LNMICC Promotes Nodal Metastasis of Cervical Cancer by Reprogramming Fatty Acid Metabolism

Chunliang Shang, Wei Wang, Yuandong Liao, Yili Chen, Tianyu Liu, Qiqiao Du, Jiaming Huang, Yanchun Liang, Junxiu Liu, Yunhe Zhao, Luyan Guo, Zheng Hu, and Shuzhong Yao

Abstract

Cancer spread to lymph nodes predicts poor survival but underlying mechanisms remain little understood. In this study, we show that overexpression of the long noncoding RNA LNMICC associates with lymph node metastasis of primary cervical cancer, where it serves as an independent high-risk factor in patient survival. Functional investigations demonstrated that LNMICC promoted lymph node metastasis by reprogramming fatty acid metabolism, by recruiting the nuclear factor NPM1 to the promoter of the fatty acid binding protein FABP5. We also found that the prometastatic effects of LNMICC were directly targeted and suppressed by miR-190. Our results establish a new mechanism of lymph node metastasis and highlight LNMICC as a candidate prognostic biomarker and therapeutic target in cervical cancer.

Significance: These results establish the role of a novel long noncoding RNA in lymph node metastasis, with implications as a candidate prognostic biomarker and therapeutic target in cervical cancer.

Introduction

Cervical cancer is the second most common cancer among females worldwide, with approximately 527,600 new cases and 265,700 deaths in 2012 (1). Pelvic lymph node metastasis is a critical independent prognostic factor and one of the leading causes of cervical cancer death (2). However, there is still no effective and reliable means to prevent or control lymph node metastasis. It has been well recognized that the spread of cervical cancer cells to lymph nodes is not a simple physical or mechanical process but a multistep and multifactor complex process, and complex biological mechanisms participate in this event (3). Thus, this fact underscores the urgency and importance of understanding the molecular mechanisms of lymph node metastasis. In lymph node metastasis, lymphangiogenesis is an important initial step and essential event (4). In the meantime, cervical cancer cells acquire the driving force to erode the extracellular matrix of the newly developed lymphatic vessels through the activation of epithelial-to-mesenchymal transition (EMT), which is a biological process enabling polarized epithelial cell changes to a mesenchymal-like phenotype (5). The cross-talk between lymphatic endothelial cells (LEC) and cervical cancer cells is a key process in metastasis progression. However, it is currently unclear how to effectively prevent the formation of lymphangiogenesis and inhibit the occurrence of EMT in cervical cancer cells.

The reprogramming of cellular energy metabolism, which supports the unrestricted proliferation and metastatic progression of cancer cells, is widely accepted to be an emerging hallmark of cancer (6). The Warburg effect, which is an increased uptake of glucose and the switch to aerobic glycolysis, is the most commonly observed metabolic phenotype in cancer cells (7). However, it is evident that the molecular mechanisms involved in carcinogenesis are not the same as those promoting cancer cell metastasis. Growing evidence indicates that the Warburg effect may contribute to the early stages of cancer progression. Instead, the reprogramming of fatty acid (FA) metabolism in cancer cells may provide a selective advantage toward the metastatic process (8, 9). FAs are the major building blocks for energy metabolism and represent the essential components of the signal transduction network of biological membranes. De novo fatty-acid synthesis is one of the special metabolic phenotypes prevalent in cancer cells (10). Fatty acid-binding proteins (FABP), as indispensable carriers of FA uptake and transport, are proved to be critical central regulators of FA metabolism and inflammatory pathways (11). FABP5 is a small (15 kDa) member of the cytoplasmic FABP family and exhibits high affinity binding of long-chain FAs. There is now convincing evidence that FABP5 plays a crucial role in cancer progression, invasion, and metastasis through the induction of EMT and the regulation of angiogenic responses (12, 13). However, it remains largely unknown how cervical cancer cells cope with the reprogramming of FA metabolism, thus resulting in lymph node metastasis.

Long noncoding RNAs (lncRNA) are a novel class of transcripts longer than 200 nucleotides without protein-coding potential.
LncRNAs, which are emerging as pivotal regulation factors of cell biological behavior, can alter tumor growth kinetics, promote lymphangiogenesis and distant metastasis of cancer by interacting with RNA, DNA, or proteins (14, 15). In recent years, the expression of certain IncRNAs has been frequently reported to be functionally important for cervical cancer, including IncRNA-p21 (16), HOXA11-AS (17), HOTAIR (18), and ANRIL (19). In our previous study, we identified an IncRNA termed LNMICC (IncRNA associated with lymph node metastasis in cervical cancer; GenBank: HG501394.1, ENST00000518880.1) that is upregulated in cervical cancer tissues with lymph node metastasis compared to its expression in those without lymph node metastasis (20). However, to date, the exact mechanisms through which IncRNAs regulate lymph node metastasis in cervical cancer remain elusive. Current evidence demonstrates that IncRNAs play both pro- and antimetastatic roles via the regulation of EMT and angiogenesis as well as binding to certain metastatic factors (21). In addition, IncRNAs, as key transcriptional regulators of central metabolic pathways, can provide proliferating advantages through significantly enhancing the biosynthesis of FA (22). This strong evidence led us to speculate whether LNMICC promotes lymph node metastasis in cervical cancer through the FABP5-mediated reprogramming of FA metabolism. In this study, we first demonstrated that LNMICC was a valuable prognostic predictor of cervical cancer. More importantly, we found that LNMICC targeted the FABP5 promoter region through directly interaction with NPM1, thus mediating fatty acid metabolism reprogramming and finally promoting the process of lymphangiogenesis and EMT in cervical cancer. Meanwhile, our data also indicated that the function of LNMICC could be suppressed by miR-190 via directly targeting the miRNA-binding site in cervical cancer cells. Taken together, our findings provide novel insights into the mechanism of lymph node metastasis in cervical cancer.

Materials and Methods

Clinical specimens

For in situ hybridization (ISH), a total of 211 paraffin-embedded tissues of cervical cancer collected from January 2005 to December 2010 were obtained from the archives of the pathology department at the First Affiliated Hospital of Sun Yat-sen University. For total RNA isolation, another 40 patients from January 2014 to March 2016 were recruited. Based on their postoperative pathological examinations, 40 cases were respectively assigned into two groups: pelvic lymph node metastasis group and non-pelvic lymph node metastasis group. The specimens were immediately frozen in liquid nitrogen and stored at −80°C until later RNA extraction. All enrolled cervical cancer patients were matched from stage Ia2 to Ia2 with available follow-up data and underwent radical hysterectomy and lymphadenectomy. None of the patients were treated with radiotherapy or chemotherapy before surgery. Consent was informed and written consent forms were obtained from each patient. Twenty normal uterine cervical tissues as controls were collected from women who underwent hysterectomy for nonmalignant conditions. The study was approved by the Institutional Review Board of First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). Patient studies were conducted in accordance with ethical guideline: Declaration of Helsinki.

Cell culture

The human cervical cancer lines SiHa, CaSki, ME180, MS751, HeLa, and HeLa229 were purchased from ATCC and cultured according to their guidelines in a humid atmosphere with 5% CO₂ at 37°C. Human lymphatic endothelial cells (HLEC) were obtained from ScienCell Research Laboratories and maintained in the recommended endothelial cell medium (ECM; ScienCell). The cell lines were tested for authenticity in 2015 using short tandem repeat (STR) genotyping and screened for mycoplasma contamination (e-Myo Mycoplasma PCR Detection Kit; iNiRON). Primary normal cervical epithelial cells were isolated from 10 above-mentioned normal uterine cervical specimens. The detailed procedure is provided in the Supplementary Methods.

Assessment of protein-coding potential

A combination of an in vitro translation assay and a protein-coding potential assessment tool (http://lilab.research.bcm.edu/cpat/index.php) was used to evaluate the protein-coding potential of transcripts.

ISH, qRT-PCR, gel electrophoresis, Western blot analysis, and IHC

LNMICC expression was detected in paraffin-embedded tissues by ISH. The ISH probe used for detecting LNMICC-labeled digoxin was designed as 5’DigN-AATCCCTAGCTAACATGCAGT-3’DigN and synthesized by Takara Biotech Co. The ISH procedure used is described in the Supplementary Methods. RNA extraction, complementary DNA synthesis, and qRT-PCR reactions were performed as described in the Supplementary Methods. Gel electrophoresis, Western blot analysis and IHC staining were performed as described in the Supplementary Methods. The primer sequences used in this study are listed in Supplementary Table S1. Antibodies and working concentrations are presented in Supplementary Table S2.

Plasmid, lentivirus construction, and cell transfection

Detailed descriptions of plasmid, siRNA, and shRNA lentiviral vector construction and cell transfection can be found in the Supplementary Methods and in Supplementary Table S1.

Cell migration and invasion assays, endothelial cell tube formation assay, cell proliferation assay, and flow cytometry assays of cell apoptosis

Wound-healing and transwell invasion assays were performed to evaluate cell migration and invasion ability, respectively, as described in the Supplementary Methods. Lymphangiogenesis was assessed by an endothelial cell tube formation assay. Cell proliferation and cell apoptosis were analyzed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay and flow cytometry assay, respectively, according to the protocol described in the Supplementary Methods.

Quantification of neutral lipids, triglycerides, and phospholipids

The lipophilic fluorescence dye BODIPY 493/503 (Invitrogen) was used for monitoring the neutral lipid accumulation in cervical cancer cells as described in the Supplementary Methods. For quantitative estimation of triglycerides (TAG) or phospholipids (PL), the EnzyChromTM Triglyceride Assay Kits (BioAssay Systems) and EnzyChromTM Phospholipid Assay Kits (BioAssay Systems) were used according to the protocols.
Isolation of cytoplasmic and nuclear RNA
Cytoplasmic and nuclear RNA isolation was performed using the PARIS Kit (Invitrogen) according to the manufacturer’s instructions. RNA extracted from each of the fractions was analyzed by qRT-PCR to determine the levels of nuclear control transcript (HOTAIR, U6), cytoplasmic control transcript (GAPDH, β-actin), and LNMICC.

Truncating mutation and dual luciferase reporter assay
The FABP5 promoter was truncated by 100 bp and cloned into a pGL3-basic vector and sequenced. The luciferase activity was measured using the Dual-Luciferase assay system (Promega). The detailed procedure is described in the Supplementary Methods.

RNA pull-down assay, mass spectrometry, and RNA immunoprecipitation
Detailed descriptions of the RNA pull-down assay, mass spectrometry, and RNA immunoprecipitation (RIP) procedure can be found in the Supplementary Methods.

Chromatin immunoprecipitation assay
Chromatin fragments were immunoprecipitated with an anti-NPM1 antibody (103061-AP; Proteintech). The chromatin acquired prior to immunoprecipitation was amplified as the input. Normal human IgG was used as a negative control, and the Anti-Histone H3 antibody was used as a positive control. The qRT-PCR was performed using SYBRVR Premix Ex Taq (Takara). The primer sequences are listed in Supplementary Table S1.

In vivo tumor growth and metastasis assays and hematoxylin–eosin staining
Nude mice were maintained under SPF conditions in the Department of Sun Yat-sen University Animal Centered according to the institution’s guidelines. All animal studies were approved by the Animal Ethical and Welfare Committee of Sun Yat-sen University. The detailed procedures used to establish the subcutaneous xenograft tumor model and the xenograft mice metastatic model and those used for the hematoxylin and eosin staining are described in the Supplementary Methods.

Statistical analyses
All statistical analyses were performed using SPSS software package (version 13.0; SPSS, Inc.) and Prism5.0 software (GraphPad). The data are presented as the mean ± SD of at least three independent experiments. The independent sample t test was used for comparing groups for statistical differences. The Kaplan–Meier method was used for disease-free survival (DFS) and overall survival analysis, and significance was determined by log-rank test. A multivariate Cox proportional hazard model analysis was performed to identify independent prognostic factors of cervical cancer. Multivariate logistic regression was analyzed to identify the independent risks related to lymph node metastasis of cervical cancer. The relationships between LNMICC expression level and clinicopathological characteristics were tested by the χ² test or Fisher exact test. Correlations between LNMICC and FABP5 or miR-190 were analyzed by Spearman rank correlation analysis. Results were considered statistically significant at \( P < 0.05 \).

Results
LNMICC is upregulated in cervical cancer with lymph node metastasis and predicts poor prognosis
To explore whether LNMICC exerted an impact on lymph node metastasis, we first investigated the mRNA levels of LNMICC in cervical cancer tissues. The qRT-PCR results showed that LNMICC was more highly expressed at the primary site of cervical cancers with lymph node metastasis than in those without lymph node metastasis and in normal uterine cervical tissues (Fig. 1A). We then detected LNMICC expression in six cervical cancer cell lines and primary normal cervical epithelial cells using qRT-PCR and gel electrophoresis. Surprisingly, the expression level of LNMICC was significantly higher in cervical cancer cells derived from metastatic sites, including MS751, ME180, and CaSki, than cervical cancer cells derived from primary sites, including SiHa, HeLa, HeLa229, or primary normal cervical epithelial cells. (Fig. 1B; Supplementary Fig. S1A). These results suggest that LNMICC upregulation may promote lymph node metastasis in cervical cancers.

To further investigate the role of LNMICC in cervical cancer, 211 cases were examined with ISH. The patients were divided into two groups based on LNMICC expression in tumor tissues: the high-LNMICC group (ISH score >4) and the low-LNMICC group (ISH score ≤4). The results showed that 85.1% (40/47) of primary sites of cervical cancers with lymph node metastasis had high LNMICC expression (Fig. 1C; Supplementary Fig. S1B; Supplementary Table S3). Then, we analyzed the relationship between LNMICC expression and the clinicopathological characteristics of cervical cancer. A higher LNMICC expression level significantly correlated with tumor size (\( P = 0.0361 \)), stromal invasion (\( P = 0.001 \)), lymphovascular space invasion (\( P < 0.001 \)), lymph node metastasis (\( P = 0.0003 \)), recurrence (\( P = 0.0014 \)), and vital status at follow-up (\( P < 0.0001 \); Supplementary Table S3). Furthermore, Kaplan–Meier survival curves and log-rank test analyses showed that patients with high LNMICC expression had significantly decreased overall survival (OS) and DFS (Fig. 1D and E). In a xenograft mouse lymph node metastatic model, the group of mice harboring stable LNMICC-knockdown (LNMICC-KD) cells exhibited improved survival (Fig. 1F and G). In addition, univariate and multivariate analysis suggested that LNMICC expression [95% confidence interval (CI), 1.136–5.932; \( P = 0.024 \)], tumor size (95% CI, 1.132–3.170; \( P = 0.015 \)), lymphovascular space invasion (95% CI, 5.302–22.415; \( P < 0.0001 \)), and lymph node metastasis (95% CI, 1.795–6.949; \( P < 0.0001 \)) were independent prognostic factors for the OS of cervical cancer patients (Supplementary Table S4). To determine whether LNMICC is a high-risk factor for pelvic lymph node metastasis, a multivariate logistic regression analysis was performed and revealed that LNMICC, lymphovascular space invasion, and parametrical infiltration were independently associated with pelvic lymph node metastasis in patients with cervical cancer (Supplementary Table S5).

In addition, the coding potential of LNMICC was calculated using the protein-coding potential assessment tool (CPAT). The results showed that LNMICC does not have a typical protein-coding ORF that is longer than 300 nt (23), and the coding probability of LNMICC is very low (Supplementary Fig. S2A). Then, the full sequence of LNMICC was cloned into the eukaryotic expression vector pcDNA3.1 with C-terminal Flag tag. GAPDH with C-terminal Flag tag was used as positive control. Western blot analysis showed that the GAPDH with Flag-tag group could detect...
LNMICC is overexpressed in cervical cancer and associated with poor prognosis. **A**, The qRT-PCR results showed that LNMICC was more highly expressed at the primary site of cervical cancers with lymph node (LN) metastasis than in those without lymph node metastasis or normal uterine cervical tissues (n = 20/group). **B**, LNMICC expression in six cervical cancer cell lines with different metastatic potentials. NCC represents primary normal cervical epithelial cells. **C**, ISH analysis of LNMICC in specimens of normal uterine cervical tissues, cervical cancers with lymph node metastasis, and cervical cancers without lymph node metastasis. Kaplan–Meier survival curves showed poor overall survival (D) and disease-free survival (E) with high expression of LNMICC. **F** and **G**, Kaplan–Meier analysis of mice injected with MS751 or HeLa229 cells into the foot pad (n = 10/group; *, P < 0.05; **, P < 0.01; ***, P < 0.001).
the expression of Flag-tagged protein, whereas the LNMICC Flag-tagged group did not show expression of this protein (Supplementary Fig. S2B). Collectively, these observations confirmed that LNMICC had no coding capability.

LNMICC promotes cervical cancer metastasis and lymphangiogenesis in vitro

To demonstrate the potential effects of LNMICC for promoting metastasis in cervical cancer, we performed gain- and loss-of-function experiments in cervical cancer cells. According to LNMICC expression in a variety of cervical cancer cell lines (Fig. 1B), the SiHa and HeLa229 cell lines were selected for overexpression and knockdown of LNMICC. We constructed an M5751 cell line derived from a metastatic lymph node of a cervical cancer patient with LNMICC overexpression and downregulation. The qRT-PCR assay was used to confirm the efficiencies of overexpression and interference (Fig. 2A; Supplementary Fig. S3A). Transwell and wound-healing assays indicated that LNMICC overexpression increased the invasion and migration ability of SiHa and M5751 cells, whereas LNMICC knockdown significantly decreased the invasion and migration ability of HeLa229 and M5751 cells, respectively (Fig. 2B and C). Furthermore, we investigated the effect of LNMICC on the tube formation of HLECs, which is crucial for tumor lymph node metastasis. Compared with those of the corresponding control groups, the culture supernatants of LNMICC-overexpressing cells significantly promoted HLEC tube formation. In contrast, LNMICC knockdown abolished the effects of HeLa229 and M5751 cell culture supernatants in promoting tube formation (Fig. 2D). Because the lymph node metastasis burden positively correlates with primary tumor size (24), we further determined whether LNMICC promotes cervical cancer cell proliferation and tumor growth. MTT assays showed that LNMICC knockdown decreased the proliferative capacity of HeLa229 and M5751 cells (Supplementary Fig. S3B and S3C). Consistent with decreased cell proliferation, the percentage of apoptotic cells was markedly increased following siLNMICC treatment relative to that in control groups, as determined using flow cytometry (Supplementary Fig. S3D).

In addition, we analyzed the effects of LNMICC on the EMT process using Western blot analysis. The results showed a significant downregulation of E-cadherin and claudin-1 and upregulation of Snail, N-cadherin, and vimentin after LNMICC overexpression in SiHa and M5751 cells. LNMICC knockdown had the opposite effect in HeLa229 and M5751 cells, respectively (Fig. 2E and F). Vascular endothelial growth factor (VEGF)-C signaling, as the key lymphangiogenic pathway, is crucially involved in lymphangiogenesis (25). Western blot analysis revealed that the protein level of VEGF-C was markedly increased in response to LNMICC overexpression, whereas LNMICC knockdown inhibited VEGF-C expression in cancer cells (Fig. 2E and F). Taken together, these data indicated that LNMICC in cervical cancer cells contributed to lymphangiogenesis and lymph node metastasis.

LNMICC activates fatty acid metabolism in cervical cancer cells

Currently, the pivotal role of FA metabolism in cancer metastasis has been highlighted (26), and IncRNAs have been demonstrated to regulate FA metabolism via different mechanisms (27). Therefore, we further examined whether LNMICC was linked to altered FA metabolism in cervical cancer. According to the expression level of LNMICC from GSE26511, we estimate that LNMICC participates in the linolenic acid metabolism classified as omega-3 FA metabolism with NES values of 1.7127879 (P < 0.001; Fig. 3A). Interestingly, the multivariate regression analysis revealed that body mass index (BMI) ≥ 25 (kg/m²) was independently associated with pelvic lymph node metastasis in patients with cervical cancer (Supplementary Table S5), and higher LNMICC expression level significantly correlated with BMI (P = 0.0232; Supplementary Table S3). Furthermore, Kaplan–Meier survival analysis showed that BMI ≥ 25 (kg/m²) indicated a poorer OS and DFS (Fig. 3B and C). These findings led us to believe that LNMICC may be involved in reprogramming of FA metabolism in cervical cancer. To evaluate the effects of LNMICC on lipid content in cervical cancer cell lines, the levels of intracellular TAG and PLs were measured. Our data showed that LNMICC knockdown induced significantly decreased levels of intracellular TAG and PLs in HeLa229 and M5751 cells, whereas overexpression of LNMICC increased the levels of TAG and PL in SiHa and M5751 cells (Fig. 3D–G). These results were further supported by cellular staining with the lipophilic fluorescence dye RODIPY 493/503, which indicated that LNMICC knockdown decreased the intracellular contents of neutral lipids in HeLa229 and M5751 cells, whereas LNMICC overexpression had the opposite effect in SiHa and M5751 cells (Fig. 3H and I).

To demonstrate the role of LNMICC in the deregulation of FA metabolism in cervical cancer, we examined the effects of LNMICC on several key FA metabolic enzymes, including FASN, ACC1, ACOX1, CPT1A, and FABP5, in cervical cancer cells. Consequently, significant downregulation of FASN, ACC1, and FABP5 and upregulation of ACOX1 and CPT1A protein and mRNA levels were observed compared with the levels in controls in HeLa229 and M5751 cells after LNMICC knockdown (Fig. 3I and Supplementary Fig. S4A–S4D). LNMICC overexpression reverted the expression of key FA metabolic enzymes in SiHa and M5751 cells (Fig. 3K; Supplementary Fig. S4E–S4H). To provide further evidence, we measured the expression of these FA metabolic enzymes in cervical cancer tissues with different LNMICC levels using IHC staining, and obtained consistent results (Fig. 3L). Thus, we conclude that LNMICC promotes the reprogramming of FA metabolism in cervical cancer.

LNMICC promotes lymph node metastasis through FABP5-mediated FA metabolism in vitro and in vivo

Although the mode of action for IncRNAs remains to be fully understood, some IncRNAs exert their functions through interacting with proteins in the nucleus, whereas others are reported to modulate mRNA stability and translation in the cytoplasm (28, 29). According to ISH, LNMICC was located primarily in the nucleus, which was confirmed by nuclear/cytoplasm fractionation (Fig. 4A), and this result suggests that LNMICC may exert its biological function in the nucleus. As IncRNAs have been shown to exert cis-regulatory effects on nearby genes (30), we examined whether LNMICC would affect the expression of its in cis genes. Bioinformatics analysis revealed that LNMICC is located upstream of the FABP5 gene with high degree of sequence conversation, and no overlapping region was found at the RNA level in UCSC genome database (Supplementary Fig. S5A). Therefore, we first measured the expression of LNMICC and FABP5 in 40 fresh cervical cancer tissues. Scatter plot analysis showed a positive correlation between the mRNA levels of LNMICC and FABP5 (r = 0.902; P < 0.001; Fig. 4B), which was confirmed by TCGA datasets (r = 0.902; P < 0.001; Supplementary Fig. S5B).
These data and those in Supplementary Fig. S5C to SSD indicate that FABP5 was regulated by LNMICC. Giving the positional relationship of LNMICC and FABP5 on the genome, we then detected whether LNMICC bound to the promoter of the FABP5 gene. We analyzed a 1-kb locus upstream from the transcription start sites (TSS) of the FABP5 gene. Through truncating mutation and a dual luciferase reporter assay, we identified (approximately) a −400 to −200-bp
Figure 3.
Effects of LNMICC expression on the intracellular contents of lipids and key lipid metabolic enzymes in cervical cancer cells. 

A, Identification of gene sets enriched in phenotypes correlated with LNMICC (236271_at) by GSEA using GSE26511 data. Kaplan–Meier survival curves showed poor DFS (B) and OS (C) with BMI ≥25 (kg/m²). Cellular content of phospholipids (D and E) and triglycerides (F and G) was detected in the indicated cells. 

H and I, The neutral lipids content was detected by double staining with BODIPY 493/503 dye and Hoechst in the indicated cells. 

J–K, Western blot analysis for protein levels of the key lipid metabolic enzymes FASN, ACC1, ACOX1, CPT1A, and FABP5 in the indicated cells. 

L, Representative IHC images of the lipid metabolic enzymes FASN, ACC1, ACOX1, CPT1A, and FABP5 in human cervical cancer tissues. Original magnification, ×200. *, P < 0.05; **, P < 0.01.
Figure 4.

LNMCC activates FA metabolism by recruiting NPM1 to enhance FABP5 transcription. **A,** The assay of nuclear/cytoplasm fractionation showing nuclear localization of LNMCC in MS751 and HeLa229 cells. **B,** Scatter plot analysis of the correlation between mRNA expression levels of LNMCC and FABP5 in 40 fresh cervical cancer tissues. **C,** Different loci of the FABP5 promoter were constructed into pGL3 vectors and subjected to luciferase reporter assays in MS751 and HeLa229 cells. **D,** SDS-PAGE analysis with silver staining showed the proteins were pulled down by LNMCC or its antisense RNA from indicated cells. The peptides were detected by subsequent mass spectrometry. **E,** Another independent RNA pull-down experiment with cell extract. Specific bands were identified by Western blot assays using a corresponding antibody. **F,** The RIP assays showed the LNMCC retrieved by the NPM1 antibody in MS751 and HeLa229 cells. The qRT-PCR products were analyzed with gel electrophoresis (left). The mRNA levels were graphed (right). **G,** Localization of the NPM1 to FABP5 promoter in MS751 and HeLa229 cells, which were analyzed by ChIP. H3 served as a positive control. **H,** The neutral lipid content was detected by double staining with BODIPY 493/503 dye and Hoechst in the indicated cells. Scale bars, 16 μm. The levels of phospholipids (I) and triglycerides (J) were measured in the indicated cells. *, *P < 0.05; **, *P < 0.01.
segment of FABP5 promoter as a sufficient binding site for LNMICC (Fig. 4C).

To further investigate the mechanism through which LNMICC enhanced the transcription of FABP5, we performed RNA pull-down assays to identify potential proteins binding to LNMICC. The LNMICC-bound complex was separated by SDS-PAGE and then subjected to mass spectrometry (Fig. 4D). NPM1 was the only transcription factor in the binding targets of LNMICC that was identified (Supplementary Fig. S5E), and the results were further confirmed by Western blotting (Fig. 4E). RIP assays were also performed using antibodies against NPM1 in cell extracts from HeLa229 and MS751 cells, and the results showed that LNMICC was detected by qRT-PCR in NPM1-immunoprecipitated RNAs in both HeLa229 and MS751 cells (Fig. 4F). As verified in Figure 4C, we found the putative NPM1-binding sites within 400 bp upstream of the transcriptional start of FABP5. Chromatin immunoprecipitation (ChIP)-qPCR assays revealed that NPM1 directly bound to the predicted binding sites of the FABP5 promoter (Fig. 4G). These data indicated that LNMICC recruits NPM1 to activate FABP5 transcription.

To assess the cross-talk between lymph node metastasis and FA metabolism, we sought to determine whether FABP5 had a key role in LNMICC-induced lymph node metastasis in vitro and in vivo. First, we investigated the effects of FABP5 on lipogenesis in SiHa and MS751 cells. As expected, silencing of FABP5 by siRNA dramatically reversed the lipogenesis-promoting effects of LNMICC overexpression on the content of intracellular TAG, PLs, and the intensity of BODIPY staining in this system (Fig. 4H–J and Supplementary Fig. S5F). Furthermore, the invasion and migration assay data showed that FABP5 downregulation remarkably reversed the LNMICC-induced increase in cell invasion and migration in SiHa and MS751 cells (Supplementary Fig. S6A–S6C). In addition, the results from the endothelial cell tube-formation assay were consistent with those of the invasion assay. Knockdown of FABP5 expression in LNMICC-overexpressed cells inhibited the tube formation of HLECs (Supplementary Fig. S6D–S6F). Then, we explored whether LNMICC promotes EMT and lymphangiogenesis though modulating FABP5. As shown in Supplementary Fig. S6G, the expression levels of Snail, N-cadherin, and vimentin were significantly decreased after FABP5 downregulation in LNMICC-overexpressed cells. However, the expression levels of E-cadherin and claudin-1 significantly increased using the same processing conditions. Meanwhile, FABP5 inhibition after LNMICC overexpression in SiHa and MS751 cells led to reduced VEGF-C expression (Supplementary Fig. S6G).

Furthermore, we selected the high-LNMICC-expressing cell lines MS751 and HeLa229 as representatives for establishing stable LNMICC-KD cells to develop xenograft mouse metastatic models. Notably, the specimens from the foot paws of mice in the control group contained more fat vacuoles than those in the corresponding LNMICC-KD group (Fig. 5A), which suggests that lipids metabolism may facilitate the metastasis of cervical cancer cells. The subcutaneous xenograft tumor model showed that FABP5 overexpression could partially accelerate tumor growth and the increase in tumor size/weight caused by LNMICC knockdown (Fig. 5B–D). Importantly, the incidence of lymph node metastasis dramatically declined in the LNMICC-KD groups, and FABP5 overexpression could reverse this trend in a xenograft mouse lymph node metastatic model (Fig. 5E). Moreover, the results from the xenograft mouse lung metastatic model were consistent with those of the lymph node metastasis model (Fig. 5F). These results were pathologically confirmed with hematoxylin and eosin staining. Taken together, these data suggest that LNMICC may facilitate lymph node metastasis by directly modulating FABP5-mediated reprogramming of FA metabolism.

LNMICC is a direct target of miR-190

Emerging evidence suggests that miRNAs have the potential to promote the degradation of IncRNA (31). To further explore the regulatory mechanism of the lncRNA expression pattern, miRcode (32) software was used to predict the potential complementary base pairing between LNMICC and miRNAs. We transfected MS751 cells with mimics of all predicted miRNAs, and miR-190 had the most significant effect on LNMICC expression (Fig. 6A). Therefore, we selected miR-190 for further study. qRT-PCR showed that, whereas miR-190 was inhibited in SiHa and MS751 cells, LNMICC expression showed the opposite trend in these cell lines (Supplementary Fig. S7A). However, there was no obvious difference in miR-190 levels after LNMICC overexpression or knockdown (Supplementary Fig. S7B and S7C). In addition, compared with the high-metastatic MS751 cells, the low-metastatic SiHa cells displayed higher expression levels of miR-190 (Fig. 6B). A significant inverse correlation between LNMICC and miR-190 was also detected in 40 fresh cervical cancer tissues (Fig. 6C). These results suggest that LNMICC might be the downstream effector of miR-190, which might be involved in lymph node metastasis in cervical cancer.

To confirm whether miR-190–mediated LNMICC regulation occurs through direct targeting of the predicted miRNA binding site in the LNMICC sequence (Fig. 6D), a dual luciferase reporter assay was performed. As shown in Fig. 6E, cotransfection of SiHa and MS751 cells with psicheck2- LNMICC-WT vector and miR-190 mimic significantly reduced luciferase reporter activity. Conversely, these repressive effects were abrogated by site-directed mutagenesis in the LNMICC sequence. It is well known that miRNAs bind their targets and mediate translational repression and/or RNA degradation functions in an Ago2-dependent manner (33). RIP experiments showed that the LNMICC and miR-190 were detected in Ago2 immunoprecipitates from SiHa and MS751 cells, which indicates that miR-190 interacts with LNMICC in a sequence-specific manner (Fig. 6F). Collectively, these results revealed that miR-190 exerts inhibitory effects on LNMICC expression by directly binding to LNMICC.

Next, we transfected SiHa and MS751 cells with miR-190 mimic and LNMICC plasmid vector to investigate the effects of miR-190 on FA metabolism and metastasis mediated by LNMICC. Then, decreased FASN, ACC1, and FABP5 expression by miR-190 mimics was restored to basal levels by the LNMICC plasmid vector treatment, and the high expression of CPT1A and ACOX1 was also inhibited (Fig. 6F). In addition, we found that miR-190 mimics could efficiently offset the effects of accelerating lipogenesis of LNMICC overexpression on SiHa and MS751 cells, as revealed by both quantification of PLs (Fig. 6G), TAG (Fig. 6H), and neutral lipids (Fig. 6I). In addition, the restoration of miR-190 by miR-190 mimics transfection in SiHa and MS751 cells increased E-cadherin and claudin-1 and decreased Snail, N-cadherin, and vimentin, which reversed the LNMICC-stimulated mesenchymal cell phenotype (Fig. 6J). Meanwhile, restoration of miR-190 after LNMICC stimulation in SiHa and MS751 cells resulted in decreased VEGF-C expression (Fig. 6J). Moreover, we validated that FABP5 siRNA could abrogate the effects of
LNMICC in inducing cell invasion and migration and abolished LNMICC-mediated HLEC tube formation (Supplementary Fig. S7D–S7F). Those observations suggest that the effects of LNMICC overexpression on the acceleration of lipogenesis and lymph node metastasis could be diminished by miR-190, in accordance with the suppression of LNMICC expression by miR-190.

Discussion

The lymphatic system is the most common route of distant metastasis for cervical cancer. A better understanding of the molecular mechanisms underlying lymph node metastasis may assist in identifying patients at high risk for mortality and may provide an effective clinical treatment of cervical cancer. However,
LNMICC is a direct target of miR-190. A, The qRT-PCR analysis of LNMICC expression in MS751 cells transfected with miRNAs mimics, which were predicted by miRcode. B, The expression levels of miR-190 in SiHa and MS751 cell lines with different metastatic potentials. C, Scatter plot analysis of the correlation between mRNA expression levels of LNMICC and miR-190 in 40 fresh cervical cancer tissues. D, Schematic of wild-type and mutant psicheck2-LNMICC reporter constructs (left). Dual luciferase reporter assay of SiHa and MS751 cells cotransfected with the psicheck2-LNMICC-WT or -MUT reporter plasmids and miR-190 mimic (right). E, The RIP assays showed that LNMICC and miR-190 were immunoprecipitated by an Ago2 antibody in SiHa and MS751 cells. The qRT-qPCR products were analyzed with gel electrophoresis (left). The mRNA levels were graphed (right). F, Western blot analysis for protein levels of the key lipid metabolic enzymes FASN, ACC1, ACOX1, CPT1A, and FABP5 in the indicated cells. Cellular content of phospholipids (G) and triglycerides (H) was detected in the indicated cells. I, The contents of neutral lipids were detected by double staining with BODIPY 493/503 dye and Hoechst in the indicated cells. Scale bars, 16 μm. J, Western blot analysis for protein levels of EMT markers (Snail, E-cadherin, N-cadherin, claudin-1, and vimentin) and VEGF-C in the indicated cells. *P < 0.05; **P < 0.01.
Cancer Res; 78(4) February 15, 2018

Lymph node metastasis

EMT

VEGF-C

Upregulate

LNMICC

miR190

miR-190 was found to be the only miRNA identified by a genomewide association study in the loci associated with lymph node metastasis of breast cancer. Our findings support the notion that miR-190 functions as a metastasis suppressor, significantly reducing invasiveness, migration and lymphangiogenesis by regulating LNMICC expression. It has been well-documented that metastatic cancer cells require FA for providing energy, macromolecules for membrane synthesis, and lipid signals, which are critical for metastatic colonization in distant organs. As reported in previously studies, the FA receptor CD36 is a marker of metastasis-initiating cells and leads cancer cells to have a higher propensity to metastasize lymph node in a lipid metabolism-dependent manner (8, 38). More interestingly, lncRNAs have been shown to be involved in lipid metabolism in cancer. For example, the lncRNA HULC could facilitate lipid metabolism through a miR-9–mediated RXRA signaling pathway in hepatoma cells (27). In this study, we wondered whether LNMICC could also modulate FA metabolism to promote lymph node metastasis in cervical cancer. Through RNA pull-down and ChIP assays, we identified FABP5 as a central regulator of lipid metabolism that directly interacted with LNMICC by recruiting NPM1. In addition, FABP5-induced reprogramming of FA metabolism of cervical cancer cells was critical for promoting lymph node metastasis, indicating effective cross-talk between HLECs and cervical cancer cells. Surprisingly, we found that BMI ≥25 (kg/m²) was independently associated with pelvic lymph node metastasis and was associated with poor OS in our clinical data, which further supported these conclusions.

Cancer cells are prone to disseminate through the lymphatic system during the metastatic spread of cervical cancer. However, it is not clear what drives cancer cells to choose the lymphatic system for migration. Karlsson and colleagues demonstrated that EMT promotes the migration of breast cancer through the lymphatic system to lymph nodes and that HLECs secrete chemokines to attract EMT cells (39, 40). Similar to those results, we found that LNMICC overexpression could induce EMT in cervical cancer cells and activate the expression of VEGF-C to promote lymphangiogenesis via FABP5-mediated reprogramming of FA metabolism. Consistent with our study, Ohata and colleagues indicated that FABP5 facilitated the invasiveness and metastasis of hepatocellular carcinoma through the induction of EMT (13). Interestingly, these findings reveal that the cross-talk between EMT and FA metabolism will be a novel breakthrough in exploring the mechanism of lymph node metastasis.

In conclusion, LNMICC as a promising prognostic biomarker for cervical cancer, promotes lymph node metastasis through the reprogramming of FA metabolism by recruiting the NPM1 to the FABP5 promoter, and its biological function can be suppressed by miR-190. Thus, our findings provide new insights into the
mechanism of FA metabolism in the lymph node metastasis of cervical cancer and add a promising new target for the development of novel antimetastasis therapeutics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C. Shang, S. Yao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Liang, J. Liu, Y. Zhao,
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): T. Liu, Q. Du, J. Huang
Writing, review, and/or revision of the manuscript: C. Shang, W. Wang, Z. Hu, S. Yao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Liao, Y. Chen, L. Gao

References


LNMICC Promotes Nodal Metastasis of Cervical Cancer by Reprogramming Fatty Acid Metabolism

Chunliang Shang, Wei Wang, Yuandong Liao, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-17-2356

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2017/12/09/0008-5472.CAN-17-2356.DC1

Cited articles  This article cites 38 articles, 5 of which you can access for free at: http://cancerres.aacrjournals.org/content/78/4/877.full#ref-list-1

Citing articles  This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/78/4/877.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link: http://cancerres.aacrjournals.org/content/78/4/877.

Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.