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Original Research Article

MiR-494-3p mediates oxaliplatin resistance of colorectal cancer cells via PTEN/AKT pathway

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Abstract

Purpose: *To unravel the influence of miR-494-3p on the insensitivity of colorectal cancer (CRC) cells to oxaliplatin.*
Methods: *The mRNA level of miR-494-3p in oxaliplatin-resistant HT-29 cells was evaluated with reverse transcript-polymerase chain reaction (RT-PCR). The cells were treated with miR-494-3p suppressor or mimic, and then apoptotic changes were determined flow cytometrically. Resistance-related gene expressions were measured using RT-PCR and western blotting. In addition, in vivo mouse experiments were conducted.*
Results: *MiR-494-3p expression in oxaliplatin-resistant HT-29 cells was much higher than that in parental HT-29 cells, accompanied by increased levels of MRP, P-gp, and AKT phosphorylation (p-AKT), and decreased phosphatase and tensin homolog (PTEN) ($p < 0.001$). The miR-494-3p mimic suppressed oxaliplatin-induced parental HT-29 cell apoptosis, while miR-494-3p inhibitor promoted oxaliplatin-resistant HT-29 cell apoptosis and decreased the levels of p-AKT, MRP and P-gp, while upregulating PTEN ($p < 0.001$). Furthermore, AKT inhibitor had similar effects as miR-494-3p inhibitor ($p < 0.001$). Experiments using nude mice demonstrated that inhibition of miR-494-3p accentuated the sensitivity of oxaliplatin-resistant HT-29 cells to oxaliplatin ($p < 0.05$).*
Conclusion: *Suppression of miR-494-3p attenuates oxaliplatin insensitivity to CRC cells via a mechanism which may involve PTEN/AKT pathway. Therefore, miR-494-3p may be an effective target for overcoming drug resistance of CRC.*

Keywords: *MiR-494-3p, Oxaliplatin, Colorectal cancer, Cell apoptosis*

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INTRODUCTION

It is known that CRC is a common malignancy associated with colon and rectum, and its incidence is on the increase, with more than 1 million newly diagnosed CRC patients each year. Nowadays, surgical resection and systematic chemotherapy are applied for CRC treatment.

Oxaliplatin is the first platinum derivative that is effective in the treatment of CRC [1]. However, due to drug resistance, advances in chemotherapy for CRC are limited. Thus, there is need to unravel the processes involved in oxaliplatin insensitivity, and to develop effective chemotherapeutic agents for CRC.

MicroRNAs (miRNAs) are of great importance in regulating cell fate, function and tumorigenesis [2]. Studies have found that miRNAs regulate chemosensitivity in various cancers, including CRC. MicroRNA-34a, a well-characterized miRNA consistently down-regulated in CRC, is involved in drug resistance, and it mediates chemoresistance of oxaliplatin in CRC [3]. It has been observed that miR-425-5p also regulates chemoresistance in CRC cells [4]. There is deletion of phosphatase and tensin homolog (PTEN) in several cancers, and it is critically important in CRC progression [5]. Abnormal PTEN is often observed in cancers, and its mRNA can be modulated by miRNAs. It is well-known that PTEN suppresses AKT signal route. A previous study has revealed that miR-22 overcame sorafenib resistance in hepatocellular carcinoma cell through the PTEN/AKT pathway [6]. Up-regulation of miR-22 reversed the chemoresistance of paclitaxel in CRC through PTEN activation [7]. Moreover, miR-494 is markedly up-regulated in CRC, where it promotes cell metastasis in CRC through PTEN [8]. However, its influence on CRC drug resistance, and the mechanism involved are poorly understood, therefore, this study was undertaken to investigate these aspects.

EXPERIMENTAL

Cell culture

Human colorectal carcinoma cell line HT-29 was got from Cell Bank of Chinese Academy of Science (Shanghai, China). They were cultured at 37 °C in a 5 % CO_2 atmosphere in a medium of 10 % FBS and 1 % mixture of streptomycin and penicillin. During incubation, medium was replaced the next day.

Experimental design

Three groups of HT-29 cells were subjected to transfection with negative control (NC), miR-494-3p mimic (Table 1) and miR-494-3p suppressor (Table 2). Medium-treated cells were control. Efficiency values of miR-494-3p mimic and inhibitor were evaluated at 48 h after transfection, using real-time PCR.

In the next step, HT-29 cells were exposed to NC + oxaliplatin, or oxaliplatin (5 μg/mL) + miR-494-3p mimic, while oxaliplatin-resistant HT-29 cells were treated with oxaliplatin (5 μg/mL), or miR-494-3p inhibitor + oxaliplatin (5 μg/mL), or 5 μM LY294002 (AKT inhibitor, Selleck, S1105) + oxaliplatin (5 μg/mL), or miR-494-3p inhibitor + IGF-1 (100 ng/mL) + oxaliplatin (5 μg/mL). Cell apoptosis and the expressions of relative-proteins were determined at 48 h.

Table 1: Sequences of mimic and suppressor used

Variable	Sequence
Mimic	5'-UGAAACAUACACGGGAAACCUC-3'
Inhibitor	5'-GAGGUUUCCCGUGUAUGUUUCA-3'

Reverse transcript-polymerase chain reaction (RT-PCR)

TRIzol was employed for RNA extraction from HT-29 cells for analyses of mRNA and miRNA levels. After quantification, the RNA was subjected to reverse-transcription to cDNA. Then, using miRNA RT-PCR detection Kit (GeneCopoeia), with cDNA as templates, RT-PCR was done in triplicate using RT-PCR instrument (Applied Biosystems, ABI-7300, USA). The level of miR-494-3p relative to the internal reference U6, was estimated using $2^{-\triangle\triangle CT}$ procedure. The sequences of primers used are shown in Table 2. The conditions used for RT-PCR were as indicated in a previous study [9].

Table 2: Sequences of primers

Variable	Sequences
Hsa-miR-494-3p	F: 5'-CGCGTGAAACATACACGGGA-3' R: 5'-AGTGCAGGGTCCGAGGTATT-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'

Immunoblot assay

Immunoblotting was used to determine levels of CRC-related proteins. Treated HT-29 cells were subjected to total protein extraction with RIPA reagent tainted with suppressors of proteases and phosphatases. About 25 μg protein was resolved using SDS-PAGE, prior to transfer to PVDF membranes which were blocked using fat-free milk (BD Biosciences, BYL40422). The PVDF membranes were subjected to incubation overnight at 4 °C with 1° immunoglobulins against P-gp (1 : 1000, Abcam, Ab170904); MRP (1 : 50, Abcam, Ab24102), PTEN (Ab31392); Caspase-3 (Ab13847); Caspase-9 (1 : 1000, Abcam, Ab32539); AKT (CST, #4685); and p-AKT (1 : 2000, CST, #4060).

Following washing, membrane incubation with HRP-labeled 2° immunoglobulin was done at laboratory temperature for 2 h, after which band development was done using ECL (Millipore). Protein expression levels were determined using Image J software, with GAPDH as internal control.

Determination of cell apoptosis

Percentage cell apoptosis was calculated with flow cytometric analysis. Treated HT-29 cells were collected and subjected to double staining using kits for Annexin V-FITC and PI (Beyotime, C1052). Following the manufacturer's protocol, 5×10^5– 1×10^6 cells were incubated in binding buffer containing Annexin V-FITC for 15 min at 4°C in a dark chamber. Then, after addition of 5 µL PI, further incubation was carried out for 5 min, also in the dark. Cells without stain treatment served as a control. The levels of apoptosis in treated HT-29 cells were evaluated by flow cytometry.

TUNEL fluorescence

Apoptosis was determined in tumor tissue slices from nude mice using TUNEL Apoptosis measurement kit in line with the kit protocol. Apoptosis was observed by examining the resultant slides using fluorescence microscopy.

Ethics approval

The animal study was approved by the Institutional Animal Ethics Committee of HwaMei Hospital, University of Chinese Academy of Sciences (approval no. 10588), and followed international guidelines for experiments involving animals.

Statistics

This was done with GraphPad prism 7.0 software. Data are shown as mean ± SD of 3 independent experiments. Two-tailed *t*-test was for two-group comparison, while the comparison amongst several groups was done with one-way ANOVA and Tukey's post-test for multiple comparison. Statistical significance was assumed at $p < 0.05$.

RESULTS

MiR-494-3p was highly expressed in oxaliplatin-resistant HT-29 cells

In vitro, miR-494-3p concentrations in parental HT-29 cells and oxaliplatin-resistant HT-29 cells were quantified with RT-PCR. Figure 1 A shows that miR-494-3p level in oxaliplatin-insensitive HT-29 cells was significantly increased, relative to parental HT-29 cells, indicating that miR-494-3p was positively associated with oxaliplatin resistance in CRC cells. In addition, P-gp MRP, and p-AKT were elevated in oxaliplatin-insensitive cells, and PTEN was decreased, while AKT levels were unchanged (Figure 1B).

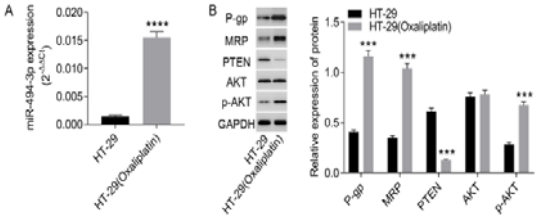

Figure 1: MiR-494-3p was highly expressed in oxaliplatin-resistant HT-29 cells (**A**) The expression levels of miR-494-3p, as quantified using RT-PCR. (**B**) Protein levels of P-gp, MRP, PTEN, pAKT, and AKT. ***$P < 0.001$, vs. HT-29

MiR-494-3p inhibition increased toxicity of oxaliplatin

The results on Figure 2 A show that miR-494-3p in HT-29 cells was down-regulated by the inhibitor, while its expression was increased by the mimic. Therefore, the inhibitor and mimic were used for subsequent experiments.

Oxaliplatin is a chemotherapy drug that inhibits tumor cell growth and arrests cells at G2-phase [10]. The results shown in Figures 2 B and C indicate that, compared to NC, miR-494-3p mimic potently inhibited oxaliplatin-induced cell apoptosis in HT-29 cells. Oxaliplatin-resistant HT-29 cells were insensitive to oxaliplatin. However, after treatment with miR-494-3p suppressor, oxaliplatin promoted the cell apoptosis of oxaliplatin-resistant HT-29 cells which was inhibited by insulin-like growth factor 1 (IGF-1). Furthermore, AKT inhibitor LY294002 had a similar effect as miR-494-3p suppressor. Thereby, it may be conjectured that the inhibition raised the toxicity of oxaliplatin to resistant HT-29 cells by promoting cell apoptosis through the AKT pathway.

MiR-494-3p suppressor mediated oxaliplatin resistance of HT-29 cells via PTEN/AKT pathway inactivation

The process underling the involvement of miR-494-3p inhibitor in mediating oxaliplatin resistance of HT-29 cells was determined. The results in Figure 3 show that high levels of P-gp and MRP in oxaliplatin-resistant HT-29 cells were significantly reduced by suppression of miR-494-

3p. In contrast, activities of caspase-3 and caspase-9, the major apoptosis executing enzymes, were increased. Moreover, the levels of PTEN were elevated by miR-494-3p inhibitor, while p-AKT levels were reduced. These data further demonstrate that miR-494-3p inhibitor attenuated oxaliplatin insensitivity in HT-29 cells by promoting apoptotic changes via inactivation of the PTEN/AKT pathway.

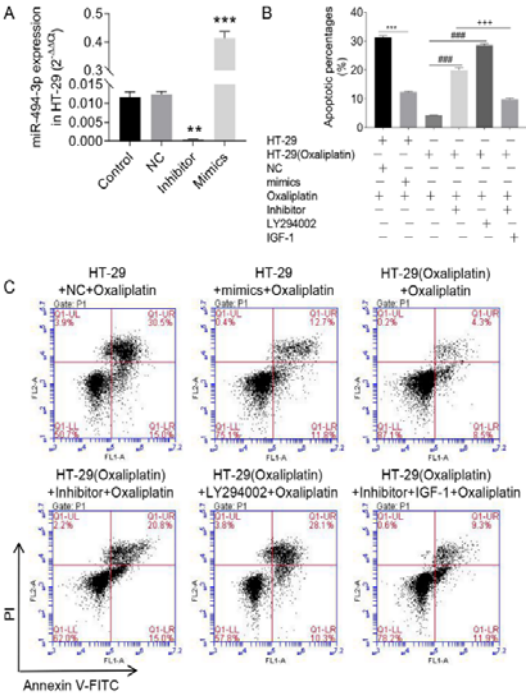

Figure 2: MiR-494-3p inhibition accentuated sensitivity of oxaliplatin-insensitive HT-29 cells to the drug (A) Effectiveness of miR-494-3p mimic and inhibitor, as detected after 48 h. (B, C) Percentages of apoptotic cells determined at 48 h after treatment with mimic, inhibitor or IGF-1

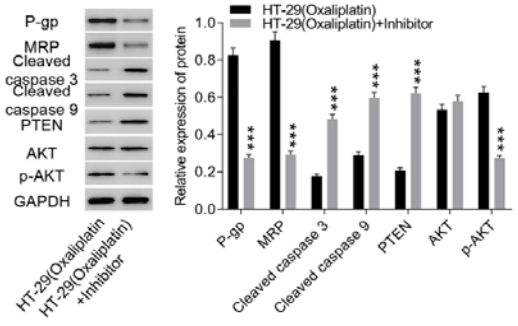

Figure 3: Levels of PTEN/AKT pathway-associated proteins in oxaliplatin-resistant cells transfected with MiR-494-3p suppressor. The inhibition reduced HT-29 cell resistance to oxaliplatin via inactivation of PTEN/AKT pathway. $^{***}P < 0.001$ vs. HT-29 (oxaliplatin)

Inhibition of MiR-494-3p promoted toxicity of oxaliplatin

The oxaliplatin-resistant cells were used to establish a subcutaneous tumor model in nude mice, followed by intraperitoneal injection of miR-494 inhibitor, alone or in combination with oxaliplatin (5 mg/kg). As shown in Figure 4 A, the results of TUNEL staining revealed that the inhibitor enhanced the sensitivity of oxaliplatin-resistant HT-29 cells to oxaliplatin by promoting apoptosis. Besides, the tumor volume and weight were significantly reduced by the suppressor (Figures 4 B - D), while the percentage survival of nude mice was significantly increased (Figure 4 E). These data are evidence indicating that suppression of miR-494-3p attenuated the oxaliplatin insensitivity of HT-29 cells.

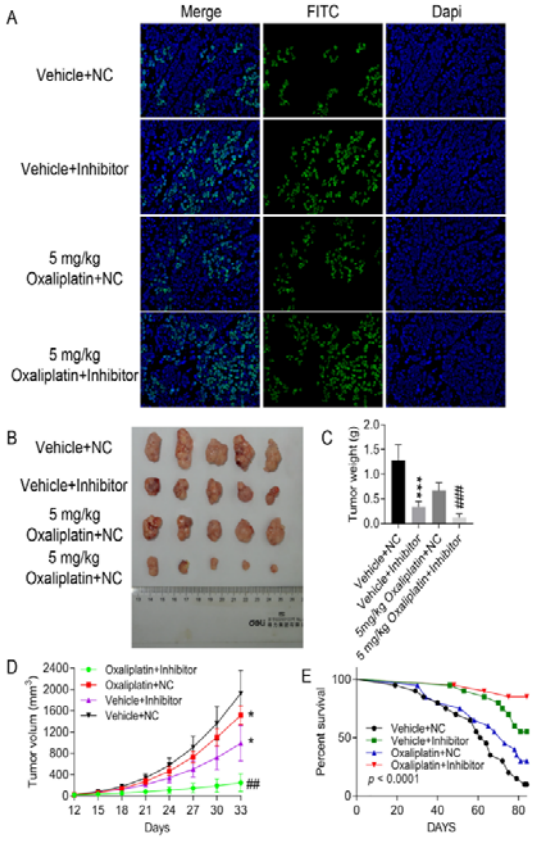

Figure 4: MiR-494-3p inhibitor promoted toxicity of oxaliplatin to the cells. Oxaliplatin-resistant cells were used to establish a subcutaneous tumor model in nude mice, followed by intraperitoneal injection of miR-494 inhibitor, alone or in combination with oxaliplatin (5 mg/kg). (A) Apoptosis, as calculated using TUNEL staining. (B) Tumor size, (C) weight and (D) volume. (E) Survival curve of nude mice in 84 days. $^{*}P < 0.05$, $^{***}p < 0.001$ vs. vehicle + NC; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. 5 mg/kg oxaliplatin

DISCUSSION

This study investigated the effect of miR-494-3p on chemoresistance of human CRC cells and its potential mechanisms. Research has found that miRNAs participate in chemoresistance of CRC: miR-451 is down-regulated in CRC and its expression circumvented the chemoresistance of CRC [11]. Elevated miR-23a was observed to enhance the chemoresistance of CRC cells [12]. In the current study, the data revealed a positive correlation between miR-494-3p and oxaliplatin insensitivity of CRC cells: suppression of miR-494-3p attenuated oxaliplatin resistance through inhibition of PTEN/AKT route.

MiR-494-3p is frequently overexpressed in many human cancers, and it is implicated in numerous cellular processes e.g., cell apoptotic and proliferative events. It serves an oncogenic function in glioma by activation of PTEN/AKT pathway [13]. In this study, miR-3p up-regulation was observed in oxaliplatin-resistant HT-29 cells, indicating that miR-494-3p expression has a positive correlation with oxaliplatin resistance of CRC cells, thereby promoting the progression of CRC. Oxaliplatin-induced cell apoptosis in parental HT-29 cells was reduced by miR-494-3p mimic, thereby suggesting that miR494-3p may act as an oncogene in CRC by inhibiting cell apoptosis. Furthermore, oxaliplatin treatment promoted apoptosis of oxaliplatin-resistant HT-29 cells by inhibition of miR-494-3p, but this effect was counteracted by IGF-1. A study has reported that IGF-1, a growth promoting factor, is positively correlated with the risk of CRC, and it increased growth CRC tumor allografts [14]. Thus, it may be inferred that miR-494-3p inhibition raised cytotoxic effect of oxaliplatin on oxaliplatin-resistant cells by promoting cell apoptosis, and the increase in apoptosis is desirable. Moreover, LY294002, an inhibitor of AKT pathway, produced a similar effect, demonstrating that the inhibitor-attenuated oxaliplatin resistance in HT-29 cells may be through inactivation of the AKT pathway. In addition, the high levels of P-gp and MRP in oxaliplatin-resistant HT-29 cells were decreased by miR-494-3p inhibitor. It is known that MRP, a novel gene encoded a human GS-X pump, is an ATP-dependent export pump which is of great importance in drug resistance [15]. Moreover, P-gp is encoded by MRP, and it is highly expressed in cancer cells, thereby causing multidrug resistance and chemotherapy failure [15]. Several studies have revealed that P-gp-mediated multidrug resistance in CRC can be reversed by methods such as cinobufagin and RNA interference [16, 17]. This suggests that the miR-494-3p inhibitor used in this study may also possibly reverse the P-gp-mediated multidrug resistance in CRC. Furthermore, it has been reported that the lack of PTEN activates AKT pathway, which further promotes tumor progression, and p-AKT is crucial for activities in PTEN/AKT pathway [18]. Moreover, PTEN is an effective suppressor of AKT, and through the induction of AKT pathway, PTEN controls several cellular events in cancer e.g., cell proliferation and apoptosis [19]. This is in agreement with the observations that PTEN, caspase-3 and caspase-9 protein levels were decreased in oxaliplatin-insensitive cells, but were increased by miR-494-3p inhibitor, and p-AKT levels decreased, whereas AKT was unchanged. These data further prove that miR-494-3p inhibitor attenuated oxaliplatin resistance of HT-29 cells, probably by PTEN/AKT pathway inactivation. Moreover, *in vivo* mouse studies demonstrated that inhibition of miR-494-3p accentuated the cytotoxic influence of the drug on oxaliplatin-resistant cells. Hence, miR-494-3p may become a molecular target for CRC resistance and therapy.

CONCLUSION

The miR-494-3p level in oxaliplatin-resistant CRC cells is up-regulated, relative to that in CRC cells, and is positively correlated with oxaliplatin insensitivity in CRC cells. Suppression of miR-494-3p attenuates oxaliplatin insensitivity in CRC cells, most likely through enhanced PTEN expression, and interrupts AKT pathway activity. Therefore, miR-494-3p is a promising target for attenuating drug resistance of CRC, thereby improving CRC patient treatment outcomes.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Authors' contributions

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jiangfan Zhu and Yisheng Cao conceived and designed the study. Yongming Yu, Zhou Wu, Zhonglei Shen and Yangyang Xie performed the experiments.

Jiangfan Zhu and Yisheng Cao wrote the manuscript. All authors read and approved the final manuscript.

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