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Correlation between Load of HPV 16 DNA in Cervical Cancer and HPV 16 DNA in Lymph Nodes

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OBJECTIVE To determine the association between viral load of human papillomavirus 16 (HPV16) DNA in the primary focus of cervical carcinoma and HPV16 DNA in pelvic lymph nodes.
METHODS The HPV16 DNA load was measured by fluorescent quantitation polymerase chain reaction (FQ-PCR) in 17 primary foci. HPV16 DNA was detected by polymerase chain reaction (PCR) using HPV16 type-specific primers in 296 pelvic lymph nodes which were from 17 cases of cervical cancer.
RESULTS The viral load of HPV16 DNA showed statistically significant differences between tumors with a diameter of < 4 cm and ≥ 4 cm ($P < 0.05$). Seven of 17 cervical cancer cases had HPV16 DNA positive lymph nodes, designated as the positive group, while the remaining 10 without positive lymph nodes was designated the negative group. The average load of HPV16 DNA showed no significant difference between the 2 groups ($P > 0.05$). The load of HPV16 in the primary lesion was not associated with that in the lymph nodes. There were 38 HPV16 DNA positive nodes in the total 296 nodes. The rate of positivity of HPV16 DNA in lymph nodes showed statistically significant differences in consideration of maximum tumor diameter, tumor differentiation, histologic type, depth of myometial infiltration and the metastatic status of the nodes, respectively ($P < 0.05$).
CONCLUSION Viral load of HPV16 in the primary cancer focus correlated with the quantity of tumor cells in the primary focus but not with the existence of HPV DNA positive lymph nodes. Detection of HPV DNA may help to find the early metastases that cannot be evaluated histopathologically, but the prognostic value of HPV positive lymph nodes needs further examination.

KEY WORDS: cervical cancer, lymph nodes, human papillomavirus, viral load.

Introduction

Persistence of human papillomavirus infection is the most important etiologic factor in cervical cancer. Although many women are infected by HPV, most of the infections can be eliminated in a short time period (9-16 months), and only a few women remain persistently infected. The high viral load of HPV is strongly associated with persistent infection and the development of cervical cancer. Viral load of HPV is being actively investigated at present. Research has indicated that integration and expression of the HPV E6/E7 gene in the host are correlated with the occurrence and the development of cervical cancer and that the same gene can be detected in the location of metastasis[1]. HPV DNA detection in pelvic lymph nodes is a helpful predictive

factor for metastases[2]. The relationship between HPV DNA load in cervical cancer and HPV DNA in lymph nodes has not been established.

In our study, viral load of HPV16 DNA was measured by FQ-PCR in 17 primary cancer foci, and HPV16 DNA was detected by PCR using HPV16 type-specific primers in 296 pelvic lymph nodes. We also analyzed the significance of HPV16 DNA load.

Materials and Methods

Specimen

The study group comprised 17 patients suffering from cervical cancer (clinical stage IA to IIB), at the Department of Obstetrics and Gynecology, the Affiliated Hospital of Medical College of Qingdao University from January to October, 2007. All of the patients underwent radical hysterectomy and pelvic lymphadenectomy. Seventeen tumors and 296 lymph nodes were analyzed. None of the patients received radiotherapy or chemotherapy before surgery.

Detection of viral load of HPV in the primary focus by FQ-PCR

HPV16 positive standard preparation (concentration 1×10^7 copies/ml) (HPV16 nucleic acid amplification fluorescence detection kit, PG Biotech, Shenzhen) was made according to serial dilutions into 1×10^7, 1×10^6, 1×10^5, 1×10^4 copies/ml. All of the FQ-PCR reactions were performed in 20 μl reaction mixtures containing 17.7 μl of HPV16 reaction mixture (including primers labeled by FAM, dNTP, $MgCl_2$), 0.2 μl Taq DNA polymerase, 0.06 μl UCG (eliminates contamination in the reaction system) and 2 μl of template DNA. A 5-min at 37°C incubation was followed by 40 cycles of amplification in a FQ-PCR analyzer (ABI7500, USA). Each cycle included a denaturation step at 95°C for 5 s and a primer-annealing and chain elongation step at 60°C for 40 s. The negative control was provided by the kit in every reaction. Each concentration of positive standard preparation was repeated 3 times. The average was obtained, and the FQ-PCR standard curve was drawn. The viral load of the primary tumor was detected and analyzed in accordance with the reaction system and reaction conditions. Each sample was repeated 3 times, and the viral load was calculated by the standard curve.

Detection of HPV 16 DNA in lymph nodes by PCR

The quality of extracted DNA was assessed by PCR targeting a 146 bp fragment of the GADPH gene. The viral load in the lymph nodes was detected by PCR using HPV16 specific primers. The HPV16 primers used were previously designed and described in the reference literature[3] and synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd (Table 1). All of the PCR reactions were performed in 20 μl reaction mixtures containing 10 μl 2 × Premix Taq™ Hot Start Version (TaKaRa Biotechnology (Dalian) Co, Ltd.), which was composed of TaKaRa Taq™ HS, PCR Buffer, dNTP Mixture, and Mg^{2+}; 1 μl upstream primers, 1 μl downstream primers, 6 μl H_2O and 2 μl template DNA. A 3-minute denaturation step at 94°C was followed by 35 cycles of amplification in a PCR thermocycler (Mastercycler Gradient PCR, Germany). Each cycle included a denaturation step at 94°C for 30 s, a primer-annealing step at 55°C for 45 s and a chain elongation step at 72°C for 45 s. The final elongation step was prolonged for 5 min to ensure complete elongation of the amplified DNA. During the detection stage, 5 μl samples of PCR product were subjected to 2% agarose gel electrophoresis (120 V, 30 min) and stained by ethidium bromide. Transilluminated gel images were digitalized and analyzed by means of the Gel Doc System (Bio-Rad, USA). The products of amplification were compared with molecular weight standards to discern the accuracy of them.

Statistical analysis

The *t*-test was used to compare the viral load among the different clinical and histopathologic types of cervical cancer. The chi-square test was used for analyzing the rates of HPV16 DNA positive lymph nodes, and Fisher's exact test used when data was insufficient. The level of statistical significance was set at $P < 0.05$, and statistical software SPSS14.0 was used for statistical analysis.

Results

Drawing of the standard curve by FQ-PCR

The logarithmic value of the HPV16 positive standard is represented on the X-axis, and the cycle-number (Ct) from the serial dilutions is represented on the Y-axis. The software generated the standard curve automatically after each reaction. Specifically, Ct has a linear relationship with the logarithmic value of the template copy number. If the Ct of the unknown samples is known, the copy number of the initial template can be determined according to the standard curve (Fig.1).

Table 1. Primer sequences and primer size.

Gene	Primer sequences	Primer size (bp)
HPV16	5-'TGTGCTGCCATATCTACTTCAGAAACTAC-3' 5-'TAGACCAAAATTCCAGTCCTCCAAA-3'	186
GAPDH	5-'TGTGCTGCCATATCTACTTCAGAAACTAC-3' 5-'TAGACCAAAATTCCAGTCCTCCAAA-3'	146

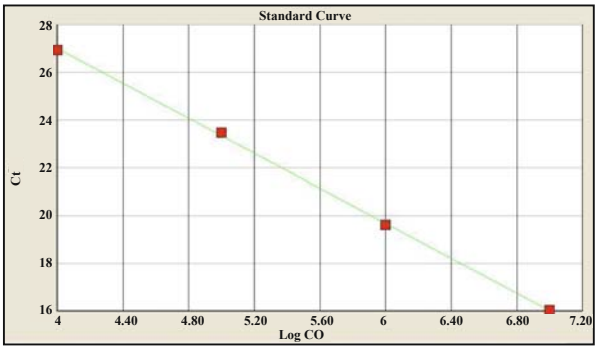

Fig.1. Standard curve of HPV 16 DNA by FQ-PCR.

Detection of load of HPV16 DNA in the primary focus

The Ct value of the 17 primary foci was 21.04 ± 5.07, and the log value of the viral load was 5.52 ± 0.98. The load of HPV16 DNA showed no statistically significant differences with regard to different FIGO stage, tumor histologic grade, depth of cervical infiltration and the metastasis of lymph nodes by cervical cancer ($P > 0.05$). However, it had statistical significance between tumor with a diameter of < 4 cm and > 4 cm (Table 2).

Detection of HPV 16 DNA in lymph nodes

There were 38 HPV16 positive pelvic lymph nodes out of the total 296 lymph nodes. In the 17 with metastatic disease, the positive rate of HPV16 was 88.24% (15/17); in the 279 lymph nodes without metastases, the positive rate of HPV16 was 8.24% (23/279). Seven of 17 cases of cervical cancer had HPV16 positive lymph nodes, designated the positive group, and the remaining 10 were designated the negative group. The viral load of HPV16 DNA had no significant differences between the 2 groups ($P > 0.05$). The *GADPH* gene was positive in all samples, so the quality of extracted DNA was sufficient (Figs.2,3).

The correlation between HPV16 DNA in lymph nodes and the clinical and histopathologic features

The positive rate of HPV16 in lymph nodes correlated with tumor diameter, degree of tumor differentiation, histologic type, depth of myometial infiltration and the presence or absence of metastasis in the lymph nodes ($P < 0.05$, Table 3).

Table 2. The correlation between load of HPV 16 DNA in primary focus and clinical and histopathological features.

Characteristics	*n*	Ct ($\bar{x}$ ± SD)	*P*	$\log^{copies}$ ($\bar{x}$ ± SD)	*P*
Age (years)					
< 40	6	23.37 ± 3.48	0.281	5.88 ± 0.94	0.287
≥ 40	11	25.61 ± 4.18		5.33 ± 1.00	
Clinical stage					
I	13	23.95 ± 2.31	0.107	5.77 ± 0.62	0.065
II	4	27.64 ± 7.05		4.74 ± 1.59	
Tumor diameter (cm)					
< 4	10	26.47 ± 3.93	0.036*	5.13 ± 1.00	0.044*
≥ 4	7	22.46 ± 2.87		6.09 ± 0.68	
Tumor differentiation					
Poor differentiated	5	24.82 ± 1.28	0.999	5.39 ± 0.52	0.727
Moderately**	12	24.82 ± 4.75		5.58 ± 1.14	
Histological type					
SC	15	25.05 ± 4.17	0.535	5.51 ± 1.03	0.895
AC	2	23.11 ± 2.16		5.62 ± 0.83	
Depth of myometrial invasion					
< 2/3	9	24.88 ± 1.58	0.948	5.47 ± 0.45	0.809
≥ 2/3	8	24.75 ± 5.79		5.59 ± 1.40	
Lymph nodes metastasis					
No	12	23.97 ± 3.05	0.182	5.68 ± 0.84	0.341
Yes	5	26.86 ± 5.55		5.16 ± 1.30	
No. of excision lymph nodes					
< 20	11	24.76 ± 4.92	0.935	5.46 ± 1.17	0.744
≥ 20	6	24.93 ± 1.57		5.64 ± 0.57	

* Have statistical significance; **Moderately/well differentiated; SC: squamous cancer; AC: adenous cancer.

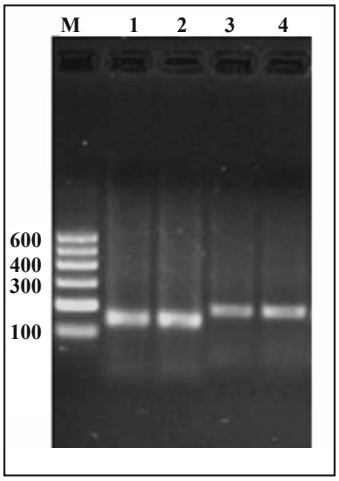

Fig.2. M, DNA MAKER DL600; 1-2, GADPH; 3-4, HPV16 DNA in lymph nodes.

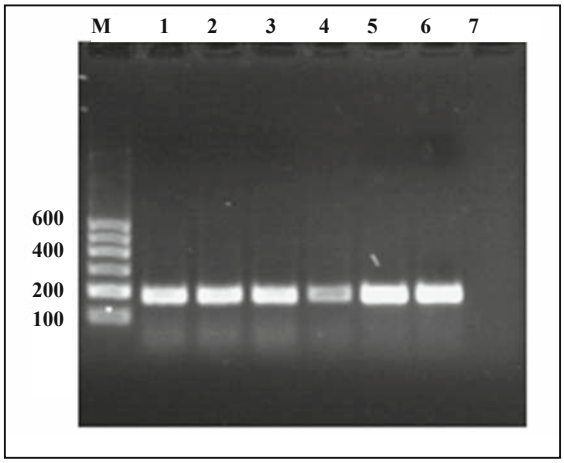

Fig.3. M, DNA MAKER DL600; 1-6, HPV16 DNA in lymph nodes; 7, negative comparison.

Table 3. The correlation between HPV16 positive lymph nodes and clinical and histopathological features.

Characteristics	*n*	HPV+ (%)	χ^2	*P*
Age (years)				
< 40	73	8 (10.96)	0.306	0.580
≥ 40	223	30 (13.45)		
Clinical stage				
I	230	30 (13.04)	0.039	0.843
II	66	8 (12.12)		
Tumor diameter (cm)				
< 4	193	18 (9.33)	6.112	0.013*
≥ 4	103	20 (19.42)		
Tumor differentiation				
Poor differentiated	82	0 (0)	16.705	0.000*
Moderately**	214	38 (17.76)		
Histological type				
SC	269	38 (14.13)	3.205	0.000*
AC	27	0 (0)		
Depth of myometrial invasion				
< 2/3	183	15 (8.20)	9.228	0.002*
≥ 2/3	113	23 (20.35)		
Lymph nodes metastasis				
No	194	8 (4.12)	38.205	0.000*
Yes	102	30 (29.41)		
No. of excision lymph nodes				
< 20	159	19 (11.95)	0.242	0.623
≥ 20	137	19 (13.87)		

* Have statistical significance; **Moderately/well differentiated.

Discussion

Cervical cancer is the most common gynecologic malignancy. More than 200 million women die due to cervical cancer worldwide every year. Persistent HPV infection and the development of cervical cancer are strongly associated. HPV is the only cancer-inducing virus confirmed in human malignancy. There is no known case of cervical cancer in absence of infection with HPV.

Correlation between viral load of HPV and cervical cancer

The existent states of HPV DNA in benign lesions and in malignant disease are different. The episomal form of HPV is detected in HPV related benign lesions and the integrated form of HPV in cervical cancer. The chromosomal binding site is weak and located in the E1/E2 region[3]. Laboratory studies have confirmed that the encoded production of HPV E2 is a negative protein regulator and that it represses the transcription of E6 and E7. The binding leads to uncontrolled expression of the E6 and E7 genes and is responsible for the ability of HPV to transform cells. The E6 and E7 genes exert their transforming function by interrupting cell differentiation and inducing DNA synthesis. They accomplish this by interacting with the cellular tumor

suppressor gene products p53 and pRB[4]. Zheng et al.[5] found that the episomal form of HPV16 was detected in most CIN I, but that the episomal form and the integrated form coexisted in 42.9% of CIN I. The mixed form of HPV16 exists in most of CIN II and CIN III, but the integrated form was found in 68.6% of cases of cervical cancer.

It has been reported that the load of high risk HPV increases with the severity of the cervical lesion. In a recent large study, 5,548 women were engaged as study subjects, and HPV DNA was semi-quantitatively determined by hybrid capture II (HC II). Cen et al.[6] found that significant differences in the high risk HPV infection rate existed between chronic cervicitis and cervical cancer, and CIN ($P < 0.01$), and between CIN I and cervical HPV infection, CIN II, CIN III, and cervical squamous cell carcinoma ($P < 0.01$). You et al.[7] reported the risk of CIN II, CIN III, and cervical cancer increased by 1.325 fold with the log of the HPV load increase.

Integration of HPV into the chromosome of the host cell is important in the process of carcinogenesis[8]. Moberg et al.[1] considered that the high viral load of HPV enhanced the integration of HPV. If the viral load increased, it followed that the replication number of the virus would rise, too, and that the viral load would persistently exist[9].

Gao et al.[10] reported that HPV DNA copies were associated with the quantity of tumor cells. He found that HPV DNA copies were much higher in lymph nodes with metastases than in lymph nodes without but that both had fewer copies than the primary tumor. Further, the study found that the difference between them was significant ($P < 0.01$). Our research supported this viewpoint, and in our investigation the viral load showed statistical significance among tumors with a diameter < 4 cm and > 4 cm ($P < 0.05$). However, there were no statistically significant differences in regard to different FIGO stage, tumor histologic grade, depth of cervical infiltration and the metastasis of lymph nodes by cervical cancer ($P > 0.05$). The diameter of the primary focus reflects the numbers of tumor cells, so we presumed the load of HPV correlated intimately with the growth of cervical cancer.

Correlation between viral load of HPV16 in the primary focus and HPV16 positive lymph nodes

Chen et al.[11] reported that HPV DNA detected in lymph nodes may originate from circulating necrotic tumor cells or those cells internalized by scavengers, which are easier to detect when the viral load per tumor cell is high. However, our results did not agree with these findings. Seven of 17 cases of cervical cancer had HPV16 positive lymph nodes, designated as the positive group, and the remaining 10 were designated as the negative group. The load of HPV16 showed no significant differences between the 2 groups. We thus found that the viral load in the primary lesion was not associated with the HPV16 in the lymph nodes. In our study, the small sample size and the relative accuracy in the methodology might explain the negative results. Furthermore, Häfner[5] was not able to prove that HPV in lymph nodes come from circulating necrotic tumor cells. mRNA disintegrates in necrotic tumor cells, but Häfner detected HPV mRNA in lymph nodes. So whether HPV comes from necrotic tumor cells or not needs further research.

Research has indicated that the integration and expression of the HPV E6/E7 gene in the host correlated with the occurrence and development of cervical cancer, and the same gene can be detected in areas of metastasis[1]. The presence of HPV DNA in histologically negative pelvic nodes is considered as a sub-clinical metastatic spread[12]. Our results indicated that the distribution patterns of lymph node metastases and HPV DNA in lymph nodes were very similar. Among the variables, clinical stage (II), tumor diameter (≥ 4 cm), histologic type (squamous carcinoma), depth of myometrial invasion (≥ 2/3 the myometrial thickness), pelvic lymph node cervical cancer metastasis, and positive rates of HPV16 in lymph nodes were significantly higher ($P < 0.05$).

Correlation between HPV16 in lymph nodes and prognosis

The time of follow up was short in our trial, so we did not summarize the prognosis of patients who had HPV DNA in lymph nodes. In our study, 2 of the 7 patients who had HPV16 positive lymph nodes died in 1 year after surgery. Lukaszuk et al.[13] found that the presence of lymph node HPV DNA, in addition to FIGO stage and primary lesion volume, were independent parameters which correlated with survival and mortality risk. In the trial by Juretzka et al.[14], all 4 patients with micrometastatic disease had poor prognostic features, including lymphovascular space invasion ($n = 3$) and > 4 cm size primary tumor ($n = 2$); in these patients adjuvant radiotherapy was deemed as necessary.

However, there are substantive disputes on whether HPV positive lymph nodes is an unfavorable prognostic factor at present. Chen et al.[11] found that the patient group with HPV positive lymph nodes was associated with a significantly higher viral load in the primary tumor. Out of the 7 patients who had no histologic evidence of lymph node metastasis but had HPV DNA positive lymph nodes, none developed tumor recurrence. In the paper by Fule et al.[15], HPV present in lymph nodes showed no correlation with the survival rate of the patient. Gillison et al.[16] posed the opinion that in non-cervical malignancies such as anal, oropharyngeal, vulvar, and vaginal cancer, HPV-associated cancers had a better prognosis, when compared with their HPV-negative counterparts . In the research by Begum[17], the presence of HPV16 in basaloid squamous cell carcinoma was significantly associated with increased overall survival, even though patients with HPV16-positive carcinomas were more likely to present with lymph nodes

metastases.

To conclude, HPV infection is an essential factor in the development of cervical cancer, and the virus is being widely investigated at present. Although viral load in the primary lesion was not associated with viral load in lymph nodes in our study, advanced research concerning HPV in nodes may help to predict lymph nodes metastasis and help to formulate individualized therapeutic regimens. Decreasing the viral load of HPV and promoting the regression of the disease also have significance in the prevention cervical carcinogenesis.

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