European Review for Medical and Pharmacological Sciences 2021; 25: 6853-6861

LINC00958/miR-3174/PHF6 axis is responsible for triggering proliferation, migration and invasion of endometrial cancer

Q.-Y. LI[1], J.-Q. SHEN[1], J.-H. LI[1], D.-F. DAI[1], M. SAEED[2], C.-X. LI[1]

[1]Department of Human Anatomy, Medical College of Qinghai University, Xining, China
[2]Faculty of Animal Production and Technology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan.

Qinyuan Li and Jiqiang Shen contributed equally

Abstract. – OBJECTIVE: To reveal the role of LINC00958 in the progression of endometrial cancer (EC) and the underlying molecular mechanism.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was conducted to detect relative level of LINC00958 in EC specimens and cell lines. Its prognostic potential in EC was analyzed by Kaplan-Meier method. After in vitro knockdown of LINC00958, cell proliferative, migratory and invasive abilities in KLE and Ishikawa cells were evaluated by Cell Counting Kit-8 (CCK-8), 5-Ethynyl-2'-deoxyuridine (EdU) and transwell assay. Dual-Luciferase reporter assay was carried out to identify the LINC00958/miR-3174/PHF6 axis, and their expression interaction was determined by Pearson correlation test. The role of miR-3174 in influencing LINC00958-induced phenotype changes of EC cells was determined through rescue experiments.

RESULTS: LINC00958 was abnormally upregulated in EC specimens and cell lines, which was unfavorable to the prognosis of EC. Knockdown of LINC00958 reduced proliferative, migratory and invasive rates in KLE and Ishikawa cells. MiR-3174 shared a binding site in the 3'-untranslated region (3'-UTR) to that of LINC00958, which was lowly expressed in EC specimens and negatively linked to LINC00958 level. Overexpression of miR-3174 partially abolished the role of LINC00958 in accelerating the malignant phenotypes of EC cells. PHF6 was the downstream target of miR-3174 and it was upregulated in EC specimens.

CONCLUSIONS: LINC00958 is upregulated in EC specimens, which is a prognostic factor of EC. It stimulates EC to proliferate, migrate and invade through the miR-3174/PHF6 axis.

Key Words:
Endometrial cancer, LINC00958, MiR-3174, PHF6.

Introduction

Endometrial cancer (EC) is a commonly diagnosed malignant tumor in the female reproductive system, with the fourth highest incidence among female tumors that is secondary to colorectal cancer, lung cancer, and breast cancer[1]. Epidemiological data proposed that the vast majority of EC occurs after menopause, and about 40% of cases are related to obesity. Other risk factors include hyperestrinism, hypertension, and diabetes[2]. Surgical treatment is the main strategy for EC. Nevertheless, the scope and effectiveness of lymphadenectomy are highly controversial[3]. It is of great significance to fully reveal the molecular mechanism of EC, and to provide new targets for the clinical diagnosis and treatment.

Long non-coding RNAs (lncRNAs) do not encode proteins and display vital biological functions in almost every aspect of cell biology[4,5]. Through epigenetic and post-transcriptional pathways, they are also involved in tumor cell behaviors[6]. It is reported that lncRNA SNHG1 accelerates proliferative, migratory and invasive capacities of cervical cancer cells[7]. By targeting microRNA-1290 (miR-1290), lncRNA CCAT1 induces proliferation and metastasis of ovarian cancer[8]. Serving as a ceRNA, lncRNA CDC6 exerts the miRNA sponge effect on miR-215 and thus aggravates the deterioration of breast cancer[9].

MiRNAs are small-chain, noncoding RNAs[10] that participate in the carcinogenesis, tumor progression and metastasis either as oncogenes or anti-cancer genes[11]. MiRNA-9 is able to suppress invasiveness of human liver cancer cells and secretion of E-cadherin[12]. MiRNA-223, through targeting the tumor-suppressor gene EPB41L3, leads to invasiveness and metastasis

Corresponding Author: Changxing Li, MD; e-mail: lcx1535@163.com

of gastric cancer[13]. High level of miR-210 is an alarm signal for the poor survival of breast cancer patients[14].

Vallone et al[15] reported the upregulation of LINC00958 in EC samples. In the present study, we aim to elucidate the role of LINC00958 in the malignant progression of EC and the molecular mechanism.

Patients and Methods

Specimen Collection

Fifty EC tissues and fifty healthy endometrium tissues were collected during surgery. Samples were frozen in liquid nitrogen and preserved at -80°C. None of recruited EC patients received preoperative chemotherapy or radiotherapy. Patients with other tumors that migrated to the uterus were excluded. Tumor staging of EC was determined based on Union for International Cancer Control (UICC) criteria. Their clinical and follow-up data were completely recorded. This study was approved by the Research Ethics Committee of Medical College of Qinghai University and complied with the Helsinki Declaration. Informed consent was obtained from patients.

Cell Culture

Human endometrial stromal cell line (hESC) and EC cell lines (KLE, Ishikawa, HEC-1-A and HHUA) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin.

Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China) and RiboBio (Guangzhou, China). Adherent cells were cultured to 70-80% density and transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell transfection, the cells were collected for verifying transfection efficacy and functional experiments.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells and tissues were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using AMV reverse transcription kit (TaKaRa, Otsu, Shiga, Japan), followed by qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were served as the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. LINC00958: F: 5′-CCATTGAAGATACCACGCTGC-3′, R: 5′-GGTTGTTGCCCAGGGTAGTG-3′; miR-3174: F: 5′-GTCAGGGATGGCAACTTTATCCACT-3′, R: 5′-GGAACCTGAAGGTCCGAGTCA-3′; PHF6 F: 5′-CAGCCACCCGAGATTGAGCA-3′, PHF6 R: 5′-TAGTAGCGACGGGCGGTGTG-3′; GAPDH: F: 5′-TGACGTGCCGCCTGGAGAAC-3′, R: 5′-CCGGCATCGAAGGTGGAAGAG-3′; U6: F: 5′-CTCGCTTCGGCAGCACA-3′, R: 5′-AACGCTTCACGAATTTGCGT-3′. With U6 GAPDH and as endogenous controls, the relative mRNA expression was calculated by using $2^{-\Delta\Delta}$Ct method.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At 0, 24, 48, 72 and 96 h, optical density at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells were pre-inoculated in a 96-well plate with 1×10^4 cells/well. They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100, and 30-min reaction in 400 μL of 1×ApollorR. Afterwards, cells were dyed in 4',6-diamidino-2-phenylindole (DAPI) for another 30 min. Positive EdU-stained cells were calculated.

Transwell

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate, where 5×10^4 cells were applied in the upper layer of the chamber, and 600 μL of medium containing 10% FBS was applied in the bottom. After 48-h incubation, cells in the bottom were fixed, dyed in crystal violet and captured. Migratory cells were counted in 5 randomly selected fields per sample. Invasion assay was conducted using transwell chamber precoated with 100 μg Matrigel.

Dual-Luciferase Reporter Aasay

Binding sites between miR-3174 and LINC00958 or PHF6 were predicted using online tools, which were used for generating luciferase vectors. Cells pre-seeded in the 24-well plate were co-transfected with Luciferase vectors and miR-3174 mimics or NC for 48 h. Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Data processing was conducted using Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was performed for comparing differences between groups. Pearson correlation test was conducted to assess the correlation between two genes in EC tissues. Kaplan-Meier method and log-rank test were introduced for survival analysis. A significant difference was set at $p < 0.05$.

Results

Upregulation of LINC00958 in EC

QRT-PCR data revealed that LINC00958 was upregulated in EC tissues than that of adjacent normal tissues (Figure 1A). Later, differential levels of LINC00958 in EC cases divided by age, tumor staging and incidence of lymphatic metastasis were analyzed. It is shown that LINC00958 was highly expressed in EC patients older than 50 years than younger patients (Figure 1B). Stage III+IV EC patients expressed higher level of LINC00958 compared to stage I+II EC patients (Figure 1C). Meanwhile, EC patients accompanied lymphatic metastasis had higher abundance of LINC00958 in comparison to non-metastatic ones (Figure 1D). Kaplan-Meier curves were depicted based on follow-up information of recruited EC patients aiming to clarify the prognostic value of LINC00958. Low survival rate was determined in EC patients expressing high level of LINC00958, indicating that LINC00958 was a risk factor for the poor prognosis of EC (Figure 1E).

Knockdown of LINC00958 Inhibited EC Cells to Proliferate, Migrate and Invade

In vitro level of LINC00958 was consistently higher in EC cell lines than that of the human endometrial stromal cell line (Figure 1F). Since KLE and Ishikawa cells expressed the most differential level of LINC00958 among the four EC cell lines, they were utilized in the following experiments. We generated three LINC00958 siRNAs, and the

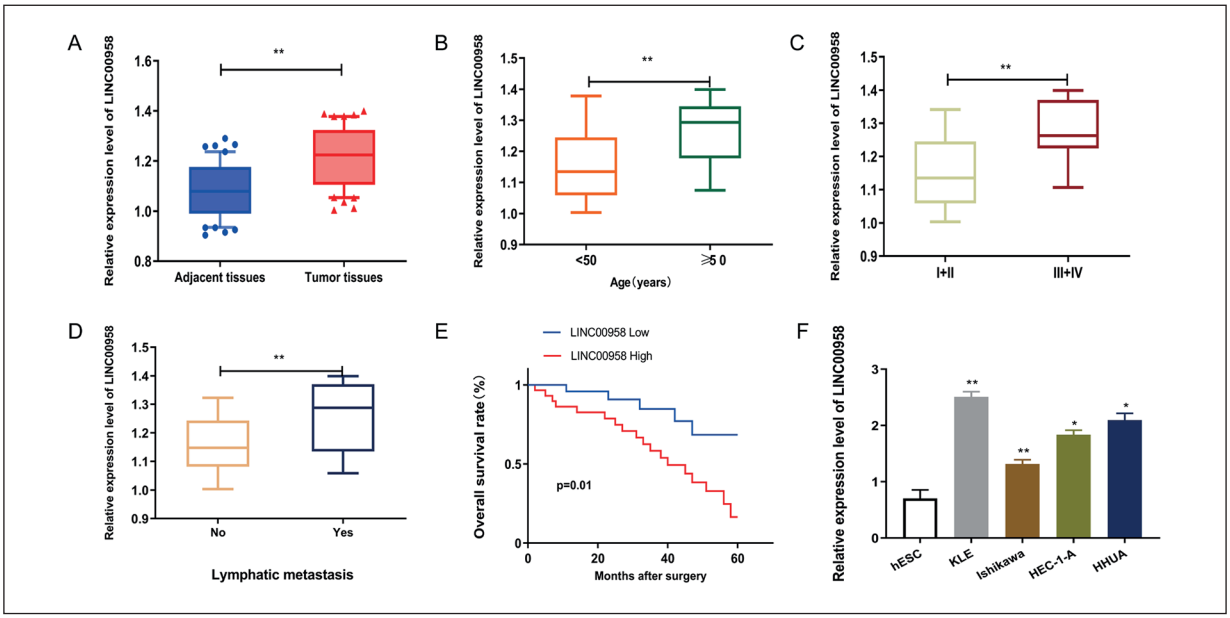

Figure 1. Upregulation of LINC00958 in EC. **A,** LINC00958 levels in EC and adjacent tissues; **B,** LINC00958 levels in EC patients older and younger than 50 years; **C,** LINC00958 levels in stage I+II and stage III+IV EC cases; **D,** LINC00958 levels in EC patients either with lymphatic metastasis or not; **E,** Overall survival in EC patients expressing high or low level of LINC00958; **F,** LINC00958 levels in EC cell lines. *p<0.05; **p<0.01.

third one was utilized since it displayed the best transfection efficacy in KLE and Ishikawa cells (Figure 2A). After knockdown of LINC00958, both viability and EdU-positive rate decreased in EC cells, indicating the inhibited proliferative potential (Figure 2B, C). Transwell assay uncovered that transfection of si-LINC00958 in EC cells reduced numbers of migratory and invasive cells (Figure 2D, E).

MiR-3174 Could Bind to LINC00958

Potential miRNAs that could bind to LINC00958 were predicted using Lncbase (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2/index-predicted) and StarBase (http://starbase.sysu.edu.cn/index.php). After cross-match analysis, three candidates (hsa-miR-3174, hsa-miR-490-3p and hsa-miR-3942-5p) were searched (Figure 3A). Only

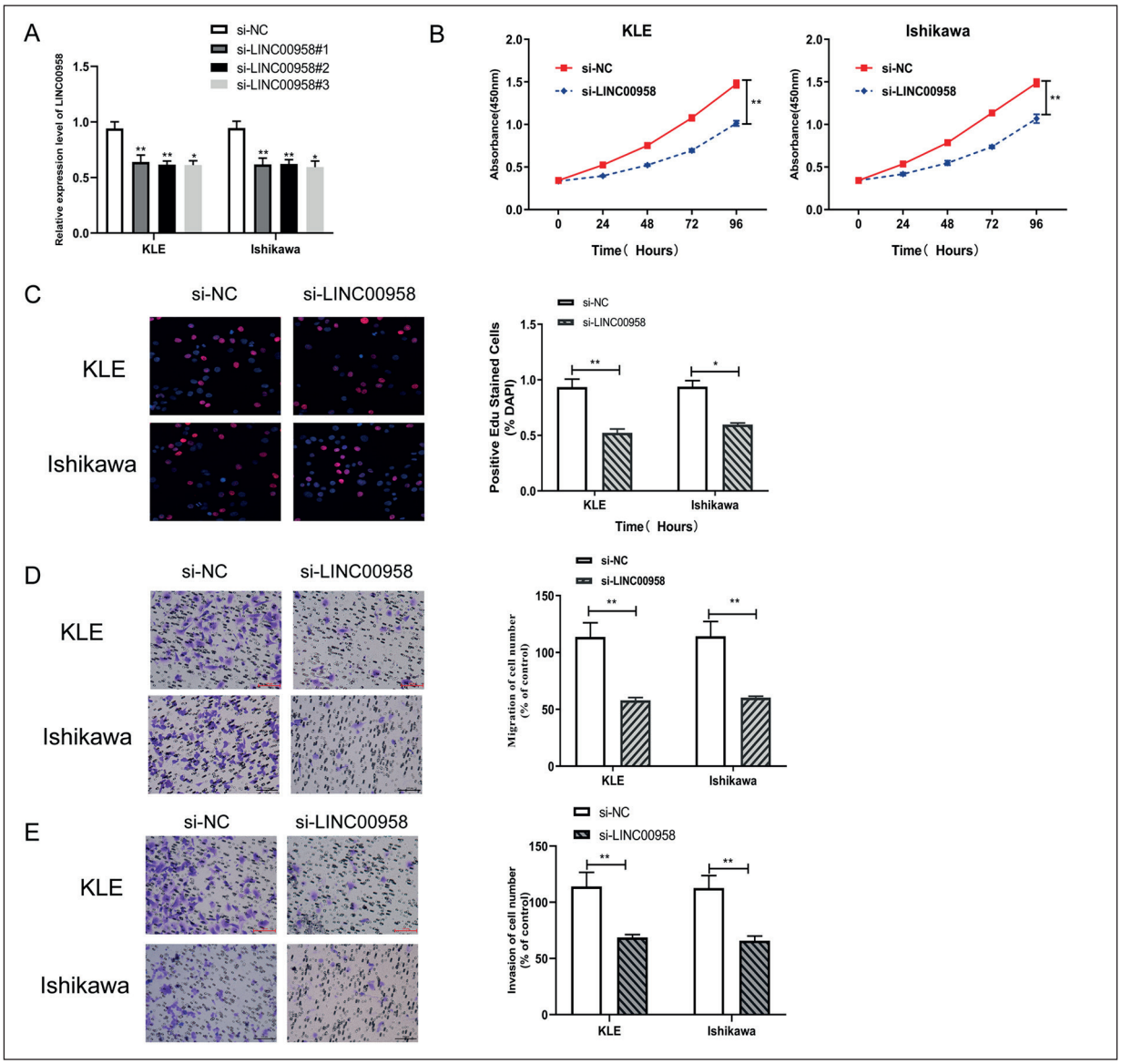

Figure 2. Knockdown of LINC00958 inhibited EC cells to proliferate, migrate and invade. **A,** Transfection efficacy of LINC00958 siRNAs in KLE and Ishikawa cells; **B,** Viability in KLE and Ishikawa cells with LINC00958 knockdown; **C,** EdU-positive rate in KLE and Ishikawa cells with LINC00958 knockdown (magnification 20×); **D,** Migration in KLE and Ishikawa cells with LINC00958 knockdown (magnification 200×); **E,** Invasion in KLE and Ishikawa cells with LINC00958 knockdown (magnification 200×). *p<0.05; **p<0.01.

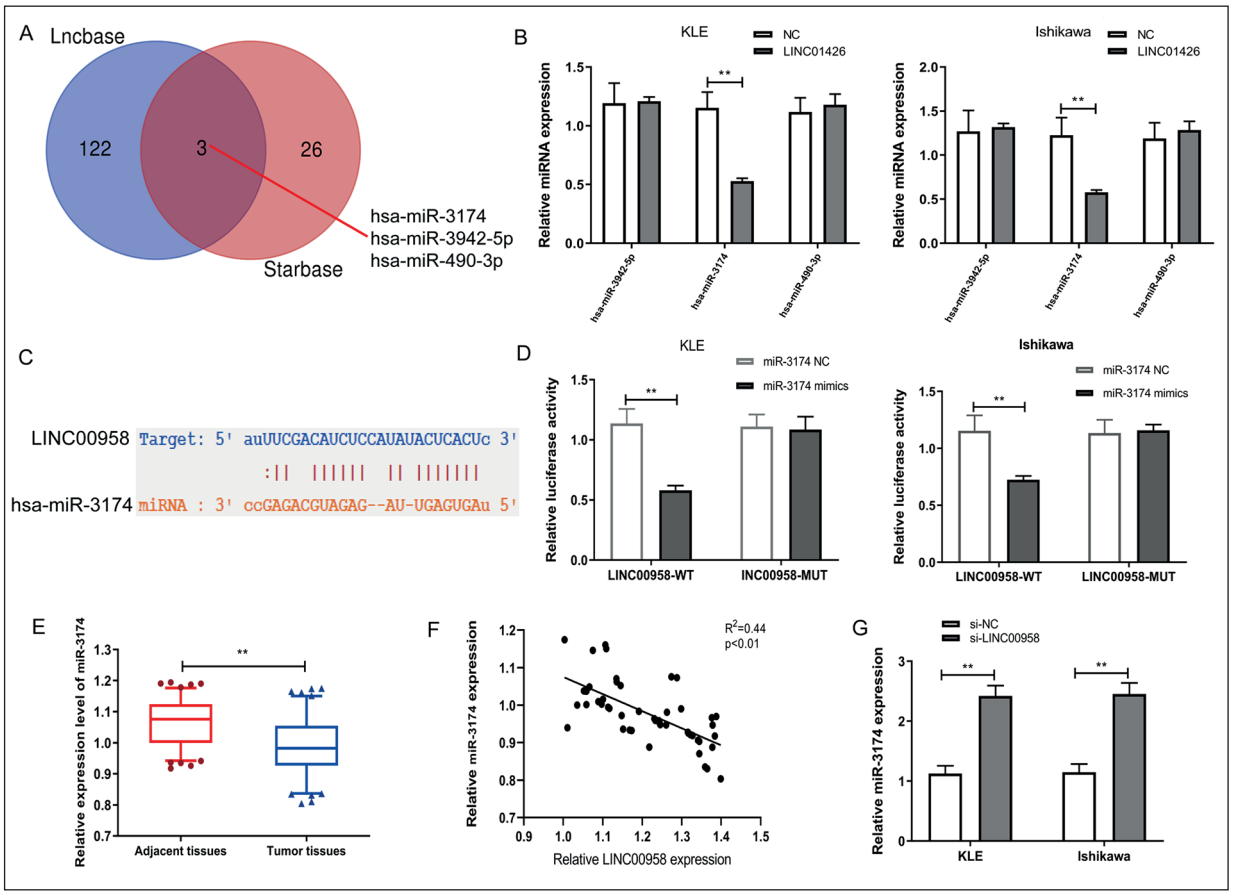

Figure 3. MiR-3174 could bind to LINC00958. **A,** Bioinformatic prediction of potential miRNAs that could bind to LINC00958 using Lncbase and Starbase; **B,** Relative levels of three candidate miRNAs in KLE and Ishikawa cells overexpressing LINC00958; **C,** Binding site in miR-3174 3'UTR that could bind to LINC00958; **D,** Luciferase activity in wild-type and mutant-type LINC00958 vectors regulated by miR-3174; **E,** MiR-3174 levels in EC and adjacent tissues; **F,** A negative correlation between miR-3174 and LINC00958; **G,** MiR-3174 level in KLE and Ishikawa cells with LINC00958 knockdown. **p*<0.05; ***p*<0.01.

miR-3174 was markedly downregulated in KLE and Ishikawa cells overexpressing LINC00958 (Figure 3B). According to the predicted binding site in miR-3174 3'UTR, Luciferase vectors were generated. Overexpression of miR-3174 was only able to decline Luciferase activity in LINC00958-WT, rather than the mutant-type one, confirming the binding between miR-3174 and LINC00958 (Figure 3C, D). MiR-3174 was lowly expressed in EC tissues, which had a negative correlation to LINC00958 expression (Figure 3E, F). As expected, knockdown of LINC00958 upregulated miR-3174 in EC cells (Figure 3G).

Overexpression of LINC00958 Reversed the Role of MiR-3174 in Regulating EC Cell Behaviors

Transfection of miR-3174 mimics effectively upregulated miR-3174 in KLE and Ishikawa cells, and the upregulated miR-3174 was further reduced by co-overexpressed LINC00958 (Figure 4A). Compared with those overexpressing miR-3174, EC cells co-overexpressing miR-3174 and LINC00958 had higher viability and EdU-positive rate (Figure 4B, 4C). Similar results were yielded in Transwell migration and invasion assay as well (Figure 4D, 4E). It is concluded that the role of miR-3174 in suppressing proliferative, migratory and invasive potentials in EC cells could be abolished by LINC00958.

PHF6 Was the Target Gene of MiR-3174

Using StarBase and TargetScan, PHF6 was analyzed as the target gene of miR-3174 with the highest binding grade (Figure 5A). Later, it was observed to bind miR-3174 3'UTR (Figure

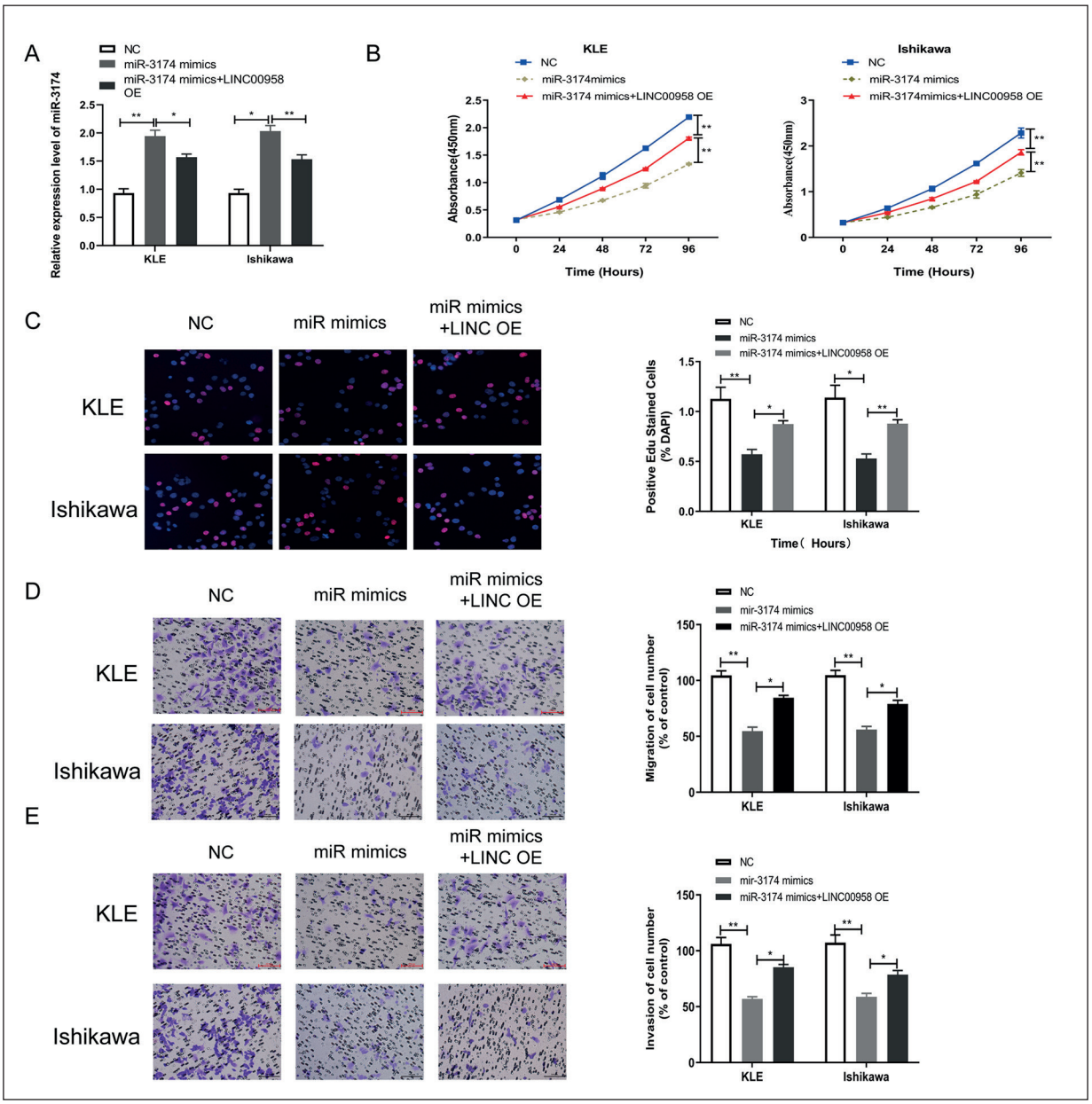

Figure 4. Overexpression of LINC00958 reversed the role of miR-3174 in regulating EC cell behaviors. **A,** Transfection efficacy of miR-3174 mimics and LINC00958 OE in KLE and Ishikawa cells; **B,** Viability in KLE and Ishikawa cells co-regulated by miR-3174 and LINC00958; **C,** EdU-positive rate in KLE and Ishikawa cells co-regulated by miR-3174 and LINC00958 (magnification 20×); **D,** Migration in KLE and Ishikawa cells co-regulated by miR-3174 and LINC00958 (magnification 200×); **E,** Invasion in KLE and Ishikawa cells co-regulated by miR-3174 and LINC00958 (magnification 200×). *p<0.05; **p<0.01.

5B, 5C). Compared with adjacent tissues, PHF6 was highly expressed in EC tissues (Figure 5D). Pearson correlation test uncovered that PHF6 was negatively correlated to miR-3174 level and positively correlated to LINC00958 level (Figure 5E).

Discussion

In recent years, with the increasing incidence of obesity and metabolic diseases, the incidence of EC is on the rise[16]. Early-stage EC patients usually have a high survival rate. However, ap-

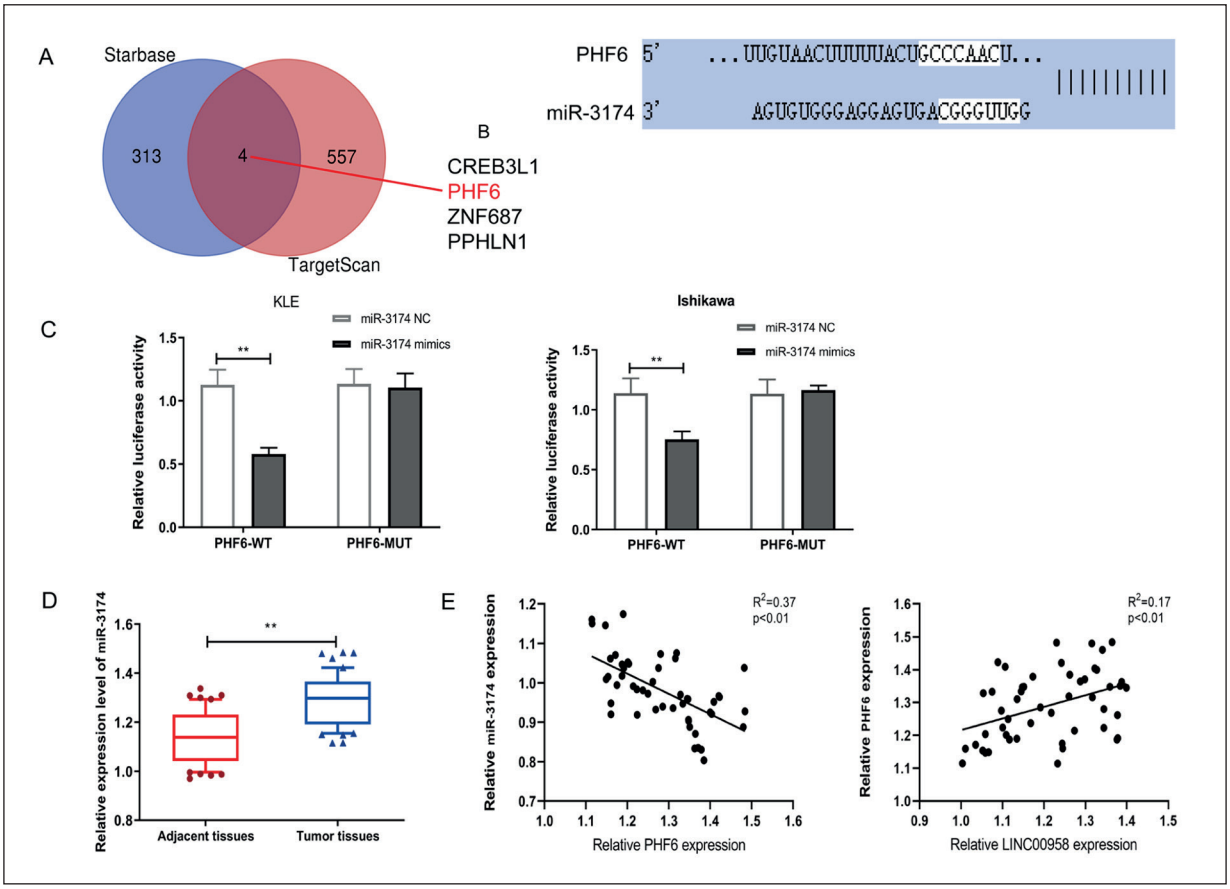

Figure 5. PHF6 was the target gene of miR-3174. **A,** Bioinformatic prediction of potential genes that could bind to miR-3174 using Starbase and TargetScan; **B,** Binding site in PHF6 3'UTR that could bind to miR-3174; **C,** Luciferase activity in wild-type and mutant-type PHF6 vectors regulated by miR-3174; **D,** PHF6 levels in EC and adjacent tissues; **E,** A negative correlation between PHF6 and miR-3174 (left), and a positive correlation between PHF6 and LINC00958 (right). *p<0.05; **p<0.01.

proximately 30% of patients are diagnosed in advanced stage and their 5-year survival is 77%[17,18]. Our findings showed that LINC00958 was abnormally upregulated in EC specimens. Its level was correlated to age, tumor staging, lymphatic metastasis and postoperative survival of EC patients. It is suggested that LINC00958 may be an oncogene in EC. On this basis, experimental research was conducted to reveal its biological role in the progression of EC.

By interacting with proteins, mRNAs or miRNAs, lncRNAs act as guidance molecules, scaffolds and sponges, thus forming complex networks that are functional in regulating cancer phenotypes[19]. LINC00958 is a cancer-relevant lncRNA. It mediates radiotherapy sensitivity by the miRNA-5095/RRM2 axis in cervical cancer[20]. Knockdown of LINC00958 protects pancreatic cancer from the malignant progression by binding miRNA-330-5p, and thus, downregulating PAX8[21]. Furthermore, LINC0095 acts as a ceRNA for miR-203 to regulate the expression of CDK2 for accelerating glioma progress[22]. In the present study, *in vitro* knockdown of LINC00958 markedly reduced proliferative, migratory and invasive rates of KLE and Ishikawa cells. A binding site was predicted in miR-3174 3'UTR that paired to LINC00958 sequences. Later, their binding relationship was indicated by dual-luciferase reporter assay. As the target gene being verified, miR-3174 was negatively correlated to LINC00958 and notably, it was able to reverse the regulatory effect of LINC00958 on EC cell phenotypes.

Through complementary base pairing, miRNAs bind to 3'UTR of the target mRNAs, resulting in translation inhibition or degradation of them[23]. PHF6 was proved as the target gene of miR-3174. PHF6 is a nuclear protein involved

in chromatin-mediated transcriptional regulation and is highly conserved among vertebrates, with a homology of 97.5% between human and mouse[24]. PHF6 is initially discovered as a single gene mutated in the X-linked intellectual disability, and its mutation is later identified in leukemia[25]. Serving as an oncogene, biological functions of PHF6 have been detected in other types of malignant tumors[26,27]. In addition, the function of LINC00958 in cell apoptosis, cell cycle and nude mice xenograft were not performed in our research. Notably, our findings only showed that PHF6 was the target gene binding miR-3174. Its potential role in EC progression should be analyzed in the future study. In short, we demonstrated the role and mechanism of LINC00958 in EC and provided a new therapy target in the future.

Conclusions

LINC00958 is upregulated in EC specimens, which is a prognostic factor of EC. It stimulates EC to proliferate, migrate and invade through the miR-3174/PHF6 axis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding support

This work was financed by the Qinghai University School of Medicine Young and Middle-aged Scientific Research Fund Project (Natural Science)(#. 2020-kyy-8) and the Qinghai Fundamental Scientific and Technological Research Plan (#. 2018-ZJ-730, 2019-SF-134).

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