RESEARCH ARTICLE

Preliminary Research on the Expression, Purification and Function of the Apoptotic Fusion Protein, Siva1

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Abstract

The objective of the present study was to investigate cloning, expression, and functions of the recombinant protein, Siva1. Siva1 gene was synthesized by RT-PCR from HCT116 cells. Plasmids were cleaved with the restriction endonuclease, BamH1/Sal1 and products were connected to pQE30, which underwent cleavage by BamH1/Sal1. The recombinant plasmid, pQE30-Siva1, was identified after digestion with restriction endonucleases followed by transformation into E. coli M15. Expression of Siva1 was induced by IPTG and identified by SDS-PAGE following purification with affinity chromatography. The results showed that size of Siva1 was 12 kDa, consistent with the molecular weight of the His-Siva1 fusion protein. Functional test demonstrated that Siva1 significantly inhibited the invasion and migration of HCT116 cells. It may thus find clinical application for control of cancers.

Keywords: Siva1 - cloning - expression - HCT116 cells - invasion - migration

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Introduction

Colorectal cancer is one of the most common cancers worldwide. It has been shown that colorectal cancer is the third leading cancer among malignancies and the fourth leading cause of death (Siegel et al., 2013). Studies show that the factors potentially involved in the pathogenesis of colorectal cancer are activating transcription factor 1 (Huang et al., 2012), hedgehog signaling pathway (Wang et al., 2012), matrix metalloproteinase-7 (Yang et al., 2012) as well as calpain-10 SNP43 and SNP19 polymorphisms (Hu et al., 2013) et al. However, the exact mechanisms underlying the initiation and development of colorectal cancer remain unknown. Therefore, it is imperative to investigate the pathogenesis of colorectal cancer and to develop effective measures to inhibit the occurrence and development of colorectal cancer.

Siva1 (an apoptosis-related protein) consists of 175 amino acids and includes a N-terminal domain formed by amino acids 1-48, a death domain homologous region formed by amino acids 49-114, a RING finger domain formed by amino acids 115-145, and a zinc finger domain formed by amino acids 146-175. Siva1 is a multifunctional protein and plays important roles in cell apoptosis. As a target gene of p53, Siva1 can inhibit the anti-apoptotic effect of bcl-XL, leading to the apoptosis of cells. In neurons, Siva1 has been shown to be involved in p53-induced apoptosis. In addition, Siva1 may also inhibit NF-kappaB activity. In patients with colorectal cancer, the transcription of Siva1 and p53 were down-regulated significantly (Okuno et al., 2001) and treatment with topetecan increased the transcription of Siva1 in colorectal cancer cells in a p53-dependent manner (Fortin et al., 2004). Tumor suppressor factors (p53 and E2F1) can directly activate Siva1 transcription in neurons. (Fortin et al., 2004; Li et al., 2011) showed that Siva1 promoted the generation and stability of microtubules and blocked the assembly of focal adhesions, cell migration, and the epithelial-mesenchymal transition.

In the present study, the Siva1 gene was synthesized by RT-PCR from colorectal cancer cells (HCT116 cells), and the His-Siva1 prokaryotic expression vector was constructed and transformed into Escherichia coli. The His-Siva1 fusion protein was isolated and purified by affinity chromatography. In addition, the function of Siva1 was also preliminarily investigated. It was shown that Siva1 significantly inhibited the invasion and migration of HCT116 cells. Our findings provide basic evidence that Siva1 might inhibit the occurrence and development of colorectal cancer.

Materials and Methods

Plasmids and strain

pMD18-T vector (TaKaRa) (Dalian, China), pQE30 vector, E. coli DH5α, and E. coli M15 (homemade?our

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laboratory) were used in the present study.

Main reagents

Taq DNA polymerase, BamHI, SalI, T4 DNA ligase (TaKaRa), Ni-NTA agarose (Thermo Scientific), M-MLV reverse transcriptase, Trizol (Invitrogen) and oligo (dT) 15 (Wuhan Beinglay Biotech Co., Ltd.) were used in the present study.

Cloning of the siva1 gene

The HCT116 cells were harvested and passaged. Total RNA was extracted with Trizol according to the manufacturer's instructions and dissolved in 8 μl of RNase-free water. DNA digestion was performed according to the manufacturer's instructions. After addition of oligo (dT) 15 and M-MLV, reverse transcription was performed according to the manufacturer's instructions.

The sequence of the Siva1 gene was obtained from GenBank (gene No. 10572). On the basis of the pQE30 vector, Primer 5.0 was used to design primers with restriction sites of BamHI and SalI for reverse transcription. The primers were as follows: P1, 5' gggccc ggatccatgcccaagcggagctgccc 3'; and P2, 5' gggcccgtcgactcaggtctcgaacatggcacagct 3'. The products (6 μl) were collected after reverse transcription and mixed with P1, P2, dNTP, and TaqDNA polymerase. PCR was performed as follows: pre-denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s; extension at 72°C for 30 s; and a final extension at 72°C for 10 min. After agarose gel electrophoresis and recycling, the products were connected into a pMD18 vector, and identified by digestion with BamHI/SalI and sequencing.

Construction and identification of the prokaryotic recombinant pQE30- Siva1 expression vector

As described previously (Sam et al., 2002; Shen, 2004), pMD18-T- Siva1 was digested with the restriction endonuclease, BamHI/SalI. The 333-bp segment was cloned into the BamHI/SalI sites of *p* pQE30, followed by transformation of E. coli DH5a. Identification was facilitated by digestion with the restriction endonuclease, BamHI/SalI. The recombinant plasmid was named pQE30-Siva1.

Induction expression of Siva1 in prokaryotic cells

After identification, the recombinant plasmid, pQE30-Siva1 was transformed into competent E. coli M15, and screening was performed with LB agar plates containing kanamycin and ampicillin as described previously (Sam et al., 2002; Shen, 2004). Incubation was performed at 37°C overnight until new colonies were observed. A single colony was selected and inoculated into liquid LB medium (2 ml) containing ampicillin and kanamycin, followed by incubation at 37°C overnight at 200 rpm. On the 2nd day, 1 ml of cell solution was added to 100 ml of liquid LB medium containing ampicillin and kanamycin, followed by incubation at 37°C and centrifugation at 200 rpm. Cells in the logarithmic growth phase (OD600 = 0.6) were harvested and treated with 0.1 mmol/L IPTG at 19°C for 12 h for induction.

Purification of Siva1

After induction, the cell solution was centrifuged at 4°C for 10 min at 8000 g. The pellets were re-suspended in 5 ml of lysis buffer (50 mmol/L Tris-HCl [pH 8.0], 300 mmol/L NaCl, 1% Triton, and 20 mmol/L imidazole), followed by sonication. The resultant mixture was centrifuged at 10 000 g for 15 min at 4 °C. The supernatant was harvested and transferred into 0.5 ml of Ni-NTA agarose, followed by incubation at 4°C for 2 h. The unbinding proteins were removed by washing in lysis buffer, and elution was done with eluant (50 mmol/L Tris-HCl, 300 mmol/L NaCl, and 200 mmol/L iminazole[pH 8.0]). The His-Siva1 was harvested and underwent dialysis with dialysate (50 mmol/L Tris-HCl, 300 mmol/L NaCl, and 10 mmol/LDTT [pH 7.4]) for 24 h. After centrifugation, the protein was collected and used for investigation of function.

Effect of Siva1 on the migration of HCT116 cells

HCT116 cells were digested with trypsin, then re-suspended in a mixture (1% FBS, 10 ng/ml EGF, and 0.5-10 μmol/L fusion protein) or PBS (control). The resultant mixture treated with 10 μmol/L Siva1 or PBS was seeded into a flask coated with 5 μg/ml of fibronectin, followed by incubation at 37°C for 3 h in an environment with 5% CO_2 A Nikon Biostation IM-Q was used to trace the migration of these cells. The images were captured once every 10 min for a total of 6 h, and at least 60 cells were observed in each group. The experiments were performed three times. NIS-Elements AR was used for image analysis according to a previous report (Wu et al., 2011).

Effects of Siva1 on the invasion of HCT116 cells

Serum-free DMEM was used to dilute Matrigel 30-fold. Then, 100 μl of this mixture was added to a Transwell chamber, followed by incubation at 37°C for 6 h until the Matrigel solidified. HCT116 cells were digested with trypsin and thrice-washed in DMEM containing 1% FBS. These cells were re-suspended in DMEM containing 1% FBS at a density of 5×10^5 cells/ml. Then, 100 μl of the cell suspension was added to the upper chamber; and 600 μl of DMEM containing 10% FBS and 10 μmol/L Siva1 or PBS (control) was added to the lower chamber. The experiments were performed in quintuplicate. The Transwell chamber was incubated at 37°C for 12 h in an environment with 5% CO_2. The Transwell chambers were removed and humidified swabs were used to remove the cells in the upper chamber, which were fixed in paraformaldehyde at room temperature for 10 min, followed by 0.1% crystal violet staining at room temperature for 30 min. After twice-washing in PBS and air-drying, the cells were observed under a light microscope. Five fields were randomly selected and the cells were counted, followed by averaging. This experiment was performed according to previous reports (Wu et al., 2011).

Results

Construction and identification of pQE30-Siva1 expression vector

Total RNA was extracted from HCT116 cells and reversely transcribed into cDNA. P1 and P2 primers

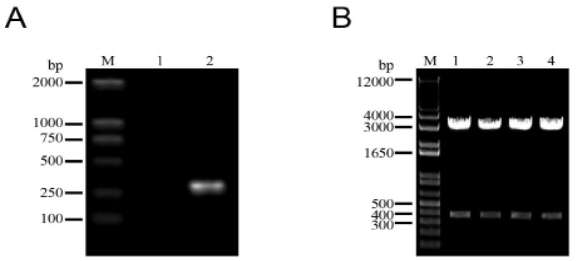

Figure 1. Construction of pQE30-Siva1. A: Amplification of Siva1 with P1 and P2 primers by PCR. M: DL2000 marker; 1: negative control; 2: Siva1 sequence. B: Identification of pQE30-Siva1 by digestion. M: O'Gene Ruler 1kb DNA ladder 1, 2, 3, 4: segment after digestion of pQE30-Siva1 with BamHI and SalI

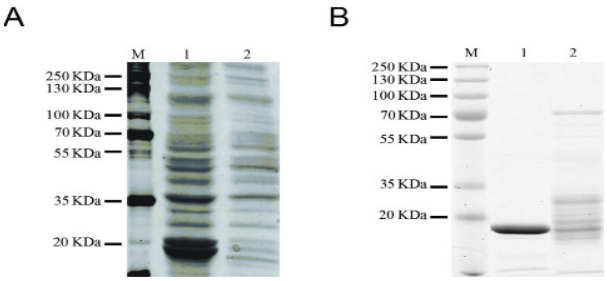

Figure 2. Expression and Purification of Fusion Protein. A: Expression of fusion protein. M: Protein marker; 1. Total protein of recombinant E. coli M15 after IPTG induction; 2. Total protein of recombinant E. coli M15 without induction. B: Purification of fusion protein 1. Purified Siva1; 2. Total recombinant protein without purification

were used for amplification of the Siva1 sequence by PCR (Figure 1A). The PCR products were cloned into the pMD-18T vector. After identification, the pMD-18T vector was digested with the restriction endonucleases, BamHI and SalI, and the products were cloned into the pQE30 vector, achieving pQE30-Siva1 (Figure 1B).

Expression and purification of Siva1

The recombinant vector, pQE30-Siva1, was transformed into competent E. coli M15. After IPTG induction, samples were subjected to SDS-PAGE. The expression of Siva1 was shown in Figure 2A. Following sonication, centrifugation was performed, and the supernatant was collected. Siva1 with high purity and size of a 12 kDa molecular weight was achieved (Figure 2B), which was consistent with what was expected.

Effects of Siva1 on the invasion of HCT116 cells

In the invasion assay, HCT116 cells were treated with Siva1 at a final concentation of 0.5 μmol/L, 2 μmol/L, 5 μmol/L, and 10 μmol/L respectively, and the cells treated with PBS served as a control. A Transwell invasion assay was performed to determine the effect of Siva1 on the invasiveness of HCT116 cells. The results showed that the number of migrated cells treated with the Siva1 was significantly less than that in the control group, and the inhibitory effect increased in the concentration dependent manner, suggesting that Siva1 inhibits the invasion of HCT116 cells (Figure 3).

Effects of Siva1 on migration of HCT116 cells

HCT116 cells were seeded into flasks coated with

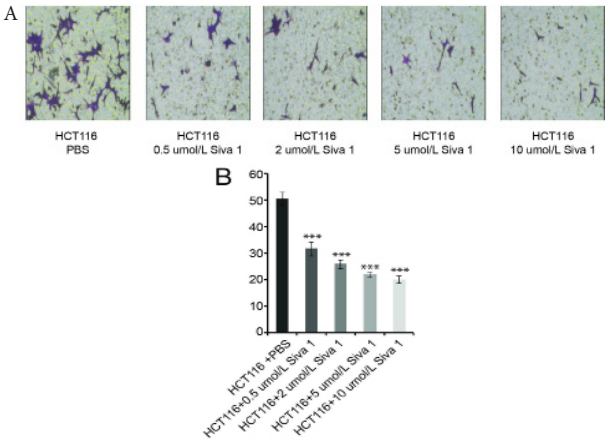

Figure 3. Siva1 Inhibits the Invasion of HCT116 Cells. A: HCT116 cells treated with Siva1 at different concentrations. B: Quantification of invasion of HCT116 cells after Siva1 treatment. Data are presented as the mean±S.E.M., n=5. ***P<0.001 vs control group

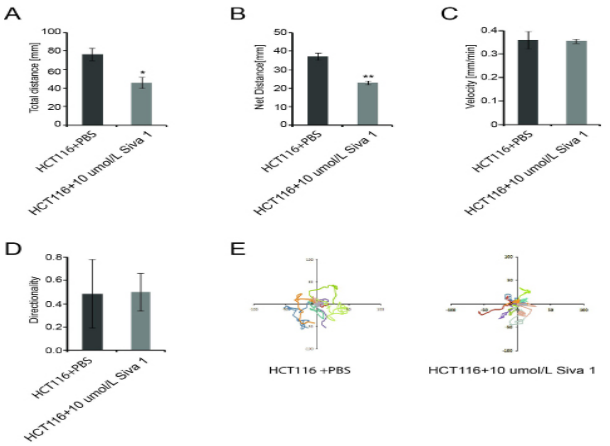

Figure 4. Siva1 Inhibits the Migration of HCT116 Cells. Total distance (A), net distance (B), velocity (C), and direction (D) of HCT116 cell migration after treatment with 10 umol/L Siva1. Data are expressed as the mean S.E.M. of > 50 cells from at least 3 independent experiments. *<0.05; **p<0.01 *vs* control group. (E) Migration tracks of HCT116 cells (n=10) treated with 10 umol/L Siva1 or PBS

5 μg/mL of fibronectin, followed by the addition of 10 μmol/L Siva1. In the control group, PBS was added. The migration of HCT116 cells was analyzed with a Nikon Biostation IM-Q. After Siva1 treatment, the total distance the HCT116 cells migrated was significantly shorter than that in the control group (45.70±5.97 mm vs. 76.52±6.79 mm), and the net distance was also markedly shorter than that in the control group (22.86±0.97 mm vs. 37.21±2.0 mm), indicating that Siva1 can dramatically inhibit the migration of HCT116 cells (Figure 4).

Discussion

We reported in the present study that Siva1 was successfully cloned, expressed and purified. The function studies showed that Siva1 significantly inhibited the migration and invasion of HCT116 cells, the colorectal cancer cells.

Colorectal cancer is one of the most common cancers worldwide. In the development of new anti-tumor drugs,

identifying the molecular and pathogenic mechanisms underlying the metastasis of cancers is crucial. Studies show that many factors potentially get involved in the pathogenesis of colorectal cancer. Wang et al revealed that positive rates and intensities of mRNA and protein expression of Hh signaling pathway related genes SHH, SMO, GLI1 were found to be significantly increased in colorectal cancer tissues, suggesting that hedgehog signaling pathway participated the pathogenesis of colorectal cancer (Wang et al., 2012). However, the exact mechanisms underlying the initiation and development of colorectal cancer remain unknown.

Many studies (Bear et al., 2002; Wu et al., 2011; Li et al., 2011) confirmed that Siva1 can inhibit the microtubule depolymerization activity of stathmin via direct protein interactions. In addition, Siva1 may also inhibit stathmin activity via modulating the phosphorylation of CaMK II at serine 16. Siva1 may promote the generation and stability of microtubules, and block the assembly of focal adhesions and inhibit the cell migration and epithelial-mesenchymal transition. In mice, there is evidence showing that Siva1 can inhibit the metastasis of cancer cells (Huang et al., 2009; Resch et al., 2009; Iorio-Morin et al., 2012). The above findings suggested that Siva1 plays an important role in the regulation of metastasis of cancers and may become a potential target for the treatment of cancers.

In order to further investigate the function of Siva1, we firstly prepared the Siva1 protein using gene engineering techniques as following process. Siva1 gene was successfully synthesized by RT-PCR from HCT116 cells, and the pQE30-Siva1 prokaryotic expression vector was constructed. The fusion protein, His-Siva1 was successfully expressed in E. coli, separated, and purified by affinity chromatography. We found that the techniques are simple and effective for preparing the Siva1 protein.

Using the Siva1 prepared, we tested its functions by observing the effects on migration and invasion of HCT116 cells. We found that Siva1 can significantly inhibit the migration and invasion of HCT116 cells. Regarding the mechanism underlying the inhibition, (Wang et al., 2013) repored that Siva1 is a specific E3 ubiquitin ligase of tumour suppressor alternative reading frame (ARF) which is one of the most frequently mutated proteins in human cancer. Siva1 promotes the ubiquitination and degradation of ARF, which in turn affects the stability of p53. Functionally, Siva1 regulates cell cycle progression and cell proliferation in an ARF/p53-dependent manner. (Li et al., 2011) found that, via the inhibition of stathmin, Siva1 enhances the formation of microtubules and impedes focal adhesion assembly, cell migration, and EMT (Wang et al., 2013). In mouse models, knockdown of Siva1 promotes cancer dissemination, whereas overexpression of Siva1 inhibits it. These results suggested that Siva1 has the property of inhibiting the cancer cell.

In summary, we successfully prepared the Siva1 protein using gene engineering techniques. The functional studies demonstrated that Siva1 inhibits the migration and invasion of colorectal cancer cell. Our findings provide the basic evidence showing that Siva1 might inhibit the occurrence and development of colorectal cancer and has potential for the development of novel anti-tumor drugs.

References

Bear JE, Svitkina TM, Krause M, et al (2002). Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell*, **109**, 509-21.

Fortin A, MacLaurin JG, Arbour N, et al (2004). The proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressors p53 and E2F1. *J Biol Chem*, **279**, 28706-14.

Huang C, Rajfur Z, Yousefi N, et al (2009). Talin phosphorylation by Cdk5 regulates Smurf1-mediated talin head ubiquitylation and cell migration. *Nat Cell Biol*, **11**, 624-30.

Iorio-Morin C1, Germain P, Roy S, et al (2012). Thromboxane A2 modulates cisplatin-induced apoptosis through a Siva1-dependent mechanism. *Cell Death Differ*, **19**, 1347-57.

Li N, Jiang P, Du W, et al (2011). Siva1 suppresses epithelial-mesenchymal transition and metastasis of tumor cells by inhibiting stathmin and stabilizing microtubules. *Proc Nat Acad Sci USA*, **108**, 12851-6.

Okuno K, Yasutomi M, Nishimura N, et al (2001). Gene expression analysis in colorectal cancer using practical DNA array filter. *Dis Colon Rectum*, **44**, 295-9.

Resch U1, Schichl YM, Winsauer G, et al (2009). Siva1 is a XIAP-interacting protein that balances NFkappaB and JNK signalling to promote apoptosis. *J Cell Sci*, **122**, 2651-61.

Siegel R, Naishadham D, Jemal A (2013). Cancer statistics, 2013. CA*: A Cancer J Clin*, **63**, 11-30.

Shen WG (2004). RNA interference and its current application inmammals J. *Chin Med J (Engl)*, **117**, 1084-91.

Sam Brooke J, Russell DW (2002). Molecular Cloning Experiment Guide, 3rd Edition. Science Publishing House, Beijing. 96-9.

Wang H, Li YY, Wu YY, Nie YQ (2012). Expression and clinical significance of hedgehog signaling pathway related components in colorectal cancer. *Asian Pac J Cancer Prev*, **13**, 2319-24.

Wang X, Zha M, Zhao X, et al (2013). Siva1 inhibits p53 function by acting as an ARF E3 ubiquitin ligase. *Nat Commun*, **4**, 1551.

Wu Z, Li X, Sunkara M, et al (2011). PIPKIgamma regulates focal adhesion dynamics and colon cancer cell invasion. *PloS One*, **6**, 24775.