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Original Article

Discrepancy in miRNA expression of HPV18 in various pathological types of cervical carcinoma and its correlation with pathology

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Abstract: Objective: This study aimed to investigate the differences in miRNA expression in HPV18 from various pathological cervical carcinomas and the correlation with pathology. Methods: A total of 93 patients with cervical carcinoma admitted to our hospital were enrolled as the observation group (OG). These patients were given surgical treatment and divided into the squamous-cell carcinoma group (n=53), adenocarcinoma group (n=26) and adenosquamous carcinoma group (n=14), according to their surgery and pathology. Para-carcinoma tissues (the distance to the lesions ≥3 cm) collected during the operation were used as the control group (CG). The positive expression rates of HPV18 in different lesion tissues were determined by immunohistochemistry. Real-time fluorescent polymerase chain reaction (PCR) was used to determine the miRNA expression of HPV18. Patients' pathological information including age, tumor type, pathological type, differentiation type and lymphatic metastasis and neoplasm staging were all used in the analysis of HPV miRNA expression under different pathological conditions. SPSS Pearson software was applied for the correlation of HPV18 expression with specific pathologic types. Results: The positive expression rate of HPV18 protein was 61.29% in the OG, which was especially higher than that of 5.38% in the CG (P<0.05). Real-time fluorescent PCR showed that levels of HPV18 miRNA in the OG (8.32±1.20) overtopped that in the CG (1.21±0.47) (P<0.05), and no statistical significance was found between HPV18 miRNA levels and patients' age or pathological types (P>0.05), but there were significant differences between HPV18 miRNA levels and tumor differentiation types, lymphatic metastasis and neoplasm staging (P<0.05). SPSS correlation analysis found that HPV18 expression was not related to age or pathological type (P>0.05), but was positively correlated with neoplasm staging and lymphatic metastasis (P<0.05) and negatively correlated with the extent of tumor differentiation (P<0.05). Conclusions: HPV18 miRNA was highly expressed in various cervical carcinomas with unobvious differences. The expression was significantly correlated with the pathology type. Elevated expression of HPV18 miRNA in patients with cervical carcinoma may reflect the severity of disease.

Keywords: HPV18, pathological types, cervical carcinoma, miRNA expression, correlation

Introduction

Cervical carcinoma is a malignant type of cancer found in women with a high incidence. The condition is usually related to biological factors such as virus infection, sexual behavior and number of vagianl deliveries. These factors interact with each other [1]. The early symptoms are inconspicuous and some patients may have vaginal bleeding, constipation, etc., which makes it difficult in the clinical diagnosis and treatment [2]. Clinically, cervical carcinoma is categorized into squamous-cell carcinoma, adenocarcinoma, and adenosquamous carcinoma on a pathological basis. Also, squamous-cell carcinoma is graded into I, II, and III on a histological differentiation basis; adenocarcinoma (accounting for 15%-20%) can be divided into mucinous adenocarcinoma and malignant adenoma [3]; whilst adenosquamous carcinoma represents 3%-5% of cervical carcinomas. Operative examination and aspiration biopsy, among other approaches, are primary methods for the treatment of cervical carcinoma and are known as the "golden standards" of clinical diagnosis. However, the poor repeatability makes it difficult to dynamically understand the patient's conditions and is therefore

an inapplicable to guide clinical treatment [4]. It is of great significance to explore non-invasive, well-reproducible diagnosis and indicators to improve patients' prognosis [5].

Human papilloma virus (HPV) plays an important role in the development of cervical carcinoma. HPV is species-specific and binds to host cells and uses them as a natural host after the human is infected [6]. Normally, 90.0% of patients infected with HPV can be cured by their own immune system, and only a small proportion continue to be infected [7]. Of the two main types of HPV: dermatotropic, and mucosotropic, the latter is much more common in clinic. Clinical studies have shown that persistent HPV16 infection increases the incidence of squamous-cell carcinoma [8]; HPV18 infection increases the incidence of adenocarcinoma; and persistent infection with HPV16/HPV18 combination leads the cause of 70.0% of cervical carcinoma. At present, the pathogenesis of cervical carcinoma has not been clarified, but active early diagnosis & treatment improves the prognosis [9]. Clinical studies pointed out [10] that HPV18 is highly expressed in cervical carcinoma tissues, which is an independent risk factor for the occurrence of this disease. Few studies have investigated the correlation of HPV18 expression with pathology types.

It can be perceived that a high incidence of cervical carcinoma in China is affected by many factors; persistent infection of HPV plays an important role in the potential development, but the correlation of HPV18 expression with clinical pathology has not been sufficiently studied [11]. In this paper, patients with cervical carcinoma were treated with surgery to find the expression of miRNA of HPV18 in different pathological types of cervical carcinoma and analyze its relationship with clinical pathology.

Material and methods

Clinical data

A total of 93 patients with cervical carcinoma admitted in our hospital from May 2017 to July 2019 were enrolled as the observation group (OG). These patients were given surgical treatment and divided into the squamous-cell carcinoma group (n=53), adenocarcinoma group (n=26) and adenosquamous carcinoma group (n=14) according to the surgery and pathology type. The research in this paper has been approved by the Hospital Ethics Committee.

Inclusion and exclusion criteria

Inclusion criteria: Patients (1) who met the diagnostic criteria for cervical carcinoma in *Obstetrics and Gynecology* [12] and had been diagnosed by surgical examination; (2) who was treated with surgery; (3) who were capable of receiving HPV18 tests and were conscious to communicate with family members/doctors.

Exclusion criteria: Patients (1) who had malignant tumors, other disease or autoimmune disease; (2) who had abnormal hepatic and renal function, bone marrow dysfunction or insufficient data in pathological files; or (3) who had a history of radiation and chemotherapy or biological immunotherapy before this research.

Instruments and equipment

Neutral formaldehyde (Shanghai Yubo Bio-tech Co., Ltd.), Rabbit Anti-human HPV18 monoclonal antibody (Shanghai Duma Bio-tech Co., Ltd.), EDTA Antigen Retrieval solution (Qingdao Jisskang Biotechnology Co., Ltd.), Electrothermal Thermostal Water Bath (Suzhou Kaiteer Instrument & Equipment Co., Ltd.), DAB Chromogenic Reagent Kit (Nanjing Saihongrui Biotechnology Co., Ltd.), Histotome (Warner, USA), and Tissue Embedding Machine (Diapath, Italy) were used in this study.

Methods

Specimen collection: All patients were given operative treatment. Lesion tissues collected were taken as the observation group (OG) and para-carcinoma tissues as the control group (CG) (the distance to the lesion ≥3 cm) during operation. Then, 4 um sections were prepared by routine paraffin embedding [13].

HPV18 protein determination: (1) Detection. IHM two-step method was used to determine the protein expression of HPV18 in tissues. Specimens were dewaxed and hydrated followed by the addition of 3% hydrogen peroxide for 8 min to deactivate endogenous enzymes. Citrate buffer was used in high-pressure antigen retrieval. Retrieval continued for 15 min

Table 1. PCR Primers with HPV18

Types	Primers	Length
β-actin	F: 5' GTGCTATGTTGCTCTAGACTTCG 3'	174
	R: 5' ATGCCACAGGATTCCATACC 3'	
HPV18	F: 5' AGGTTCCTCTCCTAGCAGATCATTCTC 3'	99
	R: 5' GAGCGGCAACTTCTGAGGTCTTAC 3'	

using reagent A. After the addition of HPV18 protein primary antibodies, the specimens were incubated overnight at 4°C. Later, HPV-18 protein secondary antibodies were applied for incubation at 37°C for 20 min. After DAB dyeing and Hematoxylin redying, the positive rates of HPV18 protein expression in tissues were recorded. (2) Assessment. The results were read and evaluated by the pathologist in our hospital. Five fields under high power lens were taken to calculate the proportion of HER2/c-erbB-2 protein positive cells that were mostly shown as tan particles. A ratio of positive cells less than 5% was recorded as negative (-), 5%-25% as +, 25%-50.0% as ++, and 50% as +++ [14, 15].

HPV18 miRNA determination: ① RNA extraction. The tissue specimens above were mixed in 500 uL Trizol and transferred to 1.0 mL centrifuge tubes, which were then rested for 5 min. With the addition of 200 uL chloroform followed by vibration within 15 s and let rest for 5 min, the mixture was centrifuged at 1216 for 15 min. The supernatant was separated. One mL of pre-cooled ethanol (75.0%) was applied before the mixture was dried at room temperature for 7 min. Absorbance at A260 was detected by ultraviolet spectrophotometer [16]. ② Detection. Real-time fluorescent PCR was used to determine the expression of HPV18 miRNA in tissues with different pathological type. The primers are shown in **Table 1**. PCR parameters and steps: 30°C for 10 min; 42°C for 30 min; 99°C for 5 min; 5°C for 5 min; 35 cycles; 72°C for 10 min. The products were subject to 1.5% Agar Gel electrophoresis, with β-actin the internal control [17].

HPV18 miRNA expression and correlation analysis under different clinical pathologies: Patients' pathological information including age, tumor types, pathological types, differentiation types and lymphatic metastasis and neoplasm staging were used in the analysis of HPV miRNA expression under different pathological conditions. SPSS Pearson software was applied in the correlation of HPV18 expression with specific pathologic types.

Statistical analysis

Statistical analysis was performed with SPSS 18.0 software. Enumeration data were expressed as n (%) and subject to χ^2 test. All the data conformed to normal distribution. One-way analysis was used for intergroup comparison. F test was adopted for comparison of among the three groups and data were expressed as mean ± SD. $P<0.05$ indicates statistically significant difference.

Results

Comparison of the general information

There was no statistically significant difference among the three groups in terms of age, body mass, course of disease, tumor staging, pathological grading, pelvic lymph node metastasis and differentiation, which were comparable ($P>0.05$), as shown in **Table 2**.

Comparison of positive expression of HPV18 in tissues

The number of cases with HPV18 protein positive expression was 57 (61.29%) in the OG, which was higher than that in the CG ($P<0.05$). In the OG, HPV18 protein positive rates in squamous-cell carcinoma, adenocarcinoma and adenocarcinoma subgroups were not statistically significant ($P>0.05$) (**Table 3**).

Immunohistochemistry results

HPV18 protein was mostly expressed in the cell membranes of the lesion tissues, and only a small proportion was expressed in the cytoplasm. Para-carcinoma tissues showed lower expression levels. The staining of squamous-cell carcinoma, adenocarcinoma and adenocarcinoma was not obviously different ($P>0.05$) (**Figure 1**).

Expression of HPV18 miRNA in different types of cervical carcinoma

Real-time fluorescent PCR showed that the expression of HPV18 miRNA in the OG was (8.32±1.20), higher than that of (1.21±0.47) in the CG ($P<0.05$). The expression of HPV18 miRNA in squamous-cell carcinoma, adenocarcinoma, and adenosquamous carcinoma sub-

Table 2. General information in the three groups

Items		Squamous-cell carcinoma (n=53)	Adenocarcinoma (n=26)	Adenosquamous carcinoma (n=14)	F	*P*
Age (years)		53.68±4.51	54.22±4.57	53.41±4.47	0.572	0.881
Body mass (kg)		59.62±5.09	60.11±5.14	59.81±5.11	1.213	0.418
Course of disease (months)		6.49±0.69	6.51±0.71	6.48±0.67	1.583	0.593
Tumor staging	I	24 (45.28)	10 (38.46)	6 (42.86)	0.796	0.791
	II	16 (30.19)	8 (30.77)	5 (35.71)		
	III	13 (24.53)	8 (30.77)	3 (21.43)		
Pathological grading	G1	21 (39.62)	10 (38.46)	6 (42.86)	0.569	0.843
	G2	20 (37.74)	9 (34.52)	5 (35.71)		
	G3	12 (22.64)	7 (26.92)	3 (21.43)		
Pelvic lymph node metastasis	Yes	20 (37.74)	12 (46.15)	8 (57.14)	0.945	0.778
	No	33 (62.26)	14 (53.85)	6 (42.86)		
Differentiation	Well-	23 (43.40)	10 (38.46)	7 (50.00)	1.424	0.693
	Moderately	17 (32.08)	8 (30.77)	4 (28.56)		
	Poorly	13 (24.53)	8 (30.77)	3 (21.43)		

Table 3. Positive expression of HPV18 in various tissues

Histologic types		Cases	HPV18 protein
OG (n=93)	Squamous-cell carcinoma	53	35 (66.04)[a]
	Adenocarcinoma	26	14 (53.85)[a]
	Adenosquamous carcinoma	14	8 (57.14)[a]
CG		93	5 (5.38)

[a]*P*<0.05 as compared with the CG.

groups were (8.53±1.23), (8.07±1.18), and (8.30±1.21), respectively, and the positive rate of HPV18 protein in different types of cervical carcinoma was not statistically significant (*P*>0.05) (**Figure 2**).

Comparison of HPV18 miRNA expression in various pathological types of cervical carcinoma

There was no significant difference between the expression of HPV18 miRNA and the age and pathological type of the patients (*P*>0.05). The level of HPV18 miRNA expression in cases at stages I-II was lower than that at stage III (*P*<0.05). The level of HPV18 miRNA in lymphatic metastasis patients was higher than that in non-lymphatic metastasis patients (*P*<0.05). Patients with the tumor well differentiated showed lower HPV18 miRNA levels than patients with the tumor moderately and poorly differentiated (*P*<0.05). One-way analysis showed that HPV18 miRNA levels in cervical carcinoma patients were not statistically correlated with age or pathological types (*t*=1.214, 1.246, *P*=0.227, 0.779, respectively), but were significantly correlated with tumor differentiation, lymphatic metastasis, and neoplasm staging (*t*=5.451, 6.392, 5.361, *P*=0.041, 0.034, 0.028, respectively) (**Table 4**).

Correlation analysis of HPV18 expression and clinical pathologies

SPSS Pearson correlation analysis indicated a positive correlation of HPV18 expression with tumor staging and lymphatic metastasis (*r*= 0.673, 0.732, *P*=0.024, 0.015, respectively) as well as a negative correlation with tumor differentiation (*r*=-0.689, *P*=0.021). There was no correlation between HPV18 expression and age or pathological types in cervical carcinoma patients (*r*=0.141, 0.096, *P*=0.793, 0.973, respectively) (**Table 5**).

Discussion

Cervical carcinoma is a malignant tumor found in women with a high incidence. Although the clinical symptoms in the early stage are not typical, it can cause vaginal bleeding and liquid flowing with the development of the disease, affecting patients' health and life [18]. Despite of the un-clarified complex etiology, it is generally considered to be associated with multiple sexual partners, HPV infection, Herpes Simplex

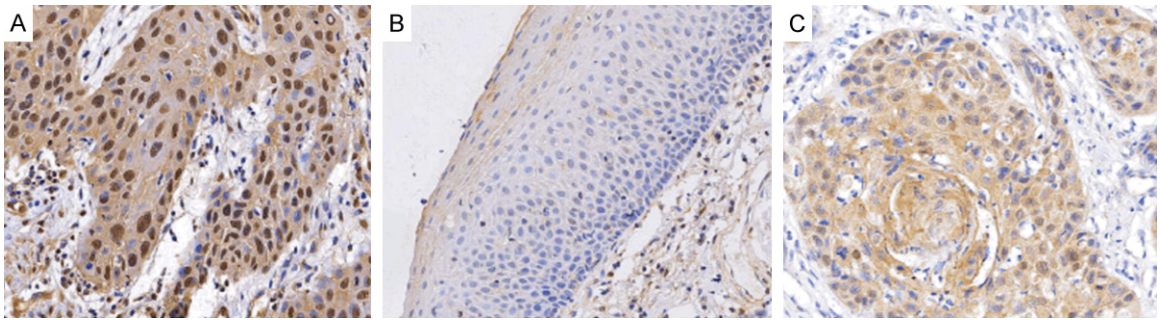

Figure 1. Expression of HPV18 protein in different types of cervical carcinoma (×200). Note: A. Immunohistochemistry of cervical squamous-cell carcinoma; B. Immunohistochemistry of cervical adenosquamous carcinoma; C. Immunohistochemistry of cervical adenocarcinoma.

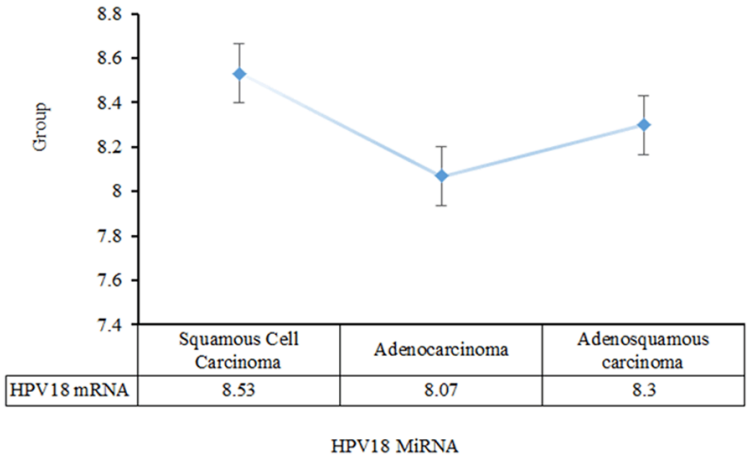

Figure 2. Expression of HPV18 miRNA in different types of cervical carcinoma.

Virus - II, and smoking. Of these factors, HPV infection has been recognized to be a risk. Patients with persistent, high-risk HPV infection have a higher incidence of cervical carcinoma [19]. Epidemiological studies suggested [20] that most patients with cervical carcinoma have a history of HPV infection. Among the various HPVs, HPV16 and HPV18 infections are the most common causes of cervical carcinoma. In this study, the positive rate of HPV18 protein in the OG was 61.29%, higher than that of 5.38% in the CG (P<0.05). Real-time fluorescent PCR revealed the expression of HPV18 miRNA in the OG (8.32±1.20) was superior to that in the CG (1.21±0.47) (P< 0.05), suggesting a highly expressed HPV18 in cervical carcinoma patients, and HPV18 directly participates in the occurrence and development of cervical carcinoma. There was no significant difference in HPV18 expression among cervical carcinoma tissues of different types. Clinical research showed that [21] cervical carcinoma is categorized into squamous-cell carcinoma, adenocarcinoma and adenosquamous carcinoma via pathology. Squamous-cell carcinomas and adenocarcinoma are common in clinic. Adenosquamous carcinoma accounts for only 3%-5%. Adenocarcinoma could be classified into ordinary endometrial carcinoma, mucinous adenocarcinoma, villioustublar adenoma, serous carcinoma, *etc*., all of which have serious impact on patients [22].

Many factors affect the occurrence and development of cervical carcinoma. At this point, skin is a natural barrier against HPV. Once the barrier is injured or damaged, HPV infection occurs and involves the basal lamina of mucosa [23]. It was found in clinical studies that [24] HPV-infected host cells may experience DNA damage and activated P53 transcription, resulting in significant changes in the proliferation cycle of cervical carcinoma cells. HPV infection also inhibits the mechanism of apoptosis and promotes continued cloning of tumor cells that lower an organism's immune response, coupled with poor vaginal environment or other reasons leading to cancer. Research by other scholars demonstrated that [25] HPV DNA could be integrated into injured cervical cells by the action of DNA-related enzymes and run a low-dose replication, which delays the expression in mucosal cells and induces DNA replication of viruses [26]. The

Table 4. Expression of HPV18 miRNA in different pathology of cervical carcinoma

Pathological tissue		Cases	HPV18 miRNA	t	P
Age	≥60 years	45	8.41±1.22	1.214	0.227
	<60 years	48	8.28±1.18		
Tumor staging	I-II	69	8.24±0.95	5.451	0.041
	III	26	8.64±1.26		
Pathological types	Adenocarcinoma	53	8.53±1.23	1.246	0.779
	Squamous-cell carcinoma	26	8.07±1.18		
	Adenosquamous carcinoma	14	8.30±1.21		
Lymphatic metastasis	Yes	40	8.68±1.32	6.392	0.034
	No	55	8.03±1.07		
Differentiation	Well-	40	8.01±1.03	5.361	0.028
	Moderately	29	8.32±1.23		
	Poorly	24	8.73±1.42		

Table 5. Correlation analysis of HPV18 expression with pathology (r, P)

Correlation	Age	Tumor staging	Lymphatic metastasis	Differentiation	Pathological types
r	0.141	0.673	0.732	-0.689	0.096
P	0.793	0.024	0.015	0.021	0.973

integrated HPV destroys and activates the E2 gene to elevate HPV18 expression, causing instability in the cytome of the host. Such actions play important roles in the occurrence and development of cancers [27]. In the present study, HPV18 miRNA levels were not significantly correlated with age or pathological types in patients with cervical carcinoma (P> 0.05), but was correlated with tumor differentiation, lymphatic metastasis and tumor staging (P<0.05), suggesting that HPV18 miRNA is highly expressed in cervical carcinoma, which reflects the severity of the disease. (1) Differentiation types. Differentiation of tumors was generally negatively correlated with the severity of the disease, reflecting the severity of the disease. Similarly, HPV18 levels hint the severity of the disease. A higher expression suggests a more serious disease. HPV18 expression is closely related to tumor classification [28]. (2) Pelvic lymph node metastasis. Pelvic lymph node metastasis affects HPV18 expression as well. Patients with pelvic lymph node metastasis are often in a severe condition and this can be even life-threatening. (3) Neoplasm staging. Tumor staging is only appropriate for malignant tumors and is used to evaluate the number and location of tumors. Clinical studies have shown that neoplasm staging reflects the severity, involvement of surrounding tissues and the extent of infiltration. Recognizing the severity helps doctors to develop good treatment plans that promise better prognosis and outcome. In general, a more advanced staging indicates a higher HPV18 expression.

Other scholars [29] confirmed that active measures taken to inhibit the integration of HPV with mitosis can suppress HPV18 genetic expression and the suppression is not changed in different types of cervical carcinoma. Clinical studies demonstrated that AURKA inhibitor MLN8237/Alisertib affects the sensitivity of precancerous lesions of the uterine cervix to drugs by specifically acting on the expression of HPV18 through the down-regulation of genetic expression in chromosome lq21 mcl. In order to uncover the relationship between HPV18 and pathology types, a correlation analysis was made in this study which showed a positive correlation of HPV18 expression with neoplasm staging and lymphatic metastasis (P<0.05) and a negative correlation with tumor differentiation (P<0.05). No relation between HPV18 expression and age or pathological types was indicated (P>0.05). These results indicate the close relationships of HPV18 and pathologies that HPV18 expression levels better reflect the severity of disease. Some studies have also pointed out that HPV18 showed differences in sensitivity in various pathological tissues of cervical carcinoma, which projects the patient's physical state. HPV infection, a potential cause of malignant cervical carcinoma, participates in cell differentiation. MicroRNA (miRNA) is a small non-coding RNA

that may bind to the targeted RNA inducing degradation of targeted RNA or inhibiting RNA translation. Other studies expressed that miRNAs are involved in multiple signaling pathways and regulate fat metabolism, proliferation and apoptosis. They contribute to the occurrence, development and turnover of tumors. For patients with suspected cervical carcinoma, HPV18 determination should be coupled with clinical diagnosis to make an early diagnosis. Patients diagnosed with cervical carcinoma should be subject to effective interventions where appropriate HPV18 monitoring helps the prognosis. Based on these measurements, prognosis evaluation is related to treatment modulation so that the patients receive personalized management. In a study involving 20 cases of cervical carcinoma [28], patients managed with effective interventions have reduced HPV18 levels and potential control of the invasion of cervical carcinoma cells by targeting MACC1. In HPV18-positive cervical carcinoma patients, HPV18 down-regulates targeted genes by DNA methylation, which offers a new target spot for cervical carcinoma treatment.

In conclusion, HPV18 miRNA is highly expressed in various types of cervical carcinoma with unapparent differences. Its expression is significantly correlated with the pathology of cervical carcinoma. An elevated HPV18 miRNA level in cervical carcinoma patients indicates the severity of the disease, for better clinical treatment.

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Disclosure of conflict of interest

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