

# Apoptosis-inducing effect of recombinant Caspase-3 expressed by constructed eukaryotic vector on gastric cancer cell line SGC7901

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## Abstract

**AIM:** To investigate the apoptosis-inducing effect of Caspases-3 expressed by constructed eukaryotic vector on gastric cancer cell line SGC7901.

**METHODS:** PCR was employed to amplify the sequences of both small and large subunits of Caspases-3. Its products were separately cloned into the *Sma* I site of pBluescript KS<sup>+</sup> to generate both plasmids pBS/SS and pBS/LS. The small subunit fragment was excised from plasmid pBS/SS with *Bam*H I and then inserted into the *Bam*H I site of plasmid pBS/LS preceding that of the large subunit to yield plasmid pBS/Rev-Caspase-3. Rev-Caspase-3 cDNA was excised with *Kpn* I + *Xba* I and then subcloned into plasmid pcDNA3.1 (+) to construct Rev-Caspase-3 eukaryotic expression vector pcDNA/Rev-Caspase-3, which was used to transiently transfect SGC7901 cell line. Cell count, MTT assay and electron microscopy were used to confirm the antiproliferation and apoptosis-inducing effect of Rev-Caspase-3 expression on gastric cancer cells.

**RESULTS:** Plasmid pBS/Rev-Caspase-3 and eukaryotic expression vector pcDNA/Rev-Caspase-3 were successfully constructed. SGC7901 cells were transiently transfected by either pcDNA/Rev-Caspase-3 or pcDNA3.1 (+) for 24, 48, 72, and 96 h respectively. Cell growth was measured by cell count and MTT assay. In cell count assay, the cell numbers were  $1.8 \times 10^6$ ,  $1.55 \times 10^6$ ,  $2.0 \times 10^6$ , and  $3.1 \times 10^6$  in the experimental group and  $2.5 \times 10^6$ ,  $3.1 \times 10^6$ ,  $4.0 \times 10^6$ , and  $5.7 \times 10^6$  in the control group at 24, 48, 72 and 96 h respectively. The growth of SGC7901 cells was suppressed by Rev-Caspase-3 in a time-dependent manner ( $P < 0.05$ ). The results of MTT assay were similar to that of cell count ( $P < 0.05$ ). The characteristics of apoptosis such as chromatin condensation, crescent formation and margination were seen and more obvious with time in the given-experimental period in the experimental group, but not easily observed in the control group.

**CONCLUSION:** The expression of Rev-Caspase-3 by the

constructed eukaryotic vector can significantly induce apoptosis of gastric cancer cell line SGC7901, which may exhibit a potential way in gastric cancer gene therapy.

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## INTRODUCTION

Apoptosis is closely related to tumor<sup>[1-10]</sup>. Although there are many factors involved in apoptotic program<sup>[11-14]</sup>, Caspases are shown to play a major role in the transduction of the apoptotic signal and the execution of apoptosis in mammalian<sup>[15-19]</sup>. Caspases belong to cysteine proteases family<sup>[20]</sup> and share several common features such as all homologous to interleukin-1- $\beta$ -converting enzyme (ICE)<sup>[21]</sup> containing conserved QACR/QGC sequence<sup>[22]</sup> existing as inactive zymogens activated by cleavages specific internal ASP residues in interdomain linkers being able to cleave their substrates ASP residues, and consisting of prodomain, small subunit and large subunit.

So far 15 members of Caspases have been reported in the literature. According to their structure and function, Caspases are divided into two classes, the initiator and executor Caspases. The initiator Caspases carry long prodomain, and can process and activate their own and other inactive Caspase zymogens when triggered by a death signal<sup>[23,24]</sup>. The executor Caspases such as Caspases-3, however, lack the long prodomain and remain to be dormant until the initiator Caspases activate them by direct proteolysis<sup>[25]</sup>. Once activated, they dismantle cell regulatory components rapidly, leading to the typical changes observed in cell apoptosis<sup>[26-28]</sup>.

Interestingly, by making small subunit fragment preceding that of the large subunit, Srinivasula *et al*<sup>[29]</sup> constructed a recombinant Caspases-3, which could simulate the three-dimensional structure of activated Caspases-3 and was capable of being catalyzed and inducing MCF-7 cell apoptosis without initiator Caspases' proteolysis. In this study, the eukaryotic expression vector of recombinant Caspases-3 was constructed and the apoptosis-inducing effect of its expression on SGC7901 cell line was observed.

## MATERIALS AND METHODS

### Subclone of both small and large subunits

Plasmid pcDNA/Caspases-3 contains all the cDNA sequences of Caspases-3 gene. It was used as template to amplify the sequences of small and large subunits of Caspases-3 by PCR. The sequences of four pairs of primers were as follows: LS-forward (P1), 5' -ATG GAG AAC ACT GAA AAC TCA G-3'; LS-reverse (P2), 5' -GTC ATC ATC AAC ACC TCA GTC T-3'; SS-forward (P3), 5' -GGA TCC ATG ATT GAG ACA GAC AGT GG-3'; SS-reverse (P4), 5' -ATC AAC TTC ATC GTG ATA AAA ATA GAG TTC-3'.

PCR was performed in 50  $\mu$ l reactive volume containing 2  $\mu$ l cDNA, 5  $\mu$ l PCR buffer, 2  $\mu$ l dNTP, 1  $\mu$ l primer, and 1  $\mu$ l Taq DNA polymerase. The samples were subjected to 30 thermal cycles for 5 min at 95  $^{\circ}$ C for pre-denaturation, for 30 s at 94  $^{\circ}$ C for denaturing, for 30 s at 56  $^{\circ}$ C for annealing, for 50 s at 72  $^{\circ}$ C for extension, and for 7 min at 72  $^{\circ}$ C for final extension after the last cycle. The PCR products were separately cloned into the *Sma* I site of pBluescript KS+ to construct pBS/SS and pBS/LS, which were confirmed by *Sma* I digestion and DNA sequencing.

#### Construction of recombinant Caspases-3 (Rev-Caspases-3)

The small subunit fragment was excised from plasmid pBS/SS with *Bam*H I and then inserted into the *Bam*H I site of plasmid pBS/LS to yield plasmid pBS/Rev-Caspases-3 with the small subunit fragment preceding that of the large subunit, which was verified with *Bam*H I+*Kpn* I digestion and PCR method. The primers were SS-forward and LS-reverse.

#### Construction of Rev-Caspases-3 eukaryotic expression vector pcDNA/Rev-Caspases-3

Eukaryotic expression vector pcDNA3.1 (+) was cleaved and linearized with *Kpn* I+*Nhe* I, meanwhile, Rev-Caspases-3 cDNA was excised from plasmid pBS/Rev-Caspases-3 with *Kpn* I+*Xba* I. Both fragments were ligated each other with T<sub>4</sub> DNA ligase to produce plasmid pcDNA/Rev-Caspases-3, which was proved with *Stu* I+*Kpn* I.

#### Cell line and cell culture

Cell line SGC7901 was derived from a moderately-differentiated gastric adenocarcinoma and characterized extensively. It was maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 200 IU·ml<sup>-1</sup> penicillin and 50  $\mu$ g·ml<sup>-1</sup> streptomycin at 37  $^{\circ}$ C in a humidified atmosphere of 5 % CO<sub>2</sub>.

#### Cell transfection, cell count and electron microscopy

Cells were seeded in a 6-well plate with  $2 \times 10^5$  cells per well 24 h prior to transfection when they were cultured to a confluency of about 90 %. Cell transfection was performed according to the manufacturers' instructions. Briefly, a transfection mixture was prepared by adding 6  $\mu$ g of plasmid DNA and 20  $\mu$ l lipofectame (GIBCO BRL) to 500  $\mu$ l serum-free RPMI1640. After incubated at room temperature for 20 min, the transfection mixture was added to the cells to be cultivated for 4 h at 37  $^{\circ}$ C when the media containing the transfection mixture was exchanged for growth medium. Cells were harvested and counted at 24, 48, 72, and 96 h after transfection. Some cells were fixed in 40 g·L<sup>-1</sup> glutaraldehyde, then post-fixed in osmium tetroxide and embedded in Epon. Ultrathin sections were prepared, stained with wronyl acetate and lead citrate, examined under a transmission electron microscope to identify the morphological changes of apoptosis.

#### MTT assay

Antiproliferation effects of recombinant Caspases-3 were measured by MTT assay. SGC7901 cells were seeded in 96-well microtitre plates with  $1 \times 10^4$  cells per well and incubated overnight in 100  $\mu$ l of culture media. Then the cells were transiently transfected with 0.3  $\mu$ g DNA of plasmid pcDNA/Rev-Caspases-3 for 24, 48, 72, and 96 h respectively. The cells were added with 100  $\mu$ l MTT (1g·L<sup>-1</sup>) and further incubated for 4 h. After supernatant was removed, the cells were added with 100  $\mu$ l DMSO per well and cultivated for 30 min. The absorbance at 490 nm was measured by a micro-ELISA reader. At the same time, the SGC7901 cells transfected with plasmid pcDNA3.1 (+) were served as control. Each assay was repeated three times.

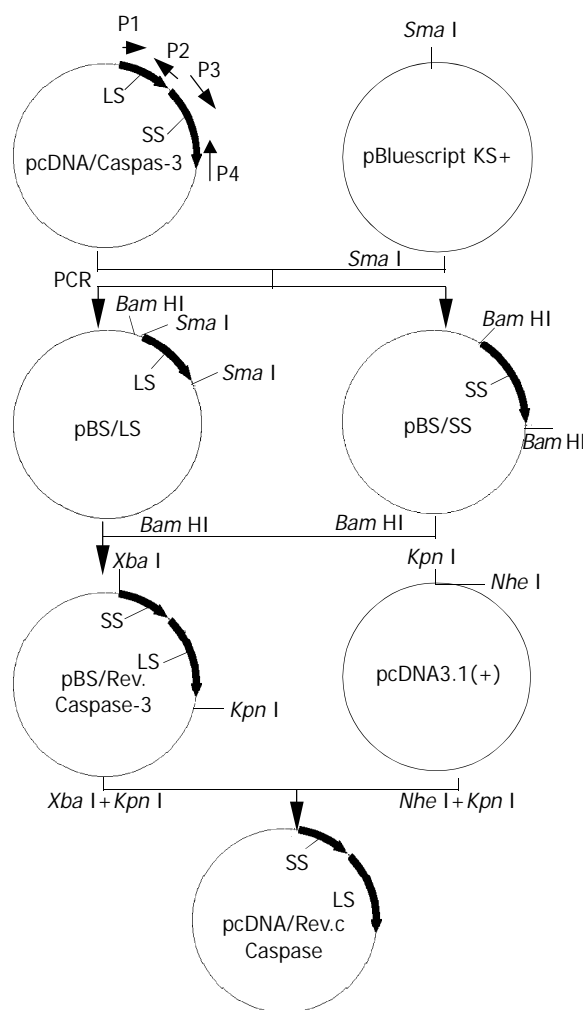
#### Statistics

Comparison of the data among the groups was carried out using the Bonferroni-Dun multiple comparisons. In each case, *P* values less than 0.05 were considered statistically significant.

## RESULTS

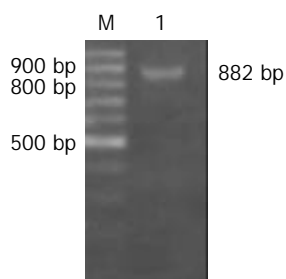
#### Construction of Rev-Caspases-3 and pcDNA/Rev-Caspases-3

The construction strategies of recombinant Caspases-3 and eukaryotic expression plasmid pcDNA/Rev-Caspases-3 are shown in Figure 1.

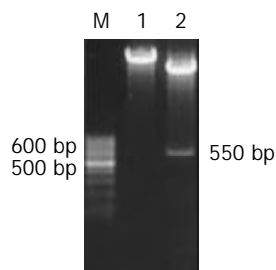


**Figure 1** Schematic map of constructions of recombinant Caspases-3 and eukaryotic expression plasmid pcDNA/Rev-Caspases-3.

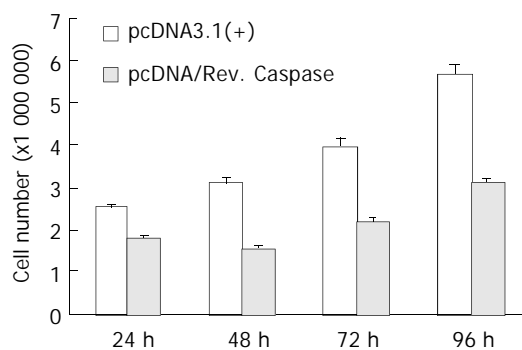
Both small and large subunit sequences of Caspases-3 were successfully amplified by PCR and subsequently cloned into *Sma* I site of pBluescript KS+ to yield pBS/SS and pBS/LS respectively, which were confirmed by *Sma* I digestion and DNA sequencing. The small subunit fragment was excised from plasmid pBS/SS with *Bam*H I and cloned into *Bam*H I site of plasmid pBS/LS to form recombinant plasmid pBS/Rev-Caspases-3 with the small subunit fragment located in-frame 5' to that of the large subunit, which was identified by PCR using SS-forward and LS-reverse as primers. The length of PCR product was 882 bp (Figure 2). Rev-Caspases-3 cDNA was excised from plasmid pBS/Rev-Caspases-3 with *Kpn* I+*Xba* I and orientatively cloned into *Kpn* I and *Nhe* I site of eukaryotic expression vector pcDNA3.1(+) to construct plasmid pcDNA/Rev-Caspases-3, which was proved by the digestion of *Stu* I+*Kpn* I with a 550 bp fragment release (Figure 3).



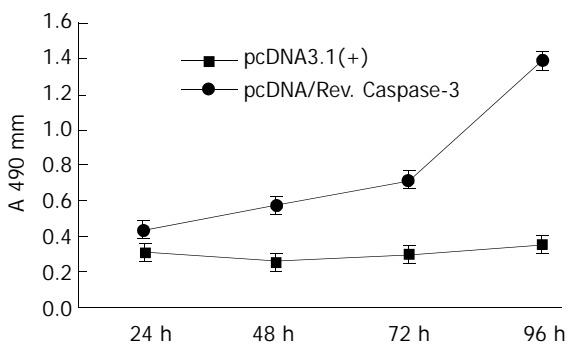
**Figure 2** Identification of plasmid pBS/Rev-Caspase-3 by PCR with SS-forward and LS-reverse as primers. M: 100 bp ladder DNA marker, 1: PCR product, 882 bp.



**Figure 3** Enzyme digestion analysis of plasmid pcDNA/Rev-Caspase-3. M: 100 bp ladder DNA marker, 1: pcDNA/Rev-Caspase-3 cut with *Stu* I, 2: pcDNA/Rev-Caspase-3 cut with *Stu* I+*Kpn* I.



**Figure 4** Comparison of cell count between SGC7901 cells transfected with pcDNA3.1(+) and pcDNA/Rev. Caspase.



**Figure 5** Comparison of MTT assays between SGC7901 cells transfected with pcDNA3.1(+) and pcDNA/Rev.Caspase-3.

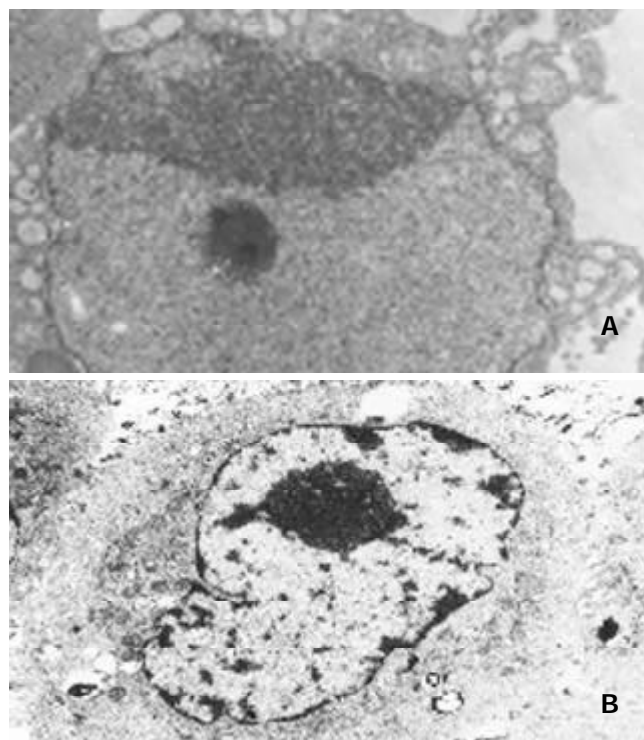
#### Effect of Rev-Caspases-3 on SGC7901 cell growth

SGC7901 cells were transiently transfected by either pcDNA/Rev-Caspases-3 (the experimental group) or pcDNA3.1 (+) (the control group) for 24, 48, 72, and 96 h respectively. Cell growth was measured by cell count and MTT assay. In cell count assay, the cell numbers were  $1.8 \times 10^6$ ,  $1.55 \times 10^6$ ,  $2.0 \times 10^6$ ,

and  $3.1 \times 10^6$  in the experimental group and  $2.5 \times 10^6$ ,  $3.1 \times 10^6$ ,  $4.0 \times 10^6$ , and  $5.7 \times 10^6$  in the control group at 24, 48, 72, and 96 h respectively. The growth of SGC7901 cells was suppressed by Rev-Caspases-3 in a time-dependent manner (Figure 4,  $P < 0.05$ ). The results of MTT assay were similar to that of cell count (Figure 5,  $P < 0.05$ ).

#### Induction of apoptosis in SGC7901 by Rev-Caspases-3

The ultrastructural characteristics of apoptosis such as chromatin condensation, crescent formation, and margination were seen by electron microscopy in the experimental group, which were more obvious with time in the given-experimental period and not easily observed in the control group (Figure 6).



**Figure 6** Electron microscopy of SGC7901 cells transfected with pcDNA3.1 (+) or pcDNA/Rev.Caspases-3. A: SGC7901 transfected with pcDNA/Rev. Caspases-3. Notice the chromatin condensation, crescent formation, original margination ( $\times 5\ 000$ ); B: SGC7901 transfected with pcDNA3.1 (+), Notice no characteristics of apoptosis ( $\times 5000$ ).

#### DISCUSSION

Caspases belong to cysteine protease family. So far, more than 15 members of Caspases have been reported in the literature, and most of them play a major role in apoptosis, participating in the initiation and execution of programmed cell death<sup>[30-33]</sup>. Based on their structure and function, Caspases are classified into two groups, initiator (such as Caspases-2,-8 and 10) and executor Caspases (such as Caspases-3, -6 and 7)<sup>[33-35]</sup>. Structurally, Caspase is made up of prodomain, large and small subunits, of which prodomain is located in N-terminus, small subunit in C-terminus and large subunit between. Initiator Caspases bear a long prodomain, whereas the executor Caspases do not<sup>[36-38]</sup>.

Caspases are synthesized as harmless inactive zymogens and generally activated by cleavages specific internal ASP residues present in interdomain linkers, which manifests as a hierarchical process. When triggered by a death signal, the initiator Caspases are recruited through their long prodomain by specialized adaptor molecules to form the death-inducing signaling complex (DISC) or Apaf. Because of the trimeric

nature of the DISC, three Caspase molecules are brought close, which will be useful to activate initiator Caspases<sup>[39-40]</sup>. When activated, the initiator Caspase can activate itself and other inactive caspase zymogens including executor Caspases that are activated by direct proteolysis. The activated executors then rapidly dismantle important cellular components, leading to cell apoptosis.

The three-dimensional structure of activated Caspases-3 has been shown that the C-terminus of large subunit and the N-terminus of small subunit are separated far from each other, whereas the N-terminus of large subunit and C-terminus of small subunit are closed to each other<sup>[41,42]</sup>. Based on these observations, Srinivasula *et al*<sup>[29]</sup> successfully engineered recombinant Caspases-3 and -6 precursors by switching their subunits order at a gene level, in which the small subunit fragment was fused in frame N terminal to that of the large subunit. Thus the N terminus of small subunit and the C terminus of large subunit are free, while the C terminus of the small subunit and the N terminus of the large subunit are connected each other through a linker. These recombinant molecules could be activated spontaneously to induce apoptosis in MCF-7 cells independent of initiator Caspases. Jia *et al*<sup>[43]</sup> have also confirmed the apoptosis-inducing effect of recombinant Caspases-3 on HeLa cells.

Gastric carcinoma is one of the most common causes of malignancy-related death in China<sup>[44-48]</sup>. In the present study, to test the effect of recombinant Caspases-3 on gastric cancer cell, we constructed a eukaryotic expression vector of constitutively active recombinant Caspases-3 and used it to transiently transfect gastric cancer cell SGC7901. The results showed that the expressed recombinant Caspases-3 could inhibit the growth of SGC7901 in a time dependent manner. In addition, the apoptosis-inducing effect of recombinant Caspases-3 on SGC7901 was also evident. This study demonstrates the possible use of recombinant Caspases-3 in gastric cancer gene therapy. But the effects of growth inhibition and apoptosis induction conducted by recombinant Caspases-3 on other cell lines of gastric cancer or on gastric cancer cell *in vivo* need to be further investigated.

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