Overexpression of X chromosome-linked inhibitor of apoptosis by inhibiting microRNA-24 protects periodontal ligament cells against hydrogen peroxide-induced cell apoptosis

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Abstract: Hydrogen peroxide (H$_2$O$_2$), a common oral clinical drug for the tooth bleaching, induces severe cell apoptosis of periodontal ligament cells (PDLCs). The excessive cell apoptosis of PDLCs impairs periodontal tissue damage and repair. However, the underlying mechanism is incompletely understood. Here, we showed that microRNA-24 (miR-24) played an important role in regulating H$_2$O$_2$-induced cell apoptosis of PDLCs. We found that miR-24 expression was increased in PDLCs in response to H$_2$O$_2$ treatment. Down-regulation of miR-24 obviously rescued H$_2$O$_2$-induced cell apoptosis in PDLCs. By bioinformatic analysis, X chromosome-linked inhibitor of apoptosis (XIAP) was identified as a candidate target gene of miR-24, which was further verified by the dual-luciferase reporter assay. Furthermore, the protein expression level of phosphatase and tensin homolog deleted on chromosome ten was significantly decreased by miR-24 silencing, whereas the phosphorylation of Akt was remarkably increased by miR-24 silencing. In addition, the gene silencing of XIAP significantly reduced Akt activity and blocked the protective effect of the miR-24 inhibitor against H$_2$O$_2$-induced cell apoptosis. Overall, our findings suggest that miR-24 plays an important role in regulating the cell survival of PDLCs through targeting XIAP.

Key words: Periodontal ligament cells, X chromosome-linked inhibitor of apoptosis, miR-24.

Introduction

Periodontal ligament cells (PDLCs) are considered important participants of periodontal regeneration (1). The number of PDLCs in periodontal tissues is maintained in homeostasis via cell proliferation and cell apoptosis, which contributes to the repair of damaged tissue (2). The cell apoptosis of PDLCs results in periodontal tissue damage and impairs the repair of periodontal tissues (3). Various biological factors such as growth factors and hormones, and physical factors as well as chemical drugs cause cell apoptosis of PDLCs that accounts for periodontitis and other dental diseases (3-6). Hydrogen peroxide (H$_2$O$_2$) is a common oral clinical drug used for the tooth bleaching that directly acts on the gingival and periodontal tissue (7). However, H$_2$O$_2$ is a type of strong oxidant that can induce the production of excessive reactive oxygen species (ROS) which causes DNA damage, leading to cell apoptosis (8,9). A previous study reported that H$_2$O$_2$ induces severe cell apoptosis of PDLCs (10). However, the mechanism of H$_2$O$_2$-induced cell apoptosis of PDLCs is not fully understood.

X chromosome-linked inhibitor of apoptosis (XIAP) is a member of the inhibitor of apoptosis protein (IAP) family which is the mostly widely studied member in many diseases such as cancer (11). XIAP is widely expressed in many tissues and can directly interact and inhibit caspase-3/7/9 (12). XIAP possesses the E3 ubiquitin ligase activity that can promote the polyubiquitination and thus protein degradation of caspase (12). Thus, XIAP has a positive effect on regulating cell survival. XIAP is aberrantly expressed in numerous human cancers and the overexpression of XIAP inhibits the cell apoptosis of cancers and increases the resistance to chemotherapy (13-15). It has been reported that XIAP inhibits cell apoptosis of vascular endothelial cells during inflammatory stimuli (16). Zhu et al. demonstrated that XIAP overexpression attenuates oxidative stress and inhibits cell apoptosis after hypoxia-ischemia or cerebral irradiation via upregulating mitochondrial antioxidants (17). In the rat hippocampus suffering mitochondrial antioxidants (17). In the rat hippocampus suffering ischemia, overexpression of XIAP inhibited caspase-3 activity and increased neuron survival (18). The stabilization of XIAP by Pseudomonas aeruginosa protected Kupffer cells against tumor necrosis factor-alpha-induced cell death (19). Moreover, staurosporine-induced apoptotic cell death in oligodendroglial cells was rescued by XIAP overexpression (20). Considering the protective role of XIAP against the harmful stimuli-induced cell apoptosis, XIAP may serve as a potential molecular target for developing novel drugs that protect periodontal tissues against oxidative stress insults.

In recent years, a group of short and non-coding RNAs, called microRNAs (miRs), have been proposed as a new class of regulatory molecules for gene expression (21,22). It has been found that miRs negatively regulate gene expression through binding the 3’-untranslated region (UTR) of messenger RNA (mRNA) inducing mRNA cleavage, or destabilization and translational repression (21,22). Thus, miRs were reported to participate in the pathologies of various diseases such as cancer and cardiovascular disease (23,24). However, the role of miRs in regulating PDLCs remains largely unexplored. Li et al. reported that miR-21 and miR-101 participate in osteogenic differentiation of PDLCs through targeting and regulating periodontal ligament-
associated protein-1 (25). Cyclic stretch and compression forces has been reported to alter the expression of miR-29 in PDLCs associated with extracellular matrix gene expression (26). miR-24 is specific miR that is extensively associated with cell survival. It has been reported that miR-24 is significantly upregulated in cardiac endothelial cells after cardiac ischemia and the suppression of miR-24 limits myocardial infarct size and preserves cardiac function (27). Elevated expression of miR-24 results in reduced stress resistance and induces cell apoptosis in smooth muscle cells (28). In gastric cancer cells, miR-24 was suggested as a tumor suppressor that inhibited cell proliferation and promoted cell apoptosis (29). In contrast, miR-24 was reported to protect cardiomyocytes against myocardial ischemia (30). The overexpression of miR-24 promoted cell proliferation in several cancer cells (31,32). It seems that miR-24 has a dual role in regulating cell survival. Indeed, the precise function of miRs is rarely known due to their potential regulation of multiple target genes. In the present study, we aimed to explore the potential role of miR-24 in regulating cell apoptosis of PDLCs in response to $H_2O_2$-induced oxidative stress.

Materials and Methods

Cell culture

Primary normal human PDLCs were isolated from periodontal ligament explants, as described previously (33). Periodontal ligament explants were obtained from the impacted third molars at Department of Prosthodontics, Yan'an University Affiliated Hospital (Yan'an, China). The preparation of clinical samples was conducted according to the guideline of The Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by Institutional Human Experiment and Ethics Committee of Yan'an University with the understanding and written consent of each subject. The tissues were minced into smaller portions (mm$^3$), and then digested in dispase (4 mg/ml; Gibco, Grand Island, NY, USA) and type 1 collagenase (3 mg/ml; BioBasic, Toronto, Canada) for 1 h at 37°C. Single-cell suspensions were obtained using the limiting dilution technique (34,35). The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum (FBS; Gibco), 100 μM $\text{-ascorbate-2-phosphate}$ (Sigma, St. Louis, MO, USA), 2 mM $\text{-glutamine}$ (Gibco), and 1% streptomycin/penicillin (Sigma). The cells were maintained in a humidified atmosphere containing 5% CO$_2$ at 37°C and the medium was refreshed every 2 days. Cells at the same passage (passage 4) were used in the subsequent experiments.

Cell treatments and cell transfection

PDLCs were exposed to $H_2O_2$ at various doses (31.25, 62.5, 125, 250, 500 and 1000 μM) and incubated for 24 h. MiR-24 inhibitor and miR-scrambled control (GenePharma, Inc., Shanghai, China) were transfected into cells with a final concentration at 50 nM by using Lipofectamine 2000 reagent (Invitrogen) for another 24 h. Then, cells were transfected with miR-24 inhibitor (50 nM) followed by exposure to $H_2O_2$ for another 24 h. Thereafter, the medium was refreshed and 15 μl of 5 mg/ml MTT per well was added before further culturing for 4 h. To dissolve the formazan dye crystals, 150 μl dimethylsulfoxide was added per well. After gently shaking for 15 min, the optical density value at 490 nm by a Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Each assay was performed in triplicate.

Cell apoptosis assay

Cell apoptosis was measured using an annexin V/propidium iodide (PI) double staining kit (Beyotime Biotechnology, Haimen, China) according to the manufacturer’s instructions. Briefly, cells were harvested by centrifugation, digested with trypsin (2.5 g/l) and washed with phosphate buffered saline (PBS). Then, cells were collected and re-suspended in 200 μl of binding buffer followed by the addition of 10 μl Annexin V stock solution for 30 min at 4°C. Afterward, 5 μl of PI solution was added for 5 min in the dark, at room temperature. The apoptotic cells including early apoptosis and late apoptosis were quantified at each experimental treatment by fluorescence-activated cell sorting scanning (BD Biosciences, San Jose, CA, USA).

Caspase-3 activity assay

Cells were lysed in ice-cold PBS and the supernatants were harvested. The protein concentrations in each sample were measured and equivalent amount of proteins were incubated with acetyl-Asp-Glu-Val-Asp $\text{-nitrilotriamide}$ (Ac-DEVD-pNA; Sigma) containing 10 mM dithiothreitol (DTT) and 10% glycerol for 6 h at 37°C. The absorbance at wavelength of 405 nm was measured using a spectrophotometer (Hitachi F-2000, Tokyo, Japan).

Quantitative PCR analysis

Total RNAs from different cell samples were extracted using miRNasy Mini Kit (Qiagen, Dusseldorf, Germany) that was reverse transcribed to complementary DNA (cDNA) with M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA) or one-step primerscript miRNA cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer’s instructions. The qPCR analysis was performed by using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) and the data were analyzed by using the $2^{-\Delta\Delta C_{t}}$ method. $\beta$-actin and U6 SnRNA were used as the internal reference genes.

Western blot analysis

Equivalent amounts of protein (50 μg) from each cell sample were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Bio-Rad,
Hercules, CA, USA). Nonspecific binding sites on the membrane were blocked by 3% skimmed milk for at 37°C for 1 h. Then, the membranes were incubated with the following primary antibodies at 4°C overnight: mouse anti-human XIAP monoclonal antibody (1:800; sc-55552), rabbit anti-human PTEN polyclonal antibody (1:500; sc-9145) and mouse anti-human β-actin monoclonal antibody (1:600; sc-47778) purchased from Santa Cruz Biotechnology; and rabbit anti-human pAkt polyclonal antibody (1:1000; #9611) purchased from Cell Signaling Technology (Danvers, MA, USA). Afterward, goat anti-mouse or rabbit secondary antibodies conjugated to horseradish peroxidase (1:1,000; Beyotime Biotechnology) were added and incubated with the membrane for 1 h at room temperature. Finally, the protein bands were detected using the enhanced chemiluminescence method.

Dual-Luciferase reporter assay
The cDNA fragment of XIAP 3'-UTR containing the predicted binding sites was amplified and subeloned into pGL3 dual-luciferase miRNA target expression vectors (Promega, Madison, WI, USA). For dual-luciferase reporter assay, human embryo kidney 293 (HEK293) cells were co-transfected with pGL3 construct vectors (100 μg/ml) and miR-24 mimics (50 nM) (GenePharma, Inc., Shanghai, China) by using Lipofectamine 2000 reagent (Invitrogen). After transfection for 48 h, the relative luciferase activity was measured by using the dual-luciferase reporter detection system (Promega).

Statistical analysis
Data were reported as mean ± standard deviation. One-way analysis of variance was used to analyze the statistical differences using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Statistical difference was significant at p < 0.05.

Results

Mir-24 is upregulated in PDLCs in response to H₂O₂ treatment
To explore whether miR-24 was involved in oxidative stress induced by H₂O₂ in PDLCs, we performed qPCR analysis to examine the expression pattern of miR-24 in PDLCs exposed to various doses of H₂O₂ (31.25, 62.5, 125, 250, 500 and 1000 μM). As shown in Fig. 1A, miR-24 was significantly induced in a dose-dependent manner with exposure to H₂O₂. Next, we also found that the expression of miR-24 was persistently induced post-time challenge in PDLCs treated with 250 μM of H₂O₂ for 24, 48 and 72 h (Fig. 1B). The results indicated that miR-24 was increased in PDLCs in a dose- and time-dependent manner in response to H₂O₂ treatment, suggesting a possible role of miR-24 involved in oxidative stress induced by H₂O₂ in PDLCs.

Inhibition of MiR-24 rescues H₂O₂-induced cell apoptosis in PDLCs
Considering the high and persistently upregulated expression of miR-24 during H₂O₂ treatment, down-regulation of miR-24 may contribute to relieve cell injury induced by H₂O₂. For the down-regulation of miR-24, we used chemically synthesized miR-24 inhibitor and transfected them into PDLCs. Twenty-four hours after transfection, we detected the expression of miR-24 by qPCR analysis. The results showed that the expression of miR-24 was significantly decreased with miR-24 inhibitor treatment (Fig. 2A). The results of the MTT assay showed that miR-24 inhibition significantly reversed the cell growth arrest induced by H₂O₂ (Fig. 2B). Furthermore, cell apoptosis increased by H₂O₂ treatment was also markedly reduced by miR-24 inhibitors, as detected by flow cytometry (Fig. 2C and D). In addition, the caspase-3 activity assay exhibited that the activity of caspase-3 was also significantly decreased by miR-24 inhibition (Fig. 2E). In summary, these results implied that down-regulation of miR-24 showed protective effect against H₂O₂-induced cell apoptosis in PDLCs.

MiR-24 directly targets the 3'-UTR of XIAP
To identify the direct target gene of miR-24, we performed bioinformatics analysis and found that the 3'-UTR of XIAP contained the predicted binding sites for miR-24 (Fig. 3A). To verify XIAP as a direct target gene of miR-24, we performed a dual-luciferase reporter assay. The results showed that the relative luciferase activity was markedly reduced by miR-24 mimics or increased by miR-24 inhibitor in pGL3-XIAP-3'-UTR vectors transfected cells (Fig. 3B). Mutations in the pre-
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Predicted miR-24 target sites apparently abolished the effect of miR-24 inhibitor or mimics on relative luciferase activity. The results confirmed that XIAP was indeed a target gene of miR-24.

**Inhibition of MiR-24 upregulates the expression of XIAP**

To validate the regulation of XIAP by miR-24, we tested the expression of XIAP in miR-24 transfected PDLCs. qPCR results showed that the mRNA expression level of XIAP was significantly increased by miR-24 inhibitor (Fig. 4A). These findings were also validated at the protein level as detected by Western blot analysis (Fig. 4B), which demonstrated that the protein expression level of XIAP was also significantly upregulated by miR-24 inhibitor (Fig. 4C). These results further confirmed that miR-24 targeted and regulated XIAP.

**Inhibition of MiR-24 results in down-regulation of PTEN and up-regulation of Akt**

To further understand the underlying mechanism of miR-24 in regulating cell survival, we further determined the effect of miR-24 on the pro-survival Akt signaling pathway which linked with XIAP (37,38). We first detected the protein levels of phosphatase and tensin homolog deleted on chromosome ten (PTEN), a well-known negative regulator of Akt phosphorylation, in response to miR-24 inhibitor treatment. The results from Western blot analysis (Fig. 5A) showed that the protein expression was significantly upregulated by H$_2$O$_2$ treatment which was markedly reduced by miR-24 inhibitor treatment in PDLCs (Fig. 5B). Interestingly, the phosphorylated Akt (pAkt) protein level was apparently reduced by H$_2$O$_2$ treatment, which was remarkably reversed by miR-24 inhibitor treatment (Fig. 5C and D). The results implied that miR-24 might improve cell survival through regulating the pro-survival Akt signaling pathway.

**Knockdown of XIAP impairs the protective effect of MiR-24 inhibitor**

To verify the effect of miR-24 inhibitor on cell survival through regulating XIAP, we determined the effect of miR-24 on the anti-apoptotic effect in PDLCs (Fig. 6A-C). As expected, the motivating effect of miR-24 inhibitor on pAkt was obviously blocked by XIAP gene silencing (Fig. 6D and E). Further data also showed that the gene silencing of XIAP significantly abrogated the
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protective effect of miR-24 inhibitor against H$_2$O$_2$-induced cell growth inhibition (Fig. 6F). Overall, these results confirmed that the miR-24 inhibitor regulated the cell survival of PDLCs through XIAP.

Discussion

In this study, we have elucidated that miR-24 is a predictor for cell apoptosis in PDLCs which is highly expressed under H$_2$O$_2$-induced oxidative stress. Interestingly, inhibiting the increased expression of miR-24 by miR-24 inhibitor significantly rescued PDLCs from H$_2$O$_2$-evoked cell injury. The potential underlying mechanism was later found in which inhibiting miR-24 upregulated the expression of XIAP, an anti-apoptotic protein that promoted cell survival under H$_2$O$_2$-induced oxidative stress.

The harmful effect of H$_2$O$_2$ for cells has been verified by several studies (6,10,39,40). Treatment with H$_2$O$_2$ produces a considerable cytotoxic effect on PDLCs (39). It was reported that H$_2$O$_2$ treatment inhibited cell viability and induced the high activity of caspase-3 and caspase-9 in human dental pulp cells (6). H$_2$O$_2$-induced cytotoxicity in PDLCs was significantly reversed by an-oxidant treatment with vitamin C (40). More recently, XIAP was found decreased in PDLCs with H$_2$O$_2$ exposure and overexpression of XIAP significantly inhibited cell apoptosis through upregulating serval pro-survival signaling pathway (10). Here, we found that XIAP was a target gene of miR-24 which was regulated by miR-24. The data showed that miR-24 was increased by H$_2$O$_2$ exposure which might account for the reduction
in XIAP expression level. Therefore, inhibiting miR-24 should promote XIAP expression and inhibit cell apoptosis. As expected, miR-24 inhibitor which silenced the miR-24 expression significantly increased the expression of XIAP and promoted cell survival against H$_2$O$_2$ exposure.

Recent studies have proposed a dual role of miR-24 in regulating cell survival. Fiedler et al. have found that miR-24 expression is upregulated in cardiac endothelial cells after cardiac ischemia and induces cell apoptosis through targeting the endothelium-enriched transcription factor GATA binding protein 2 and the p21-activated kinase 4 (27). miR-24 is enriched in renal endothelial and tubular epithelial cells after renal ischemia/reperfusion and silencing of miR-24 results in a significant reduction in cell apoptosis (41). Overexpression of miR-24 results in reduced stress resistance leading to increased cell apoptosis in smooth muscle cells (28). Furthermore, miR-24 serves as a tumor suppressor that inhibits cell proliferation and promotes cell apoptosis in gastric cancer cells (29). miR-24 inhibits cell proliferation of bladder cancer cells by targeting the forkhead box protein M1 (42). It was also found that miR-24 reduced the cellular viability of sporadic breast tumor cells in the presence of doxetaxel (43). In contrast, there are also studies that indicate that miR-24 promotes cell survival rather than inhibits cell survival. It was reported that miR-24 protected cardiomyocytes against myocardial ischemia through regulating the pro-apoptotic gene, BCL2L11 (30). Wang et al. revealed that miR-24 inhibited the initiation of apoptosis by blocking cytochrome c release and Bax mitochondria translocation in mouse cardiomyocytes (44). miR-24 promotes cell proliferation of human breast cancer via targeting p27Kip1 (32). Overexpression of miR-24 increases the cell proliferation of non-small cell lung cancer by inhibiting nuclear apoptosis-inducing factor 1 (31). These studies point out a positive role of miR-24 in regulating cell survival. The apparent contrary roles of miR-24 in regulating cell survival due to its potential regulation on multiple target genes. In addition, these roles were reported in different cell types. Different cells have different characteristics and behaviors. Even same cell type can have different behaviors according to passages and related phase of differentiation. Nevertheless, we found that miR-24 negatively regulated cell survival of PDLCs through targeting XIAP, an anti-apoptotic protein. Thereby, inhibiting miR-24 significantly increased the expression of XIAP and promoted cell survival of PDLCs. Our results were consistent with the findings of the Xie et al. which indicated that miR-24 promoted cell apoptosis through directly targeting and modulating XIAP expression (45).

On the other hand, besides miR-24, various miRs have been found to be capable of regulating XIAP expression. miR-618 was reported to suppress anaplastic thyroid cancer through targeting and inhibiting XIAP (46). XIAP expression was down-regulated by miR-200c, which promoted cell apoptosis in breast cancer cells (47). In addition, miR-519d, miR-130a and miR-7 are also found to be capable of regulating XIAP expression (48-50). These findings raised the possibility that modulation of the expression of XIAP regulates cell survival and apoptosis. Our results further verified that the modulation of XIAP expression by miR-24 inhibitor showed a regulatory effect on cell survival of PDLCs under H$_2$O$_2$ treatment.

In the present study, we further found that miR-24 inhibitor not only increased XIAP expression but also upregulated Akt pro-survival signaling pathway. Our data revealed that miR-24 inhibition significantly inhibited the protein expression level of PTEN which was an inhibitor of Akt phosphorylation. A previous study has demonstrated that overexpression of XIAP promotes the polyubiquitination and protein degradation of PTEN, thus leading to Akt activation (38). In line with the findings, Yi et al. reported that silencing of XIAP inhibited cell proliferation via regulating the PTEN/Akt signaling pathway (37). Furthermore, the reduced expression of XIAP was found to be concomitant to reducing pAkt expression, indicating a possible link between XIAP and Akt activity (51). Additionally, XIAP is a substrate of Akt and could be phosphorylated via Akt to inhibit its ubiquitination and degradation (52).

Taken together, we found that the suppression of miR-24 prevented H$_2$O$_2$-induced cell apoptosis in PDLCs through upregulating XIAP and the Akt signaling pathway. The results were further confirmed by XIAP silencing experiments in which the gene silencing of XIAP obviously abrogated the protective effect of the miR-24 inhibitor against H$_2$O$_2$-induced cell apoptosis and blocked Akt phosphorylation. Our results suggested an important role of miR-24 in regulating cell survival of PDLCs in response to a tooth bleaching agent such as H$_2$O$_2$. These findings may be valuable for understanding the mechanism of H$_2$O$_2$-induced cell apoptosis in PDLCs. However, the precise role and molecular mechanism of miR-24 in regulating cell apoptosis of PDLCs remain to be further investigated.

References

