Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading

Construction and identification of recombinant plasmid pUIS3-BLC$^+$

Yingzi Lin[1,2,3*], Shixiang Bao[1,2], Guanghong Tan[3], Fengying Huang[3], Huiqin Huang[1,2]

[1]*Agriculture School of Hainan University, Haikou 571101, China*
[2]*Biological Technology Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China*
[3]*Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical University, Haikou 571101, China*

ARTICLE INFO

Article history:
Received 7 September 2010
Received in revised form 17 September 2010
Accepted 3 October 2010
Available online 20 October 2010

Keywords:
BLC
Plasmodium berghei
Transgene
Knockout

ABSTRACT

Objective: To construct and identify recombinant plasmid pUIS3-BLC$^+$. **Methods:** The cDNA of B-lymphocyte chemoattractant (BLC) was amplified from the total RNA of spleen tissues by PCR method, and were inserted into plasmid of *Plasmodium berghei* with UIS3 knockout by digestion of restrictive endonuclease and T7 ligation. The recombinant plasmids were screened, and then underwent restriction enzymatic digestion and DNA sequencing. Then the confirmed plasmid was further transfected into COS-1 cells by lipofectamine and the BLC expression was tested by RT-PCR and Western blotting. **Results:** The cDNA of BLC gene was correctively amplified by RT-PCR and the recombinant plasmid pUIS3-BLC$^+$ was constructed successfully, which was confirmed by restriction enzymatic digestion and DNA sequencing. RT-PCR and Western blotting also showed the BLC gene expression in COS-1 cells. **Conclusions:** The recombinant plasmid pUIS3-BLC$^+$ has BLC expression in COS-1 cells, and is useful for further study on BLC transgene and UIS3 gene knockout in *Plasmodium berghei*.

1. Introduction

Malaria is a parasitic disease, leading to severe harm to human health and social development. Generally, the safe, cheap and effective vaccine is considered as the key in controlling and eliminating malaria. So WHO, United Nations Development Programme, World Bank etc. have listed the development of vaccine against malaria as one of prior vaccine projects[1]. Mueler *et al* found that sporozoites of *Plasmodium* with UIS3 gene knockout displayed detoxification action. After infecting animal models, these sporozoites stayed in hepatocells not in red blood cells. They can be used as detoxification vaccine, and have immuno-protective effect on mice with malaria[2]. Based on Mueler's research, this study transferred exogenous immunoenhancing gene into detoxified sporozoites of *Plasmodium berghei*. It could strengthen immune ability of these sporozoites and may become more effective vaccine model. As a speific chemokine of B cells , B-lymphocyte chemoattractant (BLC) can stimulate B-lymphocyte receptor-1 expression, guide the migration and homing of B cells, promote immune response by attracting B cells and CD4$^+$ T cells[3,4]. In our study, cDNA of BLC gene was amplified, and inserted into plasmid of *Plasmodium berghei* gene with knockout. It is proved BLC can be expressed in eukaryocyte, and this could pave the way for development of immunoenhancing detoxified vaccine.

2. Materials and methods

2.1. Materials and reagents

Spleen tissues were from three-week BALB/c mice in Hainan Provincial Key Laboratory of Tropical Medicine. Plasmid of *Plasmodium berghei* gene with UIS3 knockout pAKM19 was provided by Matuschewsk. COS-1 cells were from Hainan Provincial Key Laboratory of Tropical Medicine. Lipofectamine was from Gibcol BRL. PT-PCR kit (TaKaRa RNA PCR Kit ver 2.1), restrictive endonuclease *Hind* Ⅲ, EcoR Ⅰ, *Bam* Ⅰ, T4 DNA ligase, standard nucleic acid marker and standard protein marker were purchased from TaKaRa Company. Trizol Reagent was from Invitrogen Co. Plasmid extraction kit (QIQGEN plasmid mini kit), gel recycle kit (QIAquick gel extraction kit) and anti-mice BLC antibody were from Sigma Co. Horseradish peroxidase labeling anti-rat IgG antibody was from Dako Company, DAB from Zhongshan Biological Technological Company in Beijing, and Tris-Cl, SDS, glycine from Bio RAD Co.

2.2. Methods

*Corresponding author: Pro.Yingzi Lin, Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical University, Haikou 571101, China.
Tel: +86-898-66893200
Fax: 86-898-66893200
E-mail: linyingzi@263.net
Foundation Project: Supported by National Natural Science Fund of China (No. 30660177)

2.2.1. Primer design and synthesis

According to BLC sequence of mice from genetic library (NM-018866.2), the primer was designed by Oligo 6.01 as following:

F 5′-ACAGCTAGCATGAGGCTCAGCACAGCA AC-3′

R5′-CGACTCGAGTCAGGCAGCTCTTCTCTTAC-3.

Nhe Ⅰ site (GCTAGC) and three protective bases were inserted into 5′ end of forward primer, while X*ho* Ⅰ site (CTCGAC) and three bases were added into 5′ end of reverse primer. The length of PCR product was 372 bp.

2.2.2. Total RNA extraction and RT-PCR

A total of 500 mg spleen tissue from mice were grinded and added with TRIzol Reagent based on the instruction. With the reverse primer as reactant, reverse transcription reaction was conducted under following condition: 30 ℃ 10 min, 50 ℃ 30 min, 99 ℃ 5 min, 5 ℃ 5 min. Then PCR was performed as following: 94 ℃ 30 sec, 60 ℃ 90 sec. 72 ℃ 150 sec, 30 cycles, 72 ℃ 10 min.

2.2.3.Construction of BLC transgenic plasmid

Based on the instruction, amplified cDNA of BLC and pAKM19 with UIS3 knockout were treated by double enzyme digestion of Nhe Ⅰ and X*ho*. Responding fragments were recycled by 1% agarose electrophoresis. Under the ligation of T4 DNA, vector and target gene fragment were put together overnight at 16 ℃. Competent cells were transformed into *Escherichia coli* JM109, and monoclones were screened for amplification and culture. Then plasmid was extracted based on QIQGEN plasmid mini kit protocol for further identification.

2.2.4. Identification and sequencing

After amplification and extraction, positive clones were digested by restrictive endonuclease such as Nhe Ⅰ and X*ho*, or other enzymes like *Bam* Ⅰ. These clones with correct fragment were selected for DNA sequencing (Jikang Biological Technological Company in Shanghai).

2.2.5. Transfection into COS-1 cells

According to instruction from Jiao *et al*[5] and manual for lipofectamine transfection from Gibcol BRL Co., recombinant plasmid was transfected into COS-1 cells. COS-1 cells were resuspended in complete medium, inoculated into 6-well plate at 1.5×10^6-2×10^6 cells/well, then cultured at 37 ℃ for 18-24 h until 60%-80% fusion. Liposome was used as mediator. Two solutions were prepared in sterile tubes: A solution including 2 μg DNA and 100 μL culture solution free of serum, and B solution including 6 μL lipofectamine and 50 μL culture solution free of serum. Then two solutions were mixed, and incubated at room temperature for 30 min to obtain DNA-liposome complex. Cells were washes twice with Dulbecco's modified Eagle's medium (DMEM) free of serum. Then 0.8 mL free-serum DMEM was mixed with complex, and the mixture was dropped into COS-1 cells. These cells were incubated under 5% CO_2, 37 ℃ for 8-14 h. Two mL complete medium were added. After incubation for 48 h, cells or supernatants were harvested.

2.2.6. BLC expression assay by Western blotting and RT-PCR

Cell lysis solution or supernatant was mixed with 2×SDS, boiled at 100 ℃ for 5 min. After SDS/PAGE electrophoresis, isolated proteins were transferred on the PVDF membrane, and analyzed by Western blotting with anti-mice BLC antibody as the first antibody and anti-rat IgG antibody as the secondary[6]. While BLC mRNA expression was tested by RT-PCR as 1.2.2.

3. Results

3.1. RT-PCR result

The expected length of amplified product of BLC gene by PCR was 372 bp. And 1% agarose gel electrophoresis analysis showed the consistent length from 300 bp to 400 bp (Figure 1A).

3.2. Construction, identification and sequencing of recombinant plasmid

After enzyme digestion with Nhe Ⅰ and X*ho* Ⅰ, BLC fragment in 327 bp length and pAKM19 liner fragment in 3 400 bp were obtainable in prediction. And there could be two fragments in 1 000 bp and 3 000 bp length, respectively under *Bam* Ⅰ digestion. The analysis exhibited the same result (Figure 1B). While the blast comparison showed amplified cDNA of BLC and the open reading frame of pAKM19 were correct (Figure 2). It proved the successful construction of the recombinant plasmid, named as pUIS3-BLC$^+$.

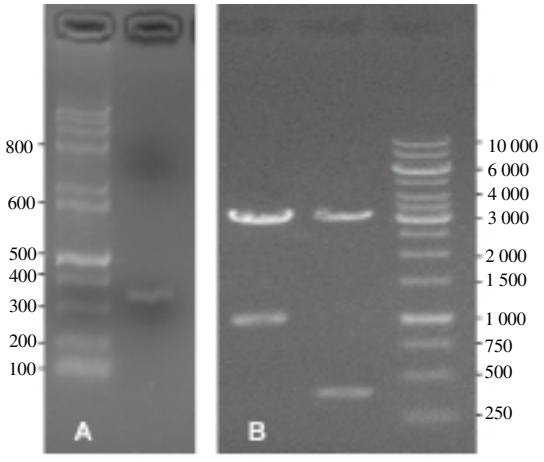

Figure 1. Amplification of BLC by RT-PCR and identification of plasmid pUIS3-BLC$^+$ by enzymatic digestion.

3.3. Identification of BLC mRNA expression in tansfected eukaryocyte by pUIS3-BLC$^+$

RT-PCR revealed that BLC mRNA expression in transfected COS-1 cells by pUIS3-BLC$^+$ were half of β-actin expression, while no expression was available in un-transfected COS-1 cells (Figure 3A). It indicated recombinant plasmid pUIS3-BLC$^+$ had BLC gene expression in eukaryocyte.

3.4. Identification of BLC protein expression in tansfected eukaryocyte by pUIS3-BLC$^+$

Western blotting analysis showed a new protein zone with molecular weight about 9.8 kD only in transfected COS-1

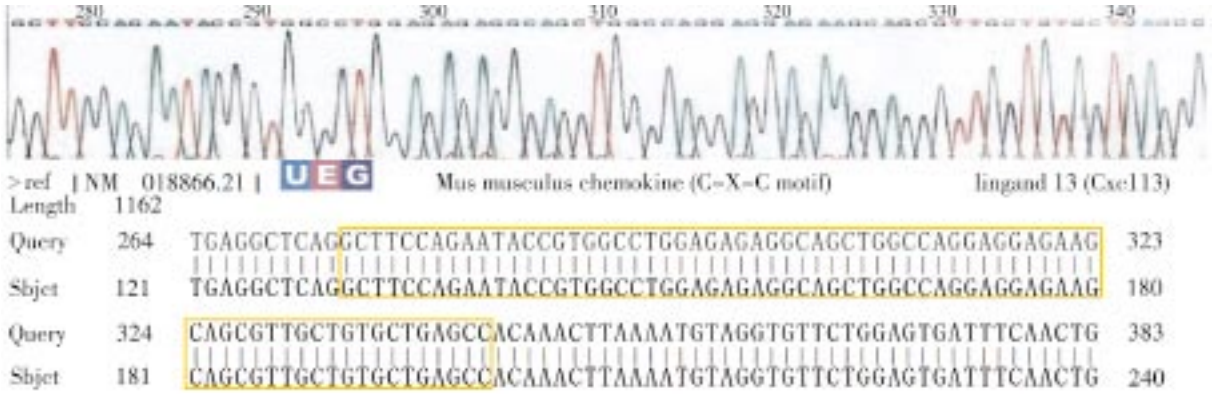

Figure 2. Identification of plasmid pUIS3-BLC$^+$ by sequencing and blasting.

cells (Figure 3B), indicating pUIS3-BLC$^+$ also had BLC protein expression in eukaryocyte.

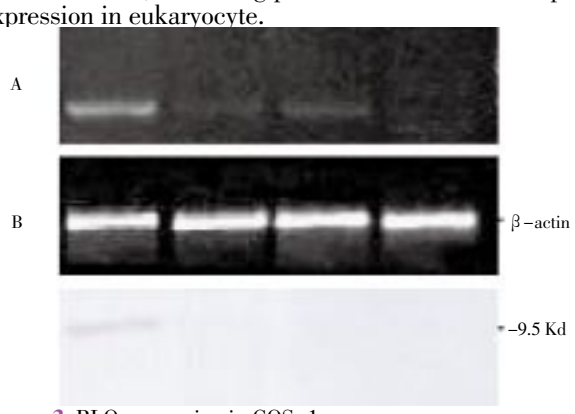

Figure 3. BLO expression in COS-1.
A: RT-PCR, B:Western blotting.

4. Discussion

Chemokines refer to a series of secretory single-chain proteins, which have molecular weight 8-12 kD, at least two CYS conservative sequences and chemotaxis effect on leucocyte[7]. According to amount and location of CYS in primary structure of chemokine, they are classified into four types: CXC chemokine (α, CXCL, SCYA), CC chemokine (β, CCL, SCYB), C chemokine (γ, XCL, SCYC) and CX3C chemokine (δ, CX3CL, SCYD). BLCs belong to CXC type, and come from lymphatic organs including spleen, thymus, lymph node, appendix etc.[8,9]. It is reported BLCs play important role in formation of germinal center and induction of organism into specific immune response[8]. The expression of chemokine in tumor tissue can promote immune response in tumor by autocrine or paracrine, and provide strong and lasting anti-tumor immunity to organism. Nowadays, malaria is a severe disease due to tropical parasites. It leads to over 10 million deaths every year, and brings serious harm into human health[10]. Development of new-style and effective vaccine is the main strategy for eradicating or controlling malaria. However, researches on vaccine against malaria for more than 100 years suggest strengthening intensity and prolonging time of immune response are bottleneck problems in the development. At present, it has been reported that it is possible to delay the growth of *Plasmodium* by inactivating expressive gene during some period with gene engineering technology. Muelar *et al* found that sporozoites of *Plasmodium* with UIS3 gene knockout displayed detoxification action. After infecting animal models, these sporozoites stayed in hepatocells not in red blood cells. They can be used as detoxification vaccine, and have immuno-protective effect on mice with malaria[2]. According to Mueler's study, immunoenhancing BLC gene was transferred into sporozoites of *Plasmodium berghei* with UIS3 gene knockout to constructed recombinant plasmid encoding BLC gene in our study.

BLC gene was amplified and cloned by RT-PCR, then inserted into 3′ end of recombinant plasmid of *Plasmodium berghei* with UIS3 gene knockout. The homologous sequence in the recombinant plasmid guarantees the transgenosis of BLC. After restrictive endonuclease digestion and DNA sequencing, blast comparison proves the correct open reading frame. RT-PCR and Western blotting assay show gene and protein of BLC can be expressed in transfect COS-1 cell, indicating after transferring into *Plasmodium* recombinant plasmid expression is available in infected animal host. This study paves the way for further development of new-style malaria vaccine with detoxification because of UIS3 knockout, and immunoenchancement because of BLC.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* 2005; **434**(7030): 214-7.
[2] Mueller AK, Labaied M, Kappe SH, Matuschewski K. Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. *Nature* 2005; **433**(7022): 164-7.
[3] Legler DE, Loetscher M, Roos RS, Clark-Lewis I, Baggiolini M, Moser B. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CCR5. *J Exp Med* 1998; **87**(4): 655-60.
[4] Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. *Trends Immunology* 2001; **22**(2): 83-7.
[5] Jiao JG, Li YN, Wang H, Liu Q, Cao JX, Bai RZ, et al. A plasmid DNA vaccine encoding the extracellular domain of porcine endoglin induces anti-tumour immune response against self-endoglin-related angiogenesis in two liver cancer models . *Dig Liver Dis* 2006; **38**(8): 578-87.
[6] Hang FY, Li YN, Wang H. A fusion protein containing murine vascular endothelial growth factor and tissue factor induces thrombogenesis and suppression of tumor growth in a colon carcinoma model. *J Zhejiang Univ Sci B* 2008; **9**(8): 602-9.
[7] Moser B, Loetscher P. Lymphocyte traffic control by chemokines. *Nat Immunol* 2001; **2**(2): 123-8.
[8] Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 2000; **406**(6793): 309-14.
[9] Dilloo D, Bacon K, Holden W, Zhong S, Burdach A, Zlotnik M. Combined chemokine and cytokine gene transfer enhances antitumor immunity. *Nat Med* 1996; **2**(10): 1090-5.
[10] Aregawi M, Cibulskis R, Otten M, Williams R, Dye C. *World malaria report 2008*. Geneva: World Health Organization; 2008.