

Original Article

APRIL-NF- κ B-Snail signaling regulates E-cadherin in colon carcinoma

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Abstract: E-cadherin is a member of transmembrane glycoproteins that mediates calcium-dependent cell-cell adhesion in epithelial tissues. E-cadherin, which blocks tumor cell invasion by restraining cells and preventing cell movement, is negatively regulated by the transcriptional repressor Snail, and stabilization of Snail is dependent on activation of NF- κ B signaling. Provided that a proliferation-inducing ligand (APRIL) is highly expressed in colorectal cancer (CRC) tissues and cell lines, in this study, we tested the hypothesis that APRIL-p65 NF- κ B-Snail signaling regulates E-cadherin in CRC. We found that expression of Snail was inversely related to that of E-cadherin in CRC tissues and colon carcinoma cell line T84. In addition, APRIL treatment induced Snail at protein level only but not mRNA level, and COP9 signalosome subunit 2 (CSN2) that acts to block ubiquitination and degradation of Snail at both mRNA and protein levels. Similarly, depletion of p65 by siRNAs led to up-regulation of Snail at protein level only, but not at mRNA level in T84 cells. Moreover, silencing of Snail resulted in decrease of E-cadherin at both protein and mRNA levels. These results suggest that APRIL-p65 NF- κ B-Snail signaling plays a critical role in regulation of E-cadherin and provide insights into mechanisms for paracrine inflammation-induced metastasis in CRC.

Keywords: APRIL, NF- κ B, Snail, E-cadherin, colon carcinoma

Introduction

Colorectal cancer (CRC) represents a major public health problem worldwide [1]. Local invasiveness and distant metastasis are the leading causes of mortality associated with CRC. Despite the progress in the diagnosis and treatment of CRC, the mechanisms underlying tumor metastasis remain poorly defined. Recent advances in large-scale sequencing and other genomic analyses of human colorectal tumors have offered new opportunities for further characterization of molecular mechanisms of CRC proliferation and progression [2, 3]. By characterizing and classifying common and rare mutations, Wood et al. have shown that there were 38 pathways dysregulated in CRC with particular frequency [2]. Of note, NF- κ B signaling pathway was deemed as a novel one because it had not been previously reported.

NF- κ B signaling pathway plays an essential role in proliferation, development, and survival in both normal and a variety of cancer tissues

[4-6]. NF- κ B is an inducible dimerized transcription factors consisting of five Rel family members of DNA-binding proteins: p50/p105 (NF- κ B1), p52/p100 (NF- κ B2), c-Rel, RelA (p65) and RelB. As a well-defined pathway, the canonical NF- κ B signaling is activated following degradation of I κ B α , which leads to nuclear translocation of NF- κ B complexes, typically, p65/p50 heterodimers. The degradation of I κ B α is mediated through I κ B kinase associated phosphorylation. The canonical NF- κ B signaling pathway regulates a number of biological processes including dendritic cell activation, B lymphocyte survival and maturation, and lymphoid organogenesis.

The canonical NF- κ B activation is triggered in response to numerous signals, which include those mediated by innate and adaptive immune receptors. These immune receptors typically include TLR, TNFR, BCR, and TCR [7]. APRIL (a proliferation-inducing ligand, or TNFSF13) is a member of the tumor necrosis factor (TNF) superfamily and a cytokine that can stimulate

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cellular proliferation [8-10]. APRIL is highly expressed in cancer tissues such as colon carcinoma and pancreatic cancer [11]. Previous studies have revealed that aberrant activation of APRIL is closely associated with both hematological malignancies and solid tumors [9, 12-14]. A recent study has provided some mechanistic insights into how APRIL, via the PI3K/Akt pathway, increases invasiveness of CRC by increasing MMP-2 and MMP-9 expression [11]. APRIL can activate the canonical NF- κ B signaling by cross-linking to the TNF receptor TACI (TNFRSF13B) [9].

It is well documented that a major factor promoting cell migration and invasion in solid tumors is disruption of E-cadherin [15-18]. E-cadherin is a member of transmembrane glycoproteins that mediates calcium-dependent cell-cell adhesion in epithelial tissues. E-cadherin inhibits tumor cell invasion likely restraining cells and preventing cell movement. Dorudi et al showed that loss of E-cadherin was predominantly observed for CRC patients with liver and lymph node metastasis, further supporting that inhibition of E-cadherin is closely associated with progression, invasions and metastasis of CRC [19]. Several transcriptional repressors of E-cadherin have been identified, including the zinc finger factors Snail, which has been described as a direct repressor of E-cadherin expression during development and carcinogenesis [20]. Indeed, in breast cancer, NF- κ B (p65/p50) is essential for the inducible expression of COP9 signalosome 2 (CSN2), which, in turn, prevents the ubiquitination and degradation of Snail [21].

In the present study, we tested the hypothesis that APRIL-NF- κ B (p65/p50)-Snail signaling pathway plays an important role in regulation of E-cadherin expression in CRC. Human CRC tissues were stained immunohistochemically for assessing expression of Snail and E-cadherin. Human colon carcinoma cell line (T84) was used to evaluate the effects of knockdown of p65 or Snail on Snail and E-cadherin, respectively. Our data suggest that induction of APRIL could be related to tumorigenesis and metastasis.

Materials and methods

Cancer tissues and cell cultures

Colon cancerous tissues were collected after patients received and signed the informed con-

sent. The protocol was approved by the ethic committee of the Second Hospital of Jilin University, Changchun, China. Human colon carcinoma cells T84 were purchased from ATCC. The cells were maintained in Dulbecco's modified Eagle's medium and supplemented with 10% FBS at 37°C and 5% CO₂.

Antibodies and reagents

Antibodies included p65 (Cell Signaling, MA, USA), GAPDH (Cell Signaling), CSN2 (Sigma-Aldrich, MO, USA), TACI (Abcam, MA, USA), Snail (Abcam, MA, USA), and E-cadherin (Santa Cruz Biotech, TX, USA). Recombinant APRIL was purchased from R&D systems (MN, USA) and the cells received APRIL treatment at 1 μ M for 24 hrs. FlexiTube siRNAs were purchased from QIAGEN (CA, USA).

Immunohistochemistry

Human colon cancer samples were fixed in buffered formalin for 24 h, dehydrated in 70% ethanol, paraffin-embedded, and sectioned. Following incubation with primary antibodies as indicated, the sections were washed with Tris-buffered saline and Tween 20 (TBST) and incubated with biotinylated secondary anti-rabbit or anti-mouse antibody diluted in TBS. Sections were washed again with TBST and secondary antibodies were detected using an ABC reagent for 30 min.

Two independent pathologists evaluated intensity of staining on the scale of 0 to 4 (0 corresponds to the absence staining and 4 is highest degree of staining).

Immunofluorescent staining

As recently described [22], T84 cells were first fixed with 4% paraformaldehyde in PBS and permeabilized for 10 minutes in 0.5% Triton X-100 in PBS at room temperature. Cells were then washed with PBS and incubated with appropriate antibodies as indicated at room temperature for 1 hr. Cells were washed with PBS containing 0.1% BSA and incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG or 532 anti-mouse IgG) for 1 hr at room temperature. The cells were then counterstained with DAPI (Invitrogen) and visualized under a fluorescence microscope.

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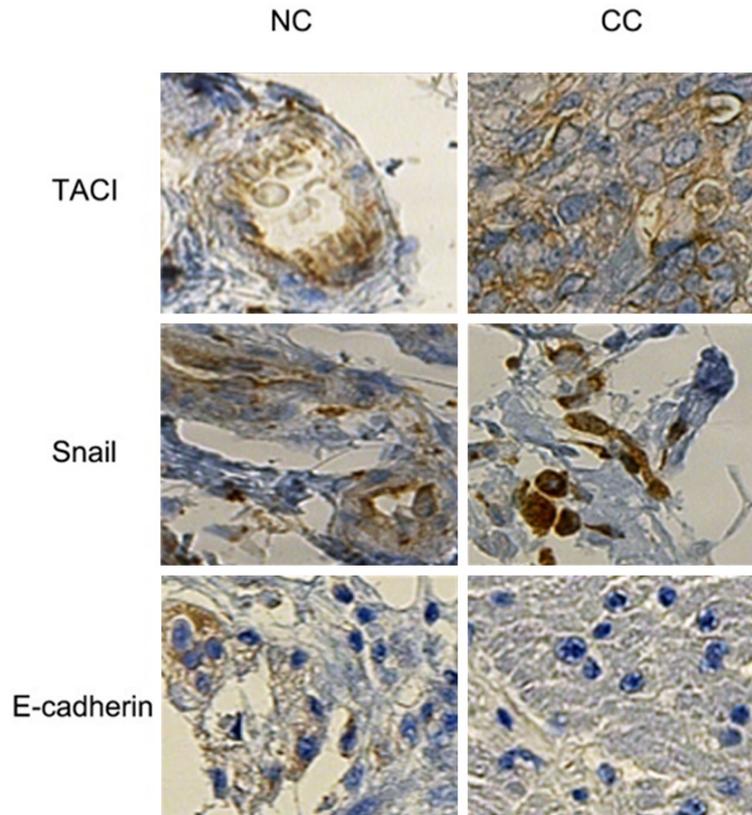


Figure 1. Immunohistochemical staining of TAC1, Snail, and E-cadherin in colon tissues. Representative image from 25 specimens. NC, adjacent non-cancerous tissues, CC, colon adenocarcinoma. Original magnification, $\times 200$.

Quantitative real-time RT-PCR (RT-qPCR) and primer sequences

Total RNA was extracted from colon cancer tissues or T84 cells with use of Trizol (Invitrogen). Reverse transcription of RNA was carried out with 2.5 μg of total RNA using a SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). The resulting cDNA was subjected to real-time PCR using SYBR green dye (Qiagen) on an ABI 7300 real-time PCR machine (Applied Biosystem) as previously described.

Primers for qPCR (Forward/Reverse): Snail, 5'-ACCCACATCCTTCTCACTG-3'/5'-TACAAA-AACCCACGCAGACA3'; CSN2, 5'-AAACAGTGG-AGGAGCATGG-3'/5'-TTCCGTTTTCTACAAT-TTCAA-3'; E-cadherin, 5'-GGCTCAAGCTATCCTT-GCAC-3'/5'-GTGCAGTGGCTCATGTCTGT-3'; GAPDH, 5'-GTCAGTGGTGGACCTGACCT3'/5'-TGCTGATGCCAAATTCGTTG-3'.

Chromatin immunoprecipitation (ChIP)

T84 cells treated with or without APRIL were cross-linked with 1% formaldehyde for 5 min

and the reactions were terminated by the addition of 1 \times Glycine. Cells were lysed in ChIP lysis buffer with freshly added 1 \times protease inhibitor cocktail (Roche Applied Science), and then sonicated to shear chromatin into ~ 300 bp fragments. Chromatin was immunoprecipitated with p65 ChIP grade antibody. Real-time PCR was performed for CSN2 promoter region encompassing the NF- κ B enhancer with a non-transcriptional factor-binding site as the control. PCR products were quantified by using SYBR green dye (Qiagen) and fold enrichment was determined by the method of cycle threshold (CT) in the formula of $2^{-\Delta\Delta\text{CT}}$.

Statistical analysis

Each experiment was repeated three times. Student t test and one-way ANOVA were used to compare 2 and ≥ 3

groups, respectively. $P < 0.05$ was considered statistically significant.

Results

Expression of TAC1, Snail and E-cadherin in colon carcinoma and adjacent non-cancerous tissues

In a total of 25 cases, 15 were males (60%) and 10 were females (40%). The mean age was 55.2 ± 11.5 . 10 were moderately differentiated (low grade) and 15 were poorly differentiated carcinoma (high-grade). No statistically significant difference was observed between the groups in terms of age ($P > 0.05$). The mean tumor diameter was 6.5 cm.

The representative staining images of TAC1, Snail, and E-cadherin in both colon carcinoma and adjacent non-cancerous tissues were shown in **Figure 1**. For the total 25 samples, the staining intensity of TAC1, Snail, and E-cadherin was 1.7 ± 0.52 , 2.1 ± 0.75 , and 1.8 ± 0.11 in low-grade adenocarcinoma, and $1.5 \pm$

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Table 1. The staining intensity of TACI, Snail, and E-cadherin in colon tissues

	Intensity		
	TACI	Snail	E-cadherin
Adenocarcinoma (N= 25)			
Low Grade (10)	1.7 ± 0.52	2.1 ± 0.75*	0.8 ± 0.11*
High Grade (15)	1.5 ± 0.83	2.8 ± 0.63*	0.5 ± 0.23*
Non-cancerous (N =25)	1.8 ± 0.42	1.5 ± 0.32	1.9 ± 0.57

Data are presented as mean ± SD. * $p < 0.05$.

0.83, 2.8 ± 0.63 , and 0.5 ± 0.23 in high-grade adenocarcinoma, respectively (**Table 1**). When the staining intensity between low-grade or high-grade adenocarcinoma was compared with the adjacent non-cancerous tissues in term of Snail or E-cadherin, there was statistically significant difference. It should be noted that Snail is predominantly localized in the nuclei in adenocarcinoma, but in cytoplasm in non-cancerous tissues.

These results show that expression of Snail is inversely related to that of E-cadherin in CRC.

Expression of TACI, Snail and E-cadherin in colon carcinoma cell line

We next determined to express TACI, Snail, and E-cadherin in colon carcinoma cell line T84 by using immunofluorescent staining. We chose T84 cell line because interferon-gamma, a key inflammatory cytokine, has been found to reduce expression of E-cadherin in T84 [23]. As shown in **Figure 2**, robust expression of TACI was observed in T84 cells. Snail was predominantly localized in the nucleus, consistent with Snail as a transcriptional repressor. Similar to results of immunohistochemistry, E-cadherin expression was from negative to low. These results further demonstrate an inverse correlation of Snail and E-cadherin in colon carcinoma cell line.

Depletion of p65 blocks APRIL-induced stabilization of Snail in T84 cells

It has been previously reported that Snail is stabilized by the inflammatory cytokine TNF α through the activation of the NF- κ B pathway, and that NF- κ B is required for the induction of CSN2 that prevents the ubiquitination and degradation of Snail in breast cancer [21]. Here we hypothesize that APRIL activates p65/p50 NF- κ B signaling pathway to induce expression

of CSN2, which in turn stabilizes Snail in CRC (**Figure 3A**).

T84 cells were transfected with siRNAs for silencing of p65, with a non-targeting siRNA as the control. As shown in **Figure 3B**, APRIL treatment led to an increase of CSN2 at both mRNA and protein levels, however, Snail was increased at only protein level, but not mRNA level. Similarly, depletion of p65 down regulated

CSN2 at mRNA and protein levels, and Snail at only protein level but not at mRNA level. Next we used ChIP to determine a direct interaction between p65 and CSN2 gene promoter. In **Figure 3C**, we showed that APRIL treatment resulted in ~3 fold increase of p65 occupancy at CSN2 gene promoter.

Taken together, these results suggest that APRIL-p65/p50 NF- κ B-CSN2 pathway stabilizes Snail in CRC.

Snail represses transcription of E-cadherin in T84 cells

Snail is a transcriptional repressor for E-cadherin in varied types of cancer [20, 24, 25]. In order to further confirm that APRIL-Snail pathway plays a role in regulation of E-cadherin transcription in CRC (**Figure 4A**), we transfected T84 cells with siRNA targeting degradation of Snail mRNA. As shown in **Figure 4B**, APRIL treatment led to decrease of E-cadherin at both protein and mRNA levels. As expected, depletion of Snail de-repressed expression of E-cadherin.

These results further support that Snail directly targets E-cadherin inhibit its transcription in CRC.

Discussion

In this study, we investigated the role of APRIL-TACI mediated inflammation in down-regulation of E-cadherin in CRC. We showed that APRIL stimulates activation of p65 NF- κ B signaling pathway, which in turn translocates into nucleus of colon carcinoma cell to associate with gene promoter of CSN2. We further showed that increased/stabilized Snail, most likely a result of increased expression of CSN2, repressed expression of E-cadherin. Our study provides novel insights into the regulation of inflammation-initiated invasion and metastasis

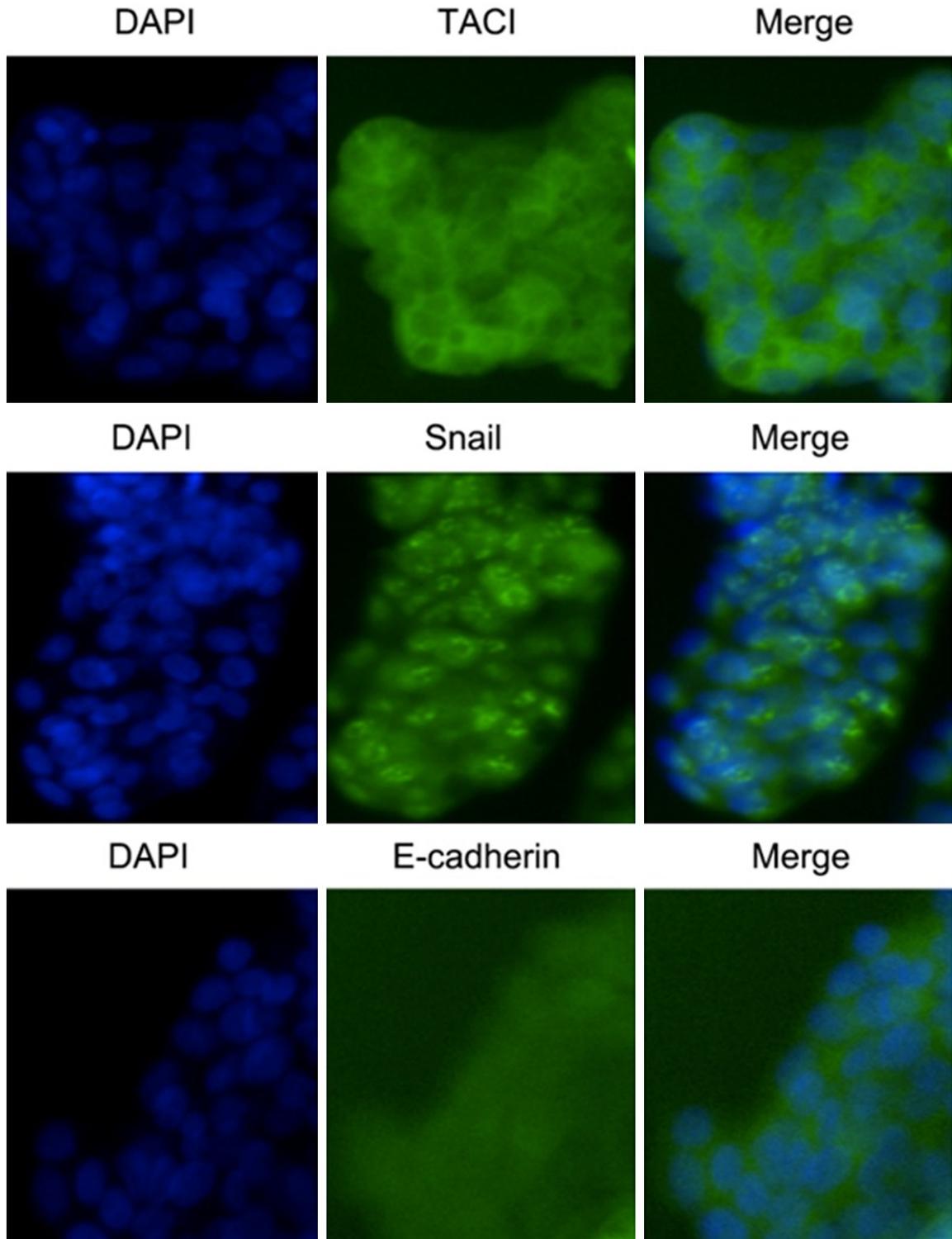


Figure 2. Immunofluorescence staining of TACI, Snail, and E-cadherin in colon carcinoma cell line T84. The staining method was detailed in Materials and Method. Original magnification, $\times 200$.

of malignancies and suggests that paracrine APRIL-mediated stimulation of Snail and in turn

repression of E-cadherin may play a critical role in invasiveness and metastasis in CRC.

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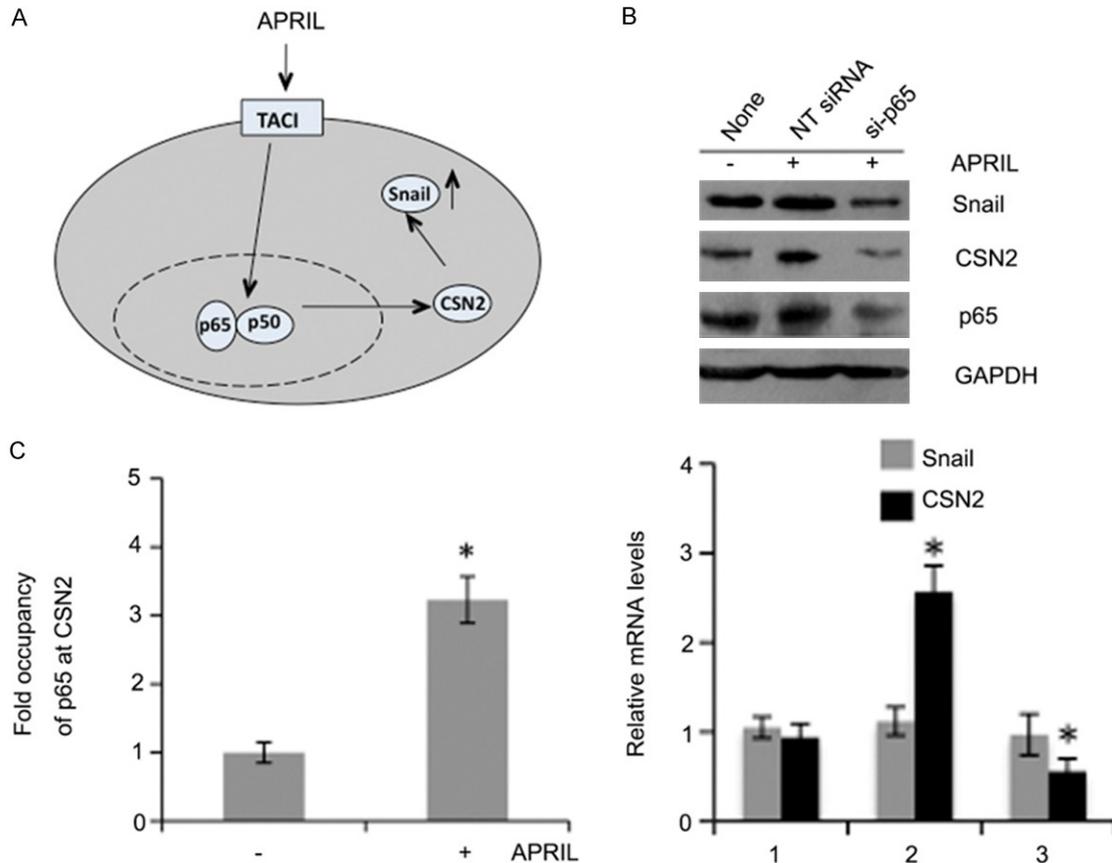


Figure 3. APRIL-NK- κ B(p65)-CSN2 stabilizes Snail in CRC. A. Schematic presentation for the pathway for APRIL-induced stabilization of Snail in CRC. APRIL cross-links to TACI to cause nuclear translocation of p65/p50 heterodimers, which in turn, promotes transcription and translation of CSN2. Increased CSN2 hence stabilizes Snail in the cytoplasm. B. T84 cells were transfected with p65 siRNAs (si-p65) for 48 hrs with non-targeting (NT) siRNAs as the control. The cells were harvested for analysis of Western blot (top panel) or RT-qPCR (Bottom panel) (N = 3 independent experiments). *P < 0.01. C. ChIP was performed to investigate interaction between p65 and CSN2 gene promoter with use a ChIP grade antibody of human p65 (N = 3 independent experiments). *P < 0.01.

The intracellular cohesion among malignant epithelial cell needs to down-regulated for invasive behavior [26]. In addition to a gene that encodes a protein with a high homology to neural cell adhesion molecule, which is deleted in 70% CRC, E-cadherin is a major candidate responsible for maintaining the cohesiveness and epithelial integrity of CRC [15, 16, 19]. Here we show that E-cadherin is inversely correlated to grade of tumor, consistent with previously reported results in paraffin-embedded materials, because use of these materials has been shown to facilitate the morphological analysis of stained tissues; despite of that CRC is a heterogeneous syndrome. Furthermore, a robust expression of E-cadherin was observed in adjacent non-cancerous tissues. Together with previous studies showing that lymph node

metastasis occur with greater frequency where staining E-cadherin was negative, this study supports the role of inflammation in invasion and spread of CRC.

Our study supports the mechanism of Snail regulation by NF- κ B in the process of inflammation-induced cancer metastasis. Despite TNF α , a canonical cytokine for stimulation of NF- κ B signaling [21], we have for the first time shown that APRIL, a highly expressed cytokine in colon carcinoma, induces activation of p65 NF- κ B signaling pathway in colon carcinoma, which in turn stabilizes Snail through the induction of CSN2 that functions by blocking phosphorylation and ubiquitination of Snail. This is an important as well as interesting finding because it suggests that paracrine-induced inflammation may play a critical role in promoting tumor

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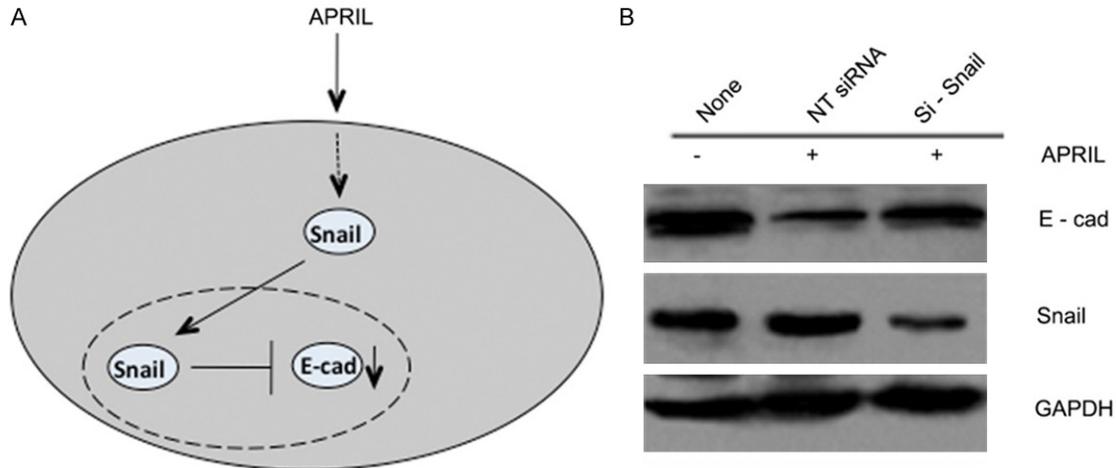


Figure 4. APRIL-induced stabilization of Snail down-regulates E-cadherin in CRC. A. Schematic presentation for the pathway for APRIL-induced stabilization for repression of E-cadherin. B. T84 cells were transfected with Snail siRNAs (si-Snail) for 48 hrs with non-targeting (NT) siRNAs as the control. The cells were harvested for analysis of Western blot (top panel) or RT-qPCR (Bottom panel) (N = 3 independent experiments). E-cad, E-cadherin. *P < 0.01.

growth and metastasis. These results are consistent with the previous observations that the introduction of LPS in mice greatly enhanced lung metastasis of breast cancer cells, and inhibition of Snail blocked metastasis in breast cancer [21, 27].

It has been reported that knockdown of APRIL in colorectal cancer cells prevented malignancy and tumor growth and metastasis in the liver [28]. Our results add a novel mechanism to previously proposed one that APRIL activates PI3K/Akt pathway to mediate these processes of CRC. Wang et al have shown that APRIL-mediated regulation of cell cycle regulatory proteins is dependent on PI3K and Akt, and PI3K/Akt mediates the effects of APRIL on invasiveness, likely by enhancing MMP-2 and MMP-9 expression [11].

In summary, we showed that APRIL triggered activation of p65 NF- κ B signaling to stabilize

Snail. Knockdown of Snail expression depressed expression of E-cadherin, an important molecule for controlling tumor cell migration, invasions, and lymph node metastasis. Because APRIL has also been found to activate PI3K/Akt signaling pathway to induce remarkable changes in cellular behaviors, including tumor cell proliferation, invasion and metastasis, APRIL may be a potential novel therapeutic target in treatment of metastatic CRC.

Disclosure of conflict of interest

None.

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