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LncRNA HOTAIR promotes colon cancer development by down-regulating miRNA-34a

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Abstract. – OBJECTIVE: To investigate the characteristics of long non-coding RNA (lncRNA) HOTAIR in colon cancer, and to further explore its function in the development of colon cancer and its potential regulatory mechanisms.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was performed to detect HOTAIR expression in 72 colon cancer tissues along with adjacent normal tissues. Meanwhile, the relationship between HOTAIR level and colon cancer pathological parameters and patient prognosis were analyzed, respectively. QRT-PCR further verified the HOTAIR expression in colon cancer cells. Besides, knockdown and overexpression of HOTAIR models were constructed using lentivirus in HT29 and HCT-116 colon cancer cell lines. Cell counting kit-8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EDU) and cell colony formation assays were used to analyze the effects of HOTAIR on biological function of colon cancer cell. In addition, dual luciferase reporter gene assay was performed to explore the underlying mechanisms.

RESULTS: QRT-PCR results showed that HOTAIR expression in colon cancer was significantly higher than that in normal tissues. The incidence of distant metastasis was higher in patients with high expression of HOTAIR while their survival rate was lower than that of patients with low HOTAIR expression. Meanwhile, cell proliferation, invasion as well as migration ability of the cells in HOTAIR knockdown group was significantly decreased than those in the negative control group. QRT-PCR results showed that mRNA levels of miR-34a and HOTAIR in colon cancer tissues were negatively correlated. Besides, luciferase reporter gene assay revealed that overexpression of miR-34a significantly attenuated the luciferase activity of the wild-type HOTAIR vector group without attenuating the mutant HOTAIR vector group ($p>0.05$) In addition, the recovery experiment also found a mutual regulation between HOTAIR and miR-34a, together they could affect the malignant progression of colon cancer.

CONCLUSIONS: HOTAIR expression was significantly increased in colon cancer, which was in association with distant metastasis and poor prognosis of colon cancer. In addition, HOTAIR may promote malignant progression of colon cancer by regulating miR-34a.

Key Words:
HOTAIR, MiRNA-34a, Colon cancer, Proliferation.

Introduction

Colon cancer is a common malignant tumor around the world. Due to its concealed characteristics and rapid progression, most patients are in advanced stage at first time of diagnosis. More than 120,000 people are newly diagnosed with colon cancer each year, with a mortality rate of more than 33%, which make colon tumor a serious threat to human health[1-3]. The occurrence of colon cancer is a complex process, including genetic and epigenetic changes. Hence, it is of great importance to clarify the molecular mechanisms of tumorigenesis and progression, so as to find out new molecular markers for early diagnosis, prognosis and therapeutic evaluation[4,5]. Long non-coding RNAs (LncRNAs) are RNA molecules with a length of more than 200 nt but without protein encoding ability, which can be localized in the cytoplasm or nucleus. LncRNAs are mostly transcribed by RNA polymerase II and have epigenetic features similar to those of the encoded protein genes[6-8]. LncRNAs are involved in the regulation of multiple processes, whose expression and dysfunction can lead to many diseases, including tumors[9]. LncRNA can act as oncogene or tumor suppressor gene in tumors, and participate in the regulation of tumor proliferation, metastasis, autophagy and apoptosis, etc.[9,10]. It has been reported[11-13] that many lncRNA abnormalities are closely related to the progression of colon cance. Although some tumor-associated lncRNAs have similar expression and function in different tumors, we need to map colon cancer-specific lncRNA expression profiles to screening for new lncRNAs specifically expressed in colon cancer[14]. LncRNAs have different regulatory functions and mechanisms in colon cancer. Clarifying the

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molecular mechanism of colon cancer carcinogenesis and metastasis can help to establish reasonable prevention and treatment programs and to improve long-term survival and quality of life[15]. Various studies have revealed that lncRNA HOTAIR is highly expressed in many tumors, which is involved in tumor invasion and metastasis. Besides, its expression level was closely related to the pathological grade, clinical stage and prognosis of some tumors. However, its expression in colon cancer has not been reported[16-18]. MicroRNA (MiRNA) expression could affect tumorigenesis and is also closely related to tumor invasion and metastasis[19-21]. Sun et al[21] have revealed that miRNA can affect the connection of tumor cells by changing the expression of specific genes, and further affect the metastasis and infiltration of tumor cells. MiRNAs are involved in the invasion and metastasis of many common tumors, which are shown as promoting or inhibiting the metastatic infiltration of tumor cells[22]. In recent years, researches[23,24] have revealed that miR-34a has been studied in various tumors to varying degrees, but in colon cancer, its role has not been reported. Therefore, the aim of this study was to explore the characteristics of LncRNA HOTAIR in colon cancer and its underlying mechanism.

Patients and Methods

Patients and Colon Cancer Samples

In this study, 72 pairs of colon tissues and corresponding adjacent non-tumor tissues were selected from surgically treated colon cancer cases and stored at -80°C. The collection of clinical specimens was approved by the Ethics Monitoring Committee of Suzhou First People's Hospital and all participating patients have signed informed consent.

Cell Lines and Reagents

The human colon cancer cell lines (HT29, HCT8, HCT-116) and the normal human intestinal epithelial cell line FHC were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Among these cells, HT29 and MDA-MB-231 were cultured in Dulbecco's modified MEM medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin while HCT-116 was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA). All of them were cultured in an incubator at 37°C with 5% CO_2 and passaged with trypsin when grown to 80-90% confluence.

Transfection

The negative control (NC or Anti-NC) and the lentivirus containing HOTAIR overexpression and knockdown sequences (HOTAIR or Anti-HOTAIR) were provided by Shanghai Gene Pharma Company (Shanghai, China). After seeded in 6-well plates and grown to a cell density of 40%, the cells were infected with lentivirus and harvested 48 hours later for quantitative Real-time polymerase chain reaction (qRT-PCR) analysis and cell function experiments.

Cell Proliferation Assays

After 48 h of infection, cells were suspended and seeded into a 96-well plate at 2000 cells per well. After 6 h, 24 h, 48 h and 72 h, the cells were added with cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) reagent according to the manufacturer's instructions. After incubation for 2 hours, optical density (OD) value of each well was measured by the microplate reader.

Colony Formation Assay

48 h after infection, cells were collected and 200 cells were seeded in a 6-well plate and cultured for 2 weeks. The medium was changed after one week, then the medium was changed twice a week. After 2 weeks, the cells were washed twice with phosphate-buffered saline (PBS) and fixed in methanol for 20 minutes, then stained with 0.1% crystal violet staining solution for 20 minutes. Lastly, cells were observed and photographed under a light-selective environment.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

The EDU proliferation assay (RiboBio, Guangzhou, China) was performed to demonstrate the influence of HOTAIR on cell proliferation. Cells were added with 50 μm EDU and incubated for 2 h, then stained with Apolo and 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China), then the number of EDU-positive cells was detected by fluorescence microscopy. The positive rate of EDU was shown as the ratio of the number of EDU positive cells to the total DAPI chromogenic cells (blue cells).

QRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). QRT-PCR reactions were performed using SYBR Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan). The primers were listed below: HOTAIR: F: 5'-GGGTGGCTCACTCTTCTGGC-3', R: 5'- TGGCCTTGCCCGGGCTTGTC-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC- 3'; miRNA-34a: F: 5'-UGGCAGUGUCUUAGCUGGUUGU-3'; U6: F: 5'-CGCAAGGATGACACGCAAATTC-3'. Each sample was subjected to a three-hole replicates and the experiment was repeated twice. Bio-Rad PCR instrument (Hercules, CA, USA) was used to analyze and process the data with the software iQ5 2.0. The GAPDH and U6 genes were used as internal controls.

Dual Luciferase Reporter Assay

The transcription factor expression plasmid was co-transfected with the reporter plasmid into cells. Activation of the target promoter by the transcription factor indicated that the luciferase gene was expressed, and the amount of luciferase expression was proportional to the intensity of the transcription factor. Then a specific luciferase substrate was added to react with the luciferase to generate fluorescence. Finally, the intensity of the fluorescence was measured to determine whether the transcription factor interacted with the target promoter fragment.

Statistics Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Data were shown as mean ± standard deviation ($\overline{x}\pm s$). Student's *t*-test and one-way ANOVA were performed to analyze the differences between two groups or multiple groups, respectively. One-way ANOVA test was followed by Post-Hoc Test (Least Significant Difference). The difference was statistically significant at $p<0.05$; $^{*}p<0.05$.

Results

HOTAIR Was Highly Expressed in Colon Cancer Tissues and Cell Lines

To investigate the function of HOTAIR in colon cancer, we collected 72 pairs of colon cancer tumor tissues and adjacent non-tumor tissues. The results of qRT-PCR revealed that the expression of HOTAIR increased in colon cancer tissues when compared with the normal controls (Figure 1A, 1B). These results indicated that HOTAIR might act as an oncogene in colon cancer. Meanwhile, we examined HOTAIR level in colon cancer cell lines. Among them, HOTAIR was found highly expressed in HT29 and HCT-116; therefore, these two cell lines were used for subsequent experiments (Figure 1C). The above results revealed that HOTAIR has a high expression both in colon cancer tissues and cells.

HOTAIR Expression Was in Correlation With Distance Metastasis and Overall Survival in Colon Cancer Patients

Based on the mRNA level of HOTAIR in 72 colon cancer tissues and paracancerous tissues, we divided patients into high HOTAIR expression group and low HOTAIR expression group. And we analyzed the relationship between HOTAIR expression and age, sex, clinical stage, lymph node or distant metastasis of colon cancer patients, respectively. As shown in Table I, HOTAIR high expression was positively correlated with distant metastasis of colon cancer, but not with age, gender, clinical stage and lymph node metastasis. Besides, in order to study the relationship between HOTAIR expression and the prognosis of patients with colon cancer, we collected relevant follow-up data. Kaplan–Meier survival curves revealed that HOTAIR high expression was significantly associated with poor prognosis of colon cancer. The higher the expression of HOTAIR, the worse the prognosis (Figure 1D). Those results indicated that HOTAIR expression was in correlation with distance metastasis and overall survival in colon cancer patients.

Knockdown of HOTAIR Inhibited Cell Proliferation

To explore the function of HOTAIR in colon cancer, we constructed HOTAIR overexpression and knockdown model using lentiviral vector. After transfecting the HOTAIR lentiviral vector in the HT29 and HCT-116 cell lines, the qRT-PCR assay was performed to verify the interference efficiency, and the difference was proved statistically significant (Figure 2A). Cell proliferation was detected by CCK-8, EDU, and cell cloning assays in HOTAIR overexpressing and knocking down

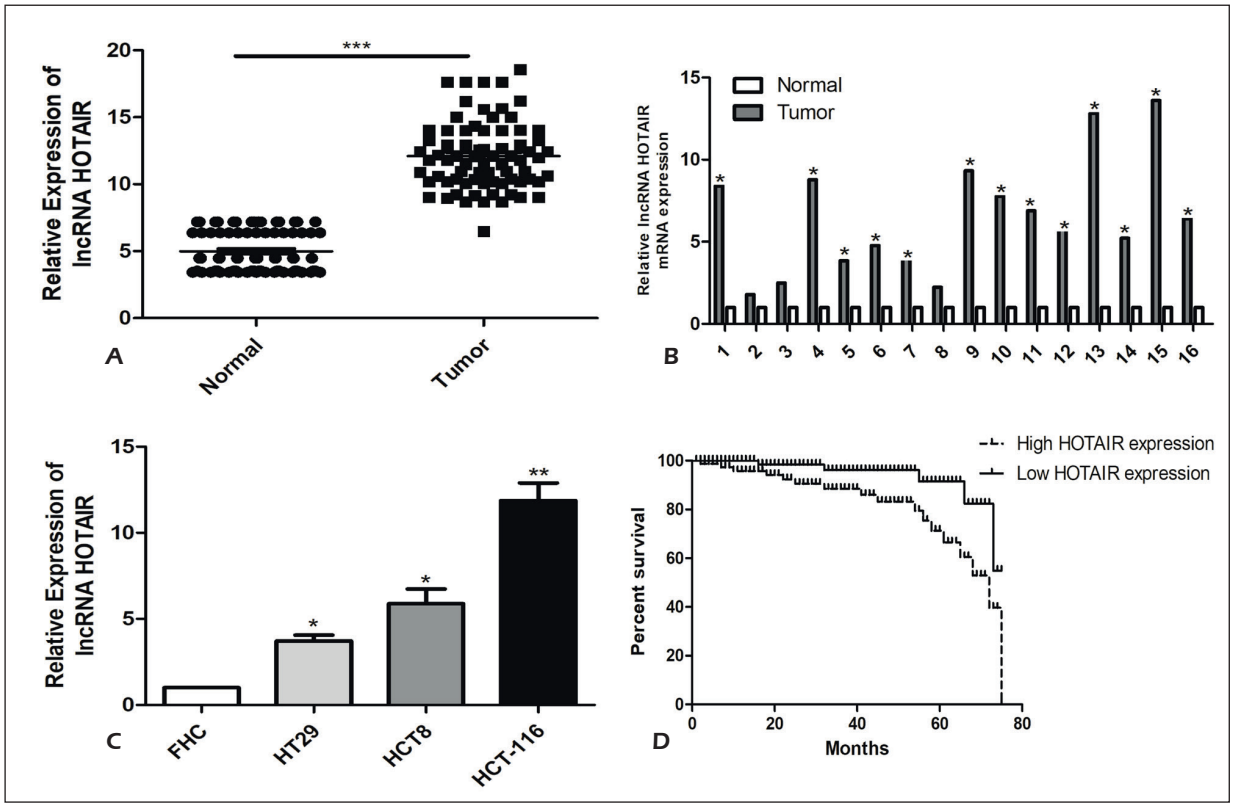

Figure 1. HOTAIR is highly expressed in colon cancer tissues and cell lines. **A**-**B**, qRT-PCR detection of HOTAIR expression differences in colon cancer tissues and adjacent non-tumor tissues; **C**, qRT-PCR detection of HOTAIR expression levels in colon cancer cell lines; **B**, HOTAIR-based colon Kaplan Meier survival curve of cancer patients. Data are expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

cell lines, respectively. The results demonstrated that cell proliferation in HOTAIR overexpression group was significantly increased compared with that in the NC group, while the opposite result was observed in the HOTAIR silent group (Figure 2B, 2C, 2D).

Table I. Association of lncRNA HOTAIR and miR-34a expression with clinicopathologic characteristics of colon cancer.

Parameters	Number of cases	FOXC2-AS1 expression		*p*-value	miR-107 expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.234			0.517
<60	31	18	13		12	19	
≥60	41	18	23		19	22	
Gender				0.346			0.475
Male	36	20	16		14	22	
Female	36	16	20		17	19	
T stage				0.096			0.079
T1-T2	41	24	17		14	27	
T3-T4	31	12	19		17	14	
Lymph node metastasis				0.147			0.054
No	44	25	19		15	29	
Yes	28	11	17		16	12	
Distance metastasis				0.032			0.025
No	41	25	16		13	28	
Yes	31	11	20		18	13	

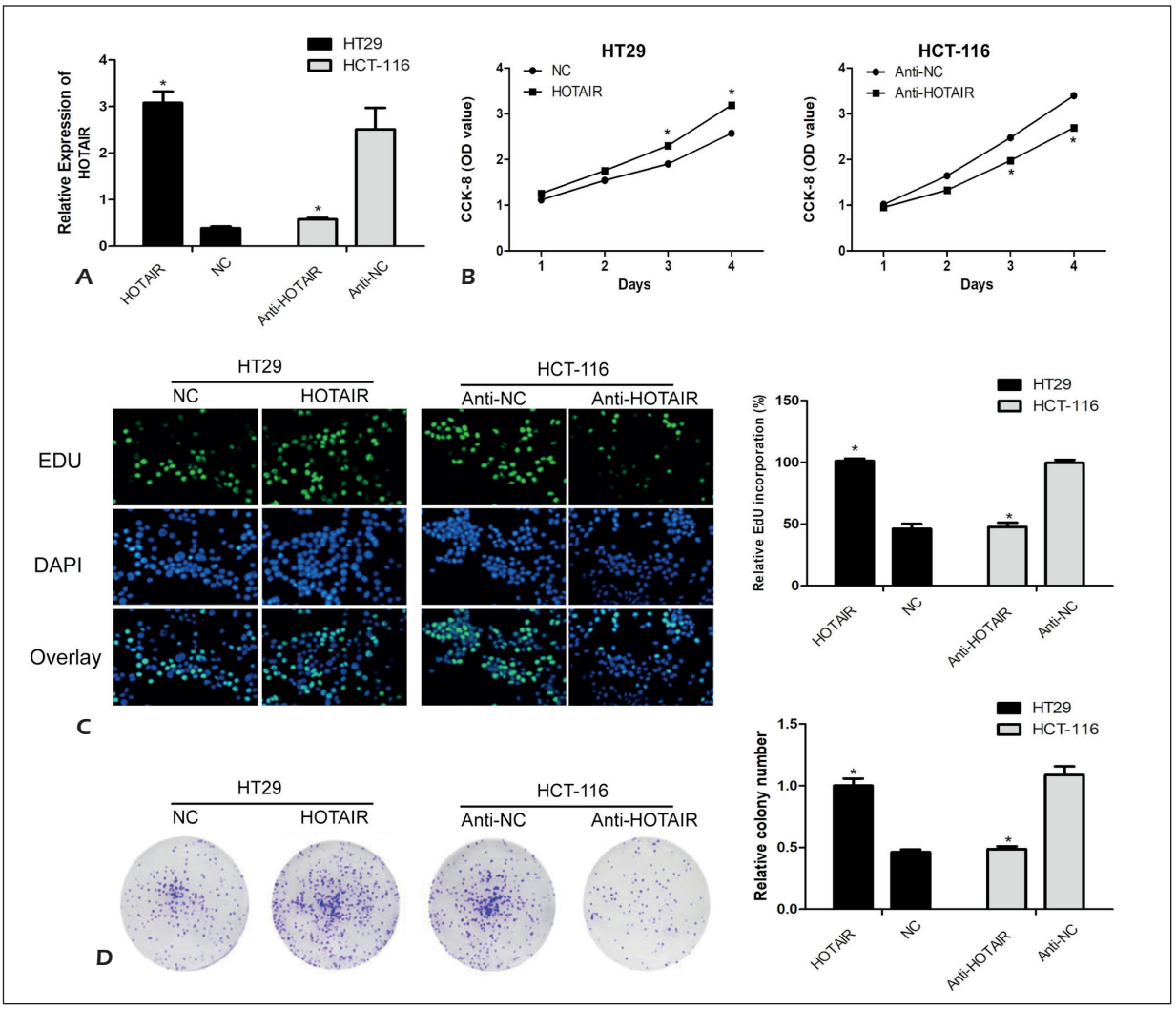

Figure 2. HOTAIR affects the proliferation of colon cancer cells. **A**, qRT-PCR was performed to verify the interference efficiency of HOTAIR transfected with HOTAIR overexpression vector in HT29 cell line and HOTAIR knockout vector in HCT-116 cell line; **B**, CCK-8 assay was performed to evaluate the cell proliferation in HT29 and HCT-116 cell lines. **C**, EDU assay was applied to detect the proliferation of colon cancer cells in HT29 and HCT-116 cell lines; **D**, cell cloning assay applied to detect the proliferation of colon cancer cells in HT29 and HCT-116 cell lines. Data are expressed as mean ± SD, *p<0.05.

Dual Luciferase Reporter Assay Verified the Direct Targeting of HOTAIR and miR-34a

To further verify the targeting of miR-34a to HOTAIR, we cloned the HOTAIR sequence into the luciferase reporter plasmid pmirGLO, and constructed the mutation vector pmirGLO-HOTAIR-mut. After pmirGLO-HOTAIR-wt or pmirGLO-HOTAIR-mut plasmid and miR-34a were co-transfected into HT29 and HCT-116 cells for luciferase reporting assay. We found that overexpression of miR-34a significantly attenuated the luciferase activity of the wild-type HOTAIR vector (p<0.05) without attenuating that of the mutant vector. All those results further demonstrated that HOTAIR can be targeted by miR-34a through this binding site (Figure 3A).

MiR-34a was Lowly Expressed in Colon Cancer

Next, we detected miR-34a expression in 72 pairs of colon cancer tissues and their corresponding adjacent tumor-free tissues as well as colon cancer cell lines by qRT-PCR. The result of qRT-PCR suggested that miR-34a was lowly expressed in colon cancer tissues (Figure 3B). Similarly, compared with FHC, miR-34a was also found significantly lower in cell lines (Figure 3C).

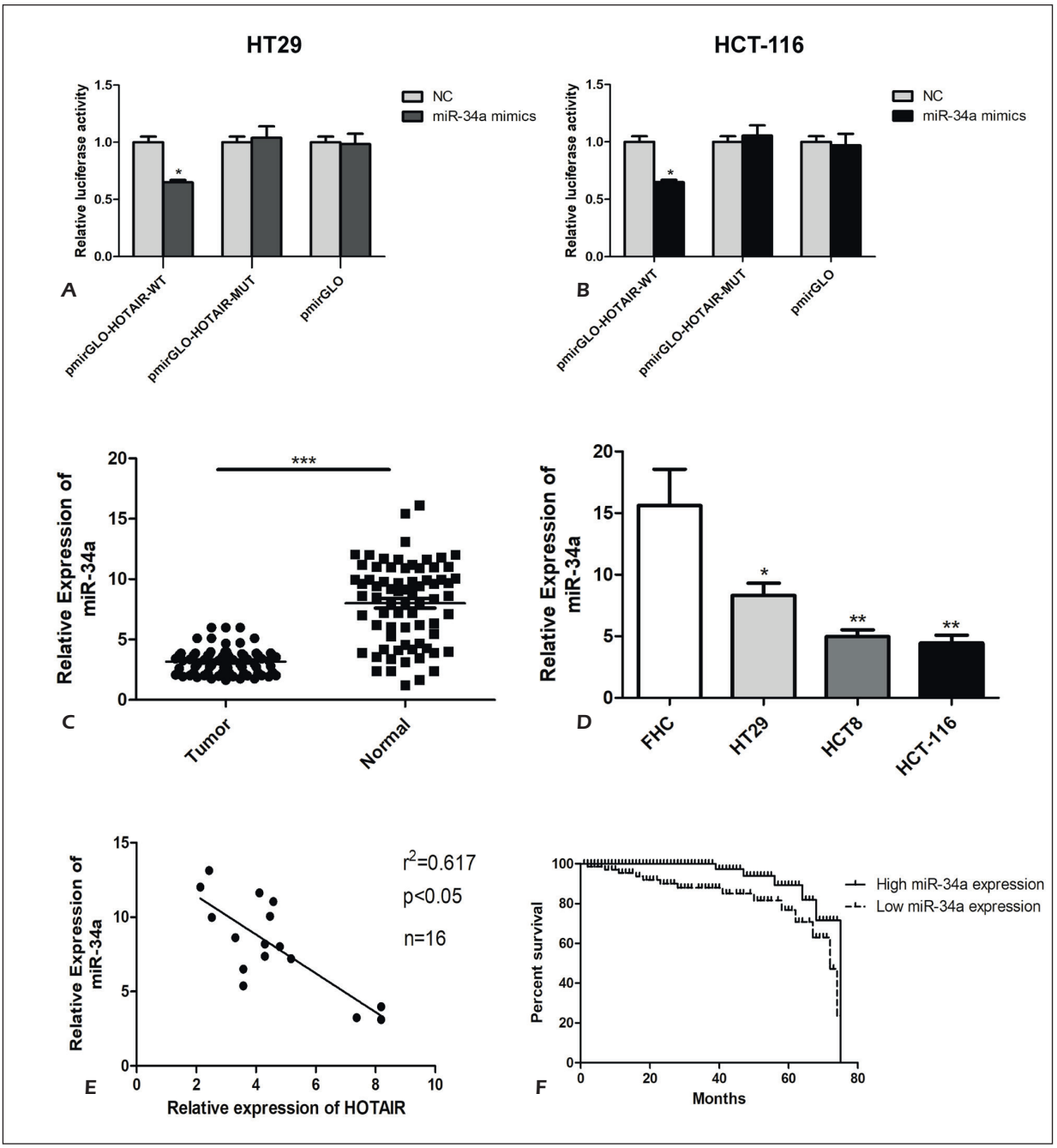

Figure 3. HOTAIR direct targeting of miR-34a. **A**, Dual luciferase reporter assay demonstrates the direct targeting of HOTAIR and miR-34a. The double luciferase reporter gene assay in HT29 and HCT-116 cell lines showed that overexpression of miR-34a significantly attenuated luciferase activity in wild-type HOTAIR vectors ($p<0.001$) without attenuating mutants Vector ($p>0.05$) or luciferase activity of empty vector ($p>0.05$); **B**, qRT-PCR detection of differential expression of miR-34a in colon cancer tissues and adjacent non-tumor tissues; **C**, The expression of miR-34a in colon cancer cell lines was detected by qRT- PCR; **D**, The expression level of HOTAIR and miR-34a was negatively correlated in colon cancer tissues; E, Kaplan Meier survival curve of colon cancer patients based on miR-34a expression. Data are expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Therefore, we selected 16 pairs of samples in colon cancer tissues and detected the expressions of HOTAIR and miR-34a. QRT-PCR results revealed that HOTAIR was negatively in correlation with miR-34a expression (Figure 3D). In addition, we analyzed the pathology and prognosis of miR-34a in colon cancer. As shown in Table I, low expression of miR-34a was positively cor-

related with distant metastasis of colon cancer, but not with age, gender, clinical stage, and lymph node metastasis. Subsequently, in order to further investigate the relationship between miR-34a expression and the prognosis of colon cancer patients, we collected relevant follow-up data. The Kaplan-Meier survival curve displayed that low expression of miR-34a was in association with poor prognosis of colon cancer (Figure 3E).

HOTAIR Modulated miR-34a Expression in Colon Cancer

In order to further explore how HOTAIR promoted the malignant progression of colon cancer, we performed relevant bioinformatics analysis and found a possible relationship between HOTAIR and miR-34a. Furthermore, to figure out the interaction between HOTAIR and miR-34a in colon cancer cells, we overexpressed miR-34a in a HOTAIR overexpressed cell line and silenced miR-34a in a cell line with HOTAIR knockdowned. The transfection efficiency of miR-34a was analyzed by qPCR (Figure 4A). Subsequently, we performed CCK-8, EDU, and cell cloning assays and found that miR-34a could counteract the effect of HOTAIR on colon cancer cell proliferation (Figure 4B, 4C and 4D). All above results indicated that HOTAIR could modulate miR-34a expression in colon cancer cells or tissues.

Discussion

LncRNAs are a class of endogenous RNAs with more than 200 nucleotides in length and have no protein coding function[6-8]. LncRNAs can regulate gene expression at many levels in a variety of ways, such as interfering with the binding of transcription factors to the promoter, or affecting the transcription process of the target gene. Besides, lncRNAs can react with the protein-binding partner and affect the localization of regulatory proteins in subcellular cells. Moreover, lncRNAs are capable of binding to a transcriptional repressor complex or serving as precursors for non-coding RNAs, structural components of mitotic spindles, and cytoskeletal components by affecting RNA processing[13-15]. Through these pathways, lncRNAs can perform various biological functions, including chromatin remodeling, gene imprinting, splice regulation, cell cycle regulation, miRNA degradation and translational regulation[9,10]. Yuan et al[9] have revealed that abnormally expressed lncRNA affects the occurrence and development of tumors by regulating various aspects of epigenetics. HOTAIR, as a classical lncRNA, affects the overall apparent modification of tumor cells[24]. HOTAIR is highly expressed in tumor tissues of nearly 25% of breast cancer patients, and its expression level can effectively predict breast cancer metastasis and prognosis. *In vivo* experiments confirmed that overexpression of HOTAIR can promote breast cancer metastasis, and part of its mechanism is to change targeting genes of polycomb proteins[25,26]. In addition, down-regulation of HOTAIR is considered as an indication of liver cancer cell metastasis and disease progression, showing its oncogene characteristics and thus serving as a potential tumor marker[27]. Therefore, this study focuses on whether HOTAIR can also affect the malignant progression of colon cancer. HOTAIR possesses trans-regulatory ability[25]. Studies have shown that HOTAIR expression is up-regulated in various tumors, and its abnormal expression is closely related to tumor prognosis, including liver cancer, gastric cancer, lung cancer, kidney cancer, etc.[26,27]. Scholars[28-30] have displayed that HOTAIR is overexpressed in breast cancer tissues and negatively correlated with tumor differentiation, suggesting that HOTAIR can be used as an important molecular marker for judging the malignancy of breast cancer. However, its specific mechanism in colon cancer still remains elusive. In this study, through lentivirus vector, we constructed overexpression or knockdown model in colon cancer cell lines, and applied bioinformatics analysis to explore the difference in expression of lncRNA between HT29 and HCT-116 cells. The lncRNA HOTAIR was selected as a candidate to explore the association between lncRNA and malignant progression of colon cancer. Finally, it was demonstrated that the up-regulation of HOTAIR can indeed accelerate the malignant progression of colon cancer. In addition, HOTAIR expression in colon cancer tissues was notably enhanced which was then proved positively correlated with distant metastasis and poor prognosis of colon cancer. Therefore, we speculate that HOTAIR may promote tumor progression in colon cancer. However, the expression of miR-23a was found negatively correlated with poor prognosis, so it was considered to play a part in tumor suppression in colon cancer. Subsequently, CCK-8, EDU and cell cloning experiments were performed to determine the cell proliferation ability after overexpression and knockdown of HOTAIR in colon cancer cell. All those results revealed that HOTAIR have a can-

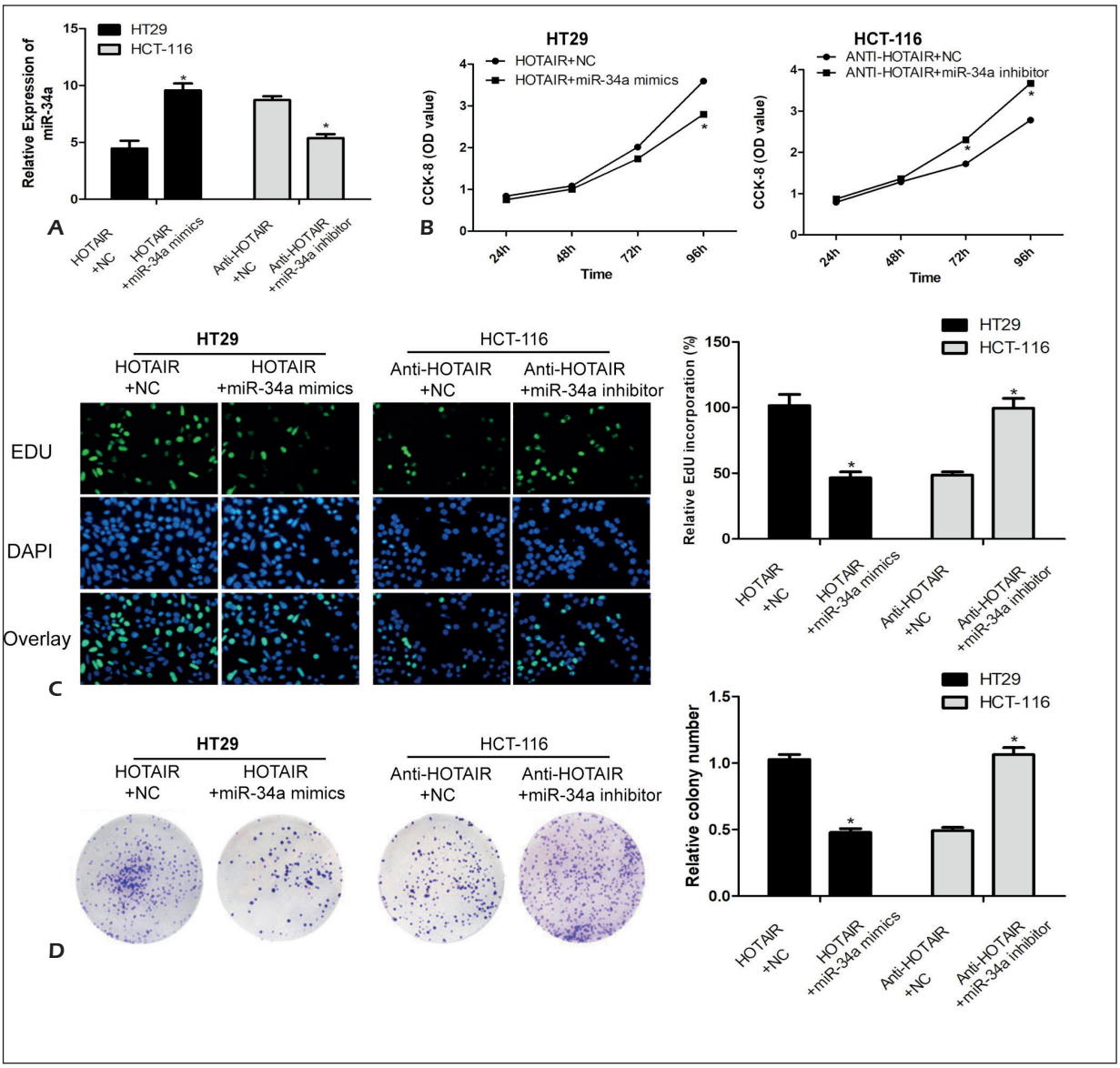

Figure 4. HOTAIR regulates the expression of miR-34a in colon cancer tissues and cell lines. **A**, Detection of miR-34a expression levels in HOTAIR and miR-34a co-transfected cell lines by qRT-PCR; **B**, CCK-8 assay was performed to detect cell proliferation of HOTAIR and miR-34a co-transfection cells; **C**, EDU assay was performed to detect cell proliferation of HOTAIR and miR-34a co-transfection cells. **D**, cell-cloning assay was performed to detect cell proliferation of HOTAIR and miR-34a co-transfection cells. Data are expressed as mean ± SD, *$p<0.05$.

cer-promoting effect in colon cancer and could significantly enhance the proliferation of colon cancer cells. LncRNA can play a part in promoting or inhibiting cancer progression through competing with miRNA for binding to their common mRNA. Previous studies have predicted by bioinformatics analysis that miRNA-34a may interact with lncRNA HOTAIR. In this study, miRNA-34a was found lowly expressed in colon cancer tissues and could inhibit angiogenesis and cell invasion in colon cancer cells. Therefore, we used bioinformatics methods to analyze the binding site of miRNA-34a in HOTAIR sequence. Our results verified the direct binding of HOTAIR to downstream miRNA-34a through molecular biology experiments such as bioinformatics and dual luciferase reporting assay. Additionally, the HOTAIR vector lacking the miRNA-34a binding site could not attaching miRNA-34a, which further confirmed the combination of HOTAIR

and miRNA-34a. In order to further investigate the regulatory relationship between HOTAIR and miRNA-34a expression in colon cancer cell lines, we performed recovery experiments and found that miRNA-34a could counteract the effect of HOTAIR on cell functions. Evidence suggested that the transcriptional activity of HOTAIR may be regulated by miRNA-34a. However, this conjecture requires further validation. All of the above findings suggested that there may be a positive feedback regulation loop in colon tumor cells. LncRNA HOTAIR inhibits miRNA-34a expression, which in turn promotes cell migration and metastasis of colon tumor.

Conclusions

We showed that lncRNA HOTAIR level is significantly increased in colon cancer, and this elevation is associated with distant metastasis and poor prognosis of colon cancer. Additionally, HOTAIR may promote the proliferation of colon cancer *via* modulating miRNA-34a expression.

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Conflict of Interests

The authors declared no conflict of interest.

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