

Cloning, expression and cytotoxicity of granulin A, a novel polypeptide contained in human progranulin

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Summary In our previous study, we isolated an antitumor polypeptide, CS5931, from the sea squirt *Ciona savignyi*; it shares high homology with *Ciona intestinalis* Granulin A (GRN A). However, little is known about the anticancer effect of GRN A. In the present study, GRN A was cloned and expressed in the yeast *Pichia pastoris*. The polypeptide was purified to almost homogeneity using a Ni-NTA column. MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] assay reveals that GRN A displays potent cytotoxicity to several human cancer cells. The polypeptide induces cell apoptosis as analyzed by morphological observation and flow cytometry assays. This study provides evidence that GRN A possesses potential to be developed as a novel anticancer agent.

Keywords: Granulin A, cloning, expression, anticancer activity

1. Introduction

Granulins (GRN), also known as epithelins, consists of a family of cysteine-rich peptides with diverse functions. All members of the GRN family contain 12 cysteine residues, arranged in highly conserved positions, and the cysteine residues form intramolecular disulfide bridges, resulting in characteristic tightly packed structures (1). The GRN family includes GRN A, B, C, D, E, F and G (2). GRN A is an acid- and heat-stable polypeptide with low molecular mass. The polypeptide contains 12 cysteine residues, and peptides bearing this domain are found in a wide range of organisms, from eubacteria to mammals (3,4). Studies have shown that GRN A plays a critical role in epithelial homeostasis, tumorigenesis, and in reproductive,

immunological, and neuronal functions (5,6).

In our previous study, we isolated a novel antitumor polypeptide termed CS5931 from the sea squirt *Ciona savignyi*, CS5931 shares high homology with *Ciona intestinalis* GRN and is conserved during evolution (7). The polypeptide shows a specific inhibition effect on the growth of several tumor cells *in vitro* (8). Prediction of 3D structure of the polypeptide revealed that CS5931 consisted of six disulfide bonds and two beta-hairpins, similar to human GRN A (9,10). Therefore, there is potential that GRN A may also display anticancer activity. However, little is known about the antitumor effect of GRN A as well its mode of action. Additionally, since the content of GRN A in tissue is very low, developing a novel approach for obtaining a sufficient amount of the polypeptide is promising.

Pancreatic cancer, one of the most common malignant tumors in man, is the fourth most common cause of cancer-related death in industrialized countries (11). Despite advances in surgical and nonsurgical treatments, it remains a tumor with poor prognosis; the overall 5-year survival rate is only 3-5% owing to the lack of symptoms and screening techniques for early detection, aggressive metastatic behavior, and resistance to conventional chemotherapy and radiotherapy regimens (12). Therefore, there is an urgent need to develop novel agents and alternative strategies for

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treatment of pancreatic cancer (13).

In the present study, we developed a highly effective approach for expressing the polypeptide in the yeast *Pichia pastoris*; the fermentation conditions were optimized, and the cytotoxicity as well as the proapoptotic activity of the polypeptide are also presented.

2. Materials and Methods

2.1. Materials

Gel Extraction Kit and Plasmid Mini Kit I were purchased from Omega (Norcross, GA, USA), and plasmid pGAPZ α A and *P. pastoris* SMD1168H were a gift from the Academy of Pharmaceutical Science of Shandong Province (Jinan, China). Protein markers and PCR Mix were products of KeyGEN (Nanjing, China), while T4 DNA ligase, restriction enzymes, DNA molecular markers and Zeocin were obtained from Thermo (Waltham, MA, USA).

2.2. Cell lines and culture

PANC-28 cells, BEL-7402 cells, and HCT-116 cells were purchased from ATCC (Manassas, VA, USA). Cell culture medium DMEM (Dulbecco's modified eagle medium) and RPMI (Roswell Park Memorial Institute) -1640 were purchased from Hyclone (Logan, Utah, USA).

2.3. Cloning of GRN A

Oligonucleotide primers for the amplification of the GRN A were designed based on the genomic sequence, and their sequences are as follows: Forward primer: 5'-CCGGAATTC GATGTGAAATG TGACATGAG-3'; Reverse primer: 5'-CTAGTCGATA CCCTGTTCCACAG GTACCCTTCT-3'. The restriction sites for *Eco* RI and *Xba* I are indicated by underlines. The PCR (Polymerase Chain Reaction) amplification conditions were initiated at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 30 s, and a final extension at 72°C for 10 min (14). The amplified DNA fragment was purified using Gel Extraction Kit, and digested with *Eco* RI and *Xba* I. The fragment was ligated into the pGAPZ α A plasmid pretreated with *Eco* RI and *Xba* I. The synthesized vector, pGAPZ α A/GRN A was transformed into *E. coli* DH-5 α , and the transformants were selected on low salt LB medium supplemented with 25 μ g/mL zeocin (15).

2.4. Expression of recombinant GRN A

The expression plasmid pGAPZ α A/GRN A was linearized with *Bsp* HI and transformed into the genome of the yeast *P. pastoris* SMD1168H by electroporation using the Gene pulser Xcell system (Bio-Rad,

California, USA) at 1.5 kV, with a 0.2 cm cuvette (16). Transformants were selected on YPDs (yeast extract peptone dextrose solid medium) plates (1% yeast extract, 2% peptone, 2% dextrose) containing 100 μ g/mL zeocin (17). Protein expression was examined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Western blotting analysis was also used to check the expressed protein. Briefly, the purified GRN A polypeptide was resolved on 15% SDS-PAGE. The gel was then semi-dry electroblotted onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA) at 2.5 mA/cm² for 20 min. The membrane then was then incubated in blocking buffer (5% fat milk in PBS) for 1 h at room temperature, washed with PBST three times for 10 min each, and incubated in PBST overnight at 4°C with anti-his monoclonal antibody (anti-His 1:1,000, Santa Cruz Biotechnology, Delaware, CA, USA). After washing the membrane with PBS, an HRP-conjugated antibody (goat anti-rabbit) was used as the secondary antibody and incubated for 1 h. The membrane was processed using the enhanced chemiluminescence method (Thermo, Waltham, USA), and the protein band was visualized by Gel imaging (Bio-Rad, Gel Doc XR+, Hercules, CA, USA).

2.5. Optimization of the expression conditions

2.5.1. Inoculation amount

The expression of GRN A in *P. pastoris* was determined by SDS-PAGE analysis, and the transformant with highest expression activity of GRN A was inoculated in YPD medium and cultured overnight at 30°C, 200 rpm. Inoculated culture was added to the YPD medium according to the proportions of 3, 5, 7, and 10%. After incubation for 96 h at 30°C, the culture medium was collected by centrifugation at 10,000 \times g for 20 min, and analyzed using SDS-PAGE.

2.5.2. Effect of temperature and culture time on the expression of GRN A

The temperature of the expression conditions of *P. pastoris* was set to 25 and 30°C, while the culture time was 48, 72, 96, 120 h respectively. SDS-PAGE analysis and MTT assay were performed to determine the amount and cytotoxicity of GRN A.

2.6. Expression and purification of recombinant GRN A

Transformant was inoculated in YPD medium and cultured at 25°C, 200 rpm overnight. After culture for 96 h, the supernatant was collected by centrifugation (10000 \times g, 10 min, 4°C) and purified using a Ni²⁺ chelating Sepharose column (GE Healthcare, Marlborough, MA, USA). After washing with 50 mM imidazole 2 times, the polypeptide was eluted using

imidazole at a concentration of 250 mM (18). The eluted polypeptide was dialyzed in dialysis buffer TE (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA) at 4°C. The concentration of purified recombinant GRN A polypeptide was quantified by BCA (bicinchoninic acid) protein assay Kit (KeyGen, Nanjing, China) and further analyzed using SDS-PAGE on 15% polyacrylamide gel, and stained with Coomassie brilliant blue R250.

2.7. MTT assay

MTT assay was performed to evaluate the anti-proliferative effects of recombinant GRN A against human liver cancer cells BEL-7402, human colon carcinoma HCT116 and human pancreatic cancer cells PANC-28. Briefly, cells were plated on to 96-well-plates and incubated at 37°C in humidified air atmosphere with 5% CO₂. After incubation for 24 h, cells were treated with various concentrations of recombinant GRN A. After culture for another 48 h, 20 µL of MTT [5 mg/mL MTT in PBS (phosphate buffered saline)] (Sigma, St. Louis, MO, USA) was added to each well and the cells were incubated for an additional 4 h (19). DMSO (dimethyl sulphoxide) 150 µL was added to each well to dissolve the reduced MTT crystals. The MTT-formazan product dissolved in DMSO was estimated by measuring the absorbance at 570 nm with a micro plate reader (Biotech, power wave, USA). The cell proliferation rate was calculated using the following formula: Relative inhibition rate (%) = $(OD_{\text{control}} - OD_{\text{treated}}) / OD_{\text{control}} \times 100\%$ (20).

2.8. Cell morphological observation

Human pancreatic cancer cells PANC-28 were seeded into 6-well culture plates with or without recombinant GRN A. After incubation for 24 h, the cells were fixed with 4% paraformaldehyde for 1 h at 4°C, washed with PBS, and incubated with 5 µg/mL of DAPI (21) (4',6-diamidino-2-phenylindole) (Beyotime Institute of Biotechnology, Shanghai, China) for 10 min in the dark. Cell morphology was observed under a fluorescence microscope (Olympus, Tokyo, Japan). Apoptosis was defined by the appearance of chromatin condensation.

2.9. Flow cytometry analysis

Annexin V-FITC (fluorescein isothiocyanate) and PI (propidium iodide) double-staining assay was performed to distinguish apoptotic from necrotic cells. Cells (2.5×10^5) were seeded in 6-well tissue culture plates and incubated for 24 h at 37°C. GRN A at several concentrations was directly added into 6-well plates and incubated for an additional 48 h. The cells were harvested and resuspended in PBS buffer. Apoptotic cells were identified using Annexin V-FITC Apoptosis

Detection kit (KeyGen, Nanjing, China) according to the manufacturer's instructions. Then the cells were examined by Cytomics FC 500 flow cytometer (Beckman Coulter, CA, USA).

3. Results

3.1. Expression of recombinant GRN A

In order to screen out the multiple copy colonies, the effect of zeocin concentration on GRN A production was studied. Zeocin was added at concentrations of 100, 300, 500, and 1,000 µg/mL respectively. Our results showed that a high yield of GRN A was found in 100 µg/mL of zeocin (data not shown). Random selection of positive clones from different batches of electroporation was beneficial to screen out the multiple copy colonies. SDS-PAGE revealed that the recombinant polypeptide was found in the supernatant of culture medium with a molecular weight around 15 kD (Figure 1A). Of note, the theoretical molecular weight of GRN A was 6.9 kD; it is conceivable that the six paired disulfides of GRN A influenced the electrophoretic mobility and the SDS-PAGE molecular weight (9). To further confirm the expression of GRN A, Western blotting analysis was performed (Figure 1B). The results showed that a clear band appeared at the same position corresponding to SDS-PAGE analysis (Figure 1B). The results indicated that the polypeptide GRN A was successfully expressed in the yeast *P. pastoris*.

3.2. Optimization of expression conditions

We first studied the effect of inoculated amount on the

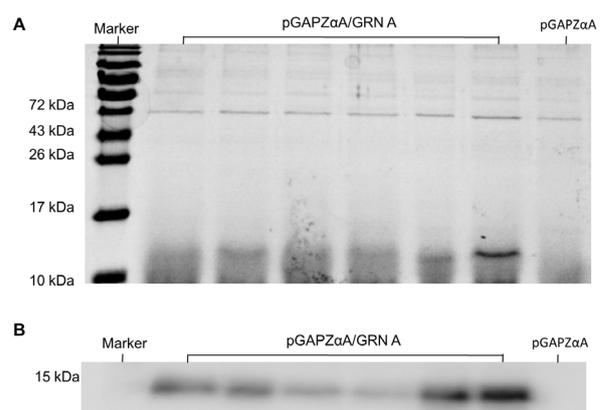


Figure 1. Expression of recombinant GRN A. (A) SDS-PAGE analysis of GRN A expression. pGAPZαA/GRN A and pGAPZαA were transformed in the yeast *P. pastoris*, and induced by zeocin. After culture for 96 h, the culture medium was collected using centrifugation. Lane 1~6 represents the expression of GRN A in different clones. Lane 7 represents the result in the strain transfected with the parent plasmid pGAPZαA. (B) Western blotting analysis of GRN A expression. Lane 1~6 represents the expression of GRN A in different clones. Lane 7 represents the result in the strain transfected with the parent plasmid pGAPZαA.

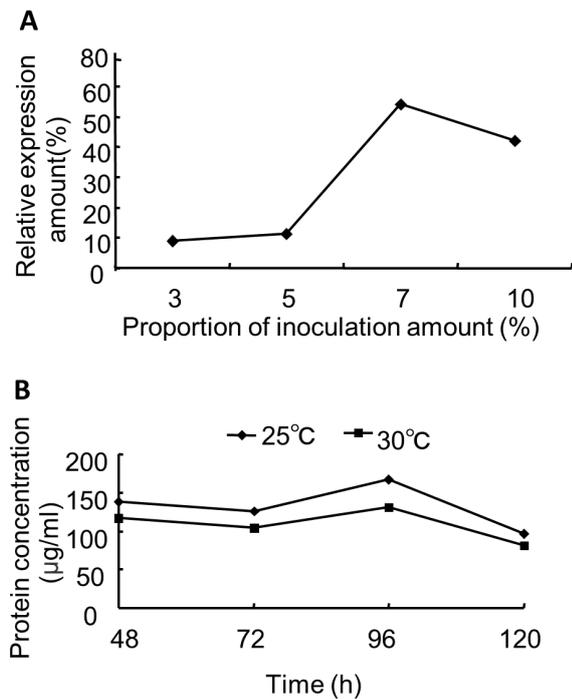


Figure 2. Optimization of expression conditions of the yeast *P. pastoris*. (A) Effect of inoculation amount on GRN A expression. The proportion of inoculation amount was 3, 5, 7, 10% respectively, and the expression of GRN A was determined by SDS-PAGE analysis. (B) Effect of temperature and fermentation period on GRN A production. The inoculated medium was cultured at 25 and 30°C for 48, 72, 96, 120 h respectively. The expression amount was analyzed using SDS-PAGE analysis.

expression of GRN A. Inoculated culture was added with proportions of 3, 5, 7, 10% respectively to the YPD medium. After culture for 96 h, the expression of GRN A was analyzed by 15% SDS-PAGE. Maximum expression of the polypeptide was obtained when the proportion of inoculation culture was 7% (Figure 2A).

Next, the effect of temperature on GRN A expression was investigated at 25 or 30°C respectively. The expression amount of GRN A was higher when the strains were cultured at 25°C than at the high temperature, 30°C (Figure 2B). Additionally, the expression of GRN A was also time-dependent and the highest production was obtained when incubated at 25°C for 96 h (Figure 2B).

3.3. Recombinant GRN A displays potent cytotoxicity to several cancer cells

GRN A was expressed at the optimized conditions, and purified using Ni-NTA column. The polypeptide was purified to almost homogeneity with a band at molecular weight of 15 kDa (Figure 3A). Using the optimized conditions, 5 mg of GRN A was obtained in one liter of medium. MTT assay was conducted to check the cytotoxicity of the purified polypeptide (Figure 3B). GRN A displayed potent cytotoxicity to several human cancer cells. The IC_{50} of GRN A

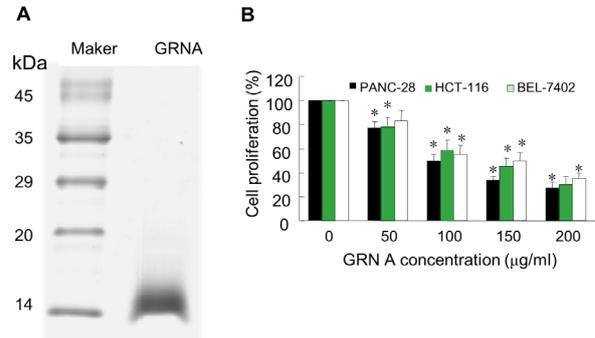


Figure 3. Purification of GRN A and its cytotoxicity on human cancer cells. (A) SDS-PAGE of purified recombinant GRN A. (B) Recombinant GRN A inhibits the growth of tumor cells. Human liver cancer cells BEL-7402, human colon carcinoma HCT116 and human pancreatic cancer cells PANC-28 were treated with certain concentrations of recombinant GRN A for 48 h. The cell inhibitory rate was determined by MTT assay as described in Materials and methods section. The experiments were repeated three times and the results are presented as mean \pm SD; * $p < 0.01$.

for PANC-28, HCT-116 and BEL-7402 cancer cells is 85.6, 100.4, and 90.9 μ g/mL respectively (Figure 3B). The results revealed that the recombinant polypeptide possesses potential to be developed as a novel anticancer agent for treatment of human cancer. Compared with other cancer cells, GRN A displayed higher cytotoxicity on PANC-28 cancer cells (Figure 3B). Therefore in our next experiments the pancreatic cancer cells were used for further study.

3.4. GRN A induces cell apoptosis

PANC-28 cells were treated with GRN A, and stained with DAPI. The cell morphology was observed by fluorescence microscope. As shown, treatment of the cancer cells with GRN A resulted in the production of nucleus pyknosis, condensation (Figure 4A, arrow). To further confirm the apoptosis induction effect of GRN A, Annexin V-FITC/PI double staining was performed (Figure 4B), a dose-dependent increase in the percentage of total apoptotic cells was observed in cells treated with GRN A; the percentage of total apoptotic cells was 9.64% in untreated cells (Figure 4C), whereas the percentages of total apoptotic cells increased to 18.52, 44.13, and 69.5% in PANC-28 cells treated with 50, 100, and 150 μ g/mL of GRN A respectively (Figure 4C). These results suggest that the recombinant polypeptide is able to inhibit tumor cell growth via the apoptotic pathway.

4. Discussion

Peptides play crucial roles in many physiological processes and a lot of peptides have been used clinically to treat human disorders. For many years, researchers have been searching for anticancer agents from the human body, since peptides from humans display little immunogenicity. Several peptides from human sources

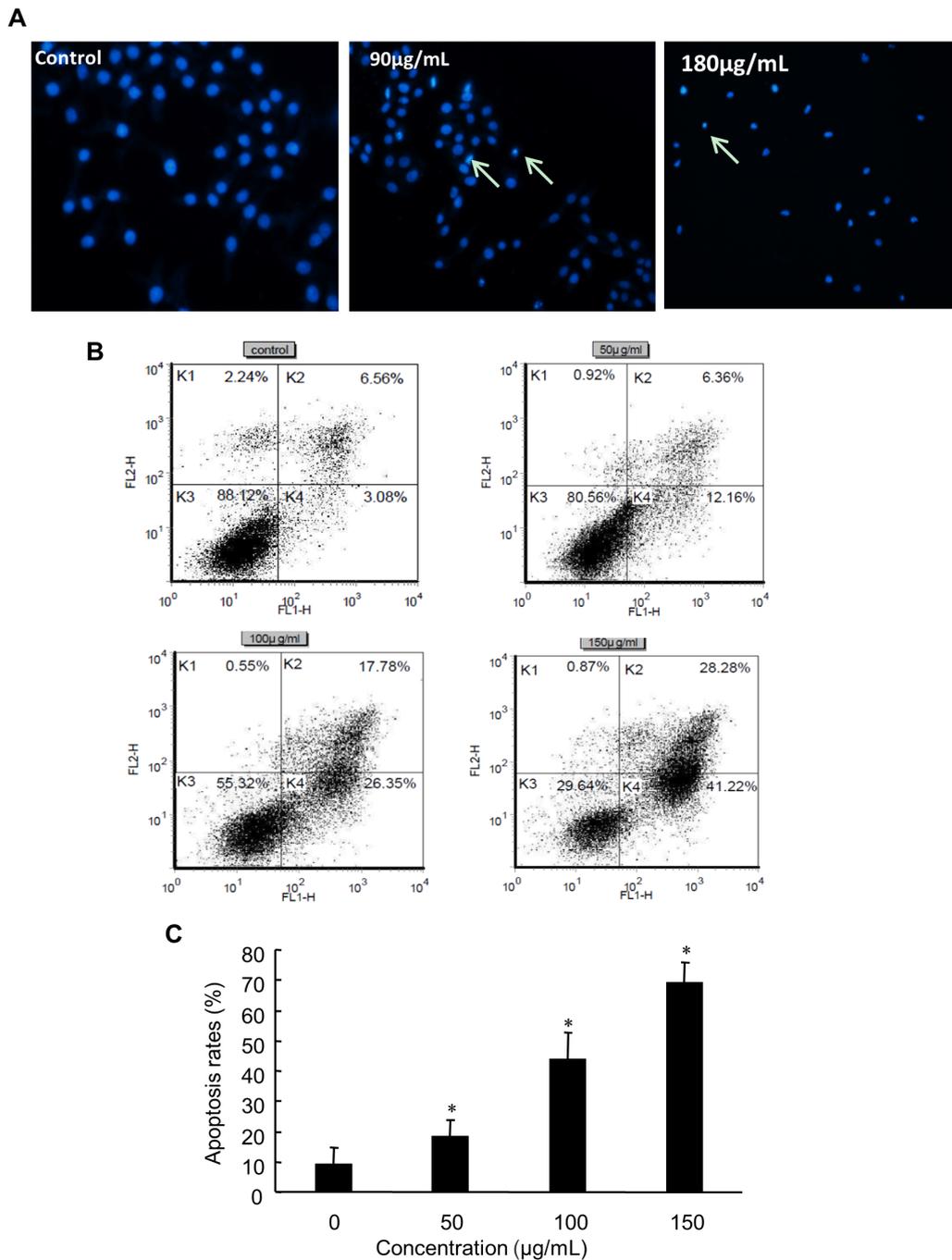


Figure 4. GRN A induces cell death via apoptotic pathway. (A) Morphological observation. PANC-28 cells were treated without (control) or with 90 µg/mL and 180 µg/mL GRN A for 24 h respectively. After staining with DAPI, the cell morphology was observed using a fluorescence microscope. The arrow indicates nuclei condensation. (B) Flow cytometry analysis. PANC-28 cells were treated without (control) or with certain concentrations of GRN A (50, 100, 150 µg/mL) and the cells were stained using Annexin V/PI double staining and analyzed by flow cytometry. The proportion of cell number is shown in each quadrant. The proportion of viable cells is shown in K3 quadrant (Annexin V-FITC⁻, PI⁻), while the early apoptotic cells is shown in K4 quadrant (Annexin V-FITC⁺, PI⁻). The K2 quadrant (Annexin V-FITC⁺/PI⁺) represents the late apoptotic/necrotic cells, while the necrotic cells are shown in K1 quadrant (Annexin V-FITC⁻, PI⁺). (C) represents the quantity of apoptotic ratio in cells treated as above. The percentage of total apoptotic cells was calculated from at least three separate experiments. All statistical significance was determined by a paired *t*-test; **p* < 0.01 versus control.

have been successfully used clinically for treatment of human cancer, such as interferons and interleukins (22-24). However, these kinds of peptides usually do not inhibit cancer cell growth directly; they affect the growth of tumors *via* an immune effect. In the present study, we found that the GRN A possesses the ability

to inhibit proliferation of cancer cells directly. This result provides primary evidence that GRN A has the potential to be developed as a novel kind of anticancer agent. Study is on-going in our laboratory to confirm the antitumor activity of GRN A in nude mice bearing human tumors.

Since the structure of GRN A contains multiple disulfide bonds, the correct folding of the polypeptide is important for maintaining the activity of GRN A. In the beginning, we tried to use a prokaryotic system to express GRN A, but the expressed polypeptide did not display any activity on human cancer cells. The results suggested that the right folding of GRN A is needed for obtaining an active polypeptide. It is well established that the apoptotic pathway is a main target for chemotherapeutic anticancer agents, and most anticancer drugs used clinically display tumor inhibition effect *via* the apoptotic pathway. The results of the present study confirmed that GRN A displays cytotoxicity to several human cancer cells (Figure 3B), and induced cell death *via* the apoptotic pathway in human pancreatic cells (Figures 4A-4C). It is well known that pancreatic cancer is very difficult to cure; the overall 5-year survival rate is only 3-5%. This study provides primary evidence that GRN A possesses the possibility to be a novel anticancer agent, especially on human pancreatic cancer. The study also provides a basis for further study of the function of GRN A both *in vitro* and *in vivo*.

In conclusion, GRN A was cloned and expressed in *P. pastoris* (Figures 1A and 1B). The polypeptide was purified to homogeneity using Ni-NTA column (Figure 3A). MTT assay confirmed that GRN A displays potent cytotoxicity to several human cancer cells (Figure 3B). Morphological observation as well as cytometry analysis reveals that the polypeptide is able to induce cell death *via* the apoptotic pathway (Figures 4A-4C). In recent years, peptides as drugs have attracted great attention due to their high efficiency and low resistance. However, up until now, there are no peptides inhibiting cancer cell growth directly, found from the human body. This study provides a novel strategy to search for anticancer agents.

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