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Role of miR-214 in Sensitization of Human Colorectal Cancer Cells to Doxorubicin by p53 Targeting

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Abstract

Background: The upregulation of miR-214 is reported to reverse chemotherapeutic resistance or sensitivity in many human malignancies.
Objectives: This study aimed to investigate the potential function of miR-214 in the apoptosis induction by targeting p53 in human colorectal cancer cells (CRC) in combination with doxorubicin (DOX).
Methods: miR-214 mimics were transfected to HT-29 CRC cells. Following that, DOX was utilized to treat the transfected cells. additionally, apoptosis, migration, and cell viability were evaluated by flow cytometry, scratch-wound motility, and MTT assays, respectively. Furthermore, qRT-PCR was employed to evaluate the expression level of miR-214 and p53.
Results: miR-214 transfection significantly inhibited the cell proliferation rate ($P<0.05$), harnessed migration ($P<0.05$), and induced apoptosis ($P<0.05$) in the HT-29 cells after 48 h. Furthermore, more effectiveness was observed in combination with DOX. Additionally, miR-214 transfection resulted in a reduction in p53 expression offering that might be a potential target for miR-214.
Conclusion: In conclusion, miR-214 sensitizes HT-29 cells to doxorubicin by targeting p53 indicating the significant role of this miRNA in colorectal cancer chemotherapy.

Keywords: Apoptosis, Colorectal cancer, Doxorubicin, miR-214, p53

1. Background

Colorectal cancer is considered one of the most common and lethal human malignancies with a high incidence rate worldwide, which imposes a great burden on human societies (1). The clear molecular mechanisms underlying the initiation/progression of colorectal cancer are still not well-understood (2). However, some risk factors, such as inappropriate lifestyle, smoking, alcohol consumption, physical inactivity, and in some cases, genetic predisposition are introduced for colorectal cancer (3). Despite the development of various effective combination therapies consisting of surgery, chemotherapy, and radiotherapy, 50% of patients with colorectal cancer experience tumor recurrence (4). It is suggested that the development of multidrug resistance is one of the main reasons for the failure of therapeutic strategies and tumor recurrence (5). Therefore, there is an urgent need for designing and developing novel strategies to combat drug resistance in patients with colorectal cancer (5).

Recent decades have witnessed an increase in the attention to the importance of micro RNAs (miRNAs) as a big family of small non-coding RNAs in the pathogenesis of colorectal cancer (6,7). miRNAs play a critical role in various biological and physiological events through the regulation of target gene expression (8). These tiny RNA molecules bind to the 3' untranslated region (3'UTR) of the target genes; moreover, mRNAs suppress the expression of targets at the post-transcription levels (9). Furthermore, miRNAs are involved in controlling cellular proliferation, growth, apoptosis, differentiation, and migration using this mechanism (9). It should be noted that miR-214 is one of the recently recognized miRNAs with major significances in the initiation/progression of colorectal cancer (10,11). Previous studies have reported a tumor repressor function for miR-214 in colorectal cancer. Decreased expression levels of miR-214 in colorectal cancer are demonstrated to be related to increased cellular proliferation and induced cell survival (12,13).

More importantly, miR-214 is also associated with chemotherapy response, and the upregulation of this miRNA is reported to reverse drug resistance to various chemotherapeutics in numerous human malignancies (14-16). To our knowledge, there have been no studies evaluating the effects of miR-214 overexpression on the resistance to doxorubicin (DOX) in colorectal cancer. Development of resistance to DOX is reported to decrease its efficacy and limit its application in the treatment of colorectal cancer despite its high cytotoxic effects against colorectal cancer cells.

2. Objectives

This study aimed to assess the effects of miR-214

transfection into HT-29 colorectal cancer cells and its efficacy in increasing the cytotoxicity of DOX against HT-29 cell proliferation and invasion.

3. Methods

3.1. Cell culture and transfection

The HT-29 colorectal cancer cells were obtained from the Chinese Academy of Sciences. Subsequently, the cells were cultured in an RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 0.1 mg/ml streptomycin. Afterward, the cells seeded on a 6-well plate overnight were transfected with miR-214 mimics using JetPEI reagent (Polyplus-transfection, Strasbourg, France) following the manufacturer's protocol. Moreover, C. Elegance miRNA (MISSION2® miRNA, Sigma-Aldrich Co.) was used as negative controls for miRNA transfections with no sequence homology to the human gene (Table 1).

3.2. MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine cell viability following treatment with DOX (Sigma Aldrich) and a combination of DOX with miR-214. Initially, 1×10^4 cells per well were seeded at 96-well plates and treated subsequently with different concentrations of syringic acid (up to 5 µM) alone or in combination with 10 nM miR-214. After 48 h, media-containing drugs were removed and MTT containing medium was added. After incubation for 4 h at 37˚C, the formed formazan crystals were solubilized using dimethylsulfoxide. The absorbance of each well was measured at 570 nm with a microplate reader.

3.3. qRT-PCR

This method was used to evaluate the mRNA expression levels of target genes, including miR-214 and p53. For this purpose, the total RNAs of all experimental groups were extracted using a Trizol RNA extraction kit. Following that, the quality and quantity of extracted total RNAs were analyzed using the NanoDrop spectrophotometric method. In the next step, the total RNAs were reversely transcribed to complementary DNA (cDNA) by a commercial cDNA synthesis kit. The cDNA was subjected to qPCR by specific primers and SYBR Green Master Mix on Rotor-Gene™ 6000 system. The primer sequences of target genes were presented in Table 1.

3.4. Annexin-V flow cytometry

Flow cytometric analysis of apoptosis was also performed to determine the effect of miR-214 transfection on the cytotoxicity of DOX in HT-29 cells. After treatment, the cells were harvested, washed twice with cold PBS, and resuspended in 500 µl of binding buffer, followed by the addition of 5 µl annexin V-FITC and 5 µl propidium iodide. After incubation for 15 min at dark and room temperature, they were analyzed by a flow cytometer.

3.5. Wound healing assay

The wound-healing assay was applied in order to evaluate the effects of miR-214 transfection on the DOX-mediated suppression of cellular invasion in HT-29 colorectal cancer cells. For this purpose, the cells were seeded into 24-well plates and scraped with pipette tips. After washing with PBS, the cells were treated with miR-NC, miR-214, and DOX combined with miR-214. They were then evaluated under a phase-contrast microscope for 24, 48, and 72 h post-induction of injury. Migrated cells were measured and quantified using Image J software.

3.6. Statistical analysis

The analyzed data were demonstrated as mean±SD. Moreover, one-way ANOVA, post hoc Tukey, and Dunnett tests were applied to compare the mean values between experimental groups. A p-value less than 0.05 was considered statistically significant.

Table 1. Primer sequences utilized in the present study

Primer name	Forward/ Reverse	Sequences
p53	F	5'-TCTTCCTGCCCACCATCTACTC-3'
	R	5'-TGCAGCCTGTACTTGTCCGTC-3'
β–actin	F	5´- TCCCTGGAGAAGAGCTACG -3´
	R	5´- GTAGTTTCGTGGATGCCACA -3´
U6 snRNA	Target sequence	5´GCUCGUUCGGCAGCACACAUAUACUAAAAUUGGAACGAUACAGAGAGAAGAUUAGCA UGGCCCCUGCGCAAGGAUGACACGCAAAUUCGUGAAGCGU UCCAUAUUUUU-3´
Has-miR-214	Target sequence	5'-UCA UCU CGC CCG CAA AGA CCC A-3'
C. elegans miRNA	Target sequence	5'-CGGUACGAUCGCGGCGGGAUAUC-3'

4. Results

4.1. miR-214 transfection led to the significant overexpression of miR-214 in HT-29 cells

The expression level of miR-214 was evaluated by qRT-PCR to assess the efficacy of miR-214 transfection in HT-29 cells. The obtained results showed that transfection with 5-20 nM miR-214 mimics+JetPEI reagent resulted in a significant increase in the expression levels of miR-214 in HT-29 cells, compared to non-treated control cells ($P<0.05$; Figure 1). In addition, miR-NC showed no significant effect on the expression levels of miR-214.

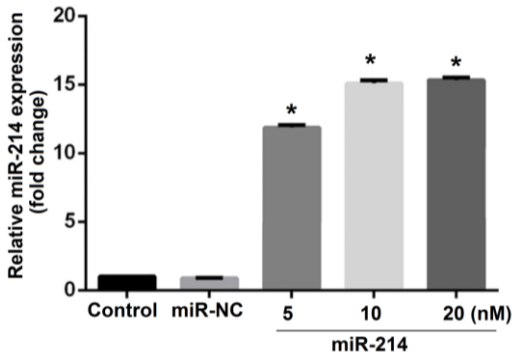

Figure 1. Relative miR-214 expression in HT-29 cells 24 after transfection. The data are represented as mean±SD (n=3), *P<0.01

4.2. Transfection of miR-214 potentiated DOX mediated suppression of cellular proliferation

The MTT assay was applied following transfection with miR-214 to investigate the effects of this miRNA on the increasing DOX-mediated cytotoxicity in HT-29 cells. As shown in Figure 2a, the cells were treated with 0-5 µM concentrations of DOX. The cytotoxic effects of DOX were dose-dependent in the HT-29 cell lines. The cell treatment with 0-0.35 µM DOX did not exert significant cytotoxic effects; however, concentrations more than 0.35 µM of DOX resulted in significant inhibition in HT-29 cell proliferation rate. The IC50 value for DOX was 0.69 µM in HT-29 cells. In addition, treatment of HT-2 cells combined with various concentrations of DOX and 10 nM miR-214 led to a significant suppression in the proliferation of HT-29 cells (Figure 2b). The IC50 value for DOX decreased to 0.29 µM when combined with miR-214. Therefore, it led to enhanced cytotoxic effects of DOX in lower concentrations in combination with miR-214.

4.3. Effects of miR-214 transfection in combination with DOX on the p53 expression levels

The P53 protein, as "genome guardian", is considered one of the most important tumor suppressor genes involved in the apoptosis and development of drug resistance. To evaluate the importance of the p53 in the miR-214 mediated

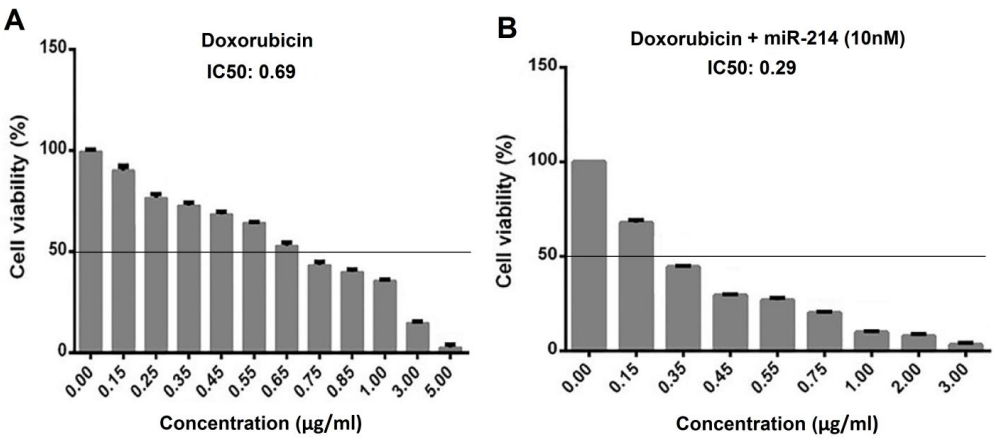

Figure 2. Effect of combination therapy on HT-2cell viability

increase in the DOX cytotoxicity, the expression levels of this gene were evaluated in cells treated with DOX alone or in combination with miR-214. As shown in Figure 3, both treatments (DOX alone or in combination with miR-214) resulted in a significant increase in the expression levels of p53 (P<0.05). In addition, miR-NC did not alter the expression levels of p53 in HT-29 cells, compared to controls. Therefore, it is suggested that the positive effects of miR-214 in increasing anti-cancer effects of DOX may be mediated by the upregulation of p53.

4.4. HT-29 cells transfection with miR-214 mimic increased DOX mediated apoptosis

Annexin-V flow cytometry analysis in HT-29 cells treated with DOX alone or in combination with miR-

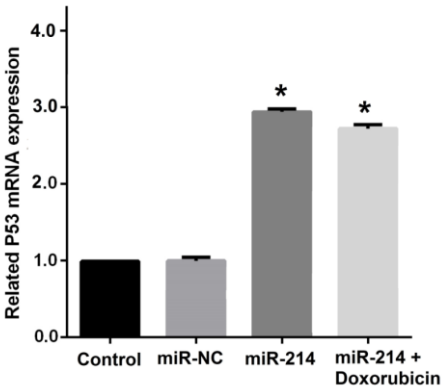

Figure 3. Analysis of p53 gene expression levels by a quantitative reverse transcription-polymerase chain reaction. The results were expressed as mean±SD (n=4); *P<0.05

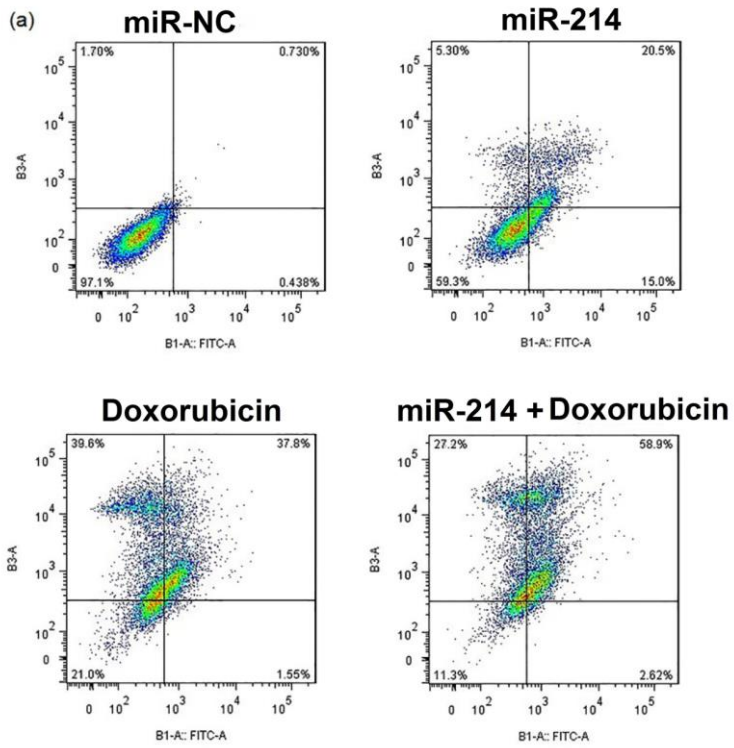

Figure 4. Fraction of apoptotic cells was analyzed using the V-FITC/PI assay in HT-29 cells under different treatments

214 mimic showed that DOX significantly increased apoptosis rate in HT-29 cells and shifted to the right in apoptosis diagram, compared to non-treated control cells (P<0.05; Figure 4). More importantly, the combination of DOX with miR-214 exerted a more potent stimulatory effect on apoptosis in such a way that cells treated with DOX+miR-214 showed higher levels of apoptosis, compared to cells treated with DOX alone (P<0.05). Therefore, miR-214 was effective in increasing DOX-mediated apoptosis in HT-29 cells.

4.5. Combination of DOX and miR-214 enhanced invasion of HT-29 cells

Wound healing assay was used to evaluate the effect of DOX and miR-214 on the invasion of HT-29 cells. After 72 h, the cell treatment with 0.69 μM DOX led to significant suppression in the invasion of HT-29 cells, compared to cells with no treatment (P<0.05; Figure 5). However, the combination of DOX with miR-214 had a more inhibitory effect on the invasion of HT-29 cells, compared to cells treated with DOX (P<0.05; Figure 5). Therefore, the

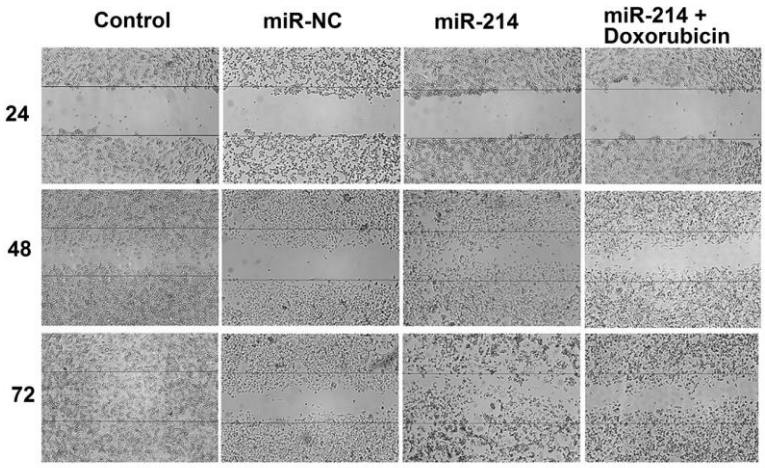

Figure 5. miR-214 alone or in combination with doxorubicin inhibited cell migration in HT-29 cells. Wound healing assay was performed to estimate cell migration in each condition.

combination of DOX and miR-214 had more efficacies in suppressing the invasive behavior of HT-29 colorectal cancer cells.

5. Discussion

According to the results of this study, miR-214 transfection led to an increase in the DOX-mediated anti-proliferative, pro-apoptosis, and anti-metastasis effects in HT-29 cells. In other words, the cells treated with the combination of DOX and miR-214 mimics had lower proliferation rates, higher levels of apoptosis, and decreased invasive phenotype, compared to cells treated with DOX alone. In addition, miR-214 transfection led to the significant upregulation of p53 in HT-29 cell, which demonstrated that miR-214 mediated increase in the DOX cytotoxicity may be p53 dependent. miR-214 is one of the most important tumor suppressor miRNAs in colorectal cancer that was commonly demonstrated to be downregulated in colon tissue of patients with colorectal cancer. Moreover, its downregulation was associated with increased cellular proliferation and metastasis (17,18). In a study conducted by Sun et al., a decrease was found in the expression levels of this miRNA in the colon tumor tissue, compared to normal adjacent tissue (19). In addition, they showed that transfection with miR-214 mimic resulted in a significant suppression in cellular proliferation, epithelial-mesenchymal transition, and metastasis in HCT116 and RKO colorectal cancer cells (19). Long et al. indicated the downregulation of miR-214 in colorectal cancer tissues, compared to healthy tissues. Additionally, they observed that an increase in the expression levels of this miRNA significantly suppressed the colorectal cancer cell line growth (13). miR-214 was also indicated to promote apoptosis o f SW620 colorectal cancer cell lines through suppressing cellular proliferation and invasion (20). More importantly, miR-214 is reported to play a critical role in regulating the cellular response to chemotherapeutic agents, such as 5-fluorouracil (5-FU), cisplatin, and DOX. In bladder cancer, upregulation of miR-214 led to reversing cisplatin resistance (21).

In a study performed by Yang et al., it was found that miR-214 suppressed cell proliferation and growth, followed by an increase in the 5-FU-mediated cell apoptosis (22). Regarding the breast cancer cells, Zhang et al. reported that the transfection of cancer cells with miR-214 resulted in a significant induction in apoptosis and sensitization to cancer cells to DOX (23). The combination treatment of HT-29 colorectal cancer cells with DOX and miR-214 led to increased cytotoxicity of DOX through induction of apoptosis and inhibition of cellular invasion in this study, which was consistent with the results of the abovementioned studies.

Therefore, miR-214 may play a major role in the colorectal cancer cells' response to DOX, and miR-214 downregulation may be involved in the development of drug resistance against DOX in HT-29 colorectal cancer cells. P53 is a key target of various miRNA, including miR-214 the modulating expression levels of which control various aspects of cancer initiation/progression. In a study performed by Wu et al., it was reported that miR-214 modulated colorectal cancer cell proliferation and apoptosis through targeting p53 (24). In ovarian cancer cells, miR-214 targeted p53 and regulated ovarian cancer cells' stemness (25). In addition, miR-214 was also reported to play a critical function in the regulation of breast cancer cell invasion by modulating the expression levels of p53 (26). In the same line, Zhang et al. reported that miR-214 mediated overexpression in p53 levels sensitized breast cancer cells to doxorubicin by promoting apoptosis (23). Similarly, it was found that colorectal cancer cells transfection with miR-214 mimic increased expression levels of p53; accordingly, p53 may be involved in the colorectal cancer cells' sensitivity to DOX.

6. Conclusion

The results obtained from this study showed the positive effects of miR-214 in sensitizing HT-29 colorectal cancer cells to DOX through increasing apoptosis, inhibiting cellular invasion, and targeting p53. This may indicate the significant role of miR-214 in colorectal cancer chemotherapy. However, further studies are required to elucidate the clear underlying mechanisms of miR-214-mediated enhanced chemotherapy response in colorectal cancer.

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Footnotes

Authors' Contribution: L.W and P.J: Performed experiments and co-wrote the paper; X C.Q: Analyzed data; X.L and L.W: Designed experiments and co-wrote the paper; C.W: Supervised the research

Conflict of Interests: The authors have no conflict of interest to declare.

Ethical Approval: The study protocol was approved by the Second People Hospital of Dezhou.

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Informed consent: Informed consent was not necessary for this *in vitro* study.

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