Recurrence-Associated Long Non-coding RNA LNAPPCC Facilitates Colon Cancer Progression via Forming a Positive Feedback Loop with PCDH7

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Long non-coding RNAs (lncRNAs) gradually show critical regulatory roles in many malignancies. However, the lncRNAs implicated in colon cancer recurrence are largely unknown. In this study, we searched the lncRNAs associated with metastasis and recurrence of colon cancer using GEO datasets. We focused on a novel lncRNA long non-coding RNA associated with poor prognosis of colon cancer (LNAPPCC), which is highly expressed in colon cancer. Increased expression of LNAPPCC is positively associated with metastasis, recurrence, and poor survival of colon cancer patients. LNAPPCC promotes colon cancer cell proliferation, migration, and in vivo xenograft growth and liver metastasis. Mechanistic investigations revealed that LNAPPCC binds EZH2, represses the binding of EZH2 to PCDH7 promoter, downregulates histone H3K27me3 level in PCDH7 promoter, and activates PCDH7 expression. Intriguingly, we also found that PCDH7 activates ERK/c-FOS signaling, increases the binding of c-FOS to LNAPPCC promoter, and activates LNAPPCC expression. Therefore, LNAPPCC and PCDH7 form a positive regulatory loop via EZH2 and ERK/c-FOS. The positive correlations between the expression of LNAPPCC, PCDH7, phosphorylated ERK, and phosphorylated c-FOS are detected in colon cancer tissues. Furthermore, depletion of PCDH7 or the adding of ERK inhibitor abolished the oncogenic roles of LNAPPCC in colon cancer. In summary, this study identified a novel lncRNA LNAPPCC that is highly expressed in colon cancer and associated with poor prognosis of colon cancer patients. LNAPPCC exerts oncogenic roles in colon cancer via forming a positive feedback loop with PCDH7. Targeting LNAPPCC/EZH2/PCDH7/ERK/c-FOS signaling axis represents a potential therapeutic strategy for colon cancer.

INTRODUCTION

Colon cancer, which originates from malignant transformation of colonic epithelial cells, has become a severe public health threat due to the high incidence and mortality worldwide.1,2 There were 1,096,601 estimated new cases and 551,269 estimated deaths of colon cancer in 2018 globally.3 In 2019 cancer statistics in the United States, colon cancer ranked third in both incidence and mortality.3 Despite great advances that have been made in surgical resection, chemotherapy, immunotherapy, and so on, the prognosis of about 30% of colon cancer patients is still very poor due to recurrence and/or metastasis.4–6 Therefore, revealing the critical molecular events underlying colon cancer recurrence and metastasis to develop more efficient prognostic biomarkers and therapeutic targets is urgently demanded.6,7

Long non-coding RNAs (lncRNAs) gradually show critical regulatory roles in many malignancies. However, the lncRNAs implicated in colon cancer recurrence are largely unknown. In this study, we searched the lncRNAs associated with metastasis and recurrence of colon cancer using GEO datasets. We focused on a novel lncRNA long non-coding RNA associated with poor prognosis of colon cancer (LNAPPCC), which is highly expressed in colon cancer. Increased expression of LNAPPCC is positively associated with metastasis, recurrence, and poor survival of colon cancer patients. LNAPPCC promotes colon cancer cell proliferation, migration, and in vivo xenograft growth and liver metastasis. Mechanistic investigations revealed that LNAPPCC binds EZH2, represses the binding of EZH2 to PCDH7 promoter, downregulates histone H3K27me3 level in PCDH7 promoter, and activates PCDH7 expression. Intriguingly, we also found that PCDH7 activates ERK/c-FOS signaling, increases the binding of c-FOS to LNAPPCC promoter, and activates LNAPPCC expression. Therefore, LNAPPCC and PCDH7 form a positive regulatory loop via EZH2 and ERK/c-FOS. The positive correlations between the expression of LNAPPCC, PCDH7, phosphorylated ERK, and phosphorylated c-FOS are detected in colon cancer tissues. Furthermore, depletion of PCDH7 or the adding of ERK inhibitor abolished the oncogenic roles of LNAPPCC in colon cancer. In summary, this study identified a novel lncRNA LNAPPCC that is highly expressed in colon cancer and associated with poor prognosis of colon cancer patients. LNAPPCC exerts oncogenic roles in colon cancer via forming a positive feedback loop with PCDH7. Targeting LNAPPCC/EZH2/PCDH7/ERK/c-FOS signaling axis represents a potential therapeutic strategy for colon cancer.

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The advances of genome and transcriptome high-throughput sequencing have found that the human genome encodes more non-coding RNAs than mRNAs.8–10 Among these non-coding RNAs, long non-coding RNA (lncRNA) is a class of transcripts more than 200 nucleotides in length with limited protein coding potential.11–15 Increasing evidence has demonstrated that lncRNAs are frequently involved in the initiation and progression of various cancers, including colon cancer.16–21 For example, upregulated lncRNA ITIH4-AS1 promotes proliferation and metastasis of colon cancer via activating JAK/STAT3.22 lncRNA LINC02418 is highly expressed and may be a diagnostic biomarker for colon cancer.23 lncRNA CALIC accelerates colon cancer metastasis via upregulating AXL.24 Although several lncRNAs have been identified to be aberrantly expressed and play corresponding biological roles in colon cancer, the lncRNAs with a predictive value of recurrence and metastasis of colon cancer are still lacking.

In the present study, using gene expression signatures of colon cancers from GEO datasets (https://www.ncbi.nlm.nih.gov/gds/), we...
identified a novel lncRNA (GenBank: AK095791), which may predict metastasis and poor prognosis of colon cancer. Therefore, we termed this lncRNA as long non-coding RNA associated with poor prognosis of colon cancer (LNAPPCC). We further investigated the expression pattern and clinical significance of LNAPPCC in colon cancer. Furthermore, gain- and loss-of-function experiments were undertaken to explore the roles of LNAPPCC in colon cancer. Mechanistic investigation identified a positive feedback loop between LNAPPCC, PCDH7, ERK, and c-FOS, which mediate the oncogenic roles of LNAPPCC in colon cancer.

RESULTS
LNAPPCC Is Highly Expressed in Colon Cancer and High Expression of LNAPPCC Is Correlated with Metastasis and Poor Prognosis of Colon Cancer Patients

To search the lncRNAs involved in colon cancer metastasis, we compared the differentially expressed genes from GSE37892 data, which includes 93 non-metastatic and 37 metastatic colon cancer tissues using GEO2R. Among these differentially expressed genes shown in Table S1, LNAPPCC (probe ID: 232113_at) is the most remarkably upregulated lncRNA with the largest fold-change (FC) value in metastatic colon cancer tissues compared with non-metastatic colon cancer tissues (Figure 1A). Analyzing the provided clinicopathological characteristics of these 130 colon cancer patients from GSE37892 data, we found that LNAPPCC expression intensity is remarkably increased in colon cancer cell lines HT-29, SW620, HCT-15, and HCT116 (Figure S1C). Kaplan-Meier survival analysis was undertaken using GSE39582 and GSE17536 data to detect the correlation between LNAPPCC expression intensities and recurrence free survival from GSE39582 data. n = 577, p = 0.0233 by log-rank test. (E) Kaplan-Meier survival analysis of the correlation between LNAPPCC expression intensities and overall survival from GSE17536 data. n = 177, p = 0.0137 by log-rank test. (F) Kaplan-Meier survival analysis of the correlation between LNAPPCC expression intensities and disease specific survival from GSE17536 data. n = 177, p = 0.0005 by log-rank test. (G) Kaplan-Meier survival analysis of the correlation between LNAPPCC expression intensities and recurrence free survival from GSE17536 data. n = 145, p = 0.0002 by log-rank test.

LNAPPCC Facilitates Colon Cancer Cell Proliferation and Migration

To observe the potential functions of LNAPPCC in colon cancer, we constructed LNAPPCC stably overexpressed HCT116 and SW620 cells via stable transfection of LNAPPCC mammalian expression plasmid pcDNA/LNAPPCC (Figures 2A and 2B). Cell counting Kit-8 (CCK-8) assays revealed that cell proliferation of both HCT116 and SW620 cells was remarkably increased by LNAPPCC
LNAPPCC were searched based on the following criteria: (1) the target genes of LNAPPCC in colon cancer. The candidate targets of LNAPPCC were identified as 13 members (Table S2). The expression of PCDH7 was previously reported as a polycomb group target gene.28 EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2), which catalyzes trimethylation of histone H3 lysine 27 (H3K27me3).29,30 H3K27me3 is a repressive chromatin marker and represses target gene transcription.31 Intriguingly, an interaction between LNAPPCC and EZH2 was predicted by the online RNA-protein interaction prediction (RPISeq)32 (http://pridb.gdcb.iastate.edu/RPISeq/) with random forest classifier value of 0.7 and support vector machine classifier value of 0.98 (greater than 0.5 was considered positive). Therefore, we hypothesized that whether the interaction between LNAPPCC and EZH2 regulates the transcription of PCDH7. RNA pull-down assays using biotin-labeled LNAPPCC were undertaken in SW620 and HCT116 cells. As shown in Figures 4A and S3A, EZH2 was remarkably enriched in biotin-labeled LNAPPCC group. Furthermore, RNA immunoprecipitation (RIP) assays using EZH2-specific antibody revealed that LNAPPCC was specifically enriched in EZH2 antibody group (Figure 4B). HOTAIR and GAPDH mRNA was used as positive and negative controls, respectively, for EZH2 binding (Figures S3B and S3C). Because EZH2 was reported to bind G-rich motif,33 we next scanned for G-rich motif in LNAPPCC and identified a G-rich motif in 685–715 nt of LNAPPCC (Figure 4C). RIP assays showed that the mutation of the G-rich motif abolished the enrichment of LNAPPCC by EZH2 (Figure 4D). These data showed that LNAPPCC interacts with EZH2. Next, we investigated whether the interaction between LNAPPCC and EZH2 modulates the binding of EZH2 to PCDH7 promoter and the chromatin structure at PCDH7 promoter. Chromatin immunoprecipitation (ChIP) assays showed that ectopic expression of LNAPPCC repressed the binding of EZH2 to PCDH7 promoter and reduced H3K27me3.

The Expression of LNAPPCC Is Significantly Positively Correlated with that of PCDH7, Whose High Expression Is Also Associated with Metastasis and Poor Prognosis of Colon Cancer Patients

Next, we investigated the molecular mechanisms mediating the oncogenic roles of LNAPPCC in colon cancer. The candidate targets of LNAPPCC were searched based on the following criteria: (1) the targets are differentially expressed between metastatic and non-metastatic colon cancer tissues from GSE37892 data; (2) the expression of the targets is correlated with LNAPPCC in colon cancer tissues from GSE37892 data; and (3) the expression of the targets is correlated with LNAPPCC in colon cancer tissues from GSE17536 data. Following the above criteria, we identified 13 candidates (Table S2). Among these candidates, we noted PCDH7 (probe ID: 228640_at), which has been reported to modulate tumor metastasis.25–27 Consistent with LNAPPCC, PCDH7 is also remarkably increased in metastatic colon cancer tissues compared with non-metastatic colon cancer tissues from GSE37892 data (Figure 3A). The expression of PCDH7 is remarkably positively correlated with LNAPPCC from both GSE37892 and GSE17536 data, with R value of 0.7844 and 0.6865, respectively (Figures 3B and 3C). Furthermore, Kaplan-Meier survival analysis showed that consistent with LNAPPCC, high expression of PCDH7 is remarkably correlated with poor overall survival, poor disease specific survival, and more cancer recurrence from GSE17536 data (Figures 3D–3F). The positive correlation between the expression of PCDH7 and LNAPPCC was further confirmed in GSE39582 data, which includes 566 cases with R value of 0.7268 (Figure 3G). In our own cohort, the expression of PCDH7 is also remarkably positively correlated with LNAPPCC with R value of 0.7308 (Figure 3H).

| Variable                  | LNAPPCC |       |       | p Value |
|---------------------------|---------|-------|-------|---------|
| Age                       |         |       |       |         |
| >60                       | 16      | 19    |       | 0.442   |
| ≤ 60                      | 15      | 12    |       |         |
| Sex                       |         |       |       | 0.445   |
| Male                      | 13      | 16    |       |         |
| Female                    | 18      | 15    |       |         |
| Pathology T stage         |         |       |       |         |
| T1-2                      | 10      | 3     |       | 0.029   |
| T3-4                      | 21      | 28    |       |         |
| Pathology N Stage         |         |       |       | 0.030   |
| N0                        | 25      | 17    |       |         |
| N1-2                      | 6       | 14    |       |         |
| Pathology M stage         |         |       |       | 0.038   |
| M0                        | 29      | 23    |       |         |
| M1                        | 2       | 8     |       |         |

p value was calculated by Pearson chi-square test.

Table 1. Correlation between LNAPPCC Expression and Clinicopathological Characteristics
level at PCDH7 promoter (Figure 4E). Conversely, silencing of LNAPPCC increased the binding of EZH2 to PCDH7 promoter and upregulated H3K27me3 level at PCDH7 promoter (Figure 4F).

*p16, which was reported to be a target gene of EZH2, was used as control (Figures S3D and S3E). Then, the effects of LNAPPCC on PCDH7 expression were detected. The mRNA and protein levels of PCDH7 were remarkably increased in LNAPPCC stably overexpressed HCT116 and SW620 cells (Figures 4G and 4H). Conversely, the mRNA and protein levels of PCDH7 were remarkably decreased in LNAPPCC stably silenced HCT116 cells (Figure 4I). Therefore, these findings suggested that LNAPPCC activates PCDH7 expression via reducing the binding and repressive roles of EZH2 on PCDH7 promoter.

PCDH7 Promotes Colon Cancer Cell Proliferation and Migration

To investigate whether PCDH7 is a mediator of the oncogenic roles of LNAPPCC in colon cancer, we next examined the potential roles of PCDH7 in colon cancer. PCDH7 stably overexpressed HCT116 cells were constructed via stable transfection of PCDH7 mammalian expression plasmid pReceiver/PCDH7 (Figure 5A). CCK-8 and EdU incorporation assays both revealed that cell proliferation of HCT116 cells was remarkably increased by PCDH7 overexpression (Figures 5B and 5C). Transwell assays revealed that cell migration of HCT116 cells was also remarkably increased by PCDH7 overexpression (Figure 5D). PCDH7 stably silenced HCT116 cells were further constructed via stable transfection of two independent PCDH7-specific shRNAs (Figure 5E). Both CCK-8 and EdU incorporation assays revealed that cell proliferation was remarkably repressed by silencing of PCDH7 (Figures 5F and 5G). Transwell assays revealed that cell migration was also remarkably repressed by silencing of PCDH7 (Figure 5H). Therefore, these findings suggested that consistent with LNAPPCC, PCDH7 also plays oncogenic roles in colon cancer.

PCDH7 Increases LNAPPCC Expression via Activating ERK/c-FOS Signaling

Due to the significant positive correlation between the expression of PCDH7 and LNAPPCC in colon cancer tissues, we next investigated whether PCDH7 could modulate LNAPPCC expression inversely. The expression of LNAPPCC in PCDH7 stably overexpressed and silenced HCT116 cells was detected by quantitative real-time PCR. Intriguingly, LNAPPCC was remarkably increased in PCDH7 overexpressed cells and decreased in PCDH7 silenced cells (Figure 6A;
These data suggested that PCDH7 also upregulates LNAPPCC. Previous reports showed that PCDH7 activates mitogen-activated protein kinase (MAPK) signaling in lung cancer.34,35 In this study, we further investigated whether PCDH7 also activates MAPK signaling in colon cancer. As shown in Figures 6B and S4B, ectopic expression of PCDH7 induced ERK and c-FOS activation, and conversely, silencing of PCDH7 repressed ERK and c-FOS activation in colon cancer cells. Searching LNAPPCC promoter, we identified a c-FOS binding site located at 460 bp upstream of LNAPPCC transcription initiation site (Figure 6C). Luciferase reporter assays showed that c-FOS increased LNAPPCC promoter activity (Figure 6D). ChiP assays showed that ectopic expression of PCDH7 increased the binding of c-FOS to LNAPPCC promoter, whereas silencing of PCDH7 decreased the binding of c-FOS to LNAPPCC promoter (Figure 6E; Figure S4C). To investigate whether the upregulation of LNAPPCC by PCDH7 is dependent on the activation of ERK/c-FOS signaling, we treated PCDH7 stably overexpressed HCT116 cells with ERK inhibitor GDC-0994. The results showed that inhibition of ERK activation abrogated the upregulation of LNAPPCC caused by PCDH7 overexpression (Figure 6F). Thus, these findings suggested that PCDH7 upregulates LNAPPCC expression via activating ERK/c-FOS signaling. Combined with the findings that LNAPPCC activates PCDH7 expression, our data demonstrated that LNAPPCC/PCDH7/ERK/c-FOS form a positive feedback loop in colon cancer (Figure 6G). Via activating PCDH7/ERK/c-FOS axis, ectopic expression of LNAPPCC increased the binding of c-FOS to LNAPPCC promoter, whereas silencing of LNAPPCC decreased the binding of c-FOS to LNAPPCC promoter (Figure 6H; Figure S4D), which further support the LNAPPCC/PCDH7/ERK/c-FOS positive feedback loop. The correlations between LNAPPCC expression, PCDH7 expression, ERK activation, and c-FOS activation were further analyzed in colon cancer tissues. In our cohort containing 62 colon cancer cases, we found that the colon cancer tissues with strong phosphorylated ERK staining have higher expression of LNAPPCC and PCDH7 than the colon cancer tissues with weak phosphorylated ERK staining (Figures 6I and 6J). The colon cancer tissues with strong phosphorylated c-FOS staining also have higher expression of LNAPPCC and PCDH7 than the colon cancer tissues with weak phosphorylated c-FOS staining (Figures 6K and 6L).

**ERK Inhibitor or Depletion of PCDH7 Abrogates the Roles of LNAPPCC in Promoting Colon Cancer Cell Proliferation and Migration**

To examine whether LNAPPCC exerts oncogenic roles via the LNAPPCC/PCDH7/ERK/c-FOS feedback loop in colon cancer, we treated LNAPPCC stably overexpressed HCT116 cells with ERK inhibitor GDC-0994. The results showed that the pro-proliferative roles of LNAPPCC were abolished by ERK inhibitor (Figures 7A and 7B). Transwell assays revealed that the pro-migratory roles of LNAPPCC were abolished by ERK inhibitor (Figure 7C). In addition, PCDH7 was stably silenced in LNAPPCC stably overexpressed HCT116 cells via stable transfection of PCDH7-specific shRNA into LNAPPCC overexpressed HCT116 cells (Figure 7D).
that the pro-proliferative roles of LNAPPCC were abolished by the silencing of PCDH7 (Figures 7E and 7F). Transwell assays revealed that the pro-migratory roles of LNAPPCC were also abolished by the silencing of PCDH7 (Figure 7G). Therefore, these findings suggested that LNAPPCC promotes colon cancer cell proliferation and migration via the positive LNAPPCC/PCDH7/ERK/c-FOS feedback loop.

LNAPPCC Facilitates Colon Cancer Xenograft Growth and Liver Metastasis in a PCDH7-Dependent Manner

To investigate the in vivo roles of LNAPPCC in colon cancer growth and metastasis, we subcutaneously injected LNAPPCC stably overexpressed HCT116 cells with or without PCDH7 silencing into nude mice. As shown in Figures 8A–8C, overexpression of LNAPPCC remarkably promoted colon cancer xenograft growth, which was abrogated by the silencing of PCDH7. Overexpression of LNAPPCC and PCDH7 in subcutaneous xenografts derived from LNAPPCC overexpressed HCT116 cells were confirmed (Figure 8D). Furthermore, the downregulation of LNAPPCC and PCDH7 in subcutaneous xenografts derived from PCDH7-silenced HCT116 cells were also confirmed (Figure 8D). Ki67 staining of subcutaneous xenografts revealed that overexpression of LNAPPCC promoted colon cancer cell proliferation in vivo, which was abolished by the silencing of PCDH7 (Figure 8E). Phosphorylated ERK and c-FOS staining revealed that overexpression of LNAPPCC activated ERK and c-FOS in vivo, which was also abolished by the silencing of PCDH7 (Figures 8F and 8G; Figures S5A and S5B). In addition, LNAPPCC stably overexpressed HCT116 cells with or without PCDH7 silencing were intrasplenically injected into nude mice to construct liver metastasis model. As shown in Figures 8H–8J, overexpression of LNAPPCC remarkably promoted colon cancer liver metastasis, which was abrogated by the silencing of PCDH7. To confirm the roles of LNAPPCC in colon cancer xenograft growth and liver metastasis, we subcutaneously injected LNAPPCC stably silenced HCT116 cells into nude mice. LNAPPCC knockdown remarkably repressed colon cancer xenograft growth (Figures 8C–8E). Downregulation of LNAPPCC and PCDH7 in subcutaneous xenografts derived from LNAPPCC-silenced HCT116 cells were confirmed (Figure S5F). Ki67 staining of subcutaneous xenografts revealed that LNAPPCC knockdown repressed colon cancer cell proliferation in vivo (Figure S5G). Phosphorylated ERK and c-FOS staining revealed that LNAPPCC knockdown inactivated ERK and c-FOS in vivo (Figures S5H–S5K). In addition, LNAPPCC stably silenced HCT116 cells were intrasplenically injected into nude mice to construct liver metastasis model. As shown
in Figure S5L–S5N, LNAPPCC knockdown remarkably inhibited colon cancer liver metastasis.

**DISCUSSION**

Until now, surgical resection is still the most efficient therapeutic strategy for most colon cancer. However, approximately 30% of colon cancer patients may suffer postoperative recurrence or metastasis. Therefore, identifying predictive biomarkers for colon cancer recurrence and/or metastasis may be beneficial to the early intervention. In this study, we first identified that lncRNA LNAPPCC is remarkably associated with colon cancer recurrence and metastasis using multiple cohorts. Our findings demonstrated that LNAPPCC is highly expressed in colon cancer tissues and cell lines. Increased expression of LNAPPCC is remarkably positively associated with metastasis, recurrence, and poor survival of colon cancer patients.

The gene encoding LNAPPCC is located at chromosome 11q24.1. LNAPPCC has one exon and 2,445 nucleotides in length. Except for the aberrant expression of LNAPPCC in colon cancer, LNAPPCC was also found to play critical roles in colon cancer. Gain-of-function experiments demonstrated that LNAPPCC promotes colon cancer cell proliferation and migration in vitro and colon cancer xenograft growth and liver metastasis in vivo.

Molecular mechanisms underlying the roles of IncRNAs are complex and various. Different subcellular locations of IncRNAs implies different molecular mechanisms. For cytoplasmic IncRNAs, they could directly bind proteins, microRNAs (miRs), and/or mRNAs and change the modification, stability, location, and roles of the interacted partners. For nuclear IncRNAs, one of the main mechanisms is to bind epigenetic modification machineries, modulate epigenetic modification and chromatin structure of target genes, and therefore modulate the expression of target genes. Among the epigenetic modification machineries, PRC2, which catalyzes H3K27me3 and represses target genes expression, is frequently involved in the roles of IncRNAs. Several IncRNAs, such as HOTAIR, ANCR, H19, SYISL, AFAP1-AS1 have been reported to bind EZH2, the catalytic subunit PRC2, change genomic location of EZH2, and therefore regulate the expression of target genes. In this study, using bioinformatic analysis and experimental verification, we also found that LNAPPCC binds to EZH2. Although we identified the interaction between LNAPPCC and EZH2, whether the interaction is direct or indirect needs further investigation. In multiple cohorts, we found a remarkably positive correlation between the expression of LNAPPCC and PCDH7. PCDH7 has
been reported to be a PRC2 target. In this study, we further found that via interacting with EZH2, LNAPPCC suppresses the binding of EZH2 to PCDH7 promoter, reduces H3K27me3 level at PCDH7 promoter, and thus upregulates PCDH7 expression. PCDH7, also known as protocadherin 7, belongs to the cadherin superfamily. PCDH7 has been reported to have oncogenic roles in lung cancer and breast cancer via activating MAPK or PLCβ-Ca2+/CaMKII/S100A4 signaling.26,34 In this study, we found that PCDH7 also plays oncogenic roles in colon cancer via activating MAPK-c-FOS signaling. Except PCDH7, whether LNAPPCC regulate the expression of other genes via interacting with EZH2 needs further investigation.

Except for the positive regulation of PCDH7 by LNAPPCC, we further found that PCDH7 could positively regulate LNAPPCC via activated ERK and c-FOS signaling. LNAPPCC was identified as a transcription target of c-FOS. Therefore, LNAPPCC, PCDH7, ERK, and c-FOS form a positive feedback loop. In clinical colon cancer tissues, we also found significant positive correlation between LNAPPCC expression, PCDH7 expression, ERK phosphorylation level, and c-FOS phosphorylation level. The positive feedback loop could amplify the effects of participants. Several feedback loops have been reported to be employed by cancer cells to exert their malignant behaviors, such as ERIC/E2F, miR-200a/HDAC4, SNHG14/miR-5590-3p/ZEB1, NF90/lncRNA-LET/miR-548k, and so on.52 Functional rescue experiments showed that inhibition of ERK activation or silencing of PCDH7 abrogated the oncogenic roles of LNAPPCC in colon cancer, which suggested that the LNAPPCC/PCDH7/ERK/c-FOS feedback loop at least partially mediates the oncogenic roles of LNAPPCC in colon cancer.

In summary, this study identified a novel oncogenic lncRNA LNAPPCC, which is upregulated in colon cancer and correlated with metastasis, recurrence, and poor prognosis of colon cancer patients. LNAPPCC facilitates colon cancer cell proliferation and migration in vitro and colon cancer growth and liver metastasis in vivo via activating the LNAPPCC/PCDH7/ERK/c-FOS positive feedback loop. Our findings suggested LNAPPCC as a potential predictive biomarker for colon cancer metastasis and recurrence. Our results also suggested that targeting LNAPPCC/EZH2/PCDH7/ERK/c-FOS feedback loop represents a potential therapeutic strategy for colon cancer.

Cell Culture and Treatment
Normal human colon mucosal epithelial cell line NCM460 and colon cancer cell lines HT-29, SW620, HCT-15, and HCT116 were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). NCM460 cells were grown in Dulbecco's modified Eagle's medium (DMEM ( Gibco)). HT-29 and HCT116 cells were grown in McCoy's 5a medium (Sigma). SW620 cells were grown in L-15 medium ( Gibco). HCT-15 cells were grown in RPMI-1640 medium (Gibco). All cells were cultured in the above described medium complemented with 10% fetal bovine serum (Gibco) in a moist air with 5% CO2 at 37°C. Where indicated, cells were treated with 1 μM GDC-0994 (Selleck) for the indicated time.

Quantitative Real-Time PCR
Total RNA was extracted from tissues and cells using RNAiso Plus (Takara) following the provided protocol. Subsequently, the RNA was reverse-transcribed into first strand complementary DNA (cDNA) with PrimeScript 1st strand cDNA Synthesis Kit (Takara). The cDNA was then used to undertake quantitative real-time PCR with TB Green Premix Ex Taq II (Takara) on StepOne Plus System (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an endogenous control. The primer sequences used for quantitative real-time PCR were as follows: for LNAPPCC: 5'-TTGGGACACAGAGGACA-3' (sense) and 5'-GGGAAATCGTGCGTGACATTAAG-3' (antisense); for PCDH7: 5'-ATCTACCACAGGCAACA-3' (sense) and 5'-CTAATACCGTCCATTCCA-3' (antisense); for GAPDH: 5'-GGGACACAGAGGACA-3' (sense) and 5'-GGGAAATCGTGCGTGACATTAAG-3' (antisense); for β-actin: 5'-GGGAAATCGTGCGTGACATTAAG-3' (sense) and 5'-TGTTGTTGGTGCAAGGGTCTGG-3' (antisense); and for U6: 5'-GCTTCGGCAGCACATATACTAAAAT-3' (sense) and 5'-CGCTTCAGGAATTGTCGAGTC-3' (antisense). The relative expressions levels of RNAs were calculated by the 2−ΔΔCt method.

Isolation of Cytoplasmic, Nuclear, and Poly(A)-Positive RNA
Cytoplasmic and nuclear fractions were isolated and harvested by the cytoplasmic and nuclear RNA purification kit (Norgen). The RNA present in the subcellular fraction was detected by quantitative real-time PCR. Poly(A)-positive RNA was enriched by the GenElute mRNA Miniprep Kit (Sigma) and then detected by quantitative real-time PCR.

5' and 3' Rapid Amplification of cDNA Ends (RACE)
The transcriptional initiation and termination sites of LNAPPCC were determined by 5' and 3' RACE analyses with SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer’s manual. The primer sequences used for quantitative real-time PCR were as follows: for 5' RACE, 5'-CTTCTCTCTCTTCTCTCC-3' (sense) and for 3' RACE, 5'-GTGAGGGTCTCTTCCTT-3' (antisense); and for 5' and 3' RACE, 5'-CCAGGAGGATGCGGCTTG-3' (antisense) and 5'-CGCTTCAGGAATTGTCGAGTC-3' (antisense). The relative expressions levels of RNAs were calculated by the 2−ΔΔCt method.

Plasmids Construction and Transfection
Full-length LNAPPCC was PCR-amplified from cDNA with the primers 5'-GGGTTACCTTGTGATTGCGAGTCTCATCGT-3' (sense) and 5'-TCCATTCCA-3' (antisense)
and 5'-GGAATTCTCAGCAAGGAATGTCTTTAACAAG-3' (antisense). Subsequently, the PCR products were subcloned into the KpnI and EcoR I sites of pCDNA3.1(+) backbone to construct LNAPPCC mammalian expression plasmid pcDNA/LNAPPCC. G-rich motif mutated LNAPPCC expression plasmid pcDNA/LNAPPCC-Mut (mutant) was synthesized by GenScript. Furthermore, the PCR products were also subcloned into the KpnI and EcoR I sites of pSPT19 backbone to construct LNAPPCC in vitro transcription plasmid pSPT19/LNAPPCC. LNAPPCC promoter was PCR-amplified from DNA with the primers 5'-GGGGTACCATTGTTAGATGGGATCAGG-3' (sense) and 5'-CCGCTCGAGGGACTATCAAAGACTGGA-3' (antisense). Subsequently, the PCR products were subcloned into the KpnI and EcoR I sites of pSPT19 backbone to construct LNAPPCC in vitro transcription plasmid pSPT19/LNAPPCC. LNAPPCC promoter was PCR-amplified from DNA with the primers 5'-GGGGTACCATTGTTAGATGGGATCAGG-3' (sense) and 5'-CCGCTCGAGGGACTATCAAAGACTGGA-3' (antisense). Subsequently, the PCR products were subcloned into the KpnI and Xho I sites of pGL3-Basic vector (Promega) to construct firefly luciferase reporter containing LNAPPCC promoter. PCDH7 mammalian expression plasmid pReceiver/PCDH7 was purchased from GeneCopoeia (EX-Y4988-M02). c-FOS mammalian expression plasmid pReceiver/c-FOS was purchased from GeneCopoeia (EX-B0247-M02). The corresponding empty plasmid pReceiver-M02 was used as negative control. The shRNA suppressing PCDH7 expression (shPCDH7) was purchased from GeneCopoeia (HSH062195-mH1). The corresponding shRNA scrambled control was purchased from GeneCopoeia (CSHCTR001-mH1). Another shRNA targeting PCDH7 (shPCDH7-2) was purchased from GenePharma. Plasmids transfection was undertaken using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s manual.

Figure 6. PCDH7 Upregulates LNAPPCC Expression via Activating ERK/c-FOS Signaling

(A) LNAPPCC expression levels in PCDH7 stably overexpressed and control HCT116 cells. (B) ERK and c-FOS phosphorylation levels in PCDH7 stably overexpressed and control HCT116 cells. (C) Schematic outlining the predicted binding site of c-FOS on LNAPPCC promoter. (D) After transient co-transfection of firefly luciferase reporter containing LNAPPCC promoter, pRL-TK (encoding renilla luciferase), and c-FOS overexpression plasmid into HCT116 cells, dual luciferase reporter assays were performed. Results were shown as relative ratio of firefly luciferase activity to renilla luciferase activity. (E) ChIP assay using c-FOS specific antibodies was undertaken to detect the effects of PCDH7 overexpression on the binding of c-FOS to LNAPPCC promoter. (F) LNAPPCC expression levels in PCDH7 stably overexpressed HCT116 cells treated with 1 \( \mu \)M GDC-0994 for 48 h. (G) A schematic model of the LNAPPCC/PCDH7/ERK/c-FOS positive feedback loop. (H) ChIP assay using c-FOS specific antibodies was undertaken in LNAPPCC stably overexpressed and control HCT116 cells to detect the effects of LNAPPCC overexpression on the binding of c-FOS to LNAPPCC promoter. (I and J) LNAPPCC (I) and PCDH7 (J) expression levels in 62 colon cancer tissues with strong or weak p-ERK staining intensity. The median p-ERK staining intensity was used as the cutoff. p = 0.0081 (I) and p = 0.0093 (J) by Mann-Whitney test. (K and L) LNAPPCC (K) and PCDH7 (L) expression levels in 62 colon cancer tissues with strong or weak p-c-FOS staining intensity. The median p-c-FOS staining intensity was used as the cutoff. p = 0.0012 (K) and p = 0.0041 (L) by Mann-Whitney test. For (A, D, E, F, and H), data are presented as mean ± SD based on at least three independent biological repeats. *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant, by Student’s t test (A, D, E, and H) or one-way ANOVA followed by Dunnett’s multiple comparisons test (F).
Cell Proliferation Assays
Cell proliferation was evaluated by CCK-8 and EdU incorporation assays. For CCK-8 assay, 2,000 indicated colon cancer cells/well were seeded into 96-well plates and cultured for 1, 2, 3, or 4 days. At indicated time, each well was added with 10 μL of CCK-8 reagent (Dojindo Laboratories) and incubated for another 2 h. Subsequently, the absorbance values at 450 nm were collected using a standard microplate reader. EdU incorporation assay was undertaken using the Cell-Light EdU In Vitro Kit (RiboBio) following the manufacturer’s manual. The images of EdU-positive cells were collected using a fluorescence microscope (Carl Zeiss).

Cell Migration Assay
Cell migration was evaluated by Transwell migration assay. 50,000 indicated colon cancer cells were plated in the upper chamber of 24-well Transwell inserts (BD Biosciences) supplemented with serum-free medium. Lower chambers were added with medium supplemented with 10% fetal bovine serum (GIBCO). The inserts were further incubated in 5% CO2 at 37°C for another 48 h. Then, the cells remaining on the upper chambers were removed, and the migratory cells were fixed and stained by crystal violet. The number of migratory cells was counted using a fluorescence microscope (Carl Zeiss).

RNA Pull-Down Assay
LNAPPCC was in vitro transcribed and biotinylated from pSPT19/LNAPPCC using Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Roche) according to the manufacturer’s manual. 3 μg of biotinylated and refolded LNAPPCC was incubated with 1 mg protein extracts from indicated colon cancer cells and Dynabeads Myone Streptavidin T1 beads (Invitrogen) overnight. Subsequently, the beads were washed three times. After streptavidin bead pull-down, the enriched proteins were detected by western blot.

Western Blot
Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime) added with protease inhibitor (Selleck) and phosphatase inhibitor (Selleck). The extracted or enriched proteins were separated by 12% SDS-PAGE. After being transferred into polyvinylidene fluoride (PVDF) membrane (Millipore), the blots were incubated with primary antibodies against EZH2 (1:500; 07-689; Millipore), PCDH7 (1:200; ab139274; Abcam), phospho-ERK1/2 (1:1,000; #4370; Cell Signaling Technology), ERK1/2 (1:1,000; #4695; Cell Signaling Technology), phospho-c-FOS (1:1,000; #5348; Cell Signaling Technology), c-FOS (1:1,000; #2250; Cell Signaling Technology), or GAPDH (1:10,000; T0004; Abfinity). After three washes, the blots were further incubated with goat anti-mouse immunoglobulin G (IgG) H&L (IRDye 680RD) preadsorbed secondary antibody (1:10,000; ab216776; Abcam) or goat anti-rabbit IgG H&L (IRDye 800CW) preadsorbed secondary antibody (1:10,000; ab216775; Abcam). The bands were visualized using an Odyssey Infrared Imaging System (LI-COR).
The western blot images were obtained using Odyssey infrared scanner (Li-Cor).

RIP

RIP experiment was undertaken with the EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore) and EZH2 specific antibody (5 µg; 07-689; Millipore) following the manufacturer’s manual. Enriched RNA was measured by quantitative real-time PCR.

ChIP

ChIP experiment was undertaken with the EZ-Magna ChIP A/G chromatin immunoprecipitation kit (Millipore) and specific antibodies against EZH2 (5 µg; 07-689; Millipore), H3K27me3 (5 µg; ab6002; Abcam), or Phospho-c-FOS (5 µg; #5348; Cell Signaling Technology) following the manufacturer’s manual. Enriched DNA was measured by quantitative real-time PCR. The primer sequences were as follows: for PCDH7 promoter: 5'-TTTGTTAAATTGC CGTTGGGG-3' (sense) and 5’-TCTTGACGCTCTCTCC-3' (antisense); for LNAPPCC promoter: 5'-ATAA CACCCAAAACGTAC-3' (sense) and 5'-GGCC CAATAATAGAAA-3' (antisense); for p16 promoter: 5'-GCCCGAGCTCTCCTCCT-3' (sense) and 5'-CCCTGTCCTCCTCAAATCCTC-3' (antisense).

Luciferase Reporter Assay

c-FOS overexpression plasmid, firefly luciferase reporter containing LNAPPCC promoter, and pRL-TK plasmid that expressed renilla luciferase were co-transfected into HCT116 cells. 48 h after transfection, the luciferase activities were measured by Dual-Luciferase Reporter Assay System (Promega). The relative firefly luciferase activity was normalized to renilla luciferase activity.

Xenograft Experiment

Indicated colon cancer cells (5 x 10⁶) were subcutaneously injected into 5- to 6-week-old athymic BALB/c nude mice. Subcutaneous xenograft growth was recorded every 7 days with a caliper, and tumor volume was calculated as 0.5 x a x b² (a, longest diameter; b, shortest diameter). At the 28th day after injection, the mice were sacrificed and the subcutaneous tumors were resected, photographed, and weighed. For liver metastasis experiment, indicated colon cancer cells (2 x 10⁶) were intrasplenically injected into 5- to 6-week-old athymic BALB/c nude mice. At the 35th day after injection, the mice were sacrificed and the livers were resected. The metastatic nodules were detected by hematoxylin and eosin (H&E) staining. Xenograft experiments were approved by the Ethics Committee of Hainan General Hospital.
Immunohistochemistry (IHC)

Subcutaneous xenograft and human colon cancer tissues were formalin-fixed and paraffin-embedded. Paraffin sections were used to perform Ki67, phospho-ERK1/2, ERK1/2, phospho-c-FOS, c-FOS IHC staining with primary antibodies against Ki67 (1:200; ab16667; Abcam), phospho-ERK1/2 (1:400; #4370; Cell Signaling Technology), ERK1/2 (1:250; #4695; Cell Signaling Technology), phospho-c-FOS (1:100; ab63444; Abcam), or c-FOS (1:1,000; ab208942; Abcam). After washes, the sections were further incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody and visualized with 3,3-diaminobenzidine.

Statistical Analysis

Statistical analysis was undertaken using GraphPad Prism 6 software. Comparisons were calculated using Mann-Whitney test, Student’s t test, and one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test, Spearman correlation analysis, or Kruskal-Wallis test followed by Dunn’s multiple comparisons test as indicated in the figure legends. p value < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.03.017.

AUTHOR CONTRIBUTIONS

B.W., T.L., and Z.L. designed the study; T.L., Z.L., H.Wan, X.T., H. Wang, F.C., and M.Z. contributed to acquisition, analysis, and interpretation of the data; B.W. and T.L. drafted the manuscript; all authors gave final approval of the work.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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Supplemental Information

Recurrence-Associated Long Non-coding RNA
LNAPPCC Facilitates Colon Cancer Progression
via Forming a Positive Feedback Loop with PCDH7

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Supplementary figure legends

**Figure S1** Characteristics of LNAPPCC. A, LNAPPCC expression intensities in colon cancer tissues with age ≤ 60 (n = 35) or > 60 (n = 95) from GSE37892 data. $P = 0.2016$ by Mann-Whitney test. B, LNAPPCC expression intensities in colon cancer tissues with tumor location of left (n = 72) or right (n = 57) from GSE37892 data. $P = 0.2023$ by Mann-Whitney test. C, LNAPPCC expression levels in normal human colon mucosal epithelial cell line NCM460 and colon cancer cell lines HT-29, SW620, HCT-15, and HCT116. ****$P < 0.0001$ by one-way ANOVA followed by Dunnett's multiple comparisons test. D, The levels of LNAPPCC in purified cytoplasmic or nuclear RNAs. β-actin and U6 serve as cytoplasmic and nuclear control, respectively. E, The levels of LNAPPCC in purified polyadenylated RNAs. β-actin and U6 serve as poly(A) positive and negative control, respectively. F, LNAPPCC full-length sequence was determined by 5' and 3' RACE. G, The codon substitution frequency scores of LNAPPCC was less than zero by PhyloCSF calculation on UCSC Genome Browser (GRCh38/hg38). H, Putative proteins possibly encoded by LNAPPCC as predicted by NCBI ORF Finder.

**Figure S2** LNAPPCC promotes NCM460 cell proliferation and migration. A, LNAPPCC expression levels in LNAPPCC stably overexpressed and control NCM460 cells. B, Cell proliferation of LNAPPCC stably overexpressed and control NCM460 cells was examined by CCK-8 assays. C, Cell proliferation of LNAPPCC stably overexpressed and control NCM460 cells was examined by EdU incorporation assay. Scale bars = 100 µm. D, Cell migration of LNAPPCC stably overexpressed and control NCM460 cells was examined by transwell migration assay. Scale bars = 100 µm. Data are presented as mean ± SD based on at least three independent biological repeats. **$P < 0.01$, ***$P < 0.001$ by Student’s t test.
**Figure S3** LNAPPCC binds EZH2 and activates PCDH7 expression. A, The in vitro transcribed LNAPPCC used for RNA pull-down assays was verified by RNA electrophoresis. B, RIP assay using EZH2 specific antibody was undertaken to detect the binding between EZH2 and HOTAIR. C, RIP assay using EZH2 specific antibody was undertaken to detect the binding between EZH2 and GAPDH mRNA. D, ChIP assay using EZH2 and H3K27me3 specific antibodies was undertaken in LNAPPCC stably overexpressed and control SW620 cells to detect the effects of LNAPPCC overexpression on the binding of EZH2 to p16 promoter and H3K27me3 level at p16 promoter. E, ChIP assay using EZH2 and H3K27me3 specific antibodies was undertaken in LNAPPCC stably silenced and control HCT116 cells to detect the effects of LNAPPCC silencing on the binding of EZH2 to p16 promoter and H3K27me3 level at p16 promoter. Data are presented as mean ± SD based on at least three independent biological repeats. **P < 0.01, ns, not significant, by Student’s t test.

**Figure S4** PCDH7 downregulates LNAPPCC expression via inactivating ERK/c-FOS signaling. A, LNAPPCC expression levels in PCDH7 stably silenced and control HCT116 cells. B, ERK and c-FOS phosphorylation levels in PCDH7 stably silenced and control HCT116 cells. C, ChIP assay using c-FOS specific antibodies was undertaken in PCDH7 stably silenced and control HCT116 cells to detect the effects of PCDH7 silencing on the binding of c-FOS to LNAPPCC promoter. D, ChIP assay using c-FOS specific antibodies was undertaken in LNAPPCC stably silenced and control HCT116 cells to detect the effects of LNAPPCC silencing on the binding of c-FOS to LNAPPCC promoter. Data are presented as mean ± SD based on at least three independent biological repeats. **P < 0.01 by Student’s t test (A,C) or one-way ANOVA followed by Dunnett's multiple comparisons test (D).

**Figure S5** LNAPPCC knockdown represses colon cancer xenograft growth and liver metastasis. A, ERK IHC staining in subcutaneous tumors derived from LNAPPCC stably overexpressed and...
concurrently PCDH7 stably silenced HCT116 cells. B, c-FOS IHC staining in subcutaneous tumors derived from LNAPPCC stably overexpressed and concurrently PCDH7 stably silenced HCT116 cells. C-E. LNAPPCC stably silenced HCT116 cells were subcutaneously injected into nude mice. Subcutaneous tumor volumes were measured every 7 days (C). Subcutaneous tumor weights were measured at the 28th day after injection (D). Image of subcutaneous tumors excised at the 28th day after injection (E). F, The expression of LNAPPCC and PCDH7 in subcutaneous tumors derived from LNAPPCC stably silenced HCT116 cells. G, Ki67 IHC staining in subcutaneous tumors derived from LNAPPCC stably silenced HCT116 cells. H, p-ERK IHC staining in subcutaneous tumors derived from LNAPPCC stably silenced HCT116 cells. I, ERK IHC staining in subcutaneous tumors derived from LNAPPCC stably silenced HCT116 cells. J, p-c-FOS IHC staining in subcutaneous tumors derived from LNAPPCC stably silenced HCT116 cells. K, c-FOS IHC staining in subcutaneous tumors derived from LNAPPCC stably silenced HCT116 cells. L-N, LNAPPCC stably silenced HCT116 cells were intrasplenically injected into nude mice. At the 35th day after injection, liver metastases of indicated colon cancer cells were detected by HE staining. Scale bars = 1000 µm. Data are presented as mean ± SD based on n = 6 mice in each group. *P < 0.05, **P < 0.01, ns, not significant, by Kruskal-Wallis test followed by Dunn's multiple comparisons test (A,B) or Mann-Whitney test (C-N).