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

Huijuan Chai1,2, Min Liu1, Ruiqing Tian1, Xin Li1, and Hua Tang1*

1Tianjin Life Science Research Center and Basic Medical School, Tianjin Medical University, Tianjin 300070, China
2Department of Physiology, Tianjin Medical University, Tianjin 300070, China
*Correspondence address. Tel/Fax: +86-22-23542503; E-mail: htang2002@yahoo.com

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 involves 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

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 can negatively regulate gene expression 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]. Kovalechuk 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.

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

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% CO2.

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-ΔΔCT method [23].

Plasmid construction
The BNIP2 3’UTR was amplified from SW620 genomic DNA using primers that contained EcoRI and BamHI tails at the 5’ and 3’ ends, respectively. BNIP2 3’UTR primers were as follows: sense, 5’-CGCGATCCAGTAAG-3’; antisense, 5’-CGGAATTCTC AAAAATCCGGAGTTC-3’. BNIP2 mutant (mut) 3’UTR was cloned using the following primers containing EcoRI and BamHI tails: sense, 5’-CTCTCAGTATG GCCCTGACAAAAATACGTCC-3’; and antisense, 5’-GAATGATTGTTGTATGAGGGCATCATGAGAC-3’. Both the wild-type and the mutant version of BNIP2 3’UTR were digested with EcoRI and BamHI 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 BamHI and EcoRI 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 BglII and EcoRI tails: sense, 5’-GCGAGATCTAGTTGTGCAAATCTATGC-3’ and 3’-GCGAATTCTAACCATAGAACGTTTC-3’. 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).

EGFP reporter assay
Cells were seeded in 24-well plates. Twenty-four hours later, cells were transfected with miR-20a ASO (5’-CTACCTGCACCTAAGCAGCTTTATT-3’), control ASO (ctrl ASO, 5’-GCTGATTTGTGAAATCTATGC-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), TargetScan Release 4.0 (http://www.targetscan.org), and miRBase targets (http://microrna.sanger.ac.uk/cgi-bin/targets/v4/search.pl).
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'-CGTGACATTTGCGGTGGAC-3' and antisense 5'-CTAGAAGCATTTGCGGTGGAC-3'. PCR conditions were as follows: initial denaturation at 94°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-diphenyl tetrazolium 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 Quant 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...
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 chemotherapy 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-
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.
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 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
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
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.

Acknowledgements

We are grateful to Dalin Ren from the College of Public Health of Tianjin Medical University (Tianjin, China), for technical assistance in fluorescent detection. We also thank the faculty of the National Foundation of Cancer Research for technical assistance in quantitative RT-PCR.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (No. 30873017) and Natural Science Foundation of Tianjin (Nos. 08JCZDJC23300 and 033182911).

References