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ORIGINAL ARTICLE

LncRNA NEAT1 Impacts Cell Proliferation and Apoptosis of Colorectal Cancer via Regulation of Akt Signaling

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Abstract Long noncoding RNA (lncRNA) have been reported to modulate oncogenesis and be used to be target for tumor. The role of lncRNA NEAT1 (nuclear paraspeckle assembly transcript 1, Gene ID: 283131) in colorectal cancer (CRC) keeps unknown. This work was to investigate the pattern of lncRNA NEAT1 (NEAT1) expression in CRC and its functional value and biological significance. NEAT1 expression was analyzed in 56 cancer tissues and cell lines in CRC cases. Results showed that NEAT1 was significantly overexpressed in CRC cells and tissues. Clinicpathologic detection verified that high NEAT1 expression associated with bulk in CRC. The serum contents of NEAT1 were observably elevated comparing with healthy cases ($P < 0.05$). The levels of NEAT1 were elevated in distinguishing CRC from normal ($ROC^{AUC} = 0.9471$; $P < 0.01$). Moreover, Kaplan–Meier analysis found that NEAT1 elevation led to adverse survival ($P < 0.05$). Further experiments illustrated that of NEAT1 knockdown signally inhibited growth and facilitated apoptosis. Importantly, we confirmed that Akt signaling pathway was inactivated after loss of NEAT1 in CRC. Taken together, this work support the first evidence that NEAT1 can be used to be a promising biomarker and target for novel treatment for human CRC.

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Introduction

Colorectal cancer (CRC) represents the most common malignancy characterized by an adverse clinical outcome [1]. Radical surgery might be the only hope for curing CRC in the stage of precursor lesions [2]. However, even after surgical resection, radiotherapy and chemotherapy obtain little benefit [3]. Previous studies have identified that a few molecular markers were correlated with prognosis, but the mechanism of CRC is still unqualified [4]. Therefore, there is urgent need to recognize oncogenesis-associated biomarker, which is helpful for developing novel treatment in view of CRC.

Long noncoding RNA (lncRNA) is identified as a sort of RNA molecules, which is longer than 200 nucleotides lacking an significant reading frame and takes participate in tumorigenesis [5, 6]. Concretely, lncRNA SPRY4-IT1 has been related to gastric cancer [7], PANDAR is reported to contribute to HCC [8] and LUNAR1 responsible for diffuse large B–cell lymphoma [9]. However, contributions of lncRNA to CRC still remain unclear.

LncRNA NEAT1 (nuclear paraspeckle assembly transcript 1, Gene ID: 283,131) locates at 11q13.1. It funtions as a transcriptional regulator for some genes involved in cancer progression [10]. Li, et al. demonstrated that NEAT1 promoted glioma cell viability and mobility [11]. Zeng, et al. showed that NEAT1 inhibition lessened cell differentiation in acute promyelocytic leukemia [12]. Wang, et al. demonstrated that NEAT1 promoted carcinogenesis in laryngeal squamous cell cancer [13]. However, the functional role and underlying mechanism of NEAT1 in CRC is largely elusive.

This study evaluated the pattern of NEAT1 expression in CRC and its diagnostic value. We showed that NEAT1 elevation expression predicted poorer survival. We also illustrated that loss of NEAT1 expression repressed growth and resulted in apoptosis in vitro. Furthermore, NEAT1 was found to promote CRC carcinogenesis by regulating Akt signaling. In conclusion, this work supported the first evidence that NEAT1 was a significant biomarker, and recognized as a potential target for CRC.

Materials and Methods

Patients

CRC tissues and corresponding normal tissues tested for qRT-PCR were acquired from 56 CRC patients who underwent surgeries between June 2010 and June 2013. Tissue specimens were conserved in liquid nitrogen until use. Meanwhile, 2 ml samples of blood were drawn from each patient, and then centrifuged and stored at −80 °C before future research. All clinicopathologic information of patients is represented in Table 1.

Cell Culture and Transient Transfection

Human CRC cells (HCT116, LoVo, HT29 and SW480) and normal colonic epithelial cells (HCoEpiC) (American Type Culture Collection, Manassas, VA, USA) were cultivated in Dulbecco minimum essential medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) tinting with 15% fetal calf serum (FCS) (Gibco) containing 2 mL-glutamine. Small interfering RNA (siRNA) targeting NEAT1 were purchased from Sangon Biotech (Shanghai, China). The sequences of siRNA were as following: siRNA targeting NEAT1 (si-NEAT1): 5′-AGGAGACGACTTACTAGAT-3′ [13]; scrambled siRNA used as a negative control (si-NC): 5′-UACUGUCUAGUCGCCGUAC-3′. siRNAs were transferred into cells using lipofectamine 2000 (Thermo Fisher Scientific) on the basis of the manufacturer's recommendation.

Table 1 Relation of NEAT1 with clinical features of CRC patients

Characteristic	*n*	NEAT1 expression		*P*
		low	high	
Gender				
Male	26	11	15	0.793
Female	30	14	16	
Age (years)				
≤ 60	31	13	18	0.788
> 60	25	12	13	
Bulk (cm)				
≤ 5	29	17	12	0.035*
> 5	27	8	19	
T stage				
T0–2	17	10	7	0.411
T3–4	39	15	24	
N stage				
N0	35	12	23	0.056
N1	21	13	8	
M stage				
M0	32	13	19	0.590
M1	24	12	12	

*$P < 0.05$

Real-Time Quantitative PCR

RNA was drawn using Trizol (Invitrogen, Carlsbad, CA) as the manufacture's procedure. The first strand cDNA was compounded using Tianscript RT kit (Tiangen biotech, Beijing, China). Expressions of mRNA were detected using ABI 7300 system (Applied Biosystems, Waters, USA). GAPDH was employed to be the control. The primers used for target genes were as following: NEAT1: forward, 5′-GUCUGUGUGGAAGGAGGAATT-3′, and reverse, 5′-UUCCUCCUUCCACACAGACTT-3′ [11]; GAPDH: forward, 5′-CAGCCAGGAGAAATCAAACAG-3′, and reverse, 5′- GACTGAGTACCTGAACCGGC-3′ [14].

MTT Assay

Cells after transfection were gathered for cell growth assay (MTT assay) following the manufacturer's recombination and previous study [15]. In brief, cells were grown in 96-well plates containing 100 μl RPMI-1640 per well. After transfection for 24, 48 and 72 h respectively, 10 μl of MTT was accessed into each well, then the medium was undocked after incubation for 4 h and subsequently added with 150 μl DMSO per well. The results represent as the average of three independent replicates.

Flow Cytometer Detection for Cell Apoptosis

Flow cytometer detection was employed to determine the impact of NEAT1 on CRC cell apoptosis as previous report [16].

Western Blotting

Cells were dissociated in RIPA lysis buffer (P0013D) (Beyotime, Haimen, China) and PMSF (ST506) (Beyotime). Split products were centrifuged at 12,000 rpm, and then supernatants were gathered. A Bradford Protein Assay Kit (P0006) (Beyotime) was used to analyzed protein concentration, and proteins were loaded to 10% SDS-PAGE

electrophorese, then proteins after separation were transferred onto PVDF membranes (Sigma, St. Louis, MO). Then PVDF membranes were obstructed with 5% skim milk (Guangming, Shanghai, China) and incubated with human Bcl-2 antibody (1:1000 dilution, #15071), Bax (#2772) (1:1000), phospho-Akt (Ser473) (#8200) (1:1000) and GAPDH (#8884) (1:1000) (Cell Signaling Technology Beverly, MA, US) for 2 h at room temperature. Then, specimens were hatched with secondary antibody conjugated with HRP (#7074) (1:1500) (Cell Signaling). Signals were detected using an HRP chemiluminescent kit (Thermo) using optional CCD camera and image processing system (Bio-Rad, CA, USA).

Statistical Analysis

Spearman's rank correlations were employed to explore the association between two variables. The independent-samples *t*-test was carried out to analyze continuous variable. Diagnostic value of NEAT1 was tested by area under the receiver operating characteristic curve (AUC). Survival curves were constructed and differences among groups were calculated using the Kaplan-Meier method. The value of *P* less than 0.05 was considered to have statistical significance.

Results

NEAT1 Is Overexpressed in CRC

The levels of NEAT1 expression in all four CRC cell lines was significantly elevated than NEAT1 expression of normal colonic epithelial cells (Fig. 1a). As NEAT1 was upregulated in CRC cell lines, our interest focused on the NEAT1 expression pattern in CRC tissues. qRT-PCR demonstrated that NEAT1 expression in CRC tissues was overexpressed compared with normal cases (Fig. 1b). Furthermore, we found that the levels of NEAT1 was positively associated with bulk ($P = 0.035$) (Table 1). However, no statistical association was observed between NEAT1 and gender, age, T, N and M stage ($P = 0.793$, 0.788, 0.411, 0.056 and 0.590, respectively) (Table 1). These results verified that elevation of NEAT1 expression may lead to CRC carcinogenesis.

Diagnostic Performance of Using NEAT1 as a Biomarker

Serum is an easy-to-access and simple specimen which owns predictive values for CRC. Importantly, we showed that serum contents of NEAT1 expression in patients with CRC were elevated comparing with the normal ($P < 0.05$, Fig. 2a). AUC achieved up to 0.9471 ($P < 0.001$, Fig. 2b).

Prognostic Significance of NEAT1 in CRC Patients

To explore the prognostic value of NEAT1, we correlated NEAT1 expression with clinical outcome of CRC patients by Kaplan-Meier analysis (Fig. 3). In our specimens, NEAT1 overexpression significantly related with improved overall ($P < 0.05$; Fig. 3a) and disease-free survival ($P < 0.05$; Fig. 3b).

NEAT1 Knockdown Restrains Cell Growth and Causes Apoptosis

Because NEAT1 was overexpressed in CRC compared with normal, we speculated that the biological functions of NEAT1 may take participate in controlling cell proliferation. To verify this hypothesis, MTT assays were used to analyze the effect of NEAT1 on of CRC cell growth. We found that loss of NEAT1 restrained cell growth compare with control in HCT116 cells ($P < 0.01$; Fig. 4a & b). These data support that NEAT1 is involved in the CRC cell growth. To investigate potential mechanism of NEAT1 in the growth of CRC, we analyzed cell apoptosis in HCT116 cells using flow cytometer. The results suggested that NEAT1 knockdown caused a prominent accumulation of apoptotic cell population ($P < 0.01$; Fig. 4c). Taken together, all the results proved that loss of NEAT1 could dramatically hold-up CRC cell growth.

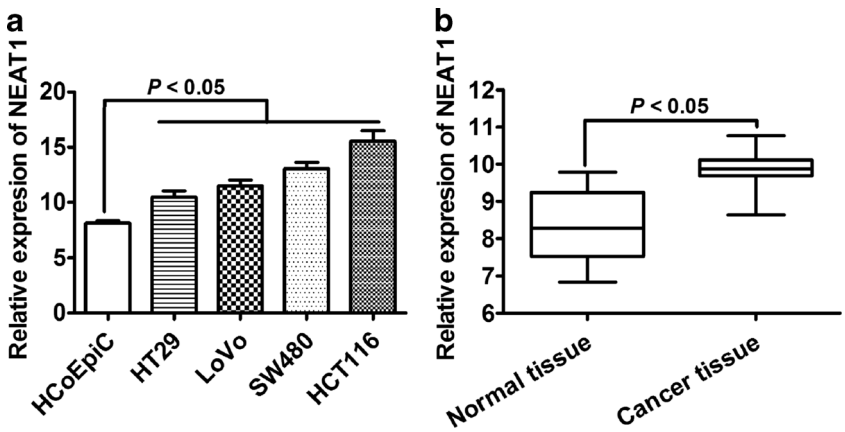

Fig. 1 Levels of NEAT1 expression in CRC. **a** Relative expression of NEAT1 in four CRC cell lines (HCT116, HT29, SW480 and LoVo) and human normal colonic epithelial cells (HCoEpiC) detected by qRT-PCR. **b** Expression of NEAT1 in CRC tissues and corresponding normal ones. ($P < 0.05$)

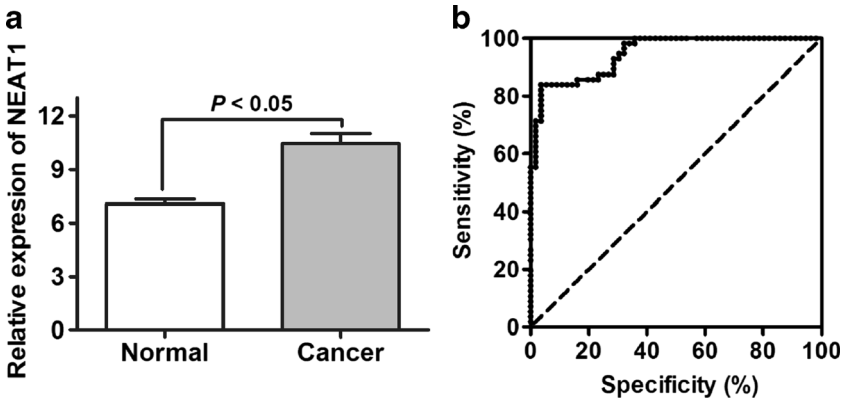

Fig. 2 Diagnostic performance of using NEAT1 as a biomarker. **a** Relative levels of serum NEAT1 from CRC patients and normal cases ($P < 0.05$). (B) ROC curve for using NEAT1 in distinguishing CRC patients from normal cases ($P < 0.01$)

Bcl-2 and Bax Is potentially Modulated by NEAT1

We inspected the changes of Bcl-2 and Bax, which is responsible for cell growth and apoptosis. The results suggested that the knockdown of NEAT1 led to Bcl-2 downregulation and Bax upregulation in HCT116 cells ($P < 0.05$; Fig. 5a). Further, loss of NEAT1 generated a conspicuous Bcl-2 reduction and Bax induction in HCT116 cells compared with control (Fig. 5b).

NEAT1 Regulates Akt Signaling in Vitro

The Akt signaling pathway functions as a pivotal modulator in cell growth and apoptosis [17]. In present study, NEAT1 was responsible for cell proliferation and apoptosis in HCT116 cells. We surmised that NEAT1 might lead to these cellular processes by suppressing the Akt signaling pathway. To verify this hypothesis, we detect the impact of NEAT1 on Akt signaling pathway in HCT116 cells. We found that NEAT1 knockdown restrain phosphorylation of Akt at Ser473 using western blot (Fig.5c). These results endorse that NEAT1 regulates Akt signaling.

Discussion

CRC is primary cause for cancer-related mortality all over the world. Accumulating evidences have demonstrated that lncRNAs participate in many malignancies [6–9]. Dysregulation of NEAT1 is a common event found in various cancers including laryngeal squamous cell cancer [13] and glioma [11]. To our best knowledge, few literatures focused on the role of NEAT1 in CRC up to now. Thus, further study about CRC-related lncRNAs in CRC may be helpful for this deadly disease. This work demonstrated the pattern of NEAT1 expression in CRC carcinogenesis and its potential prognostic and diagnostic significance in CRC patients, and further investigated its effect and mechanism in CRC cells.

In present study, this work identified NEAT1 as a promising biomarker for CRC. Expression of NEAT1 in the CRC tissues was significantly elevated than that in the corresponding normal tissues, and its elevation was associated with the bulk. The area of NEAT1 under the ROC curve achieved 0.9471, suggesting its diagnostic value.

Additionally, we supported the first evidence that upregulation of NEAT1 in CRC correlated with adverse outcome. Then, we explored the biology of NEAT1 in CRC, and showed that loss of NEAT1 expression modulating siRNA targeting NEAT1 could inhibit cell growth in vitro. The prominent ability of NEAT1 to promote tumorigenesis support that it plays an oncogenic role in CRC. Therefore, targeting NEAT1 may represent a favorable therapeutic strategy for CRC treatment.

Next, we explored potential target genes participating in step-wises. We have showed that Bcl-2 was downregulated and Bax upregulated by knockdown of NEAT1. It has

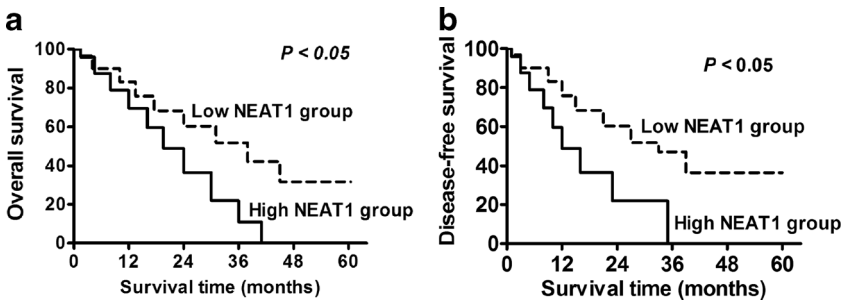

Fig. 3 Prognostic significance of NEAT1 in CRC patients. CRC patients' survival (OS and DFS) were analysed by Kaplan–Meier methodbased on the levels of NEAT1 expression ($P < 0.05$, long-rank)

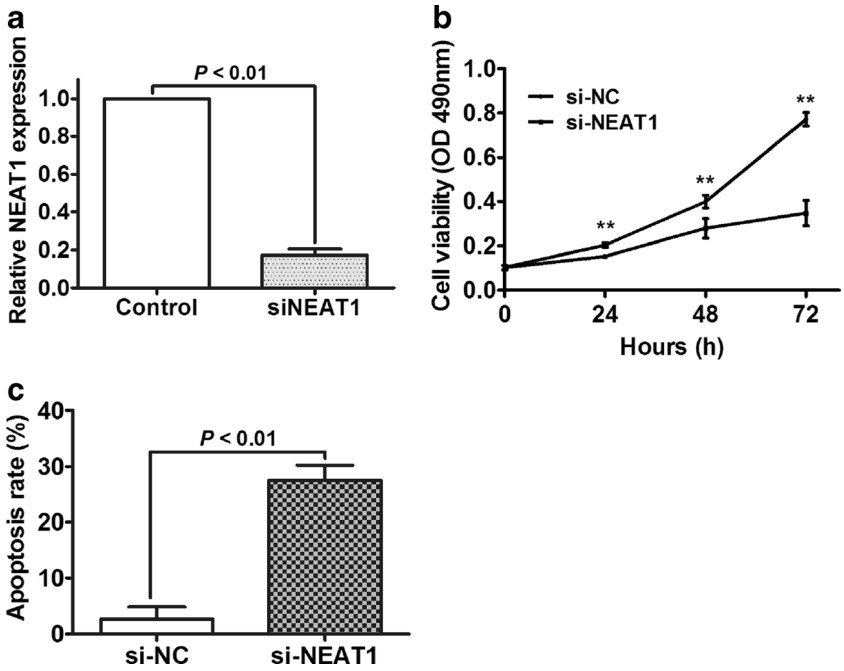

Fig. 4 Impact of NEAT1 on cell growth and apoptosis in CRC. **a** Expression of NEAT1 in HCT116 cells, after transfection with si-NC or si-NEAT1, was detected by qRT-PCR. Data showed that siRNA NEAT1 exhibited significant inhibition effect ($P < 0.01$). **b** Cell proliferation analysis. CRC cells were detected for 24, 48 and 72 h after transfection. $^{**}P < 0.01$ was defined to be statistically significant between NEAT1 knockdown and control. **c** Rate of apoptosis cell population was counted by flow cytometry. Results were representative of mean ± SD in triplicate ($P < 0.01$)

been confirmed that Bcl-2 and Bax is dysregulated in various cancers [18]. Bcl-2 and Bax functions as a regulator whose activity is required for cell apoptosis [19]. It has been shown to alter cell apoptosis, was observed frequently in various tumors and might lead to tumorigenesis [20]. Previous studies have demonstrated that lncRNAs might regulate Bcl-2 and Bax expression in cancers [21, 22]. Here we support a preliminary exploration that NEAT1 regulate Bcl-2 and Bax, resulting in to the induction of growth and reduction of apoptosis.

Furthermore, we investigated the potential mechanism by which NEAT1 resulted in CRC growth. Our results indicated that Akt signaling pathway was involved in modulating the impact of NEAT1 on induction of CRC cell growth, suggesting that NEAT1 increased the activation of Akt in this process. Akt activation implicates in regulating other gene expression and cellular behaviors in various tumors especially in CRC, therefore, approaches to block this signaling could be potential value for CRC treatment [23]. On account of the fact that less data have reported the association between NEAT1 and

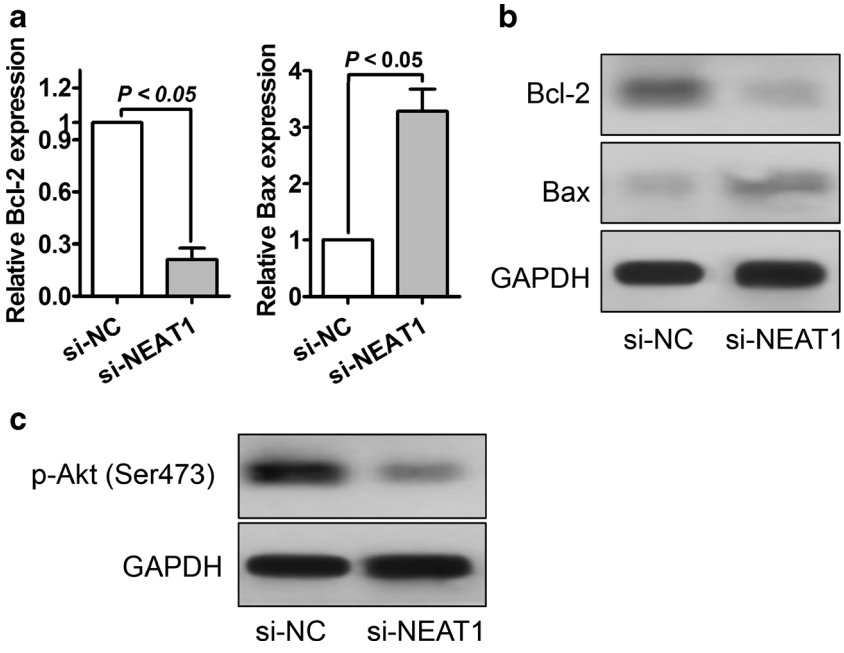

Fig. 5 NEAT1 expression is associated with Bcl2 and Bax in CRC cells after transfection with si-NC or si-NEAT1. **a** Analysis of Bcl-2 and Bax mRNA using qRT-PCR ($P < 0.05$). Western blot assays showed Bcl-2, Bax **b** and phospho-Akt (Ser473) **c** in vitro

Akt signaling pathway, we believed that Akt activation could be partly responsible for oncogenic mechanism of NEAT1.

In conclusion, we showed the first evidence that NEAT1 as an oncogene important for CRC. Firstly, our results demonstrated that NEAT1 expression was elevated in CRC cell lines and tissues. Then, our clinical data suggested that NEAT1 may be used to be a novel predictor for CRC. Moreover, NEAT1 knockdown in CRC cells not only resulted in growth arrest and cell apoptosis, but also decreased Bcl-2 and increased Bax expression. Ultimately, NEAT1 displays oncogenic activity is related with the modulation of Akt signaling pathway. Taken together, our results verify that NEAT1 may be served as a potential target for cancer therapeutics by regulating Akt signaling pathway.

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Compliance with Ethical Standards

Conflict of Interest None

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