Original Article

B7-H3 enhances colorectal cancer progression by regulating HB-EGF via HIF-1α

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Background: B7-H3 (or CD276) represents an important costimulatory molecule expressed in many malignant solid tumors, including colorectal cancer (CRC). The receptor of B7-H3 is not known, and the intracellular function of B7-H3 remains obscure. Herein, we report that B7-H3 upregulated the epidermal growth factor heparin-binding epidermal growth factor (HB-EGF), likely by regulating hypoxia-inducible factor 1α (HIF-1α) and thereby promoting the progression of CRC.

Methods: Lentiviral transfection was performed on CRC cells to establish stable low-B7-H3 expression cells. A mechanistic analysis with an Agilent human gene expression profiling chip was conducted on them. Clinical data and specimens were collected to detect the connection between B7-H3 and HB-EGF in CRC. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to detect the messenger RNA (mRNA) level of B7-H3, HB-EGF, and HIF-1α. Chromatin immunoprecipitation (ChIP) quantitative real-time PCR was conducted. The protein level of HIF-1α and the phosphatidylinositide 3-kinases (PI3K)-protein kinase B (AKT) pathway were detected by western blot. HIF-1α was recovered by lentiviral transfection, and the HB-EGF mRNA levels, proliferation, invasion, and angiogenesis ability were detected.

Results: B7-H3 promoted tumor progression through HB-EGF and the PI3K-AKT pathway. As B7-H3 was downregulated, HB-EGF levels were significantly reduced simultaneously, a growth trend that was shown by both CRC cell lines and cancer tissues. In addition, B7-H3 and HB-EGF had significant associations with tumor-node-metastasis (TNM) stage and lymph node metastasis in 50 CRC patients. The binding ability of HIF-1α to the HB-EGF promoter region was significantly decreased in the shB7-H3 RKO group. Western blot revealed that PI3K, AKT, and mammalian target of rapamycin (mTOR) protein amounts and p-AKT and p-mTOR phosphorylation were also downregulated in shB7-H3 RKO cells, suggesting that B7-H3 may regulate HIF-1α via PI3K-AKT signaling. After recovery of the HIF-1α level by lentiviral transfection, the HB-EGF mRNA levels, proliferation, invasion, and angiogenesis in CRC cells

recovered as well.

Conclusions: B7-H3 may transmit intracellular signals through PI3K-AKT-mTOR-HIF-1α signaling, upregulating HB-EGF. As the final transcription factor of the pathway, HIF-1α regulates the transcription of the HB-EGF gene, thereby promoting HB-EGF expression, which eventually mediates cell proliferation, invasion, and angiogenesis and promotes the progression of CRC.

Keywords: Colorectal cancer (CRC); B7-H3; heparin-binding epidermal growth factor (HB-EGF); hypoxia-inducible factor 1α (HIF-1α); chromatin immunoprecipitation (ChIP)

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Introduction

Colorectal cancer (CRC) represents the most predominant cancerous neoplasm within the spectrum of gastrointestinal malignancies, and ranks third among deadliest cancers (1). Although significant advancements have been achieved in CRC management thanks to novel surgical techniques and chemotherapeutic interventions, treatment is still not ideal in patients with middle and advanced stages and those with metastasis or recurrence (2). Targeting the mechanisms of tumor metastasis and recurrence has become an ideal means to eradicate tumors.

B7-H3 (or CD276) represents an important costimulatory molecule expressed in many malignant solid tumors (3). The high expression of B7-H3 in tumors makes it a promising therapeutic target. However, due to the unclear receptors for B7-H3, current approaches mainly focus on cancer antibody-based therapy. B7-H3 antibody-drug conjugates and B7-H3 targeted chimeric antigen receptor T/NK cells (4,5), which deliver drugs or T/NK cells to precisely kill B7-H3-positive tumor cells, are currently the safest and most effective treatment strategies, but none have yet entered clinical or large-scale application. Further research on B7-H3's pro-tumor mechanisms, localization, function, and molecular pathways in cancer cells will potentially expand the possibilities for targeted therapies aimed at B7-H3. As a transmembrane protein, B7-H3 is expressed in the pro-B7-H3 form on the membrane of various tumors, and further cleaved by matrix metalloproteinase (MMP) to yield soluble B7-H3 that performs cellular functions (6). In CRC, B7-H3 promotes the tumor progression (7,8) and is associated with recurrence and overall survival (9,10). Highly B7-H3 expression is associated with enhanced lymph node metastasis and poor tumor differentiation (11).

The receptor of B7-H3 is currently unknown, but its effects on tumor progression are obvious, mainly promoting proliferation and invasion (3), inducing aberrant angiogenesis (4,12), and inhibiting anticancer immune response (13).

In this article, we demonstrate that heparin-binding

Highlight box

Key findings

- Our research shows that B7-H3 and heparin-binding epidermal growth factor (HB-EGF) are all highly expressed in colorectal cancer (CRC) and show the same expression tendency. B7H3 may transmit intracellular signals through phosphatidylinositide 3-kinases (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR)-hypoxia-inducible factor 1α (HIF-1α) signalling, thus upregulating HB-EGF, eventually mediating cell proliferation, invasion, and angiogenesis and promoting the progression of CRC.

What is known and what is new?

- Previous study shows that B7-H3 has a lot of functions in non-immune effects in the progression of tumor. B7-H3 may affect the levels of protein HIF-1α by influencing reactive oxygen species (ROS). About the epidermal growth factor (EGF) family, B7-H3 can regulate the level of vascular endothelial growth factor (VEGFA) through nuclear factor-kappa-B (NFKB).
- Our study shows that B7-H3 regulates another important EGF family factor HBEGF in CRC, and HIF-1α is in this regulatory mechanism play as the translate factor of HB-EGF. B7-H3 may also regulate the expression of HIF-1α in a different pathway.

What is the implication, and what should change now?

- Our study has expanded the understanding of the mechanisms underlying the progression role of B7-H3 in CRC. It provides new therapeutic insights for tumor therapy and the regulatory mechanisms of tumor metabolism and broadens the non-immunological pro-tumor mechanisms of B7-H3.

epidermal growth factor (HB-EGF) in CRC cancer cells showed the same expression pattern as B7-H3.

HB-EGF, an epidermal growth factor (EGF), is expressed in many malignant tumors (14). It binds and induces epidermal growth factor receptors (EGFRs) 1 and 4, thus activating downstream signaling pathways, including the mitogen-activated protein kinase (MAPK), phosphatidylinositide 3-kinases (PI3K), and signal transducer and activator of transcription (STAT) pathways, which control multiple cellular processes such as growth, survival, angiogenesis, and differentiation (15-17). In CRC cells, HB-EGF induces tumor cell angiogenesis and progression (18) and mediates cetuximab resistance (19). However, the regulatory mechanism of HB-EGF in CRC cells is not clear.

Our research utilized RNA chips to preliminarily screen and analyze changes in the expression levels and biological functions of various molecules after reducing B7-H3 levels in CRC. Then, we analyzed clinical data to investigate the relationships among B7-H3, HBEGF, and clinicopathological features. Finally, we explored the role and mechanisms of B7-H3 in promoting tumor growth through the regulation of HBEGF. Here, we uncovered a pivotal role for B7-H3 in CRC progression, likely by modulating the expression of HB-EGF via PI3K-protein kinase B (AKT)-mammalian target of rapamycin (mTOR)-hypoxia-inducible factor 1α (HIF-1α) signaling. HIF-1α level restoration in the shB7-H3 group further demonstrated the importance of B7-H3 in angiogenesis, invasion, and proliferation in CRC cells. We present this article in accordance with the MDAR reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-384/rc).

Methods

Cell culture

DLD-1, HT-29, HCT-116, SW-480, SW-620, and RKO lines were obtained from the Institute of Clinical Immunology of Jiangsu Province. HCT-116, SW-480, SW-620, and RKO cells underwent culture in Dulbecco's modified Eagle medium (DMEM) (D5796, Hyclone, Logan, UT, USA); Roswell Park Memorial Institute (RPMI) 1640 (R8758, Hyclone) was utilized for DLD-1 and HT-29 cells. Cell culture was carried out in a humid atmosphere with 5% CO_2 at 37 ℃, in growth media containing 10% fetal bovine serum (FBS) (16140071, Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (C0222, Beyotime, Nanjing, China). The Institute for Cardiovascular Science of Soochow University generously provided human umbilical vein endothelial cells (HUVECs). HUVECs were maintained in EBM-2V medium (CC-3162, Lonza, Walkersville, MD, USA) containing 10% FBS and 1% penicillin/streptomycin in a humid atmosphere containing 5% CO_2 at 37 ℃. More detailed information of these cell lines is provided in Table S1.

Patient data and samples

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee of the First Affiliated Hospital of Soochow University, [2022] No. 098. Informed consent was taken from all the patients. The patient samples utilized in the present investigation were procured from the Department of General Surgery and the Department of Gastroenterology, The First Affiliated Hospital of Soochow University, Suzhou, China.

A cohort of 50 patients administered surgical intervention for colon cancer and 20 patients with colon polyps resected via colonoscopy from January to December 2020 were included.

The diagnosis of colon carcinoma or colon adenoma was confirmed via postsurgical pathological analysis. None of the patients had previously undergone any form of cancer therapy, such as chemotherapy, radiation therapy, targeted therapy, or immunotherapy, prior to surgery. CRC patients comprised 30 males and 20 females, aged 40–81 years (mean, 61.4 ±17.8 years). In terms of pathology, they included 16 and 34 stage I–II and III–IV cases, respectively, based on the 8th edition of tumor-node-metastasis (TNM) staging criteria (20); of these cases, 34 had lymph node metastasis (LNM). CRC specimens and paired adjacent normal tissues were resected as described in a previous study (21). Each tissue specimen was divided into two portions, 1 of which underwent fixation with 10% neutral formalin, paraffin-embedding, and immunohistochemistry (IHC); the other was preserved at a temperature of –80 ℃ for quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Transfection experiment

Lentiviruses for silencing B7-H3 and overexpressing

HIF-1α, and the respective negative controls were obtained from Shanghai GeneChem (GeneChem, Shanghai, China). The lentivirus for B7-H3 silencing harbored the anti-puromycin and green fluorescent protein (GFP) sequences and may be detected as fluorescein isothiocyanate (FITC) positive by flow cytometry (FCM). Meanwhile, the lentivirus overexpressing HIF-1α harbored the 3-FLAG and anti-puromycin sequences.

Lentiviruses were used to infect DLD-1 and RKO cells in the exponential growth phase (MOI for RKO, 40; MOI for DLD-1, 50). Following 72 hours of transfection, transfection efficiency was assessed by detecting GFP expression by FCM or 3-FLAG expression by western blot.

Puromycin screening and single cell cloning

Cells in the interference group were screened in the culture environment with optimal puromycin concentration (RKO, 3 μg/mL; DLD-1, 2 μg/mL). The cell status was observed at regular intervals until uninfected cells were killed by puromycin, and the medium was replaced with normal medium.

Then, cell suspensions were diluted and cultured, and inoculated into 96-well plates at 1 cell per well. Following 3–5 days of culture, monoclonal cell lines in 96-well plates appeared as single cell clusters, which were gradually digested and transferred to 6-well plates and 10 cm petri dishes when confluent.

FCM

The collected cells underwent resuspension in 0.5 mL Hanks (14175, Thermo Fisher Scientific, Waltham, MA, USA) containing 1% phosphate-buffered saline (PBS). Subsequently, cell suspensions were placed on ice and adjusted to 2×10^6 cells per 100 mL, followed by centrifugation in preparation for antibody staining. DLD-1 cells were stained with PE/Cy7-conjugated anti-human B7-H3 (1:300, No 351008, BioLegend, San Diego, CA, USA). RKO cells underwent staining with APC-linked anti-human B7-H3 (1:500, No 351006, BioLegend). Staining was carried out at 4 ℃ for 20 minutes shielded from light, which was followed by resuspension and FCM [Becton-Dickinson and Co. (BD), Franklin Lakes, NJ, USA]. FlowJo V10.0 (FlowJo, BD, USA) was utilized for data analysis.

IHC

The procedures of IHC were performed as described in a previous study (21). The antibodies used were Anti-CD276 antibody (ab105922, Abcam, Waltham, MA, USA) and anti-HB-EGF antibody (ab218019, Abcam). Experiments were repeated 3 times in each patient's one single specimen and similar results were obtained.

Western blot analysis

Protein extraction and concentration evaluation used the RIPA lysis buffer (P0013B, Beyotime, Shanghai, China) and the BCA kit (A045-3, Jiancheng Bioengineering, Nanjing, China), respectively, as directed by the respective manufacturers. Equal amounts of total protein (40 μg) underwent separation by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto a polyvinylidene fluoride (PVDF) membrane (Millipore Sigma, Burlington, NC, USA). Blocking was carried out with 5% skim milk for 1 hour at an ambient temperature. Next, primary antibodies were added for incubation at 4 ℃ overnight: Anti-CD276 antibody (1:500, ab105922, Abcam), Anti-PI3K antibody (1:1,000, ab191606, Abcam), AKT1 mAb (1:1,000, 2938, CST, Danvers, MA, USA), Phospho-AKT (Ser473) mAb (1:2,000, 4060, CST), Anti-mTOR antibody (1:1,000, ab32028, Abcam), Phospho-mTOR mAb (1:1,000, 5536, CST), Anti-HIF-1α antibody (1:2,000, ab51608, 1:2,000), and Anti-beta-Actin Polyclonal Antibody (1:5,000, bs-0061R, Bioss, Boston, MA, USA). Subsequently, the membranes were subjected to a 1-hour incubation with horseradish peroxidase (HRP)-linked goat anti-rabbit secondary antibodies (1:5,000, ab7090, Abcam) at ambient. Finally, detection was carried out with an enhanced chemiluminescence kit (32106, Thermo Fisher Scientific) in triplicate assays.

qRT-PCR

Total RNA extraction from cell and tissue samples used the TRIzol (15596026, Thermo Fisher Scientific) method. Reverse transcription was carried out with a reverse transcription kit (K1691, Thermo Fisher Scientific). qRT-PCR was performed based on the instructions of MonAmp ChemoHS qPCR Mix (No. MQ00401, Monad Biotech, Suzhou, China). Data normalization utilized human-actin

expression. Amplification was performed at 95 ℃ (10 min), followed by 40 cycles at 95 ℃ (10 sec), 60 ℃ (10 sec), and 72 ℃ (30 sec). The following primers were employed (all human species): B7-H3, 5'-GTGGGGCTGTCTGTCTGTCTCAT-3' (sense) and 5'-GCTGTCAGAGTGTTTCAGAGGCT-3' (antisense); HB-EGF, 5'-ATCGTGGGGCTTCTCAT GTTT-3' (sense) and TTAGTCATGCCCAACTTC ACTTT-3' (antisense); HIF-1α, 5'-GTTAGTTCA ATTTTGATCCCCTTTCT-3' (sense) and GCTACTGCAATGCAATGGTTTAA-3' (antisense). The $2^{-\Delta\Delta t}$ method (22) was employed for data analysis.

Cell proliferation assay

CRC cells (3×10^3) underwent seeding into 6-well plates for 7–10 days. After removing the medium, the cells underwent a 30-minute fixation with 2 mL methanol and overnight staining with crystal violet (C0121, Beyotime). A microscope (DM2500, Leica, Wetzlar, Germany) was utilized for imaging, followed by analysis with Image-Pro Plus (Media Cybernetics, Rockville, MD, USA).

Cell invasion assay

CRC cells were collected and resuspended at 5×10^4 per 200 µL and added into 8-µm pore superior chambers in 24-well plates (BD Biosciences, South San Francisco, CA, USA) after Matrigel (No. 356234, Corning, Shanghai, China) coating. Following 24 hours of culture, cells underwent a 30-minute fixation with 2 mL methanol and a 20-minute crystal violet staining. Finally, cells that had traversed the membrane were captured using an inverted microscope (DM2500, Leica) and analyzed with the Image-Pro Plus software.

Conditioned medium (CM) and endothelial tube formation assay

CRC cells (1×10^6) underwent overnight culture in 6-well plates, followed by culture with freshly prepared medium without FBS. After a 24-hour incubation period, the CM was utilized for tube formation assay involving HUVECs.

A total of 3×10^4 HUVECs were added to 96-well plates precoated with Matrigel. These cells then underwent culture in the conditioned culture medium for a duration of 4 hours at 37 ℃ under 5% CO_2. Tubules were captured using a microscope (STELLARIS 5, Leica) and assessed using Image-Pro Plus.

Agilent RNA microarray and data analysis

The Agilent human gene expression profiling 4×44K chip was utilized (Agilent, Santa Clara, CA, USA). shB7-H3 RKO and control RKO cells were collected for total RNA extraction with Ribo Pure Kit (AM1924, Thermo Fisher Scientific). Next, total RNA was obtained with RNeasy® Mini Kit (74106, QIAGEN, Tegelen, Netherlands) to achieve a ratio of A260/A280 close to 2.0. Then, complementary RNA (cRNA) was synthesized with AffinityScript One-Step RT-PCR Kit (600188, Agilent) and labeled with Cy3-CTP (ab97170, Abcam). After another purification step, the concentration, fluorescence, and labeling efficiency of the cRNA were detected with NanoDrop2000C. For the microarray assay, cRNA sample fragmentation and chip hybridization were then conducted; after 3 washes, the microarrays were scanned with an Agilent high resolution scanner, Type C. The data were analyzed by Agilent Feature Extraction. Genes with $P<0.05$ were considered differentially expressed genes (DEGs).

Chromatin immunoprecipitation (ChIP) assay

ChIP utilized an EZ-Magna ChIP A/G kit (CB29719008, Millipore) as directed by the manufacturer. Normal IgG and anti-HIF-1α antibody (51608, Abcam) served as negative and positive control antibodies, respectively. The obtained DNA was eventually assessed by qRT-PCR. ChIP-qRT-PCR utilized the following primers (all human species): HB-EGF, 5'-TCACTGTCCTGACCCAAAGAA-3' (sense) and 5'-AAAGCCAACCCAGCCCTG-3' (antisense). The qPCR steps were as described above for qRT-PCR.

Statistical analysis

Statistical evaluation utilized GraphPad Prism 10.0 (GraphPad Software, San Diego, CA, USA) and SPSS 26.0 (IBM Corp., Armonk, NY, USA). Enumeration data were expressed as rate and compared by the χ^2 test. The correlation between the messenger RNA (mRNA) levels of B7-H3 and HB-EGF was assessed by the Spearman's method. Continuous data were expressed as mean and compared by *t*-test. The whole experiments

were repeated 3 times and similar results were obtained; no results were removed in analysis. A P value <0.05 was deemed to indicate statistical significance.

Results

B7-H3 has high expression in CRC cells, promoting proliferation, invasion, and angiogenesis

To determine B7-H3 expression on CRC cells, we assessed six human CRC cell lines, namely, DLD-1, HCT-116, HT-29, SW-480, SW-620, and RKO. FCM showed that all these human CRC cells expressed high levels of B7-H3 on the membrane (*Figure 1A*). We then utilized lentiviral transfection and single cell cloning to develop shB7-H3 RKO and DLD-1 cell lines. FCM demonstrated membrane B7-H3 was overtly and stably suppressed in both cell groups (*Figure 1B*).

We next conducted proliferation and invasion assays in RKO and shB7-H3 RKO cells. The results demonstrated greatly suppressed proliferative and invasive abilities in shB7-H3 cells (*Figure 1C,1D*). The endothelial tube formation assay was conducted to investigate the tube formation ability of HUVECs, which could participate in each link of the angiogenesis process directly or indirectly (23). The results showed significantly less branch points and markedly reduced tube lengths in the shB7-H3 group (*Figure 1E*). The latter findings suggest that B7-H3 plays an important role in the proliferation, invasion, and angiogenesis of CRC cells.

B7-H3 promotes CRC by increasing HB-EGF levels

Human EGF plays important roles in cancer angiogenesis and progression (24). To elucidate the mechanisms of progression, invasion, and angiogenesis triggered by B7-H3 in RKO cells, we used Agilent Human Gene Expression Microarray to analyze DEGs between shB7-H3 RKO and control RKO cells. Totally, 147 and 98 upregulated and downregulated DEGs (fold change ≥2 and P<0.05) were obtained, respectively (*Figure 2A,2B*). The results demonstrated that HB-EGF was downregulated in the shB7-H3 group, which suggested B7-H3 may promote tumor progression through HB-EGF.

To further investigate the function of B7-H3, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were carried out. GO analysis showed that biological process and molecular function items triggered by B7-H3 were mainly enriched by protein phosphorylation and vasculature development (*Figure 2C*), indicating that B7-H3 may work by promoting angiogenesis via intracellular signaling. KEGG analysis revealed B7-H3 may affect the PI3K-AKT pathway (*Figure 2D*). Meanwhile, B7-H3 and HB-EGF mRNA levels in the shB7-H3 and control groups of DLD-1 and RKO cells were detected by qPCR, and HB-EGF was simultaneously downregulated in the shB7-H3 groups (*Figure 2E*).

The above data suggested B7-H3 might have a biological function in promoting the occurrence and progression of CRC by upregulating HB-EGF through intracellular signal transduction.

B7-H3 and HB-EGF have high expression levels in CRC tissues and are correlated with clinicopathological parameters

Cancer specimens from 50 CRC patients as well as their clinicopathological parameters and specimens from 20 colorectal adenoma patients were collected. Then, the microarrays results were verified by measuring B7-H3 and HB-EGF levels in CRC cells and cancer tissue samples.

The results of IHC revealed that B7-H3 and HB-EGF amounts were markedly elevated in cancer tissues compared with adenoma and normal tissue samples (*Figure 3A*). The results of qPCR demonstrated that B7-H3 and HB-EGF mRNA amounts were overtly elevated in cancer tissue specimens compared with normal tissue samples (*Figure 3B*). Besides, the mRNA levels of these two molecules were significantly correlated in tumor tissues (*Figure 3C*).

We then conducted a statistics analysis based on clinicopathological and IHC data. The chi-square test was carried out for each parameter, considering both B7-H3 and HB-EGF. We found that B7-H3 and HB-EGF amounts were both associated with TNM stage and LNM in those patients (*Table 1*).

B7-H3 promotes HE-BGF expression through PI3K-AKT-mTOR-HIF-1α signaling to induces the progression of CRC

The downstream and transcription factors of B7-H3 are numerous. To investigate which one may be involved

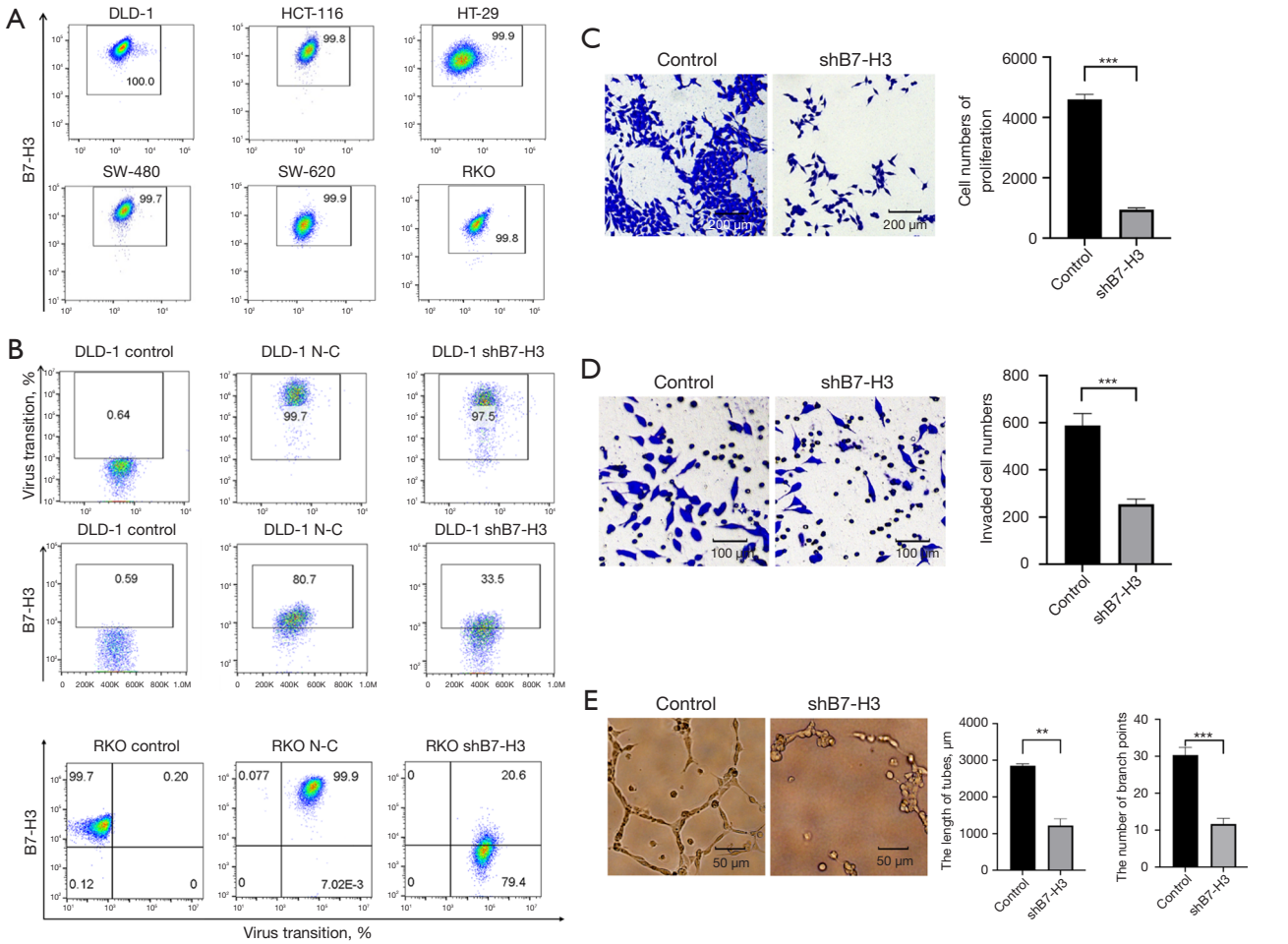

Figure 1 The expression level and the tumor progression role of B7-H3 in CRC. (A) B7-H3 expression is elevated on the membrane of 6 human colorectal cancer cell lines as detected by FCM. (B) The transfection rate of the lentivirus and B7-H3 expression on the membrane of RKO and DLD1 cells. In RKO cells, PE-CY7 was used for B7-H3, and FITC for the lentivirus. In DLD1 cells, APC was used for B7-H3, and FITC for lentivirus. (C,D) Proliferative and invasive abilities were significantly decreased in shB7-H3 RKO cells. Cells were observed after staining with crystal violet. (E) CM from control RKO and shB7-H3 RKO cells were placed in 24-well plates after Matrigel coating, followed by seeding with HUVECs. Following incubation for 4 h, a microscope was utilized to identify capillary-like structures directly and with no stain. Tube length and the amount of the branch points determined with Image pro6.0. The experiments were repeated three times and similar results were obtained (biological replicates). ***, P<0.001, **, P<0.01 *vs.* Control. N-C, negative control; CRC, colorectal cancer; FCM, flow cytometry; CM, conditioned medium; HUVECs, human umbilical vein endothelial cells.

in B7-H3-related HB-EGF overexpression, we started with the regulation mechanism of HB-EGF. A recent study reported HIF-1α as a critical transcription factor of HB-EGF in hepatocellular carcinoma (25). HIF-1α is a regulator of homeostasis response to cellular hypoxia (26), which was also involved in the development of many malignancies and highly correlated with tumor metastasis, prognosis, chemotherapy drug resistance, and other factors (27-29). Our data showed that HIF-1α amounts were decreased with decreasing B7-H3 levels. In order to investigate whether B7-H3 regulates HB-EGF via HIF-1α, we detected HIF-1α mRNA and protein amounts in Control and shB7-H3 RKO cells. The results revealed HIF-1α was overtly downregulated in the shB7-H3 group (*Figure 4A*,*4B*). To determine whether HIF-1α plays a role as a transcription factor in CRC cells, a ChIP-q-PCR assay was performed. The results showed that, in the shB7-H3 group, the binding

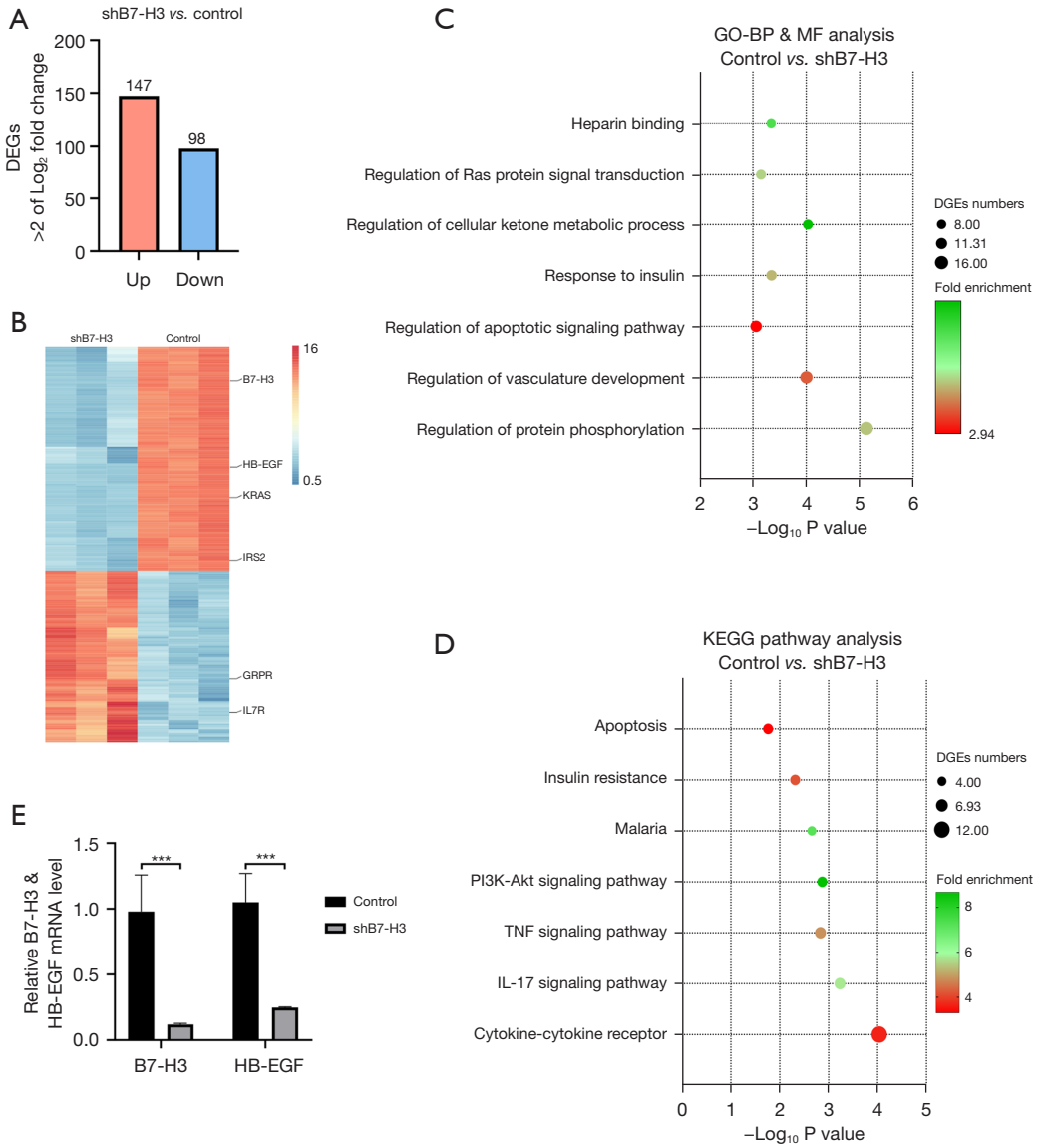

Figure 2 B7-H3's correlated genes and biological function in CRC cells. (A) Numbers of upregulated and downregulated genes in control RKO cells compared with the shB7-H3 group. (B) Heatmap of DEGs found in shB7-H3 and control RKO cells. (C,D) Biological process, molecular function and signaling pathways involving DEGs, based on GO and KEGG analyses. (E) B7-H3 and HB-EGF mRNA levels detected in the shB7-H3 and control groups. The experiments were repeated three times and similar results were obtained (technical replicates). ***, P<0.001 *vs.* Control. DEGs, differentially expressed genes; GO, Gene Ontology; BP, biological process; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; HB-EGF, heparin-binding epidermal growth factor; CRC, colorectal cancer.

of HIF-1α to the transcription factor site of HB-EGF was significantly reduced (*Figure 4C*).

We next overexpressed HIF-1α by lentiviral transfection to recover the HIF-1α level in shB7-H3 RKO cells (Oe-HIF-1α shB7-H3 RKO cells), and qPCR showed that HB-EGF was significantly upregulated with HIF-1α recovery (*Figure 4D*). Moreover, the proliferative and invasive abilities of Oe-HIF-1α shB7-H3 RKO cells were also increased significantly (*Figure 4E,4F*). The CM from Oe-HIF-1α shB7-H3 RKO cells also enhanced the tube formation ability of HUVECs (*Figure 4G*).

Finally, we detected the expression levels of the

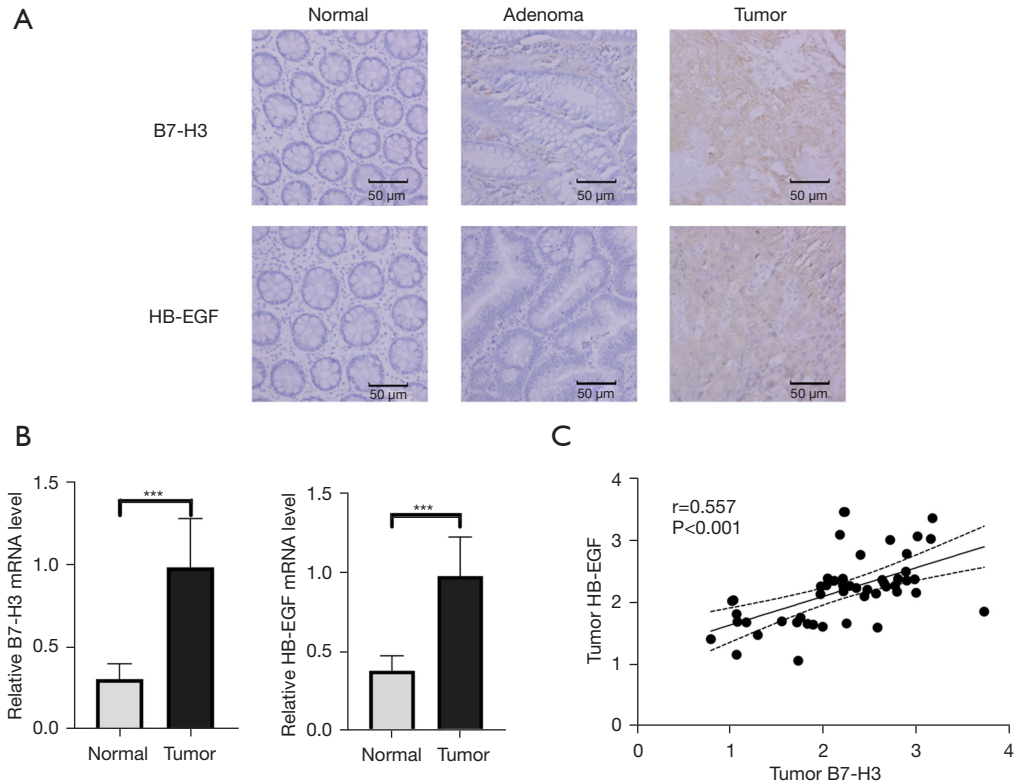

Figure 3 B7-H3 and HB-EGF in CRC patients. (A) IHC analysis of normal, colorectal tumor, and colorectal adenoma tissues. High B7-H3 and HB-EGF amounts were detected in tumor tissue specimens, as observed under light microscopy, n=50. (B) qPCR showed markedly B7-H3 and HB-EGF mRNA amounts in 50 tumor tissue samples in comparison with normal tissue samples, n=50. (C) Spearman analysis showed a significant association of B7-H3 and HB-EGF mRNA levels in 50 tumor tissue samples, n=50. The experiments were repeated three times and similar results were obtained (biological replicates). ***, P<0.001 *vs.* Normal. HB-EGF, heparin-binding epidermal growth factor; CRC, colorectal cancer; IHC, immunohistochemistry; qPCR, quantitative polymerase chain reaction; mRNA, messenger RNA.

effectors of the PI3K-AKT-mTOR pathway. The results showed that in the shB7-H3 RKO group (*Figure 4H*), PI3K, AKT, and mTOR protein amounts were significantly decreased, as well as the phosphorylation of AKT and mTOR, which indicates B7-H3 may regulate HIF-1α via PI3K-AKT-mTOR signaling to promote HB-EGF.

Discussion

B7-H3 is a vital factor in CRC which not only functions as a marker of CRC prognosis and clinicopathological stage (9-11), but also promotes CRC in many ways (7,8,12). In CRC, B7-H3 serves as a significant biomarker for survival (10). As an immune checkpoint protein, B7-H3 can inhibit the function of anti-tumor immune cells in many ways (30). And in the tumor tissues with high expression of B7-H3, there is a significant decrease in immune cells infiltration and the number of $CD8^+$ T cells in the tumor microenvironment (31,32), further exacerbating poor prognosis. In fact, B7-H3 also shows a variety of abilities that promote tumor progression through non-immunological mechanisms. B7-H3 can enhance tumor cell glucose metabolism through the promotion of the Warburg effect, thereby increasing their proliferative activity (33). Furthermore, B7-H3 enhances tumor invasiveness by promoting the MMP9, VEGFA, JAK/STAT3, or inducing epithelial-mesenchymal transition (EMT) (34-36). This leads to higher rates of lymph node metastasis and later tumor staging in B7-H3-positive patients, consistent with findings from our third section of research.

Furthermore, B7-H3 plays a significant hindering role in cancer treatment. In CRC, it promotes high expression of BRC33 in tumor cells, thereby counteracting the DNA

Table 1 Associations of B7-H3 and HB-EGF levels with clinicopathological parameters in colon cancer

Clinicopathological parameters	N	B7-H3 expression (n)				HB-EGF expression (n)			
		Negative	Positive	χ^2	P value	Negative	Positive	χ^2	P value
Gender				0.123	0.48			0.487	0.34
Male	30	12	18			12	18		
Female	20	9	11			10	10		
Age (years)				0.363	0.38			1.919	0.14
>65	19	7	12			6	13		
≤65	31	14	17			16	15		
Tumor size (cm)				0.192	0.44			0.152	0.46
<3	22	11	11			9	13		
≥3	28	10	18			13	15		
Differentiation				0.911	0.34			3.181	0.08
Poor or intermediate	34	13	21			15	19		
Well-differentiated	17	8	9			7	10		
TNM stage				4.059	0.004			12.25	0.001
I–II	16	10	6			11	5		
III–IV	34	11	23			11	23		
LNM				4.059	0.004			12.25	0.001
Without	16	10	6			11	5		
With	34	11	23			11	23		

HB-EGF, heparin-binding epidermal growth factor; TNM, tumor-node-metastasis; LNM, lymph nodes metastasis.

damage effects of 5-FU (37). In pancreatic cancer, B7-H3 promotes the production of anti-apoptotic protein Survivin, antagonizing the effects of gemcitabine therapy (38). In other malignancies, B7-H3 employs various mechanisms to resist multiple chemotherapy drugs (39-41), greatly diminishing the effectiveness of chemotherapy and becoming a crucial factor leading to poor prognosis.

Our FCM data revealed that B7-H3 is significantly upregulated in CRC cells. As our preliminary results showed, B7-H3 enhanced the proliferative and invasive abilities of RKO cells. Additionally, CM obtained from Control RKO cells showed a significantly enhanced promoting effect than CM from shB7-H3 RKO cells on the formation ability of HUVECs, which participates in almost every link of tumor angiogenesis (23). The results obtained in this study consistently demonstrated the significant involvement of B7-H3 in the pathogenesis of CRC.

In order to elucidate the promoting effect triggered by B7-H3 in CRC, we conducted a microarray analysis. DEGs (fold change ≥2 and P<0.05) were extracted and analyzed, and HB-EGF was markedly downregulated in shB7-H3 RKO group, which suggested HB-EGF could be the molecule affected by B7-H3. We proceeded to test this hypothesis by examining B7-H3 and HB-EGF amounts in CRC cells. As shown above, B7-H3 and HB-EGF were both downregulated in shB7-H3 DLD-1 and shB7-H3 RKO cells, and similar findings were obtained in CRC tumor tissues. In order to estimate the effect exerted by HB-EGF in CRC patients, we compared the patient data related to B7-H3 and HB-EGF. The results showed HB-EGF had the same expression trend as B7-H3 in patients with TNM stage and LNM, which can be considered a prognostic factor in CRC. Thus, we concluded that B7-H3 may exert tumor-promoting effects through HB-EGF.

Belonging to the EGF family, HB-EGF can activate EGF-receptor to promote tumor progression in various malignant solid tumors (24,42,43).

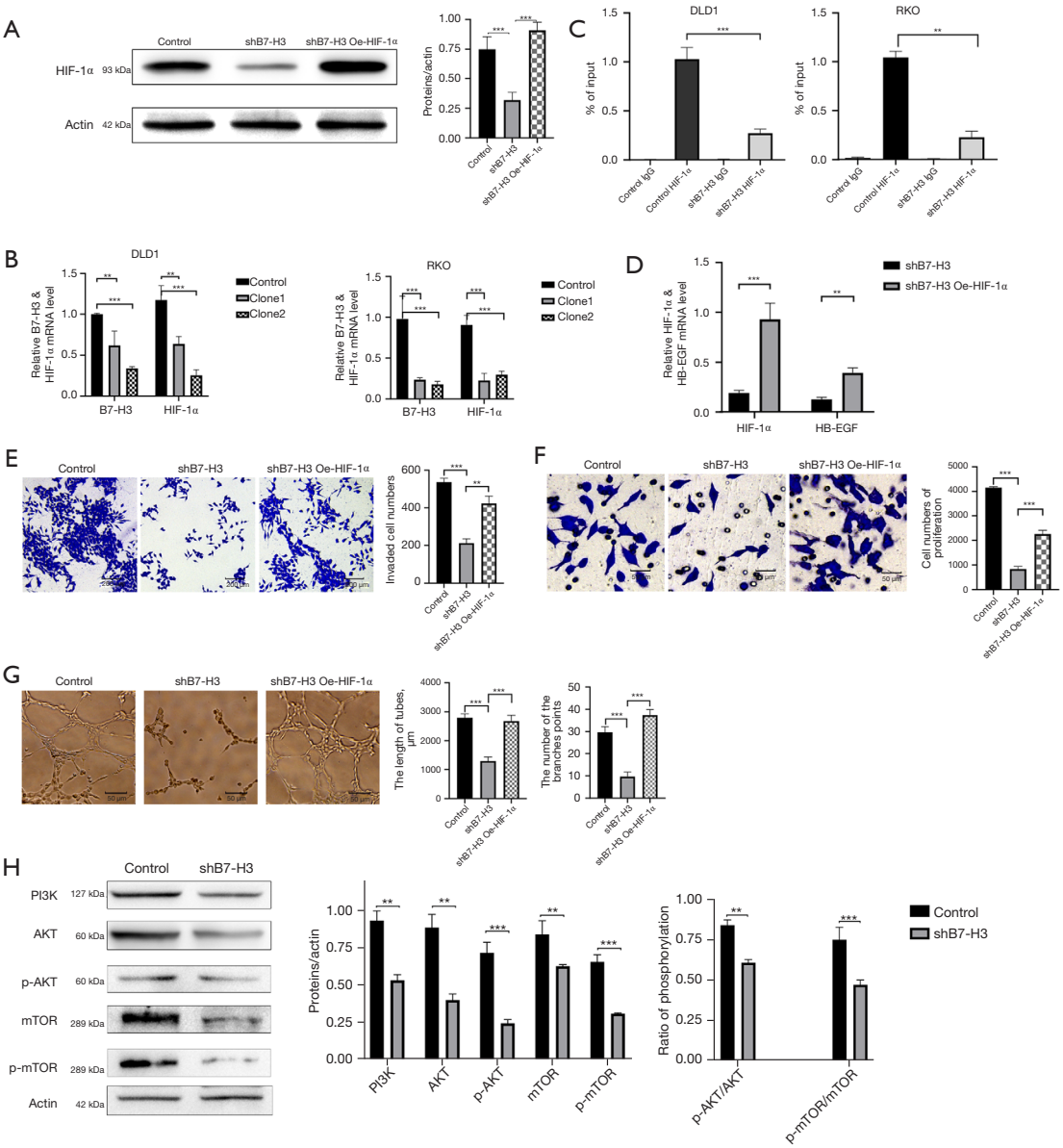

Figure 4 B7-H3 promotes HB-EGF expression through HIF-1α. (A) HIF-1α was significantly downregulated in the shB7-H3 RKO group, as detected by western blot. The HIF-1α ratio was determined with ImageJ 5.0. (B) B7-H3 and HIF-1α amounts showed synchronous declines in shB7-H3 DLD-1 and RKO cells. The more obvious the decline of B7-H3 levels, the more obvious that of HIF-1α amounts. (C) ChIP-qRT-PCR indicated a statistically significant reduction in HIF-1α binding to the HB-EGF promoter in shB7-H3 DLD-1 and RKO cells, compared with the control group. (D) After restoration of HIF-1α level in shB7-H3 RKO cells, HB-EGF mRNA levels were significantly increased. (E,F) Proliferative and invasive abilities were significantly increased after recovered HIF-1α level. Cells were observed after staining with crystal violet. (G) CM from control RKO, shB7-H3 RKO and shB7-H3 RKO-Oe-HIF-1α groups were placed in 24-well plates after Matrigel coating, followed by seeding with HUVECs. Following incubation for 4 h, a microscope was utilized to identify capillary-like structures directly and with no stain. Tube length and the amount of the branch points determined with Image pro6.0. (H) PI3K, AKT, and mTOR protein amounts and AKT and mTOR phosphorylation were significantly decreased in the shB7-H3 RKO group. The experiments were repeated 3 times and similar results were obtained (technical and biological replicates). ImageJ 5.0 was utilized for analysis. ***, P<0.001, **, P<0.01 *vs.* Control. HIF-1α, hypoxia-inducible factor 1α; Oe, overexpression; IgG, immunoglobulin G; shB7-H3 Oe-HIF-1α, shB7-H3 CRC cells overexpressed HIF-1α; HB-EGF, heparin-binding epidermal growth factor; ChIP, chromatin immunoprecipitation; qRT-PCR, quantitative real-time polymerase chain reaction; CM, conditioned medium.

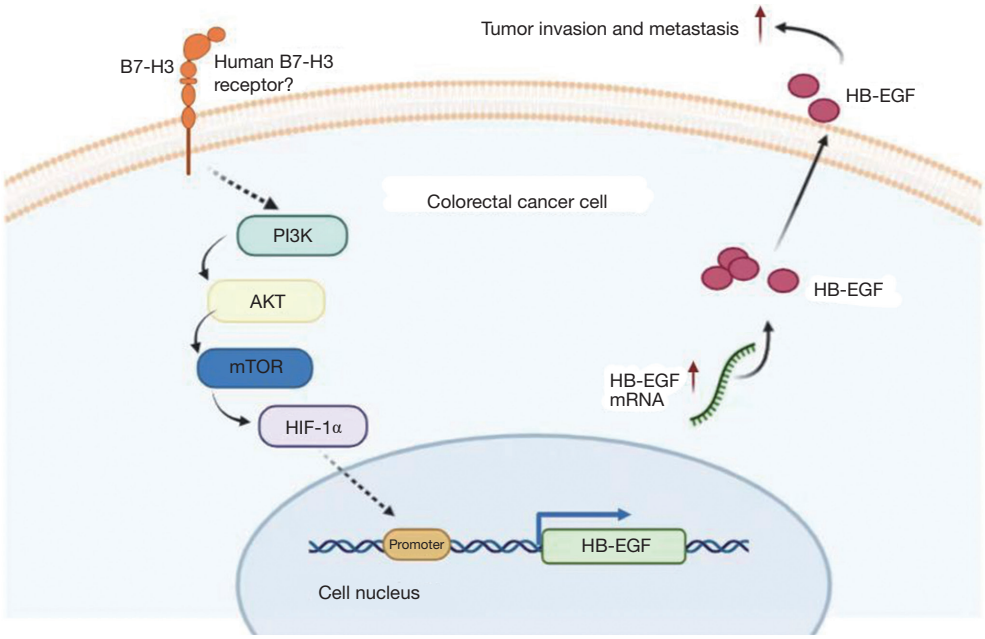

Figure 5 Mechanism by which B7-H3 promotes tumor progression and angiogenesis by enhancing HB-EGF expression. HB-EGF, heparin-binding epidermal growth factor; HIF-1α, hypoxia-inducible factor 1α.

Due to the decline of HB-EGF expression, shB7-H3 RKO cells had reduced proliferation, invasion, and angiogenesis than the Control group. However, after the rescue of HB-EGF expression in the shB7-H3 group by overexpressing HIF-1α, the abilities to perform these events also recovered. Therefore, we believe HB-EGF induces CRC cell proliferation, invasion, and angiogenesis.

Previous studies have reported HB-EGF is regulated by ADAM9/17 and MMP7/9 by facilitating the shed and cleavage (44-46). A recent study demonstrated that TMPRSS4 transcriptionally regulates HB-EGF through HIF-1α in hepatocellular carcinoma, and HIF-1α could interact with the translational region of MMP9 and HB-EGF and amplify the transcription of both genes, thereby enhancing the expression of HB-EGF (25). Since HIF-1α gene expression was also found to be affected by B7-H3 in the above microarray data, we decided to assess if HIF-1α was also involved in the regulatory mechanism of B7-H3. With decreasing B7-H3 expression, HIF-1α was also downregulated. Next, we performed a ChIP-qPCR assay, which allows the analysis of *in vivo* interactions of proteins with genomic DNA. The results showed that HIF-1α functions as a transcription factor, with a binding ability in CRC cells. Besides, in the shB7-H3 RKO group, this binding ability had a relatively lower level, mainly due to HIF-1α downregulation.

We thus conducted a HIF-1α rescue experiment by transfecting the overexpression lentivirus in shB7-H3 RKO cells and then detected HB-EGF mRNA levels as well as cell proliferation, invasion, and angiogenesis in presence of CM. The results showed that with HIF-1α level restoration, the proliferative and invasive abilities increased significantly, and the CM also had a significant promoting effect on tube formation in HUVECs, which refers to the angiogenic ability. To date, we have determined that B7-H3 indeed enhances HB-EGF expression by stimulating HIF-1α (*Figure 5*).

HIF-1α is susceptible to proteolytic degradation in normoxic conditions (47). Previous research has indicated that B7-H3 can influence the stability of HIF-1α protein within cells through the JAK-STAT3-ROS pathway (48). This indeed represents one of the potential mechanisms which B7-H3 may regulate HIF-1α in CRC cells. However, in our study, we observed alterations in HIF-1α not only at the protein level but also at the mRNA level. This suggests that, in addition to factors influencing HIF-1α protein degradation, there may be other regulatory mechanisms affecting the intracellular levels of HIF-1α. Our research identified significant changes in the PI3K-

AKT pathway, and there is evidence suggesting that in CRC, HIF-1α acts as a downstream regulatory factor of the AKT-mTOR pathway, influencing the glucose metabolism levels in CRC cells (49).

As these findings aligned well with the results of our KEGG analysis, we subsequently focused on the PI3K-AKT pathway. PI3K-AKT signaling is a ubiquitous pathway in cells, of which abnormal activation is considered a common activation of tumor development (50). Recent studies have also shown that B7-H3 also fulfills the intracellular function of the PI3K-AKT pathway (49,51). In this work, PI3K, AKT and mTOR protein amounts and AKT and mTOR phosphorylation levels were assessed in RKO cells, which were all significantly decreased after B7-H3 knockdown, indicating that the PI3K-AKT-mTOR pathway could be the intracellular mechanism involving B7-H3.

Conclusions

Collectively, this study revealed that B7-H3 represents a transmembrane protein with an essential promoting role in the progression and angiogenesis of CRC cells. Targeting HBEGF as a therapeutic target may potentially produce synergistic anti-tumor effects with B7-H3 targeted therapies. Moreover, B7-H3 and HB-EGF are potential prognostic factors in CRC patients as they both are associated with TNM stage and LNM in CRC patients. B7-H3 induces HIF-1α expression, thereby upregulating HB-EGF. The differential expression of the PI3K-AKT-mTOR pathway indicates that B7-H3 may exert intracellular signaling effects through this pathway. However, due to condition limitation, the effects of protein changes on potential cell membrane receptors of B7-H3 were not detected. Further studies are needed to identify the actual receptors of B7-H3 and to verify the uniqueness of PI3K-AKT-mTOR signaling, with a step-by-step validation.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-384/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-384/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee of the First Affiliated Hospital of Soochow University, [2022] No. 098. Informed consent was taken from all the patients.

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