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Research Article

The effect of *Clostridium perfringens* enterotoxin (Cpe) gene expression on apoptosis in the DU145 prostate cancer cell

Golnoosh Rezaeizadeh[1], Saeid Abedi [1]*

1. Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran

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ABSTRACT

Prostate cancer is one of the most common kinds of malignancy in men, with a significant morbidity and mortality rate. The aim of this research was to investigate the effects of Cpe on the expression of *bak*, *bax*, *fas*, *bcl2*, *cyclin D1*, and *cyclin E* genes on DU145 prostate cancer cell lines. In the present study, the pBudCE4.1-*cpe* recombinant vector and empty plasmid were individually transformed into the DU145 cell line using the Lipofectamine 2000 protocol. The presence of each vector was checked by PCR. The *cpe* gene expression in transfected DU145 was assessed. Expression of apoptotic genes (*fas*, *bcl2*, *bak*, and *bax*) and cell cycle progression genes (*cyclin E* and *cyclin D1*) was maintained in transfected and untransfected DU145 cells. Statistical analyses revealed that the expression of *bak*, *bax*, and *fas* were considerably higher in cells transfected with a recombinant vector ($P < 0.05$). *bcl2* and *cyclin E* and *cyclin D1* genes expression decreased significantly in vector transfected DU145 cells in compare with the cells transfected by an empty plasmid or untransfected ones. Cpe expression could suppress DU145 growth by affecting cell apoptosis. The expression of *cpe* in the DU145 cell line was tested for the first time and confirms its probable effect on similar cells. According to the findings of this study, *cpe* gene in a recombinant vector might be a candidate vaccine for the treatment of prostate cancer.

1. Introduction

Prostate cancer is a widespread and potentially fatal tumor that has a significant impact on males of various ages and geographical areas globally (Ferlay et al., 2021). The vast amount of documentation verifies the imbalanced impact of prostate cancer on individuals who are 65 years old and above. Medical science has made significant progress in developing therapeutic approaches for this cancer type, including hormone therapy, targeted drugs like enzalutamide, and more invasive treatments such as chemotherapy. Nonetheless, the goal of successfully eliminating the disease in its advanced stage remains elusive (Jewett et al., 2003; Alimirah et al., 2006).

Resistance to cancer therapy in patients with advanced malignancies necessitates the development of new medications. In 1890, for the first time, live bacteria were used by physicians to treat cancer patients (Nauts 1980). Since then, bacteria derived agents such as enzymes, secondary metabolites, proteins or peptides, and toxins have been among the most widely used bacterial products for cancer treatment (Liu et al., 2014). Bacterial toxins that can bind to tumor cell antigens are commonly

*Corresponding authors: Saeid Abedi
Email address: saied_abedi83@yahoo.com

used in cancer treatment. Investigating the effect of bacterial toxins on gene expression in cancer cells is one way to find out the effectiveness of a toxin in cancer treatment (Yaghoobi et al., 2015). In some studies, the identified bacterial toxin genes were inserted into vectors and transfected into cancerous cells in order to kill the cells (Sedighi et al., 2019) or control the cancer in them. These agents can inhibit protein synthesis, and regulate cellular processes which lead to cell growth control (Sharma et al., 2022).

Several studies have demonstrated that *Clostridium perfringens*, an anaerobic Gram-positive bacterium, possesses a toxin that exhibits significant anticancer properties. *C. perfringens* enterotoxin (CPE), which is mainly known for causing foodborne diseases, is produced by approximately 5% of *C. perfringens* strains. The present study demonstrates that *Clostridium perfringens* isolates possess notable anticancer properties (McCann et al., 2015; Riedel et al., 2019). About 70% of *C. perfringens* type A strains that cause food poisoning have a *cpe* gene on the chromosome flanked by the insertion sequences (ISs), including IS1469 and IS1470, while the remaining 30% of food and non-food poisoning type A strains have *cpe* on the plasmid family, pCPF5603 or pCPF4969 (Rood et al., 2018). A IS1469 sequence is present 1.3 kb upstream of the *cpe* gene, and two IS1470 sequences-one of which is present 3 kb upstream of the *cpe* gene and the other positioned 1.2 kb downstream of the *cpe* gene are present and proximally connected with this chromosomal *cpe* gene. Although it hasn't been established, it has been suggested that the two IS1470 sequences that border the chromosomal *cpe* gene belong to a transposon. In the type A chromosomal *cpe* strain NCTC8239, PCR has discovered circular forms harboring the *cpe* gene that may be transposition intermediates, supporting that theory (Brynestad and Granum 1999). Chromosomal *cpe* containing type A strains differ from other *C. perfringens* strains phylogenetically and also carry this putative transposon. The majority of chromosomal *cpe* type A strains produce a variant small acid soluble protein that binds tightly to spore DNA, which gives these chromosomal *cpe* strain spores much stronger resistance against food environment stresses, like heating, than exhibited by spores of other *C. perfringens* strains. This helps to explain their strong association with food poisoning (Li and McClane 2006). The receptors for Cpe are mostly claudin-3 and claudin-4, which are found in a lot of cancerous tissues, like the ovary, pancreas, breast, and prostate. Claudin-3 and claudin-4 are expressed in primary human prostate cancer tissues (Maeda et al., 2012). Claudin-4 is found on the cell membranes of all of the cancer cell lines, but it is concentrated at tight junctions in prostate epithelial cells. Cpe binds to these receptors and activates the calpain protein to induce apoptosis and cell death (Landers et al., 2008; Oliveira et al., 2007). Studies with chimeric claudins revealed that a claudin's ability to bind Cpe depends on the ECL-2 region. A pentapeptide sequence inside the ECL-2 region was found by peptide mapping experiments to be crucial for Cpe binding. An Asn residue in this pentapeptide is a crucial yes/no determinant for Cpe binding, according to site-directed mutagenesis, with nearby residues regulating the affinity of Cpe binding. (Note that some claudin receptors, such as claudin 4, have a high affinity for Cpe while other receptors, like claudin 8, have a lower affinity (Robertson et al., 2010; Hrestha et al., 2013). DU145 cell line is one of the cancerous cell lines that had been extracted from a central nervous system metastasis originating from primary prostate adenocarcinoma during a parietal-occipital craniotomy. These cells are hormone-insensitive and do not express prostate-specific antigen (PSA) (Deb et al., 2021). In this study, we designed a recombinant vector by inserting the *cpe* gene into the pBudCE4.1 eukaryotic expression plasmid. The expression of targeted genes in the DU145 cell line after successful transfection with this vector was shown. Then, the effects of *cpe* gene expression on the host cell-cycle and apoptotic genes were investigated.

2. Materials and Methods

2.1. Recombinant plasmid construction

The recombinant vector (with the 972 bp *cpe* gene designed to inserted at *XbaI/BamHI* sites of pBudCE4.1 plasmid genome) and an empty pBudCE4.1 plasmid was ordered to Generay Biotechnology (China). The plasmid includes zeocin resistance gene, which could be used in *E. coli* and mammalian transfected cell

selection. The company confirmed presence of the *cpe* gene by DNA sequencing and *BamH1/XbaI* enzymatic digestion (fig 1).

2.2. Vector extension & Confirmation

The NovaBlue K12 strain of *E. coli*, which was chemically competent, was employed to amplify the recombinant plasmids and vector using the calcium chloride heat shock method. The efficacy of the extracted vectors was evaluated using NanoDrop analysis, with readings taken at the wavelengths of 260 and 280 nm, as described by Russell and Sambrook (2001). The extraction of plasmids was carried out by using a commercially available kit manufactured by Yekta Tajhiz Azma (YTA) in Iran. Confirmation of the recombinant plasmids was performed by using Polymerase Chain Reaction (PCR) in a collective volume of 25 µl, wherein 50 ng of the recombinant vector was incorporated.

2.3. MTT estimate

In order to measure the metabolic activity of cells, the MTT assay, a well-known colorimetric technique, was employed in this study (Twentyman and Luscombe, 1987; Rahimzadeh Torabi et al., 2021). DU145 cells, both transfected and non-transfected, were inoculated in 96-well culture plates at a concentration of 5,000 cells per well. During the experimental period, different concentrations of zeocin (0, 35, 70, 140, 280, 560, and 1120 µg/mL) were added to each well for assessment. The viability of the cells was evaluated using the MTT assay at three specific time points after the incubation period: 24, 48, and 72 hours. The quantification of absorbance was performed within the wavelength range of 490 nm to 620 nm, utilizing a Stat Fax 2100 ELISA microplate reader.

2.4. Cell line transfection

The DU145 cell line prostate cancer was bought from Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in RPMI 1640 media (Bioldea, Tehran, Iran) with 10% fetal bovine serum (Saba company Shahrekord, Iran) and 100 units of Penicillin G/Streptomycin antibiotics per 1 mL in a tissue culture flask and incubated at 37 ºC with 5% CO_2 (Freshney 1994). DU 145 cells were transfected in a 6-well plate by 2.5 mg/well plasmid, total volume of 300 mL RPMI 1640 (free of antibiotic and FBS), and 7.5 mg/well in accordance with the manufacturer's protocol (Invitrogen, USA). The plate was kept at 37 ºC with 5% CO_2 for 4 hours. Then, FBS was added to the wells, and the plates were kept there for another 24 hours. The experiment was then continued for 72 hours after adding 280 mg/mL of zeocin (Merk company, Germany) to each well to select the transfected cells from the non-transfected cells. Eventually, the cells were separated from the bottom of each well using 0.25% Trypsin-EDTA enzyme (GIBCO). By this process three groups of cells were isolated: normal DU145cells, transformed DU145 with pBudCE4.1-*cpe*, and transformed DU145 with empty pBudCE4.1 plasmid.

2.5. Complementary DNA synthesis

The total RNA was collected out from both transfected and non-transfected DU145 cells in accordance with the extraction kit guidelines provided by the manufacturer (RNX-plus kit, Sinaclone, Tehran, Iran). The Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used for the generation of cDNA from 1000 ng of RNA per sample. Analysis through RT-PCR and q-PCR was conducted using the generated cDNA that was generated, with specific primers provided in Table 1.

2.6. RT-PCR and Q- PCR

In this research, the reverse transcriptase polymerase chain reaction (RT-PCR) technique was employed to ascertain the expression levels of the *cpe* gene within transfected DU145 cells. Quantitative PCR (Q-PCR) was also conducted to compare the gene expressions of fas, bcl2, bak, bax, cyclin D1, and cyclin E in both transfected and non-transfected DU145 cells. The real-time quantitative polymerase chain reaction (qPCR) analysis was performed using the Rotor-Gene 6000 instrument manufactured by Corbett Life Science, located in Concorde, NSW, Australia. This instrument was operated in conjunction with the SYBR® Premix Ex Taq™ II kit supplied by TaKaRa, a company based in Japan.

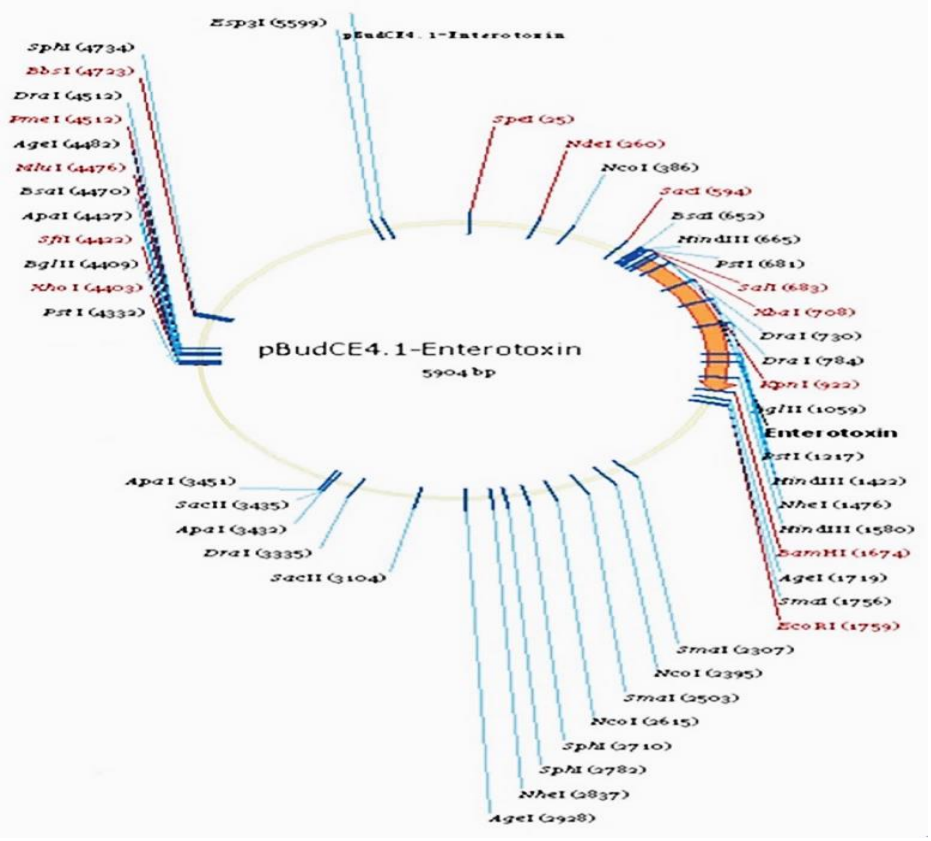

Figure 1. The constructed recombinant vector containing the 972 bp *cpe* gene.

The utilization of gene-specific primers is characterized by comprehensive and explicit specifications. The amplifications for each target gene were performed in duplicate, utilizing a reaction volume of 20 µL. The thermal cycling procedure began with an initial denaturation phase at 95 ℃ for a duration of 4 minutes. This was followed by a sequence of 40 cycles, wherein each cycle consisted of a 15-second denaturation step at 95°C, a 20-second annealing step at gene-specific temperatures, and a 20-second extension step at 72 °C. Following post-amplification, the verification of PCR product specificity was conducted through melting curve analysis. The gene coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as the internal reference gene for all numerical assessments. The relative gene expression was determined by applying the comparative ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001).

2.7. Statistical analysis

All experiments were performed in 3 replicates and collected data were analyzed by SPSS software version 25. In addition, the Kruskal–Wallis H test was conducted to investigate the differences. All experiments were performed in 3 replicates and collected data were analyzed by SPSS software version 25. In addition, the one-way ANOVA was conducted to investigate the differences.

3. Results

3.1. Vector verification

DNA sequencing and *BamH1/XbaI* enzymatic digestion were used (Dongjing, China) to confirm the accuracy of recombination (fig 2).

Table1. The primer sequence of genes involved in the q-PCR analysis

Accession number	Product length (bp)	Annealing temperature (˚C)	Sequence	Primer's name	Gene
MG45685	217	60	5′-GGAACCCTCAGTAGTTTCAAGTC-3′ 5′-ATTTGGACCAGCAGTTGTAGATAC-3′	F-Cpe R-Cpe	*cpe*
NM_002046	183	65	5′-GCCAAAAGGGTCATCATCTCTGC-3′ 5′-GGTCACGAGTCCTTCCACGATAC-3′	F-GAPDH R-GAPDH	*GAPDH*
KM114217	168	65	5′-CAATTCTGCCATAAGCCCTGTC-3′ 5′-GTCCTTCATCACACAATCTACATCTTC-3′	F-FAS R--FAS	*FAS*
NM_001188	234	65	5′-CGTTTTTTACCGCCATCAGCAG-3′ 5′-ATAGCGTCGGTTGATGTCGTCC-3′	F-BAK R-BAK	*BAK*
AH003247	237	64	5′-TGTTGGATCTCTGTGTCCTGG-3′ 5′-TCTATGTCGCACCACTGATACCC-3′	F-Cyclin E R-Cyclin E	*Cyclin E*
Abdian et al. (2015)	125	64	5′-AAGTTGCAAAGTCCTGGAGCC-3′ 5′-TCGGCTCTCGCTTCTGCTG-3′	F-CyclinD1 R-CyclinD1	*Cyclin D1*
NM_001291431	154	65	5′-AGGTCTTTTTCCGAGTGGCAGC-3′ 5′-GCGTCCCAAAGTAGGAGAGGAG-3′	F BAX R BAX	*Bax*
NM_000633.3	245	65	5′-GACGACTTCTCCCGCCGCTAC-3′ 5′- CGGTTCAGGTACTCAGTCATCCAC-3′	F BCL 2 R BCL 2	*BCL2*

3.2. DU145 cell transfection and MTT results

The DU145 cell line was transfected with either recombinant pBudCE4.1-cpe or empty pBudCE4.1 plasmid. Both cells were exposed to different doses of Zeocin. The MTT results revealed that almost all of the normal DU145 cells died after 72 hours at the concentration of 280 µg/mL (90%), whereas the most transfected DU145 cells stayed alive at the same concentration (Dead cells: 13%) ($p<0.05$) (figs 3,4).

3.3. RT-PCR results

After extraction of RNA from the DU145 cells transfected with pBudCE4.1-*cpe* or empty plasmid pBudCE4.1 and syntesising cDNA, the RT-PCR was done to evaluate the presense of targeted gene in them. A band 217 bp on gel agarose electrophoresis (1%) confirmed the succesful expresion of *cpe* gene in DU145 transfected with pBudCE4.1-*cpe* vector (Fig 5).

3.4 Evaluation of the expression of genes involved in the cell cycle and apoptosis

Q-PCR was used to measure the mRNA expression levels of *bak*, *bax*, *fas*, *bcl2*, *cyclin D1*, and *cyclin E* genes in DU145 cells transfected with pBudCE4.1-*cpe* or empty pBudCE4.1plasmid compared to normal cells (control). While there was no significant difference between gene expression levels in the cells transfected with empty pBudCE4.1plasmid and normal cells, data showed higher expression levels of *bak*, *bax*, and *fas* genes in DU145 transfected with pBudCE4.1-*cpe*. The expression of *bak*, *bax* and *fas* genes in pBudCE4.1-*cpe* transfected cells were 2.28, 18.11 and 10.17 times more than non-transfected cell respectively and the results was statistically significant ($p<0.05$). Meanwhile significant decreased level of *BCL2* gene expression were observed in the pBudCE4.1-*cpe* transfected cells compared to the other two groups ($p<0.05$). The statistical analysis illustrated that the *cyclin E* and *cyclin D1* gene expression was reduced in the DU145 transformed with the recombinant vector compared with the cells transformed with empty plasmid or normal DU145 cells ($p < 0.05$). (Fig 6 A and B).

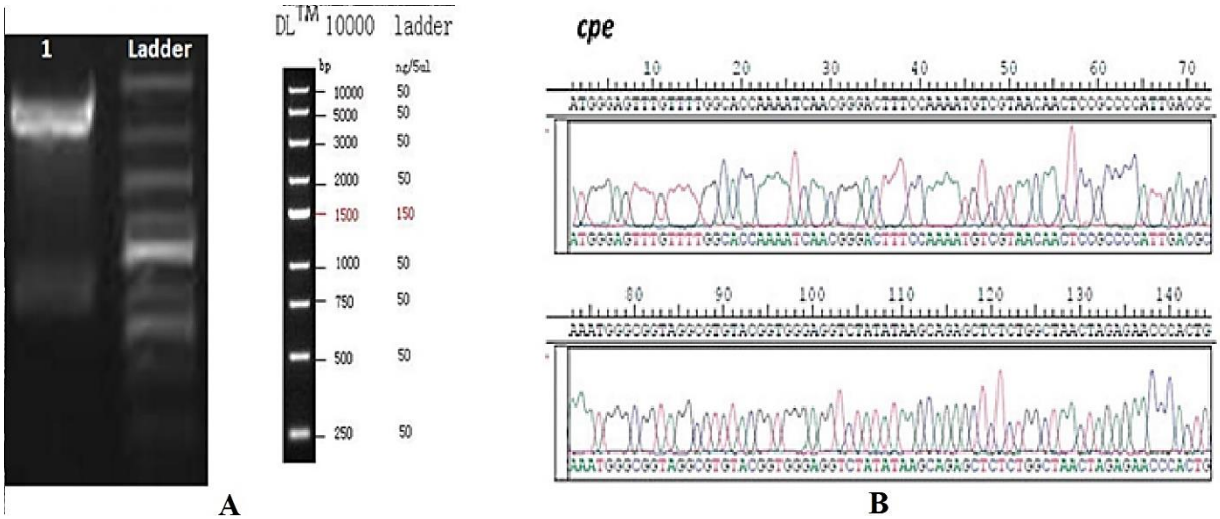

Figure 2. A) Recombinant vector pBudCE4.1-*cpe* digested by *BamHI/XbaI*. Line 1: 972 bp *cpe* gene band and the remaining part of the vector. B) DNA sequencing diagram of the *cpe* gene in the recombinant vector.

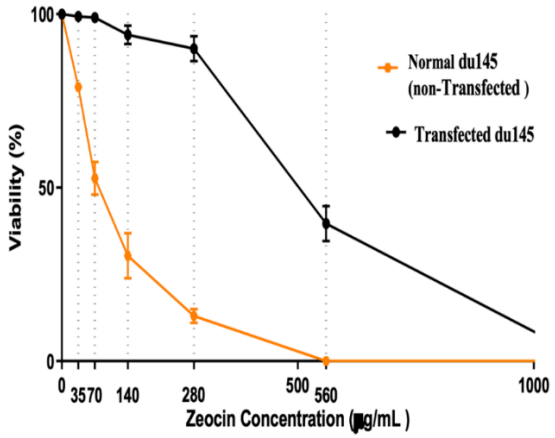

Figure 3. Evaluation of the viability of transfected and non-transfected DU145 cells in presence of different Zeocin concentrations by MTT.

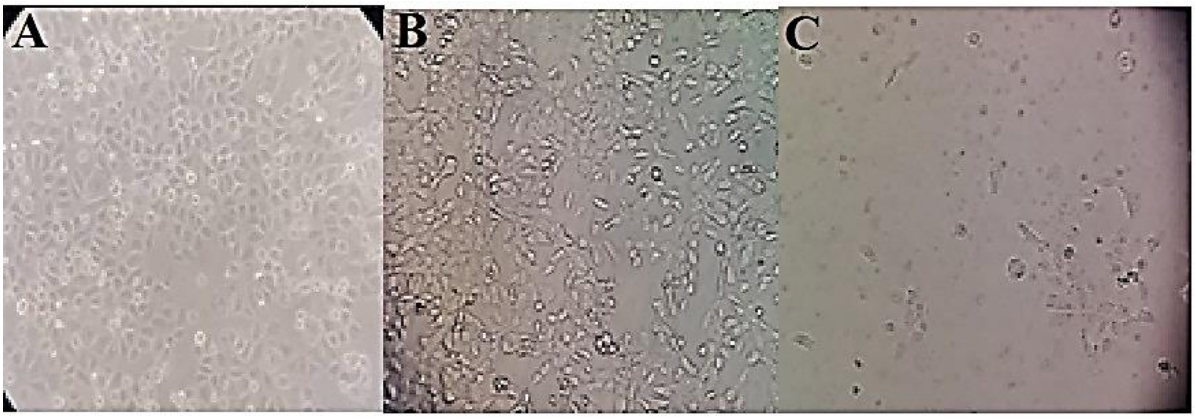

Figure 4. Microscopic view of the cells after exposure to dose of 280 mg/ml Zeocin. A). Normal DU145 cells, B) DU145 transfected with pBud-CE4.1-*cpe*, C) DU145 transfected with empty vector

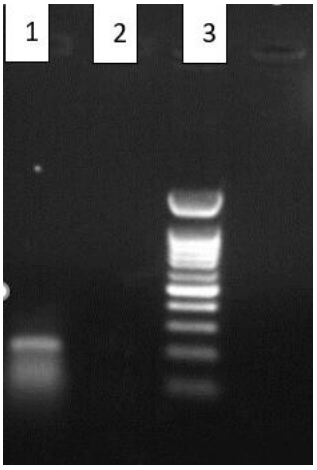

Figure 5. The results of RT-PCR products electrophoresis. Column 1: amplification of *cpe* (217 bp) in pBudCE4.1-*cpe* transformed DU145. 2: Non-presence of *cpe* in pBudCE4.1 transformed cell. 3:100bp molecular marker

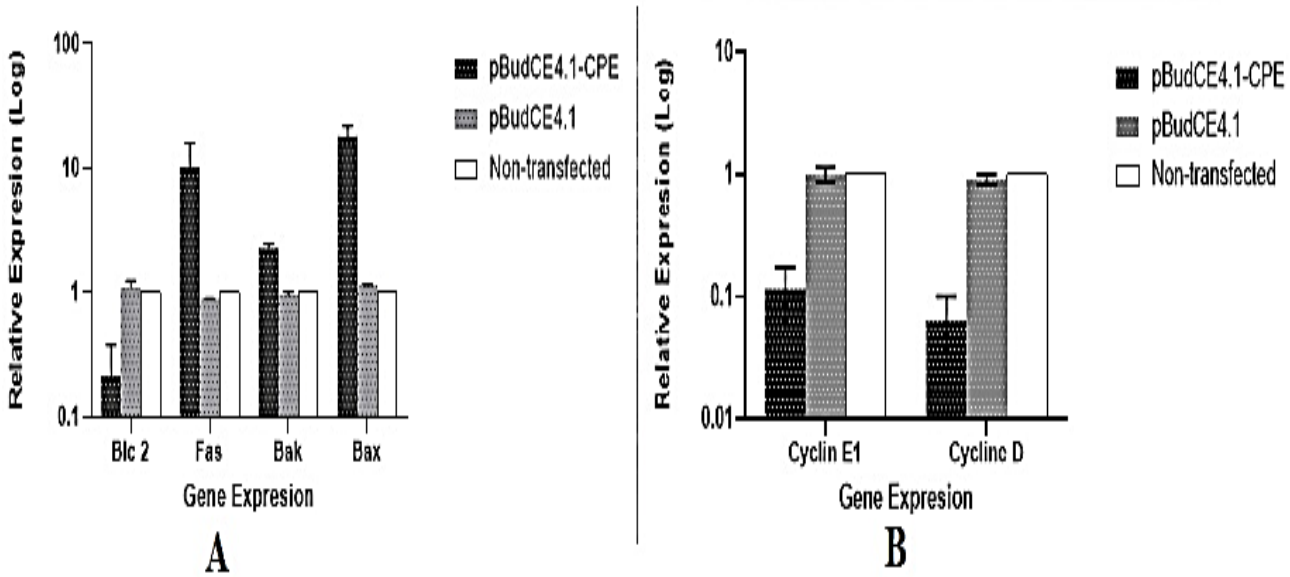

Figure 6. The apoptosis genes expression and the cell-cycling genes expression. A) The apoptosis genes expression in non-transformed cells, pBudCE4.1 transformed DU145, and pBudCE4.1-*cpe* Transformed DU145. B) The cell-cycling genes expression in pBudCE4.1-*cpe* Transformed DU145, pBudCE4.1 transformed DU145 and non-transformed cells. ($p < 0.05$).

4. Discussion

Prostate cancer is the second most common tumor in males globally (behind lung cancer). The global incidence and mortality rates of prostate cancer increase with age, with the average age at diagnosis being 66 years.

Recent investigations into prostate cancer therapy have identified novel medicines, medication sequences, and combinations (Aurilio et al., 2020). In recent years, there has been an increase in the use of bacterial toxins in cancer therapy or prevention. Some of these toxins are used in recombinant vectors to

produce recombinant vaccines (Khoshnood et al., 2022). Pathogenic bacteria such as *Escherichia coli*, *Aggregatibacter actinomycetemcomitans*, *Haemophilus ducreyi*, *Shigella dysenteriae*, *Campylobacter* spp., and *Helicobacter* spp. can produce cytolethal distending toxins (CDTs), a group of bacterial toxins that are a promising tool for vaccine development and treatment approaches for different cancers (Jinadasa et al., 2011; Cortes-Bratti et al., 2001). In this study, we tried to evaluate the effects of *Clostridium perfringens* enterotoxin (Cpe) expression by the pBudCE4.1 vector on DU145 apoptosis. The recombinant plasmid, pBudCE4.1-*cpe*, was amplified in the NovaBlue strain of *E. coli*. The DU145 cell line was transfected with these plasmids using Lipofectamine 2000, and the cells were selected for resistance to the antibiotic zeocin because the pBudCE4.1 plasmid codes for zeocin resistance agents. RT-PCR confirmed the successful expression of desired genes in transfected DU145.

We evaluated apoptosis gene expression in transfected and non-transfected cells. Pro-apoptotic (*bax*, *bak*, *fas*) and anti-apoptotic (*bcl-2*) genes, as well as *cyclin D1* and *cyclin E* genes, were found to be expressed in cancer cell apoptosis (Fauvet et al., 2005). Our findings indicated that *bak*, *bax*, and *fas* expressions in DU145 transfected with pBudCE4.1-*cpe* were notably increased when compared to normal cells and DU145 transfected with pBudCE4.1 ($p < 0.05$) while *BCL-2* was down regulated in the same condition. Since the tumor's destruction by apoptosis is a common mechanism of cancer therapy (Maeda et al., 2012). Abedi et al. used a recombinant vector coding for the *cpe* and *PSCA* genes to induce apoptosis in pc3 cancer cell. They found that *bak*, *fas*, *bax*, and *p53* gene expression was highly increased in PC3 cells transfected with the pBudCE4.1-*cpe*-PSCA recombinant vector (Abedi et al., 2020). In a study, the impact of *S. aureus* toxin on the expression of *bcl-2*, *bax*, and *fas* in PC3 cell lines was examined. The results showed that *S. aureus*-toxin significantly affected the expression level of apoptotic-related genes in the PC3 cell line. The mRNA expression of the *bax* and *fas* genes increased significantly in toxin-treated PC3 cells compared to the control group. On the other hand, *bcl2 mRNA* expression, decreased significantly as compared to PC3 cells transformed with an empty pcDNA3.1 (+) plasmid. Some bacterial toxins appear to be an effective strategy for treating some cancers (Sadeghi and Doosti 2020). *Cyclin D1 and E* expression were also studied in our research on DU145 transfected with recombinant vector or empty plasmid. Overexpression of the *cyclin D1* releases a cell from its traditional controls and causes transformation to a malignant composition (John et al., 2017). *cyclin E* expression is commonly deregulated in many cancer types, including breast, lung, lymphoma, leukemia and osteosarcoma (Leach et al., 1993; Iida et al., 1997; Fukuse et al., 2000). Our results show that *cyclin E*, and *cyclin D1* expression was lower in DU145 cells transformed with the recombinant vector compared to those transformed with an empty plasmid and normal Du145 cells ($p < 0.05$). Some researches emphasis on the role of bacterial toxins on Cyclins gene expression. Welsh et al. (2001) found that *Clostridium difficile* toxin A could control *cyclin* D1 in murine fibroblasts.

Conclusions

In this study, the findings indicate for the first time that the *cpe* toxin gene as a recombinant vector of pBudCE4.1-*cpe* can have an effect on the expression of genes involved in apoptosis like *bcl2*, *bak*, *fas* and *bax* and the cell cycle genes including *cyclin E*, and *cyclin D1* genes that could stop the growth of DU145 prostate cancer cells and thus inhibit the growth and proliferation of cancer cells. According to the findings of this study, *cpe* gene in a recombinant vector might be a candidate vaccine for the treatment of prostate cancer.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Authors Contribution

Two authors contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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