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Original Articles

MicroRNA-34a induces a senescence-like change via the down-regulation of SIRT1 and up-regulation of p53 protein in human esophageal squamous cancer cells with a wild-type p53 gene background

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ABSTRACT

MiR-34a has been reported as a non-coding RNA universally expressed in normal old cells and a probable suppressor of diverse cancer cells; however, this miRNA's expression and anti-tumor mechanism in esophageal squamous cancer cells (ESCC) remains unclear. We explored these questions in three human ESCC lines, KYSE-450, KYSE-410, and ECa-109, with wild-type p53 and mutant p53 backgrounds. Through a specific stem-loop RT primer for miR-34a, we examined the relevant expression level of miR-34a in these three cell lines using real-time reverse transcription PCR (qRT-PCR). We found that the expression level of miR-34a induced by the DNA damage agent adrmycin (ADR) was both p53- and time-dependent. Following incubation with miR-34a, cellular growth inhibition was exhibited differently in the three cell lines harbored with different p53 backgrounds. Furthermore, the MTT assay demonstrated an miR-34a-related cytotoxic effect in cell growth. Senescence-associated β-galactosidase (SA-β-Gal) staining was used to examine senescence-like phenotypes induced by miR-34a. Mechanistic investigation suggested that the down-regulation of Sirtuin1 (SIRT1) and up-regulation of p53/p21 contributed to the anti-tumor mechanism of miR-34a in wild-type p53 ECa-109 cells, while neither of the apoptosis-related proteins PARP and caspase-3 caused significant changes. In summary, our findings indicated that the intrinsic expression of miR-34a was relatively low and was expressed differently among different p53 backgrounds and ADR treatment times. The anti-tumor effect of miR-34a was primarily dependent on the regulation of SIRT1 and p53/p21 protein, not apoptosis-associated proteins.

Introduction

Esophageal carcinoma (EC), one of the most lethal human tumors, is the eighth most common cancer and the sixth most common cancer mortality cause worldwide [1]. In China, EC is characterized by the presence of esophageal squamous cancer cells. Although many patients with esophagus cancer undergo many different types of therapies, the long-term survival rate has not been significantly improved [2,3]. Therefore, there is a significant need for developing novel therapies to improve the outcomes in patients with EC.

It has been reported that miR-34a, an endogenous, small, non-coding RNA, plays pivotal roles in cellular senescence and tumor suppression [4,5]; however, the precise mechanisms of miR-34a in tumor suppression may differ in various types of cancers and have not been well-elucidated. For example, on the one hand, previous studies have shown that miR-34a is highly expressed in normal hearts and spleens, while it has low and different expression levels in diverse cancer cells [6]. On the other hand, miR-34a directly regulates the expression of such factors as Bcl-2 [7], CD44 [8], proteins in the E2F pathway [9,10], various cyclins and CDKS [11,12], SIRT1 (Sirtuin 1) [7,13,14], and PNUTS (phosphatase-1 nuclear targeting subunit) [6,15] to inhibit tumor cell growth, and this inhibitory effect may be p53-dependent or p53-independent in different cancers [13,16,17]. To date, relative evidence of miR-34a in esophagus cancer cells has not been well-documented.

In the present study, we aimed to explore the relative expression level, inhibitory effect and underlying mechanism of miR-34a

Abbreviations: EC, esophageal carcinoma; SA-β-Gal, senescence-associated β-galactosidase; SIRT1, Sirtuin1; ADR, adrmycin; PCR, polymerase chain reaction; PNUTS, phosphatase-1 nuclear targeting subunit; DMSO, dimethylsulfoxide.

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in esophageal squamous cancer cells (ESCC). Although it has been reported that p53 status may affect treatment effect for miR-34a in colon cancer [10], to the best of our knowledge, there have been no studies on the inhibitory roles of miR-34a in ESCC, especially for different p53 gene backgrounds; therefore, ESCC harbored with different backgrounds of p53 status, including KYSE-450 ($p53^{H179R}$), KYSE-410 ($p53^{R337C}$) and ECa-109 (wild-type p53), were the candidates for this study.

Materials and methods

Human esophagus squamous cancer cell lines

The human esophagus squamous cancer cell (ESCC) lines KYSE-450, KYSE-410 and ECa-109 were purchased from BoGu Biological Science and Technology Co., Ltd. in Shanghai, China. KYSE-450 and KYSE-410 cells are reported to harbor mutations in the *p53* gene, while ECa-109 contains wild-type *p53*. All three cell lines were cultured in RMPI medium 1640, which was supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg streptomycin. The cells were incubated at 37 °C in a humidified incubator with 5% CO_2.

ADR was purchased from Sigma and was dissolved in 2D water. For the expression experiments, KYSE-450, KYSE-410 and ECa-109 were seeded at 6×10^5 cells per 60-mm dish with 100 ng/ml ADR in the culture medium. The cells were collected, and total RNA was extracted at 0, 24, 48 and 72 h thereafter.

Isolation of RNA and real-time RT-PCR for miR-34a

Total RNA was extracted from the three cell lines using a Takara MiniBEST Universal RNA Extraction Kit (Takara, Dalian, China), according to the manufacturer's instructions, and quantified with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Complementary DNA was reverse-transcribed with a specific RT primer (miR-34a: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACACAACC-3′, U6 as an internal control: 5′-TGGTGTCGTGGAGTCG-3′). The real time polymerase chain reaction (PCR) primers for mature miR-34a or U6 were designed as follows: miR-34a F: 5′-GGTGTGGGCTGGCAGTGTCTT-3′ and R: 5′-CCAGTGCAGGGTCCGAGGTAT-3′; U6 F: 5′-CTCGCTTCGGCAGCACA-3′ and R: 5′-AACGCTTCACGAATTTGCGT-3′. Real-time PCR was performed with SYBR Premix Ex Taq™ (Tli RNaseH plus) (Takara, Dalian, China) using an Applied Bio-Rad CFX96 Sequence Detection system (Applied Biosystems). The expression level of miR-34a was defined from the threshold cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with reference to the expression of U6 small nuclear RNA.

Transient transfection of cells with miR-34a and cell proliferation assay

KYSE-450, KYSE-410 and ECa-109 were seeded at a density of 1.0×10^4 cells in 6-well plates for 24 h, and then the culture medium was changed and supplemented with 20 nM of either miR-34a (Takara, Dalian, China) or control miRNA premixed with HiPerfect transfection reagents according to manufacturer's instruction (Qiagen, Valencia, CA) 5–15 min in advance. Each condition was performed in triplicate. After transfection, the cells were observed under an inverted microscope and counted every day for four days. The average number of cells was determined at each time point in triplicate by trypan blue staining. The cells without any drugs in the culture medium were used as the blank controls.

Western blot assay

Each cell line was treated with miR-34a (20 nM) at 0, 24, 48, and 72 h. The cells were then collected, and proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China). Approximately 40 μg of protein was separated via 10% SDS–PAGE and electrophoretically transferred onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk in TBS-T (Tris-buffered saline/0.1% Tween-20, pH 7.4) at room temperature for 1 h and were incubated overnight with primary rabbit anti-human antibodies to p53 (1:1000), p21 (1:1000), SIRT-1 (1:1000) and β-actin (1:1000) (Abcam, USA). A horseradish peroxidase-conjugated antibody against rabbit IgG (1:5000, Abcam, USA) was used as a secondary antibody. The bands on the PVDF membrane were visualized with enhanced chemiluminescence substrates in an imaging apparatus.

SA-β-Gal staining for senescence-like cells

To observe whether miR-34a induced the senescence phenotype in the three cell lines, SA-β-Gal staining was used to examine cell growth changes. Four days after the three cells were treated with 20 nM miRNA (miR-34a or control miRNA), KYSE-450, KYSE-410 and ECa-109 were fixed and stained using an SA-β-Gal kit (Sigma, USA) for senescence-like cells according to the manufacturer's instructions. Then, cell morphology and staining were observed under an inverted microscope.

MTT assay for assessing cell growth treated with miR-34a

Cell viability was determined by the semi-automatic 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay according to the manufacturer's instructions. The cell growth inhibition rate curves for the three cell lines were constructed. KYSE-450, KYSE-410 and ECa-109 cells (5×10^3) were seeded in a 96-well microtiter plate and incubated overnight. Each cell line was treated with miR-34a in doses varying from 5 nM to 100 nM. After incubating for 72 h, the cells were incubated with MTT (0.5 mg/ml) for 4 h. The formazan precipitate was dissolved in 200 μl of dimethylsulfoxide (DMSO), and the absorbance at 490 nm was measured by a benchmark microplate reader (Bio-Rad, CA). At least three independent experiments were performed, and the averages were calculated. The cell growth inhibition rate was calculated with the equation: inhibition rate = $(OD_{control} - OD_{treatment})/OD_{control}$. The results were expressed as the percentage of inhibition relative to control cells (considered as 100%).

Statistical analysis

Statistically significant differences for continuous variables were determined using a one-way analysis of variance (ANOVA) with the least significant difference (LSD) test for the normally distribution data and the Kruskal–Wallis H test for the skewed distribution data. The chi-square test was used to determine the significance of the rate variables. All testing was performed using GraphPad Prism 5 software. A difference with a P value of less than 0.05 was considered to be significant.

Results

Induction of miR-34a in response to low-dose ADR in the three cancer cell lines

Endogenous expression levels of miR-34a in the three cell lines were analyzed at the indicated time points by quantitative real-time RT-PCR. The expression level of miR-34a was increased 3-fold or greater in ECa-109 cells after a 72-h incubation with ADR (100 ng/ml) compared to no ADR treatment's ($P < 0.01$), and miR-34a expression was increased in a time-dependent manner after ADR treatment. The other two types of esophageal squamous cancer cells, however, harbored mutant *p53* genes and exhibited no change in the expression of miR-34a (Fig. 1). These results suggest that the

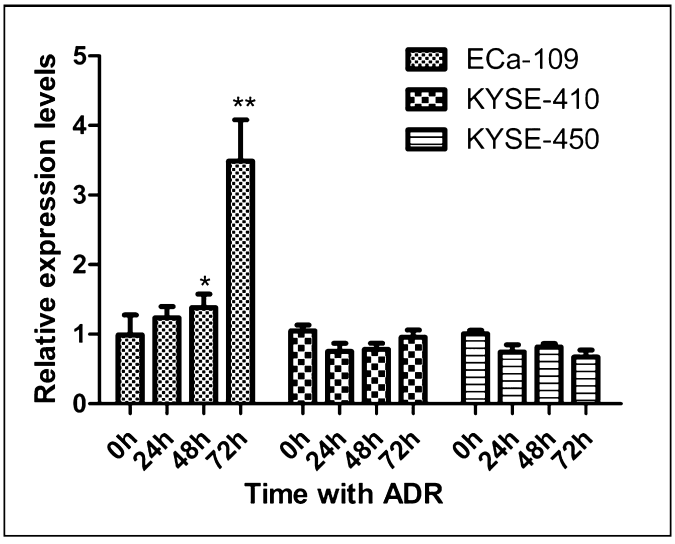

Fig. 1. Induction of miR-34a expression in three esophageal squamous cancer cell lines treated by the DNA damage agent ADR at different time points. KYSE-410, KYSE-450 and ECa-109 cells were incubated in the presence of ADR (100 ng/ml) for 0, 24, 48 and 72 h, and then relative expression levels of miR-34a were analyzed at the indicated time point using quantitative real-time RT-PCR. The differences between the expression level of miR-34a and time with ADR were significant at 48 h in ECa-109 (*$P < 0.05$), and the expression level of miR-34a at 72 h was increased 3-fold or greater compared with that at 0 h in ECa-109 (**$P < 0.01$). The variation was a time-dependent trend; however, miR-34a did not present similar changes after treatment with ADR in KYSE-410 and KYSE-450.