Original Paper

Knockdown of MiR-20a Enhances Sensitivity of Colorectal Cancer Cells to Cisplatin by Increasing ASK1 Expression

Luyao Zhang Liang He Hua Zhang Yan Chen

Department of Gastrointestinal Surgery, First Hospital of Jilin University, Jilin, China

Key Words

Colorectal cancer • Cisplatin • MiR-20a • ASK1 • ROS • JNK

Abstract

Background/Aims: Platinum-based chemotherapy is one of the most important strategies for treatment of colorectal cancer. To improve the therapeutic efficiency, adjuvant drugs were sought to sensitize colorectal cancer cells to platinum-based agents such as cisplatin. As previous research has shown that miRNAs are associated with chemosensitivity, we aimed to alter miRNA regulation in colorectal cancer cells to increase their chemosensitivity. **Methods:** MTT assays were performed to determine the viability of HT29, SW480, and LoVo cells. Quantitative real time polymerase chain reaction (qRT-PCR) was performed to examine the expression of miR-20a in these cell lines. Regulation of the miR-20a/ASK1 axis was confirmed by western blotting and luciferase reporter assays. After treatment with miR-20a inhibitor (anti-miR-20a) and cisplatin, production of reactive oxygen species (ROS), mitochondrial membrane potential, and apoptosis were measured by flow cytometry. Activation of ASK1, Bcl-xl, JNK, and caspase-9, -7, and -3 was detected by western blotting. **Results:** miR-20a was overexpressed in colorectal cancer cell lines. Furthermore, knockdown of miR-20a increased the sensitivity of colorectal cancer cells to cisplatin treatment *in vitro* and *in vivo*. We demonstrated that the ASK1 gene was the target of miR-20a, and knockdown of miR-20a increased the expression of ASK1 in colorectal cancer cells. As cisplatin treatment induced production of ROS, knockdown of miR-20a enhanced ROS signaling through promoting the phosphorylation of ASK1. Phosphorylation of JNK and the subsequent mitochondrial apoptosis were triggered by the combination of cisplatin and anti-miR-20a. **Conclusions:** Knockdown of miR-20a enhanced sensitivity of colorectal cancer cells to cisplatin through the ROS/ASK1/JNK pathway.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Introduction

Due to changes in diet and increasing stress, the incidence of colorectal cancer (CRC) has shown a gradually rising trend in recent years. At present, CRC is the third most common malignancy, and a notable cause of death worldwide [1, 2]. Despite advances in tumor-specific systemic treatment, surgical resection, radiotherapy, and chemotherapy are still the standard treatments for CRC. Among these treatments, surgical resection represents the most effective strategy. However, for patients who are diagnosed with CRC at an advanced stage, tumors are unresectable. To reduce tumor progression and metastasis and prolong the CRC patient's life, systemic chemotherapy is required to kill the tumor cells [3-5]. Unfortunately, some CRC cells show low sensitivity to chemotherapeutic drugs, and drug resistance usually occurs during the course of chemotherapy [6, 7]. Strategies to increase the chemosensitivity of CRC cells should be explored.

Platinum-based chemotherapy is one of the most important strategies for treatment of CRC. The platinum-based chemotherapeutic drug cisplatin is widely used and shows a broad spectrum of anticancer activity [8]. Uptake of cisplatin causes damage to cellular DNA, and thus induces apoptosis of cancer cells. In the apoptotic pathway dependent on the cisplatin treatment, the generated reactive oxygen species (ROS) are a key mediator because high level of cellular ROS finally induces collapse of mitochondria and subsequent apoptosis [9-11]. Although cisplatin is widely used for CRC treatment, resistance of CRC cells to the drug is a major obstacle [12, 13]. Adjuvant treatments should be explored to potentially solve this problem.

MicroRNAs (miRNAs) are a class of small and non-coding RNA molecules that negatively regulate genes by partial base pairing to the 3' untranslated regions (3' UTRs) of targeted mRNAs [14, 15]. MiRNAs are important for regulating biologic processes in various cancers including CRC. Cell proliferation, the cell cycle, and apoptosis are regulated by miRNAs, and dysregulation induces tumorigenesis, metastasis, and apoptosis resistance in cancers [16, 17]. Recent studies have demonstrated the importance of miRNAs in changing the drug sensitivity of cancer cells [18-20]. Here, we explore the potential effects of miR-20a on cisplatin treatment in CRC.

Materials and Methods

Cell culture

Human normal colon epithelial cell line FHC and human CRC cell lines HT29, SW480, and LoVo were supplied by American Type Culture Collection (Rockville, MD, USA) and cultured at 37°C in a humidified incubator containing 5% CO₂ according to the manufacturer's recommendations.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA). cDNA was synthesized using One Step PrimeScript miRNA cDNA synthesis kit (TaKaRa, Dalian, China) following the manufacturer's instructions. qRT-PCR of miR-20a was performed using SYBR Premix Ex Taq (TaKaRa) and the primer (5'-TAAAGTGCTTATAGTCAGGTAG-3'). U6 was used as a control RNA to determine the relative expression of miR-20a.

Oligonucleotides, plasmid construct, and cell transduction

Apoptosis signal-regulating kinase 1 (ASK1) small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Human mature miR-20a (5'-UAAAGUGCUUAUAGUCAGGUAG-3'), 2'-O-methyl-modified anti-miR-20a (5'-CUACCUGCACUAUAAGCACUUUA-3'), and negative control oligonucleotides (NCOs; 5'-UGAGCUGCAUAGAGUAGUGAUUA-3') were purchased from GenePharma Co., Ltd. (Shanghai, China). Recombinant ASK1 plasmid overexpression was performed by inserting the open reading frame region of human ASK1 gene into the pcDNA3.1 eukaryotic expression vector (Invitrogen).

For transfection, cells were seeded into six-well plates overnight. Subsequently, the cells were transfected using Lipofectamine 2000 (Invitrogen) with 50 pmol/ml oligonucleotides and 2 µg/ml plasmid in Opti-MEM (Gibco, Waltham, MA, USA) according to the manufacturer's protocol.

Cell viability assays

Transfected cells were seeded at 2000 cells/well in 96-well plates. After 24 h, cells were incubated with cisplatin for 48 h. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The relative cell viability of each treatment group was normalized to the NCO group. The half-maximal inhibitory concentration (IC50) values of cisplatin in HT29, SW480, and LoVo CRC cells were determined relative to a cell viability curve.

Luciferase reporter assay

3' UTR fragment of human ASK1 gene containing the seed region paired to miR-20a was cloned into pMIR-REPORT™ Luciferase miRNA Expression Reporter Vector (Life Technologies, Waltham, MA, USA), and the recombinant plasmid was termed pMIR-wild ASK1. The mutant 3' UTR of ASK1 (GCACUUA to GCAAGUUA) was created using a site-directed mutagenesis kit (TaKaRa) with pMIR-wild ASK1 as the template. For luciferase reporter assays, LoVo cells were co-transfected with RNAs (50 pmol/ml), recombinant pMIR luciferase reporter vector (2 µg/ml), and Renilla luciferase pRL-TK vector (100 ng/ml, Promega, Madison, WI, USA). Following transfection for 48 h, the cells were collected and lysed. Luciferase activities was then measured by dual luciferase assay (Promega) according to the manufacturer's protocol. Relative luciferase activity was calculated as the ratio of firefly/Renilla fluorescence.

Western blot analysis

After the designated treatment, cells were lysed in RIPA buffer (Cell Signaling Technology, Beverly, MA, USA). In addition, for detection of released cytochrome c and Smac/DIABLO in cytoplasm, cellular mitochondria were removed using Mitochondria/Cytosol Fraction Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's protocol. Equal amounts of proteins were loaded and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, proteins were transferred to polyvinylidene fluoride membranes. Subsequently, the membranes were incubated for 1 h in blocking buffer (containing 5% skim milk) before incubation with antibodies against human NADPH p47, cytochrome c, Smac/DIABLO, phosphorylated c-Jun N-terminal kinase (JNK), phosphorylated Bcl-2, cleaved caspase-9, -7, and -3, and GAPDH (all purchased from Cell Signaling Technology), and ASK1 and phosphorylated ASK1 (both purchased from Santa Cruz Biotechnology) at 4°C overnight. The membranes were then probed with appropriate secondary antibodies and the protein signal was detected using an enhanced chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Assessment of ROS, mitochondrial membrane potential, and apoptosis

After the designated treatment, cells were collected and washed with phosphate-buffered saline. For assessment of ROS, mitochondrial membrane potential and apoptosis, cells were stained with dihydroethidium (Molecular Probes, Waltham, MA, USA), 5, 5',6, 6'-tetrachloro-1, 1',3, 3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Molecular Probes), or annexin V (Sigma Aldrich; Merck KGaA, Darmstadt, Germany). Subsequently, these cells were analyzed by flow cytometry.

In vivo study in tumor-bearing mice

LoVo cells were transfected with empty lentivirus (LV-empty) or lentivirus carrying miR-20a antisense nucleotide sequence (LV-anti-miR-20a) (Genechem Co., Ltd., Shanghai, China). Next, 5×10⁶ transfected cells were resuspended in serum-free optimum medium and injected subcutaneously into 4-week-old immunodeficient nude BALB/c mice (Shanghai Super-B&K Laboratory Animal Corp., Ltd., Shanghai, China). From 3 days after inoculation, cisplatin (10 mg/kg body weight) was administered to the mice by intraperitoneal injection twice a week. Tumor size was measured every 3 days. On day 28, the tumor-bearing mice were sacrificed. The animal care and experimental protocols were approved by the Animal Care Committee of Shanghai Tenth People's Hospital, School of Medicine, Tongji University, Shanghai, China.

Statistical analysis

Data were analyzed using SPSS 15.0 and expressed as the mean $\pm$ standard deviation. One-way analysis of variance was used to determine the differences between groups. Differences were considered statistically significant when P values were <0.05 . All experiments were independently repeated at least 3 times.

Results

MiR-20a inhibitors sensitize CRC cells to cisplatin treatment

The anti-tumor effect of cisplatin on CRC cell lines HT29, SW480, and LoVo was investigated using MTT assays. Fig. 1A shows that the cytotoxicity of a single administration of cisplatin was dose dependent. MiR-20a has been reported to be responsible for resistance to chemotherapy in some cancers [21, 22]. To explore the potential role of miR-20a in CRC, relative expression of miR-20a in normal colon epithelial cell line FHC and CRC cell lines was measured. The results showed significant upregulation of miR-20a in CRC cells compared with the normal colon cells (Fig. 1B), indicating that miR-20a was overexpressed in CRC. To explore the role of miR-20a in changing cisplatin sensitivity to CRC, we transfected HT29, SW480, and LoVo cell lines with miR-20a mimics and inhibitors (Fig. 1C). We found that miR-20a negatively regulated the sensitivity to cisplatin in CRC. Knockdown of miR-20a by its antisense oligonucleotide (anti-miR-20a) significantly decreased the IC₅₀ of cisplatin in CRC cells (Fig. 1D). This demonstrates the effect of anti-miR-20a on sensitizing CRC cells to cisplatin treatment.

Anti-miR-20a sensitizes cisplatin-induced cytotoxicity in CRC through the ROS pathway

The results shown in Fig. 1A indicate that sensitivity of LoVo cells to a single administration of cisplatin was midway between the sensitivity of the other two cell types. Furthermore, the IC₅₀ of cisplatin in LoVo cells can be markedly reduced by anti-miR-20a transfection (Fig. 1D). Therefore, next we examined the signaling pathway of anti-miR-20a and cisplatin co-treatment in this CRC cell line. As shown in Fig. 2A, anti-miR-20a significantly promoted cisplatin-induced death of LoVo cells. However, addition of N-acetylcysteine (NAC), a powerful ROS scavenger [23], significantly suppressed the cytotoxicity of anti-miR-20a and cisplatin co-treatment in LoVo cells. This indicated that generation of ROS was essential for death of LoVo cells induced by anti-miR-20a and cisplatin co-treatment. However, we did not observe a clear change in the expression of NADPH oxidase, which is a key producer of ROS [24] (Fig. 2B). Furthermore, although cisplatin treatment induced significant generation of

Fig. 1. Anti-miR-20a sensitizes CRC cells to cisplatin treatment. A: MTT assays were performed to test the viability of HT29, SW480, and LoVo cells to different concentrations of cisplatin. B: Relative expression of miR-20a in normal colon epithelial cell line FHC and CRC cell lines HT29, SW480, and LoVo was determined by qRT-PCR analysis. * $P < 0.05$ vs. FHC cells. C: Effect of miR-20a and anti-miR-20a on cellular levels of miR-20a in HT29, SW480, and LoVo CRC cells. * $P < 0.05$ vs. NCO group. D: Effect of miR-20a and anti-miR-20a on cisplatin sensitivity in CRC cells. * $P < 0.05$ vs. NCO group.

Fig. 2. Anti-miR-20a sensitizes cisplatin-induced cytotoxicity in CRC through the ROS pathway. A: Effect of NAC on cytotoxicity of anti-miR-20a and cisplatin (2 μ M) co-treatment in LoVo cells. * $P < 0.05$ vs. NCO+cisplatin group, # $P < 0.05$ vs. anti-miR-20a+cisplatin (2 μ M) group. B: Western blot analysis was performed to investigate the effect of anti-miR-20a and cisplatin (2 μ M) on the expression level of NADPH oxidase in LoVo cells. C: Flow cytometric analysis was performed to investigate the effect of anti-miR-20a and cisplatin (2 μ M) on the cellular level of ROS in LoVo cells.

Fig. 3. ASK1 is the target of miR-20a in CRC. A: Complementary sequence of ASK1 mRNA 3' UTR paired with miR-20a. B: Effect of miR-20a and anti-miR-20a on the protein level of ASK1 was investigated by western blot analysis. C: After co-transfection with pMiR-reporters and miR-20a mimics/inhibitors in LoVo cells, luciferase activities of empty pMiR-reporter (pMiR-control), pMiR-reporter containing wild-type ASK1 3' UTR (pMiR-wild ASK1), and pMiR-reporter containing mutant ASK1 3' UTR (pMiR-mutant ASK1) were measured by dual-luciferase reporter assay. * $P < 0.05$ vs. NCO group.

ROS in LoVo cells, transfection with anti-miR-20a did not influence the cellular level of ROS (Fig. 2C). Taken together, these results suggest that anti-miR-20a may target downstream signaling molecules of the ROS pathway to sensitize cisplatin-induced cytotoxicity in CRC.

MiR-20a targets ASK1 in CRC

To explore the potential mechanism by which anti-miR-20a sensitized CRC cells to cisplatin, TargetScan, miRanda, and PicTar databases were used to search the predicted genes targeted by miR-20a. Among these, ASK1 mRNA was predicted to be the potential target of miR-20a because the 3' UTR of ASK1 mRNA contains a complementary sequence paired with miR-20a (Fig. 3A). Furthermore, ASK1 is a key mediator in the ROS-dependent cell death pathway [25]. To validate the effect of miR-20a on ASK1 expression, we transfected the LoVo cells with miR-20a mimics or inhibitors before detection of ASK1 protein using western blot analysis. We found that overexpression of miR-20a reduced the expression of ASK1, whereas the anti-miR-20a clearly increased the protein level of ASK1 in LoVo cells (Fig. 3B). Furthermore, we found that luciferase activity was decreased significantly in LoVo cells co-transfected with miR-20a and pMiR-reporter contained wild-type ASK1 3' UTR (pMiR-wild ASK1), whereas the luciferase activity of pMiR-wild ASK1 was increased due to the introduction of anti-miR-20a (Fig. 3C). These results demonstrate that ASK1 was the target of miR-20a, and knockdown of anti-miR-20a upregulated the expression of ASK1 in CRC.

Anti-miR-20a promotes cisplatin-induced cytotoxicity in CRC through the ROS/ASK1/JNK pathway

The above results suggested that anti-miR-20a promoted cisplatin-induced cytotoxicity in CRC through the ROS pathway. We thus investigated the potential role of cellular ROS in activation of ASK1. As shown in Fig. 4A, although single treatment with anti-miR-20a did not

Fig. 4. Anti-miR-20a promotes cisplatin-induced cytotoxicity in CRC through the ROS/ASK1/JNK pathway. A: Effect of anti-miR-20a, cisplatin (2 μ M), NAC (2 mM), and siRNA-ASK1 on phosphorylation of ASK1 in LoVo cells. B: Effect of NAC (2 mM) and siRNA-ASK1 on inhibition of anti-miR-20a and cisplatin (2 μ M) co-treatment-induced cytotoxicity against LoVo cells. *P<0.05 vs. NCO+cisplatin group, #P<0.05 vs. anti-miR-20a+cisplatin group. C: Effect of SP600125 (50 μ M) on inhibition of anti-miR-20a and cisplatin (2 μ M) co-treatment-induced cytotoxicity against LoVo cells. *P<0.05 vs. NCO + cisplatin group, #P<0.05 vs. anti-miR-20a + cisplatin group. D: After treatment with siRNA-ASK1, SP600125 (50 μ M), anti-miR-20a, and cisplatin (2 μ M), phosphorylation of JNK was investigated by western blot analysis. E: Effect of plasmid-ASK1 on cisplatin-induced (2 μ M) phosphorylation of JNK. F: Effect of plasmid-ASK1 on cisplatin-induced (2 μ M) cytotoxicity against LoVo cells. *P<0.05 vs. NCO+cisplatin group.

induce significant phosphorylation of ASK1, it promoted cisplatin-dependent phosphorylation of ASK1 as anti-miR-20a upregulated the expression of total ASK1. However, addition of NAC significantly suppressed the phosphorylation of ASK1 induced by anti-miR-20a and cisplatin co-treatment in LoVo cells. This indicated that transfection with anti-miR-20a augmented the cisplatin-induced production of ROS, which subsequently enhanced the ASK1 activation. Furthermore, results of the MTT assays showed that knockdown of ASK1 significantly abolished the enhancement of anti-miR-20a effect on cisplatin-induced death of LoVo cells (Fig. 4B). We thus demonstrated the importance of the ROS/ASK1 pathway in cytotoxicity of anti-miR-20a and cisplatin co-treatment in CRC. As JNK is an important substrate of ASK1 [26], we next investigated the role of JNK in anti-miR-20a-enhanced cell death. We found that addition of the JNK specific inhibitor SP600125 [27] significantly protected the LoVo cells from cisplatin and anti-miR-20a co-treatment (Fig. 4C). Furthermore, we observed that combination of cisplatin and anti-miR-20a induced phosphorylation of JNK. However, knockdown of ASK1 by its specific siRNA decreased the cellular level of phosphorylated JNK in LoVo cells (Fig. 4D). Furthermore, direct overexpression of ASK1 by transfection with ASK1 plasmid was also able to enhance cisplatin-dependent phosphorylation of JNK (Fig. 4E) and death of LoVo cells (Fig. 4F). Taken together, these results suggest that anti-miR-20a promoted cisplatin-induced cytotoxicity in CRC through the ROS/ASK1/JNK pathway.

Combination of cisplatin and anti-miR-20a induced mitochondrial apoptosis through the ROS/ASK1/JNK pathway

Activation of JNK induces phosphorylation of Bcl-2, thus suppressing the anti-apoptotic function of Bcl-2 [28]. As shown in Fig. 5A, combination of cisplatin and anti-miR-20a was found to induce significant phosphorylation of Bcl-2 in LoVo cells. However, disruption of the ROS/ASK1/JNK pathway by NAC, siRNA-ASK1, or SP600125 inhibited the phosphorylation of Bcl-2. This indicated that inactivation of Bcl-2 induced by cisplatin and anti-miR-20a co-treatment was dependent on the activation of the ROS/ASK1/JNK pathway. Suppression of Bcl-2 has been reported to promote cisplatin-induced apoptosis through the mitochondrial pathway [29]. We therefore investigated whether combination of cisplatin and anti-miR-20a induced mitochondrial apoptosis in LoVo cells. The results of flow cytometric analysis showed that transfection with anti-miR-20a did not induce direct mitochondrial collapse. However, transfection was able to enhance mitochondrial damage induced by cisplatin treatment (Fig. 5B). Combination of cisplatin and anti-miR-20a induced significant release of the apoptosis inducers cytochrome c and Smac/DIABLO [30, 31] from mitochondria into cytoplasm (Fig. 5C). Furthermore, the markers of mitochondrial apoptosis caspase-9, -7, and -3 were triggered in LoVo cells after co-treatment with cisplatin and anti-miR-20a (Fig. 5D), followed by induction of apoptosis (Fig. 5E). Taken together, these results indicated that anti-miR-20a treatment promoted cisplatin-induced mitochondrial apoptosis. However,

Fig. 5. Mitochondrial apoptosis induced by cisplatin and anti-miR-20a co-treatment.

A: Phosphorylation of Bcl-2 in LoVo cells treated with anti-miR-20a, cisplatin (2 μ M), NAC (2 mM), siRNA-ASK1, and SP600125. B: After treatment, LoVo cells were stained with JC-1, followed by analysis of mitochondrial membrane potential by flow cytometry. C: After treatment, mitochondria in LoVo cells were removed, and the released cytochrome c and Smac/DIABLO in cytoplasm were detected by western blot analysis. D: After treatment, cleaved caspase-9, -7, and -3 were detected in LoVo cells by western blot analysis. E: After treatment, apoptosis of LoVo cells was analyzed by flow cytometry. * $P < 0.05$ vs. NCO+cisplatin group, # $P < 0.05$ vs. anti-miR-20a+cisplatin group.

Fig. 6. Introduction of anti-miR-20a enhances anti-tumor effect of cisplatin in CRC in vivo. A: Tumor growth in mice following cisplatin treatment (10 mg/kg, twice a week). Tumor volumes were measured every 3 days after inoculation. B: Mice were killed 28 days after inoculation, and the tumor was weighed. * $P < 0.05$ vs. LV-empty+cisplatin group. C: Expression of miR-20a and ASK1 in tumor tissues was measured by qRT-PCR and western blotting, respectively. * $P < 0.05$ vs. LV-empty group, # $P < 0.05$ vs. LV-empty+cisplatin group. D: Phosphorylation levels of ASK1 and JNK were determined in LV-empty- and LV-anti-miR-20a-transfected LoVo tumors following cisplatin treatment (10 mg/kg, twice a week).

the promotion of anti-miR-20a can be disrupted by NAC, siRNA-ASK1, or SP600125. We thus demonstrated that combination of cisplatin and anti-miR-20a induced mitochondrial apoptosis through the ROS/ASK1/JNK pathway.

Introduction of anti-miR-20a enhances the anti-tumor effect of cisplatin in CRC in vivo

Nude mice were inoculated with LoVo cells transfected with LV-empty or LV-anti-miR-20a, followed by treatment with cisplatin (10 mg/kg body weight) twice a week. Then, 28 days after inoculation, mice were killed and the tumors were removed. As shown in Fig. 6A and 6B, tumors were more sensitive to cisplatin treatment after introduction of anti-miR-20a compared with the control tumors. In the resected tumors we observed lower expression levels of miR-20a and higher expression levels of ASK1 in the LV-anti-miR-20a group compared to the LV-empty group (Fig. 6C). Furthermore, following cisplatin treatment, higher levels of phosphorylated ASK1 and JNK were observed in the LV-anti-miR-20a transfected tumors compared to the LV-empty group (Fig. 6D). These results demonstrate that introduction of anti-miR-20a was able to enhance the anti-CRC effect of cisplatin *in vivo*.

Discussion

Platinum-based chemotherapy is a common treatment for CRC. However, drug resistance usually occurs during the course of platinum-based chemotherapy [32], which remains a major obstacle for effective treatment of CRC. It has been reported that some miRNAs are able to change the drug sensitivity of cancer cells [18-20]. In the present study, we focused on miR-20a which may be associated with cisplatin sensitivity in CRC. miR-20a has been reported to be a tumor promoter in many cancers. Overexpression of miR-20a in these cancers enhances tumor growth and metastasis [33, 34]. More importantly, previous research indicates that miR-20a induces cisplatin resistance in some cancer [35, 36]. However, the relationship between miR-20a and chemosensitivity to cisplatin in CRC should be explored. Here, we found that miR-20a was significantly overexpressed in CRC cell lines compared with a normal colon epithelial cell line. Moreover, our data revealed that cellular miR-20a negatively regulated the chemosensitivity to cisplatin in CRC. *In vitro*, transfection with anti-miR-20a increased the cytotoxicity of cisplatin against HT29, SW480, and LoVo CRC cells, and *in vivo*, knockdown of miR-20a enhanced the anti-tumor effect of cisplatin in CRC-bearing mice. We thus demonstrated that adjuvant treatment with anti-miR-20a sensitized CRC cells to cisplatin therapy.

ROS are intermediate products that are released under stress conditions. Excessive levels of ROS in cells have been reported to trigger apoptosis pathways, because ROS can induce oxidative damage to lipids, proteins, and DNA [37-39]. Therefore, ROS has been found to influence the chemosensitivity of cancer cells. Moreover, previous studies demonstrated that cisplatin-induced cytotoxicity against cancers is closely related to ROS accumulation [40-42]. ASK1 acts as a serine/threonine protein kinase and participates in multiple biologic processes [43]. Upon accumulation of ROS in cancer cells, ASK1 is activated, leading to apoptotic cell death [25, 26, 44]. Therefore, ASK1 is a key intermediate in the ROS-dependent pathway of apoptosis.

In this study, we found that cytotoxicity of anti-miR-20a and cisplatin co-treatment in CRC was dependent on the generation of ROS. However, although anti-miR-20a markedly enhanced the anti-tumor effect of cisplatin on CRC, it did not change the cellular level of ROS. We demonstrated that ASK1 was the target of miR-20a. Treatment with anti-miR-20a was found to enhance ROS-dependent cell death by increasing the expression of ASK1, which is a downstream signaling ROS molecule.

As part of the bridging function of ASK1, ROS-dependent activation of ASK1 causes phosphorylation of JNK, which is a stress-activated protein kinase belonging to the MAPK family [45, 46]. Previous research has demonstrated that the phosphorylation of JNK triggered by ROS induces phosphorylation of Bcl-2 [28]. Thus, activation of JNK signaling in cancer cells facilitates mitochondrial apoptosis by causing loss-of-function of Bcl-2 [29]. Consistent with these findings, our data showed that anti-miR-20a treatment was able to enhance ROS/ASK1 signaling mediated by cisplatin through increased ASK1 expression. Combination of cisplatin and anti-miR-20a markedly increased the activation of JNK and subsequent mitochondrial apoptosis in CRC cells. Overall, we have demonstrated that combination with anti-miR-20a promoted cisplatin-induced mitochondrial apoptosis through the ROS/ASK1/JNK pathway in CRC.

In summary, we found that miR-20a decreased the chemosensitivity of CRC cells to cisplatin. Hence, the present findings support a novel strategy to enhance the efficiency of platinum-based chemotherapy by combination with miR-20a inhibitors. However, further studies are required to explore the clinical applications of miR-20a inhibitors for chemotherapy of CRC.

Disclosure Statement

The authors declare to have no conflicts of interest.

References

- 1 Siegel RL, Miller KD, Jemal A: Cancer statistics, 2015. *CA Cancer J Clin* 2015;65:5-29.
- 2 Bai Z, Wang J, Wang T, Li Y, Zhao X, Wu G, Yang Y, Deng W, Zhang Z: The MiR-495/Annexin A3/P53 Axis Inhibits the Invasion and EMT of Colorectal Cancer Cells. *Cell Physiol Biochem* 2017;44:1882-1895.
- 3 Sun C, Wang FJ, Zhang HG, Xu XZ, Jia RC, Yao L, Qiao PF: miR-34a mediates oxaliplatin resistance of colorectal cancer cells by inhibiting macroautophagy via transforming growth factor- β /Smad4 pathway. *World J Gastroenterol* 2017;23:1816-1827.
- 4 Loree JM, Kopetz S: Recent developments in the treatment of metastatic colorectal cancer. *Ther Adv Med Oncol* 2017;9:551-564.
- 5 Martini G, Troiani T, Cardone C, Vitiello P, Sforza V, Ciardiello D, Napolitano S, Della Corte CM, Morgillo F, Raucci A, Cuomo A, Selvaggi F, Ciardiello F, Martinelli E: Present and future of metastatic colorectal cancer treatment: A review of new candidate targets. *World J Gastroenterol* 2017;23:4675-4688.
- 6 Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Pitot HC, Alberts SR: A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. *J Clin Oncol* 2004;22:23-30.
- 7 Zhang R, Xu J, Zhao J, Bai J: Knockdown of miR-27a sensitizes colorectal cancer stem cells to TRAIL by promoting the formation of Apaf-1-caspase-9 complex. *Oncotarget* 2017;8:45213-45223.
- 8 Xie T, Li Y, Li SL, Luo HF: Astragaloside IV Enhances Cisplatin Chemosensitivity in Human Colorectal Cancer via Regulating NOTCH3. *Oncol Res* 2016;24:447-453.
- 9 Ou Y, Zhai D, Wu N, Li X: Downregulation of miR-363 increases drug resistance in cisplatin-treated HepG2 by dysregulating Mcl-1. *Gene* 2015;572:116-122.
- 10 Raftery L, Sanoff H, Goldberg R: Colon Cancer in Older Adults. *Semin Oncol* 2008;35:561-568.
- 11 Li X, Wang H, Wang J, Chen Y, Yin X, Shi G, Li H, Hu Z, Liang X: Emodin enhances cisplatin-induced cytotoxicity in human bladder cancer cells through ROS elevation and MRP1 downregulation. *BMC Cancer* 2016;16:578.
- 12 Ma MZ, Chen G, Wang P, Lu WH, Zhu CF, Song M, Yang J, Wen S, Xu RH, Hu Y, Huang P: Xc- inhibitor sulfasalazine sensitizes colorectal cancer to cisplatin by a GSH-dependent mechanism. *Cancer Lett* 2015;368:88-96.
- 13 Ju WC, Huang GB, Luo XY, Ren WH, Zheng DQ, Chen PJ, Lou YF, Li B: X-linked inhibitor of apoptosis-associated factor 1 (XAF1) enhances the sensitivity of colorectal cancer cells to cisplatin. *Med Oncol* 2014;31:273.
- 14 Fateh A, Feizi MAH, Safaralizadeh R, Azarbarzin S: Importance of miR-299-5p in colorectal cancer. *Ann Gastroenterol* 2017;30:322-326.
- 15 Xie M, Qin H, Luo Q, Huang Q, He X, Yang Z, Lan P, Lian L: MicroRNA-30a regulates cell proliferation and tumor growth of colorectal cancer by targeting CD73. *BMC Cancer* 2017;17:305.
- 16 Xie T, Huang M, Wang Y, Wang L, Chen C, Chu X: MicroRNAs as Regulators, Biomarkers and Therapeutic Targets in the Drug Resistance of Colorectal Cancer. *Cell Physiol Biochem* 2016;40:62-76.
- 17 Zhai F, Cao C, Zhang L, Zhang J: miR-543 promotes colorectal cancer proliferation and metastasis by targeting KLF4. *Oncotarget* 2017;8:59246-59256.
- 18 Ma J, Dong C, Ji C: MicroRNA and drug resistance. *Cancer Gene Ther* 2010;17:523-531.
- 19 Liu X, Xie T, Mao X, Xue L, Chu X, Chen L: MicroRNA-149 increases the sensitivity of colorectal cancer cells to 5-fluorouracil by targeting forkhead box transcription factor FOXM1. *Cell Physiol Biochem* 2016;39:617-29.
- 20 Zhao D, Chen Y, Chen S, Zheng C, Hu J, Luo S: MiR-19a regulates the cell growth and apoptosis of osteosarcoma stem cells by targeting PTEN. *Tumour Biol* 2017;39:1010428317705341.
- 21 Zhang Y, Zheng L, Ding Y, Li Q, Wang R, Liu T, Sun Q, Yang H, Peng S, Wang W, Chen L: MiR-20a Induces Cell Radioresistance by Activating the PTEN/PI3K/Akt Signaling Pathway in Hepatocellular Carcinoma. *Int J Radiat Oncol Biol Phys* 2015;92:1132-1140.
- 22 Du Y, Zhu M, Zhou X, Huang Z, Zhu J, Xu J, Cheng G, Shu Y, Liu P, Zhu W, Wang T: miR-20a enhances cisplatin resistance of human gastric cancer cell line by targeting NFKBIB. *Tumour Biol* 2016;37:1261-1269.
- 23 Jiang K, Wang W, Jin X, Wang Z, Ji Z, Meng G: Silibinin, a natural flavonoid, induces autophagy via ROS-dependent mitochondrial dysfunction and loss of ATP involving BNIP3 in human MCF7 breast cancer cells. *Oncol Rep* 2015;33:2711-2718.

- 24 Hong D, Bai YP, Shi RZ, Tan GS, Hu CP, Zhang GG: Inhibitory effect of reinoside C on vascular smooth muscle cells proliferation induced by angiotensin II via inhibiting NADPH oxidase-ROS-ENK1/2-NF-kappaB-AP-1 pathway. *Pharmazie* 2014;69:698-703.
- 25 Liang T, Zhang X, Xue W, Zhao S, Zhang X, Pei J: Curcumin induced human gastric cancer BGC-823 cells apoptosis by ROS-mediated ASK1-MKK4-JNK stress signaling pathway. *Int J Mol Sci* 2014;15:15754-15765.
- 26 Zheng R, You Z, Jia J, Lin S, Han S, Liu A, Long H, Wang S: Curcumin enhances the antitumor effect of ABT-737 via activation of the ROS-ASK1-JNK pathway in hepatocellular carcinoma cells. *Mol Med Rep* 2016;13:1570-1576.
- 27 Li HY, Zhang J, Sun LL, Li BH, Gao HL, Xie T, Zhang N, Ye ZM: Celastrol induces apoptosis and autophagy via the ROS/JNK signaling pathway in human osteosarcoma cells: an *in vitro* and *in vivo* study. *Cell Death Dis* 2015;6:e1604.
- 28 Stander XX, Stander BA, Joubert AM: Synergistic anticancer potential of dichloroacetate and estradiol analogue exerting their effect via ROS-JNK-Bcl-2-mediated signalling pathways. *Cell Physiol Biochem* 2015;35:1499-1526.
- 29 Low SY, Tan BS, Choo HL, Tiong KH, Khoo AS, Leong CO: Suppression of BCL-2 synergizes cisplatin sensitivity in nasopharyngeal carcinoma cells. *Cancer Lett* 2012;314:166-175.
- 30 Xiong S, Mu T, Wang G, Jiang X: Mitochondria-mediated apoptosis in mammals. *Protein Cell* 2014;5:737-749.
- 31 Mendoza FJ, Henson ES, Gibson SB: MEKK1-induced apoptosis is mediated by Smac/Diablo release from the mitochondria. *Biochem Biophys Res Commun* 2005;331:1089-1098.
- 32 Giaccone G: Clinical perspectives on platinum resistance. *Drugs* 2000;59:9-17.
- 33 Xu T, Jing C, Shi Y, Miao R, Peng L, Kong S, Ma Y, Li L: microRNA-20a enhances the epithelial-to-mesenchymal transition of colorectal cancer cells by modulating matrix metalloproteinases. *Exp Ther Med* 2015;10:683-688.
- 34 Babu KR, Muckenthaler MU: miR-20a regulates expression of the iron exporter ferroportin in lung cancer. *J Mol Med (Berl)* 2016;94:347-359.
- 35 Du Y, Zhu M, Zhou X, Huang Z, Zhu J, Xu J, Cheng G, Shu Y, Liu P, Zhu W, Wang T: miR-20a enhances cisplatin resistance of human gastric cancer cell line by targeting NFKB1B. *Tumour Biol* 2016;37:1261-1269.
- 36 Liu Y, Han S, Li Y, Liu Y, Zhang D, Li Y, Zhang J: MicroRNA-20a contributes to cisplatin-resistance and migration of OVCAR3 ovarian cancer cell line. *Oncol Lett* 2017;14:1780-1786.
- 37 Sosa V, Moline T, Somoza R, Paciucci R, Kondoh H, ME LL: Oxidative stress and cancer: an overview. *Ageing Res Rev* 2013;12:376-390.
- 38 Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB: Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. *J Cell Sci* 2007;120:4155-4166.
- 39 Simon HU, Haj-Yehia A, Levi-Schaffer F: Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 2000;5:415-418.
- 40 Rossetti IB, Chagas LR, Costa MS: Photodynamic antimicrobial chemotherapy (PACT) inhibits biofilm formation by *Candida albicans*, increasing both ROS production and membrane permeability. *Lasers Med Sci* 2014;29:1059-1064.
- 41 Li X, Wang H, Wang J, Chen Y, Yin X, Shi G, Li H, Hu Z, Liang X: Emodin enhances cisplatin-induced cytotoxicity in human bladder cancer cells through ROS elevation and MRP1 downregulation. *BMC Cancer* 2016;16:578.
- 42 Ma J, Yang J, Wang C, Zhang N, Dong Y, Wang C, Wang Y, Lin X: Emodin augments cisplatin cytotoxicity in platinum-resistant ovarian cancer cells via ROS-dependent MRP1 downregulation. *Biomed Res Int* 2014;2014:107671.
- 43 Kanamoto T, Mota M, Takeda K, et al: Role of apoptosis signal-regulating kinase in regulation of the c-Jun N-terminal kinase pathway and apoptosis in sympathetic neurons. *Mol Cell Biol* 2000;20:196-204.
- 44 Fujino G, Noguchi T, Matsuzawa A, Yamauchi S, Saitoh M, Takeda K, Ichijo H: Thioredoxin and TRAF family proteins regulate reactive oxygen species-dependent activation of ASK1 through reciprocal modulation of the N-terminal homophilic interaction of ASK1. *Mol Cell Biol* 2007;27:8152-8163.
- 45 Palit S, Kar S, Sharma G, Das PK: Hesperetin Induces Apoptosis in Breast Carcinoma by Triggering Accumulation of ROS and Activation of ASK1/JNK Pathway. *J Cell Physiol* 2015;230:1729-1739.
- 46 Song JJ, Rhee JG, Suntharalingam M, Walsh SA, Spitz DR, Lee YJ: Role of glutaredoxin in metabolic oxidative stress. Glutaredoxin as a sensor of oxidative stress mediated by H₂O₂. *J Biol Chem* 2002;277:46566-46575.