Adipocyte-derived CCDC3 promotes tumorigenesis in epithelial ovarian cancer through the Wnt/β-catenin signalling pathway

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1. Introduction

Among gynaecological malignancies, ovarian cancer is the leading cause of mortality worldwide. Epithelial ovarian cancer (EOC) is the most common histological subtype of ovarian cancer, accounting for more than 90% of ovarian malignancies [1]. Unlike other tumours that disperse through the blood stream, EOC mainly disseminates throughout the peritoneum, and the omentum is often the most-frequent metastasis site [2]. The omentum is a visceral adipose tissue that is involved in fat storage, immune regulation, and tissue regeneration. The omentum adipocyte cells interact with cancer cells and promote their migration and metastasis via the secretion of adipokines, growth factors, and hormones [3], suggesting its decisive role in the colonisation of the omentum in ovarian cancer [4].

Coiled-coil domain is a specific structural protein involved in several pathophysiological processes [5,6]. Coiled-coil domain-containing (CCDC) proteins are expressed abnormally and serve as cancer promoters in multiple malignancies [7–13]. CCDC3, also known as fat/vessel-derived secretory protein (Favine) and encoded by CCDC3 (NCBI: NP_028804), is highly conserved among species [14]. It is expressed in adipose tissues and endothelial cells and its expression is particularly upregulated in human omental tissue [14,15]. Growing evidence supports that CCDC3 is closely associated with the regulation of lipid metabolism in the liver [16], suppression of the pro-inflammatory response in endothelial cells [17], and promotion of tumour cell migration in cervical cancer [18].

To date, the biological role of CCDC3 as an adipocyte-secretory protein in EOC remains elusive. Given the specificity of CCDC3 expression in omental adipose tissue and the relevance of omentum in EOC, we speculated that CCDC3 secreted by adipocytes in the omentum may promote omental metastasis by interacting with cancer cells in EOC. Our study describes a novel, canonical function of CCDC3 and
highlights the therapeutic potential of targeting canonical CCDC3 functions in EOC.

2. Material and methods

2.1. Cell culture

Rat bone mesenchymal stem cell (BMSCs, Procell, Cat no: CP-R131) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 U/mL penicillin and 0.1 mg/mL streptomycin (P/S), and 10% foetal bovine serum (FBS) before differentiation. The human ovarian carcinoma cell line SKOV3 (Procell, Cat no: CL-0215) was cultured in DMEM supplemented with P/S and 10% FBS. All the cells were cultured at 37 °C with 5% CO₂.

2.2. Adipogenesis of BMSCs

Inducers including 1 μM dexamethasone, 5 mg/L insulin, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Cat no: I7018, Sigma, St. Louis, MO, USA) were added into the medium in which BMSCs were cultured when the cells reached about 80% confluency. After two days of culture, the medium was replaced with DMEM supplemented with 50 U/mL P/S, 10% FBS, and 5 mg/L insulin. The medium was changed every two days until differentiated adipocytes were developed. All the cells were cultured at 37 °C with 5% CO₂.

2.3. Oil red O (ORO) staining of BMSC lipids

BMSC-derived adipocytes were rinsed gently with phosphate-buffered saline, and subsequently stained with fresh diluted ORO (Sigma, St. Louis, MO, USA) with final concentration of 60% to evaluate their lipid content. Briefly, the cells were stained with ORO solution for 10 min at 60 °C and then washed in 75% ethanol solution. Then, the samples were thoroughly washed under the running tap water for 2 min followed by counterstaining with Gill’s haematoxylin solution (Serv- icebio, Cat no: G1004) for 30 s. The samples were mounted using an aqueous solution. The prepared slides were visualised using an Axiovert microscope (Perkin Elmer) at × 200 magnification. The relative steatosis area is expressed as the percentage of ORO-stained areas.

2.4. CCDC3 overexpression and knockdown

Rat Ccdc3 cDNA was synthesised and cloned into a cloning expression vector pcDNA3.1 (+) at the BamHI-HindIII site to generate a CCDC3 overexpression construct, so did the empty vector to a negative control (NC) of overexpression. Different siRNAs (siRNA1, siRNA2, and siRNA3) targeting three distinct sequences of rat Ccdc3 gene were purchased from Jima Pharmaceutical Company (Shanghai, China). The siRNA1 was used for knocking down CCDC3 in the subsequent experiments, and its sequence was 5'-GGUGAACCAAAAAUCUAATT'-3' and 5'-AUUGAGUUUUUGUCCACCTT'-3'. An siRNA construct expressing a scrambled sequence of 5'-UUCUCCGAAGUUCUAGUTT'-3' and 5'-ACGUGACACGUUGAGAATT'-3', which does not target any known genes, was used as the NC of siRNA (Supplementary Table 1).

2.5. Conditioned medium (CM) experiments

BMSC-derived adipocytes were transiently transfected with CCDC3 overexpression construct, control overexpression, siRNA1 and control siRNA by using Lipofectamine 2000 Transfection Reagent (11668019, Invitrogen™) according to the manufacturer’s protocol. Cells in the CM were collected and lysed 48 h after transfection. The supernatant of transfected BMSC-derived adipocytes was collected and concentrated by ultrafiltration centrifuge tube after filtration with 0.2 μm microporous membrane. The presence of CCDC3 in the CM was confirmed using the supernatant by real-time quantitative polymerase chain reaction (RT-qPCR) and western blotting with the monoclonal CCDC3 antibody (Thermo Fisher Scientific, Cat no: PA5-49634).

2.6. SKOV3 cells co-cultured with CM

Wnt/β-catenin signalling agonist SKL2001 (Cat no: S1180) and inhibitor XAV-939 (Cat no: S8320) were purchased from Selleck (Shanghai, China). Different CMs were applied to receiving SKOV3 cells at approximately 80% confluency. Sixteen hours after incubation, the receiving SKOV3 cells were treated with 20 μM SKL2001 and XAV-939 for 48 h, respectively. SKOV3 cells were co-cultured with nine groups of CM, including blank CM, CCDC3 enriched CM, NC of CCDC3 enriched CM, CCDC3 siRNA CM, NC of CCDC3 siRNA CM, CCDC3 enriched CM plus agonist, CCDC3 enriched CM plus inhibitor, CCDC3 siRNA CM plus agonist, and CCDC3 siRNA CM plus inhibitor. The blank group refers to the co-culture of SKOV3 cells with the CM without any additional substance. The abbreviations and definitions of each group are shown in Table 1. Subsequently, cells were harvested for RT-qPCR, western blotting, and cell proliferation, migration, and invasion assays.

2.7. RNA extraction and RT-qPCR

Total RNA was extracted using MiniBEST Universal RNA Extraction Kit (Takara, Cat no: 9767), following the manufacturer’s instructions. cDNA was generated using the Reverse Transcriptase Kit (Primescript RT Reagent Kit with gDNA Eraser perfect Real time), following the manufacturer’s instructions. RT-qPCR was performed using an ABI12K Real Time PCR System. The sequences of the primers used are listed in Table 2. Relative expression levels of the candidate genes were calculated using the expression level of GAPDH as the reference using the 2^-ΔΔCT method. All primers were synthesised by Tsingke Biotechnology (Beijing, China). All the experiments were conducted in triplicate.

2.8. Western blotting

Cultured cells were lysed with lysis buffer containing radio-immunoprecipitation assay buffer (RIPA buffer). The proteins concentration in the cell lysates was quantified using the DC™ protein assay (Bio-Rad). Equal amounts of proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes. For the extraction of specific

| Table 1 | Definition of nine groups of conditioned medium (CM). |
|---------|---------------------------------------------------|
| Group          | Abbreviation         | Definition                                   |
| Blank CM       | Blank                | CM without any additional substance.         |
| CCDC3-enriched CM | CCDC3                | BMSC-derived adipocytes transfected with CCDC3 overexpression construct |
| Negative control | NC CCDC3             | BMSC-derived adipocytes transfected with empty vector |
| CCDC3-enriched CM plus Wnt/β-catenin agonist | Ccdc3 + agonist     | CCDC3-enriched CM treated with SKL2001 |
| CCDC3-enriched CM plus Wnt/β-catenin inhibitor | Ccdc3 + inhibitor   | CCDC3-enriched CM treated with XAV939 |
| CCDC3 siRNA CM | Ccdc3 siRNA          | BMSC-derived adipocytes transfected with siRNA targeting CCDC3 |
| Negative control | NC siRNA            | BMSC-derived adipocytes transfected with control siRNA |
| CCDC3 siRNA CM plus Wnt/β-catenin agonist | Ccdc3 siRNA + agonist | CCDC3 siRNA CM treated with SKL2001 |
| CCDC3 siRNA CM plus Wnt/β-catenin inhibitor | Ccdc3 siRNA + inhibitor | CCDC3 siRNA CM treated with XAV939 |
nuclear protein, such as β-catenin, a nuclear protein extraction kit was used (Beyotime, Cat no: P0028). The membranes were incubated with the following antibodies: anti-CCDC3, anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-fibronectin, anti-β-catenin, anti-c-myc, and anti-cyclin D1 (Supplementary Table 2). Bands were imaged using a ChemiDocTM XRS + scanner (Bio-Rad).

2.9. Proliferation assays

SKOV3 cells were seeded in 96-well plates at a density of 5000 cells/well, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed after 24, 48, and 72 h to evaluate cell viability and proliferation. Specifically, MTT was first prepared as a stock solution of 5 mg/mL in phosphate-buffered saline (PBS, pH 7.2) and filtered. At the end of the treatment period (24, 48, and 72 h), 10 μL of stock solution of 5 mg/mL in phosphate-buffered saline (PBS, pH 7.2) was used (Beyotime, Cat no: P0028). The membranes were incubated with MTT for 4 h at 37 °C, discard the supernatant was discarded, 150 μL of DMSO (MTT Sigma M5655-1G) was added to each well, shaked for 10 min to dissolve the crystals, and then the 96-well plate was read by an absorbance reader (Thermo MULTISKAN MK3) at 490 nm for absorbance density values to calculate the proliferation.

2.10. Cell invasion and migration assays

To measure the cell invasion ability, transwell chambers (8-μm pore size) were coated with Matrigel (50 μL/well with a solution containing four volumes of serum-free medium to one volume of Matrigel solution) in 24-well plates. Cell migration assay was performed with transwell chambers (8-μm pore size) coated with serum-free medium in 24-well plates. Cells at a density of approximately 1.5 × 10^5 cells/well were seeded into the upper chamber of the insert. After incubation for 48 h at 37 °C with 5% CO2, any cells that had invaded the lower cavity were fixed by 4% paraformaldehyde solution (Beyotime, Cat no: P0099) for 10 min and then stained with 5% crystal violet solution (Beyotime, Cat no: C0121) for 10 min. The cells were counted in three randomly selected visual fields using an inverted microscope (IX71, Olympus). The quantification analysis was carried on using the Image J software (Fiji Software). Cell numbers were counted at random three views, the average number of migratory and invasive cells were shown as histogram, respectively. All the experiments, including the quantification analysis, were performed in triplicate.

2.11. Bioinformatic analysis

2.11.1. Data acquisition

The raw data of CCDC3 mRNA expression in 359 ovarian cancer samples were extracted from TCGA (https://portal.gdc.cancer.gov/), and the data set was TCGA ovarian cancer dataset (TCGA-OV). All the samples were EOC.

2.11.2. Kaplan–Meier survival curve analysis

The relationship between CCDC3 mRNA expression (high vs. low) and the survival of patients with ovarian cancer was assessed using the online tool Kaplan–Meier plotter (kmplot.com/analysis) [19]. The log-rank p-value and hazard ratio (HR) with 95% confidence interval (CI) were calculated.

2.11.3. Co-expression analysis

The association between CCDC3 and the expression level of other genes was measured using Pearson’s correlation analysis. Pairwise genes with a correlation coefficient larger than 0.3 (p < 0.05) were considered to be co-expressed. Data are presented using a circle diagram and heatmap using R.

2.11.4. Correlation between CCDC3 expression level and tumour-infiltrating immune cells

The CIBERSORT [20] package in R was used to estimate the infiltration score of 22 tumour-infiltrating immune cells. The chi-square test was performed to analyse the correlation between the CCDC3 expression level (high vs. low) and immune cells infiltration. A Spearman correlation coefficient with p < 0.05 was considered statistically significant.

2.11.5. Drug sensitivity analysis

The pRRophetic package in R was used to predict the drug sensitivity of each tumour sample. The ridge regression method was applied to estimate the concentration of IC50 of each specific therapeutic drug. GDS2 (https://www.cancerrxgene.org/), the largest pharmacogenomics database available nowadays, was used as the training set for 10-fold cross-validation to evaluate the performance of the constructed regression mode. Default values were selected for all parameters, and batch effect was removed.

2.11.6. GSVA and GSEA

The GSVA [21] package in R was utilised to estimate the signalling pathways associated with CCDC3 expression. The clusterprofiler [22] package in R was used to perform GSEA of CCDC3 with RNA-sequencing data. The statistical significance of the category enrichment was assessed using the p-value computed from 1000 permutations. The population was divided into two groups according to the CCDC3 mRNA expression level (high vs. low). The acceptable level of significance was p ≤ 0.01. The gene set “c2.cp.kegg.v6.20symbols.gmt” was selected as the reference gene set (MSigDB, http://software.broadinstitute.org/gsea/msigdb/index.jsp).

2.11.7. GeneMANIA analysis

To better understand the function of CCDC, a protein–protein interaction (PPI) network was constructed using GeneMANIA (http://www.genemania.org), an online tool commonly used to identify functions and interactions and visualise the potential PPIs.

2.11.8. Statistical analysis

Analysis of variance (ANOVA, one-way), Wilcoxon test, and the Student’s t-test were employed to compare the variables. Survival curves were evaluated by the log-rank test using the Kaplan–Meier method. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using R (R Core Team, version 3.6).

### Table 2

Sequences of primers.

| Name    | Sequence (5'→3') | Size   |
|---------|----------------|--------|
| GAPDH   | TCAAGAAGTGGTGAAACAGG | 115bp  |
| Reverse | TCAAAGGTGGAGGTGGTGGT |        |
| E-cadherin | TACACTTCTACATGCCCCAG | 220bp  |
| Reverse | AGTTCTCGGTCCTCAGACG |        |
| N-cadherin | CGATCCCAATGCGCTCAATG | 185bp  |
| Reverse | TGCCCTCATGTGTGTAAGT |        |
| vimentin | TTGAGCAAGGATTGAATC | 157bp  |
| Reverse | AGGTAGGCTTGGGAAACA |        |
| Fibronectin | TACGTCCTGTTTTCTCTCGG | 201bp  |
| Reverse | AAATCTGCTCCCACCTCCTC |        |
| β-catenin | CTTCACCTGACAGATCAAGTC | 98bp   |
| Reverse | CCTCTCACCTCCTCCTCCTTAG |        |
| c-myc   | CATACATGCTTCGTCAGAG | 109bp  |
| Reverse | GAGTCTGAGCTGCTGCAAGT |        |
| cyclin D1 | GGAGGAAAGGAGAAGGCGG | 192bp  |
| Reverse | TGAGTTGCTGGTGTAGAAGC |        |
| CCDC3   | TCTCAACTCACTGCTGTCG | 152bp  |
| Reverse | GAGAAATGACGCAGCCCTG |        |

### Sequences of primers.

| Name        | Name   | Sequence (5'→3') | Size   |
|-------------|--------|----------------|--------|
| cyclin D1   |        | GTTCTGAGTGGTGTTGTG | 192bp  |
| Reverse     |        | TGGAGTCTGGTGTAGAAGC |        |
| CCDC3       |        | TCTCAACTCACTGCTGTCG | 152bp  |
| Reverse     |        | GAGAAATGACGCAGCCCTG |        |
3. Results

3.1. High CCDC3 expression level is associated with poor clinical outcomes

To validate the prognostic value of CCDC3 expression level in EOC patients, the log-rank test was performed. As shown in Fig. 1, patients with high CCDC3 expression level had dramatically worse overall survival (OS) and progression-free survival (PFS) than those with low CCDC3 expression level in stage III to IV EOC (OS: HR, 1.82, 95% CI, 1.44–2.29, \( p < 0.001 \); PFS: HR, 1.8, 95% CI, 1.47–2.19, \( p < 0.001 \); Fig. 1a and b). Consistent results were observed when the population was extended to patients with stage I to IV EOC, demonstrating that high CCDC3 expression level is associated with worse prognosis than low CCDC3 expression level (OS: HR, 1.84, 95% CI, 1.50–2.27, \( p < 0.001 \); PFS: HR, 1.81, 95% CI, 1.50–2.19, \( p < 0.001 \); Fig. 1c and d). Taken together, these findings suggest that CCDC3 expression level is a suitable biomarker for EOC prognosis.

3.2. CCDC3-related biological signatures in EOC

To elucidate the function of CCDC3 in EOC, a gene set variation analysis (GSVA) was performed to reveal the specific signalling pathways associated with CCDC3 in EOC. High CCDC3 mRNA expression level was associated with Wnt/β-catenin, Notch, angiogenesis, and Hedgehog signalling pathways, while low CCDC3 expression was associated with tumour necrosis factor-α (TNF-α)/nuclear factor kappa-B (NF-κB), interferon-γ response, and inflammatory response pathways. Similar results were obtained from a gene set enrichment analysis (GSEA), which showed CCDC3 was positively correlated with Notch and Hedgehog signalling pathways, and basal cell carcinoma (Fig. 2a and b). CCDC3 interacted with up to 20 proteins. Importantly, the Wnt4 protein was one of the significant hubs involved in multiple functional processes associated with CCDC3 (Fig. 2c and d).

Genes associated with CCDC3 mRNA expression in EOC were searched in The Cancer Genome Atlas (TCGA) database. Using Pearson’s correlation analysis (\(|r| \geq 0.3\)), the top 10 co-expressed genes that

![Fig. 1. Kaplan–Meier analysis of OS and PFS of patients with epithelial ovarian cancer (EOC). (A) Overall survival (OS) curve of patients with EOC based on coiled-coil domain containing 3 (CCDC3) expression level in TNM stage III/IV; (B) Progression-free survival (PFS) curve of patients with EOC based on CCDC3 expression level in TNM stage III/IV; (C) OS curve of patients with EOC based on CCDC3 expression level; (D) PFS curve of patients with EOC based on CCDC3 expression level. EOC patients with CCDC3 high expression level show significantly poorer OS and PFS rates than those with CCDC3 low expression level (\( p < 0.001 \)).](image-url)
positively correlated with CCDC3 mRNA expression level were identified (Supplementary Figs. 1a and b). Among them, Stathmin-1 (STMN1) is a well-known gene with poor prognosis in ovarian cancer [23] (Supplementary Fig. 1c). Additionally, based on the relationship between CCDC3 with the IFN-γ response suggested by GSVA, we further explored the correlation between CCDC3 mRNA expression level with tumour-infiltrating immune cells in EOC. The results showed that CCDC3 mRNA expression was positively correlated with M2 macrophages and naïve B cells (p < 0.05, respectively), while negatively correlated with activated CD4 memory T and memory B cells (p < 0.01 and p < 0.05, respectively; Supplementary Fig. 1d).

A drug sensitivity analysis was also performed. Data were downloaded from the Genomics of Drug Sensitivity in Cancer (GDSC) database and subdivided into low vs. high groups according to the CCDC3 mRNA expression level. The results demonstrated that samples with low CCDC3 mRNA expression level had relatively lower IC50 values of Mitogen-activated protein kinase (MEK) inhibitors compared with those with high CCDC3 expression level (low vs. high, AZD-6244, 3.06 vs. 3.10, p < 0.001; CI 1040, 2.63 vs. 2.66, p = 0.0046, Supplementary Fig. 1e), which indicates they are more sensitive to the MEK inhibitors. In contrast, the IC50 values of angiogenesis inhibitor and cisplatin in the samples with high expression of CCDC3 were significantly lower than those with low expression (low vs. high, AMG.706, 4.15 vs. 4.21, p < 0.001; Cisplatin, 3.28 vs. 3.44, p = 0.022, Supplementary Fig. 1e).

### 3.3. Differentiation of BMSC-derived adipocytes

Given the prognostic value of CCDC3 and its interaction with carcinogenic process in EOC found using the above-described analyses, we assessed the functional role of CCDC3 in modulating the proliferation, invasion, and migration of EOC cells. For this, BMSCs were cultured and induced to differentiate to mature adipocytes. The maturation of adipocytes was confirmed using ORO staining (Fig. 3a).

### 3.4. CCDC3 CM promotes proliferation, migration, and invasion of EOC cells

SKOV3 cells were co-cultured with nine groups of CM obtained from BMSC-derived adipocytes (Table 1). CCDC3 expression was confirmed using RT-qPCR and western blotting with the concentrated supernatant collected from CM (Fig. 3b); cell viability was measured using the MTT assay. The viability, as well as the migration and invasion abilities of EOC cells were markedly increased in the CCDC3 CM compared with that in the NC or blank CM (Fig. 3c–e). Taken together, these results suggest that CCDC3 mediates tumour invasiveness.
3.5. Silencing CCDC3 inhibits EOC cell proliferation, migration, and invasion

To explore the effect of CCDC3 on the tumorigenic activity of EOC cells, CCDC3 expression was transiently silenced using three different siRNAs. Among them, siRNA1 markedly knocked down the expression of CCDC3 (Fig. 3b). A similar attenuation in cell proliferation, and in particular, migration and invasion, was observed after silencing CCDC3 with siRNA-1 (Fig. 3c–e), confirming that CCDC3 exerts pro-metastatic effects in ovarian cancer cells.

3.6. CCDC3 affects the Wnt/β-catenin pathway in EOC cells

In line with the results of our bioinformatics analyses, which...
indicated that CCDC3 could be associated with the Wnt/β-catenin pathway in ovarian cancer, we further explored the effects of CCDC3 on the Wnt/β-catenin pathway in EOC cells using western blotting and RT-qPCR. In EOC cells co-cultured with CCDC3 CM, the protein and mRNA expression levels of N-cadherin, vimentin, fibronectin, β-catenin, c-myc, and cyclin D1 were drastically increased, while that of E-cadherin was markedly decreased compared with when the cells were co-cultured with NC or blank CM (Fig. 4a). In contrast, in SKOV3 cells co-cultured with CCDC3 siRNA CM, the expression level of N-cadherin, vimentin, fibronectin, β-catenin (nuclear), c-myc, and cyclin D1 were significantly downregulated, whereas E-cadherin expression was upregulated compared with when the cells were co-cultured with NC or blank CM (Fig. 4a).

In addition, a Wnt/β-catenin signalling agonist (SKL2001) or inhibitor (XAV-939) was added to EOC cells co-cultured with different CMs to further confirm the interaction of CCDC3 and the Wnt/β-catenin pathway. EOC cells proliferation was further promoted or inhibited when agonist or inhibitor were added compared with CCDC3 CM alone, respectively (Fig. 4b). Similarly, upregulation and downregulation of cell viability were observed when agonist and inhibitor were added, respectively, compared with CCDC3 siRNA CM alone (Fig. 4b). The migration and invasion abilities of EOC cells were consistently further increased or decreased after the addition of the agonist or inhibitor compared with those of cells co-cultured with CCDC3 CM alone, respectively (p < 0.0001, Fig. 4c and d). These abilities of EOC cells in the CCDC3 CM plus inhibitor were still observably higher than those of the cells co-cultured with NC or blank CM (p < 0.05, Fig. 4c and d). The migration and invasion abilities of EOC cells were also further decreased after culturing with CCDC3 siRNA CM with the inhibitor compared with when culturing with CCDC3 siRNA CM alone (p < 0.05, Fig. 4c and d). After the addition of agonist in the CCDC3 siRNA CM, these activities of EOC cells were improved than that without agonist, but only the improvement of migration was significant (p < 0.05, Fig. 4c and d). Furthermore, even with the addition of agonist, the migration of EOC cells in the CCDC3 siRNA CM was still significantly lower than that in the NC siRNA CM (p < 0.001, Fig. 4c and d). These results demonstrate that increased and decreased CCDC3 expression dramatically activated and suppressed the Wnt/β-catenin signalling, respectively, and correspondingly promoted or inhibited EOC cell proliferation and migration/invasion, respectively. Taken together, the overexpression of CCDC3 promoted EOC progression mediated by Wnt/β-catenin, and CCDC3 knockdown reversed the EMT in ovarian cancer.

4. Discussion

EOC is a threat to human health globally owing to its late diagnosis and metastatic capability, and new therapeutic approaches are urgently needed. As EOC is a peritoneal malignancy that rarely metastasizes and metastatic capability, and new therapeutic approaches are urgently investigated. A recent study showed that CCDC3 is a downstream member of the p63 network and regulates liver lipid metabolism by inhibiting de novo lipogenesis [16]. Another study reported that CCDC3 inhibits the expression of endothelial genes induced by TNF-α through repressing NF-κB activation, and thus, participates in the pathological mechanisms involved in obesity and atherosclerosis [17]. These findings suggest that CCDC3 plays a major role in lipid metabolism. More importantly, in subjects with abdominal obesity, the expression of CCDC3 was specifically increased in adipose tissue in the omentum rather than in subcutaneous fat [15]. It is, therefore, possible that CCDC3 could be a crucial factor, if not the only one, causing the progression and metastasis of EOC to the omentum.

In the present study, analysing data from independent databases, we demonstrated that CCDC3 overexpression is associated with poor prognoses of EOC, regardless of the tumour stage. However, owing to the lack of suitable datasets, we were unable to analyse the correlation between CCDC3 expression levels and clinicopathological features of patients with ovarian cancer. Nevertheless, our results raise the possibility of exploring CCDC3 as a candidate biomarker for EOC prognosis.

Using several bioinformatic analyses, we investigated the biological signatures substantially related to CCDC3 in EOC. The raw data extracted from TCGA were bulk mRNA sequencing data of tumor samples, which are composed of tumour cells and stromal cells (including adipocyte and endothelial cells, etc.). In this case, we can not determine whether a certain transcriptional feature belongs to tumor cells or stromal cells, unless single cell sequencing is done. In fact, there is a lot of crosstalk between tumor cells and stromal cells in tumor microenvironment, and these signaling are the key to tumor progression. CCDC3 is not only co-expressed with many genes that promote tumour development, such as CAMK1D in breast cancer [31], MAP7D2 in colorectal cancer [32], SLITRK2 in breast cancer [33], JCAD in liver cancer [34], and SKIDA1 in EOC [35], but is also probably closely related to the suppressive tumour immune microenvironment. Intriguingly, STMN1 [23], a known poor prognosis in ovarian cancer was significantly co-expressed with CCDC3. Although the underlying relationship between them needs to be further explored, this finding validates the prognostic value of CCDC3 in EOC to some extent. In addition, CCDC3 interacts with multiple crucial signalling pathways involved in tumour progression and development, including the Notch, Wnt, TNF-α, and angiogenesis signalling pathways. The relationship between CCDC3 and TNF-α/NF-κB has been previously identified by Azad et al. [17]. They demonstrated that CCDC3 overexpression suppresses the TNF-α-induced NF-κB activation in endothelial cells. Antiangiogenic therapy has been controversial in EOC. The addition of bevacizumab to standard chemotherapy did not significantly improve the overall survival of patients with advanced EOC, although PFS was prolonged for four months [36]. Tyrosine kinase inhibitors targeting vascular endothelial growth factor, pazopanib and nintedanib, showed similar results [37,38]. As a vessel-derived secretory protein, CCDC3 may also play a role in promoting angiogenesis. Endothelial cells may up-regulate the expression of CCDC3 through VEGF pathway, so the tumours with high CCDC3 expression are more dependent on this pathway and more sensitive to antiangiogenic drugs. This was observed in the results of our bioinformatic analyses but the underlying mechanisms remain to be confirmed. MEK inhibitors have been approved by the United States Food and Drug Administration as a breakthrough therapy in previously treated patients with recurrent low-grade serous ovarian cancer. The overexpression of CCDC3 was found to be related to the resistance of tumor to MEK inhibitors in our bioinformatics analysis, but more studies are needed to confirm the correlation between them.

Among these signalling pathways, we focused on the Wnt/β-catenin signalling pathway given that its role in the proliferation and metastasis
Fig. 4. Coiled-coil domain containing 3 (CCDC3) conditioned medium (CM) affects the WNT/β-catenin pathway in SKOV3 cells. (A) Western blot analysis of the expression levels of E-cadherin, N-cadherin, vimentin, fibronectin, β-catenin, c-myc, and cyclin D1 in SKOV-3 cells. GAPDH was used as the loading control. (B) The proliferation activity of SKOV3 cells is upregulated and downregulated when a WNT/β-catenin agonist and inhibitor are added to CCDC3-enriched CM or CCDC3 siRNA CM, respectively. (D) The migration of SKOV3 cells is upregulated and downregulated when a WNT/β-catenin agonist and inhibitor are added to CCDC3-enriched CM or CCDC3 siRNA CM, respectively. (E) The invasion of SKOV3 cells is upregulated and downregulated when a WNT/β-catenin agonist and inhibitor are added to CCDC3-enriched CM or CCDC3 siRNA CM, respectively. (*p < 0.05, **p < 0.01, ***p < 0.001).
of EOC has been well established. Similar to CCDC3 overexpression, CCDC3 CM increased the cellular proliferation, invasion, and migration abilities of EOC cells, suggesting that CCDC3 is a paracrine molecule that facilitates cancer progression and metastasizes. The expression of EMT marker proteins, including N-cadherin and nuclear β-catenin, was dramatically upregulated in EOC cells treated with CCDC3 CM and, correspondingly, cancer cell motility was significantly enhanced. These findings suggest that the activation of the Wnt/β-catenin signalling pathway and the initiation of the EMT underlie the pro-metastatic role of CCDC3.

Our results are in line with those of Zhang et al., who also found that CCDC3 is associated with increased migratory behaviour and EMT in cervical cancer cells [18]. CCDC3 can bind to several proteins through the C-terminal coiled-coil domain. Hence, it is possible that CCDC3 interacts with components of the Wnt/β-catenin signalling pathway, either by directly binding to its undetermined receptors or by forming heterodimers or hetero-oligomers with other ligands by indirectly binding to their receptors. In addition, the viability of EOC cells was further promoted by adding an agonist of Wnt/β-catenin to CCDC3 CM, supporting the interaction of CCDC3 with the Wnt/β-catenin signalling pathway. Interestingly, when the Wnt/β-catenin inhibitor was added to CCDC3 CM, the viability of EOC cells was not significantly reduced, whereas in the absence of CCDC3, cell viability decreased significantly. This finding implies that overexpression of CCDC3 may play a pro-tumour role not only through the activation of Wnt/β-catenin pathway and it is more likely to drive tumour progression than activation of the Wnt/β-catenin pathway. Thus, inhibiting the Wnt/β-catenin pathway alone is insufficient to suppress tumour progression when CCDC3 is overexpressed. Dual inhibition of CCDC3 and Wnt/β-catenin may be an effective treatment strategy for EOC with peritoneal metastasis in the future.

It is well known that the Wnt/β-catenin pathway plays a tumour-promoting role in EOC [39,40]. Aberrant Wnt/β-catenin signalling in EOC is essential for initiation of the EMT, a cellular process in which epithelial cells lose intercellular adhesion and acquire the stromal characteristics of migration and invasion [41,42]. By forming a complex with β-catenin at the adhesion junction, E-cadherin helps to maintain a low level of cytosolic/nuclear β-catenin. Thus, a decline in E-cadherin level accompanied by the nuclear accumulation of β-catenin leads to enhanced invasion and migration of cancer cells [43,44]. In addition, the Wnt/β-catenin pathway promotes the EMT by suppressing E-cadherin expression by directly or indirectly upregulating the expression of transcription factors known as mesenchymal inducers [45] and remodels extracellular matrix through upregulation of the activities of matrix metalloproteinases [46–49].

There are a few limitations in our study. First, the relationship between CCDC3 expression and clinicopathological features was not confirmed in clinical samples or database. Second, the in vitro studies were done using a single cell line of ovarian cancer. It is important to validate experimental findings in multiple cell lines in future. The biological function of CCDC3 in EOC, including its regulation of tumor angiogenesis and suppression of tumor immune microenvironment need to be further studied.

5. Conclusions

To the best of our knowledge, this is the first study that identified and characterised CCDC3 as a promoter of tumorigenesis of EOC. Silencing CCDC3 reduced the adhesive and invasive capacities of EOC cells. Therefore, targeting CCDC3 is a promising therapy for metastatic ovarian cancer. Our future research directions include: (i) identifying the receptor(s) in EOC cells that mediate the CCDC3 paracrine effect; (ii) determining the mechanism underlying the role of CCDC3 in tumour angiogenesis; and (iii) developing antibody- and/or peptide-based therapeutic approaches to target CCDC3 in vitro and in vivo.

Currently available therapeutic regimens are ineffective against advanced EOC. In this pre-clinical and mechanistic study, we identified the prognostic value of CCDC3 in EOC and provided novel insights into its key role in promoting the tumorigenesis of EOC through the Wnt/β-catenin signalling pathway. Our results support that CCDC3 can serve as an efficacious therapeutic target to treat EOC, which may lead to the discovery of a novel therapeutic strategy.

Ethics approval and consent to participate

The authors state that they have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

Consent for publication

All authors read and approved the final manuscript.

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Credit Author Statement

Feng Jin: contribute equally to this article. Data curation, Formal analysis, Writing – original draft preparation, Shanshan Peng contribute equally to this article. Resources, Validation, Writing- Reviewing and Editing, Chen Li: Software, Visualization. Li Wang: Investigation. Shubin Wang: Supervision. Fen Wang: Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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List of abbreviations

BMSC Bone mesenchymal stem cell
CCDC Coiled-coil domain-containing
CM Conditioned medium
CI Confidence interval
DMEM Dulbecco’s modified Eagle’s medium
EMT Epithelial-mesenchymal transition
EOC Epithelial ovarian cancer
FBS Foetal bovine serum
GDSC Genomics of Drug Sensitivity in Cancer
GSEA Gene set enrichment analysis
GSVA Gene set variation analysis
HR Hazard ratio
NC Negative control
OS Overall survival
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101507.

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