

## RESEARCH COMMUNICATION

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

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### 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

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### Introduction

Gastric carcinoma is one of the most common tumors in the world. Although its incidence rate has decrease in 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<sub>2</sub> 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 DH5 $\alpha$ , 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

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**Table 1. Primers Used in Plasmid Construction**

APRIL 828 - 848 loci	
	5'-TGCTGTCAACCAGAGGGCAACTGAGAGTTTTGGCCACTGACTGACTCTCAGTTCCTCTGGTTGA-3'
	5'-CCTGTCAACCAGAGGAACTGAGAGTCAGTCAGTGGCCAAAACCTCTCAGTTGCCCTCTGGTTGAC-3',
	named 828 shRNA.
1197 - 1217 loci	
	TGCTGTTTCGGACACCATATCCTTGGGGTTTTGGCCACTGACTGACCCCAAGGATGGTGTCCGAA-3'
	5'-CCTGTTTCGGACACCATCCTTGGGGTCAGTCAGTGGCCAAAACCCCAAGGATATGGTGTCCGAAC-3',
	named 1197 shRNA
negative control	
	5'-TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3'
	5'-CAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCAGTACATTTTCGTCC-3',
	named APRILshRNA

**Table 2. Primers Used in Real-time Analysis**

APRIL	5'-GGTATCCCTGGCAGAGTCGTC-3' , 5'-CTGTCACATCGGAGTCATC-3'
$\beta$ -actin	5'-CAGTCGGTTGGAGCGAGCAT-3' , 5'-GGACTTCCTGTAACAACGCATCT-3'
ABL1	5'-CACTCTTGAAGTGGGCGAATGTC-3' , 5'-GCAGGCGTGTCTGTGAAATAC-3'
FYN	5'-TCCAGTTGACTCTATCCAGCGAG-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'-GGAGATGAAGTTTTCCGGTCCGT-3' , 5'-CAGTTGGGGAAGGTCTTGGTG-3'
JNK	5'-TACAGAGCACCCGAGGTCATCC-3' , 5'-GTGGCAAACCATTTCTCCCATA-3'
MEK	5'-GTTCAAGGTCTCCACAAGCC-3' , 5'-AACGCACCATAGAAGCCAC-3'
nf-kb	5'-GAGGATTTTCGTTTCCGTTATGTATG-3' , 5'-CAGATTTTGACCTGAGGGTAAGACT-3'
p38	5'-ACCAAATTCTCCGAGGTCTAAAGTA-3' , 5'-CATCATCTGTGTGCCGAGCC-3'

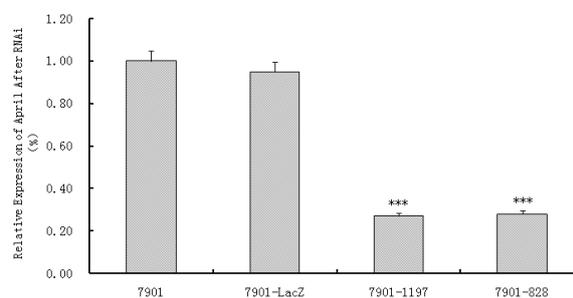
kit. In the amplification conditions, an initial pre-denaturation step was done at 95 °C for 2 min. This was followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The APRIL genes were amplified, and the gene products were quantitatively analyzed. The primers sequences before and after transfection 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  $\mu$ g/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  $\times$  g for 5 min, they were suspended in PBS and counted.  $5-10 \times 10^3$  cells were selected. After centrifugation again, they were resuspended with Annexin V-FITC. The solution was treated by pancreatin, and then



**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

mixed with propidium iodide (50 g/ml). The stained cells were analyzed with the flow cytometer (FACSCanto2, BD, USA).

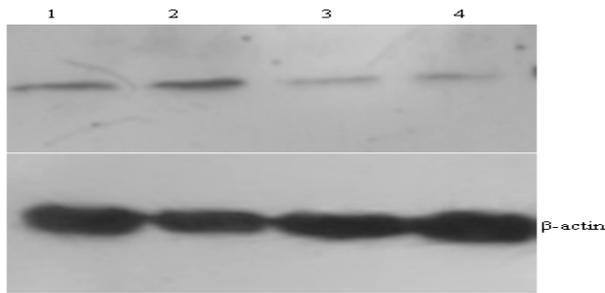
#### Statistical analysis

Data were presented by means  $\pm$  standard error of means ( $\bar{x} \pm s$ ). Differences between groups were analyzed using t test. P < 0.05 was considered significant.

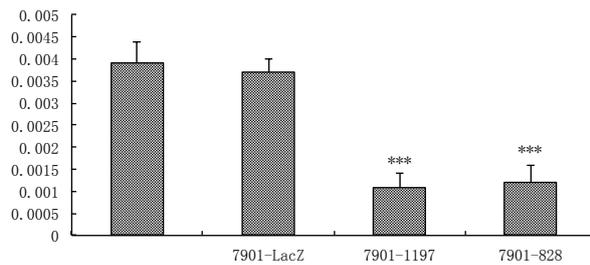
## Results

#### The APRIL gene expression before and after transfection

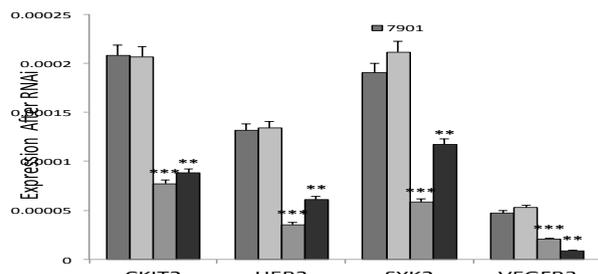
In order to exclude the non-targeting silence effect of shRNA, two pairs of primers targeting at APRIL 1197 and



**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  $\beta$ -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 (\* $P < 0.05$ )**

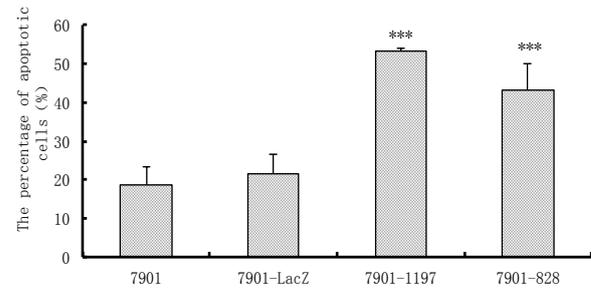
828 loci were designed in the current study. The results showed that the expression of the targeted genes decreased by 73% and 71% after transfection, whereas that of the negative control didn't display an obvious change, which indicated that the silencers designed in the current study had specificity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



**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

and other genes were not found.

#### Apoptotic analysis

Apoptosis was analyzed using flow cytometry. After transfection, the apoptotic rate of the transfected group was  $18.5 \pm 4.69\%$ , the apoptotic rate of the negative control was  $21.6 \pm 4.98\%$ , the apoptotic rate of the 828 locus silencing group was  $46.3 \pm 0.82\%$ , and that of the 1197 locus silencing group was  $53.07 \pm 6.65\%$ , 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 immunocytes, 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 gastric 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.

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