACAGTAACATGG R: GCAAAGTAGAAAAGGGCGACAAC
BCL2	F: GCGTCAACAGGGAGATGTCA R: GCATGCTGGGGCCATATAGT

Green RT-qPCR Kit (Qiagen, Germany). The synthesized cDNA served as the template for this reaction; 30 µL of the total RNA was obtained. Considering the capacity of reverse transcription kit and concentration of NanoDrop RNA samples, in each vial, 1 µg of the total RNA of each sample was converted to cDNA. The real time PCR reaction components were 100 ng template, 12.5 µL MasterMix (Applied Biosystems, Foster City, CA, USA), 10 pmol forward and reverse primers, and deionized water to increase the final reaction volume to 25 µL. The temperatures and timings were adjusted according to the instructions provided in the kit **(Table 2)**. Pre-denaturation was performed at 95°C for 10 minutes, 95°C for 20 seconds, 58°C for 20 seconds and 72°C for 20 seconds.

Table 2. qRT-PCR amplification protocol PCR

Cycle	Cycle point
Hold	Hold @ 95°C, 10 min 0 s
Cycling (40 repeats)	Step 1: Hold @ 95°C, 20 s
	Step 2: Hold @ 58°C, 20 s
	Step 3: Hold @ 72°C, 20 s, acquiring to Cycling A
Melting	Ramp from 67°C to 95°C
	Hold for 90 s on the 1st step
	Hold for 5 s on next steps, Melt A

After completion of each cycle, the results were interpreted using amplification and melting peak curves. For this purpose, after each cycle, the melting peak was recorded and if it matched the range of the Corbett's melting temperature of the gene, it was considered positive. Since the melting temperature is affected by the ionic concentration of the environment as well, values of 1°C higher and lower are also considered acceptable. A negative control was also considered to assess possible contamination. For this purpose, qRT-PCR with the same concentrations, temperatures, and timings was performed without a template (no template sample). Negative result of the reaction ensured no contamination. On the other hand, in order to ensure the quality and quantity of the samples, positive controls were considered in two steps. First, the optical density of the template was measured by NanoDrop and next, the expression of the reference gene was evaluated. Template dilution was also performed for quality control of qRT-PCR. Next, the CT values of *BAX* and *BCL2* genes as well as the positive reference housekeeping gene were recorded. To assess the difference in relative expression of genes in the study groups, the ΔΔCT method was applied. First, the CT values of *BAX* and *BCL2* genes as well as the reference housekeeping gene were extracted for each sample and the two values were subtracted from each other to determine the ΔCT. The mean of ΔCT values was calculated for each group. The two values were subtracted to determine ΔΔCT. 2 squared by -ΔΔCT was calculated to assess the difference in gene expression according to the melting peak curve. The normally distributed data were analysed and compared using ANOVA.

RESULTS

ANOVA was applied to compare the $2^{-\Delta\Delta CT}$ value for the *BCL2* gene expression among the groups. *BCL2* showed significant down-regulation in PDT group ($p<0.001$). The difference in the $2^{-\Delta\Delta CT}$ value for the *BCL2* gene in PDT and control groups was 0.0692, which indicated significant down-regulation of *BCL2* in the PDT group ($p=0.01$). The mean $2^{-\Delta\Delta CT}$ value for *BCL2* gene in MB group was 0.322, which indicated down-regulation of this gene after treatment with MB. However, this reduction was not significant ($p>0.05$). The mean $2^{-\Delta\Delta CT}$ value for *BCL2* gene in laser group was 0.358, which indicated a reduction in expression of *BCL2* gene in laser group; however, this reduction was not significant either ($p>0.05$) **(Fig. 1)**.

Using ANOVA for comparing the $2^{-\Delta\Delta CT}$ value of *BAX* gene among the groups revealed that *BAX* overexpression in PDT group compared with the control group ($p<0.001$). The mean $2^{-\Delta\Delta CT}$ value for *BAX* in PDT group was 49.35, which indicated its significant overexpression in this group ($p<0.05$). The mean $2^{-\Delta\Delta CT}$ value for *BAX* in MB group was 42.925, which indicated its overexpression; but it was not statistically significant ($p>0.05$). The mean $2^{-\Delta\Delta CT}$ value for *BAX* in laser group was 1.582, which indicated its overexpression after laser treatment; however, it was not significant either ($p>0.05$).

Based on flow cytometry analysis, cell apoptosis rate of PDT and also MB group was significantly higher than that of control group ($p<0.05$). The cell apoptosis of group L was higher than that of the control group, but this value wasn't statistically significant ($p>0.05$). The flow cytometry results are illustrated in **Fig. 2**.

DISCUSSION

Evidence shows that treatment modalities that can cause down-regulation of *BCL2* as an anti-apoptotic protein and upregulation of *BAX* as a pro-apoptotic protein can

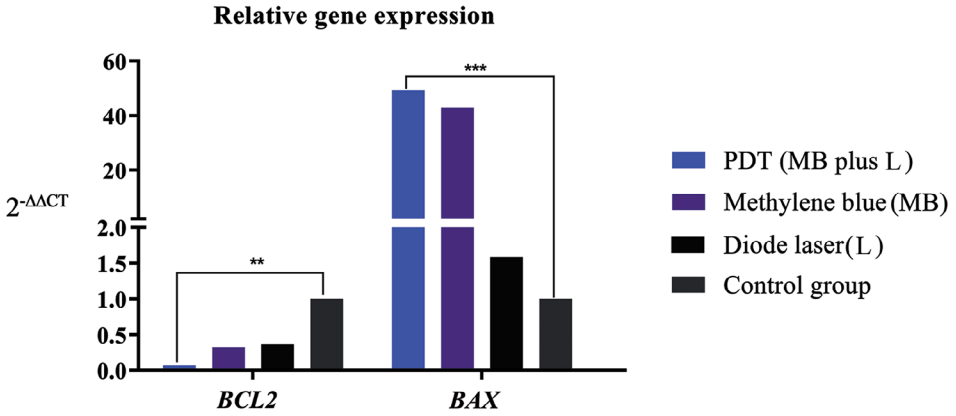

Figure 1. Analysis of relative *BAX* and *BCL2* genes expression revealed the significant difference in *BAX* and *BCL2* between PDT (MB plus L) group in comparison with control group but there are no statistically significant differences between other groups. *** ($p<0.001$), ** ($p=0.01$).

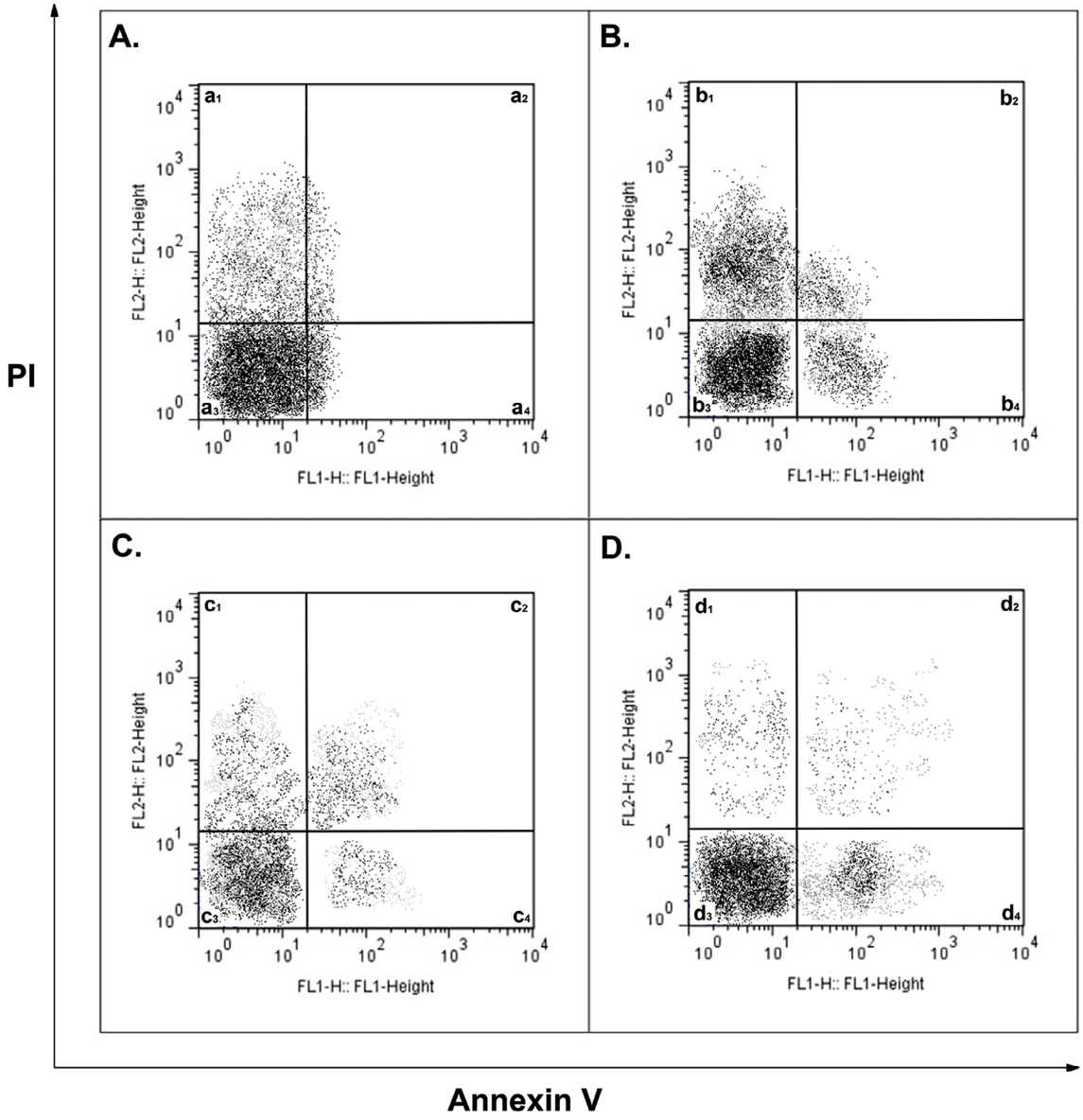

Figure 2. Results of the flow cytometry for evaluation of cell apoptosis rate. Assessment of the apoptosis rate in HN-5 cells in **A**) control group, **B**) MB group, **C**) Laser group, and **D**) PDT group.

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be effective against HNSCC. This study assessed the effect of PDT on HNSCC cell apoptosis and the expression of pro-apoptotic *BAX* and anti-apoptotic *BCL2* in HNSCC cell line. The results showed that PDT with diode laser and MB as a photosensitizer caused down-regulation of *BCL2* and overexpression of *BAX* in HNSCC cell line and also cell apoptosis based on flow cytometry. This finding indicated that PDT using MB can be used to induce apoptosis in HNSCC cell line. Reactive oxygen species released in the process of PDT can potentially induce the initiation of apoptosis.[21] Results of flow cytometry analysis confirm that PDT induces apoptosis in the HNSCC cell line. V dos Santos et al. evaluated *BCL2* targeted therapy in HNSCC and demonstrated that *BCL2* inhibitors as anti-tumour agents can be used for treatment of HNSCC. The natural *BCL2* inhibitors include EGCG, chelerythrine, antimycin A and gossypol, which have the potential for treatment of HNSCC.[22]

Kaomongkolgit et al. evaluated the cytotoxic effects of alpha-mangostin on HNSCC cell line for assessment of apoptosis. They assessed the expression of *BCL2* and *BAX* by real time PCR and western blot for evaluation of apoptosis.[23] They also assessed the morphological changes of the cells and performed single-stranded DNA immunofluorescence and DNA fragmentation analysis to evaluate apoptosis. The medication they used showed excellent apoptotic effects on the HNSCC cell line and caused down-regulation of *BCL2* and upregulation of *BAX*. Their results in assessment of apoptosis were similar to our findings, showing that medications that induce apoptosis cause down-regulation of *BCL2* and upregulation of *BAX*.

Garg et al. assessed the effect of PDT with erythrosine on malignant and premalignant oral epithelial cells in vitro, aiming to assess the effect of erythrosine as a photosensitizer for PDT on oral malignancies. They evaluated the susceptibility of H357 malignant cells and DOK dysplastic oral mucosal cell line to PDT with erythrosine photosensitizer.[24] They showed that high-dose PDT caused apoptosis and necrotic responses in both DOK and H357 cells. Their results were in line with our findings showing that PDT with a suitable photosensitizer would induce apoptosis and necrosis in cancer cells.[24] Kofler et al. assessed the effect of PDT with MB and 660 nm low-level diode laser on SCC. They reported that PDT with MB and diode laser significantly inhibited the growth of SCC colonies.[7] Their results were in accordance with ours and confirmed the potential of PDT with MB and diode laser to eliminate SCC cells. Coutinho-Camillo et al. assessed the expression of *BAX*, *BCL2,* and their family in relation to oral SCC. They indicated that absence of vascular invasion indicated upregulation of *BAX*.[25] Also, upregulation of *BCL2* had a direct association with tumour formation at the floor of the mouth. They added that the expression rate of these two genes in oral SCC can be used as a tumour predictor marker. Since *BAX* is a tumour suppressor and a pro-apoptotic factor and *BCL2* is a proto-oncogene and an anti-apoptotic factor, the rate of expression of these two genes can serve as a marker to predict the treatment outcome of SCC. In the present study, PDT caused upregulation of *BAX* and down-regulation of *BCL2*.

Induction of apoptosis (but not necrosis) in SCC cell line is among the most efficient, targeted, controlled treatment strategies for SCC with minimal side effects. Cell death should be shifted towards apoptosis instead of necrosis in cancer in order to minimize side effects. By doing so, we can prevent the unwanted death of highly proliferative normal cells such as the oral mucosal cells. Our results indicated that PDT can help us achieve this goal. Thus, it can be recommended as an adjunct to surgery especially in case of presence of residual tumoural involvement at the surgical margins or when the involved margins cannot be completely resected by surgery. Future animal studies followed by clinical trials are required to assess the efficacy of PDT for management of SCC in the clinical setting.

CONCLUSIONS

Considering the down-regulation of *BCL2* and overexpression of *BAX* after PDT with a 660-nm diode laser and MB with 3.2 μg/mL concentration, this modality can be effectively used for induction of apoptosis in HNSCC cell line.

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Оценка воздействия фотодинамической терапии, опосредованной метиленовым синим, на экспрессию генов *BCL2* и *BAX* на уровне мРНК и апоптоз клеточной линии плоскоклеточного рака головы и шеи

Шади Кахалиан[1], Мариям Коопаие[1], Неда Хакимиха[2], Саджат Колахдооз[3]

[1] *Кафедра оральной медицины, Медицинский факультет, Тегеранский университет медицинских наук, Тегеран, Иран*

[2] *Лазерный исследовательский центр дентальной медицины, Исследовательский институт дентальной медицины, Тегеранский университет медицинских наук, Тегеран, Иран*

[3] *Универсальная научно-образовательная и исследовательская сеть (USERN), Тегеран, Иран*

Адрес для корреспонеднции: Мариям Коопаие, Кафедра оральной медицины, Медицинский факультет, Тегеранский университет медицинских наук, Тегеран, ул. „Северный Каргар", п.индекс 14399-55991, п. ящик 14395 -433, Иран; Email: mariakoopaie@gmail.com; Тел.: 0098-9120330580

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Резюме

Цель: Это исследование было направлено на оценку влияния фотодинамической терапии (ФДТ) на апоптоз клеток плоскоклеточного рака головы и шеи (HNSCC) с помощью проточной цитометрии и оценку экспрессии генов *BAX* и *BCL2*.

Материалы и методы: В этом исследовании *in vitro* использовали клеточную линию HNSCC человека (HN5; NCBI. C196), и после культивирования клеток они были разделены на четыре группы: контрольная (группа C), клетки, облученные диодным лазером с длиной волны 660 нм, мощность 150 mW power и плотность энергии 45 J/cm^2 (группа L), клетки, обработанные метиленовым синим (группа MB), и клетки, обработанные с помощью ФДТ (группа MB плюс L). Затем РНК экстрагировали и подвергали количественной полимеразной цепной реакции с обратной транскрипцией (qRT-PCR) для оценки экспрессии генов *BCL2* и *BAX*. Анализ проточной цитометрии проводили для оценки апоптоза. Данные были проанализированы с использованием ANOVA.

Результаты: ФДТ вызвала значительное снижение экспрессии *BCL2* (p<0.001) и значительную сверхэкспрессию *BAX* (p<0.05), а ФДТ индуцировала апоптоз клеточной линии HNSCC. В остальных группах изменения экспрессии этих генов не были достоверными (p>0.05).

Заключение: Принимая во внимание подавление *BCL2* и сверхэкспрессию BAX после ФДТ с использованием диодного лазера с длиной волны 660 нм и MB с концентрацией 3.2 μg/mL и результаты проточной цитометрии, предполагается, что этот метод может быть введён для индукции апоптоза клеточной линии HNSCC.

Ключевые слова

апоптоз, *BAX*, *BCL2*, плоскоклеточный рак головы и шеи (HNSCC), фотодинамическая терапия, qRT-PCR