MicroRNAs Contribute to the Anticancer Effect of 1’-Acetoxychavicol Acetate in Human Head and Neck Squamous Cell Carcinoma Cell Line HN4

Haibin WANG,1,1 Li SHEN,2 Xinning LI,1 and Minglei SUN1

1Department of Oral and Maxillofacial Surgery, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China
2Nursing College, Zhengzhou University, Zhengzhou 450052, China

Received May 27, 2013; Accepted September 10, 2013; Online Publication, December 7, 2013 [doi:10.1271/bbb.130389]

1’-Acetoxychavicol acetate (ACA), extracted from rhizomes of tropical ginger, possesses antitumor properties against a wide variety of malignancies. MicroRNAs have been found to act as oncogenes and as tumor suppressor genes in the development of cancer. The purpose of this study was to investigate the miRNA involved in the molecular mechanisms of ACA action on tumor inhibition. It was found that ACA significantly inhibited the growth of human head and neck squamous cell carcinoma cell line HN4 and induced cell apoptosis. Further studies indicated that ACA downregulated the expression of miR-23a in HN4 cells. Transfection with anti-miR-23a inhibited the proliferation of HN4 cells and induced cell apoptosis. In addition, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was confirmed to be the target of miR-23a. Taken together, our findings suggest that ACA might have anticancer effects against human head and neck cancer through downregulation of miR-23a, which can repress tumor suppressor PTEN.

Key words: 1’-acetoxychavicol acetate; head and neck squamous cell carcinoma; microRNA-23a; PTEN

Head and neck squamous cell carcinoma (HNSCC), with a low 5-year survival rate, is the fifth most common malignant tumor worldwide.1,2 Combination chemotherapy is one of the therapeutic approaches to head and neck squamous cell carcinoma commonly used, but it is limited by severe side effects, complications, and resistance. Hence, novel effective and safe therapeutic agent are sought.

1’-Acetoxychavicol acetate (ACA), extracted from rhizomes of Zingiberaceae (Languas galang and Alpinia galanga), is associated with diverse medicinal properties, including anti-allergic,3 anti-inflammatory,4 anti-ulceration,5 antioxidant and anti-tumor activities.5,7 There is a wealth of experimental evidence suggesting that ACA possesses antitumor properties against a wide variety of malignancies.8 Previous studies have found that ACA suppressed carcinogenesis chemically induced, including oral,9 skin,10 colon,11 cholangio and esophageal carcinogenesis in rodent models.12,13 Some of the proposed mechanisms of ACA-induced tumor apoptosis include potentiation of a dual mitochondrial- and Fas-mediated caspase in myeloid leukemic cells and inhibition of nuclear factor-kappaB activation in multiple myeloma cells.14,15 Recent studies have revealed that ACA suppresses human prostate tumor angiogenesis and growth by targeting the Src-FAK-Rho GTPase pathway.16 More recently, it was reported that it potentiates the cytotoxic effect of cisplatin (CDDP) in cervical cancer cells, and microRNAs (miRNAs) have been found to be significantly differentially expressed upon administration of ACA and/or CDDP.17

MicroRNAs are small, highly conserved, single-stranded non-coding RNA molecules. They regulate gene expression post-transcriptionally by binding to the 3’-untranslated region of mRNA, which is then translationally repressed or directly degraded.18,19 Recent studies have found that miRNAs play important roles in human carcinogenesis,20–22 including head and neck carcinogenesis.23,24

In the present study, we measured the alteration of miRNA expression in human head and neck squamous cell carcinoma cell line HN4 after ACA treatment. In addition, we investigated the potential relationship between miR-23a and the expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN). Our results suggest that miR-23a plays an important role in the anti-cancer effects of ACA on HN4 cells.

Materials and Methods

Cell culture. Human head and neck squamous cell carcinoma cell line HN4 was acquired from the Oral Oncology Laboratory of the Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). Both HN4 and 293T cell line were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen).

Cell viability assay. The growth inhibitory effect of ACA (D.L-1’-acetoxychavicol acetate, 99.3% purity, LKT Laboratories, St. Paul, MN) on HN4 cells was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (1 × 104) were seeded in each well in a 96-well plate and incubated for 24 h. ACA was added in a dilution series of 0.977, 1.953, 3.9, 7.81, 15.625, 31.25, 62.5, 125, 250 μM. After treatment for 24 h, 20 μL of MTT (Amresco, Solon, OH) per well was added to 100 μL of culture medium and the plates were incubated for 4 h. Then the culture medium with MTT was discarded. DMSO (150 μL/well) was added to the plates and this was mixed vibrationally. The absorbance was measured at 570 nm by microplate reader. IC50 value (the concentration causing 50% cell growth inhibition) was calculated with GraphPad Prism 5 software. Each experiment was performed independently three times.

1 To whom correspondence should be addressed. Fax: +86-371-66970906; E-mail: hbwang@zzu.edu.cn
Quantitative real-time PCR for miRNA and mRNA. For miRNAs, total RNA was isolated from ACA-treated and untreated HN4 cells with Trizol reagent (Invitrogen) and a miRcute miRNA extraction kit (TIANGEN, Beijing, China). Quantitative real-time PCR of the mature miRNA was done by a miScript System (Qiagen, Gaithersburg, MD), which included specific primers for miRNAs, following the manufacturer’s instructions. The reverse transcription (RT) reaction system contained 4 μL of 5 × miScript RT Buffer, 1 μL of miScript Reverse Transcriptase Mix, and 15 μL of RNase-free water. The mixture was incubated for 60 min at 37°C and for 5 min at 95°C. The PCR reaction system contained 10 μL of SYBR Green PCR Master Mix, 2 μL of specific primer, 2 μL of miScript universal primer, 5 μL of RNase-free water and 1 μL of cDNA template. The procedure for PCR was as as follows: 95°C for 15 min, followed by 45 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. Each reaction was run in triplicate. The expression of PTEN mRNA was also detected by quantitative real-time PCR. PCR was done using the SYBR Premix Ex Taq (Takara, Japan), following the manufacturer’s instructions. U6 snRNA and GAPDH were used as internal controls for normalization of miRNA and mRNA expression respectively. The fold change for miRNA and mRNA was determined by the comparative threshold cycle (Ct) method.

Prediction of miRNA targets. Three miRNA target prediction programs, PicTar, miRanda, and TargetScan, were used to identify the targets of miR-23a.

miRNA transfection. HN4 cells were plated in 6-well plates (8 × 10^5 cells/well) for 24 h. The miR-23a mimic, miR-23a inhibitor and their controls were purchased from GenePharma Company (Shanghai, China). Transfection of miR-23a mimic, miR-23a inhibitor and their controls into HN4 cells was done with Lipofectamine 2000 (Invitrogen), following the manufacturer’s protocol. U6 snRNA and GAPDH were used as internal controls for normalization of miRNA and mRNA expression respectively. The fold change for miRNA and mRNA was determined by the comparative threshold cycle (Ct) method.

Western blot analysis. HN4 cells were lysed and total proteins were extracted. Proteins were separated by 10% SDS-PAGE and transferred electrophoretically to immobilon PVDF membranes (Millipore, Billerica, MA). The membranes were incubated with PTEN rabbit monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), poly(ADP-ribose) polymerase (PARP) rabbit monoclonal antibody (Abcam, Cambridge, UK), or β-actin antibody (Santa Cruz), and then with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Santa Cruz). Specific proteins were detected by chemiluminescence. Each band was quantified with Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, Maryland). All the experiments were performed in triplicate.

Reporter vector construction. A fragment of PTEN 3′ UTR containing the putative target site for miR-23a was designed as oligonucleotides, either as wild-type (wt) or as mutant by site-directed mutagenesis of the miR-23a binding site. The synthesized oligonucleotide sequences with flanking XhoI and NotI restriction enzyme cohesive ends were annealed and ligated into psiCHECK 2 Vector (Promega, Madison, WI). The sense oligonucleotide sequences were as follows:

wt 5′ tccagGCACGTGGATATTTTCTTCTTGGAATGTAAAGGTGCTGAAATgc 3′; mut 5′ tccagGCACGTGGATATTTTCTTCTTGGAATGTAAAGGTGCTGAAATgct gc 3′.

Luciferase reporter gene assay. 293T cells were seeded in a 12-well plate 24 h before transfection. PsiCHECK-PTEN-wt and psiCHECK-PTEN-mut plasmids (Life Tech, Carlsbad, CA) were co-transfected with 50 pmol/L final concentration of miR-23a mimics or negative controls with Lipofectamin 2000. After 48 h, the activities of firefly and Renilla luciferase were measured by the Dual-Luciferase reporter assay system (Promega, Madison, WI) as described by the manufacturer. Three independent experiments were done.

Flow cytometry analysis. HN4 cells were plated in culture dishes and incubated for 24 h. ACA was added at final concentrations of 7.81, 17.7, and 31.25 μM. After treatment for 24 h, cell apoptosis was measured with an Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA) following the manufacturer’s protocol. To evaluate the effects of miR-23a on HN4 cells, transfection of miR-23a mimic, miR-23a inhibitor and their negative controls into HN4 cells was performed in the presence and the absence of ACA for 24 h. Then cell apoptosis was analyzed by flow cytometry. Each experiment was performed in triplicate.

Statistical analysis. Numerical data were expressed as mean ± SD and compared by Student’s t-test. All statistical analyses were performed with software package SPSS11.0. Statistical significance was set at p < 0.05.

Results

ACA treatment downregulated miR-23a expression in HN4 cells
To evaluate miRNAs that expressed differently in the ACA-treated and untreated control cells, quantitative real-time PCR was performed for miR-23a, miR-26b, miR-148a, and miR-198. MiR-23a was downregulated and miR-26b was upregulated in the ACA treatment groups as compared to the controls. ACA did not significantly influence the expression of miR-148a or miR-198 in HN4 cells (Fig. 1). Based on the results as well as published reports that miR-23a is associated with certain types of tumors, we speculate that miR-23a plays a role in the anti-cancer effects of ACA.

Cell viability assay
The MTT assay revealed that ACA can significantly inhibit the growth of HN4 cells, and the inhibition ratio increased markedly in a dose-dependent manner. The IC50 of ACA at 24 h was 17.7 μM (Fig. 2). To assess the role of miR-23a in cell viability, HN4 cells were transfected with miR-23a inhibitor or a negative control

![miRNAs Contribute to the Anticancer Effect of ACA in HN4 Cells](image_url)

Fig. 1. Effect of ACA on MicroRNA Expression in HN4 Cells. HN4 cells were treated with 17.7 μM ACA for 24 h. Relative expression of the microRNA in the HN4 cells was evaluated by quantitative real-time PCR normalized to U6 snRNA. Each experiment was performed in triplicate. Data are expressed as mean ± SD.
for 48 h. As shown in Fig. 3, the absorbance of the miR-23a inhibitor group was lower than the negative control group, and the difference was statistically significant ($p < 0.05$). Our results indicate that anti-miR-23a inhibited the proliferation of HN4 cells.

**miR-23a mediated ACA induction of apoptosis in HN4 cells**

Cell apoptosis was analyzed by FCM. The results indicated that the percentages of apoptotic cells in the ACA-treated groups significantly increased with increasing concentrations of ACA compared to the control group (Fig. 4A). In addition, the expression of cleaved PARP, a marker of apoptosis, was analyzed with Western blot. As shown in Fig. 4B, the protein expression of cleaved PARP was increased by ACA in a dose-dependent manner compared to control.

To determine whether miR-23a played a role in ACA-induced apoptosis, the rate of apoptosis was detected by FCM in HN4 cells transfected with miR23a mimic, miR23a inhibitor and their controls in the presence and the absence of ACA. The results indicated that apoptosis increased in the cells transfected with miR-23a inhibitor compared to control (Fig. 5A). Furthermore, transfection with the miR-23a mimic attenuated ACA induction of apoptosis in HN4 cells (Fig. 5B).

**PTEN was a target of miR-23a**

Based on three miRNA databases, PicTar, miRanda, and TargetScan, targets of miR-23a were predicted. Among the candidate genes, PTEN, which contained the binding site of miR-23a in its 3' UTR, was chosen for further analysis because its function is known to be associated with apoptotic biological processes (Fig. 6A).

To confirm PTEN as a direct target of miR-23a, luciferase reporter assay was performed. As expected, luciferase activity was decreased in HN4 cells co-transfected with the psicheck-PTEN-wt vector and the miR-23a mimics as compared to control (Fig. 6B). On the other hand, co-transfected with psicheck-PTEN-mut vector and miR-23a mimics did not lead to reductions in luciferase activity in the HN4 cells. These results indicate that PTEN expression is directly inhibited by miR-23a.

**miR-23a regulated PTEN expression**

To investigate the interaction between miR-23a and PTEN, we examined PTEN expression by real-time PCR and Western blot. The results indicated that transfection with the miR-23a mimics led to significant reductions in PTEN mRNA and protein levels in the HN4 cells as compared to the negative controls (Fig. 7). After ACA treatment, miR-23a was downregulated, while the PTEN mRNA and protein levels increased in the HN4 cells, suggesting that ACA upregulates PTEN expression via downregulation of miR-23a (Fig. 8).

**Discussion**

Among the various molecular mechanisms associated with malignancy, dysregulation of miRNA has recently become an area of interest. MiRNAs can act as oncogenes or tumor suppressor genes in the development of cancer. Numerous studies have reported that ACA has potent growth-inhibitory and pro-apoptotic effects on a variety of tumor cell lines, without any adverse effects on normal cells.25) In the current study, we found that ACA significantly inhibited the growth of HN4 cells in a dose-dependent manner, and that the percentages of apoptotic cells in the ACA-treated groups increased significantly with increasing concentrations of ACA as compared to the control group. In addition, protein expression of apoptosis marker-cleaved PARP was increased by ACA in a concentration-dependent manner as compared to control. Since miRNAs are regarded as key regulators of gene expression in human cancers, we aimed to determine whether ACA would alter miRNA expression in human head and neck squamous carcinoma cells. Alteration of miRNA ex-
Fig. 4. Effect of ACA on the Apoptosis of HN4 Cells.

A. HN4 cells were treated with the indicated concentrations of ACA for 24 h. Percentages of apoptotic cells were analyzed by FCM. Each experiment was performed in triplicate. The right lower quadrant, which represents annexin V stained cells, indicates apoptotic rates. Data are expressed as mean ± SD. *p < 0.05; **p < 0.01 compared to the control group. B. HN4 cells were treated with the indicated concentrations of ACA for 24 h. The expression of cleaved PARP was detected by Western blot, and β-actin served as loading control. Data are expressed as mean ± SD (n = 3). *p < 0.05; **p < 0.01 compared to the control group.
pression after ACA treatment might reveal the molecular mechanism of ACA action in tumor inhibition.

Based on published reports on miRNAs associated with HNSCC, we chose miR-23a, miR-26b, miR-148a, and miR-198 for further study. The results obtained from the quantitative real-time PCR indicate that miR-23a was downregulated and miR-26b was upregulated in the ACA treatment groups as compared to control. Based on these results and published reports that miR-23a is associated with certain types of tumors, miR-23a was chosen for supplementary study. Anti-miR-23a significantly inhibited the growth of HN4 cells after transfection on MTT assay, and the cells transfected with miR-23a inhibitor had increased apoptotic rates as compared to control. Furthermore, transfection with the miR-23a mimic attenuated ACA induction of apoptosis in HN4 cells. This indicates that miR-23a might be involved in the mechanism of ACA-induced apoptosis in HN4 cells.

Fig. 5. Effect of MiR-23a on ACA-Induced Apoptosis in HN4 Cells.
A, Determination of apoptosis of HN4 cells after transfection with miR-23a inhibitor and with control by flow cytometry. Data are expressed as mean ± SD (n = 3). *p < 0.05 compared to the negative control group. B, HN4 cells were transfected with miR-23a mimic or negative control, and after 48 h 17.7 μM ACA was added. Apoptosis of HN4 cells after the various treatments were analyzed by FCM. Data are expressed as mean ± SD (n = 3). *p < 0.05 compared to those treated with ACA alone.

Fig. 6. Interaction of MiR-23a with the 3’ UTR of PTEN mRNA.
A, TargetScan predicts the pairing of miR-23a with the target region in PTEN 3’ UTR. B, Luciferase activity assay of 293T cells co-transfected with luciferase reporter vector containing wt-PTEN-3’ UTR or mut-PTEN-3’ UTR and miR-23a mimic compared to those co-transfected with negative control. Data shown are luciferase activity (mean ± SD, n = 3). **p < 0.01 compared to negative control.
human head and neck squamous carcinoma cells. Several studies have indicated that miR-26b acts as a tumor suppressor in some types of tumors through different target genes. In this study, ACA upregulated the expression of miR-26b in HN4 cells. We assume that miR-26b plays roles in the anti-cancer effects of ACA. This should be elucidated in further studies. It was recently reported that ACA synergistically potentiates the cytotoxic effects of CDDP through alteration of specific miRNAs in cervical carcinoma cells. This may help to delineate the molecular mechanism of ACA antitumor action and indicate potential therapeutic strategies.

Although miR-23a belongs to the miR-23a/C24-27a/C24-24-2 cluster, it can be transcribed independently of it. Upregulation of miR-23a expression has been reported for various human tumors, including oral squamous cell carcinoma, glioblastoma, gastric adenocarcinoma, hepatocellular carcinoma and cholangiocarcinoma. It has been revealed that miR-23a functions as a growth promoting and anti-apoptotic factor in hepatocellular carcinoma cells and gastric adenocarcinoma cells. Recently, mir-23a was identified as a oncogenic miRNA in glioma cells. In the present study, the target genes of mir-23a were predicted by three algorithms (PicTar, miRanda, and TargetScan), and PTEN was chosen for further study. To test our hypothesis, we examined the regulatory effects of miR-23a on the expression of PTEN by real-time PCR and Western blot. The results indicated that overexpression of miR-23a led to significant reductions in PTEN mRNA and protein levels in the HN4 cells. Moreover, direct binding of miR-23a to the PTEN 3' UTR was confirmed by luciferase reporter assay, attesting that PTEN is a direct target of miR-23a. PTEN is tumor suppressor. Although PTEN activity and protein expression is reduced in most tumors, the rates of genetic mutation of PTEN are low. Therefore, the loss of PTEN activity or protein expression might occur through a transcriptional repression governed by an oncogene. In a recent study, Tbx3, which was upregulated in the tissue samples of HNSCC patients, repressed PTEN transcription. We found that mir-23a regulated PTEN. In our studies, ACA treatment downregulated miR-23a expression and increased the expression of the target gene PTEN in the HN4 cells, which indicates that ACA induces apoptosis and inhibits the growth of HN4 cells by upregulating PTEN expression via downregulation of miR-23a.
In conclusion, this study indicates that ACA can induce apoptosis and inhibit the growth of human head and neck squamous carcinoma HN4 cells by down-regulating the expression of miR-23a, which can directly target tumor suppressor PTEN. The findings can help to delineate the molecular mechanism of ACA action in tumor inhibition, and it forms a basis for further studies.

References