The long non-coding RNA ENST00000547547 reduces 5-fluorouracil resistance of colorectal cancer cells via competitive binding to microRNA-31

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Abstract. Colorectal cancer (CRC) is one of the most common cancers and the third leading cause of cancer-related deaths due to its rapid progression and poor prognosis. 5-Fluorouracil (5-FU)-based chemotherapies are the standard treatment for locally advanced CRC. However, a considerable percentage of CRCs have inherent or acquired 5-FU resistance, which critically impedes clinical outcomes. In the present study, we reported that the expression level ENST00000547547 was downregulated in 5-FU-resistant CRC cells in comparison with the parental cells, While rising with the treatment of 5-FU in parental cells. Overexpression of ENST00000547547 promoted 5-FU-induced cell apoptosis and reduced the chemoresistance of 5-FU *in vitro*. Moreover, we found that ENST00000547547 was a target of miR-31, as confirmed by dual-luciferase reporter assay and RNA immunoprecipitation assay. Notably, miR-31 was upregulated in 5-FU-resistant CRC cells, and knockdown of miR-31 increased the chemosensitivity of 5-FU-resistant CRC cells. Furthermore, we demonstrated that ENST00000547547 reduced the chemoresistance of 5-FU via competitive binding to miR-31 in 5-FU-resistant CRC cell lines. Collectively, our findings revealed that ENST00000547547 reduced chemoresistance in 5-FU of 5-FU-resistant CRC cells through competitive binding to miR-31 and has the potential to serve as a therapeutic target in CRC patients.

Introduction

Colorectal cancer (CRC) is the third most common cancer and one of the leading causes of cancer-related deaths worldwide. According to WHO GLOBOCAN 2012, 1.4 million new cases of CRC were diagnosed, and 0.7 million patients succumbed to CRC, which accounted for 9.7% of the total cancer incidence and 8.5% of the cancer-related deaths worldwide (1). 5-Fluorouracil (5-FU), firstly synthesized by Heidelberger *et al* in 1957, has been widely employed for the treatment of solid cancers (2,3). The 5-FU-based chemotherapy is the standard treatment for locally advanced CRC, particularly for middle and distal rectal cancers, which improves the response rate, local control and overall survival (4,5). However, a considerable percentage of CRCs have inherent or acquired resistance to 5-FU-based chemotherapy, which is a responsible for the failure of the treatment (6,7). Thus, a deeper understanding of the molecular characteristics underlying 5-FU resistance is imperative.

In recent years, numerous studies have indicated that long non-coding RNAs (lncRNAs), generally longer than 200 nucleotides in length, have emerged as new effective regulators in tumor initiation and progression (8-10). lncRNAs generally act as key molecules in chromatin remodeling, transcription, and post-transcriptional processing (11).The roles that lncRNAs play in drug resistance have also been extensively reported (12-15). Lan *et al* demonstrated that silencing of lncRNA ANRIL inhibited the development of multiple-drug resistance in gastric cancer cells (16). Liu *et al* reported that overexpression of lncRNA PVT1 in ovarian cancer cells promoted cisplatin resistance through the regulation of apoptotic pathways (17). Wang *et al* indicated that increased expression of lncRNA UCA1 improved cell migration and invasion, and induced cisplatin resistance in human ovarian cancer cells (18). In CRC, several lncRNAs were found to be effective regulators of tumorigenesis, such as PRNCR1, MALAT-1, HOTAIR and AK027294, which partly or totally affected cell proliferation, metastasis, cell cycle progression, apoptosis and epithelial-mesenchymal transition (19-22). In 2015, a microarray analysis identified 2,662 differentially expressed lncRNAs in 5-FU-resistant CRC cells when compared with those in parental cells, suggesting that lncRNAs may play significant roles in the 5-FU resistance of CRC cells (23). However, a relative functional study has rarely been reported.

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lncRNA ENST00000547547 is a 434-bp transcript on human chromosome 12q15 (RP11-611E13.3-001) and it has been found to be significantly downregulated in drug-resistant CRC cells. A bioinformatic analysis indicated that ENST00000547547 contains a potential binding site for microRNA-31 (miR-31). miR-31 is a broadly conserved miRNA expressed in a variety of tissues and types of cells, that has been revealed to be upregulated in various human cancers, including breast, tongue and head-and-neck squamous tumors (24). Wang *et al* demonstrated that suppression of miR-31 in HCT116 cells increased its sensitivity to 5-FU at an early stage, and the downregulation of miR-31 affected cell migration and invasion (25). However, the potential association between ENST00000547547 and miR-31, and its functional role in 5-FU resistance of CRC remain largely unknown.

In the present study, we used 5-FU-resistant CRC cell models to determine the potential roles of ENST00000547547 and miR-31 in the regulation of 5-FU resistance. Following a functional study, we applied a dual-luciferase reporter assay and an RNA immunoprecipitation assay to examine the relationship between ENST00000547547 and miR-31. Finally, the functional role of the their interaction in 5-FU resistance was investigated using co-transfection cell models. Our results strongly indicated that ENST00000547547 reduces 5-FU resistance via competitive binding to miR-31 in 5-FU-resistant CRC cell lines and may provide new therapeutic targets for patients that exhibited 5-FU resistance.

Materials and methods

Cell culture and reagents. Human CRC cell lines HCT116 and LoVo, and 5-FU-resistant CRC cell lines LoVo/5-FU and HCT116/5-FU were purchased from Auragene (Changsha, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). The cultures were maintained at 37˚C in humidified air with 5% CO_2. 5-FU (F6627) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

RNA extraction and qRT-PCR analyses. Total RNA was extracted from frozen cells with TRIzol reagent (Invitrogen Life Technologies), and cDNA was synthesized using random primers and a Reverse Transcription kit (Takara Biotechnology, Dalian, China). The amount and quality of RNA were determined based on absorbance ratios using NanoDrop ND-1000. Quantitative RT-PCR (qRT-PCR) analyses were performed using SYBR-Green qPCR Mix (Toyobo, Osaka Prefecture, Osaka, Japan) according to the manufacturer's instructions. The expression levels of target genes were normalized to the transcription level of β-actin, and U6 was used for normalization of miR-31 expression. The data were collected and calculated using an ABI 7300 instrument (MK3; Thermo Fisher Scientific). Primers for qRT-PCR were synthesized by Invitrogen (Carlsbad, CA, USA) and the sequences were: ENST00000547547 sense, 5'-TTTTCTAAGGCACCA ACT-3' and antisense, 5'-CCAAA TGCTCTAAGGGA-3'; miR-31 primer was obtained from FulenGen company (HmiRQP0401); ABCB9 sense, 5'-GCTCTGG GAGAGACCTTCCT-3' and antisense, 5'-GAGCGGAAGAGA CAGTTTCG-3'; β-actin sense, 5'-AGGGGCCGGACTCGTC ATACT-3' and antisense, 5'-GGCGGCACCACCATGTAC CCT-3'.

Transfection. Cells were transfected using transfection reagent Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. They were harvested at 48 h after transfection. For co-transfection (pre-miR-31 and pCDNA3.1-ENST00000547547 as an example) the cells were initially transfected with NC or pre-miR-31 using Lipofectamine 2000, according to the protocols. Then, the cells were transfected with pCDNA3.1-ENST00000547547 or the mutant one using Lipofectamine 2000, according to the manufacturer's instructions.

Cell proliferation assay. The cell proliferation assay was conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sangon Biotech, Shanghai, China). Forty-eight hours after transfection, these cells and controls were seeded into 96-well plates at an initial density of 5,000 cells/well. After 24 h of culture, these cells were exposed to various concentrations of 5-FU (0, 5, 10, 20, 40, 60, 80 and 100 μM) for 48 h, and then they were treated with 10 μl MTT by adding it into each well. The cells were incubated at 37˚C for another 4 h, then the medium was removed carefully, and 150 μl dimethyl sulfoxide (DMSO) solution (MP Biomedicals, LLC, Santa Ana, CA, USA) was added to lyse the cells. Finally, the absorbance was assessed at 570 nm with a microplate reader Multiskan MK (Thermo Fisher Scientific). The survival rate was calculated using the equation: (mean absorbance of the drug wells/mean absorbance of the control wells) x 100%.

Flow cytometric analysis. Cells were harvested 48 h after 5-FU treatments (20 μM), and were washed three times with cold phosphate-buffered saline (PBS). Then, these cells were stained with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI; Keygentec, Nanjing, China) for 10 min in the dark at room temperature. The cells were then examined by flow cytometry (BD Biosciences, Frankin Lakes, NJ, USA).

Western blot analysis. Proteins were extracted from cells with RIPA lysis buffer (Auragene, Changsha, China), which was supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland) and phenylmethylsulfonyl fluoride (PMSF) (Auragene, Changsha, China). Equal amounts (30 μg) of proteins were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 3% bovine serum albumin (BSA)-Tris-buffered saline with Tween-20 (TBST) with gentle shaking at room temperature for 90 min and was continuously probed with indicated primary antibodies with gentle shaking at 4˚C overnight. The membranes were washed, and incubated with specific secondary antibodies at room temperature for 1 h. A β-actin antibody was used as a control, and the ABCB9 (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bcl-2 (1:1,000), Bax (1:500) (both from Abzoom, Dallas, TX,

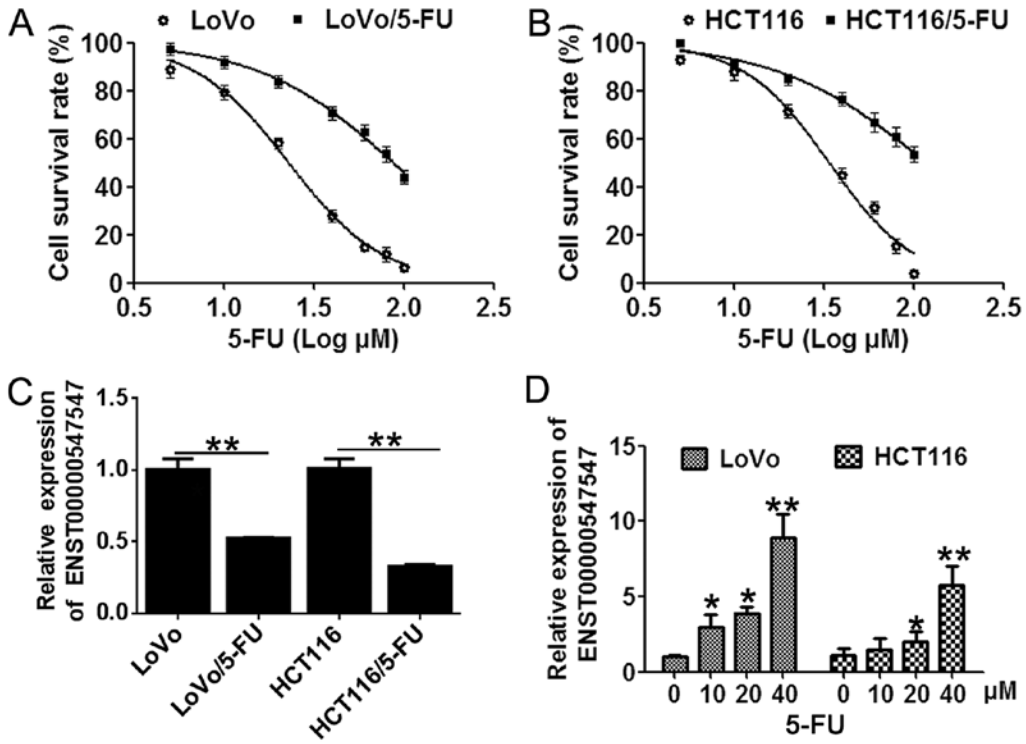

Figure 1. ENST00000547547 is downregulated in 5-FU-resistant CRC cell lines. (A and B) Parent and 5-FU-resistant CRC cells were exposed to various concentrations of 5-FU for 48 h, and the cell survival rate was determined by MTT assay. (C) The transcriptional levels of ENST00000547547 in parent and 5-FU-resistant CRC cells were determined by qRT-PCR analysis. (D) Each cell line was exposed to various concentrations of 5-FU for 48 h. Transcriptional levels of ENST00000547547 in CRC cell lines were determined by qRT-PCR analysis. Data represent the mean ± SD from three independent experiments; *P<0.05, **P<0.01.

USA), AGO2 (1:500) and IgG (1:1,000) (both from Abcam, Cambridge, MA, USA) antibodies were used for each group.

Bioinformatics analysis. miR-31 was predicted as a target of ENST00000547547 using bioinformation tool, miRcode. In addition, ABCB9 was reported to be targeted by miR-31 (microRNA.org) (26).

Dual-luciferase reporter assay. The fragments from ENST00000547547 containing the predicted miR-31 binding site were synthesized and cloned into the luciferase construct psi-CHECK2. The resulted vector ENST00000547547-3'UTR-psi-CHECK2 was called the reporter vector ENST00000547547-3'UTR. The corresponding mutant was called the reporter vector Mut-ENST00000547547. The pre-miR-31 or negative control pre-Con was co-transfected with the reporter vectors using transfection reagent (Invitrogen Life Technologies). Forty-eight hours after transfection, firefly and *Renilla* luciferase activities in cell lysates were assessed using the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA).

RNA immunoprecipitation (RIP) assay. The RIP assay for ENST00000547547 and miR-31 was carried out with the EZ-Magna RIP kit (Millipore) using 5 mg of goat anti-AGO2 antibody (Santa Cruz Biotechnology, Inc.), according to the manufacturer's instructions. The IgG-MS2-based RIP assay was carried out using the Anti-IgG Affinity Gel (Sigma, St. Louis, MO, USA). The co-precipitated RNAs were extracted with TRIzol reagent (Invitrogen) and detected by qRT-PCR.

Statistical analysis. All statistical analyses were performed using SPSS 20.0 (IBM SPSS, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data are expressed as the mean ± standard deviation (SD) from at least three independent experiments. The paired t-test was used for statistical analyses between two groups, and one-way analysis of variance (ANOVA) was used for statistical analyses among three groups. P<0.05 was considered to indicate a statistically significant result.

Results

ENST00000547547 is downregulated in 5-FU-resistant CRC cell lines. In the present study, to confirm the chemosensitivity of 5-FU in 5-FU-resistant CRC and parent cells, an MTT assay was used for evaluation. It revealed that the cell survival rates of 5-FU-resistant CRC cell lines LoVo/5-FU (Fig. 1A) and HCT116/5-FU (Fig. 1B) were significantly higher than the parental cell lines when exposed to 5-FU. The IC_{50} values of 5-FU in LoVo/5-FU and HCT116/5-FU were 87.01 and 117.7 μM. However, the IC_{50} values of 5-FU in LoVo and HCT116 cells were 22.81 and 33.79 μM, which indicated 5-FU-resistant CRC cell lines were successfully constructed.

Then, we investigated the expression of ENST00000547547 in parental and 5-FU-resistant CRC cell lines by qRT-PCR (Fig. 1C). The results revealed that the expression level of ENST00000547547 was significantly downregulated in 5-FU-resistant CRC cell lines LoVo/5-FU and HCT116/5-FU compared with parental cells. Moreover, as shown in Fig. 1D, the expression of ENST00000547547 was elevated correlated

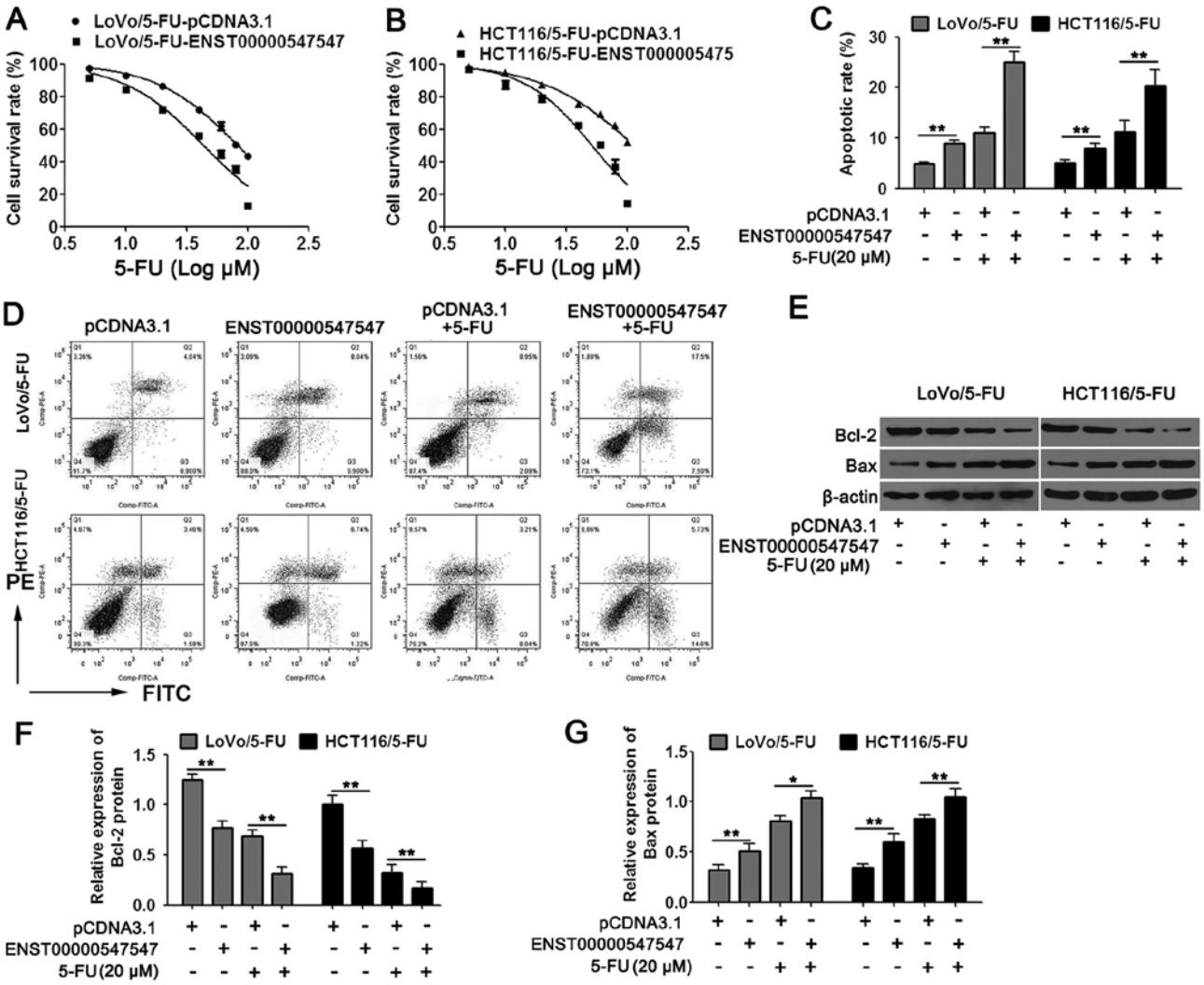

Figure 2. Overexpression of ENST00000547547 reduces the 5-FU resistance of CRC cell lines. (A and B) The cell survival rate was determined by MTT assay. All cell lines were exposed to various concentrations of 5-FU for 48 h. (C and D) Flow cytometric assays were used to analyze the cell apoptosis in ENST00000547547 overexpressing CRC cell lines with or without 5-FU treatment. (E-G) The protein levels of BCL-2 and BAX were assessed by western blot analysis. All experiments were performed in biological triplicates; $^{*}P<0.05$, $^{**}P<0.01$.

to the increasing dosage of 5-FU treatment in CRC cell lines. Collectively, these results indicated that ENST00000547547 was downregulated in 5-FU-resistant CRC cell lines and that it may play a role in 5-FU resistance.

Overexpression of ENST00000547547 reduces the 5-FU resistance of CRC cell lines. To determine the potential biological function of ENST00000547547 in the 5-FU resistance of CRC cell lines, we applied overexpression vector pCDNA3.1-ENST00000547547 to upregulate the expressions of ENST00000547547 in HCT116/5-FU and LoVo/5-FU cells. The resistance of 5-FU was evaluated based on survival rate by MTT assay (Fig. 2A and B). The IC_{50} values of 5-FU in ENST00000547547-overexpressed LoVo/5-FU and HCT116/5-FU cells were 43.21 μM and 51.31 μM and the IC_{50} of 5-FU in LoVo/5-FU-pCDNA3.1 and HCT116/5-FU-pCDNA3.1 were 82.35 μM and 114.2 μM. It was suggested that ENST00000547547 overexpression reduced 5-FU resistances in CRC cells. To further determine whether ENST00000547547 had an effect on 5-FU-induced cell apoptosis in 5-FU-resistant cells, flow cytometric assays were performed. The results revealed that overexpression of ENST00000547547 in HCT116/5-FU and LoVo/5-FUcells with 5-FU or without 5-FU treatment had higher apoptotic rates in comparison with the control cells (Fig. 2C and D). The Bcl-2 family members Bcl-2 and Bax are important anti- or pro-apoptotic regulators, respectively (27). To investigate whether the pro-apoptotic effect of ENST00000547547 was related to the Bcl-2 family, the expressions of Bcl-2 and Bax were examined. The western blot results revealed that Bcl-2 expression was decreased in ENST00000547547-overexpressed cells, whereas the expression of Bax was increased compared with the control groups (Fig. 2E-G). Collectively, these findings indicated that overexpression of ENST00000547547 reduced the 5-FU resistance of CRC cell lines, and this decreased 5-FU resistance related to the pro-apoptotic effect of ENST00000547547 on CRC cells.

ENST00000547547 directly binds to miR-31 and suppresses its expression. To investigate the relationship between ENST00000547547 and miR-31, we performed dual-luciferase reporter and RIP assays. As shown in

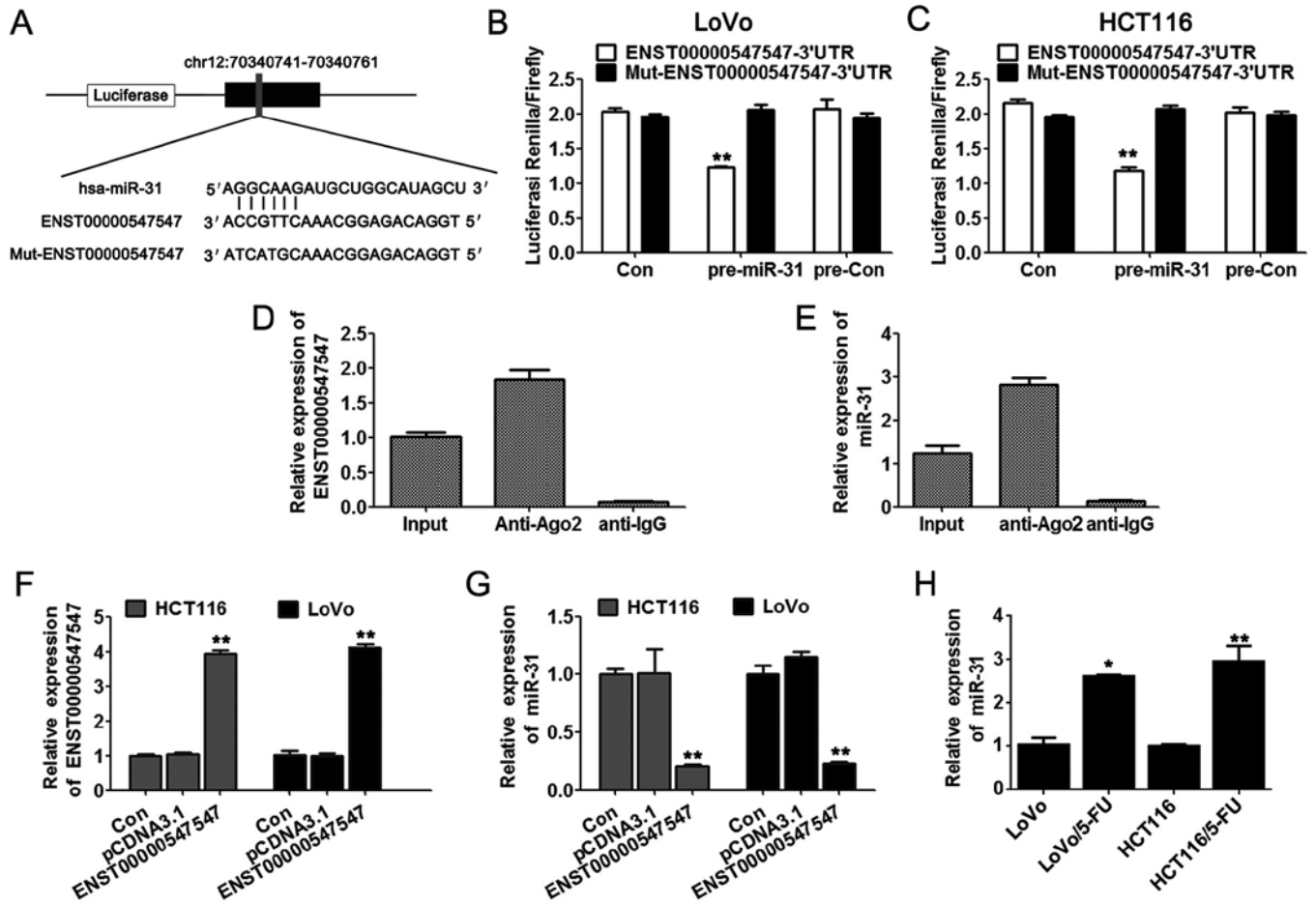

Figure 3. ENST00000547547 directly binds to miR-31 and suppresses its expression. (A) ENST00000547547 RNA contains a site complementary to miR-31, and the mutated form of ENST00000547547 sequence is revealed. (B and C) Luciferase activity was detected in CRC cells after transfection with miR-31 mimics (pre-miR-31) or miR-31 (negative control; pre-Con), and transfection with luciferase constructs of ENST00000547547-psi-CHECK2 or Mut-ENST00000547547-psi-CHECK2. Cells without miRNA transfection were used as a blank control (Con). (D and E) RIP analysis was performed in (D) LoVo and (E) HCT116 cells after transfection with miR-31. IgG was used as a negative control. The ENST00000547547 mRNA level was detected in the substrate of the RIP assay by qRT-PCR. (F and G) The expression of ENST00000547547 and miR-31 in the Con, pCDNA3.1 and pCDNA3.1-ENST00000547547 groups was examined by qRT-PCR. (H) The expression of miR-31 in CRC cells in parent and 5-FU-resistant CRC cells was examined by qRT-PCR. Data represent the mean ± SD from three independent experiments; *P<0.05, **P<0.01.

Fig. 3A, ENST00000547547 had a putative binding site for miR-31. Based on this bioinformatic prediction, psiCHECK2 containing the putative binding sequence and the mutant one were constructed for luciferase assay. The results indicated that miR-31 suppressed the luciferase activity of ENST00000547547-3' in comparison with the negative control, but it had little influence on the mutant both in HCT116 and LoVo cells (Fig. 3B and C). It is suggested that ENST00000547547 interacts with miR-31 via this putative binding site. Recent studies have shown that lncRNAs act as molecular sponges regulating the miRNA activity associatedwith micro-ribonucleoprotein complexes (miRNPs), which play critical roles in cytoplasmic miRNA assembly (28). Ago2 is a core component of the RNA-induced silencing complex (29). For further confirmation, we applied RIP assay to examine the interaction between ENST00000547547 and miR-31 in (Fig. 3D and E). The expression of ENST00000547547 was significantly enriched with anti-AGO2 in comparison with the input and anti-IgG, strongly indicating a direct interaction between ENST00000547547 and miR-31. Furthermore, we used the ENST00000547547-overexpressed cell lines to investigate whether the expression of miR-31 was regulated by ENST00000547547 (Fig. 2F). We found that the expression of miR-31 was significantly suppressed in the ENST00000547547-overexpressed CRC cell lines LoVo and HCT116 (Fig. 3G). To assess the role of miR-31 in the 5-FU-resistant cell lines, we examined the expression of miR-31 in LoVo/5-FU and HCT116/5-FU cells. As shown in Fig. 3H, the expression of miR-31 was significantly upregulated in LoVo/5-FU and HCT116/5-FU cells compared with their parental cell lines. Collectively, these results indicated that ENST00000547547 could directly bind to miR-31 to suppress its expression, and miR-31 may play an opposing role in 5-FU resistance compared with ENST00000547547.

Knockdown of miR-31 increases the 5-FU sensitivity of CRC cell lines. To explore the role of miR-31 in the 5-FU resistance of CRC cell lines, the miR-31 inhibitor was used for the knockdown of miR-31. As shown in Fig. 4A and B, with various concentrations of 5-FU treatments, both the

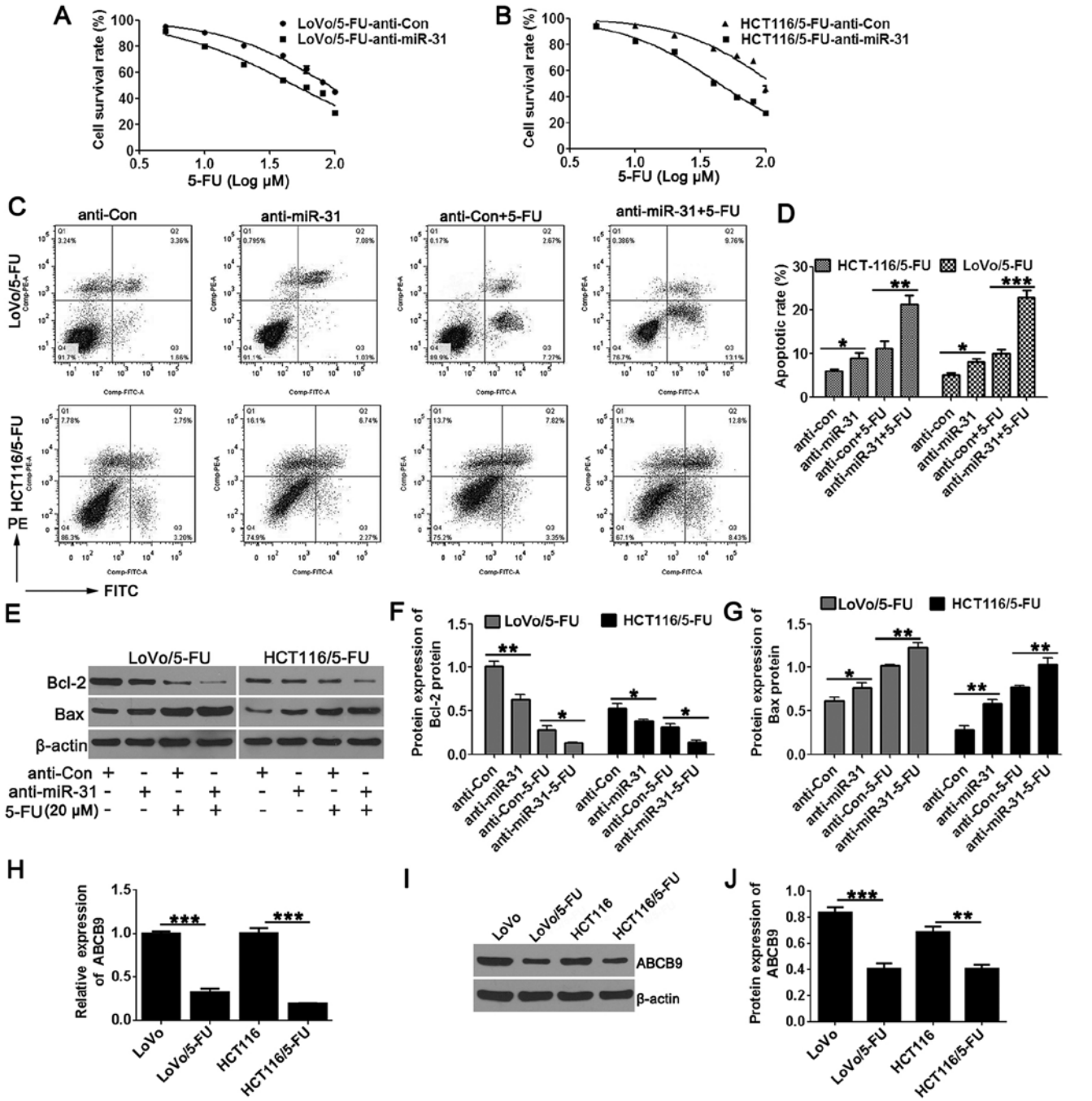

Figure 4. Knockdown of miR-31 increases the 5-FU sensitivity of CRC cell lines. (A and B) The cell survival rate of (A) LoVo/5-FU and (B) HCT116/5-FU cells transfected with a miR-31 inhibitor and a blank inhibitor vector was determined by MTT assay with treatments of 5-FU. (C and D) The apoptotic rates in the miR-31-downregulated CRC cell lines with or without 5-FU treatment were assessed by flow cytometric assays with Annexin V-FITC. (E-G) Western blot analyses were performed to detect the protein expression of Bax and Bcl-2 in the miR-31-downregulated CRC cell lines with or without 5-FU treatment. (H-J) The expression of ABCB9 was examined by (H) qRT-PCR and (I and J) western blot analysis. All experiments were performed in biological triplicates; *P<0.05, **P<0.01, ***P<0.001.

LoVo/5-FU (Fig. 4A) and HCT116/5-FU (Fig. 4B) transfected with miR-31 inhibitor vectors exhibited lower cell survival rates and reduced 5-FU resistance compared with the negative control. In addition, the IC_{50} values of 5-FU in the downregulated miR-31 LoVo/5-FU (43.79 μM) and HCT116/5-FU (48.72 μM) cells were much lower than that of LoVo/5-FU (112.2 μM) and HCT116/5-FU (87.01 μM) cells. Furthermore, apoptosis assays were performed to investigate whether miR-31 played an antagonistic role in apoptosis compared with ENST00000547547. The results revealed that the apoptotic rates of HCT116/5-FU and LoVo/5-FU cells were significantly improved by knockdown of miR-31 in comparison with the negative control after 5-FU treatment (Fig. 4C and D). Furthermore, using western blot analysis, we found that the expression of anti-apoptotic regulator Bcl-2 and pro-apoptotic regulator Bax were correspondingly affected by knockdown of miR-31 in HCT116/5-FU and LoVo/5-FU cells (Fig. 4E-G). Additionally, since the ATP-binding cassette, sub-family B, member 9 (ABCB9) is a transporter which was reported to be targeted by miR-31, involved in cisplatin-induced apoptosis (26),

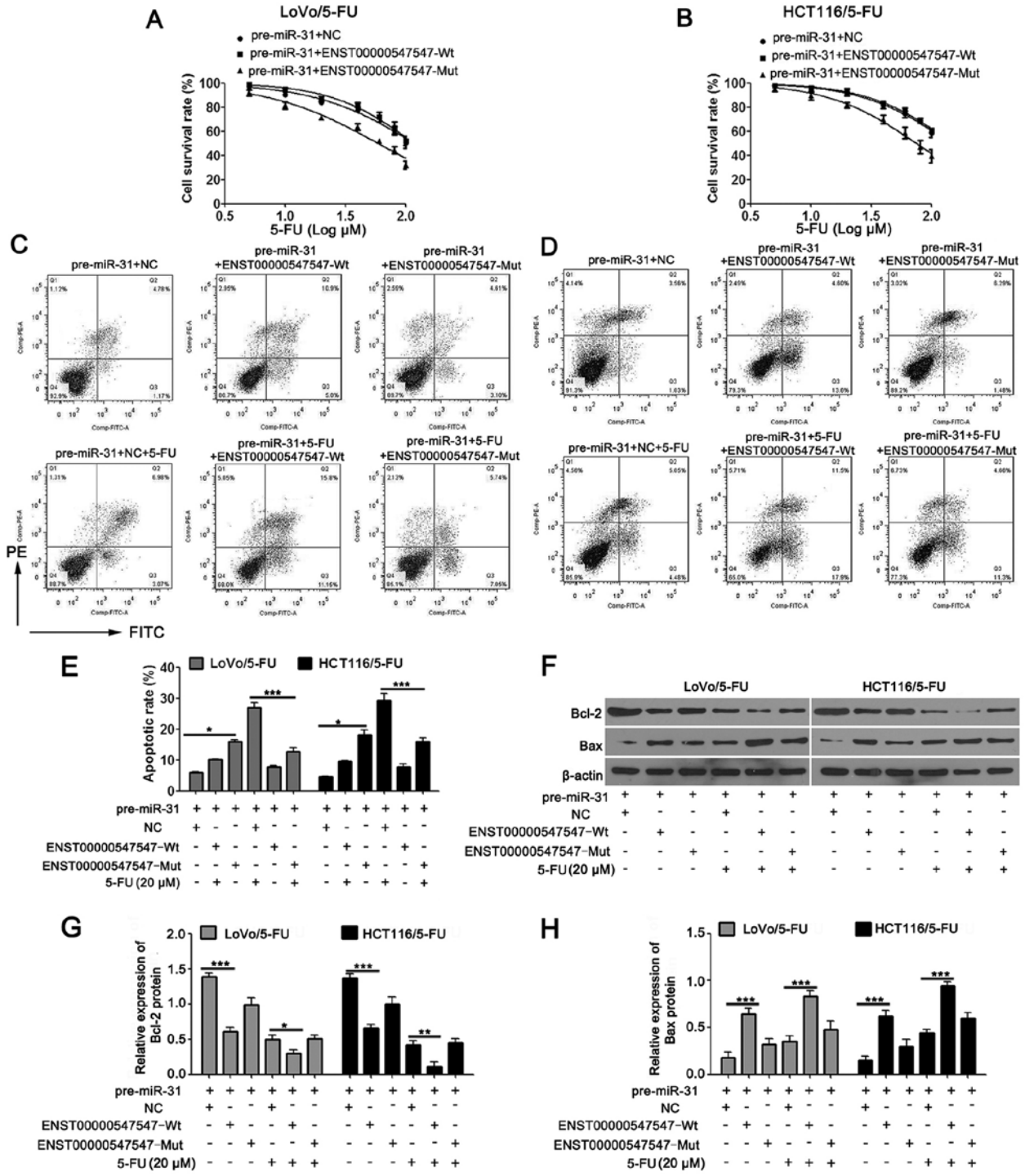

Figure 5. ENST00000547547 reduces the 5-FU resistance via competitive binding to miR-31. (A and B) Cell survival rates were calculated using MTT analysis. (C-E) The apoptosis analysis was conducted in the transfected (C) LoVo/5-FU and (D) HCT116/5-FU cells in miR-31-overexpressing cells in ENST00000547547-Wt or ENST00000547547-Mut with or without exposure to 5-FU for 48 h. (F-H) The protein levels of Bcl-2 and Bax in HCT116/5-FU and LoVo/5-FU groups were assessed by western blot analysis. The graphs are representative from three independent experiments and display the means ± SD; *P<0.05, **P<0.01, ***P<0.001.

we examined the expression of ABCB9 using qRT-PCR and western blot analysis, respectively. The results revealed that ABCB9 was markedly downregulated in HCT116/5-FU and LoVo/5-FU cells (Fig. 4H-J). These data indicated that knockdown of miR-31 increased the 5-FU sensitivity of CRC cells at least partly by upregulation of apoptosis, suggesting that miR-31 plays an antagonistic role in comparison with ENST00000547547 in the regulation of 5-FU resistance.

ENST00000547547 reduces the 5-FU resistance via competitive binding to miR-31. To determine the effect of the interaction between ENST00000547547 and miR-31 in 5-FU resistance, we co-transfected them into HCT116/5-FU and LoVo/5-FU cells. The MTT results indicated that ENST00000547547 overexpression attenuated the positive effect of miR-31 on 5-FU resistance both in LoVo/5-FU (Fig. 5A) and HCT116/5-FU cells (Fig. 5B), whereas the ENST00000547547

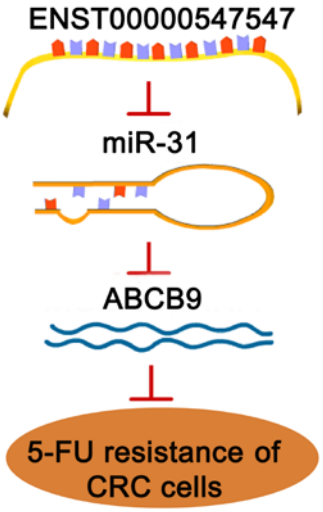

Figure 6. Schematic diagram of mechanism in the present study. ENST00000547547 promotes ABCB9 expression by acting as a sponge of miR-31 and reduces the 5-FU resistance of CRC cells.

with a mutant miR-31 binding site had little influence on the function of miR-31. To further explore whether the interaction between ENST00000547547 and miR-31 affected apoptosis of HCT116/5-FU and LoVo/5-FU cells under 5-FU treatment, we performed flow cytometric assays in these co-transfected CRC cell lines (Fig. 5C-E). The results demonstrated that overexpression of ENST00000547547 suppressed the anti-apoptotic effect of miR-31 via competitive binding to it in LoVo/5-FU and HCT116/5-FU cells. In contrast, the Mut-ENST00000547547 failed to reduce the anti-apoptotic effect of miR-31. Moreover, the expression of anti-apoptotic regulator Bcl-2 was reduced and pro-apoptotic regulator Bax was significantly improved in LoVo/5-FU and HCT116/5-FU cells that were transfected with pre-miR-31 and ENST00000547547 (Fig. 5F-H). Collectively, these results indicated that ENST00000547547 reduces the 5-FU resistance via competitive binding to miR-31 in CRC cells (Fig. 6).

Discussion

5-Fluorouracil (5-FU) is one of the most widely used anticancer agents for decades now, and 5-FU-based chemotherapy is recommended as the standard treatment for colorectal cancer (CRC) (30). It is well known that 5-FU enhances ROS production and induces marked apoptosis in 5-FU-sensitive cancer cells (31). However, the main obstacle to effective therapy with 5-FU is drug resistance (32). A large number of altered gene expression was found in the 5-FU-resistant CRC cells, and the microarray analyses of lncRNA and microRNA expression profiles indicated that 5-FU-based chemotherapeutic resistance in CRCs was associated with the changes of lncRNA and microRNA expression (33-35). In the present study, we found that the expression of ENST00000547547 was downregulated and miR-31 exhibited significant upregulation in the 5-FU-resistant CRC cells compared to the parent cells. In addition, ENST00000547547 reduced chemoresistance of 5-FU-resistant CRC cells through competitive binding to miR-31.

In the present study, we demonstrated for the first time that ENST00000547547 expression was downregulated in 5-FU-resistant CRC cell lines in comparison with the parental cell lines. In a previous study, the dysregulations of lncRNAs were found to be involved in the regulation of proliferation, apoptosis and drug sensitivity in human cancers (36). Cheng *et al* reported that overexpression of lncRNA HULC promoted proliferation and invasion, and it inhibited cell apoptosis in gastric cancer cells (9). Whereas, Pickard and Williams found that the expression of lncRNA GAS5 was positively correlated to cell responses to apoptotic stimuli, including chemotherapeutic agents, and the expression of GAS5 was directly proportional to the extent of cancer cell death (37). Similar to the functions of GAS5, our results indicated that overexpression of ENST00000547547 promoted apoptotic rates and improved the 5-FU sensitivity in 5-FU-resistant CRC cell lines, suggesting that ENST00000547547 played a positive role in enhancing the 5-FU sensitivity of 5-FU-resistant CRC cells.

The functions of microRNAs in tumor development and drug sensitivity have been extensively studied in recent years. Borralho *et al* demonstrated that miR-143 was downregulated in colon cancer, and upregulation of miR-143 was associated with increased sensitivity to 5-FU in CRC cells (32). Conversely, Valeri *et al* found that overexpression of miR-21 markedly reduced sensitivity to 5-FU and undermined the therapeutic efficacy of 5-FU in CRC cells (6). In a previous study, Xu *et al* found that expression of miR-31, along with miR-21, miR-96 and miR-135b, was upregulated in the CRC tissues compared to normal mucosal tissues (38). In addition, Wang *et al* indicated that suppression of miR-31 alone had no effect on cell proliferation. Notably, it improved the 5-FU sensitivity of CRC cell line HCT116 at an early stage (25). In the present study, our results demonstrated that knockdown of miR-31 reduced 5-FU resistance of both 5-FU-resistant CRC cell lines HCT116/5-FU and LoVo/5-FU, and upregulated apoptosis of these CRC cells, suggesting that miR-31 plays an antagonistic role in the regulation of 5-FU sensitivity, compared with ENST00000547547.

The RNA transcripts, including mRNAs, circular RNAs and lncRNAs have been identified as competing endogenous RNAs, which function as molecular sponges to inhibit the endogenous suppressive effect of their interacting microRNAs on target genes (39,40). In hepatocellular carcinoma, Deng *et al* reported that upregulation of lncRNA CCAT1 was correlated with tumor size, microvascular invasion and poor prognosis, and it promoted hepatocellular carcinoma progression by competitively binding to microRNA let-7 (41). In gastric cancer, Zhou *et al* revealed that miR-141 formed complementary base pairing with lncRNA H19, and the interaction between them induced translational suppression of a RLuc-H19 reporter gene (42). In CRC, particularly in drug resistance, the importance of the interaction between lncRNAs and microRNAs has rarely been addressed. Our results demonstrated that transcript levels of ENST00000547547 and miR-31 were negatively correlated in CRC cells. In addition, we indicated a direct interaction between ENST00000547547 and miR-31 using dual-luciferase reporter and RIP assays. Moreover, we clarified that by competitive binding to miR-31, ENST00000547547 inhibited the effect of miR-31, which was reducing the 5-FU sensitivity and apoptosis of CRC cells, and ENST00000547547 played a positive role in promoting 5-FU sensitivity of CRC cells.

Although the present study still has some short comings it may still provide some direction for further research. Firstly, we only demonstrated that one lnRNA reduced resistance to one drug. However, in our preliminary experiment, we found that oxaliplatin also upregulated the expression of ENST00000547547, only its effect was lower than that of 5-FU (data not shown). Secondly, we only found that Bax and Bcl-2 played an important role in the regulation of cell apoptosis of 5-FU-resistant CRC cells. Further apoptosis makers and mechanisms need to be examined. In addition, knockdown of miR-31 may upregulate the expression of lncRNA ENST00000547547 due to the competitive relationship of ENST00000547547/miR-31/ABCB9, however this needs to be ascertained by further experiments (43-45). Furthermore, animal experiments and the mechanism of 5-FU resistance in CRC for patients receiving clinical treatment also need to be researched in the future.

In conclusion, the present study demonstrated for the first time that lncRNA ENST00000547547 expression was downregulated in 5-FU-resistant CRC cells, and its overexpression was associated with the reduction of 5-FU resistance. Knockdown of miR-31 improved 5-FU sensitivity and the apoptotic rate of 5-FU resistance of CRC cells. Furthermore, ENST00000547547 promoted 5-FU sensitivity of 5-FU-resistant CRC cells by directly binding to miR-31. Collectively, our findings indicated that lncRNA ENST00000547547 may be a positive prognostic factor for 5-FU-based chemotherapy and is a potential target for CRC therapy.

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