RETRACTED ARTICLE: Upregulation of long non-coding RNA OGFRP1 facilitates endometrial cancer by regulating miR-124-3p/SIRT1 axis and by activating PI3K/AKT/GSK-3β pathway

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ABSTRACT

We planned to investigate the possible influences of long non-coding RNA (opioid growth factor receptor pseudogene 1) OGFRP1 in endometrial cancer and its potential regulatory mechanism. We measured the level of OGFRP1 in endometrial cancer tissues and evaluated the influences of OGFRP1 dysregulation on the tumour cell biological processes of endometrial cancer cells. Further, the regulatory relationships between OGFRP1 and miR-124-3p, between miR-124-3p and Sirtuin1 (SIRT1) were, respectively, investigated. The interaction between OGFRP1 dysregulation and activation of PI3K/AKT/GSK-3β pathway was revealed by Western blotting. OGFRP1 was up-regulated in endometrial cancer tissues and cells. OGFRP1 suppression inhibited the malignant behaviour (inhibited cell viability, promoted cell apoptosis, and suppressed cell migration and invasion) of the Ishikawa cells via negatively regulating miR-124-3p. SIRT1 was a target gene of miR-124-3p, and miR-124-3p regulated tumour growth and metastasis by the down-stream signal of SIRT1. Moreover, suppression of OGFRP1 restrained the activation of PI3K/AKT/GSK-3β signals in the Ishikawa cells via miR-124-3p/SIRT1 axis. Our experiments revealed that upregulation of OGFRP1 may enhance the progression of endometrial cancer by regulating miR-124-3p/SIRT1 axis and by activating PI3K/AKT/GSK-3β pathway. OGFRP1 may be of significance in illustrating the biology of endometrial cancer.

Introduction

Endometrial cancer remains to be a lethal gynaecologic malignancy with a tremendous increase in the incidence over recent years [1]. Although the survival rate is high if patients were diagnosed at an early stage, the percentage of patients who are diagnosed at a relative advanced stage is still high, which is about 30% [2]. The prognosis remains poor in patients at the advanced stage or with a high risk of recurrence [3]. Moreover, the molecular mechanisms of endometrial cancer have been poorly illustrated.

Long non-coding RNAs (lncRNAs), some non-coding RNAs with over 200 nucleotides in length, is widely pointed out as pivotal players in many biology of various diseases [4–6]. Aberrant expression of lncRNAs has been widely discovered in many cancers and is considered as a character in cancer [7–9]. Several lncRNAs, including HOTAIR, BANCR, and FER1L4, have been identified to be crucial in the progression of endometrial cancer [10–12]. OGFRP1, a newly reported IncRNA, has been shown to induce autophagy and growth inhibition in human coronary artery endothelial cells [13]. Moreover, dysregulation of OGFRP1 was demonstrated to be pivotal in the biology of non-small cell lung cancer (NSCIC) [14] and hepatocellular carcinoma [15]. However, OGFRP1 in endometrial cancer still remain incomplete reported. In addition, Dong et al. pointed out that miR-124 was lowly expressed in tumour tissues of endometrial cancer [16]. However, there was no study focusing on investigating the regulatory pattern between OGFRP1 and miR-124 in endometrial cancer.

During this research, we elucidated the function and possible mechanism of OGFRP1 in endometrial cancer. We determined the expression of OGFRP1 in endometrial cancer and assessed the effect of OGFRP1 dysregulation on the malignant behaviour of endometrial cancer cells. Further, we analyzed the regulatory relationships between OGFRP1 and miR-124-3p, and those between miR-124-3p and sirtuin1 (SIRT1). Moreover, the interaction between OGFRP1 dysregulation and activation of PI3K/AKT/GSK-3β pathway was also revealed. All of these data may offer a theoretical basis for designing novel strategies for illustrating the biology of endometrial cancer.

Materials and methods

Patients

Between April 2015 and July 2018, 48 patients diagnosed with endometrial cancer were recruited. Table 1 shows the characteristics of endometrial cancer patients. Forty-eight...
tumour tissues from endometrial cancer patients and an equal number of matched normal endometrial tissues were collected during an initial hysterectomy. After extraction, the collected tissues were immediately frozen in liquid nitrogen and then stored at −80 °C for total RNA extraction.

### Cell lines

The human endometrial cancer cell lines HHUA, KLE, Ishikawa, and ECC-1 and normal endometrium (NE) cells were acquired from the authoritative organization of American Type Culture Collection (ATCC). These cells were cultured in medium named as Eagle’s Minimum Essential Medium (Gibco, Darmstadt, Germany), which were mixed with 15% foetal bovine serum (FBS), 100 U/mL of penicillin (Gibco), and 100 μg/mL of streptomycin (Gibco) by incubating at 37 °C in a humidified atmosphere with 5% CO₂.

### Cell transfection and treatment

The Ishikawa cells (2 × 10⁵) were first cultured in 6-well plates and then performed a 24 h incubation at 37 °C. These cells were transfected with siRNA against OGFRP1 (si-OGFRP1#1, si-OGFRP1#2), miR-124-3p mimic (50 nM), or shRNA against SIRT1 (sh-SIRT1) using Lipofectamine 3000 transfection reagent (Life Technologies, Gaithersburg, MD). TGF-β1 (10 ng/mL; Sigma, St. Louis, MO) was added in the 96-well plates equipped with cells for a 4 h incubation at 37 °C. After taking away the cultured medium, dimethyl sulfoxide (DMSO, 100 μL; Sigma, St. Louis, MO) was added in for melting the precipitated formaldehyde. After shaking for 15 min, cell viability was measured via reading the absorbance at 570 nm (A570) using a spectrophotometer (μQuant universal microplate, BioRad, Hercules, CA).

### Cell viability assay

Approximately 24 h post-transfection, the cells (10⁴ cells/well) were cultured into 96-well plates for the subsequent analysis using MTT assay. Briefly, MTT (0.5 mg/mL; Sigma, St. Louis, MO) was added in the 96-well plates equipped with cells for a 4 h incubation at 37 °C. After taking away the cultured medium, dimethyl sulphoxide (DMSO, 100 μL; Sigma, St. Louis, MO) was added in for melting the precipitated formaldehyde. After shaking for 15 min, cell viability was measured via reading the absorbance at 570 nm (A570) using a spectrophotometer (μQuant universal microplate, BioRad, Hercules, CA).

### Cell apoptosis assay

Approximately 24 h post-transfection, the cultured cells were harvested and washed by PBS buffer. The cells were stained with fluorescein isothiocyanate-labelled annexin V and propidium iodide following the protocol recommended for Annexin V-FITC Kit (Sangon Biotech., Shanghai, China). The percentage of apoptotic cells was assessed using a flow cytometry (FACSCalibur, BioRad, Hercules, CA).

### Cell migration and invasion assays

Transwell assay was used for the assessment of cell biological processes of migration and invasion. For invasion, the transwell chambers (8 μm pore size; Corning co. Ltd., Maine, NY) were pre-coated with Matrigel (Becton-Dickinson, Franklin Lakes, NJ). Briefly, after a 24-h incubation, cells were cultured in serum-free RPMI 1640 medium for 24 h. Subsequently, the cells were seeded in the upper chamber and RPMI 1640 medium mixed with 10% FBS was added in the lower chamber. After incubating for another 48 h, transwell chambers were fixed with methanol and stained with Giemsa. Cell migration and invasion were analyzed by counting the migrated and invaded cells under a microscope (IX83, Olympus Corporation, Tokyo, Japan).

### Luciferase reporter assay

We used PCR analysis to amplify the sequences of binding site between 3'-UTR of SIRT1 mRNA and miR-124-3p and then inserted the obtained sequences into pGL3 vector (Promega, Madison, WI) to construct the wild type (WT) luciferase reporter vector pGL3-SIRT1. Moreover, Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to synthesize the mutant luciferase reporter vector pGL3-SIRT1-MUT with point mutations in the seed sequence. Cells at a density of 1 × 10⁶ per well were co-transfected with the following vectors, including 50 pmol of miR-124-3p inhibitor (or control miRNA), 1 μg of pGL3-SIRT1-WT/pGL3-SIRT1-MUT, and 1 μg of

### Table 1. The characteristics of endometrial cancer patients.

| Total no. | 48 |

| Median age, years (range) | 56.8 (39–76) |

| Pathological tumour stage |  |

| I | 22 (45.8%) |
| II | 12 (25%) |
| III | 8 (16.7%) |
| IV | 6 (12.5%) |

| Differentiation |  |

| G1 | 17 (35.4%) |
| G2 | 16 (33.3%) |
| G3 | 15 (31.3%) |

| Lymphatic metastasis |  |

| (+) | 15 (31.25%) |
| (–) | 30 (62.5%) |
| Unknown | 3 (6.25%) |
Renilla luciferase expression construct pRL-TK (Promega, Madison, WI), using Lipofectamine 3000 reagent following the manufacturer’s protocol. The luciferase activity of Renilla group was used as a control. Approximately a 48 h incubation, luciferase activity in each group was assessed using the dual-luciferase reporter assay system (Promega, Madison, WI).

Western blot
Cells were harvested and lysed with the purchased cell lysis buffer (Beyotime, Haimen, China). After centrifugation, the total protein was extracted. The protein samples (50 μg/lane) were subjected to 12% SDS-polyacrylamide gel electrophoresis and then we transferred the obtained protein band signals onto PVDF membranes (Millipore, Billerica, MA). Then, we performed the immunoblotting by incubating the membranes with the primary antibodies (1:1000 dilutions) and subsequently with the recommended secondary antibodies (1:5000). The obtained protein signals were then observed by an Imaging Analysis System (Odyssey Infrared, LI-COR, Lincoln, NE). The primary antibodies including β-actin, Bax, Bcl-2, pro- and cleaved caspase-3, pro- and cleaved caspase-9, SIRT1, N-cadherin, E-cadherin, vimentin, snail, PI3K, p-PI3K, AKT, p-AKT, GSK-3β, and p-GSK-3β were obtained from Abcam (Cambridge, UK). β-Actin was used as the internal control.

Statistical analysis
All experiments in this research were conducted three times independently. Data were expressed as the mean ± standard deviation (SD). Statistical analyses were evaluated using SPSS 20.0 (IBM, Armonk, NY). The significant differences among groups were evaluated by two-tailed or one-way ANOVA followed by Turkey’s post hoc analysis. p < .05 reveals a statistically significance.

Results
OGFRP1 upregulates in endometrial cancer
The mRNA level of OGFRP1 in endometrial cancer tissues was significantly more than in non-tumour tissues (p < .01, Figure 1(A)). Furthermore, OGFRP1 was highly expressed in endometrial cancer cells (HHUA, KLE, Ishikawa, and ECC-1) compared with NE cells (p < .01, Figure 1(B)). We selected the Ishikawa cells for further experiments because OGFRP1 expression was the highest among the four endometrial cancer cell lines (Figure 1(B)).

OGFRP1 suppression inhibits the biological performance of the Ishikawa cells
The expression of OGFRP1 was suppressed by transfecting the Ishikawa cells with si-OGFRP1 to explore the role of OGFRP1 in endometrial cancer development. OGFRP1 expression in si-OGFRP1#1 or si-OGFRP1#2 was markedly less than that in si-NC (p < .05, Figure 1(C)). si-OGFRP1#2 was selected for the subsequent experiments due to higher transfection efficiency compared with si-OGFRP1#1. We further evaluated the influences of OGFRP1 down-regulation on the Ishikawa cell biological processes including viability, apoptosis, migration and invasion. As shown in Figure 1(D), OGFRP1 suppression markedly reduced the viability of the Ishikawa cells (p < .05). The percentage of cell apoptosis was significantly increased after OGFRP1 suppression (p < .001, Figure 1(E)). Besides, we analyzed the level of apoptotic proteins including caspase-3, caspase-9, Bcl-2, and Bax. OGFRP1 down-regulation promoted the levels of pro-apoptotic proteins including cleaved caspase-3, cleaved caspase-9, and Bax, while it suppressed the anti-apoptotic protein Bcl-2 (Figure 1(E)). Furthermore, OGFRP1 down-regulation markedly decreased the Ishikawa cell migration and invasion (p < .01, Figure 1(F-G)). The levels of EMT-related proteins including E-cadherin, N-cadherin, vimentin, and snail were also examined. OGFRP1 suppression promoted E-cadherin expression; however, it restrained the level of N-cadherin, snail, and vimentin (Figure 1(H)), which indicates that OGFRP1 downregulation inhibited EMT in the Ishikawa cells.

SIRT1 is a functional target gene of miR-124-3p
Several potential targets of miR-124-3p were identified using TargetScan software, and the interaction pattern between miR-124-3p and SIRT1 was examined to elucidate the possible mechanism of miR-124-3p. The predicted binding sequence between miR-124-3p and SIRT1 is shown in Figure 3(A). Luciferase experiment showed that SIRT1 3’UTR was bounded by miR-124-3p (p < .05, Figure 3(B)), which indicates that SIRT1 is a functional target of miR-124-3p. Besides,
at mRNA and protein levels, the SIRT1 expression in the miR-124-3p mimic group was markedly lower compared with the mimic control group, while its expression in miR-124-3p inhibitor group was markedly higher compared with the inhibitor NC group ($p < .01$, Figure 3(C,D)), which indicates the negative association between miR-124-3p and SIRT1.

Figure 1. OGFRP1 was upregulated in endometrial cancer tissues (A) and cells (B). All experiments were repeated at least three times. **$p < .01$ compared to controls. OGFRP1 suppression inhibited the malignant behaviours of the Ishikawa cells. (C) The OGFRP1 level in the Ishikawa cells after transfection with siRNAs. (D) Viability of the Ishikawa cells after transfection. (E) Apoptosis and the expression of apoptosis proteins after transfection in the Ishikawa cells. (F) Migration of the Ishikawa cells after transfection. (G) Invasion of the Ishikawa cells after transfection. (H) The expression of EMT-markers after transfection. All experiments were repeated at least three times. *$p < .05$, **$p < .01$, and ***$p < .001$ compared to controls.
Figure 2. OGFRP1 suppression inhibited the malignant behaviours of the Ishikawa cells via negative regulation of miR-124-3p. (A) The OGFRP1 level in the Ishikawa cells after transfection with siRNAs. (B) miR-124-3p expression in endometrial cancer tissues and non-tumour tissues. (C) miR-124-3p expression in endometrial cancer cells and normal endometrium (NE) cells. (D) miR-124-3p expression in the Ishikawa cells after transfection. (E–I) The Ishikawa cells were cotransfected with si-OGFRP1 and miR-124-3p inhibitor. (E) Viability of the Ishikawa cells after transfection. (F) Apoptosis and the expression of apoptosis proteins after transfection in the Ishikawa cell. (G) Migration of the Ishikawa cell after transfection. (H) Invasion of the Ishikawa cells after transfection. (I) Expression of EMT-markers after transfection. All experiments were repeated at least three times. *p < .05, **p < .01, and ***p < .001 compared to controls.

Figure 3. SIRT1 was verified as a functional target of miR-124-3p. (A) The predicted binding sequence between miR-124-3p and SIRT1. (B) Luciferase reporter assay showing that SIRT1 3’UTR could be targeted by miR-124-3p. (C and D) The SIRT1 expression in the Ishikawa cells by transfection with miR-124-3p mimic, miR-124-3p inhibitor, and their controls. All experiments were repeated at least three times. *p < .05, **p < .01, and ***p < .001 compared to controls.
miR-124-3p regulates endometrial cancer growth and metastasis by targeting SIRT1

To further verify the possible influences of miR-124-3p in endometrial cancer development was adjusted by targeting SIRT1, SIRT1 was successfully suppressed in the Ishikawa cells (p < .001, Figure 4(A)). The combined influences of miR-124-3p inhibition and SIRT1 suppression were then investigated. The suppression of miR-124-3p in the Ishikawa cells increased cell viability (p < .05, Figure 4(B)). Meanwhile, it inhibited cell apoptosis by increasing BCL-2 level but decreasing the expression of Bax, cleaved caspase-3, and cleaved-caspase-9 (Figure 4(C)). Cell migration and invasion were promoted by inducing EMT through decreasing E-cadherin but increasing the expression of N-cadherin, snail, and vimentin (p < .05, Figure 4(D,F)). Further, miR-124-3p inhibition and SIRT1 suppression at the same time reversed the effects of the suppression of miR-124-3p on the Ishikawa cell viability, apoptosis, invasion and migration (p < .05, Figure 4(B-F)), which indicates that miR-124-3p regulated the malignant behaviour of the Ishikawa cells by targeting SIRT1.

OGFRP1 suppression inhibits the activation of the PI3K/AKT/GSK-3β pathway in the Ishikawa cells via miR-124-3p/SIRT1 axis

PI3K/AKT/mTOR pathway was reported to be activated by the up-stream mediators in endometrial cancer pathogenesis [17]. A recent study has shown that IncRNA DLEU1 enhances the progression of endometrial cancer through activation of PI3K/AKT/GSK-3β pathway [18]. Moreover, OGFRP1 down-regulation was shown to inhibit hepatocellular carcinoma by regulating AKT/mTOR and Wnt/β-catenin signals [19]. We investigated whether activation of PI3K/AKT/GSK-3β pathway is a key mechanism that mediates the role of OGFRP1 in endometrial cancer. Our experiments discovered that the suppression of OGFRP1 significantly decreased the protein levels of p/t-PI3K, p/t-AKT, and p/t-GSK-3β in the Ishikawa cells, which was neutralized by the inhibition of OGFRP1 and miR-124-3p at the same time (Figure 4(G)). Additionally, the concurrent inhibition of OGFRP1, miR-124-3p, and SIRT1 remarkably decreased the expression levels of these proteins (Figure 4(H)). These findings indicate that the activation of PI3K/AKT/GSK-3β pathway may be a subsequent mechanism mediating the role of OGFRP1/miR-124-3p/SIRT1 axis in the Ishikawa cells (Figure 4(I)).

Discussion

Emerging evidence points out that IncRNAs are pivotal players in endometrial carcinogenesis [20]. Understanding the crucial IncRNAs involved in endometrial cancer may facilitate designing effective therapeutic strategies. This research discovered that OGFRP1 was up-regulated in endometrial cancer tissues and cells, and down-regulation of OGFRP1 inhibited the malignant behaviour including metastasis and apoptosis of endometrial cancer cells. Consistent with previous findings [15,21], our results also imply that OGFRP1 may serve as an oncogene in endometrial cancer and play important roles in tumour progression.

Increasing studies have suggested the pivotal roles of IncRNAs in various kinds of diseases, and IncRNAs act as endogenous miRNA sponges and promote these diseases [22-24]. In line with the previous findings that OGFRP1 could sponge miR-124-3p in NSCLC cells [21], OGFRP1 negatively regulated miR-124-3p expression and down-regulation of OGFRP1 inhibited the malignant behaviour of the Ishikawa cells by negatively regulating miR-124-3p in this experiment. Accumulating evidence indicates that miR-124-3p undertakes a tumour suppressor and is associated with the progression of various cancers, such as females-related cancers including breast cancer [25] and cervical cancer [26], bladder cancer [27], and gastric cancer [28]. This research showed that miR-124-3p expression was dramatically decreased in endometrial cancer tissues and cells. Take into the key role of miR-124-3p in other cancers consideration, we confirmed that miR-124-3p may play a tumour suppressive role in endometrial cancer, and upregulation of OGFRP1 may promote endometrial cancer by inhibiting miR-124-3p.

SIRT1, a NAD-dependent histone deacetylase, is reported to play a dual role, including modifying histone protein via the following deacetylation manners, including deacetylation of lysine residues K26 on histone H1, lysine residues K9 on histone H3, and lysine residues K16 on histone H4, acting both as an oncogene and as a tumour suppressor [29]. Previous studies have confirmed that dysregulation of SIRT1 is associated with the malignant phenotypes of various cancers, such as prostate cancer [30], gastric cancer [31], and cervical cancer [32]. Moreover, it has been reported that SIRT1 can contribute to endometrial tumour growth by promoting lipogenesis [33] and is involved in regulating progestin resistance in endometrial cancer [34]. On the other point, Brennan et al pointed out that the down-regulated miR-124 was produced by the SIRT1-mediated histone deacetyrase [35]. In this study, SIRT1 was determined as a functional target of miR-124-3p, and SIRT1 suppression could changeover the influences of miR-124-3p inhibitor on the Ishikawa cell biological processes including viability, apoptosis, migration, and invasion. Therefore, we speculate that miR-124-3p may regulate the cell proliferation and metastasis of endometrial cancer by SIRT1.

Furthermore, PI3K/AKT/GSK-3β pathway, a pivotal signal transduction pathway, regulates cell proliferation, apoptosis, and migration [36]. Aberrant activation of this pathway occurs in many malignant tumours including endometrial cancer [17,37]. In addition, the downregulation of OGFRP1 has been revealed to inhibit hepatocellular carcinoma via the regulation of AKT/mTOR and Wnt/β-catenin signals [19]. Koga et al. pointed out that the PI3K/AKT/GSK-3β signals is crucial to SIRT1 induction by stress reaction of endoplasmic reticulum and consequently regulates hepatocellular injury [38]. In this study, suppression of OGFRP1 restrained the activation of PI3K/AKT/GSK-3β signals in the Ishikawa cells, which was neutralized after inhibition of miR-124-3p, and further the concurrent inhibition of OGFRP1 inhibited this pathway. We thus speculate that the activation of PI3K/AKT/GSK-3β
Figure 4. miR-124-3p regulated the malignant behaviours of the Ishikawa cells through targeting SIRT1. (A) SIRT1 expression in the Ishikawa cells after transfection with shRNAs. (B-F) The Ishikawa cells were cotransfected with miR-124-3p inhibitor and sh-SIRT1. (B) Viability of the Ishikawa cells after transfection. (C) Apoptosis and the expression of apoptosis proteins after transfection in the Ishikawa cells. (D) Migration of the Ishikawa cells after transfection. (E) Invasion of the Ishikawa cells after transfection. (F) Expression of EMT-markers after transfection. (G and H) Suppression of OGFRP1 inhibited the activation of PI3K/AKT/GSK-3β pathway in the Ishikawa cells, which was mediated by miR-124-3p/SIRT1 axis. Western blot showing the expression of p/t-PI3K, p/t-AKT and p/t-GSK-3β in the Ishikawa cells after different transfections. (I) The mechanismchart of OGFRP1 in regulating the progression of endometrial cancer in this study. All experiments were repeated at least three times. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to controls.
pathway may be a subsequent mechanism that mediates the role of OGFRP1/miR-124-3p/SIRT1 axis in endometrial cancer.

Taken together, our experiments revealed that the upregulation of OGFRP1 may promote the development of endometrial cancer through regulation of miR-124-3p/SIRT1 axis and by activating PI3K/AKT/GSK-3β pathway. OGFRP1 may serve as a marker for the diagnosis and treatment of endometrial cancer.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**References**

[1] Morice P, Leary A, Creutzberg C, et al. Endometrial cancer. Lancet. 2016;387:1094–1098.

[2] Su L, Wang H, Miao J, et al. Clinico-pathological significance and potential drug target of CDKN2A/p16 in endometrial carcinoma. Scientific Rep. 2015;5:13238.

[3] Zhou M, Zhang Z, Zhao H, et al. A novel IncRNA-focus expression signature for survival prediction in endometrial carcinoma. BMC Cancer. 2018;18:39.

[4] Iyer MK, Niknafs YS, Malik R, et al. The landscape of long noncoding RNAs in the human transcriptome. Nat Genet. 2015;47:199.

[5] Boon RA, Jander S, Kostic AD, et al. Reactivation of epigenetically silenced miR-124 reverses the epithelial-to-mesenchymal transition and inhibits invasion in endometrial cancer cells via the direct repression of IGGAP1 expression. Oncotarget. 2016;7:20260–20270.

[6] Slomovitz BM, Coleman RL. The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. Clin Cancer Res Off J Am Assoc Cancer Res. 2012;18:5856.