RESEARCH ARTICLE

Matineh Rahmani Ghaleh Shahrokhi [1]
Abbas Doosti [2]

Evaluating the effects of *Staphylococcus aureus* Enterotoxin B on the expression of BAX, p53, Caspase3, and Bcl-2 genes in gastric cancer cell line

Authors' addresses:
[1] Department of Biology, Faculty of Basic Sciences, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.
[2] Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.

Correspondence:
Abbas Doosti
Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Postal Box 166, Shahrekord, Iran
Tel.: +98-3813361001
Fax: +98-3813361001
e-mail: abbasdoosti@yahoo.com

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ABSTRACT

Gastric cancer is known as the third leading cancer in the world and the second cause of cancer death. According to previous studies, *Staphylococcus aureus* (*S. aureus*) enterotoxin B has a major role in the induction of apoptosis in various cancers. Thus, the present study was conducted to evaluate the expression of apoptosis-related genes after gastric cancer cell treatment with enterotoxin B.

In this experimental study, the pcDNA3.1 (+)–*seb* recombinant plasmid was introduced into the AGS cells by the lipofection method after amplification in *E. coli* strain TOP10F and extraction. After 10 days of treatment with neomycin antibiotic, total cellular RNA was extracted and cDNA was constructed to perform the Real-Time Polymerase Chain Reaction (PCR) for apoptotic genes of p53, BAX, caspase3, and Bcl-2, as well as the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as a reference gene.

The results showed a significant increase in the expression of BAX, and p53 genes and a significant decrease in the expression of Bcl-2 and caspase3 genes. Compared to the AGS cells, which did not receive the *seb* gene, the cells containing the toxin gene progressed more towards apoptosis.

According to the findings of the study, it can be concluded that enterotoxin B expressed in gastric cancer cells increases the expression of pro-apoptosis genes and reduces the expression of anti-apoptotic genes. According to the evidence, this toxin can act as an anticancer agent in the AGS cell line.

Key words: Gastric Cancer, Enterotoxin B, p53, Caspase3, BAX, Bcl-2, RT-qPCR.

Introduction

Gastric cancer, occurring in both males and females is the third leading cause of cancer-related death and the fourth most frequent malignant tumor worldwide (Gong et al., 2018; Li et al., 2018). The incidence rate of gastric cancer is higher in developing countries compared to developed countries (Jemal et al., 2011). Previous studies have shown that a majority of the patients with gastric cancer are regularly diagnosed in the late stages of the disease (Sitarz et al., 2018). In spite of great advances in surgical and general therapies, the clinical effect of the patients with gastric cancer remains to be developed (Cao et al., 2018; Gu et al., 2018). It is well known that genetic and epigenetic dysregulations are related to the onset and progression of gastric cancer (Palanisamy et al., 2010; Cheng et al., 2013).

Bacterial toxins have numerous therapeutic potentials for cancer therapy. Various studies (*in -vitro* and *in -vivo*) have proved the effective cell-killing potential of these toxins for cancer cells. A number of bacterial toxins such as *S. aureus* alpha-toxin (α-toxin), *Clostridium Perfringes* Enterotoxin (CPE), and *streptolysin O* (SLO) of *Streptococcus pyogenes* are most attractive for their application in cancer therapy (Forbes, 2010). Bacterial toxins are the most obvious cytotoxic agents because these genes are native to bacterial physiology.

Cytolysin A (HlyE) is a toxin that acts by making pores in the mammalian cell membranes and inducing apoptosis. Apoptosis induced by bacterial toxins during infection is common and is now regarded as a critical function in disease processes (Pahle et al., 2017). Examples are cytotoxin-associated gene-A (CagA) and vacuolating cytotoxin A (VacA) (*Helicobacter pylori*) (Moodley et al., 2012); cytolethal distending toxin ((CDT) was initially isolated from both *E. coli* and *Campylobacter jejuni* strains) (Smith & Bayles, 2006). The Avirulence protein A (AvrA), a protein toxin that influences eukaryotic cell pathways by altering ubiquitination and acetylation of target proteins to modulate

inflammation, epithelial cells apoptosis and proliferation in *Salmonella typhi* strains (Liuet et al., 2010). As a major human pathogen, *S. aureus* also causes apoptosis during infection. In some diseases, like atopic dermatitis and sepsis, the *S. aureus* influences the severity and outcomes of the diseases by inducing apoptosis (Zhang et al., 2017).

S. aureus is the second most common cause of food poisoning after the Salmonella. Within a few hours, the organisms in the jejunum produce the emetic enterotoxins that induce vomiting and secretion of water and electrolytes into the bowel as well as immune stimulation. These intermediate-sized polypeptides (28–34 000 MW) cross the epithelial cell membranes and enter the paracellular space where the Intraepithelial Lymphocytes (IEL) reside (Roberts et al., 2000).

The host immune system may conceivably enable *S. aureus* infection at the time of apoptosis, and this process in tissue cells can also stimulate the immune response through cytokine production and T cell differentiation. Therefore, the pathogenesis *S. aureus* can be influenced by the apoptosis (Torchinsky et al., 2010). Several toxins of *S. aureus* such as Staphylococcal Enterotoxins (SEs) and α-toxin have pro-apoptotic activities. SEs include at least 10 members, namely SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SER, and SET, which can cause food poisoning in some cases (Pearson & Murphy, 2017).

SEs are the superantigens that concurrently bind to certain VB chains of the T-cell receptor (TCR) on CD4+ or CD8+ T cells and the class II Major Histocompatibility Complex (MHC) on antigen-presenting cells (Fisher et al., 2018). Even in picogram masses, they bind and activate such T cells leading to the proliferation, production of Interferon-c (IFNc), and cytotoxic activity. In the vacancy of MHC class II, as in knockout mice (Chen et al., 2017), binding between the SEs and the TCR is of a lower affinity; so, there is an expansion of fewer TCR-VB clones and higher concentrations of the SEs are required for proliferation. An unknown structure on target cells may take the place of MHC class II by binding to the SEB and inducing lytic activity by effector cells (Mager et al., 2016).

The Bcl-2 family of proteins is closely associated with the regulation of apoptosis. While Bcl-2 and Bcl-x represent significant inhibitors of apoptosis, the BAX subfamily consisting of BAX, Bak as well as the BH3-only subfamily induces programmed cell death (Yang & Korsmeyer. 1996). This is also true for the apoptosis induced by cytotoxic treatment (Kale et al., 2018). Among the members of the Bcl-2 family, BAX has been proposed to be a key regulator of the apoptotic machinery, transcriptionally activated by upstream molecules, such as the tumor suppressor p53 (Miyashita & Reed, 1995). However, BAX-induced apoptosis through p53 independent mechanisms in response to anticancer agents has been found in colorectal cell line studies as well (Ravi & Bedi, 2002).

The p53 plays an essential role in cell cycle arrest and initiation of apoptosis. It is a tumor suppressor gene that is inactivated in the development of many malignancies including gastric cancer. The expression rate of p53 determined by immunohistochemistry has been described as 13-54% in gastric cancer (Karim, 2014). A cell without mutation does not show immunohistochemical staining of p53 because there is no such growth in the cell (Pietrantonio et al., 2013). Prognostic role of p53 expression in gastric cancer has been studied in many investigations. Some studies have suggested that the patients without p53 expression have longer survival because non-functional p53 expression in GC tissues was usually associated with more lymph node metastasis and a poor prognosis (Ye et al., 2012). Opposite to these studies, some reports have shown that p53 expression is not associated with survival and other clinical-pathological parameters (Tsujitani et al., 2012).

On the other hand, it has been suggested that the regulation of the cellular suicide program in response to cellular stress depends on the dynamic cooperation between Bcl-2 and BAX. Consequently, higher related BAX levels might result in apoptotic cell death, whereas higher related Bcl-2 levels might inhibit this program in response to genotoxic stress (Oltvai et al., 1994).

An emerging family of Ced-3/Ice-like cysteine proteases (caspases) has been also recognized and several studies have indicated their contribution in executing the process of cell death itself. The caspases are divided into three subfamilies based on their degrees of homology and by phylogenetic analysis; the ICE-related family includes Ice/ caspase-1 itself with its spliced isoforms, TX/ICH-2/ICE rel-II/caspase-4, TY/ ICE-rel-III/caspase-5 ,and ICH-3/caspase-11; the CPP32-related family includes CPP32/Yama/Apopain/caspase-3, Mch-2/caspase-6, Mch-3/ICE LAP3/CMH-1/caspase-7, Mch-4/caspase-10, and Mch 5/FLICE/MACH/caspase-8, and the third group includes ICH-1/caspase-2, and ICE-LAP6/Mch6/caspase-9 (Schmitt et al., 2004).

Accordingly, the present research carried out to investigate the expression of BAX, p53, caspase3, and Bcl-2 genes in AGS cell line transfected with a recombinant vector containing a gene that encodes the Staphylococcal Enterotoxin type B (SEB). To increase our knowledge of the inhibition process of anti-apoptosis genes, we selected p53, Caspase3, BAX, Bcl-2 genes for evaluation of *Staphylococcus aureus* Enterotoxin B effects in cancer cells.

Materials and Methods

Recombinant plasmid preparation and confirmation

The mammalian expression vector, pcDNA3.1(+) containing SEB encoding gene was purchased from GenRay Biotechnology (Dongjing town Songjiang, Shanghai China) and an empty pcDNA3.1(+) (Invitrogen) plasmid was used in this program as scramble plasmid. Both plasmids were transformed into two separate competent *E. coli* strain TOP10F cells by heat shock method and grown under ampicillin (50 µg.mL^{-1}) treatment. The recombinant and empty plasmids were extracted using the Plasmid MiniPrep Purification Kit (Qiagen, Germany) according to the manufacturer's protocol. The recombinant vector (pcDNA3.1(+)-*seb*) was analyzed by specific-*seb* primers. Also to confirm the presence of *seb* gene in the recombinant plasmid, this vector was digested using the restriction enzymes *BamHI* and *EcoRV* (both from New England BioLabs, USA), and digested products were electrophoresed on 1% agarose gel.

Cell transfection

AGS (ATCC® CRL-1739™, human gastric cell line) was purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were cultured in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS) (Gipco, MD, USA), 100 U.ml^{-1} penicillin, and 100 µg.ml^{-1} streptomycin (Thermo Fisher Scientific) at 37ºC in a humidified atmosphere containing 5% CO_2.

The transfection of AGS cells was carried out in a 6-well plate according to the instructions for the Lipofectamine 2000™ reagent (Invitrogen, USA). 2 µg of pcDNA3.1(+)-*seb* and 2 µg of the scramble pcDNA3.1(+) were transfected separately into AGS cell line. Also, there was one group of AGS cell that cultured in the same condition in a 6-well plate without any transfection, as the control group.

RNA isolation and cDNA synthesis

Cells were collected after transfected with a recombinant plasmid and empty plasmid for 10 days. Total RNA was extracted with RNX-Plus reagent (SinaClon, Iran) and then RNA was dissolved in sterile RNase-free water. The concentration and purity of RNA were measured using a spectrophotometer (Thermo Scientific™ NanoDrop 2000). The complementary DNA (cDNA) synthesis was performed using the PrimeScript™ First-Strand cDNA Synthesis kit (Yekta Tajhiz Azma, Tehran, Iran).

To confirm the *seb* gene expression after lipofection, a PCR test was applied by using of *seb* specific primers. PCR was performed in a 20 µl mixture containing 1 µl cDNA, $MgCl_2$ 2 mM, dNTPs 0.2 mM, *Taq* DNA polymerase 0.5 unit (SinaClon, Iran), and 0.5 µM of each primer (SEB-F/R, table 1). The reaction mixture was heated at 95°C for 3 min. Amplification was performed in an Eppendorf® Thermal Cycler (Germany), at 94°C (1 min), 65°C (1 min), 72°C (1 min) for 30 cycles, followed by a final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel.

RT-qPCR analysis

Real-time RT-PCR was carried out using specific primers (Table 1) and Super SYBR Green 2x (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's instruction. The RT-qPCR analysis were performed by specific primers of SEB, p53, caspase3, Bcl-2 and BAX genes on AGS cDNA. Also, RT-qPCR for the GAPDH gene was performed in order to the determination of cell to cell sticking.

All samples were amplified in triplicate in the same PCR condition and amplification for each target gene was performed separately. No template controls (NTC) containing DNAse-free water instead of template cDNA were also included in each run. RT-qPCR was performed with a Rotor-Gene 6000 Corbett (Qiagen, Germany). The thermal cycling conditions were: 94°C for 4 min, and 45 cycles of 94°C for 20 s, 64-65°C for 20 s and 72°C for 25 s. After the RT-qPCR run, a melting curve analysis was used to ensure the correct amplification of the expected amplicons. Standard curves for GAPDH were generated using a serial dilution of cDNA. GAPDH was monitored as a reference gene and specific gene expression levels were normalized with respect to GAPDH transcript and calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Statistical analysis

All data were presented as mean ± SEM. The statistical analysis was performed by SPSS version 24.0 (SPSS Inc, Chicago IL, USA). Independent t and LSD tests were conducted to compare significant correlations. The differences with a p-value of less than 0.05 were considered as the statistical significance level.

Results

Confirmation of recombinant plasmid

The presence of *S. aureus seb* gene in pcDNA3.1 (+)-*seb* recombinant vector was confirmed through the Polymerase Chain Reaction (PCR) and *BamHI*/*EcoRV* restriction enzyme digestion. Therefore, a fragment of 171 bp was observed as PCR product on 1% agarose gel (Fig. 1), and two 5389 bb and 813 bp fragments were observed after digestion for pcDNA3.1 (+) plasmid and *seb* gene, respectively (Fig. 2).

Results of expression of seb gene

The AGS cells transfected with the recombinant pcDNA3.1 (+)-*seb* expression vector were harvested 10 days post-transfection. The Real–Time quantitative Polymerase Chain Reaction (RT-qPCR) results showed that 171 bp fragment was amplified for *seb* gene, suggesting that the

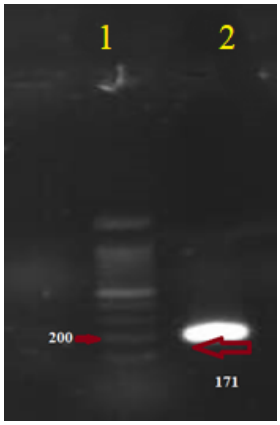

Figure 1. *Confirmation of the presence of seb gene by the PCR assay. Lane 1: 100 bp DNA markers; Lane 2: PCR product for seb gene.*

recombinant plasmid was successfully transfected into the AGS cells.

qPCR assay

The expression of the p53, caspase3, Bcl-2, and BAX genes in the AGS cell line transfected with pcDNA3.1 (+)-*seb* was evaluated compared to the control groups (cells transfected with scramble vector). All the data were normalized by the Glyceraldehyde-3-Phosphate Dehydrogenase *(GAPDH*) housekeeping gene.

It was found that the expression of p53 significantly increased in the AGS cells in pcDNA3.1 (+)-*seb* transformed cells compared to the control group (p=0.014, Fig. 3(a)). Also, the results of the statistical analysis showed a remarkable increase in the BAX gene expression in *seb* -treated AGS cells compared to the controls using the RT-qPCR assay (p=0.004, Fig. 3(b)).

Comparing the expression of the caspase3 (p=0.037) and Bcl-2 (p=0.029) genes between the pcDNA3.1 (+)-*seb* transformed cells and control cells showed a statistically significant decrease in the expression of the mentioned genes in the *seb* -treated cells. Results regarding the difference in the mRNA expression (caspase3 and Bcl-2) in the pcDNA3.1(+)-*seb* transfected AGS cells compared to controls are presented in Figs.3(c) and (d), respectively.

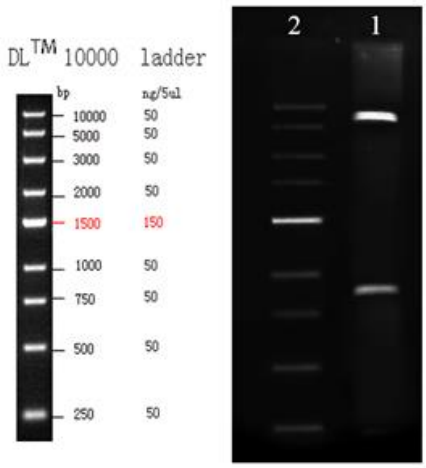

Figure 2. *Confirmation of the presence of the recombinant plasmid pcDNA3.1 (+)-seb by the restriction endonuclease analysis. Lane 1: restriction endonuclease analysis with BamHI/EcoRV for pcDNA3.1 (+)-seb (recombinant vector); Lane 2: 1 kb DNA markers.*

Discussion

Gastric cancer is one of the most frequent cancers in the world and is the second most common cause of cancer death (Parkin et al., 2005). Prevalence of gastric cancer varies among different countries (Torres et al., 2013). It is recognized as a progressive disease. Surgical resection is considered as the unique possible curative therapy for gastric cancer but it reappears in a significant number of the patients notwithstanding adjuvant or neoadjuvant chemotherapy/chemoradiotherapy. Long-term survival cannot be achieved by palliative chemotherapy in cases with inoperable or metastatic disease (Cunningham et al., 2006). All three therapy modalities administered in the patients with gastric cancer have potentially dangerous side effects. Protection of patients from the negative effects of the therapy and individualizing the therapy are among the important targets in the treatment of gastric cancer today. Prognostic factors determined at the time of diagnosis help in assessing the intensity of the therapy and are also directive in targeted-therapy (Sezer et al., 2013).

The use of bacterial toxins in cancer therapy has increased in recent years. Most of these therapies are based on the use of bacterial toxins in the recombinant vectors (Pahle & Walthe, 2016). Given the basic function of bacterial enterotoxins in tumor regression, it is rational to hypothesize that the anticancer properties of the bacterial enterotoxins may partially be related to the alteration of the cell signaling genes involved in cancer development and progression. SEs are a family of structurally related proteins produced by *S. aureus*, termed as superantigens. These proteins bind to the MHC Class II molecules on the cells and induce T lymphocytes clonally (Jung et al., 2013). It has been revealed that *S. aureus* Enterotoxin type B (SEB) may feature anticancer and anti-metastatic advantages due to its ability to modify the cell immunity processes as well as cancer cell signaling pathways (Reis et al., 2012).

Herein, a study was done at the molecular levels to evaluate the effect of the recombinant plasmid on the apoptosis of the AGS cancer cell line. The pcDNA3.1 (+) mammalian expression vector was used to insert the encoding

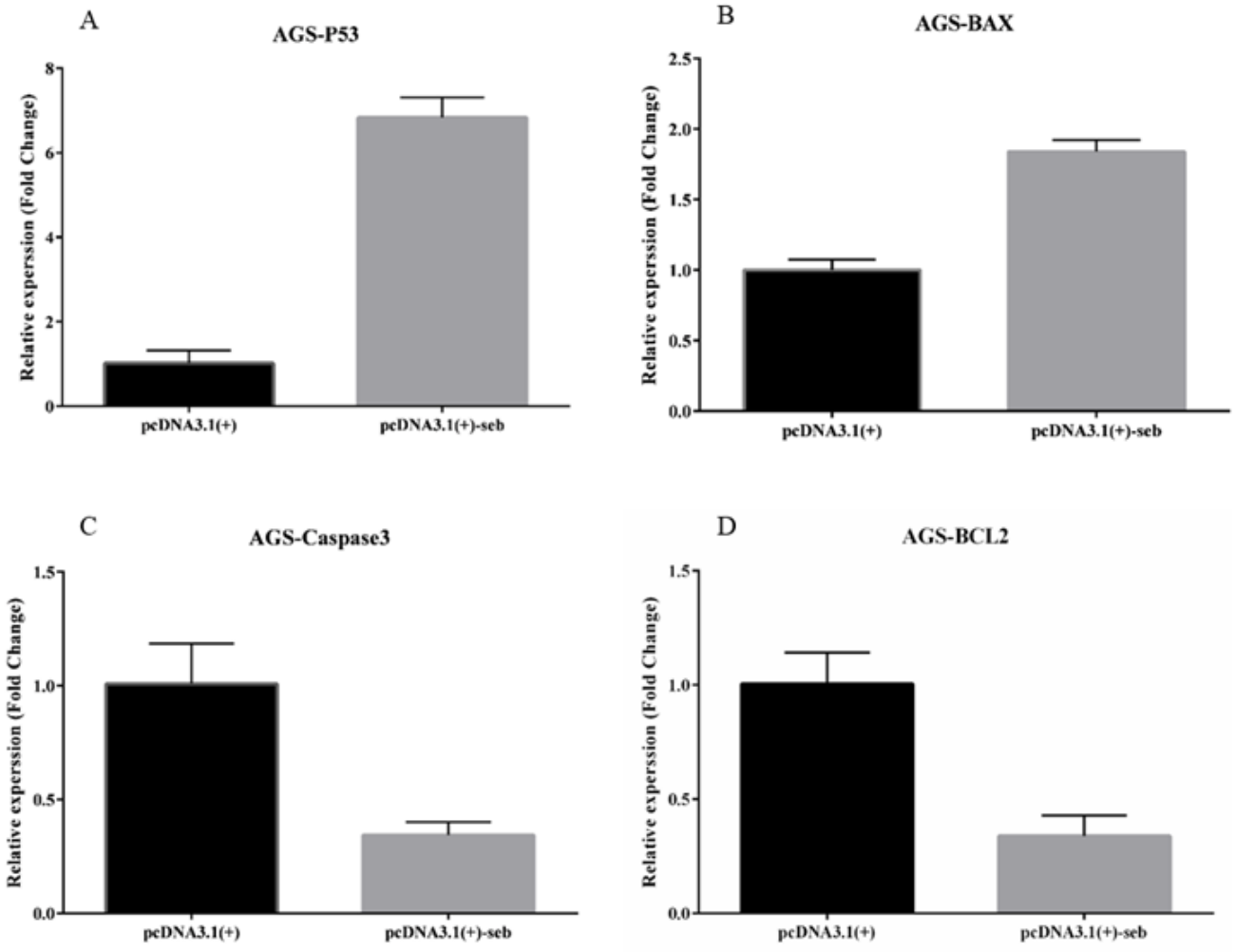

Figure 3. *A) The mRNA level of the p53 gene was evaluated in the AGS cells. The p53 expression was significantly upregulated in the seb -treated cells compared to the controls (transfected with scramble vector). B) The mRNA level of the BAX gene was evaluated in the AGS cells. The BAX expression was significantly upregulated in the seb -treated cells compared to the controls (transfected with scramble vector). C) The mRNA level of the caspase3 gene was evaluated in the AGS cells. The caspase3 expression was significantly downregulated in the seb -treated cells compared to the controls (transfected with scramble vector). D) The mRNA level of the Bcl-2 gene was evaluated in the AGS cells. The data obtained from the RT-qPCR assay were normalized versus the GAPDH (reference gene). The Bcl-2 expression was significantly downregulated in the seb -treated cells compared to the controls (transfected with scramble vector).*

gene of SEB.

The recombinant plasmid called pcDNA3.1 (+)-*seb* was constructed and amplified in a TOP10F chemically competent cell.

Gastric cancer cell line (AGS) was transfected by the pcDNA3.1 (+)-*seb* and pcDNA3.1 (+) (scramble plasmid) and then, the results of RT-qPCR assay confirmed the positive expression of *seb* gene in the transfected cells. AGS cells lipofected by the pcDNA3.1 (+)-*seb* showed more cell death compared to the controls, which was statistically significant ($P<0.05$). Therefore, our finding showed the appropriate role of the pcDNA3.1 (+)-*seb* on cell death. In addition, the expression of BAX and p53 genes increased in the *seb* -treated cells in the AGS cell line. Also, the findings showed that the expression of caspase3 and Bcl-2 genes significantly decreased in the AGS cells transfected with the pcDNA3.1(+)-*seb* compared to those transfected with empty pcDNA3.1(+) vector ($P<0.05$).

Dohlesten et al., (1993) have investigated the effect of SEA toxin on the colon cancer cells and suggested that the toxin causes reactivation of the monoclonal antibodies directing T cells to digest the colon cancer cells. These SEA-activated T cells exhibit antitumor activity and inhibit cancer cell growth (Dohlesten et al., 1993).

Ejtehadifar et al., (2017) have conducted a study on the U266 cell line co-cultured with the mesenchymal stem cells

Table 1. *Sequence of primers for RT-qPCR.*

Genes	Primer names	Sequences (5'----3')	Annealing temp (ºC)	Product length (bp)
Seb	SEB-F	AGGACACAAAGCTGGGCAACTAC	65	**171**
	SEB-R	TACGCTTGTCTGTCTGGTGGGAG		
p53	p53-F	TGCGTGTGGAGTATTTGGATGAC	64	**170**
	p53-R	CAGTGTGATGATGGTGAGGATGG		
caspase3	caspase3-F	ATAAAAGCACTGGAATGACATCTCG	64	**173**
	caspase3-R	ACTGCTCCTTTTGCTGTGATCTTC		
Bcl-2	Bcl2-F	GACGACTTCTCCCGCCGCTAC	65	**245**
	Bcl2-R	CGGTTCAGGTACTCAGTCATCCAC		
BAX	BAX-F	AGGTCTTTTTCCGAGTGGCAGC	65	**234**
	BAX-R	GCGTCCCAAAGTAGGAGAGGAG		
GAPDH	GAPDH-F	GCCAAAAGGGTCATCATCTCTGC	64	**183**
	GAPDH-R	GGTCACGAGTCCTTCCACGATAC		

and concluded that the SEB toxin could increase the expression of Interleukin 10 (IL-10), Interleukin 6 (IL- 6), and IKappa Kinase beta (IKKb) and reduce the viability and proliferation rate of U266 cells. This toxin was reported to have no effect on the Transforming Growth Factor beta (TGF-β) gene. Finally, they reported that SEB could be a suitable candidate for reducing the proliferation and survival of the cancer cells and altering the gene expression in the U266 cells (Ejtehadifar et al., 2017). Yu et al., in a study (2013) have investigated the effects of S. aureus toxins including SEB and α-toxin on the ECV304 cells. They showed the apoptosis induction and increased expression of Tumor Necrosis Factor alpha (TNF-α) and also activation of caspase3 and 8 in the ECV304 cells. Their findings suggested that SEB and α-toxin induced apoptosis through the extrinsic death pathway (Yu et al., 2013). In the present study, the effects of SEB were investigated on the AGS cell line, hence; our findings represented the same results regarding the apoptosis induction. These results indicated that S. aureus SEB toxin arrests the progression of the cell cycle that leads to activation of the apoptosis through pro-apoptotic genes.

Conclusion

The results of this study showed that the expression of BAX and p53 genes increased in the AGS cancer cell line transformed by the recombinant plasmid pcDNA3.1 (+)-*seb* that expressed SEB toxin of *S. aureus*. On the other hand, the expression of caspase3 and Bcl-2 genes was downregulated in the *seb* -treated AGS cells. According to the findings of the study, the SEB toxin of *S. aureus* could be a helpful candidate for application as a therapeutic bacterial toxin in cancer therapy in the future.

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