Original Article
Long non-coding RNA derived miR-205-5p modulates human endometrial cancer by targeting PTEN

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Abstract: Objective: This study was to investigate the roles of lncRNA associated competitive endogenous RNAs (ceRNAs) network in the endometrial cancer (EC). Methods: StarbaseV2.0 online software was used to predict the most probable lncRNAs which contain miR-205-5p binding site and are competent to interact with miR-205-5p. Then, lncRNAs which had decreased expression in EC compared with normal endometrium and conformed to the polyadenylated characteristics of lncRNAs were selected and then lncRNAs associated with miR-205-5p-PTEN network were identified. Results: Two novel genes RP11-395G23.3 and LA16c-313D11.11 associated with endometrial cancer were identified and proved to be non-coding RNAs. They were more effective ceRNAs associated with the miR-205-5p-PTEN network. Conclusion: Our results suggest that lncRNAs harbor MREs (miRNA response elements) and play important roles in the post-transcriptional regulation in EC.

Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy and ranks the fourth most common cancer in women [1]. The majority of EC (72%) is diagnosed at an early stage (stage I-II); however, 28% of EC patients have regional or distant metastasis at initial diagnosis (20% in stage III and 8% in stage IV) [2]. Endometrial endometrioid cancer (EEC) is the dominant subtype of EC and accounts for approximately 80-90%. Despite recent advances in the surgical treatments and chemoradiotherapy, a lot of patients will develop recurrence and distal metastasis after these treatments [3]. Key mutational events have been characterized in EEC, but the underlying molecular mechanisms involving oncogenic or tumor suppressive factors remain poorly elucidated [4, 5]. Therefore, it is imperative to identify new therapeutic targets and develop effective strategies for the therapy of EC, which are dependent on an increased understanding of the molecular mechanisms involved in the pathogenesis of EC.

In recent years, long non-coding RNAs (lncRNAs) have been found to be of crucial functional importance in the pathogenesis of some diseases. LncRNAs are defined as endogenous cellular RNAs of more than 200 nucleotides in length with a myriad of molecular functions (such as modulation of alternative splicing, chromatin remodeling and RNA metabolism) [6-9]. A variety of studies have demonstrated that lncRNAs are involved in the development of different types of cancer [10-12]. Many lncRNAs have been identified as being cancer-specific [13-15] and thus may be employed as novel biomarkers or therapeutic targets for cancers.

Some lncRNAs play important roles in the regulation of gene expression by acting as competing endogenous RNAs (ceRNAs). The ceRNA hypothesis was proposed by Salmena and colleagues [16]. It describes a complex post-transcriptional regulatory network mediated by miRNAs: by sharing one or more miRNA response elements (MREs), protein-coding and noncoding RNAs compete for binding to miRNAs and then regulate each other's expression. That

was supported by numerous findings [17-21]. In addition, further investigation shows that lncRNAs are more effective ceRNAs without any interference with translation [16]. Linc-MD1, a lncRNA, can regulate the myoblast differentiation by competing for binding to miR-133 and miR-135 [22]. However, the roles of lncRNA associated-ceRNAs in the oncogenesis are not fully understood and the lncRNA-miRNA network in the EC remains unknown and needs further investigation.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression has been reported to reduce in EC [23], suggesting that it has a critical role in the pathogenesis of EC [24-27]. Previous studies have identified PTEN as one of direct targets of miR-205-5p [28, 29] and the miR-205-5p expression significantly increased in EC as compared to normal tissues. Kaplan-Meier survival analysis also reveals that high miR-205-5p expression is associated with a poor overall survival [30].

In this study, two novel genes RP11-395G23.3 and LA16c-313D11.11 associated with miR-205-5p-PTEN network were successfully identified in the EC. Our results may be helpful for the understanding of molecular mechanisms underlying the pathogenesis of EC as well as the potential role of non-coding RNA in EC.

Materials and methods

Ethics statement

Ethical approval for this study was obtained from the Obstetrics & Gynecology Hospital of Fudan University, China. All the subjects provided written informed consent for the collection of samples and subsequent analysis. This study was conducted according to the principles and guidelines expressed in the declaration of Helsinki.

Subjects

A total of 30 primary EC tissues and 30 normal endometrial tissues (NE) were recruited into this study. NE was obtained from women who underwent a hysterectomy or endometrial curettage for endometrial-unrelated diseases (such as uterine myoma or prolapse).

Detection of miRNA expression

RNA extracted from samples, RNA-tailing and primer extension, and real-time quantification of miR-205-5p were conducted as described

previously. Briefly, total RNA (1 μg) was polyadenylated with ATP and poly(A) polymerase (PAP) at 37°C for 1 h in a 20-μL reaction mixture following the manufacturer's instructions of the Poly(A) Tailing Kit (Ambion, USA). After phenol-chloroform extraction and ethanol precipitation, RNAs were reverse-transcribed using the specific RT primer and PrimerScript Reverse Transcriptase (Takara, Dalian, China) and the quantitative RT-PCR was performed using 2×SYBR Premix Ex Taq™ mixture (Takara, Dalian, China) with rat 5S ribosome RNA as the internal reference RNA. The primers were designed using a primer designing software package (miR-205-5p forward: 5'-TCCACCGG-AGTCTGTCTCAT; reverse: 5'-GCTGTCAACGATA-CGCTACG). PTEN mRNA expression was examined using specific primers (forward: 5'-ACC-AACTGAAGTGGCTAAAGAG; reverse: 5'-GGTCCA-GAGTCCAGCATAAAA). GAPDH mRNA was used as an internal control.

Western-blot assay

The sample lysate was centrifuged at 10,000×g for 10 min at 4°C and the supernatant was collected for Western blot assay. Total protein was separated by SDS-PAGE on 10% polyacrylamide gels and then transferred onto Hybond-P polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membrane was then incubated with anti-Pten primary antibody (1:10,000; Cell Signaling, CA, USA) overnight at 4°C. Protein bands were visualized using IR Dye 800 conjugated secondary antibody of rabbit IgG (Rockland, Philadelphia, USA). Images were captured and band density was analyzed using Odyssey Infrared Imaging System (LI-COR Biosciences, USA).

LncRNA prediction and expression detection

The has-miR-205-5p miRNA and its target lncRNA were predicted by bioinformatics based on the fact that target gene has the conserved or non-conserved sites matching the seed region of corresponding candidate miRNA to form RNA secondary structure. The starBase 2.0V software was sued to predict the lncRNA for miR-205-5p. The expression of each lncRNA was normalized to that of rRNA U6 and the relative expression was calculated with the comparative CT method.

Construction of plasmids

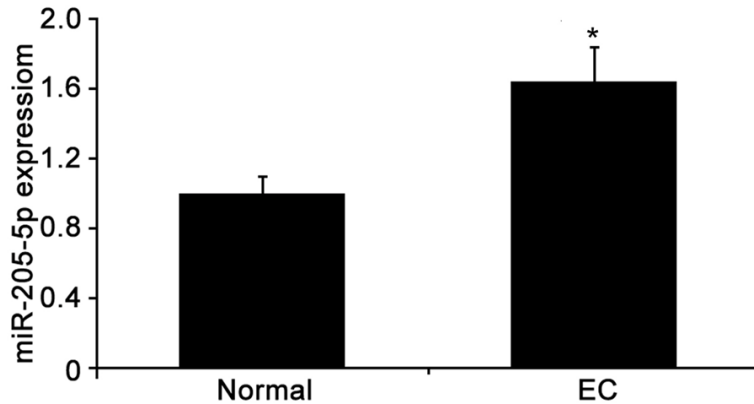According to the DNA sequence of pre-miR-205-5p and the multiple clone site of pSuper

Figure 1. miR-205-5p expression in EC tissues (*P<0.05). miR-205-5p significantly increased in EC tissues as compared to NE tissues.

EGFP1, the PCR primers designed to amplify this miRNA and the PCR fragments after BamH I and Hind III digestion were subcloned into the pSuper EGFP1 vector, and the resultant recombinant was named pSuper-205-5p. The fragment containing the miR-205-5p binding site of candidate lncRNA was subcloned into the XbaI restriction site downstream of the Firefly luciferase gene of pGL3-Basic Vector (Promega) as pGL3-3'UTR. pSuper EGFP1 was used as the internal control for the following transfection experiments.

Luciferase assay

The pRL-TK vector (Promega, USA) containing Renilla luciferase gene was used as an internal control reporter vector. The constructed luciferase reporter plasmids and the pRL-TK vectors were co-transfected into the HEK293T cells at a ratio of 50:1. Luciferase activity was measured at 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions. Firefly luciferase activity was normalized to the renilla luciferase activity.

Statistical analysis

SAS version 8.2 was used for the statistical analysis. Comparisons of data from quantitative RT-PCR and Western blot assay were done with analysis of variance (ANOVA). All the data are shown as means ± standard deviation (SD) from three experiments. Two-tailed Students' test was employed for comparisons between two groups. A value of $P<0.05$ was considered statistically significant.

Results

Identification of endogenous lncRNA for miR-205-5p in EC

miRNAs are important regulators in the EC. Previous studies have identified that miR-205-5p determines the radio-resistance of human nasopharyngeal carcinoma by directly targeting PTEN. Thus, the expressions of miR-205-5p and PTEN were detected in EC tissues (n=30) and NE tissues (n=30). Results showed that miR-205-5p significantly increased in EC tissues (**Figure 1**), and the mRNA and protein expressions of PTEN markedly reduced in EC tissues as compared to that in NE tissues (**Figure 2**). These indicate that miR-205-5p regulates PTEN expression at post-transcriptional level by inducing mRNA cleavage, which was consistent with previously published.

LncRNAs are a large group of non-encoding RNAs and represent a new group of regulators in the complex mammalian gene regulatory networks, but they remain poorly characterized. Recent studies suggest that lncRNAs have the binding site of miRNAs and may interact with miRNAs to influence the functions of miRNA. To understand which lncRNA may act as endogenous sponge RNAs to interact with miR-205-5p in EC, starbaseV2.0 online software was employed to predict the most probable lncRNAs of miR-205-5p. Using the default parameter settings, 29 putative target lncRNA for miR-205-5p were identified (**Figure 3**). Then, RT-PCR was performed for the detection of 29 lncRNAs expression, and 21 were found to be polyadenylated (**Figure 4**). Meanwhile, quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expressions of these lncRNAs. Results indicated that, as compared to NE tissues, 8 lncRNAs showed up-regulated expression, while 21 lncRNAs displayed down-regulated expression in EC tissues (**Figure 5**).

Recently, the ceRNA hypothesis is proposed and assumes that a large number of non-coding RNA may function as molecular sponges for miRNAs and hence functionally liberate other RNA transcripts targeted by aforementioned active miRNAs. Thus, altering the expression of
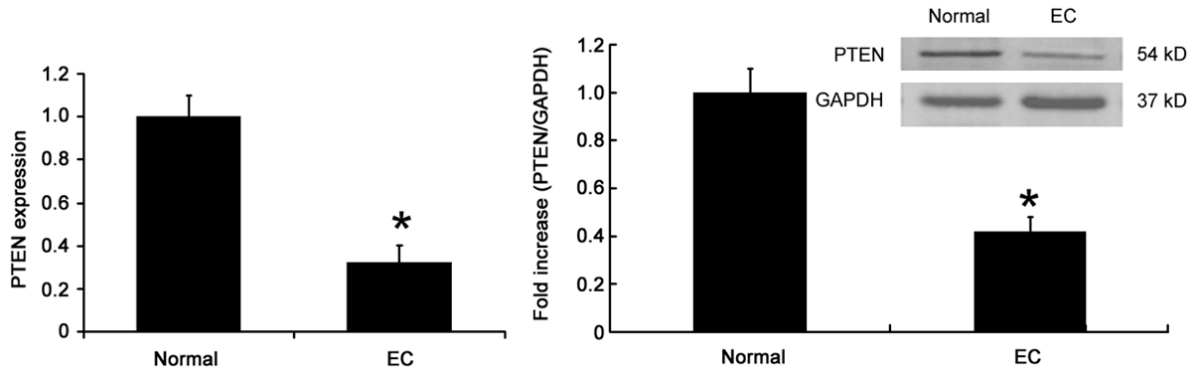
Figure 2. PTEN expression in EC patients (*P<0.05). The mRNA and protein expressions of PTEN markedly reduced in EC tissues when compared with NE tissues.

Figure 3. Prediction of lncRNA interacted with miR-205-5p. Using the default parameter settings, starbaseV2.0 identified 29 putative target lncRNA for miR-205-5p.
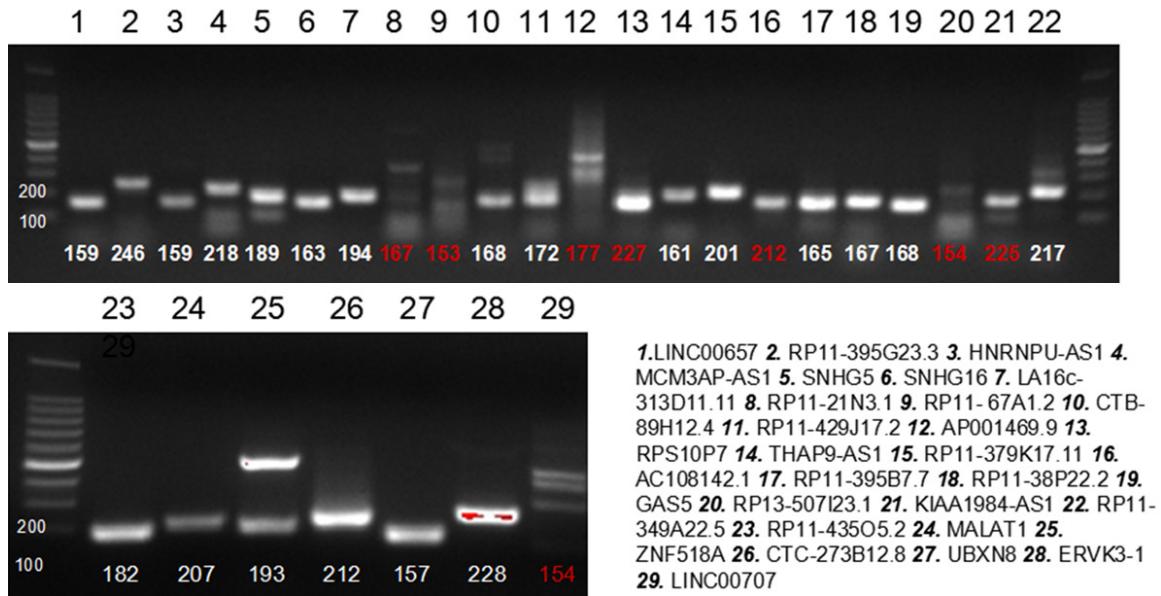
Figure 4. PolyA qualitative analysis of candidate lncRNA (strips marked with red number have not a PolyA tail). RT-PCR was performed for these 29 lncRNAs expression and 21 were found to be polyadenylated.

Figure 5. Expression of candidate lncRNA in NE tissues and EC tissues. qRT-PCR was used to detect the expression of 29 lncRNAs. Results indicated that, when compared with NE tissues, 8 lncRNAs displayed up-regulated expression, while 21 lncRNAs showed down-regulated expression in EC tissues.
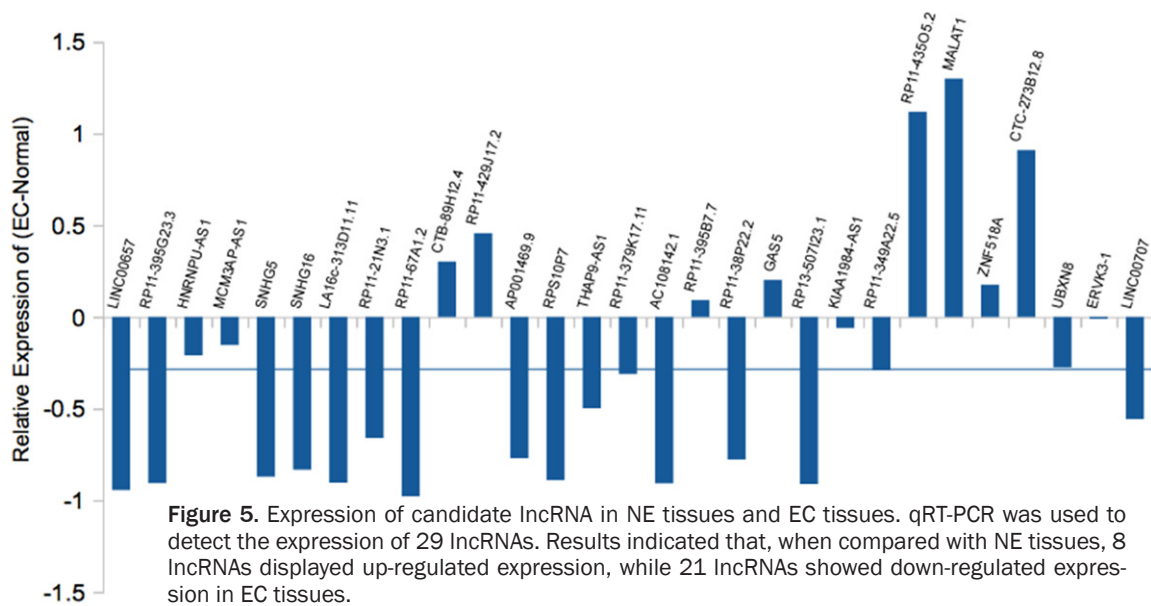
an individual ceRNA would have repercussions on the target genes with which it shares the MREs. As proposed by the ceRNA hypothesis, down-regulated ceRNA decreases the cellular concentrations of particular MREs and can improve the repression of other transcripts that contain the same MREs. According to the PTEN expression in EC, we speculated that lncRNA with the same MREs of miR-205-5p should have a similar expression profile to PTEN. Based on this hypothesis and above findings,

13 lncRNAs with decreased expression were selected as the candidate lncRNAs: LINC00657, RP11-395G23.3, HNRNPU-AS1, MCM3AP-AS1, SNHG5, SNHG16, LA16c-313D11.11, THAP9-AS1, RP11-379K17.11, RP11-38P22.2, RP11-349A22.5, UBXN8 and ERVK3-1.

miR-205-5p targets lncRNAs-RP11-395G23.3 and LA16c-313D11.11 in EC

Bioinformatic analysis revealed above 13 candidate lncRNAs contained one conserved tar-

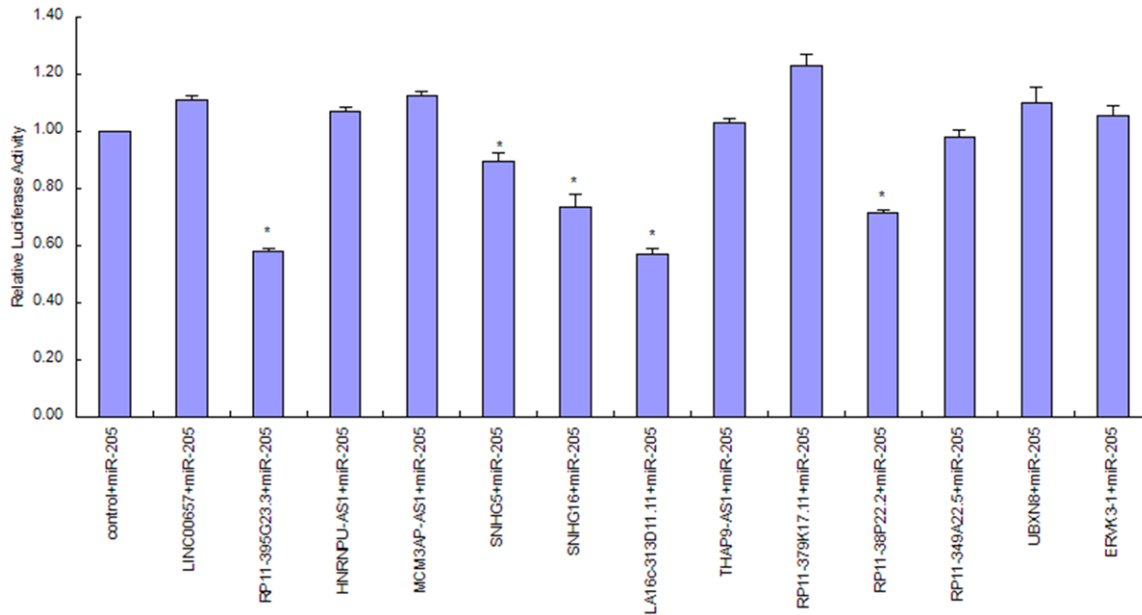**Figure 6.** Luciferase assay of candidate lncRNA (*P<0.05). Luciferase assay revealed that miR-205-5p over-expression could significantly reduce the Renilla luciferase activities of RP11-395G23.3, SNHG5, SNHG16, LA16c-313D11.11, and RP11-38P22.2.

get site of miR-205-5p. To verify the interaction between lncRNAs and miR-205-5p, the binding sites of 13 lncRNAs were cloned into luciferase reporter PGL3 vector. The constructs with miR-205-5p mimics were co-transfected into Hep3B cells. Luciferase assays revealed that miR-205-5p over-expression could significantly reduce the Renilla luciferase activities of RP11-395G23.3, SNHG5, SNHG16, LA16c-313D11.11 and RP11-38P22.2 (**Figure 6**). To confirm this putative target site of miR-205-5p, deletion assay was performed. Luciferase assay indicated that miR-205-5p over-expression had no influence on the deleted RP11-395G23.3 and LA16c-313D11.11 lncRNAs as compared to the wild-type counterparts. Furthermore, the over-expression of RP11-395G23.3 (RP11-395G23.3 wild-type) and LA16c-313D11.11 (LA16c-313D11.11 wild-type) resulted in down-regulated expression and activity of miR-205-5p.

However, no significant change was observed between cells transfected with PGL-3 empty vector and RP11-395G23.3 without miR-205-5p target site (RP11-395G23.3-Del-205), which may be ascribed to a higher expression of miR-205-5p in EC tissues. These reveal that RP11-395G23.3 may inhibit both expression and activity of miR-205-5p via this putative binding

site at post-transcription level in both normal and cancer tissues. Same results were also found in LA16c-313D11.11.

Discussion

LncRNAs have been recognized as a regulator of gene expression, and the deregulation of lncRNAs have been reported to be correlated with the carcinogenesis and cancer progression. Pathway and gene ontology analyses demonstrate that these deregulated transcripts are involved in multiple signal pathways, biological processes, cellular components and molecular functions. Recently, accumulating evidence on lncRNA has indicated that dysregulation of lncRNA may not only affect the regulation of the eukaryotic genome, but is helpful for the growth of malignant cells, resulting in the progressive and uncontrolled growth of cancer cells [31, 32]. Cancer susceptibility candidate 2 (CASC2), an lncRNA whose gene is mapped to chromosome 10q26, was first identified as a down-regulated gene in EC and to act as a tumor suppressor gene as well. Genomic and cDNA sequence comparisons reveal the presence of three alternatively spliced CASC2 transcripts (CASC2a, CASC2b and CASC2c) that share the first three exons but contain different downstream exons [33]. Exogenous expression

of CASC2a in undifferentiated EC cells may significantly inhibit the clonal growth [34]. ASLNC04080 is an lncRNA with the most significantly up-regulated expression in the microarray assay. It is highly expressed in 22 out of 24 EC tissues and HEC-1-B cells. ASLNC04080 expression is correlated with 19 coding genes, and may contribute to the pathogenesis and progression of EC by co-regulating with coding genes. The expression inhibition of ASLNC04080 in HEC-1-B cells may inhibit cell proliferation, increase cell apoptosis, and arrest cells in G1 phase [35]. MALAT1 transcription is regulated by the Wnt/β-catenin signaling pathway via binding to TCF promoter, and PCDH10 may decrease MALAT1 by modulating this pathway. Clinically, MALAT1 expression is associated with multiple parameters in patients with EEC [36]. A recent study of Huang et al showed that HOTAIR expression was higher in EC cells and EC tissues than in NE tissues, and the down-regulation of HOTAIR resulted in a significant inhibition of EC cell proliferation, migration, and invasion and also arrested cells in G0/G1 phase [37]. HOTAIR can regulate gene expression in many ways, including chromosome remodeling, transcription, and posttranscriptional modification [38]. The HOTAIR functions are linked to cancer progression, metastasis, and prognosis. He et al [39] found that HOTAIR expression in EC tissues was significantly up-regulated as compared to normal tissues. Furthermore, HOTAIR depletion significantly suppressed the EC tumorigenesis *in vivo* [37]. HOTAIR is aberrantly expressed and hormonally regulated in EC and may either directly or indirectly regulate a number of genes whose products are critical for the EC growth and regression as well as their cellular transformation [40].

Emerging evidence suggests that lncRNAs may participate in the ceRNA regulatory network and act as endogenous miRNA sponges to bind to miRNAs and then regulate their function [22, 41]. Zhou et al [42] found that Linc-RoR was a ceRNA and acted as a miR-145 "sponge" to inhibit the miR-145 mediated differentiation of endometrial tumorspheres. These suggest that linc-RoR has an important role in the endometrial carcinogenesis.

In this study, starbaseV2.0 online software was used to predict the most probable lncRNAs for miR-205-5p. Then, the sequence was blasted and lncRNAs containing miR-205-5p binding site and competent to interact with miR-205-5p were selected. The expressions of these lncRNAs were verified with two methods-quantitative and qualitative PCR (qPCR and RT-PCR). The lncRNAs with decreased expression in EC tissues as compared to NE tissues and being conformed to the polyadenylated characteristics of lncRNAs were screened. Finally, 13 lncRNAs (LINC00657, RP11-395G23.3, HNRNPU-AS1, MCM3AP-AS1, SNHG5, SNHG16, LA16c-313D11.11, THAP9-AS1, RP11-379K-17.11, RP11-38P22.2, RP11-349A22.5, UBXN8 and ERVK3-1) were selected as the candidate lncRNAs. on the expressions of these 13 lncRNAs were detected in EC tissues. Results showed that these 13 lncRNAs might be associated with miR-205-5p-PTEN network. We speculate that these 13 lncRNAs may act as endogenous sponge RNAs to interact with miR-205-5p to suppress miR-205-5p and maintain the totipotency of cells. Linc-MD1 (a long noncoding RNA, also known as muscle differentiation 1) is one of the first lncRNAs having a role in the myogenesis, and able to control the progression of muscular cells from early to late phases in the muscle differentiation by functioning as a ceRNA. Through competing for the binding of miR-133 and miR-135, it regulates the expressions of mastermind-like protein 1 (MAML1) and myocyte-specific enhancer factor 2C (MEF2C) [22]. The linc-MD1 expression is up-regulated before miR-133 during the muscle differentiation.

Luciferase assay and biotin–avidin pull-down assay confirmed that miR-205-5p could bind to RP11-395G23.3 and LA16c-313D11.11 directly via the putative MRE. MRE was identified to be a highly conserved sequence, suggesting that MRE may be an important functional sequence element.

In conclusion, we for the first time identify two novel genes RP11-395G23.3 and LA16c-313D11.11 associated with the pathogenesis of EC which are proven to be noncoding RNAs. They are effective ceRNAs associated with miR-205-5p-PTEN network. Further studies are required to investigate the roles and functions of RP11-395G23.3 and LA16c-313D11.11 in EC, which may improve our understanding of the molecular mechanisms involved in the pathogenesis of EC and is helpful for the identification of new diagnostic and therapeutic targets for the treatment of EC.

Disclosure of conflict of interest

None.

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