Elevation of miR-27b by HPV16 E7 inhibits PPARγ expression and promotes proliferation and invasion in cervical carcinoma cells

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Abstract. MicroRNAs (miRNAs) have been reported to be involved in multiple biological pathways that can influence tumor progression and metastasis. High-risk human papillomavirus (HR-HPVs) is aetiologically correlated to cervical cancer. Recently, miRNAs were reported to be regulated by virus and play pivotal roles in HPV-related tumor progression. However, the underlying mechanism remains poorly understood. In the present study, we report that HPV16 E7 upregulated miR-27b to promote proliferation and invasion in cervical cancer. The results showed that PPARγ, as a target of miR-27b, played a significant role in suppressing cervical cancer progression by downregulating the sodium-hydrogen exchanger isoform 1 (NHE1). It was also shown that the inhibition of miR-27b diminished the ability of HPV16 E7 to suppress PPARγ or activate NHE1 expression. In addition, we observed high expression of miR-27b and NHE1, but low expression of PPARγ in HPV16-positive cervical cancer tissues. In summary, the present study revealed that miR-27b is upregulated by HPV16 E7 to inhibit PPARγ expression and promotes proliferation and invasion in cervical carcinoma cells.

Introduction

Cervical cancer, with an estimated global incidence of 528,000 cases and approximately 266,000 deaths every year, is one of the most common types of cancer among women worldwide (1). It has been well recognized that persistent infection with high-risk human papillomaviruses (HR-HPVs), such as HPV16, HPV18 and HPV31, is the most important risk factor of cervical cancer (2). E6 and E7, two oncoproteins encoded by HR-HPVs, are believed to be crucial for the development and progression of cervical cancer. E6 deregulates host genes by inactivating the tumor suppressor p53, which serves as genome safeguard via inducing DNA repair, cell cycle arrest and apoptosis (3). E7 can destabilize another tumor suppressor RB1, to release the E2F family, leading to deregulation of cell cycle progression (4).

MicroRNAs (miRNAs) are a class of small non-coding endogenous RNAs of 21-25 nucleotides in size that modulate gene expression. Mature miRNAs may inhibit translation by interacting preferentially with 3'-untranslated region (3'-UTR) of target mRNAs (5). Recent studies show that miRNAs mediate important biological activities such as cellular proliferation, differentiation and apoptosis. Thus, dysregulated miRNA expression is linked to the development of a number of diseases, including human cancers (5,6). miRNAs can act as oncogenes or tumor suppressors by regulating different pathways (7-9). Accumulating evidence indicates that the expression of cellular miRNA are deregulated in response to virus infection (10,11). However, the function of miRNAs involved in virus-mediated cervical carcinogenesis remains largely undefined.

Peroxisome proliferator-activated receptor gamma (PPARγ) belongs to the nuclear receptor superfamily (12). It functions as a transcription factor or regulator of gene expression. Thus, PPARγ has been implicated to modulate various biological process and play a significant role in several diseases, such as obesity, diabetes, atherosclerosis and a variety of cancers (13). Due to the ability of promoting apoptosis and differentiation as well as inhibiting proliferation and growth, PPARγ is suggested to function as a tumor suppressor (14-17). Cervical cancer tissues, in particular, were found to express lower levels of PPARγ than normal cervical tissues (18). Therefore, PPARγ is considered to exert antitumor roles in cervical cancer. However, the detailed function of PPARγ in cervical cancer has not been well elucidated.

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A previous report indicated that the activation of PPARγ inhibits growth of breast cancer cells by repressing NHE1 expression (19). Na^+/H^+ exchanger isoform 1 (NHE1) is a pH regulator that mediate the electroneutral exchange of extracellular Na^+ for intracellular H^+ across the cell membrane (20). NHE1 activation results in cytosolic alkalinization, which is commonly regarded as an early phenotype to most carcinoma cells (20,21). It has been shown that HPV16 E7 stimulates NHE1 activity to alkalinize pHi in NIH3T3 cells (21). Although both HPV16 E7 and PPARγ have been implicated to be associated with NHE1 activity, the correlation between HPV16 E7, PPARγ and NHE1 has not been confirmed and the underlying mechanism remains unknown.

In the present study, we found that miR-27b was upregulated by HPV16 E7 to suppress the expression of PPARγ and increase the level of NHE1. Furthermore, we observed that miR-27b enhanced the proliferation and invasion of cervical cancer cells. Consequently, the HPV16 E7-miR-27b-PPARγ-NHE1 pathway is established and its role in HPV-related carcinogenesis in cervical cancer cells is shown.

Materials and methods

Tissue samples and cell lines. Clinical samples were obtained from six HPV16-positive cervical cancer patients treated at the Guangzhou Nanfang Hospital in China. All of the samples were collected with informed consent of patients and all of the experiments were approved by the Internal Review and Ethics Boards of Nanfang Hospital.

HPV16-positive human cervical carcinoma cell lines CaSki and SiHa, and HPV-negative cervical cancer cell line C33A, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). CaSki cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37˚C and 5% CO_2. SiHa and C33A cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% FBS at 37˚C and 5% CO_2.

Transfection. All siRNAs, hsa-miR-27b mimics and hsa-miR-27b inhibitors were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The full-length of HPV16 E6 and E7 cDNA was subcloned into the pEGFP vector (Invitrogen, Grand Island, NY, USA). The target sequences of siRNAs were as follows: siRNA-198 (22) targeting HPV16 E6 and E7: 5'-GCA CAC ACG UAG ACA UUC G-3'; PPARγ: 5'-GAG GGC GAT CTT GAC AGG AAA-3'. Cells were seeded into 6-well plates and grown to 50-60% confluence. Then cells were transfected with the respective siRNAs, plasmids, mimics or inhibitors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The RNA level was assessed by real-time PCR at 48 h after transfection and protein level was assayed by western blot analysis at 72 h after transfection.

RNA isolation and real-time PCR. Total RNA was extracted by TRIzol (Takara, Shiga, Japan) according to the manufacturer's instructions and then reverse transcribed using PrimeScript™ RT reagent kit (Takara) to generate cDNA. cDNA was amplified using SYBR® Premix Ex Taq™ (Takara). For real-time PCR analysis of HPV16 E6, HPV16 E7, PPARγ, NHE1 mRNAs, the following primers were used: PPARγ: forward, 5'-CTC TCC GTA ATG GAA GAC CAC T-3' and reverse, 5'-TCT GCA ACC ACT GGA TCT GTT C-3'; NHE1: forward, 5'-GCC TTC TCT CTG GGC TAC CT-3' and reverse, 5'-CTT GTC CTT CCA GTG GTG GT-3'; β-actin: forward, 5'-TGG CAC CCA GCA CAA TGA A-3' reverse, 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'. β-actin was used as a loading control. For real-time PCR analysis of hsa-miR-27b, cDNA was prepared from 2000 ng total RNA using All-in-One™ miRNA First-Strand cDNA Synthesis kit (GeneCopoeia, Guangzhou China) and was amplified using All-in-One™ miRNA qPCR kit (GeneCopoeia). U6 was used as miRNA reference gene. Primers for hsa-miR-27b were purchased as kits from GeneCopoeia. Each reaction was done in triplicate and $2^{-\Delta\Delta CT}$ method was used to calculate fold changes (23).

Western blot analysis. A total of 50 μg of total cellular protein was extracted by Lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), separated on 12% sodium dodecyl sulfate-polyacrylamide gels, transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4˚C with anti-PPARγ (1:1,000; Proteintech Group Inc., Chicago, IL, USA), anti-NHE1 (1:1,000; Proteintech Group Inc.) and anti-β-actin (1:1,000; Cell Signaling Technology, Danvers, MA, USA) and subsequently incubated with secondary anti-rabbit goat peroxidase antibody (1:10,000; Bioworld, Visalia, CA, USA). Protein was detected using ECL reagents (Thermo Fisher Scientific, Waltham, MA, USA). β-actin was used to demonstrate equal loading.

Cell proliferation assay. *In vitro* proliferation activities were measured by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 5,000 cells/well with 100 μl complete culture medium and allowed to adhere overnight prior to transfection as described above. At 24, 48, 72 and 96 h after transfection, the media was removed and cells were treated with 10% CCK-8 in basic media for 1-4 h at 37˚C. The absorbance of all wells was recorded at 450 nm. All samples were run in triplicate independently.

Transwell invasion assay. Cell invasion assay was conducted in 24-well plates using a Transwell invasion system (Corning, Corning, NY, USA) following the manufacturer's protocols. Approximately 200,000 cells in serum-free media were added into the top chamber, and bottom chamber was filled with media containing 30% FBS.

Statistical analysis. All statistical analyses were undertaken by one-way ANOVA or Student's t-test using Graphpad Prism 6.01. (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered statistically significant.

Results

HPV16 E7 promotes miR-27b expression in cervical cancer cell lines. In order to understand the way miRNAs are involved in HPV16 oncogene-induced carcinoma progression, we

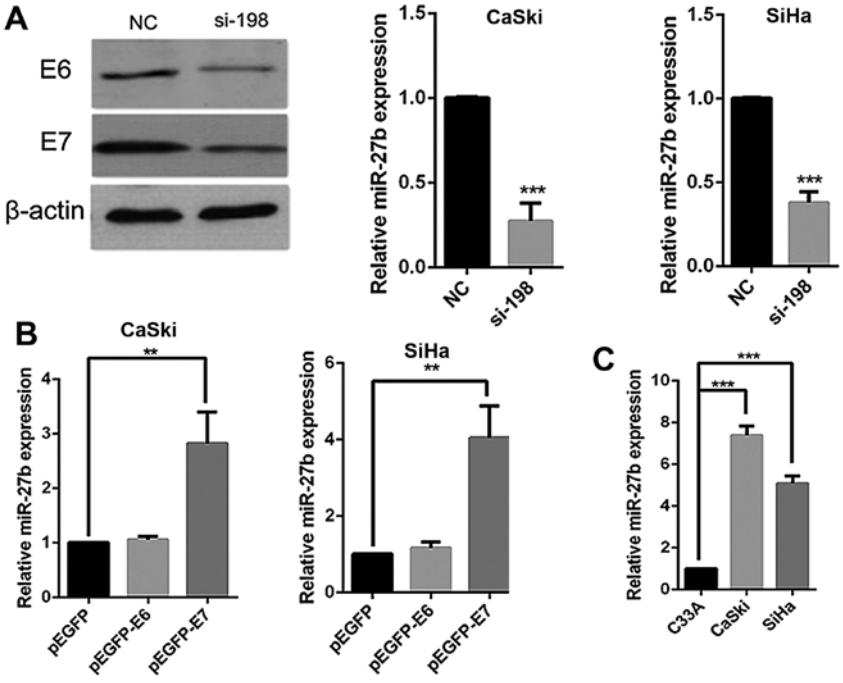

Figure 1. The effects of HPV-16 E7 on miR-27b expression in cervical cancer cells. (A) The Caski or SiHa cells were transiently transfected with siRNA-198 for 48 h. The expression of HPV16 E6 and E7 in CaSki cells was determined by western blot analysis. β-actin served as the loading control. miR-27b expression level in CaSki and SiHa cells was determined by real-time PCR analysis. (B) CaSki and SiHa cells were transfected with plasmids expressing HPV16 E6 or E7 and the expression of miR-27b was analyzed by real-time PCR. (C) The basal level of miR-27b in C33A, CaSki and SiHa cells was measured by real-time PCR. Values are presented as mean ± SD and were repeated three times with similar results (**P<0.0, ***P<0.001).

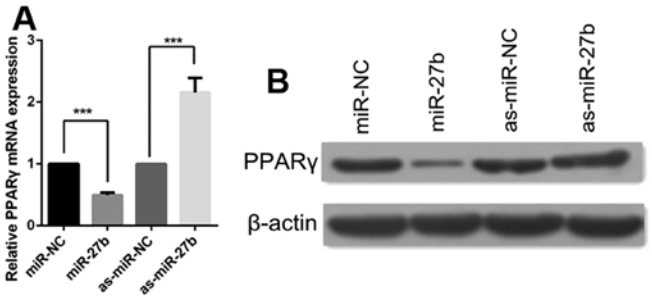

Figure 2. The effect of miR-27b on PPARγ in CaSki cells. The cells were transfected with miR-27b mimics (miR-27b) or inhibitors (as-miR-27b). The mRNA level of PPARγ was determined by real-time PCR (A) and PPARγ protein expression level was determined by western blot analysis (B). Values are presented as mean ± SD and were repeated three times with similar results (**P<0.01, ***P<0.001).

knocked down the expression of E6 and E7 using a reported siRNA-198 (22) in the HPV16-positive human cervical cancer cell line, CaSki, and then miRNA microarray analysis was used to search miRNAs regulated by HPV16 E6 and E7. The results showed that knockdown of HPV16 E6 and E7 led to significant downregulation of miR-27b in CaSki cells (data not shown), which was validated by real-time PCR (Fig. 1A). Similar results were also observed in the HPV16-positive cervical cancer cell line SiHa (Fig. 1A). We then transfected CaSki and SiHa cells with E6 or E7 plasmids to determine which viral oncogene regulated the expression of miR-27b. As shown in Fig. 1B, an increase of miR-27b was only observed in cells transfected with HPV16 E7 plasmids, not E6 plasmids, indicating that it was HPV16 E7 that induced miR-27b expression in cervical cancer cells. Furthermore, the basal levels of miR-27b in CaSki and SiHa cells were also higher than HPV-negative C33A cells (Fig. 1C), which was consistent with a recent report (24).

miR-27b represses PPARγ expression in cervical cancer cells. Given the fact that PPARγ was one of the direct targets of miR-27b in adipocytes and macrophages (25,26), we examined whether miR-27b also regulated endogenous PPARγ levels in cervical cancer by transfecting miR-27b mimics or inhibitors into CaSki cells. As shown in Fig. 2, the overexpression of miR-27b reduced both mRNA and protein levels of PPARγ. The opposite result was observed after inhibiting miR-27b expression. Together, these results suggest that PPARγ is also targeted by miR-27b in cervical cancer cells.

PPARγ inhibits tumor progression in cervical cancer cells. PPARγ has been shown in breast cancer cells to directly downregulate NHE1, which is a well characterized oncogenic pH regulator (19). To examine whether PPARγ also affected the expression of NHE1 in cervical cancer cells, CaSki cells were transfected with PPARγ siRNA, followed by the measurement of NHE1 mRNA and protein levels using RT-PCR and western blot analysis. The results indicated that the inhibition of PPARγ was able to increase both mRNA and protein expression level of NHE1 in cervical cancer cells (Fig. 3A). PPARγ has been reported to be involved in tumor cell proliferation and invasion (13). To investigate the effects of PPARγ on proliferation of cervical cancer cells, CaSki cells were transfected with siRNA targeting PPARγ and the changes in cell proliferation were analyzed

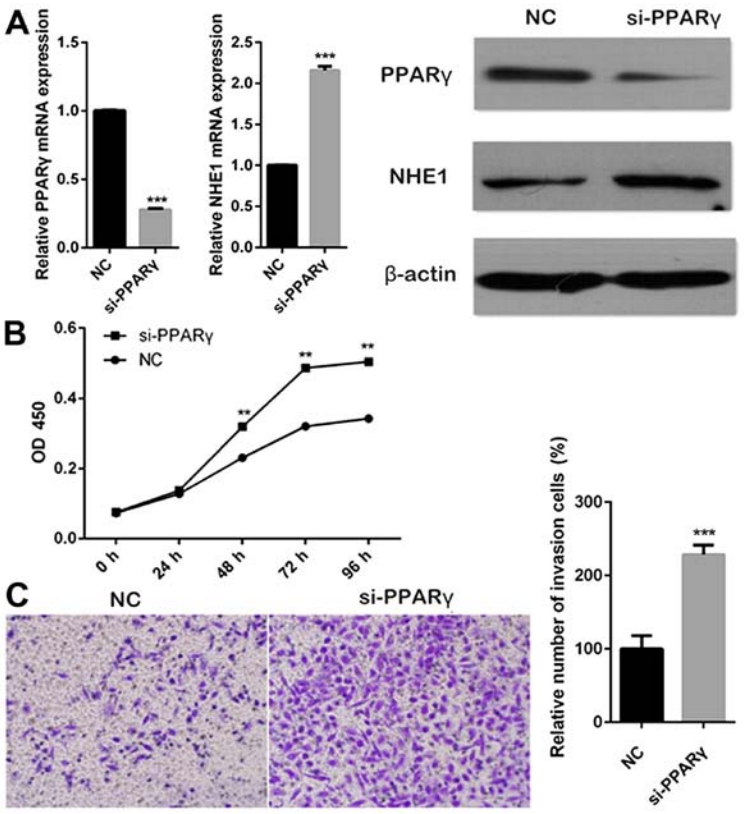

Figure 3. PPARγ negatively regulates carcinogenesis in cervical cancer cells. The cells were transiently transfected with PPARγ siRNA. (A) PPARγ and NHE1 mRNA and protein expression levels in CaSki cells were measured by real-time PCR and western blot analysis, respectively. β-actin served as the loading control. (B) The proliferation of CaSki cells was determined by CCK-8 assay. (C) The invasion of SiHa cells was analyzed by Transwell assay. Values are presented as mean ± SD and were repeated three times with similar results (**P<0.01, ***P<0.001).

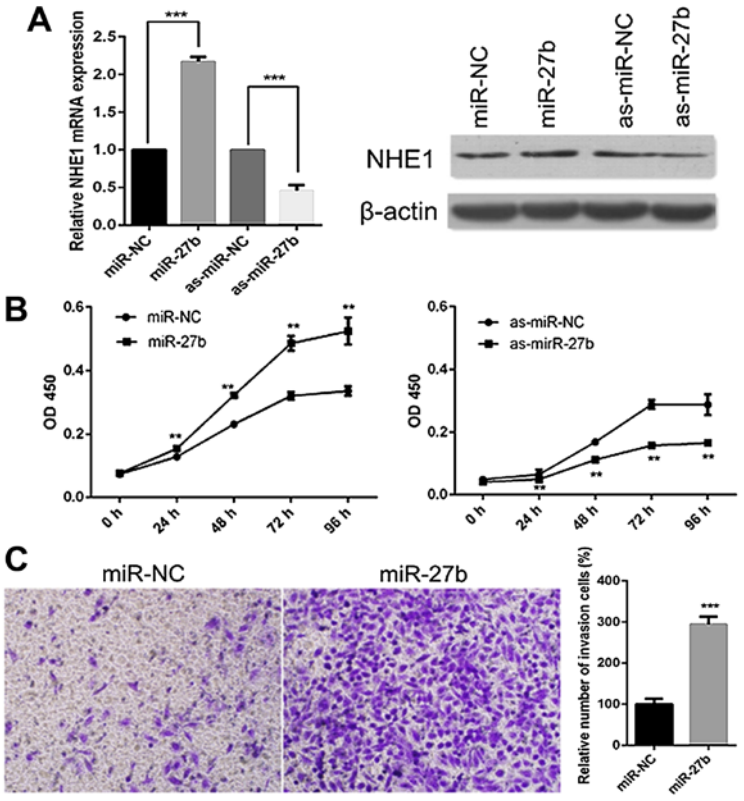

Figure 4. miR-27b promotes tumorigenesis in cervical cancer cells. The cells were transiently transfected with miR-27b mimics or inhibitors. (A) NHE1 mRNA and protein expression levels in CaSki cells were measured by real-time PCR and western blot analysis, respectively. β-actin served as the loading control. (B) The proliferation of CaSki cells was determined by CCK-8 assay. (C) The invasion of SiHa cells was analyzed by Transwell assay. Values are presented as mean ± SD and were repeated three times with similar results (**P<0.01, ***P<0.001).

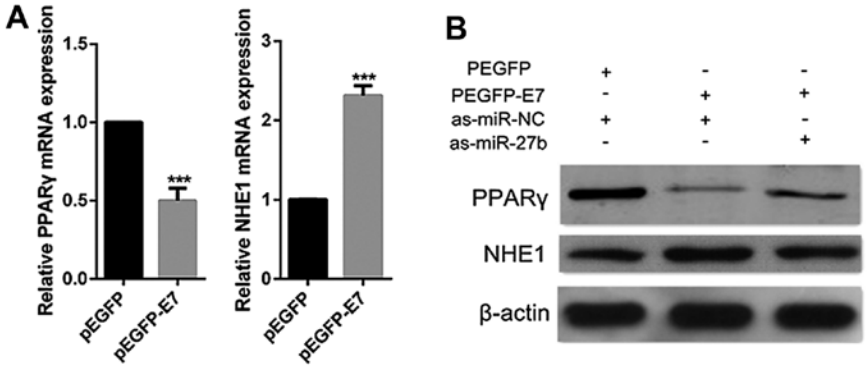

Figure 5. PPARγ repression and NHE1 activation by HPV16 E7 are associated with promotion of miR-27b. (A) CaSki cells were transiently transfected with HPV16 E7 plasmids. PPARγ and NHE1 mRNA expression levels were measured by real-time PCR. β-actin served as the loading control. (B) CaSki cells were co-transfected with HPV16 E7 plasmids and miR-27b inhibitors or control oligos. PPARγ and NHE1 protein expression level were measured by western blot analysis. β-actin served as the loading control. Values were presented as mean ± SD and were repeated three times with similar results (**P<0.01, ***P<0.001).

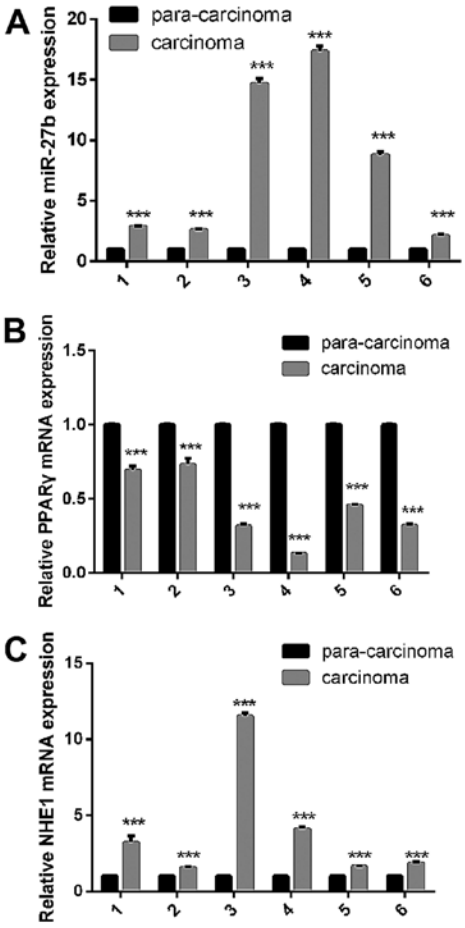

Figure 6. The expression of miR-27b, PPARγ and NHE1 in HPV16-positive cervical cancer tissues. Total RNAs were isolated from 6 paired cervical tumor and adjacent normal cervical tissues. The relative levels of (A) miR-27b, (B) PPARγ and (C) NHE1 were determined by real-time PCR. Values were presented as mean ± SD and were repeated three times with similar results (**P<0.01, ***P<0.001).

using the CCK-8 assay. The results showed that inhibition of PPARγ promoted proliferation of CaSki cells (Fig. 3B). We then determined the roles of PPARγ in invasion ability of cervical cancer cells. SiHa cells were transfected with PPARγ siRNA, followed by Transwell assay. As shown in Fig. 3C, knockdown of PPARγ resulted in the induction of invasion.

miR-27b has a tumor-promoting role in cervical cancer cells. Since PPARγ, the target of miR-27b, has been indicated as a tumor suppressor in cervical cancer in the present study, we then investigated the roles of miR-27b in cervical cancer cells by transfecting miR-27b mimics or inhibitors into CaSki or SiHa cells. In accordance with the results of PPARγ inhibition, the miR-27b overexpression significantly increased the levels of NHE1, while miR-27b repression led to a reduction of NHE1 both at mRNA and protein levels (Fig. 4A). Next, we assessed the effects of miR-27b on the proliferation of CaSki cells. CCK-8 assay revealed that overexpression of miR-27b enhanced the proliferation, whereas miR-27b inhibition reduced the proliferation of CaSki cells (Fig. 4B). Furthermore, we tested the ability of miR-27b in invasion of SiHa cells. As seen in Fig. 4C, the treatment with miR-27b mimics significantly increased the number of SiHa cells that passed through the Transwell chamber. These results suggest the tumor-promoting role of miR-27b in cervical cancer.

HPV16 E7 inhibits PPARγ expression and promotes NHE1 expression via promotion of miR-27b in cervical cancer cells. HPV16 E7 acts as an oncogenic promoter and is considered to be crucial for human cervical tumorigenic process (27). HPV16 E7 upregulates miR-27b which suppresses the expression of PPARγ, it is therefore hypothesized that HPV16 E7 could inhibit PPARγ expression. To confirm this hypothesis, CaSki cells were transfected with E7 plasmids. Decrease of PPARγ expression was observed in HPV16 E7 overexpressed cells by real-time PCR and western blot analyses (Fig. 5). Besides, NHE1 was reported previously to be upregulated by HPV16 E7 (21,28). In the present study, we also observed that the overexpression of HPV16 E7 led to increase of NHE1 (Fig. 5). However, when cells were co-transfected with HPV16 E7 plasmids and miR-27b inhibitors, the inhibition of miR-27b dramatically abolished the ability of HPV16 E7 to suppress PPARγ or induce NHE1 expression (Fig. 5B). Taken together, these data indicate that HPV16 E7 modulates the expression of PPARγ and NHE1 through miR-27b regulation.

Increase in expression of miR-27b and NHE1, and decrease in expression of PPARγ are observed in HPV16-positive cervical cancer tumor samples. We then measured the expression of miR-27b, PPARγ and NHE1 by real-time PCR using six pairs of HPV16-positive cervical cancer tissue samples. The results showed that the levels of miR-27b and NHE1 were significantly higher in cervical tumor tissues than in adjacent normal cervical tissues, while PPARγ expression was decreased in cervical tumor tissues (Fig. 6). Again, these clinical data provide strong support for our hypothesis that the reduced expression of PPARγ and enhanced expression of miR-27b and NHE1 are likely associated with the positive status of HPV16.

Discussion

Cervical cancer is one of the most highly malignant and lethal types of cancer in women worldwide. HR-HPV infection has been widely recognized as a leading cause of cervical cancer (11). Two viral oncogenes, HR-HPV E6 and E7, have been considered to play a critical role in HPV-associated cervical cancer pathogenesis. They deregulate multiple genes that are essential for host cell biological processes (27). MicroRNAs represent a class of non-coding RNAs that regulate gene expression at the post-transcriptional level. Substantial numbers of cellular miRNAs exhibited altered expression due to HPV infection (11). Thus, it is conceivable that aberrant expression of miRNAs is associated with HPV E6 and E7. Recent reports have suggested that HPV E7 deregulates the expression of miRNAs such as miR-15a/miR-16-1, miR-203 and miR-15b (11,29,30). Similarly, in the present study, we found that miR-27b was upregulated by HPV16 E7. The miR-27 family is shown to be induced upon inflammation in macrophages and could inhibit adipocyte differentiation (25,26,31). However, the roles of miR-27b in tumor development are controversial. It has been suggested that miR-27b acts as a tumor suppressor. A recent report has shown that miR-27b inhibits tumor progression and angiogenesis in colorectal cancer by targeting VEGFC (32). It is also shown that miR-27b targets LIMK1 to inhibit growth and invasion of NSCLC cells (33). However, several lines of evidence have suggested that miR-27b functions as an oncogene. One report showed that miR-27b could promote the proliferation and invasion of breast cancer cells by inhibiting the expression of ST-14 (34). It has been also found that the inhibition of miR-27b promotes apoptosis and negatively regulates the growth and invasion of glioma cells (35). Nevertheless, the expression condition and detailed roles of miR-27b in cervical cancer are poorly understood, except that miR-27b has been previously reported to be at much higher levels in HPV16-positive cervical carcinoma cells than in HPV-negative cervical carcinoma cells (24). According to the results of the present study, it is suggested that higher basal level of miR-27b in HPV16-positive cells is contributed to HPV16 E7. Subsequently, we found that miR-27b expression levels were higher in cervical cancer tissues than adjacent normal tissues and miR-27b enhanced proliferation and invasion of cervical cancer cell lines, indicating that miR-27b serves as an oncogene in cervical cancer.

It has been previously reported that miR-27b targets PPARγ directly in adipocytes and macrophages (25,26). Similar results were found in the present study that miR-27b suppressed PPARγ at mRNA and protein levels in CaSki cells. PPARγ is a member of nuclear receptor superfamily and is suggested to be involved in cancer progression (13). PPARγ probably acts as a tumor suppressor in various types of cancer (13). The activation of PPARγ interferes with proliferation and invasion in glioblastoma, as well as reduces growth and expansion of brain tumor stem cells (36,37). It is also suggested that PPARγ activators induce differentiation and apoptosis in non-small-cell lung cancer cells (38). In gastric cancer cell lines PPARγ promoted apoptosis and induced G1 cell cycle arrest (39,40). Anticancer effects of PPARγ ligands have also been reported in pancreatic cancer cells as PPARγ activation represses cell growth and attenuates the capacity of migration and invasion (41,42). Regarding the roles of PPARγ in cervical cancer, one report shows that PPARγ is less abundant in cervical carcinoma tissues than in normal cervical tissues (18), which is confirmed in the present study. Another study showed that treatment with PPARγ agonist *in vitro* induced apoptosis and suppressed proliferation of cervical cancer cells (43). In this study, we found that the inhibition of PPARγ by siRNA promoted proliferation and invasion of cervical cancer cells, implicating the antitumor roles of PPARγ in cervical cancer. In addition, we observed that miR-27b, which was positively regulated by HPV16 E7, inhibited the expression of PPARγ. Furthermore, the overexpression of HPV16 E7 suppressed the expression of PPARγ depending on the existence of miR-27b. These findings suggest a link between HPV16 E7 and PPARγ whereby HPV16 E7 is able to repress the expression of PPARγ through the promotion of miR-27b.

NHE1 is an integral membrane transport protein involved in regulating cytoplasmic pH. The activation of NHE1 could result in intracellular alkalinization and extracellular acidification. This dyregulation in pH by NHE1 takes place very early in cancer progression and subsequently drives behavior such as enhanced proliferation and growth, dyregulation of cell cycle progression, resistance to apoptosis, facilitation of migration and invasion, which is essential for the development and maintenance of transformed phenotype (20). Therefore, NHE1 is well characterized to be an oncogene. In the present study, we found that the levels of NHE1 were significantly higher in cervical cancer tissues than those in normal adjacent tissues. It has been reported that NHE1 enhanced proliferation, migration and invasion in cervical cancer cell lines (44-46), suggesting the carcinogenic role of NHE1 in cervical cancer. A recent study showed that NHE1 is directly targeted by PPARγ in breast cancer cells (19). Similarly, in this study, the repression of PPARγ resulted in significant increase of NHE1, indicating that PPARγ also inhibited NHE1 in cervical cancer cells. The findings also illustrated ectopic expression of miR-27b induced NHE1 expression, while suppression of miR-27b led to the downregulation of NHE1. As for the relationship between E7 and NHE1, our results showed that HPV16 E7 could upregulate NHE1. Actually, the induction of NHE1 expression by HPV16 E7 was previously reported (21,28). HPV16 E7 may activate NHE1 through a PKA-RhoA-induced inhibition of p38α (28). Nevertheless, the present study revealed another mechanism underlying HPV16 E7-mediated upregulation of NHE1 whereby HPV16 E7 upregulates NHE1 via inducing the expression of miR-27b.

Numerous studies have focused on exploiting the clinical potential of PPAR or NHE1 in anti-cancer treatment (13,47). The intelligent use of optimized compounds derived from PPAR agonist and the development of NHE1 inhibitors could be avenues for anti-cancer drug design. In the present study, we observed the levels of miR-27b were consistently correlated with those of NHE1 but inversely correlated with PPARγ in cervical cancer tissues. Besides, miR-27b suppresses the expression of PPARγ and upregulates the levels of NHE1, suggesting that miR-27b may be a promising therapeutic target for treatment of cervical cancer.

In a conclusion, our findings demonstrate that HPV16 E7 upregulates miR-27b, which in turn decreases the expression of PPARγ to enhance cervical cancer progression. We believe that an understanding of the roles of E7-miR-27b-PPARγ-NHE1 pathway holds much promise for the development of future treatments for cervical cancer.

Acknowledgements

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