

## Cloning, Expression, and Dimer formation Assay of GANAB cDNA in Lung Cancer Cells

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### Keywords:

Glucosidase II; Misfolded; cDNA

## 1. Abstract

**1.1. Background:** Glucosidase II is an endoplasmic reticulum heterodimer enzyme, a neutral glucosidase, involved in the trading and folding of newly synthesized glycoproteins in the endoplasmic reticulum. The destruction of GANAB leads to the accumulation of misfolded glycoproteins and the induction of unfolded protein responses. GANAB is also a key regulator of glycosylation. The encoded GANAB protein has an increased expression level in lung tumor tissues, but its function in lung cancer is not yet clear. The cloned sequence is the mutant gene of GANAB gene (NM\_198334.3) in the Genbank database, and its role in lung cancer.

**1.2. Methods:** In this study, the GANAB gene was obtained by homologous cloning, and the correct gene sequence was verified by sequencing by bioinformatics analysis; and the cDNA of the GANAB gene was cloned by RT-PCR. Aiming at high expression level, use the T base of pMD19-T Vector and the A base of the RT-PCR amplified cDNA to match the GANAB cDNA, and use the pcDNA3.1 plasmid to construct a high expression GANAB plasmid, and then use the sensory cell plasmid to purify. Western blot analysis was used to confirm the expression of the protein.

**1.3. Results:** The cDNA fragments of 1.8 kbp and 1.0 kbp were isolated by RT-PCR experiment. Use pMD19-T Vector to connect the GANAB cDNA fragment to construct a 5.5 kbp cDNA sequence, and use the pcDNA3.1 plasmid to construct a GANAB plasmid to obtain a 8.2 kbp cDNA sequence. Western blot analysis confirmed the expression of the protein.

**1.4. Conclusion:** In this study, we cloned the GANAB gene in

A549 cells and successfully introduced the forced expression into A549 cells. The cloning and protein expression of GANAB gene are of great significance to the study of the key effects of lung cancer adhesion, invasion and metastasis.

## 2. Introduction

Neutral alpha-glucosidase AB (GANAB), an enzyme encoded by the GANAB gene in the human body. The alpha subunit glucosidase II encoded by this gene is a member of the 31 group of glycosyl hydrolases. Cytogenetic location of GANAB gene: 11q12.3, the heterodimeric GANAB separates glucose residues from immature glycoproteins in the endoplasmic reticulum, and plays a role in protein folding and quality control [1,2]. The encoded GANAB protein has an increased expression level in lung tumor tissues and reacts under ultraviolet radiation, and mutations in this gene can cause polycystic kidney and liver diseases. Related pathways of GANAB gene include protein metabolism, transport to the Golgi apparatus, and so on [3]. The gene ontology related to this gene is annotated with hydrolase activity, hydrolysis of carbonyl compounds and so on. An important homolog of this gene is glucosidase II $\beta$ , which is encoded by GANC [4]. The two subunits of GANAB and GANC play an important role in the completion of the normal function of glucosidase II. The GenBank database shows that there are 10 mutants in the GANAB gene. GANAB is related to the occurrence of lung cancer by negatively regulating the migration and invasion of cells. This down-regulation further supports the correlation between GANAB expression and the aggressiveness and low survival rate of lung cancer. Since the N-glycosylation of a variety of cellular proteins may change the invasion and metastasis ability of tumors, GANAB may participate in the

occurrence of lung cancer by regulating the N-glycosylation of specific target proteins [5,6].

Most secreted proteins and transmembrane proteins translocate in the endoplasmic reticulum, and undergo protein folding process and quality control in the endoplasmic reticulum. Glucosidase II is involved in glycoprotein processing and promotes protein folding by catalyzing the hydrolysis of glucose residues in glycans bound to proteins [7,8].

A common feature of malignant tumors is that the glycosylation modification of cells has undergone significant changes. In the process of tumor occurrence and development, the glycosylation modification of certain proteins will change with the progress of the disease. The change of glycosylation form of glycoprotein on the surface of tumor cells plays an important role in the process of tumor cell adhesion, invasion and metastasis. Therefore, searching for the characteristic changes and functional research of GANAB gene can provide important information for the early diagnosis, progress monitoring, prognostic evaluation and the search for therapeutic targets of lung cancer[9,10]. The study of GANAB on the changes of the characteristic sugar chain structure of lung cancer cell membrane and its regulation mechanism, this research has important value for the clinical diagnosis and treatment of lung cancer.

### 3. Materials and Methods

#### 3.1. Plasmids

pcDNA3.1 Vector is a gift from Professor OKIO HINO (Juntendo University, Japan) and pMD19-T Vector is a gift from Professor Zhang Chenyu.

#### 3.2. Cloning of GANAB specific fragments

**3.2.1. Extraction of total RNA from A549 cells, 1. PCR amplification of GANAB cDNA and purification and recovery:** The total RNA of A549 cells was extracted using the Trizol extraction kit from Invitrogen. The extracted RNA was analyzed by formaldehyde denaturing gel electrophoresis and quantitative analysis.

In this experiment, AMV reverse transcription kit was used to synthesize cDNA from mRNA extracted from A549 cells. Primer primer 6.0 software was used to design primers. The primers were designed according to the cDNA (NM\_198334.3) sequence of the GANAB gene published in the Genbank database. Both ends of the primer are added with restriction bases for restriction site and restriction site respectively. The designed pair of primers are as follows: P1 fragment upstream primer: 5'-AACTCTGCAC AAGATGGCGG-3'; Downstream primer: 5'-GTTAGCCAC-CAGGCCCT-3'. The upstream primer of the P2 fragment: 5'-AGGGCCTGGTGGGCTAAC-3'; the downstream primer: 5'-AACTCTGCAC AAGATGGCGG-3'. The primers were synthesized by Beijing SBS Genetech Co., Ltd.

The synthesized first-strand cDNA is used as a template, and the

designed P1 and P2 are used as primers for amplification. The PCR amplification conditions were: 94°C pre-denaturation for 1 min, 94°C for 30s, 55°C for 30s, 72°C for 3min, 35 cycles. Take 5µL of PCR product and perform 1% agarose gel electrophoresis under 100V voltage for 40 min. DNA bands of corresponding sizes are excised from the gel, digested, purified, electrophoresed and quantitatively analyzed. Then the P1 cDNA fragment was digested with HindIII and NsbI enzymes. The P2 cDNA fragment was digested with NsbI and NSacII enzymes, and then electrophoresed on a 1% agarose gel. The corresponding size DNA bands were excised from the gel, digested, purified, electrophoresed and quantitatively analyzed.

**3.2.2. Connection of PCR product and pMD19-T vector:** The recovered and purified PCR products P1 and P2 were cloned into pMD19-T vector to construct recombinant plasmids. The constructed plasmid was introduced into the sensory cells, and then cultured with 200µg/ml Amp cold day medium to select the plasmid-introduced sensory cells, and cultured overnight. Then the DNA is extracted and the extracted recombinant plasmid is used as a template and P1 and P2 are used as primers for PCR amplification and identification. The recombinant plasmid was entrusted to Shanghai Sangon Biotech Co., Ltd. for sequencing and identification. pMD19-T Vector Primer: F: 5'-CGCCAGG-GTTTTCCCAGTC-3'; R: 5'-GAGCGGATAACAATTTCCACACAGGAAA-3'. The sequences of all structures were confirmed by Sanger sequencing.

**3.2.3. Construction of pcDNA3.1-GANAB DNA plasmid:** Total DNA was extracted from A549 lung cancer cells, and then GANAB cDNA fragments were cloned and purified using GANAB's specific Primer. In the second step, use pMD19-T Vector to insert the cloned GANAB cDNA fragment into the EcoR V enzyme digestion position, and then use the sensory cell dh5α to amplify the recombinant GANAB expression plasmid, and then perform gel electrophoresis to extract from the gel The P1 fragment was digested with HindIII and NsbI digestive enzymes; the P2 fragment was extracted from the gel and then digested with NsbI and SacII digestive enzymes. At the same time, pcDNA3.1 plasmid was digested by HindIII and SacII. Then, insert P1 and P2 GANAB cDNA between the Hind III and SacII digestive enzyme sites of pcDNA3.1 Vector. Amplify the recombinant GANAB expression plasmid with DH5α (Introin in the United States) of the constructed plasmid, and use specific primers to sequence it firmly. The primers are as follows: F: 5'-CGGAATTCTACTCTATATACATGCTCCATCC-3', R: 5'-CGTCTAGATCACAGACCAA-GAGAGAAAGGAAAC-3'. The sequences of all structures were confirmed by Sanger sequencing.

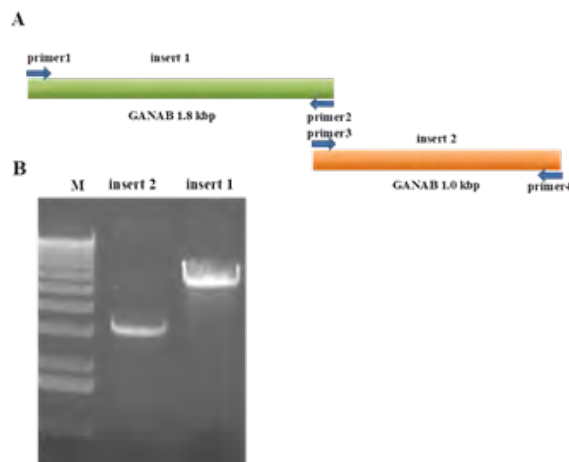
**3.2.4. Verification of the protein expression of the constructed plasmid:** A549 cells are seeded in a 6-well plate at a concentration of 106 cells/well, and transfected when the cells have grown

to 30%-50%. The experiment is divided into 2 groups: pcDNA3.1-GANAB group and mock group. Prepare two sterile 1.5 ml EP tubes, add 250  $\mu$ l RPMI1640 medium and 5  $\mu$ l lipofectamin 2000 to each; then, add 5  $\mu$ l pcDNA3.1-GANAB to one EP tube, and mock plasmid to the other EP tube, and mix them well at room temperature. After standing for 5 minutes, transfer to 4°C for storage. Then discard the supernatant of the cultured cells in the 6-well plate, add 1ml of serum-free RPMI1640 medium to each well, then add the prepared plasmid mixture to each well, mix it, and incubate at 37°C, 5% CO<sub>2</sub>. Cultivate in the box for 4-6h. Discard the transfection solution, add 1.5ml of RPMI1640 medium containing 10% FBS to each well and incubate for 48 hours, wash twice with 1 $\times$ PBS, add 750 $\mu$ l RIPA buffer to fully lyse; then use western blotting to detect the expression of GANAB protein. Condition. (Primary antibody: Secondary antibody:)

## 4. Results

### 4.1. PCR amplification of human GANAB gene cDNA

Synthesize the cDNA of human GANAB gene with the GANAB gene (NM\_198334.3) sequence in Genbank as a template. In this experiment, the cDNA of the GANAB gene was divided into two fragments for cloning, namely the P1 fragment and the P2 fragment. After using 1% agarose gel electrophoresis to excise the 1800bp and 1000bp specific amplified bands, the size of our amplified GANAB cDNA is 2834 bp (Figure 1).



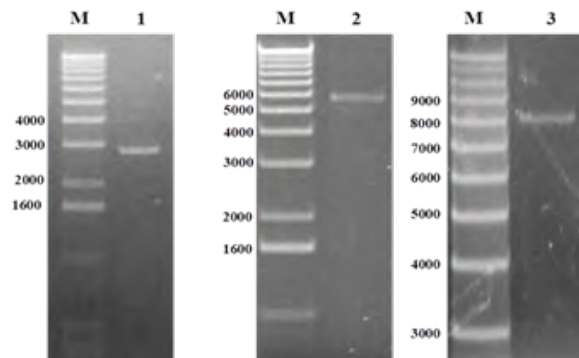
**Figure 1:** PCR amplification of ganab cDNA sequence. A. The ganab cDNA sequence is divided into insert 1 and insert 2. B. Results of PCR amplification of ganab cDNA sequence insert 1 and insert 2.

### 4.2. Cloning of pMD19-T GANAB plasmid

The amplified GANAB cDNA P1 and P2 fragments were cloned using pMD19-T plasmid. Sequencing verification using T vector specific primers. After the ligation is successful, the plasmid is digested with EcoR V enzyme, and after electrophoresis on a 1% agarose gel, there should be a single DNA band around 3.8 kbp and 2.8 kbp. Then use a DNA sequencer to sequence the results consistent with the involved sequence. It is proved that the GANAB gene cDNA P1 and P2 fragments are successfully connected with the T vector (Figure 2).

### 4.3. Construction of GANAB expression vector

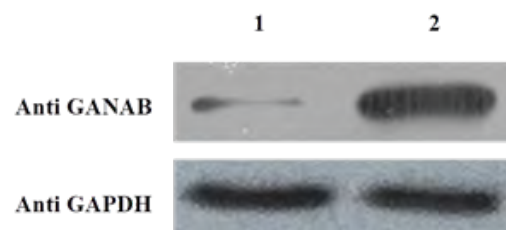
Total RNA was extracted from A549 cells, and specific primers were used to amplify the P1 and P2 fragments of GANAB cDNA, and then the P1 and P2 fragments were inserted into the EcoR V site of the pMD19-T plasmid. The GANAB coding sequence was cut out and cloned into the Hind III/Sac II site of pcDNA3.1. In order to establish the correct reading frame, the resulting plasmid was digested with Sac II, and then Hind III/NsbI digested GANAB cDNA P1 and NsbI/Sac II digested GANAB cDNA P2 fragments were added to reconnect. This plasmid is called pcDNA3.1-GANAB. The constructed plasmid pcDNA3.1-GANAB was digested and cut with Hind III enzyme, and a 8.2 kbp fragment was observed by 1% agarose gel electrophoresis. Then use a DNA sequencer to sequence the results consistent with the involved sequence. Prove that the construction of pcDNA3.1-GANAB was successful (Figure 2).



**Figure 2:** M: Marker X; 1: GANAB cDNA insert 2.8 kbp; 2: pMD19-T Vector + GANAB 5.5 kbp; 3: pcDNA3.1 GANAB plasmid 8.2 kbp.

### 4.4. Construction of the protein expression of the plasmid

A549 cells were transfected with pcDNA3.1-GANAB plasmid and mock plasmid. Incubate in a 37°C, 5% CO<sub>2</sub> incubator for 4-6 hours. Discard the transfection solution, add 1.5ml of RPMI-1640 medium containing 10% FBS to each well and incubate for 48 hours, wash twice with 1 $\times$ PBS, add 750 $\mu$ l RIPA buffer to fully lyse; then use western blotting to detect the expression of GANAB protein. Condition. (Primary antibody: Secondary antibody:.) (Figure 3).



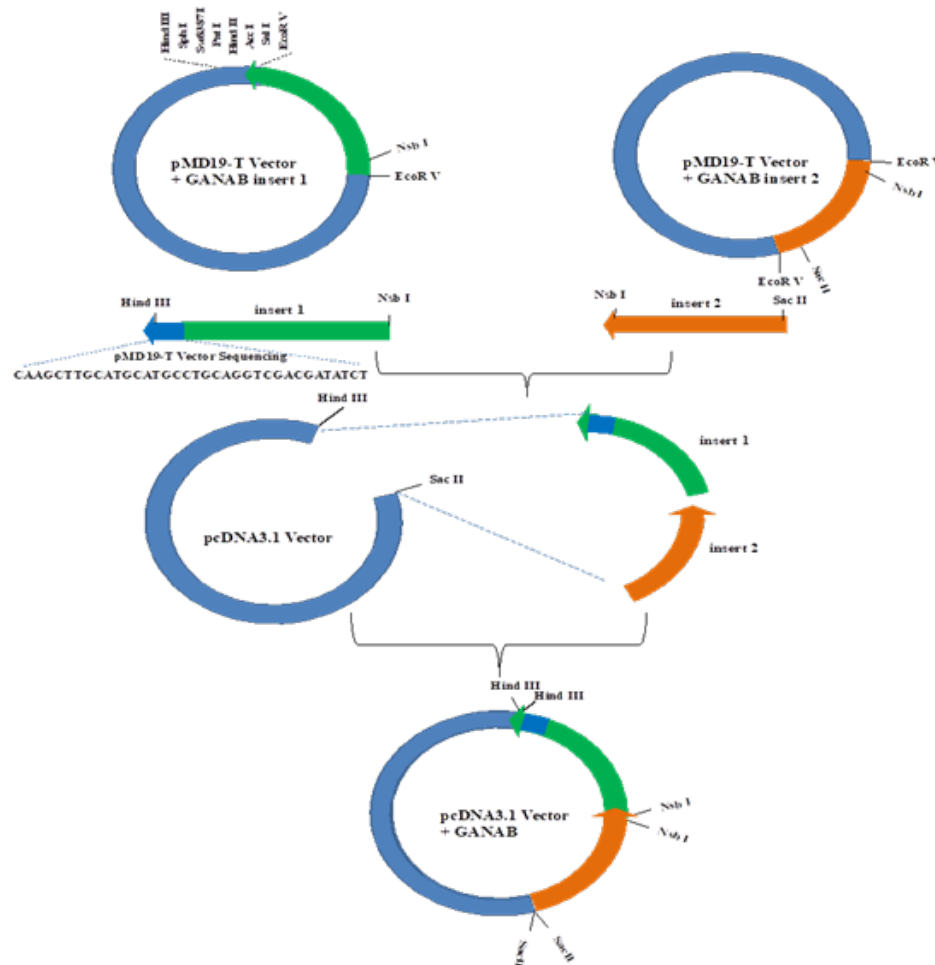
**Figure 3:** Expression of ganab plasmid. 1: Expression of pcDNA3.1 mock plasmid; 2: Expression of pcDNA3.1-ganab plasmid.

### 5. Discussion

GANAB, also known as glucosidase II- $\alpha$  subunit, is a neutral glucosidase that participates in the trading and folding of newly synthesized glycoproteins in the endoplasmic reticulum. The destruction of GANAB leads to the accumulation of misfolded glycoproteins and the induction of unfolded protein responses. GANAB is also a key regulator of glycosylation. The absence of GANAB caused the inhibition of the biosynthesis of new N-glycosylated proteins on the cell surface. According to reports, the lack of glucosidase II is related to polycystic liver disease. In polycystic liver disease, hepatic cystine cannot bind to GANAB during carbohydrate processing, leading to changes in cell proliferation and differentiation. Except for polycystic liver disease, GANAB has not been reported to be related to other diseases. In this study, it was found that the encoded GANAB protein has increased expression levels in lung cancer tissues and cells. GANAB is related to the occurrence of lung cancer by negatively regulating the migration and invasion of cells. This down-regulation further supports the correlation between GANAB expression and the aggressiveness and low survival rate of lung cancer. Since the N-glycosylation of a variety of cellular proteins may change the invasion and metastasis ability of tumors, GANAB may participate in the occurrence of lung cancer by regulating the N-glycosylation of specific target

proteins [11-14]. The GenBank database shows that there are 10 mutants in the GANAB gene.

The rapid increase in the number of cloned genes has led to an increase in the ability to recognize sequence homology between different genes, whether within the same species (homologs) or between species (homologs). Easier identification and cloning of gene family member genes in electrophoresis provides the potential to greatly facilitate their physical cloning. Our research report cloned the previously described human neutral  $\alpha$ -glucosidase AB gene (GANAB), which is responsible for the catabolism and regulation of carbohydrate chain degradation. These enzymes are mainly identified as members of glycoside hydrolase (GH) family 13 and 31. The GH13 family contains a variety of glucoside processing enzymes [15-17]. The combination of human gene research and cell analysis shows that this study obtained the GANAB gene through homologous cloning, and performed bioinformatics analysis on the correct gene sequence verified by sequencing; and through RT-PCR, Analyzed the mRNA expression pattern of GANAB gene, and verified the protein expression of this gene by plasmid overexpression experiment and western blot experiment (Figure 4). It is expected to lay the foundation for further research on the molecular mechanism and biological function of the gene expression regulation.



**Figure 4:** Cloning of ganab gene. PcDNA3.1-GANAB expression plasmid was constructed with pmd19-t and pcDNA3.1 plasmids.

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