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Carbonyl reductase 4 suppresses colorectal cancer progression through the DNMT3B/CBR4/FASN/mTOR axis

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Abstract

Lipid metabolism is implicated in the initiation and progression of human colorectal cancer (CRC). Carbonyl reductase 4 (CBR4), a member of the carbonyl reductase family, plays a role in the biosynthesis of fatty acids. However, its involvement in CRC remains poorly understood. In this study, we aim to explore the function of CBR4 in CRC. Our findings indicated that the expression of CBR4 was significantly reduced in CRC tissues. Functional analyses revealed that CBR4 functions to inhibit cell proliferation, colony formation, migration, invasion, and tumor growth in vivo. Mechanistically, CBR4 interacts with fatty acid synthase (FASN), activating the ubiquitin-proteasome pathway, which leads to a reduction in FASN expression, thereby inhibiting the mTOR pathway and curtailing CRC development. Orlistat, a known FASN inhibitor, demonstrated anti-cancer properties both in vitro and in vivo. Additionally, DNMT3B, a DNA methyltransferase, contributed to the down-regulation of CBR4 by inducing methylation in the promoter region. In summary, our findings suggest that the DNMT3B/CBR4/FASN/mTOR signaling pathway is crucial in the advancement of CRC, and elucidate the potential mechanism by which enzymatic carbonyl reduction and lipid metabolism may be connected to CRC progression, offering a novel therapeutic strategy for its clinical management.

Introduction

Colorectal cancer (CRC) is widely recognized as the third most prevalent cancer and the second leading cause of cancer-related fatalities globally [1, 2]. In recent years, there has been a rapid increase in its incidence [3, 4]. Each year, over 2 million individuals worldwide receive a diagnosis of CRC, with approximately 1 million fatalities attributed to the disease last year [5]. The primary treatment options for CRC encompass surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy [6, 7]. Nonetheless, surgical intervention is only appropriate for patients diagnosed at an early stage. For those with advanced CRC, the effectiveness of current treatment approaches remains inadequate [8, 9].

Altered lipid metabolism is among the most significant metabolic alterations observed in cancer, where increased lipid synthesis or uptake plays a crucial role in the rapid proliferation of cancer cells and tumor development. Research indicates that enzymatic carbonyl reduction may facilitate cancer progression due to its direct involvement in lipid metabolism [10]. In mammals, carbonyl reduction plays a pivotal role in the phase I metabolism of xenobiotics, primarily carried out by families of

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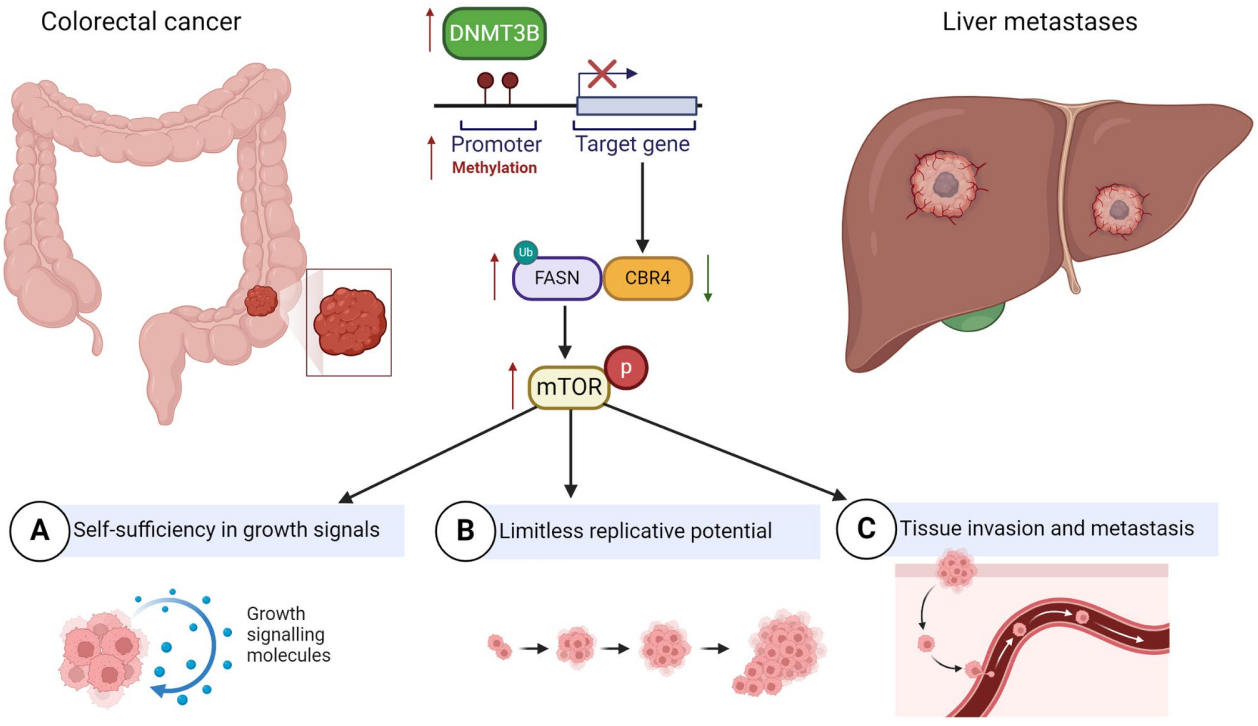

Keywords Colorectal cancer, CBR4, FASN, DNA methylation, mTOR

aldo-keto reductases (AKR) and short-chain dehydrogenase/reductases (SDR) [11]. Carbonyl reductase (CBR), a member of the SDR superfamily, is currently classified into three identified SDR carbonyl reductases: carbonyl reductase 1 (CBR1), carbonyl reductase 3 (CBR3), and carbonyl reductase 4 (CBR4) [12, 13]. Notably, CBR4 functions as a tetrameric protein [14] and is involved in fatty acid biosynthesis [15]. It is well understood that continuous de novo fatty acid synthesis is critical to meet the biosynthetic needs of cancers [16]. Dysregulation of genes associated with lipid metabolism contributes to the onset and progression of colorectal cancer (CRC), making them potential molecular targets for CRC treatment [17, 18]. Increasingly, novel drug development focuses on the heightened metabolic traits of tumors to enhance clinical outcomes. Thus, CBR4 may hold significant clinical relevance in CRC.

In the current study, we discovered that CBR4 exhibited a marked downregulation and was notably linked to unfavorable prognosis in colorectal cancer (CRC). Experimental investigations in both cell cultures and animal models have demonstrated that CBR4 functions as a tumor suppressor in CRC. Mechanistically, CBR4 is known to interact with FASN, leading to a reduction in FASN expression, the deactivation of the mTOR signaling pathway, and a decline in lipid metabolism, ultimately inhibiting CRC progression. This research aims to elucidate the role of the CBR4/FASN/mTOR axis in CRC concerning lipid metabolism, with the potential to identify new molecular markers for prognosis, diagnosis, and treatment of CRC.

Methods

Human CRC cell lines and tissues

All cells were sourced from the Shanghai Cell Bank of the Chinese Academy of Sciences. HCT116 and RKO cell lines were grown in RPMI-1640 (Biological Industries, Israel), while SW480 cells were maintained in Leibovitz's L-15 (Procell, Wuhan, China). The 293T cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Procell). Each type of cell was supplemented with 10% fetal bovine serum (FBS, Yeasen Biotechnology, Shanghai, China) and 1% penicillin-streptomycin solution (New Cell & Molecular Biotech, Suzhou, China). All cells were incubated in a cell incubator (Thermo) set to 37 ℃ with 5% CO2 and saturated humidity. CRC tissues, along with matched adjacent normal tissues, were collected from the Jiangsu Province Hospital, with informed consent obtained from each participant.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA from CRC cell lines and tissues was extracted using TRIzol (Vazyme, Nanjing, China). Subsequently, we conducted reverse transcription to produce cDNA

for the quantitative real-time polymerase chain reaction (qPCR) utilizing a specific cDNA synthesis kit (Vazyme). Following reverse transcription, the qPCR assay was performed using Hieff Universal Blue SYBR Green Mix (Yeasen). The relative expression of genes was determined by the 2-ΔΔCt method, with GAPDH serving as the internal control for standardization. The final analysis was carried out using GraphPad Prism 9 software. The primers utilized in this investigation are detailed in Supplementary Table 1.

Stable transfection of cell lines

Plasmids for the knockdown and overexpression of CBR4 were obtained from Genomeditech in Shanghai, China. Short hairpin RNA (shRNA) targeting DNMT1, DNMT3A, and DNMT3B was sourced from Tsingke Biotechnology in Beijing, China. Gifts of DNMT3B overexpression plasmids were provided by Professor Duan Ma from Fudan University. All knockdown plasmids were developed using the PLKO1 vector, whereas the overexpression plasmids utilized the PLVX vector. The specific short hairpin targets employed in this research are listed in Supplementary Table 2. Subsequently, lentivirus was packaged within 293T cells through the application of PEI MAX transfection reagent from Polysciences, USA. In brief, 40 μL of the PEI-MAX transfection agent combined with 1.25 μg of PAX2, 3.75 μg of PDM2G, and 5 μg of the target genes were mixed into 1.5 mL of serum-free DMEM. After a 15-minute incubation, this mixture was introduced to 293T cells in 5.5 mL of serum-free DMEM. Following 6 h, 0.7 mL of FBS was added, and the lentivirus was harvested after 48 h using a 0.45 μm filter. A combination of 3 mL of lentivirus and 3 mL of complete medium was applied to CRC cells for a duration of 24 h. Stably transfected cells were selected following screening with puromycin (5 μg/ml, Yeasen) and confirmed using qPCR and western blot analyses.

Cell proliferation assays

Cell proliferation was assessed using CCK-8, EdU, and colony formation assays. In the CCK-8 assay, 5 × 10^3 CRC cells were plated in 100 μL of the corresponding culture medium within 96-well plates and then incubated with 10 μL of CCK-8 reagent for 2 h. The absorbance was measured at 450 nm utilizing a microplate reader. For the EdU assay, cells in the logarithmic growth phase were inoculated into 96-well plates at a density of 1 × 10^5 cells per well and cultured until they reached the logarithmic phase. An appropriate volume of 50 μM EdU medium was prepared by diluting the EdU solution with the culture medium, and 100 μL of this solution was added to each well for a 2-hour incubation. Afterward, each well received 2 mg/mL glycine following incubation with 4% paraformaldehyde at room temperature for 30 min. After washing with PBS and treating with 0.5% Triton X-100, 100 μL of 1×Apollo® staining solution and 100 μL of 1×Hoechst 33,342 reaction solution were added to each well. Finally, images were captured using a fluorescence microscope. In the colony formation assay, 1 × 10^4 cells were planted in a 6-well plate, cultured for one week, and subsequently fixed with 4% paraformaldehyde and stained with 0.25% crystal violet. The results from the EdU and colony formation assays were quantified using ImageJ software.

Cell migration and invasion assays

In the cell migration assays, 2 × 10^5 cells were placed into 8 μm micropore inserts that contained no FBS. For assays assessing cell invasion, 4 × 10^5 cells were introduced into 8 μm micropore inserts that had been coated with 50 μL of diluted matrigel (Yeasen) and also lacked FBS. A solution of 30% FBS was then added to the 24-well plates. After a period of 48 h, the bottoms of the inserts were fixed using 4% paraformaldehyde for 15 min and subsequently stained with 0.5% crystal violet for 25 min, followed by washing with PBS. Images were captured randomly using a microscope and analyzed with Image J software.

Western blot

Protein from CRC cells and tissues was extracted utilizing RIPA lysis buffer (Beyotime, Nantong, China) supplemented with 1mM PMSF (Beyotime) while kept on ice for 25 min. Tumor tissue extraction necessitated the homogenization of samples using an ultrasonic grinder. Subsequently, protein concentrations were determined through the Bradford assay (Beyotime). Samples were combined with 1× SDS protein loading buffer (Yeasen) and boiled for 5 min at 100 °C (Yeasen). After electrophoresis, the membranes (Millipore, USA) were subjected to blocking with 8% skimmed milk (Beyotime) for 1 h. Primary antibodies (as listed in Supplementary Table 3) were then applied to the membranes for overnight incubation at 4 °C. Following three washes with TBST buffer, membranes underwent incubation with secondary antibodies for 1 h at room temperature. After three additional washes with TBST, signals of bands were detected using the Enhanced Chemiluminescent Reagent kit (New Cell & Molecular Biotech).

Co-immunoprecipitation

To extract proteins, 1 ml of NP-40 lysate buffer (Beyotime) was mixed with CRC cells in a 10 cm dish, following standard western blot procedures. After a 2-hour incubation at 4 °C with 2 μg of primary antibody, 20 μl of protein A/G magnetic beads (Beyotime) were added and the mixture was inverted overnight. The next day, following three washes with NP-40 lysate buffer, the samples were

incubated in 20 µl of 2×SDS-PAGE sample loading buffer (Beyotime) and subjected to boiling at 100 °C for 5 min in preparation for subsequent western blot analysis.

Animal assays

Subcutaneous injections of 1 × 10^6 CRC cells from each group were performed into the flanks of 4–6 week old BALB/c nude mice to establish animal models. To evaluate the impact of orlistat on tumor growth in vivo, orlistat dissolved in oil (10 mg/kg/day) was administered intragastrically after the cells were transplanted into the mice. After a three-week period, the mice were euthanized, and tumors were harvested for volume and weight measurements. Subsequently, tumor tissues were fixed, and slides were prepared for immunohistochemical analysis of Ki67, CBR4, and FASN. All animal experiments received approval from the Institutional Animal Care and Use Committee at Nanjing Medical University.

Statistical analysis

Each assay was conducted independently at least three times. Data analysis was performed using GraphPad Prism 8.0 software. Results are expressed as mean ± SD. The Student's t-test was utilized to determine significant differences in pairwise comparisons, with P values < 0.05 indicating statistical significance.

Results

CBR4 is down-regulated in CRC

Initially, we observed that the level of CBR4 mRNA in colorectal cancer (CRC) was significantly lower than that in the control group (Fig. 1A), and this level decreased with increasing tumor stage (Fig. 1B). Furthermore, the CBR4 protein levels in CRC tissues were also lower compared to the control group (Fig. 1C), and although the expression of CBR4 protein decreased with advancing tumor stage, this reduction was not statistically significant (Fig. 1D). Survival analysis indicated that lower levels of CBR4 were associated with poorer prognosis (Fig. 1E). Data from western blot analysis revealed that CBR4 expression in tumor tissues was significantly lower than in adjacent tissues (Fig. 1F and G), with further reductions observed in CRC tissues exhibiting distant metastasis (Fig. 1H and I). We tested several CRC cell lines (HCT116, RKO, SW480) and reached the same conclusion (Fig. 1J). Finally, we validated the trend of low CBR4 expression in paracancerous tissues and metastatic tumors using immunohistochemistry (Fig. 1K).

Over-expression of CBR4 reduces CRC cell proliferation, migration and invasion

To investigate the potential tumor inhibitory role of CBR4 in colorectal cancer (CRC), stable CBR4-overexpressing RKO and SW620 cell lines were established through stable transfection. The efficiency of this overexpression was validated using quantitative polymerase chain reaction (qPCR) and western blot analysis (Fig. 2A and B). In cell counting kit-8 (CCK-8) and colony formation assays, the upregulation of CBR4 was associated with a significant inhibition of cell proliferation (Fig. 2C and F). Furthermore, in transwell assays, the increased expression of CBR4 resulted in reduced cell migration and invasion (Fig. 2G and H). Collectively, these findings suggest that CBR4 exerts an anti-cancer effect in CRC.

CBR4 interacts with FASN and promotes its ubiquitin proteasome pathway

Through the STRING database, we identified several proteins that may interact with CBR4 (Fig. 3A). The CPTAC database revealed that the expression of FASN in colorectal cancer (CRC) was significantly higher than that in the control group (Fig. 3B). We confirmed this finding using various cell lines, yielding consistent results (Fig. 3C). In contrast to CBR4, FASN exhibited a pronounced expression trend in paracancerous tissues and metastatic tumors (Fig. 3D and G). The reliability of the interaction between CBR4 and FASN was further validated through co-immunoprecipitation (CO-IP) experiments (Fig. 3H). To explore the relationship between CBR4 and FASN more thoroughly, we first observed that the overexpression of CBR4 reduced the stability of FASN (Fig. 3I). Following treatment with MG132, the level of FASN increased significantly (Fig. 3J). CO-IP experiments involving FASN and ubiquitin indicated that CBR4 enhances the binding of FASN to ubiquitin, facilitating its degradation (Fig. 3K). Subsequent experiments demonstrated that CBR4 promotes the ubiquitin-mediated degradation of FASN primarily at the protein level, rather than at the mRNA level (Fig. 3L and M).

Knockdown of CBR4 promotes CRC cell proliferation, migration and invasion by improving the level of FASN

To further investigate the anti-cancer effect of CBR4 in colorectal cancer (CRC), we established an HCT116 cell line with stable CBR4 knockdown, and the efficiency of this knockdown was verified using Western blot analysis (Fig. 4A). In CCK-8 and colony formation assays, the down-regulation of CBR4 was found to promote cell proliferation (Fig. 4B and D). The Transwell assay revealed that CBR4 knockdown enhances the migration and invasion of HCT116 cells (Fig. 4E and F), thereby supporting the tumor-inhibitory role of CBR4 in CRC. However, following treatment with orlistat, a fatty acid synthase (FASN) inhibitor, this trend was reversed, leading to a reduction in the proliferation, migration, and invasion of tumor cells (Fig. 4H and M).

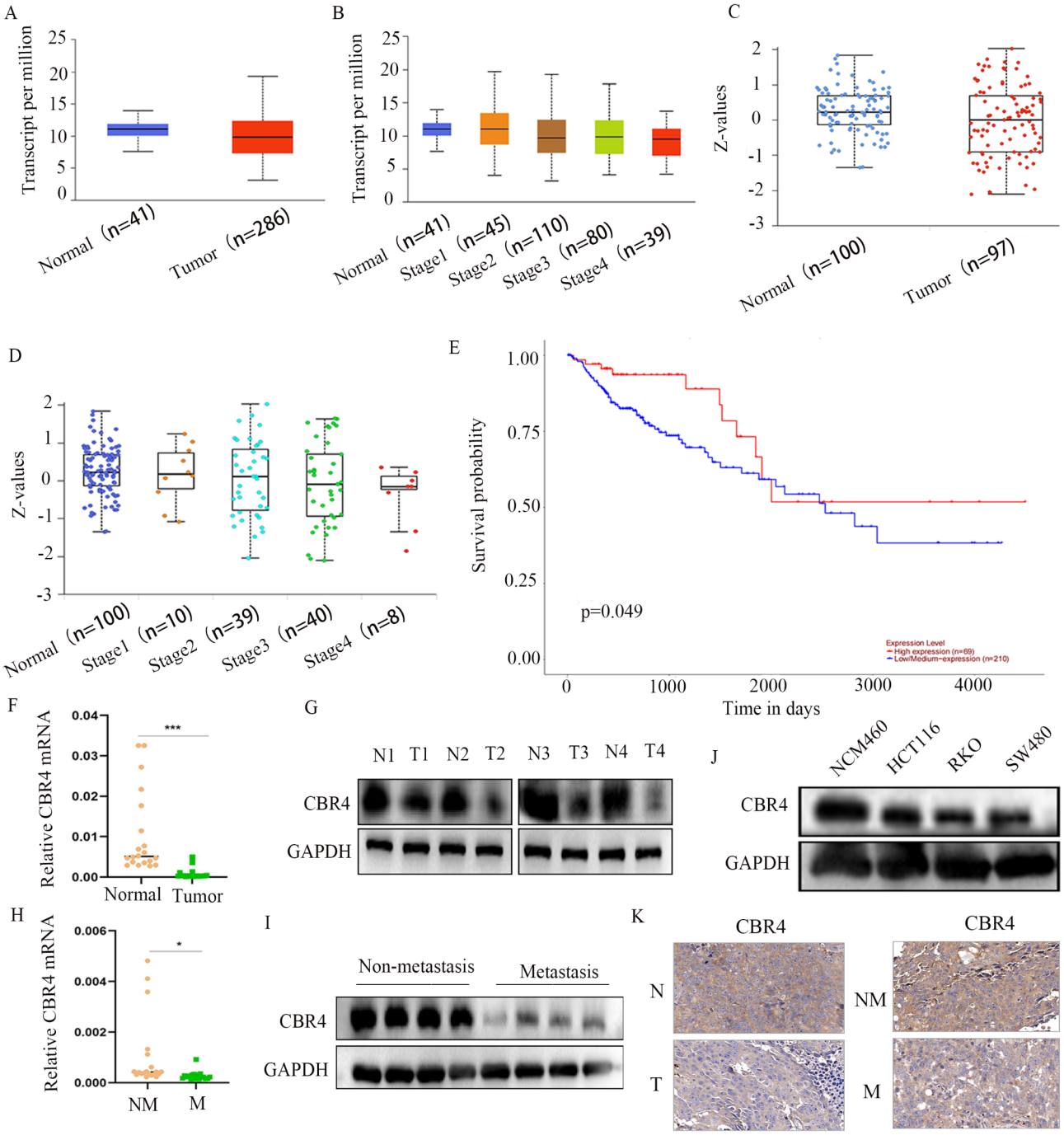

Fig. 1 CBR4 is downregulated in CRC. (**A**) CBR4 was down-regulated in colorectal cancer (CRC) compared to normal tissues, as indicated by the TCGA database ($p = 2.261700E-02$). (**B**) The expression of CBR4 decreased with increasing tumor stage in the TCGA database (Stage 1 vs. Stage 4, $p = 1.285680E-02$). (**C**) CBR4 protein levels in CRC tissues were lower than those in the control group, according to the CPATC database ($p = 2.000459E-02$). (**D**) CBR4 protein levels decreased as tumor stage increased, as reported in the CPATC database ($p > 0.05$). (**E**) Low expression of CBR4 was associated with shorter survival probabilities in CRC cases, as shown in the TCGA database. (**F**-**G**) Quantitative PCR and western blot analyses were conducted to assess the RNA and protein levels of CBR4 in CRC and normal tissues. (**H**-**I**) The RNA and protein levels of CBR4 decreased as tumor metastasis progressed. (**J**) Levels of CBR4 protein were compared between various CRC cell lines and normal cells. (**K**) Immunohistochemical staining of CBR4 was performed on CRC tumor tissues (T), paracancerous tissues (NT), metastatic tumors (M), and non-metastatic tumors (NM) from patients, with a magnification of ×100. (*$P < 0.05$; ***$P < 0.001$)

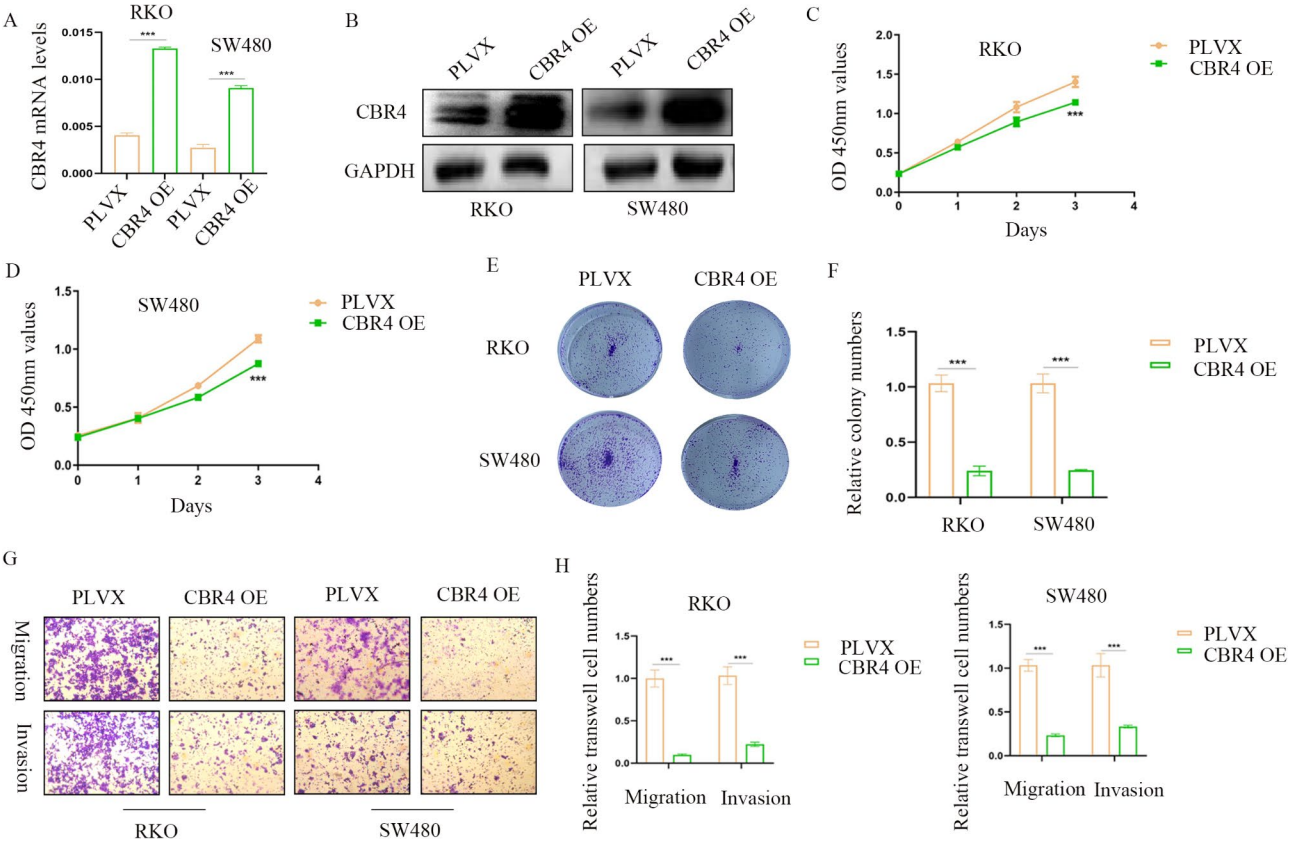

Fig. 2 Over-expression of CBR4 suppresses cell proliferation, migration and invasion. (**A**) mRNA levels of CBR4 were analyzed in both over-expressing and control colorectal cancer (CRC) cells. (**B**) Western blot analysis was conducted to assess the changes in protein levels of CBR4 in over-expressing versus control CRC cells. (**C**-**D**) The over-expression of CBR4 resulted in a significant inhibition of proliferation in RKO (**C**) and SW480 (**D**) cells, as determined by the CCK8 assay. (**E**-**F**) The up-regulation of CBR4 led to a reduction in the number of colonies formed. (**G**-**H**) CBR4 was found to suppress migration and invasion in RKO and SW480 cells, with a magnification of ×100. (***$P < 0.001$)

CBR4 suppresses mTOR pathway mediated by FASN

Subsequently, we analyzed the differential gene expression in tumor cells following the knockdown of CBR4, resulting in the identification of a series of genes with both relatively high and low expression levels (Fig. 5A). Through KEGG and GO analyses, we observed that the expression of the mTOR signaling pathway in the CBR4 knockdown group significantly differed from that in the control group (Fig. 5B and C). Western blot results indicated that the overexpression of CBR4 inhibited the mTOR pathway (Fig. 5D), whereas the downregulation of CBR4 activated the mTOR pathway (Fig. 5E). Furthermore, the inhibition of FASN reversed the activation of the mTOR pathway induced by CBR4 silencing, thereby supporting the tumor suppressor role of CBR4 through its inhibition of mTOR via FASN (Fig. 5F).

DNMT3B regulates CBR4 via DNA methylation

Subsequently, we investigated the downregulation mechanism of CBR4 in colorectal cancer (CRC). Our findings revealed that the methylation level of CBR4 in tumor tissue was significantly higher than that in normal tissue (Fig. 6A) and was positively correlated with tumor stage (Fig. 6B). Following treatment with the DNA methylation inhibitor 5-aza, we observed an increase in both mRNA and protein levels of CBR4, along with a decrease in FASN expression and inhibition of the mTOR pathway (Fig. 6C and F). We also knocked down the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B, verifying the efficiency of the knockdown through qPCR (Fig. 6G and H). Notably, the knockdown of DNMT3B significantly elevated the levels of CBR4 (Fig. 6I and K). Conversely, overexpression of DNMT3B inhibited CBR4 expression and promoted FASN expression (Fig. 6L and N).

Over-expression of CBR4 and knockdown of FASN inhibits tumor growth in vivo

We further verified the roles of CBR4 and FASN in vivo using tumor xenograft models. Specifically, RKO and SW480 cells with CBR4 overexpression and control vector were implanted into nude mice. Notably, the weights and volumes of tumors with the CBR4 overexpression vector were significantly reduced (Fig. 7A and F). A liver

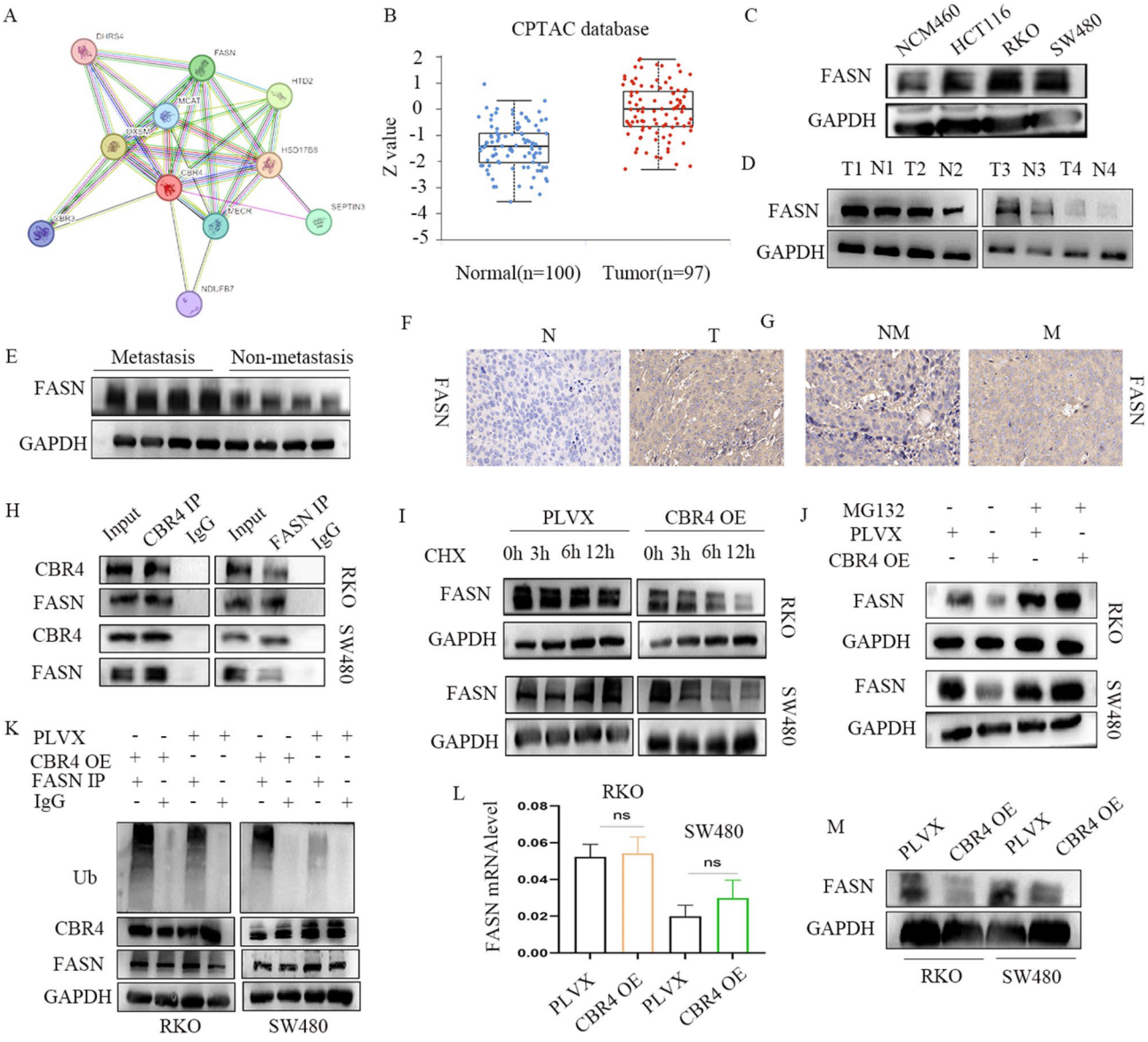

Fig. 3 CBR4 destabilizes FASN by activating its ubiquitin-proteasome pathway. (**A**) Proteins that could potentially interact with CBR4 were identified from the STRING database. (**B**) FASN levels in colorectal cancer (CRC) patients were significantly elevated compared to the control group, as indicated by data from the CPTAC database ($p = 6.11443254489116E-20$). (**C**) The protein levels of FASN were assessed across various CRC cell lines and normal cells. (**D**) The protein levels of FASN were compared between CRC tissues and normal tissues. (**E**) The protein levels of FASN were evaluated in CRC cases with and without metastasis. (**F**-**G**) Immunohistochemical staining of FASN was conducted in CRC tissues, paracancerous tissues, metastatic tumors, and non-metastatic tumors from patients, with a magnification of ×100. (**H**) Co-immunoprecipitation (Co-IP) analysis demonstrated the interaction between CBR4 and FASN in RKO and SW480 cells. (**I**-**J**) Western blot analysis was performed to evaluate FASN levels in RKO and SW480 cells, comparing CBR4 over-expressing groups to control groups, following treatment with CHX (10 μmol/L) and MG132 (10 μmol/L). (**K**) Co-IP analysis was conducted to assess the interaction between FASN and ubiquitin in CBR4 over-expressing and control groups of RKO and SW480 cells. (**L**-**M**) The mRNA and protein levels of FASN were measured in both CBR4 over-expressing and control groups of RKO and SW480 cells

metastasis model was established through tail vein injection, revealing that liver metastases with CBR4 overexpression were markedly suppressed (Fig. 7G and I). Immunohistochemical staining indicated that CBR4 negatively regulated the expression of FASN and decreased Ki67 in vivo, further confirming the anti-tumor effect of CBR4 (Fig. 7J). Western blot analysis demonstrated that CBR4 overexpression inhibited the mTOR pathway (Fig. 7K). Conversely, knockdown of CBR4 accelerated tumor growth and liver metastasis while activating the mTOR pathway, an effect that could be reversed by orlistat (Fig. 7L and R). In summary, our results underscore the essential role of CBR4 in FASN-mediated colorectal cancer progression both in vitro and in vivo.

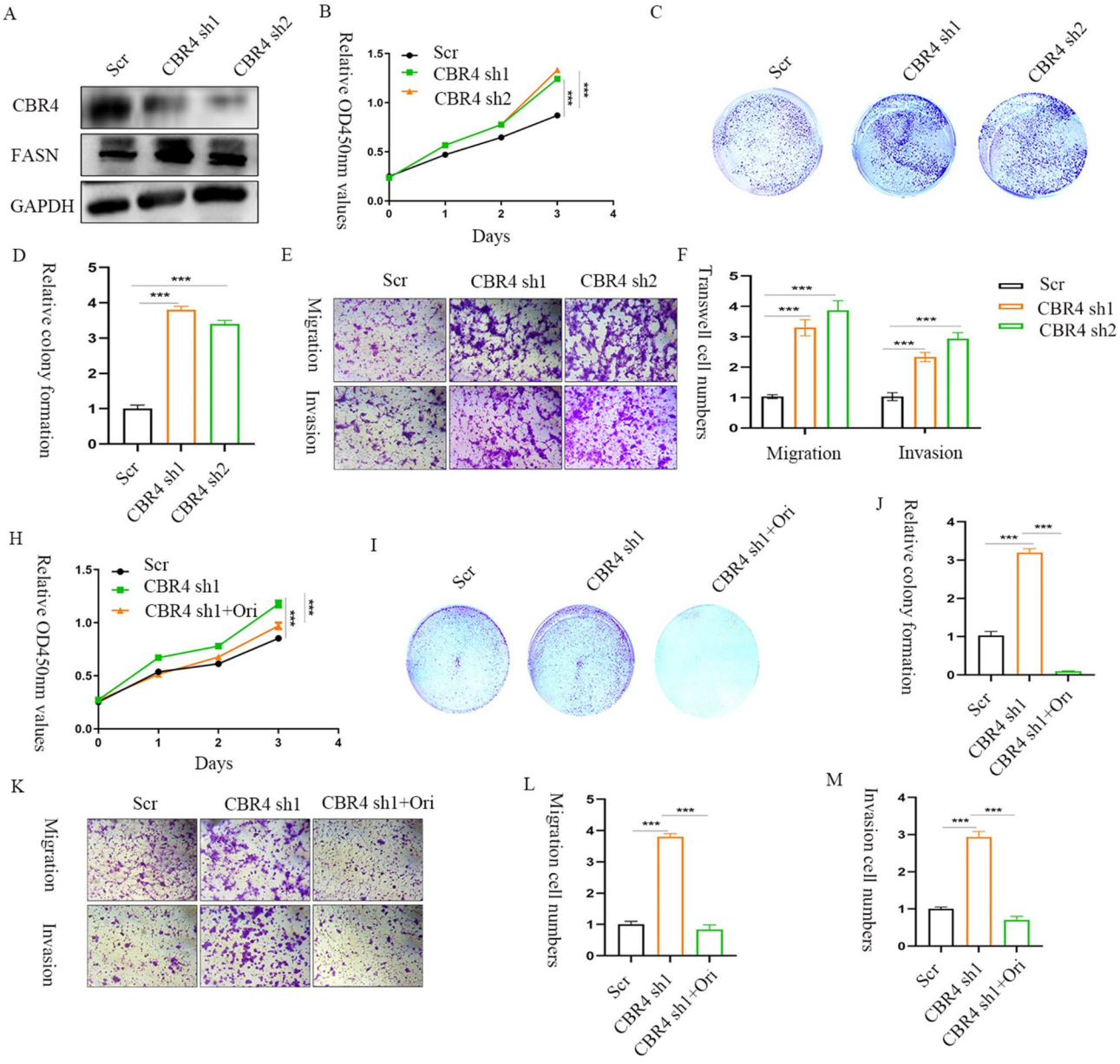

Fig. 4 Orlistat rescues malignant biological behaviors of CRC cells promoted by knocking down of CBR4. (**A**) Western blot analysis was conducted to assess the protein levels of CBR4 in both knockdown and control HCT116 cells. (**B**) CCK-8 assay results indicated that silencing CBR4 enhanced the proliferation of HCT116 cells. (**C**-**D**) Down-regulation of CBR4 resulted in an increased number of colonies. (**E**-**F**) Silencing CBR4 promoted cell migration and invasion in HCT116 cells, with a magnification of × 100. (**H**-**J**) The proliferation of HCT116 cells was subsequently inhibited following treatment with orlistat. (**K**-**M**) Similarly, the migration and invasion of HCT116 cells were also inhibited after treatment with orlistat, at a magnification of × 100. (***$P < 0.001$)

Discussion

Lipids play crucial roles as fundamental components of biofilms and as structural elements of cells. Additionally, they are involved in energy storage and metabolic processes, functioning as significant signaling molecules in various cellular activities. Proper regulation of lipid metabolism—including lipid uptake, synthesis, and hydrolysis—is vital for the maintenance of cellular homeostasis. During the progression of tumors, the nutrient availability in the tumor microenvironment undergoes continuous fluctuations [19, 20]. On one hand, reprogramming of lipid metabolism allows tumor cells to enhance energy production, aiding their survival in nutrient-deficient environments. Conversely, the signaling molecules produced through this lipid reprogramming can activate pathways associated with tumors. Consequently, tumor cells utilize lipid metabolism to support rapid proliferation and metastasis [21, 22]. Modulating lipid metabolism is emerging as a promising strategy in anti-cancer therapies.

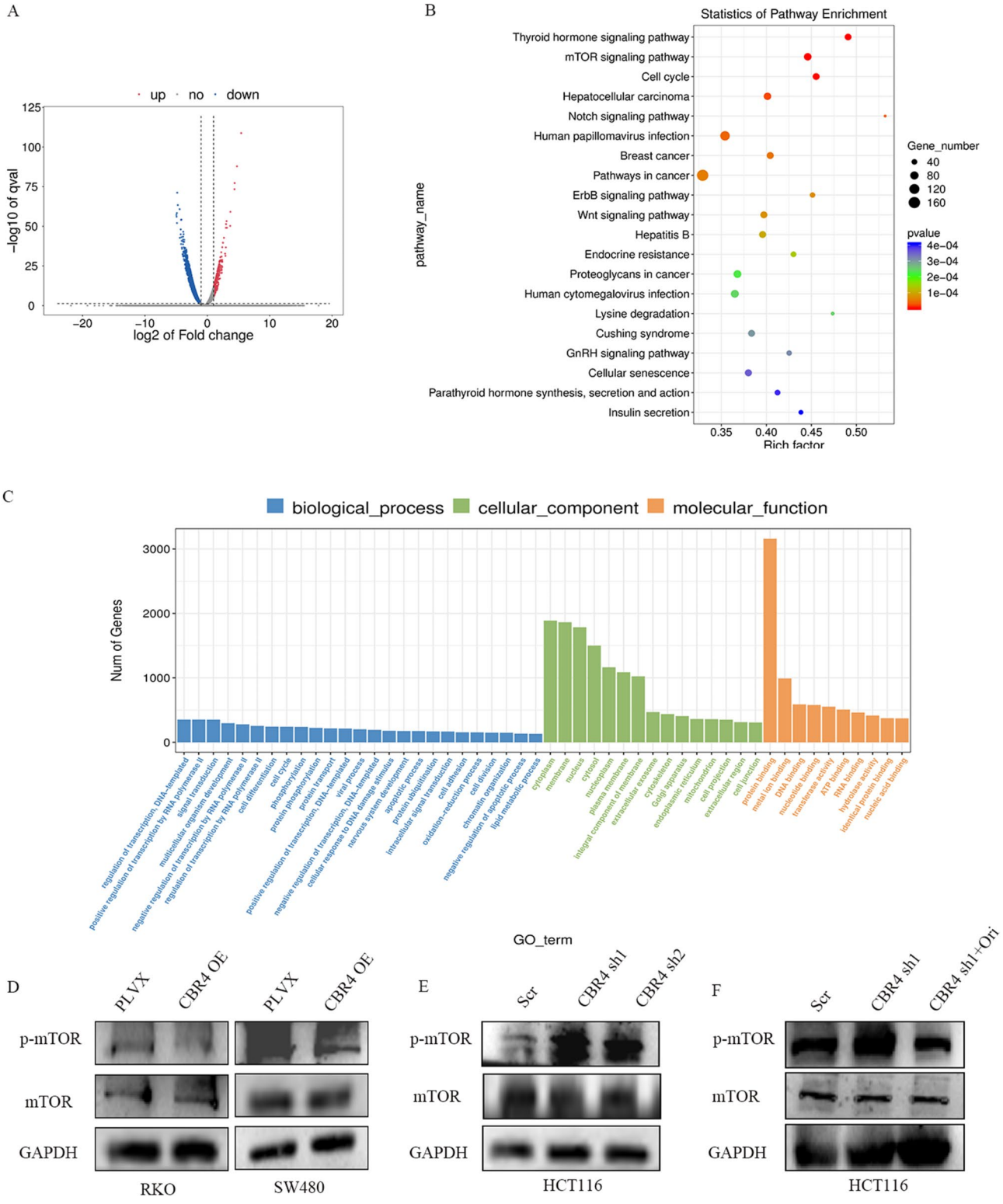

Fig. 5 CBR4 suppresses mTOR pathway mediated by FASN. (**A**) Volcano plot illustrating genes with relatively high and low expression levels in CBR4 knockdown colorectal cancer (CRC) cells. (**B**-**C**) KEGG and Gene Ontology (GO) analyses of differentially expressed genes between CBR4 knockdown and control CRC cells. (**D**-**F**) Western blot analysis of mTOR and phosphorylated mTOR (p-mTOR) in groups with CBR4 overexpression, CBR4 downregulation, and CBR4 downregulation combined with orlistat treatment

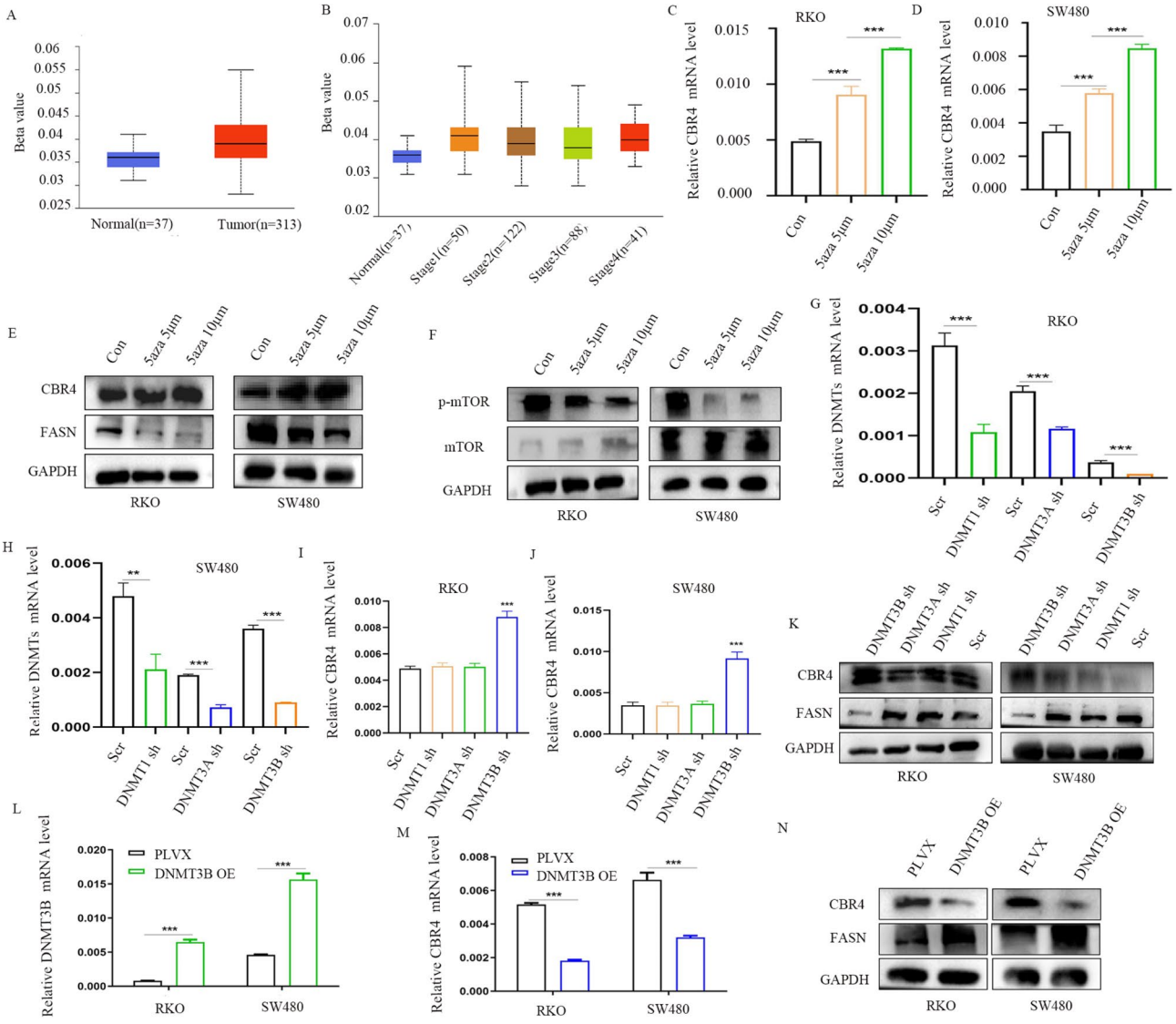

Fig. 6 DNMT3B regulates CBR4 via methylation. (**A**) The methylation level of CBR4 in CRC and normal cells ($p = 3.231300E-04$). (**B**) The methylation level of CBR4 in different stages of CRC(Stage1-vs-Stage3 $p = 2.749800E-02$). (**C**-**E**) The mRNA and protein levels of CBR4 in RKO and SW480 cells after treatment with DNA methylation inhibitor 5aza. (**F**) The levels of mTOR/p-mTOR in RKO and SW480 cells after treatment with 5aza. (**G**-**H**) mRNA levels of DNMT3A, DNMT3B and DNMT1 in knockdown and control CRC cells. (**I**-**K**) mRNA and protein levels of CBR4 in DNMT3A, DNMT3B, DNMT1 knockdown and control CRC cells. (**L**) mRNA levels of DNMT3B in over-expression and control CRC cells. (**M**-**N**) mRNA and protein levels of CBR4 in DNMT3B over-expression and control CRC cells. (***$P < 0.001$)

Fatty acid synthase (FASN) is a crucial protein that plays a significant role in the regulation of lipid metabolism. It is known that both solid tumors and hematologic malignancies often exhibit excessive expression of FASN. Currently, several targeted therapies aimed at FASN are undergoing preclinical trials. The use of FASN inhibitors in conjunction with signal transduction inhibitors and immunotherapy holds promise for the treatment of various tumors, which has garnered increasing interest [23]. Thus, understanding the molecular mechanisms of colorectal cancer (CRC) in relation to FASN could provide a valuable therapeutic target. This study investigates the interplay between CBR4 and FASN in CRC. Our findings indicate that CBR4 expression is decreased in CRC and exhibits tumor-suppressive effects through its interaction with FASN. Additionally, the expression of CBR4 is influenced by the DNA methyltransferase DNMT3B. Notably, the knockdown or inhibition of FASN markedly slows tumor development both in vivo and in vitro. The CBR4 gene, situated on chromosome 4q32.3 [24], is part of the SDR superfamily [25], which plays essential roles in lipid metabolism. Recently, CBR4 has been recognized as an integral component of the mitochondrial fatty acid synthesis (FAS) pathway in humans [15].

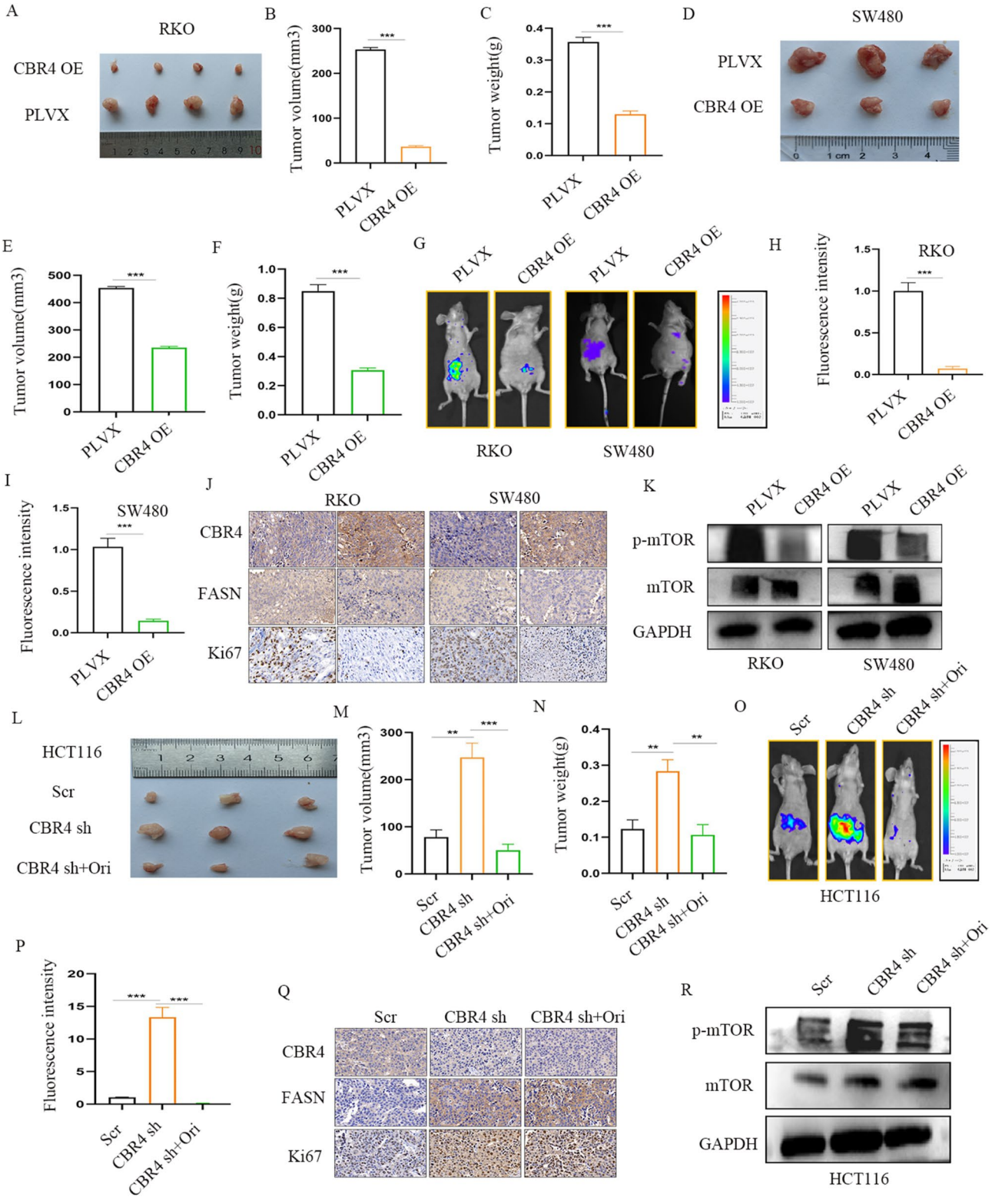

Fig. 7 (See legend on next page.)

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Fig. 7 CBR4 suppresses tumor growth via regulation of FASN in vivo. (**A**-**F**) Primary tumor samples were obtained from mice subcutaneously injected with RKO and SW480 cells, which were transfected with CBR4 over-expression and control cell groups (**A**, **D**). The relative tumor volumes (**B**, **E**) and weights (**C**, **F**) were measured at the endpoint. (**G**-**I**) Fluorescence intensity was assessed in a liver metastasis model with or without CBR4 over-expression. (**J**) Representative immunohistochemistry images illustrate the expression of CBR4, FASN, and Ki67 in both CBR4 over-expression and control groups, with a magnification of ×73. (**K**) Western blot analysis was conducted to examine mTOR and p-mTOR levels in CBR4 over-expression and control groups. (**L**-**N**) Primary tumor samples were also obtained from mice subcutaneously injected with HCT116 cells transfected with CBR4 knockdown, CBR4 knockdown + orlistat treatment, and control cell groups (**L**). The relative tumor volumes (**M**) and weights (**N**) were recorded at the endpoint. (**O**-**P**) The fluorescence intensity in the liver metastasis model was evaluated for CBR4 knockdown, CBR4 knockdown + orlistat treatment, and control cell groups. (**Q**) Representative immunohistochemistry images depict the expression of CBR4, FASN, and Ki67 in CBR4 down-regulation, CBR4 down-regulation + orlistat treatment, and control groups, with a magnification of ×73. (**R**) Western blot analysis was performed on mTOR and p-mTOR in CBR4 down-regulation, CBR4 down-regulation + orlistat treatment, and control groups. (**$P < 0.01$, ***$P < 0.001$)

Immunoprecipitation and mass spectrometry analyses have identified FASN as a protein that interacts with CBR4. Furthermore, CBR4 promotes the degradation of FASN by engaging its ubiquitin-proteasome pathway. Orlistat serves as a potent and long-lasting FASN inhibitor, effective in reversing the aggressive behavior of cells caused by CBR4 knockdown, indicating that CBR4 influences cancer progression through its impact on FASN.

The DNA methyltransferase (DNMT) family comprises a set of conserved enzymes that modify DNA and are crucial for epigenetic regulation of genes, which includes DNMT1, DNMT2, and DNMT3. The DNMT3 subfamily contains three members: DNMT3A, DNMT3B, and DNMT3L [26, 27]. Research has demonstrated that DNA methylation mediated by DNMT3B plays a vital role in both the development and progression of cancer. For instance, DNMT3B influences colorectal cancer (CRC) development by modulating FLI1 through hypermethylation of DNA [28]. Additionally, DNMT3B facilitates the progression of esophageal squamous cell carcinoma by affecting the promoter methylation of microRNA149 [29]. Furthermore, the methylation of FAM111B mediated by DNMT3B enhances glycolysis, tumor growth, and metastasis in thyroid papillary tumors [30]. Our findings also indicate that the level of CBR4 is influenced by DNMT3B-mediated methylation. In conclusion, the irregular uptake, synthesis, and breakdown of lipids are intricately linked to the initiation and advancement of tumors. Current preclinical and clinical findings suggest that various targeted lipid metabolism strategies exhibit significant anti-cancer effects. Nevertheless, developing tumor treatment approaches focused on lipid metabolism, which rely on the reprogramming mechanisms of lipid metabolism, still encounters numerous obstacles. A comprehensive understanding of the irregularities in tumor lipid metabolism could offer novel insights and methods for tumor treatment. This study has uncovered that DNMT3B can mediate the hypermethylation of the CBR4 promoter, leading to a decrease in CBR4 levels, a reduction in the ubiquitin degradation of FASN, a rise in FASN expression, and activation of the mTOR pathway, thereby facilitating CRC progression. Our research highlights the critical role of the DNMT3B/CBR4/FASN/mTOR axis in the advancement of CRC and suggests it as a potential therapeutic target for managing the disease.

Supplementary Information

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Supplementary Material 1

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Author contributions
YM and JX: conceptualization, methodology, software; ZJ, CT, WW, HC and WB: data curation, writing-original draft preparation; ZJ, CT and WW: visualization, investigation; YM and JX: writing-reviewing and editing; HC and WB: software, validation; YM and JX: supervision. All authors read and approved the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Animal study was approved by Institutional Animal Care and Use Committee (IACUC)of Nanjing Medical University. The discarded tumor tissue was obtained with patients inform and was approved by The First Affiliated Hospital with Nanjing Medical University (Jiangsu Province Hospital).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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