Abstract. Berberine, a natural isoquinoline alkaloid derived from Berberis species, has been reported to have anticancer effects. However, the mechanisms of action in human colorectal cancer (CRC) are not well established to date. In the present study, the cell cytotoxicity effect of berberine on human CRC cells, as well as the possible mechanisms involved, was investigated. The results of the cell viability and apoptosis assay revealed that treatment of CRC cells with berberine resulted in inhibition of cell viability and activation of cell apoptosis in a concentration-dependent manner. To reveal the underlying mechanism of berberine-induced anti-tumor activity and cell apoptosis, RNA-sequencing followed by reverse-transcription quantitative PCR were performed. In addition, RNA immunoprecipitation, chromatin immunoprecipitation and western blot analysis were used to identify the functional regulation of CASC2/ EZH2/BCL2 axis in berberine-induced CRC cell apoptosis. The data revealed that IncRNA CASC2 was upregulated by berberine treatment. Gain- or loss-of-function assays suggested that IncRNA CASC2 was required for the berberine-induced inhibition of cell viability and activation of cell apoptosis. Subsequently, the downstream antiapoptotic gene BCL2 was identified as a functional target of the berberine/CASC2 mechanism, as BCL2 reversed the berberine/CASC2-induced cell cytotoxicity. IncRNA CASC2 silenced BCL2 expression by binding to the promoter region of BCL2 in an EZH2-dependent manner. In summary, berberine may serve as an important therapeutic target to improve the anticancer effect of berberine.

Introduction

Colorectal cancer (CRC) is one of the most malignant cancer types worldwide. In developed countries, ~100 million people suffer from CRC each year, and the mortality rate has increased in recent years (1,2). Metastasis is the major cause of mortality in patients with CRC and increases the risk of tumor recurrence (3). Currently, chemotherapy or radiotherapy following surgical resection are the main treatment options (4). The most commonly used first line chemotherapeutic drugs are 5-fluorouracil, oxaliplatin and irinotecan (5). Although progress in diagnosis and treatment of cancer has been made in the past decade, the overall survival rate for patients with CRC remains unfavorable and a relatively high proportion of patients become chemoresistant (6). Therefore, early screening and novel therapeutic approaches are essential for improved prognosis of patients with colorectal cancer.

Herbal medicines have long been accepted as useful adjuvant therapeutic agents for a number of diseases. The use of berberine as an alternative medicine is widespread in the developed world (7). Berberine is a natural isoquinoline alkaloid derived from Berberis species, including Coptis chinensis, which has been used to treat intestinal infections, particularly bacterial diarrhea, for thousands of years in China (8). Additionally, it has been reported to have antitumor effects in various types of cancer including melanoma, glioma, lung and breast cancer and hepatocellular carcinoma (9-12). Berberine may exhibit an anticancer effect in CRC (13,14); however, the underlying functional mechanism has not been fully elucidated.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs >200 nucleotides in length and serve an important role in regulating gene expression (15). They can regulate target gene expression level in the cytoplasm by serving as microRNA (miRNA/miR) sponges (16), and silence or activate target genes through modification at the promoter region (17,18). lncRNAs may directly interact with signaling pathways to serve as prognostic predictors or therapeutic targets for different types of cancer (19,20).
Furthermore, previous reports indicated that IncRNAs may participate in chemoresistance (21,22). However, their roles in the function of berberine are poorly understood. The identification of IncRNAs involved in the function of berberine may enhance human cancer treatment and aid to overcome chemoresistance.

In the current study, the regulatory function of IncRNA cancer susceptibility 2 (CASC2) in the tumor-suppressive role of berberine in CRC was investigated. The results revealed that berberine suppressed cell viability and promoted apoptosis of human CRC cancer cell lines in a concentration-dependent manner. Furthermore, berberine treatment increased the endogenous level of IncRNA CASC2, and this upregulation of IncRNA CASC2 promoted berberine-induced cell cytotoxicity by interacting with enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) and silencing the expression of BCL2.

Materials and methods

Ethics statement and tissue samples. The study included 60 patients with CRC [male/female ratio, 36/24; age range, 42-73 (median age, 56)] who underwent partial or total surgical resection at the West China Second University Hospital (Chengdu, China) between June 2014 and July 2016. Paired cancer tissues and normal control tissues (>2 cm distal from the cancer area) were collected by surgical resection. Tissue specimens were stored in liquid nitrogen during transportation. The current study was approved by the West China Second University Hospital. All participants signed informed consent prior to using the tissues for scientific research.

Cell culture. Five human CRC cell lines (HT-29, HCT116, SW480, SW620 and LoVo) and one human fetal normal colonic cell line (FHc) were purchased from the American Tissue Culture Collection. Human CRC cells were cultured in DMEM (BioWhittaker; Lonza Group, AG) containing 10% FBS (Thermo Fisher Scientific, Inc.) and 10% FBS (Thermo Fisher Scientific, Inc.) in a humidified incubator at 37˚C and 5% CO2 penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Human CRC cells were grown in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 5 µg/ml insulin, 100 ng/ml hydrocortisone and additional levels of detected RNAs were normalized to GAPDH using the comparative 2−ΔΔCt method (23).

Expression profile analysis of IncRNAs. Total RNA was extracted from HT29 cells treated with berberine (50 µM for 48 h) or normal culture medium using the RNeasy Plus Mini kit (Qiagen, Inc.) according to the manufacturer's protocol. The 20 µl RT reactions were performed using a PrimeScript® RT reagent kit (Takara Biotechnology co., ltd., dalian, China) and incubated for 30 min at 37˚C and 5 sec at 85˚C. Subsequently, 2 µl of diluted RT product was mixed with 23 µl reaction buffer (Takara Inc.) and incubated for 30 min at 37˚C and 5 sec at 85˚C. The primer sequences are presented in Table i. All reactions were performed using the Thermal Cycler (Takara Biotechnology co., ltd., dalian, China) and incubated for 45 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The expression levels of detected RNAs were normalized to GAPDH using the comparative 2−ΔΔCt method (23).

Cell viability assay. The cell viability following treatment with berberine was assayed using the MTT assay (Dojindo Molecular Technologies, Inc.). In brief, 3×104 cells were seeded into a 96-well plate and treated with 0-100 µM berberine (cat. no. B3251; Sigma-Aldrich; Merck KGaA) or PBS control for 12-72 h. The cells were treated with the 10 µl MTT reagent and incubated for an additional 2 h at room temperature. Purple formazan was then dissolved by adding 150 µl DMSO (Sigma-Aldrich; Merck KGaA) to each well. The optical density at a wavelength of 450 nm was subsequently measured with a spectrophotometer (Thermo Fisher Scientific, Inc.).

Apoptosis assay. Cells in 6-well plate(1-5×10⁶/ml) were collected following berberine treatment (50 µM) or transient transfection for 48 h, and washed with PBS and 0.025% trypsin-EDTA...
to obtain single-cell suspensions. Cells were fixed in ice-cold 70% ethanol (60 min at 4°C) and stained with an Annexin V FITC and propidium iodide solution kit (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature. Apoptosis was detected using a flow cytometer (BD FACSCalibur™; BD Biosciences) and analysed using Modfit LT version 5.0 software (Verity Software House Inc.).

Cell migration and invasion assay. Cell migration was evaluated by performing a wound-healing assay. The cells were seeded at a density of 5x10^5 cell/well into six-well plates. After 12 h of treatment with 50 µM berberine and/or transfection with si-cASC2, the layer of cells was scratched using a sterile 20 µl pipette tip to form wounds. The non-adherent cells were washed away with culture medium, and the cells were cultured for 48 h followed by measurement of the gap area. Cell invasive ability was evaluated using a Transwell invasion assay using Boyden chambers (8 µm pore size; BD Biosciences). The membranes were coated with Matrigel. Cells in serum-free media were placed into the upper chamber. Media containing 10% FBS was added to the lower chamber. Following 24 h of incubation at 37°C, the cells that had invaded through the membrane were fixed with 100% methanol for 20 min at room temperature and stained with 0.1% crystal violet for 10 min at room temperature and 3 random fields were imaged using an inverted microscope at 10x magnification (leica Microsystems, Inc., Buffalo Grove, IL, USA).

RNA immunoprecipitation (RIP). HT29 and HCT116 cells were rinsed with cold PBS and fixed using 1% formaldehyde for 10 min. Following 10 min centrifugation at 1,5000 x g and 4°C, cell pellets were collected and resuspended in NP-40 lysis buffer (Beyotime institute of Biotechnology). The RIP assay was performed using the Magna RIP™ rna-Binding Protein Immunoprecipitation kit (EMD Millipore) according to the manufacturer's protocol. Total RNA was immunoprecipitated overnight at 4°C with an antibody against EZH2 (1:50, cat. no. ab186006; abcam) or negative control IgG (1:50, cat. no. 12-370; EMD Millipore). Two sites in the GaPdH promoter region were used as a control for the identification of interactions between GaPdH and Bcl2. Precipitated DNA was recovered and analyzed by RT-qPCR.

Chromatin immunoprecipitation (ChIP). An EZ-Magna ChIP kit (EMD Millipore) was used according to the manufacturer's protocol. Briefly, HT29 cells (1x10^6/ml) were treated with 4% paraformaldehyde and incubated for 10 min at 4°C to generate DNA-protein cross-links. Cell lysates were then sonicated for with a 10 sec on and 10 sec off mode for 12 cycles to generate chromatin fragments of 200-300 bp and immunoprecipitated overnight at 4°C with an antibody against EZH2 (1:50, cat. no. ab186006; Abcam) or negative control IgG (1:50, cat. no. 12-370, EMD Millipore). The magnetic bead bound complexes were immobilized with a magnet and unbound materials were washed off. RNA was extracted and analyzed by RT-qPCR.

Western blot and antibodies. RIPA assay buffer (Sigma Aldrich; Merck KGaA) was used to extract proteins from

### Table I. Primer and siRNA sequences.

#### A. RT-qPCR primers

| Name | Sequence (5'-3') |
|------|------------------|
| CASC2 (Forward) | GCCACTTTGACCATCTCCACATCA |
| CASC2 (Reverse) | GCAGGAGCGCACATCA |
| IncRNA control (Forward) | GCCAGGACATGCTAGGCTG |
| IncRNA control (Reverse) | GCCAGGACATGCTAGGCTG |
| β-actin (Forward) | ATCGTCCCAGGAGATTCAG |
| β-actin (Reverse) | ATCGTCCCAGGAGATTCAG |

#### B. RIP-qPCR primers

| Name | Sequence (5'-3') |
|------|------------------|
| BCL2-A (Forward) | GAGGAGGCACATCGCAGATCC |
| BCL2-A (Reverse) | GAGGAGGCACATCGCAGATCC |
| BCL2-B (Forward) | AGTCCCAACAACAGATAGGG |
| BCL2-B (Reverse) | AGTCCCAACAACAGATAGGG |
| GAPDH-D (Forward) | ATCGTCCCAGGAGATTCAG |
| GAPDH-D (Reverse) | ATCGTCCCAGGAGATTCAG |
| GAPDH-E (Forward) | ATCGTCCCAGGAGATTCAG |
| GAPDH-E (Reverse) | ATCGTCCCAGGAGATTCAG |

#### C. ChIP-qPCR primers

| Name | Sequence (5'-3') |
|------|------------------|
| BCL2-A (Forward) | GAGGAGGCACATCGCAGATCC |
| BCL2-A (Reverse) | GAGGAGGCACATCGCAGATCC |
| BCL2-B (Forward) | AGTCCCAACAACAGATAGGG |
| BCL2-B (Reverse) | AGTCCCAACAACAGATAGGG |
| GAPDH-D (Forward) | ATCGTCCCAGGAGATTCAG |
| GAPDH-D (Reverse) | ATCGTCCCAGGAGATTCAG |
| GAPDH-E (Forward) | ATCGTCCCAGGAGATTCAG |
| GAPDH-E (Reverse) | ATCGTCCCAGGAGATTCAG |

#### D. siRNA

| Name | Sequence (5'-3') |
|------|------------------|
| si-CASC2-1 | UGAAAAGAGCCGUGAGCUA |
| si-CASC2-2 | AAATAAAGATGTTGGGAATG |
| si-CASC2-3 | CUGCAAGGGCCGCAUGAUAGA |
| si-EZH2 | AUCAGGCUCUGUCAGAACUCUU |
| si-NC (GFP) | GCCUACGAGGAGGCGCCACC |

siRNA, small interfering RNA; RT, reverse transcription; qPCR, quantitative PCR; CASC2, cancer susceptibility 2; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; RIP, RNA immunoprecipitation; ChIP, chromatin immunoprecipitation; NC, negative control.
the cells. Protein concentration was measured using a bicinchoninic acid assay (Sigma Aldrich; Merck KGaA). The same quantity of protein (25 µg) was transferred onto PVDF membranes following the separation process by performing a routine SDS-PAGE with 10% gel. The membrane was blocked with 5% (5 g/100 ml) nonfat dry milk in TBS plus Tween buffer for 2 h at room temperature. The membrane was incubated with primary antibodies against EZH2 (1:1,000; cat. no. ab186006; Abcam), BCL2 (1:1,000; cat. no. ab32124; Abcam) and β-actin (1:1,000; cat. no. ab8227; Abcam) overnight at 4˚C. Following primary incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no. 7074; cell Signaling Technology, Inc.) for 1 h at room temperature. Protein bands were detected using ECL reagent (Amersham; GE Healthcare). Gray analysis by image analysis was performed using the software Gel-Pro Analyzer (version 4.0; United States Biochemical) after scanning.

Statistical analysis. Data are presented as the median and interquartile range. The Mann-Whitney U test was used for the comparison of datasets containing two independent groups, and the Wilcoxon signed-rank test was used for comparison of paired samples. The Kruskal-Wallis test followed by the Bonferroni post hoc analysis was used for evaluating the differences among multiple groups. The Spearman's test was used to analyze the correlation between CASC2 and BCL2 mRNA expression. Statistical analysis was performed using GraphPad Prism (version 6; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Berberine suppresses cell viability and promotes apoptosis in CRC cells. The inhibitory effects of berberine on the cell viability of CRC cell lines was investigated. The MTT assay indicated that berberine decreased the *in vitro* cell viability of two CRC cell lines in a dose-dependent manner, with a half maximal inhibitory concentration of 43.77 µM for HT29 and 56.44 µM for HCT116 at 48 h post-treatment (Fig. 1A). Flow cytometry analysis was subsequently used to detect cell apoptosis. The percentage of apoptotic cells was significantly increased following treatment with 50 µM berberine for 48 h compared with controls (Fig. 1B). To investigate whether the increased cell apoptosis was due to activation of apoptotic signaling pathways, the expression levels of several apoptotic proteins were detected. Western blotting revealed that the expression levels of cleaved caspase-3 and -9 were upregulated by the treatment of berberine at 50 µM for 48 h (Fig. 1C). The effect of berberine on migration and invasion capacity of HT29 cells were assessed by performing scratch and Transwell assays, respectively. The results revealed that berberine treatment did not have a significant effect on cell migration and invasion compared with controls (Fig. 1D).

Expression level of lncRNA CASC2 in CRC cell lines is increased following berberine treatment. The aforementioned dysregulated lncRNA(s) were validated using qPCR. Compared with the RNA sequencing data, only lncRNA CASC2 showed a statistically significant increased expression level in HT29 cells following berberine treatment compared with controls. Expression of the other five lncRNAs showed little difference between the two group of cells when analyzed by RT-qPCR (Fig. 3A-F). Therefore, the functional role of CASC2 was
investigated further. Previous reports indicated that lncRNA CASC2 may be involved in cell apoptosis, proliferation and cell cycle regulation in cancer (25,26). Therefore, berberine may decrease CRC cell viability and promote apoptosis through the upregulation of lncRNA CASC2. To investigate this hypothesis, the expression level of lncRNA CASC2 in CRC cell lines was determined. The results revealed that the expression level of lncRNA CASC2 was decreased in the five CRC cell lines used in the present study compared with the normal colon epithelial cell line FHC (Fig. 3G). In addition, CASC2 was downregulated in primary CRC tissues when compared with non-tumor tissues (Fig. 3H). Furthermore, lncRNA CASC2 was upregulated in HT29 cells following treatment with berberine for 48 h in a dose-dependent manner (Fig. 3I).

BCL2 is required for the functional effect induced by berberine and lncRNA CASC2. As the berberine/CASC2 signaling pathway affected apoptosis in CRC cell lines, the expression of apoptosis-targeted genes was investigated. Significant differences in several apoptosis-related targets regulated by lncRNA CASC2, including BCL2, which is a well-known antiapoptotic protein (27,28), were identified (Table III). Thus, lncRNA CASC2 may promote apoptosis via targeting the anti-apoptotic gene, BCL2. The expression level of BCL2 in CRC tissue samples was determined using RT-qPCR. A significant negative correlation between the expression of BCL2 and lncRNA CASC2 was identified (Fig. 5A). Moreover, overexpression of CASC2 decreased the expression of BCL2 at the transcript and protein levels, as demonstrated by RT-qPCR and western blot analysis (Fig. 5B). BCL2 mRNA and protein levels were decreased in HT29 cells treated with berberine in a dose-dependent manner (Fig. 5C). However, transfection with si-CASC-1 reversed the berberine-induced decrease of BCL2
Figure 2. High-throughput IncRNA sequencing identified 64 IncRNAs with a >2-fold difference between HT29 cells treated with berberine-containing medium and cells cultured in berberine-free medium were identified. R package was used to establish a heat map. B, berberine (50 µM)-treated HT29 cells; C, control HT29 cells.
mRNA and protein levels (Fig. 5D), suggesting that berberine exerted its proapoptotic effect via a CASC2-BCL2 signaling mechanism.

**EZH2 associates with IncRNA CASC2, which subsequently suppresses BCL2 expression.** A RIP assay was performed to identify whether there was a direct interaction between IncRNA CASC2 and EZH2. CASC2 was pulled down by EZH2 while no enrichment was identified when using an IgG antibody as a control (Fig. 6A). Moreover, no enrichment was identified when using the EZH2 antibody to pull down β-actin or IncRNA control, suggesting that the association between EZH2 and CASC2 was specific (Fig. 6B). Furthermore, the role of EZH2 in the regulation of BCL2 expression was evaluated. si-EZH2 was used to silence EZH2 in HT29 and HCT116 cells (Fig. 6C). Knockdown of EZH2 in HT29 and HCT116 cells upregulated the expression level of BCL2 (Fig. 6D and E). A ChIP assay was performed to detect the effect of IncRNA CASC2 on histone modification in the BCL2 gene promoter in HT29 cells. A set of detection sites were used to design specific primers for the amplification of BCL2 promoter sequences pulled down by anti-EZH2 or anti-H3K27me3 antibodies (Fig. 6F). Overexpression of CASC2 significantly increased the enrichment of BCL2 (detection site A, B and C) when using the anti-EZH2 and anti-H3K27me3 antibodies, while no significant change in the enrichment level was identified for GAPDH (detection site D and E; Fig. 6G and H). In addition, IncRNA CASC2 had little effect on the enrichment of BCL2 when using the anti-IgG antibody (Fig. 6I). To conclude, the current study revealed that IncRNA CASC2 downregulated
Table III. Long non-coding RNA CASC2 regulated target genes associated with apoptosis.

| Gene symbol | Gene name                          | Location     | Fold change (p-CASC2/negative control, all P<0.005) |
|-------------|------------------------------------|--------------|-----------------------------------------------------|
| BCL2        | BCL2 apoptosis regulator           | Chr18q21.33  | 0.008                                               |
| BCL2A1      | BCL2 related protein A1            | Chr15q25.1   | 0.06                                                |
| PARP2       | Poly (ADP-ribose) polymerase 2     | Chr14q11.2   | 0.19                                                |
| BIRC3       | Baculoviral IAP repeat containing 3| Chr11q22.2   | 0.52                                                |
| BAX         | BCL2-associated X, apoptosis regulator | Chr19q13.33 | 25.39                                               |
| MCL1        | MCL1 apoptosis regulator, BCL2 family member | Chr1q21.2 | 19.31                                               |
| BAK1        | BCL2 antagonist/killer 1           | Chr6p21.31   | 10.48                                               |
| CASP9       | Caspase 9                          | Chr1p36.21   | 9.56                                                |
| CASP3       | Caspase 3                          | Chr4p35.1    | 7.38                                                |

CASC2, cancer susceptibility 2.

Figure 4. Berberine inhibited cell growth and increased apoptosis by increasing the expression level of CASC2. (A) The expression level of CASC2 was significantly increased in HT29 and HCT116 cells transfected with the p-CASC2 vector. ***P<0.001 vs. p-Vector group. (B) MTT assay was performed to investigate the effects of CASC2 on cell viability. The cell viability was significantly decreased in cells transfected with the p-CASC2 vector. *P<0.05 vs. p-Vector group. (C) Apoptosis was significantly increased in HT29 and HCT116 cells overexpressing CASC2. *P<0.05 vs. p-Vector group. (D) Western blotting revealed that the overexpression of CASC2 increased the expression level of cleaved caspase-3 and -9. (E) Three siRNAs against CASC2 were generated and validated by performing reverse transcription-quantitative PCR. *P<0.05, ***P<0.001 vs. si-NC group. (F) Berberine treatment (50 µM, 48 h) significantly inhibited the viability of HT29 and HCT116 cells; however, this was partly nullified by knockdown of CASC2. *P<0.05, as indicated. (G) Knockdown of CASC2 partially reversed the inhibition of cleaved caspase-3 and -9 in HT29 and HCT116 cells caused by berberine treatment. CASC2, cancer susceptibility 2; siRNA, small interfering RNA; NC, negative control.
Figure 5. BCL2 was identified as a direct target of berberine and CASC2 in CRC. (A) The expression levels of long non-coding RNA CASC2 and BCL2 transcripts in primary CRC tissues were determined via reverse transcription-quantitative PCR and the correlation between their expression was determined using Spearman's correlation test. (B) Enhanced expression of CASC2 increased BCL2 expression at the mRNA (left panel) and protein (right panel) levels. *P<0.05 compared with the p-vector group. (C) BCL2 mRNA and protein was decreased in HT29 treated with berberine in a dose-dependent manner. *P<0.05 vs. respective control group. (D) Transfection with si-CASC-1 reversed the berberine-induced decrease of BCL2 at the mRNA (left panel) and protein levels (right panel). *P<0.05, as indicated. CASC2, cancer susceptibility 2; CRC, colorectal cancer; siRNA, small interfering RNA; NC, negative control.

Figure 6. lncRNA CASC2 decreased the expression level of BCL2 by binding with EZH2. (A) RIP experiments were performed to verify the direct interaction between EZH2 and lncRNA CASC2 by using an anti-EZH2 antibody. Primers used for amplifying CASC2 were generated to verify the enrichment of lncRNA CASC2 pulled down by the anti-EZH2 antibody. ***P<0.001 vs. control IgG group. (B) lncRNA control or β-actin was used as a negative control for CASC2. A RIP assay was performed using EZH2 antibody. **P<0.001 vs. total RNA group. (C) The silencing effect of siRNA against EZH2 was determined via quantitative PCR. **P<0.01 vs. si-NC group. BCL2 (D) mRNA and (E) protein levels were measured in cells with EZH2 knockdown. *P<0.05 vs. si-NC group. (F) Chromatin immunoprecipitation analysis of HT29 cells overexpressing CASC2 was performed with generated primers to detect the amplification at the Bcl2 promoter (detection sites A, B and C) and GAPDH promoter (detection sites D and E) regions. Measurement of BCL2 sequences with (G) EZH2, (H) H3K27m3 and (I) IgG is presented as relative to total input. *P<0.05 compared with the p-Vector group, respectively. lncRNA, long non-coding RNA; CASC2, CASC2, cancer susceptibility 2; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; RIP, RNA immunoprecipitation; siRNA, small interfering RNA; NC, negative control; H3K27m3, H3 lysine 27 trimethylation.
BCL2 expression level in CRC by binding with the promoter region of BCL2 in an EZH2-dependent manner.

Discussion

Patients with advanced colon cancer that develop resistance to chemotherapy currently have limited therapeutic options in the clinic; therefore, many patients turn to alternative treatments (29). In recent years, the interest in herbal remedies has grown rapidly in the industrialized world, as these drugs are increasingly considered to be effective and safe alternatives to synthetic drugs. The present study focused on berberine, one of the few well-established plant products supported by clinical trials (30,31), which is the standardized extract from Coptis chinensis. The results obtained in the current study indicated that berberine inhibited the viability and promoted apoptosis of CRC cell lines. High throughput RNA sequencing identified lncRNA CASC2 as a potential functional target of berberine, and that CASC2 is involved in the chemopreventive functions of berberine. The antiapoptotic protein Bcl2 was investigated and it was revealed that IncRNA CASC2 exerted its effect by suppressing BCL2 expression via EZH2.

Alkaloids are used in traditional medicine for the treatment of many diseases. These compounds are synthesized in plants as secondary metabolites and have several effects on cellular metabolism (32). The medicinal alkaloid isolated from Coptis chinensis, berberine, has attracted attention in the scientific community (33). For example, Piyanuch et al (34) demonstrated that berberine upregulated the expression levels of growth differentiation factor 15 and activating transcription factor 3 in colorectal cancer. Liu et al (35) suggested that berberine may exert antitumor effects in CRC by regulating miR-429. Chuang et al (36) indicated an anticancer role of berberine via the suppression of cell viability of hepatocellular cancer cells and the upregulation of the protein expression of multiple tumor suppressor genes. The role of berberine in CRC was further explored by Liu et al (14), which revealed that berberine inhibited the invasion and metastasis of CRC cells via the prostaglandin-endoperoxide synthase 2/prostaglandin E2 mediated Janus kinase 2/STAT3 signaling pathway. However, the mechanism underlying the cytotoxic effect of berberine and whether one or a cluster of lncRNAs are involved in this process remains unknown. The present study suggested that berberine suppresses the viability of CRC cell lines and promotes cell apoptosis in a dose-dependent manner. Moreover, RNA sequencing indicated that a number of lncRNAs may be important regulators of the berberine-dependent pathway.

During the past years, miRNAs and lncRNAs have been investigated as potential diagnostic predictors or therapeutic targets for a number of different diseases (37). lncRNAs may have novel regulatory roles in cancer and may serve as potential prognostic and therapeutic targets in clinical practice (38). However, the specific functional association between IncRNAs and berberine in cancer is not well known. Therefore, the current study sought to identify and validate specific IncRNAs, which may be important for the antitumor effects exhibited by berberine. By combining RNA sequencing array with RT-qPCR validation, IncRNA CASC2 was identified as an IncRNA that was regulated by berberine treatment. Furthermore, the gain- and loss-of-function assays revealed that berberine exerted its anticancer effect by upregulating lncRNA CASC2 expression.

lncRNA CASC2, a novel human IncRNA mapping to 10q26 in humans, has been originally characterized as a downregulated gene acting as a tumor suppressor gene in endometrial cancer (39), and recently in other cancers (25,40,41). Exogenous expression of CASC2 significantly inhibited the growth of undifferentiated endometrial cancer cells (39). Fan et al (42) revealed that CASC2 promoted the apoptosis of hepatocellular carcinoma cells by targeting miR-24. Ba et al (43) reported that the decreased expression of IncRNA CASC2 facilitated osteosarcoma growth and invasion by regulating miR-181a. Huang et al (44) demonstrated that IncRNA CASC2 inhibited CRC cell proliferation and tumor growth by sponging miR-18a and silencing the STAT3 gene. The aforementioned studies demonstrated a decreased expression of CASC2 in cancer tissues compared with normal tissues. The results obtained in the current study revealed that CASC2 was downregulated in CRC cells compared with normal epithelial cells, consistent with the aforementioned studies. Furthermore, the current study investigated the regulatory mechanism that may account for the role of CASC2 following treatment with berberine. The antiapoptotic gene, BCL2, was identified as a functional target of CASC2. Gain- and loss-of-function experiments revealed that berberine exerted its proapoptotic effect via a CASC2-BCL2 signaling mechanism.

Antiapoptosis is established as an important factor in the development of drug resistance, and disruptions of the apoptotic pathway have been demonstrated to suppress cell cytotoxicity and promote drug resistance (45). BCL2 was identified as the most dysregulated protein following silencing of IncRNA CASC2 in the current study. BCL-2 family proteins, which include BCL-2, BCL-XL, BCL-W, MCL1 apoptosis regulator BCL2 family member and BCL2 related protein A1, share structural homology in the BCL2 homology 1, 2, 3 and 4 domains. BCL2 is an important cell death regulator, and is involved in the control of the release of cytochrome c from mitochondria in the intrinsic apoptotic pathway (46-48). Thus, the current study investigated whether IncRNA CASC2 promoted apoptosis via antiapoptotic gene, BCL2. Currently, the specific mechanism by which IncRNA CASC2 regulates BCL2 remains unclear. Consequently, the current study focused on EZH2, which is frequently associated with other IncRNAs and affects cancer progression by altering H3K27 trimethylation and silencing transcription. IncRNAs may exert their function by recruiting RNA-binding portions, including EZH2, thus leading to gene methylation and chromatin modifications. EZH2 is often overexpressed in different types of human cancer and may promote cell proliferation, invasion and tumor angiogenesis. The RIP experiment in the current study revealed that IncRNA CASC2 was physically associated with EZH2 in CRC cells. It is known that EZH2 enhances methylation of H3K27, leading to gene silencing involved in cancer progression and cell apoptosis (49). This accords with the ChIP results of the present study, which indicated that overexpression of CASC2 increased binding of EZH2 at the promoter region of the BCL2 gene, causing elevated H3K27me3 formation, and potentially inhibiting the antiapoptotic effect of BCL2.
The present study had limitations. The cytotoxic effect of berberine requires further validation in in vivo models. Experimental conditions for the treatment of CRC tumors with berberine in mice, including the concentration, duration and administration route of berberine are to be determined. The diagnostic and prognostic functions of CaSC2 expression in patients with CRC receiving berberine treatment remain unclear. Future experiments focused on the clinical importance of CaSC2 in berberine treatment are required.

In conclusion, the current study revealed that berberine decreases cell viability and promote apoptosis of CRC cell lines in vitro. Moreover, mechanistic research suggested that berberine exerted its anticancer effect in CRC by increasing the expression level of lncRNA CaSC2 and suppressing BCL2 in an EZH2-dependent manner. Thus, berberine may be a potential alternative treatment drug for patients with CRC, and lncRNA CaSC2 may serve as an important therapeutic target to improve the anticancer effect of berberine.

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Availability of data and materials

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

Authors' contributions

WD and LM acquired the data, and created a draft of the manuscript; YC and YL collected clinical samples and performed the experimental assays; PC, HX and XW analyzed and interpreted the data, and performed statistical analysis; WD, LM, YC and HX reviewed the manuscript, and produced the figures and tables. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Clinical Research Ethics Committee of the West China Second University Hospital. All participants signed informed consent prior to using the tissues for scientific research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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