MiR-499 Enhances the Cisplatin Sensitivity of Esophageal Carcinoma Cell Lines by Targeting DNA Polymerase β

Yuanyuan Wang  a  Jianfang Feng  b  Wenzhao Zang  a  Yuwen Du  a  Xiaonan Chen  a  Qianqian Sun  a  Ziming Dong  a,c  Guoqiang Zhao  a,c

a) College of Basic Medical Sciences, Zhengzhou University, Zhengzhou, China, b) Medical College of Henan University of Science and Technology, Luoyang, China, c) Collaborative Innovation Center of Cancer Chemoprevention, Henan, China

Key Words
Esophageal carcinoma • miR-499 • Cisplatin sensitivity • DNA polymerase β

Abstract
Background: Human DNA polymerase β (DNA polymerase β, polβ) is a small monomeric protein essential for short-patch base excision repair (BER). It plays an important role in regulating the sensitivity of tumor cells to chemotherapy. Methods: Luciferase reporter and western blot assays were used to determine whether polβ is a major target of miR-499. CCK-8, colony-forming survival and in vivo tumor growth assays were conducted to evaluate if miR-499 can potentially enhance the cisplatin sensitivity and therefore inhibit the proliferation of esophageal cancer (EC) cells. Flow cytometry and immunofluorescence microscopy assays were performed to evaluate whether miR-499 enhance the cisplatin sensitivity and the corresponding apoptosis in EC cells. Results: polβ was pinpointed as a target gene of miR-499. Additionally, we identified that miR-499 can enhance cisplatin’s function of inhibiting proliferation and of promoting apoptosis in EC9706 and KYSE30 cell lines. Conclusions: We first investigated whether miR-499 modulates polβ, and observed the influence of miR-499 up-regulation on the sensitivity of EC cell lines to cisplatin treatment. Our study paves the way for more insightful understanding and application of chemotherapy in esophageal cancer in the future.

Introduction
MicroRNAs (miRNAs), typically 18-25 nucleotides long, constitute a class of small non-coding single-stranded RNAs (ssRNAs) that are highly conserved and endogenously expressed across many species. These miRNAs regulate gene expression by targeting complementary
sequences primarily located within the 3’untranslated regions (UTRs) of mRNAs [1-6]. Although the full extent of miRNA biological functions have yet to be elucidated, they have been suggested to function as intrinsic regulators of many cellular processes, including cell invasion, differentiation, proliferation and apoptosis [7-11]. The aberrant expression of miRNAs has been linked to the development and progression of several cancers and was shown to have prognostic significance for certain types of cancer, such as lung and esophageal cancer, neuroblastoma and lymphocytic leukemia [12-14].

The altered expression miRNAs has previously been observed in esophageal cancer (EC), a major cause of cancer-related death worldwide [15-18]. This observation indicates that the deregulation of miRNAs may play a role in the carcinogenesis of EC. Presently, the effects of miR-499 on the apoptosis and the metastasis of EC cells are not exactly elucidated.

Human DNA polymerase β (DNA polymerase β, polβ), a small monomeric protein of 335 residues, is found in the nuclei of mammalian cells [19]. Polβ, a member of the DNA polymerases family, is essential for short-patch base excision repair (BER) [20-22]. BER is one of the major pathways of DNA repair that repairs DNA damage caused by oxidation [23]. Polβ is also involved in the meiotic recombination [24] and the repair of breaks in double-stranded DNA through the process of nonhomologous end joining [25]. There are also studies that have revealed that polβ plays an important role in DNA single-strand breaks (SSB) resulting from rays and alkylating agents [26-28]. Polβ expression is increased in many cancer cells [29]. More specifically, the overexpression of polβ results in aneuploidy and tumorigenesis in nude immunodeficient mice [30]. Moreover, polβ heterozygous mice exhibit increased single-stranded DNA breaks, chromosomal aberrations, and mutagenicity compared to normal animals [31]. Based on bioinformatic analyses we hypothesized that human polβ 3’UTR contains the putative binding sites of miR-499, and then miR-499 could enhance the cisplatin sensitivity of EC cell lines by targeting polβ.

In the present study, we first investigated whether miR-499 modulates polβ, and then observed the influence of miR-499 up-regulation on EC cell lines’ sensitivity to cisplatin.

Materials and Methods

Cell culture

Human EC cell lines (EC9706 and KYSE30) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Both cell lines were maintained in RPMI 1640 with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) and incubated at 37°C/5% CO₂.

Luciferase reporter assay

The human polβ 3’UTR fragment containing putative binding sites for miR-499 was amplified by PCR from human genomic DNA. The mutant polβ 3’UTRs were obtained by overlap extension PCR. The fragments were cloned into a pmirGLO reporter vector (Promega, Madison, WI, USA), downstream of the luciferase gene, in order to generate the recombinant vectors pmirGLO-WT and pmirGLO-MUT.

For the luciferase reporter assay, EC9706 and KYSE30 cells were trypsinized and resuspended in PBS for electroporation with BTX ECM 2001 electroporator; Then 5 × 10⁶ cells of each group were mixed with miRNA (miR-499 agomir or scrambled-miR-499 negative control; GenePharma, Shanghai, China) and reporter vectors (pmirGLO-WT reporter vectors or pmirGLO-MUT reporter vectors), and were electroporated with square pulse condition. Electroporated cells were incubated in 2 ml RPMI 1640 supplemented with 10% FBS for 24 hour. Luciferase activities were measured with a Dual-Luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions, at 24h post-transfection. Experiments were repeated three times.

RNA isolation and RT-PCR

EC9706 and KYSE30 cell lines were plated at a density of 1.5 × 10⁵ cells/well in 6-well plates 24 h prior to transfection. Once the cells reached approximately 50% confluence, transfection was conducted using BTX ECM 2001 electroporator following the manufacturer’s instructions. Each cell line was separated
into three groups: a non-transfected blank group (Blank), a scrambled miR-499 transfected negative control group (NC), and a miR-499 agomir transfected group (miR-499).

RNA was extracted using the Total RNA Kit I (R6834-01; Omega) from the aforementioned three groups of cells. Approximately 1 µg of total RNA were used for the reverse transcriptase (RT)-PCR with (0.5 µg) oligo (dt) primer for first strand synthesis to generate cDNA. Next, the cDNAs were amplified using the following primers:

Polβ upstream: 5’ GTGCAGATGCAGTGGTGACA 3’;
Polβ downstream: 5’ CAGTTTTGGCTGTTTGGTTGATT 3’;
β-actin upstream: 5’ TTCACTTCTTCAGTTCTGCCATCT 3’;
β-actin downstream: 5’ CCAAGCCTTTCTCAGTCCCAAA 3’.

The following thermal cycling conditions were used: denaturation at 94 °C for 5 min followed by 36 cycles of denaturation at 94 °C for 35 s, annealing at 56 °C for 30 s, and extension at 72 °C for 35 s. PCR-amplified fragments were separated on a 1.5 % agarose gel.

Western blot assay

Total proteins from the three groups of transfected cells (Blank, NC and miR-499) were extracted using a RIPA buffer containing phenylmethanesulfonyl fluoride (PMSF). A BCA protein assay kit (Beyotime, Haimen, China) was used to determine the protein concentrations. Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were later transferred onto polyvinyl difluoride (PVDF) membranes. After blocking, the membranes were incubated overnight at 4°C with diluted (1:300) primary antibody (polyclonal rabbit anti-polβ; Santa Cruz, USA). Following extensive washes, the membranes were incubated with diluted (1:3000) horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz). Signals were determined using the DAB detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). β-tubulin (Santa Cruz, USA) served as the endogenous reference.

Cell growth assay

Cell growth assay was performed using a cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. The three groups of cells (Blank, NC and miR-499) were seeded into a 96-well plate at a density of 1 × 10⁴ cells/well, with six replicate wells per group. Next, the cells were respectively treated with 2ìM cisplatin (Sigma-Aldrich, UK; dissolved in 0.15M NaCl, stored at -20°C) during the logarithmic growth phase. After cells were cultured for four consecutive days, the optical density (OD) was measured at a wavelength of 450 nm (OD₄₅₀) to estimate viable cell numbers. The cell survival rate was calculated according to the absorbance value inferred from living cell numbers in each of the three different treatment groups.

Colony-forming survival assay

The three groups of cells (Blank, NC and miR-499) were respectively treated with six different concentration cisplatin (0ìM, 2ìM, 4ìM, 6ìM, 8ìM and 10ìM) for 2 h during the logarithmic growth phase. Then the cells were washed, trypsinized, counted and plated into 10-cm dishes containing DMEM supplemented with 10% FBS, at a plating number of 200 cells per dish. The formation of colonies, usually occurring in approximately two weeks, could be visibly observed. The newly formed colonies were fixed with ethanol and stained with crystal violet, and the colonies with >50 cells were scored as surviving colonies [32, 33]. The cloning efficiency was calculated by dividing the average number of colonies per dish by the amount of cells plated.

In vivo tumor growth assay

The three groups of EC9706 cells (Blank, NC and miR-499) were respectively transfected with a luciferase-expressing lentivirus containing an independent open-reading frame of GFP. After 72 h, cells were examined by fluorescence microscopy to confirm infection. Then the EC9706-Luc cells (5 × 10⁶ cells) were subcutaneously inoculated into the armpit of right forelimb of 6-week-old female BALB/c nude mice purchased from The Laboratory Animal Unit of the University of Zhengzhou. All three groups (6 mice per group) were intraperitoneally (i.p.) treated with 2 mg/kg cisplatin weekly for 4 weeks. Before the mice were anesthetized with Forane, an aqueous solution of luciferin (150 mg/kg) was intraperitoneally injected at 10 min prior to imaging. The mice were placed into the light-tight chamber of a CCD camera system.
(Xenogen). The luminescent area of the xenograft tumor was defined as the region of interest (ROI) and the total signal in the ROI (photon/sec/m²) was quantified using Living Image software 3D (Xenogen). All procedures involving mice were performed in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Flow cytometry assay

The three groups of cells (Blank, NC and miR-499) were respectively treated with 0.2μg/ml of cisplatin for 2 h during the logarithmic growth phase. Cells were resuspended at a density of 1 × 10⁶ cells/ml in 1 × binding buffer. After being double stained with FITC-Annexin V and propidium iodide (PI) using the FITC Annexin V Apoptosis Detection Kit I (BestBio, Shanghai, China), the cells were analyzed using a FACSscan® flow cytometer (BD Biosciences) equipped with Cell Quest software (BD Biosciences).

Immunofluorescence microscopy

Immunofluorescence was performed to observe the number of γ-H2AX foci of different groups. After being treated with 0.2μg/ml of cisplatin for 2 h, the three groups of cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100. After blocking in PBS containing 5% BSA for 1 h, the cells were incubated with anti-γ-H2AX primary antibodies and stained with FITC-conjugated secondary antibodies. Then the cells were stained with DAPI (4′,6-diamidino-2-phenylindole). Cells were imaged with fluorescence microscope (Olympus), and data were collected and analyzed with CellSens software.

Statistical analysis

Statistical testing was conducted with the assistance of SPSS 17.0 software. All data are expressed in the format of means ± standard deviation (SD). One-way ANOVA and LSD tests were used to analyze the data. Results were considered significant when P-values were < 0.05.

Results

Polβ is identified as a target gene of miR-499 in EC9706 and KYSE30 cell lines

Bioinformatics analysis by TargetScan and miRanda predicted that the 3’UTR of polβ contained binding sites for miR-499 (Fig. 1A).

To verify whether polβ is a direct target of miR-499, we first used a Dual-Luciferase reporter system containing either the wild-type or the mutant 3’UTR of polβ. Co-transfection with miR-499 significantly suppressed the luciferase activity of the reporter containing the wild-type 3’UTR (P < 0.05, Fig. 1B). Our results indicate that miR-499 negatively regulates polβ expression by directly binding to the putative binding sites in the 3’UTR.

RT-PCR and western blot assays were employed to detect the expression levels of polβ mRNA and protein after transfection with miR-499 agomir in EC9706 and KYSE30 cells. The expression level of polβ protein in the miR-499 group was found to be significantly lower (P < 0.05, Fig. 1C) than in the Blank and NC groups. However, there were no significant differences in polβ mRNA expression levels among the three groups (P > 0.05, Fig. 1D).

MiR-499 enhanced the proliferation inhibition effect of cisplatin in EC9706 and KYSE30 cells

The levels of miR-499 after transfection in EC9706 and KYSE30 cells are shown in Figure 2A. Expression levels of miR-499 in the miR-499 group were significantly higher (P < 0.05, Fig. 2A) than in the Blank and NC groups.

The cell survival rate curves are presented in Fig. 2A. Cell viability decreased with increased chemotherapy dose. The miR-499 group’s viability declined more significantly, and the decline has statistical differences with the Blank and NC groups at different chemotherapy doses (P < 0.05, Fig. 2B).
polβ was identified as a target gene of miR-499 in EC9706 and KYSE30 cell lines. (A) The putative miR-499 binding sequences for the polβ 3'UTR. (B) Co-transfection with miR-499 significantly suppressed the luciferase activity of the reporter containing the wild-type 3'UTR \( (P < 0.05) \). Control: scrambled-miRNA; miR-499: miR-499 agomir; wt-polβ: wild-type pmirGLO-polβ; mut-polβ: mutant pmirGLO-polβ. (C) In both the EC9706 and KYSE30 cells, the expression of polβ protein was significantly reduced in the miR-499 group compared to the NC and Blank groups \( (P < 0.05) \). (D) In both EC9706 and KYSE30 cells, there were no significant differences in the polβ mRNA expression levels among the three groups \( (P > 0.05) \). miR-499: cells transfected with miR-499 agomir; NC: cells transfected with scrambled miR-499 negative control; Blank: non-transfected cells. \* \( P < 0.05 \) compared to the control group.

miR-499 enhanced the effect of proliferation inhibition of cisplatin in EC9706 and KYSE30 cells. (A) Expression levels of miR-499 in the three treatment groups. Expression levels of miR-499 in the miR-499 group were significantly higher \( (P < 0.05, \text{Fig. 2A}) \) than in the Blank and NC groups. (B) In both EC9706 and KYSE30 cells, CCK8 assay results demonstrated that the survival rate of cells in the miR-499 group had
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The clonogenic survival fraction decreased with increased chemotherapy dose. The miR-499 group declined more significantly, and the decline has statistical differences with the Blank and NC groups at 4μM, 6μM, 8μM and 10μM of cisplatin (P < 0.05, Fig. 2C). There were no significant differences in clonogenic survival rate between the Blank and NC groups (P > 0.05, Fig. 2C).

The results of tumor growth assay are shown in Fig. 2C. The bioluminescence signal of the miR-499 group cells was relatively weaker than Blank and NC groups (P > 0.05, Fig. 2D).

However, there were no significant differences in bioluminescence signal between the Blank and NC groups. These results suggest that miR-499 enhanced the proliferation inhibition effect of cisplatin in EC9706 and KYSE30 cells.

**MiR-499 enhances cisplatin's apoptosis effect on EC9706 and KYSE30 cells**

Our flow cytometry results indicated no significant differences among the miR-499, NC and Blank groups without chemotherapy treatment (P > 0.05; Fig. 3). However, post treatment with 5μM or 10μM of cisplatin, the apoptosis levels of the miR-499 group increased much more significantly when compared to the NC and Blank groups (P < 0.05; Fig. 3).

**MiR-499 decreases the DNA repair capacity of EC9706 and KYSE30 cells**

Immunofluorescence microscopy assay was performed to observe the number of γ-H2AX foci of the different groups after chemotherapy. The immunofluorescence microscopy images of EC9706 and KYSE30 cells exhibit strongly enhanced γ-H2AX expression in the miR-499 group compared with the Blank and NC groups (Fig. 4A, 4B).
Discussion

EC is a major cause of cancer-related death worldwide. Each year, approximately 30 million people worldwide die from esophageal cancer. Although the diagnosis and treatment of EC have advanced, the disease progresses quickly and has a poor prognosis caused by invasion and early stage metastasis [34, 35]. Due to the potential difficulties an operation would impose on both the patient and the surgeon, most advanced patients choose to undergo palliative treatments instead. Chemotherapy is one of the main palliative treatments of EC. However, the clinical efficacy of chemotherapy is not satisfactory, as evidenced by a 5-year survival rate of only 10-30% and a local tumor uncontrolled rate and recurrence rate reaching up to 60-80% [36, 37]. Therefore, improving the efficacy of chemotherapy is the current focus of researchers. In recent years, some reports have found a variety of genes whose expression products can affect tumor chemotherapy; examples include cell cycle regulatory genes, apoptotic genes, and DNA damage repair proteins [38-40].

The DNA damage repair system exists in all cells, and has been demonstrated to be an important mechanism leading to the tumor cell’s resistance or insensitivity to chemotherapy. Polβ is a key enzyme in the DNA damage repair system. This system serves as a crucial factor in maintaining genome integrity and stability, in addition to modulating the chemotherapy sensitivity of tumor cells. Not surprisingly, polβ has become a research hot spot worldwide.

miRNAs have been estimated to regulate up to 30% of human genes and control a variety of cellular processes [15, 41]. Recent studies have shown that miRNAs are dysregulated in various cancers, and that their expression is relevant to a diverse array of tumors [42-45].

In this study, we conducted a variety of assays including the luciferase reporter, RT-PCR and western blot. Through these assays we found that miR-499 could bind to the putative binding sites within the polβ mRNA 3’UTR and thereby reduce the expression of polβ. We also discovered that miR-499 could inhibit the proliferation, induce the apoptosis and decrease, the DNA repair capacity of EC cell lines by targeting polβ. Furthermore, using the CCK-8,
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in colony-forming survival and tumor growth assays, we found that miR-499 could inhibit the proliferation of EC9706 and KYSE30 cells after chemotherapy. Flow cytometry assay further showed that miR-499 could induce apoptosis in EC cells post chemotherapy. In addition, the result of the immunofluorescence microscopy assay demonstrated that miR-499 suppressed the DNA repair capacity of EC cells after chemotherapy.

This study confirms our hypothesis that miR-499 could enhance the cisplatin sensitivity of EC cell lines by targeting polβ. These findings provide new insights on the future of esophageal cancer treatment. With this in mind, potential drugs could be developed with a focus on enhanced sensitivity of EC patients to chemotherapy.

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Disclosure Statement

The authors have declared that no competing interest exists.

References


