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Original Article

miR-20a targets BNIP2 and contributes chemotherapeutic resistance in colorectal adenocarcinoma SW480 and SW620 cell lines

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Chemotherapy is an important treatment for colorectal adenocarcinoma cancer; however, colorectal adenocarcinoma cells often develop resistance to chemotherapeutic drugs, leading to relapse and poor patient prognosis. The development of drug resistance is often a multifactor process, which involved several genes and cellular mechanisms. microRNAs are endogenous small non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. In the present study, we investigated the possible role of microRNAs in regulating drug sensitivity of colorectal adenocarcinoma cells SW620 and SW480. Using microRNA expression arrays and quantitative reverse transcriptase (RT)-PCR, we found that SW620 cells exhibited elevated miR-20a expression compared with SW480 cells. In addition, these two cell lines displayed different sensitivities to the chemotherapeutic drugs fluorouracil, oxaliplatin, and teniposide. Modulation of miR-20a altered the sensitivity of SW620 and SW480 cells to these drugs; knockdown of miR-20a sensitized SW620 cells to chemotherapeutic agents, whereas overexpression of miR-20a in SW480 cells resulted in chemoresistance. Endogenous *BNIP2* mRNA and BNIP2 protein levels were inversely related to miR-20a levels as detected by quantitative RT-PCR and western blot analysis. Fluorescence reporter assays showed a direct interaction between miR-20a and the *BNIP2* 3′UTR. Taken together, our findings suggested that miR-20a may play a role in colorectal adenocarcinoma cancer cell drug resistance and may be a therapeutic target against chemotherapy drug resistance in colorectal adenocarcinoma.

Keywords microRNA-20a; chemotherapeutic resistance; colorectal adenocarcinoma; BNIP2

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Introduction

Resistance of cancer cells to chemotherapy continues to be a major clinical obstacle to the successful treatment of cancer, including colorectal cancer [1–3]. Drug resistance of cancer cells is linked to mutational events (genetic hypothesis) and to non-mutational alterations of gene function (epigenetic hypothesis) including microRNAs (miRNAs) [4,5]. miRNAs are a recently discovered class of non-coding RNAs that regulate gene expression either by degrading target mRNA or by a mechanism similar to small-interfering RNA-mediated gene silencing. miRNAs regulate a large number of genes by interacting with complementary sites in the 3′ untranslated region (3′UTR) of target genes [6]. Extensive studies have shown that miRNAs play an important role in cellular processes including cell proliferation [7], differentiation [8], and apoptosis [9,10]. miRNAs have been shown to affect cancer initiation, progression, classification, diagnosis, and prognosis [11,12], including colorectal cancer [13]. Studies have also revealed that miRNAs are involved in drug resistance of cancer cells. For example, miR-21 antisense oligonucleotide (ASO) increased the susceptibility of cholangiocarcinoma cells to gemcitabine [14], indicating that miRNAs-based therapy could be effective combined with chemotherapy. In addition, it has been determined that miR-24 down-regulated expression level of the chemoresistance enzyme dihydrofolate reductase, which led to methotrexate resistance [15]. Kovalchuk *et al*. [16] reported that miR-451 down-regulated *MDR1* and induced resistance to doxorubicin in breast cancer cells, and Xia *et al*. [17] demonstrated that miR-15b and miR-16 modulated multidrug resistance by targeting *Bcl2* in gastric cancer cells. The expression levels of miRNA are also altered in colon cancer [18–20]. However, little is known about the relationship between miRNAs and drug resistance in colorectal cancer.

Using miRNA array assays and quantitative reverse transcriptase (RT)-PCR, we showed that colorectal adenocarcinoma cell lines SW620 and SW480 displayed different miR-20a expression levels. Colorectal adenocarcinoma cell lines, SW480 and SW620, are derived from the same patient and thus share a common background [21]. So these two cell lines provide a very good model to study whether the aberrant miR-20a expression is related to the cell sensitivity to chemotherapeutic agents. Drug sensitivity assays demonstrated that SW480 and SW620 cells were differently sensitive to fluorouracil (5-FU), oxaliplatin (L-OHP), and teniposide (VM-26). We sought to determine the significance of the differential expression of miR-20a in the two cell lines about their sensitivity to chemotherapeutic agents. Here we also showed that the aberrant expression of miR-20a was involved in chemotherapeutic sensitivity of SW620 and SW480 cells to 5-FU, L-OHP and VM-26; miR-20a overexpression resulted in resistance to these chemotherapy agents, while miR-20a knockdown led to sensitization. Moreover, we showed that miR-20a down-regulated both *BNIP2* mRNA and BNIP2 protein levels. These results improved our understanding of the molecular mechanisms behind drug resistance in colorectal cancer cells and indicated that miR-20a might be a therapeutic target for drug resistance in colorectal adenocarcinoma.

Materials and Methods

Cell lines and culture conditions

SW620 and SW480 cells were maintained using α-MEM medium (Sigma, St. Louis, USA) containing 10% fetal bovine serum and 100 IU/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Quantitative RT-PCR for miRNAs

Total RNA was extracted from the cultured cells using Trizol reagent (Invitrogen, Carlsbad, USA) and RNA molecules smaller than 200 nucleotides in size were purified using the mirVana miRNA isolation kit (Ambion, Austin, USA) according to the manufacturer's instructions. As described previously [22], the expression level of mature miRNAs was determined by real-time PCR analysis following stem-loop RT. The primers of miR-20a used for stem-loop RT-PCR (**Table 1**) were obtained from Sun Biotech (Canyon Country, USA) or AuGCT, Inc. (Beijing, China); M-MLV RT was obtained from Promega (Madison, USA). PCR cycles were as follows: initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 40 s. The relative amount of miR-20a was normalized to U6 snRNA. The fold change about miR-20a expression was calculated using the $2^{-\triangle\triangle CT}$ method [23].

Plasmid construction

The *BNIP2* 3′UTR was amplified from SW620 genomic DNA using primers that contained *Eco*RI and *Bam*HI tails at the 5′ and 3′ ends, respectively. *BNIP2* 3′UTR primers were as follows: sense, 5′-CGCGGATCCAGCAAGTACAGATGCAAG-3′; antisense, 5′-CGGAATTCTCAAACAACTACCGAGTTC-3′. *BNIP2* mutant (mut) 3′UTR was cloned using the following primers containing *Eco*RI and *Bam*HI tails: sense, 5′-CTCTCACTATGGCCTCATAACCAAAATACTTC-3′; and antisense, 5′-GAAGTATTTTGGTTATGAGGCCATAGTGAGAG-3′. Both the wild-type and the mutant version of *BNIP2* 3′UTR were digested with *Eco*RI and *Bam*HI restriction endonucleases and then gel purified. The wild-type *BNIP2* 3′UTR or the mutant one was ligated into the pcDNA3.1(+)/EGFP control vectors by using *Bam*HI and *Eco*RI sites located immediately downstream of the stop codon of EGFP.

To construct the miR-20a-expressing vector, primary miR-20a (pri-20a) sequences were amplified by PCR using primers with *Bgl*II and *Eco*RI tails: sense, 5′-GCGAGATCTAGTTGTGCAAATCTATGC-3′, antisense, 5′-GGCGAATTCTAACCATAGAACAGTGTTC-3′, and subsequently cloned into pcDNA3.1(+) vector using the *Bgl*II and *Eco*RI sites.

EGFP reporter assay

Cells were seeded in 24-well plates. Twenty-four hours later, cells were transfected with miR-20a ASO (5′-CTACCTGCACTATAAGCACTTTATT-3′), control ASO (ctrl ASO, 5′-GTGGATATTGTTGCCATCA-3′), EGFP reporter vector pcDNA3.1(+)/EGFP-*BNIP2* 3′UTR or pcDNA3.1(+)/EGFP-*BNIP2* 3′UTR mut. The yeast fluorescent (RFP) expression vector pDsRed2-N1 (Clontech, Mountain View, USA) was also transfected into the cells to normalize fluorescence values. Seventy-two hours after transfection, cells were lysed with radioimmunoprecipitation assay lysis buffer (RIPA lysis buffer, 50 mM Tris–HCl, pH 7.2, containing 150 mM NaCl, 1% Triton X-100, and 0.1% SDS), and EGFP and RFP fluorescence levels were quantified using a F-4500 Hitachi spectrophotometer (Kyoto, Japan).

Bioinformatics method

The following online miRNA target prediction algorithms were used to predict miR-20a target genes: PicTar (http://pictar.bio.nyu.edu/cgi-bin/PicTar_vertebrate.cgi), Target Scan Release 4.0 (http://www.targetscan.org), and miRBase targets (http://microrna.sanger.ac.uk/cgi-bin/targets/v4/search.pl).

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Table 1 Primers used for stem-loop RT-PCR for miR-20a and U6 snRNA

Primer	Sequence (5′→3′)
miR-20a RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCTG
miR-20a-F	GCCCGCTAAAGTGCTTATAGTG
miR-20a-R	CCAGTGCAGGGTCCGAGGT
U6 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAAC
U6-F	TGCGGGTGCTCGCTTCGGCAGC
U6-R	CCAGTGCAGGGTCCGAGGT

F, forward primer; R, reverse primer.

Semi-quantitative RT-PCR

BNIP2 and *β-actin* (control) mRNA levels were measured by semi-quantitative RT-PCR. Total RNA was isolated using Trizol reagent and reverse transcribed to cDNA using M-MLV RT. The cDNA was then PCR amplified using primers for *BNIP2* and *β-actin*. The *BNIP2* primers were the same as those used in vector construction. The primers of *β-actin* were as follows: sense 5′-CGTGACATTAAGGAGAAGCTG-3′ and antisense 5′-CTAGAAGCATTTGCGGTGGAC-3′. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. PCR products were resolved on 1% agarose gel. LabWorks™ Image Acquisition and Analysis software (UVP, Upland, Wisconsin, USA) was used to detect band intensity. All the primers were purchased from Sun Biotech or AuGCT, Inc.

Western blot analysis

Cells were transfected with miR-20a ASO or control ASO. Forty-eight hours later, total cellular extracts were prepared by incubation in RIPA buffer for 30 min at 4°C followed by centrifugation at 10,000 *g* for 10 min at 4°C. Total protein extracts were collected from the supernatant. Proteins were separated on a 10% SDS–PAGE gel and transferred to polyvinylidene nitrocellulose membrane. The membrane was incubated with antibody against BNIP2 (dilution 1:400) and GAPDH (control; dilution 1:1000) overnight at 4°C. Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibody followed by ECL plus chemiluminescent detection (GE Healthcare Biosciences, New York, USA). Luminescence was detected by exposure to chemiluminescent film. LabWorks™ Image Acquisition and Analysis software was used to assess band intensity. Anti-BNIP2 antibody was purchased from Saier, Inc. (Tianjin, China), and anti-GAPDH antibody was purchased from Sigma.

5-FU, L-OHP, and VM-26 cytotoxicity

Cytotoxicity assays were carried out in α-MEM culture media containing 10% FBS, 100 IU/ml penicillin, and 100 μg/ml of streptomycin. About 6000–8000 cells were plated in each well of 96-well plates in 90 μl of medium. Twenty-four hours later, various concentrations of 5-FU, L-OHP, and VM-26 diluted in medium were added to each well in a total volume of 10 μl. After another 48 h of incubation, MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5-dipheny ltetrazolium bromide] was added to a final concentration of 0.5 mg/ml. Four hours later, the reduced insoluble MTT or formazan was solubilized in 100 μl dimethyl sulfoxide, and the absorbance of each well was determined at 570 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tech Instruments, Winooski, USA). Wells containing cells without chemotherapeutic agent and wells containing medium alone were used as positive and negative controls, respectively. All experiments were repeated three times.

Statistical analysis

Student's *t*-test was performed to assay statistical significance. *P*-values <0.05 were considered statistically significant.

Results

Aberrant miR-20a expression levels and differential sensitivity of SW480 and SW620 cells to 5-FU, L-OHP, and VM-26

Using miRNA expression array assays, we previously found that miR-20a was one of the most differently expressed miRNAs in SW480 and SW620 cells (data not shown). Now using real-time PCR, we found that miR-20a had a higher expression in SW620 cells than in SW480 cells [**Fig. 1(A)**]. miR-20a belongs to the miR-17–92 miRNA cluster, which is present on the chromosome 13 [24]. This cluster is induced by the c-Myc proto-oncogene [25] and amplified in B-cell lymphomas [7]. Schetter *et al.* [13] found that increased expression of miR-20a was associated with the therapeutic outcome of colon adenocarcinoma cancer. miR-20a has been revealed to regulate E2F and to play an antiapoptotic role in PC3 prostate cancer cells treated with DOX [26]. However, little is known

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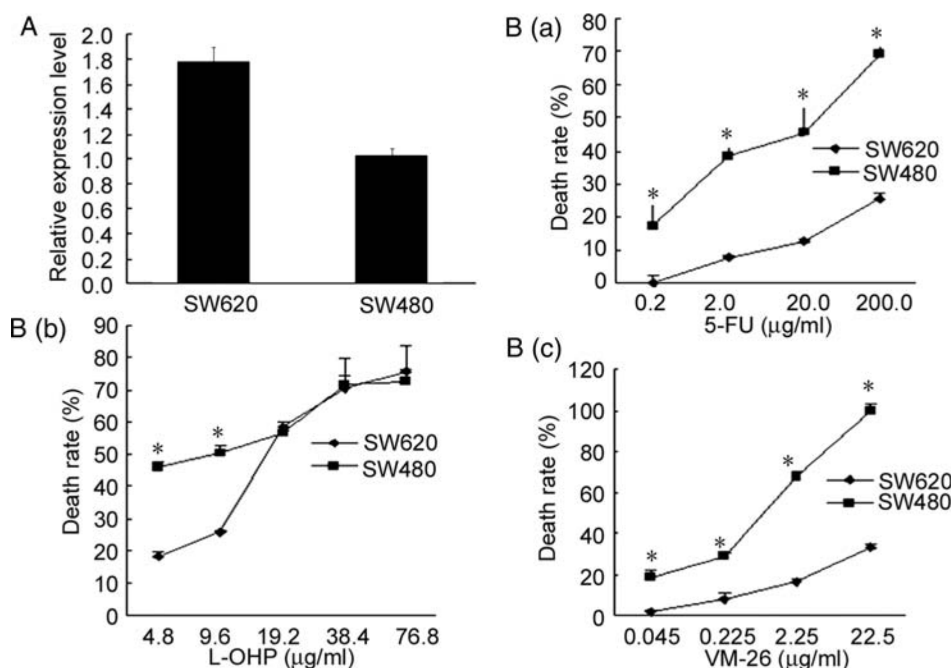

Figure 1 Differential miR-20a expression levels and chemotherapeutic sensitivity of SW620 and SW480 cells (A) Higher miR-20a expression level in SW620 cells compared with SW480 cells. The expression levels of miR-20a in SW620 and SW480 cells were determined by quantitative RT-PCR. Relative levels of miR-20a were determined by normalization with U6 snRNA. Relative miR-20a expression levels are shown as fold change in SW620 cells compared with those in SW480 cells, which are set to 1. (B) Differential chemotherapeutic sensitivity of SW620 and SW480 cells to 5-FU, L-OHP, and VM-26. SW480 and SW620 cells were plated in 96-well plates and incubated with various concentrations of 5-FU (a), L-OHP (b), and VM-26 (c) for 48 h. Cell viability was assessed using an MTT-based cytotoxicity assay. Data are represented as percentage death relative to untreated control. Statistical significance of differences of the two cell lines at different VM-26 concentrations were determined by Student's *t*-test (*$P < 0.05$).

about miR-20a and its effect upon chemotherapeutic sensitivity of colorectal adenocarcinoma cancer.

Using the MTT cytotoxicity assay, we set out to detect the drug sensitivity of SW480 and SW620 cells to 5-FU, L-OHP, and VM-26, which are the most used cancer chemotherapy agents. In stark contrast to the higher level of miR-20a in SW620 cells than that in SW480 cells, the MTT assay revealed that SW620 cells showed much less death compared with SW480 cells at the various concentrations of 5-FU, L-OHP, and VM-26 used in this experiment [**Fig. 1(B)**], indicating that SW620 cells, with higher levels of miR-20a, are more resistant to chemotherapy agents.

Association between miR-20a expression and 5-FU, L-OHP, and VM-26 sensitivity in SW620 and SW480 colorectal adenocarcinoma cell lines

Specific ASO is commonly used to knockdown endogenous miRNA [27]. We synthesized a miR-20a ASO and then transfected it into SW620 cells to knockdown endogenous miR-20a. In addition, a miR-20a expressing vector, pcDNA3.1(+)/pri-20a, was constructed and transfected into SW480 cells to overexpress miR-20a [**Fig. 2(A)**]. To determine the effect of miR-20a expression on cancer cell drug sensitivity, we analyzed cell survival after being treated with varying concentrations of 5-FU, L-OHP, and VM-26. Control ASO and control vector were used to normalize results. Knockdown of miR-20 by ASO in SW620 cells resulted in increased cell death compared with control cells [**Fig. 2(C,E,G)**], demonstrating that inhibition of miR-20a-sensitized SW620 cells to these chemotherapeutic agents. In contrast, SW480 cells that overexpress miR-20a showed a decrease of cell death [**Fig. 2(B,D,F)**], indicating that overexpression of miR-20a conferred resistance to 5-FU, L-OHP, and VM-26. High expression levels of miR-20a result in chemotherapeutic resistance, while low expression levels lead to sensitivity. Taken together, these results indicated that miR-20a was involved in 5-FU, L-OHP, and VM-26 sensitivity of colorectal adenocarcinoma cancer cells.

miR-20a regulates BNIP2 expression at both mRNA and protein levels

miRNAs physically function through their interaction with the 3′UTR of their target genes; therefore, we sought to determine the functional target gene of miR-20a that could modulate chemotherapy sensitivity. We used miRanda, PicTar, and TargetScan, which were widely used bioinformatic algorithms, to predict miR-20a candidate targets. We concluded that miR-20a must target a proapoptotic or anti-

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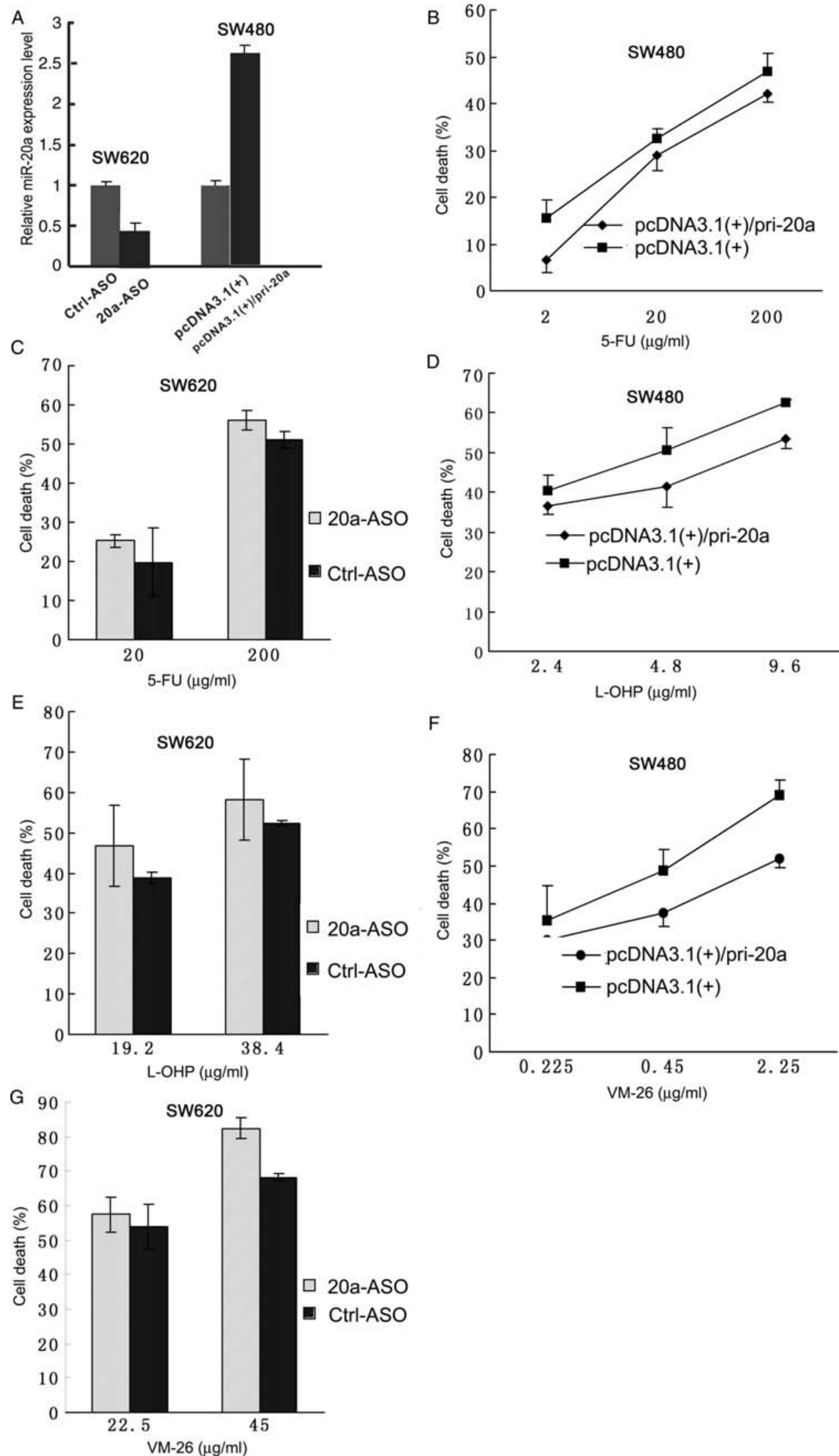

Figure 2 Modulation of miR-20a expression alters chemotherapeutic sensitivity of SW480 and SW620 cells (A) Real-time PCR was performed to detect the miR-20a level in SW620 and SW480 cells treated with miR-20a ASO or pcDNA3.1(+)/pri-20a. SW480 cells (B,D,F) were transfected with pcDNA3.1(+)/pri-20a or pcDNA3.1(+), and SW620 cells (C,E,G). Cells were transfected with miR-20a ASO or control ASOs (ctrl-ASO). Cell viability was determined using an MTT-based cytotoxicity assay after incubation with 5-FU, L-OHP, and VM-26 for 48 h. Data are represented as the percentage relative to untreated control. $*P < 0.05$.

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survival factor in order to promote survival when cells were treated with apoptosis-inducing drugs such as 5-FU, L-OHP, and VM-26. Bioinformatic analysis predicted the presence of miR-20a binding sites in the 3′UTR of the *BNIP2* mRNA [**Fig. 3(A)**]. Furthermore, previous studies [28] have revealed that *BNIP2* is a proapoptosis factor. To confirm our hypothesis of a functional interaction between

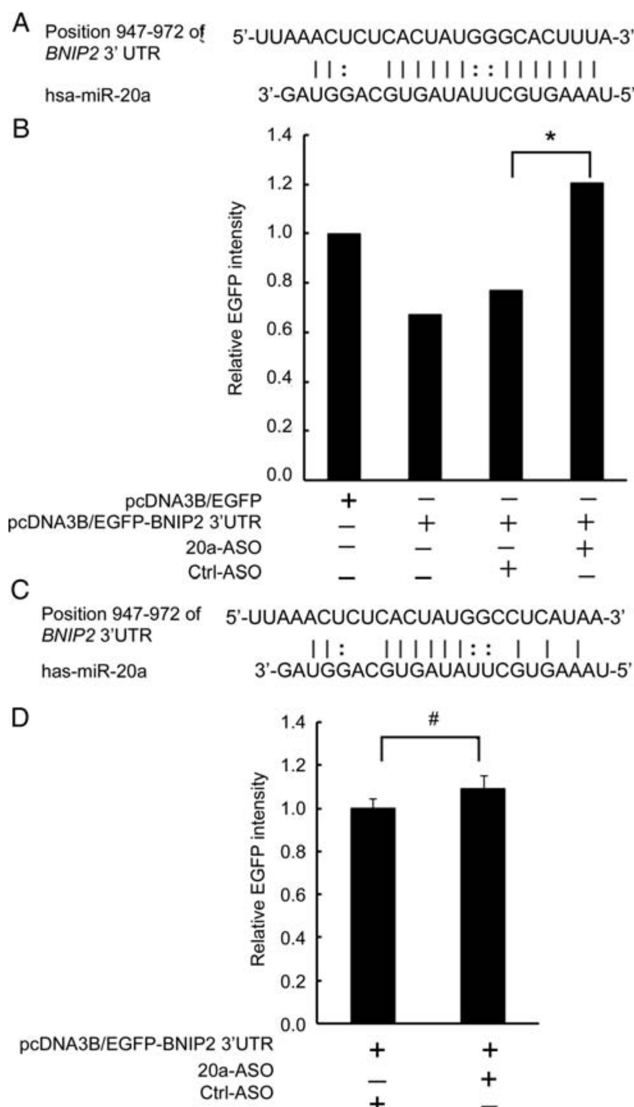

Figure 3 miR-20a directly targets *BNIP2* (A) Complementary site for miR-20a in 3′UTR of *BNIP2* predicted by computer algorithms. (B) Inhibition of EGFP fluorescence reporter expression by miR-20a ASO transfection in SW620 cells. SW620 cells were transfected with pcDNA3.1(+)/EGFP-*BNIP2* 3′UTR and either miR-20a ASO or control oligonucleotides. Seventy-two hours post-transfection, EGFP fluorescence intensity was detected, and the relative expression was determined using SW620 cells transfected with the pcDNA3.1(+)/EGFP vectors as controls. EGFP levels relative to controls are shown. $^{*}P < 0.05$. (C) The mutant version of BNIP2 3′UTR with a mutant miR-20a-binding site. (D) EGFP expression with the BNIP2 3′UTR mutant fluorescence reporter. SW620 cells were transfected with pcDNA3.1(+)/EGFP-*BNIP2* 3′ UTR mut vector as well as miR-20a ASO or control oligonucleotides. EGFP level was detected as described above. $^{\#}P > 0.05$.

miR-20a and *BNIP2* 3′UTR, we designed and implemented an EGFP fluorescence reporter assay that could test the functional interaction between the two *cis* elements. The *BNIP2* 3′UTR containing the putative miR-20 complementary sequence was amplified by PCR and cloned into pcDNA3.1(+)/EGFP, just at the downstream of the stop codon of EGFP, resulting in fluorescence reporter construct pcDNA3.1(+)/EGFP-*BNIP2* 3′UTR. pcDNA3.1(+)/EGFP-*BNIP2* 3′UTR mut, a mutant version of the fluorescence reporter vector in which the miR-20a-binding sequences were mutated, was also constructed [**Fig. 3(C)**]. Both constructs were independently transfected into SW620 and SW480 cells together with either miR-20a ASO or pcDNA3.1(+)/pri-20a. Seventy-two hours post-transfection, EGFP protein levels were detected using a fluorescence spectrophotometer. As shown in **Fig. 3(B)**, a nearly 1.6-fold increase was observed from pcDNA3.1(+)/EGFP-*BNIP2* 3′UTR after miR-20a knockdown in SW620 cells. In contrast, mutation of the miR-20a target site restored EGFP expression to near control levels [**Fig. 3(D)**], indicating that binding of miR-20a to the *BNIP2* 3′UTR was necessary and sufficient to inhibit EGFP expression.

The above data indicated a direct interaction between miR-20a and the *BNIP2* 3′UTR that is capable of down-regulating gene expression of an upstream gene. To determine whether miR-20a affects endogenous *BNIP2* expression in colorectal adenocarcinoma cells, semi-quantitative PCR and western blot were used to detect the expression levels of endogenous *BNIP2* mRNA and BNIP2 protein, respectively, under conditions of miRNA-20a knockdown or overexpression. As shown in **Fig. 4**, transfection of SW620 cells with miR-20a ASO resulted in an increased expression of *BNIP2* mRNA and BNIP2 protein, while miR-20a overexpression in SW480 cells led to the opposite effect. Altogether, these results demonstrated that endogenous *BNIP2* is under the regulation of miR-20a, and that *BNIP2* is a functional miR-20a target in colorectal adenocarcinoma cancer.

Discussion

Resistance to cancer drugs is akin to a multifactor polygenetic disease that involves multiple major mechanisms, such as decreased uptake of water-soluble drugs, increased repair of DNA damage, altered metabolism of drugs, increased energy-dependent efflux of chemotherapeutic drugs that diminish the ability of cytotoxic agents to kill cancer cells, and reduced apoptosis under the function of chemotherapeutic agents [29]. Both genetic and epigenetic hypotheses about drug resistance have recently been proposed, and the latter is much more thoroughly investigated. It has recently been shown that miRNAs are involved in

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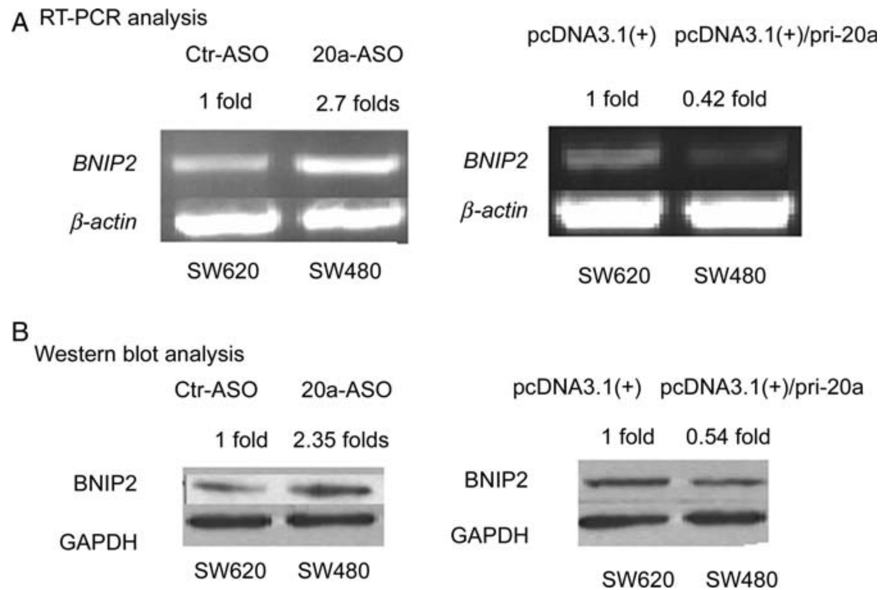

Figure 4 miR-20a regulate endogenous BNIP2 expressions at both the mRNA and protein levels (A) Levels of *BNIP2* mRNA in SW620 and SW480 cells transfected with miR-20a ASO and pcDNA3.1(+)/pri-20a, respectively. Cells were transfected with miR-20a ASO or pcDNA3.1(+)/pri-20a along with the corresponding control vectors. At 48 h post-transfection, RNA was extracted, and *BNIP2* mRNA level was detected by semi-quantitative PCR. *BNIP2* mRNA band intensity is expressed as fold difference relative to *β-actin* mRNA. (B) BNIP2 protein levels in SW620 and SW480 cells transfected with miR-20a ASO and pcDNA3.1(+)/pri-20a, respectively. Cells were transfected with miR-20a ASO or pcDNA3.1(+)/pri-20a along with the corresponding control vectors. At 48 h post-transfection, cells were lysed, and BNIP2 protein was detected by western blot analysis. BNIP2 band intensity is shown as fold difference relative to GAPDH control.

chemotherapeutic resistance in prostate carcinoma [26], colangiocarcinoma [14], breast cancer [16], and gastric cancer [17]. Here we demonstrate the involvement of miRNAs in the development of drug resistance in colorectal cancer cells and experimentally determined a potential mechanism of action.

Colorectal adenocarcinoma SW620 cells showed higher miR-20a expression levels compared with SW480 cells as determined by quantitative RT-PCR. Drug-sensitivity assays demonstrated that SW480 and SW620 cells were differentially sensitive to 5-FU, L-OHP, and VM-26. Using miR-20a specific ASOs, 20a-ASO, and the miR-20a-expressing vector, pcDNA3.1(+)/pri-20a, we found that miR-20a knockdown sensitized SW620 cells, while miR-20a overexpression led to resistance of SW480 cells to the chemotherapeutic agents. The fact that high miR-20a levels result in drug resistance, whereas low miR-20a levels result in drug sensitivity indicates that miR-20a may regulate drug resistance in colorectal adenocarcinomas.

miRNAs regulate their target genes by interacting with the 3′UTR of mRNAs. Perfect or nearly perfect base pairing between a miRNA and its target mRNA induces target cleavage, whereas imperfect base pairing mainly induces translational silencing of the target but can also reduce the amount of target transcript [30]. Computational algorithms, based on the base pairing of miRNA and target 3′UTR, have been widely used to predict miRNA targets [31]. The computational algorithms, PicTar, TargetScan, and miRBase Targets, were used to predict putative miR-20a targets. Based on these algorithms, *BNIP2* was predicted to be a target gene of miR-20a. Since computational prediction of miRNA targets are not fully reliable [32], we developed an EGFP fluorescence assay to verify if miR-20a could modulate the activity of a transcript containing the 3′UTR of *BNIP2*. miR-20a overexpression led to decreased expression of EGFP, while knockdown of miR-20a led to increased EGFP expression, indicating that miR-20a could interact directly with *BNIP2* 3′UTR. To explore whether miR-20a affects endogenous *BNIP2* expression in colorectal adenocarcinoma cancer cells, we performed semi-quantitative RT-PCR and western blots to detect *BNIP2* mRNA and BNIP2 protein levels, respectively, in colorectal adenocarcinoma cancer cells. Both *BNIP2* mRNA and BNIP2 protein levels were up-regulated after miR-20a knockdown. In contrast, both were down-regulated under conditions of miR-20a overexpression. These results indicated that *BNIP2* is a functional miR-20a target in colorectal adenocarcinoma cancer cells.

BNIP2 is a BH3-only member of the BCL-2 family of proteins [33,34]. BH3-only proteins play important roles in mitochondrion-mediated apoptosis [35], which is the main mechanism involved in chemotherapeutic agent-induced apoptosis [36]. Belcredito *et al*. [28] found that *BNIP2* functioned as a proapoptotic factor in estrogen neuroprotection. Furthermore, caspase-mediated cleavage of BNIP2 is

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crucial for its proapoptotic activity [37]. We have concluded that miR-20a down-regulated the expression of the proapoptotic factor *BNIP2*, leading to an imbalance of anti-apoptosis and pro-apoptosis factors, resulting in the blockage of events leading to apoptosis. Colorectal adenocarcinoma cells have a survival advantage when treated with the chemotherapeutic agents 5-FU, L-OHP, and VM-26.

In conclusion, we demonstrated for the first time that miR-20a functions as an inducer of chemotherapeutic resistance in colorectal adenocarcinoma by targeting *BNIP2* and miR-20a may be a target to reverse chemotherapeutic resistance. Further investigations on miR-20a regulation of *BNIP2* will hopefully shed light on the mechanistic details of this regulatory network.

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