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ORIGINAL ARTICLE

Construction and identification of the recombinant lentiviral expression vector targeting human Bax inhibitor-1 gene

Hai-qing Luo[1], Wang-tao Zhong[2], Fu-mei Liu[3], Zhong-yi Cui[3], Ke-yuan Zhou[3], Xiang-yong Li[3]

[1]Center of Oncology, [2]Department of Neurology, The Affiliated Hospital of Guangdong Medical College, Guangdong; [3]Institute of Biochemistry and Molecular Biology, Guangdong Medical College, Zhanjiang, Guangdong, China

Summary

Purpose: *The aim of this study was to construct a recombinant lentiviral expression vector targeting human BAX inhibitor-1(BI-1) gene and observe its expression in NIH3T3 cells.*

Methods: *Human BI-1 gene was amplified by polymerase chain reaction (PCR), and then cloned into the vector pLCMV-IG using DNA recombinant technique. After the inserted sequences in the recombinant plasmids were identified by PCR, and double digesting and DNA sequencing analysis, the recombinant lentivirus was packaged and administered into NIH3T3 cells. The BI-1 mRNA and protein expression were examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting.*

Results: *PCR double digesting analysis and DNA sequencing confirmed that the BI-1 DNA sequences were successfully inserted into the lentiviral vectors. After transfection with the recombinant lentivirus, BI-1 expression in NIH3T3 cells was significantly increased at both mRNA and protein levels.*

Conclusion: *The lentiviral vector expressing BI-1 has been successfully constructed, which allowed for the subsequent analysis of the role of BI-1 in cell growth and transduction.*

Key words: *BI-1, NIH3T3, recombinant lentiviral vector*

Introduction

BI-1, a recently discovered apoptosis inhibitor, is located in chromosome 7 in rats, chromosome 15 in mice and chromosome 12 in humans [1]. It is one of the few conservative cell death inhibitors existing in both animals and plants. Previous studies revealed that BI-1 was closely related with the growth and metastasis of various cancers [2-4]. The lentiviral vector system is shown to be a gene delivery method derived from human immunodeficiency virus 1 (HIV-1), which has been widely applied in the genetic treatment against malignant tumors and hereditary diseases [5].

The present study was designed to establish recombinant vectors highly expressive of the BI-1 gene via the lentiviral vector system and to evaluate the effect on BI-1 expression after infecting mouse fibroblast NIH3T3 cells. This could provide a basis for analyzing the effect of BI-1 on cell growth and proliferation.

Methods

Materials

The lentiviral vector system was donated by Dr. Yangchao Chen from the Chinese University of Hong Kong. The system consisted of transfer vector pLIG and 3 packaging vectors pSL3, pSL4 and pSL5. pLIG containing the reporter gene of green fluorescent protein (GFP) could simultaneously express GFP alone during expression of endogenous genes, which could be applied in rapid screening of recombinant lentivirus. The eukaryotic cells expressing plasmid pU6 were constructed in our laboratory. *E.coli* XL-blue was stored in our laboratory. EcoR I, Sal I, Cal I restriction enzymes, T4 DNA ligase, Trizol and RT-PCR were Fermentas products (Beijing, China). The remaining agents were purchased from Geneseed Biotech Company (Guangzhou, China).

Correspondence to: Xiang-yong Li, MD, PhD. Institute of Biochemistry and Molecular Biology, Guangdong Medical College, Zhianjiang, Guangdong 524023, China. Tel: +86 759 2387448, E-mail: xyli75@126.com, hqluo@126.com
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BI-1 gene cloning

According to the cDNA sequence of wild-type BI-1 in combination with lentiviral vector structure, a pair of specific oligonucleotide primers containing EcoR I and Sal I restriction enzyme sites were designed: forward primer (5'-CGA ATT CGC CAC CAT GAA CATA TTT GAT CGA AAG A -3'), reverse primer (5'-CGC GTC GAC TCA TTT CTT CTC TTT CTT CTT A -3'). Full-length BI-1 cDNA fragment was amplified by PCR using HEK293 cells cDNA as template under the following reaction conditions: pre-denaturalization at 95 ^{o}C for 5 min, denaturalization at 95 ^{o}C for 30 sec, annealing at 55 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min for 25 cycles and a final extension at 72 ^{o}C for 10 min. PCR products were examined and electrophoresed on 1% agarose gel and then extracted and purified for subsequent analysis.

Construction, screening and identification of lentiviral plasmid expressing BI-1

The digestion of the PCR product and the lentiviral transfer vector pLIG were performed by EcoR I and Sal I restriction enzymes. The extraction and purification of spliced products were conducted using a gel extraction kit. A portion of 1μL purified pLIGG vector sequence was obtained and mixed with 10 μL of purified BI-1, 1.0μL of T4 DNA ligase, 2.0μL of 10×T4 DNA ligase buffer and sterile distilled water up to 20.0μL, centrifuged for few secs and kept at 16 ^{o}C overnight.

The obtained products were inserted into fresh competent bacteria *E.Coli* XL-blue which were cultured on an LB plate containing 100 mg/L ampicillin at 37 ^{o}C overnight (about 16 h) until single colonies were observed. Ten monoclonal colonies were randomly selected for subsequent PCR amplification and identification. PCR identification conditions: forward primer (5'-CCT CTT CTG GTG GAT GCT TTG-3') and reverse primer (5'-GCC TCG CTC TGT TGA TGT GA-3'). PCR amplification conditions: 95 ^{o}C 5min, 95 ^{o}C 30sec, 55 ^{o}C 60sec, 72 ^{o}C 60sec, 72 ^{o}C 7 min for 25 cycles. The PCR-amplified products were identified by agarose gel electrophoresis. The clonal products qualified for initial PCR screening were identified by EcoR I and Sal I and prepared for sequence analysis by Shanghai Bioengineering Company (Shanghai, China).

Package and condensation of recombinant lentivirus expressing BI-1

293T cells at a density of 6×10^{8}/L were inoculated in a 100 mm-culture plate and cultured overnight. The cultured cells were transfected when the confluence reached 70-80%. The culture medium was changed 2 h prior to transfection. DNA solution containing each type of lentiviral plasmid (pLIG vector: 15μg, pSL3 vector 4μg, pSL4 vector 8 μg and pSL5 vector 6μg) was evenly mixed with an equivalent amount of Opti-MEM, adjusted to 2.5 mL, and incubated at room temperature for 5 min. A portion of 100μL LipofectamineTM 2000 was mixed with Opti-MEM and incubated at room temperature for 5 min. The DNA and LipofectamineTM 2000 dilutions were mixed and incubated at room temperature for 20 min, then used as transfected compounds, transferred into 293T cell culture solution, incubated for 8 h, the culture media containing transfected compounds were discarded and then rinsed by PBS. Finally, 10 mL of culture medium containing 10% fetal bovine serum (FBS) were added, incubated for another 48 h and the supernates were harvested for later use. After centrifugation at 4 ^{o}C at 4000 g for 10 min, the supernatants were infiltrated by 0.45 μm-filter and centrifuged at 4 000 g until the required viral concentration was obtained.

The lentivirus expressing BI-1 was named lent-BI-1, and the lentivirus expressing EGFP (named lenti-EGFP) was used as control.

Expression level of recombinant Lenti- BI-1 in NIH3T3 cells

NIH3T3 cells at logarithm growth phase were plated on 24-well plate (2×10^{4} cells/well). At 70% confluence, the cells were infected by recombinant lenti-BI-1 and lenti-EGFP, respectively. Meanwhile, a working solution containing 6μg/μl polybrene was supplemented to increase the infection rate. The following day, the culture medium containing virus was discarded and replaced by complete medium. The illumination of GFP was examined under fluorescence microscope at 48 h post-infection. The expression of BI-1 at both protein and RNA levels in infected cells were analyzed by RT-PCR and Western blot, respectively. RT-PCR reaction: forward primer of BI-1 (5'-CCTCTTCTGGTGGATGC TTTG-3'), reverse primer (5'-GCCTCGCTCTGTTGAT-GTGA-3'); GAPDH (as internal standard) forward primer (5'-CCGCCGAGCCACATCGCTC-3'), reverse primer (5'-ATGAGCCCCAGCCTTCT CCAT-3'); PCR reaction conditions: 95 ^{o}C 5min, 95 ^{o}C 30sec, 55 ^{o}C 60sec, 72 ^{o}C 60sec, for 25 cycles and 72 ^{o}C 7min. Mouse-anti-human BI-1 polyclonal antibody was used as primary antibody (1:500) and goat-anti-mouse IgG was utilized as secondary antibody (1:800) for Western blot. The protein band was detected by chemoluminescence reagent (ECL) and the integrated optical density (IOD) of each protein band was measured. IOD values were adjusted by internal standard actin. Protein expression was compared among all samples.

Statistics

The results from different treatment groups were compared using two-tailed Student's t-test. A p value <0.05 was considered to be statistically significant.

Results

PCR identification of positive cloning

The positive clones on the LB plates were used as a template and amplified to 714 bp band by PCR and empty plasmids as controls, as shown

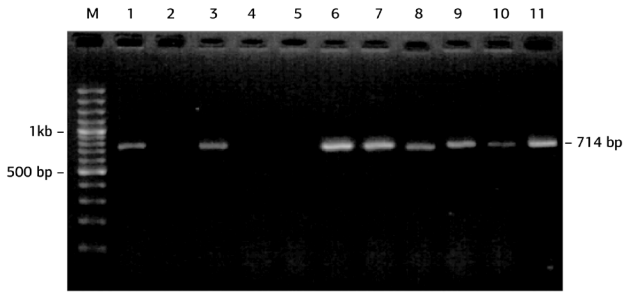

Figure 1. Agarose gel electrophoresis of positive clone of recombinant plasmid pLIG-BI-1. M 100bp marker; 1-10: recombinant plasmid monoclone; 11: positive control.

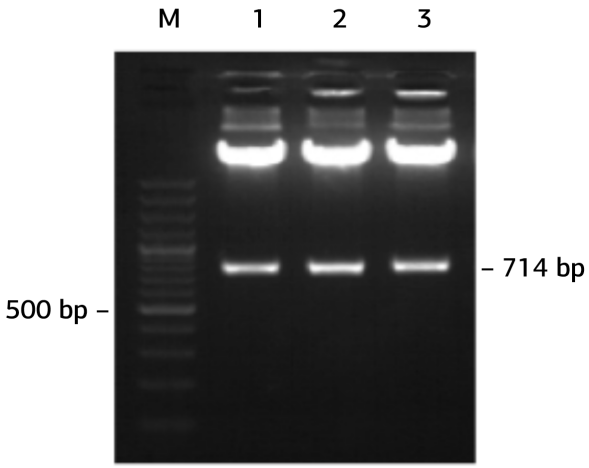

Figure 2. Agarose gel electrophoresis of EcoRI/SalI endonuclease fragments of recombinant plasmids pLIG-BI-1. M: 1kb marker; 1-3: recombinant plasmid monoclone.

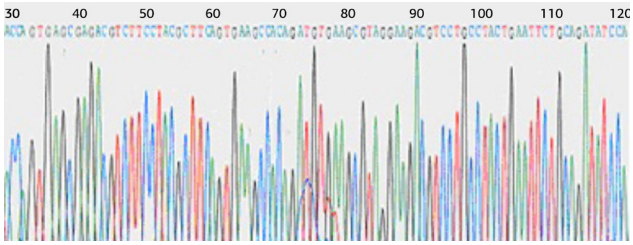

Figure 3. Partial sequencing map of recombinant plasmid.

in Figure 1. The results were in accordance with estimated values and showed that BI-1 was linked to plasmids transfected with pU6 lentiviral, indicating that the recombinant plasmid pLIG- BI-1 was successfully constructed.

Identification of recombinant plasmid with DNA endonuclease and sequence

Endonuclease testing revealed that recombinant plasmid pLIG-BI-1 produced a 714 bp-fragment after treatment with EcoR I and Sal I, as shown in Figure 2. The outcomes conformed to the theoretical values. Subsequent DNA sequencing analysis (Figure 3) showed that the inserted sequence was in accordance with that of BI-1 released by GenBank, suggesting the accurate sequence of BI-1 gene inserted into pLIG-BI-1 and the successful construction of recombinant plasmid pLIG-BI-1.

Package and identification of recombinant lentiviral vector

The plasmids for lentiviral package were mixed with the constructed vectors pLIG- BI-1 and then co-transfected with 293T cells. The package of lentivirus was successfully conducted by showing apparent fluorescence and vigorous cellular growth under a fluorescence microscope at 24 h post-transfection. At 48 h after transfection, the supernatant was harvested for lentiviral titering. The viral titer was 4×10^5 TU/mL. The virus suspension was prepared for use or stored at -80 ^{O}C.

Expression of BI-1 of recombinant lentiviral lenti-BI-1 in NIH3T3 cells

As shown in Figures 4 and 5, the expression of BI-1 at both RNA and protein levels was significantly increased in NIH3T3 cells 48 h after infection by recombinant lenti-BI-1. However, there were no or few expression of the target gene or protein in untreated and control groups. Compared with untreated or control, the expression of BI-1 in lenti-BI-1 cells group had changed dramatically. These results indicated that recombinant plasmid pLIG-BI-1 can effectively enhance the expression of BI-1 in infected NIH3T3 cells.

Discussion

BI-1, as a new type of antiapoptotic factor, has gradually become a novel target of gene therapy for cancer. Previous findings indicate that BI-1 does not belong to any gene family related to programmed cell death (PCD). In addition, BI-1 is a novel regulator in the pathway of cell death regulated by bcl-2 and bax. BI-1 can block the progress of cell apoptosis by inhibiting mitochondria-related apoptosis pathways. Compared with healthy subjects, patients with cancer such as breast cancer , lung adenocarcinoma and gastric cancer [6,7] showed 4-10 fold upregulation of BI-1 expression [8], suggesting that overexpression of the BI-1 gene closely correlates with multiple cancers. Equally, our previous studies detected a significantly high level of BI-1 in na-

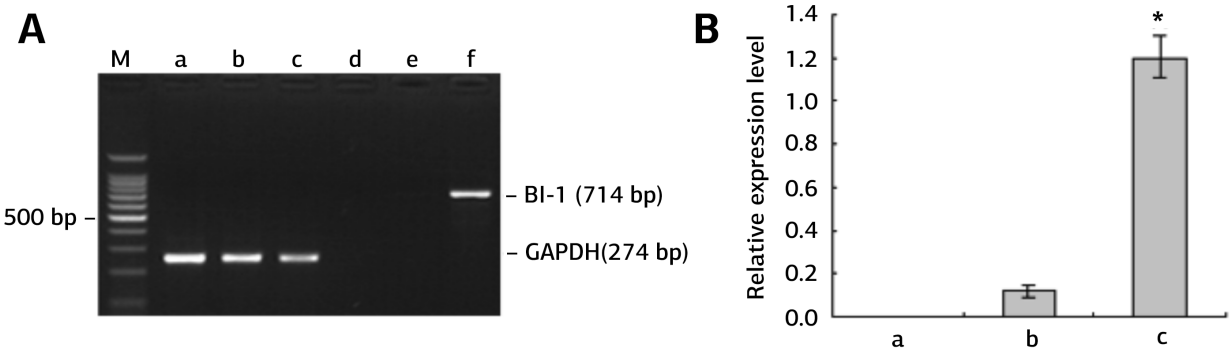

Figure 4. Effect of recombinant lentiviral Lenti-BI-1 on the mRNA expression of BI-1 in NIH3T3 cells. **A**: Gel electrophoresis of PCR products of BI-1 gene in different treated cell groups. **B**: Relative expression level of BI-1 mRNA in different treated cells. **M**: 100 bp DNA marker; **a,d:** untreated group; **b,e:** lenti-EGFP; **c,f:** lenti-BI-1. The results in Figure 4B are the mean ± SD; N=3, *p<0.01 vs control.

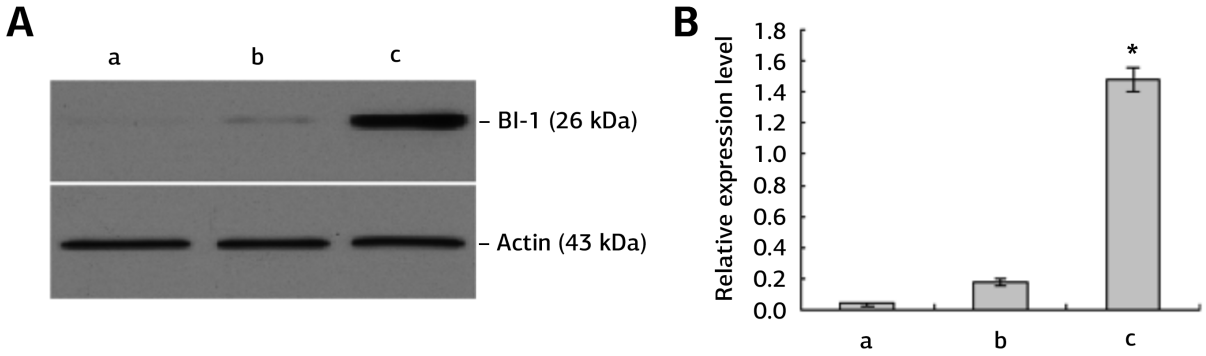

Figure 5. Effect of recombinant lentiviral Lenti-BI-1 on the protein expression of BI-1 in NIH3T3 cells. **A**: Expression of BI-1 protein in 3 cell groups. **B**: Relative expression level of BI-1 protein in different treated cells. **a**: untreated group; **b**: lenti-EGFP; **c**: lenti-BI-1. The results in Figure 5B are the mean ± SD; N=3, *p<0.01 vs control.

sopharyngeal cancer cells. Moreover, cancer cells showed relatively evident cell apoptosis after BI-1 gene silencing [9,10]. These results were consistent with the findings of Grzmil et al. [6] that the expression and function of BI-1 in breast cancer patients characterizes it as novel regulator in the pathway of cell apoptosis and may be of vital importance in gene therapy of cancer, and therefore may serve as a potential new target for carcinoma treatment.

Compared with traditional non-viral shuttle vectors, viral vectors have been widely employed as gene shuttle vectors in current studies due to their high delivery efficiency, rapid and durable effect and low cost. Commonly used viral vectors include retroviral vectors (such as lentiviral vector), adenoviral vectors, adeno-associated viral vectors and herpes simplex viral vectors. Lentiviral vector is a gene shuttle vector modified from human immunodeficiency virus, whose biggest advantage is to integrate endogenous genes into the genome of cells at division and quiescent phases (non-division phase). At present, lentiviral vector has the highest transfection efficacy among all vectors. Besides, lentiviral vector also has a variety of advantages such as delivering large-length gene fragments, lasting effect, low risk of host immune reactions, etc [11]. Lentiviral vector has been widely employed as the primary vector in experimental researches [12] and contains the potential to serve as the most common vector for developing RNAi-mediated gene therapy for cancer [13].

In the present study, we successfully constructed lentiviral vectors expressing BI-1 gene verified by PCR, endonuclease identification and DNA sequencing. The treated lentiviral vectors were used to transfect NIH3T3 cells, and significant upregulation of BI-1 expression at both mRNA and protein levels were spontaneously detected. All these results suggested that the constructed lentiviral vectors specifically enhanced the expression level of BI-1 in NIH3T3 cells, providing fundamental evidence to subsequent analysis of the effect of overexpression of BI-1 on cell growth and proliferation.

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