P53-microRNA interactions regulate the response of colorectal tumor cells to oxaliplatin under normoxic and hypoxic conditions

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Abstract. Oxaliplatin (OXA)-containing regimens are used as first-line chemotherapy in colorectal cancer (CRC). However, OXA resistance remains a major challenge in CRC treatment. CRC cells that adapt to hypoxia can potentially develop OXA resistance, and the underlying molecular mechanisms still need to be further investigated. In the current study, the OXA drug sensitivity of two CRC cell lines, HCT116 ($TP53^{WT}$) and HT29 ($TP53^{MT}$), was compared under both normoxic and hypoxic conditions. It was found that under normoxic condition, HCT116 cells showed significantly higher OXA sensitivity than HT29 cells. However, both cell lines showed remarkable OXA resistance under hypoxic conditions. It was also revealed that P53 levels were increased after OXA and hypoxia treatment in HCT116 cells but not in HT29 cells. Notably, knocking down $P53^{WT}$ decreased normoxic but increased hypoxic OXA sensitivity in HCT116 cells, which did not exist in HT29 cells. Molecular analysis indicated that $P53^{WT}$ activated microRNA (miR)-26a and miR-34a in OXA treatment and activated miR-23a in hypoxia treatment. Cell proliferation experiments indicated that a high level of miR-23a decreased OXA sensitivity and that a high level of miR-26a or miR-34a increased OXA sensitivity in HCT116 cells. Additionally, it was demonstrated that miR-26a, miR-34a and miR-23a affect cell apoptosis through regulation of MCL-1, EZH2, BCL-2, SMAD 4 and STAT3. Taken together, the present findings revealed the dual function of P53 in regulating cellular chemo-sensitivity and highlighted the role of P53-miR interactions in the response of CRC cells to OXA chemotherapy under normoxic and hypoxic conditions.

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide. In China, the incidence and mortality of CRC are increasing every year (1). Surgical resection is the mainstay of potentially curative treatments for CRC; however, prognosis is generally poor due to locoregional recurrence with resection alone (2).

Cytotoxic chemotherapy is another mainstay of treatment for CRC patients. Oxaliplatin (OXA) is a third-generation chemotherapy drug of the diamino-cyclohexane platinum family (3,4). Due to potent *in vitro* cytotoxicity and *in vivo* antitumor activity, OXA-containing regimens are effectively used as first-line chemotherapy in CRC. However, *de novo* and acquired OXA resistance remains a major challenge in CRC treatment (5). The acquisition of OXA resistance in CRC is multi-factorial and includes the following: cellular transport and detoxification systems (copper transporters, solute carrier transporters and ATP-binding cassette transporters), OXA-induced DNA adduct repair and alterations in key cell death-related genes and/or tumor suppressors (p53, Bcl-2 family and MMP7) (6-9).

Hypoxia is a common feature of the tumor microenvironment that activates the expression of numerous genes associated with cell growth, angiogenesis, metastasis and drug resistance (10-13). Cells adapting to hypoxia have been demonstrated to reduce the cytotoxicity of numerous drugs, such as OXA and 5-fluorouracil (5-FU) (14,15). Therefore, elucidating the underlying mechanisms of hypoxia-induced drug resistance and developing more effective therapeutic regimens to overcome hypoxia-induced drug resistance are clinical priorities.

Accumulating evidence has shown that microRNAs (miRs) play an important role in acquired drug resistance in colorectal carcinoma (CRC). miRs can act as hypoxia sensors and their levels are altered consistently in CRC cells (16). Nijhuis *et al* (16) indicated that treatment with miR-21 and miR-30d antagonists sensitized hypoxic CRC cells to 5-FU. Xu *et al* (17) indicated that hypoxia-inducible transcription factor 1α (HIF-1α)-mediated suppression of miR-338-5p conferred OXA resistance in CRC cells.

P53 is a stress-inducible transcription factor that regulates numerous downstream genes, such as p21, Bax and GADD45, to exert regulatory functions in multiple signaling processes (18,19). TP53 mutation occurs in ~40-50% of CRC (20). The TP53 mutation status is closely related to the progression, drug resistance and outcome of CRC (21,22). However, the effect of TP53 mutation on drug resistance in CRC cells, particularly hypoxic CRC cells, and the role of miRs during this process remain to be elucidated.

The aim of the present study was to investigate how p53 affects hypoxia-induced OXA resistance by regulating miR expression in CRC.

Materials and methods

Cell lines and cell culture. Human CRC cell lines HCT116 ($TP53^{WT}$) and HT29 ($TP53^{MT}$, c.818G>A (23), STR profiled) were purchased from Stem Cell Bank, Chinese Academy of Sciences. Cells were cultured in DMEM (HyClone; Cytiva) supplemented with 10% fetal bovine serum (Shanghai ExCell Biology, Inc.) and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Sangon Biotech Co., Ltd.) and were maintained in a humidified 37˚C incubator with 5% CO_2. Cells were passaged when they reached ~80% confluency and were regularly tested with Mycoplasma Test Kit (Shanghai Yeasen Biotechnology Co., Ltd.) to ensure the absence of mycoplasma contamination.

For OXA chemotherapy, OXA (MilliporeSigma) solution was prepared with cell culture medium and the working concentration is 5, 10, 20, 40 and 60 μM. Cells were cultured in OXA for 24 h before other experiments were carried out. For the hypoxic culture, cells were cultured at 37˚C in a humidified O_2 (1%)/CO_2 (5%)/N2 (94%) incubator (Thermo Fisher Scientific, Inc.) for 24 h (17). Echinomycin (1 nM; MedChemExpress) dissolved in dimethyl sulfoxide (DMSO; MilliporeSigma) was added to cell culture medium for 24 h to inactivate HIF-1α (24).

Plasmid construction and cell transfection. For overexpressing $P53^{WT}$, the full-length CDS of human $TP53^{WT}$ gene was synthesized and cloned into pcDNA3.1-neo vector. For knocking down P53, short hairpin (sh)RNA was synthesized and cloned into RNA interference vector pSilencer3.1-neo. The sequences of TP53 siRNA and scrambled siRNA were CAC CATCCACTACAACTACAT (25) and GGATTTCGAGTCG TCTTAA. Stable cell lines were selected by G418 (800 μg/ml; Thermo Fisher Scientific, Inc.).

For plasmid transfection, HCT116 and HT29 cells were seeded in six-well plates 24 h prior to transfection in complete medium until they reached 40~60% confluency. Plasmid DNA was complexed with Lipofectamine 3000 and P3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Transfection media was removed and replaced with new media at 7 h post-transfection. miR-23a, -26a, -34a, -133a, -107a, -205 listed in Table I and NC-mimics were synthesized (Guangzhou RiboBio Co., Ltd.) and transfected into cells at a concentration of 10 nmol/ml with lipofectamine 3000. All the subsequent experimentations were carried out at 48 h post-transfection.

Cellular growth inhibition assay. Normal or transfected cells (at a density of 5,000 per well) in 100 μl complete medium were seeded in one well of 96-well plates. After culturing for 24 h, cells were treated with OXA and/or hypoxia for another 24 h, and 20 μl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MTT; Beyotime Biotechnology) was added to each well and incubated at 37˚C for 4 h. After removing the medium, the blue formazan was dissolved with 200 μl DMSO, and absorbance at 550 nm was measured. The cellular growth inhibition rate was defined as (1-OD_{550} of the experimental group)/OD_{550} of the control group x100%.

Expression analysis of miRs and reverse transcription-quantitative polymerase chain reactions (RT-qPCR). Total RNA of the OXA or hypoxia treated HCT116 cells was isolated with RNAzol (Sigma-Aldrich; Merck KGaA). A total of 31 miRs were selected and examined to analyze the effect of miRs on cellular chemoresistance (15 miRs) or cellular response to hypoxia (16 miRs).

RT-qPCR was conducted to detect the enrichment of relevant miRs. RNA was reverse transcribed to cDNA using Mir-X miRNA First-Strand Synthesis kit according to the manufacturer's instructionσ (Takara Biotechnology Co., Ltd.). Mir-X TB Green RT-qPCR kit (Takara Biotechnology Co., Ltd.) was used to conduct RT-qPCR reaction according to the manufacturer's instruction. U6 snRNA expression was used as endogenous control. The 5' primer of U6 is CGCTTCGGCAGCACATAT AC. PCR was performed on an ABI 7500 qPCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions consisted of 10 sec at 95˚C followed by 40 cycles at 95˚C for 5 sec and 60˚C for 35 sec. The abundance of miR was calculated using the formula of $2^{-\Delta\Delta Cq}$ (26). The primer sequences for amplification of miRs are listed in Table I.

Western blot analysis. Cells were lysed with lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) complemented with protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Cell lysates were centrifuged at 12,000 x g at 4˚C for 20 min to remove cell debris and insoluble material. Protein concentration was quantitated using the Bradford protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of lysate (25 μg) were loaded per lane and proteins resolved by 5-10% SDS-PAGE gel, semi-dry transferred to 0.45-μm polyvinylidene fluoride membranes (EMD Millipore). The membranes were incubated in 5% skim milk in Tris-buffered saline Tween-20 (TBST; 10 mM Tris-Base, 150 mM NaCl, 0.05% Tween-20; pH 7.4) for 1 h at room temperature, followed by incubation with primary antibody in 5% skim milk in TBST at 4˚C overnight. The membranes were then washed for 3x5 min in TBST, and then incubated in TBST-diluted secondary antibodies for 45 min at room temperature, followed by another 3x5 min washes with TBST. Protein-antibody binding was detected with ECL Western Blotting Substrate (Beijing Solarbio Science & Technology Co., Ltd.) followed by exposure of the membranes to X-ray film (Kodak). Protein expression levels were determined semiquantitatively by densitometric analysis with the Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.). The following antibodies were used: anti-p53 (1:1,000; cat. no. sc-47698; Santa Cruz Biotechnology, Inc.), anti-BCL-2

Table I. miRs and primer sequences.

Chemoresistance-related miRs	Primer sequence (5'→3')	(Refs.)
hsa-miR-149-5p	TCTGGCTCCGTGTCTTCACTCC	(27)
hsa-miR-150-5p	TCTCCCAACCCTTGTACCAGTG	(27)
hsa-miR-34a-5p	TGGCAGTGTCTTAGCTGGTTGTT	(30,31)
hsa-miR-34c-5p	AGGCAGTGTAGTTAGCTGATT	(36)
hsa-miR-519d-3p	CAAAGTGCCTCCCTTTAGAGT	(36)
hsa-miR-204-5p	ACTCGTGGACTTCCCTTTGT	(36)
hsa-miR-143-3p	TGAGATGAAGCACTGTAGCT	(31,40)
hsa-miR-153-3p	TTGCATAGTCACAAAAGTGAT	(31)
hsa-miR-27a-3p	TCACAGTGGCTAAGTTCCG	(31)
hsa-miR-218-5p	TTGTGCTTGATCTAACCATGT	(31)
hsa-miR-520a-5p	CTCCAGAGGGAAGTACTTTCT	(31)
hsa-miR-503-5p	TAGCAGCGGGAACAGTTCTGCAG	(46)
hsa-miR-195-5p	CTGACCTATGAATTGACAGCC	(49)
hsa-miR-133a-3p	TTTGGTCCCCTTCAACCAGCT	(50)
hsa-miR-26a-5p	TTCAAGTAATCCAGGATAGGCT	(52,53)

Chemoresistance-related miRs	Primer sequence (5'→3')	(Refs.)
hsa-miR-27a-3p	TCACAGTGGCTAAGTTCCG	(28)
hsa-miR-34a-5p	TGGCAGTGTCTTAGCTGGTTGTT	(29)
hsa-miR-210-3p	CTGTGCGTGTGACAGCGGCTGA	(32-35)
hsa-miR-107	AGCAGCATTGTACAGGGCTATCA	(37)
hsa-miR-205-5p	TCCTTCATTCCACCGGAGTCTG	(38)
hsa-miR-338-5p	AACAATATCCTGGTGCTGAGTG	(17)
hsa-miR-23a-3p	ATCACATTGCCAGGGATTTCC	(39)
hsa-miR-224-5p	TCAAGTCACTAGTGGTTCCGTT	(41)
hsa-miR-107	AGCAGCATTGTACAGGGCTATCA	(42)
hsa-miR-103a-3p	AGCAGCATTGTACAGGGCTATGA	(42,43)
hsa-miR-19a-3p	TGTGCAAATCTATGCAAAAC	(44)
hsa-miR-590-5p	GAGCTTATTCATAAAAGTGCA	(45)
hsa-miR-675-5p	TGGTGCGGAGAGGGCCCACA	(47,48)
hsa-miR-145-5p	GTCCAGTTTTCCCAGGAATC	(51)
hsa-miR-27b-3p	TTCACAGTGGCTAAGTTCTG	(51)
hsa-miR-26a-5p	TTCAAGTAATCCAGGATAGGCT	(51)

miR, microRNA; hsa, *Homo sapiens*.

(1:1,000; cat. no. 15071), anti-MCL-1 (1:1,000; cat. no. 39224), anti-EZH2 (1:1,000; cat. no. 5246), anti-STAT3 (1:1,000; cat. no. 9139), anti-SMAD4 (1:1,000; cat. no. 46535; all from Cell Signaling Technology, Inc.), anti-β-ACTIN (1:2,000; cat. no. sc-8432) and HRP-conjugated secondary antibody (1:3,000; cat. no. sc-2357; both from Santa Cruz Biotechnology, Inc.).

Statistical analysis. Statistical analysis was performed using SPSS software (version 19.0; IBM Corp.). Each experiment was repeated at least three times. Statistical significance was assessed by comparing the mean ± SD using an unpaired Student's t-test or ANOVA test followed by Fisher's Least Significant Difference, Bonferroni or Sidak post-hoc tests. $^{*}P<0.05$ was considered to indicate a statistically significant difference.

Results

Hypoxia reduces OXA sensitivity in CRC cells. The response of HCT116 and HT29 cells to OXA was investigated under both normoxia and hypoxia. Both cell lines were treated with 5, 10, 20, 40 and 60 μM OXA and it was found that the cellular growth inhibition rates increased with the increase of drug concentration (Fig. 1A). Taking the 20 μM OXA treatment group as an example, the cellular growth inhibition rate of HCT116 cells was 56.6% under normoxic condition and 27.7% under hypoxic condition (Fig. 1B; $P<0.01$), while the cellular growth inhibition rate of HT29 cells was 35.7% under normoxic condition and 25.5% under hypoxic condition (Fig. 1B; $P<0.05$). The cellular growth change rate of HCT116

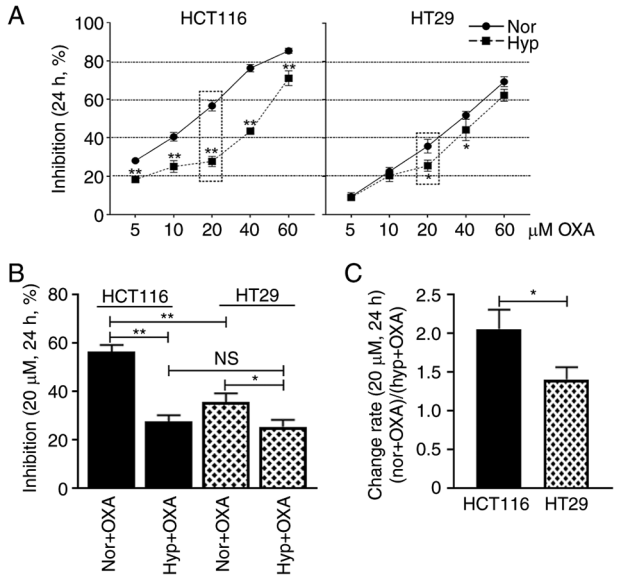

Figure 1. Hypoxia reduces OXA sensitivity in CRC cells. HCT116 and HT29 cells were treated with 5, 10, 20, 40 and 60 μM OXA for 24 h and the cell proliferation under normoxia and hypoxia was measured. (A) Cellular growth inhibition rates. Black dotted box: 20 μM OXA treatment group. (B) The cellular growth inhibition rates of HCT116 and HT29 cells in the 20 μM OXA treatment group were shown separately. It was clear that HCT116 cells were more sensitive to OXA than HT29 cells under normoxic condition. (C) Growth inhibition change rate indicated that hypoxia generated a greater impact on the OXA sensitivity of HCT116 cells than HT29 cells. *P<0.05 and **P<0.01. Dotted boxes, a moderate dose of 20 μM OXA was selected as a standard dose and used in the following experiments. OXA, oxaliplatin; CRC, colorectal cancer; Nor, normoxia; Hyp, hypoxia.

cells was 2.06 and of HT29 cells was 1.41 (Fig. 1C; P<0.05). These data suggested that HCT116 cells were more sensitive to OXA than HT29 cells under normoxic condition. Additionally, although hypoxia decreases the OXA sensitivity, it generated greater impact on HCT116 cells than HT29 cells.

TP53 status affects CRC cell drug sensitivity. TP53 status is the typical difference between HCT116 cells and HT29 cells. P53 expression was detected after both cell lines were treated with OXA and hypoxia and it was found that the P53 expression level of HCT116 cells increased significantly and of HT29 cells did not change considerably (Fig. 2A and B). P53 was then knocked down and cell drug sensitivity to 20 μM OXA was measured (Fig. 3A). Under normoxic condition, the cellular growth inhibition rate of HCT116 cells was 57.3% in the EV group and 40.8% in the shP53 group (Fig. 3B, left panel; P<0.01), while under hypoxic condition, the cellular growth inhibition rate of HCT116 cells was 28.6% in the EV group and 38.4% in the shP53 group (Fig. 3B, left panel; P<0.05). These data suggested that $P53^{WT}$ plays different roles in HCT116 cell drug sensitivity, enhancing drug sensitivity under normoxic conditions but reducing drug sensitivity under hypoxic conditions. However, knocking down TP53 did not change HT29 cell response to OXA under either normoxia or hypoxia (Fig. 3B, right panel). The growth inhibition change rate also reflected this phenomenon (Fig. 3C).

P53 affects expression of miRs after OXA and hypoxia treatment. P53-miR interaction plays pivotal role in regulating CRC cell drug sensitivity (31). To directly reveal how P53 and miRs affect chemoresistance under hypoxia, 31 miRs that have been demonstrated to be correlated with chemoresistance or hypoxia in CRC were selected. By using RT-qPCR, the expression of miRs after HCT116 cells were treated with OXA or hypoxia was analyzed, and it was found that the expression level of miR-143, 26a, 34a, 133a, 149 and 195 were significantly increased after HCT116 cells were treated with OXA (Fig. 4A), while miR-23a, 27a, 107, 19a, 145, 205, 210 and 590 were significantly increased after HCT116 cells were treated with hypoxia (Fig. 4B). However, after TP53 was knocked down, the expression levels of miR-26a, 34a, 133a, 23a, 107 and 205 were decreased accordingly (Fig. 4C and D), indicating that these miRs were P53-induced miRs or interact with P53.

miRs affect HCT116 cell OXA sensitivity depending on $P53^{WT}$. The effects of these P53-related miRs were analyzed by overexpressing them in HCT116 cells. Cellular growth inhibition assay showed that miR-23a decreased OXA sensitivity, whereas miR-26a and miR-34a increased OXA sensitivity. However, miR-133a, 107 and 205 did not affect cell drug sensitivity (Fig. 5).

Then the association between miRs and P53 status was analyzed. The expression levels of miRs in HT29 cells ($TP53^{MT}$) were first detected and it was identified that OXA- or hypoxia-treatment did not upregulate miR-26a, 34a and 23a (Fig. 6A). However, transfection of miR-23a decreased cellular growth inhibition rate and transfection of miR-26a and 34a increased cellular growth inhibition rate, which was similar as they were functioning in HCT116 cells (Fig. 6B). Notably, after introducing exogenous $P53^{WT}$ to HT29 cells (Fig. 6C), it was revealed that miR-26a, 34a and 23a can be induced by OXA or hypoxia treatment (Fig. 6D). These data suggested that the effect of these 3 miRs on CRC cell drug sensitivity depends on $P53^{WT}$.

Hypoxia suppresses the expression of miR-26a and miR-34a. It can be observed from the aforementioned experiments that miR-23a, 26a and 34a are all driven by P53. However, it was strange that with hypoxia upregulating the level of P53, it only induced the expression of miR-23a instead of affecting the level of miR-26a and miR-34a. To address this issue, the level of miR-26a and miR-34a was examined after CRC cells were treated with hypoxia for 2, 8, 16, 24 and 48 h. It was demonstrated that hypoxia significantly suppressed the expression levels of these two miRs in both HCT116 and HT29 cells (Fig. 7A and B). However, administration of HIF-1α inhibitor echinomycin reversed the inhibition effect of hypoxia on miRs in HCT116 cells but not in HT29 cells (Fig. 7A and B). These data suggested that hypoxia and $P53^{WT}$ synergistically altered expression of miRs.

miRs regulate cellular apoptosis-related factors and modulate drug sensitivity. The possible molecular mechanism of how miR-23a, 26a and 34a modulate OXA sensitivity was further investigated. According to previous studies, miR-26a and 34a may activate BCL-2, MCL-1, EZH2, SMAD 4 and STAT3 (54-58). In the present study, it was also demonstrated that miR-26a could decrease the expression levels of MCL-1, EZH2 and BCL-2 (Fig. 8A) and miR-34a could decrease the expression levels of SMAD4, STAT3 and BCL-2 (Fig. 8B). However, high level of miR-23a could reverse the OXA-induced

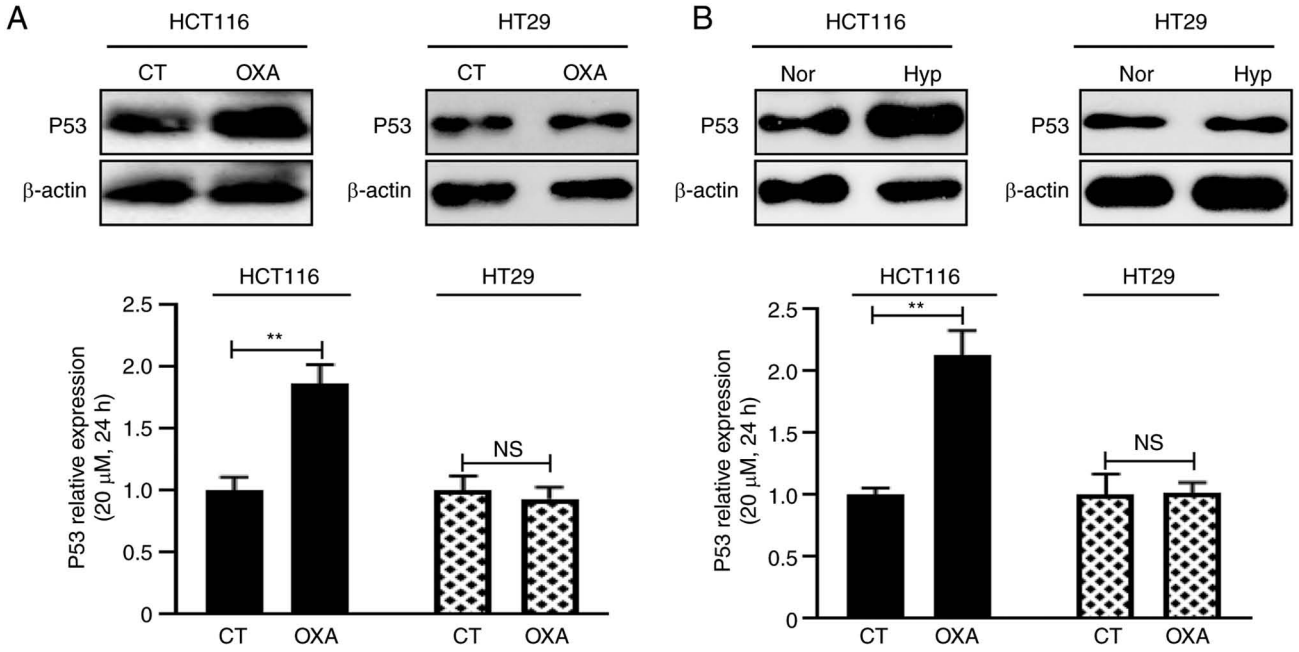

Figure 2. Western blot analysis of P53 protein expression levels in HCT116 and HT29 cells treated with OXA or hypoxia. (A) After treated with 20 μM OXA, the P53 level of HCT116 cells increased significantly and of HT29 cells did not change considerably. Upper panel, western blot band; histograms, the relative band density. (B) After treated with hypoxia, the P53 level of HCT116 cells increased significantly and of HT29 cells did not change considerably. Upper panel, western blot band; histograms, the relative band density. **P<0.01. Nor, normoxia; Hyp, hypoxia. OXA, oxaliplatin; Nor, normoxia; Hyp, hypoxia; NS, not significant.

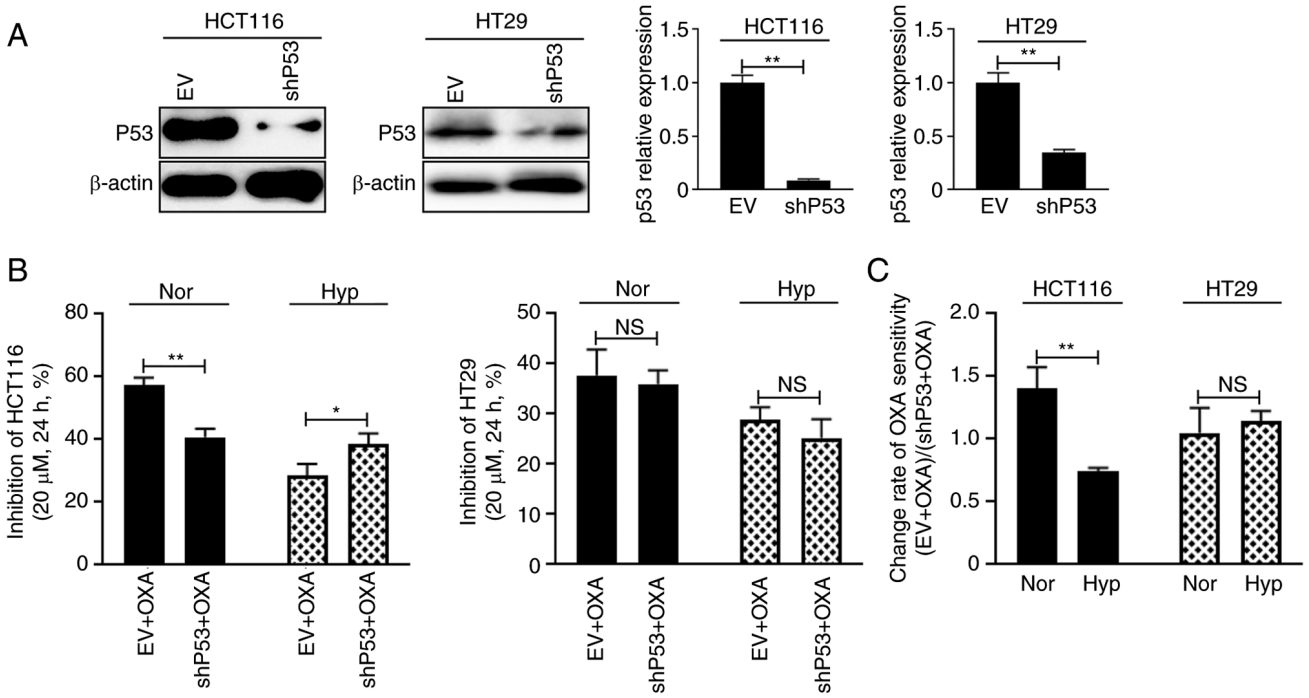

Figure 3. P53^{WT} affects HCT116 cell drug sensitivity. (A) Western blotting results showed the knocking down efficacy of P53. (B) Knocking down P53 enhanced HCT116 cell drug sensitivity under normoxic condition but reduced cell drug sensitivity under hypoxic condition. However, knocking down P53 did not change HT29 cell response to OXA under either normoxia or hypoxia. (C) Cellular growth inhibition rate also reflected that knocking down P53 affects the response of HCT116 cells to OXA under hypoxia. *P<0.05 and **P<0.01. OXA, oxaliplatin; NS, not significant; Nor, normoxia; Hyp, hypoxia.

suppression of MCL-1 and BCL-2 (Fig. 8C). These data possibly explain why P53^{WT}-induced miRs promote OXA sensitivity under normoxic conditions but hypoxia enhances OXA resistance in CRC cells.

Discussion

Since OXA is a major antitumor drug for CRC chemotherapy, finding useful biomarkers and potential molecular mechanisms

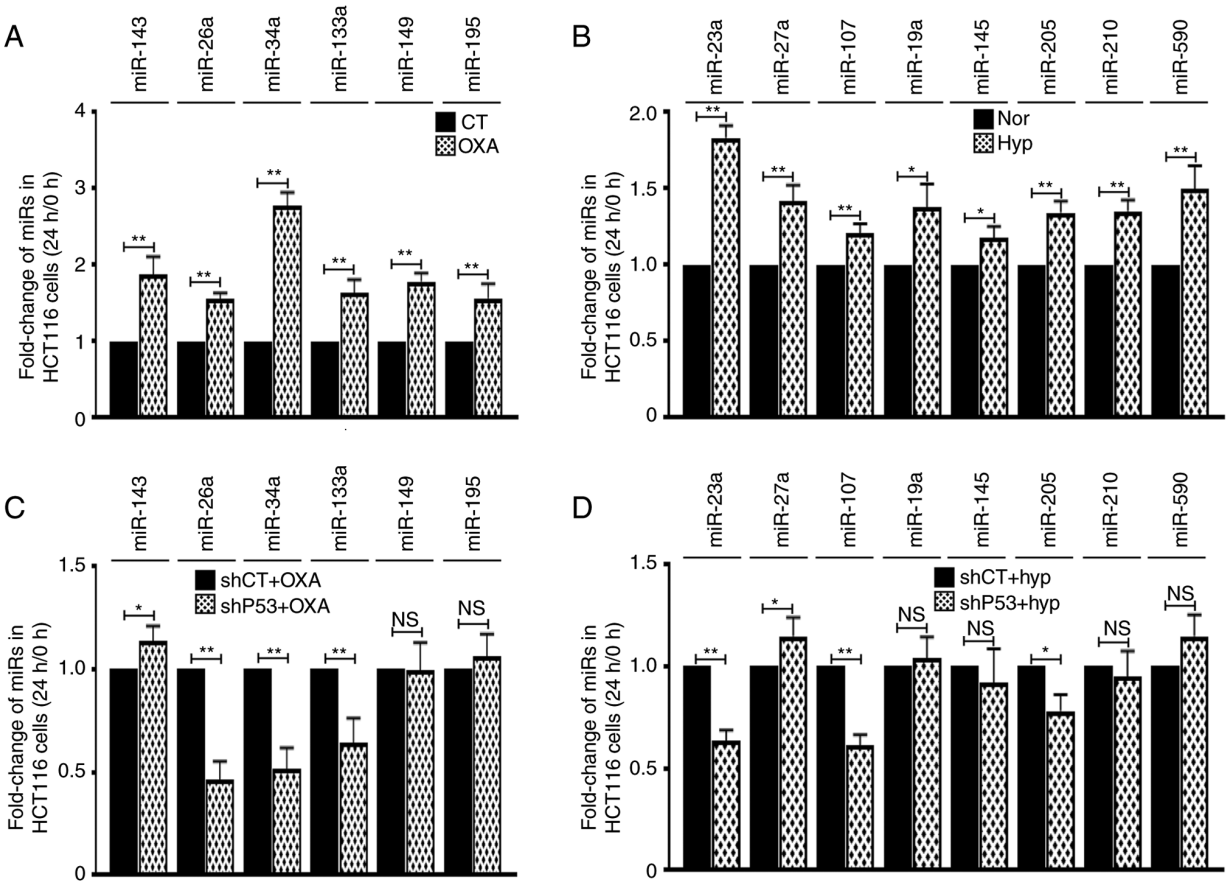

Figure 4. Reverse transcription-quantitative PCR is applied to detect miR expression level changes in HCT116 cells treated with OXA or hypoxia and to find the P53-related miRs in P53-knocked down HCT116 cells. (A) The expression level of miR-143, 26a, 34a, 133a, 149 and 195 increased significantly after HCT116 cells were treated with 20 μM OXA for 24 h. (B) The expression level of miR-23a, 27a, 107, 19a, 145, 205, 210 and 590 increased significantly after HCT116 cells were treated with hypoxia for 24 h. (C) With $P53^{WT}$ knocked down, the expression levels of miR-26a, 34a and 133a (in bold type) were decreased in the OXA treatment group. (D) With $P53^{WT}$ knocked down, the expression levels of miR-23a, 107 and 205 (in bold type) were decreased in the hypoxia treatment group. *P<0.05 and **P<0.01. OXA, oxaliplatin; miR, microRNA; NS, not significant; Nor, normoxia; Hyp, hypoxia.

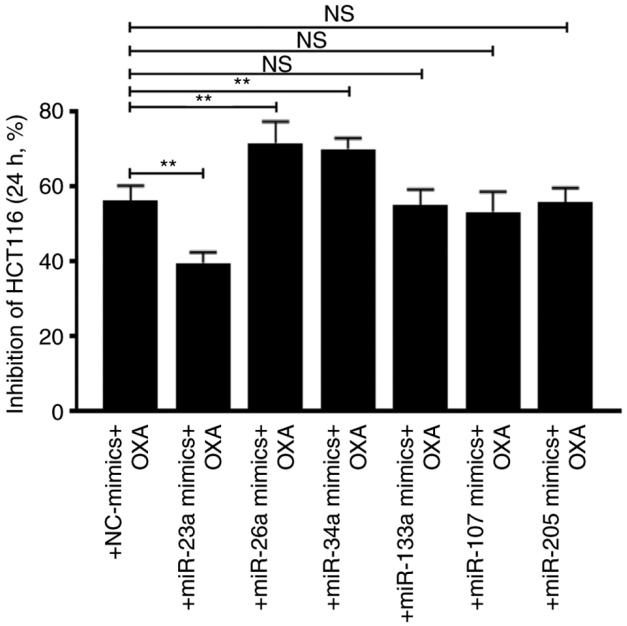

Figure 5. miR23a, 26a, 34a, 133a, 107 and 205 are overexpressed in HCT116 cells. miR-23a decreased cell growth inhibition, whereas miR-26a and miR-34a increased cell growth inhibition. However, miR-133a, 107 and 205 did not affect cell growth inhibition. **P<0.01. miR, microRNA; OXA, oxaliplatin; NS, not significant; NC, negative control.

of OXA resistance is significant for adjusting the treatment regimen for patients with CRC. The hypoxic tumor microenvironment has a pivotal influence on behavior of tumor cells. Impaired drug penetration into hypoxic regions of tumors and adaptive cellular response to hypoxia are considered to account for the reduction of cytotoxicity of numerous drugs in multiple cancer types (59-61).

In the present study, the OXA sensitivity of HCT116 and HT29 cells was firstly examined. The cell proliferation inhibition results clearly showed that hypoxia affects the OXA sensitivity of both cell lines. Notably, hypoxia caused a greater impact on HCT116 cells than on HT29 cells. Since the major difference between these two cell lines is the TP53 genotype (HCT116 is $TP53^{WT}$ and HT29 is $TP53^{MT}$), it was hypothesized that P53 may play a key role in hypoxia-induced OXA resistance. To test this hypothesis, the P53 expression level was determined after HCT116 and HT29 cells were treated with 20 μM OXA or hypoxia and it was found that the $P53^{WT}$ level in HCT116 cells was significantly increased, while the $P53^{MT}$ level in HT29 cells did not change considerably. P53 was then knocked down and drug sensitivity was examined. Surprisingly, it was found that P53 had dual effects on regulating the OXA sensitivity of HCT116 cells: It promoted OXA sensitivity under normoxic conditions and reduced OXA sensitivity under hypoxic conditions.

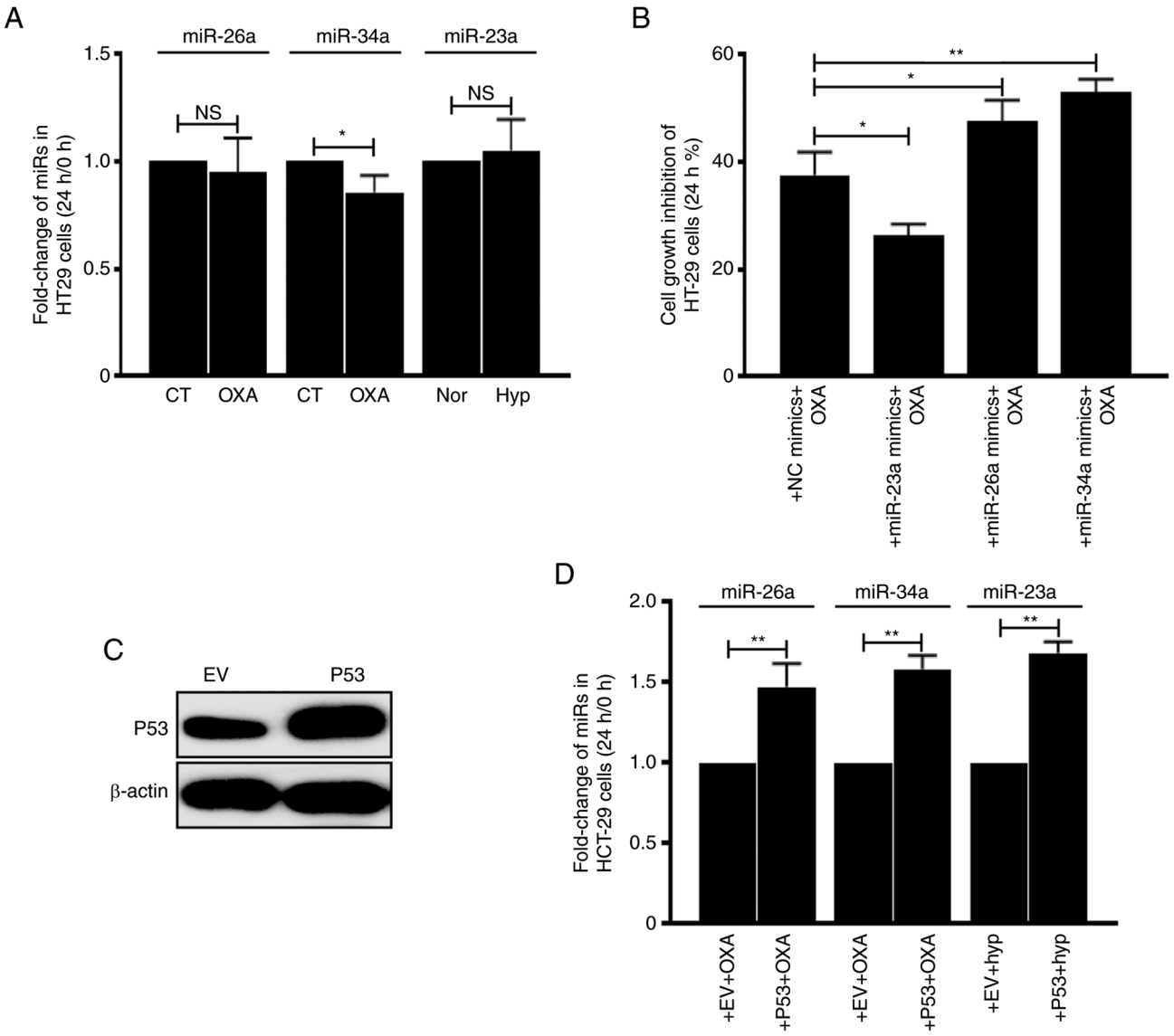

Figure 6. miRs affect colorectal cancer cell drug sensitivity depending on $P53^{WT}$. (A) Reverse transcription-quantitative PCR results indicated that OXA or hypoxia did not promote the expression level of miR-26a, 34a and 23a in HT29 cells. (B) The effect of miRs on HT29 cell drug sensitivity was measured by cell growth inhibition assay. The results indicated that miR-23a decreased cell growth inhibition, whereas miR-26a and 34a increased cell growth inhibition. (C) $P53^{WT}$ was introduced in to HT29 cell and confirmed by western blotting. (D) After introducing exogenous $P53^{WT}$ to HT29 cells, miR-26a, 34a and 23a could be induced by OXA or hypoxia treatment. $^{*}P<0.05$ and $^{**}P<0.01$. miR, microRNA; NS, not significant; Nor, normoxia; Hyp, hypoxia; EV, empty vector.

miRs are critical transcriptional mediators and epigenetic regulators in multiple biological activities, including tumorigenesis, angiogenesis, cell senescence, metabolism and drug resistance (62-66). There is a great number of miRs in cells and thousands of human genes are miR targets. Compared with transcriptional regulation, miR regulation is fast and flexible, and miRs may regulate cellular behaviors without affecting basic biological activities. Previous studies have indicated that there are numerous P53-dependent miRs (67,68). Most of these miRs have P53 response elements in their promoter region, such as miR-145, -34s, -202, -1204, -1206, -10b and -23b (69-72). This suggests that although P53 is upregulated by OXA and hypoxia, it may activate different miR groups under these two stimuli, which can explain the distinct effects of P53 on OXA sensitivity under normoxic and hypoxic conditions. By referring to previous research findings, 31 OXA- or hypoxia-induced miRs were selected. Since numerous of these miRs are P53 dependent (Table I) (73-76), they are probably involved in the regulation of resistance by P53. RT-qPCR results indicated that among these miRs, 6 miRs were induced by OXA, and 8miRs were induced by hypoxia. The following P53 deprivation experiments indicated that miR-26a-5p, miR-34a-5p and miR-133a-3p levels were associated with P53 in OXA treatment conditions, while miR-23a-3p, miR-107 and miR-205-5p were altered in hypoxia treatment conditions. Further miR overexpression experiments indicated that among the aforementioned six miRs, only miR-23a-3p (decreases OXA resistance), miR-26a-5p (increases OXA sensitivity) and miR-34a-5p (increases OXA sensitivity) were involved in the OXA response. These three miRs were then introduced into $TP53^{MT}$ HT29 cells, and a similar phenomenon was observed. $P53^{WT}$ restoration experiments in HT29 cells clearly indicated that miR-26a-5p, miR-34a-5p and miR-23a-3p are P53 dependent. Taking these data together, it could be concluded

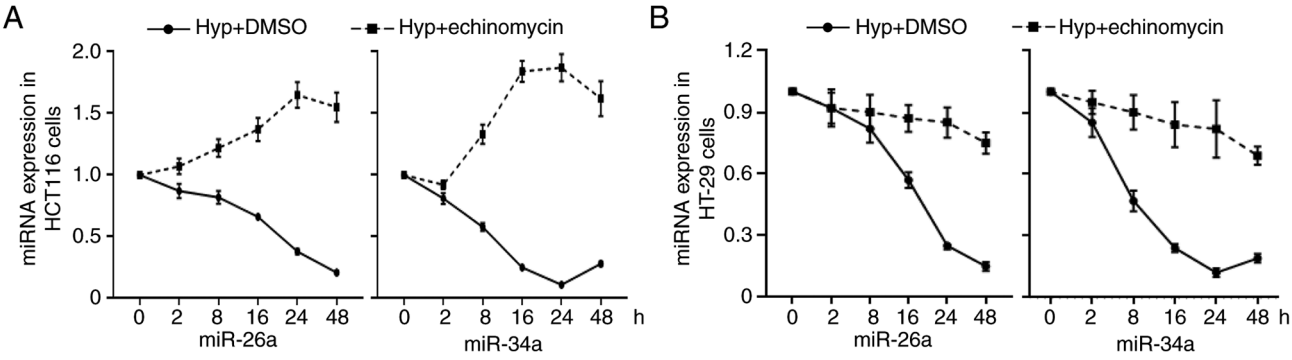

Figure 7. Hypoxia suppresses expression of miR-26a and miR-34a. Expression levels of miR-26a and miR-34a in hypoxia-treated HCT116 and HT29 cells were analyzed by reverse transcription-quantitative PCR. (A and B) The results indicated that hypoxia significantly suppressed the expression of miR-26a and miR-34a in both (A) HCT116 and (B) HT29 cells. However, administration of echinomycin reversed the hypoxic inhibition effect in (A) HCT116 but not in (B) HT29 cells. miR, microRNA; Nor, normoxia; Hyp, hypoxia.

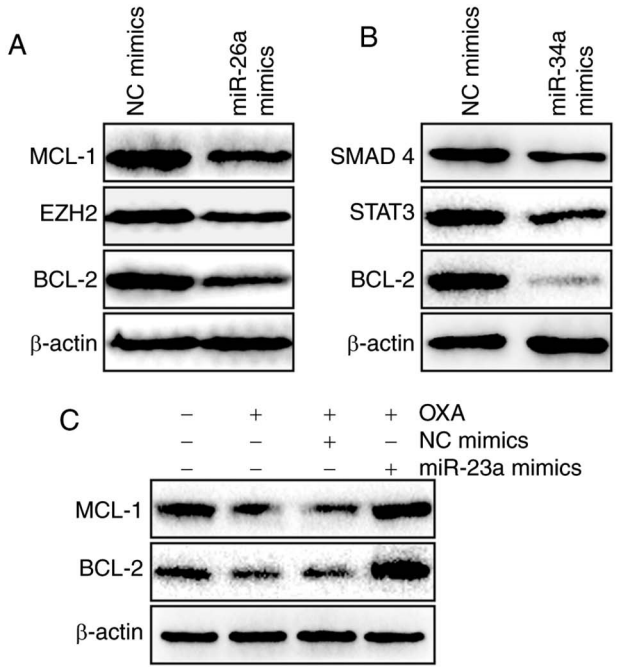

Figure 8. miRs regulate cell apoptotic factors and modulate drug sensitivity. (A) miR-26a decreased the expression levels of MCL-1, EZH2 and BCL-2 and (B) miR-34a decreased the expression levels of SMAD4, STAT3 and BCL-2. (C) However, overexpressing miR-23a may reverse the inhibition of MCL-1 and BCL-2 that were induced by miR-23a/34a. These data possibly explain how $P53^{WT}$-induced miRs promote drug sensitivity and why hypoxia enhances drug resistance in CRC cells. miR, microRNA; OXA, oxaliplatin; NC, negative control.

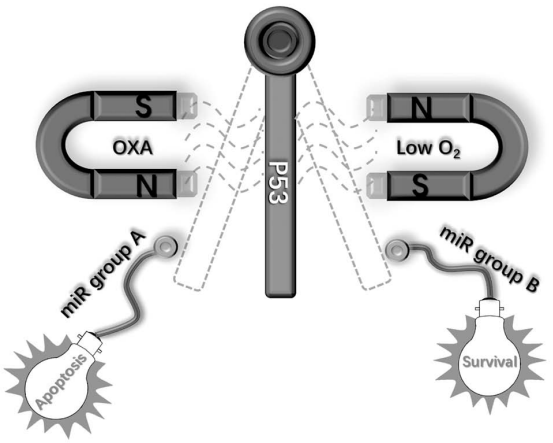

Figure 9. Schematic of the molecular mechanisms of p53-miRs cross-talking regulating OXA chemosensitivity in hypoxia: OXA and low O_2 as two stress conditions drive P53 to modulate different miR groups and thus activate distinct cellular signaling pathways. miR, microRNA; OXA, oxaliplatin.

that in $P53^{WT}$ HCT116 cells, $P53^{WT}$ protein plays two roles in regulating cell sensitivity to OXA: Under normoxic conditions, OXA stimulates $P53^{WT}$ expression and therefore induces miR-26a-5p and miR-34a-5p, which renders cells sensitive to OXA; by contrast, under hypoxic condition, although $P53^{WT}$ is also upregulated, it induces miR-23a and decreases cell sensitivity to OXA.

Hypoxia activates HIF signaling pathways in cancer cells, which can transactivate a wide variety of transcripts including miR transcripts (77). To further confirm that miR-26a-5p and miR-34a-3p are specifically induced by hypoxia, echinomycin was used to inactivate HIF-1α, and it was found that miR-26a-5p and miR-34a-3p could not be sustained without HIF-1α in both HCT116 and HT29 cells. These data indicated that although $P53^{WT}$ can be used to regulate these two miRs under hypoxic conditions, HIF-1α is necessary for their stable expression.

miRs regulate drug sensitivity through modulation of numerous cellular apoptosis- and gene transcription-related factors. For example, Zhou *et al* (78) reported that miR-26a inhibits bladder cancer cell proliferation through inhibition of EZH2; Li *et al* (79) showed that knocking down EZH2 promotes OXA-induced cell cytotoxicity in OXA-resistant HT29 cells; Gao *et al* (55) reported that miR-26a inhibits breast cancer cell proliferation through repression of MCL-1; Yang *et al* (56) reported that miR-26a decreases Bcl-2 expression and that suppression of miR-26a causes cisplatin resistance in human non-small cell lung cancer. These findings supported our statement that $P53^{WT}$-induced miR-26a overexpression promotes OXA sensitivity in HCT116 cells. Similarly, miR-34a may promote OXA sensitivity through interactions with SMAD4, STAT3 and BCL-2 (58,80-82). The underlying molecular mechanisms for the association between $P53^{WT}$-induced miR-23a overexpression and hypoxia-mediated acquired OXA

resistance were also investigated. Western blot results clearly showed that OXA suppressed the expression of the tumor antiapoptotic molecules MCL-1 and BCL-2. However, administration of miR-23a mimics restored their levels. Numerous studies have reported the oncogenic and promoting drug resistance property of miR-23a in cancers (83-86). Jin *et al* (39) reported that hypoxia led to an upregulation of miR-23a in CRC cells. Xu *et al* (87) found that sinomenine exerts an antitumor effect by downregulating miR-23a, and transfection of miR-23a-3p increased the level of BCL-2 in PC3 cells. Zhang *et al* (88) also revealed that miR-23a-3p inhibited the expression of BAX, promoted the expression of BCL-2 and inhibited the apoptosis of U937 cells. These investigations were in line with the present findings.

In summary, in the present study, the role of P53 in OXA-induced cellular apoptosis in CRC was investigated and it was identified that under normoxic conditions, P53 may promote cell apoptosis through activation of miR-26a and miR-34a, whereas under hypoxic conditions, P53 may induce OXA resistance through the activation of miR-23a. The present findings revealed the dual function of P53 in regulating cell apoptosis and highlighted the role of P53-miR interactions in the response of CRC cells to OXA under normoxic and hypoxic conditions (Fig. 9). These findings may provide deep insight into the molecular mechanism of antitumor drug resistance and a novel idea to overcome drug resistance in clinical cancer treatment. However, to deeply understand the crosstalk between P53 and the miR group, the identification of more research targets, particularly through data mining and bioinformatics analysis, is still needed.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TZ and DS contributed to the conception and design of the study. JZ and CL contributed to the acquisition, analysis and interpretation of data. LS analyzed the data and was involved in performing the experiments. DS drafted the work. TZ revised it critically for important intellectual content. All authors confirm the authenticity of all the raw data and read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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