Curcumin inhibits cervical cancer cell proliferation, migration and invasion by suppressing the PI3K/AKT signaling pathway *via* the miR-29b/KDM2A axis

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Abstract: In this study, we aimed to examine whether curcumin exerted its anti-tumor effects by regulating miR-29b/KDM2A in cervical cancer cells. The cell viability, migration and invasion were estimated in HeLa cervical cancer cells treated with curcumin. The effects of microRNA-29b (miR-29b) on biological behaviors of HeLa SiHa cells were also assessed. Potential target genes of miR-29b were predicted and confirmed using a luciferase reporter assay, and the effects of curcumin and miR-29b on the PI3K/AKT signaling pathway were analyzed. Curcumin treatment inhibited cell proliferation, migration and invasion of HeLa cells (P<0.05). The miR-29b expression was promoted by curcumin treatment in HeLa cells (P<0.01), and miR-29b depletion could restore the effects of curcumin on cell proliferation, migration and invasion of HeLa cells (P<0.05). KDM2A was proved as a direct target gene of miR-29b, and the activity of the PI3K/AKT signaling could be regulated by curcumin and miR-29b (P<0.05). All the data revealed that curcumin played a protective role in cervical cancer. The proliferation, migration and invasion of cervical cancer cells were inhibited by curcumin through the miR-29b/KDM2A/PI3K/AKT pathway.

Keywords: Curcumin; Proliferation; Migration; Invasion; MiR-29b; Cervical cancer

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1. Introduction

Cervical cancer is one of the most common malignancies diagnosed in females worldwide, with relative high rates of morbidity and mortality[1,2]. This malignancy has regional activity, which is prevalent in the developing countries, and it becomes the leading cause of cancer-related deaths in women[3]. It has been reported that genetic susceptibility, viral infection and some environmental factors are the risk factors, which contribute to the tumorigenesis of cervical cancer[4]. The mortality rate of cervical cancer has decreased in recent years due to the progresses in cancer diagnosis and therapeutic modalities, such as surgery, radiotherapy and chemotherapy[5]. Some chemotherapeutic drugs used for patients with cervical cancer present good response, especially in the cases with advanced disease. However, the overall survival of this malignancy remains low[6]. Therefore, it is of great importance to identify safe and efficient therapeutic strategies for cervical cancer treatment.

Curcumin refers to a phytochemical polyphenol extracted from rhizome of the turmeric Curcuma genus plant, Curcuma and turmeric. The chemical structure of curcumin is shown in Figure 1. It has been extensively studied and reported to be characterized by significant pharmacological effects, good tolerance and low toxicity[7]. Therefore, curcumin becomes one of the hottest research spots in various human diseases[8]. Accumulating evidence has revealed that curcumin plays a critical role during antioxidant, anti-inflammatory, anti-infection, anticoagulant, would-healing, atherosclerosis and anti-tumor activities[9–11]. Curcumin has been

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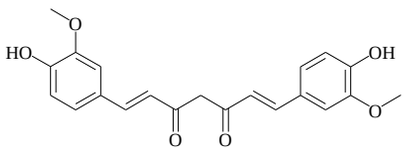

Figure 1. The structure of curcumin.

highlighted for its suppressive effects on cell proliferation, migration and invasion in different human cancers, including cervical cancer[12]. However, the mechanisms underlying the anti-tumor effects of curcumin remain largely unexplored.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules with critical regulatory function on gene expression at the post-transcriptional level[13]. It is generally considered that miRNAs are closely related with various biological processes, such as cell proliferation, differentiation, migration, invasion, cell cycle and apoptosis[14]. Emerging studies have reported that miRNAs are involved in tumor development through regulating some signaling pathways, such as PI3K/AKT, JAK/STAT and MAPK/ERK[15–17]. In recent studies, curcumin has been demonstrated to exert its anti-tumor activities by regulating miRNAs[18]. However, the effects of curcumin on miRNAs when it exhibits its inhibitory effects in cervical cancer remain unclear.

To improve the application of curcumin in clinical practices, the mechanisms underlying the anti-tumor effects of curcumin need to be analyzed for patients with cervical cancer. In the present study, we aimed to determine whether the inhibitory effects of curcumin on cell proliferation, migration and invasion were associated with aberrant expression of miR-29b and its related signaling pathway.

2. Materials and methods

2.1. Cell culture and curcumin treatment

Human cervical cancer lines HeLa and SiHa were purchased from American Type Culture Collection (ATCC, Manassas, VA). The normal cervical epithelial cells (NCECs) were collected from the normal cervical tissues. All the cells were maintained in DMEM (Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) at 37 ºC in a humidified incubator containing 5% CO_2. For curcumin treatment, HeLa cells were cultured overnight, and then treated with various curcumin concentrations (5, 10, 20 and 40 μM) for different time periods (12 and 24 h). The curcumin used was dissolved in DMSO (Sigma-Aldrich, USA).

2.2. Cell proliferation assay

To estimate the effects of curcumin on cell proliferation, MTT assay was performed in the present study. The cervical cells were seeded in 96-well plates at a cell density of 2×10^4 cells/well and cultured in a humidified incubator with 5% CO_2 at 37 ºC. Briefly, MTT (Sigma-Aldrich, USA, 0.5 mg/mL) was added into the wells, followed by incubation for 1 h. DMSO (Sigma-Aldrich, USA) was added to each well, followed by agitation for 10 min. The viable cells were counted by measuring the absorbance at 490 nm using an enzyme-linked immune monitor (Thermo Fisher Scientific).

2.3. Cell migration and invasion assay

The effects of curcumin on cell migration and invasion were examined using Transwell analysis. The wells without Matrigel were used for cell migration assay, and those with Matrigel were used for cell invasion analysis. The upper chambers were full of serum-free DMEM medium, and the lower chambers were filled with medium supplemented with 10% FBS. The cells were seeded into the upper chambers at a density of 2×10^4 cells/well and cultured in humidified incubator with 5% CO_2 at 37 ºC for 24 h. To evaluate the cell migration and invasion abilities, the cell number in the lower chambers was counted by an inverted microscope.

2.4. Cell transfection

MiR-29b mimic, miR-29b inhibitor and their corresponding negative controls (mimic-NC and inhibitor-NC) were synthesized in RiboBio (Guangzhou, China). MiR-29b expression in the cervical cancer cells was increased by transfection of miR-29b mimic, and it was decreased using miR-29b inhibitor. The transfection was carried out using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

2.5. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA, including miRNAs, in the cervical cancer cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The M200Pro Eliasa (Tecan Australia) was adopted to estimate the purity and concentration of RNA. Reverse transcription was conducted to synthesize cDNA from the purified RNA with a PrimeScript RT reagent Kit (TaKaRa).

To determine the expression levels of miR-29b, qRT-PCR was performed with a SYBR Premix Ex TaqTM II kit (TaKaRa) and the ViiATM 7 real-time fluorescent quantitative PCR system (American Life Tech). In all reactions, U6 was selected as the internal control with the primers as follows: F: 5′-CTCGCTTCGGCA-GCACATATACT-3′, R: 5′-ACGCTTCACGAATTTGC-GTGTC-3′. The primer sequences for miR-29b were as follows: F: 5′-GCGCTAGCACCATTTGAAAT-3′ and R: 5′-CAGTGCAGGGTCCGAGGT-3′. The final relative expression level of miR-29b was calculated with $2^{-\Delta\Delta Ct}$ method and normalized to U6.

2.6. Luciferase reporter assay

The potential target genes of miR-29b were predicted by TargetScan, and lysine (K)-specific demethylase 2A (KDM2A) was found to contain the complementary sequence in its 3′-UTR for miR-29b. To confirm the interaction between miR-29b and KDM2A, a luciferase reporter assay was conducted. The wild-type (WT) and mutant-type (MT) of the 3′-UTR of KDM2A were cloned into the firefly luciferase reporter vectors, and then separately co-transfected into HeLa cells with miR-29b mimic, inhibitor or the negative controls using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The luciferase activity was determined after 48-h incubation by a SecrePair Dual-Luciferase Reporter System (Promega Corporation, Mannheim, Germany).

2.7. Western blotting analysis

The cervical cancer cells were lysed, and the cell lysate was collected, and then centrifuged at 12 000 g at 4 ºC for 10 min. The concentration of the proteins was determined using a BCA protein assay kit (Sigma-Aldrich, USA). Equal amounts of proteins were subjected to 12% SDS-PAGE and subsequently transferred onto a PVDF membrane. The membrane was firstly blocked with TBS supplemented with 5% non-fat milk, followed by incubation with primary antibodies, including anti-PI3K (1:1500, Santa Cruz, CA, USA), anti-p-AKT (1:1000, Santa Cruz, CA, USA) and anti-AKT (1:1000, Santa Cruz, CA, USA). Subsequently, the blot was incubated with secondary antibodies at room temperature for 2 h. The immunoreactive bands were visualized by the enhanced chemiluminescence (Thermo Fisher Scientific, USA).

2.8. Statistical analysis

All statistical analyses were performed using SPSS 21.0 software (SPSS Inc., Chicago, IL) and GraphPad Prism 5.0 software (GraphPad Software, Inc., USA). Data used were expressed as mean±SD. The differences

were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA). A difference was considered statistically significant with a $P<0.05$.

3. Results

3.1. Curcumin suppresses proliferation of cervical cancer cells

The cervical cancer cells were treated with different concentrations of curcumin (5, 10, 20 and 40 μM) for different time periods (12, 24 and 36 h), and the proliferation of these cells was estimated using MTT assay. Figure 2 shows that the cell viability of HeLa and SiHa cells treated with 10, 20 or 40 μM for 12, 24 or 36 h was significantly suppressed in a dose- and time-dependent manner compared with the control group (all $P<0.05$).

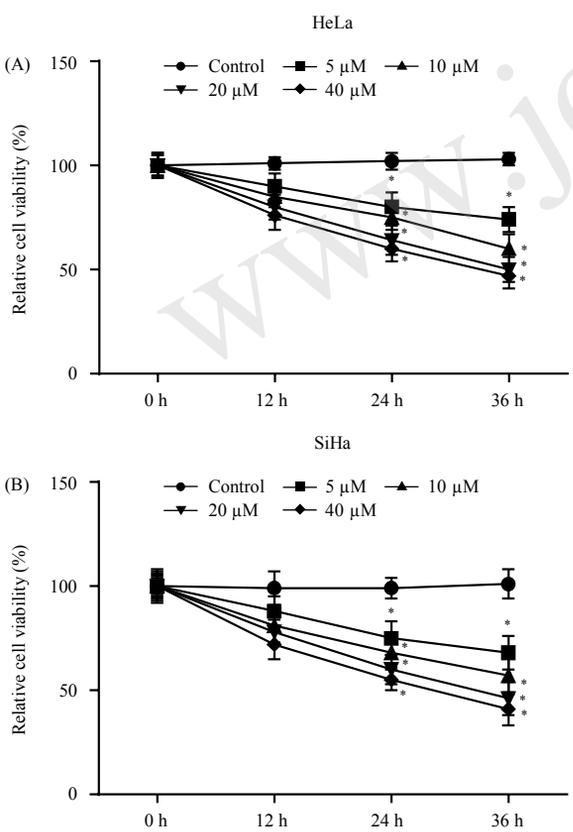

Figure 2. Effects of curcumin on cell proliferation of HeLa (A) and SiHa (B) cells ($^*P<0.05$).

3.2. Curcumin inhibits migration and invasion of cervical cancer cells

After the treatment with different concentrations of curcumin (5, 10, 20 and 40 μM) for 24 h, the cell migration and invasion of HeLa and SiHa cells were evaluated using Transwell analysis. Figure 3A shows that the migration ability of HeLa and SiHa cells was inhibited by curcumin in a dose-dependent manner compared with the control group (all $P<0.05$). Similarly, the invasion ability of the cervical cancer cells was also suppressed by curcumin in a dose-dependent manner (all $P<0.05$, Fig. 3B).

3.3. MiR-29b suppresses proliferation, migration and invasion of cervical cancer cells

MiR-29b expression in cervical cancer cells was measured by qRT-PCR, and the results revealed that the expression of miR-29b was decreased in both two cervical cell lines compared with the NCEC cells ($P<0.001$, Fig. 4A).

In order to investigate the effects of miR-29b on cell proliferation, migration and invasion of cervical cancer cells, miR-29b mimic was adopted to induce the expression of miR-29b in the cervical cancer cells. Figures 4B and 4C indicate that the miR-29b expression was markedly higher in the HeLa and SiHa cells transfected with miR-29b mimic than that in the control groups ($P<0.001$), suggesting that the cells over-expressing miR-29b were successfully established. Following the MTT and Transwell analyses, cell viability, migration and invasion were significantly suppressed by the up-regulated expression of miR-29b in both HeLa and SiHa cell lines (all $P<0.05$, Fig. 4D–4G).

3.4. MiR-29b expression is promoted by curcumin

To examine the effects of curcumin on the expression of miR-29b, the expression of miR-29b was determined

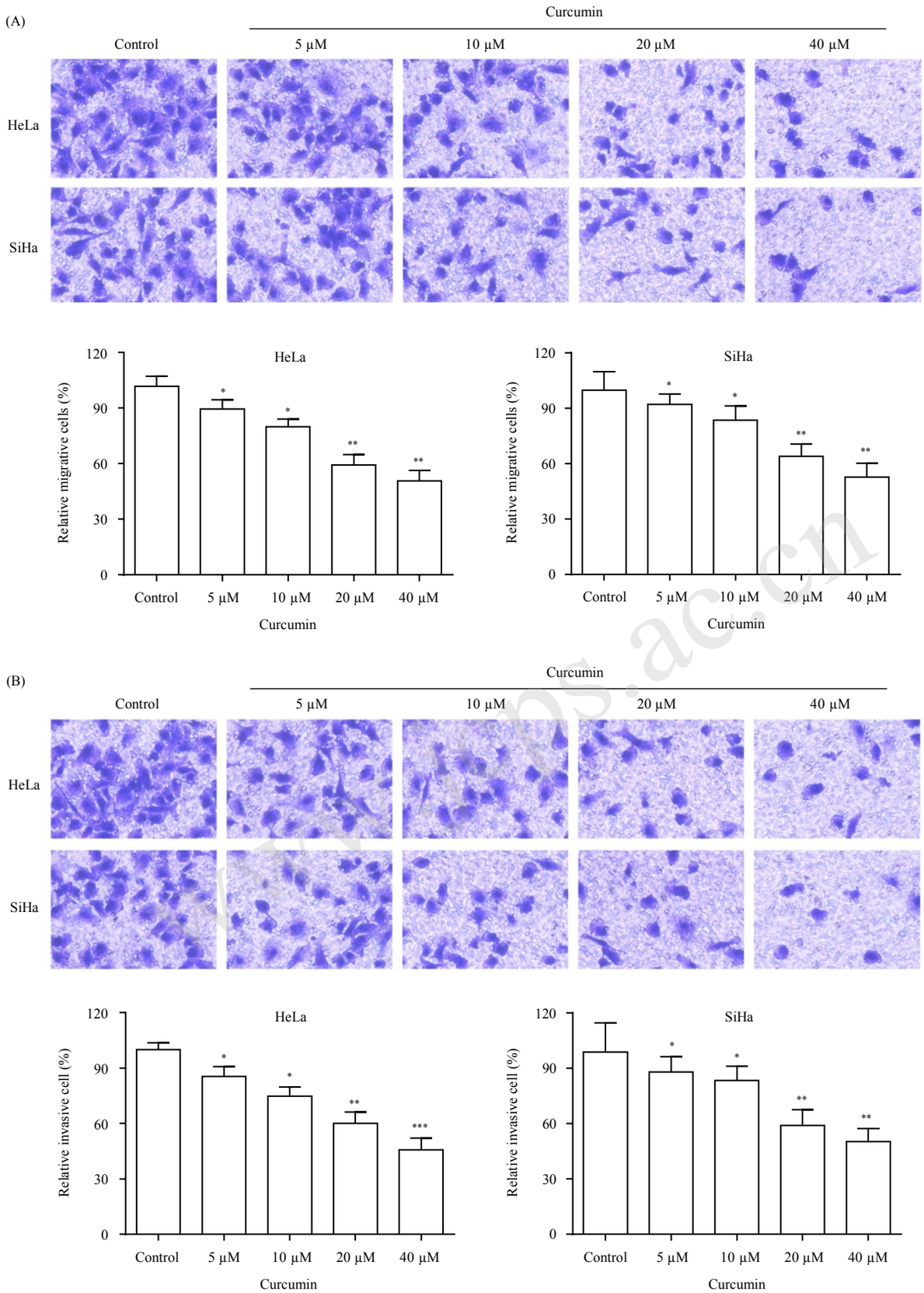

Figure 3. Effects of curcumin on cell migration and invasion of HeLa and SiHa cells. The abilities of migration (A) and invasion (B) of cervical cancer cells were inhibited by curcumin treatment ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

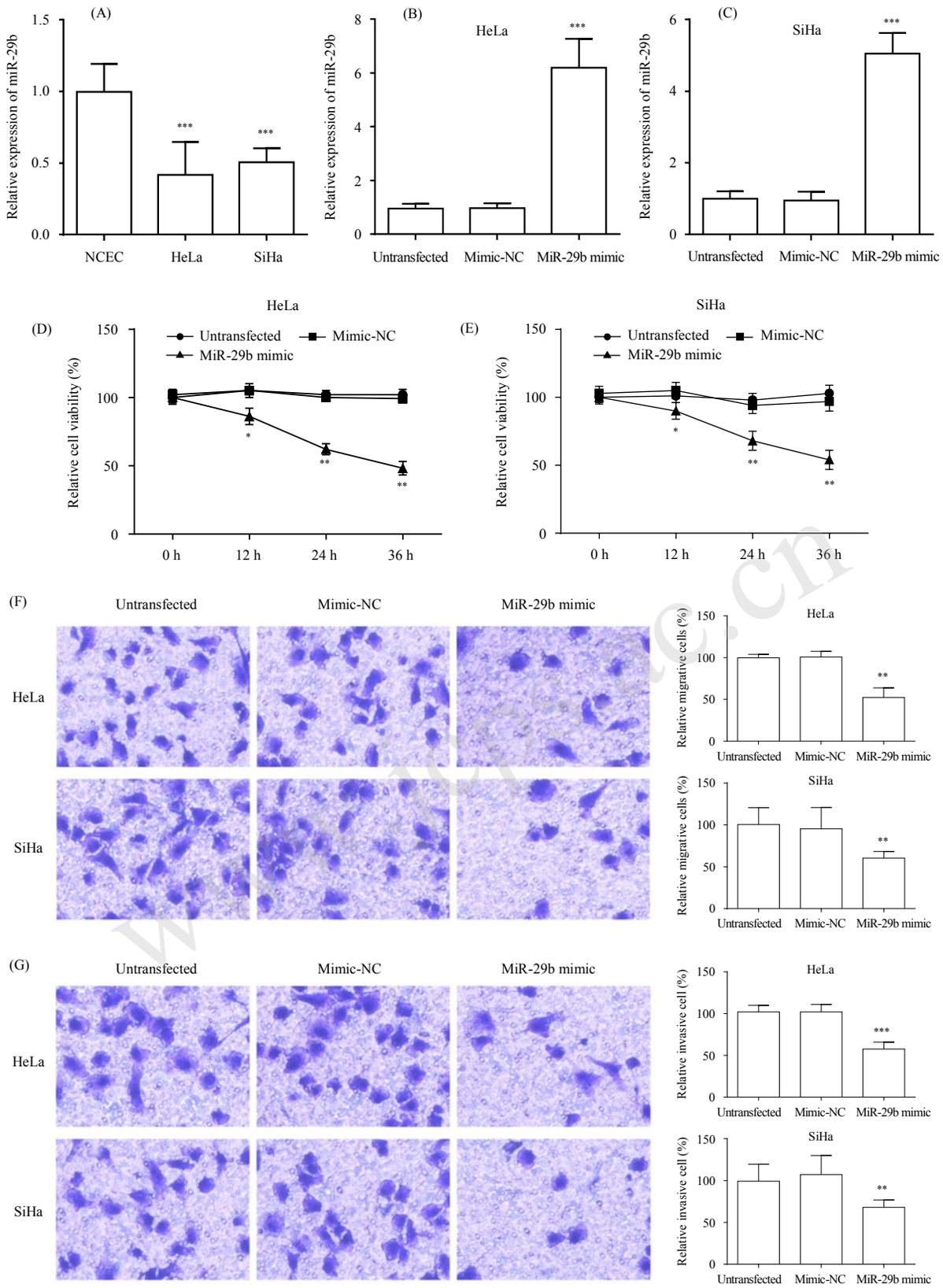

Figure 4. MiR-29b expression in HeLa and SiHa cells and its effects on cancer cell biological behaviors. (A) The expression of miR-29b was significantly down-regulated in HeLa and SiHa cells compared with the NCEC cells ($^{***}P$<0.001). (B) and (C) The miR-29b expression was successfully increased by using miR-29b mimic in HeLa and SiHa cells ($^{***}P$<0.001). (D)–(G) Cell proliferation (D and E), migration (F) and invasion (G) were all suppressed by over-expression of miR-29b in cervical cancer cells ($^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001).

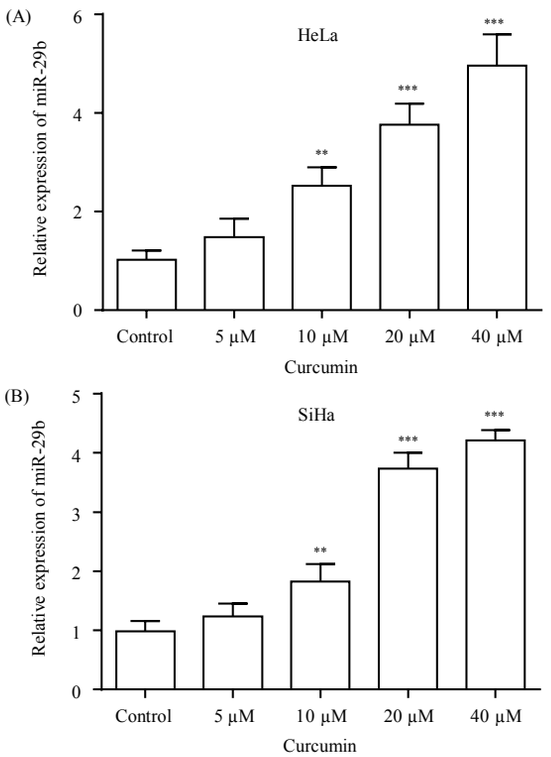

Figure 5. Curcumin treatment promotes the expression of miR-29b in HeLa (A) and SiHa (B) cells ($^{**}P$<0.01, $^{***}P$<0.001).

using qRT-PCR in both HeLa and SiHa cells following the treatment with different concentrations of curcumin (5, 10, 20 and 40 μM) for 24 h. The analysis results showed that the miR-29b expression was elevated in the cells treated with 10, 20 and 40 μM curcumin compared with the control group (all P<0.01, Fig. 5).

3.5. Depletion of miR-29b inhibits the effects of curcumin on cell proliferation, migration and invasion

To determine whether miR-29b was involved in the effects of curcumin on cell biological behaviors of HeLa cells, miR-29b inhibitor was transfected into the HeLa cells following the treatment with 20 μM curcumin for 24 h. Figures 6A and 6B show that the miR-29b expression was down-regulated using the miR-29b inhibitor in HeLa and SiHa cells compared with controls (P<0.01). By the *in vitro* regulation of miR-29b, the decreased cell proliferation, migration and invasion induced by curcumin treatment were all restored by the depletion of miR-29b (all P<0.05, Fig. 6C–6F).

3.6. KDM2A is a direct target gene of miR-29b

A complementary sequence of miR-29b was found in the 3'-UTR of KDM2A (Fig. 7A). By a luciferase reporter analysis, we found that the relative luciferase activity of the WT group was decreased by the over-expression of miR-29b, while it was increased by the depletion of miR-29b (all P<0.05, Fig. 7B). However, no significant change was observed in the luciferase activity in the MT group (all P>0.05).

3.7. Curcumin suppresses the PI3K/AKT pathway

It is generally considered that PI3K/AKT signaling pathway plays a critical role in the tumor development and progression in cervical cancer. Therefore, we investigated whether PI3K/AKT pathway was regulated by curcumin in HeLa cells. According to the Western blotting analysis, the protein expressions of PI3K and AKT as well as the phosphorylation of AKT were all suppressed following the treatment of curcumin (10, 20 and 40 μM) for 24 h (all P<0.05, Fig. 8A).

3.8. PI3K/AKT pathway is regulated by miR-29b expression

In order to confirm the role of miR-29b during the regulation of PI3K/AKT following the treatment of curcumin in HeLa cells, the key proteins were estimated in the cells transfected with miR-29b inhibitor. Figure 8B shows that the effects of curcumin (20 μM) on the protein expressions of PI3K and AKT as well as the phosphorylation of AKT were significantly declined by the reduction of miR-29b (all P<0.05).

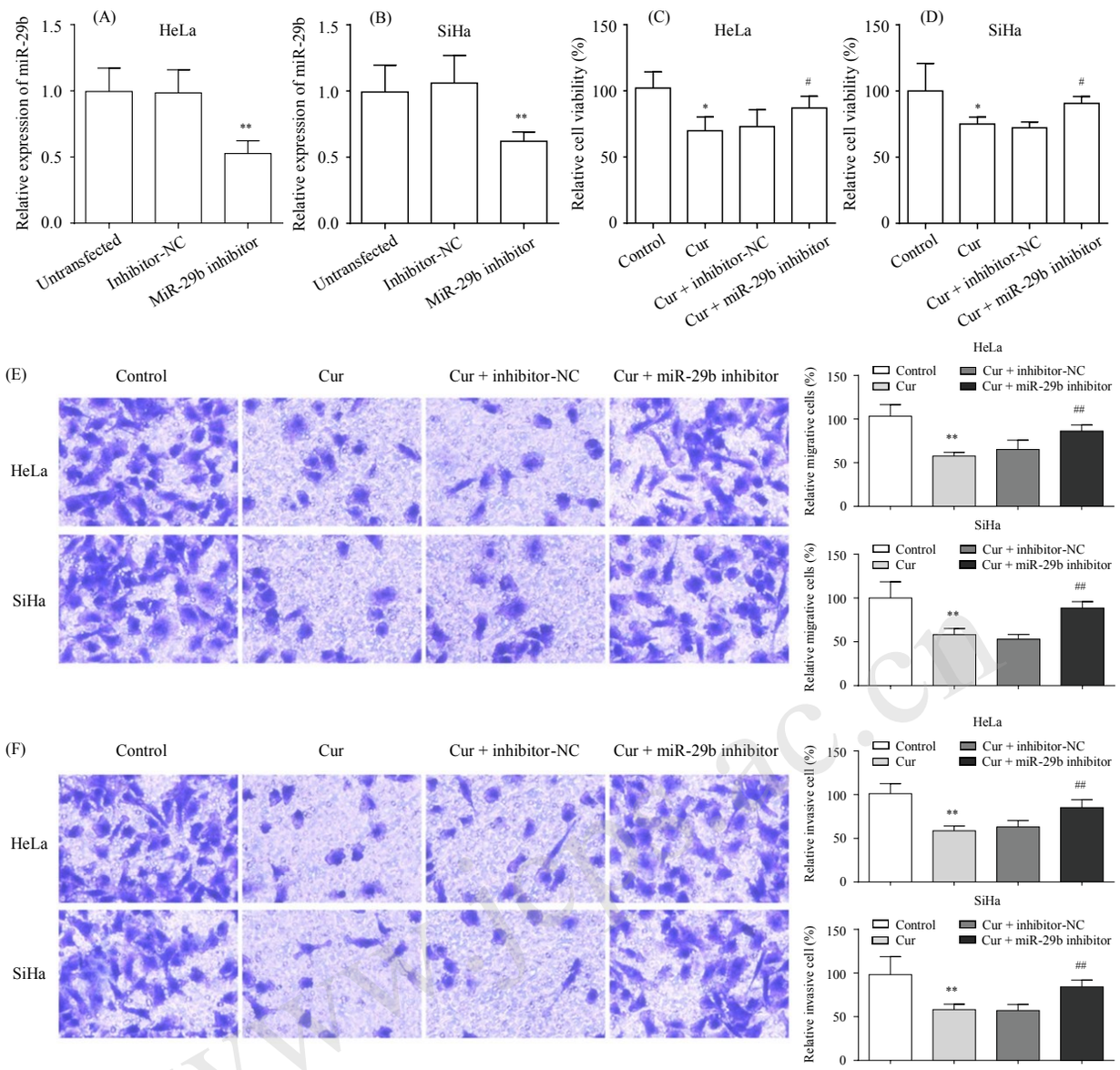

Figure 6. Effects of miR-29b on anti-tumor activity of curcumin. (A)and (B) MiR-29b expression was successfully decreased by using miR-29b inhibitor in HeLa and SiHa cells ($^{**}P$<0.01). (C)–(F) Depletion of miR-29b could restore the anti-effects of curcumin on cell viability (C and D), migration (E) and invasion (F) in HeLa and SiHa cells ($^{*}P$<0.05, $^{**}P$<0.01 compared with the control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared with the curcumin treatment).

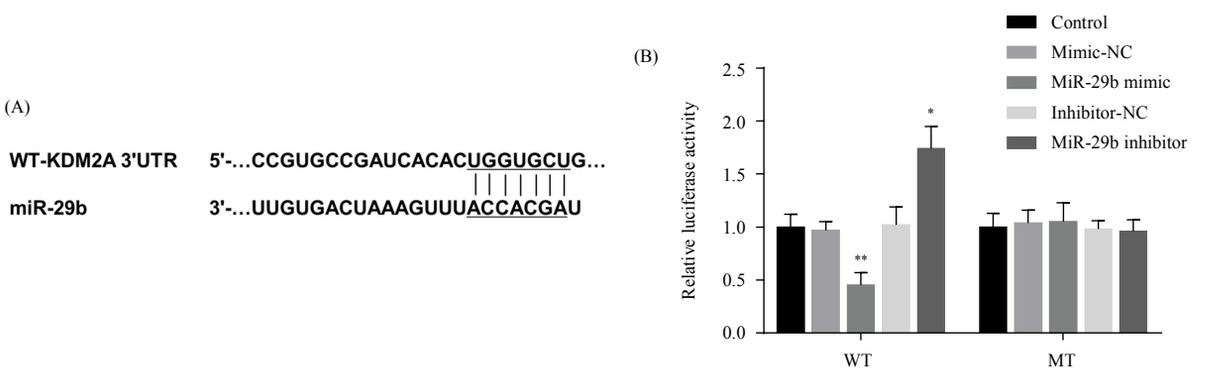

Figure 7. KDM2A was a direct target gene of miR-29b in HeLa cells. (A) A complementary sequence of miR-29b was found in the 3'-UTR of KDM2A. (B) Results of the luciferase reporter assay to confirm the interaction between miR-29b and KDM2A ($^{*}P$<0.05, $^{**}P$<0.01).

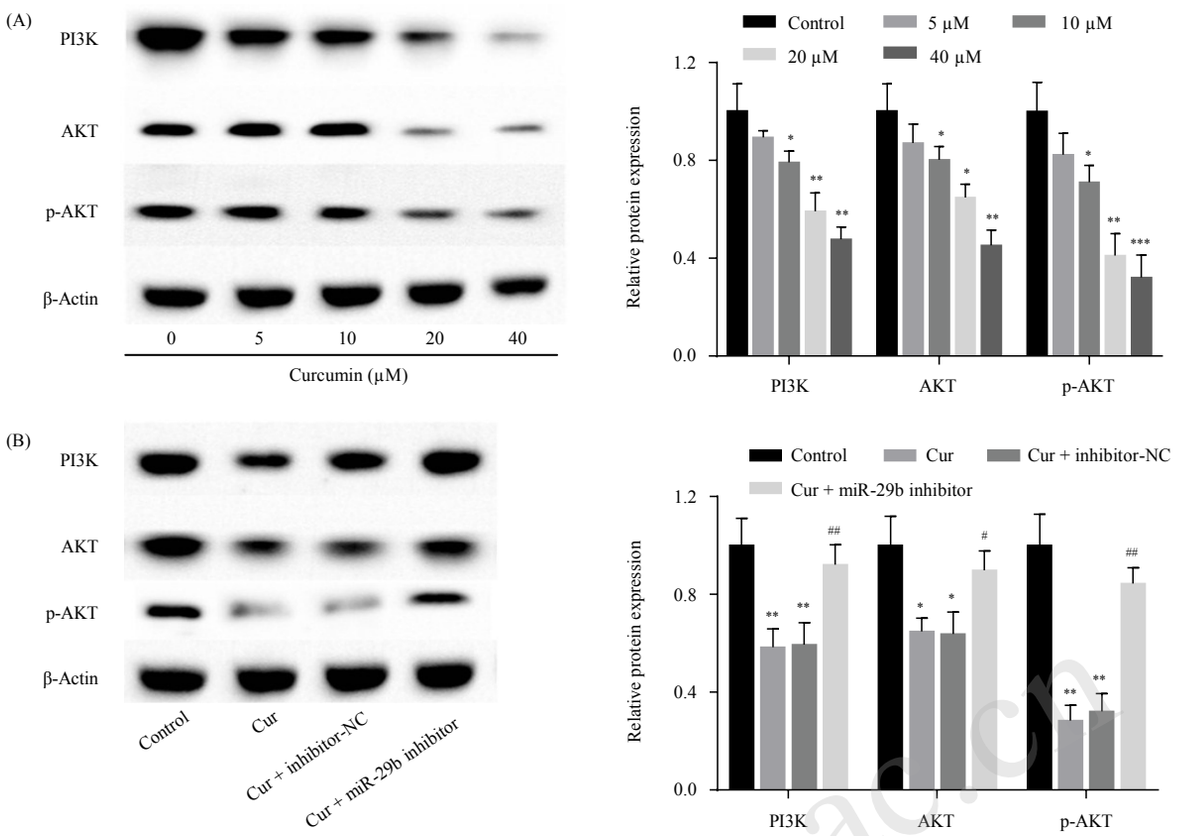

Figure 8. Effects of curcumin (A) and miR-29b (B) on the expressions of key proteins related to PI3K/AKT pathway ($^{*}P$<0.05, $^{**}P$<0.01 compared with the control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared with the curcumin treatment).

4. Discussion

Cervical cancer represents a serious healthy burden among women worldwide with the increased incidence and mortality. Although advances in various therapeutic strategies, the outcomes of cervical cancer remain not ideal[19]. Curcumin is generally considered to have significant anti-cancer effects on different human malignancies[20]. For example, a study scheduled by Lim et al.[21] has reported that the cell proliferation of colon cancer cells is markedly inhibited by curcumin through targeting cyclin-dependent kinase 2 (CDK2). Similarly, curcumin has been proved to suppress the proliferation of breast cancer cells by decreasing the expression of Flap endonuclease 1 (Fen 1) mediated by Nrf2[22]. Moreover, cell proliferation and invasion are suppressed by curcumin in non-small cell lung cancer *via* Wnt/β-catenin signaling pathway[23]. In cervical cancer, curcumin has also the anti-tumor effects by promoting cell apoptosis through induction of DNA damage[24]. In the current study, we investigated the effects of curcumin on biological behaviors of cervical cancer cells and found that cell proliferation, migration and invasion were significantly suppressed by curcumin in a dose- and time-dependent manner.

A growing body of evidence has demonstrated that the anti-tumor effects of curcumin are caused by regulating expressions of miRNAs. In the study by Li et al.[25], curcumin is found to suppress the proliferation of breast cancer cells by modulating miR-19 expression and PTEN/AKT/p53 pathway. MiR-192-5p is up-regulated during the anti-cancer effects of curcumin on cell

proliferation in human non-small cell lung cancer[26]. In addition, the inhibitory effects of curcumin on cell growth and invasion of pancreastic cancer cells are exerted through up-regulation of miR-7[27]. All these miRNAs involved in the anti-tumor effects of curcumin have been proved to participate in the tumor progression of human cancers. MiR-29b is an extensively investigated miRNA with down-regulated expression and inhibitory effects in diverse human malignancies, such as colorectal carcinoma[28], tongue squamous cell carcinoma[29] and breast cancer[30]. The observably down-regulated miR-29b has also been detected in cervical cancer, indicating its potential suppressive role[31]. Therefore, we aimed to examine whether curcumin suppressed cell progression by regulating the expression of miR-29b in the present study.

In the present study, we confirmed that the expression of miR-29b was significantly decreased in cervical cancer cells compared with the normal cells. The cell proliferation, migration and invasion were proved to be inhibited by over-expression of miR-29b, indicating that miR-29b acted as a potential tumor suppressor and could inhibit the tumor progression of cervical cancer. Furthermore, the effect of curcumin on expression of miR-29b was explored, and we found that the miR-29b expression was elevated in the cervical cancer cells by using curcumin. To further investigate whether miR-29b was involved in the anti-tumor effects of curcumin in cervical cancer cells, the miR-29b expression was silenced using miR-29b inhibitor. The decreased cell proliferation, migration and invasion caused by curcumin were restored by the depletion of miR-29b. The above-mentioned data suggested that curcumin exerted its anti-tumor effects by upregulating of miR-29b.

The tumor suppressive role of miR-29b has been reported in several other malignancies. For example, Liu et al.[32] have found that miR-29b is involved in the oxaliplatin-resistance of colorectal carcinoma through targeting SIRT1. In pancreatic cancer, miR-29b can suppress the proliferation, migration and invasion of tumor cells by targeting SOX12[33]. Another study by Kong et al.[34] has also demonstrated the inhibitory effects of miR-29b on gastric cancer cells by targeting KDM2A. KDM2A has been described as an oncogene by Ou et al.[35] in cervical cancer. However, it remains unclear whether miR-29b can regulate KDM2A in cervical cancer. In the present study, luciferase reporter assay indicated that KDM2A served as a target of miR-29b in cervical cancer cells, suggesting that miR-29b exerted its biological function in cervical cancer by targeting KDM2A. A recent study by Lu et al.[36] has indicated that KDM2A can facilitate the tumor progression of ovarian cancer by the regulation of the PI3K/AKT signaling. In addition, the PI3K/AKT signaling serves as a key role in the development and progression of various human cancers[37]. Many studies have reported that miRNAs are involved in the tumor progression by regulating the PI3K/AKT pathway in diverse cancers, including cervical cancer. For instance, the cell viability, metastasis and invasion of cervical cancer cells are suppressed by miR-383 through down-regulating the PI3K/AKT signaling pathway[38]. MiR-125b inhibits cell growth and enhances cell apoptosis of cervical cancer cells by targeting the PI3K/AKT/mTOR signaling pathway[39]. Additionally, PI3K/AKT pathway has also been demonstrated to be involved in the anti-tumor effects of curcumin in some cancers, such as thyroid cancer[40] and pancreatic cancer[41]. In this study, we found that the activity of PI3K/AKT signaling was decreased by the treatment of curcumin in cervical cancer cells, suggesting that PI3K/AKT signaling was involved in the anti-tumor effects of curcumin. Besides, the suppression of PI3K/AKT induced by curcumin was restored by inhibition of miR-29b in cervical cancer cells. Therefore, we considered that curcumin might exert its anti-tumor effects by inhibiting the PI3K/AKT mediated by miR-29b/KDM2A axis in cervical cancer.

In conclusion, the data of this study revealed the effects of curcumin on cell proliferation, migration and invasion of cervical cancer, and its anti-tumor effects might be performed by the regulation of miR-29b/ KDM2A/PI3K/AKT pathway. We considered that the molecules related to curcumin might be the potential targets for the treatment of cervical cancer. However, some limitations in the mechanisms were included in the present research, and further studies are needed to confirm the molecular mechanisms underlying the protective effects of curcumin.

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姜黄素通过miR-29b/KDM2A介导PI3K/AKT信号通路抑制宫颈癌细胞的侵袭和迁移

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摘要: 研究姜黄素通过调控miR-29b/KDM2A抑制宫颈癌细胞侵袭和迁移的作用机制。用姜黄素处理HeLa宫颈癌细胞, 观察细胞活力、细胞迁移和细胞侵袭情况。我们还评估了microRNA-29b (miR-29b)对HeLa SiHa细胞生物学行为的影响。利用荧光素酶报告基因实验预测和确认miR-29b的潜在靶基因,分析姜黄素和miR-29b对PI3K/AKT信号通路的影响。姜黄素对HeLa细胞的增殖、迁移和侵袭均有抑制作用(P<0.05)。在HeLa细胞中,姜黄素可促进miR-29b的表达(P<0.01),而miR-29b的下调可恢复姜黄素对HeLa细胞增殖、迁移和侵袭的影响(P<0.05)。KDM2A被证实是miR-29b的直接靶基因,而姜黄素和miR-29b可以调控PI3K/AKT信号通路的活性(P<0.05)。姜黄素对宫颈癌具有有效的保护作用。姜黄素可通过miR-29b/KDM2A/PI3K/AKT通路抑制宫颈癌细胞的增殖、迁移和侵袭。

关键词: 姜黄素; 扩散; 迁移; 入侵; MiR-29b; 宫颈癌