Adipocyte-derived CCDC3 Promotes Tumorigenesis in Epithelial Ovarian Cancer Through the Wnt/β-catenin Signalling Pathway

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Abstract

**Background:** Epithelial ovarian cancer (EOC) is a highly aggressive disease whose unique metastatic site is the omentum. Coiled-coil domain containing 3 (CCDC3) is an adipocyte-derived secreted protein that is specifically elevated in omental adipose tissue. However, its function is still unknown.

**Results:** Overexpression of CCDC3 was associated with poor prognosis of EOC. CCDC3 interacted with multiple key signalling pathways, including the Wnt/β-catenin pathway. EOC cellular proliferation, migration, and invasion were promoted in vitro when co-cultured with conditioned medium containing CCDC3, and this tumour-promoting effect was induced by activating the Wnt/β-catenin pathway. Furthermore, the epithelial-mesenchymal transition of EOC cells was reversed after CCDC3 silencing.

**Conclusions:** Our results support that CCDC3 promotes EOC tumorigenesis through the Wnt/β-catenin pathway and that CCDC3 may serve as a novel prognostic biomarker and therapeutic target for metastatic EOC.

Background

Among gynaecological malignancies, ovarian cancer is the leading cause of mortality. 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-secretary 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.
Results

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 Figure 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: hazard ratio (HR), 1.82, 95% confidence interval (CI), 1.44–2.29, \( p < 0.001 \); PFS: HR, 1.8, 95% CI, 1.47–2.19, \( p < 0.001 \); Figure 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 is (OS: HR, 1.84, 95% CI, 1.50–2.27, \( p < 0.001 \); PFS: HR, 1.81, 95% CI, 1.50–2.19, \( p < 0.001 \); Figure 1c and d). Taken together, these findings suggest that CCDC3 expression level is a suitable biomarker for EOC prognosis.

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, KRAS, 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 (Figure 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 (Figure 2c).

Genes associated with CCDC3 mRNA expression in EOC were searched in The Cancer Genome Atlas (TCGA) database. Using Pearson's correlation analysis (|r| ≥ 0.3), the top 10 co-expressed genes that positively correlated with CCDC3 mRNA expression level were identified (Additional Figure 1a and b). 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; Additional Figure 1c).

Mitogen-activated protein kinase (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. KRAS mutation status have been reported to predict the response rate of MEK inhibitors in EOC (19). Since a positive relationship between CCDC3 mRNA expression and the KRAS
signalling pathway was observed in GSVA, a drug sensitivity analysis was 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 MEK inhibitors compared with samples 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; roscovitine, 4.91 vs. 4.93, p = 0.0024). This indicated that the expression level of CCDC3 mRNA is related to the sensitivity of EOC to MEK inhibitors (Additional Figure 1d).

**Differentiation of bone mesenchymal stem cell (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 oil red O (ORO) staining (Figure 3a).

**CCDC3 conditioned medium (CM) promotes proliferation, migration, and invasion of EOC cells**

SKOV3 cells were co-cultured with eight groups of CM obtained from BMSC-derived adipocytes: CCDC3 CM; negative control (NC) CCDC3; CCDC3-agonist CM; CCDC3-inhibitor CM; si-CCDC3 CM; NC si-CCDC3; si-CCDC3-agonist CM; and si-CCDC3-inhibitor CM (Table 1). CCDC3 expression was confirmed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) with cells collected from CM and western blotting (Figure 3b); cell viability was measured using the MTT assay. The viability of cells in the CCDC3 CM group was markedly increased compared with that in the NC and blank groups (Figure 3c). In addition, CCDC3 CM consistently increased the *in vitro* migration and invasion abilities of SKOV3 cells compared with when control CM was added (Figure 3d). Taken together, these results suggest that CCDC3 mediates tumour invasiveness.

**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 (Figure 3b). Among them, siRNA1 markedly knocked down the expression of CCDC3. A similar attenuation in cell proliferation, and in particular, migration and invasion, was observed after silencing CCDC3 with all siRNAs (Figure 3c and d), confirming that CCDC3 exerts pro-metastatic effects in ovarian cancer cells.
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 and blank CM (Figure 4a). In contrast, in SKOV3 cells co-cultured with si-CCDC3 CM, the expression level N-cadherin, vimentin, fibronectin, β-catenin, c-myc, and cyclin D1 was significantly downregulated, whereas E-cadherin expression was upregulated compared with when the cells were co-cultured with NC and blank CM (Figure 4a).

In addition, a Wnt/β-catenin signalling agonist (SKL2001) or inhibitor (XAV-939) was added to EOC cells co-cultured with different CM to further confirm the interaction of CCDC3 and the Wnt/β-catenin pathway. EOC cell proliferation was further promoted or inhibited when CCDC3-agonist or -inhibitor CM were added compared with CCDC3 CM, respectively (Figure 4b). Similarly, upregulation and downregulation of cell viability were observed when si-CCDC3-agonist CM and si-CCDC3-inhibitor CM were added, respectively, compared with when si-CCDC3 CM was added (Figure 4b). The migration and invasion abilities of EOC cells were consistently increased after the addition CCDC3 CM, and further increased after the addition of the agonist compared with those of cells co-cultured with NC or blank CM (p < 0.001, Figure 4c). The migration and invasion abilities of EOC cells were slightly decreased when cells were cultured with si-CCDC3-inhibitor CM, but still significantly higher than those of the cells co-cultured with NC or blank CM (p < 0.05, Figure 4c). The migration and invasion abilities of EOC cells were significantly decreased in si-CCDC3 CM compared with those of cells co-cultured with NC or blank CM (p < 0.005 and p < 0.001, respectively; Figure 4c). These viabilities were further decreased after culturing with si-CCDC3 CM with the inhibitor compared with when culturing with NC or blank CM (p < 0.001, Figure 4c). 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.

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 outside the adipocyte-rich abdominal cavity (20), it has been suggested that the omentum adipose cells play specific roles in favouring the establishment of EOC metastasis in this tissue (4). Interactions between adipocytes and tumour cells have been found in multiple solid malignancies, including EOC. Obesity contributes to the metastasis of ovarian cancer and is negatively correlated with the survival of patients with ovarian cancer (21). In EOC, adipocytes not only accelerate tumour growth via adipocyte-specific lipolysis but also promote tumorigenesis and development by secreting adipokines (22),
hormones (23), and growth factors (3). Additionally, some adipose-derived proteins, such as fatty acid-binding proteins 4 and 5, affect the fatty acid oxidation pathway to enhance cancer progression by mediating lipid transfer between adipocytes and cancer cells (24).

CCDC3 is an adipocyte/endothelial cell-derived secretory protein that belongs to the CCDC family. The biological functions of this family of proteins have been investigated extensively, including their involvement in tumour metastasis, invasion, and other pathophysiological processes (18). For example, overexpression of CCDC34 is associated with angiogenesis in oesophageal squamous cell cancer (25). CCDC109B is highly expressed in gliomas and reportedly related to tumour progression (26). The role and significance of CCDC3, which is mainly expressed in adipose tissue and aorta, have not been thoroughly 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. CCDC3 is not only co-expressed with many genes that promote tumour development, such as CAMK1D in breast cancer (27), MAP7D2 in colorectal cancer (28), SLITRK2 in breast cancer (29), JCAD in liver cancer (30), and SKIDA1 in EOC (31), but is also probably closely related to the suppressive tumour immune microenvironment. In addition, CCDC3 interacts with multiple crucial signalling pathways involved in tumour progression and development, including the Notch, Wnt, TNF-α, and KRAS 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. Additionally, another study suggested that mutations in KRAS help predict the efficacy of MEK inhibitors (32). In a randomised phase III clinical trial in which the efficacy of the MEK inhibitor binimetinib was compared with that of chemotherapy in low-grade serous ovarian cancer, patients with KRAS mutations benefited more from binimetinib treatment (19). The interaction between CCDC3 and the KRAS pathway may explain CCDC3-induced resistance to MEK inhibitors, which was observed in the results of our bioinformatics analyses. Nonetheless, the underlying mechanism remains to be determined.
Among these signalling pathways, we focused on the Wnt/β-catenin signalling pathway given that its role in the proliferation and metastasis of EOC has been well established. Similarly 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 metastases. The expression of EMT marker proteins, including N-cadherin and 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 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 (33, 34). 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 (35, 36). 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 (37, 38). 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 (39) and remodels extracellular matrix through upregulation of the activities of matrix metalloproteinases (40-43).

There are a few limitations in our study. The relationship between CCDC3 expression and clinicopathological features was not confirmed in clinical samples or database. The biological function of CCDC3 in EOC, including its regulation of tumor angiogenesis and suppression of tumor immune microenvironment need to be further studied.

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.

**Methods**

**Cell culture**

Rat 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.

**Adipogenesis of BMSCs**

Inducers including 1 μM dexamethasone, 5 mg/L insulin, and 0.5 mM IBMX (all purchased from Sigma) 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.

**ORO staining of BMSC lipids**

BMSC-derived adipocytes were rinsed gently with phosphate-buffered saline, and subsequently stained with fresh diluted ORO with final concentration of 60% (Sigma) 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 (Servicebio, Cat no: G1004) for 30 s. The samples were mounted using an aqueous solution. The prepared slides were visualised using an Axiosvert microscope (Perkin Elmer) at ×200 magnification. The relative steatosis area is expressed as the percentage of ORO-stained areas.
**CCDC3 overexpression and knockdown**

Rat *Ccdc3* cDNA was synthesised and cloned into a cloning expression vector pcDNA3.1 (+) at the *BamHI*-*XhoI* site to generate a CCDC3-expression vector. CCDC3-packaged plasmids were transiently transfected into BMSC-derived adipocytes for the generation of the CCDC3-containing CM (hereinafter referred to as CCDC3 CM). Different siRNAs (siRNA1, siRNA2, and siRNA3) targeting three distinct sequences of rat *Ccdc3* gene (Additional Table 1) were purchased from Jima Pharmaceutical Company. These siRNAs were used for knocking down *CCDC3* and an NC siRNA was used. BMSC-derived adipocytes were transfected with siRNA1 for the generation of knocked down CCDC3-containing CM (hereinafter referred to as si-CCDC3 CM).

**SKOV3 co-cultured with CM**

SKOV3 cells were co-cultured with eight groups of CM: CCDC3 CM; NC of CCDC3 CM (NC CCDC3); CCDC3 CM treated with 20 μM SKL2001 (Selleck, S1180) for 48 h (hereinafter referred to as CCDC3-agonist CM); CCDC3 CM treated with 5 μM XAV939 (Selleck, S8320) for 48 h (CCDC3-inhibitor CM); si-CCDC3 CM; NC of si-CCDC3 CM (NC si-CCDC3); si-CCDC3 CM treated with 20 μM SKL2001 for 48 h (si-CCDC3-agonist CM); and si-CCDC3 CM treated with 5 μM XAV939 for 48 h (si-CCDC3-inhibitor CM). Subsequently, cells were harvested for RT-qPCR, western blotting, and cell proliferation, migration, and invasion assays.

**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. Real-time 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 $2^{-\Delta \Delta CT}$ method. All primers were synthesised by Tsingke Biotechnology (Beijing, China). All the experiments were conducted in triplicate.

**Western blotting**

Cultured cells were lysed with lysis buffer containing radioimmunoprecipitation 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. 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 (Additional Table 2). Bands were imaged using a ChemiDoc™XRS+ scanner (Bio-Rad).
Proliferation assays

SKOV3 cells were seeded in 96-well plates at a density of 5,000 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 MTT solution was added to each well. After incubation for 4 h at 37 °C, discard the supernatant, add 150 µl DMSO to each well, shake for 10min to dissolve the crystals, and then the 96-well plate was read by an enzyme-linked immunosorbent assay (ELISA) reader at 490 nm for absorbance density values to determine the cell viability. All the experiments were conducted in triplicate.

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, P0099) for 10 min and then stained with 5% crystal violet solution (Beyotime, C0121) for 10 min. The cells were counted in three randomly selected visual fields using an inverted microscope (IX71, Olympus). All the experiments were performed in triplicate.

Bioinformatic analysis

Data acquisition

The raw data of CCDC3 mRNA expression in 359 ovarian cancer samples were extracted from TCGA (https://portal.gdc.cancer.gov/).

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) (44). The log-rank p-value and HR with 95% CI were calculated.

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

The CIBERSORT (45) 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.

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. GDSC (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.

GSVA and GSEA

The GSVA (46) package in R was utilised to estimate the signalling pathways associated with CCDC3 expression. The clusterprofiler (47) 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 1,000 permutations. The population was divided into two groups according to the CCDC3 mRNA expression level (high vs. low). The acceptable level of significance was \( p \leq 0.01 \). The gene set “c2.cp.kegg.v6.2.symbols.gmt” was selected as the reference gene set (MSigDB, http://software.broadinstitute.org/gsea/msigdb/index.jsp).

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.

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).

List Of Abbreviations

BMSC Bone mesenchymal stem cell

CCDC Coiled-coil domain-containing
Declarations

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

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.
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Authors’ contributions

FW and SBW designed the study. FJ and SSP performed the bioinformatic analyses. FW and FJ performed the in vitro experiments. CL and LW performed the statistical analyses. FW wrote the manuscript. FJ, SSP, CL, LW, and SBW reviewed and edited the manuscript. All authors read and approved the final manuscript.

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References


Tables

Table 1  Definition of eight groups of conditional medium (CM)
Table 2 Sequences of Primers
### Name | Sequence 5'-3' | Size
---|---|---
**GAPDH** | Forward: TCAAGAAGGTGGTGAGTGAAGCGG | 115bp
| Reverse: TCAAGGTGGAGGAGTGG | 
**E-cadherin** | Forward: TCACATCCTACACTGCCCAG | 229bp
| Reverse: AGTGTCCCTGTCCAGTAGC | 
**N-cadherin** | Forward: CGATCCCAATGCCCTCAATG | 185bp
| Reverse: TGCCTTCCATGTCTGTAGCT | 
**Vimentin** | Forward: TTGAACGCAAAGTGGAATC | 157bp
| Reverse: AGGTCAGGCTTGGAAACA | 
**Fibronectin** | Forward: TAGCTTTTGTGTCTCTGGG | 201bp
| Reverse: AAACCTCTGCTCCACATCCT | 
**β-catenin** | Forward: CTTCATCGACGATCCAGTC | 98bp
| Reverse: CCTTCATCCCTCCTGTTTAG | 
**c-myc** | Forward: CATACATCCTGTCGCTCAAG | 109bp
| Reverse: GAGTTCCGTAAGCTGTTCAAGT | 
**Cyclin D1** | Forward: GCGAGGAACAGAAGTGCG | 192bp
| Reverse: TGGAGTTGCTCGTGTAGATGC | 
**CCDC3** | Forward: TCTAACACTACCTGCCTGG | 152bp
| Reverse: GGAGAAGTAGCCCAGACCT | 

### Figures

#### Figure 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 \((p < 0.001)\).
Figure 2

Analysis of signalling pathways and protein interactions associated with coiled-coil domain containing 3 (CCDC3). (A) Gene set variation analysis of CCDC3 in The Cancer Genome Atlas (TCGA) epithelial ovarian cancer (EOC) dataset; (B) Gene set enrichment analysis of CCDC3 in TCGA-EOC dataset; (C) Protein–protein interaction network of CCDC3.

Figure 3

Coiled coil domain-containing 3 (CCDC3) conditioned medium affects the proliferation and migration and invasion abilities of SKOV3 cells. (A) Adipogenesis of rat bone mesenchymal stem cells (BMSCs) evaluated using oil red O staining of their lipid content. (B) BMSC-derived adipocytes were stably transduced with plasmids encoding CCDC3 or siRNAs targeting CCDC3 to generate CCDC3-containing CM and si-CCDC3 CM, respectively. The presence of CCDC3 in the CM was confirmed using reverse transcription-quantitative polymerase chain reaction with cells collected from CM and western blotting. (C) Significant increase and decrease in the proliferation activity of SKOV3 cells is observed in CCDC3-containing CM and si-CCDC3 CM, respectively. (D) Significant increase and decrease in migration and invasion activities of SKOV3 cells is observed in CCDC3-containing CM and si-CCDC3 CM, respectively (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 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 CM, respectively. (D) The migration and invasion activities of SKOV3 cells is upregulated and downregulated when a WNT/β-catenin agonist and inhibitor are adding to CCDC3 CM, respectively.

Supplementary Files

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