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Expression and clinicopathological significance of the lncRNA HOXA11-AS in colorectal cancer

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Abstract. HOXA11 antisense RNA (HOXA11-AS) is a long non-coding RNA (lncRNA) that is important in determining cancer progression. HOXA11-AS was recently identified as a novel biomarker in lung cancer progression. However, its role in colorectal cancer (CRC) remains poorly understood. The present study aimed to analyze lncRNA HOXA11-AS expression in CRC and investigate a possible association between HOXA11-AS and clinicopathological factors. HOXA11-AS expression was examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in 84 CRC tissues and adjacent non-cancerous tissues, in addition to 3 CRC cell lines and 1 human normal colorectal cell line. The results demonstrated that HOXA11-AS expression was decreased in the CRC tissues and cell lines compared with that of the controls ($P<0.05$). Clinicopathological analysis indicated that low HOXA11-AS expression was significantly correlated with tumor size, advanced tumor-node-metastasis stage, lymph node metastasis and carcinoembryonic antigen level of patients with CRC ($P<0.05$). Furthermore, the areas under the curve (AUC) were 0.613 and 0.628 for HOXA11-AS, indicating that the lncRNA is able to distinguish CRC tissue from non-cancerous tissue, and CRC tissue with lymph node metastasis from CRC without lymph node metastasis. Therefore, HOXA11-AS may function as a potential biomarker and target for novel therapeutic strategies to treat CRC.

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer in males and the second most prevalent in females worldwide, with ~55% of CRC cases occurring in developed countries (1). In the USA, there are ~132,700 new CRC cases every year and the mortality rate remains significant at ~8.4% (2). In China, the incidence of CRC has rapidly increased, and in 2011, it was the fifth leading cause of cancer-associated mortality (3). Despite recent advances in treatment and experimental oncology, the prognosis of patients diagnosed with CRC remains poor; the 5-year survival rate for metastatic colorectal cancer is <20% (2) A number of molecular predictors have been systematically analyzed to determine whether they may be used for the diagnosis and prognosis of CRC (4). However, none are accurate enough for clinical use, and thus have not been adopted by practitioners.

Long non-coding RNAs (lncRNAs) are >200 nucleotides long and lack the ability to code proteins (5). Until recently, lncRNAs were not considered to possess any useful biomedical functions (6). However, due to developments in technology and genomics, an increasing number of studies have demonstrated that lncRNAs are important in regulating gene expression (7). Previous studies have indicated that lncRNAs are associated with post-transcriptional regulation (8), chromatin remodeling (9), stem cell function (10), behavior of colonic epithelia (11) and cancer prognosis (12).

HOXA11-AS is a lncRNA located in the HOXA gene cluster. This gene cluster consists of protein-coding genes and genes for non-coding RNA, and certain HOXA lncRNAs may function as biochemical markers in several forms of cancer (13,14). HOXA11-AS was first discovered in a mouse embryonic cDNA library using a probe from the sense HOXA11 cDNA sequence (15). The lncRNA is transcribed from the opposite strand of the protein coding gene HOXA11; HOXA11-AS is highly conserved and the 5.1 kb transcript is located on chromosome 7p15.2 (16).

It has been demonstrated that HOXA11-AS has the ability to repress HOXA11 mRNA expression by transcriptional interference, which is essential for embryo implantation and endometrial development (17,18). Furthermore, previous studies have indicated that HOXA11-AS may function as a biomarker for lung cancer metastasis and poor patient prognosis (19), and that aberrant expression of HOXA11-AS may be linked to the malignant characteristics of bladder cancer (20).

However, the association between HOXA11-AS expression and CRC development has not yet been thoroughly investigated. Therefore, the present study aimed to measure HOXA11-AS expression in CRC tissues, investigate the

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association between HOXA11-AS levels and clinicopathological features, and determine whether HOXA11-AS could serve as a viable biomarker for CRC prognosis.

Materials and methods

Patient samples. All CRC tissues and adjacent non-cancerous tissues were collected from 84 patients who underwent primary surgical resection between January 2014 and January 2015 at the Department of Colorectal and Anal Surgery, The First Affiliated Hospital of Guangxi Medical University (Nanning, China). No patients received radiotherapy or chemotherapy prior to surgery. The CRC and adjacent healthy tissues were immediately preserved in liquid nitrogen and stored at -80˚C until total RNA extraction. The clinicopathological factors of patients are presented in Table I. The present study was approved by the Research Ethics Committee of the First Affiliated Hospital of Guangxi Medical University and written informed consent was obtained from all patients.

Cell lines and culture conditions. Normal human colorectal CCD-18Co cells and human CRC HCT8, HCT116 and RKO cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Wisent Biotechnology, Nanjing, China) supplemented with 10% fetal bovine serum (Excell Bio, Shanghai, China), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in 5% CO_2.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the frozen tumor tissues, adjacent non-cancerous tissues and cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's protocol. Next, a total of 3 μg RNA for each sample was reverse transcribed into cDNA using DNase I (#EN0521; Thermo Fisher Scientific, Inc.) and a RevertAid First Strand cDNA Synthesis kit (#K1622; Thermo Fisher Scientific, Inc.). qPCR was performed using the StepOnePlus™ Real-Time PCR system (ABI7500; Thermo Fisher Scientific, Inc.) and SYBR® Green PCR Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The PCR cycles were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All reactions were run in triplicate using HOXA11-AS specific primers. β-actin expression served as the endogenous control, and all samples were normalized to human β-actin according to the $2^{-\Delta\Delta Cq}$ method (21). HOXA11-AS expression level was determined by RT-qPCR using the following primer sequences: HOXA11-AS, forward, 5'-TGCCAAGTTGTACTTACTACGTC-3', and reverse, 5'-GTTGGAGGAGTAGGAGTATGTA-3'; β-actin, forward, 5'-GCACCACACCTTCTACAATGAGC-3', and reverse, 5'-GGATAGCACAGCCTGGATAGCAAC-3'.

Statistical analysis. All data were analyzed by SPSS software v16.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between groups were performed using the χ^2 test or two-tailed Student's *t*-test. Receiver operating characteristic (ROC) curves were constructed to evaluate the diagnostic value of HOXA11-AS levels by plotting sensitivity vs. 100% specificity. In all cases, P<0.05 was considered to indicate a statistically significant difference.

Table I. Association between HOXA11-AS expression and clinicopathological features of colorectal cancer.

		HOXA11-AS expression		
Characteristics	N	Low	High	P-value
Gender				0.943
Male	52	37	15	
Female	32	23	9	
Age, years				0.685
<50	20	15	5	
≥50	64	45	19	
Tumor diameter, cm				0.016[a]
<5	38	18	14	
≥5	46	42	10	
Tumor site				0.437
Colon	51	13	38	
Rectum	33	11	22	
Tumor differentiation				0.685
Well and moderate	64	19	45	
Poor	20	5	15	
Depth of invasion				0.890
T1-T2	50	36	14	
T3-T4	34	24	10	
Lymphatic metastasis				0.035[a]
Negative	34	20	14	
Positive	50	40	10	
Venous invasion				0.754
Absent	69	50	19	
Present	15	10	5	
Perineural invasion				1.000
Absent	76	54	22	
Present	8	6	2	
TNM				0.035[a]
I-II	34	20	14	
III-IV	50	40	10	
CEA, ng/ml				0.015[a]
<5	53	33	20	
≥5	31	27	4	
CA199, U/ml				0.770
<37	69	50	19	
≥37	14	9	5	
Obstruction				0.153
Absent	12	6	6	
Present	72	54	18	

[a]P<0.05. All data were analyzed using the χ^2 test or continuity correction test. HOXA11-AS, HOXAII antisense RNA; TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; CA, carbohydrate antigen.

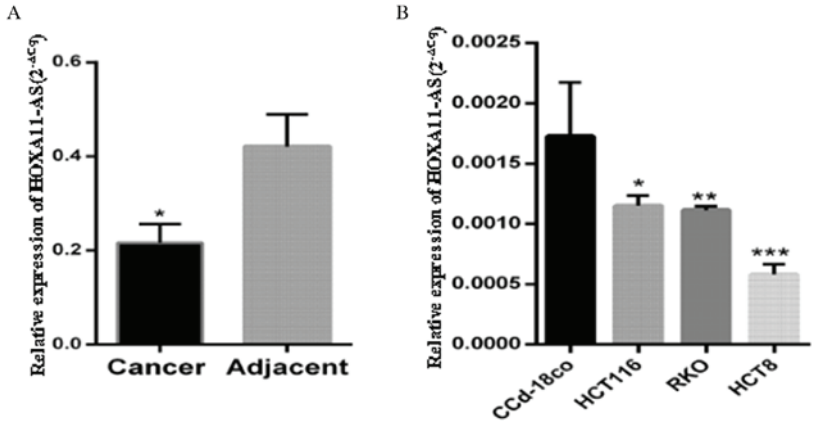

Figure 1. Relative expression of HOXA11-AS ($2^{-\Delta\Delta Cq}$). (A) RT-qPCR was performed to detect levels of HOXA11-AS expression in CRC tissues and paired normal colorectal tissues (P=0.0112 vs. paired normal tissues). (B) CRC cells (HCT8, RKO and HCT116) and one normal colorectal tissue cell line (CCD-18Co) were analyzed by RT-qPCR. HOXA11-AS expression was significantly decreased in the CRC cells compared with the CCD-18Co cells (*P<0.05, **P<0.01 and ***P<0.001 vs. normal colorectal cells). HOXA11-AS, HOXA11 antisense RNA; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Results

HOXA11-AS was downregulated in the CRC tissues and cell lines. HOXA11-AS expression was measured in 84 paired CRC and normal colonic mucosa tissues and observed to be significantly lower in the CRC tissues compared with the paired normal colonic mucosa tissues (P<0.05; Fig. 1A). Furthermore, HOXA11-AS expression was measured in 3 CRC cell lines (HCT8, RKO and HCT116) and 1 normal colorectal tissue cell line (CCD-18Co), and was observed to be significantly lower in the CRC cell lines compared with the normal colorectal tissue cells (P<0.05; Fig. 1B).

HOXA11-AS expression significantly differed between the CRC tumor tissues and adjacent normal tissues. A ROC curve was constructed by grouping all tumor samples to determine whether HOXA11-AS was able to distinguish CRC tissue from normal tissue, thus functioning as a biomarker for CRC. As presented in Fig. 2A, the area under the curve (AUC) of this ROC was 0.6130 [95% confidence interval (CI), 0.5277-0.6983; P<0.05], and the cut-off value for HOXA11-AS was 0.1908. Subsequently, a ROC was constructed using two groups: CRC samples with lymphatic metastasis and CRC samples without lymphatic metastasis. The AUC of this ROC was 0.628 (95% CI, 0.5052-0.7507; P<0.05; Fig. 2B). Each ROC curve suggests that HOXA11-AS has potential diagnostic value in CRC.

Association between HOXA11-AS expression and clinicopathological factors in CRC. To investigate the possible correlation between HOXA11-AS expression and clinicopathological factors, data from all 84 patients were collected and compared. As presented in Table I, decreased expression of HOXA11-AS was observed in patients with advanced tumor-node-metastasis (TNM) stage, lymphatic metastasis, large tumor size and a higher carcinoembryonic antigen (CEA) level compared with other patients in the corresponding groups (P<0.05). Moreover, Spearman's Rank correlation coefficient demonstrated that HOXA11-AS expression level was significantly correlated with advanced TNM stage (r=-0.23; P<0.05), lymphatic metastasis (r=-0.23; P=0.035), tumor size (r=-0.264; P<0.05) and CEA level (r=-0.265; P<0.05) (Fig. 3). No significant associations were observed between HOXA11-AS expression and other patient clinicopathological features, including age, tumor location, gender, histological grade, distant metastasis, obstruction, depth of invasion, perineural invasion, venous invasion and carbohydrate antigen 199 level (P>0.05; Table I).

Discussion

lncRNAs are non-coding RNAs that are important in cancer metastasis and carcinogenesis (22). Previous studies have indicated that the abnormal expression of certain well-defined lncRNAs, including HOX transcript antisense RNA, maternally expressed 3 and metastasis associated lung adenocarcinoma transcript 1, are strongly correlated with CRC development and progression (23-25). Therefore, the role of lncRNAs in the development and progression of CRC is gaining increasing attention (26).

A number of previous studies have investigated how HOXA11-AS expression affects different types of cancer. A gene microarray analysis indicated that HOXA11-AS was downregulated in bladder cancer, suggesting that it may function as a diagnostic marker for patients with this disease. In addition, HOXA11-AS expression has been demonstrated to be significantly lower in human epithelial ovarian cancer (EOC) tissues compared with normal ovarian tissues, and its upregulated expression may reduce cell migration, invasion, survival and proliferation in EOC cells (27). Therefore, such evidence indicates that HOXA11-AS may serve an important role in carcinogenesis.

In the current study, HOXA11-AS levels were investigated in 84 pairs of CRC tissues and adjacent normal tissues by RT-qPCR. The data obtained demonstrated that the relative expression level of HOXA11-AS was markedly reduced in the tumor tissues compared with the adjacent normal tissues. Furthermore, HOXA11-AS expression was significantly reduced in the CRC HCT8, HCT116 and RKO cells compared with the normal colon CCD-18Co cells. The decreased expression of HOXA11-AS in the CRC tissues and colorectal cells suggests that HOXA11-AS may function as a tumor suppressor in CRC.

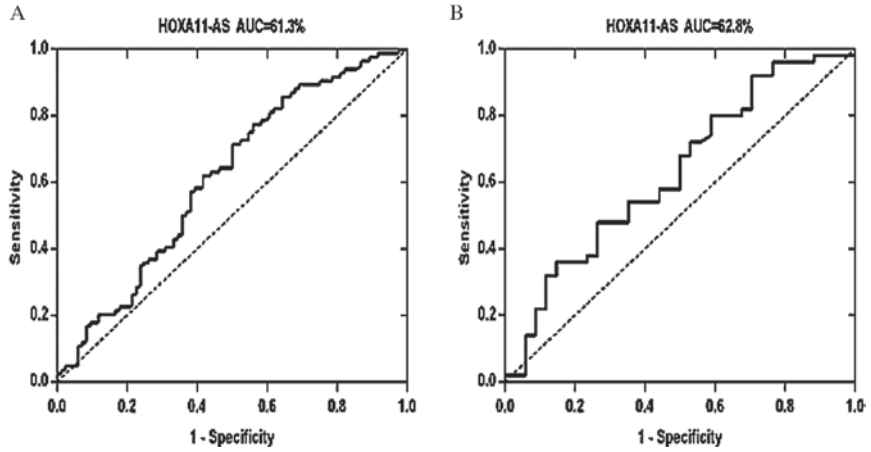

Figure 2. HOXA11-AS expression levels are significantly different between CRC tumor tissues and adjacent normal tissues. (A) A ROC curve of patients with CRC based on HOXA11-AS expression was constructed to distinguish CRC tissue from normal tissue. (B) A ROC curve of patients with CRC based on HOXA11-AS expression in lymph node metastasis and non-lymph node metastasis. Each curve demonstrates different levels of HOXA11-AS identified in CRC tissue. HOXA11-AS, HOXA11 antisense RNA; CRC, colorectal cancer; ROC, receiver operating characteristic; AUC, area under the curve.

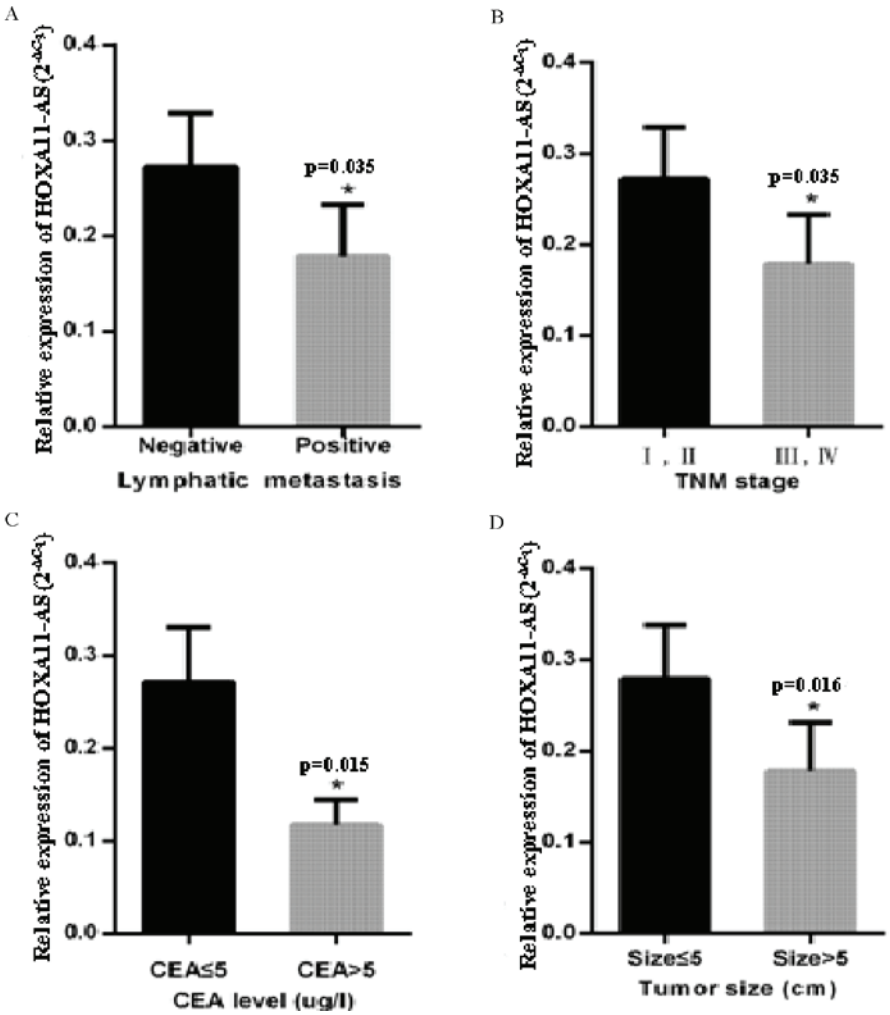

Figure 3. Relative expression of HOXA11-AS ($2^{-\Delta\Delta Cq}$). HOXA11-AS expression was positively correlated with (A) lymphatic metastasis (r=-0.23; P=0.035), (B) advanced TNM stage (r=-0.23; P=0.035), (C) CEA levels (r=-0.265; P=0.015; μg/l) and (D) tumor diameter (r=-0.264, P=0.016). HOXA11-AS, HOXA11 antisense RNA; TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen.

Previous studies have indicated that abnormal expression of lncRNAs is associated with several clinicopathological parameters of human cancer, including CEA level, lymph node metastasis, tumor size, TNM stage and distant metastasis (28-30). In the current study, the association between HOXA11-AS expression and different clinicopathological parameters of CRC

tissues was investigated. The results demonstrated that low HOXA11-AS expression was significantly correlated with lymph node metastasis, advanced TNM stage, tumor size and the CEA level of patients with CRC; however, HOXA11-AS expression was not associated with any other clinicopathological features. As lymph node metastasis indicates the likelihood of tumor migration, this suggests that HOXA11-AS may be involved in suppressing CRC tumorigenesis and metastasis.

However, the results of the current study did not exhibit a correlation between HOXA11-AS expression and distant metastasis or depth of invasion. This may be due to the lack of samples available for subgroup analysis. A recent study of 29 pairs of lung cancer samples indicated that HOXA11-AS expression levels were higher in patients with lymph node metastasis and stage III lung cancer (19), which is inconsistent with the results of the present study. This may be attributed to the small number of samples studied, or may be explained by the notion that the progression of different tumors may be regulated by different expression of the same lncRNA (31,32). Further research is required, however, taken together these results suggest that the function and contribution of HOXA11-AS may be tumor dependent.

In the current study, a ROC curve was constructed to distinguish CRC tissues from normal tissues. The AUC area calculated from the ROC was 0.613, suggesting that HOXA11-AS expression possesses promising diagnostic value in distinguishing CRC tissues from normal tissues. HOXA11-AS expression levels also differed between the CRC tissues with lymph node metastasis and the CRC tissues without lymph node metastasis with an AUC area of 0.628, thus suggesting that the lncRNA may be used to distinguish between the two groups. However, this was based on a limited number of patients and future studies investigating larger patient samples are required.

In conclusion, the results of the current study demonstrated that HOXA11-AS expression was significantly decreased in CRC tissues and certain CRC cell lines. Low HOXA11-AS expression was significantly correlated with lymph node metastasis, advanced TNM stage, tumor size and CEA level of patients with CRC. These findings suggest that HOXA11-AS may function as a potential prognostic indicator and an adjuvant therapy target in patients with CRC. However, the molecular mechanisms of HOXA11-AS involved in malignant phenotypes of CRC require further study.

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