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ORIGINAL ARTICLE

MicroRNA-21 regulates the sensitivity of diffuse large B-cell lymphoma cells to the CHOP chemotherapy regimen

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Abstract Numerous studies have demonstrated that microRNA-21 (miR-21), as an oncogene, is involved in the occurrence of many types of tumor and the sensitivity of tumor cells to chemotherapeutic drugs. In the present study, we investigated whether miR-21 is involved in regulating the sensitivity of the diffuse large B-cell lymphoma (DLBCL) cell line CRL2631 to the cyclophosphamide, vincristine, Adriamycin, and prednisone (CHOP) chemotherapeutic regimen. Knockdown of miR-21 with antisense oligonucleotides significantly increased the cytotoxic effects of the CHOP regimen in CRL2631 cells. A luciferase reporter assay showed that PTEN is a target gene of miR-21 in CRL2631 cells, and subsequent experiments demonstrated that miR-21 impacts the PI3K/AKT signaling pathway through the regulation of PTEN, thereby affecting cellular sensitivity to the CHOP chemotherapeutic regimen. Furthermore, knockdown of NF-κB decreased miR-21 expression and sensitized CRL2631 cells to CHOP treatment. These results provide evidence that it may be possible to overcome microRNA-based DLBCL drug resistance.

Keywords miR-21 · Diffuse large B-cell lymphoma · Drug resistance · PTEN · NF-κB

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common histological type of non-Hodgkin lymphoma, accounting for 30–40 % of non-Hodgkin lymphoma cases in adults [1]. The combined chemotherapy represented by the cyclophosphamide, vincristine, Adriamycin, and prednisone (CHOP) regimen is the standard chemotherapeutic regimen for treating DLBCL. However, nearly half of the patients treated with the CHOP regimen stop responding to treatment and become drug resistant. Therefore, drug resistance is a major obstacle to the successful treatment of DLBCL [2, 3]. Studies have suggested that abnormalities in diverse, complicated signaling pathways, including NF-κB, CDKs, BCL-2, and P53, are involved in drug resistance [4], but the exact mechanism underlying DLBCL drug resistance is still unclear.

microRNA (miRNA) includes a group of endogenous, non-coding, regulatory RNAs with lengths of approximately 20 nucleotides. miRNAs regulate gene expression through either sequence-specific translation inhibition or mRNA degradation, and they participate in a series of important biological processes, such as cell development, proliferation, differentiation, and apoptosis [5]. Studies have demonstrated the abnormal regulation of microRNA expression in many human tumors [6]. Increasing evidence has suggested that microRNAs are involved in chemotherapeutic drug resistance in

tumor cells [7]. In particular, as an oncogene, microRNA-21 (miR-21) is highly expressed in many cancers, including DLBCL, and is involved in tumorigenesis and resistance to chemotherapeutic agents [8]. However, the role of miR-21 in the drug resistance of DLBCL has not been reported. In this study, we discovered that the downregulation of miR-21 in the DLBCL cell line CRL2631 could significantly increase cell sensitivity to the CHOP regimen, and our experiments confirmed that miR-21 affected the PI3K/AKT pathway through the regulation of PTEN to exert these biological effects. In addition, knockdown of NF-κB decreased miR-21 expression and sensitized the cells to CHOP treatment. Our results provide a new perspective for a better understanding of the mechanism of drug resistance in DLBCL cells.

Materials and methods

Cell culture

The DLBCL cell line CRL2631 was obtained from the American Type Culture Collection (ATCC). The cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10 % fetal bovine serum (FBS). The HEK-293T cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10 % FBS, 100 g/mL L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Gene knockdown and transduction

The miR-21 antisense oligonucleotide (ASO) and the control oligonucleotide were purchased from Invitrogen. The miR-21 ASO sequence was 5′-TCAACATCAGTCTG ATAAGCTA-3′, and the control ASO sequence was 5′-GTGGATATTGTTGCCATCA-3′. The ASOs were transfected into cells with Lipofectamine 2000 according to the manufacturer's instructions. The ASOs were dissolved in Opti-MEM culture medium (Invitrogen) without antibiotics at a final working concentration of 200 nM. A SignalSilence® NF-κB p65 siRNA kit was purchased from Cell Signaling Technology (CST), and the transfection was performed according to the manufacturer's protocol. The cells were prepared for further analysis 48 h after transfection. The transfection efficiency was evaluated by fluorescence microscopy by calculating the percentage of fluorescein-labeled cells. The transfection efficiency was approximately 80 %. PTEN expression was achieved using the ViraPower™ Lentiviral Expression System (all reagents and vectors were purchased from Invitrogen) according to the manufacturer's instructions, which is summarized as follows: the PTEN gene was initially cloned in the Gateway entry clone vector (containing the coding sequence and the Kozak sequence) and was then subcloned into the pLenti6.2/V5DEST vector through an LR recombination reaction to construct the pLenti6.2/V5-GW/PTEN vector. The PTEN constructed vector and a control vector, pLenti6.2/V5-GW/lacZ, were separately transfected into 293T cells with a packaging plasmid to prepare the lentivirus. Forty-eight hours after lentiviral transduction, 15 μg/mL blastocidin was added to select for resistant cells.

Detection of cell viability and caspase activity

In vitro CHOP treatment was performed similar to published reports [9–11]. Briefly, the cells were cultured in 96-well plates with RPMI-1640 medium containing 10 % FBS. After 24 h, various concentrations of cyclophosphamide, vincristine, Adriamycin, and prednisone (CHOP) were added. The composition of the 4 drugs in a ratio of 80/5.5/0.16/11.1 was determined based on the ratio used in clinical application [9]; all drugs were purchased from Sigma. Seventy-two hours after drug treatment, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for an additional 4 h. The OD of the samples was determined using a microplate spectrophotometer, and each experiment was performed in triplicate. Caspase activity was assayed using Caspase Colorimetric Assay Kit (KeyGEN, Nan Jing, China). Briefly, cells were lysed in lysis buffer on ice for 20 min. After centrifugation, the supernatants were incubated with the caspase substrate at 37 °C for 4 h in a reaction buffer according to the manufacturer's instructions and then read on a 96-well plate reader at 405 nm. The percentage of A405 values for the samples versus those for control samples indicated the percentage of caspase activity.

Quantitative PCR

The total cellular RNA was extracted using the RecoverAll kit (Ambion Inc., Austin, Texas) according to the manufacturer's instructions. The detection of miR-21 expression was performed with the ABI 7500 quantitative PCR system using the TaqMan microRNA Assay (Applied Biosystems, Foster City, CA) according to the product manual, and U6 was used as a control [12]. The quantitative detection of PTEN mRNA was performed using the SYBR Green PCR kit (Qiagen), and β-actin was used as a corresponding control. The forward and reverse primers of PTEN were 5′-GAGGGATAAAACACCATG-3′ and 5′-AGGGGTAGG ATGTGAAC CAGTA-3′, respectively. The forward and reverse primers of β-actin were 5′-CAGAGCCTCGC CTTTGCC-3′ and 5′-GTCGCCCACATAGGAATC-3′, respectively. All PCR reactions were repeated 3 times concurrently.

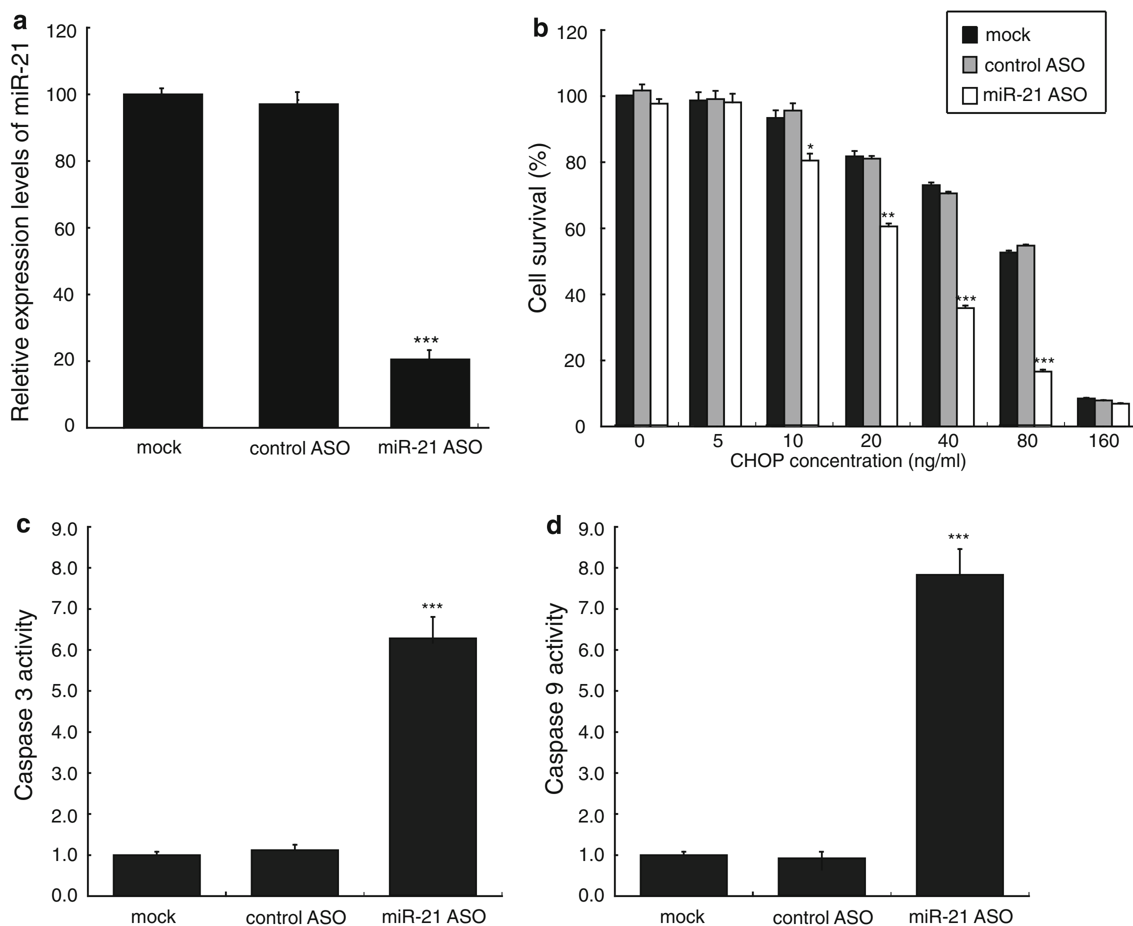

Fig. 1 miR-21 knockdown significantly increases the drug sensitivity of CRL2631 cells to the CHOP regimen. **a** CRL2631 cells were mock transfected, transfected with miR-21 ASOs or control ASOs. miR-21 expression was detected using real-time quantitative PCR. **b** After transfection with mock, miR-21-ASOs or control ASOs, CRL2631 cells were treated with different concentrations of the CHOP drugs. The cell viability was determined by the MTT method. **c** Activity of caspase 3 was determined by Caspase Colorimetric Assay when cells were exposed to 40 ng/mL CHOP. **d** Activity of caspase 9 was determined by Caspase Colorimetric Assay when cells were exposed to 40 ng/mL CHOP. The results were from 3 independent experiments. The data are presented as the mean ± SE. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

Western blot analyses

After treatment, the cells were washed with PBS twice, lysed with a cell lysis buffer containing protease inhibitors, and stored at −80 °C until use. A total of 50 μg of protein was loaded, run on a gel, and transferred to a PVDF membrane. After blocking with 5 % non-fat milk, the membrane was incubated with a primary antibody overnight followed by incubation with a secondary antibody for 2 h at room temperature. The NF-κB, PTEN, p-AKT, and AKT antibodies were purchased from Cell Signaling Technology, and the β-actin antibody was from Sigma. The protein signal was developed with ECL (Pierce Biotechnology). The protein bands were quantified with the ImageJ 1.33 software.

Detection of luciferase activity

The full length PTEN 3′UTR sequence was PCR amplified using human genomic DNA as a template. The forward and reverse primers were 5′-TCGCTCGAGATTTTTTTTTATC AAGAGGG-3′ and 5′-TCGGCGGCCGCGACAAGAATG AGACTTTAATC-3′, respectively. The resulting PCR product was inserted into the *Xho*I and *Not*I sites in the multiple cloning site of the luciferase reporter vector, psi-CHECK2 (Promega), to construct the PTEN-3′UTR luciferase reporter system. Mutations in 3 bases were introduced into the miR-21 binding site of the PTEN3′-UTR reporter plasmid using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The forward and reverse primers were 5′-ACTTGTGGCAACAGTTTACTTTGCAGTTG-3′ and

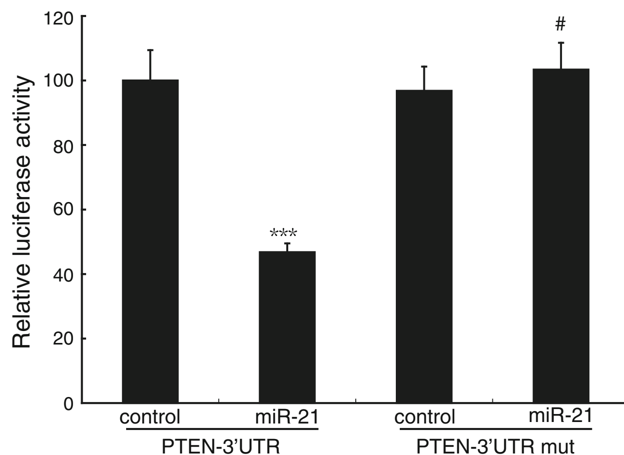

Fig. 2 PTEN is a target gene of miR-21 in the CRL2631 cell line. The target effect of miR-21 on the PTEN-3′UTR in CRL2631 cells was detected using the luciferase reporter system. CRL2631 cells were co-transfected with the miR-21-expressing plasmid, the luciferase reporter plasmid, or the control plasmid. The results were from 3 independent experiments. The data are presented as the mean ± SE. ***$P < 0.001$; $^{\#}P > 0.05$

5′-CAACTGCAAAGTAAACTGTTGCCACAAGT-3′, respectively. The miR-21-expressing plasmid was constructed as previously described [13]. The CRL2631 cells were cultured in 96-well plates at 5,000 cells per well; after 24 h, 100 ng of the plasmid mixture (90 ng of the miR-21-expressing plasmid and 10 ng of the reporter plasmid) was transfected into cells with the FuGENE6 reagent (Roche). After 48 h, the firefly and *Renilla* luciferase activities were determined using the Dual-Glo Luciferase assay kit (Promega); the luminescence was quantified on a Tecan Spectrafluor Plus machine.

Statistical analyses

The results were analyzed using the SPSS 13.0 software. The data were presented as the mean ± standard error (SE). The comparison of the data from more than 2 groups was analyzed using one-way ANOVA and Tukey's test, and the comparison of the data between 2 groups was analyzed with Student's *t* test. $P < 0.05$ was considered significant.

Results

MiR-21 knockdown significantly increases the sensitivity of CRL2631 cells to the CHOP regimen

Studies have shown that the expression of miR-21 is increased in DLBCL cells [14]. To determine whether miR-21 regulates the sensitivity of DLBCL cells to the CHOP chemotherapeutic regimen, CRL2631 cells were either mock transfected or transfected with control ASOs or miR-21 ASOs and were subsequently treated with various concentrations of CHOP. As shown in Fig. 1a, transfection with miR-21 ASOs, instead of control ASOs and mock transfection, significantly reduced miR-21 expression ($P < 0.001$). MTT assay revealed that, compared with the control group, miR-21 ASOs significantly reduced cell survival at CHOP concentrations above 10 ng/mL (Fig. 1b). In addition, we examined the activation of caspase cascade. As shown in Fig. 1c, d, caspase 3 and caspase 9 activity were significantly increased in miR-21 ASO transfection group compared to control ASO when cells were exposed to 40 ng/mL CHOP ($P < 0.001$). These findings suggested that the downregulation of miR-21 expression alters the sensitivity of CRL2631 cells to the CHOP regimen.

PTEN is a target gene of miR-21 in the DLBCL cell line, CRL2631

Our previous studies demonstrated that PTEN is a miR-21 target gene in the K562 leukemia cell line [13]; therefore, we hypothesized that PTEN might also be a miR-21 target gene in CRL2631 cells. To test this hypothesis, the miR-21-expressing plasmid and the PTEN-3′UTR luciferase reporter were co-transfected into CRL2631 cells; co-transfection with the control vector was also performed. As shown in Fig. 2, miR-21 significantly reduced the luciferase activity of the PTEN-3′UTR plasmid ($P < 0.001$), but co-transfection with the mutant PTEN 3′UTR plasmid did not ($P > 0.05$).

miR-21 significantly affects PI3K/AKT signaling through the regulation of PTEN

We examined the relationship between the change in miR-21 expression and the change in PTEN mRNA and protein levels. The results showed that miR-21 knockdown in CRL2631 cells using miR-21 ASOs increased the expression level of PTEN protein (Fig. 3a, b; $P < 0.001$) but did not affect the level of PTEN mRNA (Fig. 3c, $P > 0.05$). Because PTEN is an important negative regulator of PI3K/AKT, we hypothesized that miR-21 downregulated PI3K/AKT signaling through an increase in PTEN protein expression and thus increased the cytotoxic effects of chemotherapeutic drugs. As shown in Fig. 3a, d, miR-21 knockdown significantly decreased the level of AKT phosphorylation in CRL2631 cells ($P < 0.001$).

PTEN plays a key role in regulating the sensitivity of CRL2631 cells to the CHOP regimen

Previous studies have suggested that PTEN regulates the sensitivity of a variety of tumors to chemotherapeutic drugs and radiation [15, 16]; however, its role in the regulation of

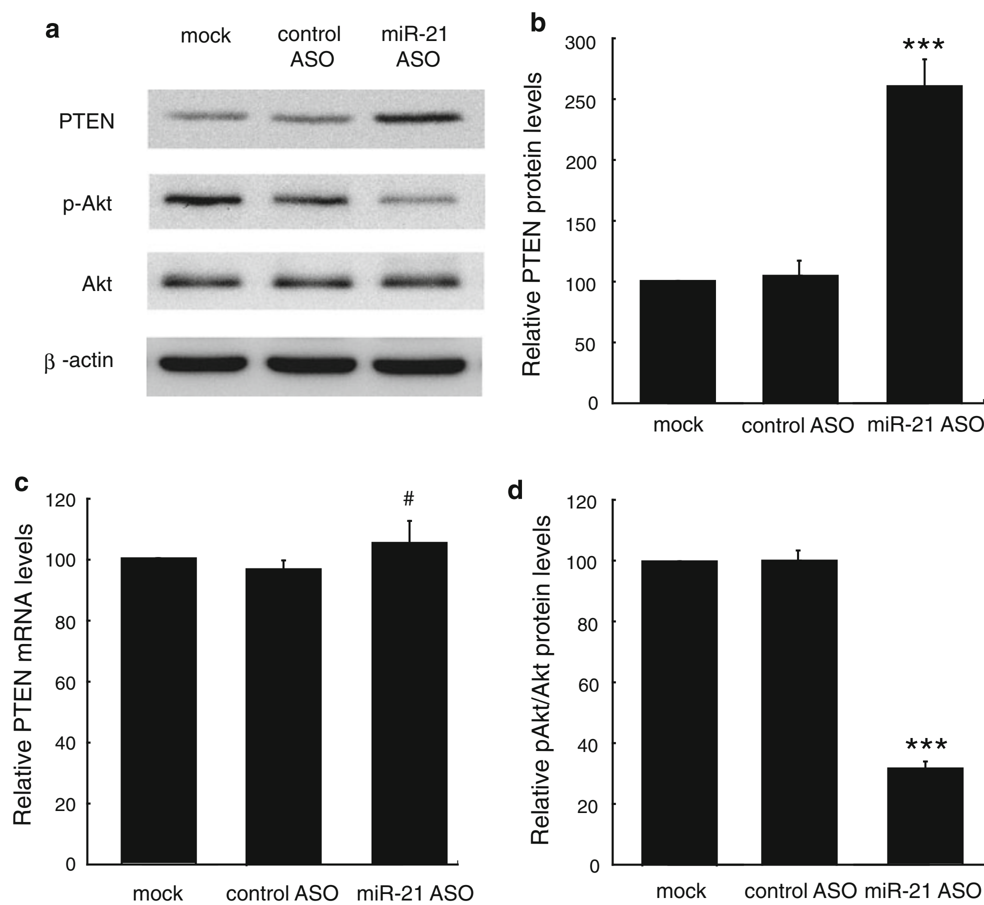

Fig. 3 miR-21 affects the PI3K/AKT signaling in CRL2631 cells through the regulation of PTEN. **a** A representative Western blot shows the changes in PTEN, pAKT, and AKT protein levels in CRL2631 cells after transfection with mock, control ASOs or miR-21 ASOs. **b** The statistical analyses show the changes in PTEN, pAKT, and AKT protein levels in CRL2631 cells after transfection with mock, control ASOs or miR-21 ASOs. **c** Real-time quantitative PCR detection of changes in PTEN mRNA levels in CRL2631 cells after transfection with mock, control ASOs or miR-21 ASOs. **d** Statistical analyses show the changes in the pAKT/AKT protein levels in CRL2631 cells after transfection with mock, control ASOs or miR-21 ASOs. The results were from 3 independent experiments. The data are presented as the mean ± SE. ***$P < 0.001$; #$P > 0.05$

the sensitivity of the DLBCL cell line, CRL2631, to the CHOP regimen is still unclear. To elucidate the relationship between PTEN and CHOP-induced cytotoxicity, the PTEN, control plasmid or mock was expressed in CRL2631 cells prior to treatment with different concentrations of CHOP. The results showed that the transduction of the PTEN plasmid, similar to the effects of the miR-21 knockdown, significantly increased the expression level of PTEN protein (Fig. 4a) and, compared to the control plasmid, significantly reduced the cell survival rate (Fig. 4c) and the phosphorylation level of AKT (Fig. 4b). These results suggest that miR-21 participates in the regulation of the sensitivity of CRL2631 cells to the CHOP chemotherapy regimen via the regulation of PTEN expression.

NF-κB is a key upstream signal of miR-21 in CRL2631 cells

By bioinformatics analysis, we found there were four potential NF-κB binding sites located in the promoter region of miR-21. We next investigated whether NF-κB could regulate miR-21 expression and chemotherapeutic resistance in CRL2631 cells. As shown in Fig. 5b, inhibition of NF-κB activity significantly downregulates miR-21 expression. Moreover, NF-κB inactivation showed similar CHOP-induced cytotoxicity with miR-21 ASO (Fig. 5c) which suggests that miR-21 was transcriptionally regulated by NF-κB and contributed to the drug resistance in CRL2631 cells.

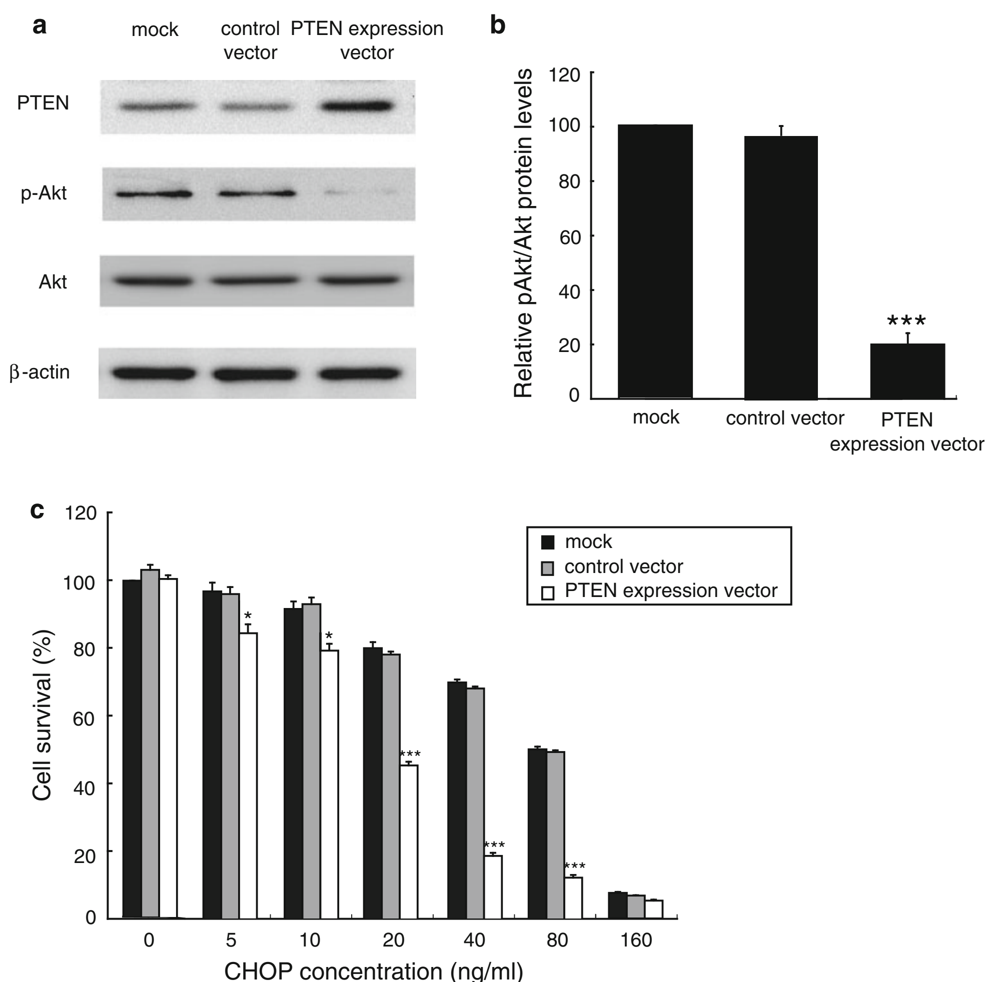

Fig. 4 PTEN is a key molecule regulating the sensitivity of CRL2631 cells to the CHOP regimen. **a** A representative Western blot shows the changes in the protein levels of PTEN, pAKT, and AKT in CRL2631 cells after transduction with mock, the PTEN-expressing or control plasmid. **b** Statistical analyses demonstrate the changes in the pAKT/AKT protein levels in CRL2631 cells after transduction with mock, the PTEN-expressing or control plasmid. **c** After transduction with mock, the PTEN-expressing or control plasmid, CRL2631 cells were treated with different concentrations of the CHOP regimen drugs. The cell viability assay was performed using an MTT assay. The results were from 3 independent experiments. The data are presented as the mean ± SE. $*P < 0.05$; $***P < 0.001$

Discussion

This study showed for the first time that the sensitivity of DLBCL cells to the classical CHOP chemotherapeutic regimen is associated with miR-21 expression. Through the targeted regulation of PTEN expression, miR-21 altered the PI3K/AKT signaling pathway and regulated the survival of DLBCL cells.

Many studies have shown that, as an oncogene, miR-21 is overexpressed in many cancers, such as pancreatic cancer [17], glioma [18], breast cancer [19], prostate cancer [20], leukemia [21], and lymphoma [14]. Recently, increasing evidence has indicated that miR-21 is involved in the resistance of tumor cells to many chemotherapeutic drugs via diverse pathways [22–24]. It has been shown that miR-21 is highly expressed in DLBCL cell lines and clinical patient samples and can be used as an independent prognostic indicator for DLBCL patients [14], suggesting that miR-21 may be involved in the regulation of the sensitivity of DLBCL cells to chemotherapy. In this study, we confirmed that the downregulation of miR-21 in DLBCL cell lines significantly increased the cytotoxic effect of the CHOP regimen on CRL2631 cells.

Many important tumor suppressor genes, including PDCD4 [25], MARCKS [26], LRRFIP1 [24], and TPM1 [27], have been confirmed as target genes of miR-21. We have focused on the PTEN gene. Meng et al. [28, 29] first demonstrated that PTEN is a target gene of miR-21 in hepatocellular carcinoma and cholangiocarcinoma. Our previous studies have indicated that miR-21 targets PTEN and induces the drug resistance of leukemia cells to daunorubicin [11]. Studies have also suggested that the loss of PTEN function is associated with a poor response to treatment and reduced survival in DLBCL patients [30]. Animal studies confirmed that the loss of the PTEN allele in mice results in the development of lymphoma and in drug resistance to conventional chemotherapeutic drugs [31]. PTEN is an important negative regulator of the PI3K/AKT signaling pathway; therefore, the loss of PTEN gene function or low levels of PTEN expression can lead to abnormal amplification of the PI3K/AKT signaling pathway, resulting in tumor development, metastasis, and resistance to chemotherapy.

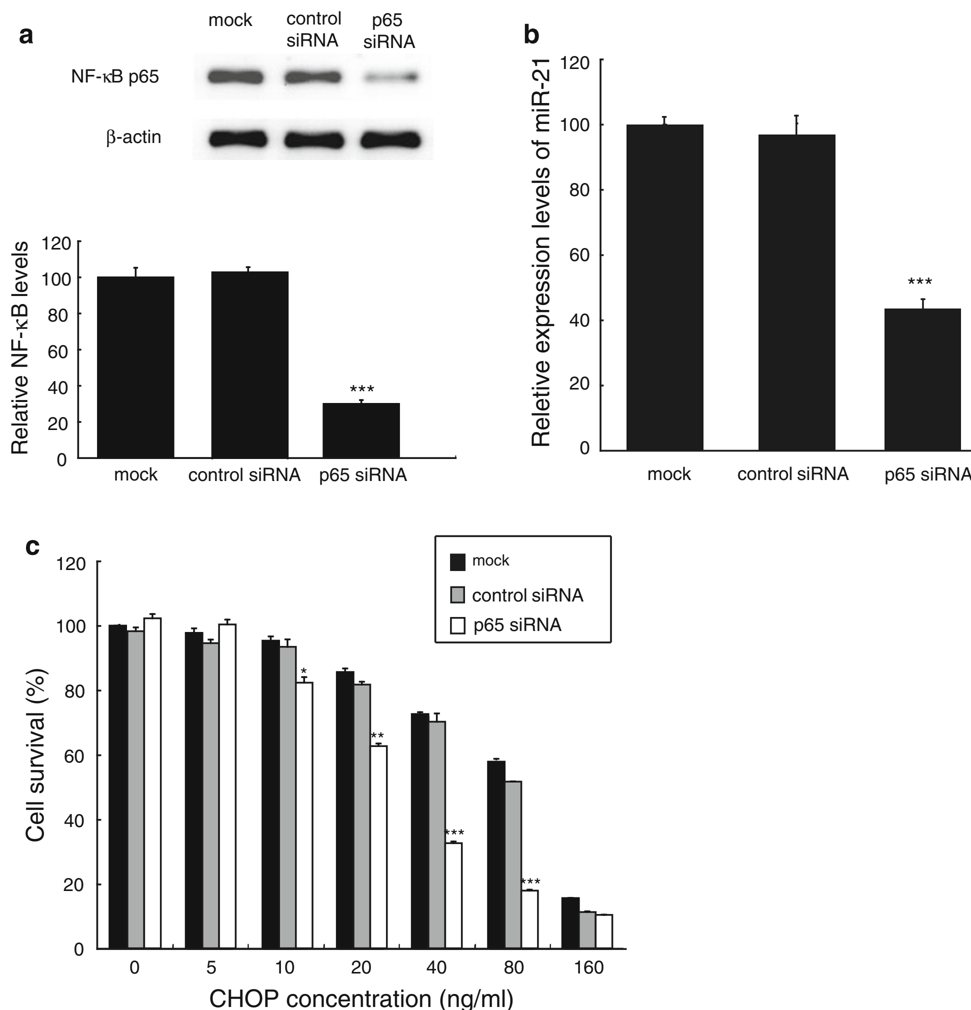

Fig. 5 NF-κB may be an upstream signal of miR-21 in CRL2631 cells. **a** Western blot and statistical analyses show the changes in NF-κB levels after transfection with mock, control scramble or p65 siRNA. **b** Real-time quantitative PCR detection of changes in miR-21 expression in CRL2631 cells after transfection with mock, control scramble or p65 siRNA. **c** After transfection with mock, control scramble or p65 siRNA, CRL2631 cells were treated with different concentrations of the CHOP regimen drugs. The cell viability assay was performed using an MTT assay. The results were from 3 independent experiments. The data are presented as the mean ± SE. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

The abnormal activation of the PI3K/AKT signaling pathway is an important mechanism of resistance to chemotherapeutic drugs in hematological malignancies [32]. Uddin et al. [33] showed that the PI3K signaling pathway is activated in DLBCL cell lines, that AKT phosphorylation levels are higher in drug-resistant cell lines than in sensitive ones, and that suppression of PI3K induces apoptosis in DLBCL cells. Consistent with these studies, we confirmed that PTEN was a target of miR-21 and regulated the sensitivity of the DLBCL cell line, CRL2631, to the CHOP chemotherapeutic regimen. More importantly, similar to the biological effects of miR-21 knockdown, overexpression of PTEN significantly decreased the survival of drug-treated cells. To the best of our knowledge, this is the first time that a connection among miR-21, PTEN expression, and the sensitivity of DLBCL cells to chemotherapy has been shown. We found that both miR-21 knockdown and PTEN overexpression reduced AKT kinase activity. In lymphoma cells, AKT regulates its downstream signaling molecules to promote cell growth and inhibit apoptosis. These downstream molecules include mTOR and BCL-2 [32, 34]; however, whether these AKT downstream molecules participate in drug resistance requires further investigation.

DLBCL cells are notable for the high expression of the transcription factor NF-κB which has been shown enhancing the transcriptional activity of several microRNAs [35]. Several lines of evidence have shown that constitutive activation of the NF-κB pathway may contribute to CHOP resistance in DLBCL. Ramzi et al. [36] reported that the DLBCL mice model treated with CHOP expressed higher level of NF-κB and inactivated NF-κB activity sensitized the mice to CHOP chemotherapy in vivo. Another research found that rituximab

decreased resistance to CHOP regimen in DLBCL patients by NF-κB inhibition [37]. In this study, we confirmed that inhibition of NF-κB increased the cell sensitivity to CHOP. More important, inactivation of NF-κB decreased miR-21 expression. It is possible that functioning downstream of NF-κB, miR-21 is involved in chemoresistance in DLBCL. Surprisingly, bioinformatic analysis revealed that there were several NF-κB binding sites located in the promoter region of miR-21 gene. In addition, recent study found that NF-κB directly binds to miR-21 promoter and is involved in breast cancer cell invasion [38]. Thus, our study provides evidence that knockdown of NF-κB may antagonize chemotherapy resistance through decreasing miR-21 expression.

In summary, our studies demonstrate that the miR-21 expression level in DLBCL cell lines is relatively high, and miR-21 knockdown can significantly downregulate the expression level of PTEN protein and increase the sensitivity of DLBCL cell lines to the CHOP chemotherapeutic regimen. These results will aid in the development of individualized treatment plans for DLBCL patients with abnormal expression of miR-21 or PTEN, thus increasing the efficacy of the CHOP regimen. More importantly, these results provide potential therapeutic targets for overcoming DLBCL drug resistance based on the NF-κB/miR-21/PTEN/PI3K/AKT signaling pathway. However, the role of miR-21 in DLBCL drug resistance must be confirmed with animal studies and detection in clinical samples.

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Conflict of interest The authors declare that they have no conflict of interest.

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