Long Noncoding RNA NEAT1 Promotes Tumorigenesis in H. Pylori Gastric Cancer By Sponging miR-30a To Regulate COX-2/BCL9 Pathway

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Abstract

Background: Helicobacter pylori (H. pylori) is a carcinogenic factor for gastric cancer. Our previous study demonstrated that H. pylori decreased the expression of microRNA(miRNA)-30a to promote the tumorigenesis in gastric cancer. However, the upstream regulatory mechanism of miR-30a hasn't well-elucidated. In this study, we found the long non-coding RNA (IncRNA) NEAT1 may sponge miR-30a to regulate COX-2/BCL9 pathway.

Methods: The expression of NEAT1 was detected in gastric cancer tissues and tumour adjacent tissues by fluorescence in-situ hybridization(FISH) analysis and RT-qPCR. LncRNA-miRNA interaction networks were constructed using the RNAhybird and Starbase v.2.0. and then validated using dual-luciferase assay. The effects of NEAT1 dysregulation on the proliferative, migratory and invasive abilities of H. pylori filtrate infected gastric cancer cells were observed by cell counting kit-8 (CCK-8), colony formation, wound healing test and transwell assays. Western blot and RT-qPCR were performed to detect protein and RNA expression. The Immunohistochemistry(IHC) was carried out to analyze the location and expression of COX-2 and BCL9.

Results: FISH and RT-qPCR demonstrated that the expression of NEAT1 was up-regulated in gastric cancer tissues, especially in H. pylori gastric cancer tissues, and the expression of NEAT1 is negatively correlated with miR-30a(miR-30a-3p, miR-30a-5p).

The proliferation, migration and invasion of H. pylori filtrate infected gastric cancer cells could be largely enhanced by the up-regulation of NEAT1, while the downregulation of NEAT1 decreased these abilities, and miR-30a could reverse the effect of NEAT1 on these abilities. Dual-luciferase assay identified that NEAT1 directly targeted miR-30a(miR-30a-3p, miR-30a-5p).Due to miR-30a(miR-30a-3p, miR-30a-5p) negatively regulated the expression of downstream COX-2 and BCL9, NEAT1 was identified to indirectly upregulate the expression of COX-2 and BCL9.IHC showed that the expression of COX-2 and BCL9 were increased in H. pylori gastric cancer tissues.

Conclusion: The study demonstrated that IncRNA NEAT1 may act as a promoter of tumorigenesis in H. pylori gastric cancer, by sponging miR-30a(miR-30a-3p, miR-30a-5p) to regulate COX-2/BCL9 pathway.

1. Background

Gastric Cancer is one of the most frequently diagnosed digestive cancers, accounting for over million new cases globally in 2018. Incidence rates of gastric cancer are markedly elevated in Asian countries, especially in China. Compelling evidences supported that Helicobacter pylori (H. pylori) is the first class carcinogen leading to gastric adenocarcinoma, so it is directly linked to the development of gastric cancer. Early diagnosis of gastric cancer for radical cure largely improved the overall survival of patients, but due to early stage gastric cancer being asymptomatic, or else unspecified symptoms, most
gastric cancer patients are already in advanced stages when they are diagnosed. Thus, it is urgent to identify valuable markers of diagnose gastric cancer at early stage.

Non-protein-coding RNAs (ncRNAs) were found to participate in genome encoded-transcripts and almost all cellular processes. ncRNAs are classified as long noncoding RNAs (lncRNAs), and small ncRNAs, such as microRNAs (miRNAs). The interaction of lncRNAs, miRNAs, and mRNAs are critically implicated in various diseases, including cancer. Multiple ncRNAs act as oncogenes or tumour suppressor genes during carcinogenesis. Therefore, ncRNAs represent efficient diagnostic biomarkers of cancer.

miR-30a has been identified to have tumour suppressor properties, the double-stranded precursor generated two single-stranded miRNAs, including miR-30a-3p and miR-30a-5p, which respectively played in two different manners in regulating the proliferation and migration of *H. pylori*-infected gastric cancer cells. miR-30a-3p targeted the 3'UTR of COX-2 mRNA and regulated nuclear translocation of β-catenin, miR-30a-5p targeted the 3'UTR of BCL9 mRNA to affect TCF/LEF promoter activity, and regulate downstream gene expression of β-catenin.

LncRNA nuclear enriched abundant transcript 1 (NEAT1) is aberrantly expressed in different types of cancer. High levels of NEAT1 may act as a biomarker of prostate cancer patients, and NEAT1 promotes cervical cancer cell invasion and the progression of sarcoma metastasis. But NEAT1 is downregulated in acute promyelocytic leukaemia. It was predicted that NEAT1 sponge miR-30a by bioinformatics analysis. Hence, our study aimed at evaluating the function of NEAT1 in *H. pylori* gastric cancer, which might be valuable for the diagnosis and treatment of gastric cancer.

2. Materials And Methods

2.1 Clinical samples

Gastric cancer tissues and matching adjacent tissues were isolated from 38 patients (15 with *H. pylori* infection history, 23 without *H. pylori* infection history, between stage II and stage IV). None of the patients had other malignant tumours or undergo chemotherapy or radiotherapy. All patients signed the informed consent. Sample collection and usage was performed in accordance with the relevant guidelines. The study was approved by ethics committee of Shuguang Hospital. All the specimens were immediately flash-frozen in liquid nitrogen.

2.2 Preparation of *H. pylori* filtrate

*H. pylori* strain NCTC11637 (containing cacA and cagA gene) was obtained from Renji Hospital (Shanghai, China). *H. pylori* was cultured under micro-aerophilic conditions (12% CO₂ and 5% O₂) at 37°C, with Columbia agar plates (Oxoid, Basingstoke Hampshire, UK) containing 5% sheep blood. For further experiments, *H. pylori* was used to prepare the bacterial lysate as previously described. *H. pylori* suspended in phosphate buffered saline (PBS), the suspension mixture kept in an ice bath, and then
pulse sonicated for 5 min at 50% capacity. Removing the bacterial debris by centrifugation at 10000 rpm for 15 min, and the collected supernatants was sterilized by passing through a 0.22 µm cellulose acetate filter. BCA protein quantitative method (Beyotime, Shanghai, China) determined the protein concentration. The lysate were kept at -80°C.

2.3 Cell culture

Human MKN45 and SGC-7901 gastric cancer cell lines were commercially obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in RPMI 1640 medium containing 10% fetal bovine serum, 1% streptomycin and penicillin, at 37°C, 5% of CO₂, and saturated humidity. MKN45 and SGC-7901 cell treated with *H. pylori* filtrate at a concentration of 500 µg/mL.

2.4 Cell transfection

Cell transfection was performed using the HilyMax kit (DOJINDO, Japan) according to the manufacturer’s instructions. The NEAT1 overexpression vector (pcDNA3.1-NEAT1, p-NEAT1), NEAT1-targeting siRNA (siNEAT1), miR-30a-3p mimics or inhibitor, miR-30a-5p mimics or inhibitor, were commercially provided by Genomeditech (Shanghai, China). Empty pcDNA3.1 vector, mimics control and non-targeting siRNAs were used as negative controls. The miRNA mimics or inhibitors, and the siNEAT1 were introduced into cells at a final concentration of 50 nM. The empty pcDNA3.1 vector and p-NEAT1 vector were introduced into cells at a final concentration of 1ug/mL.

2.5 Reverse transcription and quantitative real-time PCR

Total RNA was reverse transcribed using a PrimeScript RT Reagent Kit (Vazyme, Nanjing, China) and a miRNA qPCR Quantitation Kit (Tiangen, Beijing, China) to synthesize cDNA. Quantification of NEAT1 expression was normalized to GAPDH expression, and miR-30a-3p, miR-30a-5p expression was normalized to U6 expression using the 2^−ΔΔCt method. RT-qPCR was performed using the following primers: lncRNA NEAT1 sense, 5'- GAGTGGCTGGAGTGGATGGTATTG-3' and antisense, 5'- AACTTCCTCCTCTCTCTTGAAC-3'; miR-30a-3p sense, 5'-ACACTCCAGTGGGCTTTGAGTCGGATG-3' and antisense, 5'-CTCAACTGGCTGGAGTCGGATG-3'; miR-30a-5p sense, 5'-ACACTCCAGTGGGCTTTGAGTCGGATG-3' and antisense, 5'-CTCAACTGGCTGGAGTCGGATG-3'; U6 sense, 5'-CTCGCTTCGGAATTTGC-3' and antisense, 5'-AACGCTTCAGAATTTGCGGTTCCATC-3'; GAPDH sense, 5'-GGTGGTCTCCTCTGACTTCAGGA-3' and antisense, 5'-AACGCTTCAGAATTTGCGGTTCCATC-3'.

2.6 Cell viability, migration and invasion assays

Cell viability was tested by CCK-8 and colony formation. 5 × 10^3 cells were grown in 96-well plates, 10 µL CCK-8 reagent was added into every chamber according to the standard protocol (Beyotime, Beijing, China). Subsequently, the absorbance at 450 nm was measured by a platereader (Thermo Fisher Scientific, USA). For colony formation assay, 100 cells were grown in 12-well plates for 2 weeks. Then, the cells were fixed with ethanol and stained with crystal violet (Beyotime, Beijing, China). Wound healing test
and transwell assay evaluated the invasive and migratory abilities of MKN45 and SGC-7901 cells. Cells in the bottom chamber were stained using crystal violet and counted by optical microscope.

2.7 Dual-luciferase reporter assay

RNAhybird and Starbase v.2.0 predicted that NEAT1 targeted both miR-30a-3p and miR-30a-5p. Dual-luciferase assays verified the predicted relationships between NEAT1 and miR-30a-3p or miR-30a-5p. Luciferase reporters containing the wild-type 3’-UTRs of NEAT1 (which contain the predicted binding site of miR-30a-3p or miR-30a-5p), or mutant 3’-UTRs of NEAT1 (the predicted binding site of miR-30a-3p or miR-30a-5p were mutated), miR-30a-3p mimics or inhibitor, miR-30a-5p mimics or inhibitor, or mimics-control, were constructed by Genomeditech. The constructed reporters were co-transfected into MKN45 and SGC-7901 cells with Renilla reporters. For luciferase assay, cells in 24-well plates were co-transfected with miR-30a (miR-30a-3p or miR-30a-5p) mimics or inhibitor and 200 ng/well luciferase reporter constructs, 5ng/well SV-Renilla luciferase plasmid was applied as the internal control. After 24 hours transfection, the luciferase activity was detected using the Dual Luciferase Reporter Assay kit (Promega, USA). Firefly luciferase activities were normalized to Renilla luciferase activity.

2.8 Western blot

The protein expressions of COX-2 and BCL9 in MKN45 and SGC-7901 cells were evaluated by Western blot. In details, cells lysed in 1×RIPA buffer (Beyotime, Beijing, China), lysates were quantified, electrophoresed and transferred onto PVDF membranes, and blocked in 10% skimmed milk for 1.5h, then incubated with an antibody against COX-2 or BCL9 (1:1000 dilution, Abcam, UK) for 12 hours at 4°C. After incubation with secondary antibody (Beyotime, Beijing, China), protein bands were visualized by ECL detection kit (Millipore, USA).

2.9 Fluorescence in-situ hybridization

The fluorescence in-situ hybridization (FISH) assay analyzed the location and expression of NEAT1. Twenty five formalin-fixed paraffin-embedded (FFPE) gastric cancer tissues (13 with H. pylori infection history, 12 without H. pylori infection history) were detected.

Tissues were cut into 3µm thick paraffin section, deparaffinize slides in xylene and ethanol solutions. Apply Proteinase K reagent to fully cover the slides, then incubated at 37°C for 10 min. Apply 50µl hybridization mix (including RNA probes) to the slides, and then apply a sterile coverslip to each section. Start a hybridization program for 1h in a hybridizer. Wash the slides with 5× SSC buffer. Block the slides into a humidified chamber and incubate with blocking solution for 15 min. Remove the blocking solution, and incubate the anti-FAM/CY3 for 60 min. The freshly prepared AP substrate was applied to the sections, and then was incubated for 2h at 30°C. Incubate the slides in KTBT buffer to stop the reaction. Finally, 300µl DAPI (Beyotime, Beijing, China) was added to the sections, followed by fluorescence microscopy (Nikon, Eclipse Ci) evaluation. The RNA probes of NEAT1 (Genomeditech, China) was labeled with Spectrum-Red.

2.10 Immunohistochemistry
The Immunohistochemistry (IHC) was carried out to analyze the location and expression of COX-2 and BCL9. Twenty five formalin-fixed paraffin-embedded (FFPE) gastric cancer tissues (13 with \textit{H. pylori} infection history, 12 without \textit{H. pylori} infection history) were detected. Tissues were cut into 3µm thick paraffin section. After deparaffinization, sections were blocked of endogenous peroxidase and transferred in retrieval solution. Sections then incubated with an antibody against COX-2 or BCL9 (1:100 dilution, Abcam, UK) at 37°C for 30 min. After incubation with secondary antibody (Beyotime, Beijing, China), the DAB was used to illuminate the positive staining signals, then counterstained with hematoxylin. The positive staining signals were analyzed by Image J.

2.11 Statistical analysis

SPSS 24.0 software package (SPSS Inc) and Excel 2019 were used for statistical analyses. Student’s t test was evaluated to analyse any significant differences. $P<0.05$ indicates a statistically significant difference.

3. Results

3.1 Expression of NEAT1 and miR-30a in gastric cancer tissues

Our previous study has proved that miR-30a affected the \textit{H. pylori}-induced gastric cancer, and bioinformatics analysis by RNAhybird and Starbase v.2.0 has revealed that IncRNA NEAT1 could effectively bind to the two strand of miR-30a, including miR-30a-3p and miR-30a-5p. Hence, we detect the expressions of NEAT1, miR-30a-3p, and miR-30a-5p in gastric cancer tissues to determine if these genes are correlated with \textit{H. pylori} gastric cancer. FISH detected the expression of NEAT1 and demonstrated that NEAT1 was overexpressed in gastric cancer tissues, especially in \textit{H. pylori} infected gastric cancer tissues. Besides, compared to non-\textit{H. pylori} infected tumour adjacent tissues, NEAT1 was also overexpressed in \textit{H. pylori} infected tumour adjacent tissues (Fig. 1A). Real time-qPCR also demonstrated that NEAT1 was significantly overexpressed in \textit{H. pylori} infected gastric cancer tissues and slightly overexpressed in \textit{H. pylori} infected tumour adjacent tissues, which confirmed the results of FISH (Fig. 1B). On the contrary, miR-30a-3p or miR-30a-5p were decreased in \textit{H. pylori} infected gastric cancer tissues and \textit{H. pylori} infected tumour adjacent tissues (Fig. 1C, D). Besides, Real time-qPCR further demonstrated that the miR-30a-3p and miR-30a-5p were decreased in gastric cancer tissues and tumour adjacent tissues while NEAT1 was overexpressed (Fig. 1E, F).

3.2 NEAT1 promotes the proliferative, migratory and invasive abilities of gastric cancer cells

In our preliminary study, we found NEAT1 was expressed in the gastric cancer cell lines including MKN45 and SGC-7901. Herein we further found that, NEAT1 expression was greatly elevated in MKN45 and SGC-7901 cells upon \textit{H. pylori} filtrate treatment for 24h (Fig. 2A), demonstrating the promotion of \textit{H. pylori} on
NEAT1 expression. However, the detailed function of NEAT1 in gastric cancer is still unclear. Both CCK-8 and colony formation assay demonstrated that upregulation of NEAT1 facilitated the proliferation of MKN45 and SGC-7901 cells while downregulation of NEAT1 inhibited MKN45 and SGC-7901 cells proliferation (Fig. 2B, C). Additionally, wound healing test and transwell assay determined the aberrant expression of NEAT1 influenced the migratory and invasive abilities of MKN45 and SGC-7901 cells. We found both the migration and invasion of MKN45 and SGC-7901 cells were promoted by the upregulation of NEAT1 and suppressed by the downregulation of NEAT1 (Fig. 2D, E).

### 3.3 NEAT1 directly targets miR-30a

Bioinformatic analysis revealed that IncRNA NEAT1 could effectively bind to the two strand of miR-30a (miR-30a-3p or miR-30a-5p), and the predicted binding sites of NEAT1 to miR-30a-3p or miR-30a-5p were shown in Fig. 3A-B. In MKN45 and SGC-7901 cells, the dual-luciferase assay validated that miR-30a-3p mimics or miR-30a-5p mimics suppressed the expression of a reporter plasmid carrying the wild-type 3′-UTR sequence of NEAT1 in MKN45 and SGC-7901 cells, but not the mutant sequence in MKN45 cells and SGC-7901 cells. In addition, miR-30a-3p inhibitor or miR-30a-5p inhibitor could slightly increase the expression of a reporter plasmid carrying the wild-type 3′-UTR sequence of NEAT1, but the difference without statistical significance (Fig. 3C). Overexpression of NEAT1 obviously decreased the expression of miR-30a-3p or miR-30a-5p in MKN45 cells and SGC-7901 cells, but silencing NEAT1 significantly increased miR-30a-3p or miR-30a-5p expression in MKN45 and SGC-7901 cells (Fig. 3D, E).

### 3.4 Effect of NEAT1 on miR-30a and downstream COX-2/BCL9 pathway

In previous reported study, we have demonstrated that the downstream mRNA targets of miR-30a-3p or miR-30a-5p were the 3′-UTR of COX-2 or BCL9 respectively. We subsequently identified the protein expressions of COX-2 or BCL9 were positively correlated with NEAT1. In MKN45 and SGC-7901 cells, both the protein expressions of COX-2 and BCL9 were promoted by transfection with p-NEAT1, and decreased by transfection with si-NEAT1 (Fig. 4A). Compared with the NC group, co-transfection of miR-30a-3p inhibitor and si-NEAT1 or co-transfection of miR-30a-3p mimics and p-NEAT1 did not change the expression of COX-2 significantly (Fig. 4B, 4C). Meanwhile, co-transfection of miR-30a-5p inhibitor and si-NEAT1 or co-transfection of miR-30a-5p mimics and p-NEAT1 did not change the expression of BCL9 significantly (Fig. 4B, 4C).

### 3.5 miR-30a reverses the effect of NEAT1 on the proliferation, invasion, migration of H. pylorifiltrate infected gastric cancer cells
Since p-NEAT1 promoted the proliferation of *H. pylori* filtrate infected MKN45 and SGC-7901 cells, while si-NEAT1 inhibited it, herein we explored if miR-30a affect the regulatory effect of NEAT1 on gastric cancer cells proliferation, migration and invasion. The *H. pylori* filtrate infected MKN45 and SGC-7901 cells were divided into seven groups based on transfections with empty pcDNA3.1 plasmid, p-NEAT1, si-NEAT1, co-transfection of miR-30a (miR-30a-3p, miR-30a-5p) mimics and p-NEAT1, co-transfection of miR-30a (miR-30a-3p, miR-30a-5p) inhibitor and si-NEAT1. The CCK-8 assay showed that co-transfection of miR-30a (miR-30a-3p, miR-30a-5p) mimics and p-NEAT1 did not change the *H. pylori* filtrate infected MKN45 and SGC-7901 cells proliferation significantly. In addition, co-transfection of miR-30a (miR-30a-3p, miR-30a-5p) inhibitor and si-NEAT1 did not change the *H. pylori* filtrate infected MKN45 and SGC-7901 cells proliferation significantly neither (Fig. 5A). Similarly, transwell assay also showed that co-transfection of miR-30a (miR-30a-3p, miR-30a-5p) mimics and p-NEAT1 could slightly reduce the promoting effect of p-NEAT1 on *H. pylori* filtrate infected MKN45 and SGC-7901 cells invasion and migration, while co-transfection of miR-30a (miR-30a-3p, miR-30a-5p) inhibitor and si-NEAT1 slightly reduce the inhibiting effect of si-NEAT1 on *H. pylori* filtrate infected MKN45 and SGC-7901 cells invasion and migration(Fig. 5B-E).

### 3.6 Immunohistochemical detection of COX-2/BCL9 protein in gastric cancer tissues

Lastly, using above gastric cancer tissues with or without *H. pylori* infection, as well as the adjacent tumour tissues, we evaluated the expressions of COX-2 and BCL9 protein by IHC staining. Positive COX-2/BCL9 immunostaining was mainly localized in the cytoplasm of gastric cancer tissue cells. According to the positive expression area, compared with non-*H. pylori* infection gastric cancer tissue, both COX-2 and BCL9 protein expressions were increased in the *H. pylori* infected gastric cancer tissues(Fig. 6A-B). All above findings suggested that NEAT1 may accelerate tumorigenesis in *H. pylori* gastric cancer, by sponging miR-30a (miR-30a-3p, miR-30a-5p) to regulate COX-2/BCL9 pathway(Fig. 6C).

### Discussion

The precise pathological mechanisms that *H. pylori* promotes gastric cancer development are less well defined. Evidences have proved that *H. pylori* genotypes are related to gastric cancer risk\(^\text{17}\). The *H. pylori* strains that contain the cag pathogenicity island (PAI) are more likely to induce gastric cancer\(^\text{18}\), CagA thread through the host cell membrane into cytosol\(^\text{19}\), and exert multiple effects on promoting cell proliferation, migration and invasion through relative genes activation, such as COX-2\(^\text{20}\), and stimulating signaling pathways such as the β-catenin/WNT pathways\(^\text{21-23}\). Thus, we chosed *H. pylori* strain NCTC11637 (containing cacA and cagA gene) in this study.

IncRNAs participate in the transcriptional regulation processes, and miRNAs are an important link of IncRNAs and mRNA. IncRNAs compete with miRNAs, as called “miRNA sponging”\(^\text{24}\), miRNAs negatively
regulate mRNA expression by targeting mRNA for directing translational inhibition\textsuperscript{25}, and each miRNA is able to target multiple genes. The reciprocal regulatory mechanism is involved in many biological processes, including cancer development\textsuperscript{26-28}.

In our previous study has proved that miR-30a affected the \textit{H. pylori}-induced gastric cancer, the mechanism by which downregulates miR-30a is explored in this research. Researches found NEAT1 promote gastric cancer progression, and it was mainly mediated by targeting multiple miRNA related pathway, such as miR-365a-3p/ABCC4\textsuperscript{29},miR-17\textsuperscript{30}, miR-335-5p/ROCK1 \textsuperscript{31}, and miR-506/STAT3\textsuperscript{32}. Here, we observed the tumour-promoting effect of NEAT1 in \textit{H. pylori} gastric cancer by sponging miR-30a to regulate COX-2/BCL9 pathway.

We determined the expression of NEAT1 in the \textit{H. pylori} infected gastric cancer tumour tissues and adjacent tissues of patients by fish and RT-qPCR. The results showed that NEAT1 were up-regulated in gastric cancer tumour tissues as compared to tumour-adjacent tissues, which is consistent with previous studies\textsuperscript{33-35}, and the expression of NEAT1 significantly increased in \textit{H. pylori} infected tumour tissues. Unexpectedly, the expression level of NEAT1 in a small portion in \textit{H. pylori} infected gastric cancer tumour tissues was lower than the non-\textit{H. pylori} infected specimens, we hypothesized that it largely resulted from the individual differences. Besides, we also found the expressions of NEAT1 were inversely correlated with expression of miR-30a (miR-30a-3p and miR-30a-5p).

Subsequent studies showed that the expression of NEAT1 was significantly elevated after \textit{H. pylori} filtrate infected MKN45 and SGC-7901 cells. Overexpression of NEAT1 facilitated the proliferation, migration and invasion of \textit{H. pylori} filtrate infected MKN45 and SGC-7901 cells while downregulation of NEAT1 inhibited these abilities

Since downregulation of miR-30a in gastric cancer specimens corresponded to the upregulation of NEAT1 as bioinformatics analysis predicted, dual-luciferase reporter assay also verified it. Besides, transfection of si-NEAT1 increased the expression of miR-30a (miR-30a-3p and miR-30a-5p), while transfection of p-NEAT1 decreased the expression of miR-30a (miR-30a-3p and miR-30a-5p).

Previous study has revealed that miR-30a regulate the COX-2/BCL9 pathway, this study verified that NEAT1 could indirectly regulate the protein expression of COX-2 and BCL9 in a miR-30a-dependent manner. To further investigate if miR-30a influence the regulatory effect of NEAT1 in \textit{H. pylori} filtrate infected gastric cancer cell, we found co-transfection of p-NEAT1 and miR-30a(miR-30a-3p and miR-30a-5p) mimics or co-transfection of si-NEAT1 and miR-30a(miR-30a-3p and miR-30a-5p) inhibitor didn’t significantly change the cell proliferation and invasion, migration of \textit{H. pylori}-infected MKN45 and SGC-7901 cells, which indicated miR-30a(miR-30a-3p and miR-30a-5p) was negatively associated with the biological function of NEAT1 in \textit{H. pylori}-infected gastric cancer cell. Besides, the IHC staining positive expression area showed that the protein expressions of COX-2 and BCL9 in \textit{H. pylori} infected gastric cancer tissues were increased compared with non-\textit{H. pylori} infected gastric cancer tissues.
Conclusion

Our experiments results indicate that IncRNA NEAT1 is elevated in gastric cancer tissues, especially in *H. pylori* infected gastric cancer tissues. Aberrant expression of NEAT1 in *H. pylori* infected gastric cancer cells affects the proliferation, migration, invasion of gastric cancer cells. The cancer-promoting activity of NEAT1 mainly realized by sponging miR-30a and subsequently upregulating the expression of COX-2 and BCL9.

Abbreviations

RT-qPCR: Real-time quantitative polymerase chain reaction; siRNAs: Small interference RNAs; PBS: Phosphate-buffered saline; FBS: Foetal bovine serum; IF: Immunofluorescence; IHC: Immunohistochemistry; FISH: Fluorescence in situ hybridization; ncRNA: Non-coding protein-coding RNA; LncRNAs: Long noncoding RNAs; miRNAs: microRNAs; PAI: Pathogenicity island; *H. pylori*: Helicobacter pylori; RPMI1640: Roswell Park Memorial Institute 1640; SD: Standard deviation

Declarations

Ethics approval and consent to participate

The experiments using the clinical samples were approved by the ethics committee of Shuguang Hospital.

Consent for publication

Not applicable.

Availability of data and material

All datasets and materials supporting the conclusion for this study are included in the article.

Competing interests

None.

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Authors’ contributions
QL, QJ, XL, CH supervised the project. XWR and NNL performed the experiments. RJ, YYY, ZZZ analyzed the experiments. GH, NLS, RC and ZYW provided clinical samples. LHZ wrote the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

(A) FISH was performed to observe the expression of lncRNA NEAT1 in the tumour adjacent tissues. (B) Real time-qPCR results of lncRNA NEAT1 expression. (C) Real time-qPCR results of micro-RNA miR-30a-3p expression. (D) Real time-qPCR results of micro-RNA miR-30a-5p expression. (E) Pearson's correlation analysis showed that miR-30a-3p expression was negatively correlated with expression of NEAT1. (F) Pearson's correlation analysis showed that miR-30a-5p expression was negatively correlated with
expression of NEAT1. *P<0.05, **P<0.001 compared with the non-H. pylori infected tumour group, #P<0.05 compared with the non-H. pylori infected tumour adjacent group.

Figure 2

LncRNA NEAT1 enhances the proliferative, colony-forming, migratory and invasive abilities of gastric cancer cells. (A) NEAT1 expression was elevated upon H. pylori filtrate infected MKN45 and SGC-7901 cells for 24h. (B) CCK-8 assay showed that NEAT1 promoted the proliferation of H. pylori filtrate infected
MKN45 and SGC-7901 cells. (C) Colony formation assay showed that NEAT1 promoted the colony forming ability of MKN45 and SGC-7901. (D) Wound healing test showed that the migration of H. pylori filtrate infected MKN45 and SGC-7901 cells were promoted by the upregulation of NEAT1 and suppressed by the downregulation of NEAT1. (E) Transwell test showed that the invasion and migration of H. pylori filtrate infected MKN45 and SGC-7901 cells were promoted by the upregulation of NEAT1 and suppressed by the downregulation of NEAT1.*P<0.05, **P<0.001 compared with the NC group.

Figure 3

The miRNA target of NEAT1 is miR-30a. (A) Predicted binding sites and mutant sites of miR-30a-3p on the NEAT1 transcript. (B) Predicted binding sites and mutant sites of miR-30a-5p on the NEAT1 transcript. (C) Dual-luciferase reporter assay demonstrated that both miR-30a-3p and miR-30a-5p can target NEAT1 and significantly inhibit the expression of NEAT1. Here, miR-30a-3p and miR-30a-5p mimics or mimics control were separately transfected into MKN45 and SGC-7901 cells with pmirGLO-NEAT1 (wild-type) or
pmirGLO-NEAT1 (mutant). **P < 0.001 compared with cells co-transfected with pmirGLO-NEAT1 (wild-type) and mimics control. (D) Expression of miR-30a-3p in MKN45 and SGC-7901 cells was negatively regulated by NEAT1. (E) Expression of miR-30a-5p in MKN45 and SGC-7901 cells was negatively regulated by NEAT1. *P < 0.05 compared with MKN45 and SGC-7901 cells transfected with empty pcDNA3.1 plasmid (NC group).

Figure 4
NEAT1 regulate the downstream COX-2/BCL9 pathway by miR-30a. (A) Protein expressions of COX-2, BCL9 were increased by upregulation of NEAT1 and decreased by downregulation of NEAT1 in H. pylori filtrate infected MKN45 and SGC-7901 cells. (B) Protein expression of COX-2 were negatively regulated by miR-30a-3p, co-transfection of miR-30a-3p mimics and p-NEAT1 or co-transfection of miR-30a-3p inhibitor and si-NEAT1 didn’t change the protein expression of COX-2 significantly in H. pylori filtrate infected MKN45 and SGC-7901 cells. (C) Protein expression of BCL-9 were negatively regulated by miR-30a-5p, co-transfection of miR-30a-5p mimics and p-NEAT1 or co-transfection of miR-30a-5p inhibitor and si-NEAT1 didn’t change the protein expression of BCL-9 significantly in H. pylori filtrate infected MKN45 and SGC-7901 cells. *P<0.05, **P<0.001 compared with the NC group.
(A) CCK-8 assay showed that NEAT1 promoted the proliferation of H. pylori filtrate infected MKN45 and SGC-7901 cells, but miR-30a suppressed the effect of NEAT1 on the proliferation of H. pylori filtrate infected MKN45 and SGC-7901 cells. *P<0.05, **P<0.001 compared with the NC group. (B-E) Transwell assay showed that NEAT1 promoted the invasion and migration of H. pylori filtrate infected MKN45 and
SGC-7901 cells, but miR-30a suppressed the effect of NEAT1 on the invasion and migration of H. pylori filtrate infected MKN45 and SGC-7901 cells. *P<0.05, **P<0.001 compared with the NC group.

**Figure 6**

Protein expression of COX-2, BCL9 in gastric cancer tissues. (A) Expression of COX-2 in nearly 18% of the cells in tumour adjacent tissues and 19% in non-H. pylori infected gastric cancer tissues, 32% in H. pylori infected gastric cancer tissues. (B) Expression of BCL9 in nearly 18% of the cells in tumour adjacent
tissues and non-H. pylori infected gastric cancer tissues, 28% in H. pylori infected gastric cancer tissues. *P<0.05, compared with the adjacent group. (C) A schematic mechanism underlying interactions of NEAT1/miR-30a/ COX-2/BCL9 in promoting tumorigenesis in H. pylori gastric cancer.