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Original Article

MicroRNA-135b regulates apoptosis and chemoresistance in colorectal cancer by targeting large tumor suppressor kinase 2

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Abstract: Colorectal cancer remains the third most common cause of death from cancer worldwide. MicroRNA emerges as a good area of research for current cancer therapy. Here, we identified miR-135b to be a contributor to anti-apoptosis and chemoresistance in colorectal cancer. We observed high levels of miR-135b in colorectal cancer cell lines and clinical tissues, compared to colorectal epithelium cell line and noncancerous tissues. Furthermore, enforced expression of miR-135b attenuated doxorubicin-induced apoptosis in colorectal cells. (Doxorubicin alone can trigger significant apoptosis). In elucidating the molecular mechanism by which miR-135b participate in the regulation of apoptosis and chemoresistance in colorectal cancer, we discovered that large tumor suppressor kinase 2 (LATS2) is a direct target of miR-135b. The role of miR-135b was confirmed in colorectal tumor xenograft models. The growth of established tumors was suppressed by an inhibition of miR-135b expression and enhanced apoptosis was further assessed by TUNEL assay. Taken together, our results reveal that miR-135b and LATS2 axis may be a novel therapeutic target for colorectal cancer.

Keywords: miR-135b, chemoresistance, colorectal cancer, LATS2

Introduction

Colorectal cancer (CRC) is the third most common cause of death from cancer [1]. Chemotherapeutic agents serve as important adjuvant therapies for CRC treatment. However, cytotoxicity and multidrug resistance (MDR), which cause cancer cells to become resistant to a broad spectrum of chemotherapeutics, are major obstacles for clinical usage, highlighting the necessity for the development of novel drugs. MicroRNAs (miRNAs), which are a series of small noncoding RNAs of 19 to 22 nucleotides, are involved in many important cellular processes such as development, differentiation, proliferation, cell cycle progression, apoptosis, inflammation, and stress response [2-4]. Since expression alterations of some miRNAs are associated with a series of genetic events, their expression patterns may either have pro- or anti- cancer effects [5-7]. Direct blockade of miRNAs may serve as a therapeutic intervention against a cascade of events driving oncogenesis, and may improve therapeutic efficacy in chemo-resistant patients [8].

Elevated expression of miR-135b has been reported in a variety of cancers [9-11]. Microarray analysis and quantitative reverse transcription-PCR (qRT-PCR) studies have demonstrated that miR-135b upregulation is far more robust in non-small cell lung cancer (NSCLC) [9] and in head and neck squamous cell carcinoma (HNSCC) [10]. Studies in CRC also demonstrate

a distinct increase in expression of miR-135b in both adenomas and carcinomas compared with normal epithelium [11]. MiR-135b expression was up-regulated from normal tissue to polyp to carcinoma, suggesting miR-135b deregulation may be involved in an early event of increasing dysplasia in CRC. MiR-135b is located on 1q32.1 and encoded in the first intron of LEMD1 gene. 1q32.1 was frequently shown to gain DNA copy number in CRC progression [12], and LEMD1 gene was reported to be highly expressed in CRC compared with control tissue [13]. Furthermore, previous studies indicate that miR-135b promote colorectal tumor transformation and progression [14, 15]. We have also found that inhibiting miR-135b expression can induce CRC cells to undergo apoptosis [16], but the exact mechanisms involved, and the relationship of miR-135b expression to CRC cell chemoresistance remains unclear.

In this study, we focused on the function of miR-135b in CRC cells with respect to apoptosis and chemoresistance, pushing miRNA-based therapeutics closer to clinical usage. We found that miR-135b could regulate CRC cell proliferation, apoptosis and chemoresistance through negatively regulating LATS2 expression. These results were confirmed in human CRC samples and xenograft tumor models. Our results identify a novel regulatory pathway for apoptosis involving miR-135b and possibly provide valuable insight into cancer therapy.

Materials and methods

Reagents and cell culture

Doxorubicin was purchased from Sigma (St. Louis, MO, USA). Anti-LATS2 antibody was obtained from Santa Cruz Biotechnology (Texas, USA). Anti-cleaved caspase-3 antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Human colorectal cancer cell line SW480, LOVO, COLO205 and HT29 were obtained as we described previously [16]. The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Cancer samples

A total of 18 patients - 10 males (ages ranging from 42 to 79 years, with a mean age of 58 years) and 8 females (ages ranging from 46 to 67 years, with a mean age of 59 years) diagnosed with CRC by colonoscopy were enrolled in this study from Beijing Military General Hospital (Beijing, China). These patients were randomly selected from the patient pool of the hospital's gastrointestinal biobank; none of the subjects had undergone chemotherapy or radiotherapy. During colonoscopy, four or five samples from cancer tissues and adjacent non-cancer tissues, respectively, were obtained and stored in liquid nitrogen. All tissue samples were reviewed and evaluated by a gastrointestinal pathological expert Jianqiu Sheng. The study was approved by the ethics committee of the Beijing Military General Hospital. Informed consent was obtained from all study subjects.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Cell viability was determined by MTT assay, which was performed as we described previously [17]. The growth characteristics of cells were assessed in the same culture medium. Cells were seeded into 96-well plates at a density of $3x10^3$/well and cultured at 37°C. After 48 hours, trypan blue-positive and blue-negative cells were counted on a hemocytometer. TUNEL assay was performed to detect the apoptotic cell death using a kit from Roche Applied Science (Hamburg, Germany). The procedures were followed as per the instructions in the kit. The samples were imaged using a laser scanning confocal microscope (Zeiss LSM 510 META, Carl Zeiss, Jena, Germany).

Cell transfection with miRNA duplexes or miRNA inhibitors

The hsa-miR-135b duplexes were synthesized by GenePharma Co. Ltd (Shanghai, China). MiR-135b mimic sequence was 5'-UAUGGCUUUUCAUUCCUAUGUGA-3'. Mimic control sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. Chemically modified antisense oligonucleotides (antagomirs) were used to inhibit endogenous miR-135b expression. The antagomir sequence was 5'-UCACAUAGGAAUGAAAAGCCAUA-3'. The antagomir control sequence was 5'-CAGUACUUUUGUGUAGUACAA-3'. All the bases were 20-O-methyl-modified (GenePharma Co. Ltd). Cells were transfected with miRNA duplexes (100

nM) or antagomirs (100 nM) using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions.

Reporter constructions and luciferase assay

The fragment of LATS2 3'-UTR containing one miR-135b binding site was amplified by PCR. The forward primer was 5'-CTGGAATTCGAAGTGTGAGCAAGGTGATG-3', and the reverse primer was 5'-ACGACTAGTGACTTGAGTATGCCACTCAC-3'. PCR product was cloned downstream of the stop codon of the luciferase gene of pGL3 vector (Promega, Madison, WI, USA) to generate reporter vector containing miR-135b binding sites. To generate LATS23'-UTR-Mut, the mutations (wild-type LATS23'-UTR site: GCCA, LATS2 3'-UTR-Mut: CAGT) were produced using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The binding site of miR-135b was mutated simultaneously to generate LATS2 3'-UTR-Mut.

Luciferase assay in HEK-293 cells was performed as we described previously [18]. Cells in 24-well plates were co-transfected with 200 ng per well luciferase reporter constructs, 400 ng per well miR-135b mimics, or control mimics using Lipofectamine 2000 (Invitrogen). SV-Renilla luciferase plasmids of 5 ng per well served as the internal control. Cells were harvested at 24 hours after transfection and the luciferase activity was detected using the Dual Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instructions. 30 ml of protein samples were analyzed in a luminometer. Firefly luciferase activity was normalized to Renilla luciferase activity.

LATS2 RNA interference (RNAi)

Small interfering RNA (siRNA) oligonucleotides specific for LATS2 were designed using Ambion's siRNA design tool, and purchased from GenePharma Co. Ltd (Shanghai, China). The LATS2 siRNA sense sequence is 5'-TCACCTCGCCCAATAACAA-3'; the LATS2 siRNA antisense sequence is 5'-TTGTTATTGGGCGAGGTGA-3'. The scramble LATS2 siRNA sense sequence is 5'-TCACGCTAACCAATCCCAA-3'; the scramble LATS2 siRNA antisense sequence is 5'-TTGGGATTGGTTAGCGTGA-3'. The specificity of the oligonucleotides was confirmed by comparing them with all other sequences in Genbank using Nucleotide BLAST. Transfection of siRNAs was performed using LipofectamineTM 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions.

Immunoblotting

Immunoblotting was performed by having cells washed with phosphate-buffered saline, and then lysed in extraction-buffer (Biosource, Camarillo, CA) [16]. Protein concentration was determined with BCA (Pierce, Rockford, IL). An aliquot of sample (25 mg) was separated on a 10% SDS-PAGE, and then transferred to a nitrocellulose membrane. Blots were probed using corresponding primary antibodies. Then, horseradish peroxidase-conjugated secondary antibodies were used, and then visualized with chemiluminescence. The bands detected in Western blotting were analyzed with Totallab 2.0 software.

Quantitative real-time PCR (qRT-PCR)

Stem-loop qRT-PCR was carried out as described on an Applied Biosystems ABI Prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) [16]. The total RNA was extracted with Trizol (Invitrogen., Carlsbad, CA). RNA concentrations were measured with a spectrophotometer (NanoDrop™ 2000) and RNA integrity was analyzed with gel electrophoresis. Before cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis Kits (Fermentas., Vilnius, Lithuania), total RNA (one mg) was treated with DNase I (Invitrogen., Carlsbad, CA) to remove DNA contamination. The RT primer for miR-135b was: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAT. Mature miR-135b levels were measured using SYBR Green Real-Time PCR Master Mix (Toyobo) according to the manufacturer's instructions. The sequences of miR-135b primers were: forward, 5'-GCTTATGGCTTTTCATTCCT-3'; reverse, 5'-GTGCAGGGTCCGAGGT-3'. The levels of miR-135b analyzed by qRT-PCR were normalized to that of U6. The sequences of U6 primers were: forward, 5'-CTCGCTTCGGCAGCACA-3'; reverse, 5'-AACGCTTCACGAATTTGCGT-3'. Quantitative detection of LATS2 was performed using the same strategy. The primers used for LATS2 were: forward, 5'-CTGGAATTCGAAGTGTGAGCAAGGTGATG-3'; reverse, 5'-ACGACTAGTGACTTGAGTATGCCACTCAC-3'. The mRNAs levels were normalized to that of β-actin and the sequences were: forward, 5'-CATGTACGTTGCTATCCAGGC-3'; reverse, 5'-CTCCTTAATGTCACGCACGAT-3'.

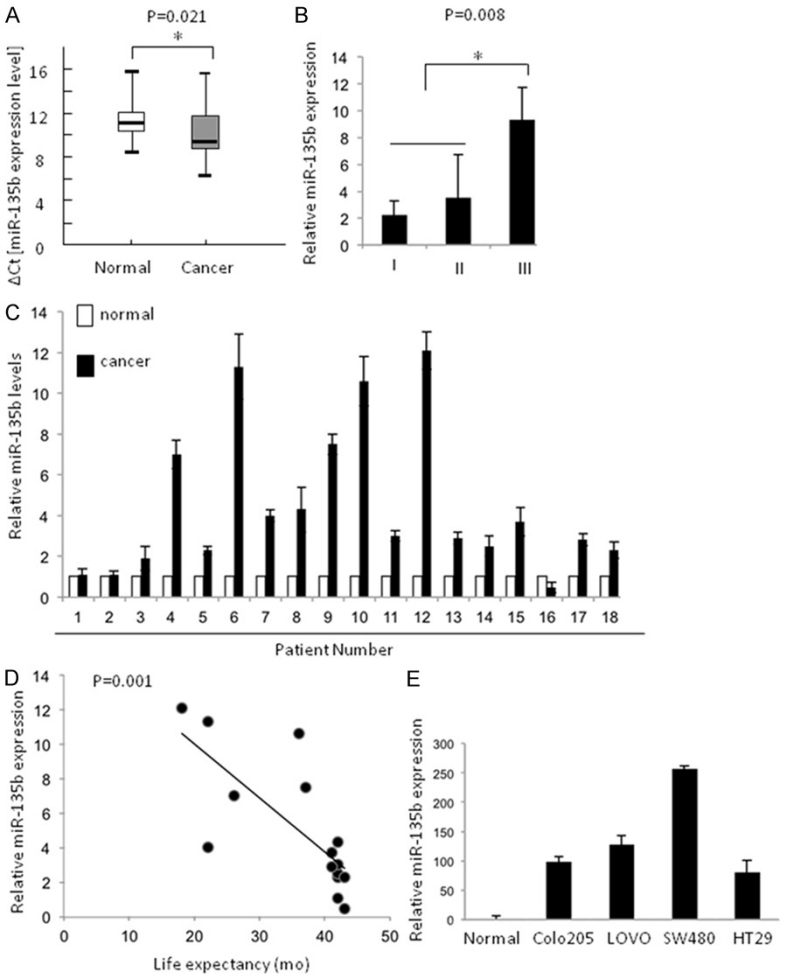

Figure 1. MiR-135b was upregulated in colorectal cancer cell lines and cancer tissues. A. MiR-135b expression levels in 18 pairs of human colorectal cancer tissues and matched normal tissues. The boxes represent lower and upper quartiles separated by the median, and the excluding point was marked as a dot. B. Box plots showed that the expression of miR-135b was higher in advanced carcinomas (stage III) than in early carcinomas (stage I and II). C. The expression level of miR-135b in normal tissue of each patient was assigned as one. D. A positive correlation between miR-135b expression and patients' life expectancy was indicated. E. Real-time polymerase chain reaction (PCR) analysis of miR-135b levels in normal tissues and four colonic cancer cell lines. Each determination was performed in triplicate. The mean ± SD is shown. The miRNA abundance was normalized against an endogenous U6 RNA control.

Subcutaneous tumor xenograft model

For the experiments in **Figure 6A**, approximately 1×10^7 SW480 cells were injected subcutaneously into the right flanks of female BALB/c nude mice (4-5 weeks old) [18]. When tumors reached an average volume of 400-500 mm^3, the mice were randomly divided into 2 groups with 5 mice in each group. According to the experimental design, intratumoral injection of miR-135b antagomir or antagomir control was administrated every 3 days. The tumor size was measured with a caliper every 3 days. The tumor volume was calculated using the formula volume = length × width^2/2. The mice were killed at day 18 after the first injection, and the tumors were separated for further analysis.

Histology

We collected xenograft tumors and fixed them in 4% paraformaldehyde overnight. Then the tumors were embedded in paraffin and cut into 6 μm thick sections. TUNEL staining was performed according to manufacturer's instructions (Roche Applied Science). The total nuclei were stained using DAPI. 400× magnification photos were taken using a laser scanning confocal microscope, and 10 random fields were quantified by an investigator who was blind to the treatment.

Statistical analysis

All statistical analyses were performed using the SPSS 13.0 statistical software package. The results are expressed as mean ± S.D. of at least three independent experiments. The differences among experimental groups were evaluated by one-way analysis of variance. Correlation analysis was performed with Pearson's test. Paired data were determined by two-tailed Student's t-test. $P<0.05$ was considered statistically significant.

Results

MiR-135b was upregulated in colon cancer cell lines and cancer tissue

Elevated expression of miR-135b has been reported in a variety of cancers [9-11, 14-16,

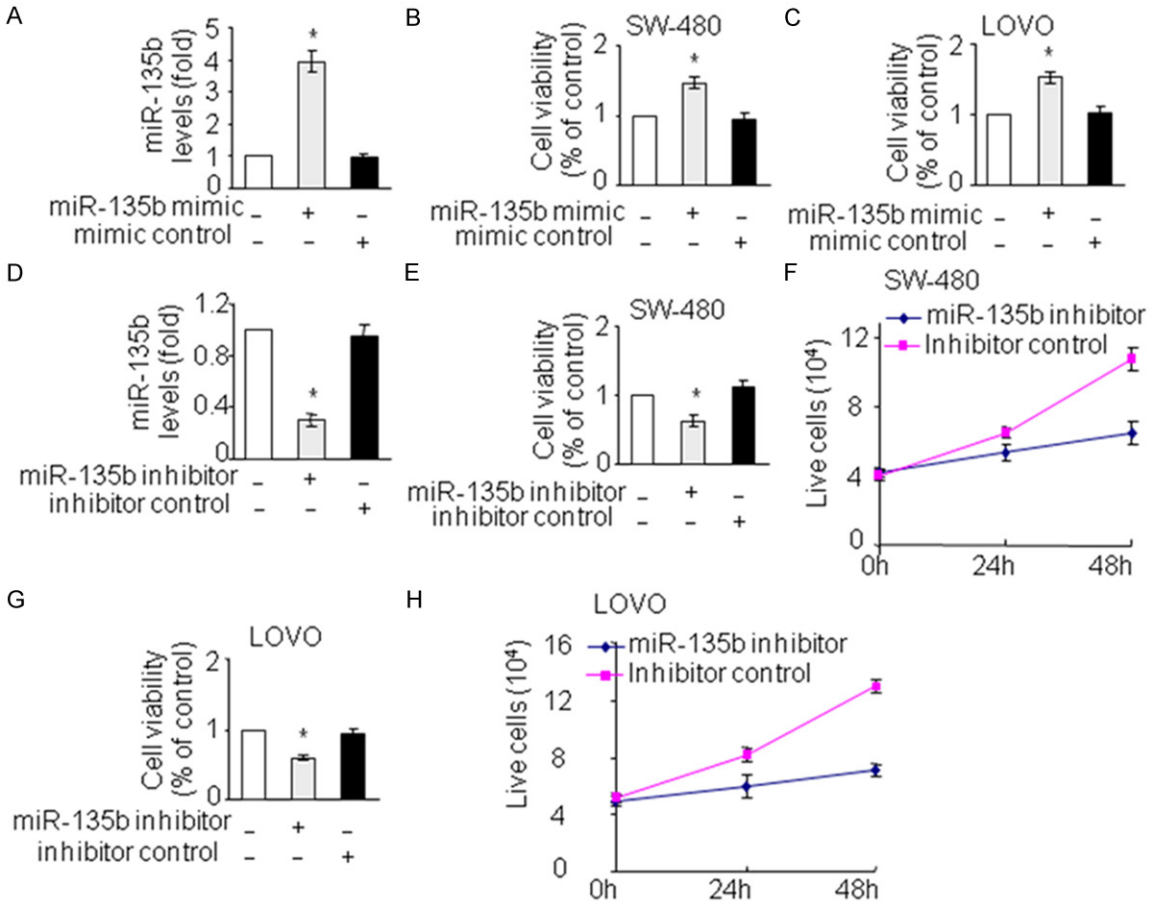

Figure 2. MiR-135b induced proliferation in CRC cells. (A, B) Enforced expression of miR-135b increases cell proliferation in SW-480 cells. SW-480 cells were transfected with miR-135b mimics or control mimics, and the levels of miR-135b were detected by qRT-PCR (A) 24 hours after transfection. Cell proliferation was detected by MTT assay 48 after transfection (B). (C) Enforced expression of miR-135b increases cell proliferation in LOVO cells. LOVO cells were treated as in B. and cell proliferation was detected by MTT assay. (D-F) Inhibition of endogenous miR-135b prevents cell proliferation in SW-480 cell. SW-480 cells were transfected with miR-135b antagomir or antagomir control, and the levels of miR-135b were detected by qRT-PCR (D) 24 hours after transfection. Cell proliferation was detected by MTT assay 48 after transfection (E). Trypan blue exclusion assay was performed to determine the number of live cells in the indicated time after transfection (F). (G, H) inhibition of endogenous miR-135b prevents cell proliferation in LOVO cells. LOVO cells were treated as in D, and cell proliferation and live cell number were detected as described above. The results are expressed as the mean ± SD; n=3, *p<0.05 compared with the control.

19-21]. We have also reported that inhibiting miR-135b expression can induce CRC cells to undergo apoptosis [16]. In this study, we analyzed 18 pairs of human colorectal cancer tissues and matched adjacent noncancerous tissues to explore the role of miR-135b in human colorectal cancer. RNAs were isolated from biopsied tumor tissues and benign tissues of patients with CRC. Analysis of miR-135b with real-time PCR revealed that miR-135b levels were significantly upregulated in CRC tissues compared with benign tissues (**Figure 1A**). Furthermore, we detected that the miR-135b expression was higher in advanced carcinomas (stage III) than in early carcinomas (stage I and II) (**Figure 1B**). An obvious upregulation (>2 folds) was observed in 15 of 18 patients (**Figure 1C**). Out of all the patients, 15 received chemotherapy after the surgery. With a follow up of 3.5 years, 6 patients total had passed. There was a positive correlation between miR-135b expression and response to chemotherapy and therefore patients' life expectancy (**Figure 1D**). In parallel, miR-135b expression was also upregulated in all four collected colorectal cancer cell lines, compared to its expression in the first patient's benign tissue, in which relative miR-135b level was not upregulated (**Figure**

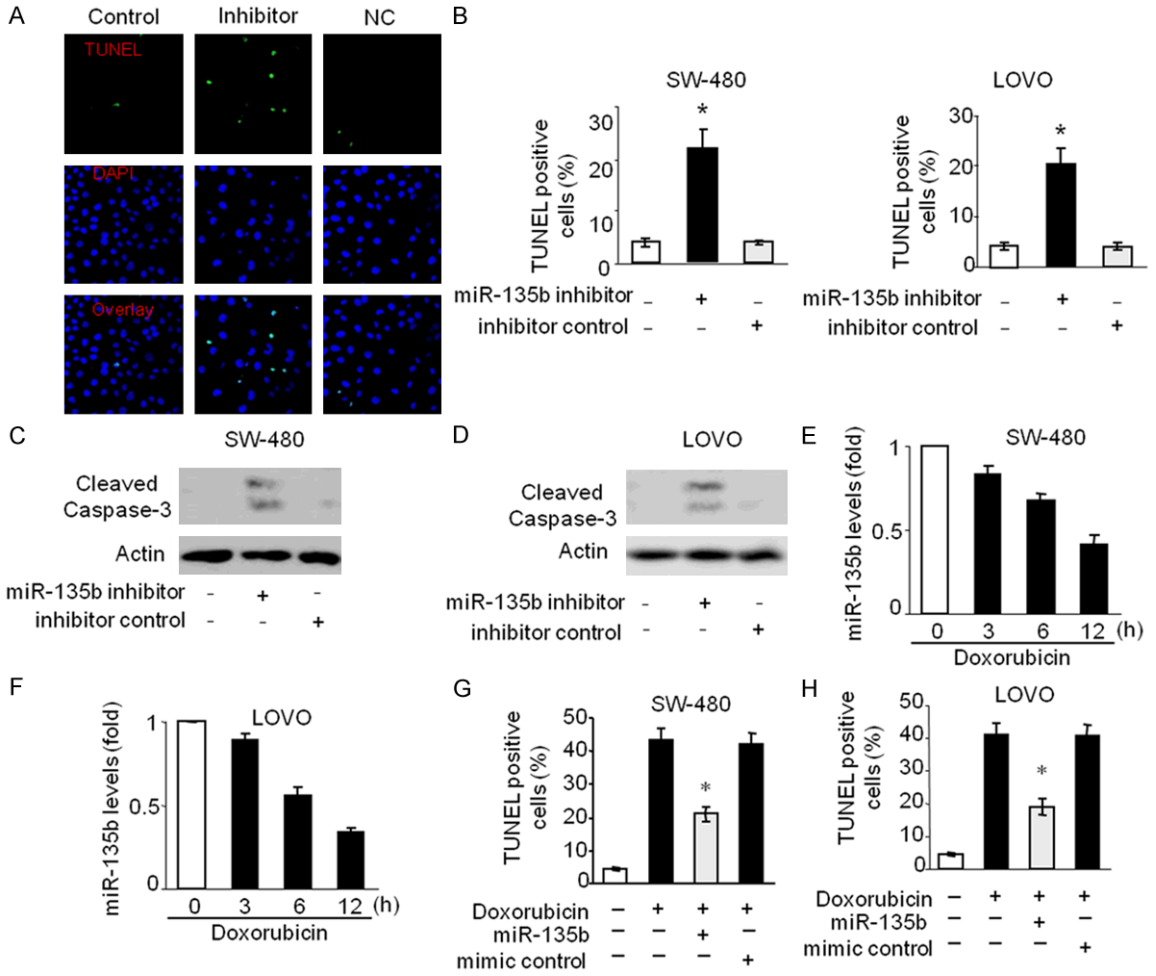

Figure 3. miR-135b regulates apoptosis and drug-resistance. (A and B) Inhibition of endogenous miR-135b induced apoptosis in SW480 (A) and LOVO cells (B), detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Green, Representative photos showed TUNEL positive cell (A, left). TUNEL-positive nuclei; blue, 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei, scale bar=50 μm. (C and D) Cleaved caspase-3 was analyzed by Western blot in SW480 (C) and LOVO cells (D) transfected with miR-135 antagomir. (E and F) MiR-135b levels were detected in SW480 (E) and LOVO cells (F) treated with 2 mM doxorubicin (DOX) at the indicated time.*p<0.05 compared with 0 h. (G and H) Ectopic expression of miR-135b attenuated doxorubicin-induced apoptosis in SW480 (G) and LOVO cells (H) after 2 μM doxorubicin treatment for 36h. The results are expressed as the mean ± SD; n=3, *p<0.05 compared with the control.

1E). These results imply a role of miR-135b in the pathogenesis and chemoresistance of CRC.

MiR-135b promotes the proliferation of CRC cells

MiR-135b could regulate ECM formation and somatic cell reprogramming and may be a powerful tool in dissecting the intracellular and extracellular molecular mechanisms of reprogramming [22]. It has been reported that miR-135b could enhance cancer cell invasive and migratory abilities [9, 20] and radioresistance [19]. To explore the potential role of miR-135b in the pathogenesis and chemoresistance in human colorectal cancer, we first detected the effect of miR-135b on proliferation in CRC cells. SW-480 (**Figure 2A**) and LOVO cells (data not shown) were transiently transfected with miR-135b mimics or control mimics and the expression levels of miR-135b were confirmed by quantitative RT-PCR (qRT-PCR). MTT assay revealed that miR-135b overexpression in SW-480 and LOVO cells promoted an increment in cell proliferation (**Figure 2B** and **2C**). To better understand the effect of endogenous miR-135b on CRC cells, we attempted to compare the cell proliferation before and after inhi-

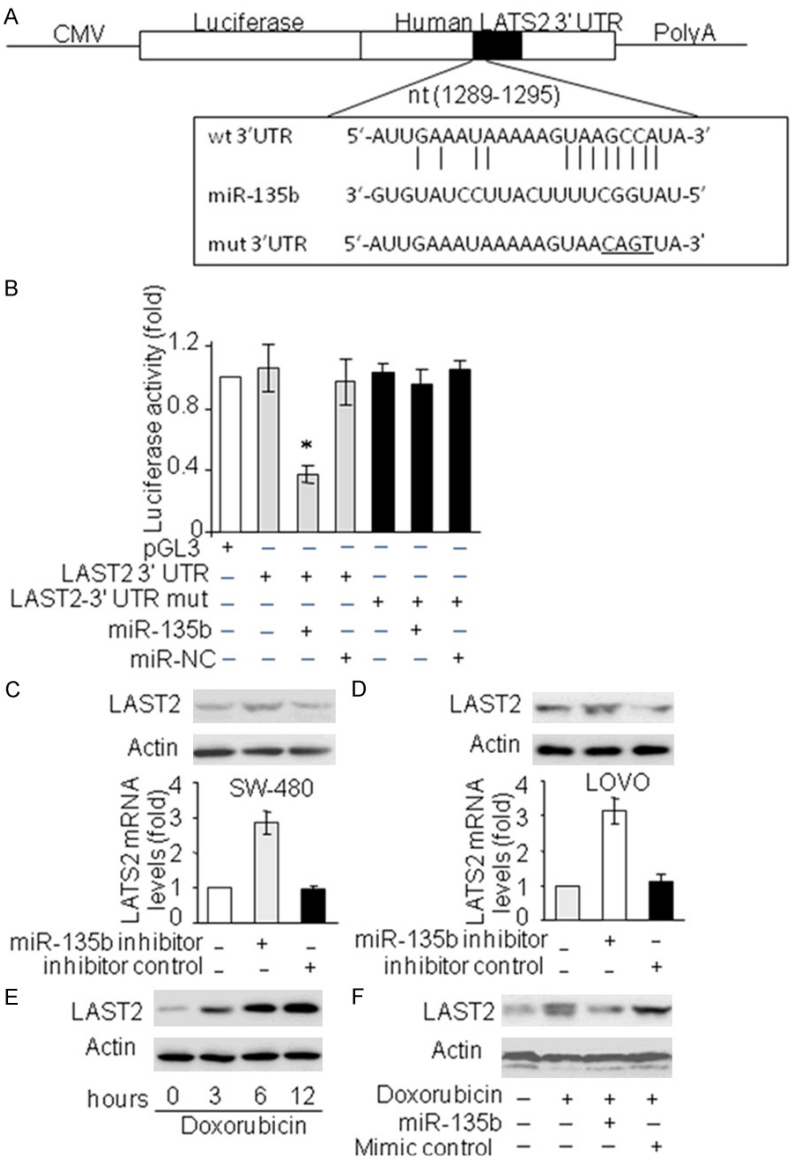

Figure 4. LATS2 is a direct target of miR-135b. (A) Schematic diagram of the reporter constructs containing one putative miR-135b binding site in human LATS2 3'-UTR. Mutations introduced into miR-135b binding sites were underlined. (B) Luciferase activity detected in HEK-293 transfected with miR-135b mimics or control mimics, along with luciferase reporter constructs as indicated. (C and D) LATS2 mRNA levels and protein levels in SW480 (C) and LOVO cells (D) inhibit miR-135b. (E) MiR-135b levels in SW480 (E) and LOVO cells (data not shown) after 2 mM doxorubicin (DOX) treatment as the indicated time.*p<0.05 compared with 0 h. (F) Overexpression of endogenous miR-135b attenuated increase of LATS2 protein levels upon doxorubicin (DOX) treatment for 12 h in SW480 (F) and LOVO cells (data not shown). Error bars represent S.D. *P<0.05. Mut, mutated; Wt, wild type; NC, negative control.

bition of miR-135b. MiR-135b antagomir was transfected into SW-480 to inhibit the endogenous miR-135b. MiR-135b levels were reduced by its specific antagomir (**Figure 2D**). Inhibition of the endogenous miR-135b led to a significant reduction in cell proliferation (**Figure 2E** and **2F**). Similar results were obtained in LOVO cells (**Figure 2G** and **2H**).

MiR-135b regulates apoptosis and chemoresistance in CRC cells

MiR-135b promotes the proliferation of CRC cells, but the role of miR-135b in CRC cell apoptosis and chemoresistance is not fully understood. We next investigated the function of endogenous miR-135b on apoptosis and chemoresistance. To characterize the function of endogenous miR-135b on cell survival, miR-135b antagomir was transfected into SW-480 and LOVO cells. TUNEL assay demonstrated that miR-135b inhibitor introduced significant apoptosis compared to the control group (**Figure 3A** and **3B**). To further confirm the effect of miR-135b on apoptosis in colorectal cancer cell lines, cleaved caspase-3 was detected in SW-480 and LOVO cell lines. These results suggest that cleaved caspase-3 was activated in both cell lines transfected with miR-135b inhibitor compared with control inhibitor (**Figure 3C** and **3D**). Doxorubicin, a commonly used DNA damage agent, can induce CRC cells to apoptosis. When exposed to doxorubicin (2 mM), miR-135b levels were markedly decreased in SW-480 (**Figure 3E**) and LOVO cells (**Figure 3F**). The inhibition of miR-135b expression induced by doxorubicin treatment led us to consider whether doxorubicin induced apoptosis was correlated with miR-135b inhibition. To characterize the function of

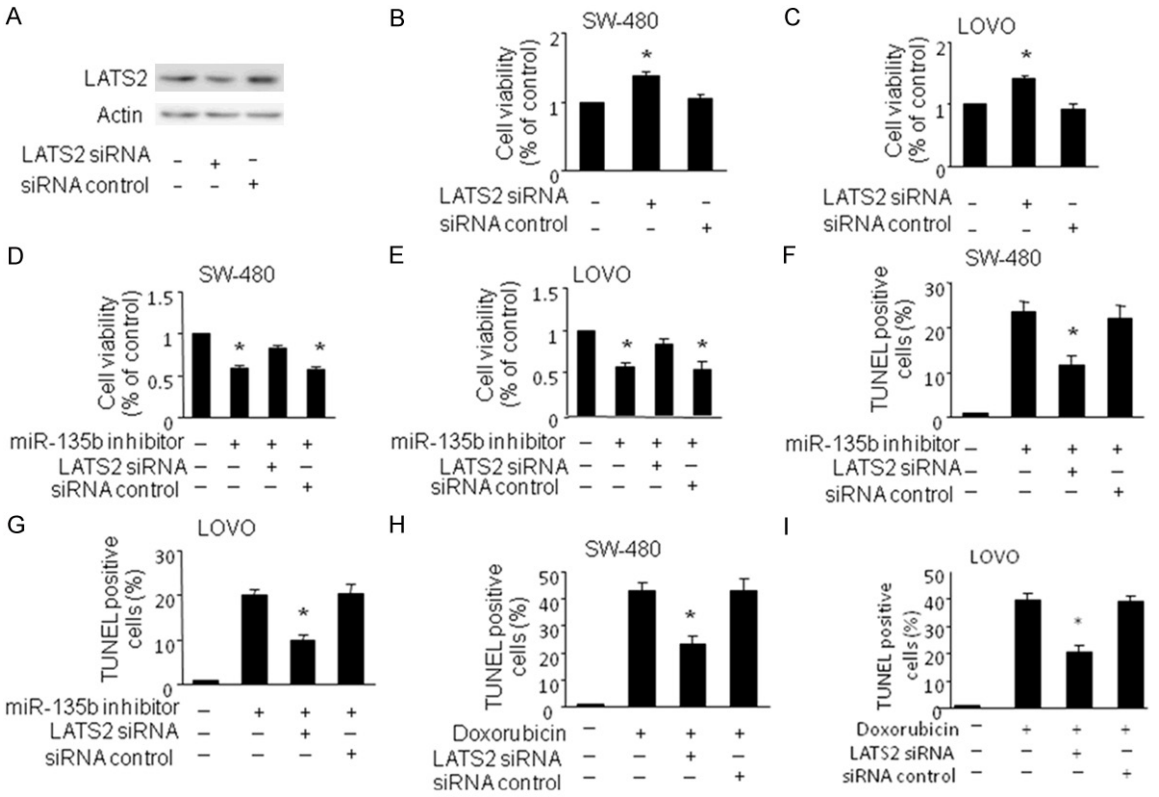

Figure 5. MiR-135b regulates chemotherapeutic resistance through LATS2. (A-C) Knockdown of endogenous LATS2 using its small interfering RNA (siRNA) significantly reduces LATS2 protein expression (A), and promotes SW480 (B) and LOVO cell (C) proliferation. In production of miR-135b inhibitor and knockdown of endogenous LATS2, cell proliferation was not reduced significantly (D and E) and a limited amount of cells undergoing apoptosis was observed (F and G). Knockdown of endogenous LATS2 attenuated apoptosis in SW480 (H) and LOVO cells (I) treated with doxorubicin for 36 h, compared with siRNA control group. Error bars represent S.D. *P<0.05.

endogenous miR-135b in the mediation of doxorubicin-induced apoptosis, miR-135b mimics were transfected into SW480 and LOVO cells, and apoptosis was obviously attenuated (**Figure 3G** and **3H**). Taken together, these results suggest that miR-135b has an effect on apoptosis and chemoresistance in colorectal cancer cells.

LATS2 is a target of miR-135b

To elucidate the possible mechanisms of how miR-135b regulates apoptosis and chemoresistance, we performed a bioinformatic analysis on TargetScan and found that human LATS2 mRNA contains a binding site for miR-135b (**Figure 4A**). To verify whether miR-135b directly targets LATS2, we cloned LATS2 3'-UTR containing miR-135b binding sites downstream of the luciferase reporter gene (LATS2 3'-UTR-WT) to examine luciferase translation. MiR-135b mimics or control miRNA mimics were transfected into HEK-293 cells to perform the luciferase assays. MiR-135b overexpression induced a decrease in the luciferase activity (**Figure 4B**). We also generated mutated luciferase constructs (LATS2 3'-UTR-Mut), and introduced these constructs into the miR-135b binding site of LATS2 3'-UTR. We found that the binding site was responsible for the role of miR-135b in regulating LATS2 expression (**Figure 4B**). Furthermore, we attempted to investigate if miR-135b modulates LATS2 expression. Reduced expression of miR-135b resulted in a significant elevation of both LATS2 mRNA and protein levels in SW480 and LOVO cells (**Figure 4C** and **4D**). Interestingly, doxorubicin treatment can inhibit the expression of miR-135b (**Figure 3E** and **3F**), but can elevate LATS2 protein levels (**Figure 4E**). This effect is attenuated when transfecting miR-135b mimics into cancer cells (**Figure 4F**). Thus, our data indicate that miR-135b is able to target LATS2 directly, and that LATS2 may contribute to chemoresistance.

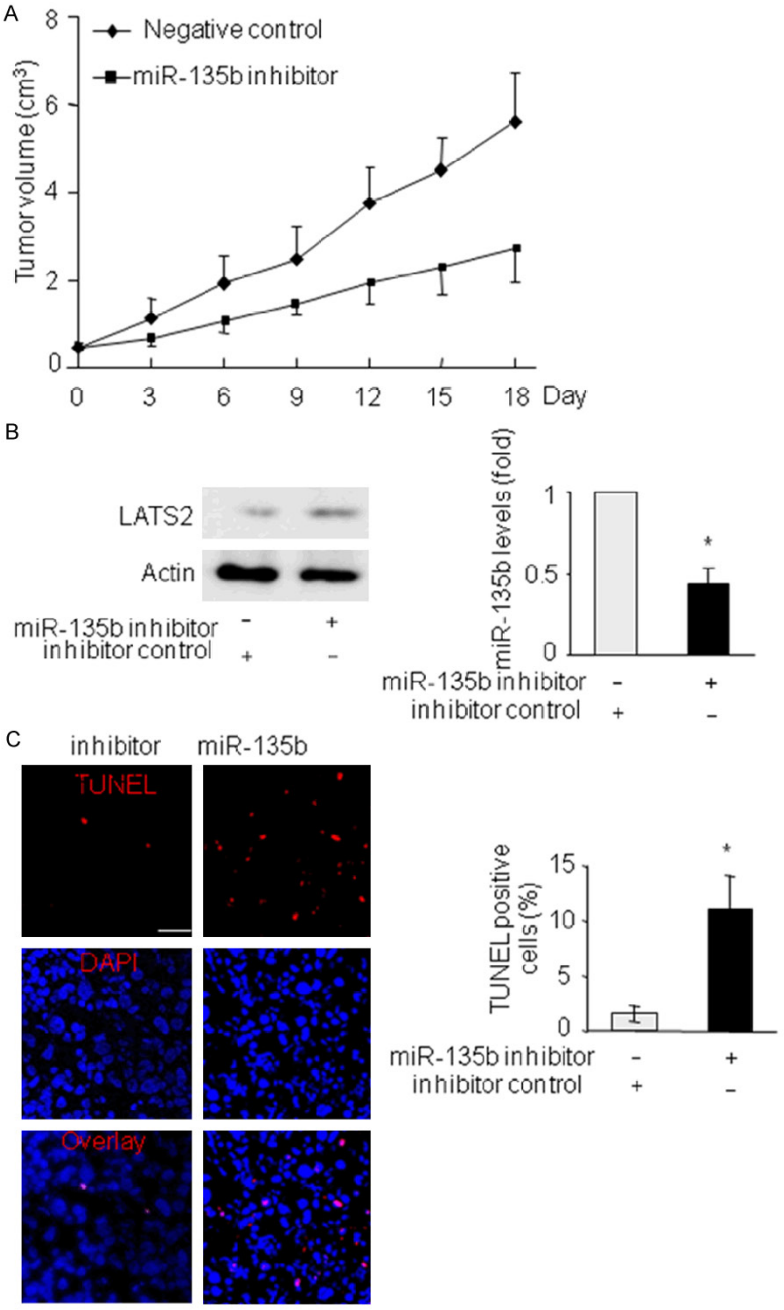

Figure 6. MiR-135b inhibition enhances apoptotic effect *in vivo*. A. A total of 1×10^7 SW480 cells stably inhibiting miR-135b or negative control were injected subcutaneously into BALB/c nude mice. Photographs show morphology of tumors at day 18, n=5 each group. B. Immunoblot of LATS2 protein (left) and miR-135b expression levels (right) in generated xenograft tumors compared with control are shown. C. Detection of apoptosis by TUNEL assay in sections of xenograft tumors, n=6 each group. *P<0.05 compared with control. Red, TUNEL-positive nuclei; blue, DAPI-stained nuclei, scale bar=50 μm. Error bars represent SD.

MiR-135b regulates chemotherapeutic resistance through LATS2

Studies have reported that LATS2 functions as a tumor suppressor and belongs to the Hippo (Hpo) signaling pathway [23], which has been shown to suppress tumor growth by inhibiting TAZ [24, 25]. After doxorubicin treatment, we found a strong elevation of LATS2 protein levels, which is contrary to the alteration of miR-135b levels (**Figure 4E** and **4F**). We then wondered whether miR-135b regulates chemoresistance in colorectal cancer cells by targeting LATS2. Knockdown of endogenous LATS2 significantly reduces LATS2 protein expression (**Figure 5A**), mimicking the effects of miR-135b upregulation, promoting cell proliferation (**Figure 5B** and **5C**). To evaluate the effect of miR-135b-LATS2 on cell proliferation and apoptosis, SW-480 and LOVO cells were transiently transfected with miR-135b antagomir or endogenous LATS2 was knocked down. MTT assay revealed that addition of miR-135b antagomir and endogenous LATS2 was knocked down could not reduce cell proliferation (**Figure 5D** and **5E**). TUNEL assay revealed that when miR-135b antagomir was added and endogenous LATS2 was knocked down, a limited amount of cells undergoing apoptosis (**Figure 5F** and **5G**). Furthermore, knockdown of endogenous LATS2 mimics the result of miR-135b upregulation to attenuate doxorubicin-induced apoptosis (**Figure 5H** and **5I**). These results show that LATS2 contributes to chemoresistance, which is regulated by miR-135b.

MiR-135b inhibition enhances apoptotic effect in vivo

Having demonstrated that miR-135b significantly regulates apoptosis and chemoresis-

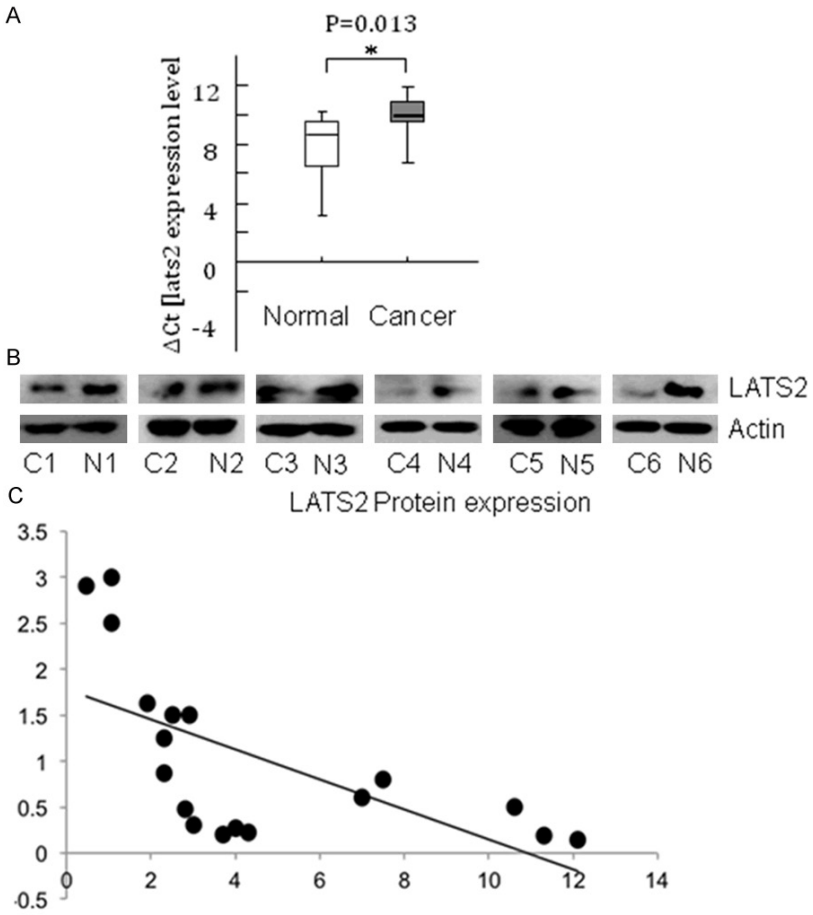

Figure 7. LATS2 is deregulated in human colonic carcinomas according to miR-135b expression. (A) LATS2 mRNA expression levels in 18 pairs of human colorectal cancer tissues and matched normal tissues. The boxes represent lower and upper quartiles separated by the median, and the excluding point was marked as a dot. (B) Immunoblot of LATS2 proteins in six representative pairs of normal colorectal tissues (N) and matched cancer tissues (C). (C) The correlation analysis showed that the expression of miR-135b was negatively correlated with the expression of LATS2 mRNA in normal and matched cancer tissues. Error bars represent S.D. *P<0.05.

tance of colorectal cancer cells in vitro, we further investigated the role of miR-135b in xenograft models. First, we tested whether miR-135b alone could inhibit colorectal tumor growth, SW480 cells stably inhibition of miR-135b or negative control was established. Inhibition of miR-135b was confirmed by qRT-PCR (**Figure 2A**). The stable cells, SW480-miR-135b- or SW480-negative control, were injected subcutaneously into nude mice. Xenograft tumor formation was monitored over 18-day time course. Compared with controls, miR-135b inhibition resulted in a smaller tumor size (**Figure 6A**), suggesting that miR-135b promotes colorectal tumor growth in vivo. The mice were killed at day 18. As expected, we found significantly decreased miR-135b levels in SW480-miR-135b-generated tumors, accompanied by the upregulation of LATS2 (**Figure 6B**). Next, the TUNEL assay (**Figure 6C**) in xenograft models was used to show that a reduction of miR-135b levels and a remarkable elevation of LATS2 expression may contribute to enhanced apoptosis. In summary, our data indicate that inhibition of miR-135b increases colorectal cancer apoptosis by upregulating LATS2 expression in vivo.

LATS2 is deregulated in human colonic carcinomas according to miR-135b expression

To further explore the potential clinical relevance of miR-135b-mediated signaling pathway in human colorectal cancer, we determined the expression levels of LATS2 in human colorectal tissues. Results revealed that LATS2 mRNA levels were significantly decreased in colorectal cancer (**Figure 7A**). This observation was further confirmed by immunoblot of LATS2 protein in some of these clinical samples. Six representative results were shown here (**Figure 7B**). According to our data, a negative correlation between miR-135b expression and LATS2 mRNA levels was found (**Figure 7C**). These findings are consistent with the results in colorectal cancer cell lines, and suggest that miR-135b-LATS2 axis may be involved in the development and chemoresistance of colorectal cancer.

Discussion

Therapeutic resistance is a major challenge to effective cancer treatment, especially in colo-

rectal cancer, which is why it is imperative to investigate new modalities for CRC therapy. Our current study identified miR-135b as a regulator of apoptosis and chemoresistance in CRC cells treated with chemotherapy. We found that miR-135b was expressed at a higher level in several CRC cell lines and human CRC tissues. MiR-135b expression was negatively correlated with patients' response to chemotherapy and therefore adversely affected life expectancy. Enforced expression of miR-135b can also attenuate doxorubicin-induced apoptosis in colorectal cancer cells. Lastly, we also discovered a miR-135b-controlled apoptotic pathway involving LATS2.

Elevated miR-135b expression has been identified in various cancers in previous studies [9-11, 14-16, 19-21]. In colorectal cancer, higher expression levels of miR-135b were observed [14-16, 21] which was consistent with our findings. MiR-135b promotes proliferation potential and tumor growth in colorectal cancer [14], head and neck squamous cell carcinoma (HNSCC) [10], and osteosarcoma (OS) [20]. It promotes invasion and migration in non-small-cell lung cancer [9] and colorectal cancer [15]. MiR-135b's ability to regulate ECM formation and somatic cell reprogramming, and may be a powerful tool to dissect the intracellular and extracellular molecular mechanisms of reprogramming [22]. These investigations imply that miR-135b is a potential regulator of somatic cell reprogramming, a definite onco-miRNA. Our results display miR-135b tumor-suppressing activity in the colorectal cancer xenograft model. Based on our experiments, inhibition of miR-135b could be a possible therapy strategy for colorectal cancer. However, the mechanism of miR-135b involvement in apoptosis signaling pathway is not fully understood. We next showed that miR-135b increases anti-apoptosis and attenuates anticancer drug-induced apoptosis by directly targeting LATS2.

LATS2 (large tumor suppressorkinase2) plays a central role in mediating Hippo growth-inhibitory signaling [26, 27]. Mutation and deregulation of this signaling pathway has been found in several human cancers [28-31]. LATS2 can regulate mitotic progression, YAP activation [32], retinoblastoma protein (pRB) activity [33] and p53 activity, leading to cell cycle arrest and inhibition of tumor growth [34, 35]. In the present study, we found that miR-135b promotes colorectal cancer cells proliferation and anti-apoptosis by targeting LATS2. Several other miRNAs have been found to target LATS2 in different types of cancer cells. MiR-93 promotes tumor angiogenesis and metastasis by suppressing LATS2 in human breast carcinoma cells [36]. MiR-31 acts as an oncogenic miRNA in lung cancer by targeting LATS2 [37]. MiR-181b causes ovarian cancer cell growth and invasion by targeting LATS2 [38]. MiR-372 regulates cell cycle and apoptosis in gastric cancer cell lines through direct regulation of LATS2 [39]. MiR-373 regulates LATS2 and stimulates proliferation in human esophageal cancer [29]. This indicates that LATS2 may undergo post-transcriptional regulation by miRNAs to maintain its low levels in many malignant tumors. The specific miRNAs targeting LATS2 may be cell-type dependent during tumorigenesis. Increased expression of miR-135b has been found in various cancers, such as lung carcinoma, head and neck cancer, glioblastoma, and osteosarcoma [9-11, 14-16, 19-21]. Whether miR-135b functions as an oncogenic miRNA by targeting LATS2 in these cancers needs to be further explored.

We found that expression of miR-135b represses LATS2 levels, leading to increased proliferation and chemoresistance. Since the effects of LATS2 on tumor cell chemoresistance are not known, we conducted a series of experiments to confirm the role of LATS2 in mediating miR-135b functions associated with proliferation, apoptosis and chemoresistance. Using siRNA to silence LATS2 expression, we showed that LATS2 plays an important role in cell proliferation and apoptosis. Knockdown of endogenous LATS2 can mimic the result of miR-135b upregulation to attenuate doxorubicin-induced apoptosis. Results are also confirmed in human CRC samples and xenograft tumor models. Nevertheless, the function of miR-135b in regulation of Hippo signaling pathway in colorectal cancer cells needs to be further explored. The relevance of miR-135b expression levels to clinicopathologic features and prognosis of CRC patients should also been studied with a larger study population.

Current therapeutic effects have their own limitations given their toxicity and chemoresistance. Meanwhile, miRNAs have been demonstrated to regulate multiple tumor-suppressor genes, and to participate in cancer progres-

sion, thereby emerging as a potential candidate for therapeutic intervention for innovative cancer therapies [8]. Such examples include work by Q Li et al who identified a novel regulatory pathway for apoptosis and chemotherapy involving RUNX3, miR-185, and ARC, and suggested that miR-185 might be a potential therapeutic target for gastric cancer [40]. Another example is knocking down miR-21 in the HXO-RB44 cell to inhibit cancer progression in retinoblastoma. 8-mer tiny seed-targeting anti-miR-21 (t-anti-miR-21) is another strategy for mir-21-based therapeutics and drug discovery [41]. Tamoxifen along with administration of anti-miR-181b or anti-miR-221 has led to apparent reduction in breast tumor size [42]. A combination of miR-34 and let-7 treated the aggressive Kras; p53 non-small cell lung cancer mouse model can suppress tumor growth and lead to survival advantage [43]. In our present investigation we also found that miR-135b can attenuate doxorubicin-induced apoptosis in colorectal cancer cells.

Taken together, combination therapy using suppressed miR-135b expression and lower dose chemotherapeutic drugs may have an effective therapeutic effect against colorectal cancer, with decreased host toxicity. Contrary to other miRs that may be involved in physiological conditions, miR-135b has a low basal expression in normal epithelium. For these reasons, it would be quite possible to develop a strategy to silence miR-135b for effective cancer therapy. We report here that miR-135b decreases the apoptosis and chemosensitivity of colorectal cancer cells in vitro and in vivo. It exerts oncomiR function through negatively regulating LAST2. Our results suggest that miR-135b might be a novel and innovative therapeutic target for colorectal cancer.

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Abbreviations

LATS2, large tumor suppressor kinase 2; qRT-PCR, quantitative reverse transcription PCR; UTR, untranslated region; miRNA, microRNA.

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