Int J Clin Exp Pathol 2017;10(4):4470-4478
www.ijcep.com /ISSN:1936-2625/IJCEP0036042

Original Article

Long non-coding RNA NEAT1 promotes metastasis via enhancing ZEB2 by sponging miR-662 in colorectal cancer

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Received April 25, 2016; Accepted November 18, 2016; Epub April 1, 2017; Published April 15, 2017

Abstract: Recent studies reported that long non-coding RNA NEAT1 was dysregulated expression in some cancers and also closely related with malignant phenotype of lymphatic metastasis and TNM stage. However, the detailed effect mechanism of NEAT1 still remains mysterious. NEAT1 and ZEB2 were up-regulated in CRC, meanwhile, miR-662 presented down-regulated in CRC tissues, compared with adjacent normal intestinal mucosal tissues (all $P<0.05$); Correlation analysis showed that NEAT1 expression was negatively with miR-662 in CRC tissues, miR-662 expression was negatively with ZEB2 and NEAT1 was positively with ZEB2 (all $P<0.01$). Either knowing-down NEAT1 or over-expressing miR-662 inhibited invasion and migration of colon cancer cells. Both knowing-down NEAT1 and over-expressing miR-662 could suppress expression of EMT relative molecules of ZEB2, Fibonectin and SNAIL. Luciferase report assay, RIP test and RNA Pull-Down confirmed that NEAT1 could directly sponge miR-662 and miR-662 could directly target ZEB2. Consequently, NEAT1 can directly sponge miR-662 which can directly target ZEB2, to promote invasion and migration of colon cancer cells. NEAT1-miR-662-ZEB2 axis is formed to regulate invasion and metastasis of CRC.

Keywords: Long non-coding RNA NEAT1, miR-662, ZEB2, colorectal cancer

Introduction

Recent years, studies showed that long non-coding RNAs (lncRNAs) could regulate disease development and in response to diverse signaling cues. In addition, lncRNAs have been reported to be abnormally expressed in plenty of tumors [1], such as liver cancer [2, 3], colorectal cancer [4, 5], gastric cancer [6], gallbladder carcinoma [7], cervical cancer [8], renal carcinoma [9], et al. Several lncRNAs controlling transcriptional alteration implies that difference in lncRNAs profiling between normal and cancer cells is not merely the secondary effect of cancer transformation, and that lincRNAs are strongly associated with cancer progression [10]. Thus, the differential expression of lncRNAs may be profiled to aid in cancer diagnosis and prognosis and in the selection of potential therapeutics.

Although some lncRNAs have been reported maybe to have an effect on various human diseases [11], the basis of their molecular mechanisms still remains largely unknown. The impact of lncRNAs have multiple types on various carcinomas. For example, several lncRNAs can control gene expression by directly recruiting histone-modifying enzymes to chromatin [12]; Some lncRNAs can regulate special protein activity by binding them. Furthermore, lncRNAs can sponge different microRNAs (miRNAs) indirectly to promote some special molecule expression so as to play a role in tumorigenesis and progress. Long noncoding RNA H19 competitively binds miR-17-5p to regulate YES1 expression in thyroid cancer [13]. Long noncoding RNA MEG3 is downregulated in cervical cancer and affects cell proliferation and apoptosis by regulating miR-21 [14]. Similarly, NEAT1, a long non-coding RNA, have been reported associated with some cancers. NEAT1 was dysregulated expression in non-small cell lung cancers [15]; The oestrogen receptor alpha-regulated lncRNA NEAT1 was reported to be a critical

modulator of prostate cancer [16]; What's more, NEAT1 has ever been reported to bind miR-107 to regulate CDK6 pathway in laryngeal squamous cell cancer [17].

In our study, we found that NEAT1 was up-regulated in colorectal cancer (CRC) and had ability of promoting invasion and migration of colon cancer cells. Through bioinformatics prediction, we found that NEAT1 could sponge miR-662, which was lowly expressed in CRC. miR-662 might target ZEB2, which is a relative molecule of epithelial-mesenchymal transition process. Therefore, NEAT1 might promote ZEB2 expression by sponging miR-662 in CRC.

Materials and methods

Clinical samples

A total of 20 fresh cancer tissue samples were collected from the patients with CRC of stage II/III (II stage for 8 cases and III stage for 12 cases). All these patients received radical surgical resection without preoperative chemotherapy, radiotherapy or immunotherapy at Sichuan Cancer Hospital from December 1st, 2014 and September 31th, 2015. Mean patient age was 60.01 ± 9.44 years. Prior to participation, a diagnosis of CRC was confirmed by histopathology for all patients. The adjacent normal tissue samples were gained from the normal colorectal tissues located 5 centimeters away from the tumor. TNM stage was ascertained according to the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) TNM stage system of CRC (2010, Seventh Edition). The study design and procedures were approved by our the Institute Research Ethics Committee and written informed consent was gained from each patient. All patient specimens were also permitted by Ethics Committee.

Cell culture and the treatment

Two colon cancer cell lines (SW480, HCT116) were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). SW480 were grown in RPMI Medium 1640 (Invitrogen), and HCT116 was in cultured in high-glucose DMEM (Gibco Co., Ltd., USA). The media were supplemented with 10% FBS (fetal bovine serum) (Gibco Co., Ltd., USA). All these cell lines were cultured in a humidified atmosphere at 37°C containing 5% CO_2. The gene-specific siNEAT1 (5'-GUGAGAAGUUGCUUAGAAACUUUCC-3') and non-targeting Control siNC (5'-UUCUCCGAACGUGUCACGUtt-3') were synthesized by Guangzhou RiboBio Co., Ltd. Colon cancer cells were transfected with 20 nM. Transfection were performed according to the protocol of Ribo FECT ™ CP Transfection Kit (C10511-05, RiboBio Co., Ltd, Guangzhou, China) was used to transfect colon cancer cells.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from tissue samples and cultured cells using TRIZOL reagent (Invitrogen Life Technologies). These total RNA was reverse transcribed to cDNA from 2000 ng of total RNA in a final volume of 20 μl according to the instructions of Prime-Script RT-PCR kit (TaKaRa, Dalian, China). qRT-PCR analysis were carried out by a protocol from Power SYBR Green (Takara Dalian, China). Primers were as followings, NEAT1: 5'-CTTCCTCCCTTTAACTTATCCATTCAC-3' (forward) and 5'-CTCTTCCTCCACCATTACCAACAATAC-3' (reverse); SNAI1: 5'-GGTTCTTCTGCGCTACTGCT-3' (forward) and 5'-TGCTGGAAGGTAAACTCTGGAT-3' (reverse); Fibronectin 1 (FN1): 5'-GAACAAACACTAATGTTAATTGCCC-3' (forward) and 5'-TCTTGGCAGAGAGACATGCTT-3' (reverse); GAPDH: 5'-AGAGGCAGGGATGATGTTCTG-3' (Forward) and 5'-GACTCATGACCACAGTCCATGC-3' (Reverse); Gene specific primer was 5'-ACACAGAATAAAATAACAC-3' for cDNA conversion. Each sample was analyzed in triplicate pattern.

Protein immunoblot experiment

Protein immunoblot experiments were performed as the previously described [18]. The antibodies of ZEB2, Fibonectin, SNAIL and GAPDH were all purchased from Cell Signaling Technology, Inc in BOSTON, USA.

Cell migration and invasion assays

Cell motility and invasiveness were determined by a 24 well transwell plate (8 μM pore size; Costar) [19]. 48 hs after transfection, for transwell migration assays 1×10^4 cells in 200 μl serum-free media were placed on the top chamber lined with the noncoated membrane; for invasion assays, 3×10^4 cells in 200 μl serum-free media were placed on the upper chamber of each insert coated with 200 mg/ml of Matrigel (BD Biosciences, CA, USA). The chambers were incubated in the bottom cham-

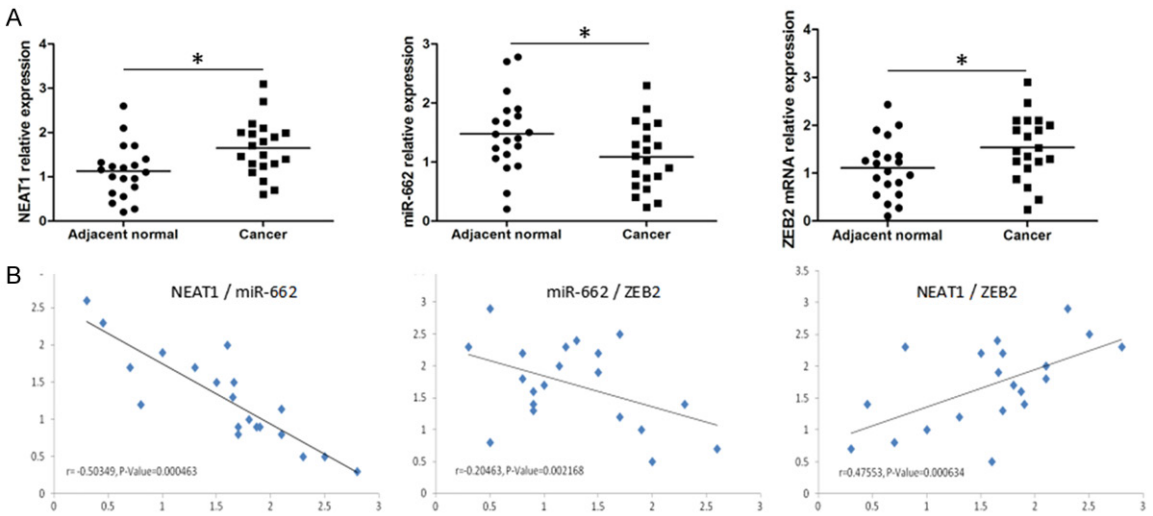

Figure 1. NEAT1, miR-662 and ZEB2 expression in colorectal cancer (CRC). A: NEAT1 and ZEB2 were highly expression in CRC, compared with adjacent normal intestinal mucosal tissue, but not miR-662. B: Correlation analysis showed that NEAT1 and ZEB2 were positively related with each other in CRC; Both NEAT1 and ZEB2 were negatively associated with miR-662, respectively.

bers with 10% FBS media for 36 hs. The migrated and invaded cells to the reverse side of chamber inserts were fixed and stained with methanol and 0.1% crystal violet. Then, the stained cells were counted under the microscope.

RNA immunoprecipitation (RIP)

The MS2bs-MS2bp-based RNA immunoprecipitation (RIP) test was performed according to previous reports [20]. HCT116 cells were transfected with pcDNA-NEAT1-MS2, pcDN-NEAT1-mut-MS2, pcDNA-NEAT1-MS2, or pcDNA-MS2. After 48 hours, cells were used to perform RIP using a GFP antibody (Roach) and the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Then the miR-662 level was analyzed by qPCR.

RNA pull-down assays

The RNA pull-down was performed as previously described [21]. HCT116 cells were transfected with biotinylated miRNA (40 nmol/L). After 48 hours, the cells were harvested and the lysates were incubated with M-280 streptavidin magnetic beads (Sigma). The bound RNAs were purified using TRIzol and the NEAT1 level was analyzed by qPCR.

Luciferase report assay

Both NEAT1 gene and 3'UTR of ZEB2 gene harboring miR-662 binding site was inserted in psi-CHECK2 plasmid, named NEAT1 WT and ZEB2 3'UTR WT. NEAT1 with four binding site mutations and ZEB2 3'UTR gene with two mismatch base mutations in miR-662 seed complementary site was inserted in psi-CHECK2-plasmid and named as NEAT1 MT and ZEB2 3'UTR MT. HCT116 cells were cultured in 96-well plates and cotransfected with negative control, miR-662 mimics, psi-CHECK2-NEAT1 WT or psi-CHECK2-NEAT1 MT (miR-662). The pRL-TK Renilla luciferase plasmid was used as internal control. 48 h after transfection, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay (Promega, USA). The results were expressed as relative luciferase activity (firefly luciferase/Renilla luciferase).

Statistical analysis

Statistics were carried out with the SPSS statistical software package, version 18.0 (SPSS Inc., Chicago, IL, USA). The Students' t test were analyzed for RT-PCR and cell migration and invasion assays. The correlation analysis among NEAT1, miR-662 and ZEB2 was probed by Spearman test. A value of $P<0.05$ indicated a significant difference.

Results

NEAT1, miR-662 and ZEB2 was up-regulated in CRC

QRT-PCR was performed to measure NEAT1, miR-662 and ZEB2 mRNA expression, respec-

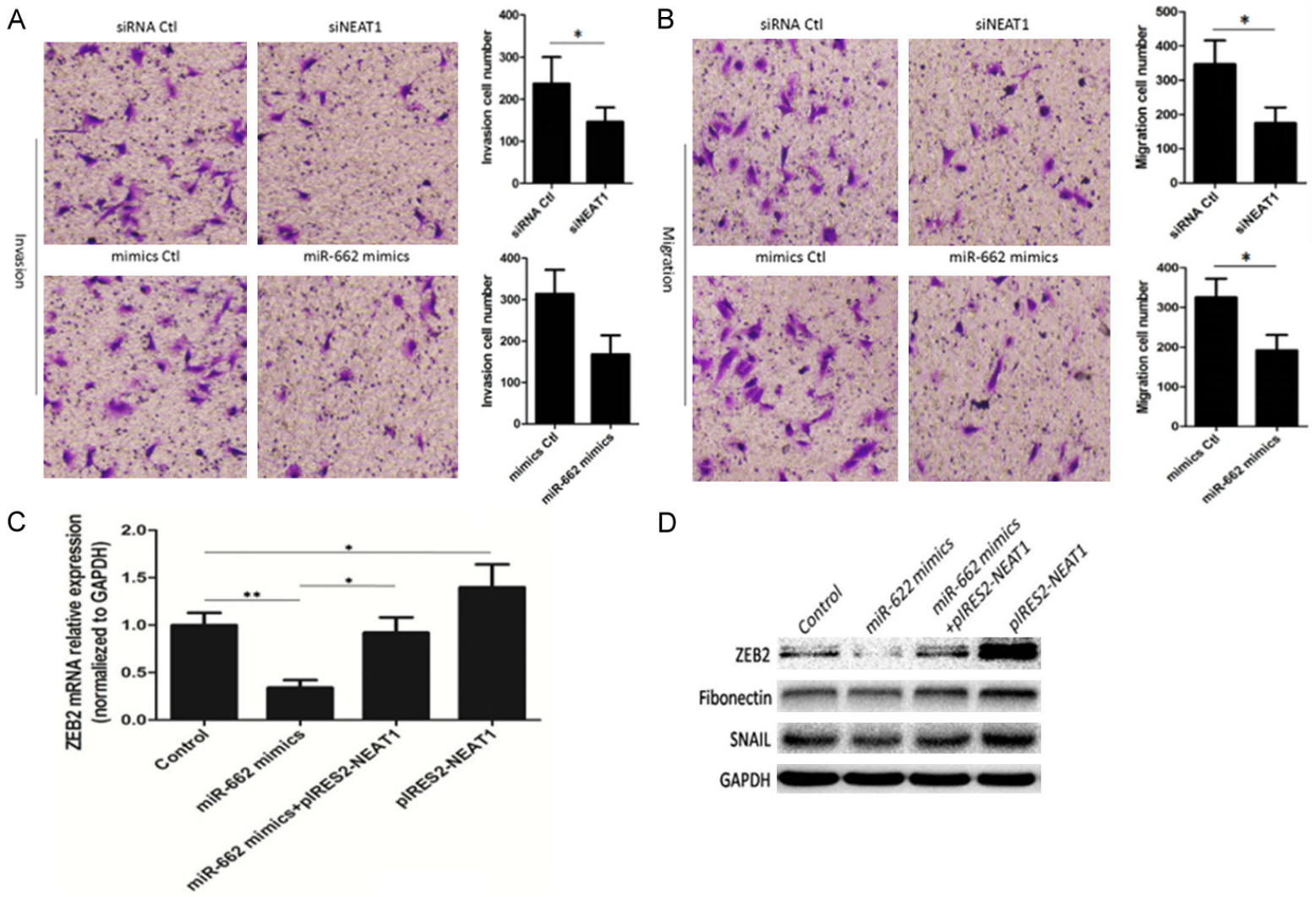

Figure 2. NEAT1 promoted invasion and migration in CRC. A: HCT116 cells were transfected with negative control, NEAT1 RNAi (siNEAT1) or miR-662 mimics. After 48 hours, transwell test indicated that both NEAT1 RNAi and miR-662 mimics reduced invasion of HCT116 cells, compared with negative control group. B: Similarly, transwell test also indicated that both NEAT1 RNAi and miR-662 mimics reduced invasion of HCT116 cells, compared with negative control group. C: HCT116 cells were transfected with negative control, NEAT1 RNAi (siNEAT1), miR-662 mimics or NEAT1 combined with miR-662. After 48 hours, qRT-PCR showed that NEAT1 promoted ZEB2 mRNA expression and miR-662 inhibited ZEB2 mRNA expression. Meanwhile, ZEB2 mRNA expression in NEAT1 combined with miR-662 group was higher than that in miR-662 group. D: Western blotting showed that NEAT1 promoted ZEB2, Fibonectin and SNAIL protein expression and miR-662 inhibited ZEB2, Fibonectin and SNAIL protein expression. Meanwhile, ZEB2, Fibonectin and SNAIL protein expression in NEAT1 combined with miR-662 group was higher than those in miR-662 group.

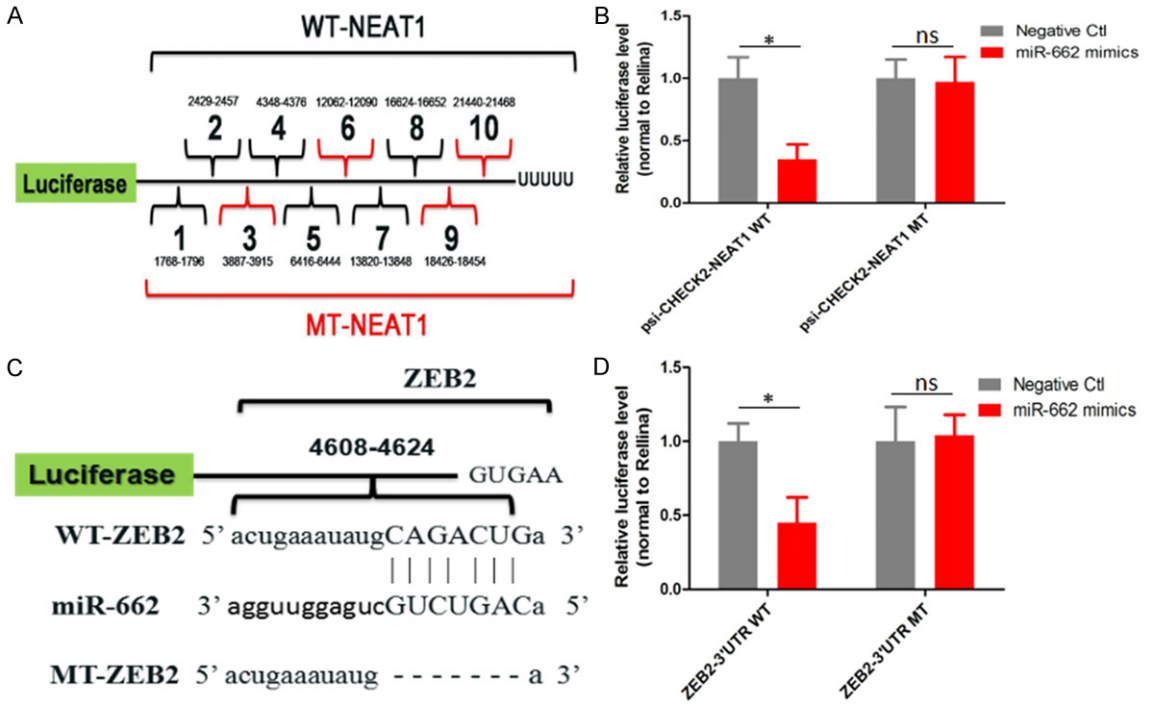

Figure 3. NEAT1 sponging miR-662 and miR-662 targeting ZEB2 were verified by dual-luciferase reporter assay. A: Mode chart of NEAT1 sponging miR-662. B: HCT116 cells were transfected with negative control, miR-662 mimics, psi-CHECK2-NEAT1 WT or psi-CHECK2-NEAT1 MT (miR-662). After 48 hours, dual-luciferase reporter assay showed that miR-662 mimics reduced fluorescence activity, compared with negative control in NEAT1 WT group, meanwhile no changes in NEAT1 MT group. C: Mode chart of miR-662 targeting ZEB2. D: HCT116 cells were transfected with negative control, miR-662 mimics, psi-CHECK2-ZEB2 3'UTR WT or psi-CHECK2-ZEB2 3'UTR MT (miR-662). After 48 hours, dual-luciferase reporter assay showed that miR-662 mimics reduced fluorescence activity, compared with negative control in ZEB2 WT group, meanwhile no changes in ZEB2 MT group.

tively. The results showed that NEAT1 and ZEB2 mRNA expression level in CRC tissues were higher than those in adjacent normal intestinal mucosal tissues (both P<0.05); miR-662 was lowly expressed in CRC tissues compared with adjacent normal intestinal mucosal tissues (P<0.05). In addition, correlation analysis indicated that showed that NEAT1 expression level was negatively associated with miR-662 expression (P<0.001), miR-662 expression level was negatively related with ZEB2 mRNA expression (P<0.001) and NEAT1 expression level was positively correlated with ZEB2 mRNA in CRC (P<0.001) (**Figure 1**).

NEAT1 promoted invasion and migration in colon cancer cells

HCT116 cells were transfected with negative control, NEAT1 RNAi (siNEAT1) or miR-662 mimics. After 48 hours, transwell test indicated that both NEAT1 RNAi and miR-662 mimics reduced invasion of HCT116 cells respectively, compared with negative control group (both P<0.05). Similarly, the results also indicated that both NEAT1 RNAi and miR-662 mimics reduced invasion of HCT116 cells, compared with negative control group (both P<0.05). HCT116 cells were transfected with negative control, NEAT1 RNAi (siNEAT1), miR-662 mimics or NEAT1 combined with miR-662. After 48 hours, qRT-PCR showed that NEAT1 promoted ZEB2 mRNA expression (P<0.05) and miR-662 inhibited ZEB2 mRNA expression (P<0.01). Meanwhile, ZEB2 mRNA expression in NEAT1 combined with miR-662 group was higher than that in miR-662 group (P<0.05). Western blotting showed that NEAT1 promoted EMT relative proteins expression of ZEB2, Fibonectin and SNAIL and miR-662 inhibited these EMT relative proteins expression. Meanwhile, ZEB2, Fibonectin and SNAIL protein expression in NEAT1 combined with miR-662 group was higher than those in miR-662 group (**Figure 2**).

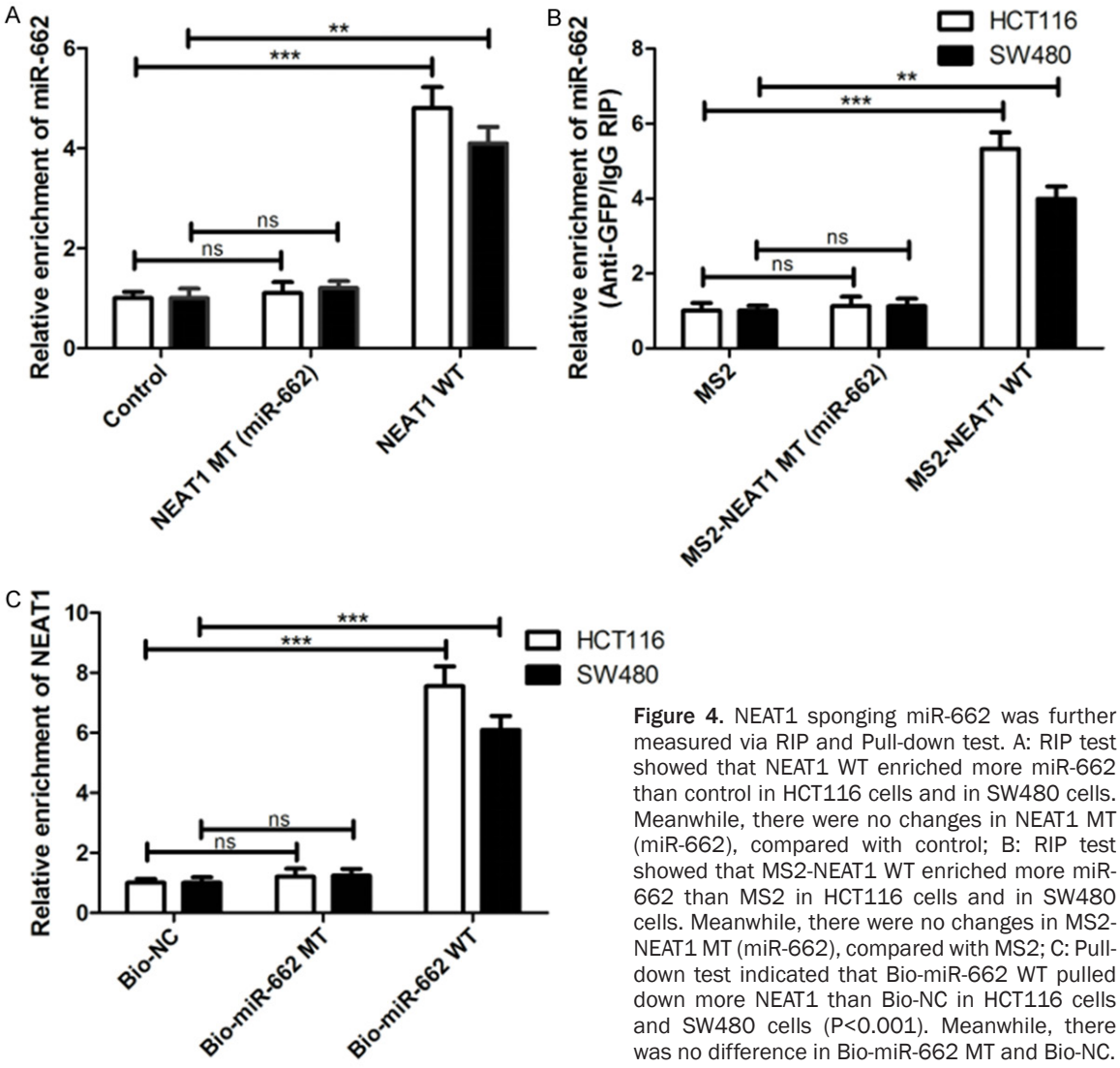

Figure 4. NEAT1 sponging miR-662 was further measured via RIP and Pull-down test. A: RIP test showed that NEAT1 WT enriched more miR-662 than control in HCT116 cells and in SW480 cells. Meanwhile, there were no changes in NEAT1 MT (miR-662), compared with control; B: RIP test showed that MS2-NEAT1 WT enriched more miR-662 than MS2 in HCT116 cells and in SW480 cells. Meanwhile, there were no changes in MS2-NEAT1 MT (miR-662), compared with MS2; C: Pull-down test indicated that Bio-miR-662 WT pulled down more NEAT1 than Bio-NC in HCT116 cells and SW480 cells (P<0.001). Meanwhile, there was no difference in Bio-miR-662 MT and Bio-NC.

NEAT1 sponges miR-662 and miR-662 targets ZEB2

Bioinformatics predicted that long noncoding RNA NEAT1 had 10 binding sequences of miR-662 by Target scan. Like mode chart of **Figure 3A**. HCT116 cells were transfected with negative control, miR-662 mimics, psi-CHECK2-NEAT1 WT or psi-CHECK2-NEAT1 MT (miR-662). After 48 hours, dual-luciferase reporter assay showed that miR-662 mimics reduced fluorescence activity, compared with negative control in NEAT1 WT group (P<0.05), meanwhile no changes in NEAT1 MT group (P>0.05). (C) Bioinformatics predicted that ZEB2 mRNA 3'UTR region could be targeted by miR-662. HCT116 cells were transfected with negative control, miR-662 mimics, psi-CHECK2-ZEB2 3'UTR WT or psi-CHECK2-ZEB2 3'UTR MT (miR-662). After 48 hours, dual-luciferase reporter assay showed that miR-662 mimics reduced fluorescence activity, compared with negative control in ZEB2 WT group (P<0.05), meanwhile no changes in ZEB2 MT group (P>0.05) (**Figure 3**).

NEAT1 sponging miR-662 was further measured via RIP and pull-down assay

In order to further verified the function of NEAT1 sponging miR-662, RIP and Pull-down test were carried out, respectively. RIP test showed that NEAT1 WT enriched more miR-662 than control in HCT116 cells (P<0.001) and in SW480 cells (P<0.01). Meanwhile, there were no changes in NEAT1 MT (miR-662), compared with control

(P>0.05); RIP test showed that MS2-NEAT1 WT enriched more miR-662 than MS2 in HCT116 cells (P<0.001) and in SW480 cells (P<0.01). Meanwhile, there were no changes in MS2-NEAT1 MT (miR-662), compared with MS2 (P> 0.05); Pull-down test indicated that Bio-miR-662 WT pulled down more NEAT1 than Bio-NC in HCT116 cells and SW480 cells (P<0.001). Meanwhile, there was no difference in Bio-miR-662 MT and Bio-NC (**Figure 4**).

Discussion

A major goal in the development of cancer therapeutics is to identify molecular targets that are specific for cancer cells [22]. The differential expression and biological importance of specific non-coding RNAs (ncRNAs) in cancer suggest that ncRNAs may be useful targets [23]. miRNAs have been effectively targeted in preclinical models by means of small molecules and exogenously introduced complementary RNA sequences [24]. Any factors influence miRNAs indirectly regulate their targeted genes so as to impact on tumor development.

miRNAs can play a role as oncogene [25-27] or anti-oncogene [28, 29] according to their targeting genes. miR-103 played a oncogene role in CRC in vitro through targeting PER3 [30]; miR-126-5p played a suppressive role in CRC by binding IRS-1 [31]. In our study, we found that miR-662 was lower expression in CRC tissues than in adjacent normal intestinal mucosal tissues. In vitro, we demonstrated that miR-662 inhibited invasion and migration of colon cancer cells. Consequently, miR-662 might play a suppressive part in CRC. Furthermore, we carried out bioinformatics prediction, the results showed us that ZEB2 might be targeted by miR-662. Next, we verified that miR-662 could directly inhibit ZEB2 expression by dual luciferase reporter gene detection system and the expressions of some EMT relative molecules were also be depressed by miR-662.

Since miR-662 could promote the expressions of EMT relative molecules of ZEB2, Fibonectin and SNAIL, what molecules could regulate miR-662? Similarly, we performed bioinformatics prediction and found that a long non-coding RNA molecule, NEAT1, has ten binding sites of sequences of miR-662. First of all, NEAT1 was found up-regulated in CRC tissues compared with adjacent normal intestinal mucosal tissues and over-expressed NEAT1 could enhance invasion and migration of colon cancer cells in vitro. Thus, NEAT1 acted an oncogene in CRC. NEAT1 was reported up-regulated in non-small cell lung cancer. NEAT1 was positively correlated with lymphatic metastasis, vascular invasion and clinical TNM stage [15], which implied that NEAT1 might be an oncogene in non-small cell lung cancer. Next, we confirmed that NEAT1 could directly sponge miR-662 to indirectly promote the expressions of some EMT relative molecules. NEAT1 level was significantly higher in laryngeal squamous cell cancer (LSCC) than in corresponding adjacent non-neoplastic tissues, and patients with neck nodal metastasis or advanced clinical stage had higher NEAT1 expression. NEAT1 knockdown significantly inhibited the proliferation and induced apoptosis and cell cycle arrest at G1 phase in LSCC cells. NEAT1 regulated CDK6 expression in LSCC cells which was mediated by miR-107. Therefore, NEAT1 plays an oncogenic role in the tumorigenesis of LSCC by binding miR-107 [17], the mechanism of which was similar to our study.

In brief, NEAT1 was over-regulated in CRC tissues compared with adjacent normal intestinal mucosal tissue; NEAT1 could directly sponge miR-662 to promote invasion and migration of colon cancer cells in vitro; miR-662 could directly target ZEB2, one of EMT relative molecule. Consequently, NEAT1-miR-662-ZEB2 axis is formed to regulate invasion and metastasis of CRC and has potential for targeted therapy.

Disclosure of conflict of interest

None.

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