LncNEN885 inhibits epithelial-mesenchymal transition by partially regulation of Wnt/β-catenin signalling in gastroenteropancreatic neuroendocrine neoplasms

Ya-Ling Wei1 | Jie Hua1 | Xiao-Yu Liu2 | Xiu-Mei Hua1 | Cheng Sun2 | Jian-An Bai3 | Qi-Yun Tang4

1Department of Gastroenterology, The First Affiliated Hospital with Nanjing Medical University, Nanjing, China
2Key Laboratory for Neuroregeneration of Jiangsu Province and Education Ministry, Nantong University, Nantong, China
3Department of Gastroenterology, The Affiliated Sir Run Run Hospital of Nanjing Medical University, Nanjing, China
4Department of General Practice, The First Affiliated Hospital with Nanjing Medical University, Nanjing, China

Correspondence: Jian-An Bai, Department of Gastroenterology, The Affiliated Sir Run Run Hospital of Nanjing Medical University, NO.109, Longmian Road, Nanjing, China. (baijianan031@163.com).
Qi-Yun Tang, Department of General Practice, The First Affiliated Hospital with Nanjing Medical University, NO.300, Guangzhou Road, Nanjing, China. (tqy831@163.com).

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It has been shown that long noncoding RNAs (lncRNAs) are involved in the carcinogenesis of multiple cancers. However, the roles of lncRNAs in gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) remain elusive. In the present study, we found that lncNEN885 was markedly decreased in human gastric NEN samples compared to adjacent normal tissues by transcriptome sequencing. Functionally, silencing or overexpression of lncNEN885 could not obviously affect cell proliferation or apoptosis in BON-1 or LCC-18 cells but could affect cell migration and invasion as well as wound-healing rates. Furthermore, dysregulation of lncNEN885 affected these biological functions by activating epithelial-mesenchymal transition through increased expression of Snail, vimentin, and N-cadherin as well as decreased E-cadherin levels in BON-1 and LCC-18 cells. Silencing of lncNEN885 could dramatically increase the phosphorylation of glycogen synthase kinase-3β and decrease the expression of adenomatous polyposis coli and Axin, with the subsequent accumulation of β-catenin. Taken together, dysregulation of lncNEN885 can regulate cell migration and invasion by activating epithelial-mesenchymal transition process partially through canonical Wnt/β-catenin signaling in GEP-NEN cells, which may be a novel biomarker for the metastasis of GEP-NENs.

KEYWORDS
BON-1 cell, epithelial-mesenchymal transition, lncNEN885, neuroendocrine neoplasm, Wnt/β-catenin signaling

1 INTRODUCTION

Neuroendocrine neoplasms (NENs) are a kind of heterogenous solid tumor distributed in the neuroendocrine system. Those common in the digestive tract, such as the stomach, small intestine, rectal, and pancreas, are named gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs). The incidence and prevalence of GEP-NENs are rising steeply. These neoplasms represent the second most common gastrointestinal malignancy in the world, with their incidence reportedly rising from 1.09/100 000 to 6.98/100 000 in the last 30 years.1,2 The survival time of GEP-NEN patients is relatively long, which indicates more metastasis and poor quality of life. Distant
metastasis, especially liver metastasis, is as common as other carcinomas, which results in more difficult treatment and worse prognosis.\(^3,4\) Thus, seeking sensitive and distinctive biomarkers for early diagnosis of metastasis is a key method toward the effective treatment of GEP-NEN patients.

Long noncoding RNAs (lncRNAs), known as RNAs longer than 200 nt without translated protein products, are vital regulators involved in tumor initiation, progression, and metastasis.\(^5,6\) Previous studies have reported that dysregulated expression of lncRNAs is frequently relevant to tumorigenesis and metastasis as well as novel effective biomarkers for prognosis in other carcinomas. For instance, lncTCF7 regulated transcription factor 7 expression and induced effective biomarkers for prognosis in other carcinomas.\(^7\) For instance, frequently relevant to tumorigenesis and metastasis as well as novel studies have reported that dysregulated expression of lncRNAs is cancer.\(^15,16\)

**2.1 | Patients and samples**

The GEP-NEN tissues and adjacent normal gastric tissues were obtained from three patients who underwent surgical resection at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). None of the patients received any local or systemic treatment before surgery. All experiments were approved by the Research Ethics Committee of Nanjing Medical University. Written informed consent was obtained from all participants.

**2.2 | Gene expression profiles**

Total RNA was extracted and purified according to the protocol described elsewhere. cDNA was prepared according to the standard Affymetrix protocol from 250 ng total RNA by using GeneChip (Genminix Informatics Ltd. Co., Shanghai, China) WT PLUS Reagent. The differentially expressed lncRNAs with statistical significance were identified with volcano plot filtering. The threshold we used to screen upregulated or downregulated lncRNAs is fold change >2 and P-value < .05.

**2.3 | Cell culture**

BON-1 and LCC-18, used as human pancreatic and colonic NEN cell lines, were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM/F12 containing 10% FBS and cultured at a constant temperature of 37°C in humidified air with 5% CO₂.

**2.4 | Overexpression of si-lncNEN885 in GEP-NEN cells**

A lentivirus overexpression RNA (Lv) and three pairs of siRNAs specifically targeting lncNEN885 were synthesized by Ribobio (Guangzhou, China). The sequences of siRNAs were as follows: si-lncNEN885-1, CCAAGGACACACTCTAAT; si-lncNEN885-2, CCC TTGACCATAACGAA; and si-lncNEN885-3, GCAGCAGAATTAATTAGCAGAC. For overexpression of lncNEN885, the cells were transfected with the lentivirus (Lv-lncNEN885) using Lipofectamine 2000 (Life Technologies). For silencing of lncNEN885 (si-lncNEN885), the cells were transfected with Lipofectamine RNAiMAX (Life Technologies, New York, NY, USA). Cells transfected with negative control RNAs (Lv-control or si-control) were used as control.

**2.5 | RNA extraction and quantitative real-time PCR**

Total RNA was extracted from cultured cells or tissues with TriPure Isolation Reagent (Roche, Penzberg, Germany) according to the manufacturer’s instructions. The first line of cDNA was synthesized using a Reverse Transcription Kit (Takara, Dalian, China). Real-time PCR analyses were undertaken with Essential DNA Green Master (Roche). The results were normalized to the expression of 18S. The primers were as follows: 18S, 5′-AGCTCCTATAGCTATAGAA-3′ (forward) and 5′-CGGTCCCTATCCATTACCTA-3′ (reverse); lncNEN885, 5′-TATGGTCTCCATCATGTTCACG-3′ (forward) and 5′-
GTGTTGTT
TCTTGTTGTTTGT-3′ (reverse); E-cadherin, 5′-GAACGCATTGCCA
CATACAC-3′ (forward) and 5′-CTCAATCTCTCCTGTTCCA-3′ (re-
verse); N-cadherin, 5′-AGGATCAACCCCATACACCA-3′ (forward) and
5′-GATGATGATGACAGGAGAAT-3′ (reverse); Snail, 5′-CACAAGCTT
AATGCCGCGCTC-3′ (forward) and 5′-CGGGATCTCAGCGGGGGA
CAT-3′ (reverse); vimentin, 5′-CTTGACCAGAAATGGGAAT-3′ (for-
ward) and 5′-TCTTGCGCTCCTGAAAAAT-3′ (reverse); and GAPDH,
5′-CGGAGTCAACGGATTGTCGTAT-3′ (forward) and 5′-AGCCT
CTCCCAT GGTGAGACG-3′ (reverse).

2.6 | Protein extraction and western blotting

The method for protein from cells and tissues was extracted
according to the manufacturer’s recommended protocol (Vazyme,
Nanjing, China). Samples from cell lysates were resolved by SDS-
PAGE and then transferred to PVDF membranes and incubated
with specific antibodies (Cell Signaling Technology, Boston, MA,
USA). The ECL chromogenic substrate was used to detect specific
bands. Actin and GAPDH were used as the inner control. Relative
protein levels were quantified by ImageJ (https://imagej.nih.gov/ij/).

2.7 | Flow cytometry

Fluorescein isothiocyanate-conjugated annexin V and propidium
iodide (AV-PI; Beyotime Institute of Biotechnology, Jiangsu, China)
analysis was carried out according to the manufacturer’s protocol. In
brief, 1 x 10^5 pretreated cells were transfected and stained with AV-
PI in the provided binding buffer. After incubation at room tem-
perature for 15 minutes in the dark, cells were analyzed immediately on a
FACS Sort Flow Cytometer (Becton Dickinson, San Jose, CA, USA).

2.8 | Apoptosis assays

Apoptotic assays were carried out with one-step TUNEL (Beyotime
Institute of Biotechnology) reactions. Briefly, the samples (tissues and
pretreated cells) were permeabilized with 0.1% Triton X-100 for 2 min-
utes at 4°C, and the TUNEL complex was added for 1 hour at 37°C.
Hoechst 33342 (Beyotime Institute of Biotechnology) was used to label
nuclei. The TUNEL-positive cells were imaged with a microscope. The
cells with deep green fluorescence were defined as apoptotic cells.

2.9 | Cell proliferation assay

Cell proliferation was assessed by CCK-8 assay (Beyotime
Institute of Biotechnology). Cells were trypsinized and plated at a
density of 2000 cells/100 μL medium per well in 96-well culture
plates. At different time points after transfection, 10 μL CCK-8 liq-
uid was added to wells and further incubated for 1 hour at 37°C.
The absorbance was measured at 450 nm.

2.10 | Cell migration, invasion, and wound-healing assays

At 24 hours after transfection, the cells in serum-free media were
seeded into the upper chamber for migration assays (8-μm pore size; 
Millipore, Darmstadt, Germany) and invasion assays with the Matri-
gel-coated (BD, Franklin Lakes, NJ, USA) filters in 24-well plates fol-
lowing the protocol described elsewhere.

For wound-healing assays, cells transfected with LncNEN885
or si-LncNEN885 were cultured in 12-well plates to approximately
90% confluence, and the monolayer was wounded using a vitreous
pin that delivered a precise scratch. Images of the extent of wound
healing were captured at 0, 24, 48, 72 hours.

2.11 | Statistical analysis

All the experiments were carried out at least three times indepen-
dently, each in triplicate. Bars represented mean ± SEM. Significance
tests were analyzed by Student’s t test using SPSS software (IBM
SPSS 20.0, Chicago, IL, USA), and a P-value < .05 was considered
significant.

3 | RESULTS

3.1 | LncNEN885 expression pattern in tissues and
dysregulation of LncNEN885

To examine whether IncRNA variation was involved in GEP-NE
progression and metastasis, we explored the expression profiles of
IncRNAs in GEP-NEs and normal neighboring noncancerous tissues
using gene chip analysis. The IncRNAs with a fold change >2 and
P-value < .05 in gene chip data were selected. We found 13 upreg-
ulated IncRNAs and 22 downregulated IncRNAs in tumor tissues
compared with the adjacent normal tissues (Figure 1A). Of them,
the expression of LncNEN885 (Enst00000414885) was remarkably
lower in tumor tissues and had not been previously reported, thus
it was chosen for further investigation. To further ascertain the
gene chip data, we evaluated the expression of LncNEN885 in
paired tumor tissues and nontumorous tissues from three patients
by quantitative real-time (qRT)-PCR and obtained similar results (Fig-
ure 1B).

Overexpression or silencing efficiency of LncNEN885 in
GEP-NEs cells was assessed by PCR assay. With Lv-LncNEN885
transfection, LncNEN885 expression was significantly upregulated
compared with the Lv-control group (Figure 1C). With si-LncNEN885
transfection, LncNEN885 expression was significantly downregulated
compared with the si-control group, especially si-LncNEN885-1 (Fig-
ure 1D). Thus, si-LncNEN885-1 was chosen for the following experi-
ments.
3.2 | Dysregulation of LncNEN885 did not influence cell proliferation

As is well known, cancer results from the imbalance between cell proliferation and apoptosis. To investigate whether dysregulation of LncNEN885 regulated cell proliferation, we undertook CCK-8 assays in BON-1 and LCC-18 cells. Unfortunately, no significant difference was found between Lv-LncNEN885 and Lv-control or si-LncNEN885 and si-control groups in either BON-1 cells (Figure 2A) or LCC-18 cells (Figure 2B).

3.3 | Dysregulation of LncNEN885 did not influence cell apoptosis

In view of the effect of LncNEN885 on the growth of cancers, we still considered that dysregulation of LncNEN885 could affect cell
apoptosis in GEP-NENs. Thus, we detected the influence of lncNEN885 dysregulation on annexin V-positive cells by observing apoptotic rates with flow cytometry (Figure 3A,B) and TUNEL assay (Figure 3C,D). The results showed that Lv-lncNEN885 or si-lncNEN885 treatment did not significantly affect cell apoptosis compared to the control group. Therefore, we concluded that dysregulation had no effect on cell proliferation or apoptosis in GEP-NENs.

3.4 Dysregulation of LncNEN885 mediated cell migration, invasion, and wound-healing rate

Migration and invasion are crucial for tumor progression and result in poor prognosis in GEP-NENs. We undertook a series of experiments to explore whether dysregulation of IncNEN885 could mediate GEP-NEN cell migration and invasion. To our surprise, overexpression of IncNEN885 dramatically attenuated the migration and invasion, and knockdown of IncNEN885 obviously enhanced the migration and invasion abilities both in BON-1 and LCC-18 cells (Figure 4A,B).

To further confirm this function of lncNEN885 in metastasis of GEP-NENs, we undertook wound-healing experiments using overexpression or silencing of IncNEN885 conditions in BON-1 cells and LCC-18 cells. The wound-healing rate in the Lv-IncNEN885 group was markedly decreased compared to the control group, from 24 to 72 hours, in BON-1 and LCC-18 cells. The wound-healing rate in the si-IncNEN885 group was markedly increased compared to the si-control group in BON-1 and LCC-18 cells.
cells, from 24 to 72 hours (Figure 4C, D). These results indicated that dysregulation of lncNEN885 might affect metastasis of GEP-NENs.

3.5 | Dysregulation of LncNEN885-mediated EMT

Epithelial-mesenchymal transition had been reported as a pivotal factor for invasion and metastasis in several cancers. Thus, we deduced that the influence of lncNEN885 dysregulation on metastasis of GEP-NENs might be relevant to EMT. Thus, we detected the expressions of EMT-related markers with PCR assays. The epithelial marker E-cadherin was increased, whereas the mesenchymal markers N-cadherin, vimentin, and Snail at protein and mRNA levels were decreased further by Lv-lncNEN885 than by Lv-control in BON-1 cells. When transfected with si-lncNEN885 or si-control, the effects were reversed (Figure 5A, C). The same phenomena were confirmed in LCC-18 cells (Figure 5B, D). These results indicated that dysregulation of lncNEN885 might affect metastasis of GEP-NENs by activating EMT.

3.6 | LncNEN885-mediated EMT partially relied on Wnt/β-catenin signaling

How did dysregulation of lncNEN885 activate EMT? The canonical Wnt/β-catenin pathway is a family of extracellular signaling involved in several cancers. The related GSK3β level could affect the phosphorylation of β-catenin, which initiated several cellular behaviors, such as EMT. Thus, we detected the levels of Wnt/β-catenin signaling with qRT-PCR and western blot assays. In the Lv-lncNEN885 group, phosphorylation of GSK3β was significantly decreased, whereas Axin and APC were significantly increased, which induced the degradation and lower level of β-catenin than in the Lv-control group. Conversely, β-catenin was accumulated following with high level of GSK3β phosphorylation and lower levels of Axin and APC expression in BON-1 cells (Figure 6A, C). The same phenomena were confirmed in LCC-18 cells (Figure 6B, C). Moreover, to further confirm that the role of lncNEN885 in EMT might be through the regulation of Wnt/β-catenin signaling, we transfected si-β-catenin in BON-1 and LCC-18 cells, and qRT-PCR
and western blot assays were undertaken to examine the levels of EMT. As shown in Figure 7, we found that after transfection with si-β-catenin, the expressions of N-cadherin and vimentin were observed to be significantly downregulated, whereas the expression of E-cadherin was obviously upregulated. Furthermore, cotransfection with si-β-catenin and Lv-lncNEN885 could enhance this phenomenon, and si-lncNEN885 could reverse the effect of β-catenin on the expression of EMT. These results showed that dysregulation of lncNEN885-related EMT partially relied on Wnt/β-catenin signaling.

4 | DISCUSSION

In recent years, IncRNAs have been a hot point in the research of developmental processes and disease states as a potentially novel and crucial layer of biological regulation. Several studies have reported that dysregulation of IncRNAs might induce multiple diseases, such as diabetes, kidney dysfunction, cardiovascular diseases, and malignant tumors. The role of IncRNAs in cancer has been the focus of this research. However, little is known about the role of IncRNAs in GEP-NENs.

In the present study, we aimed to search anomalous IncRNAs in GEP-NEN patients for early diagnosis and therapeutic targets. With transcriptome sequencing analysis, we found that the level of IncNEN885 was downregulated in human gastric neuroendocrine neoplasm tissues. LncNEN885 is a 567-bp IncRNA that is expressed at low levels in cancer tissues. However, there have been no published reports into lncNEN885 and human disease until now. Cancer is often considered a disease of deregulated cell proliferation and apoptosis. Thus, we explored the role of IncNEN885 in GEP-NENs. Regrettably, silencing or overexpression of IncNEN885 showed no significant effect on cellular proliferation or apoptosis in BON-1 or LCC-18 cells. We concluded that dysregulation of IncNEN885 might not affect cell death or growth in GEP-NENs.
In view of the metastatic features of GEP-NENs, especially the liver metastasis, we detected the influence of lncNEN885 on metastatic ability and found that overexpression of lncNEN885 could inhibit cellular migration and invasion in BON-1 and LCC-18 cells. Moreover, overexpression of lncNEN885 could inhibit the healing rate, but silencing of lncNEN885 could promote the healing rate. We concluded that dysregulation of lncNEN885 could affect the metastasis of GEP-NENs. We then wondered how lncNEN885 variation affected metastasis. Previous studies have suggested that EMT contributes to dissemination of cancer cells and is pivotal for invasion and metastasis. The assay on the level of EMT-related protein revealed that high levels of lncNEN885 could increase the expression of Snail, vimentin, and N-cadherin and decrease E-cadherin levels. Thus, we believed that dysregulation of lncNEN885 could mediate metastasis by activating EMT in GEP-NENs.

The canonical Wnt/β-catenin pathway is a family of extracellular signaling that can influence cellular differentiation, division, and survival in several cancers and other diseases. A named "destruction complex" composed of β-catenin, APC, Axin, and GSK3β often results in the phosphorylation, polyubiquitination, and proteasomal degradation of β-catenin in the absence of Wnt ligand. Phosphorylation of GSK3β can break this complex and inhibit its ability to phosphorylate β-catenin, which initiates several cellular behaviors such as EMT. In our study, we found that silencing of lncNEN885 could dramatically increase the

![Figure 6](image-url)
phosphorylation of GSK3β and decrease the expression of APC and Axin, with the subsequent accumulation of β-catenin. Furthermore, si-β-catenin could decrease the expressions of N-cadherin and vimentin and increase the expression of E-cadherin. Cotransfection with si-catenin and Lv-lncNEN885 could enhance this phenomenon, and si-lncNEN885 could reverse the effect of β-catenin on the expressions of EMT. That is, lncNEN885 induces EMT partially through canonical Wnt/β-catenin signaling.

Taken together, lncNEN885 regulates cellular migration and invasion by activating the EMT process partially through canonical Wnt/β-catenin signaling in GEP-NENs, but does not influence cellular apoptosis or proliferation. LncNEN885 could be a novel diagnostic and therapeutic biomarker for the metastasis of GEP-NENs.

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CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

ORCID

Jian-An Bai http://orcid.org/0000-0001-7822-7297

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