MicroRNA-124 inhibits proliferation, invasion, migration and epithelial-mesenchymal transition of cervical carcinoma cells by targeting astrocyte-elevated gene-1

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Received March 13, 2016; Accepted August 1, 2016

DOI: 10.3892/or.2016.5025

Abstract. MicroRNA-124 (miR-124) was reported to be attenuated in human cervical cancer (CC) specimens. However, its role in CC remains elusive. In the present study, quantitative real-time PCR (qRT-PCR) analysis demonstrated that miR-124 expression is significantly downregulated in human CC tissues and several CC cell lines. Transfection of miR-124 mimics in CC cell lines HeLa and SiHa markedly inhibits cell proliferative, migratory and invasive capacities, as well as the epithelial-mesenchymal transition (EMT) process in vitro. Further studies have identified astrocyte-elevated gene-1 (AEG-1) as a direct target gene of miR-124. miR-124 downregulated AEG-1 expression through interaction with its 3'-untranslated regions (3'-UTRs), and miR-124 expression was inversely correlated with AEG-1 levels in CC specimens. Moreover, exogenous overexpression of AEG-1 significantly rescued the miR-124-induced inhibition of cell proliferation, migration and invasion, as well as the EMT process in HeLa cells. In conclusion, these findings suggested that miR-124 was able to suppress cell proliferation, invasion and migration, as well as the EMT process in cervical carcinomas through directly targeting AEG-1. miR-124 and AEG-1 may be potential therapeutic targets for the treatment of cervical carcinoma.

Introduction

Cervical cancer (CC) is one of the most frequent gynecologic malignancies, accounting for significant morbidity and mortality in females worldwide (1). Tumor invasion and metastasis remain formidable obstacles to the effective treatment of this disease. Thus, research on the molecular mechanisms leading to invasion and metastatic dissemination of carcinoma cells are urgently needed for a more effective therapeutic strategy.

Astrocyte-elevated gene-1 (AEG-1), also known as metadherin (MTDH) and lysine-rich CEACAM1 co-isolate protein (LYRIC), is considered an important oncogene, whose expression is involved in carcinogenesis, tumor progression and chemotherapeutic resistance in several malignancies (2). Expression of AEG-1 is elevated in glioblastoma multiforme and contributes to tumor cell survival, proliferation and invasion (3). Overexpression of AEG-1 contributes to the neoplastic phenotype of bladder cancer cells by promoting survival, clonogenicity and migration (4). It is reported that AEG-1 is highly expressed in CC and associates with progression of cervical intraepithelial neoplasia and unfavorable prognosis (5,6). This suggests that AEG-1 plays a crucial role in the progression of CC. However, the mechanism of upregulation of AEG-1 in cervical cancer has not yet been fully clarified. MicroRNAs (miRNAs or miR) are endogenous non-coding small RNAs, many of which are abnormally expressed in various types of cancers and involved in tumorigenesis and tumour development. miR-124 is one of the most studied and best characterized miRNAs with pivotal roles in several malignant processes, including tumor proliferation, motility and angiogenesis (7). It has been reported that miR-124 promoter is heavily methylated in CC cell lines, thus contributing to its slight expression in human CC specimens (8,9). miR-124 was discovered to be involved in MALAT1-miR-124-RBG2 axis where it affects growth and invasion of high-risk human papillomavirus-positive CC cells (10). However, molecular mechanisms by which miR-124 achieve its effects in CC are still being elucidated.
Recent studies revealed that several miRNAs may serve as potential regulatory mechanisms of AEG-1 in human cancers, such as miR-26a (11), miR-137 (12) and miR-375 (13). Intriguingly, bioinformatic analysis showed that AEG-1 harbored a potential miR-124 binding site in its 3′-untranslated regions (3′-UTRs), suggesting that AEG-1 is a putative target of miR-124. In the present study, the expression of miR-124 was found to be inversely downregulated with the expression of AEG-1 in cervical carcinoma. Furthermore, it was shown that miR-124 can suppress CC cell proliferation, migration and invasion, as well as inhibit epithelial-mesenchymal transition (EMT) in cervical carcinoma through directly targeting AEG-1, suggesting that miR-124/AEG-1 axis has a pivotal role in the progression of CC.

Materials and methods

Ethics statements. The present study was conducted with the approval from the Ethics Committee of the Second Affiliated Hospital of Zhengzhou University. Written informed consent was obtained from all patients before study initiation.

Tissue specimens. Pair-matched tumor and adjacent normal cervical tissues from 18 CC patients were obtained from the Second Affiliated Hospital of Zhengzhou University, China. None of the patients had suffered from any other severe diseases that may affect the study. None of them received radiotherapy or chemotherapy before the tissues were obtained. All specimens were obtained during routine resection surgery, then immediately snap-frozen in liquid nitrogen, and stored at -80°C for further analysis.

Cell culture. The human CC cell lines (HeLa, SiHa, CaSKi and C33A) and normal cervical epithelial cells (End1/E6E7) were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). HeLa, SiHa and C33A cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), CaSKi cells were cultured in RPMI-1640 medium (both from Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen, Carlsbad, CA, USA). End1/E6E7 cells were cultured in KER-SFM medium (Gibco). All cells were fostered in a humidified atmosphere chamber containing 5% CO2 at 37°C.

Cell transfection. HeLa and SiHa cells were respectively seeded into 24-well plates (1x10⁴ cells/well) and grown to 70% confluency before transfection. miR-124 mimics (5′-GGCAUACACCUCUGGCCUUA-3′) and negative control mimics (NC; 5′-ACUACUGAGUGACGUA-3′) were synthesized by GenePharma (Shanghai, China) and transiently transfected with miR-124 mimic or NC. Cells were harvested after 72 h by trypsinization. Double staining with FITC-Annexin v and propidium iodide (PI) was carried out using the FITC-Annexin V apoptosis detection kit (BD Biosciences, Bedford, MA, USA). Cells were analyzed by flow cytometry.

Cell invasion and migration assays. Cell invasion and migration abilities were determined using an 8-µm-pore polycarbonate membrane Boyden chamber insert in a Transwell apparatus (Costar, Cambridge, MA, USA), with and without Matrigel (BD Biosciences), respectively. Cells were harvested and resuspended in serum-free medium after 48 h transfection. For the invasion assays, 1x10⁵ cells were prepared in 200 µl of serum-free RPMI-1640 medium then placed in the upper chamber coated with Matrigel. For the migration assays, 3x10⁴ cells were prepared in 200 µl of serum-free RPMI-1640 medium and seeded into the upper chamber of the Transwell. The lower chamber was filled with RPMI-1640 medium containing 10% FBS to induce chemotaxis. After incubation for 24 h at 37°C in a 5% CO2 incubator, the invaded or migrated cells were fixed with 100% methanol, stained in 0.5% crystal violet (Sigma), and counted under a microscope. The results were averaged over three independent experiments.

Xenograft tumor model. To evaluate in vivo tumorigenesis, a CC xenograft mouse model was used. Female BALB/c
athymic nude mice (4-6-weeks old) were purchased from the Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). All animal experiments were performed with the guide and approval of the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Zhengzhou University. HeLa cells (2x10⁶) stably transfected with miR-124 mimic or NC were injected subcutaneously into the flanks of nude mice (n=8). Once palpable tumors developed, tumor volume was measured and calculated using the formula: Volume = 0.5 x length x width². Thirty days after inoculation, the mice were euthanized and tumor weights were assessed.

Western blot analysis. Total proteins were extracted from CC samples or cells using RIPA buffer (Abcam, Cambridge, MA, USA). Protein concentrations were determined by BCA protein assay kit (Takara Bio Inc., Otsu, Japan). Twenty micrograms of protein was fractionated by 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The blots were probed with the corresponding primary antibodies specific for either AEG-1, E-cadherin, β-catenin, N-cadherin, vimentin, twist, matrix metalloproteinase-9 (MMP-9), aldo-keto reductase family 1 member C2 (AKR1C2), neurofibromin 1 (NF1) or GAPDH (Abcam). Thereafter, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (Abcam). Signals were visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, San Francisco, CA, USA), and analyzed using ImageJ software. Protein levels were determined by normalization to GAPDH.

Plasmid construction and luciferase assays. The wild-type 3’-UTR segment of AEG-1 was cloned downstream of the luciferase coding region in the psi-CHECK™ vectors (Promega, Madison, WI, USA) to generate luciferase reporter vector AEG-1 3’-UTR-wt. The corresponding mutant constructs (AEG-1 3’-UTR-mut) were created by mutating the seed regions of the miR-124-binding sites. Cells were plated into 24-well plates until 80% confluence before transfection. The wild-type or mutant AEG-1 3’-UTR plasmid was transiently co-transfected with miR-124 mimics or NC into HeLa cells. Cell lysates were harvested 24 h post-transfection and luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized by Renilla luciferase activity.

Statistical analysis. All statistical calculations were performed using SPSS software (version 19.0). Data were obtained from three repeated experiments and expressed as mean ± standard deviation (SD). Differences between groups were subjected to the Student’s-t test. Statistical significance was set at P<0.05.

Results

Downregulation of miR-124 in CC tissues and cell lines. Expression of miR-124 in CC tissues was determined and compared to that in the adjacent normal tissues. It was shown that miR-124 expression levels were significantly reduced in CC tissues (Fig. 1A; P<0.05). We further evaluated the expression of miR-124 in CC cell lines. The results showed that the expression levels of miR-124 in all four CC cell lines (HeLa, SiHa, CaSKi and C33A) were much lower than that in End1/E6E7, a normal cervical epithelial cell line (Fig. 1B; P<0.05). We next tested the association between miR-124 and clinicopathological characteristics of CC patients (Table I). Consequently, low expression of miR-124 correlated with advanced International Federation of Gynecology and Obstetrics (FIGO) stage, vascular invasion and lymph node metastasis.

Table I. Association between miR-124 expression and clinicopathological parameters in study patients.

<table>
<thead>
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<th>Expression of miR-124</th>
<th>Clinical parameters</th>
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<th>Low (n=8)</th>
<th>P-value</th>
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<td>3</td>
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</table>

When the expression level was higher than or equal to the average, it was defined as high expression; *P<0.05. HPv, human papilloma virus; FIGO, International Federation of Gynecology and Obstetrics; AEG-1, astrocyte-elevated gene-1.
ZHANG et al: miR-124 INHIBITS CERVICAL CANCER PROCESS BY TARGETING AEG-1

Figure 1. miR-124 expression is downregulated in cervical cancer. (A) Expression of miR-124 in each sample was quantitatively analyzed using RT-PCR and normalized with U6 mRNA; *P<0.05, vs. normal tissues (normal). (B) Relative miR-124 expression levels in four cervical cancer lines (HeLa, SiHa, CaSKi and C33A) and a normal cervical epithelial cell line (End1/E6E7) were determined with RT-PCR; *P<0.05, vs. End1/E6E7.

Figure 2. Overexpression of miR-124 suppresses cervical cancer progression. HeLa and SiHa cells were transiently transfected with miR-124-mimic (miR-124) or negative control mimic (NC). (A) Overexpression of miR-124 in HeLa and SiHa cells was validated using RT-PCR. (B) Cell growth curve of HeLa and SiHa cells was determined by CCK-8 assay. (C) Changes of apoptosis in HeLa and SiHa cells was determined by flow cytometric assay. (D and E) Migration and invasion abilities of HeLa and SiHa cells were measured using Transwell migration assay and Transwell invasion assay, respectively. (F) and (G) In vivo tumorigenesis assay was used to measure tumor volume (F) and tumor weight (G). (H) Expression of EMT markers and MMP-9 in HeLa and SiHa cells were evaluated by western blotting; *P<0.05, vs. NC.
These results showed that the expression of miR-124 was downregulated in human CC tissues and cells, and miR-124 may be involved in the progression of cervical carcinoma.

**Exogenous overexpression of miR-124 suppresses the proliferation, migration and invasion of CC cells.** To investigate the role of miR-124 in cervical carcinogenesis, miR-124 mimics or NC were transfected into HeLa and SiHa cells (Fig. 2A), and the biologic behavior of the cells were subsequently characterized. Cell growth curves indicated that upregulation of miR-124 significantly suppressed cell proliferation (Fig. 2B; P<0.05). Transfection of miR-124 leads to induction of apoptosis in both HeLa and SiHa cells (Fig. 2C). Overexpression of miR-124 significantly decreased the migratory and invasive capacities of HeLa and SiHa cells (Fig. 2D and E; P<0.05). Xenografted tumors in mice inoculated with miR-217 mimic infected HeLa cells grew much more slowly than those in mice inoculated with NC (Fig. 2F and G; P<0.05). These results confirm that miR-124 acts as a tumor-suppressor, and its upregulation inhibits cell proliferation, migration and invasion in CC cell lines.

**Exogenous overexpression of miR-124 inhibits EMT of CC cells.** As epithelial-mesenchymal transition (EMT) is thought to be critical for cancer cell invasion and metastasis, we next examined the expression of several EMT-related molecules (lower E-cadherin and β-catenin, higher N-cadherin, twist and vimentin), and metastasis-related molecules (higher MMP-9) to determine whether miR-124 is involved in regulation of EMT in cervical carcinomas. In both HeLa and the SiHa cells, miR-124 overexpression increased the expression level of E-cadherin and β-catenin. Simultaneously, protein levels of N-cadherin, twist, vimentin and MMP-9 showed robust downregulation in both HeLa and SiHa cells (Fig. 2H). These results indicate that exogenous overexpression of miR-124 in CC cells partially represses EMT in cervical carcinomas.

**miR-124 targets AEG-1 3’-UTR and downregulates its expression in cervical carcinomas.** Bioinformatics analysis using miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/index.php) and TargetScan 7.0 (http://www.targetscan.org/vert_70/) identified that AEG-1 harbors a potential miR-124 binding site (Fig. 3A). To verify whether AEG-1 is the direct target of miR-124, luciferase reporter vectors that contain the putative miR-124 binding sites within AEG-1 3’-UTR were constructed. The overexpression of miR-124 significantly suppressed the luciferase activity of AEG-1 containing a wild-type 3’-UTR, but did not suppress activity of AEG-1 with a mutant 3’-UTR (Fig. 3B; P<0.05). Transfection of miR-124 mimic led to a significant decrease of AEG-1 mRNA and protein expression in HeLa and SiHa cells (Fig. 3C and D; P<0.05). To further determine whether miR-124-mediated repression of AEG-1 is of clinical relevance, we measured the expression of miR-124 and AEG-1 in CC specimens and pair-matched normal tissues.

Figure 3. AEG-1 is a functional target for miR-124 in cervical cancer. (A) miR-124 and its putative binding sequences in the 3’-UTR of AEG-1. Mutations were generated in the complementary site that binds to the seed region of miR-124. (B) Luciferase reporter assay of the interaction between the 3’-UTR of AEG-1 and miR-124 was validated in HeLa cells. (C and D) AEG-1 mRNA and protein expression in HeLa and SiHa cells transfected with miR-124-mimic (miR-124) or negative control mimic (NC) were detected by quantitative RT-PCR and western blot assay, respectively. (E and F) Quantitative RT-PCR and western blot analysis revealed significantly higher AEG-1 mRNA and protein expression in human CC specimens compared with normal tissues (normal). (G) Spearman’s correlation analysis showed that miR-124 expression was inversely correlated with AEG-1 mRNA expression in CC specimens; *P<0.05, vs. NC or normal.
In contrast to the significantly lower expression of miR-124, AEG-1 showed higher levels in CC specimens when compared with the normal group (Fig. 3E and F; P<0.05). Furthermore, a negative correlation between mRNA levels of AEG-1 and miR-124 was revealed by Spearman's correlation analysis (Fig. 3G). Together, these results suggest that AEG-1 is a target of miR-124 in cervical carcinomas.

Exogenous overexpression of AEG-1 partially reverses the inhibitory effects of miR-124 on CC cells. In order to investigate the contribution of AEG-1 to migration and invasion of human CC cells, AEG-1 was force-expressed along with miR-124 in HeLa and SiHa cells. Overexpression of AEG-1 partially nullified miR-124-mediated suppression of the proliferation, migration and invasion, and reduced miR-124-induced apoptosis in both HeLa and SiHa cells (Fig. 4A-D; P<0.05). The forced expression of AEG-1 increased the average weight of the miR-124 overexpressing tumors (Fig. 3E; P<0.05). Overexpression of AEG-1 partially restored miR-124-induced downregulation of N-cadherin, twist, vimentin and MMP-9, and decreased E-cadherin and β-catenin expression in miR-124 overexpressing cells; *P<0.05, vs. control; #P<0.05, vs. miR-124.
Collectively, our results demonstrated that miR-124 exerts suppressive effects on the progression of cervical carcinomas partly by targeting AEG-1.

**Discussion**

Numerous microRNAs (miRNAs) are known to play a key role in tumorigenesis due to their ability to act as either oncopgenes or tumor suppressor genes. Various miRNAs, such as miR-34a, are considered to be ideal targets for therapeutic intervention in certain types of cancers (14). In the present study, we focused on miR-124, which is normally expressed in a variety of cancer types (7,15,16). To date, evidence shows that miR-124 gene polymorphism is closely related to susceptibility to cervical cancer (CC) (17). In the present study, qRT-PCR analysis demonstrated that miR-124 was significantly down-regulated in CC tissue samples and cancer cell lines. This finding was also verified by a study suggesting that abnormal promoter methylation and downregulation of miR-124 is a common event during cervical carcinogenesis (9).

Recently, reports also indicated multiple functions of miRNAs in cervical carcinomas. For example, miR-506 acts as a tumor suppressor in cervical carcinomas (18). The present study revealed that exogenous overexpression of miR-124 was able to markedly suppress cell proliferation and induce cell apoptosis in two CC cell lines. The introduction of miR-124 in HeLa and SiHa cells resulted in markedly decreased migratory and invasive behavior, consistent with previous studies showing that miR-124 inhibited the migration and invasion of ovarian cancer cells (19), and suppressed tumor growth and metastasis in nasopharyngeal carcinoma (20). Moreover, miR-124 overexpressed in CC cells exhibited an increasing E-cadherin and β-catenin expression, and decreasing N-cadherin, twist, vimentin and MMP-9 expression, indicating that miR-124 inhibits EMT and metastasis in CC cases. The above observations suggest that miR-124 acts as a tumor-suppressor in CC, and ectopic expression of miR-124 may be helpful for treatment of cervical carcinoma. However, the specific regulatory mechanism and signal transduction network are still poorly understood and need to be further explored.

Knockdown of AEG-1 inhibits migration and invasion in colon cancer, suppresses metastasis in liver cancer (21), and decreases protective autophagy and chemosensitization of cancer cells (22). Several miRNAs, such as miR-145 and miR-217, were found to play an important role in tumor occurrence and development by regulating the expression level of AEG-1 in human cancers (23,24). The present study revealed, for the first time, the involvement of miR-124 in tumorigenesis through targeting AEG-1 in cervical carcinoma. miR-124 downregulated AEG-1 expression through interaction with its 3'-UTR, and overexpression of miR-124 significantly suppressed AEG-1 expression in vitro. Accumulating studies have reported that AEG-1 is highly expressed in CC and is associated with cervical carcinoma progression and angiogenesis (5,25). In the present study, higher expression of AEG-1 was observed, and was inversely correlated with miR-124 levels in CC specimens. Furthermore, exogenous overexpression of AEG-1 reversed the inhibitory effects of miR-124 on EMT and metastasis in CC cells. In accordance with the effect of AEG-1 silencing (21), overexpression of miR-124 also results in downregulation of AKR1C2 and upregulation of NF1. Both AKR1C2 and NF1 were involved in the process of proliferation and migration in vitro and in vivo (26,27). Thus, the abnormal expression of AEG-1/AKR1C2/NF1 may contribute to the molecular mechanisms of inhibition of miR-124 on cell proliferation and migration in CC.

However, the present study did not clarify the specific mechanism by which AEG-1 affects EMT in CC. Both miR-124 and AEG-1 are involved in the EMT process though various signaling networks and effector targets, such as PI3K/Akt, NF-κB, Wnt/β-catenin and TGF-β pathways (28,29). According to existing research, PI3K/Akt and Wnt/β-catenin seems to be more critical in the EMT process in CC. A previous study showed that AEG-1 mediates CCL20/CCR6-induced EMT development via both ERK1/2 and Akt signaling pathways in CC (30). Another study demonstrated that AEG-1 promotes EMT via the Wnt signaling pathway in cervical squamous cell carcinoma (31). Previously it was suggested that overexpression of miR-124 significantly suppressed migration and invasion and the activity of non-canonical Wnt signaling in osteosarcoma cells (32), while AEG-1 can activate Wnt signaling to promote metastasis in tongue squamous cell carcinoma. Thus, miR-124 mediated downregulation of AEG-1 may regulate the EMT process mainly via the Wnt signaling in CC. The specific mechanism in this process remains to be elucidated in the future.

Taken together, our results indicated that miR-124 is significantly downregulated in cervical carcinoma. miR-124 may suppress CC cell proliferation, migration and invasion, as well as inhibit EMT and metastasis in cervical carcinoma through directly targeting AEG-1. Our findings suggest that the miR-124/AEG-1 link has considerable value as a potential therapeutic target in cervical carcinoma cases.

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ZHANG et al: miR-124 INHIBITS CERVICAL CANCER PROCESS BY TARGETING AEG-1


