Original Article

Spliceosome-associated factor catenin b-like 1 accelerates ovarian cancer progression by inducing expression and nuclear accumulation of yes-associated protein

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Abstract: Ovarian cancer is a gynecologic malignancy with potentially fatal consequences. The molecular pathogenesis of ovarian carcinoma remains incompletely understood. The aim of this present study was to investigate the molecular mechanisms of catenin b-like 1 (CTNNBL1) and yes-associated protein (YAP) in ovarian cancer progression. Immunohistochemical analysis and Western blotting were carried out to determine expression of CTNNBL1 in ovarian cancer tissues and adjacent non-tumor tissues. Colony formation, wound healing, and transwell assays, along with xenograft models and flow cytometry, were used to assess the effects of CTNNBL1 on cell viability, migration, invasion, tumorigenesis, and apoptosis, respectively. Western blotting analysis and immunofluorescence staining were performed to evaluate the roles of CTNNBL1 regarding expression level and location of YAP. Results revealed that spliceosome-associated factor CTNNBL1 was significantly upregulated in ovarian carcinoma tissues. It promoted proliferation, migration, and invasion of ovarian cancer cells, in vitro, and inhibited apoptosis. CTNNBL1 upregulated YAP1 expression and accelerated its translocation from the cytoplasm to the nucleus in ovarian cancer cells. In addition, HULC (Highly Upregulated in Liver Cancer) and MCAM (Melanoma Cell Adhesion Molecule), two target genes of CREB (cAMP Response Element-binding Protein), were upregulated by CTNNBL1. In a xenograft model, CTNNBL1 increased tumorigenesis, whereas tumorigenesis was significantly reduced when YAP expression was inhibited. The findings of this study suggest that CTNNBL1/YAP may represent a novel therapeutic target for prevention of ovarian cancer progression.

Keywords: CTNNBL1, YAP, HULC, MCAM, ovarian cancer

Introduction

In China, ovarian cancer is one of the most common gynecologic malignancies, with potentially lethal consequences. In 2015, 52,100 new cases and 22,500 deaths were reported [1]. High-grade serous ovarian carcinoma, an aggressive subtype of ovarian cancer, accounted for the majority of diagnoses of ovarian cancer (70%) and is associated with low survival rate [2]. Despite significant advances in research and treatment, the molecular pathogenesis of ovarian carcinoma remains incompletely characterized.

Catenin b-like 1 (CTNNBL1), a widely expressed nuclear protein, associates with the spliceosomalPrp19 complex and interacts with the CDC5L component of the complex [3, 4]. As shown by RNA interference (RNAi) screening, the CTNNBL1 gene is a putative regulator of canonical Wnt signaling pathways that acts upstream of or in parallel to β-catenin [5]. A previous study has demonstrated that depletion of CTNNBL1 in mouse embryos resulted in mid-gestational embryonic lethality [5]. Research has also shown that single nucleotide polymorphisms, in CTNNBL1 individuals with an increased body mass index and fat mass, were associated with risk of colorectal cancer in case-control studies of different populations [6-8]. Regarding ovarian cancer, Li et al. [9] reported that CTNNBL1 was significantly upregulated in patients with high-grade serous ovarian carcinomas, indicating poor prognosis, and elevated expression of CTNNBL1 promoted cell proliferation and invasion of ovarian cancer cells. However, specific molecular mech-
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The Hippo pathway is known to play an important role in regulating cell proliferation and organ size, in both Drosophila and mammals. In mammals, the Hippo pathway consists of a highly conserved kinase cascade, including upstream proteins. Together, they form a complex, including phosphorylates Yes-associated protein (YAP), a key transcriptional coactivator, thereby facilitating the localization of YAP in the cytoplasm. Consequently, YAP is not translocated to the nucleus, where it normally interacts with TEAD transcription factors and mediates expression of downstream growth-promoting and apoptosis-inhibiting genes [10, 11]. A number of reports have shown that YAP functions as an oncoprotein by interacting with several transcription factors and participating in progression of various types of cancer [12-15]. Several studies have reported aberrant activation or overexpression of the YAP oncoprotein in ovarian cancer [16-18]. Xia et al. [17] suggested that YAP promotes ovarian cancer cell tumorigenesis and that it was indicative of a poor prognosis in ovarian cancer patients. Zhang et al. [19] demonstrated that high nuclear YAP expression was positively correlated with poor prognosis in a cohort of 268 patients with invasive epithelial ovarian cancer, suggesting that YAP de-repression contributed to the genesis of ovarian clear cell carcinoma. Taken together, the literature points a close relationship between YAP and progression of ovarian cancer. However, whether CTNNBL1 can promote cell viability by activating YAP-Hippo signaling remains unclear.

The present study investigated the molecular mechanisms of CTNNBL1/YAP in ovarian cancer progression. The ultimate aim was to find a new therapeutic strategy for ovarian cancer. Evidence suggesting that CTNNBL1/YAP possesses pro-cancer effects would provide new gene therapy targets for ovarian cancer.

Material and methods

Tissue specimen preparation

Samples of matched fresh ovarian cancer and para-carcinoma tissues were obtained from patients with ovarian cancer having undergone a radical ovariectomy, without chemotherapy or radiation. Pancreatic cancer surgical specimens were cut into 1 cm³ pieces, quickly fixed in 100 g/L of formaldehyde solution, embedded in paraffin, and then stored at 4°C until used in further studies. Histological specimens were mounted on slides and reviewed by two experienced pathologists having no knowledge of the clinical data.

This study was approved by the Human Research Committee of Cangzhou Central Hospital and Chinese Anti-Cancer Association and was performed in accordance with the Helsinki Declaration.

Immunohistochemical analysis

Corresponding sections of fresh ovarian cancer and para-carcinoma tissues were incubated with the following primary antibody: anti-CTNNBL1 (diluted 1:200), (Abcam, MA, USA). IgG conjugated with peroxidase was used as the secondary antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China). In negative controls, incubation with the above antibodies was omitted. Sections were examined using an upright light microscope (Olympus, Tokyo, Japan) and analyzed using ImageJ software (National Institutes of Health, MD, USA). At least three randomly selected areas were evaluated.

Cell culture

A2780 and SKOV3 human ovarian cancer cell lines were obtained from American Type Culture Collection (USA). A2780 cells were cultured in Dulbecco's Modified Eagle's Medium (BI, USA). SKOV3 cells were grown in McCoy's 5a (modified) medium (BI). All were supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL of penicillin (Life Technologies, MA, USA), and 100 μg/mL of streptomycin sulfate (Life Technologies) and maintained at 37°C in a humidified 5% CO₂ atmosphere. The culture medium was changed every other day in all experiments.

Western blotting analysis

Total proteins from cells or tissue samples were extracted using RIPA buffer containing protease inhibitor (Beyotime Institute of Biotechnology, China) on ice. For nuclear and cytoplasmic protein extraction, CelLytic™ NuCLEAR™ extraction kit (Sigma, USA) was used, according
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to manufacturer instructions. Concentration of proteins in the samples was detected by BCA protein assay kit (Thermo Fisher Scientific, USA). The proteins were separated by 10% SDS-PAGE and electro transferred to PVDF membranes (Merck Millipore, USA). Membranes were then blocked in 5% nonfat milk for 1 hour and incubated overnight with primary antibodies at 4°C. The next day, PVDF membranes were washed with 1 × TBST, followed by incubation for 1 hour with horse radish peroxidase (HRP)-labeled secondary antibodies diluted with 5% nonfat milk. Finally, the protein bands were detected using an ECL system (GE, USA). GAPDH, histone, and tubulin were used as endogenous control for total, nuclear, and cytoplasmic proteins, respectively. Primary antibodies used in this study included the following: anti-GAPDH (1:5,000, Sigma-Aldrich, USA), anti-histone (1:5,000, Abcam), anti-tubulin (1:5,000, Abcam), anti-CTNNBL1 (1:500, Abcam), and YAP1 (1:1000, Abcam).

RNA extraction and real-time polymerase chain reaction (PCR)

RNA was extracted from A2780 and SKOV3 cells using TRIzol Reagent (Life Technologies) when cells reached confluence of 90%, according to manufacturer instructions. cDNA was synthesized by reverse transcription using ThermoScript reverse-transcription PCR system (Promega, WI, USA), according to manufacturer protocols. Quantitative real-time PCR was performed using SYBR Green PCR master mix (204143; Qiagen NV, Venlo, the Netherlands) in a total volume of 20 μL on a 7900HT fast real-time PCR system (Thermo Fisher Scientific). Primers were as follows: YAP, 5’-CGCTCTTACACGCCGTCA-3’ (forward) and 5’-AGTACTGGCTGTCGGGAGT-3’ (reverse); MCAM, 5’-GCGTCTACAAAGCTCCGGAGGA-3’ (forward) and MCAM, 5’-GAATGTGGACCCGGTTCTTCTCCTC3’ (reverse); HULC, 5’-ACCTCCAGCCGTCA-3’ (forward) and HULC, 5’-CAATTGAGGGAGGTAGGACAC-3’ (reverse); GAPDH, 5’-ATCATCCCTGCCCTTACTGG-3’ (forward) and GAPDH, 5’-GTACAGTGCCACCCACGAC-3’ (reverse).

Small interfering RNA (siRNA) and cell transfection

siRNAs against human CTNNBL1 genes (No. SR311144) and YAP genes (No. SR323110) (OriGene, MA, USA) were used to knockdown expression of CTNNBL1 and YAP, respectively. Each siRNA (37.5 nM) was transfected into ovarian cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific, MA, USA), according to manufacturer instructions. After cells were transfected with siRNA for 24 hours, the knockdown efficiency of each target gene was verified by qRT-PCR analysis. CTNNBL1 vector (No. SC109047) and its control vector (OriGene, MA, USA) were used to overexpress levels of CTNNBL1 in ovarian cells. Every 5 × 10^5 cells were transfected with 2 mg of vector using Lipofectamine 2000, according to manufacturer instructions.

Cell counting kit-8 (CCK-8) assay

Cell proliferation was measured using CCK-8 assay (Dojindo, Tokyo, Japan). Cells were seeded in 96-well plates, in triplicate, at densities of 1 × 10^3 cells per well. Cell proliferation was monitored at different times (0-96 hours). After incubation for designated times, 10 μl of CCK-8 solution were added to each well and incubated for another 2 hours. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad, USA).

Colony formation assay

Regarding colony formation assay, 2 × 10^2 A2780 and SKOV3 cells were plated into six-well plates and cultured for 14 days. The colonies were then fixed for 5 minutes with 10% formaldehyde and stained with 1.0% crystal violet for 30 seconds.

Detection of cell apoptosis

The effects of CTNNBL1 on cell apoptosis were determined by flow cytometry, as described in a previous study [20]. For assessment of apoptosis, an annexin V-fluorescein isothiocyanate (FITC) staining kit was used. A2780 and SKOV3 cells were harvested in 0.25% trypsin and washed once with phosphate-buffered saline (PBS). After centrifugation, cells were stained using an annexin V-FITC/propidium iodide apoptosis detection kit (BD Biosciences, MA, USA). Analysis of apoptotic cells was performed on a flow cytometer (BD Biosciences, MA, USA). The experiment was performed in triplicate.

Migration and invasion transwell assays

For the migration assay, A2780 and SKOV3 cells were seeded in a 24-well plate at 3.0 ×
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10⁵ per well and incubated overnight to reach a confluent monolayer of layers. A 20 µl pipette tip was used to scratch a straight line slowly across the well (wound healing assay). The well was then washed with 1 × PBS three times. The medium was replaced with serum-free medium and continued to culture. After 24 hours, the distance between the two edges of the scratch was photographed and quantitatively evaluated.

For the invasion assay, a 24-well transwell plate (8.0 µm, Corning, USA) was used to determine the invasive capability of each cell line. Briefly, the upper surface of membranes was precoated with Matrigel Basement Membrane Matrix (BD Biosciences, MA USA). A total of 1 × 10⁵ cells in serum-free medium were seeded into the upper chambers and treated with indicated drugs, according to manufacturer instructions. Complete medium supplemented with 10% fetal bovine serum was added to the lower chamber as a chemoattractant. After incubation for 48 hours, invading cells attached to the lower surface were fixed, stained, and counted. The percentage of invasive cells was calculated based on the ratio of attached stained cells to the total number of seeded cells. All experiments were performed in triplicate.

Immunofluorescence staining

Immunofluorescence staining was performed in accordance with previously described protocol [21]. Briefly, A2780 or SKOV3 cells were seeded at 10% confluence onto small glass coverslips placed in 24-well plates. After 24 hours of culturing, different treatments were performed. The coverslips were then removed, washed with PBS three times, and fixed with 4% paraformaldehyde in PBS for 15 minutes. After pushing through the cytomembrane (in 0.1% Triton, 0.1% sodium citrate for 10 minutes) and blocking in Dako blocking solution for 1 hour, cells were incubated with YAP1 primary antibodies (1:100 dilution) overnight at 4°C. After washing with PBS, cells were incubated with a 1:500 dilution of a fluorescent tag (Alexa Fluor 488; Invitrogen) and conjugated with secondary antibodies for 30 minutes in the dark. Samples were treated with DAPI (1:10,000, Invitrogen, MA, USA) for 5 minutes, washed three times with PBST, covered with an anti-fade mounting medium (Vectashield, Loerrach, Germany), and placed onto microscope slides. The slides were examined under a laser scanning microscope (TCSSP2-AOBS-MP; Leica Microsystems CMS).

Xenograft model

Eight-week-old female C57BL/6 SCID mice were obtained from Jackson Laboratory (Beijing, China) and housed in a specific pathogen-free facility. C57BL/6 SCID mice were implanted 107 cells with different treatments. Control group mice were given 200 ml phosphate-buffered saline (PBS) in their mammary fat pads. Development of solid tumors was monitored for up to 28 days post-xenotransplantation. The experimental protocol was approved by the Animal Care and Research Committee of Cangzhou Central Hospital.

Statistical analysis

Each experiment was performed in triplicate. Statistical analyses, including two-tailed tests,
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Figure 2. CTNNBL1 increased ovarian cancer cell proliferation, migration, and invasion in vitro. A. Knockdown efficiency of siRNAs-CTNNBL1 was detected by Western blotting analysis. B. Clonogenic assay of the effects of CTNNBL1 on the proliferation of ovarian cancer cells. C, D. CCK-8 analysis of the effects of CTNNBL1 on cell growth. E, F. Results of a wound healing assay, showing the effects of CTNNBL1 on cell migration of ovarian cancer cells. G. Results of a transwell assay of the influence of CTNNBL1 on cell invasion of ovarian cancer cells. H. Cell apoptosis rates in CTNNBL1 knockdown A2780 and SKOV3 cells compared to control cells analyzed by flow cytometry. The data presented are mean ± standard error (SE) and represent three independent experiments. (*P < 0.05, **P < 0.01, ***P < 0.001).
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CTNNBL1 was overexpressed in ovarian cancer tissues

Results of the immunohistochemistry analysis revealed that CTNNBL1 was significantly upregulated in ovarian cancer tissues compared with that in adjacent nontumor tissues (Figure 1A). To confirm CTNNBL1 overexpression in ovarian cancer tissues, Western blotting and RT-PCR analysis were conducted to determine CTNNBL1 expression levels. Expression of CTNNBL1 was significantly higher in ovarian cancer tissues than in adjacent nontumor tissues and normal tissues (n=3; Figure 1B). These results suggest that CTNNBL1 may play an important role in development of ovarian cancer.

CTNNBL1 promoted cell viability, migration, and invasion in ovarian cancer cells, in addition to inhibiting cell apoptosis

To investigate the functional roles of CTNNBL1 in ovarian cancer, expression of CTNNBL1 in ovarian cancer cell lines (SKOV3 and A2780) was downregulated by siRNA targeting of human CTNNBL1 genes. The effects of CTNNBL1 on colony formation ability, growth, migration, and invasion of ovarian cancer cells, as well as apoptosis, was then analyzed. As shown in Figure 2A, siRNA2-CTNNBL1 showed the highest knockdown efficiency in both A2780 and SKOV3 cells. Thus, this siRNA was used in subsequent analyses. Downregulation of CTNNBL1 dramatically decreased the colony-forming efficiency and growth of A2780 and SKOV3 cells (Figure 2B-D). Wound healing and transwell assays demonstrated that knockdown of CTNNBL1 appeared to inhibit cell migration and invasion abilities (Figure 2E-G). In addition, cell apoptosis rates were significantly elevated when cells were transfected with siRNA-CTNNBL1, compared with the control group in both A2780 and SKOV3 cells (Figure 2H). These findings suggest that CTNNBL1 promotes proliferation and migration of ovarian cancer cells, in vitro, but inhibits cell apoptosis.

CTNNBL1 upregulated YAP1 expression and accelerated nuclear accumulation of proteins

To further explore the molecular mechanisms of CTNNBL1 in ovarian cancer progression, this study examined the effects of upregulation or downregulation of CTNNBL1 expression on YAP1 expression. Figure 3A displays the trans-
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**Figure 4.** CTNNBL1 accelerated nuclear accumulation of YAP1 proteins. A. Immunofluorescence staining analysis of CTNNBL1-induced nuclear translocation of YAP1 proteins. B. Western blotting analysis of the effects of CTNNBL1 on nuclear translocation of YAP1 (extracted from the nucleus and cytoplasm) in A2780 and SKOV3 cells. Expression of the nuclear protein was normalized to that of histone and expression of the cytoplasmic protein was normalized to that of tubulin. The data presented are mean ± standard error (SE) and represent three independent experiments. (*P < 0.05, **P < 0.01, ***P < 0.001).

Infection efficiency of CTNNBL1 vector in A2780 and SKOV3 cells. Western blotting showed that protein levels of YAP1 were clearly increased in CTNNBL1-upregulated cells, compared with that in control cells, whereas YAP1 expression was markedly reduced in A2780 and SKOV3 cells when CTNNBL1 was knocked down (Figure 3B, 3C), demonstrating that CTNNBL1 positively regulated expression of YAP1 in ovarian cancer cells.

Immunofluorescence staining results showed that overexpression of CTNNBL1 induced nuclear accumulation of YAP1, whereas nuclear expression of YAP1 was decreased in the CTNNBL1 knockdown group compared with the control group (Figure 4A). Western blotting analysis of nuclear and cytoplasmic proteins extracted from A2780 and SKOV3 cells revealed that YAP1 proteins were translocated from cytoplasm to the nucleus in accordance with incubation time when CTNNBL1 was overexpressed in cells. In contrast, YAP1 proteins were translocated from the nucleus to cytoplasm in accordance with incubation time when expression of CTNNBL1 was downregu-
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**Discussion**

Ovarian cancer, one of the most common gynecologic malignancies, has the highest mortality rate among all female reproductive system cancers [22]. The high mortality has been attributed to late diagnosis of the disease and the ineffectiveness of chemotherapy. Through functional characterization studies, it has been revealed that CTNNBL1 promotes proliferation and invasion of ovarian cancer cells, in vitro. However, the underlying mechanisms by which CTNNBL1 promotes ovarian cancer development remain to be elucidated. In this present study, for the first time, it was demonstrated that CTNNBL1 accelerates cancer progression through upregulation of YAP expression and translocation to the nucleus.

Although previous studies have confirmed that CTNNBL1 is associated with the spliceosomal-Prp19 complex, it has not been considered to be part of the core, as it dissociated from the CDC5L-SPF27-PRPF19-PLRG1 complex under high salt conditions and heparin treatment [23, 24]. Unlike other members of the Prp19 complex, CTNNBL1 is not essential for cell viability [25]. However, CTNNBL1 functionally interacts with the complex by maintaining cellular levels of core proteins in the complex [4], illustrating that CTNNBL1 can exert a pro-growth function.

A recent genome-wide survey and functional brain imaging study reported that CTNNBL1 is a memory-related gene [26]. Previous studies have reported that polymorphisms in CTNNBL1 were associated with colorectal cancer and overexpression of CTNNBL1 showed a positive correlation with progression of ovarian cancer. In this study, both protein and mRNA levels of
CTNNBL1 were overexpressed in ovarian cancer tissues versus those in adjacent noncancer tissues. These findings suggest that dysregulated expression of CTNNBL1 might play a crucial role in the development and progression of ovarian cancer. Based on these findings, the function of CTNNBL1 on cell viability, migration, invasion, and apoptosis were explored after downregulation of the protein in ovarian cancer cells. Results revealed that cell viability, migration, and invasion decreased whereas cell apoptosis increased in CTNNBL1 knockdown ovarian cells.

YAP, encoded by YAP1, is a critical component of the Hippo signaling pathway [27]. Its activity relies on downstream transcription factors, including TEAD and CREB [28]. CTGF and ANKRD1 are target genes of TEAD [29, 30] and MCAM and HULC are target genes of CREB [31, 32]. Dysregulation of the Hippo pathway is common in human cancers [10, 33-35] and is correlated with poor prognosis [36]. In the present study, CTNNBL1 upregulated expression of YAP and it induced the translocation of YAP from cytoplasm to the nucleus. As YAP functions as a co-transcription factor, the loss of YAP in the nucleus decreases transcriptional activities and subsequent target gene expression of its transcription factors, such as MCAM and HULC, which play critical roles in development of cancer in humans [37, 38]. In the current study, levels of MCAM and HULC were elevated after cells were transfected with CTNNBL vector, whereas MCAM and HULC expression levels appeared to be reduced in cells with knockdown of CTNNBL1. These results suggest that CTNNBL1 may promote proliferation and migration of ovarian cancer cells by activating YAP/MCAM and HULC pathways.

Finally, this study confirmed the assumption that CTNNBL1 promotes development of ovarian cancer by activating YAP pathways, in vivo. Although CTNNBL1 promoted the growth of tumors when injected into nude mice, tumorigenesis was inhibited after injection of cells with YAP was depleted. This finding verifies the hypothesis that CTNNBL1 plays a role in development and progression of ovarian cancer by activating YAP pathways.

Taken together, the findings of this present study identify CTNNBL1/YAP as a novel regulator of cell viability, migration, and tumorigenesis in ovarian cancer cells. Knockdown of CTNNBL1 represses cell viability, migration, and invasion, as well as promoting cell apoptosis. Inhibition of YAP impedes cell tumorigenesis, regardless of overexpression of CTNNBL1 in ovarian cancer cells. Therefore, CTNNBL1/YAP might be a novel therapeutic target inhibiting the progression of ovarian cancer.

Disclosure of conflict of interest

None.

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References

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