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▣ BRIEF COMMUNICATION

Cloning and Iron Transportation of Nucleotide Binding Domain of *Cryptosporidium andersoni* ATP-Binding Cassette (*CaABC*) Gene

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Abstract: *Cryptosporidium andersoni* ATP-binding cassette (*Ca*ABC) is an important membrane protein involved in substrate transport across the membrane. In this research, the nucleotide binding domain (NBD) of *CaABC* gene was amplified by PCR, and the eukaryotic expression vector of pEGFP-C1-*Ca*NBD was reconstructed. Then, the recombinant plasmid of pEGFP-C1-*Ca*NBD was transformed into the mouse intestinal epithelial cells (IECs) to study the iron transportation function of *Ca*ABC. The results indicated that NBD region of *CaABC* gene can significantly elevate the transport efficiency of Ca^{2+}, Mg^{2+}, K^{+}, and HCO_3^{-} in IECs ($P<0.05$). The significance of this study is to find the ATPase inhibitors for NBD region of *CaABC* gene and to inhibit ATP binding and nutrient transport of *Ca*ABC transporter. Thus, *C. andersoni* will be killed by inhibition of nutrient uptake. This will open up a new way for treatment of cryptosporidiosis.

Key words: *Cryptosporidium andersoni*, cloning, iron transportation, ATP-binding cassette transporter protein

Cryptosporidiosis is a zoonotic parasitic disease caused by *Cryptosporidium* infection [1]. *Cryptosporidium* usually parasitizes the epithelial cells of the gastrointestinal tract in the host, and causes severe diarrhea. So far, *Cryptosporidium* animal models have been established [2], and the treatment of cryptosporidiosis has been studied in vitro [3-6]. However, there is still no effective drug for treating cryptosporidiosis. A possible reason is that *Cryptosporidium* have multidrug ATP-binding cassette (ABC) transporters to inhibit anti-protozoal drugs and to enter the protozoa bodies [7,8]. It was also reported that ABC transporters use the energy of ATP binding and hydrolysis to drive the transport of various substrates across the cell membrane [3-5]. *C. parvum* ABC1 (*Cp*ABC1), *C. parvum* ABC2 (*Cp*ABC2), and *C. parvum* ABC3 (*Cp*ABC3) which have been proved in *C. parvum* can transport different substrates across the cells through the energy of ATP binding and hydrolysis [9-12].

Cryptosporidium andersoni usually parasitizes the ruminants and people [13]. So far, *Ca*ABC for the transportation of nutrients and the characteristics of resistant drugs were not reported. Two transmembrane domains (TMDs) and 2 nucleotide-binding domains (NBDs) constitute the basic architecture of ABC transporters, and TMDs can provide the substrate translocation pathway across the cell membrane, while the NBDs bind and hydrolyze ATP to provide energy for active transport [14,15]. Walker et al. [16] showed that NBD region is in the conservative domain of ABC protein in *C. parvum*. However, there is no research of the existence of NBD region in *Ca*ABC protein and its exact function. In the study, our first objective was to amplify the NBD region of *CaABC* gene and to construct the *Ca*NBD eukaryotic recombinant plasmid. Our second objective was to establish a cell model carrying *Ca*NBD and to study the role of *Ca*NBD. These results will provide the basis for nutrient transport of *Ca*ABC transporter in the presence of ATPase inhibitor.

All of experimental procedures on animals were in accordance with the recommendations of the guidelines of the Chinese Association for Laboratory Animal Science. The feces of cows infected with *C. andersoni* were obtained from a cattle farm in Hefei. *C. andersoni* oocysts were separated and washed 3 times with PBS, and shocked in a vortex mixer 30 min after adding 500 µl oocyst lysate, repeatedly, then freeze-thawed in -70°C 3 times. Genomic DNA of *C. andersoni* was extracted with the DNA extraction kit (Omega, New York, USA) according to the instructions of the manufacturer. The primer of

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NBD region of *CaABC* gene was designed according to Perkins [7]. The primer with promoter ATG, terminator TAA, and enzyme cut sites *Bgl*II and *EcoR*I (TaKaRa, Dalian, China) was synthesized (Sangon Biotech, Shanghai, China). The forward primer was 5′GAAGATCTATGGTAGGTGAAACTGGTAGTGGTAAATCTAC, the reverse primer was 5′CGGAATTCTTAATCTAGAGAAGATGTAGCTTCATC.

Cloning and identification of NBD region of *CaABC* gene were performed by PCR. The product was examined using 1.0% agarose gel electrophoresis and observed with the gel imaging system (BIO-RAD, Hercules, California, USA) and extracted with gel extraction kit (Sangon Biotech, Shanghai, China) according to the instructions of the manufacturer. The product was linked to a pMD19-T clone vector (TaKaRa, Dalian, China), and transformed into *E. coli* DH5α (Sangon Biotech, Shanghai, China). The plasmid of positive colony was extracted by PCR and was sequenced. The constructed clone vector was named as pMD19-T- *Ca*NBD.

The sequencing *Ca*NBD and eukaryotic expression vector pEGFP-C1 were digested by restriction double-enzyme *EcoR*I and *Bgl*II. The products were analyzed by electrophoresis and respectively extracted with gel extraction kit according to the instructions of manufacturer. The target gene was linked to linear pEGFP-C1 vector under the action of T4 DNA ligase (TaKaRa, Dalian, China). After transfection, PCR and sequence identification, the pEGFP-C1-*Ca*NBD recombinant plasmid was obtained.

In transfection experiment, the experiment was divided into 3 groups: transfection group is the IECs that transfected a recombinant plasmid pEGFP-C1-*Ca*NBD; the control group is the IECs that transfected empty plasmid pEGFP-C1; the blank group is IEC cells without plasmid. Before transfection at 24 hr, primary IECs were cultured at 37℃, 5% CO_2 for 18-48 hr in a 24-well plate (1.5×10^5 cells/well) which was coated by 1% gelatin. A density of 80-90% IECs was transfected. Then, the cells were analyzed with the fluorescence microscope (Olympus-CK40, Tokyo, Japan). The medium in 3 groups were collected to analyze the extracellular ions concentration. The cells in 3 groups were digested with 0.25% trypsin, collected, and broken up by ultrasonic cell disruptor (VCX-500, Sonics, Danbury, Connecticut, USA). Intracellular and extracellular ion (Ca^{2+}, Mg^{2+}, K^+, and HCO_3^-) concentrations were analyzed with calcium, magnesium, potassium assay kit, and HCO_3^- reagent

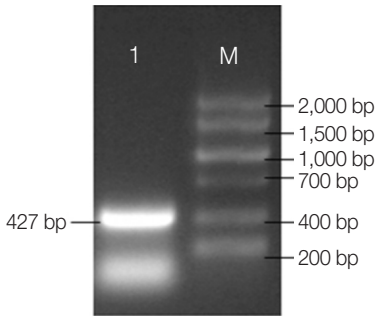

Fig. 1. Electrophoregram of PCR product of NBD region of *CaABC* gene. M: DNA marker; 1: a DNA band of NBD region of *CaABC* gene.

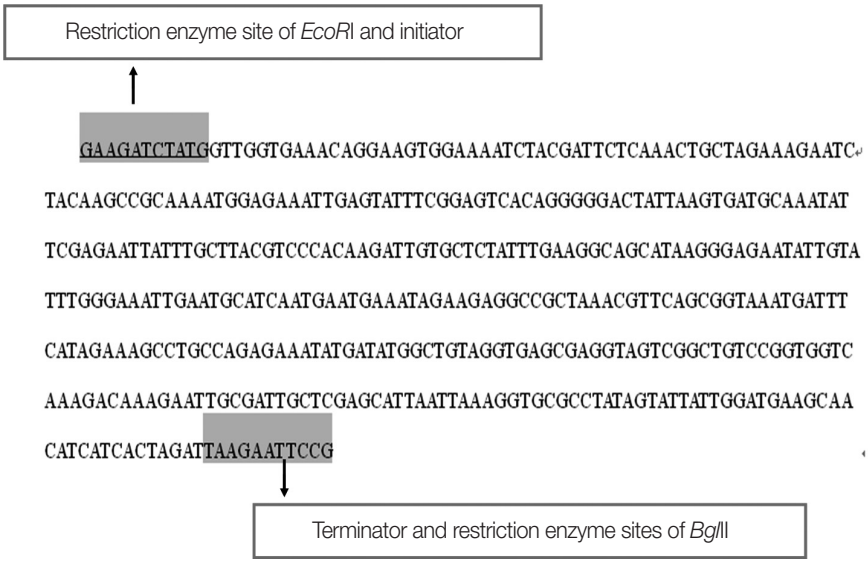

Fig. 2. Sequencing results of PCR product of the NBD region of *CaABC* gene.

kit (Jiancheng Inc., Nanjing, China) according to the instructions of manufacturer by automatic biochemistry analyzer (Hitachi-7060, Tokyo, Japan). The experiments were carried out 3 times. Statistical analyses were conducted using the SPSS version 17 software (Version 17.0; SPSS, Inc., Shanghai, China). All the values were considered significant at $P < 0.05$.

NBD region of *CaABC* gene was amplified by PCR. A DNA band about 427 bp was observed, which was in accordance with the expected result (Fig. 1). It was clear that NBD region of *CaABC* gene was successfully amplified. In order to identify NBD region of *Ca*ABC protein, the product was sequenced and analyzed. The results showed that the purpose fragment was 433 bp (Fig. 2). The restriction enzyme sites, the promoter and terminator were 22 bp. The fragment of NBD region of *CaABC* gene was 411 bp; it was more than 6 bp in comparison with *Plasmodium* glycoprotein (*Pgp1*) gene sequence.

Nucleotide sequences of NBD region of *CaABC* gene were translated into a protein with 137 amino acids: VGETGSGKSTILKLLERIYKPQNGEIEYFGVTGGLLSDANIRELFAYVPQDCALFEGSIRENIVFGKLNASMNEIEEAAKRSAVNDFIESLPEKYDMAVGERGSRLSGGQRQRIAIARALIKGAPIVLLDEATSSLD.

Amino acid sequence of NBD region of *CaABC* gene was compared with the *Pgp1* and *C. parvum* multidrug resistance-associated protein (*Cp*-MRP) by BLAST, respectively. The results showed that 9 amino acids of the Walker A motif in NBD region of *CaABC* gene in N-terminal amino acid sequence and 10 amino acids of Walker B in C-terminal amino acids were the same as *Pgp1* (Fig. 3A) and *Cp*-MRP (Fig. 3B), and a NBD region of *Ca*ABC protein family exist, which is composed of 9 amino acids. Therefore, 411 bp nucleotides can be determined as ATP binding region sequence of *Ca*ABC protein, named as *Ca*NBD.

Two DNA bands, about 4.7 kb and 433 bp, were observed in the study (Fig. 4). The former was the linear plasmid pEGFP-C1, and the latter was a purpose gene, which was in accordance with the expected result. The results proved that the recombinant eukaryotic plasmid pEGFP-C1-*Ca*NBD was suc-

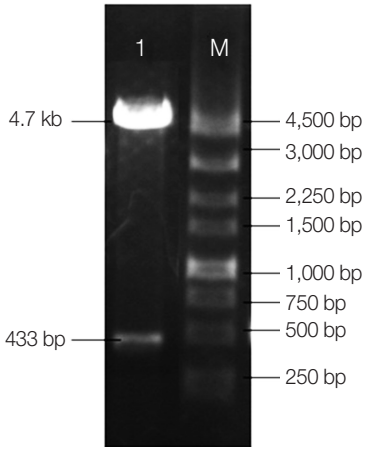

Fig. 4. Electrophoregram of double digestion of pEGFP-C1-*Ca*NBD. M: DNA marker; 1: a DNA band of pEGFP-C1-CaNBD double digestion.

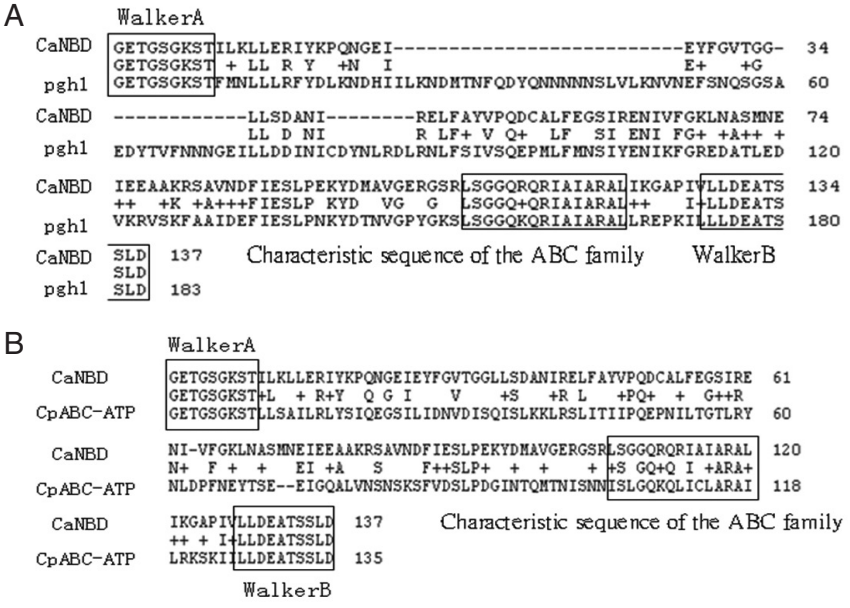

Fig. 3. Amino acid sequences of *Ca*NBD. (A) *Ca*NBD amino acid sequences compared with *Pgp1*. (B) *Ca*NBD amino acid sequences compared with *Cp*-ATP.

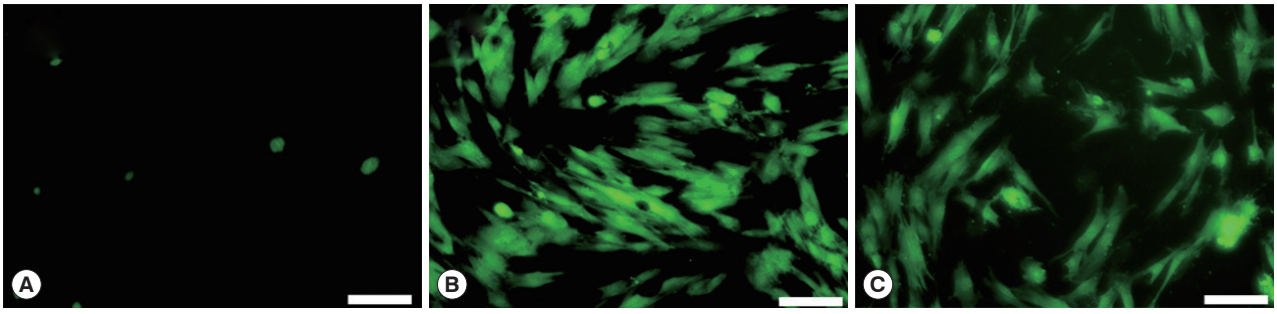

Fig. 5. Expression of EGFP in different IECs groups. (A) The blank group (IECs without plasmid). (B) The control group (IECs transfected with empty plasmid pEGFP-C1). (C) Transfection group (IECs transfected with a recombinant plasmid pEGFP-C1-*Ca*NBD). Bar = 100 μm.

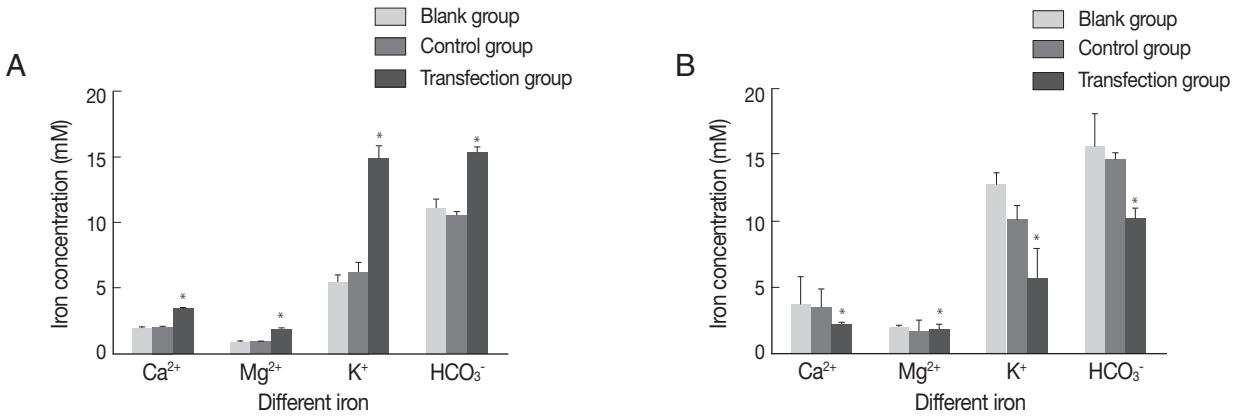

Fig. 6. Change of intracellular and extracellular ions concentration. (A) Change of intracellular ions concentration. (B) Change of extracellular ions concentration. *$P < 0.05$. Error bars represent SEM of 9 repeats.

cessfully constructed.

Mouse IECs with enhanced green fluorescent protein (EGFP) gene in the transfection group (Fig. 5C) and control group (Fig. 5B) were observed after being transfected. Mouse IECs with EGFP gene were more in the control group (Fig. 5B) compared to the transfection group (Fig. 5C). Mouse IECs in blank group (Fig. 5A) was no green fluorescence; however, auto-fluorescence in a small amount of apoptosis IECs was observed. The results showed that green fluorescence was emitted by the EGFP gene from the introduced plasmid. The eukaryotic expression vector pEGFP-C1-*Ca*NBD was successfully transfected into mouse IECs.

The intracellular Ca^{2+}, Mg^{2+}, K^{+}, and HCO_3^- concentrations in the transfection group were significantly elevated in comparison with the black group and control group ($P < 0.05$) (Fig. 6A); however, no significant difference was observed between the black group and control group ($P > 0.05$). A significantly lower level of the extracellular Ca^{2+}, Mg^{2+}, K^{+}, and HCO_3^- concentrations were present in the transfection group ($P < 0.05$) in comparison with the black group and control group (Fig. 6B). Furthermore, the extracellular Ca^{2+}, Mg^{2+}, K^{+}, and HCO_3^- concentrations in the control group were not significantly lower than in the black group ($P > 0.05$).

The ABC proteins generally consisted of 4 regional; 2 hydrophobic transmembrane binding regions and 2 NBDs [14,15]. Walker et al. [16] clarified that the NBDs existed in the conservative area of ABC transporters, which contained 2 main motifs (Walker A and Walker B) and ABC protein characteristic motifs. In this study, 9 amino acids of the Walker A motif in NBD region of *CaABC* gene in N-terminal amino acid sequence and 10 amino acids of Walker B in C-terminal amino acids were the same as *Pgp1* and *Cp*-MRP. The results were in agreement with previous studies. Therefore, the amplification of 411 bp gene is NBD region of *CaABC* gene.

*Cp*ABC protein is transport proteins located in fold structure feeding device membrane of *Cryptosporidium*, which has greatly relation with *Cryptosporidium* nutrient intake and waste drainage [17]. In this study, the recombinant plasmid pEGFP-C1-*Ca*NBD

was constructed, and imported into mouse IECs. Recombinant plasmid pEGFP-C1-*Ca*NBD was successfully imported and expressed in mouse IECs by analyzing the results of recombinant plasmid sequencing and observing IECs fluorescence. The results showed that NBD region of *CaABC* gene could express validly in mouse IECs, and the ABC protein of *Cryptosporidium* research continue to expand through the cell model.

This study also showed the changes of ion concentration in IECs after NBD domain transformation. The mechanism may be that NBD region of *Ca*ABC transporter is responsible for ATP binding and hydrolysis and regulate gated substrate channels. Thus, NBD domain transformation in IECs was used for more ATP binding; gated substrates channels (including ion channel) were widely opened. Hence, the ion concentration in IECs by NBD domain transformation changed. The amplification and expression of NBD region of *CaABC* gene will provide an important basis for ABC protein gene complete sequence amplification and study of nutrient transport and multidrug resistance in IECs. It is expected to find the inhibitor to inhibit ATP binding to NBD region and transport processes with substrates. The eventual purpose is used for drug development and treatment of cryptosporidiosis.

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CONFLICT OF INTEREST

The authors report no conflicts of interest with this study.

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