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Knockdown of astrocyte elevated gene-1 inhibits tumor growth and modifies microRNAs expression profiles in human colorectal cancer cells

Sujun Huang [a,b,1], Binwen Wu [a,*,1], Dongfeng Li [a,1], Weihong Zhou [a], Gang Deng [a], Kaijun Zhang [a], Youjia Li [a]

[a] East Department of Gastroenterology, Institute of Geriatrics, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong 510080, PR China
[b] Southern Medical University, Guangzhou, Guangdong 510515, PR China

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ABSTRACT

Astrocyte elevated gene-1 (AEG-1), upregulated in various types of malignancies including colorectal cancer (CRC), has been reported to be associated with the carcinogenesis. MicroRNAs (miRNAs) are widely involved in the initiation and progression of cancer. However, the functional significance of AEG-1 and the relationship between AEG-1 and microRNAs in human CRC remains unclear. The aim of this study was to investigate whether AEG-1 could serve as a potential therapeutic target of human CRC and its possible mechanism. We adopted a strategy of ectopic overexpression or RNA interference to upregulate or downregulate expression of AEG-1 in CRC models. Their phenotypic changes were analyzed by Western blot, MTT and transwell matrix penetration assays. MicroRNAs expression profiles were performed using microarray analysis followed by validation using qRT-PCR. Knockdown of AEG-1 could significantly inhibit colon cancer cell proliferation, colony formation, invasion and promotes apoptosis. Conversely, upregulation of AEG-1 could significantly enhance cell proliferation, invasion and reduced apoptisis. AEG-1 directly contributes to resistance to chemotherapeutic drug. Targeted downregulation of AEG-1 might improve the expression of miR-181a-2*, -193b and -193a, and inversely inhibit miR-31 and -9*. Targeted inhibition of AEG-1 can lead to modification of key elemental characteristics, such as miRNAs, which may become a potential effective therapeutic strategy for CRC.

1. Introduction

Astrocyte elevated gene (AEG)-1 was cloned as a human immunodeficiency virus (HIV)-1 and tumor necrosis factor α (TNF-α)-inducible gene in primary human fetal astrocytes by a rapid subtraction hybridization approach [1,2], which is a downstream target molecule of Ha-ras and c-myc mediating their tumor promoting effects [3]. AEG-1 is overexpression in multiple types of human cancers including breast cancer, gastric cancer, hepatocellular carcinoma, ovarian cancer, prostate cancer, nonsmall cell lung carcinoma, colorectal carcinoma and esophageal squamous cell carcinoma [4–11]. As a multifunctional oncoprotein, AEG-1 can enhance phenotypes characteristic of malignant aggressiveness, including proliferation, invasion, migration, neovascularization, chemoresistance and senescence [12]. AEG-1 plays a central role in several signaling pathway, including Ha-ras-mediated oncogenesis through the phosphatidylinositol 3-kinase (PI3K)/Akt [13], and Wnt/β-catenin signaling via ERK42/44 activation and upregulated lymphoid-enhancing factor 1/T cell factor 1 (LEF1/TCF1) [8]. AEG-1 directly contributes to the resistance of chemotherapeutic drugs [14], such as 5-fluorouracil (5-FU). AEG-1 augments expression of the transcription factor LSF that regulates the expression of thymidylate synthase (TS), a target of 5-FU, and dihydropyrimidine dehydrogenase (DPYD) that catalyzes the initial and rate-limiting step in the catabolism of 5-FU [13]. These data indicate that AEG-1 plays a central role in regulating diverse aspects of cancer pathogenesis. Targeted inhibition of AEG-1 might lead to an effective therapeutic strategy for cancer.

MicroRNAs have recently taken center stage in the field of human molecular oncology molecules by miR genes which are able to control the expression of a large number of cellular proteins

* Corresponding author. Fax: +86 20 83827712.
E-mail address: wubinwengd@aliyun.com (B. Wu).
[1] These authors contributed equally to this work.

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by targeting mRNAs of protein coding genes. Modification of miRNAs expression could be an important factor in the development and maintenance of the neoplastic state. We hypothesize that AEG-1 could be able to alter miRNAs expression pattern to promote the tumor development.

Colorectal carcinoma (CRC) is one of the most common malignancies worldwide. Song et al. [9] have reported AEG-1 expression may be associated with tumor progression in CRC and high AEG-1 expression correlates with poor overall survival in the CRC patients. Our previous study also show that AEG-1 was overexpressed in colon cancer and associated with the prognosis (submitted). However, the function of AEG-1 in pathogenesis of CRC has not been clearly known. In this study, we applied shRNA against AEG-1 and transfected AEG-1 overexpression mediated by lentivirus to investigate colon cancer cell growth in vitro. Microarray was performed to detect the modification of miR expression pattern, in order to identify AEG-1 as a molecular target for cancer therapy.

2. Materials and methods

2.1. Cell lines and culture

Human colon cancer cell lines Lovo, HT29, CaCO2, HCT116, SW1116, SW620 and SW480 (Chinese Type Culture Collection, Beijing, China) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, AUS) at 37 °C in an atmosphere of 5% CO_2 with humidity.

2.2. Vectors and retroviral infection

pMSCV/AEG-1-overexpressing human AEG-1 was generated by subcloning the PCR-amplified human AEG-1 coding sequence into pMSCV vector. The preparation of pure recombinant AEG-1 polypeptide was done according to a standard protocol. To silence endogenous AEG-1, two short hairpin RNAi oligonucleotides were cloned into the pSuper-retro-puro vector to generate pSuper-retro-AEG-1-RNAis, respectively. The following oligonucleotides for AEG-1 were synthesized through Genepharma as shown in Table 1. Recombinant retroviral vectors were produced by transient cotransfection. Viral infections were done serially, and stable cell lines expressing AEG-1 or AEG-1 RNA is were selected for 10 days with 0.5 μg/ml puromycin 48 h after infection. After 10-day selections, the cells were harvested for Western blot analysis.

2.3. Western blot analysis

Proteins were extracted after break-down of cells by SDS boiling method. Proteins were quantified by Bradford method. 50 μg of protein underwent SDS–PAGE and was transferred to PVDF membrane afterward. It was then sealed at room temperature for 1 h. The primary antibodies, rabbit anti-human AEG-1 antibody (Invitrogen, Carlsbad, CA) was added at a ratio of 1:1000, and incubated overnight at 4 °C. The membrane was washed with TBST. Then, the secondary antibody, mouse anti-rabbit IgG/HRP antibodies (Amersham Biosciences), was added at a ratio of 1:3000, and incubated at room temperature for 1 h. The membrane was washed three times and reacted with chemiluminescent agent for 5 min. It was then ECL tabletting, exposed, and displayed. The amount of each protein sample was controlled by GAPDH. Three independent experiments were performed.

Table 1
Targeted AEG-1 sequences were chemically synthesized by Genepharma (Shanghai, China).

Name	Sequences
AEG-1-RNAi1	s: GCAGCAAGGCAGTCTTTAAGT as: ACTTAAAGACTGCCTTGCTGC
AEG-1-RNAi2	s: GGAACGGTACGCTATACAACG as: CGTTGTATAGCGTACCGTTCC

2.4. Cell proliferation assay

Cells were trypsinized and plated in 96-well plates with 3.0×10^3 cells in 100 μl of the medium and allowed to attach for 24 h, then 10 μl of MTT (5 mg/ml in PBS) was added for 4 h incubation at 37 °C after 4, 24, 48, 72 h, respectively. Subsequently the formazan crystals were solubilized with 150 μl DMSO for 10 min. The absorbance was measured using a Microplate Reader (Bio-rad 680, Bio-rad, USA) with a test wavelength of 570 nm and all experiments were performed in triplicate. The cell proliferation curve was plotted using the absorbance at each time point. Three independent experiments were performed.

2.5. Colony formation assay

Cells were trypsinized, counted, and seeded for the colony forming assay in 6-well plate at 100 cells per well. After incubation for 10 days, colonies were stained with Gimsa and the numbers of positive cells counted. Colonies containing more than 50 cells were scored, and triplicates were counted in each treatment. Three independent experiments were performed.

2.6. Transwell matrix penetration assay

2×10^4 cells were plated into the top side of polycarbonate Transwell filter coated with Matrigel in the upper chamber of the BioCoat™ Invasion Chambers (BD, Bedford, MA) and incubated at 37 °C for 24 h, followed by removal of cells inside the upper chamber with cotton swabs. Migratory and invasive cells on the lower membrane surface were fixed in 1% paraformaldehyde, stained with Gimsa, and counted (Ten random 100× fields per well). Cell counts were expressed as the mean number of cells per field of view. Three independent experiments were performed.

2.7. Cell cycle and apoptosis analysis

Detection of apoptosis and cell cycle by flow cytometry was performed using the ApoScreen Annexin V Apoptosis Kit (SouthernBiotech, USA) and PI kit (Becton Dickinson, CA, USA). The transfected cells were harvested with trypsinization. The staining was performed according to the producer's manual. Flow cytometry (Becton Dickinson, CA, USA) was performed immediately after staining. Three independent experiments were performed.

2.8. MicroRNA array hybridization

Total RNA was extracted from cells by using TRIzol (Invitrogen, Carlsbad, CA) and the concentration of total RNA was evaluated by measuring the absorbance at 260 nm. We examined the expression profile of miRs by using a Human Affymetrix microarray, which is equipped with 3440 oligonucleotide probes for the detection of human miRs. Each sample was independently hybridized to one array. Hybridization signals were analyzed by using an Affymetrix GeneChip Command Console software. The hybridized signal intensities were normalized by the intensities of synthetic oligonucleotide DNA probes.

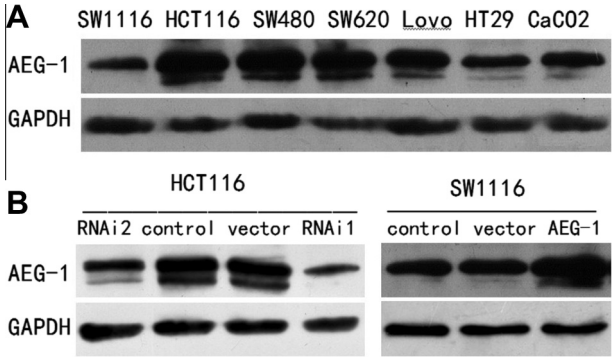

Fig. 1. AEG-1 expression in CRC cell lines. (A) AEG-1 expression was examined in CRC cell lines including SW1116, HCT116, SW480, SW620, Lovo, HT29 and CaCO2 by Western blot analysis. (B) Knockdown of AEG-1 in specific shRNAs-transduced stable HCT116 cells. AEG-1 expression decreased in HCT116 transfected with AEG-1 RNAi1 or RNAi2 compared with the control and vector group; stable AEG-1 overexpression in SW1116 was shown by Western blot analysis.

2.9. Quantitative RT-polymerase chain reaction assay for miRNA

Total RNA was extracted from cells by TRIzol (Invitrogen, Carlsbad, CA) was reverse-transcribed to cDNA by using 365 specific stem-loop reverse transcription primers. In order to examine the expression level of mature miR-181a-2*, -31, -9*, -193a and -193b in detail we performed TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA) by using the qPCR System with the following thermal cycling parameters: 94 °C for 2 min, followed by 40 cycles of amplification (94 °C for 10 s, 60 °C for 20 s, 72 °C for 30 s). The absorbance values of the SYBR Green I in each tube were detected at the end of each cycle. The melting curves of PCR products were also graphed after PCR amplification, followed by incubation at 12 °C. The expression level of the miR in each sample was measured and was normalized to U6 expression, as an internal control. Calculation of the Ct value was done by using a second derivative maximum method, and relative quantification was analyzed by a comparative Ct method. Final results illustrated in Fig. 4 were presented as log2 of N-fold change. All reactions were run in triplicate. The PCR primer pairs for miRs were obtained commercially from Ribobio Co. (Table 2).

2.10. Cell viability assay

Cells were seeded at a density of 3 × 103 cells in 96-well plates (Corning, NY, USA). After overnight culture, they were exposed to various concentrations of 5-Fu for 72 h in a CO_2 incubator. MTT assay as described above was used to detect the chemo-sensitivity of cells. Absorbance values at 570 nm of each well were expressed as percentages relative to controls, and the concentrations resulting in 50% inhibition of cell growth (IC_{50} values) were calculated. Three independent experiments were performed.

2.11. Statistical analysis

Three independent experiments were performed. Results were presented as means ± S.D. Statistical analyses were performed using the SPSS 13.0 statistical software. The two-tailed Student's *t* test or ANOVA were used to analyse. *P*-value <0.05 was considered statistically significant.

3. Result

3.1. AEG-1 expression in CRC cell lines and downregulation or upregulation of AEG-1 in vitro

AEG-1 expression was examined in CRC cell lines including SW1116, HCT116, SW480, SW620, Lovo, HT29 and CaCO2 by Western blot analysis. However, AEG-1 protein expression was higher in HCT116, SW480, SW620, and relatively lower in SW1116, Lovo, HT29 and CaCO2. Furthermore, AEG-1 expression was highest in HCT116 and lowest in SW1116 (Fig. 1A). As shown in Fig. 1B, transfection of HCT116 with AEG-1 RNAi1 or RNAi2 resulted in the knockdown of AEG-1 at translation levels in HCT116 cell line and AEG-1 RNAi1 downregulation effect seems more obvious than RNAi2, while transfection of SW1116 with stable AEG-1 plasmid upregulates the expression of AEG-1 in Western Blot.

3.2. Knockdown of AEG-1 inhibits cell proliferation, colony formation and invasion

In order to examine the role of AEG-1 on CRC cell proliferation and invasion, we constructed AEG-1 shRNA in HCT116 cell line and examined the effect of AEG-1 RNAi1. MTT assay results indicated that AEG-1-RNAi1 significantly decreases cell proliferation by 29.51% in HCT116 at 72 h after plating (Fig. 2A). This phenomenon was further confirmed by colony formation assay. As shown in Fig. 2B, the downregulation of AEG-1 could significantly decrease the colony formation of HCT116. Around 2 times more colonies were formed by control cells than that in AEG-1-RNAi1 HCT116 cells. Furthermore, AEG-1-RNAi1 significantly decreased HCT116 cell invasive ability compared to control group (Fig. 2C). The mean number of invasive HCT116 cell lines with AEG-1-RNAi1 and the control group was 25 and 245 per field of view, respectively ($P < 0.05$).

3.3. Upregulation of AEG-1 enhances proliferation, invasion and colony formation

To further investigate the biological role of AEG-1 expression in CRC progression, CRC cell line SW1116 was established to stably overexpress AEG-1. MTT assay results indicated that AEG-1-infected SW1116 cells grew faster, with 1.53-fold and 1.41-fold more cells than the control by 48 and 72 h after plating (Fig. 2D). In Transwell Matrix Penetration Assay, AEG-1 upregulation significantly increased the invasive ability of SW1116 cells compared to control group. The difference of cell invasion between SW1116 with stable AEG-1 overexpression and the control group was significant ($P < 0.01$; Fig. 2F). However, AEG-1 had no impact on colony formation (Fig. 2E). Although the colony formation rates between them were not statistically significant, increases in colony formation size were obvious.

3.4. Knockdown of AEG-1 accumulates G0/G1-phase cells and promotes apoptosis in CRC cells

Cell proliferation inhibited by knockdown of AEG-1 was shown above. To reveal mechanism involved in proliferation inhibition, we analyzed cell cycle by using flow cytometry. As shown in Fig. 3A and B, knockdown of AEG-1 resulted in accumulation in the G0/G1-phase cell and overexpress of AEG-1 resulted in increase of S phase cell. We also evaluated apoptotic levels by using flow cytometry. The apoptotic cell fraction was 7.67% and 14.97% in AEG-1RNAi1 and control group, 12.36% and 6.86% in SW1116-vector and AEG-1 group (Fig. 3C).

3.5. MicroRNA expression

To identify AEG-1-downstream genes, we performed Affymetrix microarray containing 3440 human miRNA probes to identify the comprehensive modification of miRNA expression profiles in HCT116 and SW1116 cell lines, and chose the level of 2-fold change of expression as selection criteria. The analysis of miR gene expression showed that a number of genes were affected by upregulation or downregulation of AEG-1. We analyze that there

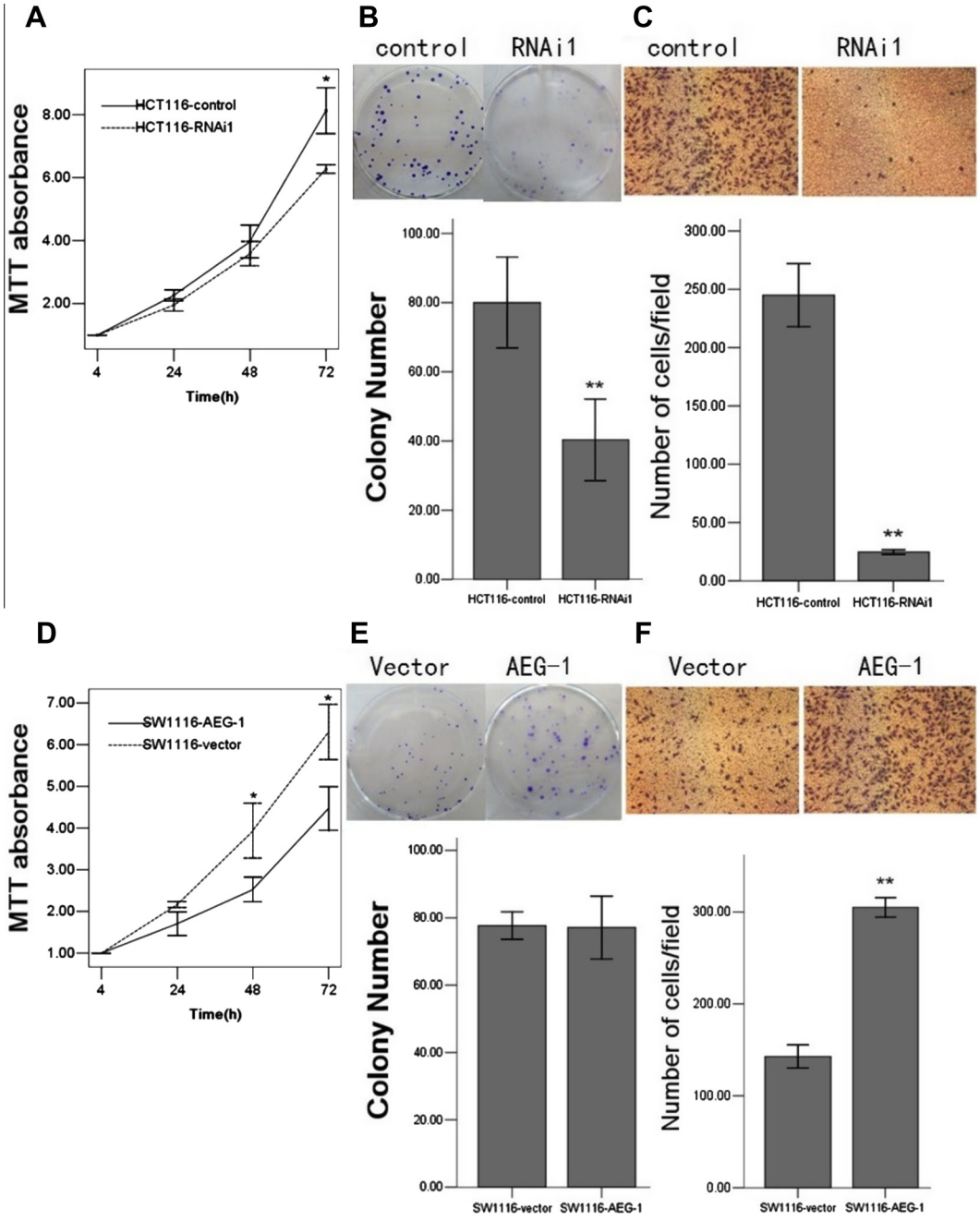

Fig. 2. Downregulation or upregulation of AEG-1 affects cell growth, invasion and tumorigenicity activity. (A) Silencing AEG-1 inhibits cell growth as determined by 3-(4,5-dimethy1-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assays. (B) Silencing AEG-1 inhibits cell growth as determined by colony formation assays. (C) Silencing AEG-1 dramatically inhibited invasion of HCT116 compared with the control group by transwell matrix penetration assay. (D) Overexpression of AEG-1 stimulates SW1116 cell proliferation as determined by MTT assays. (E) Colony formation assay shows that the upregulation of AEG-1 promotes cell growth. (F) Upregulation of AEG-1 dramatically increased invasion of SW1116 compared with the control group by transwell matrix penetration assay. Error bars represent mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

were 11 miRNAs up-regulated in HCT116 treated group and inversely down-regulated in SW1116 treated group. Oppositely, there were 8 miRNAs down-regulated in HCT116 treated group and inversely up-regulated in SW1116 treated group (data not shown). Around the 19 miRNAs, 5 miRNAs (miR-181a-2*, -31, -9*, -193a and -193b) were chosen to be validated because their expression was great difference between both cell lines after treatment.

The expression of selected miRNAs was then validated by qRT-PCR. Data were transformed in N-fold gene expression of target miRNA with respect to the expression of the same miRNA in the control sample. Both cell lines showed a opposite trend of up- or down-regulation of AEG-1 induced miRNA changes. miR-181a-2*, -193a and -193b were found to be down-regulated in SW1116-AEG-1 cells but up-regulated in HCT116-RNAi1 cells. miR-31 and -9* were found to be down-regulated in HCT116-RNAi1 cells but up-regulated in SW1116-AEG-1 cells (Fig. 4A). The 5 miRNAs all showed significant expression changes with respect to controls in both cell lines, although miR-193a did not reach the level of the 2-fold change of expression in HCT116 cells (Table 3).

qRT-PCR analysis showed that miR-181a-2*, -193b, and -193a expression were inversely correlated with AEG-1 expression, but miR-31 and miR-9* were in accordance with AEG-1 expression. Knockdown of AEG-1 by RNAi in CRC cells, similar to the 3 miRs (miR-181a-2*,-193b and -193a) overexpression, suppressed tumor

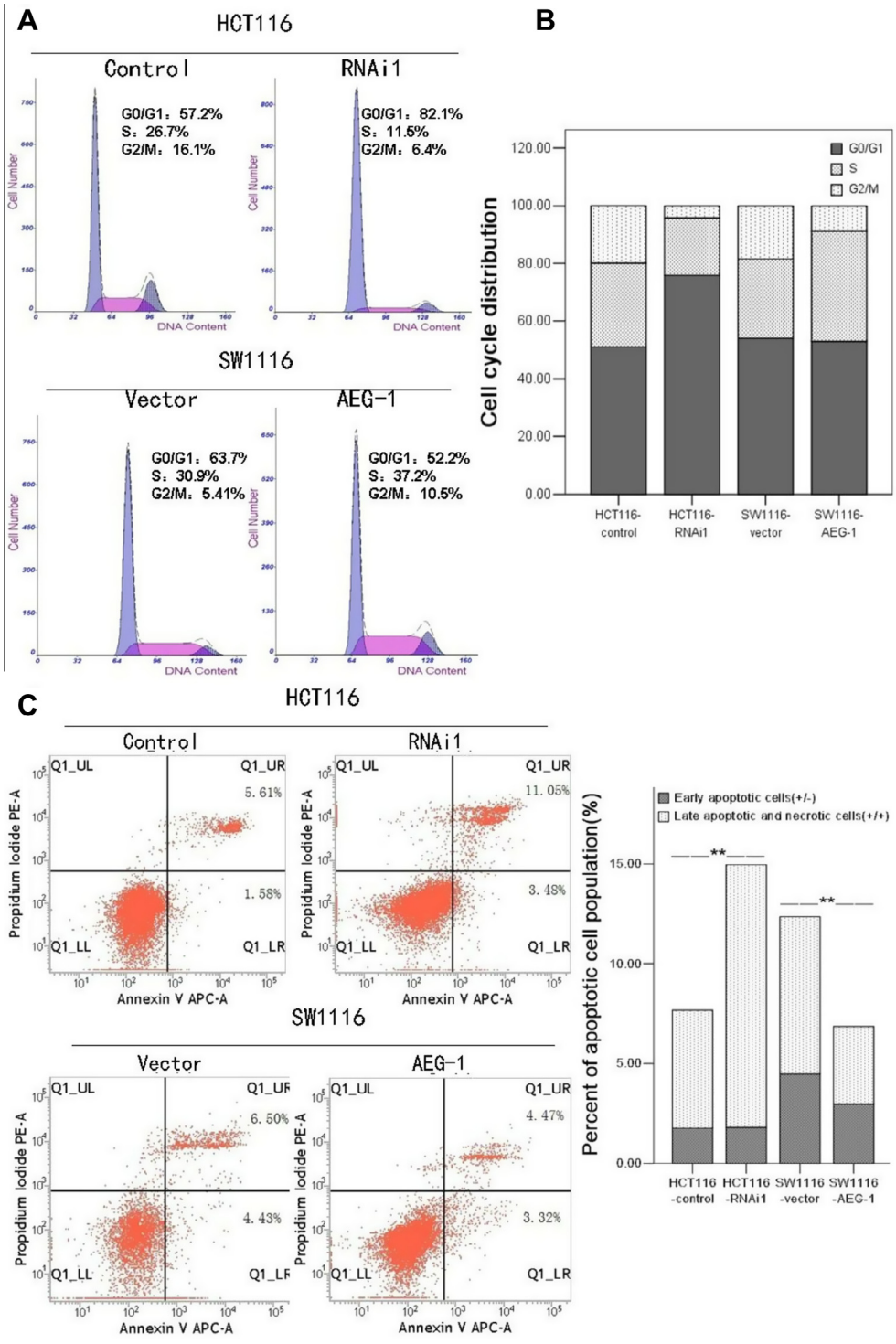

Fig. 3. Effect of AEG-1 downregulation and upregulation on the cell cycle and apoptosis in HCT116 and SW1116 cells. (A) Representative cell cycle distribution results are shown. (B) The population of G0/G1 phase was significantly increased in HCT116-RNAi1 cell compared to control group and the population of S phase was obviously increased in SW1116-AEG-1 cell compared to control group ($^{*}P < 0.05$). (C) Annexin V APC-A staning discriminates cells in early (lower right quadrant) and late apoptotic and necrotic states (upper right quadrant). The apoptosis rates were 14.97 ± 3.03% and 7.67 ± 1.21% in HCT116-RNAi1 and control group, 12.36 ± 2.06% and 6.86 ± 1.45% in SW1116-vector and AEG-1 group, respectively ($^{**}P < 0.01$). These experiments were performed in triplicate.

properties. Upregulation of AEG-1 can promote the expression of miR-31 and -9* appears to be associated with tumor development.

3.6. AEG-1 knockdown increases 5-FU cytotoxicity

In order to further examine the contribution of AEG-1 to the sensitivity to 5-FU, we determined various concentration of 5-FU on the 2 colon cancer cell lines. The growth of both HCT116 and SW1116 colon cancer cells were suppressed by the treatment of 5-Fu. From 0 to 16 uM of 5-FU, AEG-1 knockdown increased 5-FU cytotoxicity in a dose-dependent manner than control. The IC50 values of 5-Fu for HCT116 untreated and treated cells were 8.13 and 2.77 uM, respectively (Fig. 4B and Table 4). These results demonstrated that transfection of AEG-1 RNAi1 increased the

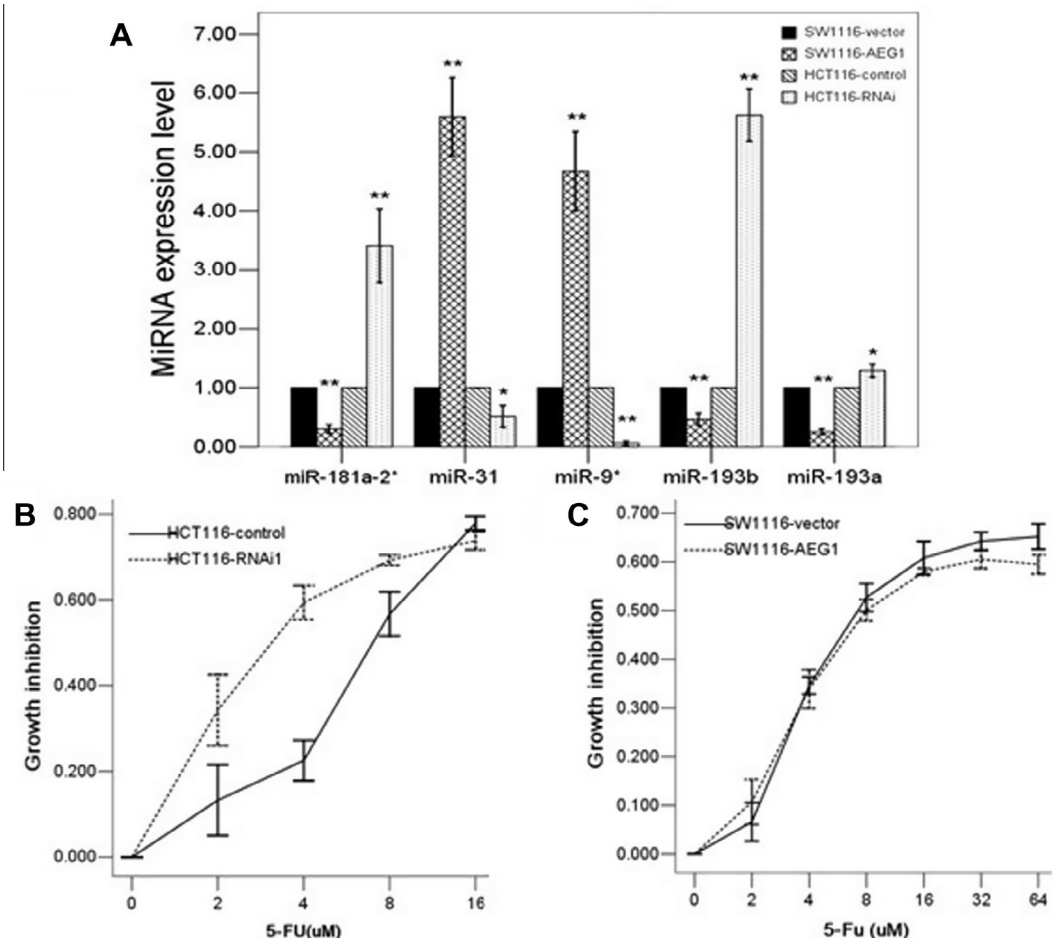

Fig. 4. The expression of 5 miRNAs was validated by qRT-PCR and AEG-1 confers resistance to 5-FU. (A) 5 miRNAs (miR-181a-2*, -31, -9*, -193a and -193b) were validated. miR-181a-2*, -193a and -193b were found to be down-regulated in SW1116-AEG-1 cells but up-regulated in HCT116-RNAi1 cells. miR-31 and -9* were found to be down-regulated in HCT116-RNAi1 cells but up-regulated in SW1116-AEG-1 cells ($^{*}P < 0.05$, $^{**}P < 0.01$). (B) HCT116-RNAi1 and control cells were treated with the indicated increasing concentrations of 5-FU. (C) SW1116-AEG-1 and control cells were treated with the indicated increasing concentrations of 5-FU. IC_{50} values were calculated.

Table 2
The sequence of 5 miRNAs.

miRNA	Sequence
miR-181a-2*	ACCACUGACCGUUGACUGUACC
miR-31	UGCUAUGCCAACAUAUUGCCAU
miR-9*	AUAAAGCUAGAUAACCGAAAGU
miR-193b	CGGGGUUUUGAGGGCGAGAUGA
miR-193a	UGGGUCUUUGCGGGCGAGAUGA

Table 3
The fold change of 5 miRNAs in 2 treated group compared to the control.

miRNA	Fold change	
	SW1116-AEG-1	HCT116-RNAi1
miR-181a	0.30	3.41
miR-31	5.60	0.52
miR-9a	4.67	0.06
miR-193b	0.46	5.62
miR-193a	0.25	1.29

Table 4
The IC_{50} values of 2 groups and their *P*-value.

		Control	Treatment	*P*-value
IC_{50} (uM)	HCT116	8.13 ± 0.66	2.77 ± 1.39	<0.05
	SW1116	14.35 ± 0.79	18.30 ± 1.91	<0.05

sensitivity of HCT116 to 5-FU. The corresponding values for SW1116 cells were 14.35 and 18.30 uM, which indicated SW1116-AEG-1 cells that expression more AEG-1 were more resistant to 5-FU compared to the control cells (Fig. 4C and Table 4). Inhibition of AEG-1 by shRNA significantly increased 5-FU-mediated killing in the HCT116 cells. It should be noted that AEG-1 RNAi had a more pronounced effect on 5-FU-mediated killing compared to control group indicating that AEG-1 controls multiple effectors mediating 5-FU killing.

4. Discussion

Carcinogenesis of CRC is the result of the progressive acquisition of multiple oncogenic factors through the accumulation of genetic or epigenetic alterations. Aberrant AEG-1 expression has been observed in multiple types of human cancers including CRC. AEG-1 expression was markedly correlated with the UICC stage, TNM classification, and histological differentiation in the CRC patients. High AEG-1 expression correlates with poor overall survival in the CRC patients, whereas patients with lower AEG-1 expression had better survival [9]. Our earlier data also have demonstrated that AEG-1 expression was increased in human colon cancer tissues compared to normal colon tissues. The expression level of AEG-1 was correlated with the clinical stage of CRC. Multivariate analysis strongly suggested that AEG-1 might be an independent and valuable biomarker for the prediction of prognosis of CRC

(submitted). In the current study, we evaluated the possibility of AEG-1 as a therapeutic target of CRC and its possible mechanism.

Numerous reports have demonstrated that oncoprotein AEG-1 is linked to the biological processes such as cancer cell survival, apoptosis, migration and invasion. To investigate whether the gain or loss function of AEG-1 is related to the progression of CRC, we adopted a strategy of ectopic overexpression or a small hairpin RNAi to upregulate or downregulate expression of AEG-1 in CRC models. After transfection with AEG-1 shRNA, protein level of the AEG-1 gene decreased, and meanwhile cell growth inhibited, colony-forming ability decreased and apoptosis increased. Meanwhile, upregulation of AEG-1 enhanced cell proliferation, invasion properties and inhibited apoptosis, but not the colony formation rates. Therefore, our data confirmed that AEG-1 served in regulating both cell proliferation and survival, which is the same as other reports. For instance, Zhang et al. [15] have reported that AEG-1 enhanced migration and invasion of CRC. Emdad et al. [16] have confirmed that AEG-1 is indeed a transforming oncogene and show that stable expression of AEG-1 in normal immortal cloned rat embryo fibroblast (CREF) cells induces morphological transformation and enhances invasion and anchorage-independent growth in soft agar.

The oncogenic role of AEG-1 in tumor development and progression has been reported to be associated with several pathways. Our reports show that knockdown of AEG-1 resulted in accumulation in the G0/G1 phase and overexpress of AEG-1 resulted in increase of S phase cell. The underlying mechanisms in CRC are still unclear, maybe associated with the PI3K/AKT signaling pathway. AEG-1 play a critical role in Ha-ras-mediated oncogenesis through the PI3K/AKT signaling pathway [3,17]. AEG-1 cooperates with Ha-ras to promote transformation and its overexpression augments invasion of transformed cells demonstrating its functional involvement in Ha-ras-mediated tumorigenesis. Kikuno et al. also revealed that aberrant AEG-1 expression as a positive auto-feedback activator of AKT and as a suppressor of FOXO3a in prostatic cancer cells [10]. We also find that AEG-1 can enhance cell invasion property in CRC which may be associated with the NF-κB pathway. Cellular motility and extracellular matrix degradation are two major determinants of cancer cell invasion. Invasion involves secretion of chemokines that help in tumor cell motility in a defined direction and proteolytic enzymes involved in extracellular matrix degradation. Emdad et al. documented that AEG-1 exerts its effects by activating the NF-κB pathway [18]. AEG-1 increases in malignant glioma and regulates in vitro invasion and migration of malignant glioma cells by activating the NF-KB signaling pathway [19]. AEG-1 can physically interact with the p65 subunit of NF-κB and modulate its function in the nucleus.

miRNAs have recently taken center stage in the field of human molecular oncology and have been increasingly recognized as key regulators in many cancers. Even a small change in miRNA expression could cause a profound effect on gene expression of hundreds of mRNAs. The 5 miRNAs whose expression was great difference between two cell lines after treatment may be performed as the effectors, contributing to tumorigenesis. miR-181a-2*, -193a and -193b were found to be down-regulated in SW1116 treated group but up-regulated in HCT116 treated group. AEG-1 inhibited the expression of the 3 miRNAs and shRNA-mediated inhibition of AEG-1 significantly increased their expression. It means that the 3 miRNAs may perform as anti-oncogens. miR-193b inhibited cell proliferation, migration and invasion has been observed in multiple types of human cancers including nonsmall cell lung carcinoma, Melanom and hepatocellular carcinoma [20–22] Some research identified that miR-193a regulating proliferation and apoptosis, and inhibiting cellular transformation by directly targeting PLAU and K-Ras. Expression of miR-193a is inversely correlated with PLAU and K-Ras in human CRC [23]. Novel miRNA (miR-181a-2*) has been reported rarely and its function remain unclear. We inferred that the 3 miRs (miR-193b,-193a, -181a-2*) involved in not only the regulation of cell proliferation and invasion, but also the chemotherapy response. miR-31 and -9* were found to be down-regulated in HCT116 treated group but up-regulated in SW1116 treated group. AEG-1 enhances the expression of miR-31 and miR-9* by 5.6 and 4.67-fold. We conclude that miR-31 and miR-9* plays oncogenetic functions in CRC. A lot of researches had confirmed that miR-31 played as oncogenetic role [24,25]. Suppression of miRNA-31 increases sensitivity to 5-FU at an early stage, and affects cell migration and invasion in HCT-116 colon cancer cells. The biochemical function of the 5 miRNAs has been characterized cancer association.

We explored the therapeutic role of AEG-1 in combination with chemotherapeutic drug, such as 5-FU. Our results suggest that 5-FU could be more effective in combination with RNAi mediated knockdown of AEG-1. Inhibition of AEG-1 by shRNA significantly increased 5-FU sensitivity indicating that targeted downregulation of AEG-1 might improve the expression of miR-181a-2*, -193b and -193a, and inversely inhibit miR-31 and -9* to mediate 5-FU killing. In our study, we demonstrate that AEG-1 confers resistance to 5-FU by inducing the expression of miR-31 and miR-9*. Inhibition of AEG-1 might be exploited as a therapeutic strategy along with 5-FU-based combinatorial chemotherapy for CRC.

In conclusion, the present study demonstrated that down-regulation of AEG-1 in colorectal cancer cells results in the inhibition of cell proliferation and colony formation in vitro. Based on these results, we conclude that AEG-1 servers as a potential molecular target for cancer treating. Also as AEG-1 directly contributes to resistance to apoptosis and sensitivity to 5-FU, AEG-1 may serve as an important molecular target for the discovery of new strategies for cancer treatment. Targeted inhibition of AEG-1 can lead to modification of key elemental characteristics, such as miR-181a-2*, -31, -9*, -193a and -193b, which may become a potential effective therapeutic strategy for CRC.

Conflict of interest

All authors read and approved the final paper and they have no competing interests.

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