Cloning human *Benchwarmer* gene (*BNCH*) harboring E164K in vector pcDNA3.1 by site-directed mutagenesis method

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Tóm tắt

Benchwarmer (*BNCH*) gene encodes an orphan transmembrane transporter belonging to the Major Facilitator Superfamily (MFS), facilitating the transport of ions, amino acids, simple sugars and recently lysolipids. The loss of *BNCH* function caused lethality in several animal models with neurodegeneration and senescence. At the cellular level, dysregulation of *BNCH* leads to adverse phenotypes of lysosome and also autophagy (i.e. dyshomeostasis, accumulation of carbohydrates and sphingolipids, and enlarged lysosome). However, the molecular function and ligand of BNCH protein remain to be unrevealed. This study aims to create a radical substitution change in human BNCH coding gene to knock out the protein functions. More specifically, lysine (K) was used to replace the glutamic acid residue 164 (E164K) which is conserved in many animals (fly, zebrafish, mouse and human) and this E164K mutation recapitulated *BNCH* mutant phenotype. In conclusion, *BNCH* harboring E164K (*BNCH**) was successfully produced by site-directed mutagenesis and cloned into pcDNA.3.1 vector. The construct was transformed into *E. coli* OmniMAX and that provides a valuable cell assay to search for the molecular ligand of BNCH.

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Từ khóa
Benchwarmer, plasmid pcDNA3.1, cloning, lysosomal membrane protein.

1 Introduction

Lysosomes are abundant cellular organelles in most eukaryotic cells, which play an important role in intracellular digestion, autophagy, and apoptosis. The functions of lysosomes depend on the presence of different hydrolytic enzymes of which optimal pH values range from 4.5 to 5.0 at the lumen of lysosome [1].

The lysosome is recently characterized as a signaling center for intracellular metabolism regulation through interaction with two kinase complexes mTORC1 and mTORC2 [2]. These two complexes regulate most cellular functions through TOR (target of rapamycin) protein kinase activity, the core enzymatic component of mTORC1 and mTORC2 [3]. Recent studies showed that mTORC1 translocates to the lysosomal surface and recognizes signals from the lysosomal lumen (amino acid/nutrients), thereby transporters in lysosomal membrane involved in cell metabolism and growth through mTORC [4-6]. For example, the Solute Carrier 38A9 transport protein (SLC38A9) was identified to transport arginine and directly activate the mTORC1, thereby regulating gene expression and protein synthesis [5]. Furthermore, various transport proteins on the lysosome membrane play a key role in the lysosome function, which supports a hypothesis that hydrolyzed products from lysosome (i.e. amino acid, carbohydrate, lipid, and nutrients) could be signals for mTOR complexes to regulate many essential functions of the cell. Remarkably, the lysosomal membrane proteins are important targets for the treatment of many diseases (i.e. lysosomal storage disorders and cancers) [7,8].

Mutated *BNCH* significantly increased the number of sphingolipids (ceramide and sphingosine) in fruit fly larvae, which suggests an essential role of *BNCH* gene in regulating sphingolipids metabolism [8]. Mutated *BNCH* greatly affected the survival of organisms, especially in the early stage of embryonic development [9]. Our unpublished data indicate that dysfunctional *BNCH* results in the deregulation of many nutrient transporters (i.e., glucose, lactate, and amino acids) in a human cell line. Furthermore, these nutrient transporters were regulated by mTOR and

ceramide biosynthesis [10,11]. These findings support a hypothesis that BNCH transports a key sphingolipid from the lumen to the cytoplasm and that transport potentially participates in regulating mTOR function.

The 164th codon of *BNCH* coding gene is conserved in various organisms (i.e., fruit flies, yeast, zebrafish, mice and human), suggesting its essential for the protein function [6]. In fruit flies, mutation of E217K (equal to the E164K mutation in human) resulted in accumulation of carbohydrate in lysosome which caused endocytic defects synaptic cell, progressive loss of function of neurons, and enhanced tau-mediated toxicity [8]. These findings suggest an important role of glutamic acid 164 in the human BNCH protein.

Identifying the functional transporters on the lysosomal membrane and more understanding of the interaction between the BNCH and the mTOR complex have revealed how the lysosome adapts to environmental cues through mTORC and provides a novel therapeutic approach for adjusting lysosomal function in human disease.

In this study, to produce a tool for investigating BNCH function, we aim to construct a loss of BNCH function version of human *BNCH* gene by introducing the radical substitution E164K. A rapid and straightforward PCR based mutagenesis method was employed to introduce E164K mutation in BNCH gene accurately. The mutant *BNCH* coding sequence was cloned into a mammalian expression vector pcDNA3.1 by restriction digestion and ligation. The produced material will be used to develop a cell-based assay to determine the target ligand of BNCH transporter.

2 Materials and Methods

2.1 Materials

The sequence of *BNCH* was retrieved from NCBI (accession number: NC_000016.10) and synthesized from Addgene (USA). Vector pDNOR322 containing *BNCH* cDNA sequence was used as a template to generate mutation in the target gene and was provided from Addgene (MA, USA). *E. coli* OmniMAX was provided by the Microbiology Department, University of Science (Vietnam National University Ho Chi Minh City) and was used to replicate recombinant vector.

Restriction enzymes (*Nde*I, *Hind*III, *Eco*RV and *Xho*I), DNA T4 ligase, Monarch® Plasmid Miniprep Kit, Phusion® Hot Start Flex DNA Polymerase kit, and OneTaq DNA polymerase kit were purchased from New England Biolab (UK). The culture media include LB-Amp medium (peptone 10 g/L, yeast extract: 5 g/L, NaCl: 5 g/L, pH = 7.0, 10 µg/mL ampicillin) and LB-Amp agar medium (LB media supplement with 1.6 % agar).

2.2 Generating E164K mutation in BNCH using PCR-based site-directed mutagenesis

The wild type *BNCH* coding sequence was previously cloned into plasmid pcDNA3.1 at *Hind*III and *Eco*RV enzymes engineered on primer A and B, respectively (as depicted in Figure 1). Method for mutagenesis was described elsewhere [12]. In more detail, the E164K was introduced in *BNCH* (*BNCH** formation procession consisted of two stages and amplified by a combination of four primers:

(A)-ttttttAAGCTTCACCATGGCCGGGTCCGAC
*Hind*III
(B)-GGGGTCGGGAAGGCCAGTTATTCCAC
Codon harboring mutation (E164K)
(C)-GTGGAATAACTGGCCTTCCCGACCCC
Codon harboring mutation (E164K)
(D)-ttttttGATATCGATGAGCACACTGGCCACGG
*Eco*RV

Primer A and B were designed to complement 3' and 5' end of *BNCH* cDNA sequence. The recognition sites for *Hind*III in primer A and *Eco*RV in primer D were compatible with those in multiple cloning sites in pcDNA3.1. Meanwhile, primer B and C are reversed complemented to each other and were designed to span E164 codon (GAG) but carry a mismatch (G to A) to introduce lysine codon (AAG) during PCR.

Stage 1: Two separate PCR assays were performed with primer pair (A and C) and primer pair (B and D) to amplify the two half of full-length *BNCH* coding sequence. PCR was performed as follow: 200 ng of the template (pcDNA3.1 carrying wild type BNCH cDNA sequence), 1.25 µL each primer (10 µM), 10 mM dNTPs, 5 µL 5X buffer, 1-unit DNA Polymerase (Phusion® Hot Start Flex DNA Polymerase) and nuclease-free water up to 50 µl. Amplification conditions were as follows: 30 s at 98 ^{0}C, followed by 35 cycles of 98 ^{0}C for 10s, 61 ^{0}C for 40 s, 72 ^{0}C for 30 s, and a final extension at 72 ^{0}C for 10 min.

The two PCR products overlap at the sequence of primer B and C and carry lysine codon instead of the glutamic acid codon for amino acid 164. PCR products were purified and used for the second PCR stage.

Stage 2: In this PCR, the two purified PCR products from stage 1, mixed with primer A and D, were used in a single PCR to obtain the full-length of mutated BNCH (BNCH*) cDNA product. In detail, 200 ng each of PCR products, 1.25 µL each primer (10 µM), 10 mM dNTPs, 5 µL 5X buffer, 1-unit DNA Polymerase (Phusion® Hot Start Flex DNA Polymerase) and nuclease free water up to 50 µL. Amplification condition was similar to stage 1. Gel agarose electrophoresis was used to check the PCR products. The bands of correct size PCR products were extracted from gel and used for the next experiment.

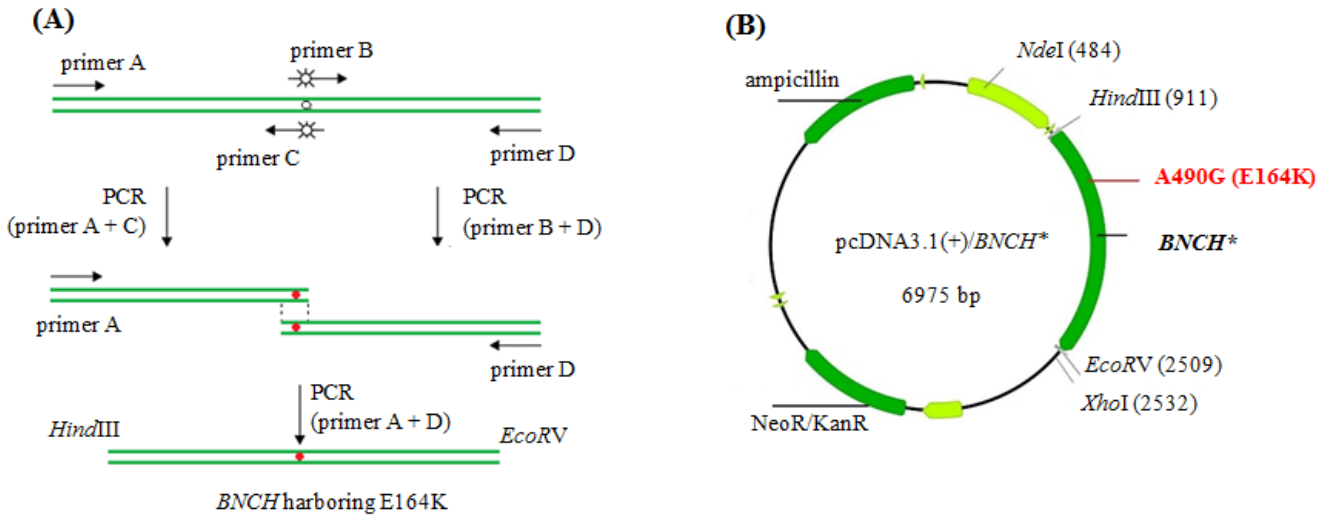

Figure *1* (A) Diagram of site-directed mutagenesis by overlap extension PCR. The small dots indicate the site of mutagenesis. Horizontal arrows are primers used for PCR. The line symbols for the target genes;
(B) Schematic of the pcDNA3.1 expression vector.

2.3 Construction of plasmid pcDNA3.1/*BNCH** and transformation into *E. coli* OmniMAX

Vector pcDNA3.1 is a 5428 bp plasmid that is commonly used to express the recombinant gene in a wide range of mammalian cell systems. The plasmid also harbors an ampicillin resistance gene which allows selection of positive bacteria during the cloning process.

The PCR product of *BNCH** and pcDNA3.1 were digested by *Hind*III and *Eco*RV. The insertion of *BNCH** into pcDNA3.1 was performed using T4 DNA ligase. The ligation products were transformed into *E. coli* OmniMAX via heat shock method. The mixture of the ligation and *E. coli* OmniMAX was incubated at 42 ^{0}C for 1 minute and cooled quickly in ice for 1 min. In the recovery step, the transformed bacteria were cultured on LB medium at 37 ^{0}C for 1 h before being cultured overnight on LB agar plate supplement with ampicillin (100 µg/mL) for selecting plasmid–containing colonies.

2.4 Colony analysis

Colony-PCR was conducted to detect the presence of recombinant *BNCH* in the bacterial cells. The PCR mixture contained 200 ng template, 0.5 µL of 10 µM each primer, 10 mM dNTPs, 5 µL buffer (5X), 1.25 units DNA Polymerase (OneTaq DNA Polymerase, New England Biolabs) and nuclease-free water up to 25 µL. Thermocycling condition was: 30 s at 94 ^{0}C, followed by 35 cycles of 94 ^{0}C for 15 s, 61 ^{0}C for 15 s, 68 ^{0}C for 15 s, and a final extension at 68 ^{0}C for 30 s. The primes were Bnch-F (5'-CTCATTGGCCTGATCTCTGAC-3') and Bnch-F (5'-CCTCAAGAAGATGGCGGTGTTCCAC-3'). Agarose gel electrophoresis was then used to analyze the PCR products.

2.5 Sequence analysis

Selected colonies with the correct size in the previous PCR experiment were grown in LB-ampicillin broth. The cell biomass was then collected to extract the plasmid DNA using Monarch Plasmid Miniprep Kit (New England Biolab).

Restriction digestion by *Nde*I and *Xho*I enzymes followed by agarose gel electrophoresis was used to confirm the plasmids carrying recombinant *BNCH**. Positive plasmids were sequenced by the Sanger method using CMV-F primer, a universal primer in pcDNA3.1 by 1st Base (Singapore). Nucleotide sequences were aligned with *BNCH* sequence to determine mutation by using Geneious Prime 2020.1.2 (https://www.geneious.com).

3 Results

3.1 Generation E164K mutation by PCR-based Site-directed mutagenesis method

The recombinant *BNCH** was successfully generated by the site-directed mutagenesis method from plasmid pcDNA3.1 carrying *BNCH* gene (wild type) (pcDNA3.1/*BNCH*). Firstly, a mutation on the *BNCH* gene by using primers A and C amplifies the PCR product size, as shown in Figure 2 (lane 1). Similarly, primers B and D were used to amplify a 1107 bp product (lane 2). Amplified products were detected on 1 % gel agarose with single sharp bands of the expected size (Fig. 2). These products were then ligated and amplified by using primer A & D (Fig. 2, lane 3). The final amplification product was purified before inserted into vector pcDNA3.1.

The amplification product covers full length (1587 bp) of *BNCH* gene (Fig. 2). Fragment *BNCH** and vector pcDNA3.1 were digested using *Hind*III and *Eco*RV. After digesting, they were ligated by T4-DNA ligase. The ligation product was transformed into *E. coli* OmniMAX, cultured on LB-Amp agar medium to screen for colonies carrying vector pcDNA3.1/*BNCH**. Recombinant plasmid pcDNA3.1/*BNCH** was extracted and examined by colony PCR and enzyme digestion.

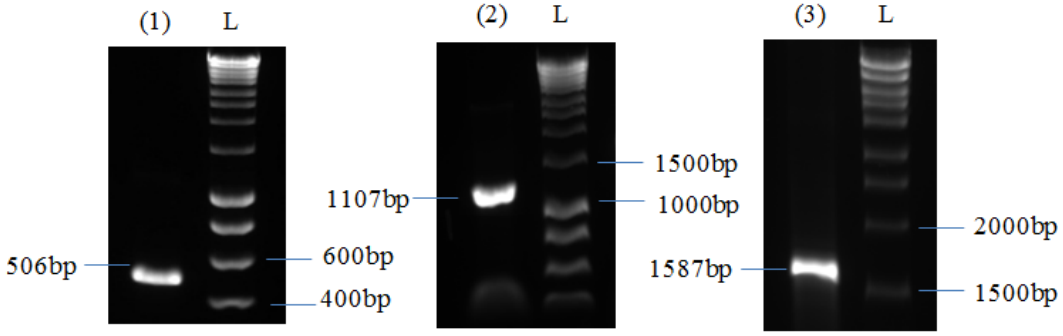

Figure 2 Electrophoresis of PCR products. Lane 1: PCR product using primer A & C; Lane 2: primer B & D; lane 3: primer A & D with purified PCR products from land 1 and 2 as templates to produce full length *BNCH**. L: 1kb DNA ladder.

3.2 Validation of pcDNA3.1/*BNCH** by colony PCR and restriction enzyme digestion

Three different methods determined cloning accuracy for the construction of plasmid pcDNA3.1/BNCH. First, colony PCR was performed on a panel of randomly selected colonies, followed by agarose gel electrophoresis analysis. The appearance of the band with size 151 bp implied that plasmid pcDNA3.1/*BNCH* was successfully constructed and transformed into *E. coli* OmniMAX (Fig. 3).

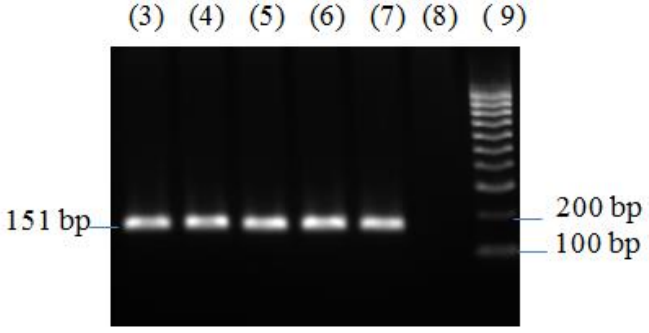

Figure 3 An agarose gel electrophoresis of Colony PCR products. Note: (3) – Positive control; (4) – (7): PCR product from five colonies; (8) – Negative control; (9) – 100bp DNA ladder.

Restriction digestion and gel analysis were then performed to assess correctly assembled plasmids. Based on the restriction enzyme map of pcDNA.1/*BNCH** (Fig. 1), the restriction enzymes *Nde*I and *Xho*I were used to digest pcDNA3.1/*BNCH** to confirm the insertion of *BNCH**. Each of the enzymes has only one restriction site in plasmid pcDNA3.1 and has no restriction site in the *BNCH* gene. Enzyme digestion result of plasmid position 484 and position 2532, respectively) yielded two products of 2048 bp and 4927 bp in length (Fig. 4). This result indicates that the target fragment was correctly inserted into pcDNA3.1.

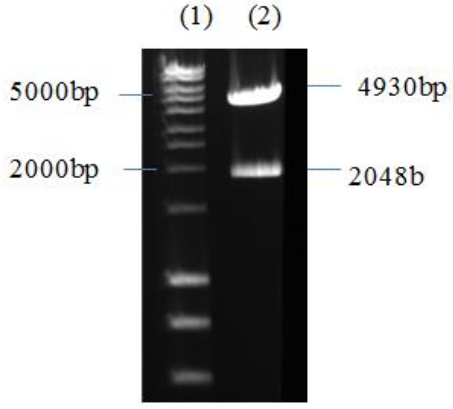

Figure 4 Restriction digestion analysis of pcDNA3.1/*BNCH**. Lanes 1: 1kb DNA ladder (Bioline); Lane 2: recombinant plasmid digested with by *Nde*I and *Xho*I enzyme.

3.3 Validation of pcDNA3.1/*BNCH** by sequencing

To confirm the E164K mutation was successfully introduced, plasmids were extracted and sequenced to examine the point mutation by Sanger sequencing. Sequencing result revealed a change of nucleotide from G → A at position 490 which caused a change from glutamic acid to lysine on amino acid residues 164 of BNCH protein (Fig. 5).

We have successfully cloned plasmid pcDNA3.1/ *BNCH* harboring E164K mutation into the *E. coli* OmniMAX. This is confirmed by using difference methods including restriction enzymes digestion, Colony PCR and Sanger sequencing.

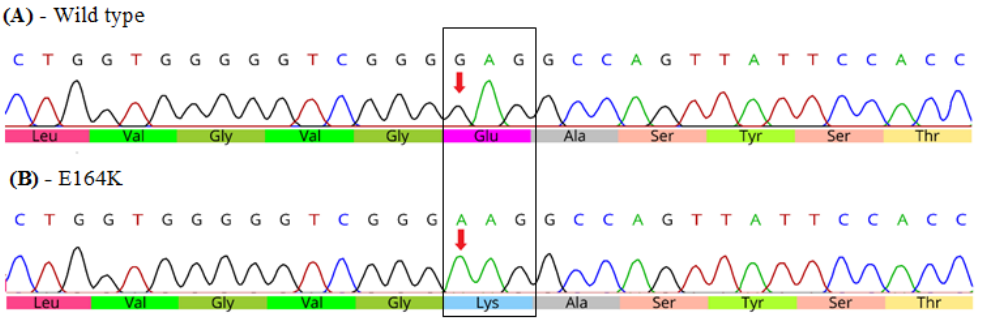

Figure 5 (A) Sanger sequencing of plasmid pcDNA3.1/BNCH wild type and (B) plasmid pcDNA3.1/BNCH harboring E164K mutation.Discussion and conclusion

BNCH was firstly reported and identified in *Drosophila* in 1996 [13] as the name *spinster* or *diphthong* [14-16]. In *Drosophila*, dysfunction of *BNCH* had defects in courtship behaviour, reduced adult life span, decreased neuronal apoptosis in the central neutral system of larvae [15]. In third instar larvae, loss of *BNCH* causes over-growth neuromuscular synaptic that increases TGF-beta signalling [16]. Overexpression of the human *BNCH* in HEK293 cell line resulted in autophagic cell death and necrosis [17], and loss of the homologue in Zebrafish led to embryonic lethality by the accumulation of substances in the yolk [18]. These findings have shown the functions of *BNCH* in nerve cell growth, apoptosis, and late endosome/lysosome. However, its role in mTORC pathway and sphingolipid metabolism remains uncharacterized.

BNCH containing the E217K mutation in *Drosophila* (E164K in human) was firstly mentioned in 2005 [8]. To date, different methods have generated mutation (i.e., CRISPR/Cas9 and site-directed mutagenesis). Site-directed mutagenesis still represents a powerful technique that allows selective engineering of gene sequences and has led to rapid advances in understanding gene expression and function. Using the PCR-based method with the availability of comfortable designed, synthetic-based on double-stranded DNA can reduce the time and steps required to obtain the same sequence changes. Moreover, the technique allows for rapid conducts at low cost due to no need for expensive or unique plasmids and DNA polymerases. This method is still the best option in analyzing the role of different amino acids in a protein's function and structure [12].

In our study, the vector pcDNA3.1(+)/*BNCH** was designed and cloned using a prokaryote system. Cloning accuracy was confirmed by colony-PCR, enzyme digestion, and sequencing. In colony PCR, the size of PCR products was amplified by primers specific for the *BNCH* gene. Enzyme digestion results showed that the fragments separated from the vector and *BNCH* gene. Finally, DNA sequencing with CMV-F primers confirmed the cloning. In previous study, we constructed vector pcDNA3.1/*BNCH* (wild type). In the future experiments, we are going to express protein BNCH (include both mutation and wild type) in mammalian cell lines to evaluate the effect of E164K on the level and pattern expression of the BNCH protein.

To summarize, we have successfully constructed the plasmid pcDNA3.1/*BNCH* harboring the E164K mutation. It provides a valuable tool for future research to deorphanize the role of *BNCH*, including its molecular ligand and its physiological function in lysosomal storage diseases.

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Tạo dòng gene *Benchwarmer* (*BNCH*) mang đột biến Glutamic acid 164 thành Lysine (E164K) trên vector pcDNA3.1

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Tóm tắt Gen *Benchwarmer* (*BNCH*) mã hóa protein vận chuyển xuyên màng thuộc họ protein Major Facilitator Superfamily. BNCH vận chuyển các ion, axit amin, đường đơn và lysolipid. Mất chức năng protein BNCH gây chết ở một số mô hình động vật do thoái hóa thần kinh và lão hóa sớm. Ở cấp độ tế bào, rối loạn chức năng gene *BNCH* dẫn đến các kiểu hình bất lợi ở lysosome và vì thế ảnh hưởng đến quá trình tự thực (bao gồm rối loạn cân bằng nội môi, tích tụ carbohydrate và sphingolipid khiến lysosome phình to). Tuy nhiên, chức năng phân tử của protein BNCH vẫn chưa được hiểu rõ. Trong nghiên cứu này, chúng tôi nhân dòng gene *BNCH* mang đột biến làm mất chức năng protein BNCH, để tạo nguyên liệu sử dụng cho nghiên cứu chức năng của BNCH. Cụ thể, codon 164 mã hóa axit glutamic 164 sẽ được thay thế bằng lysine (K) (E164K). Vị trí E164 trên gene *BNCH* được bảo tồn ở nhiều loài động vật (bao gồm ruồi, cá ngựa vằn, chuột và người) cho thấy axit amin này cần thiết về mặt chức năng. Gene *BNCH* mang đột biến E164K (*BNCH**) đã được tạo ra thành công bằng phương pháp gây đột biến hướng điểm và được gắn vào vector cDNA 3.1 để biểu hiện ở tế bào động vật. Plasmid pcDNA3.1 mang *BNCH** đột biến và *BNCH* kiểu dại sẽ cung cấp nguyên liệu để tìm kiếm chức năng phân tử của protein BNCH trong tương lai.

Từ khóa *Benchwarmer*, plasmid pcDNA3.1, tạo dòng, protein trên màng lysosome.