

Original Article



The effects of pBudCE4.1-azurin-MAM-A recombinant vector on IL-2, IL-6, IL-7, and IL-10 expressions in laboratory mice

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Abstract

Background and aims: Breast cancer is one of the most common types of malignancy in women with morbidity and mortality (15.0%) in the world. The antitumor activity of azurin protein produced by *Pseudomonas aeruginosa* has been described before. Mammaglobin-A (MAM-A) protein is especially expressed in 40%-80% of breast cancer types and this protein is a very specific molecular marker for stimulating the immune system. Accordingly, this study investigated the effects of pBudCE4.1-azurin-MAM-A recombinant vector on the induction of the immune system in laboratory mice by the real-time polymerase chain reaction (PCR) method.

Methods: The pBudCE4.1-azurin-MAM-A recombinant and empty vectors were purchased and then separately transformed into *Escherichia coli* for multiplying. Next, each plasmid was extracted and the accuracy of transformation was confirmed by the PCR. These recombinant and empty (control) vectors were separately infused into the thigh muscle of the animals and the healthy group was infused with phosphate-buffered saline. The infusion sites, blood specimens, as well as the serum of the animals were collected and examined by serological and molecular tests.

Results: Molecular and serological studies showed that the serum and expression levels of IL-2, IL-6, IL-7, and IL-10 in infused mice with pBudCE4.1-azurin-MAM-A recombinant vector significantly increased compared to healthy animals and injected mice with an empty vector ($P < 0.05$).

Conclusion: In general, the findings revealed that the pBudCE4.1-azurin-MAM-A recombinant vector can stimulate the immune system of the mouse by an increase in the expression levels of IL-2, IL-6, IL-7, and IL-10. Thus, it would be better to examine the effects of this recombinant vector as a DNA vaccine on the prevention and treatment of breast cancer.

Keywords: Azurin, MAM-A, Recombinant vector, Breast cancer

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Introduction

Breast cancer is the fifth leading cause of death with an average of 522 000 cases per year worldwide. In addition, this type of cancer includes 2% of all cancers and is the second most common type in developed countries after lung cancer (1,2). One out of every 8 to 10 women have breast cancer during their lifetime (3,4). According to the limitations of current therapies such as surgery, radiation therapy, chemotherapy, hormone therapy, and biological therapy, it is very important to find a definitive and effective treatment based on molecular methods and DNA vaccines (5).

Many bacteria produce toxins, extracellular enzymes, and pigments. Further, toxins and bacterial enzymes play an important role in their pathogenesis (6,7). Although

bacterial toxins inhibit protein synthesis, DNA replication, and cell wall synthesis in response to adverse conditions, the evidence is available regarding their useful applications (8). For example, *Clostridium* and *Bacillus* toxins have many applications in medicine, agriculture, and industry (9,10).

Bacterial agents, including enzymes, secondary metabolites, derived proteins or peptides, and their toxins have an anticancer role in cancer cells (11). For instance, some bacterial toxins can destroy tumor cells and can alter and control cell cycle processes such as cell proliferation, apoptosis, and differentiation at low concentrations (12). The most common bacterial toxins used for the production of immunotoxins are the diphtheria toxin of *Corynebacterium diphtheriae* and exotoxin A and azurin

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bacteriocin of *Pseudomonas aeruginosa*. These bacterial toxins cause a decrease in cell growth or cycle and can naturally induce apoptosis and cell death. As a supplement, therefore, such toxins can be used to improve the therapeutic effects of anticancer drugs (13). Among the important bacteria, *P. aeruginosa* is of great significance because its toxins and enzymes have therapeutic aspects. It is a Gram-negative, facultative anaerobic, rod-shaped, motile, and non-sporadic bacterium. Furthermore, *P. aeruginosa* is an opportunistic human pathogen and is particularly involved in biofilm formation at the surfaces (14). Moreover, azurin bacteriocin is a small water-soluble protein and one of the bacterial agents with anti-cancer properties that is secreted by *P. aeruginosa* (15,16). This bacteriocin has cytostatic properties, can specifically penetrate into human cancer cells, and induces apoptosis but has no apparent activity in normal cells (17,18).

Mammaglobin-A (MAM-A) protein is one of the antigens that is present on the surface of most breast cancer cells and is expressed in more than 80% of breast cancer patients. This 10.5 kDa secreted glycoprotein contains 93 amino acids and is encoded by the *SCGB2A2* gene (19,20). Additionally, MAM-A protein is a very specific molecular marker in breast cancer and a suitable target for the immune-based therapy of patients with disseminated breast cancer (21).

The application of DNA vaccines (a type of subunit vaccine) is a novel tool for immunization against bacterial, viral, parasitic, and fungal infections and cancers (5,22). The major advantages of DNA vaccines are cheapness, safety, as well as the ease of development and production, heat resistance, and the long-term safety of their use. Similarly, these vaccines are subunit and free of the risk of infection transmission and induce humoral and cellular responses (23).

The finding of DNA vaccines with anticancer property is important because these vaccines have a high potential for inducing antitumor immunity without any adverse side effects. The role of some bacterial toxins in controlling cell growth and proliferation is also remarkable. Accordingly, the present study was performed by considering the anticancer property of the azurin protein of *P. aeruginosa* and MAM-A antigen as a candidate for the development of the breast cancer vaccine. In this study, the effects of pBudCE4.1-azurin-MAM-A recombinant vector on IL-2, IL-6, IL-7, and IL-10 expressions were evaluated in laboratory mice.

Materials and Methods

Preparation of Plasmids

In this research, the recombinant pBudCE4.1-azurin-MAM-A and empty pBudCE4.1 plasmids were purchased from Generay Biotech Company (Ltd., Shanghai, China). This recombinant vector contains two different multiple

cloning sites for the insertion of azurin and MAM-A genes separately (Figure 1). The lengths of the applied azurin and MAM-A genes in the recombinant plasmid were 1287 and 1309 bp, respectively.

Bacterial transformation

The lyophilized stock of *E. coli* NovaBlue strain (Novagen Company) was prepared from the Cellular and Molecular Research Center of Shahrekord University of Medical Sciences. This bacterium was overnight-cultured at 37°C by shaking at 200 rpm. The competent cells were prepared using CaCl₂ and heat shock (42°C for 90 seconds). Then, these cells were used for the separate transformation of pBudCE4.1-azurin-MAM-A and pBudCE4.1 vectors. Next, the transformed colonies were screened on LB agar medium containing 25 µg/mL of zeocin antibiotic. The accuracy of transformation was checked by colony-polymerase chain reaction (PCR).

Plasmid Purification

The bacterial colonies containing recombinant pBudCE4.1-azurin-MAM-A and pBudCE4.1 vectors were separately cultured in 5 and 250 mL LB media (containing zeocin antibiotic) at 37°C for 18 hours in an incubator. The plasmid extraction was performed using Miniprep and Maxiprep

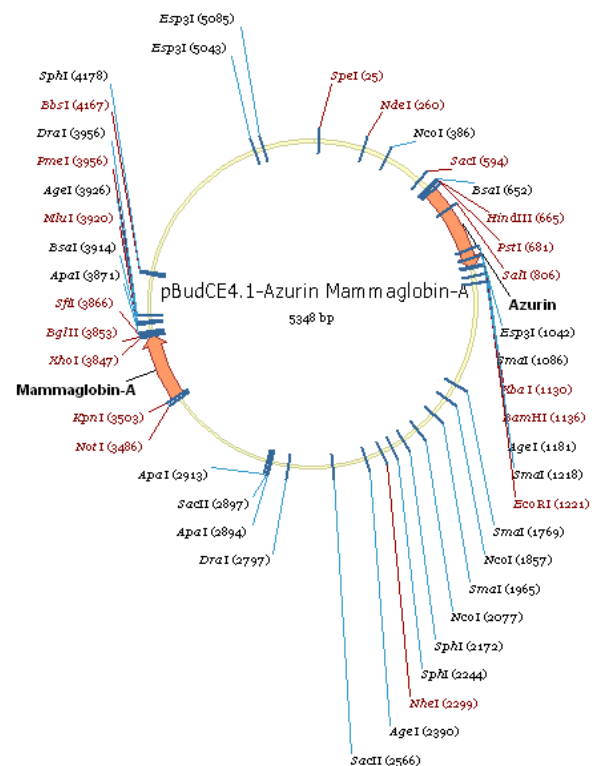


Figure 1. The schematic image of pBudCE4.1-azurin-MAM-A recombinant vector .

YTA plasmid extraction kits (Azma Equipments Company, Tehran, Iran). In addition, the purity and concentration of the extracted plasmid were measured using a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop 2000, Wilmington, DE, USA) in a ratio of absorbance at 260 nm and 280 nm. The PCR reaction was also performed to confirm the accuracy of the extracted plasmid and detect the presence of the target genes.

Amplification of target genes

The specific primers for the azurin and MAM-A (target genes) of the recombinant vector were designed using Gene Runner software, version 3.05, and the sequences of primers and their identity were analyzed in the BLAST system of the National Center for Biotechnology (Table 1). The PCR reaction was performed in 0.2 mL microtubes at a final volume of 25 µL. Each reaction contained the selected colonies or extracted plasmids (50 ng), the 1X PCR buffer, 1 µM of each forward and reverse primers, 0.2 mm dNTP mix, 2 mm MgCl₂, and 1 unit of *Taq* DNA polymerase (all manufactured by CinnaGen Company, Tehran, Iran). The negative control consisted of all the above-mentioned substances except for the transformed colony or the template DNA plasmid. Then, the microtubes were inserted into the thermal cycler (Gene Atlas G, ASTEC Company, Seoul, Korea) to perform the temperature steps of gene replication. Further, PCR reaction temperatures included several cycles as one step of denaturation at 94°C for 5 minutes, 35 repetitive cycles of 1 minute of denaturation at 94°C, annealing at 65°C (for *azurin* gene) and 66°C (for *MAM-A* gene) for 50 seconds, as well as the extension step at 72°C for 50 seconds and the final extension stage at 72°C for 10 minutes. The amplified products were electrophoresed on 1% agarose gel at 110 V for 45 minutes in the running buffer (TBE 1%). After

an appropriate ethidium bromide (2 µg/mL) staining, the gel was visualized under the UVIDoc gel documentation system (Uvitec, UK).

Animals Grouping and Infusions Program

Twenty-four female BALB/c mice with approximately 18 g weight were divided into 3 groups (A to C) according to Table 2. Groups A, B, and C included infused mice with the pBudCE4.1-azurin-MAM-A recombinant vector, empty pBudCE4.1 plasmid, and PBS recipient (healthy group), respectively. The treatment times were 15 days and the period time for inducing the immune system by the recombinant vector was set for 15 days after the last injection.

Sampling

At the end of the treatments, the mice of all groups were killed by chloroform via ethical conditions (without disturbing the animal). The tissue of the infusion site and blood specimens of each animal were collected, followed by taking the whole blood samples of each mouse directly by the aspiration from the heart of the animal. Then, the samples were individually collected in EDTA tubes and stored at -70°C for RNA extraction and molecular testing. The residues of half of the blood samples (blood clotted) were collected in a standard 1.5 mL microtube and then were centrifugated and stored at -20°C for serological tests (ELISA). The tissue of the injection site (thigh muscle) of each animal was isolated separately with a sterile scalpel and razor blades and, after RNA extraction and cDNA synthesis, was used for investigating the expression of the target genes (i.e., *azurin* and *MAM-A*) by the real-time (RT)-PCR.

Table 1. Details of Primers and Their Sequences

Gene	Primers Name	Sequences	Annealing Temperature (°C)	Product Length (bp)	Accession Number
<i>azurin</i>	Azu-F	5'-ATGCTACGTAACCTCGCTGCC-3'	65	292	M30389
	Azu-R	5'-TGTCGTCGGGCTTCAGGTAATC-3'			
<i>MAM-A</i>	Mam-F	5'-CAGCGGCTTCCTTGATCCTTG-3'	66	221	NM_002411
	Mam-R	5'-TGGCATTGTCGTCTATGAACCTTG-3'			
<i>IL-2</i>	IL-2-F	5'-TGCCTAGAAGATGAACCTGGACCTC-3'	65	229	BC116845
	IL-2-R	5'-GGCTTGTTGAGATGATGCTTTGAC-3'			
<i>IL-6</i>	IL-6-F	5'-AGAGTCCTTCAGAGAGATACAG-3'	64	246	M24221
	IL-6-R	5'-ACAACTGATATGCTTAGGC-3'			
<i>IL-7</i>	IL-7-F	5'-AGGACATGTAGTAAACAACCTCC-3'	64	220	NM_008371
	IL-7-R	5'-ATGATTGCATTACAAAAATCTG-3'			
<i>IL-10</i>	IL-10-F	5'-CTGGACAACATACTGCTAACCGACTC-3'	66	250	NM_010548
	IL-10-R	5'-TCTTCACCTGCTCCACTGCCTTG-3'			
<i>GAPDH</i>	GAPDH-F	5'-TCCCGTAGACAAAATGGTGAAGG-3'	65	261	XM_017321385
	GAPDH-R	5'-ATGTTAGTGGGGTCTCGCTCCTG-3'			

Table 2. Classification of animals and their infusion and treatment schedules

Treatment Program	Number of Mice	Group
Recombinant vector* infusion (3 times, days 0, 7, and 15) and 15 days after the last injection sampling for serological and molecular studies	8	A
Infused with empty pBudCE4.1 vector (without target genes) for 3 times (days 0, 7, and 15) and 15 days after the last injection sampling for serological and molecular tests	8	B
Infused with PBS for 3 times (days 0, 7, and 15) and 15 days after the last infusion sampling for serological and molecular tests	8	C

Note. *Recombinant vector: pBudCE4.1-azurin-MAM-A.

Expression of genes in tissue and blood samples

RNA extraction was performed on blood and tissue samples from the injection site in all treated mice using the RNX plus solution (CinnaGen Company, Tehran, Iran) according to the manufacturer's instructions. Then, 1 µg of the extracted RNA was synthesized for cDNA according to the standard protocol of the cDNA synthesis kit (Yekta Tajhiz Azma, Tehran, Iran). Furthermore, the expression of the *azurin* and *MAM-A* target genes of the recombinant vector in the tissue of the injection site was performed by the RT-PCR method according to the above-mentioned procedure. The amplified products were analyzed on 1% agarose gel electrophoresis.

Moreover, the expression levels of important cytokines and immunoglobulins in breast cancer, including IL-2, IL-6, IL-7, and IL-10 in blood samples from the animals, were compared with the normal group by qRT PCR. The details of each designed primer are shown in Table 1.

The RT PCR was performed on a Rotor-Gene 6000 machine (Corbett, Australia). Additionally, each cDNA sample was diluted 1 to 5 and the q-PCR reaction mixture in a 0.2 mL microtube was prepared in triplicate at a final volume of 13 µL (on ice) for each reaction. Each reaction consisted of 50 ng of the cDNA sample (1 µL) added to 6.5 µL of YTA 1X SYBR Green qPCR Mix (Yekta Tajhiz Azma, Tehran, Iran), 0.5 µL of each forward and reverse primers (2 µM), and 4.5 µL distilled water (dH₂O). In addition, the non-template control was inserted to evaluate the reaction contamination or primers for each step of RT PCR. The temperature conditions of RT PCR reaction consisted of a single denaturation step at 95°C for 3 minutes, 40 cycles of denaturation at 94°C for 30 seconds, primer annealing at 65°C for 3 minutes, and elongation at 72°C for 30 seconds. Finally, the melting curve analysis was performed at the end of the reaction. The relative expressions were monitored by Rotor-Gene RT analysis software, version 6.0 (Qiagen, Inc., Valencia, CA, USA) using the comparative C_ts (2^{-ΔΔC_t}) (Livak method).

ELISA test

To this end, 40 µL of the serum samples of all mice was subjected to determine the serum levels of IL-2, IL-6, IL-

7, and IL-10 cytokines using separately enzyme-linked immunosorbent assay (ELISA) kits (Hangzhou, Chin Hangzhou Eastbiopharm Company, Ltd., Hangzhou, China) according to the described method by the company. The ELISA sandwich method was used for all tests and the samples were finally measured using the Stat Fax-2100 ELISA plate reader (Awareness Technology, Palm City, FL) under 450 nm wavelength.

Statistical analysis

All experiments were repeated at least three times and data were entered and analyzed using the Statistical Package for the Social Sciences software (SPSS, Inc., Chicago, IL, USA), version 20. Further, the mean differences between the groups were calculated by an independent t-test or the analysis of variance (ANOVA) statistical methods. All graphs were prepared using GraphPad Prism, version 7 (GraphPad Software, San Diego, CA, USA). In this study, a *P* value of less than 0.05 was considered statistically significant.

Results

Verification of Bacterial Transformations

The successful transformation of the pBudCE4.1-azurin-MAM-A recombinant vector into the bacterial host was confirmed by PCR. Furthermore, the amplified fragments with lengths of 292 and 221 bp were observed on an agarose gel for *azurin* and *MAM-A* genes, respectively (Figure 2).

Expression of target genes

The expression of the target genes of the pBudCE4.1-azurin-MAM-A recombinant vector in the tissue of the injection site on the agarose gel showed the DNA bands with 292 and 221 bp lengths for *azurin* and *MAM-A* genes, respectively (Figure 3). These results indicated the successful expression of the recombinant vector in the infusion site of the animal.

Serological analysis

The evaluation of the serum levels of IL-2, IL-6, IL-7, and IL-10 by the ELISA test (Figure 4) showed that these cytokines in injected mice with the pBudCE4.1-

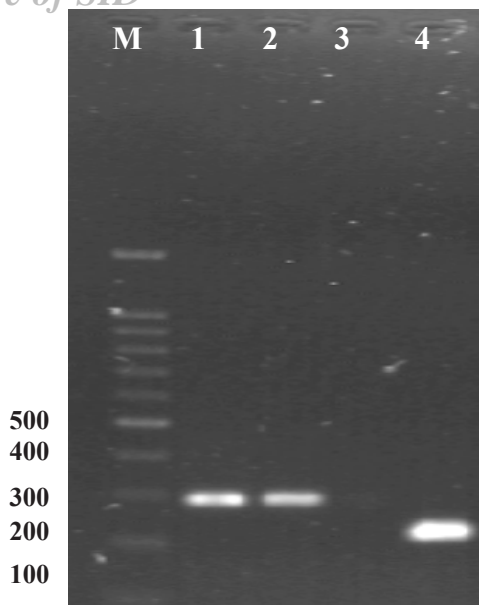


Figure 2. The amplified products of the *azurin* and *MAM-A* genes of Recombinant Plasmid by PCR on 1% Agarose Gel Electrophoresis
 Note. M: 100 bp DNA marker (Yekta Tajhiz Azma, Tehran, Iran); Lines 1 and 2: The amplified fragments for the *azurin* gene; Line 3: Negative control (no DNA); Line 4: The *MAM-A* gene band.

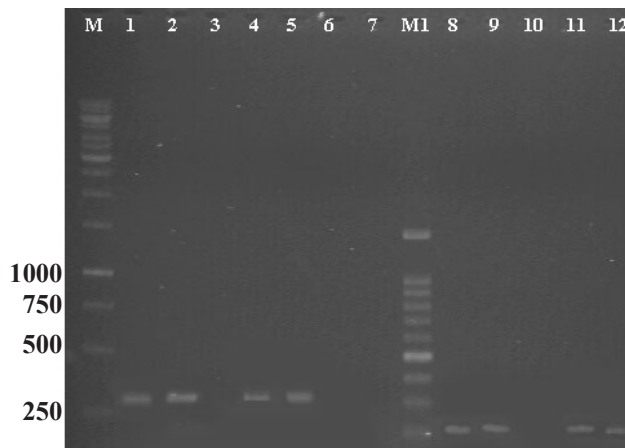


Figure 3. The expression of *azurin* and *MAM-A* genes in the tissue of the infusion site of BALB/c mice by the recombinant vector.
 Note. M and M1: 1 kb and 100 bp DNA markers, respectively (Thermo Fisher Scientific); Lanes 1, 2, 4, and 5: The amplified *azurin* gene in the tissue of infused mice with the recombinant vector; Lane 3: Injected mice with the pBudCE4.1 empty vector (control group); Lane 6: Injected mice with PBS; Lane 7: Negative control (without template DNA); Lanes 8, 9, 11, and 12: The amplified fragments of the *MAM-A* gene in the injection site of infused mice by the recombinant vector; Lane 10: Injected mouse by the pBudCE4.1 empty vector; Lane 13: Injected with PBS; Lane 14: Negation control (without DNA).

azurin-MAM-A recombinant vector (group A) increased significantly ($P < 0.05$) compared to injected mice with the pBudCE4.1 empty vector (group B, without target genes) and PBS recipient (group C, control).

Quantitation of cytokine gene expression by RT-PCR

As displayed in Figure 5, the expression level of IL-2, IL-6, IL-7, and IL-10 cytokines in infused animals with a recombinant pBudCE4.1-azurin-MAM-A plasmid (group A) increased significantly ($P < 0.05$) compared to injected mice with the empty vector (group B, without target gene delivery) and PBS (group C as a control) .

Discussion

Nowadays, due to the lack of effective therapy against breast cancer and the disadvantages of existing therapies, it is important to find new strategies for prevention, therapy, and induction of the immune system in the body against cancer cells. Due to the anti-cancer effect of the azurin protein of *P. aeruginosa* and the stimulation of the immune system by MAM-A protein, the present study investigated the immunogenicity of pBudCE4.1-azurin-MAM-A recombinant vector (encoded these two genes) in BALB/c mice.

According to the similarity of the human *MAM-A* gene sequence to the mouse *MAM-A* antigen and due to the application of this vector as a DNA vaccine in humans in the future, this gene was used in the recombinant vector to induce the immune system in an animal model.

The successful expression of azurin and MAM-A genes in the tissue of the injection site of BALB/c mice was observed by RT-PCR reaction. Additionally, the ELISA assay showed that IL-2, IL-6, IL-7, and IL-10 serum levels in recipient mice with the recombinant pBudCE4.1-azurin-MAM-A vector (group A) increased significantly ($P < 0.05$) compared to infused mice with empty vector (group B) and PBS (group C). Moreover, the RT-PCR findings demonstrated that the pBudCE4.1-azurin-MAM-A recombinant vector (group A) stimulates the immune system by increasing the expression of IL-2, IL-6, IL-7, and IL-10 cytokine genes. However, this increase in the expression of these cytokines and immune system incitement was not observed in B and C groups and control mice. The significant enhancement of the expression of these cytokines in recombinant vector recipient mice by molecular methods confirmed the findings of the ELISA test on the stimulation of the immune system of the animals.

It should be noted that IL-2 is an immunogenic cytokine in breast cancer. This cytokine plays an important role in the activation of natural killer cells (NK) and monocytes and it is a major growth factor for B and T lymphocytes (24). In addition, by inducing acute-phase proteins, IL-6 induces tissue invasion at the tumor site, as well as Th17

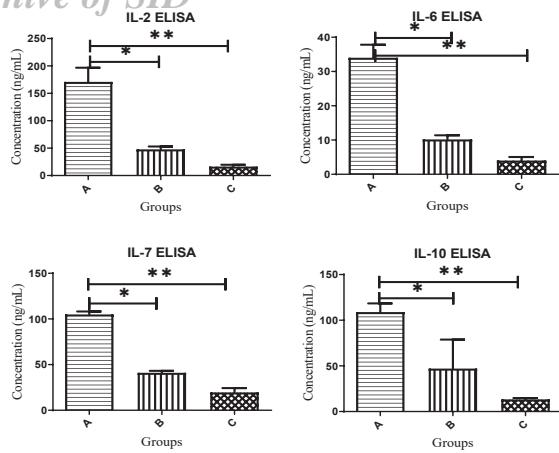


Figure 4. Comparison of the serum levels of IL-2, IL-6, IL-7, and IL-10 cytokines in the mice of each group by the ELISA test.

Note. A: The injected mice with the pBudCE4.1-azurin-MAM-A recombinant vector; B: The injected mice with empty pBudCE4.1 vector (without target genes); C: The injected mice with PBS

cells, and thus affects B cells (25). Further, IL-7 induces the immune response type 1 increases CD⁸⁺ cytotoxicity, and stimulates NK cells and lymphokine-activated killer (LAK) cells (26,27). Similarly, IL-10 promotes angiogenesis and tissue invasion, while it inhibits the growth mechanism and metastasis of breast cancer cells (28,29). Thus, the expression enhancement of these cytokines in recombinant vector recipient mice, which was observed in this study, confirms the correct function of this recombinant gene construct by the delivery of azurin and MAM-A genes in immune system stimulation.

So far, no study has evaluated the synergistic effects of the azurin gene of *P. aeruginosa* with the human MAM-A gene as a recombinant gene structure in the stimulation of the immune system in laboratory animals. The findings of the study by Yamada et al showed that azurin protein in nude mice that received human cancer cells leads to the regression of human UISO-Mel-2 tumors. Accordingly, they suggested that it can be potentially used for cancer therapy and the stimulation of the immune system (30). However, in our study, the simultaneous effects of bacterial azurin and human MAM-A as a gene structure on immune system stimulation were examined and the appropriate stimulation of cytokines was observed using molecular and serological methods.

In their clinical trial study, Tiriveedhi et al investigated the immune and biological effects of the MAM-A DNA vaccine against breast cancer. After vaccination, their findings represented a significant increase in the frequency of CD⁸⁺ T cells specific for MAM-A by using flow cytometry. There was also a significant increase in the number of MAM-A-specific IFN γ -secreting T cells. They further indicated that the MAM-A DNA vaccine was safe

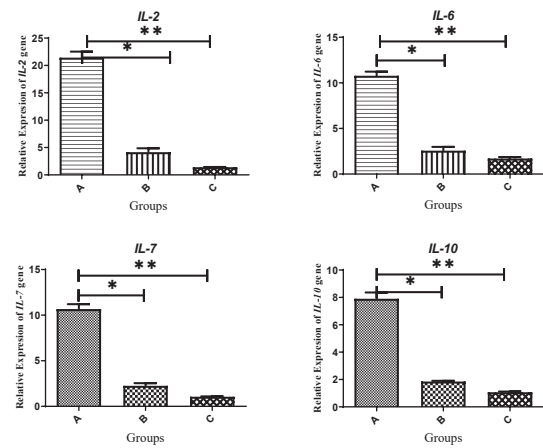


Figure 5. Comparison of cytokine gene expression levels in the BALB/c mice of each group.

Note. A: The recipient animals with pBudCE4.1-azurin-MAM-A recombinant vector; B: The infused mice with empty pBudCE4.1 vector (without target genes); C: The PBS-infused mice.

and capable of stimulating and responding to the immune system, but also suggested that further studies must be done to evaluate the potential of the DNA MAM-A vaccine in the prevention or therapy of breast cancer (31). However, the present study, to the best of our knowledge, was the first one to use MAM-A and azurin genes as gene constructs and to determine the efficacy of pBudCE4.1-azurin-MAM-A recombinant plasmid by serological (ELISA) and molecular (real-time PCR) tests. The results of our study revealed that the serum levels and mRNA expression levels of IL-2, IL-6, IL-7, and IL-10 cytokines increased significantly in injected mice with the recombinant vector (group A) compared to lack of the target genes in the group B (injected mice with empty vector) and injected with PBS (group C).

In another study, the potential effects of azurin on non-small cell lung cancer were examined and the anticancer effects of this bacterial protein were demonstrated in several cancer cell lines, especially breast cancer (32,33). The present study investigated the effects of the pBudCE4.1-azurin-MAM-A recombinant vector via the expression of the azurin bacteriocin of *P. aeruginosa* and human MAM-A on the stimulation of the immune system of BALB/c mice by molecular and serological methods. The stimulation of the mouse immune system was observed by inducing the increased expression of cytokines.

In conclusion, the findings of this study showed that this recombinant vector via expressing azurin and MAM-A genes in BALB/c mice has a potential role in stimulating the immune system of the animal by increasing the serum levels and the expression of cytokine genes. Thus, other studies are suggested to evaluate the effects of this gene construct on other experimental animals and cancer cell lines. Finally, further research on this recombinant gene

construct may promise a new strategy for the prevention and treatment of breast cancer in the near future.

Conflict of interests

The authors declare there is no conflict of interests in this study.

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Ethical Statement

The ethical approval for the use of laboratory animals was obtained from the Research Ethics Committees of the Deputy of Research and Technology of Islamic Azad University of Shahrekord Branch, Shahrekord, Iran on October 10, 2017 (ethics code: IR.IAU.SHK.REC.1397.049).

Authors Contribution

All authors contributed in all experiments. PG and AD performed all molecular and serological methods. MJ and PG infused all mice and analyzed the data. PG and AD wrote the manuscript.

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