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APRIL knockdown suppresses migration and invasion of human colon carcinoma cells

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Abstract

Objective: The purpose of this study was to investigate the function of a proliferation-inducing ligand (APRIL) gene among the metastatic colorectal cancer (CRC) disease.

Methods: Cell adhesion, cell migration and invasion behavior were detected after knockdown of APRIL expression in the colon carcinoma cell line, SW480 by RNAi and the rescue experiments were performed with recombinant human APRIL (rhAPRIL) *in vitro*.

Results: This original study indicated that knockdown of APRIL expression strongly inhibited colon carcinoma cell adhesion, cell migration and invasion *in vitro*. By contrast, reconstitution of APRIL expression with rhAPRIL in these APRILi cells, prominently restores CRC cell migration and invasion.

Conclusion: These results strongly suggest that APRIL play an important role in the tumor development of the invasive or migratory behavior of CRC cells and may be a useful therapeutic target in the malignant colon carcinomas.

Keywords: APRIL; RNAi; Invasion; Migration; Colorectal neoplasms

Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer death in the US [1]. And in China, the attack rate of colon carcinoma is increasing year by year. Despite the increased use of screening strategies such as fecal occult blood testing, sigmoidoscopy and colonoscopy, more than one-third of patients with colorectal cancer will ultimately develop metastatic disease. Although several new treatment options have become available for metastatic colorectal cancer in the past decade, most patients will eventually die of uncontrolled metastatic spread within a few years of diagnosis [2]. It is widely accepted that genic factors play key roles in the predisposition to colorectal cancer development and progression, but the category of genes involved in colorectal cancer cases are still undefined.

APRIL (a proliferation-inducing ligand, also known as TRDL-1, TALL-2, and TNFSF13) is a member of the TNF family and has been shown to be capable of inducing the proliferation of certain tumor cell lines *in vitro* and *in vivo* [3]. The encoding APRIL gene is located on human chromosome 17p13, contains six exons transcribed as three alternatively spliced mRNA of 1.8, 2.1 and 2.4 kb, and encodes a common 250-amino-acid protein [3,10]. As a cytokine of the TNF family, APRIL has pleiotropic biological effects, including stimulating tumor cell growth [3], modulating tumor cell apoptosis [16], and activating nuclear factor-kappa (NF-κB) [6,9] which functions by binding to its receptors. TACI (transmembrane activator and cyclophilin ligand interactor) and BCMA (B-cell maturation antigen) [4,13–15] are two common receptors for APRIL. However, HSPG (heparan sulfate proteoglycans), which can be blocked by heparin, is found to serve as a special receptor or a binding partner for APRIL [17,18]. BCMA and TACI are all expressed on B cells, while TACI is also detected on the surface of some T cells [7]. APRIL can promote B-cell

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proliferation by binding to BCMA and TACI receptors, but the role of APRIL in immune regulation is not well defined [4–9]. In contrast, HSPG may exist in Jurkat T cells, fibroblasts, and epithelial cell lines that do not express either BCMA or TACI [11,13]. And it is an alternative possibility that cell proliferation is induced by HSPG-dependent binding of APRIL to these BCMA- and TACI-negative cell lines [11]. Nonetheless, the biological role of APRIL does not seem to be restricted to proliferation induction [3], but to be more complex, as a proapoptotic effect of APRIL has been demonstrated as well [12]. Furthermore, overexpression in a number of tumors tissues or cell lines, such as colon carcinomas and lung cancer, suggests a regulatory role for APRIL in tumor growth [3,11].

It is increasingly understood that changes in the adhesive and migratory capabilities of tumor cells, as well as the tumor microenvironment play critical roles in malignant tumor progression and invasion [19].Therefore, in current study, we investigated the functional contribution of APRIL in determining colorectal cancer cell migration and invasion *in vitro*. Knockdown of APRIL expression by RNAi inhibited colon carcinoma cell migration and invasion *in vitro*. In contrast, reconstitution of APRIL expression with recombinant human APRIL (rhAPRIL) in the APRIL-knockdown colorectal cancer cells restored the malignant cell migration and invasion. These data further indicate that APRIL function in the tumor development of the invasive or migratory behavior of colorectal cancer cells and may be a useful therapeutic target in the malignant colon carcinomas.

Materials and methods

Recombinant plasmids, stably transfected cell line and related reagents

The pGCsi/H1/Neo/GFP plasmid vector (Genechem, Shanghai, China) was digested to line by the restriction enzymes, BamH I and Hind III (Promega, San Francisco, California, USA). Four pairs of chemically-synthesized shRNA oligonucleotides of APRIL were inserted into this plasmid through T4 DNA polymerase ligation at 16 °C for 8 h, and designated sh637, sh1450, sh1534, and sh1750 (Supplementary Table 1). The plasmids, pGEM-APRIL and pGEM-18S, were bacterial expression vectors in which the human APRIL or human 18S RNA PCR products were inserted in the poly dT sites of the pGEM-T Easy vector (Promega), respectively.

The colon carcinoma cell line, SW480 (ATCC, Manassas, USA) was seeded at 1×10^5/12 well and the next morning was transiently transfected with lipofectamine 2000 (Invitrogen, Jefferson City, MO, USA), according to the manufacturer's instructions, with sh637, sh1450, sh1534, sh1750, or negative control plasmids. After 48 h, cells were screened by neomycin G418 (Invitrogen, 600 μg/mL) to harvest the stably transfected cell line.

Analysis of APRIL mRNA level by RTFQ-PCR

Real-time fluorescence quantitative PCR (RTFQ-PCR) analysis for APRIL was performed by using a LightCycler 1.5 Instrument (Roche, Mannheim, Germany). PCR was performed in a LightCycler capillary in a 20 μL reaction volume that contained 1× DNA Master SYBR Green I, 0.2 mM dNTPs, 3.5 mM $MgCl_2$, 0.3 μM primers, and 2 μL cDNA. The PCR protocol was as follows: initial denaturation for 3 min at 94 °C, 40 cycles at 94 °C for 6 s, 60 °C for 15 s, and 72 °C for 12 s. Results were analyzed with LightCycler software, version 3.5.3.

Western blot analysis

Cells were washed with PBS, lysed in 50 μL of ice-cold protease inhibitor cocktail and RIPA buffer (Beyotime, Nantong, China). Lysates were resolved by 5% and 12% SDS-PAGE before transfer to a nitrocellulose membrane (AMC, Ann Arbor, MI, USA). Then membranes were incubated for 4 h at room temperature with anti-hAPRIL antibody (Protein Tech Group, Chicago, USA). The primary antibodies were visualized with goat anti-rabbit peroxidase-conjugated antibodies (Sigma, St. Louis, MO, USA) using an enhanced chemiluminescence (ECL) detection system. Blots were quantified by densitometry using acquisition into Adobe Photoshop (Apple, Cupertino, CA, USA) and analyzed with the Bandscan Image software.

SW480 cell adhesion assay

SW480 cells (10^6 cells/mL) stably transfected with sh1750 (hereinafter referred to as APRILi cells) or negative control shRNA (hereinafter referred to as control cells) or not were incubated in the wells coated with Human Collagen Type IV (Chemicon, USA) at 37 °C for 45 min in a CO_2 incubator. And 100 μL of 0.2% crystal violet was added to each well and incubated for 5 min at room temperature. The absorbances at 540 nm were determined when dissolved the stain cells with 100 μL of Solubilization Buffer (A 50/50 mixture of 0.1 M NaH_2PO_4, pH 4.5 and 50% ethanol) and data were present with optical density (OD).

SW480 cell migration assay

Confluent SW480 cell monolayer was incubated for 24 h in serum-free medium in 60 mm dishes, then wounded with a sterile razor blade and incubated in the treatment of stably transfected sh1750 or negative control for 48 h at 37 °C in 5% CO_2, take the wounded one incubated in medium alone as the wild-type SW480 control. Conversely, wounded APRILi cell monolayer was incubated in the presence of 1 ng/mL recombinant human APRIL (rhAPRIL, Alexis Biochemicals, Switzerland) [20] for 48 h at 37 °C in 5% CO_2. Photomicrographs were taken using 100× magnification at 4–5 locations per wound and the number of migrated cells was determined by counting nucleated cells that crossed the wound edge.

SW480 cell invasion assay

The cell invasion assay was performed in an invasion chamber (Chemicon, USA), a 24-well tissue culture plate with 12-cell culture inserts. Cell suspensions (0.5×10^6 cells/mL)

stably transfected with sh1750, control shRNA or medium alone were added to the interior of the inserts with 300 μL serum-free media, 500 μL of media containing 10% fetal bovine serum to the lower chamber and incubated in a tissue culture incubator for 72 h. Adversely, a rescue experiment which rhAPRIL was administrated to APRILi cell suspensions was practiced. Colorimetric reading of OD at 560 nm was quantitated when invasive cells on lower surface of the membrane were stained and dissolved in 10% acetic acid.

Statistics

All data were imported to SPSS 13.0, checked, and cleaned. Independent sample *t* tests were used for analyses involving two samples, and ANOVA, with post-hoc Scheffe tests, for tests involving more than two samples. All tests are two-tailed (criterion level, $P<0.05$).

Results

A prominent knockdown effect on APRIL gene occurred in SW480 after stable shAPRIL transfection

In order to exclude an off-target silencing effect mediated by specific shRNA, we employed four different sequences of shAPRIL in our present study. To evaluate the inhibition of APRIL mRNA expression, RTFQ-PCR was performed after stable transfection, the APRIL mRNA expression in SW480 cells transfected with sh637 and sh1750 were reduced by 97% and 88%, respectively, as compared with the wild-type SW480 cells and negative shRNA control transfected ones ($P<0.05$). In addition, no difference was observed among the wild-type SW480 cells and negative shRNA control, sh1450 and sh1534 transfected cells (Fig. 1a), indicating that the corresponding mRNA sequences for sh637 and sh1750, though not sh1450 or sh1534, are specific RNAi targets.

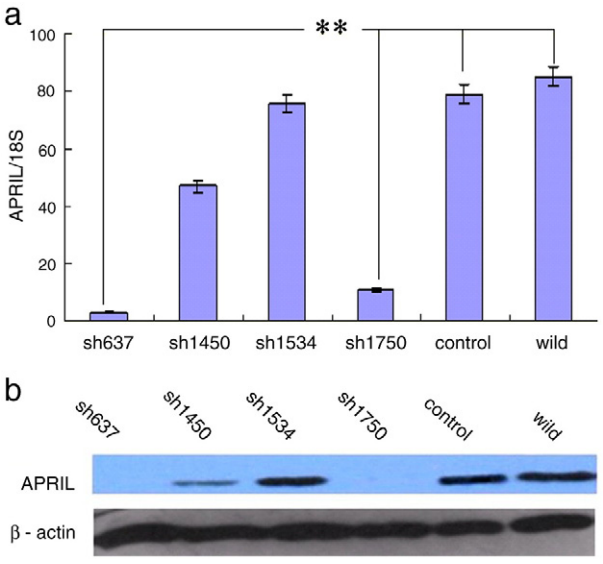

Fig. 1. Expression of APRIL mRNA (a) and protein (b) after SW480 cells transfected by i) sh637, ii) sh1450, iii) sh1534, iv) sh1750 v) negative shRNA control and vi) wild-type. Representative data from 3 independent experiments were shown. $^{**}P<0.01$. β-actin was used as a control.

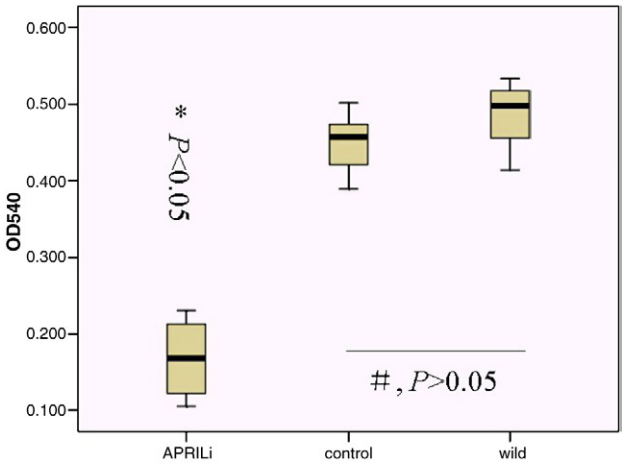

Fig. 2. Knockdown APRIL significantly depressed SW480 cell adhesion. APRILi: group of SW480 cells stably transfected with sh1750, control: negative shRNA control group, wild: wild-type SW480 cells group. * APRILi compared to wild and control.

Western blot analysis was also performed after stable transfection. The APRIL protein expression demonstrated a significant reduction in sh637 (0.25±0.9% of beta-actin) and sh1750 (1.45±1.9% of beta-actin) transfected SW480 cells respectively, as compared with the wild-type SW480 cells (38.9±3.2% of beta-actin), negative shRNA control (39.7±4.3% of beta-actin), sh1450 (34.3±4.2% of beta-actin) and sh1534 (36.3±3.7% of beta-actin) transfected ones ($P<0.05$, Fig. 1b), suggesting that sh637 and sh1750 strongly blocked APRIL expression, whereas no obvious inhibition of APRIL protein was observed in the wild-type SW480 cells, negative shRNA control and sh1450 or sh1534 transfected cells.

Knockdown APRIL depresses SW480 cell adhesion

The SW480 cell adhesion ability was investigated with the absorbance at 540 nm. The APRILi cells significantly reduced the adhesion rate compared to the wild-type SW480 or negative control cells (OD: 0.181±0.113 vs 0.409±0.113, 0.412±0.113, $P<0.05$, Fig. 2).These data demonstrated the knockdown of APRIL gene markedly depressed SW480 cell adhesion compared with the wild-type SW480 or control cells *in vitro*.

Silencing APRIL expression strongly inhibits SW480 cell migration

The effect of silencing expression of APRIL protein on SW480 cell migration was investigated with the *in vitro* migration assay (Fig. 3). Compared to the APRILi cell line, which migrated at 47.9±8.2 per day, the wild-type SW480 cells migrated at 108.4±16.1 per day ($P<0.01$) and the control cells migrated at 98.8±10.3 per day ($P<0.03$). APRILi cells that were administrated with rhAPRIL rescued their migration deficit relative to the APRIL-knockdown CRC cells (83.9±8.7 vs 47.9±8.2 per day, $P<0.05$). Taken together, these data

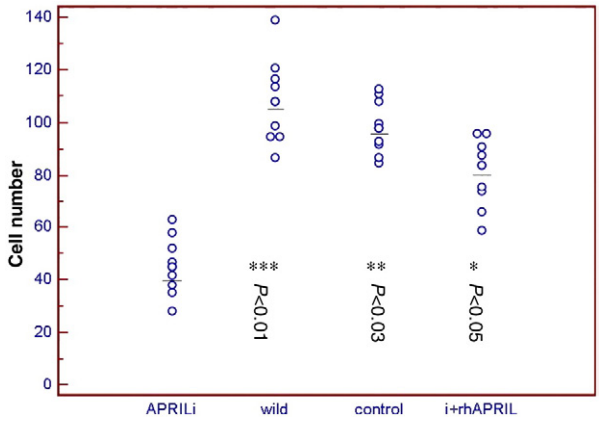

Fig. 3. Knockdown APRIL strongly inhibits SW480 cell migration. APRILi: group of SW480 cells stably transfected with sh1750, control: negative shRNA control group, wild: wild-type SW480 cells group, i+rhAPRIL: group of APRILi cells administrated with rhAPRIL. * i+rhAPRIL compared to APRILi, ** control compared to APRILi, *** wild compared to APRILi.

supported the view that knockdown of APRIL expression significantly reduced the migration rate of APRILi cells compared to the wild-type SW480 and control cells in the migration assay.

Knockdown APRIL protein depresses SW480 cell invasion

In our invasion model, APRILi cells invaded at 0.582±0.104 (OD) vs 0.954±0.229 (OD) for the control cells and 1.112 ±0.233 (OD) for the wild-type SW480 cells (Fig. 4). And APRILi cells administrated with rhAPRIL rescued their invasion deficit relative to the APRIL-knockdown CRC cells (OD: 0.922±0.112 vs 0.582±0.104, $P<0.05$). These data verified that knockdown of APRIL expression prominently reduced the invasion ability of APRILi cells compared to the wild-type SW480 and control cells *in vitro*.

Discussion

RNA interference (RNAi) is an evolutionarily conserved process of gene silencing in multiple organisms, which has become a powerful tool for investigating gene function by reverse genetics [21]. Several colon carcinoma cell lines including the SW480 cell line, have been found to overexpress APRIL gene in a previous study [3]. Herein, we constructed a short hairpin RNA (shRNA) expression vector stably knock-downed APRIL gene in SW480 cells to observe the tumor development of colorectal cancer.

APRIL, a member of the TNF superfamily, can induce cell proliferation and is overexpressed in most tumor tissues or cells [3–9]. However, relatively little is known about the role of APRIL gene in relation to cancer metastases. Therefore, in the study described herein we provided clear evidence for the ability of APRIL gene and protein to reinforce colorectal metastases of the colon carcinomas by several generally adequate extra-experiments, which knockdown APRIL protein prominently inhibited SW480 cell adhesion, migration and invasion whereas rhAPRIL restored colorectal cancer cell migration and invasion in the APRIL-knockdown cells *in vitro*. To our knowledge, the proliferation and invasion are both phenotypes ultimately need to be addressed for effective tumor management. In our study, we found that APRIL could induce not only proliferation but metastases of the tumor cell. Theses results indicated that proliferation and invasion are integral to the pathogenesis of CRC by APRIL gene.

Why is APRIL involved in invasion and metastases of CRC? Previous studies reported that APRIL has two definite receptors, BCMA and TACI [13–15] and HSPG, which served as a third receptor or co-receptor of APRIL in the colon carcinoma cell line [11,17,18]. It will be interesting to test which one participates in colorectal metastases of the colon carcinomas along with APRIL. BCMA and TACI primal function is to promote B or T cell proliferation by binding to APRIL ligand [11–15]. The HSPGs is known as a matrix protein collection that can be either extracellular (e.g. Perlecan, Agrin) or transmembrane proteins (i.e. syndecan-1–4, glypican 1–6 or CD44v3). Via their HS chain, HSPGs can bind growth factors (e.g. Wnt, TGFb, FGF1/2, IGF, VEGF and HGF), cytokines, chemokines, proteases and protease inhibitors (antithrombin) [17,22–24]. HSPGs play an important role in a wide variety of biological responses and processes such as adhesion, migration, proliferation, embryonal development, differentiation, morphogenesis, angiogenesis and blood coagulation [22–25]. For example, both syndecan-1 and CD44 variants are expressed in myeloma and, in addition to binding growth factors, promote adhesion to bone marrow stromal cells that become stimulated for IL-6 secretion [18,26]. In our unpublished data, we have detected a high level of HSPG mRNA and protein expression in SW480 cells and no alteration of the matrix metalloproteinase-2 (MMP-2), MMP-7 and MMP-9 with gelatinase spectrum analysis after APRIL gene knockdown. Therefore, a possible interpretation is that the metastasis-promoting ability of APRIL is engagement with HSPG.

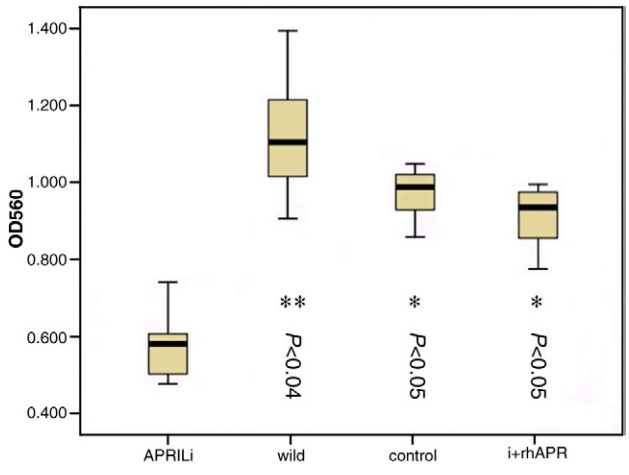

Fig. 4. Knockdown APRIL significantly depressed SW480 cell invasion ability. APRILi: group of SW480 cells stably transfected with sh1750, control: negative shRNA control group, wild: wild-type SW480 cells group, i+rhAPR: group of APRILi cells administrated with rhAPRIL. * i+rhAPR or control compared to APRILi, ** wild compared to APRILi.

In conclusion, the colorectal metastasis is an incurable progression of colorectal cancer disease, which leads patients' survival to end up [1,2]. Understanding the relative gene networks (e.g. APRIL, HSPGs) that trigger the colon carcinoma cell migration and invasion could potentially result in the development of novel anti-invasive therapies or approaches that effectively target migrating cells to increase their susceptibility to current treatment modalities.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2009.06.019.

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