Huaier promotes sensitivity of colorectal cancer to oxaliplatin by inhibiting METTL3 to regulate the Wnt/β-catenin signaling pathway

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Abstract. Colorectal cancer (CRC) ranks fifth in terms of incidence rate and mortality among malignant tumors in China. Oxaliplatin (OXA) is a first-line drug for the clinical treatment of CRC, but its antitumor effect is limited because of the development of drug resistance. The present study aimed to investigate whether the traditional Chinese medicine Huaier can regulate the Wnt/β-catenin signaling pathway by affecting the expression of METTL3, thereby promoting the sensitivity of HCT-8/L cells to OXA. The expression of METTL3 was analyzed based on the UCSC Xena and Gene Expression Omnibus databases. Silent METTL3 and overexpression METTL3 models were constructed, and Cell Counting Kit-8 and flow cytometry were used to detect the effects of Huaier on the viability and apoptosis of HCT-8/L cells. Western blotting, reverse transcription-quantitative PCR, nuclear cytoplasmic separation and immunofluorescence were used to detect the effects of Huaier on the expression of METTL3, Pgp, Wnt/β-catenin signaling pathway-related proteins, apoptosis-related proteins and related mRNA. The results demonstrated that patients with high expression levels of METTL3 had a shorter overall survival period. The expression level of METTL3 significantly increased in drug-resistant CRC cells. Silencing METTL3 promoted apoptosis of CRC cells and increased their sensitivity to OXA by inhibiting the Wnt/β-catenin signaling pathway. Huaier downregulated the expression of METTL3, thereby promoting apoptosis of drug-resistant CRC cells and increasing their sensitivity to OXA by inhibiting the Wnt/β-catenin signaling pathway.

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths, and its incidence (~9.39%) and mortality rates (~10.01%) in China have also been increasing (1,2). Most patients with CRC are already in the middle or late stage when diagnosed (3). The commonly used treatment methods for CRC include surgery, chemotherapy and targeted therapy (4). Chemotherapy is the main treatment method, and the commonly used chemotherapy drugs in clinical practice include oxaliplatin (OXA), 5-fluorouracil (5-FU), capecitabine and calcium folinate (5). Although most patients can experience favorable therapeutic effects through chemotherapy drugs, some patients succumb because of the development of drug resistance and ineffective chemotherapy within a few cycles (6,7). Targeted therapy works by blocking specific molecules involved in the growth and spread of cancer cells (8). The main types of targeted drugs are monoclonal antibodies and small molecule inhibitors, which target molecules involved in tumorigenesis and their related signaling pathways in cancer cells, inhibiting cancer development (9). In addition, a recently added treatment method in the CRC treatment regimen is immune checkpoint inhibitors. In the treatment of metastatic CRC, this treatment approach has exhibited promising clinical responses in patients with high microsatellite instability in mismatch repair genes (10).

Tumor drug-resistance mostly occurs after receiving initial chemotherapy. The MDR1 gene encodes the multidrug resistance-associated protein ABCB1, also known as P-gp, which is a member of the ABC binding cassette transporter superfamily and plays an important role in tumor resistance (11,12). OXA is a third-generation platinum-based antitumor drug, and its antitumor mechanism involves entering tumor cells, where it causes cell cycle arrest and induces cell apoptosis (13). P-gp can pump antitumor drugs that enter the cell out of the cell, thereby playing an important role in the formation of chemotherapy resistance (14).

Methyltransferase 3 (METTL3) is a key N6 methyladenosine (m6A) methyltransferase that plays a major catalytic role in m6A modification (15). It plays an important role in regulating biological processes such as cell cycle, proliferation, apoptosis, differentiation, invasion and migration, and

inflammatory response (16). METTL3 also has an antagonistic effect on 5-FU, leading to the development of drug resistance (15). This is consistent with our previous experimental results. METTL3 expression is increased in patients resistant to OXA chemotherapy. Concurrently, it was found that Huaier can inhibit the expression of METTL3. Therefore, it was decided to focus on METTL3 to explore the mechanism by which Huaier affects chemotherapy resistance in CRC.

Although there has been significant progress in the diagnosis and treatment of CRC, issues such as postoperative sequelae, chemotherapy resistance, toxic side effects, high rate of metastasis, and recurrence rates have critically affected the quality of life of patients (17,18). An increasing number of studies have revealed that traditional Chinese medicine has favorable therapeutic effects in treating cancer. Traditional Chinese medicine can exert anti-CRC effects on multiple targets and pathways, while improving the toxic side effects caused by surgical chemotherapy, radiotherapy, targeted therapy and immunotherapy, and prolonging the survival time of patients (19). Previously, research has found that Huaier has anticancer effects on various types of tumors (20-23). Huaier is a fungus that grows on various trees such as Huai Shu and Qing Tan. It contains various organic components and minerals, and has a history of over 1,600 years as a traditional Chinese medicine (24). The main component of Huaier is polysaccharide protein, which achieves antitumor effects by affecting tumor cell proliferation, apoptosis, drug sensitivity, autophagy and other aspects (25-29). It has been revealed that Huaier can inhibit the proliferation of CRC tumor stem cells by downregulating the Wnt/β-catenin signaling pathway (30). However, it is currently unclear whether Huaier can regulate the Wnt/β-catenin signaling pathway to reduce P-gp expression and thereby decrease resistance to OXA-based chemotherapy regimens in CRC through METTL3.

In the present study, the mechanism by which Huaier regulates drug resistance in CRC was further investigated. It was demonstrated that Huaier inhibited the Wnt/β-catenin signaling pathway by downregulating the expression of METTL3, thereby increasing the sensitivity of CRC to OXA. This provides a theoretical basis for the treatment of resistance to chemotherapy in CRC by utilizing the traditional Chinese medicine Huaier.

Materials and methods

Public data. The Cancer Genome Atlas (TCGA) queue data were accessed in the UCSC Xena database (https://xenabrowser.net/datapages/). The GSE28702 and GSE17536 datasets were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The GSE28702 dataset contains tumor-tissue RNA expression profile data from 42 patients with CRC who responded to the FOLFOX chemotherapy regimen and 41 patients with CRC who did not respond to this regimen. The GSE17536 dataset contains complete survival information for 177 patients with CRC.

Chemicals. Huaier was purchased from Qidong Gaitianli Pharmaceutical Co., Ltd. Huaier particles (2 grams) were dissolved in 20 ml of PRMI-1640 medium (Procell Life Science & Technology Co., Ltd.), filtered with a 0.22-μm filter (MilliporeSigma) to obtain 100 mg/ml of the original solution, and then stored at -40˚C. OXA was purchased from MedChemExpress. Wnt agonist 1 (AMBMP) was purchased from Selleck Chemicals. AMBMP is a cell permeable Wnt signaling pathway activator that induces transcriptional activity dependent on β-catenin and TCF. It stabilizes the intracellular β-catenin by disrupting the Axin/β-catenin interaction, thereby activating the Wnt signaling pathway.

Cell lines and cell culture. NCM460 cells (cat. no. JNO-H0138) were purchased from Guangzhou Jennio Biotech Co., Ltd. HCT-8 cells (cat. no. PC193) were purchased from Procell Life Science & Technology Co., Ltd. The resistant cells of HCT-8/L-OHP (HCT-8/L) were manufactured by Shanghai Aolu Biotechnology. Both cell lines were cultured in PRMI-1640 medium containing 10% fetal bovine serum (Procell Life Science & Technology Co., Ltd.) and placed at 37˚C in a 5% CO_2 incubator.

Cell transfection. Targeted METTL3 [small interfering (si)-METTL3#1 and si-METTL3#2] and negative control siRNA oligonucleotides (si-NC) were designed and synthesized by Guangzhou RiboBio Co., Ltd. The sequences were as follows: si-NC, 5'-UUCUCCGAACGUGUCACG U-3'; si-METTL3#1, 5'-CAAGUAUGUUCACUAUGAA-3'; and si-METTL3#2, 5'-GACUGCUCUUUCCUUAAUA-3'. Overexpression of METTL3 was accomplished using the expression plasmid PCMV3 synthesized by SinoBiological, with empty vector as negative control. HCT-8/L cells were inoculated into 6-well plates. After the cells adhered to the wall, they were transfected with si-NC, si-METTL3#1, si-METTL3#2, pC-NC, or pC-METTL3 (2.5 μg per well) using Lipofiter 3.0 (HanBio Biotechnology Co., Ltd.) transfection reagent at 37˚C, following the manufacturer's protocol. After 48 h, reverse transcription-quantitative PCR (RT-qPCR) and western blotting were used to detect the knockdown and overexpression efficiency.

Cell viability assay [Cell counting kit-8 (CCK-8) assay]. Cell viability was detected using CCK-8 (APeXBIO Technology LLC). In a 96-well plate, ~3,000 cells were inoculated into each well. After overnight cultivation at 37˚C and 5% CO_2, the cells were treated with different concentrations (0, 5, 10, 20, 30 and 40 μmol/l) of OXA for 24 and 48 h. Then, a mixed solution of CCK-8 (10 μl CCK-8 and 100 μl PRMI-1640 medium per well) was added and incubated in a dark incubator for 2 h at 37˚C and 5% CO_2. Finally, the absorbance was measured at 450 nm using a microplate reader. In addition, HCT-8/L cells were treated with Huaier at the following concentrations: 0, 6, 9, 12, 15 and 18 (mg/ml). All measurements were repeated three times. Cell viability was calculated as follows: [As Ab)/(Ac Ab)] x100%, where As is the absorbance of the experimental group; Ac is the absorbance of the control group; and Ab represents the absorbance of the blank group.

Immunofluorescence. Cells (~50-60% confluency) were cultivated in a 24-well plate at 37˚C and 5% CO_2. Each well contained sterile treated cover slips, allowing the cells to adhere to the cover slips overnight. The cells on the cover glass were washed for three times with phosphate-buffered saline

(PBS). Then, they were fixed with 4% paraformaldehyde at room temperature for 15 min and washed again with PBS. After penetrating with 0.1% TritonX-100 (Beijing Solarbio Science & Technology Co., Ltd.) on ice for 15 min, the cells were blocked at room temperature for 2 h with 75% bovine serum albumin (BSA; Beijing Solarbio Science & Technology Co., Ltd.), and then incubated with anti-β-catenin antibodies (1:2,000; cat. no. 8814s; Cell Signaling Technology, Inc.) at 4˚C overnight. Finally, after washing three times with PBS, the goat anti-rabbit IgG labeled with Alexa Fluor 488 (1:100; cat. no. AS053; ABclonal Biotech Co., Ltd.) was incubated at room temperature for 1 h. After 15 min of DAPI (2 μg/ml) staining at room temperature, the slides were placed under a fluorescence microscope (Olympus Corporation) for observation.

Western blotting. Total cell proteins were extracted using RIPA cell lysate (Report Biotech; http://www.ruipate.com/) containing 1% phenylmethylsulfonyl fluoride (Report Biotech) and placed on ice for 15 min. The BCA protein concentration assay kit (Shandong Sparkjade Scientific Instruments Co., Ltd.) was used to determine protein concentration, and the proteins were separated by 12% SDS-PAGE electrophoresis with a sample size of 30 μg per well. After SDS-PAGE electrophoresis, the protein sample was transferred to a PVDF membrane (MilliporeSigma) under constant pressure of 100 V. A total of 5% BSA was used to block the membrane at room temperature for 2 h. Then, an appropriate mass of primary antibodies against METTL3 (1:2,000; cat. no. A19079; ABclonal Biotech Co., Ltd.), P-gp (1:1,000; cat. no. 13342S; Cell Signaling Technology, Inc.), Wnt 3a (1:2,000; cat. no. bs-1700R; BIOSS), β-catenin (1:1,000; cat. no. 8814S; Cell Signaling Technology, Inc.), Bax (1:2,000; cat. no. A0207; ABclonal Biotech Co., Ltd.), Bcl-2 (1:2,000; cat. no. A0208; ABclonal Biotech Co., Ltd.), Caspase3 (1;2,000; cat. no. 19677-1-AP; Proteintech Group, Inc.), β-catenin (1:100,000; cat. no. AC026; ABclonal Biotech Co., Ltd.) and Histone H3 (1:2,000; cat. no. AF0863; Affinity Biosciences) was added and incubated at 4˚C overnight. Then, the HRP-conjugated goat anti-rabbit IgG (H + L) secondary antibody (1:10,000; cat. no. AS014; ABclonal Biotech Co., Ltd.) was incubated for 1 h at room temperature. Finally, the target protein was exposed and developed using an enhanced chemiluminescence (ECL) reagent (Biosharp Life Sciences) in a 1:1 ratio of solution A and solution B. Densitometric analysis was performed using Image Lab software (version 5.2.1; Bio-Rad Laboratories, Inc.).

RT-qPCR. Total RNA was extracted from the cells using TRIzol reagent (Biosharp Life Sciences), and cDNA was synthesized using SPARKscript II ALL-in-one RT SuperMix for qPCR (Shandong Sparkjade Scientific Instruments Co., Ltd.) according to the manufacturer's protocol. mRNA expression levels were detected using SYBR Green qPCR Mix (Shandong Sparkjade Scientific Instruments Co., Ltd.). The qPCR cycling conditions were as follows: Denaturation at 94˚C for 2 min, followed by 40 cycles of denaturation at 94˚C for 10 sec, primer annealing and extension at 60˚C for 30 sec. Each experiment was repeated at least three times, and the experimental data were analyzed using the $2^{-\Delta\Delta Cq}$ method (31). The specific primer sequences were as follows: METTL3 forward, 5'-GTGATCGTAGCTGAGGTTCGT-3' and reverse, 5'-GGGTTGCACATTGTGTGGTC-3'; P-gp forward, 5'-TCTATGGTTGGCAACTAACACT-3' and reverse, 5'-CTCCTGAGTCAAAGAAACAACG-3'; β-catenin forward, 5'-ATGGCTTGGAATGAGACTGCT-3' and reverse, 5'-GGGTCCATACCCAAGGCATC-3'; and GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'.

Flow cytometry. Detection of cell apoptosis was accomplished through flow cytometry. The cells (~90% confluency) were digested with trypsin, collected in centrifuge tubes, and washed twice with PBS. After centrifugation at 1,000 x g for 5 min at room temperature, the supernatant was discarded completely and staining was performed with the Annexin V-FITC/PI dual staining apoptosis detection kit (APeXBIO Technology LLC), avoiding light at room temperature until 5 min. Subsequently, apoptosis was analyzed using BD FACSCalibur flow cytometer (BD Biosciences) and modify software (BD Biosciences).

Nuclear cytoplasmic separation. Cytoplasmic proteins and nuclear proteins were separated using a nuclear protein and cytoplasmic protein preparation kit (Applygen Technologies, Inc.). After removing the cells from the incubator, they were washed 2-3 times with PBS and CEB-A reagent was added for scraping; the cells were transferred to a precooled centrifuge tube with a pipette, shook, and resuspended, and centrifuged at 12,000 x g at 4˚C for 5 min. The precipitate was retained and the supernatant was collected into a new centrifuge tube, which was the cytoplasmic protein component. Reagents CEB-A and CEB-B (Applygen Technologies, Inc.) were added to centrifuge tubes containing cell precipitates, shook and resuspended, and centrifuged at 1,000 x g at 4˚C for 5 min. All supernatants were discarded and the precipitate was retained. Then, the reagent NEB was added to the precipitate, placed it on ice for 30 min, centrifuged it at 12,000 x g at 4˚C for 5 min, and finally the supernatant was collected, which was now the nuclear protein component.

Statistical analysis. All results were based on at least three independent experiments. The results were expressed as mean ± standard deviation (SD). Unpaired Student's t-test, Welch's t-test, one-way ANOVA (followed by Bonferroni post hoc test) and Mann-Whitney U test were used to test the significance of differences. Survival analysis was conducted using the Kaplan-Meier method followed by the log-rank test. Data analysis was conducted using SPSS (version 20; IBM Corp.) and GraphPad Prism 8 (Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

METTL3 expression increases in CRC and is associated with drug resistance and poor prognosis. By downloading the TCGA queue data from the UCSC Xena database, it was found that the expression level of METTL3 in CRC tissue significantly increased, compared with that in normal tissue (Fig. 1A). Meanwhile, western blot analysis revealed a significant increase in the expression level of METTL3 in HCT-8 cells (Fig. 1B). Furthermore, it was found in the GSE17536 dataset that patients with high METTL3 expression levels had

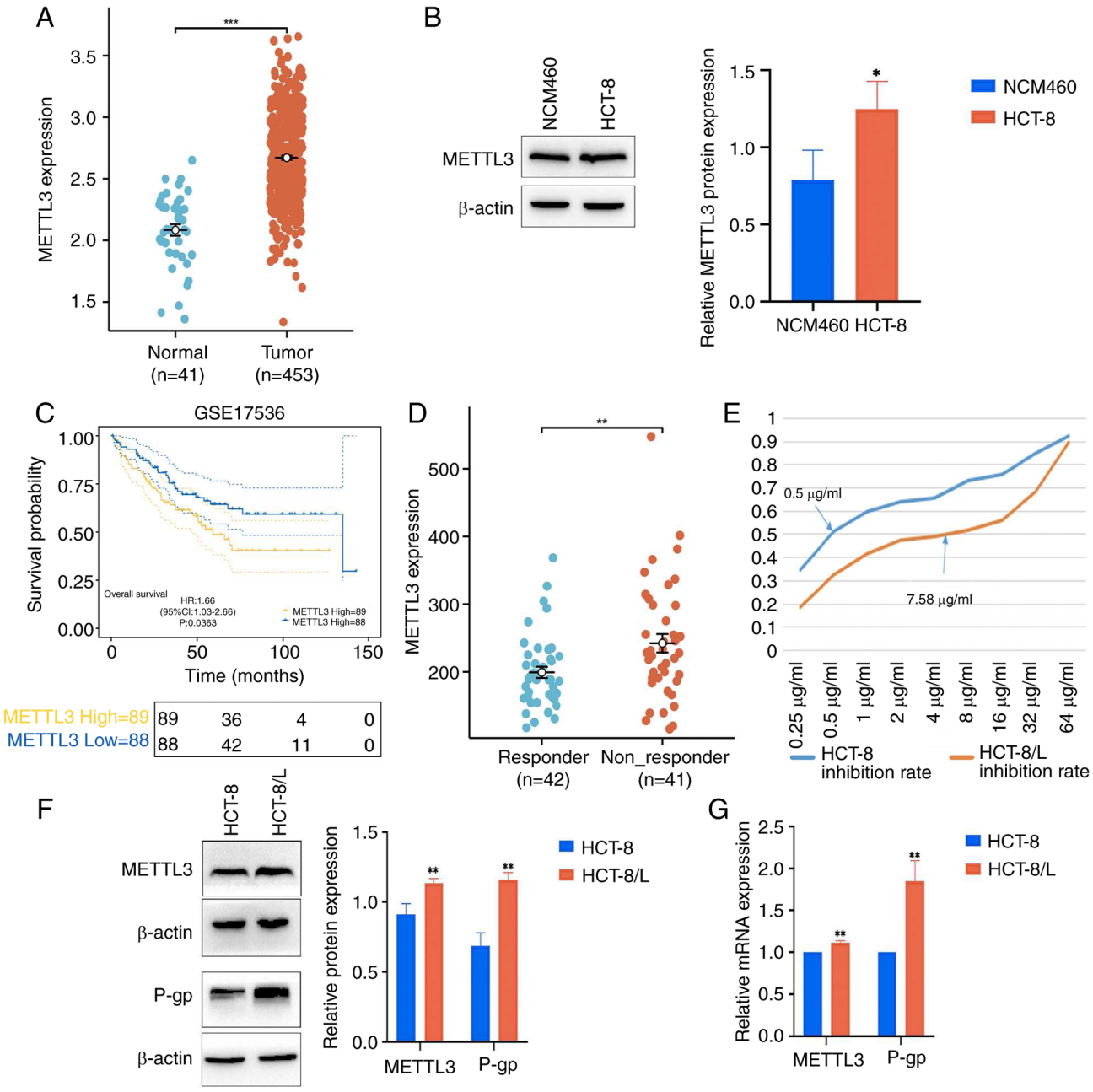

Figure 1. METTL3 expression increases in CRC and is associated with drug resistance and poor prognosis. (A) The expression level of METTL3 in normal tissues and CRC tissues (The Cancer Genome Atlas data) was evaluated. (B) Western blot detection of METTL3 expression levels in NCM460 cells and HCT-8 cells. (C) The overall survival Kaplan-Meier survival curve based on METTL3 mRNA expression in 177 patients with CRC from the GSE17536 dataset. All patients were divided into two groups based on the median level of METTL3. Log-rank test was used to calculate significance level. (D) Expression of METTL3 in tumor tissues of patients resistant and sensitive to FOLFOX chemotherapy in the GSE28702 dataset. FOLFOX is combination chemotherapy regimen consisting of leucovorin calcium, fluorouracil and OXA, and not OXA only. (E) Cell survival analysis of HCT-8 cells and HCT-8/L cells treated with different concentrations of OXA. (F) Western blotting was used to detect the expression of METTL3. (G) Reverse transcription-quantitative PCR was used to detect the expression of METTL3 mRNA. The data are expressed as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 compared with the HCT-8 group. METTL3, methyltransferase 3; CRC, colorectal cancer; OXA, oxaliplatin.

shorter overall survival time (Fig. 1C). To clarify the relationship between METTL3 and drug resistance, the GSE28702 dataset was downloaded and it was found that the expression level of METTL3 increased in FOLFOX chemotherapy-unresponsive patients, but not in FOLFOX chemotherapy-sensitive patients (Fig. 1D). The CCK-8 experiment demonstrated that after OXA treatment, the IC_{50} of HCT-8 cells was 0.5 μg/ml, and the IC_{50} of HCT-8/L cells was 7.58 μg/ml. The resistance index was ~15.16-fold, indicating high resistance (Fig. 1E). Western blotting and RT-qPCR showed that the protein expression levels and mRNA expression levels of METTL3 and P-gp in HCT-8/L cells significantly increased, compared with those in HCT-8 cells (Fig. 1F and G).

Knocking down METTL3 inhibits the Wnt/β-catenin signaling pathway and increases the sensitivity of HCT-8/L cells to OXA. Given the increased expression of METTL3 in HCT-8/L cells, it was hypothesized that regulating METTL3 can alter

the sensitivity of HCT-8/L cells to OXA. To confirm this hypothesis, a knockdown model of METTL3 was constructed using si-METTL3 siRNA in HCT-8/L cells. Western blotting and RT-qPCR showed that the protein and mRNA expression levels of METTL3 in si-METTL3#1 and si-METTL3#2 were significantly downregulated, compared with those in si-NCs (Fig. 2A and B). The CCK-8 experiment identified that knocking down METTL3 significantly decreased the activity of HCT-8/L cells and increased their sensitivity to OXA (Fig. 2C). Concurrently, western blotting and RT-qPCR demonstrated a significant decrease in both P-gp protein and mRNA expression levels (Fig. 2D and F). The effect of METTL3 on apoptosis of HCT-8/L cells was further investigated. Through flow cytometry, it was found that the apoptotic rate of the si-METTL3#1 group and si-METTL3#2 group were significantly higher than that of the si-NC group (Fig. 2E). This was further supported by the results of western blotting. Knocking down METTL3 resulted in a significant increase in Bax protein expression level and a significant decrease in Bcl-2 protein expression level. The expression levels of cleaved caspase 3/total caspase 3 were also increased (Fig. 2F). To explore the effect of METTL3 on the Wnt/β-catenin signaling pathway, western blotting was used. The results showed that the expression levels of Wnt 3a and β-catenin proteins significantly decreased after knocking down METTL3 (Fig. 2F). RT-qPCR also revealed that the expression levels of β-catenin mRNA were significantly downregulated (Fig. 2D). Furthermore, immunofluorescence revealed that knocking down METTL3 inhibited the entry of β-catenin into the nucleus (Fig. 2G). By separating the cytoplasmic and nuclear proteins of HCT-8/L cells, it was found that knocking down METTL3 resulted in no significant change in the expression of β-catenin in the cytoplasm, whereas the expression of β-catenin in the nucleus decreased (Fig. 2H). The aforementioned results indicated that knocking down METTL3 inhibits the Wnt/β-catenin signaling pathway in HCT-8/L cells, promotes cell apoptosis, and increases sensitivity to OXA.

Knocking down METTL3 increases sensitivity of HCT-8/L cells to OXA by inhibiting the Wnt/β-catenin signaling pathway. To further elucidate the impact of METTL3 on the sensitivity of HCT-8/L cells to OXA by inhibiting the Wnt/β-catenin signaling pathway, AMBMP was used in the present study. The results of flow cytometry showed that the apoptotic rate of HCT-8/L cells in the si-METTL3#1 group was significantly higher than that in the si-NC group, while knocking down METTL3 and adding AMBMP caused no significant change in the apoptotic rate of HCT-8/L cells, compared with the negative control group (Fig. 3A). The use of AMBMP has weakened this trend. The RT-qPCR results revealed that knocking down METTL3 resulted in a decrease in the mRNA expression levels of β-catenin and P-gp, while AMBMP upregulated the mRNA expression levels of β-catenin and P-gp. There was no significant difference in the mRNA expression levels of METTL3. After knocking down METTL3 and adding AMBMP, compared with the si-METTL3#1 group, the mRNA expression levels of β-catenin and P-gp both increased, while there was no significant change in the mRNA expression level of METTL3 (Fig. 3B). Western blot results identified that knocking down METTL3 inhibited the expression of Wnt 3a, β-catenin, Bcl-2 and P-gp, and enhanced the expression of Bax and cleaved caspase 3/total caspase 3; whereas AMBMP upregulated the expression of Wnt 3a, β-catenin, Bcl-2 and P-gp, and reduced the expression of Bax and cleaved caspase 3/total caspase 3. There was no significant difference in METTL3 expression. Compared with the si-METTL3#1 group, knocking down METTL3 and adding AMBMP enhanced the expression of Wnt 3a, β-catenin, Bcl-2 and P-gp, and inhibited the expression of Bax and cleaved caspase 3/total caspase 3, whereas the expression of METTL3 remained unchanged (Fig. 3C). The immunofluorescence results showed that knocking down METTL3 inhibited the entry of β-catenin into the nucleus, whereas AMBMP promoted the entry of β-catenin into the nucleus. After knocking down METTL3 and adding AMBMP, compared with the si-METTL3#1 group, the entry of β-catenin into the nucleus increased (Fig. 3D). By separating the cytoplasmic and nuclear proteins of HCT-8/L cells, it was found that knocking down METTL3 downregulated the expression of β-catenin in the nucleus, while showing no significant changes in the cytoplasm. AMBMP upregulated β-catenin in both the cytoplasm and nucleus. Knocking down METTL3 and adding AMBMP resulted in upregulation of β-catenin in both the cytoplasm and nucleus, compared with the si-METTL3#1 group. Compared with the group that was only supplemented with AMBMP, knocking out METTL3 and adding AMBMP did not significantly change β-catenin in the cytoplasm, but inhibited β-catenin in the nucleus compared with the si-NC + AMBMP group (Fig. 3E). The aforementioned results indicated that METTL3 can affect the sensitivity of HCT-8/L cells to OXA by regulating the Wnt/β-catenin signaling pathway.

Huaier reduces the expression of METTL3 and the vitality of HCT-8/L cells. CCK-8 method was used to detect the effect of Huaier on the viability of HCT-8/L cells. It was found that the activity of HCT-8/L cells decreased in a dose-dependent manner with different doses of Huaier (0, 6, 9, 12, 15 and 18 mg/ml) (Fig. 4A). After 24 h of treatment with Huaier, the IC_{50} value of HCT-8/L cells was 15.74 mg/ml, and after 48 h, the IC_{50} value of HCT-8/L cells was 12.35 mg/ml. To investigate whether Huaier affects the expression of METTL3, western blotting was used. Under different doses (0, 9, 12 and 15 mg/ml) of Huaier, the expression level of METTL3 gradually decreased with increasing concentration of Huaier (Fig. 4B). The aforementioned results indicated that Huaier can inhibit the expression of METTL3 and reduce the activity of HCT-8/L cells. The concentration of 12 mg/ml was selected for subsequent experiments with Huaier.

Huaier downregulates the expression of METTL3, inhibits the Wnt/β-catenin signaling pathway, and increases the sensitivity of HCT-8/L cells to OXA. Considering that Huaier can inhibit the expression of METTL3, it was further analyzed whether Huaier can inhibit the Wnt/β-catenin signaling pathway and increase the sensitivity of HCT-8/L cells to OXA. It was found through CCK-8 experiments that Huaier significantly increased the sensitivity of HCT-8/L cells to OXA (Fig. 5A), while western blotting and RT-qPCR showed a significant decrease in the protein and mRNA expression levels of P-gp (Fig. 5B and D). The results of flow cytometry demonstrated

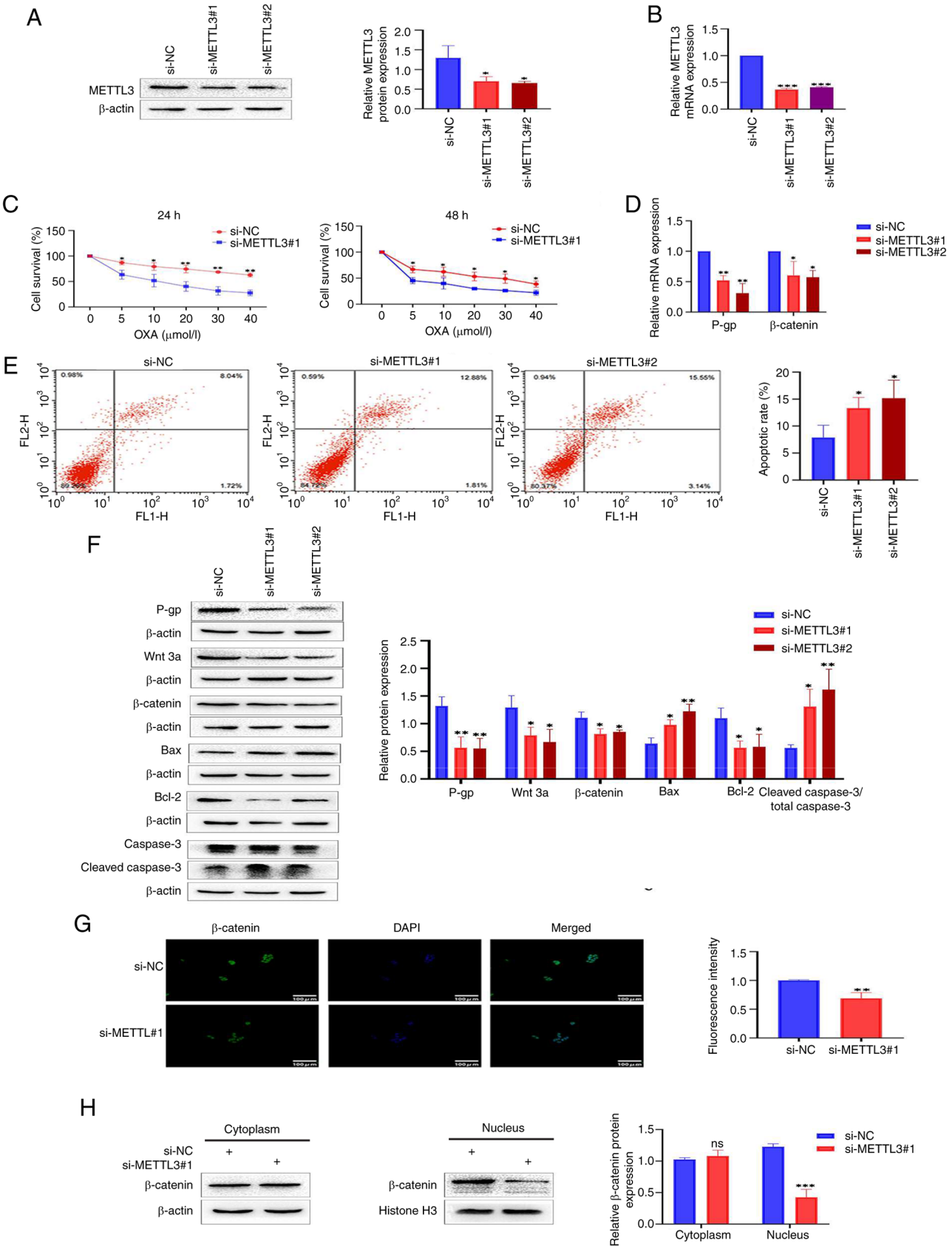

Figure 2. Knocking down METTL3 inhibits the Wnt/β-catenin signaling pathway, making colorectal cancer cells sensitive to OXA. (A) Western blot detection of METTL3 knockdown efficiency. (B) RT-qPCR detection of METTL3 knockdown efficiency. (C) Cell survival analysis of si-METTL3#1 and control si-NC treated with different concentrations of OXA. (D) Reverse transcription-quantitative PCR was used to detect the mRNA expression changes of P-gp and β-catenin after knocking down METTL3. (E) Flow cytometric analysis of cell apoptosis. (F) Western blot analysis was used to detect the expression changes of P-gp, Wnt 3a, β-catenin, Bax, Bcl-2 and cleaved caspase3/total caspase 3 proteins after knocking down METTL3. (G) Immunofluorescence analysis of β-catenin's entry into the nucleus. (H) Western blotting was used to detect the expression of β-catenin in the cytoplasm and nucleus of HCT-8/L cells. The data are expressed as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 compared with the si-NC group. METTL3, methyltransferase 3; OXA, oxaliplatin; si-, small interfering; NC, negative control.

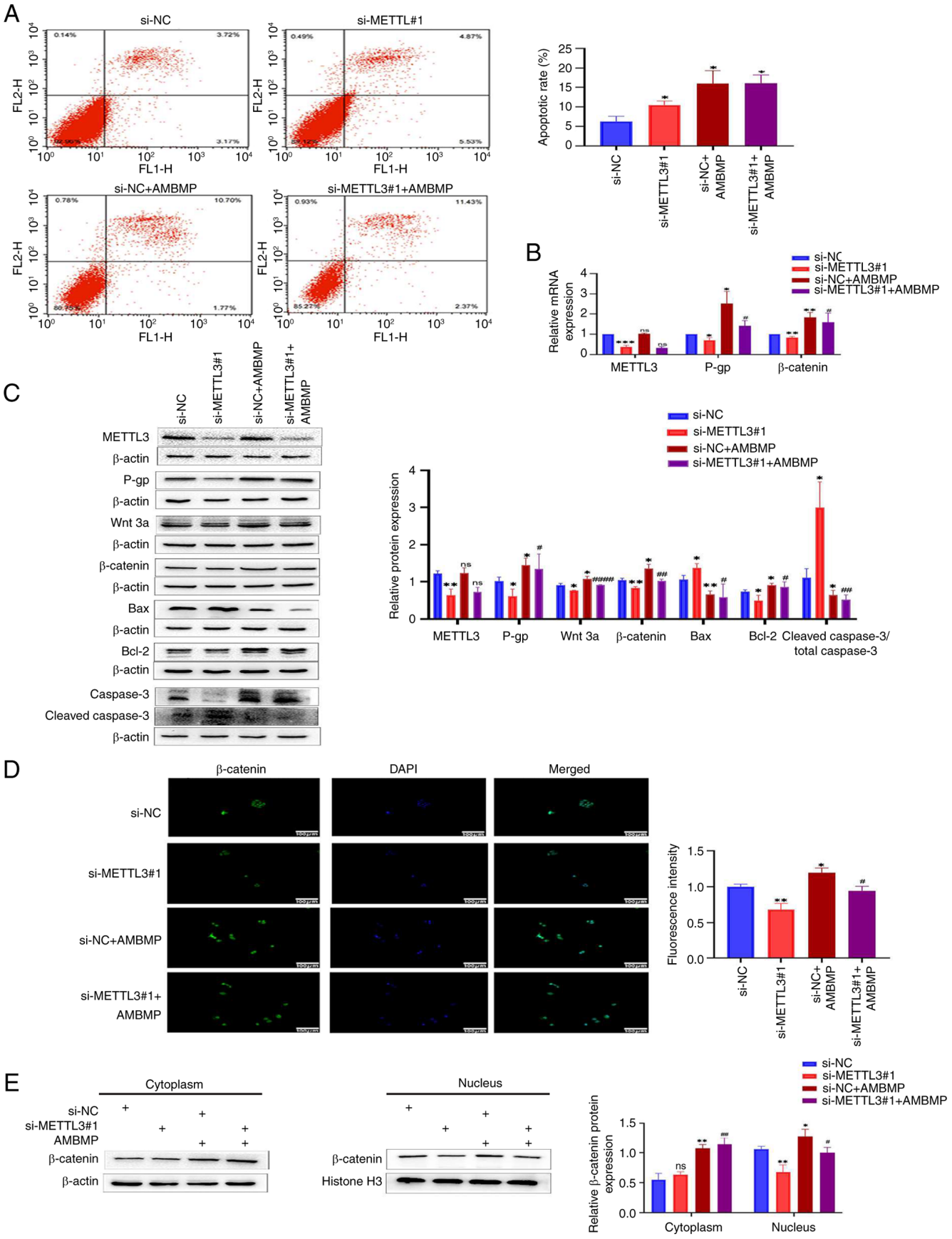

Figure 3. Knocking down METTL3 increases the sensitivity of HCT-8/L cells to oxaliplatin by inhibiting the Wnt/β-catenin signaling pathway. (A) Flow cytometric analysis of cell apoptosis. (B) Reverse transcription-quantitative PCR was used to detect changes in mRNA expression of METTL3, P-gp and β-catenin. (C) Western blotting was used to detect the protein expression changes of METTL3, P-gp, Wnt 3a, β-catenin, Bax, Bcl-2 and cleaved caspase3/total caspase 3. (D) Immunofluorescence analysis of β-catenin's entry into the nucleus. (E) Western blot analysis of the expression of β-catenin in the cytoplasm and nucleus of HCT-8/L cells. The data are expressed as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 compared with the si-NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#\#}$P<0.0001 compared with the si-METTL3#1 group. METTL3, methyltransferase 3; si-, small interfering; NC, negative control; ns, not significant.

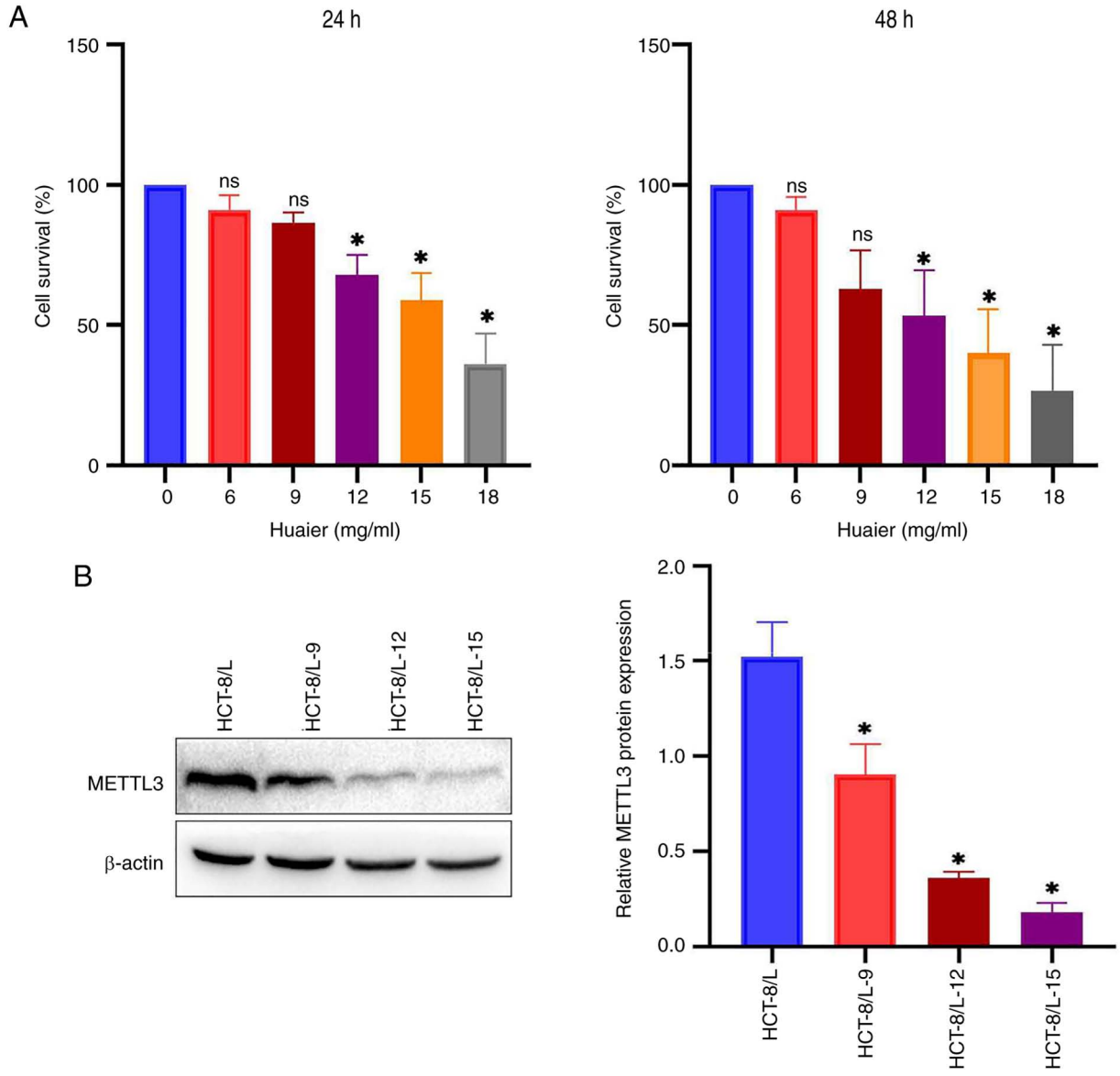

Figure 4. Huaier inhibits METTL3 expression and the activity of HCT-8/L cells. (A) Survival analysis of HCT-8/L cells treated with different concentrations of Huaier for 24 and 48 h. (B) The effect of different concentrations of Huaier on the expression of METTL3 in HCT-8/L cells. The data are expressed as the mean ± SD. *P<0.05 compared with the HCT-8/L group. METTL3, methyltransferase 3; ns, not significant.

that under the action of Huaier, the apoptotic rate of the cells significantly increased (Fig. 5C), and the results of western blot supported this finding. The expression level of Bax and cleaved caspase 3/total caspase 3 significantly increased, whereas the expression level of Bcl-2 significantly decreased (Fig. 5D).

Subsequently, it was identified using western blotting that Huaier inhibited the expression levels of METTL3, Wnt 3a and β-catenin (Fig. 5D). The RT-qPCR results revealed a significant decrease in the expression levels of METTL3 and β-catenin mRNA after the action of Huaier (Fig. 5B). Immunofluorescence experiments showed that Huaier inhibited the entry of β-catenin into the nucleus (Fig. 5E). By extracting cytoplasmic and nuclear proteins from HCT-8/L cells, it was found that Huaier inhibited the expression of β-catenin in the cytoplasm and nucleus (Fig. 5F). This indicated that Huaier downregulates the expression of METTL3, inhibits the Wnt/β-catenin signaling pathway, and increases the sensitivity of HCT-8/L cells to OXA.

Huaier suppresses the Wnt/β-catenin signaling pathway by downregulating the expression of METTL3, rendering HCT-8/L cells sensitive to OXA. To further elucidate whether Huaier inhibits the Wnt/β-catenin signaling pathway by downregulating the expression of METTL3, rendering HCT-8/L cells sensitive to OXA, a model overexpressing METTL3 in HCT-8/L cells was constructed. Western blotting and RT-qPCR showed that the protein and mRNA expression levels of METTL3 in the pC-METTL3 group significantly increased, compared with those in the pC-NC group (Fig. 6A and B). The results of flow cytometry revealed that the apoptotic rate of HCT-8/L cells in the pC-METTL3 group was significantly reduced, compared with that in the pC-NC group, while Huaier increased the apoptotic rate of HCT-8/L cells. Compared with the pC-METTL3 group, the addition of Huaier

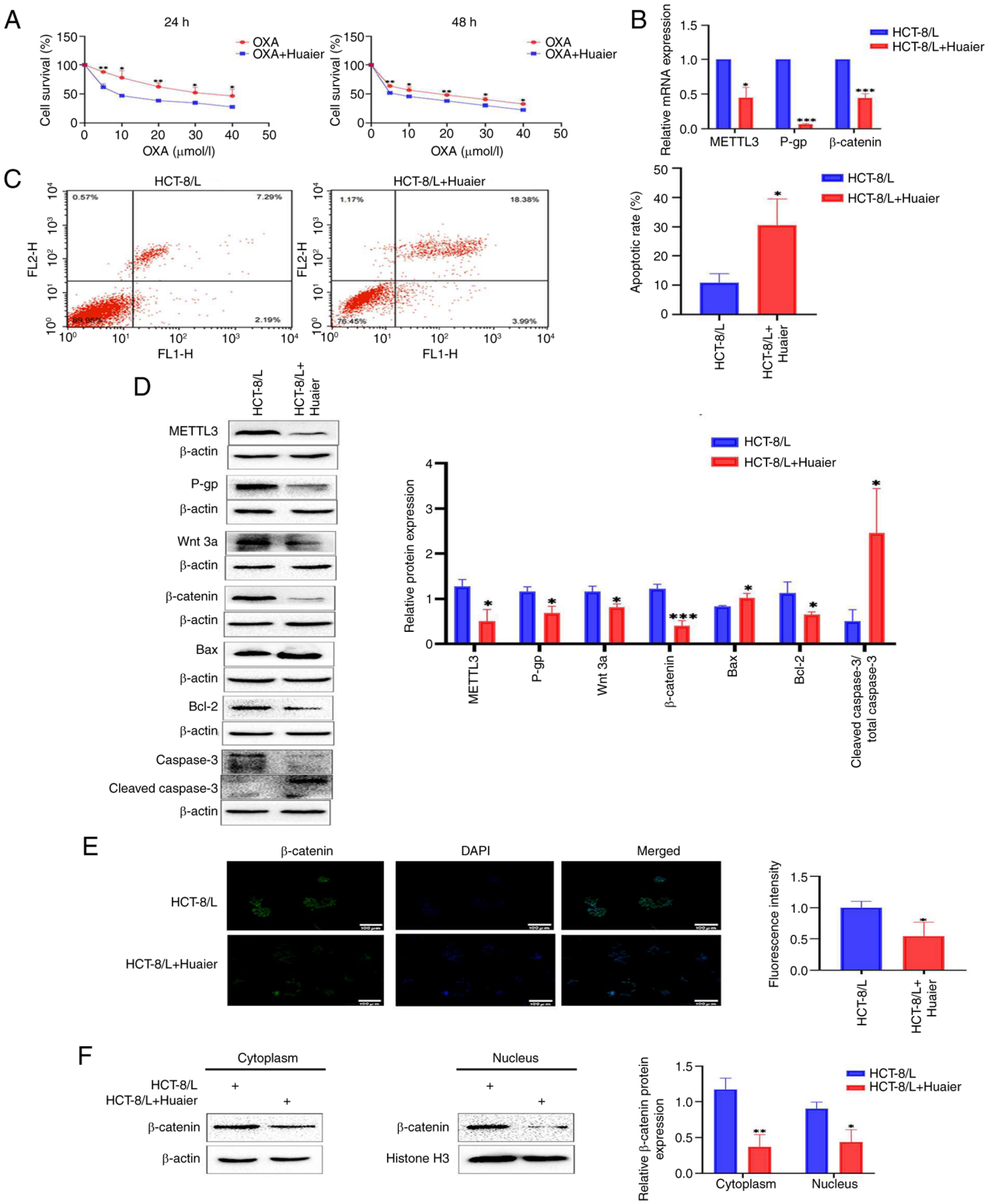

Figure 5. Huaier downregulates the expression of METTL3, inhibits the Wnt/β-catenin signaling pathway, and renders HCT-8/L cells sensitive to OXA. (A) Survival analysis of HCT-8/L cells treated with different concentrations of OXA after treatment with Huaier. (B) Reverse transcription-quantitative PCR was used to detect the effect of Huaier on the mRNA expression of METTL3, P-gp and β-catenin. (C) Flow cytometric analysis of cell apoptosis. (D) Western blot was used to detect the effects of Huaier on the protein expression of METTL3, P-gp, Wnt 3a, β-catenin, Bax, Bcl-2 and cleaved caspase3/total caspase 3. (E) Immunofluorescence analysis of β-catenin's entry into the nucleus. (F) Western blotting was used to detect the expression of β-catenin in the cytoplasm and nucleus of HCT-8/L cells. The data are expressed as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 compared with the HCT-8/L group. METTL3, methyltransferase 3; OXA, oxaliplatin.

after overexpression of METTL3 increased the apoptotic rate of HCT-8/L cells (Fig. 6C). Western blot results demonstrated that overexpression of METTL3 upregulated the expression of METTL3, Wnt 3a, β-catenin, Bcl-2 and P-gp, and inhibited the expression of Bax and cleaved caspase 3/total caspase 3. Huaier inhibited the expression of METTL3, Wnt 3a, β-catenin, Bcl-2

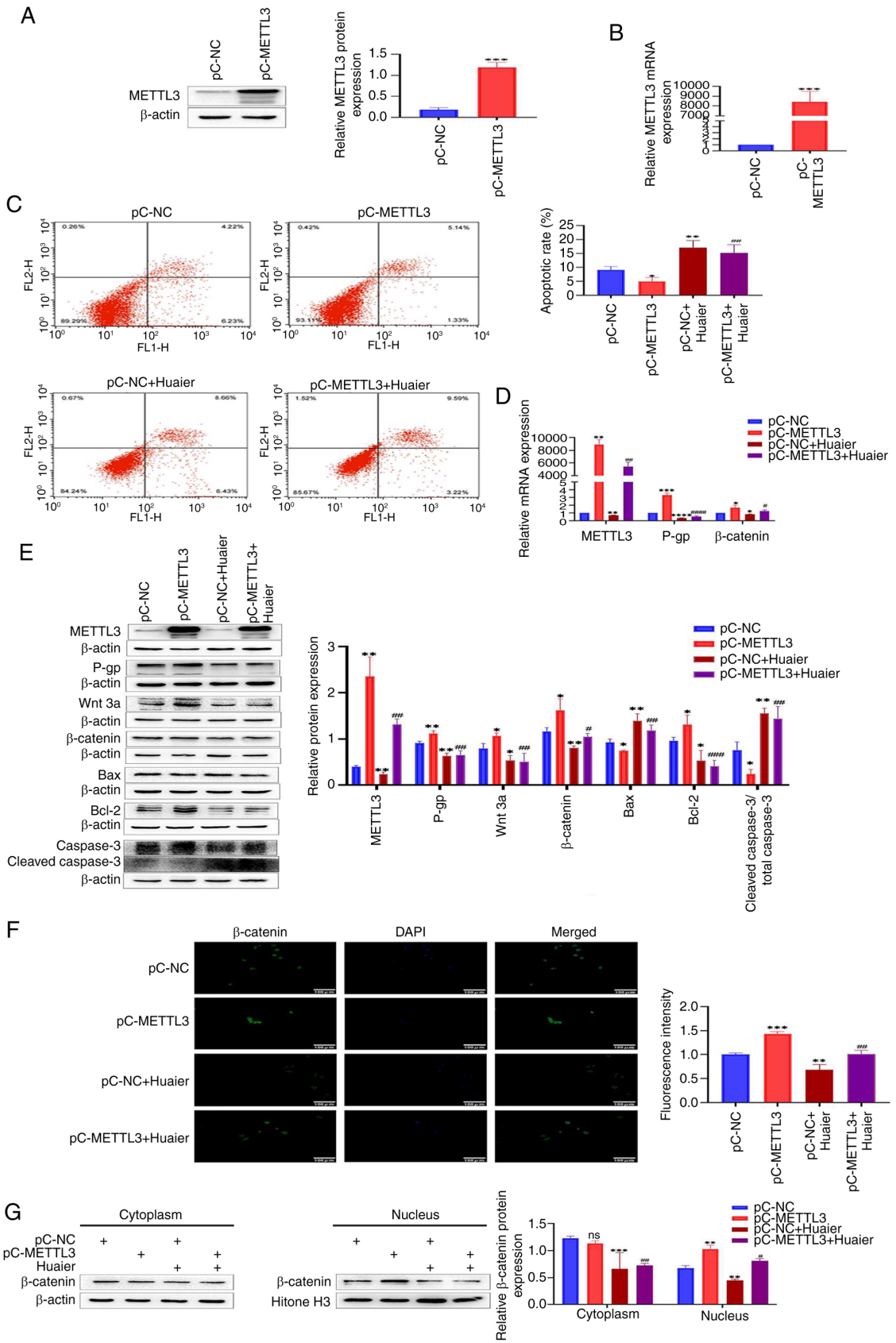

Figure 6. Huaier inhibits the Wnt/β-catenin signaling pathway by downregulating the expression of METTL3, rendering HCT-8/L cells sensitive to OXA. (A) Western blotting was used to detect the overexpression efficiency of METTL3. (B) RT-qPCR was used to detect the overexpression efficiency of METTL3. (C) Flow cytometric analysis of cell apoptosis. (D) RT-qPCR was used to detect changes in mRNA expression of METTL3, P-gp and β-catenin. (E) Western blotting was used to detect the protein expression changes of METTL3, P-gp, Wnt 3a, β-catenin, Bax, Bcl-2 and cleaved caspase3/total caspase 3. (F) Immunofluorescence analysis of β-catenin's entry into the nucleus. (G) Western blot analysis of the expression of β-catenin in the cytoplasm and nucleus of HCT-8/L cells. The data are expressed as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared with the pC-NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 and $^{\#\#\#\#}$P<0.0001 compared with the pC-METTL3. METTL3, methyltransferase 3; OXA, oxaliplatin; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; ns, not significant.

and P-gp, and enhanced the expression of Bax and cleaved caspase 3/total caspase 3. Compared with the pC-METTL3 group, adding Huaier after overexpressing METTL3 inhibited the expression of METTL3, Wnt 3a, β-catenin, Bcl-2 and P-gp, and upregulated the expression of Bax and cleaved caspase 3/total caspase 3 (Fig. 6E). The RT-qPCR results showed that after overexpression of METTL3, the mRNA expression levels of METTL3, β-catenin and P-gp were all upregulated. After the action of Huaier, the mRNA expression levels of METTL3, β-catenin and P-gp were all reduced. Compared with the pC-METTL3 group, overexpression of METTL3 followed by the addition of Huaier inhibited the mRNA expression levels of METTL3, β-catenin and P-gp (Fig. 6D). The immunofluorescence results identified that overexpression of METTL3 promoted the entry of β-catenin into the nucleus, whereas Huaier inhibited the entry of β-catenin into the nucleus. After overexpressing METTL3 and adding Huaier, compared with the pC-METTL3 group, the entry of β-catenin into the nucleus was reduced (Fig. 6F). After separating the cytoplasmic and nuclear proteins of HCT-8/L cells, it was found that overexpression of METTL3 upregulated the expression of β-catenin in the nucleus, whereas there was no significant change in β-catenin in the cytoplasm. Huaier downregulated β-catenin in both the cytoplasm and nucleus. After overexpressing METTL3, the use of Huaier resulted in downregulation of β-catenin in both the cytoplasm and nucleus compared with the pC-METTL3 group (Fig. 6G). These data indicated that Huaier can downregulate the expression of METTL3, thereby inhibiting the Wnt/β-catenin signaling pathway and rendering HCT-8/L cells sensitive to OXA.

Discussion

CRC is the third most common malignant tumor worldwide, and its incidence rate has increased in recent years (32). The mortality rate of CRC is second only to that of lung cancer (33). Most patients have a hidden onset and are already in the middle or late stage when discovered (3). Chemotherapy is a commonly used treatment for middle- and late-stage CRC in traditional Chinese medicine (34). The first-line chemotherapy drug commonly used in clinical practice for the treatment of CRC is mainly OXA. However, chemotherapy resistance developed during the treatment process has become a common cause of treatment failure. Due to the unclear mechanism of OXA chemotherapy resistance, patients with CRC lack effective treatment methods.

METTL3 is the first reported m6A methyltransferase and has been identified as the main methyltransferase involved in the methylation process (35). Peng *et al* (36) have found that METTL3 promotes the proliferation, migration and invasion of CRC, and affects the prognosis of patients with CRC. According to Li *et al* (15), inhibition of METTL3 makes CRC cells sensitive to 5-FU, and overcomes 5-FU resistance in CRC cells by downregulating the expression of RAD51-related protein 1, enhancing DNA damage accumulation, and promoting cell apoptosis.

In the present study, a database analysis was first conducted and it was found that METTL3 expression is elevated in CRC chemotherapy-tolerant patients and that high expression of METTL3 is closely related to poor prognosis in patients. HCT-8/L cells were selected as the research object and it was found that METTL3 expression was elevated in HCT-8/L cells. Knockdown of METTL3 downregulated the expression of P-gp and Bcl-2, increased the expression of Bax, promoted the sensitivity of HCT-8/L cells to OXA, and facilitated cell apoptosis. In addition, it was revealed that METTL3 has a regulatory effect on the Wnt/β-catenin signaling pathway in HCT-8/L cells. Knocking down METTL3 can inhibit the expression of Wnt3a and β-catenin and suppress the nuclear entry of β-catenin. Then, AMBMP was used to further validate the regulatory effect of METTL3 on the Wnt/β-catenin signaling pathway. It was found that compared with those in the AMBMP group, knocking down METTL3 and adding AMBMP downregulated the expression of Wnt3a, β-catenin, Bcl-2 and P-gp; increased the expression of Bax; and inhibited the nuclear entry of β-catenin. Under the action of AMBMP, there was no significant difference in the expression of METTL3. It is considered that AMBMP activates the Wnt/β-catenin signaling pathway, but as METTL3 is an upstream gene, AMBMP does not significantly affect the expression of METTL3. The aforementioned experimental results confirm that METTL3 can promote the sensitivity of HCT-8/L cells to OXA by regulating the Wnt/β-catenin signaling pathway.

Although the knockout of METTL3 cannot completely reverse drug resistance, current data at least suggest that inhibiting METTL3 can partially overcome OXA resistance in HCT-8/L cells by suppressing the Wnt/β-catenin signaling pathway. As a key methyltransferase in m6A methylation modification, METTL3 may also interact with other genes to contribute to drug resistance in CRC. Liu *et al* (37) found that METTL3-mediated m6A modification of Sec62 mRNA upregulated Sec62 expression in CRC. Subsequently, Sec62 potentiates Wnt signaling through repressing β-catenin binding to APC complex. This was consistent with the present results. However, further research is needed to clarify the specific mechanisms of the relationship between METTL3 and the Wnt/β-catenin signaling pathway, as well as its connection to chemotherapy resistance, which includes gathering more clinical tissue samples for analysis.

Since artemisinin was proven to treat malaria, traditional Chinese medicine has garnered considerable attention. Traditional Chinese medicine is the main source of natural medicines and herbal products and an important source for developing anti-CRC drugs (38). The active ingredients in traditional Chinese medicine can disrupt the living environment of cancer cells, promote cell apoptosis, enhance individual immunity and eliminate pathogens through the autoimmune system, thereby achieving anticancer effects (39-41). An increasing amount of evidence suggests that Huaier has anticancer effects on various types of tumors. Zhou *et al* (42) found that Huaier could slow down the growth of pancreatic cancer and reduce the invasion, migration, and epithelial-mesenchymal transition of pancreatic cancer cells by inhibiting the Wnt/β-catenin signaling pathway. Sun *et al* (43) reported that Huaier significantly reduced the tumor development of HT-29 CRC cell line transplanted into nude mice by downregulating the expression of PI3KR1, AKT, Wnt1, CTTNB1 and Notch genes. Cong *et al* (44) found that

Huaier can inhibit the development of cholangiocarcinoma by regulating the Twist1/FBP1/Wnt/β-catenin signaling axis.

The present study found through CCK-8 experiments that Huaier can inhibit the proliferation activity and drug resistance of HCT-8/L cells. The use of Huaier downregulated the expression of METTL3, P-gp and Bcl-2 in HCT-8/L cells; upregulated the expression of Bax; promoted the sensitivity of HCT-8/L cells to OXA; and facilitated cell apoptosis. Moreover, Huaier has a regulatory effect on the Wnt/β-catenin signaling pathway. Huaier inhibited the expression of Wnt3a and β-catenin in HCT-8/L cells and inhibited the entry of β-catenin into the nucleus. Then, an overexpression METTL3 model was constructed in HCT-8/L cells to further explore the mechanism by which Huaier affects HCT-8/L cells. It was found that after overexpression of METTL3 in Huaier, the expression of METTL3, P-gp, Wnt3a, β-catenin, and Bcl-2 decreased, whereas the expression of BAX increased. The nuclear entry of β-catenin was also inhibited, and cell apoptosis increased. The aforementioned experimental results confirmed that Huaier can suppress the Wnt/β-catenin signaling pathway and render HCT-8/L cells sensitive to OXA by downregulating the expression of METTL3.

The present study indicated that the fungus Huaier has a certain inhibitory effect on CRC; however, the current results are based on cellular experiments but have not been validated in the second cell line. Further research to verify the clinical efficacy of Huaier is needed. More in-depth research is still required regarding the safety, effectiveness, metabolism, side effects and toxicity of Huaier in patients with CRC. Meanwhile, the effect of Huaier on normal colon cells will be explored in future studies.

In summary, Huaier downregulates the expression of METTL3, inhibits the Wnt/β-catenin signaling pathway, and increases the sensitivity of OXA-resistant CRC cells to OXA. The present study provides a theoretical basis for the treatment of CRC chemotherapy-induced drug resistance with the traditional Chinese medicine Huaier.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MH and JZ conceived and designed the study. MH and ZG collected data. MH supervised the study. MH and GW analyzed and interpreted the data. MH, ZH and JZ performed statistical analysis. MH, JZ and ZH wrote the draft of the manuscript. MH and JZ critically revised the manuscript. All authors read and approved the final version of the manuscript, participated sufficiently in the work and agreed to be accountable for all aspects of the work. MH and JZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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