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Original Article

Correlation between human papillomavirus and microRNA-210 in hypoxia-associated human cervical cancer

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Abstract: Human papillomaviruses (HPVs) are relevant to the pathogenesis of cancer of the cervix (CaCx). MicroRNA 210 (miR-210) participates in various aspects of carcinogenesis. The aim of the present study was to examine the relationship between HPV and miR-210 in hypoxia-associated cervical squamous cell carcinoma (SCC). Remarkably, it was showed that expression of miR-210 was significantly increased in SCC tissues compared with normal samples ($P < 0.01$). The cancerous tissues of patients with advanced International Federation of Gynecology and Obstetrics (FIGO) stage was significantly higher than those with an early FIGO stage in the expression of miR-210 ($P = 0.02$). In addition, miR-210 was expressed at significantly higher levels in HPV-positive patients than in HPV-negative patients ($P = 0.027$). The HIF-1α protein positivity rate on immunohistochemistry was 72.5%, but no differences were observed in HIF-1α mRNA levels ($P > 0.05$). HPV16E6/E7 expressions were higher in SCC tissues than those in normal tissues in both protein and mRNA level. Furthermore, there was statistically significant evidence of an interaction between miR-210 and HPV16E6/E7 ($\varphi E6 = 0.352$, $P = 0.012$; $\varphi E7 = 0.196$, $P = 0.161$) as well as between miR-210 and HIF-1α ($rH = 0.407$, $P = 0.003$). Our findings describe a novel correlation between miR-210 and oncoprotein E6/E7 of HPV16 in hypoxia-associated cervical cancers.

Keywords: microRNA-210, human papillomavirus, E6/E7, hypoxia inducible factor-1a, cervical cancer

Introduction

Human papillomavirus (HPV) infection is very common all over the world. It is 12-18 months that infection with high-risk HPV (HR-HPV) typically lasts, which is finally cleared by the immune system [1]. However, approximately 10% of women fail to clear HPV infections, which lead to an enduring infection. Cervical cancer caused by enduring infection of oncogenic HPV is the second most common cancer in women all over the world [2]. HR-HPV is closely related to premalignant and malignant cervical lesions, especially those caused by HPV 16 and 18 [3-5]. HR-HPV, especially HPV 16, which has a long period of clearance may bring about a persistent infection. E6 and E7, of HPV16 and HPV18 can promote cell cycle dysregulation, destroy the cell growth and regulate pathways and modify the cellular environment to facilitate viral replication, so that the cell differentiation and cycle may be affected. Beyond that, in highly differentiated cells, the decrease of viral protein synthesis causes a weaker response of the host immune [6]. However, only a few HPV-infected individuals develop invasive cervical cancer [7]. Most infected individuals eliminate the virus without developing classical clinical manifestation [3]. E6 and E7 are in charge of viral oncogenesis by destroying the peace of two major cellular tumor suppressors, p53 and pRB, respectively, which are necessary for cell cycle control [8]. In addition to these well-known events, both proteins have a wide range of other targets 8, but the extent of contribution which these additional interactions make to HPV-associated carcinogenesis is not yet fully understood.

Aberrant microRNA (miRNA) expression or mutant miRNA forms have been proved in all cancers. Interestingly, a variety of cancers are

also inclined to exhibit specific signatures of dysregulated miRNAs [9-15]. Importantly, numerous studies have demonstrated that miRNAs can suppress or promote tumor growth [10, 16]. However, we know little about their role in cancer and the cause of their aberrant expression. Previous evidence indicated that the aberrant expression of miRNA plays a role in tumor progression, metastasis, and chemoradioresistance [17]. Hypoxia is a characteristic of all solid tumors that can promote tumor development. Hypoxic conditions in solid malignancies may confer resistance to conventional therapies and are associated with a poorer prognosis [18-20]. Recent work has proved that non-coding RNAs, miRNAs in particular, may be involved in the adaptive response to low oxygen in tumors. Specifically, all experimental studies demonstrated that the induction of miRNA-210 (miR-210) is an invariable feature of the hypoxic response in normal cells or in cancer cells under physiological hypoxic conditions [21]. The overexpression of miR-210, a sturdy target of hypoxia-inducible factors, has been detected in different kinds of diseases with a hypoxic component, including most of solid tumors. In cancer tissues, hypoxia inducible factor-1α (HIF-1α) is frequently overexpressed due to the fact that hypoxia is a typical feature of the tumor microenvironment [22].

One study showed that HIF-1α protein levels are increased in hypoxia when HPV oncogenes are exist in both nononcogenic and oncogenic virus types. Moreover, this increased HIF-1α induction gave rise to increases in some but not all downstream effectors of the hypoxic response, suggesting that HPV particularly manipulates aspects of the cellular hypoxic response [23]. The study also showed that miR-210 in cell lines including integrated HPV-16 DNA were expressed at much higher levels than the HPV-negative cervical carcinoma cell line C-33A cells (mean value = 5.7-fold) [24]. As such, we proposed the hypothesis that HPV oncoprotein could regulate miR-210 expression through regulating and controlling HIF-1α. We conducted this study of 51 patients with cervical cancer and 28 healthy controls to determine the expression of HPV oncoprotein E6/E7, miR-210, and HIF-1α and identify the correlation between HPV oncoproteins and miR-210 and HIF-1α levels.

Materials and methods

Patients and tissue samples

This study was approved by the Research Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, P. R. China. Written informed consent was obtained from all of the patients. All specimens were anonymized according to ethical and legal standards.

Cervical cancer and normal cervical tissue samples were obtained from the 51 patients with cervical cancer and the 28 patients with benign disease. The tissue samples in vitro were then divided into two parts: one was immediately frozen in liquid nitrogen until use, while the other was placed in 4% formalin and embedded in paraffin.

Determination of mRNA levels of miRNA-210, HPV16E6/E7, and HIF-1α using real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated using RNAiso Plus (Takara Bio Inc, Otsu, Japan) according to the manufacturer's specifications. RNA concentration and quality were assessed by the A260/A280 ratio using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The specific cDNA of miR-210 and RNU6B were synthesized from quantitative detection of mature miRNA using an All-in-One™ miRNA RT-qPCR Detection Kit (GeneCopoeia, FulenGen, Guangdong). To assay for miRNA, poly (A) tails were added to short RNAs using a One Step PrimeScript miRNA cDNA Synthesis Kit (D350A; TaKaRa) according to the manufacturer's protocol. RT-qPCR was performed using SYBR Premix Ex TaqII (Perfect Real Time) (RR716; TaKaRa). Amplification was performed for 40 running cycles of 30 s each at annealing temperature (60°C) using CFX Manager 3.0 (Bio-Rad, Hercules, CA, USA). For the data analysis, U6 RNA was used as an endogenous control. The difference between cervical cancer and normal control for microRNA-210 was calculated with the $2^{-\Delta\Delta Ct}$ method as described previously [25]. The reverse transcriptions (RT) of HPV16E6/E7 and HIF-1α were performed in a 20-μL reaction mixture including 500 ng of total RNA using a PrimeScript RT Reagent Kit (TaKaRa). Primer sequences used for the detection included

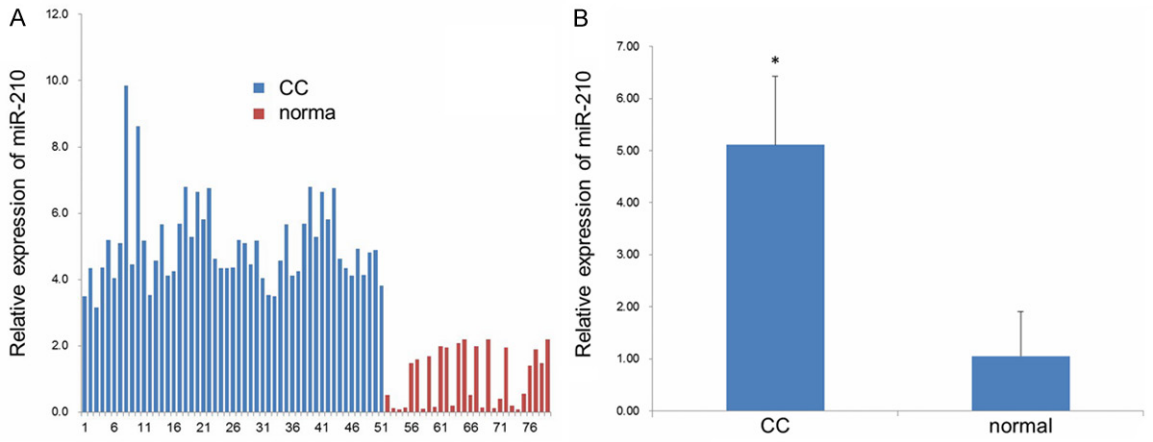


Figure 1. Expression of miR-210 was upregulated in squamous cell carcinoma (SCC) tissues. A: Overexpression of miR-210 was verified by real-time qualitative polymerase chain reaction. Seventy samples were divided into the SCC (upregulating miR-210) and normal groups. The numbers on the X axis represent the specimen numbers, while the Y axis values show the mean expression. B: The differences between the cervical cancer tissues and the normal tissues were statistically significant. The data were evaluated by independent samples t-tests. Each column represents the mean ± SD. *P < 0.01.

Table 1. Association between miR-210 expression and clinicopathological parameters in cervical cancer

		Negative	Positive	χ^2	*P*-value
IA-IIA	9	4	5	5.400	0.02
IIB-IV	42	5	37		
HPV					
HPV (+)	45	6	39	4.898	0.027
HPV (-)	6	3	3		

HPV16E6 (forward, 5'AGCGACCCAGAAAGTTACCACA3'; reverse, 5'CAAAGTCATATACCTCACGTCGCA3'), HPV16E7 (forward, 5'ACAATTAAATGACAGCTCAGAGG3'; reverse, 5'CGCACAACCGAAGCGTAGAGT3'), and HIF-1α (forward, 5'TCTCCATTACCCACCGCTGA3'; reverse, 5'ACTTTGTCTAGTGCTTCCATCGG3'), and β-actin (forward, 5'ccacgaaactaccttcaactcc3', reverse, 5'gtgatctccttctgcatcctgt3') was used as an endogenous control. RT-qPCR was performed using SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (RR820A; TaKaRa). The remaining steps were the same as miR-210.

Immunohistochemistry (IHC)

HPV16E6/E7 and HIF-1α IHC were carried out using goat polyclonal anti-human HPV16E6 antibody (HPV16 E6 [N-17]: sc-1584; Santa Cruz Biotechnology, CA, USA]; mouse monoclonal anti-human HPV16E6 antibody (HPV16 E7 [TVG710Y]: sc-264; Santa Cruz Biotechnology, CA, USA); mouse monoclonal anti-human HIF-1α antibody (HIF-1α [28b]: sc-13515; Santa Cruz Biotechnology, CA, USA), respectively. Briefly, the 4-μm sections were de-paraffinised by repeated processes through xylene and graded alcohol, and rehydrated. The antigen retrieval was established with 0.01 M citrate buffer for 20 min in a microwave at a medium heat setting [26] and then the slides were cooled to room temperature, washed in PBS (3×5 minutes). They then hatched together with hydrogen peroxide for 30 min to eliminate endogenous peroxidase activity, followed by overnight incubation at 4°C with 1:30, 1:30, and 1:50 dilutions of HPV16E6/E7 and HIF-1 primary antibodies. After continuing 30-min incubations with HRP, the tissue sections that were incubated with DAB (ZSGB Biotechnology, Beijing, China) developed coloration. The sections were counterstained with hematoxylin, dehydrated, and observed under a light microscope (CX22RFS1, OLYMPUS, Japan). As a negative control, the primary antibody was replaced by phosphate buffer saline. HPV16E6/E7 and HIF-1α proteins were mainly expressed in the nucleus. IHC score group was made up of three independent observers, containing the pathologist. All were blinded to the clinical data.

Western blot analysis

The tissue samples (0.1 g) extracts were prepared using RIPA buffer (Beyotime Institute of

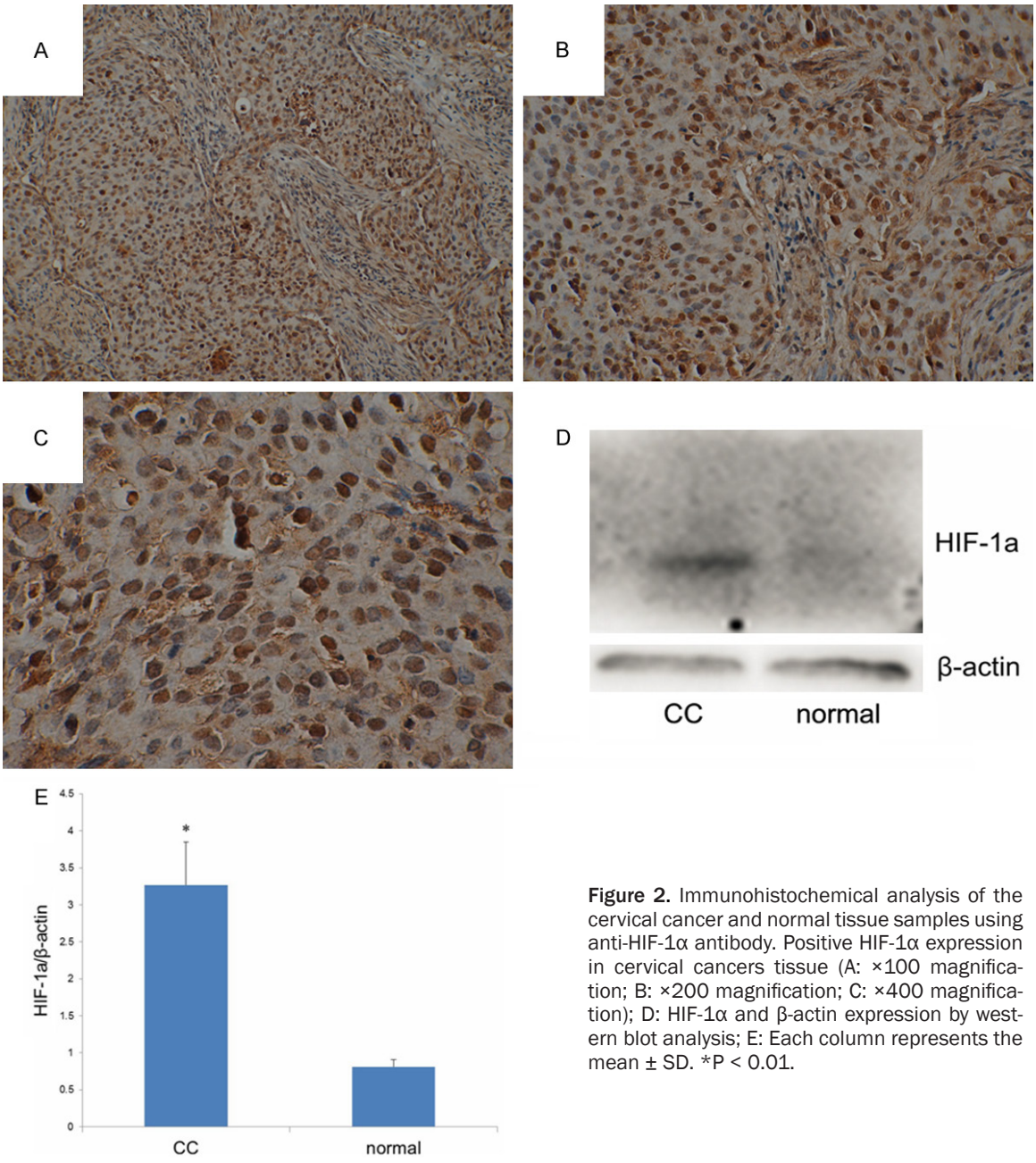


Figure 2. Immunohistochemical analysis of the cervical cancer and normal tissue samples using anti-HIF-1α antibody. Positive HIF-1α expression in cervical cancers tissue (A: ×100 magnification; B: ×200 magnification; C: ×400 magnification); D: HIF-1α and β-actin expression by western blot analysis; E: Each column represents the mean ± SD. *P < 0.01.

Biotechnology, Shanghai, China). Protein concentrations were determined using a bicinchoninic protein assay kit (Beyotime Institute of Biotechnology). The lysates were mixed with 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. The protein samples (40 μg) were then loaded on a 10% SDS-PAGE apparatus and transferred to polyvinylidene fluoride membranes. The membranes was blocked with 5% nonfat milk in Tris-buffered saline with Tween (TBST) for 2 hours and incubated overnight at 4°C with primary antibodies as described previously. The membranes were washed three times with TBST and then incubated with secondary antibodies. Finally, the immunoreactive bands were detected by chemiluminescence using mouse anti-β-actin as an endogenous control. Densitometry was performed using Fusion FX7 (VILBER LOURMAT,

Table 2. Expression of HIF-1α in cervical cancer and normal cervical tissues

Group	Cases	HIF-1a expression				Positive (%)	*P*-value
		-	+	++	+++		
Normal tissues	28	28	0	0	0	0.0	P < 0.05*
Tumor tissues	51	14	3	22	12	72.5	

HIF-1a expression was determined by immunohistochemistry and the difference between the 2 groups was estimated by a Wilcoxon rank sum test. *P < 0.05 indicates statistical significance.

France). The relative protein level in each bar graph was calculated grounded on its protein band density after being normalized to β-actin for sample loading.

Statistical analysis

The statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL). All results are expressed as mean ± SD. Student's t-test or the chi-square test was used to compare the parameters between the study groups. Correlation of the targets was assessed using Pearson's and Spearman's rank tests or cross-tabs. *P* values < 0.05 were considered statistically significant.

Results

Patient situation

The pathological diagnosis of all 51 patients with cervical cancer was cervical squamous cell carcinoma by punch biopsy or cone excision. In the present study, none of the patients recruited received radiotherapy, chemotherapy, or any other treatment before surgery. The 51 patients were 30-74 years of age (mean, 47.7 years). The clinical FIGO stages included IA-IIA (9 cases) and stage IIV-IV (42 cases). There were 45 HPV16-positive patients and six HPV16-negative patients.

Overexpression of miR-210 in SCC tissues

The expression level of miR-210 in the tumor samples and the normal cervical tissues was determined by RT-qPCR (**Figure 1**). Compared to the normal tissues, 42 of 51 (82.4%) SCC tissue samples demonstrated upregulated miR-210 levels (**Figure 1A**). The miR-210 expressions in the tumor and the normal tissues were 5.11 ± 1.32 and 1.05 ± 0.86, respectively. The difference between the two groups was statistically significant (P < 0.01) as shown in **Figure 1B**. These results show that miR-210 upregulation may have an impact on the progression of SCC.

Correlation between miR-210 expression and clinicopathological characteristics of SCC

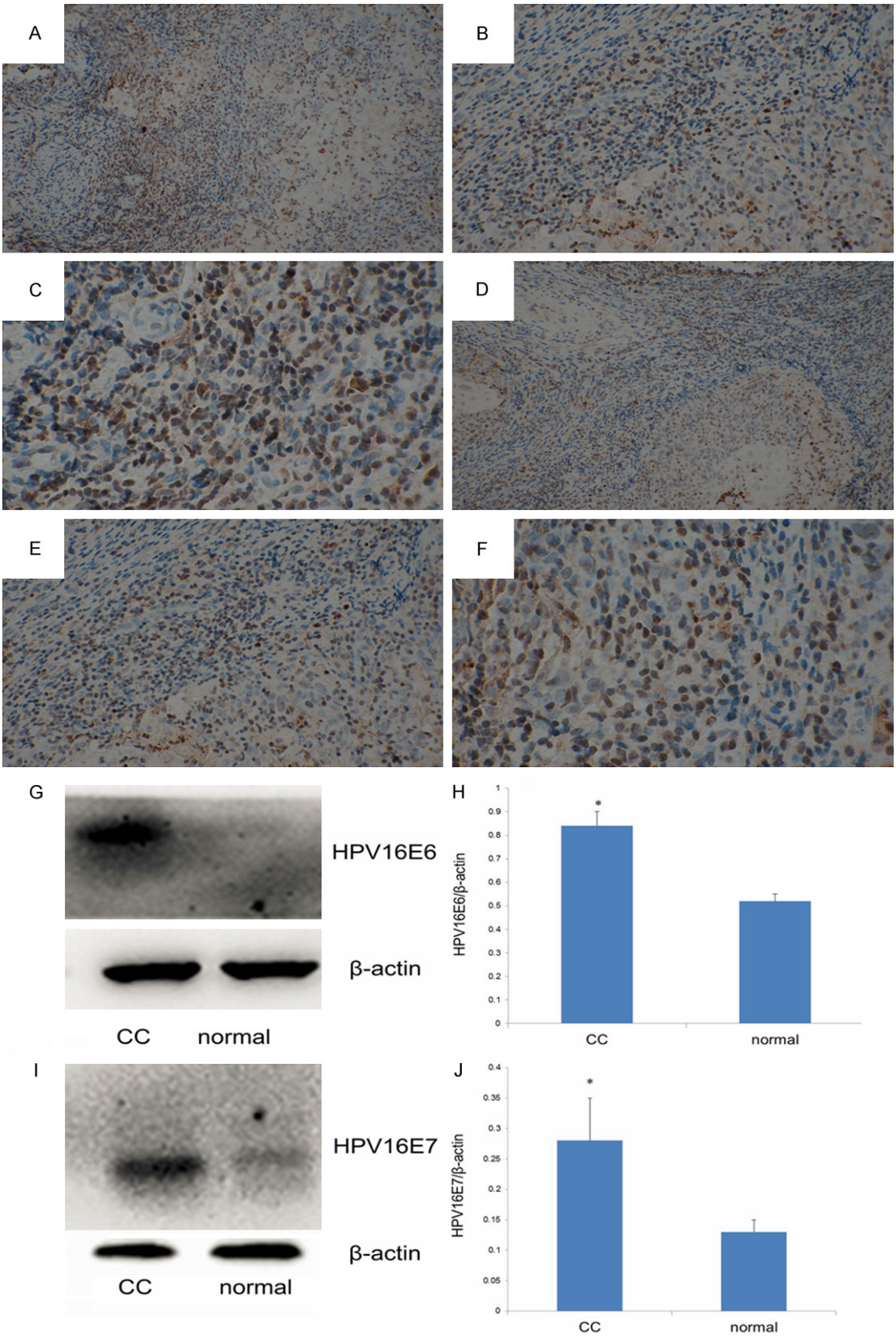
Data are presented in **Table 1**. The SCC tissues of patients with advanced FIGO stage was significantly high from those with early FIGO stage in the expression of miR-210 (P = 0.02, **Table 1**) and miR-210 was expressed at significantly higher levels in HPV-positive patients than in HPV-negative patients (P = 0.027, **Table 1**).

HIF-1α protein and mRNA levels in cervical cancer and normal tissues

The protein expression of HIF-1α was highly expressed in cervical cancer compared with normal cervical tissues by IHC.As shown in **Figure 2A-C**, HIF-1α was mainly expressed in the nucleus and occasionally in the cytoplasm. As demonstrated in **Table 2**, 37 of the 51 tumor tissue samples (72.5%) and none of the 28 normal tissue samples expressed HIF-1α. Significantly higher HIF-1α expression levels were observed in the tumor tissues than in the normal tissues on the Wilcoxon rank sum test (P < 0.05). Western blot analysis (**Figure 2D**) showed the same results (**Figure 2E**) (3.27 ± 0.58, 0.81 ± 0.10). However, HIF-1α mRNA levels were seen in no differences (P > 0.05), indicating that the upregulation of HIF-1α is due to a post-transcriptional event.

Association among HIF-1α, HPV16E6/E7, and miR-210

To determine the expression of HIF-1α and HPV16E6/E7 in correlation with the miR-210 level in cervical cancer, some analyses are performed, such as RT-qPCR, western blot analysis, and IHC analyses for HPV16E6/E7 expression in 51 cervical cancers and 28 normal samples (**Figure 3**). RT-qPCR showed that HPV16E6/E7 mRNA expression in cervical cancer sample was 147.1 and 127.6 folds higher in normal tissue, respectively. In agreement with RT-qPCR, Western blot analysis demonstrated that HPV16E6/E7 protein expressions were


A
B
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HPV16E7/β-actin
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normal

Figure 3. Expression of HPV16E6 protein (A: ×100; B: ×200; C: ×400) and HPV16E7 protein (D: ×100; E: ×200; F: ×400) in the cervical cancer tissues. (G) HPV16E6 and β-actin by western blot analysis. (H and J) Analysis of the results. (I) HPV16E7 and β-actin by western blot analysis. Each column shows the mean ± SD. *P < 0.05 compared with the control group.

Table 3. Relationship between expression of HPV16E6/E7 protein and miR-210 in cervical cancer tissues

	miR-210 (+)	miR-210 (-)	φ	*P*
E6 (+)	32	3	0.352	0.012
E6 (-)	10	6		
E7 (+)	29	4	0.196	0.161
E7 (-)	13	5		

higher in cancer tissues (E6 0.84 ± 0.06, E7 0.28 ± 0.07) than those in normal cervical tissues (E6 0.53 ± 0.03, E7 0.13 ± 0.02) (**Figure 3G-J**).

Next the correlation between miR-210 or HIF-1α with E6/E7 at the mRNA and protein levels was investigated by using the 51 cervical cancer samples with HPV16 infection and it was found that miR-210 was correlated with E6/E7 (**Table 3**) (correlation coefficient φE6 = 0.352, P = 0.012; φE7 = 0.196, P = 0.161), as was HIF-1α (rH = 0. 407, P = 0.003).

Discussion

Despite the development of advanced treatment strategies, prognosis of patients with cervical cancer remains difficult to predict. To date, tumor invasion and metastasis is the main reason for high mortality. Early detection and diagnosis primarily determine treatment outcome. Fortunately, the role of miRNA in cancer and its potential utility for tumor invasion and metastasis [27] and prognostic markers has emerged recently. Of these, miR-210, an independent marker to predict clinical outcomes in breast cancer patients [28], could exert significant influence on mitochondrial function, cell survival, and homeostasis [29]. However, our knowledge about the potential role of miR-210 in cervical cancer remains limited. To confirm whether miR-210 expression may influence cervical cancer, here an RT-qPCR assay was performed to explore the expression profile of this miRNA and research its relationship with clinicopathological features of patients with cervical cancer. The results showed that miR-210 expression levels were significantly higher in SCC tissues than in the normal cervical tissues. What's more, miR-210 expression was proven to be associated with FIGO stage and HPV status, which strongly demonstrated that it is involved in cervical cancer.

Cervical tissues demonstrate some unique features which is different from other solid tumors .That is to say, chronic hypoxia would appear in normal conditions [30, 31]. HIF-1 is widely considered as a vital mediator in cellular hypoxia which can promote angiogenesis, glycolysis, invasion, and metastasis as well as a key gene for tumorigenesis. Because of the actions of bioactive lipid mediators as well as viral infections, hypoxia, and mutations to oncogenes and tumor suppressor genes elevated HIF-1 expression. As an indirect sign of hypoxia in many tumors, HIF-1α expression and its prognostic value in many cancers, including cervical cancer, have been extensively studied [32, 33]. Here it is showed that HIF-1α expression as exposed by IHC can be observed in various intensities in 72.5% of cervical cancers but not in normal cervical epithelia. However, our finding that HIF-1α mRNA expression was not prognostic and there was no significant difference in the level of HIF-1α expression between cervical cancers and the normal cervical tissues was at odds with the IHC and western blot analysis results. A good correlation was also observed between miR-210 expression and HIF-1α in our patients, verifying miR-210 expression would be influenced by hypoxia like other results previously shown in other solid tumors [21, 28]. On the other hand, it was wondered that whether miR-210 had a positive influence on HIF-1 activity. This remains unclear.

HPV is regarded as the etiologic agent of approximately 99% of cervical cancers worldwide. In an overwhelming of patients, cervical cancer is induced by infection with high-risk HPV types (e.g., HPV 16, 18, and 33). In this study, HPV16 was mainly detected. In addition, the expression of E6/E7 has been tested in protein and mRNA levels by western blot analysis and RT-qPCR, respectively (**Figure 3**). Together with the fact that HPV may enhance the levels of HIF-1α through protein stabiliza-

tion rather than mRNA level. HPV may be a significant co-player for the development of the tumorigenic properties of HIF-1α. Recent studies have demonstrated that miRNAs might be possible markers for the occurrence and development of HPV-associated cancers and that the differences in tumor-specific miRNA signatures might be helpful for distinguishing among different cervical lesions [34]. Substantial evidence shows that HPV regulate the expression of cellular miRNA most likely to occur through viral oncoproteins [35-37]. The matter is to discover what these miRNAs are and how E6 and E7 regulate them. Thus, it should be illustrated in future studies that the effects of such miRNAs on HPV protein expression.

It was also indicated by our study that a certain relationship between miR-210 and oncoprotein E6/E7 of HPV16, but it remains unknown if this upregulation is a cause or consequence. Our results suggest the possibility that HR-HPV may get more opportunities to trigger carcinogenic development by reshaping the environment of the composition of cellular miRNA and target gene expression which is benefit to the virus. Furthermore, the data of miR-210 presented in this paper expand the known classes of transcripts within the HPV-HIF-1α target gene network. It is essential that the potential of specific miR-210 or genes caused by HPV infection should be validated by appropriate culture models.

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Disclosure of conflict of interest

None.

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