RESEARCH ARTICLE

Staphylococcus aureus enterotoxin A gene induce the changes in the expression of apoptosis-related genes in AGS cell line

Sara Afzali [1]
Abbas Doosti [2]
Mansour Heidari [1,3]
Nahid Babaei [1]
Parvaneh Keshavarz [1, 4]

Authors' addresses:
[1] Department of Molecular Cell Biology, Bushehr Branch, Islamic Azad University, Bushehr, Iran.
[2] Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.
[3] Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
[4] Cellular and Molecular Research Center, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran

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

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ABSTRACT

Gastric cancer is one of the most common malignancies worldwide and it is the fourth leading cause of cancer-related death. *Staphylococcus aureus* enterotoxin A (SEA) is one of the toxins affecting the expression of apoptotic-related genes. The aim of this research is to investigate the effects of *S. aureus* enterotoxin A on the expression of BAK, FAS, BAX, TNF-α, BCL-2 and Survivin in AGS cell lines. In this study, AGS cells were transfected with the pcDNA3.1(+)-enterotoxin A or empty pcDNA3.1(+) plasmids using Lipofectamine 2000. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Then, the mRNA expression level of BAK, FAS, BAX, TNF-α, BCL-2, and Survivin was measured by the qRT-PCR method. Our findings indicated that *Staphylococcus aureus* enterotoxin A markedly altered the expression level of the apoptotic-related gene of the AGS cell line. The mRNA expression of BAK, FAS, BAX, and TNF-α genes increased statistically significantly in enterotoxin A treated AGS cells compared with the control group. On the contrary, BCL2 and Survivin mRNA expression showed a significant decrease compared with AGS cells were transformed by empty pcDNA3.1(+) plasmid. We suggested that SEA could represent an agent that can significantly change apoptosis-related genes expression in the AGS cell line, leading to reduction of tumor cell proliferation.

Key words: *Staphylococcus aureus*, enterotoxin A, AGS, gastric cancer, apoptosis-related gene

Introduction

Gastric cancer (GC) is a deadly disease with poor overall survival statistics throughout the world (Machlowska et al., 2020) since it is the fifth leading cancer in the world and the third leading cause of cancer-related death, responsible for almost 800,000 deaths every year (Bray et al., 2018). It occurs approximately twice as frequently in men as in women, with most cases occurring after the age of 60 (Bray et al., 2018; Marqués-Lespier et al., 2016). The incidence of GC varies widely across different geographic regions, with the highest incidence observed in East Asia, some Eastern Europe and South American countries, and the lowest in North America and Africa. Globally, over 70% of GC occurs in developing countries (Jemal et al., 2011). Risk factors that affect the incidence of gastric cancer were identified and classified into nine important categories: diet, lifestyle, genetic predisposition, family history, treatment, and medical conditions, infections, demographic characteristics, occupational exposures, and ionizing radiation'(Yusefi *et al.*, 2018).

In the past, two sets of genes, including oncogenes and tumor suppressor genes, were thought to be involved in the cancer process. But the third set of genes are also involved in this process, and they are the genes that control programmed cell death or apoptosis (Aktipis & Nesse, 2013). In 2012, the number of reported cases of lung cancer (1.82 million) and breast cancer (1.67 million), and colon cancer (1.36 million) were diagnosed as the most important types of cancer and based on the forecast. According to the World Health Organization (WHO), cancer will continue to be a major human problem until 2030, and many people will die from cancer (Torre et al., 2016).

Bacterial toxins play an important role in the pathogenesis of these infectious agents, and many bacteria are able to produce toxins, extracellular enzymes, and pigments (Allison et al., 2014; Ramachandran, 2014). Although bacterial toxins appear to be harmful to humans and other hosts, researchers are now finding useful applications for these biological

products. The role of bacteria as anti-cancer agents has been known for many years (Sabzehali et al., 2017). *Staphylococcus aureus* is a gram-positive, non-spore-forming, capsule-free, immobile, aerobic, or anaerobic bacterium. Bacterial enterotoxins of various types including; they have A, B, C1, C2, D, E, and G, and F. For example, enterotoxin B is an exotoxin produced by Staphylococcus aureus (Ouyang et al., 2012). Different members of *staphylococcal* enterotoxins show their ability to induce apoptosis in a variety of ways; even a single staphylococcal enterotoxin is able to induce apoptosis in different forms in different cells (Pinchuk et al., 2010; Ghaleh & Doosti, 2019). Alpha toxins are water-soluble secretory monomers with a molecular weight of 33.3 kDa that, upon attachment to the cell, oligomerize and form heptamer pores in the plasma membrane of the host cell, and like enterotoxins in different cells apoptosis. They induce initiators from different pathways such as internal path, external path, and even caspase pathway apoptosis (Iacovache et al., 2010; Srivastava et al., 2009).

Given the limitations of therapies available in the treatment of cancers as well as the possibility of metastasis as well as the lack of definitive treatment in advanced cases of the disease, research into finding ways to control this deadly disease seems necessary. At present, considering the large evidence of interaction between bacterial toxins in cancer control from different pathways, in this study, we try to determine the anti-cancer and induction effects of apoptosis to determine the expression of genes involved in these processes in transformed cells. Therefore, we studied the effects of *Staphylococcus aureus* enterotoxin A on the expression of apoptosis-related genes in the AGS cell line.

Materials and Methods

Preparation of recombinant vector

The sequences of *S. aureus* sea gene in order to expression in mammalian cells (human AGS cell line) were obtained from GenBank (ACCESSION: IBRC C10071). Then, the gene optimized by the script online software was examined. Via Generay Biotech Co., Ltd. (Shanghai, China), the pcDNA3.1(+)-sea recombinant vector was constructed. The heat shock method was used to transform the recombinant plasmid pcDNA3.1(+) and empty plasmid (Invitrogen) into CaCl2 chemically competent *E.coli* TOP10F cells19. The transformed bacteria were plated on Luria-Bertani (LB) medium containing 100 µg/ml ampicillin. The extraction of plasmids was done by the Favorgen plasmid DNA Extraction mini kit according to the manufacturer's protocol. The pcDNA3.1(+)-sea was confirmed by BamHI/EcoRV enzymatic double digestion and PCR and digested products were electrophoresed on 1% agarose gel.

Cell culture and transfection

AGS cell line was purchased from the National Cell Bank of Iran (NCBI, Pasture Institute of Iran, Tehran). AGS cell line in a T-75 flask with DMEM and DMEM-F12, culture medium, respectively (Dulbecco's Modified Eagle's medium) (Sigma-Aldrich, St., MO, USA), supplemented with 10% fetal bovine serum (Sigma-Aldrich, St., MO, USA) and 1% Penicillin-Streptomycin (Gibco BRL, Karlsruhe, Germany), were cultured and incubated at 37 $^{\circ}$C in an environment with 5% CO_2 and 90% humidity. The medium was changed three times a week and trypsin/EDTA (Invitrogen, USA) was used to remove the cells. In order to transfect the AGS cell, they were cultured as mentioned above. When the confluence of cells reached 85%, the AGS separately were seeded in three wells of 6-well tissue culture plates (1×10^6 cells/well). Then, by use of lipofectamine 2000 reagent (Invitrogen, USA), the AGS cell in the first and second wells of the plates were transformed by pcDNA3.1(+)-*sea* (as a recombinant plasmid), and empty pcDNA3.1(+) (as control plasmid), respectively according to the manufacturer's instructions. Each transfected well was supplemented with 4 µg plasmid per each well [plasmid (µg): Lipofectamine 2000 (µl) = 1:3], in a serum-free medium. Four hours following transfection, the media were replaced with fresh medium containing 600 mg/l G418 and 10% FBS for 10 days. To accurately assess the performance of G418, there was no transfected cell in the third well. Ultimately stably a transfected clone of this cell line was selected by G418 screening. These clones were shown to express mRNA from the introduced plasmid. After transformation, the frequency of the transfected cell was more than 1 x 106 cells used initially.

RNA extraction and cDNA synthesis

Using RNX-Plus reagent (SinaClon, Iran), total RNA was extracted from transformed and untransformed AGS cells according to the manufacturer's instructions. The concentration and purity of the extracted RNAs were evaluated by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), read at wave lengths of 230 nm, 260 nm, and 280 nm. Subsequently, all RNA sequences were converted cDNA and transferred to -20° C through a cDNA synthesis kit (RevertAID First Standard cDNA Syn Kit), (Termo Scientific, Lithuania).

RT-PCR reaction and dot blotting

To determine the expression of the *sea* gene in transfected AGS, RT-PCR reaction mixture with a final volume of 25 µl containing 3 µl of cDNA, 2.5 µl of 10x buffer, 0.75 µl of 50 mM mgcl2, 0/5 µl 10 µM dNTP, 0/5µl Taq DNA polymerase, 1 µl of each primer (10 pmol/ml) and 15/75 µl ddH_2O, was

ready and the reaction within the Thermal Cycler in 30 cycles carried out. According to the program, initial denaturation for 5 minutes at 94 °C, denaturation for one minute at 94 °C, annealing for 60 seconds at 56 °C, and final extension for 5 minutes at 72 °C was carried out. Finally, electrophoresis was performed on 1% agarose gel for PCR product evaluation. The Dot blot method is used to confirm the specificity of the *sea* gene expression. Briefly, for this purpose, 8 µL of proteins extracted from AGS cells transformed with a recombinant plasmid (pcDNA3.1 (+) - sea) were dotted on nitrocellulose membrane (Millipore, USA). Non-specific binding sites were then blocked using TBS-T (Merck, Germany) for 30 min at room temperature. The membrane was washed three times with TBST, incubated for 30 min with an anti- histag HRP-conjugated monoclonal antibody (Sigma, USA) with a dilution of 1:1000. Following three washes with PBS buffer, the substrate was used for the detection. The PBS was utilized as a technical negative control in the experiment.

Real time RT-PCR

Expression of BAK, BAX, BCL2, FAS, Survivin, and TNF-α genes was analyzed by real-time PCR (Rotor gene 6000 corbett, Australia) using DNA Master SYBR Green 1 (Roche Diagnostics GmbH Mannheim, Germany). A PCR reaction mixture containing 5 mM of $MgCl_2$, 2 µl of SYBR Green 1 mix, 2-4 µl of cDNA, and a pair of primers. Seven pairs of primers were separately used: six pairs to amplify the BAK, BAX, BCL2, FAS, Survivin, and TNF-α genes, the other pair for the endogenous control gene, GAPDH. All samples were amplified in a 20 µl PCR reaction containing specific primers (Table 1)**,** GAPDH gene, and SYBR Green as an internal control and reporter, respectively. No template controls (NTC) were included in each run. Real-time runs were performed on the Corbett-Research Rotor Gene 6000 (Sydney, Australia). The amplification program consisted of 1 cycle of 95ºC with a 4 min hold, followed by 40 cycles of 95ºC with a 15 s hold, annealing temperature at 64ºC (for FAS, Survivin and GAPDH), 65ºC (BAX, BCL-2, and TNF-α) and 66ºC (BAK) with a 20 s hold, and 72ºC with a 20 s hold. Following amplification, a melting curve was run to confirm that a single PCR product was amplified. Relative quantification analysis was performed by using the comparative C_T method ($2^{-\Delta\Delta Ct}$) [20]. To verify the product sizes, the PCR products were electrophoresed on 2% agarose gel.

Statistical analysis

All data were presented as mean±SD. Paired Student T-test was performed for statistical analysis. The level of statistical significance was set at $p< 0.05$.

Results

Confirmation of recombinant plasmid

The presence of *S. aureus sea* gene in pcDNA3.1 (+)-sea recombinant vector was confirmed through the Polymerase Chain Reaction (PCR) and *BamHI/EcoRV* restriction enzyme digestion. Therefore, a fragment of 203 bp was observed as a PCR product on 1% agarose gel (Figure. 1).

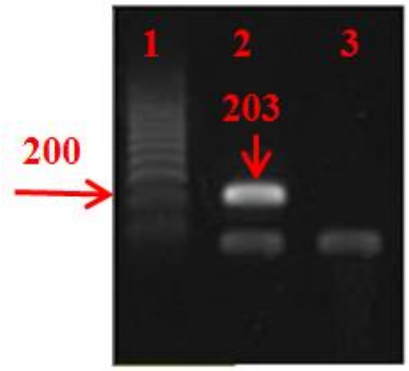

Figure 1. *Confirmation of the presence of sea gene by the PCR assay. Lane 1: 100 bp DNA markers; Lane 2: PCR product for sea gene; Lane 3: negative control.*

Results of RT-PCR analysis and expression of sea gene

To confirm the mRNA expression of *sea* gene encoding staphylococcal enterotoxin type A, AGS cells transfected with both recombinant plasmid (pcDNA3.1(+)-*sea*) and empty plasmid (pcDNA3.1(+)) were examined. The results showed that it was only in AGS cells transformed with a recombinant plasmid (pcDNA3.1(+)-*sea*) that a 203 bp fragment related to the *sea* cDNA was amplified by RT-PCR (Figure. 1). In the following, as shown, the extracted protein content appeared as a light stain indicating the binding of specific anti-his-tag monoclonal antibody interaction with the expressed protein of sea gene (Figure. 2).

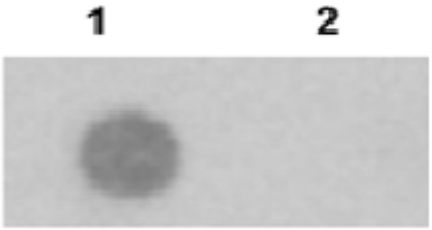

Figure 1. *(1) Result of dot blot analysis (2) negative control [PBS].*

RT-Real time PCR

Table 1. *Forward and reverse primer sequences for real time RT-PCR.*

Genes	Forward and reverse	Sequences	Annealing temperature (ºC)	Product length (bp)
Sea	Forward Reverse	5′-TATGGTTATCAATGTGCGGGTG-3′ 5′-CTTGAAGATCCAACTCCTGAACAG-3′	64	203
BCL-2	Forward Reverse	5’-GACGACTTCTCCCGCCGCTAC-3’ 5’-CGGTTCAGGTACTCAGTCATCCAC-5’	65	245
BAX	Forward Reverse	5’-AGGTCTTTTTCCGAGTGGCAGC-3’ 5’-GCGTCCCAAAGTAGGAGAGGAG-3’	65	234
FAS	Forward Reverse	5’-CAATTCTGCCATAAGCCCTGTC-3’ 5’-GTCCTTCATCACACAATCTACATCTTC-3’	64	163
BAK	Forward Reverse	5’-CGTTTTTTACCGCCATCAGCAG-3’ 5’-ATAGCGTCGGTTGATGTCGTCC-3’	66	154
TNF-a	Forward Reverse	5’-GCCTCTTCTCCTTCCTGATCGTG -3’ 5’-TTTGCTACAACATGGGCTACAGG-3’	65	184
Survivin	Forward Reverse	5′-AGAACTGGCCCTTCTTGGAGG -3′ 5′-CTTTTTATGTTCCTCTATGGGGTC -3′	64	170
GAPDH	Forward Reverse	5’-GCCAAAAGGGTCATCATCTCTGC-3’ 5’-GGTCACGAGTCCTTCCACGATAC-3’	64	183

Further examined was the expression of BAK, BAX, BCL2, FAS, Survivin, and TNF-α genes in AGS cells transformed with a recombinant plasmid (pcDNA3.1(+)-*sea*) and empty plasmid (pcDNA3.1(+)), as control. The melting curve was generated to screen for primer dimmers and to document single product formation for each gene (BCL2, TNF-a, BAX, FAS, Survivin, and BAK) in both cell lines. It was drawn based on the temperature (x axis) and dF/dT derivation (y axis). The amplification product of BCL2, TNF-α, BAX, FAS, Survivin and BAK genes was compared to the reference gene product by amplification analysis curve, and to demonstrate the efficiency of each PCR reaction, the concentration was plotted against the crossing points (Ct) to create a BCL2, TNF-α, BAX, FAS, and BAK standard curve with r =-0.99. Analysis of Real-Time PCR Ct values via $2^{-\Delta\Delta ct}$ formula showed a The mRNA expression of BAK, FAS, BAX, and TNF-α genes increased statistically significant in enterotoxin A treated AGS cells compared with the control group. On the contrary, BCL2 and Survivin mRNA expression showed a significant decrease compared with AGS cells transformed by empty pcDNA3.1(+) plasmid (Table 2 and Figure. 3).

Discussion

Cancer remains one of the major challenges of the 21st century. The increasing numbers of cases are not accompanied by adequate progress in therapy. The standard methods of treatment often do not lead to the expected effects. Therefore, it is extremely important to find new, more effective treatments (Łukasiewicz & Fol, 2018). In

Table 2. *BAX, BAK, BCL.2, FAS, Survivin and TNF-a mRNA expression in transformed AGS cell by pcDNA3.1(+)-sea.*

Cell	Parameters		Mean	Std.D	p-value
AGS	BAK	PcDNA3.1(+) PcDNA3.1(+)-sea	0.613194 1.759562	0.098750 0.043117	0.0044**
	BAX	PcDNA3.1(+) PcDNA3.1(+)-sea	0.939546 1.531788	0.009210 0.037535	0.0021**
	BCL.2	PcDNA3.1(+) PcDNA3.1(+)-sea	1.001352 0.271171	0.073553 0.038414	0.0180*
	FAS	PcDNA3.1(+) PcDNA3.1(+)-sea	0.960778 1.527580	0.136105 0.089793	0.0390*
	Survivin	PcDNA3.1(+) PcDNA3.1(+)-sea	1.001351 0.271171	0.073554 0.038414	0.0064**
	TNF-α	PcDNA3.1(+) PcDNA3.1(+)-sea	0.787540 1.552947	0.027014 0.007611	0.0007***

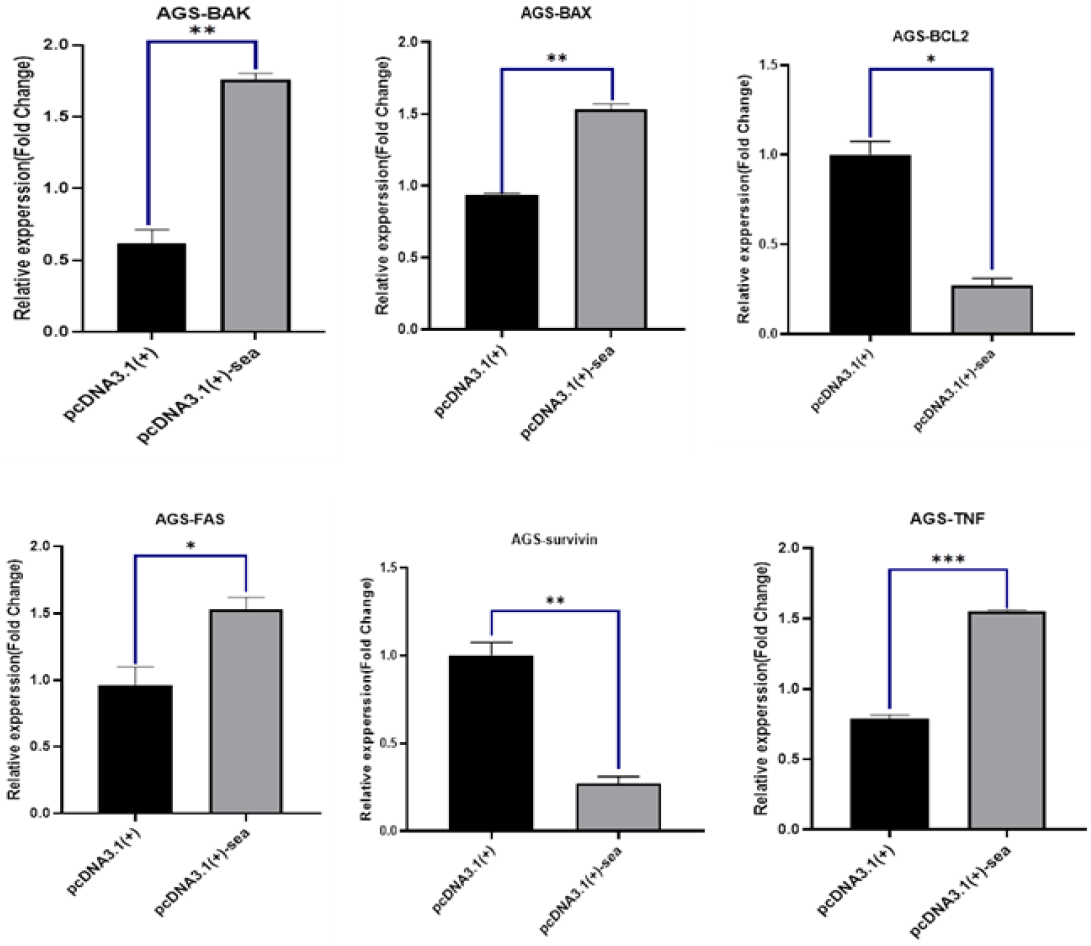

Figure 3. *BAK, BAX, FAS, TNF-α, BCL2 and Survivin mRNA expression in AGS cell transformed by pcDNA3.1(+)-sea: BAK, BAX, FAS and TNF-α mRNA expression in AGS cells transformed by pcDNA3.1(+)-sea significantly increased compared to control cells. Data are means±SE, P<0.05 vs. control. BCL2 and survivin mRNA expression in AGS cells transformed by pcDNA3.1(+)-sea was significantly reduced compared to the control cells. Data are means±SE, P<0.05 vs. control.*

recent decades, bacteria's therapeutic role has aroused attention in medicinal and pharmaceutical research. While bacteria are considered among the primary agents for causing cancer, recent research has shown intriguing results suggesting that bacteria can be effective agents for cancer treatment. In order to reduce or prevent endogenous production of antibodies against the toxin(s) or other unwanted side-effects, human-derived or human-compatible antitoxins as an adjunct to therapy with a combination of toxins can be used. State-of-the-art genetic engineering has been recently applied to bacteria therapy and resulted in greater efficacy with minimum side effects (Song *et al.*, 2018). The role of bacteria as an anticancer agent was recognized almost a hundred years ago. The German physicians W. Busch and F. Fehleisen separately observed that certain types of cancers regressed following accidental erysipelas (*Streptococcus pyogenes*) infections that occurred whilst patients were hospitalized. Independently, the American physician, William Coley, noticed that one of his patients suffering from neck cancer began to recover following an infection with erysipelas. He began the first well-documented use of bacteria and their toxins to treat end-stage cancers (Zacharski & Sukhatme, 2005). Current studies further indicate that bacterial toxins can control the differentiation, proliferation, and apoptosis of the cells (Pahle *et al.*, 2017). For example, reviews in 2003 and 2010 showed that *Salmonella* and *Clostridia* have emerged as agents with high antitumor potential (Leschner and Weiss, 2010; Minton, 2003). In 2012, treating metastatic melanoma with an auxotrophic *Salmonella typhimurium* showed insignificant tumor regression (Zhao *et al.*, 2012). Also understood is that *Staphylococcus aureus* as a major human pathogen has This various toxins like *staphylococcal* enterotoxins which have reportedly triggered apoptosis. feature turned the old pathogen into a new treatment for human health (Aziz *et al.*, 2014). In the present study, the ability of *staphylococcal* enterotoxin type A to inhibit growth and induction apoptosis in the AGS cell line was investigated by inducing changes in

the expression of genes involved in the cell death pathway. Evaluation of BAK, FAS, BAX, TNF-a, BCL-2, Survivin gene expression in AGS cells using Real Time PCR and determination of changes in their expression based on $2^{-\Delta\Delta Ct}$ (Relative Quantification) results Showed significant changes in the expression of the target genes (BAK, FAS, BAX, TNF-a, BCL-2, Survivin) in this cell line. Analysis of Ct data obtained from Real Time PCR related to the expression of BAK, FAS, BAX, TNF-a, BCL-2 genes in the AGS cell line transformed with a recombinant plasmid (pcDNA3.1 (+)-sea), reduction Significance in the expression of BCL-2 and Survivin genes with anti-apoptotic properties ($P < 0.05$) and a significant increase in expression of BAK, FAS, BAX, TNF-a genes with pro-apoptotic properties ($P < 0.05$) in cell line Transformed AGS showed. The results showed that recombinant pcDNA3.1(+) plasmid with *sea* gene was successfully expressed in the AGS cell line. In addition, our finding in this study confirmed that the product of this gene can alter the expression of the genes involved in the apoptotic process of both cell lines.

Conclusion

The results of this study showed that the expression of BAK, BAX, FAS, and TNF-α genes increased in the AGS cancer cell line transformed by the recombinant plasmid pcDNA3.1 (+)-sea that expressed SEA toxin of *S. aureus*. On the other hand, the expression of Survivin and Bcl-2 genes was downregulated in the sea-treated AGS cells. According to the findings of the study, the SEA 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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