RESEARCH COMMUNICATION

siRNA Interference with a Proliferation-Inducing Ligand Gene in the Sgr-7901 Gastric Carcinoma Cell Line

Shao-Zhong Ni[1,3], Hong-Yong Cao[3], Zheng Chen[1], Yi Zhu[1,2], Ze-Kuan Xu[1,2]*

Abstract

Objective: The present study aimed to investigate the influence of siRNA interference with a proliferation-inducing ligand (APRIL) gene on gastric carcinoma sgr-7901 cell apoptosis. Correlations between APRIL silencing and tyrosine kinase (trka) expression were also explored. Methods: Two APRIL-silencing siRNA vectors were constructed, and transfected into human gastric carcinoma sgr-7901 cells, expression before and after transfection being detected using RT-PCR and western blot analyses. The expression of 15 trka genes was detected using RT-PCR and apoptotic rates of sgr-7901 were assessed by flow cytometry. Results: The expression levels of receptor trka genes were significantly decreased, and the apoptotic rate of sgr-7901 was significantly increased after transfection ($P < 0.05$). Conclusion: APRIL gene silencing can increase the apoptotic rate of gastric carcinoma cells, and inhibit the expression of receptor trka genes. There is a correlation between the signaling pathways of APRIL and trka.

Keywords: Proliferation-inducing ligand (APRIL) gene - RNA interference - gastric carcinoma - tyrosine kinase

Asian Pacific J Cancer Prev, **13**, 1511-1514

Introduction

Gastric carcinoma is one of the most common tumors in the world. Although its incidence rate has decreasein recent years, it is still a type of malignant cancer with a high death rate, especially in East Asian countries including Korea, Japan and China (Stadtlander and Waterbor 1999; Wu et al., 2002; Hohenberger and Gretschel 2003; Jemal et al., 2006). Surgical treatment is a commonly-adopted method for gastric carcinoma patients, and has a good effect for patients at an early stage. But for those with advanced gastric carcinoma or cardia carcinoma, the prognosis is bad. Although recent years also see some new therapies adopted, such as perioperative neoadjuvant chemotherapy (Cunningham et al., 2006) and adjuvant chemoradiotherapy (Macdonald et al., 2001), the prognosis still remains poor. Transcription inhibition through short-stranded oligonucleotides (like antisense DNA and RNA) is a newly-emerging molecule-targeting treatment method (Cho-Chung et al., 1999; 2003; Stephens and Rivers, 2003). It can inhibit the expression of such genes as bcl-2 (Konturek et al., 2001), bcl-XL (Lei et al., 2005), HER2 (Bao et al., 2010) and APRIL (Russell et al., 2009). Researches in the past few years have shown that APRIL is overexpressed in those patients with colon carcinoma who are resistant to 5-FU and have a low survival rate. Base on this finding, it is proposed that APRIL is a new type of anti-5-FU gene, and can serve as a biological marker in predicting the survival rate of patients with colon carcinoma (Russell et al., 2009).

APRIL is also expressed in gastric carcinoma cells (Zhang et al., 2009). However, to the best of our knowledge, study on the expression of APRIL in gastric carcinoma has not been reported up to now. Therefore, we conduct the present study to investigate the correlation between APRIL expression and the apoptosis of gastric carcinoma cells. Meanwhile, the correlation between APRIL and receptor tyrosine kinase (trka) expression is also explored.

Materials and Methods

Cell culture

Gastric carcinoma sgr-7901 cells were cultured in RPMI1640 medium supplemented with 10% FCS in 5% CO_2 at 37 °C in a saturated humidity incubator.

Plasmid construction and transfection

Two pairs of primer were designed. The primers sequences are shown as in Table 1. After being enzyme-linked with renatured plasmids, they were transferred to escherichia coli DH5α, and the transformants were analyzed. Then, they were stably transfected into sgr-7910 cells after determination of blasticidin resistance of the targeted cells.

Real-time analysis

Total RNA was extracted using the RNA purification

*[1]Department of General Surgery, First Affiliated Hospital, , [3]Department of General Surgery, Affiliated Nanjing First Hospital, Nanjing Medical University, [2]Jiangsu Province Academy of Clinical Medicine, Institute of Tumor Biology, Nanjing, China *For correspondence: xuzekuan@yeah.net*

Table 1. Primers Used in Plasmid Construction

APRIL 828 - 848 loci
5'-TGCTGTCAACCAGAGGGCAACTGAGAGTTTTGGCCACTGACTGACTCTCAGTTCCTCTGGTTGA-3'
5'-CCTGTCAACCAGAGGAACTGAGAGTCAGTCAGTGGCCAAAACTCTCAGTTGCCCTCTGGTTGAC-3', named 828 shRNA.
1197 - 1217 loci
TGCTGTTCGGACACCATATCCTTGGGGTTTTGGCCACTGACTGACCCCAAGGATGGTGTCCGAA-3'
5'-CCTGTTCGGACACCATCCTTGGGGTCAGTCAGTGGCCAAAACCCCAAGGATATGGTGTCCGAAC-3', named 1197 shRNA
negative control
5'-TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3'
5'-CAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCGCAGTACATTTCGTCC-3', named APRILshRNA

Table 2. Primers Used in Real-time Analysis

APRIL	5'-GGTATCCCTGGCAGAGTCGTC-3' , 5'-CTGTCACATCGGAGTCATC-3'
ß-actin	5'-CAGTCGGTTGGAGCGAGCAT-3', 5'-GGACTTCCTGTAACAACGCATCT-3'
ABL1	5'-CACTCTTGAACTGGGCGAATGTC-3' , 5'-GCAGGCGTGCTCTGTGAAATAC-3'
FYN	5'-TCCAGTTGACTCTATCCAGGCAG-3' ,5'-CTTTCATATCATCCCAATCACG-3', 5'-GGCTTGTGGGTGATGTTTGAC-3'
SRC	5'-TCCTGGACTCTTGGCTCTTCTATG-3', 5'-TGAGGCAGGAGAATCGCTTGAAC-3'
VEGF	5'-TGCTGGCATAAGAATGATGGCTA-3', 5'-AGGGCAGAATCATCACGAAGT-3' , 5'-GGTCTCGATTGGATGGCAGTA-3'
C-KIT	5'-GGCGACGAGATTAGGCTGTTA-3', 5'-CTTTTCCGTGATCCATTCATTC-3'
EGFR4	5'-GGGCTGATGCGTGAGTGCTG-3', 5'-GGGGAATAGGGTCCGAAGGTC-3'
her4	5'-GGAGATAACCAGCATTGAGCACA-3', 5'-TCCAGAGGCAGGTAACGAAACT-3'
PDGFRB	5'-AATGTCTCCAGCACCTTCGTTCT-3' , 5'-GCAAAAGTATTCTCCCGTGTCTA-3'
VEGFR3	5'-GCACGAGGTACATGCCAACGA-3' , 5'-GCTGCTCAAAGTCTCTCACGAAC-3'
ERK	5'-GGAGATGAAGTTTTCGGTGCGT-3' , 5'-CAGTTGGGGAAGGTCTTGGTG-3'
JNK	5'-TACAGAGCACCCGAGGTCATCC-3',5'-GTGGCAAACCATTTCTCCCATA-3'
MEK	5'-GTTCAAGGTCTCCCACAAGCC-3' , 5'-AACGCACCATAGAAGCCCAC-3'
nf-kb	5'-GAGGATTTCGTTTCCGTTATGTATG-3', 5'-CAGATTTTGACCTGAGGGTAAGACT-3'
p38	5'-ACCAAATTCTCCGAGGTCTAAAGTA-3', 5'-CATCATCTGTGTGCCGAGCC-3'

kit. In the amplification conditions, an initial pre-denaturation step was done at 95 °C for 2 min. This was followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The APRIL genes were amplified, and the gene products were quantitatively analyzed. The primers sequences are shown as in Table 2. Their mRNA contents before and after transfection were quantitatively analyzed.

Western blot analysis

The cells were hydrolyzed with SDS-PAGE sample buffer, and the extracted total protein was quantified using the BAC kit. The protein sample was diluted by loading buffer, and then boiled for protein denaturation. After denaturation, the protein sample was fractionated on 12% SDS-PAGE at a density of 50 μg/well and electro-transferred to the PVDF membrane. The membrane was treated with 5% BSA TBST for 1 h, and incubated with APRIL antibody (Abcam, USA) at dilution of 1:2000 at 4 °C overnight. After washing, it was incubated with HRP-labeled secondary antibody for 1 h. After washing again, the membrane was colorated by enhanced chemoluminescence (Amersham Biosciences, USA).

Flow cytometry

The sgr-7901 cells were collected, hydrolyzed with pancreatin, and medium was then added. After centrifugation at 1000 × g for 5 min, they were suspended in PBS and counted. 5-10×10^3 cells were selected. After centrifugation again, they were resuspended with Annexin V-FITC. The solution was treated by pancreatin, and then mixed with propidium iodide (50 g/ml). The stained cells were analyzed with the flow cytometer (FACSCCanto2, BD, USA).

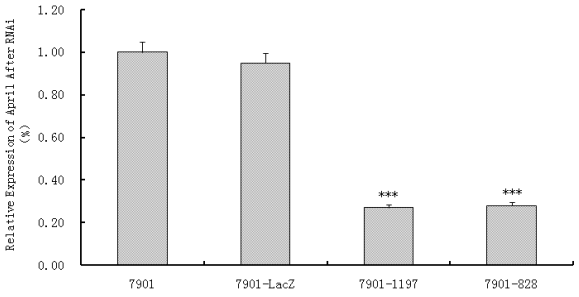

Figure 1. APRIL Gene Expression in Cells before and after Transfection by Real-time Analysis. 7901: the transfected group; 7901-LacZ: the negative control group; 7901 - 1197: the 1197 locus silencing group; and 7901 - 828: the 828 silencing group. ***P < 0.05

Statistical analysis

Data were presented by means ± standard error of means (x ± s). Differences between groups were analyzed using t test. P < 0.05 was considered significant.

Results

The APRIL gene expression before and after transfection

In order to exclude the non-targeting silence effect of shRNA, two pairs of primers targeting at APRIL 1197 and

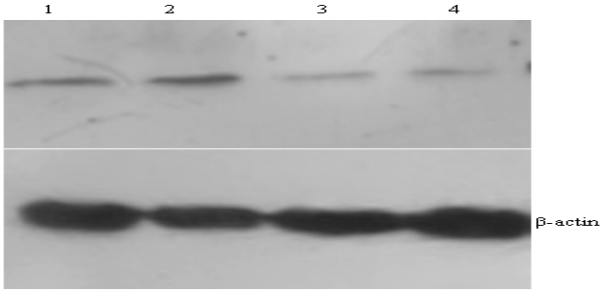

Figure 2. APRIL Protein Expression before and after Transfection by Western Blot Analysis. 1: the transfected group; 2: the negative control group; 3: the 1197 silencing group; 4: the 828 silencing group; and β-actin: the control group

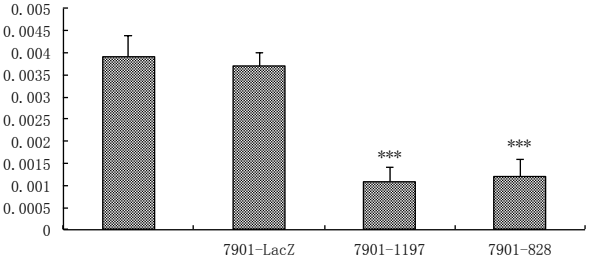

Figure 3. APRIL Protein Expression before and after Transfection by Western Blot Analysis ($^{***}P < 0.05$)

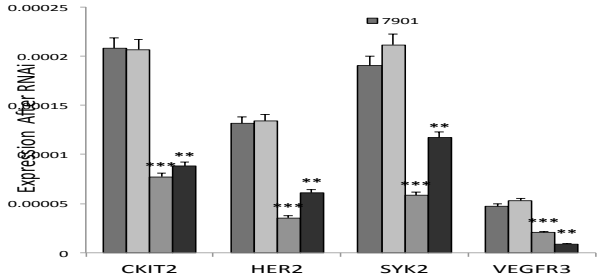

Figure 4. Changes in c-kit, Syk, Vegr3, Her4 and Pdgfrb Expression after Silencing ($^{*}P < 0.05$)

828 loci were designed in the current study. The results showed that the expression of the targeted genes decreased by 73% and 71% after transfection, whereas that of the negative control didn't display an obvious change, which indicated that the silencers designed in the current study had specificity to the targeted genes (Figure 1).

The APRIL protein expression before and after transfection

Western blot analysis showed that the protein expression at both loci decreased significantly after transfection, compared with that of the negative control ($P < 0.05$, Figures 2 and 3).

The trka gene expression before and after APRIL gene silencing

In order to determine the changes in trka gene contents before and after transfection, 15 trka genes were quantitatively analyzed in the current study. The results showed that the expression of c-kit, vegr3, her4 and pdgfrb decreased by 62.50%, 74.21%, 71.28% and 52.86% after 1197 silencing ($P < 0.05$). After 828 silencing, they decreased by 57.44%, 54.48%, 45.92% and 82.98% ($P < 0.05$) (Figure 4). But such significant changes in EGFR4 and other genes were not found.

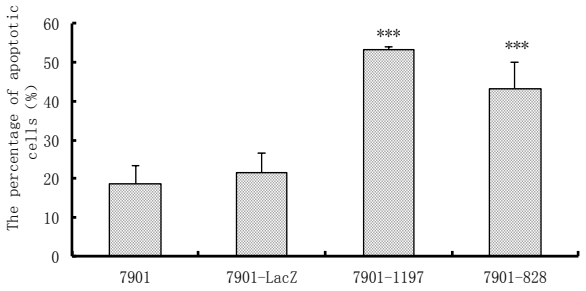

Figure 5. Analyses of Apoptotic Rates before and after Transfection. 1: the transfected group; 2: the negative control group; 3: the 828 silencing group; and 4: the 1197 silencing group

Apoptotic analysis

Apoptosis was analyzed using flow cytometry. After transfection, the apoptotic rate of the transfected group was 18.5 ± 4.69%, the apoptotic rate of the negative control was 21.6 ± 4.98%, the apoptotic rate of the 828 locus silencing group was 46.3 ± 0.82%, and that of the 1197 locus silencing group was 53.07 ± 6.65%, showing significant differences ($P < 0.05$, Figure 5).

Discussion

Gastric carcinoma is one of the most common malignant tumors in the world. Surgical therapy, radiotherapy and chemotherapy are traditional treatment methods for gastric carcinoma. These methods can achieve a satisfactory curative effect on its early stage. But when they are applied in treatment of advanced gastric carcinoma with local diffusion and metastasis, the prognosis is always poor. With the development of the understanding of the pathogenesis of tumors, gene therapies for patients with tumors have been more and more widely studied, such as suicide gene therapy, tumor suppressor gene therapy, immunogene therapy, and combined gene therapy based on single gene therapy. Because RNA interference has the merits of high efficiency, specificity, stability and hereditability, it has been widely adopted in studies on gastroenteric tumors (Li et al., 2009; Xie et al., 2009; Xu et al., 2009).

APRIL is a member of the TNF receptor family, and is formed through the processing of furin in the Golgi apparatus (Kurehara et al., 2007). It is the proliferation-inducing ligand of tumor cells as well as immnocytes, and regulates cell proliferation and survival through the intracellular environment. Unlike other members of the TNF family which exist on the cell surface, APRIL is produced in the form of an independent secretory ligand (Lopez-Fraga et al., 2001). APRIL plays an important role in immune response, and is expressed in dendritic cells, macrophages, and T and B cells (Aggarwal, 2003; Mackay and Ambrose, 2003; Mackay et al., 2003). It can increase the proliferation of T and B cells as well as the survival of T cells in vitro, and influence the response of independent B cells in the thymus in vivo (Medema et al., 2003). APRIL secreted by dendritic cells directly demonstrates the classification of APRIL in vitro, and even more, IgA in vivo is classified based on APRIL

(Litinskiy et al., 2002; Stein et al., 2002). As a member of the TNF family, APRIL is expressed in most tumors of various origins, and is related with the formation and/or maintenance of the tumor. It can obviously promote the growth of solid tumors and lymphatic tumors in vitro, tumor cells transfected with APRIL can grow fast in rats in vivo, and the inhibition of APRIL can obviously inhibit the growth of tumor cells (Hahne et al., 1998; Rennert et al., 2000; Castigli et al., 2004). The current study also shows that the inhibition at two loci of APRIL in grastric carcinoma sgr-7901 cell line can significantly increase the apoptotic rate of the cells ($P < 0.05$).

With the fast development of oncobiology as well as its related disciplines, it is gradually known that the nature of cell canceration is subjected to the unlimited proliferation of cells due to the dysfunction of its signaling transduction pathway. In the process of signaling transduction, many factors take part, such as adenylate cyclase, phospholipase c, protein kinase A, protein kinase C, serine/threonine kinases, g-protein, ATP, calcium, and so on. Among all the factors, trka (shorted for protein tyrosine kinase) is the most important substance in the process of cell signal transduction with multiple cell functions. Too high activity of trka can lead to the activation of its downstream signal pathway, which in turn leads to cell transformation, cell proliferation, the promotion of the survival of the cells, the apoptosis of its antagonistic cells, and ultimately the tumor formation (Huang and Zhu, 2005). Till now, studies on the correlation between APRIL and trka signaling pathways have not been reported, to the best of our knowledge. The current study shows that the expression of most receptor trka is significantly decreased after APRIL silencing. The result suggests that there is correlation between their signaling pathways.

References

Aggarwal BB (2003). Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol*, **3**, 745-56.

Bao W, Fu HJ, Jia LT, et al (2010). HER2-mediated upregulation of MMP-1 is involved in gastric cancer cell invasion.*Arch Biochem Biophys*, **499**, 49-55.

Castigli E, Scott S, Dedeoglu F, et al (2004). Impaired IgA class switching in APRIL-deficient mice. *Proc Natl Acad Sci*, **101**, 3903-8.

Cho-Chung YS (2003). Antisense DNAs as targeted genetic medicine to treat cancer. *Arch Pharm Res*, **26**, 183-91.

Cho-Chung YS, Park YG, Lee YN (1999). Oligonucleotides as transcription factor decoys. *Curr Opin Mol Ther*, **1**, 386-92.

Cunningham D, Allum WH, Stenning SP, et al (2006). Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *New Eng J Med*, **355**, 11-22.

Hahne M, Kataoka T, Schroter M, et al (1998). APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. *J Exp Med*, **188**, 1185-90

Hohenberger P, Gretschel S (2003). Gastric cancer. *Lancet*, **362**, 305-15.

Huang WL, Zhu XF (2005). Signal Transduction [M]. *Beijing: People'8 Medical Publishing House*, 151-99.

Jemal A, Siegel R, Ward E, et al (2006). Cancer statistics, 2006. *CA Cancer J Clin*, **56**, 106-30.

Konturek PC, Konturek SJ, Sulekova Z, et al (2001). Expression of hepatocyte growth factor, transforming growth factor alpha, apoptosis related proteins Bax and Bcl-2, and gastrin in human gastric cancer. *Aliment Pharmacol Ther*, **15**, 989-99.

Kurehara H, Ishiguro H, Kimura M, et al (2007). A novel gene, RSRC2, inhibits cell proliferation and affects survival in esophageal cancer patients. *Int J Oncol*, **30**, 421-8.

Lei XY, Zhong M, Feng LF, et al (2005). Silencing of Bcl-XL expression in human MGC-803 gastric cancer cells by siRNA. *Acta Biochim Biophys Sin*, **37**, 555-60.

Li Q, Zhang N, Jia Z, et al (2009). Critical role and regulation of transcription factor FoxM 1 in human gastric cancer angiogenesis and progression. *Cancer Res*, **69**, 3501-9.

Litinskiy MB, Nardelli B, Hilbert DM, et al (2002). DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat Immunol*, **3**, 822-9.

Lopez-Fraga M, Fernandez R, Albar JP, et al (2001). Biologically active APRIL is secreted following intracellular processing in the Golgi apparatus by furin convertase. *EMBO Rep*, **2**, 945-51.

Macdonald JS, Smalley SR, Benedetti J, et al (2001). Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Eng J Med*, **345**, 725-30.

Mackay F, Ambrose C (2003). The TNF family members BAFF and APRIL: the growing complexity. *Cytokine Growth Factor Rev*, **14**, 311-24.

Mackay F, Schneider P, Rennert P, et al (2003). BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol*, **21**, 231-64.

Medema JP, Planelles-Carazo L, Hardenberg G, et al (2003). The uncertain glory of APRIL. *Cell Death Differ*, **10**, 1121-5.

Petty RD, Samuel LM, Murray GI, et al (2009). APRIL is a novel clinical chemo-resistance biomarker in colorectal adenocarcinoma identified by gene expression profiling. *BMC Cancer*, **9**, 434.

Rennert P, Schneider P, Cachero TG, et al (2000). A soluble form of B cell maturation antigen, a receptor for the tumor necrosis factor family member APRIL, inhibits tumor cell growth. *J Exp Med*, **192**, 1677-84

Stadtlander CT, Waterbor JW (1999). Molecular epidemiology, pathogenesisand prevention of gastric cancer. *Carcinogenesis*, **20**, 2195-208.

Stein JV, Lopez-Fraga M, Elustondo FA, et al (2002). APRIL modulates B and T cell immunity. *J Clin Invest*, **109**, 1587-98.

Stephens AC, Rivers RPA (2003). Antisense oligonucleotide therapy in cancer. *Curr Opin Mol Ther*, **5**, 118-22.

Wu CW, Chi CW, Lin WC (2002). Gastric cancer: prognostic and diagnostic advances. *Expert Rev Mol Med*, **4**, 1-12.

Xie Y, Yin Y, Li L, et al (2009). Short interfering RNA directed against the E2F-1 gene suppressinggastric cancer progression in vitro. *Oncol Rep*, **21**, 1345-53.

Xu R, Sato N, Yanai K (2009). Enhancement of paclitaxel-induced apoptosis by inhibition of mitogen-activated protein kinase pathway in colon cancer cells. *Anticancer Res*, **29**, 261-70.

Zhang JF, Mao ZB, Xu Z, et al (2009). Pathological significance of expression of a proliferation-inducing ligand and its receptor mRNAs in human gastric carcinoma. *Acta Academiae Medicinae Nantong*, **29**, 98-100.