RESEARCH COMMUNICATION

Knockdown of a Proliferation-inducing Ligand (PRIL) Suppresses the Proliferation of Gastric Cancer Cells

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Abstract

Purpose: PRIL (proliferation-inducing ligand) is a newly identified member of the tumor necrosis factor (TNF) family and modulates death ligand-induced apoptosis. Here, we investigated the effect of PRIL on cellular characteristics relating to tumor progression in human gastric cancer. Method: Recombinant lentivirus containing PRIL siRNA was constructed and then infected MGC803 and SGC7901 gastric cancer cells. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colony formation and cell cycle analysis were used to study the effect of PRIL knockdown on gastric cancer cell proliferation. Results: PRIL expression in lentivirus infected cells was significantly reduced as evidenced by quantitative real-time PCR. Cell viability and colony formation of MGC803 and SGC7901 cells were significantly hampered in PRIL knock-down cells. Moreover, the cell cycle was arrested at G2/M phase, elucidating the mechanism underlying the inhibitory effect of siRNA on cell proliferation. Conclusions: Our study indicated that PRIL functions in promoting cell growth, and lentivirus-mediated PRIL gene knockdown might be a promising strategy in the treatment of gastric cancer.

Keywords: APRIL - lentivirus - gastric cancer - proliferation

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Introduction

Gastric cancer (GC), the second most common cause of cancer-related death in the world, can develop in any part of the stomach and spread throughout the stomach and to other organs. There are about 1,000,000 new diagnoses and 800,000 deaths worldwide each year (Kamangar et al., 2006). The 5-year survival rate for curative surgical resection ranges from 30-50% for patients with stage II disease and from 10-25% for patients with stage III disease. The operative mortality rate for patients undergoing curative surgical resection at major academic centers is less than 3% (http://emedicine.medscape.com/article/278744-overview). In this scenario, understanding the molecular mechanisms underlying the initiation and progression of GC is important for prevention, early diagnosis and identifying novel therapeutic and clinical targets for GC (Li et al., 2009; Yu et al., 2009).

A proliferation-inducing ligand (PRIL) (also known as TALL-2, TRDL1, or TNFSF13) is a member of tumor necrosis factor (TNF) family. It was originally identified in cell lines and primary samples from various tumor lesions in 1998 and named for its capacity to stimulate the proliferation of tumor cells (Hahne et al., 1998). In contrast to most TNF family members, APRIL is processed in the Golgi apparatus into the active soluble form by a furin convertase (Lopez-Fraga et al., 2001). APRIL is expressed by a subset of immune cells that also produce B-cell activating factor (BAFF): monocytes, macrophages, dendritic cells, neutrophils, activated B cells and T cells (Hahne et al., 1998; Nardelli et al., 2001; He et al., 2004; Chu et al., 2007) as well as in some non-immune cells such as epithelial cells and osteoclasts. Moreover, PRIL is abundantly expressed in a variety of tumor cells and tissues, such as lung carcinomas, melanoma (Roth et al., 2001; Stein et al., 2002), lymphoid malignancies (Nardelli et al., 2001; Deshayes et al., 2004; Kern et al., 2004; Moreaux et al., 2004; Chiu et al., 2007; Pelekanou et al., 2008; Yaccoby et al., 2008) and in particular gastrointestinal tumors including rectum, duodenum, colon, stomach and esophagus (Hahne et al., 1998; Kelly et al., 2000).

In this study, we report for the first time the functional role of APRIL in the proliferation of GC cells. We found that APRIL gene promotes the proliferation of GC cells by regulating the cell cycle progression.

Materials and Methods

PRIL siRNA design and construction of lentivirus vectors

DNA oligos containing the siRNA sequence (5'-aaTCCAGGATGCTGGAGTTTA-3') targeting human PRIL were chemically synthesized, annealed, and inserted into the lentiviral vector pLVTHM (Biovector Science Lab) which contains green fluorescent protein (GFP). The ligation was transformed into competent *E. coli* DH5α cells. Correct transformant was identified by restriction enzyme analysis and DNA sequencing. A nonsilencing (NS)

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siRNA sequence (5'-TTCTCCGAACGTGTCACGT-3') was used as a control.

Cell culture, lentivirus production and infection

Human gastric cancer cell lines (MGC803 and SGC7901) and 293T cells were purchased from ATCC. Cells were grown in 5% CO_2 saturated humidity at 37°C and cultured as monolayer in RPMI 1640 supplemented with penicillin/streptomycin and 10% FBS.

Recombinant lentiviral vectors were transfected into 293T cells with lentivirus packaging helper plasmids (psPAX2 and pMD2.G, both obtained from Biovector Science Lab) using Lipofectamine 2000 reagent. Lentivirus particles containing APRIL siRNA or scrambled siRNA (named Lenti-siAPRIL or Lenti-NS) were harvested 48 h post-transfection, and the virus titer was determined thereafter. MGC803 and SGC7901 cells were infected with recombinant lentivirus for 3 days at a multiplicity of infection (MOI) of 20 for next investigation.

Real-time RT-PCR

Total RNA in MGC803 and SGC7901 cells were extracted with Trizol (Invitrogen, USA). The reverse transcription was then conducted with M-MLV reverse transcriptase (Promega, USA) at 42°C for 60 min according to the manufacturer's recommendation. APRIL mRNA expression was analyzed using the SYBR green PCR master mix (TaKaRa) with the following program: 95°C for 15 s, followed by 45 cycles consisting of 5 s at 95°C, 30 s at 60°C. Primers used were as follows: APRIL forward: 5'-GGTATCCCTGGCAGAGTC-3', reverse: 5'-CTGTCACATCGGAGTCATC-3' and human β-actin forward: 5'-GGCGGCACCACCATGTACCCT-3', reverse: 5'-AGGGGCCGGACTCGTCATACT-3'. APRIL expression was determined by normalization of the threshold cycle (Ct) of APRIL to that of the control housekeeping gene β-actin. All reactions were performed in triplicate.

MTT assay

Cell viability was assessed by MTT assay as described previously (Li et al., 2010). Briefly, Lenti-siAPRIL or Lenti-NS infected gastric cancer cells were seeded in 96-well plates in complete culture medium at a density of 2,000 cells per well in triplicate. After 1, 2, 3, 4 and 5 days of incubation, 20 μl MTT (5 mg/ml; Sigma-Aldrich Corp.) was added and incubated for another 4 h at 37°C. Then 150 μl of dimethyl sulfoxide was added and thoroughly mixed for 10 min. Absorbance was measured at 570 nm using Victor3 Multilabel Counter Model 1420 (PerkinElmer). Cell growth curves were calculated as means of triplicate per group.

Colony formation assay

MGC803 and SGC7901 cells infected with lentivirus for 3 days were seeded into 6-well plates (800 cells per well) in triplicate. After culture for 14 days, the paraformaldehyde-fixed colonies were stained with Giemsa (Sigma-Aldrich Corp.) for 20 min and photographed with a digital camera. The number of colonies containing more than 50 cells was counted using an inverted microscope.

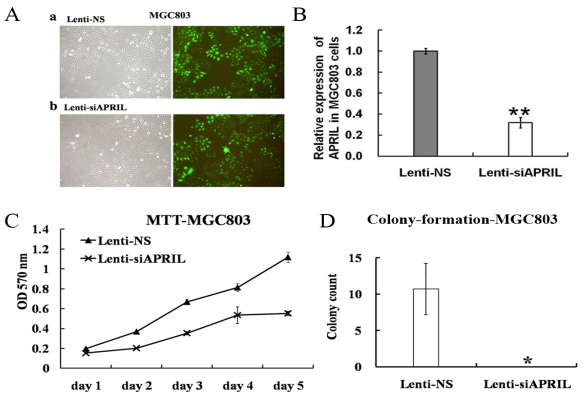

Figure 1. Effect of PRIL-siRNA-Containing Lentivirus on MGC803 Gastric Cancer Cell Proliferation. A. Phase contrast (left) and GFP expression (right) in MGC803 cells under fluorescent microscope 3 days after infection with Lenti-NS (a) and Lenti-siAPRIL (b). Magnification ×100. B. Expression of APRIL mRNA in MGC803 cells infected with lenti-siAPRIL for 3 days. Data shown was $2^{-\triangle\triangle CT}$ value in real-time PCR assay. Each experiment was performed in triplicate. C. Effect of Lenti-siAPRIL on cell viability. MGC803 cells infected with individual lentivirus for 3 days were seeded in 96-well plates and further cultured for 1, 2, 3, 4 and 5 d for MTT assay. D. Colony formation in Lenti-siAPRIL infected MGC803 cells. $^{*}P<0.05$, $^{**}P<0.01$, in comparison with control

Flow cytometric cell cycle analysis

Lentivirus infected cells were collected when the confluence was about 80%, then washed with D-Hanks solution and fixed in 70% ethanol at 4°C for 1 h. Cells were then washed with PBS and stained with 50 μg/ml of PI and 100 μg/ml of RNase (Sigma-Aldrich Corp.). After 5 min incubationat room temperature, cells were analyzed by flow cytometry in triplicate for both groups.

Results

Infection efficacy of recombinant lentivirus

The lentiviruses expressing shRNAs against human APRIL gene and negative control were constructed in this study. After exposure of recombinant lentivirus to SGC-803 cells for 3 days, more than 90% of transfectants showed evident GFP expression (Figure 1A), indicating high and stable infection of lentiviral vector system. Similar results were observed in SGC7901 cells (data not shown).

Down-regulation of PRIL mRNA expression by Lenti-siPRIL

In order to investigate PRIL knockdown efficiency, real-time RT-PCR was performed on lentivirus infected cells. As shown in Figure 1B, APRIL mRNA level in Lenti-siPRIL infected-MGC803 cells were significantly reduced by 68.1% in comparison with Lenti-NS infected MGC803 cells.

Down-regulation of PRIL resulted in inhibited GC cell viability and colony formation

As demonstrated in Figure 1C, the viability of Lenti-siPRIL infected MGC803 cells was evidently decreased

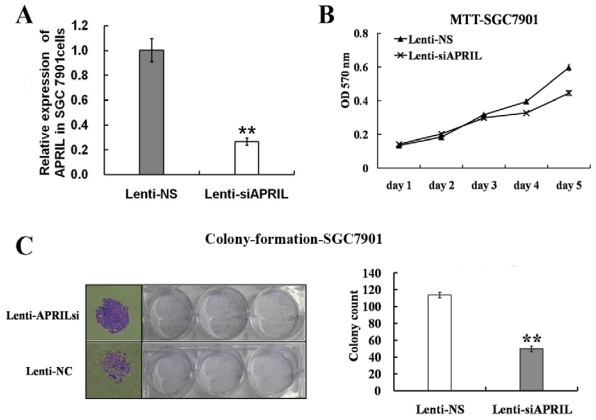

Figure 2. Effect of APRIL-siRNA-Containing Lentivirus on SGC7901 Gastric Cancer Cell Proliferation. A. Expression of APRIL mRNA in SGC7901 cells infected with lenti-siAPRIL for 3 days. Data shown was $2^{-\triangle\triangle CT}$ value in real-time PCR assay. Each experiment was performed in triplicate. B. MTT assay. Lenti-siAPRIL infection resulted in the inhibition of cell viability in SGC7901 cells after 3 days of lentivirus infection. C and D. Colony formation assay. Representative pictures of Giemsa stained colonies of SGC7901 cells (C. Left: micrograph ×50; Right: digital photo). Statistical results of colonies in each well of 6-well plates were shown (D). $^{**}P<0.01$, in comparison with control

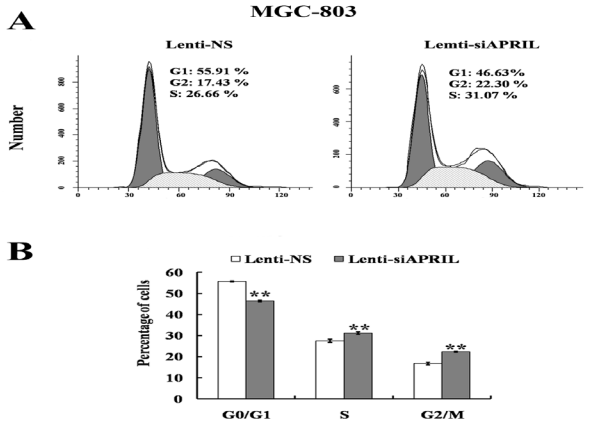

Figure 3. Effects of Lenti-siPRIL on Cell Cycles Distribution of MGC803 Cells. A DNA histogram showing cell cycle distribution by flow cytometry assay; B Statistical results of cells at G0/G1, S, and G2/M phase. $^{**}P<0.01$, in comparison with control

after 2, 3, 4 and 5 days of culture in comparison with Lenti-NS infected cells. We further examined the effect of restraining PRIL expression on in vitro tumorigenicity ability. Results from colony formation assay showed that infection of Lenti-siPRIL resulted in significant reduction in the number of MGC803 cell-formed colonies, indicating significant inhibition of tumorigenicity in MGC803 cells (Figure 1D).

These experiments were repeated using another gastric cancer cell line and gave comparable results. It was showed that APRIL mRNA level in Lenti-siAPRIL infected SGC7901 cells were reduced by 73.6% when compared with Lenti-NS infected SGC7901 cells (Figure 2A). The viability became apparent difference between Lenti-siPRIL and Lenti-NS infected SGC7901 cells on the 4th and 5th day after cell seeding (Figure 2B, $P<0.01$). Moreover, the colony formation of APRIL knock-down SGC7901 cells was significantly inhibited (Figure 2C and D).

Effect of APRIL downregulation on cell cycle

To test whether silencing of PRIL could impede cell cycle progression, flow cytometric cell cycle analysis of MGC803 cells were performed after 3 days of lentivirus infection. As shown in Figure 3, knock-down of APRIL induced increase in the percentage of cells in the S phase and G2/M phase of the cell cycle; while the proportion of cells in the G0/G1 phase was decreased significantly in Lenti-siAPRIL infected cells in comparison with control. In conclusion, the G2/M phase arrest could be responsible for the repression of cell proliferation in GC cells.

Discussion

GC still represents a great health care burden all over the world. Due to the lack of strategies for early diagnosis and detection, GC continues to have poor prognosis (Resende et al., 2010). During the course of cancer development, gene abnormality such as mutation andamplification which may be implicated in tumor survival, progression and metastasis, can be detected frequently. In human GC, a series of specific genes (oncogene and tumor suppressor gene) mutations have been observed, including the PRIL gene.

As a new member of TNF superfamily, PRIL shares about 30% identity with the B cell activation factor from the TNF family (BAFF, also known as B lymphocyte stimulator BLyS, TALL-1 or THANK). APRIL and BAFF share two receptors, B cell maturation antigen (BCMA), and transmembrane activator calcium modulator cyclophilin ligand interactor (TACI), whereas PRIL binds to a third receptor heparin sulfate proteoglycans (Marsters et al., 2000; Ingold et al., 2005; Kalled et al., 2005), and BAFF binds to a third receptor BAFF-R (Kalled et al., 2005).

In animal models, PRIL and BAFF show a tumor-promoting activity, as their overexpression induces development of B cell neoplasia (Batten et al., 2004; Planelles et al., 2004). However, APRIL tumor-promoting activity is not restricted to B cell lymphomas. Indeed, PRIL was also shown to provide a proliferative/survival signal to solid tumor cells. Although only modestly detectable in vitro (Hahne et al., 1998; Roth et al., 2001), this activity has been observed significantly in vivo by overexpressing APRIL in tumor cells (Hahne et al., 1998) or by blocking endogenous PRIL (Rennert et al., 2000).

RNA interference (RNAi) has emerged as a powerful genetic tool for studying gene functions and developing highly specific therapeutics by posttranscriptional silencing of gene expression (Fire et al., 1998; Wang et al., 2007). The effective delivery of siRNA molecules into target cells or tissues is critical for successful RNAi application. Lentiviruses are a family of retroviruses that can integrate into the genomes of not only dividing cells but also nondividing cells with high efficiency to achieve stable, long-term expression of shRNAs, and thus have become one of the favorite delivery systems

for exogenous genes transfer (Naldini et al., 1996; Fish and Kruithof, 2004).

In this study, we demonstrated that recombinant lentiviruses could efficiently express APRIL-siRNA in MGC803 and SGC7901 cells. Infection of Lenti-siAPRIL resulted in great downregulation of endogenous APRIL, and induced inhibited cell viability and colony formation, implying that APRIL modulates the proliferation of GC cells. Moreover, silencing of APRIL leads to significant increase in the proportion of S phase and G2/M phase cells, indicating that APRIL siRNA inhibits cellular proliferation by inducing a G2/M phase cell cycle arrest. APRIL may play a functional role in regulating the cell cycle progression. Further investigation of the functional role of APRIL may lead to a better understanding of the molecular mechanism of gastric cancer.

Taken together, our results showed that knockdown of APRIL by lentivirus-mediated siRNA effectively inhibits the cell viability and colony formation of GC cells by arresting cell cycle at G2/M phase. These results provide novel clues for investigating the malignant proliferation of gastric cancer.

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