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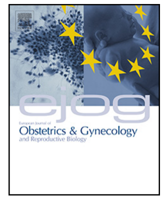
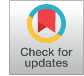


Full length article

Aberrant expression of CHL1 gene and long non-coding RNA CHL1-AS1, CHL1-AS2 in ovarian endometriosis



Chen Zhang[a], Wei Wu[b], Xue Ye[a], Ruiqiong Ma[a], Jianjun Luo[b], Honglan Zhu[c,**], Xiaohong Chang[a,*]

[a] Center of Gynecologic Oncology, Peking University People's Hospital, Beijing, China
[b] Key Laboratory of RNA Biology, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China
[c] Department of Obstetrics and Gynecology, Peking University People's Hospital, Beijing, China

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ABSTRACT

Objective(s): CHL1 (close homologue of L1 or cell adhesion molecule L1 like), also referred as CALL, is a member of the L1 gene family of neural cell adhesion molecules and belongs to immunoglobulin superfamily. This study aims to investigate the potential correlation of the CHL1 gene and the long non-coding RNAs (lncRNAs), *i.e.*, CHL1-AS1 and CHL1-AS2, and to validate the expression patterns of CHL1 and CHL1-AS2 in ovarian endometriosis (EM).

Study design: Our previous microarray analyses (GSE86534) of 4 patients with ovarian EM indicated that CHL1 was the most upregulated mRNA in ectopic endometrium (EC) compared with eutopic endometrium (EU) tissues, and that its two antisense lncRNAs CHL1-AS1 and CHL1-AS2, exhibited the same expression pattern. We used a bioinformatics-based strategy to calculate the correlation among CHL1, CHL1-AS1 and CHL1-AS2. Gene set enrichment analysis (GSEA) was performed to analyze commonly enriched gene sets for CHL1-AS1 and CHL1-AS2. Using quantitative real-time polymerase chain reaction (qPCR), we examined the expression levels of CHL1 mRNA and lncRNA CHL1-AS2 in paired tissues of EC and EU from 30 EM patients and normal endometrium (NE) tissues from 27 controls using quantitative real-time polymerase chain reaction (qPCR). We also examined the expression of CHL1 protein in EC, EU and NE tissues using western blotting and immunohistochemistry (IHC).

Results: CHL1, CHL1-AS1 and CHL1-AS2 were significantly correlated with each other given that the Pearson correlation values were > 0.9 using bioinformatic calculation. GSEA revealed that CHL1-AS1 and CHL1-AS2 were negatively associated with the same gene set "WAMUNYOKOLI_OVARIAN_CANCER_LMP". qPCR confirmed that the CHL1 and CHL1-AS2 expression levels were significantly higher in EC tissues than in EU and NE tissues, while they were not significantly different in EU compared with NE tissues. The relative expression levels of CHL1 and CHL1-AS2 in EC compared with EU tissues were positively significantly correlated (Pearson correlation coefficient = 0.421 and P value = 0.02). Elevated expression of CHL1 protein in EC tissues was detected by western blotting. IHC revealed that CHL1 protein expression levels enhanced in ectopic endometrial glands and stroma.

Conclusion(s): Our results indicate a significant correlation among CHL1, CHL1-AS1 and CHL1-AS2, which might be involved in the development of ovarian EM and serve as novel targets for future research.

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Introduction

Endometriosis (EM), a common chronic and benign disease, profoundly affects the physical and mental health of women of child-bearing age. Recently, some scholars proposed that abnormal endometrial tissue in EM patients may have the ability to undergo a 3-step procedure of pathogenesis (attachment-aggression-angiogenesis) and finally develop into EM [1]. In addition, genetic factors play a significant role in EM [2,3]. Despite extensive investigations, the pathogenesis of EM remains poorly understood.

* Corresponding author at: Center of Gynecologic Oncology, Peking University People's Hospital, No.11 Xizhimen South Str., Xicheng District, Beijing 100044, China.
** Corresponding author at: Department of Obstetrics and Gynecology, Peking University People's Hospital, No. 11 Xizhimen South Str., Xicheng District, Beijing 100044, China.
E-mail addresses: honglan_zhu@sina.com (H. Zhu), changxiaohong@pkuph.edu.cn (X. Chang).

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Therefore, understanding of the underlying mechanisms of EM from different perspectives is urgently needed, which will advance progress towards better and more effective treatments.

Long non-coding RNAs (lncRNAs) are greater than 200 nucleotides (nt) in length and have no protein-coding potential. Recent studies have demonstrated that lncRNAs engage in numerous biological processes and are involved in human diseases, including EM [4–6]. The functional mechanisms of lncRNAs are diverse, as they serve as signals, decoys or scaffolds, and act through genomic targeting, *cis* or *trans* regulation, and antisense interference [4,7]. H19, the first reported lncRNA, contributes to EM-associated infertility by regulating the H19/Let-7/IGF1R pathway [5]. However, the functions of most lncRNAs have not yet been determined. One of the most common ways to predict and investigate their functions is through analyzing their co-expressed protein-coding genes and related biological pathways [8]. For instance, Sun PR et al. predict that the lncRNA HOXA11-AS1 possibly cis-regulates the expression of HOXA10 mRNA [9].

Our previous microarray analyses (GSE86534) [10] compared the lncRNA/mRNA expression profiles of paired ectopic endometrium (EC) and eutopic endometrium (EU) samples from 4 EM patients. We found that CHL1 and its two antisense lncRNAs, CHL1-AS1 and CHL1-AS2, were significantly upregulated in EC compared with EU tissues and that the log2-fold change was 6.77, 4.11 and 2.21, respectively. CHL1 is a member of the L1 gene family of neural cell adhesion molecules and belongs to immunoglobulin superfamily. The deletion of one copy of CHL1 may be responsible for mental defects in 3p-syndrome [11]. Several studies have shown that CHL1 is involved in the progress of different cancers [12–14]. It was reported that CHL1 acted as a tumour suppressor in human neuroblastoma [12], but as a malignancy promoter in glioma [14]. CHL1-AS1 and CHL1-AS2 are antisense lncRNAs located on human chromosome 3: 405056-427478 and human chromosome 3: 237440-238542, respectively. Until now, no studies have reported the functions of CHL1-AS1 and CHL1-AS2.

In this study, we presented the expression patterns of CHL1 and CHL1-AS2 in EC, EU and normal endometrium (NE) tissues. We also investigated the possible correlation amongCHL1, CHL1-AS1 and CHL1-AS2 by bioinformatic analyses, and predicted the potential functions of these two lncRNAs by analysing their co-expressed protein-coding genes.

Materials and methods

This study was approved by the Medical Ethics Committee of Peking University People's Hospital. Written informed consent was obtained from each enrolled subject.

Patients characteristics and samples

Paired EC and EU samples were collected from 31 patients with pathologically confirmed EM at Peking University People's Hospital from July 2014 to March 2018. EU tissues were obtained from the cyst wall and EU tissues from the same patients were simultaneously collected by curettage. As control, NE samples were obtained from 30 patients undergoing surgery for tubal diseases or uterine leiomyoma during the same time period. The absence of EM was confirmed by laparoscopic surgery examination. Detailed clinical characteristics are presented in Table 1. After the samples were harvested, half of each specimen was snap-frozen in liquid nitrogen and stored at −80 °C, while the other half was fixed in 4% paraformaldehyde (PFA) and prepared for histopathological observation. The menstrual cycle was identified according to endometrial histological examination. Subjects had not taken hormone therapy for at least 3 months.

Table 1
Description of the study subjects.

Study subjects		Controls	EM
n[a]	61	30	31
Age(years)	61	40.47 ± 8.28	32.13 ± 5.39
Gravidity			
0	61	5 (16.7)	22 (71.0)
≥1		25 (83.3)	9 (29.0)
Parity			
0	61	11 (36.7)	25 (80.6)
≥1		19 (63.3)	6 (19.3)
Cycle phase			
Proliferative	59	21 (75.0)	23 (74.2)
Secretory		7(25.0)	8 (25.8)
rAFS stage			
II			1
III			15
IV			15

EM, endometriosis; rAFS, revised American Fertility Society classification.
Age is presented as mean ± SD; the other variables are presented as the number (%).
[a] n means the number of cases with complete clinical data.

Bioinformatic analyses

Gene Set Enrichment Analysis (GSEA) is a powerful computational method that interprets gene expression data and determines whether a set of genes shows a statistically significant difference between two biological states [15]. GSEA analyses reveal significant difference with a false discovery rate (FDR) q-value < 0.05 in enrichment using the Molecular Signature Database (MSigDB) Collection. The FDR q-value and normalized enrichment score (NES) were used to sort the pathways enriched in each phenotype. In this study, we performed GSEA to extract commonly enriched gene sets for CHL1-AS1 and CHL1-AS2 using our previous microarray data (GSE86534). Pearson's correlation coefficients of all mRNAs based on their expression levels in all samples were calculated as the weight for CHL1-AS1 and CHL1-AS2. A positive/negative NES with a higher value indicates higher positive/negative correlation between the lncRNA and the pathway. We also used the R language to calculate the correlation among CHL1, CHL1-AS1 and CHL1-AS2.

RNA isolation and quantitative real-time PCR validation

Total RNA was isolated from approximately 100 mg of tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The concentration of RNA was measured using a spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific, Wilmington, DE, USA). RNA purity was evaluated by the ratio of absorbance at 260 nm to that at 280 nm (A_{260}/A_{280}) and its integrity was determined by agarose gel electrophoresis and Gene Green staining (Tiangen Biotech, Beijing, China). Reverse transcription was carried out using a FastQuant RT Kit (with gDNase) (Tiangen Biotech, Beijing, China) under the manufacturer's instructions. Because primer design for CHL1-AS1 failed multiple times, we detected the relative expression ratio of CHL1 and CHL1-AS2 in this study. Recently, endometrial tissue in EM patients was found to be different from its counterpart in healthy controls [1], and therefore, we also included NE tissues. We validated the expression patterns of CHL1 and CHL1-AS2in samples from 30 ovarian EM patients and 27 controls by quantitative real-time polymerase chain reaction (qPCR). The reactions were run in a Bio-Rad CFX Connect Real-Time PCR System using Power SYBR® Green PCR Master Mix (Applied Biosystems, Austin, TX, USA) and were performed under the following conditions: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The

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Table 2
Primers for quantitative real-time PCR.

ID	Forward (5′-3′)	Reverse (5′-3′)
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
CHL1	TTACCAACCTTCAACCAA	GACATCCACAACATCAATA
CHL1-AS2	GGTGTGGTGCCATGATAC	TCCAACTCTGAGGGTAGC

primers were synthesized by Sangon Biotech (Beijing, China) and are listed in Table 2. Each sample was run in triplicate. The expression levels of CHL1 and CHL1-AS2 were normalized using GAPDH as the endogenous control and were expressed as fold changes using the $2^{-\Delta\Delta Ct}$ method. Specificity of amplification was determined by melting curve analysis and the absence of amplification in the no-template control.

Western blot analysis

Total proteins from tissues were collected under the manufacturer's instructions (CWBIOTECH, Beijing, China). Paired EC and EU tissues from 6 ovarian EM patients, and NE tissues from 6 controls were used for western blotting. The protein content was quantified using a BCA assay kit (CWBIOTECH, Beijing, China). Forty micrograms total protein was subjected to 10% SDS-PAGE gel for electrophoresis and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA) with the use of a Transblot apparatus (Bio-Rad, Hercules, CA, USA). The membranes were blocked at room temperature for 1 h with 5% bovine serum albumin, and were then incubated with the anti-CHL1 antibody (ab106269, 1:1000 dilution; Abcam) and anti-GAPDH antibody (ab181602, 1:5000 dilution; Abcam) at 4 °C overnight. Next day, the membranes were incubated with a goat anti-rabbit horseradish peroxidase-COnjugated antibody (ZB-2301, ZSGB-BIO, China) at a 1:5000 dilution for 1 h at 37 °C. Proteins were quantified using a LI-COR Odyssey Infrared Imaging System (Lincoln, NE, USA), and GAPDH was used as an internal control.

Haematoxylin-eosin staining and immunohistochemistry

To investigate the location of the CHL1 protein, we performed immunohistochemistry (IHC) in paired EC and EU tissues from 1 ovarian EM patient, and NE tissue from 1 control. Tissues were fixed in 4% paraformaldehyde (PFA), embedded in paraffin and cut into 4-μm-thick sections for haematoxylin-eosin staining and IHC. Slides were deparaffinized in xylene and rehydrated through gradient ethanol solutions. Endogenous peroxidase activity was quenched for 15 min using endogenous enzyme blocking solution. For heat–induced antigen retrieval, slides were boiled in citrate buffer (pH 6.0) in a microwave oven for 15 min and were even incubated in a humidified chamber with 5% goat serum (ZLI-9056; ZSGB-BIO, China) for 60 min at 37 °C. Since the anti-CHL1 antibody (from Abcam) that was used for western blotting failed to have satisfactory result on IHC, another antibody from Bioss was used. Slides were incubated with the antibody against CHL1 (bs-11046R, 1:400 dilution; Bioss, Beijing, China) overnight at 4 °C. For the negative control, normal rabbit immunoglobulin (IgG) isotype was used at the same concentration as the primary antibody. The following day, the sections were incubated with goat anti-rabbit antibody working solution (PV-6001; ZSGB-BIO, China) for 30 min at 37 °C. The exposure time to 3,3'-diaminobenzidine chromogen solution for all slides was 6 min. Slides were counterstained in haematoxylin (ZSGB-BIO, China) for 2 min, dehydrated through gradient ethanol solutions and xylene, and mounted using neutral gum sealant. The images were observed and captured under a microscope (Nikon Eclipse 50I, Japan). Image J software was used to semi-quantitatively analyse the area percentage of positive staining of CHL1 in tissues.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 7.0 software. Data are presented as the means ± standard deviation (SD). The Kolmogorov-Smirnov test was used to test normality. Comparisons between multiple groups were performed using a one-way analysis of variance (ANOVA) test. *P*-values of 0.05 or less were considered significant.

Results

Bioinformatic analyses

Our previous microarray data (GSE86534) demonstrated that CHL1 and its two antisense lncRNAs, CHL1-AS1 and CHL1-AS2, were significantly higher expressed in EC than in EU tissues. The genome browser view of the CHL1, CHL1-AS1 and CHL1-AS2 loci is shown in Fig. 1A. Bioinformatic analyses showed that CHL1, CHL1-AS1 and CHL1-AS2 were significantly correlated with each other for all the Pearson correlation values > 0.9 (Fig. 1B). Based on the expression profiles from the microarray, we performed GSEA to examine the enrichment of identified gene sets related to CHL1-AS1 and CHL1-AS2. GSEA analyses demonstrated that CHL1-AS1 and CHL1-AS2 were both positively related to the gene set “WAMUNYOKOLI_OVARIAN_CANCER_LMP_DN” (NES = 2.69 and 2.82, respectively, for CHL1-AS1 and CHL1-AS2), while they were negatively related with “WAMUNYOKOLI_OVARIAN_CANCER_LMP UP” (NES = -3.37 and -3.18, respectively, for CHL1-AS1 and CHL1-AS2) (Fig. 1C–F).

Increased CHL1 and CHL1-AS2 RNA expression in ectopic endometrium tissues

To verify the microarray data, we used qPCR to investigate the expression levels of CHL1 and CHL1-AS2 in 30 EM patients and 27 normal controls. We found that CHL1 and CHL1-AS2 were significantly higher expressed in EC than in EU (both $P < 0.0001$) and NE (both $P < 0.0001$) samples, while their expression levels in EU tissues were not significantly different from those in NE tissues (both $P = 0.9999$) (Fig. 2A and B). Statistical analyses indicated that the expression of CHL1 in EC tissues was positively correlated with that of CHL1-AS2 (Pearson correlation coefficient $r = 0.421$) ($P = 0.02$, Fig. 2C).

Increased CHL1 protein expression in ectopic endometrium tissues

The expression of CHL1 at the protein level was detected by western blotting and IHC. Western blotting demonstrated that the expression of CHL1 protein was higher in EC tissues than in EU ($P < 0.0001$) and NE ($P < 0.0001$) tissues, while its expression was not significantly different in EU compared with NE tissues ($P = 0.9989$) ($n = 6$, Fig. 3A and B). We further assessed the localization of the CHL1 protein in EC, EU and NE tissues using IHC, which further verified the western blotting results. We observed that the staining intensity of CHL1 protein was stronger in the cytoplasm of glandular and stromal cells in EC tissues than in EU and NE tissues. Semi-quantitative analysis showed that the area percentage of positive staining for the NE, EU and EC tissues were 9.70%, 18.53% and 27.98%, respectively (Fig. 4).

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Fig. 1. Characterization of CHL1, CHL1-AS1 and CHL1-AS2. (A) Genome browser view of CHL1, CHL1-AS1 and CHL1-AS2 loci. (B) Pearson correlation coefficients of CHL1, CHL1-AS1 and CHL1-AS2 calculated by the R language. GSEA analyses of CHL1-AS1 (C and D) and CHL1-AS2 (E and F).

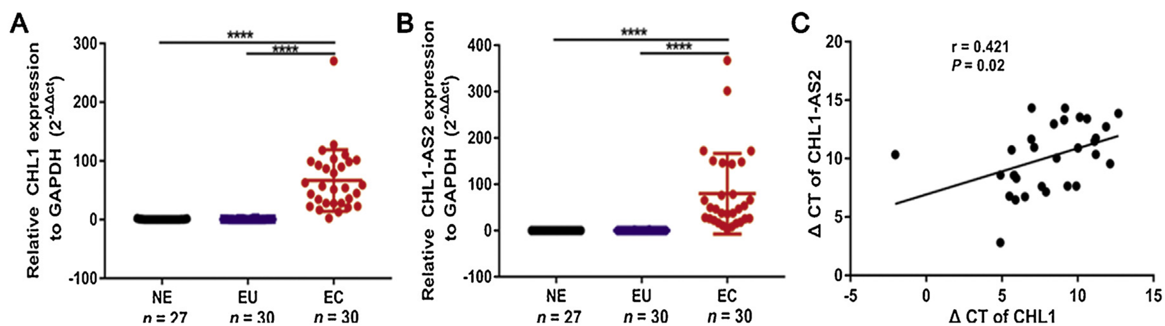

Fig. 2. Increased expression of the CHL1 mRNA and CHL1-AS2 lncRNA in ectopic tissues from patients with endometriosis. The relative expression levels of CHL1 mRNA (A) and CHL1-AS2 lncRNA (B) detected by qPCR in the paired EC and EU tissues from 30 patients with endometriosis and NE tissues from 27 patients without endometriosis. (C) The transcriptional level (Δct of gene levels/GAPDH) of CHL1-AS2 was compared with that of CHL1 in EC tissues using the Pearson correlation analysis. Data are presented as the means ± SD. ****$P < 0.0001$. qPCR, quantitative real-time polymerase chain reaction; NE, normal endometrium; EU, eutopic endometrium; EC, ectopic endometrium.

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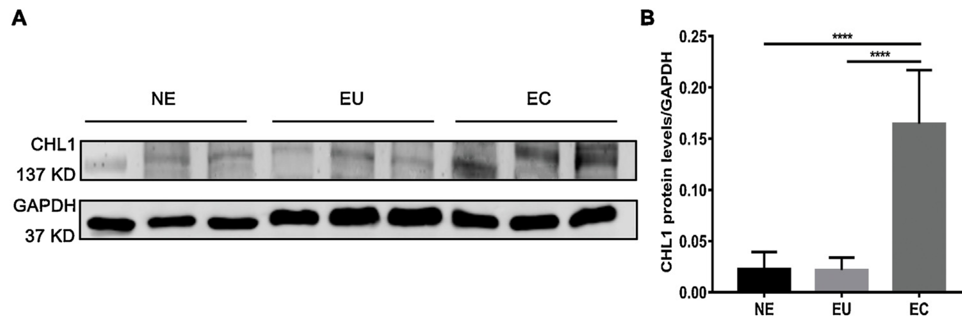

Fig. 3. Increased expression of the CHL1 protein in ectopic tissues from patients with endometriosis. (A) Representative images of CHL1 protein in NE, EU and EC tissues detected by western blotting ($n = 6$ per group). (B) Quantification of CHL1 protein levels in NE, EU and EC tissues. GAPDH was used for normalization of protein expression. The data are derived from at least 3 independent experiments and are presented as the means ± SD. ****$P < 0.0001$. NE, normal endometrium; EU, eutopic endometrium; EC, ectopic endometrium.

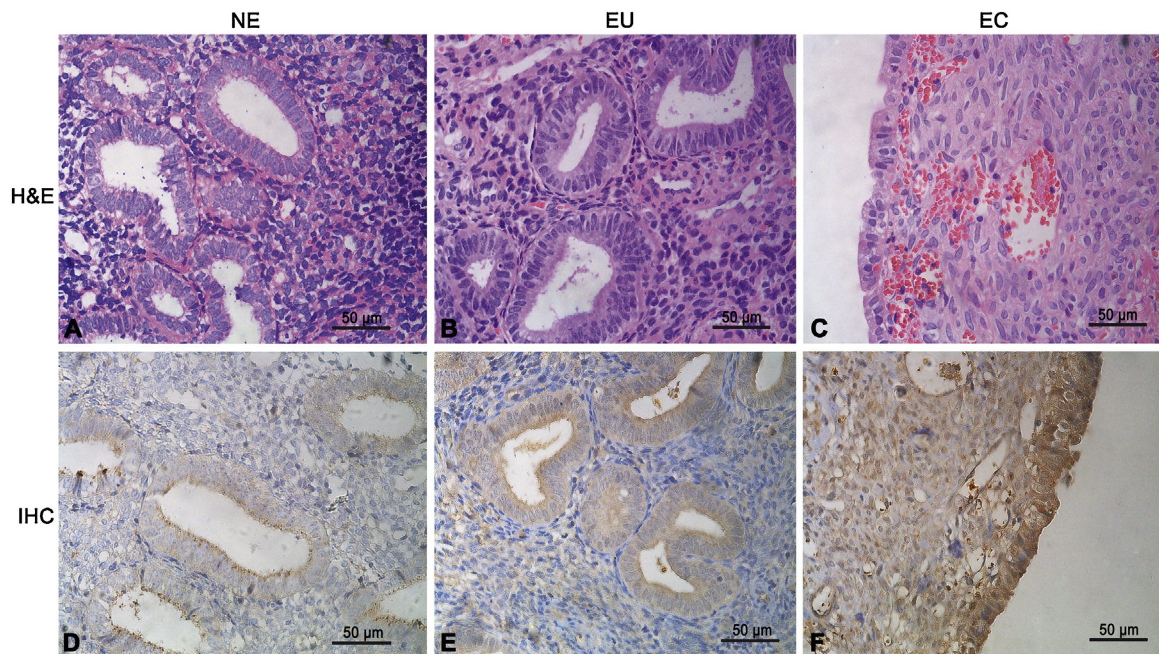

Fig. 4. The localization of CHL1 protein in normal endometrium, eutopic endometrium and ectopic endometrium tissues. Representative images of H&E staining of NE (A), EU (B) and EC (C) tissues. Representative images of immunohistochemical staining of CHL1 protein in NE (D), EU (E) and EC (F) tissues. The semi-quantitative analysis showed that the area percentage of positive staining for the NE, EU and EC tissue were 9.70%, 18.53% and 27.98%, respectively. Magnification: × 400; scale bar, 50 μm. NE, normal endometrium; EU, eutopic endometrium; EC, ectopic endometrium. H&E, haematoxylin-eosin staining; IHC, immunohistochemistry.

Discussion

The mechanism of EM genesis is complicated and our understanding is far from sufficient. This study confirmed that the CHL1 mRNA and protein expression levels were increased in EC tissues compared with EU and NE tissues. The expression level of CHL1-AS2 was in accordance with that of CHL1.

CHL1 plays a role in many diseases, and as a neural recognition molecule, may be involved in signal transduction pathways. Katic J et al. found that CHL1 mediated different types of molecular interactions to induce cerebellar neurite outgrowth and cell migration in postnatal cerebellar development [16]. CHL1 contains the integrin-binding motif Arg-Gly-Asp (RGD), which acts as a coreceptor of integrins and interacts with β1 integrins to promote cell migration and neuritogenesis [17]. It is noted that the RGD peptide, which is an integrin binding site, is present in many types of extracellular matrix proteins, such as fibronectin and tenascins, where it mediates cell-cell and cell-matrix adhesion and signal transduction [18,19]. The functions of CHL1 in tumour progression have be less well studied. CHL1 was found to be downregulated by hypermethylation in breast cancer [20], and the decreased expression of CHL1 promoted tumourigenesis and progression *in vitro* and *in vivo* [21].On the contrary, CHL1 was negatively regulated by some microRNAs, such as miR-10a and miR-21 [22,23], which resulted in the promotion of different cancers. In contrast to the functions of CHL1 in most other cancers, CHL1 was determined to function as a malignancy promoter in glioma [14]. To the best of our knowledge, nothing has been published regarding the relationship between CHL1 and EM.

CHL1-AS1 and CHL1-AS2 are two antisense lncRNAs of CHL1 mRNA. Our results validated that CHL1-AS2 expression was increased in EC tissues, which was consistent with the findings of Sun PR et al. [7]. However, little is known about the functions of CHL1-AS1 and CHL1-AS2. The functions of differentially expressed lncRNAs could be predicted by analysing their co-expressed protein-coding genes and related biological pathways. The antisense RNA transcript might

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predominantly maintain or modify chromatin structure and finally activate or suppress sense RNA gene expression [24]. For instance, DDX11-AS1, which is a divergent non-overlapping transcript of DDX11, interacted with DDX11 and enhanced its activity in the establishment of sister chromatid cohesion [25]. Another example is that BACE1-AS increased the stability of BACE1 mRNA through the formation of transient RNA duplex [26]. In this study, we found that the expression levels of CHL1-AS1 and CHL1-AS2 were positively correlated with CHL1 expression. As noted above, CHL1 participates in signal transduction pathways *via* its RGD peptide or its interaction with microRNAs and is thus involved in the development of many diseases. Consequently, we hypothesize that CHL1-AS1 and CHL1-AS2 may interact with CHL1 to achieve their functions in the progression of EM, either directly or indirectly. We also performed GSEA analyses to predict the enriched biological pathway for CHL1-AS1 and CHL1-AS2. The results indicated that CHL1-AS1 and CHL1-AS2 were negatively correlated to the gene set "WAMUNYOKOLI_OVARIAN_CANCER_LMP". As we all know, EM is a type of benign tumour despite that it has some malignant characteristics. The results of the GSEA analyses conformed to the features of EM.

This study suggests that CHL1, CHL1-AS1 and CHL1-AS2 might be involved in the development of EM. Further studies are required to investigate the underlying mechanisms of these molecules.

Conflict of interest

None declared.

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