

LINC00659 Regulates Colorectal Cancer Migration and Invasion by Targeting miR-485-5p/HOXC13 Axis

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Abstract

Background

Long noncoding RNA LINC00659 has been reported to be involved in the carcinogenesis and progression of colorectal cancer. However, the molecular mechanism remains ambiguous.

Methods

In this study, we found that HOXC13 expression was closely related with colorectal cancer and positively correlated with LINC00659 via bioinformatics analysis and clinical validation experiment. Meanwhile, miR-485-5p was identified as an overlapped target miRNA. To further dissect whether miR-485-5p and HOXC13 were involved in LINC00659 mediated colorectal cancer progression, we first established human in vitro models and demonstrated that LINC00659 could directly bind with miR-485-5p and knockdown of LINC00659 upregulated the expression of miR-485-5p. In addition, knockdown of LINC00659 inhibited the expression of HOXC13 by targeting miR-485-5p. Finally, we analyzed the effect of LINC00659/miR-485-5p/HOXC13 axis on tumor growth. Both animal model and in vitro model confirmed the anti-tumor effect of knockdown of LINC00659, which could suppress the colorectal cancer cell viability, migration and invasion by targeting miR-485-5p/HOXC13 axis.

Results

1. LINC00659 and HOXC13 are highly expressed in colorectal cancer cells.
2. miRNA-485-5p is lowly expressed in colorectal cancer cells.
3. LINC00659/miR-485-5p/HOXC13 axis is important for colorectal cancer cells.
4. LINC00659 promotes tumor growth by sponging miR-485-5p.

Conclusions

Our study uncovered a novel mechanism of LINC00659 in the progression of colorectal cancer and provided a potential strategy for the treatment and diagnose of colorectal cancer.

Background

Colorectal cancer, a common malignant tumor in the digestive tract, often resides at the junction of sigmoid colon and rectum. The incidence rate for population between 40-50 age group is the highest [1]. Despite the therapy and diagnosis methods of colorectal cancer, including radiotherapy, chemotherapy, and endoscopic techniques have made a major breakthrough in recent years, the five-year survival rate for colorectal cancer is still at low level [2, 3]. The major reasons of its poor survival rate are drug resistance and metastasis [4]. Studies have shown that more than half of colorectal cancer patients have different metastasis at the time of diagnosis, resulting in difficulty in obtaining radical cure and a

high recurrence rate [5-7]. Therefore, it is of great significance to study the regulatory mechanism of colorectal cancer progression in order to develop new therapeutic strategies and new drug targets.

Long non-coding RNA (lncRNA) is a type of non-protein-coding RNA with more than 200 nucleotides in length [8]. lncRNAs are well known to engage in epigenetic and transcriptional regulation in the nucleus by interacting with chromatin regulators [9]. For example, *yy1ncT* binds with DNA methyltransferase DNMT3B, which is required for T-mediated mesodermal commitment of human pluripotent stem cells [10]. Recently, more and more studies demonstrate that lncRNAs can modulate gene expression post-transcriptionally in the cytoplasm, and therefore, they can directly or indirectly regulate protein expression [11-13]. A number of lncRNAs have been identified to affect cancer cell proliferation, apoptosis and metastasis by sponging microRNAs (miRNAs) and indirectly regulate expression of protein-coding mRNAs [14-17]. This kind of lncRNAs was defined as competing endogenous RNAs (ceRNAs) [18]. Several lncRNAs with ceRNA activity have been reported currently. lncRNA MALAT1 regulates HMGB1 expression by sponging miR-129-5p in colorectal cancer development [19]. lncRNA SNHG7 played an oncogenic role in the progression of colorectal cancer through sponging miR-216b to upregulate GALNT1 [20]. However, the function of most lncRNAs remains largely uncovered in colorectal cancer.

In previous reports, high expression of LINC00659 in colorectal cancer has been addressed, and it was suggested to enhance colorectal cancer cell proliferation through promoting cell cycle progression [21, 22]. However, molecular mechanism is still largely unknown. In this study, we downloaded the transcriptome sequencing data of colorectal cancer from TCGA database. Then, we utilized bioinformatics to analyze differentially expressed miRNAs and mRNAs in colorectal cancer tissue compared with normal control. A LINC00659-miR-485-5p-homeobox C13 (HOXC13) axis was identified by bioinformatics and their differential expression was confirmed in clinical samples. Further, *in vitro* and *in vivo* experiments were performed to further characterize their interplay and effects in the progression of colorectal cancer. Overall, we uncovered a novel mechanism that LINC00659 regulated viability, migration and invasion of colorectal cancer cells via miR-485-5p/HOXC13 axis. It might provide a new strategy to treat and diagnose colorectal cancer patients.

Results

HOXC13 expression is associated with colorectal cancer and positively correlated with LINC00659

To investigate the differentially expressed miRNAs and mRNAs in colorectal cancer tissues, we downloaded the RNA sequencing data of 471 colorectal cancer patients and 41 normal controls from the TCGA database. Based on the criteria of $|\log FC| > 2$ and $P < 0.01$, a total of 5540 differentially expressed genes were identified, including 4101 upregulated genes and 1439 downregulated genes (**Figure 1A**). Among them, HOXC13 was significantly up-regulated in colorectal cancer compared with normal controls (**Figure 1B**). Meanwhile, the 5-year survival rate of patients with high HOXC13 expression was dramatically lower compared with those with low expression of HOXC13 (**Figure 1C**). Co-expression

analysis showed that the expression of HOXC13 was positively correlated with LINC00659 in colorectal cancer tissues (**Figure 1D**).

Besides, a total of 437 differential miRNAs, including 226 upregulated miRNAs and 211 downregulated miRNAs, were filtered (**Figure 1E**). In order to screening for the miRNAs that might connect with LINC00659 and HOXC13, we predicted the target miRNAs of LINC00659 and HOXC13 on starBase, respectively. Since LINC00659 and HOXC13 were upregulated in colorectal cancer tissues, we restricted the expression pattern of differential miRNA in colorectal cancer tissues as “down-regulation”. Finally, the overlapped targeted miRNAs of LINC00659 and HOXC13, miR-485-5p, which was downregulated in colorectal cancer was identified (**Figure 1F**). The expression level of miR-485-5p in colorectal cancer tissue was significantly lower than that in normal tissue (**Figure 1G**) and was negatively correlated with expression of LINC00659 (**Figure 1H**). Taken together, these data suggested the potential ceRNA relationships among LINC00659, miR-485-5p and HOXC13.

To further validate the results of bioinformatics analysis, the expression levels of LINC00659, miR-485-5p and HOXC13 in colorectal cancer tissues and paracancerous tissues were measured by qRT-PCR. The results showed that LINC00659 and HOXC13 were significantly up-regulated and miR-485-5p was down-regulated in colorectal cancer tissue compared with adjacent normal tissue (**Figure 1I**). These results confirmed the above bioinformatics results and demonstrated that LINC00659, miR-485-5p and HOXC13 play important roles in colorectal cancer.

LINC00659 binds with miR-485-5p directly and knockdown of LINC00659 upregulates the expression of miR-485-5p

In order to dissect whether miR-485-5p and HOXC13 were involved in LINC00659 mediated colorectal cancer progression, the *in vitro* experiments were performed. Firstly, we detected the distribution of LINC00659 in DLD-1 and SW620 cells. Subcellular fractionation analysis found that LINC00659 was mainly abundant in the cytoplasm of DLD-1 and SW620 cells (**Figure 2A**). The binding sites between LINC00659 and miR-485-5p were predicted in starBase (**Figure 2B**). Next, to confirm whether miR-485-5p was a direct target of LINC00659, luciferase reporter plasmids containing WT-LINC00659 or MUT-LINC00659 were constructed. The results demonstrated that the relative luciferase activity was significantly reduced in cells transfected with WT-LINC00659 and miR-485-5p, while there is no significant difference when cells were co-transfected with MUT-LINC00659 and miR-485-5p (**Figure 2C**). This result indicated that miR-485-5p is a target of LINC00659.

To further analyze the effects of LINC00659 and miR-485-5p on colorectal cancer cells, LINC00659 was silenced in DLD-1 and SW620 cells. The efficiency of transfection was confirmed by qRT-PCR (**Figure 2D**). Then, DLD-1 and SW620 cells were separately treated with control, sh-NC+miR-485-5p NC, sh-LINC00659+miR-485-5p NC, sh-NC+miR-485-5p inhibitor and sh-LINC00659+miR-485-5p inhibitor. The results showed that sh-LINC00659 significantly decreased the level of LINC00659, and miR-485-5p

inhibitor had no significant effects on the level of LINC00659 (**Figure 2E**). Meanwhile, sh-LINC00659 dramatically upregulated the expression of miR-485-5p, and miR-485-5p inhibitor restored the high expression of miR-485-5p induced by sh-LINC00659 to basal level (**Figure 2F**). This result suggested that LINC00659 could target and modulate the expression of miR-485-5p.

Knockdown of LINC00659 inhibits the expression of HOXC13 by targeting miR-485-5p

The binding sites between miR-485-5p and HOXC13 were predicted in Starbase and their relationships were verified by luciferase assay. The result demonstrated that miR-485-5p was able to directly target HOXC13 (**Figure 3A-3B**). The level of miR-485-5p was significantly decreased by miR-485-5p inhibitor treatment while not affected by si-HOXC13 (**Figure 3C**). After treatment with miR-485-5p inhibitor, the levels of HOXC13 mRNA and protein were up-regulated significantly, and si-HOXC13 partially reversed the effects of miR-485-5p inhibitor on the cells (**Figure 3D-3E**). This result demonstrated that miR-485-5p can target HOXC13 in colorectal cancer cells.

LncRNA/miRNA/mRNA regulatory axis has been documented many times, so we hypothesized that LINC00659/miR-485-5p may modulate downstream mRNA target of HOXC13. Western blot and qRT-PCR results showed that sh-LINC00659 inhibited the expression level of HOXC13 and miR-485-5p inhibitor promoted HOXC13 expression. Moreover, miR-485-5p inhibitor could partly abrogate the inhibitory effect of sh-LINC00659 on HOXC13 (**Figure 3F-3G**). Altogether, these data indicated that LINC00659 can sponge miR-485-5p to regulate HOXC13 expression, and promote colorectal cancer cell viability, migration and invasion.

Knockdown of LINC00659 suppresses colorectal cancer cell viability, migration, and invasion by targeting miR-485-5p/HOXC13 axis

To further investigate the functions of LINC00659/miR-485-5p, cell viability, migration and invasion experiments were conducted after silencing of LINC00659 and/or transfecting with miR-485-5p inhibitor. The result showed that sh-LINC00659 significantly inhibited cell viability, cell migration and invasion ability. On the contrary, miR-485-5p inhibitor promoted cell viability, migration and invasion ability. In addition, miR-485-5p inhibitor can partially restore the inhibitory effect of sh-LINC00659 on tumor cell viability, migration and invasion (**Figure 4A-4C**).

Further rescue experiments demonstrated that miR-485-5p inhibitor could increase cell viability, migration and invasion, and knockdown of HOXC13 showed opposite results. Besides, si-HOXC13 could partly restore the promotion effects induced by miR-485-5p inhibitor to initial level (**Figure 4D-4F**). These data demonstrated that miR-485-5p regulated cell viability, migration and invasion by directly targeting HOXC13.

Knockdown of LINC00659 suppresses colorectal tumor formation *in vivo*

To further analyze the axis of LINC00659/miR-485-5p/HOXC13 on tumor growth in a biological context, we divided the DLD-1 and SW620 cells into 4 groups respectively: sh-NC+inhibitor-NC, sh-LINC00659+inhibitor-NC, sh-LINC00659+miR-485-5p inhibitor and sh-NC+miR-485-5p inhibitor. The cells were injected into nude mice to establish a tumor-bearing nude mouse model. Results showed that sh-LINC00659 inhibited tumor volume and weight, while miR-485-5p inhibitor can partially restore the inhibitory effect of tumor growth caused by sh-LINC00659 (**Figure 5A-5C**). The results also showed that HOXC13 mRNA and protein levels in the sh-LINC00659+inhibitor-NC group were significantly decreased, while those in the sh-LINC00659+inhibitor group were dramatically elevated. In addition, miR-485-5p inhibitor could reverse the inhibitory effect of sh-LINC00659 on HOXC13 expression (**Figure 5D-5E**).

In order to investigate the downstream of LINC00659/miR-485-5p/HOXC13, the mRNA and protein expressions of MMP2, MMP9, E-cadherin and N-cadherin were determined. The results suggested that sh-LINC00659 significantly inhibited the levels of MMP2, MMP9, N-cadherin mRNA and protein expressions while enhanced the expression of E-cadherin compared with sh-NC. However, miR-485-5p inhibitor enhanced MMP2, MMP9 and N-cadherin expression, while inhibited E-cadherin expression compared with inhibitor-NC group. More importantly, miR-485-5p inhibitor partially reversed the effects of sh-LINC00659 of expression of MMP2, MMP9, E-cadherin and N-cadherin (**Figure 5F-5H**). These data proved that LINC00659 regulated the expression of HOXC13 by targeting miR-485-5p to influence downstream genes such as MMP2, MMP9, MMP-13, E-cadherin and N-cadherin expression, eventually participated in colorectal cancer development and metastasis.

Discussion

The roles of lncRNAs in colorectal cancer have been extensively investigated. Some lncRNAs can be used as markers for diagnosing colorectal cancer and evaluating its prognosis [23-25]. Moreover, it was found that lncRNAs FOXD3-AS1, KCNQ10T1, BCAR4, and ZDHHC8P1 can participate in the proliferation, apoptosis, migration and invasion of colorectal cancer cells by targeting miRNAs to regulate mRNA expression [26-28]. In previous reports, high expression of LINC00659 in colorectal cancer was suggested to be an oncogene in colorectal cancer [21, 22]. However, the molecular mechanism has not been fully uncovered. In this study, we predicted the ceRNA mechanism of LINC00659 via bioinformatics analysis and validated this axis by *in vitro* and *in vivo* experiments.

It suggested that lncRNA might regulate gene expression at post-transcriptional level, such as working as ceRNA to sponge miRNA [29], or regulating mRNA translation and splicing by base pairing [30]. To this end, we predicted all of the targeted miRNAs downstream and upstream of LINC00659 and HOXC13, respectively, and overlapped these two miRNA lists. Meanwhile, the candidate miRNA should be lowly expressed in colorectal cancer to meet the high expression of LINC00659 and HOXC13. The miR-485-5p was singled out to comply with above parameters. In addition, clinical studies showed that miR-485-5p

was down-regulated in colorectal cancer tissues, and its expression level was negatively correlated with LINC00659. It further supported that miR-485-5p was the mediator of LINC00659 and HOXC13. MiR-485-5p is a miRNA that is down regulated in a variety of tumors, including gastric cancer, breast cancer, esophageal cancer, and lung cancer. Moreover, miR-485-5p has been documented to exert roles of inhibiting tumor proliferation, migration and invasion by targeting the different mRNAs [31-34]. More importantly, miR-485-5p has been indicated to target CD147 to inhibit tumor growth in colorectal cancer [35]. Furthermore, we have also verified through subsequent cell experiments that miR-485-5p could regulate the expression of HOXC13 by directly targeting HOXC13 mRNA, to regulate the colorectal cancer cell growth.

HOXC13 is a member of the homeobox family, which binds to a specific nucleotide sequence on DNA and activates or inhibits the transcription process [36-38]. It has been reported that high expression of HOX proteins may lead to carcinogenesis [39]. The expression of HOXC13 was reported to be upregulated in several kinds of cancers, including esophageal squamous cell carcinoma, lung adenocarcinoma, breast cancer and melanoma [40-43]. Especially, aberrant expression of HOXC13 was also associated with colorectal cancer [44, 45] and knockdown of HOXC12 could significantly inhibit colorectal cancer cell growth and result in cell cycle arrest [46]. The results of bioinformatics analysis and clinical samples evaluation in our study showed that HOXC13 was significantly up-regulated in colorectal cancer, and patients with high HOXC13 expression were associated with a poor prognosis. These data suggested that LINC00659 regulates viability, migration and invasion of colorectal cancer cells by regulating HOXC13 through competing sponging miR-485-5p.

The results of our study also found that in the tumor-bearing nude mouse model, knockdown of LINC00659 inhibited tumor growth, while miR-485-5p partially restored the inhibition of sh-LINC00659. Immunohistochemistry experiments also showed that HOXC13 was mainly distributed in the nucleus. In colorectal cancer, HOXC13 might be involved in the metastasis of colorectal cancer by directly or indirectly regulating the transcriptional levels of MMP2, MMP9, E-cadherin and N-cadherin. However, the effects might also be regulated by LINC00659 and miR-485-5p. Further studies are needed on the regulatory relationship between LINC00659/miR-485-5p/HOXC13 axis and the expression of metastasis-associated genes in colorectal cancer. In addition, the hierarchical order of the axis should be validated with more experiments *in vitro* and *in vivo*.

In conclusion, LINC00659 and HOXC13 were overexpressed while miR-485-5p was decreased in colorectal cancer tissues and cells. Meanwhile, we found that LINC00659 promoted the expression of HOXC13 by sponging miR-485-5p and thus influenced downstream genes such as MMP2, MMP9, E-cadherin and N-cadherin expression, eventually participated in colorectal cancer development and metastasis.

Materials And Methods

Bioinformatics analysis

The RNA sequencing data from 471 cases of colorectal cancer tissues and 41 cases of normal colon tissues were acquired from the TCGA database (<https://cancergenome.nih.gov>). The differentially expressed RNAs (lncRNAs, mRNAs and miRNAs) were screened with the edgeR package and the Cyber-T model (T-testing) after data normalization and matrixes processing. Genes with $|\log \text{fold change (FC)}| > 2$ and $P < 0.01$ between colorectal cancer group and normal control group were regarded as differentially expressed genes and miRNAs with $|\log \text{FC}| > 1$ and $P < 0.05$ were identified as differential miRNAs. Heatmap and volcano map were used to visualize differentially expressed RNAs.

The starBase (<http://starbase.sysu.edu.cn/index.php>) was used to predict the miRNA targets of LINC00659 and HOXC13. miR-485-5p was obtained through combining the predicted miRNA datasets of LINC00659 and HOXC13, and limiting the expression pattern as lowly expression in colorectal cancer. Furthermore, the target genes of miR-485-5p were predicted in starBase, and were compared with the up-regulated genes in colorectal cancer patients identified from TCGA database. The overlapped up-regulated genes in colorectal cancer patients were collected and subjected to Gene Ontology (GO) analysis.

Clinical research

Tumor tissues and adjacent normal tissues of 30 colorectal cancer patients were collected. All patients were diagnosed by pathological biopsy. The patient was not treated with radiotherapy or chemotherapy within 3 months before enrollment. LINC00659, miR-485-5p and HOXC13 expression were measured by quantitative real-time polymerase chain reaction (qRT-PCR) in each case. The correlation between LINC00659, miR-485-5p and HOXC13 were analyzed. In addition, the five-year survival rate of patients was compared based on expression level of HOXC13. Ethical approval was obtained from the ethics committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-C-2013-008).

Cell culture

DLD-1 and SW620 colorectal cancer cell lines were obtained from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS, Thermo Fisher, Waltham, MA, USA), 50 U/mL penicillin and 50 $\mu\text{g/mL}$ streptomycin (15070063, Thermo Fisher) in incubator at 37°C.

Cell treatment

The cells were divided into five groups: control, sh-negative control (NC) + miR-485-5p NC, sh-LINC00659 + miR-485-5p NC, sh-NC + miR-485-5p inhibitor and sh-LINC00659 + miR-485-5p inhibitor to investigate the functions and relationship between LINC00659 and miR-485-5p. The cells were divided into other five groups: control, si-NC + miR-485-5p NC, si-NC + miR-485-5p inhibitor, si-HOXC13 + miR-485-5p NC and si-HOXC13 + miR-485-5p inhibitor to evaluate the functions and relationship between HOXC13 and miR-485-5p. The sh-LINC00659, miR-485-5p inhibitor, si-HOXC13 and the corresponding negative controls were

obtained from Shanghai Genepharma Company (Shanghai, China). Lipofectamine™ 2000 (Lipo2000, Thermo Fisher) was used for transfection and cells were collected for subsequent experiments at 48 h post transfection.

qRT-PCR

Trizol reagent (Thermo Fisher) was used to extract total RNAs from tissues and cells following the manufacturer's instructions. The cDNAs were prepared from isolated RNAs by iScript TMC DNA Synthesis Kit (Bio-Rad, Shanghai, China). Fast Start Universal SYBR Green Master kit (Roche, Switzerland) was used for qPCR detection. The reaction was performed according to the below procedures: Initial denaturation was followed by 40 cycles of 95°C/15 sec, 60°C/25 sec and 72°C/60 sec. GAPDH was used for normalization. The primers were synthesized by Sangon Biotech (Shanghai, China) and the sequences were listed in Table 1.

Western blot

The total proteins from cells and tissues were lysed in ice-cold RIPA buffer (catalog) supplemented with protease inhibitor cocktail (ab65621, Abcam, San Francisco, CA, USA). The proteins (20 ug per lane) were loaded and separated, followed by transferring to polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked and probed with below antibodies (HOXC13, ab168368, 35 kD; MMP2, ab37150, 72 kD; MMP9, ab73734, 21 kD; E-cadherin, ab40772, 97 kD; N-cadherin, ab18203, 130 kD; β -actin, ab8226, 42 kD; Abcam) at 4°C overnight. The second antibody IgG-H&L-HRP (ab6721, Abcam, 1:2000) was added to the membrane after the three times of washing with PBST. β -actin was used as the loading control. The protein bands were detected in ChemiDoc MP (Bio-Rad, USA). The band density was quantified with ImageJ software.

Dual-luciferase reporter assay

pGL3 vector (Promega Corporation, USA) was used to construct the luciferase reporter plasmid. The sequences containing wild-type (WT) or mutated (MUT) region of LINC00659 and HOXC13 were subcloned to the vector. The respective reporter plasmids were transfected into cells using Lipo2000 according to the instructions. Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to determine the activities of Renilla and firefly luciferases. The signals were recorded by the luminometer at 24h post transfection. The results were presented as relative luciferase activity.

Cell counting kit 8 (CCK-8) assay

The cells were seeded on 96-well plates with blank controls (medium alone). CCK-8 solution (Beyotime, Shanghai, China) was added to each well after hypoxia-reoxygenation. The plate was incubated for 2 h in the incubator at 37°C. The corresponding optical density ratio at 450nm was used to present the cell vitality by the microplate reader.

Immunohistochemistry (IHC)

The specimen blocks were cut into 4- μm -thick sections. Citrate buffer solution (0.01 mol/L) and peroxidase blocking solution (50 μL) were used for antigen retrieval and endogenous peroxidase blocking, respectively. The primary antibody (anti-HOXC13, ab55251, Abcam) was added according to the kit instructions (Bioss, USA) and incubated at 4°C for 12 h. Then, the secondary antibody was added and incubated for 10 min at 25°C. DAB (3,3'-diaminobenzidine, 100 μL) was added to the slides for 5 min, the staining images were captured under the inverted microscope (Olympus IX71, Tokyo, Japan).

Wound healing assay

The wounding healing assay was conducted to measure the cell migration. Briefly, the cells were seeded in a 6-well plates at 1×10^6 per well and cultured until the confluence reached up to 90%. Monolayers of cells were detached with a 200 μL pipette tip, followed with another 24 h culture. Images of wound recovery were captured under the inverted microscope (Olympus IX71). ImageJ software was used to analyze the leading edge gap on both sides.

Transwell assay

For invasion assay, the cells were seeded into the matrigel-coated upper chamber of Transwell apparatus (8- μm , BD Biosciences, CA, USA). The chamber at the bottom was filled with the complete medium as chemical attractant. After incubation for 48 h, a cotton swab was used to sweep the cells that did not pass through the membrane. The invaded cells were fixed with 20% methanol, and 0.2% crystal violet was used for staining. The invasion ability was calculated as the number of cells under inverted microscope.

Xenograft formation assay

4-week-old BALB/c nude mice of specific pathogen free (SPF) grade were purchased from the SLAC laboratory animal center (Shanghai, China). DLD-1 and SW620 cells were treated with sh-NC + miR-485-5p NC, sh-LINC00659 + miR-485-5p NC, sh-LINC00659 + miR-485-5p inhibitor and sh-NC + miR-485-5p inhibitor, respectively. Then, 1×10^6 cells in each group were injected into dorsal flank of nude mice respectively. The mice were sacrificed, and tumors were weighed and photographed after 28 days of injection. The animal experiment protocol was approved by the Animal Experimentation Ethics Committee of Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine.

Statistical analysis

Data were demonstrated as mean \pm standard deviation (SD) and were analyzed by Graphpad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Multiple-group comparison was conducted by one-way analysis of variance (ANOVA) with LSD post-hoc analysis. $P < 0.05$ was considered as statistical significance.

Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-C-2013-008). Written informed consent was obtained from individual or guardian participants.

Consent for publication

Not applicable.

Availability of data and material

All the data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding authors upon reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Author's contributions

H.Y, J.F, G.J, Y.Z, Y.H, Y.H and Y.X conducted experiments, data analysis, and interpretation. H.Y, J.F wrote the manuscript. C.H, and Z.W., conceived and designed the experiments, analyzed the data, and revised the manuscript. All authors approved the final version of the manuscript.

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Tables

Table 1 The sequences of primers

Primer name	Sequence (5'-3')
LINC00659-Forward	ACCCCTGAAGGACCATATCCA
LINC00659-Reverse	GGCTCGGCTGTGTCTCAAG
miR-485-5p-Forward	AGAGGCUGGCCGUGAUGAAUUC
miR-485-5p-Reverse	GAATACCTCGGACCCTGC
HOXC13-Forward	AAGCTTACGACTTCGCTGCTCCTGC
HOXC13-Reverse	GGATCCTCAGGTGGAGTGGAGATGAGGC
MMP2-Forward	TGATGGTGTCTGCTGGAAAG
MMP2-Reverse	GACACGTGAAAAGTGCCTTG
MMP9-Forward	GGAGAGAGGCCTGCTGAAAATGAC
MMP9-Reverse	CTCCTCTTGACCTGCTGTGTGCGAG
N-cadherin-Forward	GCGTCTGTAGAGGCTTCTGG
N-cadherin-Reverse	GCCACTTGCCACTTTTCCTG
E-cadherin-Forward	TTCCCTCGACACCCGATTCAAAGT
E-cadherin-Reverse	AGCTGTTGCTGTTGTGCTTAACCC
U6-Forward	CTCGCTTCGGCAGCACA
U6-Reverse	AACGCTTCACGAATTTGCGT
β -actin-Forward	GAAATCGTGCGTGACATTAA
β -actin-Reverse	AAGGAAGGCTGGAAGAGTG

Figures

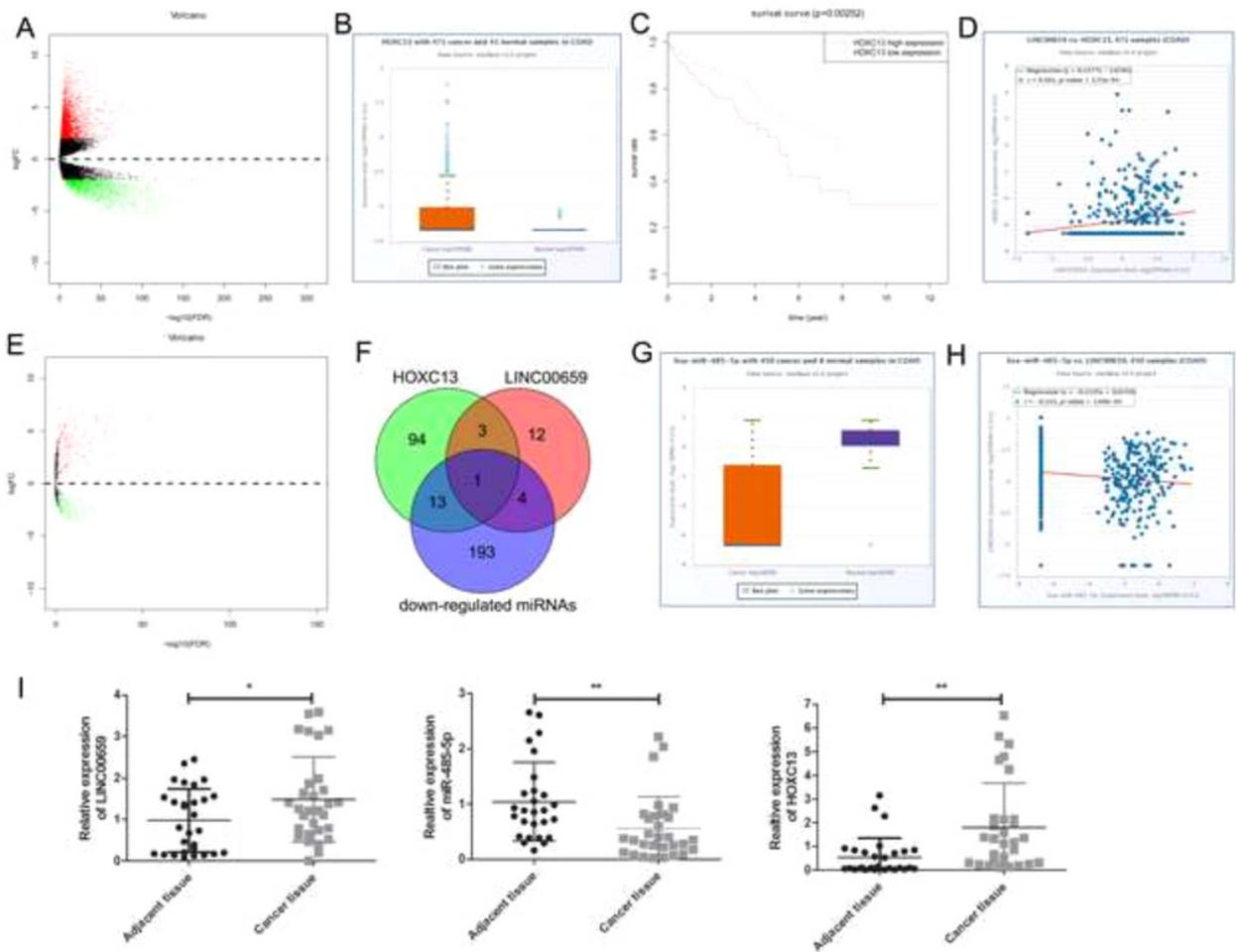


Figure 1

Bioinformatics analysis found that LINC00659 and HOXC13 were up-regulated and miR-485-5p was down-regulated in colorectal cancer. (A) Volcano plot of differentially expressed genes in colorectal cancer in the TCGA database. (B) Differential expression of HOXC13 in colorectal cancer. (C) Kaplan-Meier survival analysis of HOXC13 expression in colorectal cancer patients. (D) Correlation analysis of LINC00659 and HOXC13 in colorectal cancer. (E) Volcano plot of differential miRNAs in colorectal cancer in the TCGA database. (F) Venn diagram of down-regulated miRNAs and the targeted miRNA of LINC00659 and HOXC13. (G) Differential expression of miR-485-5p in colorectal cancer. (H) Correlation analysis of LINC00659 and miR-485-5p in colorectal cancer. (I) qRT-PCR analysis revealed that LINC00659 and HOXC13 were up-regulated and miR-485-5p was down-regulated in colorectal cancer. $n = 30$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

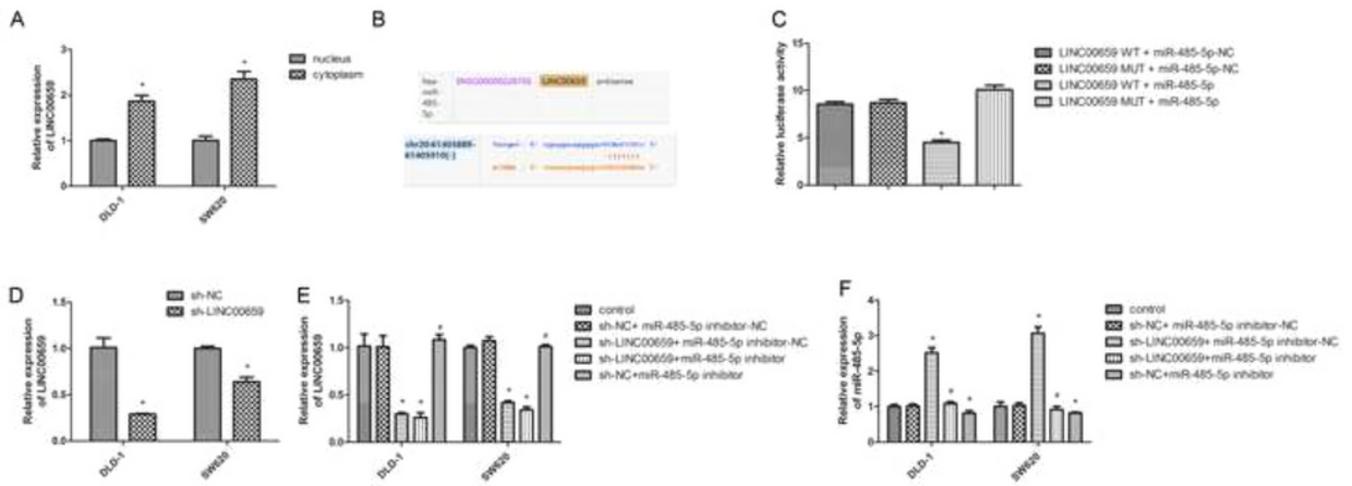


Figure 2

LINC00659 could target miR-485-5p. (A) The distribution of LINC00659 in the cytoplasm and nucleus were measured by qRT-PCR in DLD-1 and SW620 cells. (B) The binding sites between LINC00659 and miR-485-5p predicted in starbase. (C) Dual-luciferase reporter assay demonstrated that LINC00659 interacted with miR-485-5p. (D) qRT-PCR demonstrated LINC00659 was successfully knock-down in DLD-1 and SW620 cells. (E-F) Expression levels of LINC00659 and miR-485-5p were measured by qRT-PCR in different groups of cells. Bars indicated means \pm (standard deviation) SD. * $P < 0.05$ vs. control and sh-negative control (NC) + miR-485-5p inhibitor-NC group; # $P < 0.05$ vs. sh-LINC00659 + miR-485-5p inhibitor-NC group.

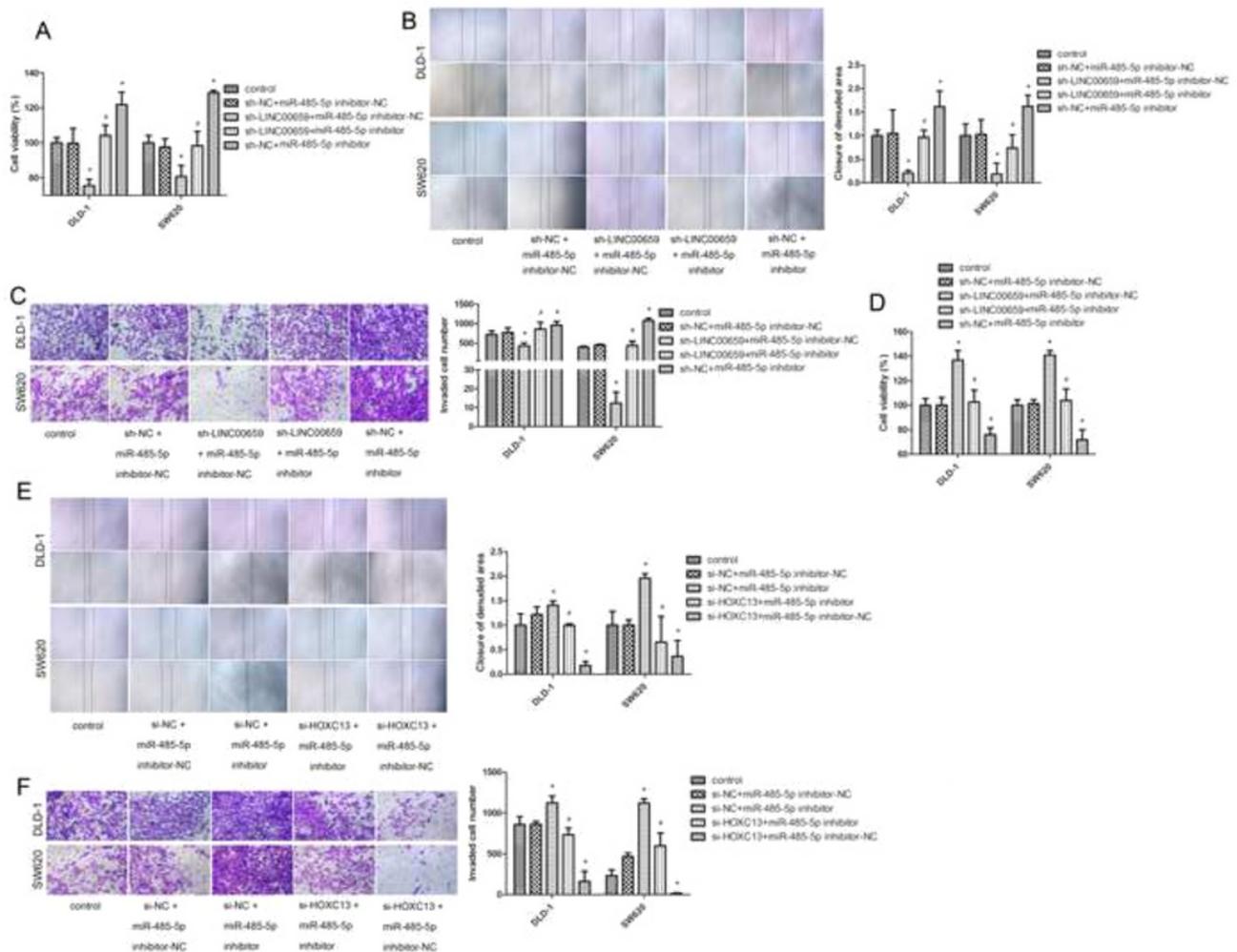


Figure 4

LINC00659 regulated colorectal cancer cell viability, migration and invasion by regulating miR-485-5p/HOXC13. (A, D) Cell viability was detected by CCK-8 assay. (B, E) Wound healing assay was used to monitor the migration ability in different groups of cells. (C, F) Transwell assay was applied to monitor the invasion ability in different groups of cells. Bars indicated means \pm (standard deviation) SD. * $P < 0.05$ vs. control and sh-NC + miR-485-5p inhibitor-NC group; # $P < 0.05$ vs. sh-LINC00659 + miR-485-5p inhibitor-NC group.

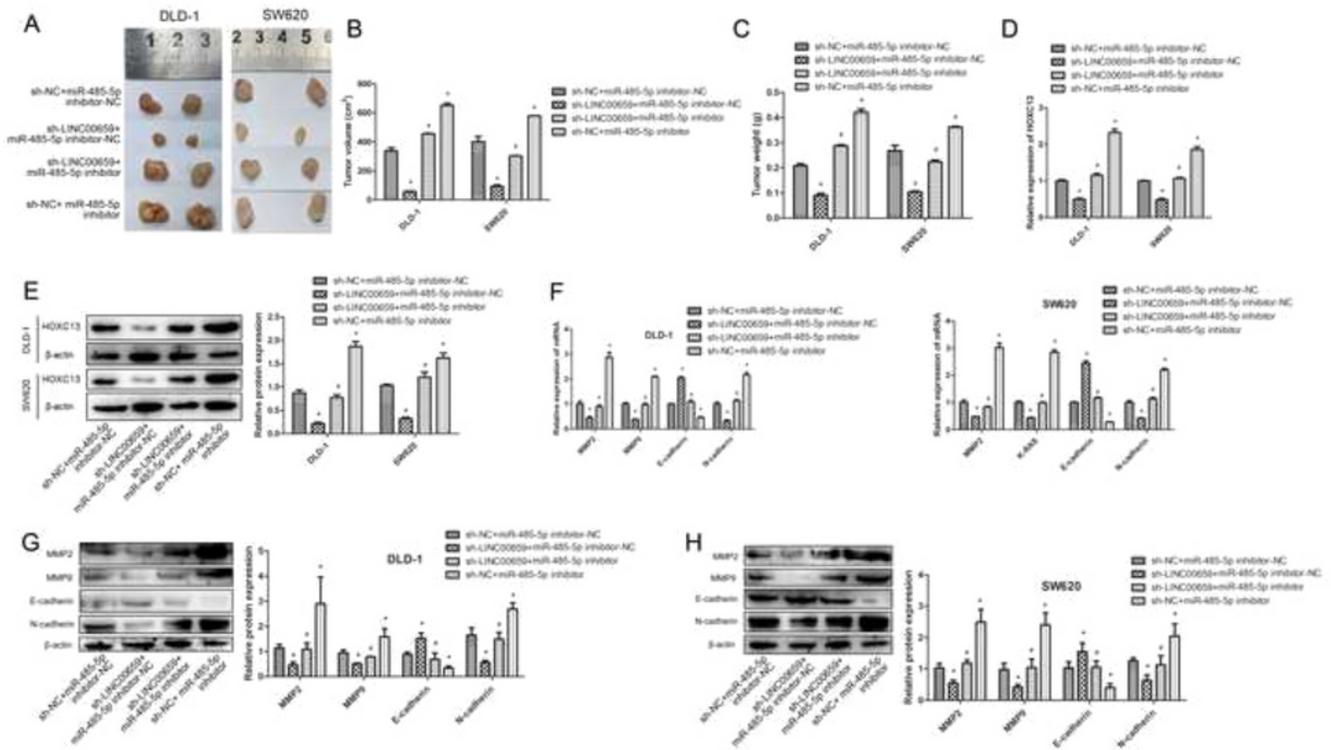


Figure 5

LINC00659 regulated the expression of HOXC13 by targeting miR-485-5p and participated in the tumor formation. (A-C) The tumor-burdened assay was used to analyze the effects of LINC00659 and HOXC13 on tumor growth. (D, E) The expression levels of HOXC13 mRNA and protein were detected by qPCR and Western blot. (F-H) The mRNA and protein expression levels of MMP2, MMP9, E-cadherin and N-cadherin were detected by qRT-PCR and Western blot. Bars indicated means \pm (standard deviation) SD. * $P < 0.05$ vs. control and sh-NC + miR-485-5p inhibitor-NC group; # $P < 0.05$ vs. sh-LINC00659+ miR-485-5p inhibitor-NC group.