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Cloning of the Ssap-NtrB prokaryotic gene into the eukaryotic expression vector pcDNA3.1 / V5 / His B vector

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Abstract

Suicide gene therapy has recently emerged as a method used in cancer treatments. These therapies utilized enzymes that are expressed in the cell. In this study, Staphylococcus saprophyticus supsp. saprophyticus Nitroreductase gene (Ssap-NtrB) was subcloned into the eukaryotic expression vector namely pcDNA3.1 / V5 / His B. For this purpose, Nitroreductase gene region was firstly amplified from the pET14B vector using PCR strategy and cloned into the pGEM-T-Easy vector. After this step, the Ssap-NtrB gene was restricted with KpnI/ApaI and was ligated into pcDNA3.1 / V5 / His B vector. Recombinant colonies were verified using KpnI/ApaI restriction enzymes. As a result, the Ssap-NtrB gene was cloned into pcDNA3.1/V5/His B vector and was readyfor use in suicide gene therapy in eukaryotic human cancer cells.

Keywords: *Ssap-NtrB, cloning, nitroreductase, expression vector, pcDNA3.1/HisB.*

Ssap-NtrB prokaryotik geninin ökaryotik ekspresyon vektörü pcDNA3.1 / V5 / His B içine klonlanması

Öz

İntihar gen terapisi son zamanlarda kanser tedavilerinde kullanılan bir yöntem olarak karşımıza çıkmaktadır Bu terapilerde hücrede ifade olan enzimlerden faydalanılmaktadır. Bu çalışma kapsamında, Staphylococcus saprophyticus supsp. saprophyticus'tan elde edilen Ssap-NtrB geninin ökaryotik ekspresyon vektörü olan

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pCDNA3.1 / V5 / His B'ye klonlanması gerçekleştirilmiştir. Bu amaçla, ilk olarak genin içinde bulunduğu pET14B vektöründen restriksiyon kesimleri ile gen bölgesi alınarak, pGEM-T-Easy vektörüne aktarılmıştır. Bu adımdan sonra seçilen uygun enzimler olan KpnI/ApaI ile kesim yapılarak hazırlanmış olan pCDNA3.1 / V5 / His B'ye ligasyonu sağlanmıştır. Kontrol kesimleri ile gen klonlamanın doğrulanması sağlanmıştır. Sonuç olarak, intihar gen tedavisinde kullanıma hazır hale getirilen Ssap-NtrB geninin klonlanması ve kontrolü gerçekleştirilmiştir. Bu sayede ökaryotik kanser hücrelerinde kullanıma hazır hale getirilmiştir.

Anahtar kelimeler : *Ssap-NtrB, nitroredüktaz, ekspresyon vektörü, pcDNA3.1/HisB.*

1. Introduction

Nitroreductases (NTR); flavoenzymes are belonging to the class of oxidoreductases. They allow the reduction of nitro groups of nitro compounds (nitrofurazone, nitrobenzene, nitrofuran, and nitrofurantoin) [1]. All nitroreductases show approximately similar biochemical features. They have a molecular weight of about 30 kDa and also have broad substrate specifity [2,3]. Nitroreductases that were first discovered in eubacteria, have conserved sequences. Although nitroreductase enzymes are found in eubacteria, nitroreductase-like enzymes can be expressed in archaea and also eukaryotes. Flavin mononucleotide (FMN) or flavin adenine nucleotide (FAD) were used by these enzymes as a prosthetic group. Also, they were reduced by nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor [3,4].

There are many nitroreductase enzymes isolated from prokaryotes and eukaryotes. Because of their importance, many nitroreductases were cloned for structural analyzes. Moreover, functional analyses have been performed. Some of these enzymes are RdxA isolated from *H. pylori* [5], NTR separated from *E. coli* [2, 4, 6, 7], *Vibrio fischeri* and FRazI [8] and YwrO *prurifed from B. amyloliquefaciens* [9]. Different nitroreductases such as NfsA and NfsB have been isolated in *E. coli* and classified into oxygen insensitive and oxygen-sensitive nitroreductases [3].

Yetis and Celik have reported that, a novel nitroreductase named Ssap-NtrB had been isolated from *Staphylococcus saprophyticus* supsp. *Saprophyticus*. It was discovered in 2012. It catalyzes the reduction of antibiotic nitrofurazone and CB1954 which is cancer prodrug of dinitrobenzamide family at very low temperatures [10]. In our previous studies, some nitro compounds were designed, synthesized and evaluated as the substrate of Ssap-NtrB [11,12]. In this study, the full-length Ssap-NtrB gene of the nitroreductase enzyme in the *E.coli* expression vector has been subcloned into a eukaryotic expression vector for ectopic expression studies. Thus, a vector expressing in the eukaryotic cell will be obtained for use in the treatment of suicide gene therapy.

2. Material and methods

2.1 Bioinformatic analysis and primer designing of Ssap-NtrB gene

The nucleotide sequence of the Ssap-NtrB (NC 007350.1) gene was screened using the NCBI tool (https://www.ncbi.nlm.nih.gov/). The primers were designed for the ORF of the Ssap-NtrB gene (671bp). The non-cutters restriction enzymes were selected using the

Restriction Mapper tool. With this strategy, the recognition sequences of the selected enzymes (KpnI/ApaI) were added to the 5 'ends of the designed primers (Table 1). Also, consensus sequences were added to 5' ends of the designed primers for enhanced PCR amplification. The primers were analyzed with the nucleotide blast tool in NCBI for searching specifity.

Table 1. DNA sequences of Ssap-NtrB primers.

	DNA sequences	**TM (^{0}C)**
Ssap-NtrB Forward Primer	5'-AAGGTACCACCATGATAAATAATAATTTTGA-3'	62
Ssap-NtrB Reverse Primer	5'-TTGGGCCCTCAACGTATTTAGTCACA-3'	58

2.2 Amplification of the Ssap-NtrB gene by polymerase chain reaction (PCR)

The Ssap-NtrB sequence was amplified by PCR from pET14B plasmid DNAusing the primers in Table 1. This sequence contained KpnI and ApaI sites for insertion into the pcDNA3.1HisB plasmid. Primers were diluted into a concentration of 100 ng/µL with the sterle water. The PCR reaction mixture consisted of 5 µL of Taq Buffer (1X), forward and reverse primers (100 pmoL/µL), plasmid DNA (50 ng), dNTP (10 mM), 4 µL of $MgCl_2$ (1 mM), 05 µL of Taq Polymerase Enzyme (2.5 U/µL) and distilled water (34.5 µL) in a reaction volume of 50 µL. The PCR conditions were 94°C for 3 min, 35 cycles of 94°C for 1 min, 60°C for 45 sec, 72°C for 1min and final extension of 72°C for 10min. Taq DNA Polymerase (Fermentas) was used for the PCR reaction.

2.3 Cloning strategy of Ssap-NtrB gene into eukaryotic expression vector

Cloning of the Ssap-NtrB was performed in a three-step procedure (Figure 1). Firstly, the PCR product was cloned into the pGEM-T Easy vector (Promega) with T: A cloning system [13,14]. Subsequently, transformation was carried out. Spesifically, a mixture of ligation was added to 200 µL of *E.coli* XL1Blue competent cells and was incubated for 40 min. After this step, cells were heat-shocked at 42°C for 2 min and then chilled on ice for 2 min. 800 µL of LB Broth medium was added and incubated for 2 hours at 37°C. At the end of incubation, bacterial suspension was spread on an X-gal and IPTG plate. Plates were incubated for 16 hours at 37°C to obtain blue colonies. Recombinants cloned into pGEM-T Easy vector were selected by the blue-white selection, white colonies were screened for recombinant. Secondly, the cloned Ssap-NtrB gene in the pGEM-T Easy vector was sub-cloned into the eukaryotic expression vector of pcDNA3.1/HisB. Specifically, the PCR product and pGEM-T Easy vector were digested with KpnI/ApaI and gel purified using Gel Purification Kit (Thermo). Ligation was completed using T4 DNA ligases according to the manufacturer's instructions (Thermo). For this purpose 3 µL of vector (1000 ng/µL), 23 µL of plasmid (780 ng/ µL), 1 µL of T4 DNA ligase Enzyme (5 U/µL) and buffer (1X) in a reaction volume of 30 µL. Ten microliters of ligation mixture were transferred into 200 µL E. coli *XL1Blue* (the competency at 1 x 10^6 colony-forming unit / µg DNA/100 µL cells) competent cell lines [13-15]. Overnight cultures were prepared from each of the resulting colonies. The plasmid DNA was purified from transformants using a Thermo™ plasmid isolation kit. In step 2, a sample of the purified plasmid DNA was verified using the restriction endonuclease method.

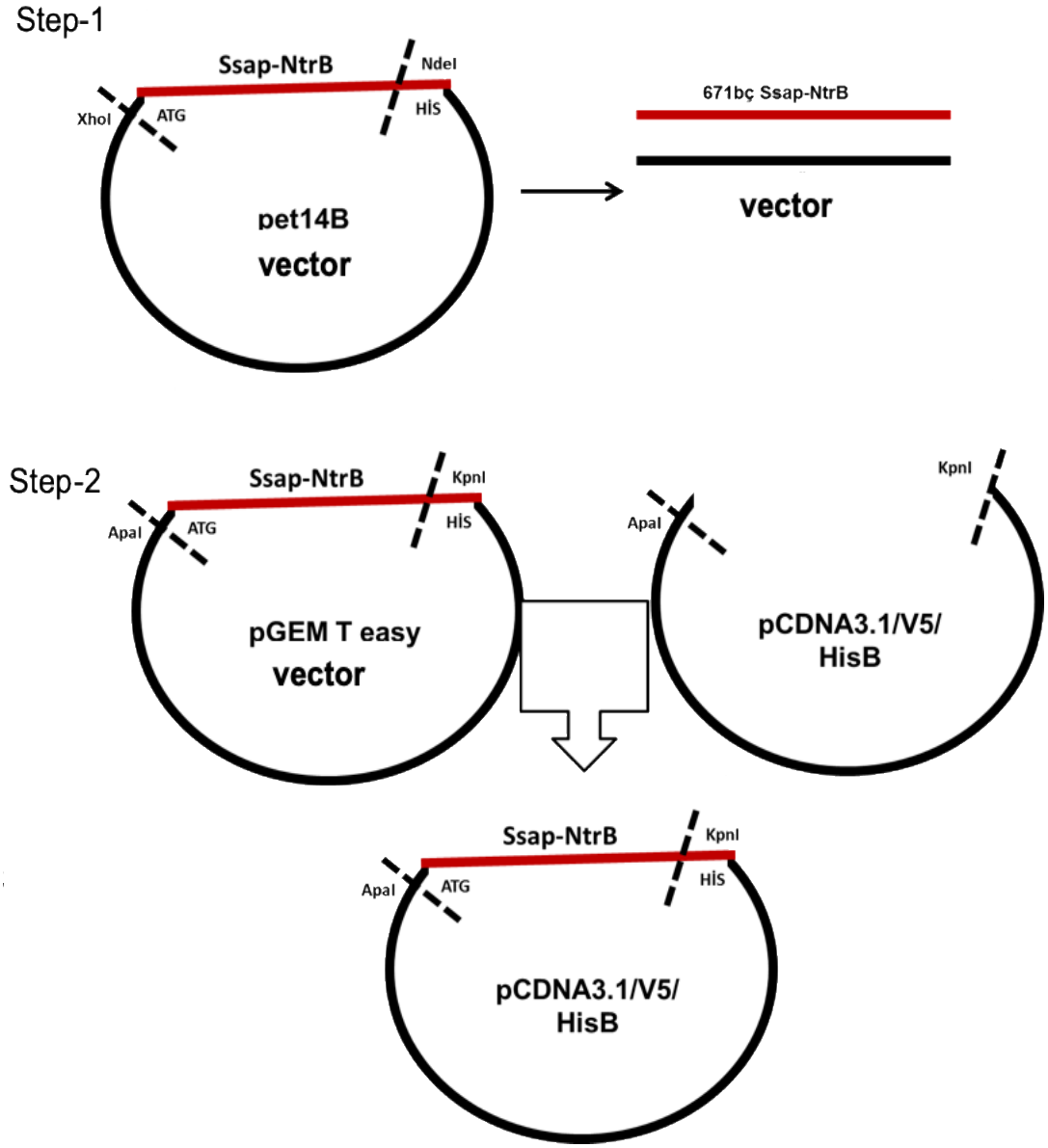

Figure 1. The pathway of the cloning of the Ssap-NtrB prokaryotic gene into the eukaryotic expression vector pCDNA3.1/V5/His B.

3. Result and discussion

Nitroreductase enzymes are an important group of enzymes that interact with prodrugs. In this field, *E.coli* Nitroreductase was originally used for the reduction of nitro compounds. However, there is a need for the discovery of new nitroreductase enzymes that have better interactions with prodrugs. Nitroreductase enzymes and prodrugs are preferred in targeted therapy, called suicide gene therapy used for cancer treatment.

In this respect, the Ssap-NtrB gene was originally cloned into a prokaryotic expression system, pET14B. This system allows the IPTG induction of Ssap-NtrB production in *E.coli*. The nucleotide sequence of *Staphylococcus saprophyticus* supsp. *saprophyticus*

revealed the presence of an open reading frame of 671 base pairs. The molecular weight of the recombinant protein was 25,567 kDa.

Prodrug potential of the new nitro-based compounds were determined with nitroreductase enzyme which was produced in the prokaryotic expression system [11, 12]. However, the important step of the study is to determine the interaction of this enzyme and nitro compounds in cancer cells. This would give an idea of its utility in the treatment of cancer. In this study, the Ssap-NtrB gene will be cloned into a eukaryotic expression vector for transfection assays. In this system, Ssap-NtrB in pET14B was used as a gene source by PCR-based gene amplification (Figure 1). For this purpose, forward and reverse primers have been designed via open reading frame of the gene. Also, two enzyme recognition sites were placed at the 5 ’ends of the primers for cloning into the vector pcDNA3.1/HisB. In the first step, the PCR product was cloned into pGEM-T Easy to allow T:A cloning and during the production of PCR products, Taq polymerase was used to allow this cloning.

As a result of PCR amplification, the region of approximately 671 base pairs was successfully amplified. PCR products were visualized on 1% agarose gel electrophoresis (Figure 2).

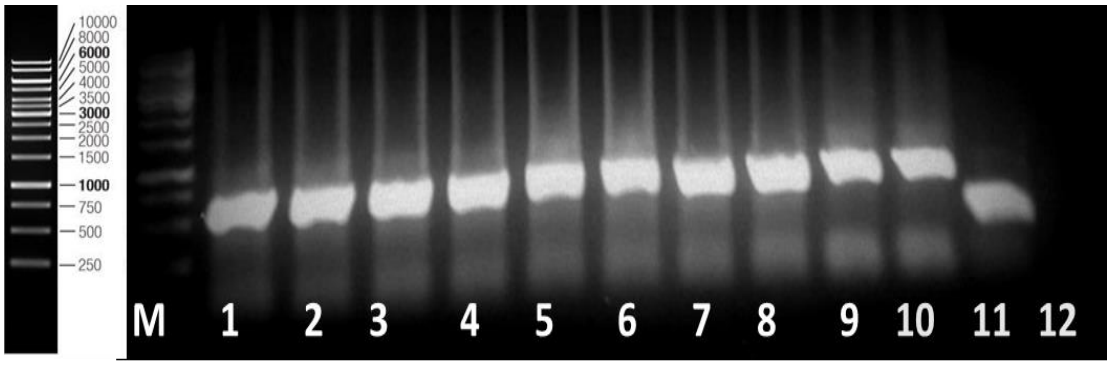

Figure 2. Amplification of the pET14B gene containing the Ssap-NtrB gene. (The PCR conditions were 94°C for 3min, 35 cycles of 94°C for 1min, 60°C for 45sec, 72°C for 1min and final extension of 72°C for 10min. In figure M: SM0311 1kb marker, 1-10: Amplification of the pET-14b gene containing the Ssap-NtrB gene, 11: Positive control and 12: Negative control).

Recombinant colonies were selected by blue-white selection, white colonies were screened for recombinant identification. Following plasmid DNA isolation, recombinant colonies were confirmed by KpnI/ApaI restriction enzyme digestion (Figure 3).

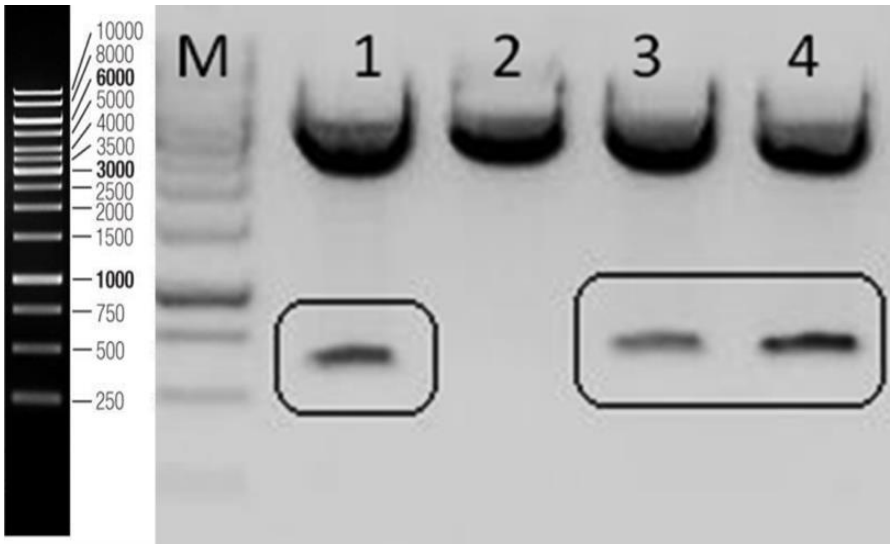

Figure 3. Cut result of pGEM-T Easy vector carrying the Ssap-NtrB gene. (M: SM0311 1kb marker, 1-4 Cut result of pGEM-T Easy vector carrying the Ssap-NtrB gene).

The restricted Ssap-NtrB gene containing 671 base pairs was ligated with the vector pcDNA3.1/HisB and the ligation mixture was transformed to XL1-blue compotent cells. The plasmid isolation was performed from colonies. Then the recombinant plasmids were checked using restriction endonuclease system (Figure 4). The KpnI/ApaI recognition sites were found in the primers and were used in cloning strategy. As a result, the gene was cloned into eukaryotic expression vector.

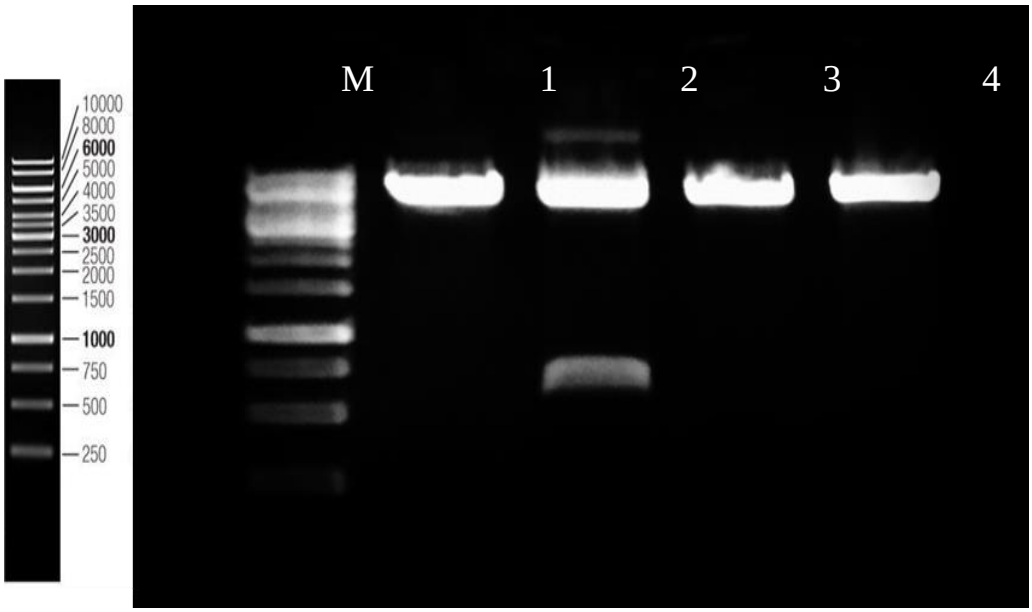

Figure 4. pcDNA3.1/HisB vector containing Ssap-NtrB gene was restricted with *KpnI*/*ApaI sites* by restriction endonuclease enzymes (M: SM0311 1kb marker, 1-4 SsapNtrB pcDNA3.1/HisB *KpnI*/*ApaI* restriction cut).

4. Conclusion

As a result, the cloning of Ssap-NtrB into pCDNA3.1/V5/HisB was achieved for using suicide gene therapy. With this vector system, the Ssap-NtrB gene could be transfected and expressed into evariety cancer cells to treatment various cancer types via GDEPT (Gene Directed Enzyme Prodrug Therapy) system.

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