Original article

The functions of azurin of *Pseudomonas aeruginosa* and human mammaglobin-A on proapoptotic and cell cycle regulatory genes expression in the MCF-7 breast cancer cell line

Payam Ghasemi-Dehkordi [a], Abbas Doosti [b], Mohammad-Saeid Jami [a,c,∗]

[a] *Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran*
[b] *Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*
[c] *Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, USA*

A B S T R A C T

Azurin protein of *Pseudomonas aeruginosa* is an anti-tumor agent against breast cancer and mammaglobin-A (MAM-A) protein is a specific antigen on the surface of MCF-7 for induction of cellular immune. The purpose of the present study was to investigate the effects of simultaneous expression of *azurin* and human *MAM-A* genes on the mRNA expression level of apoptosis-related and cell cycle genes in MCF-7 breast cancer cell line. The recombinant or empty plasmids were separately transferred into MCF-7 cells using Lipofectamine reagent. Flow cytometry was done to detect cell death and apoptosis. The expression of *azurin and MAM-A* genes were evaluated by IF assay, RT-PCR and western blot methods. Finally, apoptosis-related and cell cycle genes expression was examined in transformed and non-transformed MCF-7 cells by qPCR method. The successful expression of *azurin* and *MAM-A* genes in the MCF-7 cell were confirmed by RT-PCR, IF and western blotting. The apoptosis assay was showed a statistically significant ($p < 0.05$) difference after transfection. The expression of *BAK*, *FAS*, and *BAX* genes in transformed cells compare with non-transformed and transformed MCF-7 by pBudCE4.1 were increased statistically significant ($p < 0.05$) increases. Although, the increase of *SURVIVIN* and *P53* expressions in transformed cells were not statistically significant ($p > 0.05$). Co-expression of *azurin* and *MAM-A* genes could induce apoptosis and necrosis in human MCF-7 breast cancer cells by up-regulation of *BAK*, *FAS,* and *BAX* genes. In future researches, it must be better the immune stimulation of pBudCE4.1-azurin-MAM-A recombinant vector in animal models and therapeutic approaches will be evaluated.

© 2020 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Many bacteria produce toxins, extracellular enzymes, and pigments. Toxins and bacterial enzymes play important roles in the

Production and hosting by Elsevier

pathogenicity of bacteria. These toxins are generally classified into exotoxin and endotoxin groups (Allison et al., 2014; Ramachandran, 2014; Ezepchuk, 2014). Endotoxins are lipopolysaccharide that produced by Gram-negative bacteria, while exotoxins are usually heat-sensitive proteins that produced by specific strains of Gram-positive and Gram-negative bacteria and secreted into the surrounding environment (Lüderitz et al., 2016; Minasyan, 2017).

Although bacterial toxins inhibit protein synthesis, DNA replication, and cell wall synthesis in response to unfavorable conditions of growth, but useful applications of them have also been reported (Mathieu, 2015). For example, toxins of *Clostridium* and *Bacillus* genera have many uses in medicine, agriculture, and industry. Ophthalmologists, dermatologists, and neurologists are widely used the *Clostridium botulinum* toxin (Ferreira et al., 2016; Freedman et al., 2016). One of the important applications of

botulinum toxin (BTX or Botox) is in Botox and muscle paralysis by inhibiting the release of neurotransmitters such as acetylcholine which make the nerves of the infusion site unable to stimulate the muscle and the relaxation (Shehata et al., 2017). Also, toxic crystals of *Bacillus thuringiensis* are used as bioinsecticide against agricultural pests (Palma et al., 2014). Besides, the role of bacteria as anticancer agents has been detected about 100 years ago (Felgner et al., 2016). Bacterial enzymes, toxins, secondary metabolites, proteins or derived peptides could specifically effect on cancer cells or have anticancer roles. Bacterial toxins can be used to degrade tumor cells, or at low concentrations can control cell cycle processes such as cell proliferation, apoptosis, and differentiation (Karpiński & Adamczak, 2018). Diphtheria toxin (DT) of *Corynebacterium diphtheriae*, exotoxin A and azurin from *Pseudomonas aeruginosa* (*P. aeruginosa*) are the most common bacterial toxins that have been used to produce immunotoxins (Karpiński & Adamczak, 2018). These toxins cause a decrease in cell growth or cell cycle, and naturally, can induce apoptosis and cell death. As a result, they can be used as a supplement to improve the therapeutic effects of anticancer drugs (O'Brien-Simpson et al., 2018).

Azurin bacteriocin which is secreted by the *P. aeruginosa* bacterium is a small water-soluble protein that has a cytostatic property and can specifically penetrate the human cancer cells, especial breast cancer and induces apoptosis and has no apparent activity in normal cells (Yamada et al., 2004; Nguyen and Nguyen, 2016). *P. aeruginosa* is a Gram-negative, rod-shaped, asporogenous, and monoflagellated bacterium that is one of the most important agents in the development of hospital infections, which can create a wide range of infections in immunocompromised patients (Bhasin et al., 2015). The length of the *azurin*-encoding gene is 1287 bp (Gene Registration Number: M30389) that encodes a 14 kilo-daltons (kDa) protein containing 128 amino acids, which contain an alpha helix and eight beta-shits consisting of a β-motif and a hydrophobic moiety (Karpiński & Szkaradkiewicz, 2013). This protein is a member of the oxidation and redox proteins that there is a copper ion called cupredoxin in its structure (Fialho et al., 2012; Bernardes et al. 2013). Also, azurin reacts direct and sustained with the p53 tumor inhibitor (Yamada et al. 2004; Nguyen and Nguyen, 2016).

Breast cancer is the most common malignancy and it is the fifth significant cause of death in women worldwide (Youlden et al., 2014; Keyghobadi et al., 2015). The risk of breast cancer increases with age and depending on sex, family history, biological profile, genetic mutations, environmental agents, high-fat diet, late pregnancy (above age 30), nulliparity, early age of menarche, and late age of menopause (Asif et al., 2014; Anjum et al., 2017). The prevalence of breast cancer in women increases after the age of 35–54 and reaches a constant level after age 80 and although the incidence of breast cancer in men is rare (about 1 in 1000) but it does happen (Doosti et al., 2011; Edwards et al., 2014; Denny et al., 2017).

One of the important antigens presents in breast cancer cells is MAM-A which expressed in >80% of breast cancer cases. MAM-A is a 10.5-kDa secretion glycoprotein with 93 amino acids encoded by the *SCGB2A2* gene (Huo et al., 2013; Kim et al., 2016). This protein has a unique expression in breast cancer cells and it is a very specific molecular antigen in these cells; therefore, it is a suitable target for immunosuppressive therapy in breast cancer patients (Margan et al., 2016).

The role of some bacterial toxins like azurin bacteriocin in controlling and inhibiting the growth and proliferation of cancer cells like Michigan Cancer Foundation-7 (MCF-7) and 4 T1 breast cancer cell lines (derived from the mammary gland tissue of a human and mouse, respectively) are also significant. It should be noted that MAM-A protein is a specific antigen on the surface of the breast cancer cells and stimulates the immune system against cancer cells. The simultaneous effects of azurin and MAM-A on MCF-7 breast cancer cell have not been yet studied; therefore, the purpose of the present study was to generate of a pBudCE4.1-azurin-MAM-A gene construct containing the *azurin* gene of *P. aeruginosa* and human *MAM-A* gene and evaluation of their effects on cell cycle genes expression and apoptosis in the MCF-7 breast cancer cell line.

2. Materials and methods

2.1. Recombinant vector preparation and bacterial culture

The recombinant pBudCE4.1-azurin-MAM-A and empty pBudCE4.1 plasmids were purchased and provided from Generay Biotech Co., Ltd. (Shanghai, China). Also, the sequences of target genes were codon-optimized for better expression in a eukaryotic host by this company. The accuracy of synthesis genes and producing of this construct gene were confirmed by this company using Sanger's DNA sequencing method and *Xba*I/*Pst*I enzymatic digestion. In this study, pBudCE4.1 was used for the separate expression of *azurin* and *MAM-A* genes and this vector can express insertional genes in eukaryotic hosts. The length of inserted *azurin* and *MAM-A* genes in the recombinant plasmid were 1287 and 1309 bp, respectively. pBudCE4.1 vector has a length of 4.6 kb with strong eukaryotic promoters; including human cytomegalovirus (CMV) and human elongation factor 1 alpha (EF-1 alpha) promoters and also has two distinct sites for the simultaneous entry of two genes (MCS or multiple cloning sites). The lyophilized stock of *E. coli* strain Nova Blue (Novagen) was obtained from Cellular and Molecular Research Center of Shahrekord University of Medical Sciences and cultured in Luria-Bertani (LB) broth (Sigma, St. Louis, Mo.) and then incubated overnight at 37 °C with 200 rpm shaking.

2.2. Bacterial transformation

The recombinant pBudCE4.1-azurin-MAM-A vector and empty pBudCE4.1 were separately transformed into *E. coli* strain Nova Blue using calcium chloride (CaCl$_2$) heat-shock (for 90 s at 42 °C) treatment. These bacteria were cultured on the Low Salt LB agar medium in the presence of 25 µg/mL of zeocin. Colony-PCR screening using specific primers for *azurin* and *MAM-A* genes was performed on 20 randomized colonies for identification of plasmid transformation accuracy into *E. coli*. The specific oligonucleotide primers for *azurin* and *MAM-A* genes were designed (Table 1) using the Gene Runner software version 3.05 and the primer sequences were analyzed and compared to a query sequence of GenBank data using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI).

For mini-prep plasmid isolation, colonies contain vectors were cultured in 5 mL of LB broth containing zeocin and were incubated overnight at 37 °C. Plasmid DNA was purified from an overnight bacterial culture using the GeneJET Plasmid Miniprep Kit according to the recommendations of the manufacturer (Thermo Fisher Scientific, Freiburg, Germany). The extracted plasmid yield and quality were measured at 260 nm vs. 280 nm optical density using the NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop 2000, Wilmington, DE, USA) according to the method described by Sambrook & Russell, 2001. The confirmation of transformation and presence of target genes in the recombinant pBudCE4.1-azurin-MAM-A extracted plasmid were examined by PCR and enzymatic digestion with *Nco*I, *Kpn*I, *Apa*I, *Sma*I, and *Sac*I restriction enzymes. The PCR products and digested fragments were electrophoresed on a 2% agarose gel at constant voltage (80 V). A 1 kb DNA ladder (CinnaGen, Iran) was used as a molecular size marker. After staining of a gel by ethidium bromide (2 µg/mL),

Table 1
Detail of primers and their sequences used in this study.

Gene	Primers name	Sequences	Annealing temperature (°C)	Product length (bp)	Accession number
azurin	Azu-F	5′-ATGCTACGTAAACTCGCTGCC-3′	65	292	M30389
	Azu-R	5′-TGTCGTCGGGCTTCAGGTAATC-3′			
MAM-A	Mam-F	5′-CAGCGGCTTCCTTGATCCTTG-3′	66	221	NM_002411
	Mam-R	5′-TGGCATTGTCGTCTATGAACTCTTG-3′			
BAX	Bax-F	5′-AGGTCTTTTTCCGAGTGGCAGC-3′	65	234	NM_001291431
	Bax-R	5′-GCGTCCCAAAGTAGGAGAGGAG-3′			
BAK	Bak-F	5′-CGTTTTTTACCGCCATCAGCAG-3′	65	154	NM_001188
	Bak-R	5′-ATAGCGTCGGTTGATGTCGTCC-3′			
FAS	Fas-F	5′-CAATTCTGCCATAAGCCCTGTC-3′	65	163	KM114217
	Fas-R	5′-GTCCTTCATCACACAATCTACATCTTC-3′			
GAPDH	GAP-F	5′-GCCAAAAGGTCATCATCTCTGC-3′	65	183	NM_002046
	GAP-R	5′-GGTCACGAGTCCTTCCACGATAC-3′			
SURVIVIN	Surv-F	5′-AGAACTGGCCCTTCTTGGAGG-3′	65	170	Shen et al. 2008
	Surv-R	5′-CTTTTTATGTTCCTCTATGGGGTC-3′			
P53	P53-F	5′-TGCGTGTGGAGTATTTGGATGAC-3′	65	170	KY907146
	P53-R	5′-CAGTGTGATGATGGTGAGGATGG-3′			
CyclinD1	CyclinD1-F	5′-AAGTTGCAAAGTCCTGGAGCC-3′	65	125	Abdian et al. 2015
	CyclinD1-R	5′-TCGGCTCTCGCTTCTGCTG-3′			
ERBB2 (*HER-2*)	HER-2-F	5′-TAGACAATGGAGACCCGCTGAAC-3′	65	217	NC_000017
	HER-2-R	5′-GAGCGGTTGGTGTCTATCAGTGTG-3′			

the image was photographed under the UV light using the UVIDoc gel documentation system (Uvitec, UK).

2.3. The proliferation of target genes

Polymerase chain reaction (PCR) for amplification was carried out in a total volume of 25 µL in 0.2 mL tubes, which contained 50 ng of purified recombinant pBudCE4.1-azurin-MAM-A vector, $1 \times$ PCR buffer, 1 µM of each primer, 0.2 mM dNTPs mix, 2 mM MgCl$_2$, 1 unit of *Taq* DNA polymerase (all CinnaGen, Iran) per each reaction. Amplifications were performed on Gene Atlas Thermal Cycler (ASTEC, Seoul, Korea). The PCR reaction temperature was included the following cycles: one step of an initial denaturation at 94 °C for 5 min, 35 cycles of 1 min denaturation at 94 °C, a primer-annealing step at 65℃ (for *azurin* gene) and 66℃ (for *MAM-A* gene) for 50 s, and extension step at 72℃ for 50 s, and then a final step at 72 °C for 10 min. The amplification products were visualized on 1% agarose gel electrophoresis according to the procedure as mentioned above.

2.4. Cell line culture

MCF-7 human breast cancer was obtained from the Pasture Institute (Tehran, Iran). MCF-7 cells were cultured in T75 tissue culture flask (JET BIOFIL, Guangzhou, China) containing RPMI1640 medium (Gibco, Grand Island, NY, USA) that supplemented with 10% fetal bovine serum (FBS), and 100 units of Potassium Penicillin and 100 µg of Streptomycin Sulfate antibiotics per 1 mL of culture media (all reagents from Gibco, NY, USA). The flask was incubated in a humidified atmosphere of 5% CO$_2$ at 37 °C. The culture media were checked and added every second day to reach the cell confluence to 70–80%. Cells were passaged via discarding of the media and flask was washed twice with phosphate-buffered saline (PBS) and was dissociated by the 0.25% Trypsin-EDTA (Invitrogen) at 37 °C for 3 min. The enzyme activity was quenched with culture media supplemented by 10% FBS and was centrifuged at 1200 rpm for 5 min.

2.5. Cell cytotoxicity assay

An MTT assay was used to check the cytotoxicity level of zeocin antibiotic on MCF-7 to evaluate a proper concentration of this antibiotic for the screening of transformed cells. In brief, 20 µL of diluted MCF-7 in RPMI1640 medium was mixed by an equal volume of trypan blue (0.3% in PBS) and incubated for 5 min and cell counting was performed with a Neubauer hemocytometer under the microscope. The MCF-7 cells at a concentration of 9000 cells per each well of the 96-wells plate (SPL Life Sciences) were cultured in media supplemented by 10% FBS and incubated in a humidified CO$_2$ incubator at 37 °C for 24 h. Yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide powder (Roche Diagnostics, Penzberg, Germany) was provided in PBS at a concentration of 5 mg/mL and was stored under dark condition. Zeocin antibiotic was prepared at concentrations of 50–1000 µg/mL in media and was added to the cells of each well and was repeated triple for each concentration and the plate was kept in the CO$_2$ incubator at 37 °C. Non-treated MCF-7 (without adding zeocin in culture media) was used as a control group. The effect of zeocin on cell proliferation and its cytotoxicity was examined after 24, 48 and 72 h after treatment and were measured by MTT-colorimetric method (each well of each concentration was tested). That way, first, the media from the wells were disposed and each well was washed slowly by 100 µL PBS. Then, the prepared stock of MTT was diluted in RPMI 1640 (no phenol red) to the final concentration of 0.5 mg/mL (100 µL) and added on each well of cultured cell in 96-wells plate. The plate was incubated in a CO$_2$ incubator at 37 °C for 3.5 to 4 h in a dark condition. Media containing MTT was discarded and 100 µL of dimethylsulfoxide (DMSO, (C$_2$H$_6$OS) was added to each well and was shacked well. The resultant Formosan crystals dissolve by DMSO. The plate was incubated for 30 min and shacked for 5 min and optical density was measured at a wavelength of 490 to 620 nm by Stat Fax 2100 - ELISA microwell plate reader/mixer.

2.6. Transfection of vectors into MCF-7

In this study, 3×10^5 MCF-7 cells were cultured in each well of 6-wells plate (SPL Life Sciences) and then recombinant pBudCE4.1-azurin-MAM-A or pBudCE4.1 (empty vector as a control) were transfected into the MCF-7 cell line, separately using Lipofectamine 2000 (Invitrogen Corporation, Inc.) according to the recommendations of the manufacturer with some modification. For this purpose, 7.5 µL of Lipofectamine 2000 reagent was added in 150 µL of RPMI1640 medium without serum and antibiotics and also 2.5 µg/µL of DNA plasmid was prepared in 150 µL of serum and antibiotic-free media. Both diluted plasmid DNA and diluted Lipofectamine reagent were mixed (1:1 ratio) and was incubated for 5 min at room temperature (RT). Then, the normal media of

culture cells were discarded from each well of the 6-wells plate and washed with PBS one time to remove FBS and was replaced by media containing plasmid DNA and diluted Lipofectamine compound. 24 h later, the media was replaced by RPMI containing 20% FBS and 400 µg/mL of zeocin antibiotic (the appropriate dose obtained from the MTT test) for the screening of the transformed cells. The transfected MCF-7 cells by recombinant pBudCE4.1-azurin-MAM-A, pBudCE4.1 (empty vector) as well as treated cells by only Lipofectamine reagent (without DNA plasmid) were incubated for 3 days at 37 °C in the CO$_2$ incubator. The follow-up of cells and the screening of transformed cells from non-transformed were done in this mentioned time. Finally, after 72 h, the cells were detached from the bottom of each well of a plate using a 0.25% Trypsin-EDTA enzyme as a previously described method and were used for the next experiments.

2.7. Total RNA extraction and cDNA synthesis

Seventy-two hours after transfection, the total RNA isolation was performed on transformed and non-transformed MCF-7 (control group) using RNX plus (RNX plus^{TM} Kit Cinnagen, Tehran, Iran) according to the manufacturer's protocol. The quality and quantity of each extracted RNA specimen were measured using NanoDrop ND-2000 spectrophotometer at a wavelength of 260/280 nm as described above. Each cDNA sample was synthesized from 1 µg of total RNA according to the instruction protocol of the cDNA synthesis kit (Yekta Tajhiz Azma, Tehran, Iran).

2.8. RT-PCR and quantitative real-time PCR (q-RT-PCR)

The reverse transcriptase PCR (RT-PCR) was done to determine the expression of target genes including *azurin* and *MAM-A* genes in transformed MCF-7 by pBudCE4.1-azurin-MAM-A recombinant vector. The amplification conditions it was the same as stated above except the cDNA sample was used instead of template DNA plasmid. PCR products were analyzed on a 1.0% agarose gel electrophoresis.

Also, the quantitative real-time PCR (q-PCR) was used for investigating the proapoptotic (*BAK, FAS, BAX*, and *SURVIVIN*) and cell cycle regulatory genes (*Cyclin D1* and *P53*) expression in transformed and non-transformed MCF-7 (normal cells), as well as transformed MCF-7 by the entire vector (pBudCE4.1; control group). The gene's name and primers are shown in Table 1. Besides, the expression of *HER-2* (human epidermal growth factor receptor 2) also known as the *ERBB2* gene in transformed and non-transformed MCF-7 was evaluated. All primers except *SURVIVIN* and *CyclinD1* genes (that obtained from Shen et al., 2008; Abdian et al., 2015, respectively) were designed using Gene Runner software version 3.05 and as well as the identification of authentic of their sequences binding to the target gene site were analyzed by basic local alignment search tool (BLAST) of GenBank data. In this study, the cell cycle genes expression levels were compared to a *GAPDH* gene expression (as an internal control) and the standard curve analysis was done for this gene. Real-time PCR was performed in a Corbett Rotor-Gene 6000 rotary analyzer (Corbett, Australia). Each cDNA sample was diluted at a concentration of 1:5 and serial dilutions of 2:25, 3:125, 1:625, and 1:3125 were prepared. The q-PCR reaction mixture was performed in a triple at a final volume of 13 µL per each reaction in a 0.2 micro-tube. A 50 ng of cDNA sample (1 µL) was added into 6.5 µL 1 × SYBR Green real-time PCR master mix (Yekta Tajhiz Azma, Tehran, Iran), 0.5 µL of each forward and reverse primer (2 µM), and 4.5 µL distilled water. The following conditions were used for q-PCR: an initial heat denaturation step (hold) at 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s. Finally, the melting curve was gener-

ated at the end of the PCR amplification by increasing temperature from 72 °C to 95 °C (1 °C/s). The standard curve method was used to evaluate the relative changes in mRNAs expression. The gene expression analysis report and cycles of threshold (CTs) were generated by Rotor-Gene Real-time analysis software version 6.0 (Qiagen, Inc., Valencia, CA, USA) for calculation. The comparative CT method ($2^{-\Delta\Delta Ct}$ or Livak method) was used to analyze the expression of target genes and normalized to the reference gene.

2.9. Flow cytometry analysis

In this study, the effects of recombinant vector transformation, empty vector (pBudCE4.1), and as well as treated cells by only zeocin antibiotic on apoptosis and cells death were evaluated by FITC Annexin V Apoptosis Detection Kit I 556,547 (BD Biosciences, San Jose, CA, USA) in flow cytometric analysis. First, 3×10^5 MCF-7 cells were cultured in each well of the 6-wells plate according to the procedure as mentioned above. Then, each group in duplicate was included: transformed cells by pBudCE4.1-azurin-MAM-A recombinant vector, treated cells by zeocin antibiotic alone and transformed cells by pBudCE4.1 (empty plasmid as a control). Afterward, 100×10^3 MCF-7 cells were washed twice with cold PBS and were resuspended the cells in a 1X binding buffer provided in the kit. Then, 100 µL of cells solution was transferred into Partec flow cytometry micro-tube. After that 5 µL of FITC Annexin V and 5 µL PI were added on the cells by gently vortex mixing and was incubated for 15 min at RT (25 °C) in the dark condition and finally was analyzed by flow cytometry.

2.10. Immunofluorescence (IF) assay

To assess the transfection efficiency and the expression of azurin and MAM-A, the transformed MCF-7 by pBudCE4.1-azurin-MAM-A and entire (empty pBudCE4.1) vectors and as well as un-transformed MCF-7 cells (normal group) were fixed in 4% paraformaldehyde in PBS (pH: 7.2–7.8) at RT for 10 min and were washed three times with washing buffer (0.05% Tween 20 (Sigma, Missouri) in PBS). Also, fixation buffer 4% (2 gr Paraformaldehyde was dissolved in up to 50 mL PBS), permeabilization buffer 0.1% (10 µL Triton-X-100 was added into 9.9 mL PBS), and blocking buffer 1% (0.1 gr BSA was dissolved in 10 mL PBS) were prepared. One day before the IF assay, 10,000 cells for each group (including transformed MCF-7 by pBudCE4.1-azurin-MAM-A and empty vectors and as well as non-transformed cells as a negative control) were counted and seeded on the coverslip. The next day, the culture media were removed from the cells and was washed twice with 0.05% washing buffer. The cells were placed in a suitable volume of 4% fixation buffer for 10 min and then were rinsed three times with a wash buffer. The permeabilization buffer was placed on the cells for 15 to 20 min and then was washed three times. Blocking buffer was placed on cells for 45 min and then the cells were exposed to the primary antibodies including goat anti-azurin polyclonal antibody (STJ140126) and rabbit anti-Mammaglobin A polyclonal antibody (STJ93996) (both from St John's Labs) at a final concentration of 1:1000 for overnight at 4 °C. After three wash steps, the secondary antibodies including donkey anti-mouse IgG (NorthernLights^{TM}) NL493-conjugated antibody (NL003, R&D Systems, Canada) and Goat Anti-Rabbit IgG H&L (Cy3 $^®$) preadsorbed (ab6939, Abcam, Cambridge, U.K.) were applied at a 1:200 and 1:1000 dilution rate in PBS supplemented with 1% BSA for 45 min in RT. In all steps, the technical negative controls excluded the primary antibody were applied. Also, a solution contains secondary antibody was removed and the cell nuclei were stained with DAPI (1 µg/mL). Finally, the coverslips were removed from the chamber and washed well in distilled water. The cells were observed at desired wavelengths using a

fluorescence Nikon AZ100 Multizoom microscope (Nikon Instruments, Inc., Japan).

2.11. Western blotting

The expression proteins of *azurin* and *MAM-A* target genes in transformed MCF-7 cells by pBudCE4.1-azurin-MAM-A recombinant vector compared to the control cells (normal MCF-7 or non–transformed) were assessed by western blotting. Urea protein extraction was done by 8 M urea lysis buffer. This lysis buffer was added to a cell culture plate (1×10^6 cells) and was incubated on ice for 45 min and gently vortex. Then, the suspension was centrifuged at 13000 rpm for 10 min at 4 °C. The protein concentration in the supernatant was determined using Bradford assay at a wavelength of 595 nm according to the manufacturer's instructions. The proteins (40 µg) of each sample were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to a polyvinylidene fluoride (PVDF) membrane (Pall Corporation, Dreieich, Germany) by the semidry-blotting method. Tris-Buffered Saline (TBS) contains 5% BSA with 0.01% Tween 20 (TBST buffer) at RT for 30 min was used for blocking of the membranes. Then, the membranes were incubated overnight at 4 °C by primary antibodies (goat anti-azurin polyclonal antibody (STJ140126) and rabbit anti-MAM-A polyclonal antibody (STJ93996) were used separately) that diluted in TBST buffer contains 2% BSA. Afterward, the blots membrane was washed with TBST three times and were incubated with secondary antibodies including goat anti-rabbit HRP (IgG H&L) (ab97051, Abcam, USA) and donkey anti-goat IgG-HRP (sc-2020, Santa Cruz Biotechnology Inc.) for azurin and MAM-A, respectively in 2 h. In both transformed and non-transformed-cells the primary anti-GAPDH antibody (ab9483, Abcam, USA) was used as an internal control of western blot and detects a band of approximately 37 kDa. Also, for GAPDH the secondary antibody was goat anti-rabbit HRP (IgG H&L) (ab97051, Abcam, USA). The blots were washed by TBST buffer 3 times for 10 min and were developed on a Western Blotting Luminol Reagent (Thermo Fisher Scientific, USA). The bands were visualized by the Licor Odyssey Scanner system and finally, the band's density was quantified using Licor Odyssey Software version 5.5 (LI-COR Biosciences).

2.12. Statistical analysis

All data were transferred into Statistics programs for the Social Sciences software (SPSS, Inc., Chicago, IL, USA) version 20 and Microsoft Excell version 7.0 (Microsoft Corporation, Redmond, WA, USA) and processed for mean and standard deviation. The variance between groups was calculated by a non-parametric test (Kruskal-Wallis). All graphs were made using GraphPad Prism Software version 7 (GraphPad, San Diego, California). The differences with a *p*-value<0.05 were considered as the statistical significance level.

3. Results

3.1. Accuracy of transformation

The confirmation of the insertion of target genes (*azurin* and *MAM-A*) in pBudCE4.1-azurin-MAM-A recombinant vector for transformation examined by Generay Biotech company using enzymatic digestions (*Xba*I and *Pst*I restriction enzymes) and DNA sequencing (Fig. 1 and Fig. 2). Also, the accuracy of transformation was done on extracted vectors by PCR and restriction enzyme digestions. These methods showed the successful transfor-
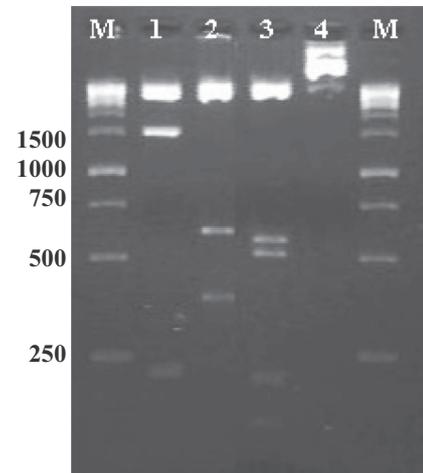
Fig. 1. The digestion patterns of purified pBudCE4.1-azurin-MAM-A recombinant vector using restriction enzymes on 1% agarose gel electrophoresis (M: 1-kb DNA size marker (CinnaGen, Iran), lane 1: digested vector by *Nco*I: 220 bp, 1471 bp (containing *azurin* gene), and 3657 bp, lane 2: digested plasmid by *Kpn*I and *Apa*I including 590 bp, 19 bp, 368 bp (containing *MAM-A* gene), and 4371 bp, lane 3: restriction fragments by *Sma*I and *Sac*I: 187 bp, 551 bp, 132 bp, 492 bp (containing *azurin* gene), and 3986 bp, line 4: uncut pBudCE4.1-azurin-MAM-A (5348 bp) recombinant vector, respectively).

mation of the vector into the host and the recombinant vector intended (Fig. 3).

3.2. MCF-7 cells culture

The normal shape of cultured MCF-7 cells in RPMI media to obtain proper cell density for the experiments is shown in Fig. 4A.

3.3. MTT assay findings

The MTT assay was done for investigation of the zeocin resistance curve on transformed- and non-transformed MCF-7. The MTT results were showed that the cytotoxicity of zeocin antibiotic at a concentration of 400 µg/mL (minimum lethal dose or LD_{min}) was the proper and effective concentration for the screening of the transformed MCF-7 from non-transformed cells. In this concentration, all normal MCF-7 cells were dead while most of the transformed MCF-7 alive (Fig. 5).

3.4. Transformation findings

The shapes of transformed cells by plasmid DNA after 72 h were shown in Fig. 4B. Moreover, the effects of Lipofectamine and zeocin antibiotic on cytotoxicity of the exposed cells were investigated for 72 h, separately (Fig. 4C and Fig. 4D).

3.5. RT-PCR analysis

The products of gene amplification after separation on agarose gel electrophoresis revealed the fragments with the length size of 292 and 221 bp for *azurin* and *MAM-A* genes, respectively in transformed MCF-7 by pBudCE4.1-azurin-MAM-A recombinant vector (Fig. 6). These fragments in transformed cells by empty vector and non-transformed MCF-7 were not observed. The RT-PCR results confirmed the transformation accuracy and expression of the recombinant vector in transformed MCF-7 cells.
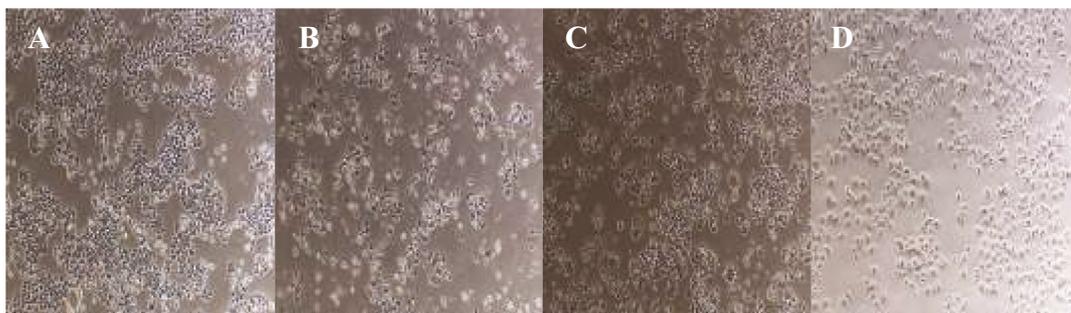
Fig. 2. A: The normal MCF-7 cells (non-transformed cells), B: transformed MCF-7 cells by pBudCE4.1-azurin-MAM-A recombinant vector using Lipofectamine 2000 reagent, C: treated cells by Lipofectamine compound only, and D: treated MCF-7 s by zeocin antibiotic.
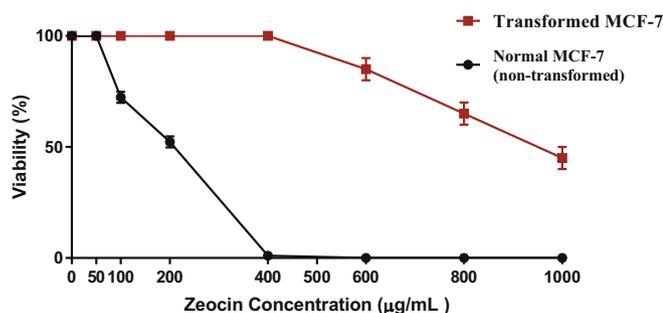

Fig. 3. Quantifying the cytotoxicity of zeocin antibiotic (zeocin resistance curve) on transformed- and non-transformed MCF-7 cells using MTT assay.

3.6. Results of apoptosis and necrosis assay

As shown in Fig. 7 the findings of the flow cytometry method showed the apoptosis and necrosis in transformed MCF-7 cells by pBudCE4.1-azurin-MAM-A recombinant vector compare to the transformed cells by empty pBudCE4.1 plasmid were increased statistically significant ($p < 0.05$). Although, in treated MCF-7 cells by a zeocin antibiotic, the apoptosis and necrosis were seen, but compared to the transfected cells by pBudCE4.1 vector not increased statistically significantly ($p > 0.05$). Also, apoptosis range in transformed cells compared to the treated cells by only zeocin was increased statistically significantly ($p < 0.05$). The mortality percentage (apoptosis and necrosis) in treated groups, including transformed MCF-7 by pBudCE4.1-azurin-MAM-A recombinant vector and empty pBudCE4.1 vector (without target genes), and as well as treated MCf-7 by a zeocin antibiotic that was measured by flow cytometry are shown in Fig. 8. The results of flow cytometry were that 24.11% of transformed MCF-7 by this recombinant vector death (necrosis and apoptosis percentages), while in the transformed cells by transfected cells by pBudCE4.1 vector 5.3% of cell death occurred and this mortality was statistically significant ($p < 0.05$). In treated normal MCF-7 by an only zeocin antibiotic (non-transformed) 15.16% of the mortality observed and compared to the transformed cells by recombinant construct was not statistically significant ($p > 0.05$).

3.7. Genes expression findings

The analysis of proapoptotic and cell cycle regulatory genes expression showed that the fold changes of *BAK*, *FAS*, *BAX*, *SURVIVIN*, and *P53* expressions in transformed MCF-7 by recombinant plasmid compare to the transformed MCF-7 by empty plasmid (pBudCE4.1) were 35.5, 12.7, 10.7, 5.4, and 3.1, respectively. Also, the expressions of *BAK*, *FAS*, and *BAX* genes in transformed cells by recombinant vector compared with other groups increased sta-
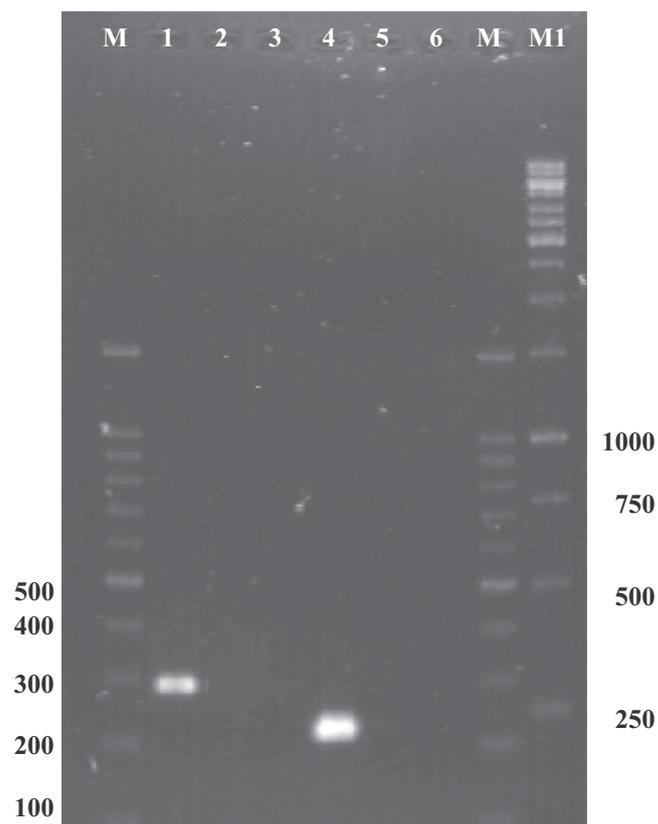

Fig. 4. The RT-PCR products in transformed MCF-7 by pBudCE4.1-azurin-MAM-A recombinant vector and non-transformed MCF-7 (control cells) for evaluating the expression of *azurin* and *MAM-A* genes on 1% agarose gel electrophoresis (lane M: 100 bp DNA molecular marker (GIBCO-BRL), lane M1: 1 kb DNA molecular ladder (Promega), lane 1: the amplified fragments of *azurin* (292 bp) in transformed MCF-7, lane 2: non-presence of *azurin* in normal MCF-7, lanes 3 and 6: negative controls, lane 4: proliferation the fragment of *MAM-A* gene (221 bp) in transformed MCF-7, lane 5: lack of expression of *MAM-A* in normal MCFs, respectively).

tistically significant ($p < 0.05$). The expression of *cyclin D1* transfected cells with a recombinant vector was showed the significantly down-regulation on this gene compare to transfected MCF-7 by empty plasmid and normal MCF-7 cells ($p < 0.05$) (Fig. 9). *HER-2* or *ERBB2* gene can influence the development of breast cancer and therefore its expression was evaluated in MCF-7 breast cancer cell line in this study. Also, the expression of *HER-2* gene in transformed MCF-7 by recombinant vector was higher (2.9 times) than transformed MCF-7 by pBudCE4.1 and non-transformed MCF-7 groups but was not statistically significant ($p > 0.05$) (Fig. 9).
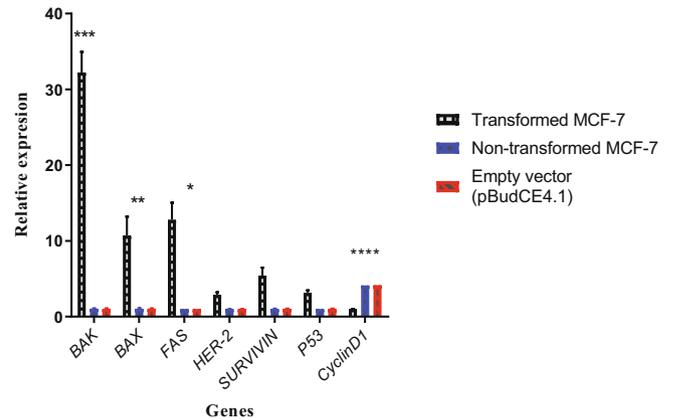
A **B** **C**
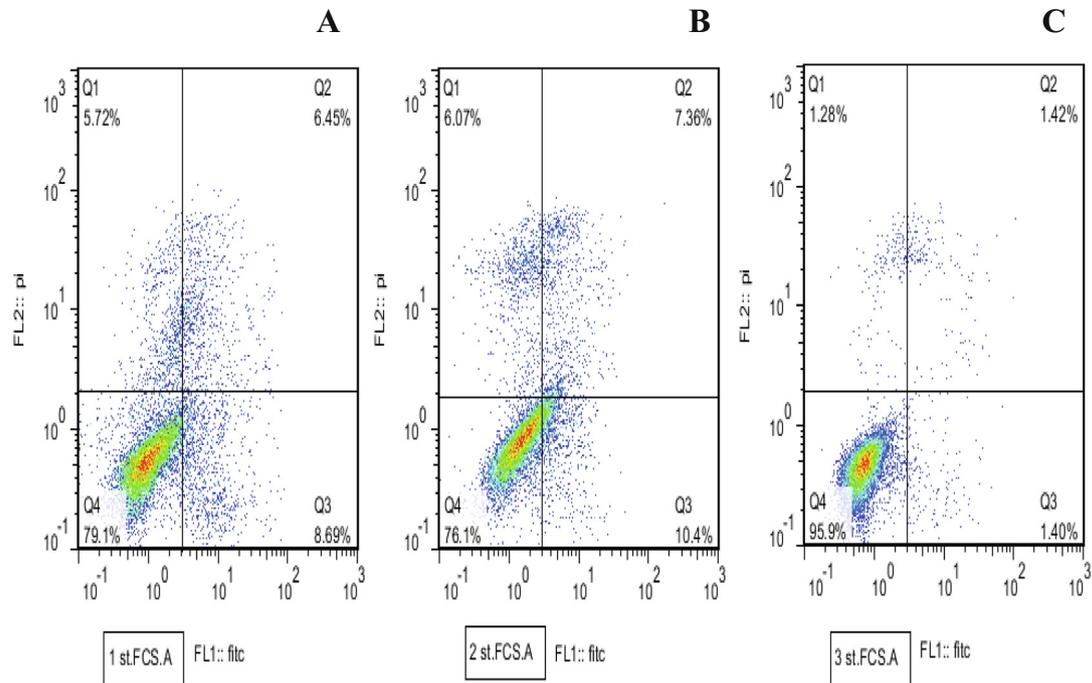
Fig. 5. The results of apoptosis assay by detection FITC Annexin V apoptosis on transformed MCF-7 by the recombinant vector (A), treated cells by zeocin antibiotic alone (B), and transformed cells by the empty vector (pBudCE4.1) (C) using flow cytometry assay.
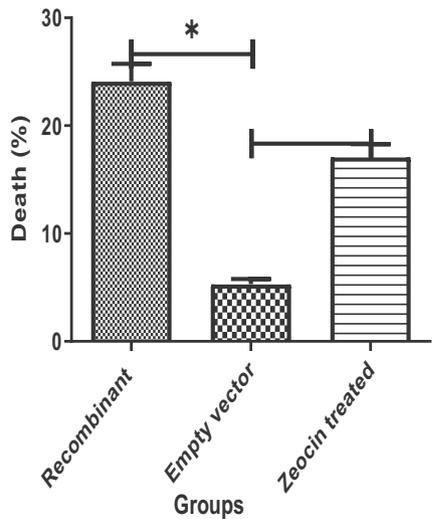
Fig. 6. The percentage of death (apoptosis and necrosis) in treated groups after detection by flow cytometry assay.


Fig. 7. The comparisons of relative cell cycling and *HER-2* genes expression in transformed, non-transformed, and transformed cells by the empty vector.

3.8. IF results

The immunofluorescence (IF) test was performed for the detection of azurin and MAM-A antibodies in the transformed MCF-7 by pBudCE4.1-azurin-MAM-A. Also, the transformed MCF-7 by pBudCE4.1 vector and un-transformed cells (normal MCF-7) were used as controls. The *azurin* and *MAM-A* expressive cells were conjugated by goat anti-azurin and rabbit anti-MAM-A polyclonal antibodies and were seen in green and red colors under the fluorescent microscope, respectively and while stained cells with DAPI solution for nuclei staining in all groups were seen in blue color (Fig. 10).

3.9. Proteins expression analysis

The proteins measurement were showed that Azurin (14 kDa) and MAM-A (10.5 kDa) proteins in transformed cells were expressed, successfully. Also, the expression of GAPDH was checked out as an internal control (approximately 37 kDa) (Fig. 11).

4. Discussion

Today, due to the lack of effective treatments against any kind of cancer and the disadvantages of existing therapies, it is very important to find new strategies to prevent and inhibition of cancer cells' propagation. According to the anti-breast cancer effect of Azurin protein from *P. aeruginosa* and stimulation of cellular immune system by MAM-A against breast cancer, the present work was performed to generate a pBudCE4.1-azurin-MAM-A recombinant
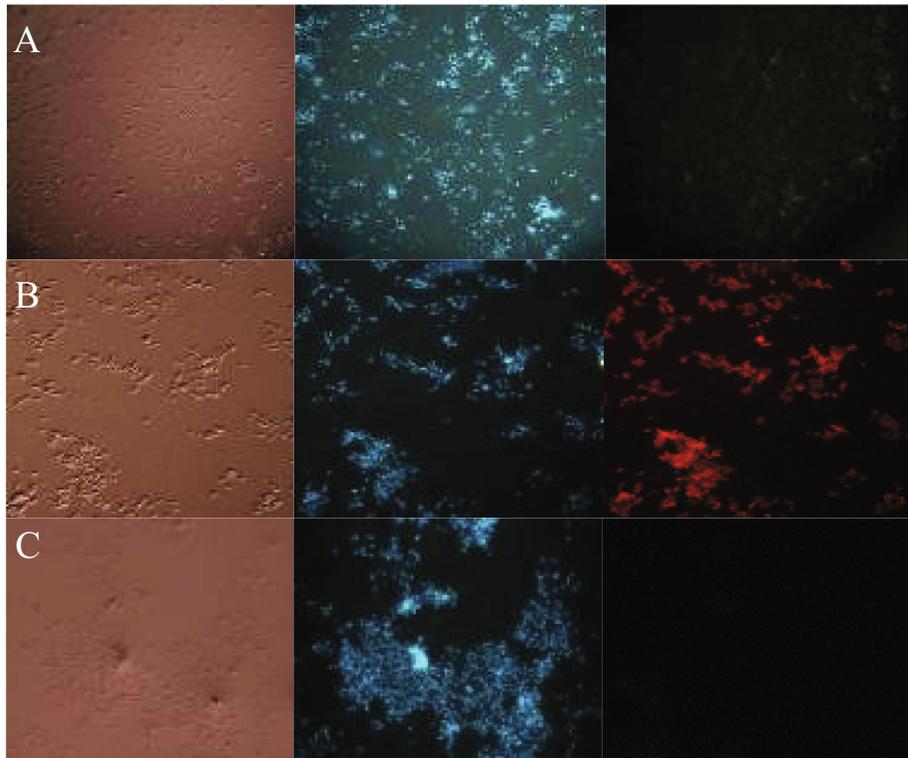
Fig. 8. The IF results (bright field and fluorescence imaging) for the detection of *azurin* and *MAM-A* genes expression in transformed MCF-7 (positive cells). A: azurin positive MCF-7 cells, B: MAM-A expressed cells, and C: empty vector (pBudCE4.1). Blue was stained cells by DAPI, green and red were positive expressed MCF-7 for azurin and MAM-A, respectively.
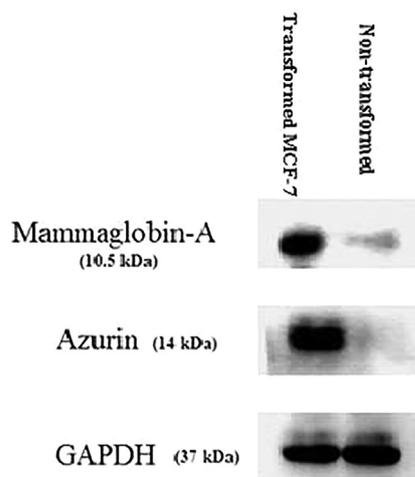
Fig. 9. The SDS-PAGE for confirmation of the expression of Azurin and MAM-A proteins in transformed MCF-7 by recombinant pBudCE4.1-azurin-MAM-A vector compares to the non-transformed MCF-7 (negative control) using western blotting. The GAPDH protein expression (internal control) was observed in both groups.

construct and investigate its effects in the MCF-7 cells. The prepared pBudCE4.1-azurin-MAM-A recombinant vector was transferred into MCF-7 using Lipofectamine reagent and after the screening of transformed cells by treatment of zeocin antibiotic; the effects of zeocin antibiotic and vectors transformation on necrosis, apoptosis, and cells death were evaluated by flow cytometry. The expression of target genes was evaluated by RT-PCR, IF assay and western blot method. Besides, the expressions of cell cycle genes were assessed by q-real-time PCR technique. The RT-PCR results were showed the successful expression of *azurin* and

MAM-A genes in transformed MCF-7. The results of flow cytometry indicated that 24.11% of transformed cells by recombinant vector had apoptosis and necrosis (death) and compare to the transformed MCF-7 by pBudCE4.1 (5.3% death) this mortality was statistically significant ($p < 0.05$). But in treated cells by the only zeocin antibiotic (non-transformed) 15.16% of death observed and compared to the transformed MCF-7 by recombinant vector were not statistically significant ($p > 0.05$). These findings showed the proper effects of the recombinant pBudCE4.1-azurin-MAM-A vector on the mortality of MCF-7. Also, the expression of these genes was approved by IF and western blotting. The bands of Azurin and MAM-A proteins in the transformed group indicated the successful expression of these proteins on SDS-PAGE. The findings of q-real-time PCR for investigation of cell cycle genes showed that the expression of *BAK*, *FAS,* and *BAX* genes in transformed cells compared with non-transformed cells and transformed MCF-7 by empty vector increased statistically significant ($p < 0.05$). *BAK, FAS,* and *BAX* are pro-apoptotic genes and increasing the expression of these genes in transformed MCF-7 indicates the proper effects of pBudCE4.1-azurin-MAM-A recombinant vector on apoptosis and death in breast cancer cells. Although, the expression of *SURVIVIN* (as an inhibitor of apoptosis) and *P53* (pro-apoptotic gene) were increased in transformed cells compared to the non– transformed and transformed MCF-7 by pBudCE4.1 but these variations were not statistically significant ($p > 0.05$). The *Cyclin-D1* expression in transformed cells by recombinant vector compare to normal and transfected MCF-7 by empty vector was decreased statistically significant ($p < 0.05$). Furthermore, the minimal increase in expression of *HER-2* gene in transformed MCF-7 compared to the other two groups was observed and this increase indicated that expressions of *azurin* and *MAM-A* genes can affect the amount of expression of this molecular biomarker in breast cancer cells. In one study, the researchers showed that cytokeratin 19, MAM-A, and HER-2

are the multi-markers of early breast cancer cells using RT-PCR assay (Ignatiadis et al., 2008). Although in our study, the low increase of HER-2 gene expression in transformed MCF-7 was observed, but this enhancement was not significant and demonstrated the usefulness of the engineered vector in breast cancer cells. MAM-A is overexpressed in 40% to 80% of primary breast cancers and can capable of eliciting MAM-A-specific CD8 T-cell responses (Tiriveedhi et al., 2014). In this study, we transformed MCF-7 breast cancer cells by MAM-A and Azurin expressing vector for inducing of the immune system in these cells and overexpression of cell cycle genes (BAK, FAS, and BAX) were observed. These findings support that MAM-A is an important biomarker for stimulation of the immune system and together Azurin protein of P. aeruginosa can prevent the expansion and growth of breast cancer cells.

In a study by Bernardes et al., 2011 an Azurin protein of P. aeruginosa was introduced as a candidate for treating non-treatable breast cancer. In their study, the association rate of Azurin protein and its interaction with cell adhesion molecules (CAMs), especially P-cadherin, in breast cancer cells was evaluated. In another study the effects of Azurin by ROS measurement, immunocytochemistry, gelatine zymography to evaluate MMP-2 activity and invasion capacity evaluated in breast and lung cancer cell lines and showed that Azurin decreased integrin subunits ($\alpha 6$, $\beta 1$, and $\beta 4$) and this protein it can introduce as a new candidate anti-cancer drug by decreasing cell adhesion through integrins (Abreu, 2013). In the present study, the co-effects of Azurin and MAM-A proteins on cell apoptosis and MCF-7 cell growth by FITC Annexin V apoptosis were evaluated and growth inhibition of breast cancer cells and increase in expression patterns of pro-apoptotic genes like BAK, FAS, and BAX were observed.

In a study of Punj and co-workers, the mechanisms of the action of azurin bacteriocin in the regression of breast cancer expressed and they showed that Azurin can act as an anticancer chemotherapeutic. Their findings indicated that azurin enters into the cytosol of MCF-7 cells and migrates to the nucleus and increased the intracellular levels of P53 and BAX, thereby triggering the release of mitochondrial cytochrome c into the cytosol and finally by activating the caspase-9 and caspase-7 lead to apoptotic process (Punj et al., 2004). Also, in our study, the significant enhancement of BAK, FAS, and BAX in transformed MCF-7 by the recombinant vector that expressed azurin and MAM-A genes were observed. Moreover, P53 was increased in transformed MCF-7 by pBudCE4.1-azurin-MAM-A vector compare to the non-transformed cells and transformed cells by pBudCE4.1 vector but this enhancement was not statistically significant ($p > 0.05$). Another study demonstrated that azurin internalizes in J774 or cancer cells in a temperature-dependent manner and this protein entry into cancer cells compared with normal cells preferentially (Yamada et al., 2005). Taylor and colleagues indicated that amino acids 50 to 67 (P18 and P28) of Azurin protein are responsible for cellular penetration, and antiproliferative and proapoptotic activity against several solid tumors and human cancer cells (Taylor et al., 2009). Van Mellaert et al. 2006 demonstrated that Azurin is not entering into the normal cells and it is safe for cancer cell treatment. In the present study, the performance of Azurin protein on inhibition of MCF-7 cancer cells growth in the transformed cell by pBudCE4.1-azurin-MAM-A recombinant vector was observed. Likewise, in this research, the over-expression of BAK, FAS, and BAX genes in transformed cells (azurin-expressed MCF-7 cells) were showed. In a study of Yang et al., 2005 in treated osteosarcoma U2OS cells by bacterial redox protein Azurin the down-regulation of Bcl-2 (an inhibitor of apoptosis) was detected, while BAX and CASPASE-3 were significantly up-regulated. Whereas, in our study, the up-regulation of BAK, FAS, and BAX genes in transformed MCF-7 by Azurin-MAM-A expression vector were seen, but the increasing

of SURVIVIN (anti-apoptosis) and P53 (pro-apoptotic gene) in transformed cells by genetically manipulated vector compare to the non-transformed and transformed MCF-7 by pBudCE4.1 were not statistically significant ($p > 0.05$). In both studies, the over-expression of BAX in treated cancer cells by azurin was viewed. These results refer that Azurin protein from P. aeruginosa inhibits the uncontrolled cell cycle progression and activates apoptotic events by enhancement of pro-apoptotic genes.

In conclusion, the pBudCE4.1-azurin-MAM-A recombinant vector prevents the growth and proliferation of MCF-7 breast cancer cells via controlling cell cycle genes and up-regulation of pro-apoptotic genes (BAK, FAS, and BAX) Cyclin-D1 (cell cycle regulatory) compare with non-transformed cells and transformed cells by the empty vector. It was suggested that the effects of pBudCE4.1-azurin-MAM-A recombinant vector on stimulation of the immune system and immunization in animal models are followed up in further studies. These findings will provide hope for therapeutic approaches to prevent the expansion of breast cancer cells and the treatment of this malignancy in the foreseeable future.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgement

Disclosure of interest

The authors declare that they have no competing interests.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abdian, N., Ghasemi-Dehkordi, P., Hashemzadeh-Chaleshtori, M., Ganji-Arjenaki, M., Doosti, A., Amiri, B., 2015. Comparison of human dermal fibroblasts (HDFs) growth rate in culture media supplemented with or without basic fibroblast growth factor (bFGF). Cell Tissue Bank. 16, 487–495.

Abreu, S.A.S., 2013. Bacterial protein azurin as a new candidate anticancer drug by decreasing cell adhesion through integrins. 2013, https://hdl.handle.net/10316/24731.

Allison, S.D., Lu, L., Kent, A.G., Martiny, A.C., 2014. Extracellular enzyme production and cheating in Pseudomonas fluorescens depend on diffusion rates. Front. Microbiol. 5, 169.

Anjum, F., Razvi, N., Maqbool, A., Jahan, N., 2017. A review of breast cancer risk factors. Univ. J. Pharmaceutic. Res. 2, 40–45.

Asif, H.M., Sultana, S., Akhtar, N., Rehman, J.U., Rehman, R.U., 2014. Prevalence, risk factors and disease knowledge of breast cancer in Pakistan. Asian Pac. J. Canc. Prev. 15, 4411–4416.

Bernardes, N., Ribeiro, A.S., Abreu, S., Mota, B., Matos, R.G., Arraiano, C.M., 2013. The bacterial protein azurin impairs invasion and FAK/Src signaling in pcadherin-overexpressing breast cancer cell models. PLoS ONE 8, e6902.

Bernardes, N., Ribeiro, A.S., Seruca, R., Paredes, J., Fialho, A.M., 2011. Bacterial protein azurin as a new candidate drug to treat untreatable breast cancers. 1st Portuguese Biomedical Engineering Meeting, Lisbon 1–4. https://doi:10.1109/ENBENG.2011.6026047.

Bhasin, S., Shukla, A.N., Shrivastava, S., 2015. Observation on Pseudomonas aeruginosa in Kshipra river with relation to anthropogenic activities. Int. J. Curr. Microbiol. App. Sci. 4, 672–684.

Denny, L., de Sanjose, S., Mutebi, M., Anderson, B.O., Kim, J., Jeronimo, J., Herrero, R., Yeates, K., Ginsburg, O., Sankaranarayanan, R., 2017. Interventions to close the

divide for women with breast and cervical cancer between low-income and middle-income countries and high-income countries. The Lancet. 389, 861–870.

Doosti, A., Dehkordi, P.G., Davoudi, N., 2011. A *p53* codon 72 polymorphism associated with breast cancer in Iranian patients. Afr. J. Pharm. Pharmacol. 5, 1278–1281.

Edwards, B.K., Noone, A.M., Mariotto, A.B., Simard, E.P., Boscoe, F.P., Henley, S.J., Jemal, A., Cho, H., Anderson, R.N., Kohler, B.A., Eheman, C.R., Ward, E.M., 2014. Annual report to the nation on the status of cancer, 1975–2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer. Canc. 120, 1290–1314.

Ezepchuk, Y.V., 2014. Biological concept of bacterial pathogenicity (theoretical review). Adv. Microbiol. 7, 535–544.

Felgner, S., Kocijancic, D., Frahm, M., Weiss, S., 2016. Bacteria in cancer therapy: renaissance of an old concept. Int. J. Microbiol. 2016, Article ID 8451728, 14 pages, https://doi:10.1155/2016/8451728.

Ferreira, M., Moreira, G., Cunha, C., Mendonça, M., Salvarani, F., Moreira, Â., Conceição, F., 2016. Recombinant alpha, beta, and epsilon toxins of *Clostridium perfringens*: Production strategies and applications as veterinary vaccines. Toxins. 8, 340.

Fialho, A.M., Salunkhe, P., Manna, S., Mahali, S., Chakrabarty, A.M., 2012. Glioblastoma multiforme: novel therapeutic approaches. ISRN Neurol. 2012, 1–10. https://doi.org/10.5402/2012/642345.

Freedman, J., Shrestha, A., McClane, B., 2016. *Clostridium perfringens* enterotoxin: action, genetics, and translational applications. Toxins. 8, 73. https://doi.org/10.3390/toxins8030073.

Huo, L., Zhang, J., Gilcrease, M.Z., Yun, G., Wu, Y., Zhang, H., Resetkova, E., Hunt, K.K., Deavers, M.T., 2013. GCDFP-15 and mammaglobin expression by immunohistochemistry is of limited utility in triple-negative breast cancer. Histopathol. 62.

Ignatiadis, M., Kallergi, G., Ntoulia, M., Perraki, M., Apostolaki, S., Kafousi, M., Mavroudis, D., 2008. Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, Mammaglobin A, and HER2 in early breast cancer. Clin. Canc. Res. 14, 2593–2600.

Karpiński, T., Adamczak, A., 2018. Anticancer activity of bacterial proteins and peptides. Pharmaceutic. 10, 54. https://doi.org/10.3329/bjp.v8i3.15704.

Karpiński, T.M., Szkaradkiewicz, A., 2013. Anti-cancer peptides from bacteria. Bangladesh J. Pharmacol. 8, 343–348.

Keyghobadi, N., Rafiemanesh, H., Mohammadian-Hafshejani, A., Enayatrad, M., Salehiniya, H., 2015. Epidemiology and trend of cancers in the province of Kerman: southeast of Iran. Asian Pac. J. Canc. Prev. 16, 1409–1413.

Kim, S.W., Goedegebuure, P., Gillanders, W.E., 2016. Mammaglobin-A is a target for breast cancer vaccination. OncoImmunol. 5, e1069940.

Lüderitz, O.T.T.O. et al., 2016. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides. Microbial Toxins. 4, 145–233.

Margan, M.M., Jitariu, A.A., Cimpean, A.M., Nica, C., Raica, M., 2016. Molecular portrait of the normal human breast tissue and its influence on breast carcinogenesis. J. Breast Canc. 19, 99–111.

Mathieu, J., 2015. Interactions between autophagy and bacterial toxins: Targets for therapy?. Toxins. 7, 2918–2958.

Minasyan, H., 2017. Sepsis and septic shock: Pathogenesis and treatment perspectives. J. Crit. Care 40, 229–242.

Nguyen, C., Nguyen, V.D., 2016. Discovery of azurin-like anticancer bacteriocins from human gut microbiome through homology modeling and molecular docking against the tumor suppressor p53. BioMed Res. Int. https://doi.org/10.1155/2016/8490482.

O'Brien-Simpson, N.M., Hoffmann, R., Chia, C.S.B., Wade, J.D., 2018. Editorial: antimicrobial and anticancer peptides. Front. Chem. 6, 13.

Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P., 2014. *Bacillus thuringiensis* toxins: an overview of their biocidal activity. Toxins. 6, 3296–3325.

Punj, V., Bhattacharyya, S., Saint-Dic, D., Vasu, C., Cunningham, E.A., Graves, J., Yamada, T., Constantinou, A.I., Christov, K., White, B., Li, G., Majumdar, D., Chakrabarty, A.M., Das Gupta, T.K., 2004. Bacterial cupredoxin azurin as an inducer of apoptosis and regression in human breast cancer. Oncogene 23, 2367–2378.

Ramachandran, G., 2014. Gram-positive and gram-negative bacterial toxins in sepsis. Virulence. 5, 213–218.

Sambrook, J., Russell, D.W., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 148–190.

Shehata, A.S., Al-Ghonemy, N.M., Ahmed, S.M., Mohamed, S.R., 2017. Effect of mesenchymal stem cells on induced skeletal muscle chemodenervation atrophy in adult male albino rats. Int. J. Biochem. Cell Biol. 85, 135–148.

Shen, C.X., Hu, L.H., Xia, L., Li, Y.R., 2008. Quantitative real-time RT–PCR detection for Survivin, Ck20 and CEA in peripheral blood of colorectal cancer patients. Jpn. J. Clin. Oncol. 38, 770–776.

Taylor, B.N., Mehta, R.R., Yamada, T., Lekmine, F., Christov, K., Chakrabarty, A.M., 2009. Noncationic peptides obtained from azurin preferentially enter cancer cells. Canc. Res. 69. https://doi.org/10.1158/0008-5472.CAN-08-2932.

Tiriveedhi, V., Tucker, N., Herndon, J., Li, L., Sturmoski, M., Ellis, M., Ma, C., Naughton, M., Lockhart, A.C., Gao, F., Fleming, T., Goedegebuure, P., Mohanakumar, T., Gillanders, W.E., 2014. Safety and preliminary evidence of biologic efficacy of a mammaglobin-A DNA vaccine in patients with stable metastatic breast cancer. Clin. Canc. Res. 20, 5964–5975.

Van Mellaert, L., Barbé, S., Anné, J., 2006. *Clostridium* spores as anti-tumour agents. Trends Microbiol. 14, 190–196.

Yamada, T., Fialho, A.M., Punj, V., Bratescu, L., Gupta, T.K., Chakrabarty, A.M., 2005. Internalization of bacterial redox protein azurin in mammalian cells: entry domain and specificity. Cell. Microbiol. 7, 1418–1431.

Yamada, T., Hiraoka, Y., Ikehata, M., Kimbara, K., Avner, B.S., Das Gupta, T.K., 2004. Apoptosis or growth arrest: modulation of tumor suppressor p53's specificity by bacterial redox protein azurin. PNAS USA 101, 4770–4775.

Yang, D.S., Miao, X.D., Ye, Z.M., Feng, J., Xu, R.Z., Huang, X., Ge, F.F., 2005. Bacterial redox protein azurin induce apoptosis in human osteosarcoma U2OS cells. Pharmacol. Res. 52, 413–421.

Youlden, D.R., Cramb, S.M., Yip, C.H., Baade, P.D., 2014. Incidence and mortality of female breast cancer in the Asia-Pacific region. Canc. Biol. Med. 11, 101–115.