Overexpressed PLAGL2 transcriptionally activates Wnt6 and promotes cancer development in colorectal cancer

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Abstract. Researchers hold the view that PLAGL2 is overexpressed in many malignancies and that it can promote tumor proliferation, migration, invasion and self-renewal; however, there is no evidence revealing a relationship between PLAGL2 and colorectal cancer (CRC). In the present study, genes that are overexpressed in CRC were screened using the COSMIC database and GEPIA database and the expression of PLAGL2 in carcinoma tissues and pericarcinomatous tissues was detected by RT-qPCR and western blot assays. A Cell Counting Kit-8 assay, a cell cycle analysis experiment and a xenograft model were used to explore the influence of PLAGL2 on CRC after knocking down PLAGL2 expression in HCT116 and SW480 cells. Using ChIP assays and Dual-Luciferase Reporter assays, the promoter regions to which PLAGL2 binds were discovered. It was observed that PLAGL2 was overexpressed in colorectal cancer and that it influenced the colorectal cancer cell cycle and promoted colorectal cancer proliferation *in vivo* and *in vitro*. The expression of some genes in the Wnt/β-catenin pathway, were downregulated after knocking down the expression of PLAGL2; Wnt6 was altered the most. PLAGL2 could bind to the promoter region of Wnt6 and promote its expression. These results indicated that PLAGL2 was overexpressed in CRC as a proto-oncogene and that it could active the Wnt/β-catenin pathway as a transcription factor by binding with the promoter region of Wnt6. PALGL2 was revealed to play an important role in colorectal cancer and may be a new therapeutic target for targeted medicine.

Introduction

According to the survey results published by the American Cancer Society (ACS), colorectal cancer (CRC) is one of the most common tumors worldwide. The incidence rate is ranked third among cancers (1). The number of deaths attributed to colorectal cancer each year still reaches 610,000 (2), ranking it second in malignant tumors (1).

Polymorphic adenoma-like protein 2 (PLAGL2) is a zinc finger protein of the PLAG gene family with 7 C2H2 zinc finger domains on the N-terminus (3-5). This structure is highly conserved, can bind DNA and enables the transcription factor PLAGL2 to activate the transcription of specific genes (6). PLAGL2 is closely related to the development of malignant tumors. PLAGL2 is important in the development of acute myeloid leukemia (7), lung cancer (8), glioma (9), prostate cancer (10) and other tumors. In colorectal cancer, Liu *et al* (11) selected 225 pairs of colon cancer tissues and 66 normal tissues for immunohistochemical studies and found that PLAGL2 expression was significantly increased in colorectal cancer. The mechanism of how PLAGL2 regulates the development of colon cancer has not been clearly elucidated.

In our previous study, causes of high PLAGL2 expression in colorectal cancer were initially addressed (12). The aim of the present study was to investigate the mechanism by which PLAGL2 affects the development of colorectal cancer.

Materials and methods

Tissue specimens. All 31 CRC tissue specimens and pericarcinomatous tissues were collected from the Third Affiliated Hospital of Central South University between January 2017 and May 2017 (Changsha, China). There are 15 female and 16 male patients and their age ranged from 35 to 70 years. All tissues were immediately flash-frozen in liquid nitrogen after resection and were then stored in liquid nitrogen. All cases were diagnosed as colorectal cancer (CRC) by pathological sections, and the patients received no chemo-, radio- or hormone therapy. All the patients signed consent forms. The present study was approved by the Institute Research Medical Ethics Committee of Central South University (Changsha, China).

Animals. BALB/C nude mice (n=24, female, 5-weeks-old and 16-20 g) were purchased from the SJA Laboratory Animal Company (Changsha, China) and housed under specific pathogen-free conditions with a 12-h light/dark cycle and autoclaved food/water were provided freely. The LV-PLAGL2-Homo-808 or LV-Vector (Shanghai GenePharma

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Co., Ltd., Shanghai, China) was stably transfected in SW480 and HCT116 cells following the manufacturer's instructions, and a suspension of transfected-cells [$5x10^6$ in 100 μl phosphate-buffered saline (PBS)] was injected into nude mice (n=6 for each group). Three groups were injected with HCT116 cells (mock, LV-Vector and LV-PLAGL2-Homo-808). The tumor volume was assessed every three days beginning 10 days after the injection and was calculated with the following formula: Tumor volume (mm^3) = (length x $width^2$)/2. All mice were sacrificed 30 days later by cervical dislocation after the mice were anaesthetized, and all animal protocols in this study were approved by the Animal Ethics Committee of Central South University (Changsha, China).

Cell culture. The human CRC cell lines SW480 and HCT116 were purchased from Wuhan Boster Biological Technology Ltd. (Wuhan, China). SW480 cells were cultured in L15 medium (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) containing 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit-Haemek, Israel). HCT116 cells were cultured in McCoy's 5A medium (Nanjing KeyGen Biotech Co., Ltd.) containing 10% FBS. All cells were cultured in a humidified incubator at 37˚C and 5% CO_2.

Plasmid and lentivirus constructs. The human CRC cell lines SW480 and HCT116 were transfected with three different vectors, which were transferred by lentivirus, targeting PLAGL2 to knock down PLAGL2 protein expression. Three short hairpin RNAs targeting human PLAGL2 and a non-targeting RNA sequence serving as a negative control were cloned into the pGPU6 vector (Shanghai GenePharma Co., Ltd., Shanghai, China). The details of the RNA sequences are presented in Table I. Virus packaging was performed in 293T cells. SW480 and HCT116 cells were cultured in 6-well plates with normal medium containing 10% FBS the day before transfection ($5x10^5$ cells/plate). After 24 h, the cells were transfected with 50 μl lentivirus with 5 μl Polybrene (5 μg/ml) and 1.5 ml medium. The cells were selected for 5 days and then used for various assays. The lentivirus and vectors were purchased from Nanjing KeyGen Biotech Co., Ltd.

Full-length human WNT6 cDNA (NM_006522.3) was cloned into the pCMV vector (Shanghai GenePharma Co., Ltd.). Virus packaging and transduction were performed as aforementioned. WNT6 was ovexpressed in HCT116 cells as PLAGL2 was knocked down. In addition, WNT6-overexpressed-cells were cultured in McCoy's 5A medium (Nanjing KeyGen Biotech Co., Ltd.) without FBS.

Western blotting (WB). Total protein was extracted by lysis in RIPA buffer (Nanjing KeyGen Biotech Co., Ltd.) containing protease inhibitor cocktail (Nanjing KeyGen Biotech Co., Ltd.) for 15 min at 4˚C and centrifuged at 16,666 x g for 15 min at 4˚C. The proteins were measured by BCA assay. Proteins (40 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes.

The membranes were blocked with 5% non-fat milk in PBS for 2 h at room temperature and were then incubated with primary antibodies at 4˚C overnight. Subsequently, the membranes were incubated with IRDye 800CW conjugated goat (polyclonal) anti-rabbit IgG (H+L) (dilution 1:15,000; cat. no. 926-32211; LI-COR Biosciences, Lincoln, NE, USA)

for 2 h at room temperature. The protein bands were visualized by Infrared fluorescence with Odyssey CLx (LI-COR Biosciences) and the excitation wavelength was 778 nm. The primary antibodies used were as follows: Anti-PLAGL2 (cat. no. ab139509) and anti-Wnt6 (cat. no. EPR9244) (both from Abcam, Cambridge, UK), anti-β-catenin (cat. no. 8480) (Cell Signaling Technology, Danvers, MA, USA) at a 1:1,000 dilution; anti-GAPDH (cat. no. ab181602; Abcam) at a 1:3,000 dilution at a 1:3,000 dilution.

Quantitative real-time PCR (RT-qPCR). Total RNA was extracted by TRIzol reagent (Invitrogen; Thermo Fisher, Scientific, Inc., Waltham, MA, USA). Real-Time Quantitative PCR (RT-qPCR) was performed with the Toyobo RT kit and KOD SYBR® qPCR kit (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocol. The primers for mRNA were produced by Sangon Biotech Co., Ltd. (Sangon Biotech, China) and the primer sequences are listed in Table II. The thermocycling conditions are listed in Table III. All mRNA expressions were standardized to GAPDH, and the mRNA levels were determined by the $2^{-\Delta\Delta Cq}$ method. The method of quantification employed by Livak and Schmittgen (13).

Cell Counting Kit-8 assay. Cell proliferation was assessed by a Cell Counting Kit-8 assay (CCK-8 Kit; Dojindo Laboratories, Kumamoto, Japan). After cells were stably transfected with lentivirus, which included the vectors to knock down the expression of PLAGL2, the cells ($4x10^3$ cells/well) were incubated in normal medium without serum at 37˚C overnight the day before and incubated in 96-well plates with 100 μl normal culture medium at 37˚C. At the indicated time-points (12, 24, 48, 72 and 96 h), 10 μl of CCK-8 solution was added into each well and then cultured for 3 h at 37˚C. The absorbance of each well at 450 nm (A450) was detected with an EnVision microplate reader (PerkinElmer, Inc., Waltham, MA, USA). All experiments were performed in triplicate.

Cell cycle analysis experiment. Cells were plated into a 6-well plate at a density of $3x10^5$ cells/well for 24 h and fixed with 2 ml 1% paraformaldehyde and stored in 70% ethanol at -20˚C. The cells were stained with 0.5 ml PI/RNase (BD Biosciences, Fraklin Lakes, NJ, USA) staining buffer for 15 min at room temperature and analyzed by flow cytometry.

Immunofluorescence. A total of $3x10^5$ cells were plated into the 24-well plates for 24 h at -20˚C and each well was covered by a glass coverslip. Cells were fixed in 4% paraformaldehyde for 24 h at room temperature and blocked in 10% BSA for 2 h at room temperature. Cells were labeled with anti-PLAGL2 antibodies (dilution 1:100; cat. no. 139509; Abcam) at 4˚C for 12 h. The following day, the cells were stained for 1 h with cy3-labeled goat anti-rabbit IgG (dilution 1:100; cat. no. KGIF010; Nanjing KeyGen Biotech Co., Ltd.) at 37˚C and slides were washed three times for 5 min with PBS. Nuclei were stained with DAPI (Nanjing KeyGen Biotech Co., Ltd.) for 5 min at 37˚C. PLAGL2 immunofluorescence was imaged using a Zeiss LSM 800 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Sequence analysis. The overexpression of PLAGL2 was analyzed by the GEPIA database (14) and COSMIC database

Table I. Sequences of the vectors.

Serial number	Sequence (5'-3')
PLAGL2-Homo -506 (LV1)	CCACCAGTGTATGTACTGTGA
PLAGL2-Homo -699 (LV2)	CTGCAGACCTTTGAGAGTACC
PLAGL2-Homo -808 (LV3)	GGCGGTTCTATACTCGTAAGG
LV-NC	TTCTCCGAACGTGTCACGT

Table II. Primer sequences for RT-qPCR.

Gene detected	Sequence (5'-3')
GAPDH	F: GAAGGTGAAGGTCGGAGT
	R: CATGGGTGGAATCATATTGGAA
PLAGL2	F: GAGTCAAGTGAAGTGCCAATGT
	R: TGAGGGCAGCTATATGGTCTC
FZD6	F: TCTGCTGTCTTCTGGGTTGG
	R: CTGTAGCTCCTGTGCTGGTT
FZD7	F: TTCTACCACAGACTTAGCCACAG
	R: CTCACTTCCAGGTCACTTCTCA
Wnt3	F: TGACTTCGGCGTGTTAGTGT
	R: GTGCATGTGGTCCAGGATAG
Wnt6	F: GGTGCGAGAGTGCCAGTTC
	R: CGTCTCCCGAATGTCCTGTT
Wnt11	F: GGAGTCGGCCTTCGTGTATG
	R: GCCCGTAGCTGAGGTTGTC

F, forward; R, reverse.

Table III. The thermocycling conditions used in qPCR.

Temperature (˚C)	Time	
95	5 min	
95	30 sec	
60	30 sec	40 cycles
72	30 sec	
72	10 min	
4	Hold	

(http://cancer.sanger.ac.uk/cosmic/). The NCBI gene browser (https://www.ncbi.nlm.nih.gov/gene/) was used to find the human Wnt6 locus (Chr2: 218,858,382-218,875,674) and the Wnt6 promoter region (Chr2: 218,858,821-218,860,072). Candidate transcription factor binding sites were predicted by means of the JASPAR database (http://jaspar.genereg.net/).

ChIP. Chromatin immunoprecipitation (ChIP) was performed using an EZ-ChIP™ ChIP kit (Merck Millipore, Darmstadt, Germany) following the manufacturer's protocol, including anti-RNA polymerase II (Merck Millipore; cat. no. 05-623B),

Table IV. Primer sequences for qPCR in ChIP assays.

Gene detected	Sequence (5'-3')
GAPDH	F: TACTAGCGGTTTTACGGGCG
	R: TCGAACAGGAGGAGCAGAGAGCGA
Wnt6-1	F: TCTCTACTCTTCTTCCCAGCCCTT
	R: TCGGGCTGTGGGACCCGCCCGCTT
Wnt6-2	F: CCAAGTCCGAGAGAGGCGAGGCGA
	R: GCGGGACTCTCGGGAGCGAGCCGT
Wnt6-3	F: AGGAGACACAGGCGCTGGCTGCCC
	R: TCAGTGCGAGCGCGGCGAGCGCAA

F, forward; R, reverse.

anti-IgG (mouse) (Merck Millipore; cat. no. 12-371B) and GAPDH primers (Merck Millipore; cat. no. 22-004). HCT116 cells ($2x10^7$) were crosslinked with 37% formaldehyde. The chromatin was cleaved into fragments between 200 and 600 bp by ultrasound. Anti-PLAGL2 was purchased from Abcam and qPCR was performed with the KOD SYBR® qPCR kit (Toyobo). Primer details are presented in Table IV. The levels of DNA were determined by the $2^{-\Delta\Delta Cq}$ method.

Generation of luciferase reporter constructs. The promoter fragments of the WNT6 gene were generated by PCR using a forward primer with a *Kpn*I (15) site inserted at the 5' end and a reverse primer with a *Bgl*II (15) site inserted at the 3' end. PCR products were digested with *Kpn*I/*Bgl*II and cloned into a pGL3 firefly luciferase basic vector (Promega Corp., Madison, WI, USA) following the manufacturer's protocol.

Cell transfection and luciferase assays. HCT116 cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells were then incubated at 37˚C for 48 h before assaying for luciferase activity using the Dual-Luciferase Reporter assay system (Promega Corp.) according to the manufacturer's instructions. Firefly luciferase activity was normalized relative to *Renilla* luciferase activity for each transfection and calculated as the fold increase over pGL3 firefly luciferase basic vector (pGL3Basic). At least two independent transfections and two replicates per assay were performed for each individual construct.

Statistical analysis. The statistical analysis was performed by using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). Data were imaged with GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the mean ± SD. Differences between groups were compared with Student's t-test (when 2 groups) and one-way ANOVA (when >2 groups) was used, followed by Student-Newman-Keuls (SNK) method for comparing each two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PLAGL2 is overexpressed in colorectal cancer. Using the Catalogue of Somatic Mutations in Cancer database (COSMIC;
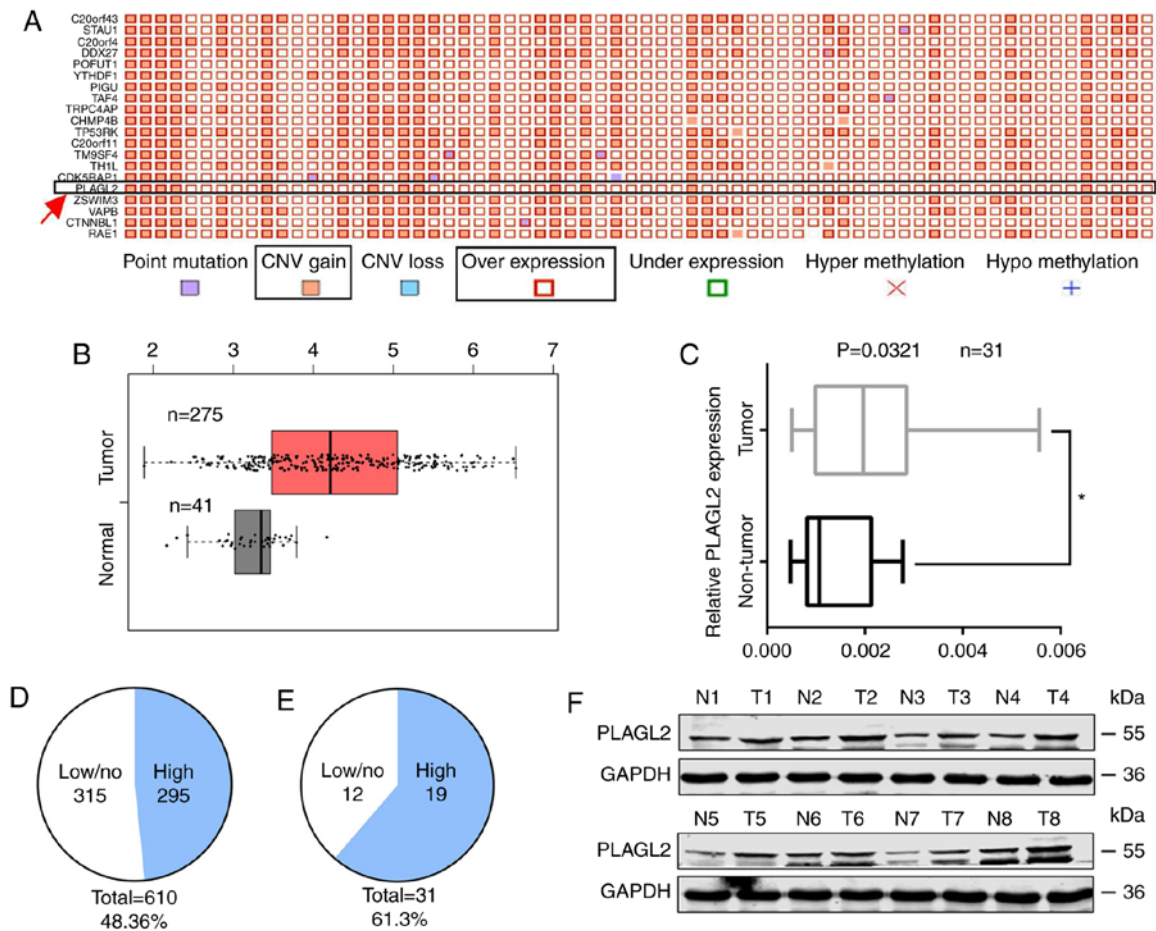
Figure 1. Expression of PLAGL2 in cancer tissues and pericarcinomatous tissues. (A) The top 20 genes that are overexpressed in CRC. This information was obtained from the COSMIC database. (B) The analysis of the mRNA of 275 tumor tissues and 41 normal tissues from the GEPIA database. (C) Expression in 31 pairs of cancer and pericarcinomatous tissues. (D and E) The percentages of the pairs with PLAGL2 overexpression. The information is from the COSMIC database and our research. (F) The western blot analysis of 8 pairs of cancer and pericarcinomatous tissues in which PLAGL2 was overexpressed in the tumor tissues. The data are expressed as the mean ± SD for 3 experiments, each performed in duplicate, *P<0.05.

http://cancer.sanger.ac.uk/cosmic/), overexpressed genes in colorectal cancer were screened and the top 20 were selected; PLAGL2 at position 16 was revealed (Fig. 1A). GEPIA analysis of the mRNA from 275 colon cancer specimens and 41 normal colonic tissues in TCGA revealed that the expression of PLAGL2 in cancer tissue was 1.235-fold that of PLAGL2 in normal tissue (Fig. 1B). The mRNA of 31 colorectal cancer tissue samples and 31 adjacent normal tissue samples was examined by RT-qPCR. The expression of PLAGL2 in cancer tissue was 2.247-fold that of PLAGL2 in the normal tissues (Fig. 1C). In the COSMIC database, ~295 gene fusion pairs exhibited PLAGL2 expression that was higher in cancer tissues than in normal tissues, accounting for ~48.36% of the total number of tissues. In the 31 pairs of tissues used in this experiment, PLAGL2 was found in cancer tissues. Approximately 19 pairs had higher expression in cancer tissues than normal tissues, accounting for ~61.3% of the total number of tissues (Fig. 1D and E). Eight of the 19 pairs of tissues in which PLAGL2 expression was higher in the cancer tissues than normal tissues were selected to detect the protein expression of PLAGL2 by western blot analysis. The expression of PLAGL2 in colorectal cancer tissue was significantly higher than that in the normal tissue adjacent to the tumor (Fig. 1F). These results indicated that PLAGL2 was overexpressed in colorectal cancer.

PLAGL2 influences the cell cycle and promotes the proliferation of colorectal cancer. Next, we sought to reveal the function of PLAGL2 in CRC. The CRC cell lines HCT116 and SW480 were selected and different vectors were applied to knock down PLAGL2. It was found that both in SW480 and HCT116 cells, the most efficient plasmid was PLAGL2-Homo-808 (LV3) (Fig. 2A and B). The PLAGL2-knockdown group (PL-LV3) was selected as the experimental group and the empty plasmid group (NC) was used as the control group for functional experiments. The results of the CCK-8 assay revealed that in SW480 cells, the growth rate of the experimental group cells was significantly lower than that of the control group cells after 48 h (P<0.05) and the difference among them was more pronounced with time (P<0.01). In HCT116 cells, from 72 h onwards, the growth rate of the experimental group of cells was significantly lower than that of the control group cells, with significant differences (P<0.05), and the differences among them were more obvious with time (P<0.01) (Fig. 2C and D). Flow cytometry was used to detect the cell cycle. The results indicated that in the HCT116 and SW80 cells, the cells in the experimental group exhibited a prolonged G1 phase and the S phase was shortened (Fig. 2E and F) when compared with the control cells. Tumor formation experiments in nude mice indicated that the tumor volume in the untreated and the
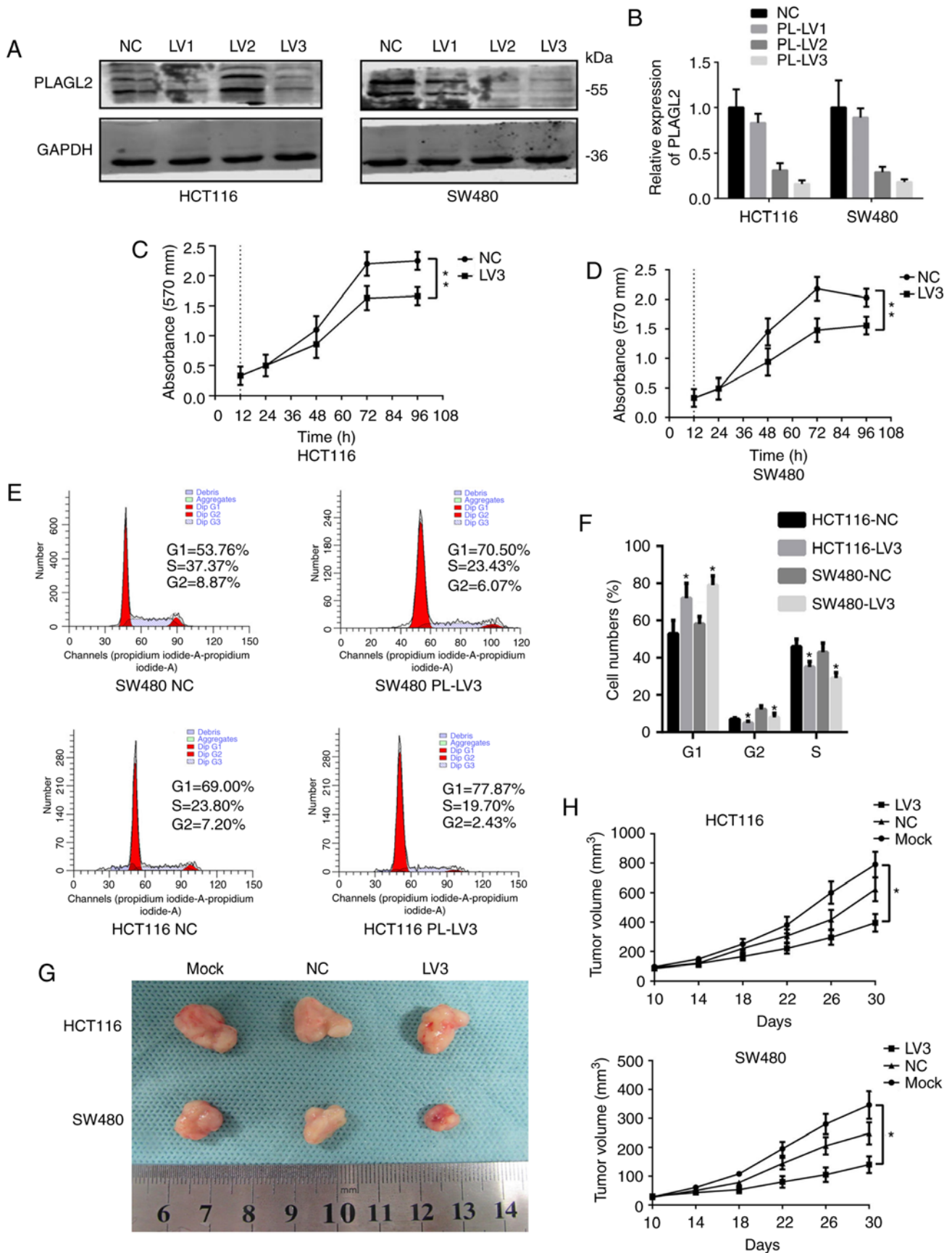
Figure 2. PLAGL2 influences the cell cycle and promotes the proliferation of colorectal cancer. (A and B) The verification of the efficiency of PLAGL2 knockdown with western blot analysis and RT-qPCR assays. (C and D) The CCK-8 assays for HCT116 and SW480 colorectal cancer cells. LV3 indicates that PLAGL2 was knocked down, and NC indicates the control group. (E and F) The cell cycle was analyzed with flow cytometry. LV3 indicates PLAGL2 was knocked down, and NC indicates the control group. (G) Tumor formation experiments in BALB/C nude mice. Mock indicates the original cancer cells, LV3 indicates PLAGL2 was knocked down, and NC indicates the control group. (H) The growth curve of the tumor in tumor formation experiments. The data are expressed as the mean ± SD for 3 experiments, each performed in duplicate, $^{*}P<0.05$.
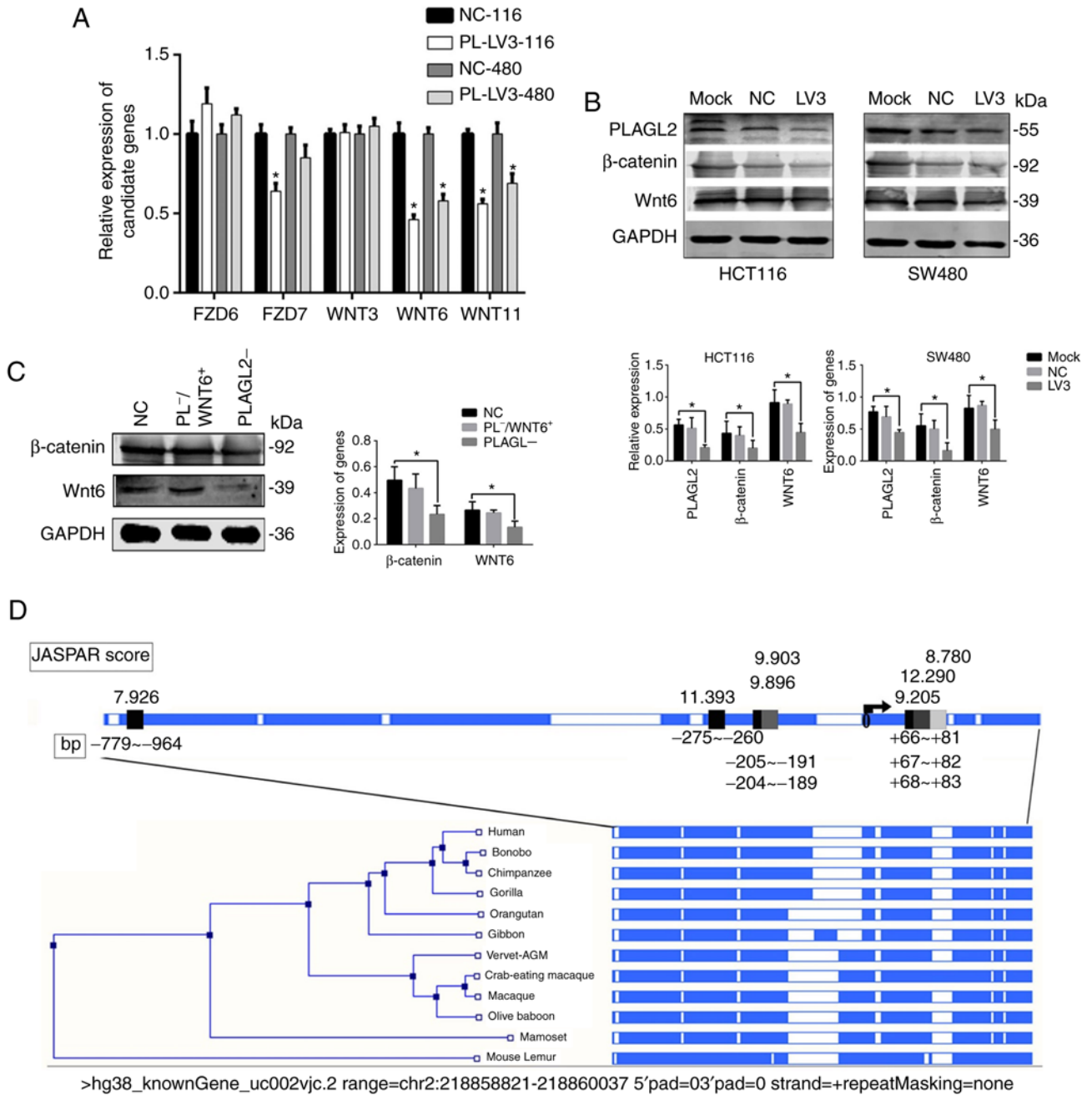
Figure 3. Screening of signaling pathway factors and prediction about the promoter region. (A) The factors in the Wnt pathway were screened and the one that was related to PLAG2 was choosen using RT-qPCR assays. (B) With western blot assays, the expression levels of PLAGL2, Wnt6 and β-catenin were revealed to have a positive correlation. Mock indicates the original cancer cells, LV3 indicates PLAGL2 was knocked down, and NC indicates the control group. (C) With western blot assays, the expression levels of Wnt6 and β-catenin were revealed to have a positive correlation. NC indicates the control group, PLAGL2⁻ indicates that PLAGL2 was knocked down, and PL⁻/WNT6⁺ indicates PLAGL2 was knocked down and WNT6 was overexpressed. (D) Prediction about the promoter region that can bind with PLAGL2, which was predicted by the JASPAR database. The data are expressed as the mean ± SD for 3 experiments, each performed in duplicate, *P<0.05.

control groups was greater than that in the experimental group (Fig. 2G), and the growth curve indicated that the growth rate of the control group was greater than that of the experimental group (Fig. 2H). These results indicated that PLAGL2 affected the development of colorectal cancer *in vitro* and *in vivo*.

PLAGL2 promotes the Wnt/β-catenin pathway. PLAGL2 plays as an important role in other tumors, where it can always activate the Wnt/β-catenin pathway, and the Wnt/β-catenin pathway is one of most active pathways in colorectal cancer. Several

key factors that are active in the Wnt/β-catenin pathway were selected and primers were designed. The mRNA of SW480 and HCT116 cells was extracted and the expression was detected with RT-qPCR. It was found that the expression of Wnt6 and Wnt11 was significantly decreased after PLAGL2 knockdown (Fig. 3A). While Wnt11 mainly regulates the Wnt/Ca²⁺ pathway, Wnt6 regulates the Wnt/β-catenin pathway. A western blot assay was used to detect changes in the expression of Wnt6 and β-catenin protein before and after PLAGL2 knockdown. The results revealed that the expression of Wnt6 and β-catenin

A >hg38_knownGene_uc002vjc. 2 range=chr 2:218858821-218874233 5'pad=03'pad=0 strand=+repeatMasking=none

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cccgccccaaactccctcattcctgggaagttttctctctttacagtcaacaaacctgtcaaacgtct
cccttccagcccgtccccattcctcagctcctctttctcccccctgcacccctctctactcttcttcc
cagcccttccttgccccgactccaggcggccgcgagcggcgggggctccaagtccgagagaggcga    Mutation 1
ggcgaggggcgaagcgggcgggtcccacagcccgaagcgcgctggggcccccgccccggcaccccgct    Mutation 2
cccgccctccgctgcgctccgcgccagccgccccggggcgcacggctcgctcccgagagtcccgctgc
ctgactgtcgcaactgccacctccctccggccccctgcccccccgcccccgcctcggccccgctcccct
cccacctccgcccccggctctgatttcttctcccgagcgagcTCCGCAGGAGACACAGGCGCTGGCTG
CCCCGTCCGCTCTCCGCCTCCGCCGCGCCCTCCTCGCCCGGGATGGGCCCCCCCGCCGCCGCCGGATC    Mutation 3
CCTCGCCTCCCGGCCGCCGCCGTTGCGCTCGCCGCGCTCGCACTGAAGCCCGGGCC
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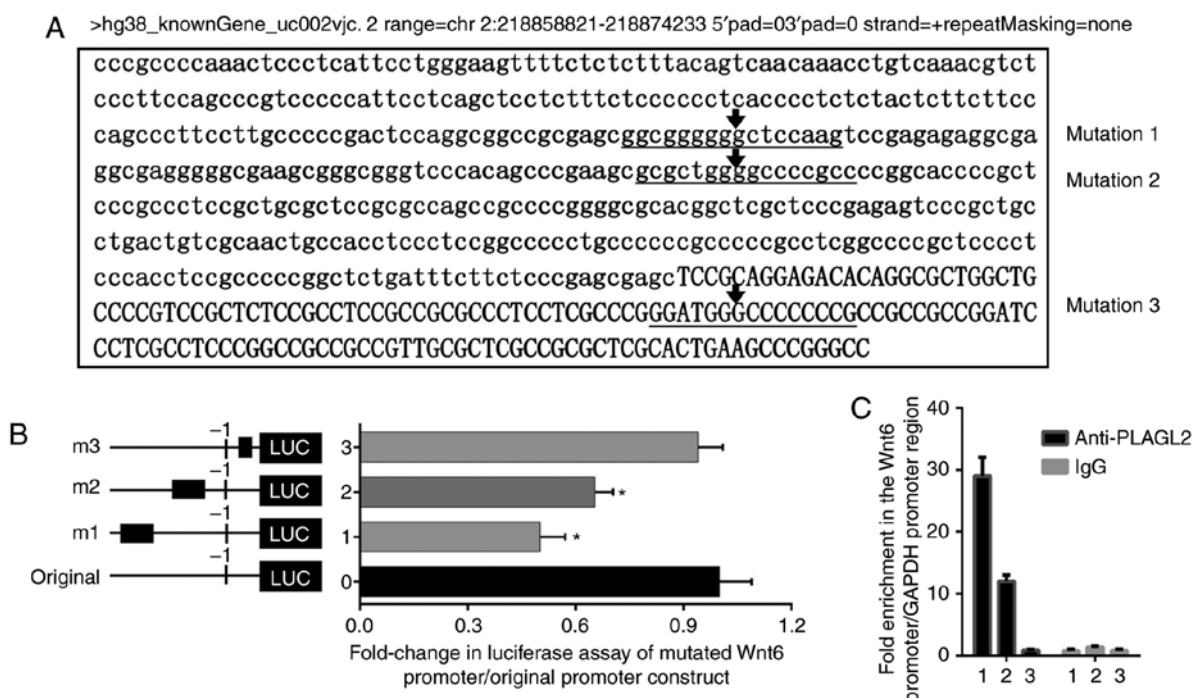
Figure 4. Verification that PLAGL2 activates Wnt6 as a transcription factor using luciferase reporter and ChIP assays. (A) Three ideal binding regions that were predicted by the JASPAR database. (B) In HCT116 cells, the changes in the activity of the promoter when three mutations were made in different promoter regions were revealed. The promoter activity was assessed using the luciferase reporter assay. (C) PLAGL2 was enriched in the Wnt6 promoter in Wnt6-expressing HCT116 cells (ChIP analysis. The data are expressed as the mean ± SD for 3 experiments, each performed in duplicate, *P<0.05.

proteins decreased with the knockdown of PLAGL2 (Fig. 3B). Wnt6 was overexpressed in HCT116 cells when PLAGL2 was knocked down and the two groups were compared. The WB assays revealed that β-catenin was increased when WNT6 was overexpressed but PLAGL2 was knock down. It revealed that PLAGL2 activated the Wnt/β-catenin pathway by promoting the expression of WNT6 (Fig. 3B). The sequence 1,000 bp upstream and 216 bp upstream of the WNT6 gene was used to make predictions using the JASPER database, indicating that the WNT6 upstream sequence was highly conserved among different species and that the conserved region had 7 possible binding domains for the PLAGL2 gene (Fig. 3C).

PLAGL2 binds to the promoter region of Wnt6 and actives it. The three-ideal binding region mutation analyses predicted by the JASPAR database were selected; the horizontal lines represent possible binding sites and mutation regions of PLAGL2 and Wnt6 (Fig. 4A). The luciferase assay results revealed that after mutation of the predicted binding regions, the promoter activity was decreased. Compared to the activity of the original promoter region, mutation 1 resulted in a ~50% reduction in promoter activity, mutation 2 resulted in a ~35% reduction, and mutation 3 resulted in a ~10% reduction (Fig. 4B). In HCT116 cells, we applied ChIP assays to detect the binding of PLAGL2 to the Wnt6 promoter region. It was revealed that PALGL2 strongly bound to the Wnt6 promoter region in HCT116 cells, with a ~28 and ~12-fold enrichment compared to the GAPDH control (Fig. 4C). The results of immunofluorescence also revealed that the PLAGL2 proteins were located in the cell nucleus (Fig. 5A). These results indicated that PLAGL2 could activate Wnt6 expression in colorectal cancer cells, activating the Wnt/β-catenin pathway.

Discussion

The polymorphic adenoma-like protein 2 (PLAGL2), located on chromosome 20q21.11, is a zinc finger protein derived from the PLAG gene family. The protein can function as a transcription factor to activate the expression of some oncogenes and cancer-promoting signaling pathways by activating promoters, thereby inhibiting cell differentiation and increasing the speed of cell self-replication. The carcinogenic effects of PLAGL2 have been found in many malignancies, including acute myeloid leukemia, neurogenic stromal tumor, lung and breast cancer (7-9,16-18). For the relationship between PLAGL2 and colorectal cancer, more studies are required. In the present study, the information we collected by screening the COSMIC database (19-21) and GEPIA database and by RT-qPCR and WB demonstrated that PLAGL2 was overexpressed in colorectal cancer tissues.

As a proto-oncogene, PLAGL2 is overexpressed in colorectal cancer, and it can be reasonably speculated that it plays a role in the development of colorectal cancer. However, PLAGL2 can act as a transcription factor in many tumors and activate some cancer-promoting pathways relating to cancer, such as the Wnt/β-catenin pathway.

The Wnt/β-catenin pathway is a very active signaling pathway in colorectal cancer, and it can promote the proliferation, cell cycle, apoptosis, invasion and migration of colorectal cancer after activation (22). β-catenin can be phosphorylated by GSK3-β and ultimately be hydrolyzed by the ubiquitin-proteasome pathway. The binding of the Wnt signaling pathway ligand to the paired frizzled-like receptors inhibits hydrolysis of the β-catenin complex, including APC, AXIN and GSK3-β, and a series of reactions leads to the stabilization of β-catenin
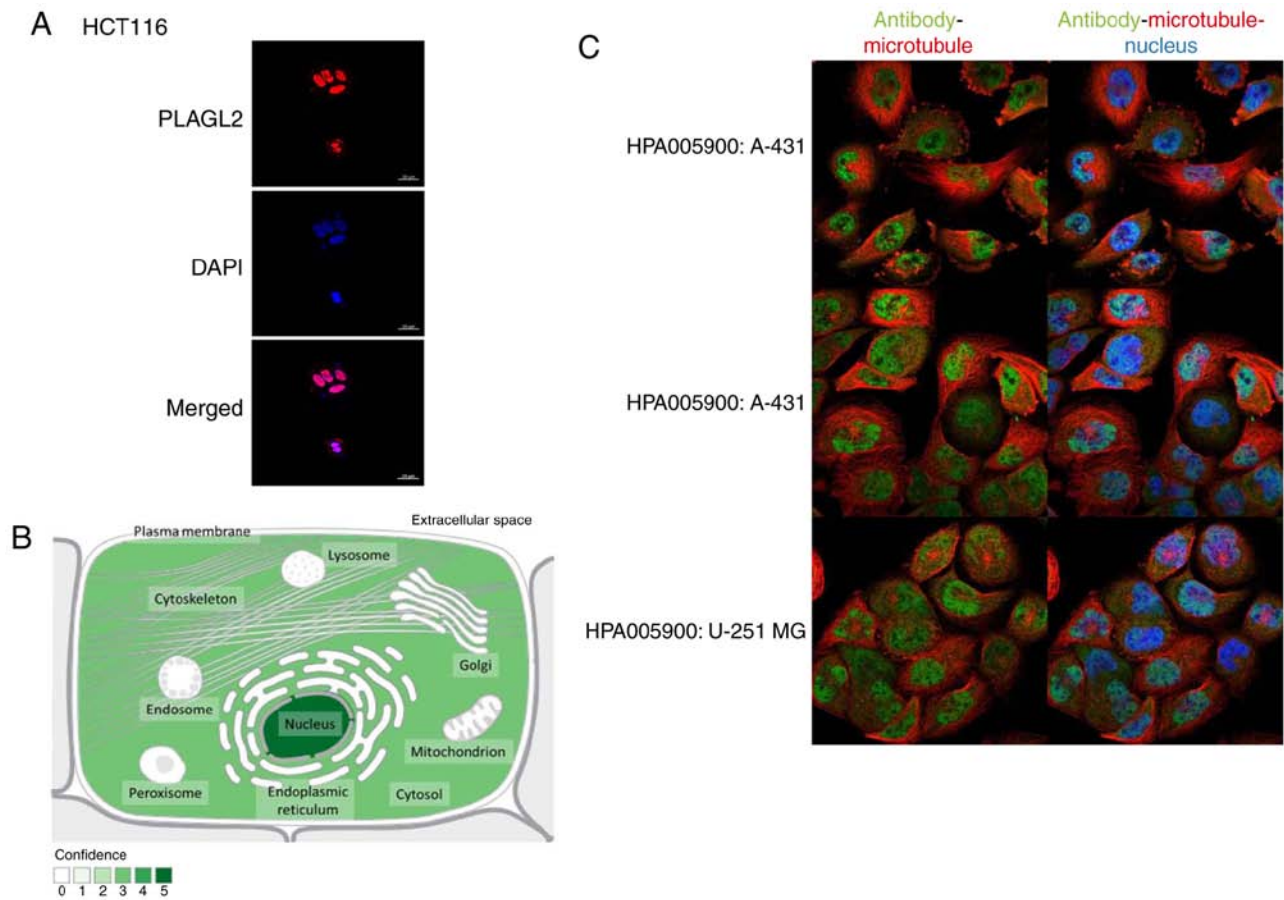
Figure 5. PLAGL2 proteins are located in cell nucleus. (A) The immunofluorescence assay revealed that the proteins PLAGL2 in the HCT116 cells were located in the cell nucleus. (B) The GeneCards database revealed the subcellular locations for the PLAGL2 gene, the confidence was 5 in the compartment nucleus and the confidence was 3 in the compartment cytosol. (C) The immunofluorescence assay results of the Human Protein Atlas database revealed that the PLAGL2 proteins were located in the nucleoplasm.

in the nucleus and eventual activation of the downstream target gene. When the Wnt/β-catenin pathway is activated, β-catenin is translocated into the nucleus and binds to the transcription factor TCF/LEF in the nucleus, which leads to the activation of expression of the downstream target genes, such as a MMP-7, CCND1, c-Myc and survivin. The activation of these downstream genes can promote the development of colorectal cancer (23-26). For example, the proto-oncogene c-Myc can be activated by β-catenin and inhibited by wild-type APC. The dependence of β-catenin-mediated c-Myc proto-oncogene expression will maintain the initial phenotype of colorectal cancer cells by inhibiting p21 expression and disrupting cell differentiation (27). β-catenin can also regulate the expression of CCND1. CCND1 is an important G1 phase cyclin, and β-catenin can increase the expression of CCND1 after it enters the nucleus. CCND1 accumulates in the nucleus and promotes cells to enter the S phase from the G1 phase. The cells enter the S phase and hydrolyze, which accelerates the cell cycle (28,29). MMP-7 which functions as a downstream target gene for the Wnt pathway is also activated when the Wnt pathway is activated. MMP-7 is a member of the MMP family, and MMPs are Zn^{2+}-dependent proteolytic enzymes that can degrade a variety of basement membrane and extracellular matrix components. It plays an important role in tumor invasion and metastasis. Survivin, a member of the apoptotic inhibitor protein family,

inhibits apoptosis and expression in tumors and enhances tumor cell viability (30). High expression of survivin was also revealed when the Wnt pathway was activated.

It was revealed that the WNT/β-catenin pathway plays an important role in colorectal cancer. This study demonstrated that PLAGL2 was related to the activation of the WNT/β-catenin pathway in colorectal cancer. Now, the question that remains is how PLAGL2 regulates the expression of the WNT/β-catenin pathway. PLAGL2 is located on chromosome 20q11.21 and contains the initiation codon ATG and the stop codon TAG, without the presence of the AATAAA polyadenylation signal. Its open reading frame encodes 496 amino acid residues. The protein consists of seven zinc finger structure consensus sequences at the amino acid terminus and a carboxylic end rich in proline and serine (4). The zinc finger structure is often found in DNA-binding proteins. It consists of a ring containing approximately 30 amino acids and a Zn^{2+} coordinated to 4 Cys or 2 Cys and 2 His on the ring, forming a structure like a finger (31). The zinc finger has a pair of cysteine residues at the N-terminus and a pair of histidine residues at the C-terminus. The zinc finger structure can specifically recognize the DNA sequence through the conserved sequence at the N-terminus, and the C-terminus has a transactivation function. The transcriptional activity of PLALG2 is

activated by binding to the bidirectional consensus sequence GRGGC(N)(6-8)GGG (32), and its protein contains seven zinc finger structures, of which zinc finger structure 3 is mainly responsible for recognizing the aforementioned. In the sequence, zinc finger structures 6 and 7 are the cores that bind to proteins (33).

It is known from the structural features of PLAGL2 that it recognizes the promoter region of genes and promotes gene transcription using the C-terminal activation functions. Some researchers have claimed that PLAGL2 can be secreted to the medium *in vitro*, but as a transcription factor, all the aforementioned processes occur in the nucleus (9,34-36). The introduction of PLAGL2 in PubMed revealed that most PLAGL2 proteins are located in the cell nucleus. The data from the GeneCards database (https://www.genecards.org/) revealed the same results (Fig. 5B). The immunofluorescence results from The Human Protein Atlas (https://www.proteinatlas.org/) revealed that the proteins of PLAGL2 are surrounded by karyotheca (Fig. 5C).

In the present study, it was demonstrated through a ChIP assay, that PLAGL2 could be combined with the promoter region of WNT6 in the nucleus. The luciferase assay revealed that PLAGL2 could activate the transcription of WNT6 by binding to the WNT6 promoter region.

Wnt6 protein expression activates the Wnt/β-catenin pathway. The activation of the Wnt/β-catenin pathway leads to the overexpression of a series of target proteins, which may play important roles in the development of colorectal cancer.

In summary, PLAGL2 is an oncogene that is active in a variety of malignancies. It is rarely expressed in normal adult tissues and is active in embryos, stem cells and tumors (5). Further research on PLAGL2 may provide a new target for the targeted therapy of colorectal cancer.

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Availability of data and materials

The bioinformatics data analysis and figures used in our study can be obtained from the following websites: GEPIA database (http://gepia.cancer-pku.cn/); COSMIC database (http://cancer.sanger.ac.uk/cosmic/); The NCBI gene browser (https://www.ncbi.nlm.nih.gov/gene/); JASPAR database (http://jaspar.genereg.net/). Genecards database (https://www.genecards.org/); The Human Protein Atlas (https://www.proteinatlas.org/). All the data and figures from these websites above are open and there are no copyright disputes. The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

Article checking and project design were carried out by GH and XL; the experiment design, the writing and the cell experiments were performed by NL and DL; the tissue experiments and the animal experiments were performed by YD and CS; the figure editing, the statistical analysis and the specimen collection was carried out by CY and CL. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and patient consent for participation

Signed consent forms were provided by each patient, and the study was approved by The Institute Research Medical Ethics Committee of Central South University (Changsha, Hunan). All animal protocols in this study were approved by the Animal Ethics Committee of Central South University (Changsha, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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